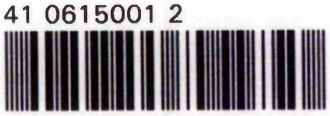


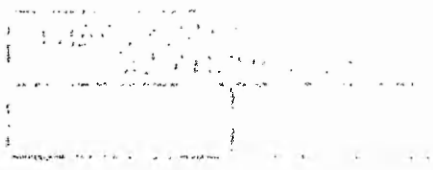
ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Partial Purification and Characterisation of Transglutaminases from Dicotyledonous Plant Tissues

Lyndsey Jane Durose

A thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

May 2001



Abstract

Pisum sativum leaf tissue transglutaminase activity was found predominantly in the soluble fraction but approximately 10% of the activity was found in the microsomal membrane fraction. Microsomal transglutaminase could not be extracted from the membranes by high concentrations of salt or at pH 10, but was released from the membrane by the addition of 0.23% (w/v) sodium deoxycholate. Incubation of the washed microsomal membranes with trypsin released an active fragment of the enzyme. Discontinuous sucrose density gradient centrifugation of a mixed *Pisum sativum* microsomal membrane preparation showed that the microsomal transglutaminase activity was enriched in the tonoplast fraction. The soluble and microsomal forms of transglutaminase activity present in *Pisum sativum* leaf tissue showed similar biochemical characteristics including calcium ion requirement, response to thiol reactive reagents, substrate kinetics and inhibition by GTP when the activities were measured in the presence of sodium deoxycholate. The microsomal transglutaminase activity could be detected in leaf tissue 10 days after germination and rose to a maximum 19 days after germination.

A calcium dependent transglutaminase from *Vicia faba* cotyledons was purified 500-fold by the calcium dependent binding and elution of the enzyme from both ion exchange and hydrophobic interaction chromatography resins. The molecular mass of the *Vicia faba* cotyledon transglutaminase was estimated to be 85 000 by SDS-PAGE and western blotting. The transglutaminase was activated at 10μM free calcium, was thiol dependent and had a pH optimum of 8.0. At sub-optimal calcium ion concentrations the activity was inhibited by GTP suggesting this enzyme could be regulated by GTP nucleotides. The *Vicia faba* cotyledon transglutaminase activity was detected 14 days after germination and reached a maximum 21 days after germination.

Acknowledgements

There are several people I wish to acknowledge for their contribution to this thesis. Sincere thanks to my supervisor, Dr. Philip Bonner for his continued support and guidance during the practical work and the preparation of this thesis. I would like to thank past and present members of the lab for their friendship and help over the past three and a half years, in particular James S., Brian, Richard, Zita and James P. Thanks to the technical staff in the biochemistry department, Jane, Gill, Janet and Rob for the provision of apparatus and general helpfulness. Thanks also to Kevin Bailey at The University of Nottingham for the protein sequencing. I wish to thank my family who have been so tolerant and supportive of me throughout the years. A huge thank you to my good friends Susy, Helen, Steph and Kirsty for keeping me entertained over the years. A special thanks to Carl for his encouragement and for being there whenever I needed him.

Declaration

This work has not been accepted in substance for any other degree and is not currently being submitted in candidature for any other degree.

This is to certify that the candidate herself carried out the work here. Due acknowledgement is made of any assistance.

Signed……..

(Candidate)

Signed……..

(Director of studies)

Contents

Publications and presentations

Publications

(1) Durose, LJ. and Bonner, PLR. (1999) The detection of a calcium activated transglutaminase in *Pisum sativum* root and leaf membranes. *J. Exp. Bot. (supplement)* **50**, 64.

(2) Durose, LJ. and Bonner, PLR. (2000) Partial purification of plant transglutaminase using calcium dependent elution from chromatographic media. *J. Exp. Bot. (supplement)* **51**, 52.

The following papers are in preparation:

(5) Durose, LJ., Griffin, M. and Bonner, PLR. Purification and characterisation of transglutaminase from *Vicia faba* cotyledons.

(6) Durose, LJ., Griffin, M. and Bonner, PLR. Soluble and membrane-associated transglutaminase in *Pisum sativum* leaf tissue.

Presentations

(1) The detection of a calcium activated transglutaminase in *Pisum sativum* root and leaf membranes (1999). Poster presented at the Society for Experimental Biology annual meeting, Heriot-Watt University, Edinburgh, UK.

(2) Partial purification of plant transglutaminase using calcium dependent elution from chromatographic media (2000). Oral presentation given at the Society for Experimental Biology annual meeting, University of Exeter, UK.

(3) Characterisation of a calcium dependent transglutaminase from *Vicia faba* cotyledons (2000). Poster presented at the sixth international conference on transglutaminases and protein crosslinking reactions, Lyon, France.

Abbreviations

AIE	affinity ion exchange chromatography
ANS	1-anilino-8-naphthalenesulfonic acid
ATP	adenosine 5'triphosphate
ATPase	adenosine triphosphatase
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propane-sulfonic acid
cDNA	DNA complementary to RNA
DEAE	diethylaminoethyl
DIECA	diethyldithiocarbamic acid
N,N'-DMC	N,N'-dimethylcasein
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E_{64}	trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane
ECL	enhanced chemiluminescence
EDC	ethyldimethylaminopropyl carbodiimide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FW	fresh weight
GPC	gel permetation chromatography
GTP	guanosine 5'triphosphate
HIC	hydrophobic interaction chromatography
HMBA	p-hydroxymercuribenzoic acid
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
IEC	ion exchange chromatography
kDa	kilodaltons
K_m	Michaelis Menten constant
LDH	lactate dehydrogenase

2-ME	2-mercaptoethanol
MES	(2-[N-morpholino] ethane sulphonic acid)
M_r	relative molecular mass
NEM	N-ethylmaleimide
NADH	nicotinamide adenine dinucleotide (reduced form)
PAGE	polyacrylamide gel electrophoresis
P_i	inorganic phosphate
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonylfluoride
PPase	inorganic pyrophosphatase
PP_i	inorganic pyrophosphate
PVDF	polyvinylidenedifluoride
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
Rubisco	ribulose 1,5-bisphosphate carboxylase-oxygenase
SDS	sodium dodecyl sulphate
SEM	standard error mean
SWR	standard working reagent
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGase	transglutaminase
TLC	thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
Tris	(tris [hydroxymethyl] amino methane)
Triton X-100	t-octylphenoxypolyethoxyethanol
Triton X-114	octylphenoxypolyethoxyethanol
Tween 20	polyoxyethylene sorbitan monolaurate
Tween 80	polyoxyethylenesorbitan
V_{max}	maximum velocity

List of tables

List of figures

List of plates

Chapter 1 – Introduction

Chapter 1 – Introduction

1.1 Introduction to transglutaminases

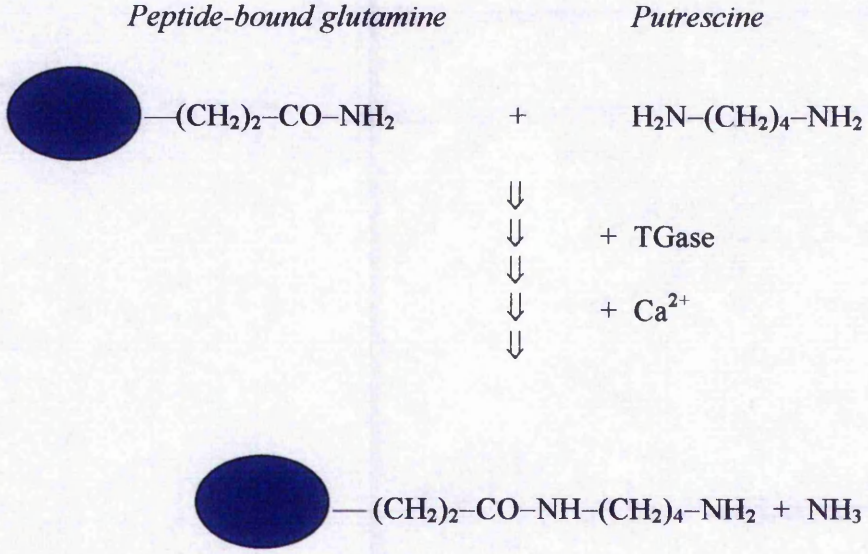
Transglutaminases are recognised by the Enzyme Commission on Nomenclature as R-glutaminyl-peptide: amine γ-glutamyltransferases (E.C. 2.3.2.13). These enzymes catalyse a Ca^{2+}-dependent acyl transfer reaction between peptide-bound glutamine and primary amine groups. This results in the post-translational modification of proteins either by the incorporation of amines or the crosslinking of proteins via ε(γ-glutamyl) lysine bridges when the ε-amino group of peptide-bound lysine serves as the acyl acceptor (figure 1A-C). In the presence of less than saturating levels of a suitable primary amine or in the absence of amine, water can act as the nucleophile with the formation of peptide-bound glutamic acid (figure 1D) (Folk and Chung, 1973).

1.1.1 Reaction mechanism

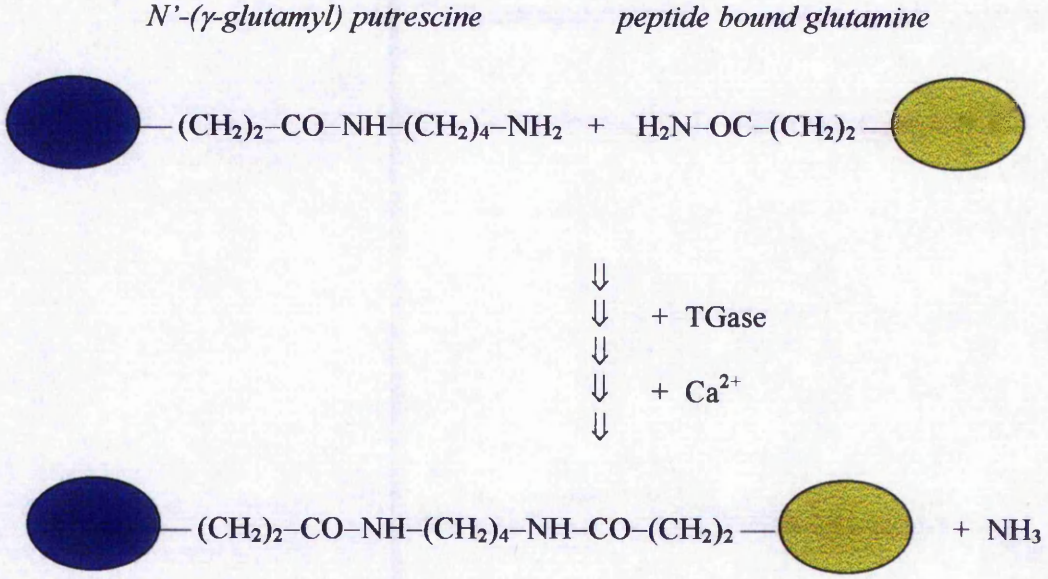
The transglutaminase catalysed reaction is a multistep process (Folk, 1983). Calcium ions bind to the enzyme and expose the active site cysteine, which reacts with the γ-carboxamide group of a suitable glutamine residue forming the γ-glutamyl thioester intermediate and releasing ammonia. The acyl intermediate reacts with a nucleophilic amine, yielding either an isodipeptide crosslink or a (γ-glutamyl) polyamine bond and releasing the reactivated enzyme (for review see Folk, 1983). The resultant bonds are covalent, stable and resistant to proteolysis, thereby increasing the resistance of tissues to chemical and physical degradation (Griffin and Smethurst, 1994). However, an enzyme, γ-glutamylamine cyclotransferase, has been partially purified and characterised in rabbit kidney and various other tissues that catalyses the conversion of γ-glutamylamines to free amines and 5-oxo-proline (Fink *et al.*, 1980). A similar enzyme has also been purified to homogeneity from *Glycine max* (soybean) seeds (Kang *et al.*, 1997).

Figure 1.1 - Reactions catalysed by transglutaminases.

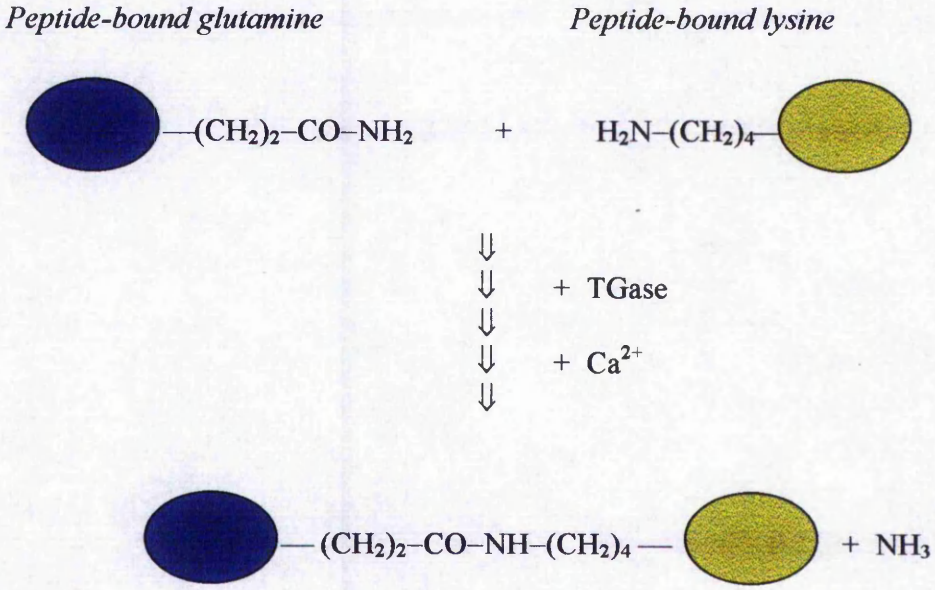
(A) An acyl-transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and a polyamine resulting in the formation of an N'-(γ-glutamyl) polyamine bond.



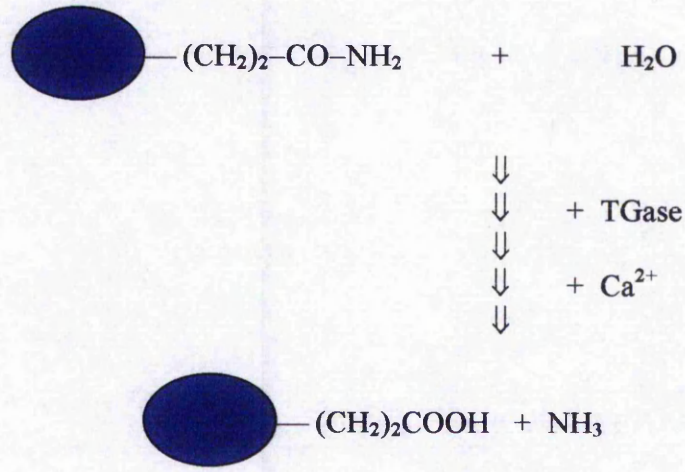
(B) A second amine group of an already covalently attached polyamine can react with a second peptide-bound glutamine residue resulting in the formation of an N',N'-bis(γ-glutamyl) polyamine crosslink in or between proteins.



(C) An acyl-transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and the ε-amino group of a peptide bound lysine residue resulting in the formation of an ε-(γ-glutamyl) lysine isodipeptide crosslink.



(D) Water can act as the nucleophile with the formation of peptide-bound glutamic acid.



1.1.2 Substrate specificity

Transglutaminases are specific for peptide bound glutamine as their acyl donor substrate, they are unable to catalyse the hydrolysis and transfer of the carboxamide group of free glutamine (Folk and Finlayson, 1977). In order to function as a substrate, the glutamine residue must be at least three amino acid residues from the amino terminus of a peptide chain (Folk and Chung, 1973).

Transglutaminases exhibit limited glutamine substrate specificity due to variations in the susceptibility of glutamine residues to catalytic modification (Gorman and Folk, 1980). The susceptibility is predicted by both the amino acid sequence (including charge) and conformation surrounding the glutamine residue (Gorman and Folk, 1981; Aeschlimann and Paulsson, 1994). Under *in vitro* conditions, factor XIIIa and tissue transglutaminase differ in their binding affinity and/or catalytic rate for the amine acceptor sites in β-casein (Gorman and Folk, 1881 and 1984) and fibrin(ogen) (Shianoff *et al.,* 1991) although they often crosslink at the same sites. The structural requirements for a glutamine residue to serve as the acyl donor is more stringent for factor XIIIa than for tissue transglutaminase (Gorman and Folk, 1981 and 1984; Fesus *et al.*, 1986).

Transglutaminases do however possess an exceptionally broad specificity for acyl acceptor substrates (Folk, 1980). The amino acid sequence surrounding a lysine residue does not have a pronounced effect on its ability to act serve as the amine donor, which explains why peptide-bound lysine and primary amines serve equally well as amine donors (Aeschlimann and Paulsson, 1994).

Several transglutaminases have been identified to date and have a wide distribution amongst tissues and physiological fluids of a large number of only distantly related organisms. Transglutaminase activity has been identified in mammals (Clark *et al.*, 1959; Chung, 1972; Birckbichler *et al.*, 1976), invertebrates (Singer *et al.*, 1992; Mehta *et al.*, 1992; Tokunaga *et al.*, 1993b), micro-organisms (Ando *et al.*, 1989; Kanaji *et al.*, 1993) and plants (Icekson and Apelbaum, 1987; Serafini-Fracassini *et al.*, 1988).

1.2 Mammalian transglutaminases

In mammals, seven forms of transglutaminase have been identified which have a wide distribution amongst the various tissues and body fluids (For review see Folk, 1980; Ichinose *et al.*, 1990; Aeschlimann and Thomazy, 2000). Proteins modified by transglutaminase can be found throughout the organism; for example, in fibrin clots formed in haemostasis and wound healing (Folk and Finlayson, 1977), in extracellular matrices (Aeschlimann and Paulsson, 1991), in seminal fluids of rodents (Williams-Ashman, 1984) and in the cornified envelope of the epidermis (Thacher and Rice, 1985; Simon and Green, 1988). There is a degree of amino acid identity between the transglutaminases identified to date. Transglutaminases share an active site consensus sequence, Tyr-Gly-Gln-Cys-Trp, which contains the active site cysteine residue (Griffin and Smethurst, 1994).

1.2.1 Factor XIII

Human plasma factor XIII is a tetramer consisting of a dimer of non-covalently associated a-subunits and two filamentous b-subunits. It is present in plasma as a proenzyme and is activated by thrombin to the catalytically active factor XIIIa during the final stages of the blood coagulation cascade (Lorand, 1986). Factor XIII also exists as a dimer of only a-subunits in platelets, placenta, prostate, hepatocytes and macrophages (for review see Ichinose *et al.*, 1990).

1.2.1.1 Factor XIII structure

The complete amino acid sequence of human placental factor XIII a-chain was established by cDNA cloning and protein sequencing techniques (Ichinose *et al.*, 1990). The a-chain consists of 731 amino acid residues with a relative molecular mass of 83 000 (Ichinose *et al.*, 1986; Grundmann *et al.*, 1986). Despite six potential N-glycosylation sites, no carbohydrate modifications have been detected (Ichinose *et al.*, 1986; Takahashi *et al.*, 1986). The cDNA sequence revealed the lack of a typical hydrophobic leader sequence for secretion (Ichinose *et al.*, 1990). The lack of carbohydrate and disulphide bonding in the structure, and the acetylation of the N-terminal serine residue are consistent with factor XIII a-chains being cytoplasmic proteins (Takahashi *et al.*, 1986). The mechanism of a-chain release into the plasma remains uncertain. Aeschlimann and Thomazy (2000) proposed that the a-chains are

slowly released into the plasma from platelets where their activity is controlled by an excess of b-subunits in the plasma.

Analysis of the three-dimensional structure of the a-subunit of factor XIII shows that the protein has a central core domain that forms the enzyme's active site and Ca^{2+} binding site, an N-terminal β-sandwich domain and two C-terminal β-barrel domains that are presumably involved in regulation of enzyme activity and specificity (Yee *et al.*, 1994 and 1996).

The primary structure of the b-chain of human factor XIII was determined by sequencing clones obtained from a human liver cDNA library (Bottenus *et al.*, 1990). It is composed of 641 amino acids with a relative molecular mass of approximately 79 700 after addition of carbohydrate (8.5%) (Bohn *et al.*, 1972). The b-chain contains a 20 amino acid leader sequence common to secretory proteins (Ichinose *et al.*, 1990).

1.2.1.2 Factor XIII activation

The active enzyme, factor XIIIa, is generated during the final stages of the blood coagulation cascade through thrombin cleavage of the peptide bond between Arg-37 and Gly-38 near the amino terminus of each of the a-chains (Lorand, 1986). In the presence of Ca^{2+} ions, the tetramer dissociates into dimers of 2 a-chains and 2 b-chains (Cooke and Holbrook, 1974; Lorand *et al.*, 1974). The Ca^{2+} ion concentration required for this b-chain dissociation is reduced to plasma Ca^{2+} levels by the presence of fibrinogen (Credo *et al.*, 1978).

The fibrin monomers produced by thrombolytic cleavage of fibrinogen are crosslinked by factor XIIIa into a highly stable matrix; the incorporation of α_2-antiplasmin by factor XIIIa renders the matrix resistant to plasmin degradation (Tamaki and Aoki, 1985). The polymerisation of fibrin monomers increases the rate of thrombin-catalysed cleavage of factor XIII further by providing binding sites for both factor XIII and thrombin (Greenberg *et al.*, 1987).

Individuals who are deficient of factor XIII experience a lifelong bleeding disorder and some wound healing problems (Duckert, 1972). The blood clots these individuals form are mechanically weak and unable to resist proteolytic degradation by plasmin (Tamaki

and Aoki, 1985). Women deficient in factor XIII also experience infertility problems due to spontaneous abortion (Board *et al.*, 1993).

1.2.2 Type I (keratinocyte) transglutaminase

Keratinocyte transglutaminase exists in several intact and proteolytically processed forms partitioned between the cytosol and the membranes (Kim *et al.*, 1995a and 1995b). The enzyme is expressed principally in the granular layer of the epidermis where its major function appears to be in the crosslinking of a number of proteins to form a tough insoluble cell envelope in the terminally differentiating keratinocytes (Griffin and Smethurst, 1994).

1.2.2.1 Structure and distribution of keratinocyte transglutaminase

Keratinocyte transglutaminase is present in proliferating keratinocytes but is more abundantly expressed in differentiating cells. It exists in several intact and proteolytically processed soluble and membrane-associated forms (Kim *et al.*, 1995a and 1995b; Steinert *et al.*, 1996a). In cultured normal human epidermal foreskin keratinocytes (NHEK cells) the soluble forms exist as a full length protein with a M_r of 106 000 with low specific activity and two proteolytically processed forms, one with a M_r of 67 000 and a second consisting of polypeptides with relative molecular masses of 67 000 and 33 000 held together by non-covalent bonds. The proteolytically processed forms have a 5- to10-fold higher specific activity compared with the full length protein. These three soluble forms account for 5% of the total activity in proliferating keratinocytes and up to 35% in terminally differentiating cells (Kim *et al.*, 1995a).

In NHEK cells the membrane-associated enzyme exists in two forms, a full length inactive zymogen with a relative molecular mass of 106 000 and an active complex of polypeptides with relative molecular masses of 67 000, 33 000 and 10 000 that is membrane-anchored by way of the 10 000 M_r polypeptide (Steinert *et al.*, 1996a). In proliferating keratinocytes the membrane-bound inactive zymogen constitutes 95% of the type I transglutaminase protein. However, in terminally differentiating keratinocytes up to 50% of the inactive zymogen is proteolytically processed to the active 67 000 /33 000 /10 000 M_r complex while still bound to the plasma membrane and this membrane-bound form accounts for most of the type I transglutaminase activity in the differentiating keratinocytes (Steinert *et al.*, 1996a).

Earlier works suggested that keratinocyte transglutaminase is peripherally associated with the cytoplasmic side of the plasma membrane via the post-translational addition of thioester-linked palmitic and myristic acid (Chakravarty and Rice, 1989; Rice *et al.*, 1990). Fatty acid acylation occurs at a cluster of five cysteine residues located near the amino terminus of the polypeptide (Chakravarty and Rice, 1989). Site directed mutagenesis of these cysteines (residues 47, 48, 50, 51 and 53) showed that several are involved in attachment of type I transglutaminase to the membrane (Philips *et al.*, 1993). It has been shown that the modification of the enzyme by myristate and palmitate is responsible for the partitioning of the enzyme between the plasma membrane and the cytosol, and the extent of fatty acid acylation correlates to the differentiation state of the keratinocytes (Steinert *et al.*, 1996b).

Keratinocyte transglutaminase is constitutively N-myristoylated during or immediately after translation. In proliferating or stationary confluent cultures of NHEK cells keratinocyte transglutaminase is also thio-esterified by an average of two S-myristoyl adducts allowing robust anchorage of the 106 000 M_r inactive zymogen to the plasma membrane. The half-life of the S-myristoyl anchor is less than that of the protein and therefore the protein can cycle off the membrane and this accounts for the small amount of soluble inactive zymogen present in proliferating cells (Kim *et al.*, 1995a; Steinert *et al.*, 1996b).

In NHEK cells induced to terminally differentiate in a high Ca^{2+} medium, newly synthesised type I transglutaminase is N-myristoylated and S-palmitoylated. S-palmitoylated transglutaminase forms a weak attachment with the membrane and since the half-life of the S-palmitoylation is much less than that of the protein there is an increased cycling of transglutaminase off the membrane (Steinert *et al.*, 1996b). The membrane-bound inactive zymogen is thought to be cleaved by proteases into the active membrane-bound 67 000 /33 000 /10 000 relative molecular mass complex (Kim *et al.*, 1995b). Some of this complex may dissociate from the 10 000 M_r anchorage segment and enter the cytoplasm as the active 67 000 /33 000 M_r complex or dissociate further to the 67 000 M_r form. The 67 000 /33 000 /10 000 M_r membrane-bound form and the 67 000 /33 000 and 67 000 M_r soluble forms account for the majority of type I transglutaminase activity in differentiating keratinocytes (Kim *et al.*, 1995a; Steinert *et al.*, 1996b).

1.2.2.2 Cornified envelope formation

During the terminal differentiation of epidermal cells, an envelope is formed beneath the plasma membrane that consists of insoluble protein as a result of disulphide bonding and ε-(γ-glutamyl) lysine isodipeptide crosslinks formed by the action of transglutaminases (Rice and Green, 1979; Thacher and Rice, 1985). These highly crosslinked envelopes are termed the cornified envelope and form an intricate part of the outermost layer of the epidermis. These terminally differentiated cells surrounded by the cornified envelope are highly cohesive and together form the major protective barrier to the environment (Green, 1979).

Several mutations of the transglutaminase I gene have been identified in families with the skin disorder lamellar ichthyosis (Huber *et al.*, 1995; Russell *et al.*, 1995; Parmentier *et al.*, 1995). In mice lacking the transglutaminase I gene cell envelope assembly was defective, the skin barrier function was markedly impaired, and the mice died within 4 to 5 hours of birth (Matsuki *et al.*, 1998). These results clearly demonstrate the importance of type I transglutaminase to the development and function of the stratum corneum.

1.2.3 Type II (tissue) transglutaminase

Type II or tissue transglutaminase is the most widely distributed form of the enzyme in mammals (Folk and Finlayson, 1977). Immunohistochemical analysis has shown expression of tissue transglutaminase in liver, muscle, kidney, lung, adrenal gland, brain, testes, pancreas, erythrocytes and uterus (Chung, 1972; Lorand and Conrad, 1984). This form of transglutaminase has been found to exist in two forms, a soluble cytosolic form and an insoluble membrane associated form (Birckbichler *et al.*, 1976; Chang and Chung, 1986; Knight *et al.*, 1990).

1.2.3.1 Tissue transglutaminase structure

The primary structure of tissue transglutaminase from guinea pig liver (Ikura *et al.*, 1988), bovine aorta (Nakanishi *et al.*, 1991), mouse macrophages (Gentile *et al.*, 1991), human endothelial cells (Gentile *et al.*, 1991) and chicken erythrocytes (Weraarchakul-Boonmark *et al.*, 1992) has been determined from the nucleic acid sequence of cDNA clones. In these tissues, tissue transglutaminase is a monomeric protein of 685 – 691 amino acids with a relative molecular mass of 76 – 85 000. Amino acid sequence

conservation between species is moderate and varies from 65% from chicken to human, to 88% from bovine to human (Aeschlimann and Paulsson, 1994). The active site cysteine is located at position 276 in guinea pig (Ikura *et al.*, 1988) and 277 in human type II transglutaminase (Gentile *et al.*, 1991).

Despite the fact that guinea pig liver tissue transglutaminase has 5 – 6 N-glycosylation sites and 17 cysteine residues, the enzyme has neither attached sugar residues (Folk and Finlayson, 1977) or disulphide bonds (Folk and Cole, 1966; Boothe and Folk, 1969). The protein also lacks a typical hydrophobic leader-sequence characteristic of secretory proteins. However, N-acetylation of the amino terminus alanine has been demonstrated (Ikura *et al.*, 1989) and this may serve as an alternative signal for protein secretion (Muesch *et al.*, 1990).

In addition to catalysing protein crosslinking, tissue transglutaminase can also bind and hydrolysis GTP and ATP (Achyuthan and Greenberg, 1987; Lee *et al.*, 1989 and 1993). In its GTP-bound form, tissue transglutaminase has reduced affinity for Ca^{2+} due to conformational changes (Bergamini, 1988; Smethurst and Griffin, 1996). The addition of Ca^{2+} reverses the inhibition by GTP suggesting that local concentrations of Ca^{2+} and GTP may help regulate the two catalytic activities *in vivo* (Achyuthan and Greenberg, 1987). GTP/GDP binding inhibits tissue transglutaminase Ca^{2+} dependent crosslinking activity; GTP-binding and GTPase activity are independent of the amino acid residues constituting the active site and crosslinking activity (Chen *et al.*, 1996). It has been shown that the transglutaminase core domain contains the GTP-binding site and GTPase activity and the N-terminal β-sandwich domain and the core domain are essential for transglutaminase crosslinking activity (Iismaa *et al.*, 1997).

1.2.3.2 Functions of tissue transglutaminase

Although tissue transglutaminase has been subject to intensive research, the physiological role for the enzyme is still not fully elucidated and might be diverse in different tissues and biological events. However, tissue transglutaminase has been implicated in a number of different cellular processes and events including, stabilization of the extracellular matrix (Aeschlimann and Paulsson, 1991), apoptotic body formation in cells undergoing programmed cell death (Tarcsa *et al.*, 1992) and GTP binding and hydrolysis in receptor signalling (Chen *et al.*, 1996).

Despite tissue transglutaminase not having a sequence characteristic of secretory proteins, it has been well documented that the enzyme is present in the extracellular space (Aeschlimann and Paulsson, 1991; Verderio *et al.*, 1998) and is expressed at the cell surface of a number of cell types, including fibroblasts, macrophages, hepatocytes and endothelial cells (Barsigian *et al.*, 1991; Bendixen *et al.*, 1993; Gaudry *et al.*, 1999). A number of studies have shown that the N-terminal β-sandwich domain of tissue transglutaminase binds to fibronectin with high affinity (Upchurch *et al.*, 1991; Yeong *et al.*, 1995; Verderio *et al.*, 1998; Gaudry *et al.*, 1999). Furthermore, an N-terminally truncated tissue transglutaminase failed to bind fibronectin *in vitro*, and in contrast to the full length protein was absent from the cell surface, suggesting that tissue transglutaminase secretion into the extracellular matrix is mediated by fibronectin binding (Gaudry *et al.*, 1999). There is increasing evidence to suggest that the cell surface expressed tissue transglutaminase is directed to distinct plasma membrane domains, facilitating matrix assembly at these sites and contributing to the stabilization of cell-surface interactions (for review see Aeschlimann and Thomazy, 2000).

Apoptosis is a form of cell death that plays important roles during embryonic development, normal cell turnover, hormone induced tissue remodelling and tumour regression (Ellis *et al.*, 1991). In contrast to necrosis, execution of apoptosis requires the activation of specific genes. Tissue transglutaminase is thought to be expressed during the final stages of the protein activation cascade leading to cell death (Tarcsa *et al.*, 1992). Activation of tissue transglutaminase results in the formation of intracellular crosslinked protein polymers that constitute the major component in an apoptotic envelope (Tarcsa *et al.*, 1992). The crosslinking process plays an important role in stabilizing the apoptotic bodies and preventing an inflammatory response by immobilizing the intracellular contents. This in turn ensures that the cells are targets for clearance by neighbouring phagocytes (Piredda *et al.*, 1997; Melino and Piacentini, 1998).

In its GTP-bound form tissue transglutaminase has been shown to transmit the α_1-adrenergic receptor signal that activates phospholipase C-δ1, which in turn could modulate various physiological processes, for example blood pressure, appetite and mood (Das *et al.*, 1993; Bylund *et al.*, 1995). Many proteins involved in signal

transduction undergo covalent modifications such as fatty acid acylation and isoprenylation (Aeschlimann and Thomazy, 2000). The presence of fatty acid anchored tissue transglutaminase (Slife *et al.*, 1987; Harsfalvi *et al.*, 1987) in the plasma membrane may regulate this enzyme's function in signalling (Aeschlimann and Thomazy, 2000).

1.2.3.3 Tissue transglutaminase associated pathologies

No inherited deficiencies are known for tissue transglutaminase but it is speculated that deficiency is lethal (Aeschlimann and Thomazy, 2000). Nevertheless, tissue transglutaminase mediated crosslinking has been implicated in a number of pathological events (Lorand and Conrad, 1984; Aeschlimann and Paulsson, 1994; Lorand, 1996).

Tissue transglutaminase in the eye lens has been shown to crosslink β-crystalin subunits (Lorand *et al.*, 1992; Groenen *et al.*, 1992), which is thought to contribute to the increased levels of ε(γ-glutamyl) lysine in cataract tissue (Lorand, 1988). Increased tissue transglutaminase activity and ε-(γ-glutamyl) lysine crosslink are present in artherosclerotic plaques (Bowness *et al.*, 1994) and there is an upregulation of tissue transglutaminase in rheumatoid arthritis but not in osteoarthritis (Weinberg *et al.*, 1991). An abundance of tissue transglutaminase ε-(γ-glutamyl) lysine crosslinks are found in fibrotic diseases such as renal interstitial fibrosis, liver cirrhosis, parasitic liver fibrosis and pulmonary fibrosis (Mirza *et al.*, 1997; Johnson *et al.*, 1997; Kunico *et al.*, 1998; Gerard *et al.*, 1999).

In recent years attention has focused on a potential role for tissue transglutaminase in the pathogenesis of neurodegenerative diseases, including the formation of neurofibrillary tangles and β-amyloid plaques in Alzheimer's disease (Selkoe *et al.*, 1982; Appelt and Balin, 1997). Proteins associated with other neurological diseases, including spinal and bulbar muscular atrophy, dentatorubral-pallidoysian atrophy, spinocerebellar ataxias and Huntington's disease exhibit polyglutamine extensions (Igarashi *et al.*, 1998; Perutz, 1999). These proteins can serve as acyl donor substrates for tissue transglutaminase, the production of ε-(γ-glutamyl) lysine crosslinks causes protein aggregate formation and results in neuronal death and progression of neurodegradation (Kahlem *et al.*, 1996 and 1998; Cooper *et al.*, 1997; Igarashi *et al.*, 1998).

1.2.4 Type III (epidermal) transglutaminase

The primary structure of epidermal transglutaminase was established by cDNA cloning and revealed a protein of 692 amino acids with a relative molecular mass of approximately 77 000 in mouse and human epidermis (Kim *et al.*, 1993). The epidermal transglutaminase is a proenzyme, which *in vitro* can be activated by a number of proteases including trypsin, thrombin, proteinase k and dispase (Kim *et al.*, 1990). However, in the epidermis the proenzyme is probably cleaved by a member of the cysteine proteases, the calpains, which are known to be present in the epidermis (Aeschlimann and Paulsson, 1994). Activation of epidermal transglutaminase occurs by proteolytic cleavage after Ser-469, producing the active enzyme (M_r 50 000) and a carboxyl terminal fragment (M_r 27 000) (Kim *et al.*, 1990 and 1993).

Epidermal transglutaminase is found in epidermal cells during advanced stages of terminal differentiation, and hair follicle cells (Martinet *et al.*, 1988; Kim *et al*, 1990 and 1993). It is likely to play a role in cornified envelope formation during the latter stages of terminal differentiation and may contribute to hair shaft formation (Kim *et al.*, 1993).

1.2.5 Type IV (prostate) transglutaminase

By molecular cloning, a cDNA coding for a major secretory protein (DP1) of rat anterior and dorsal prostate has been isolated (Ho *et al.*, 1992). Sequence analysis revealed that DP1 shares sequence identity with other transglutaminases, including the highly conserved active site region and indicated that DP1 accounted for the previously reported transglutaminase activity in the rat prostate (Williams-Ashman, 1984). Purification of the enzyme from rat coagulation gland secretion revealed a polypeptide with a relative molecular mass of 75 000 (Esposito *et al.*, 1996). The presence of myo-inositol and glycerol bound fatty acids, and the high content of mannose residues were in agreement with previous reports suggesting the presence of a lipid anchor (Seitz *et al.*, 1991). More recently, prostate transglutaminase was discovered in humans following screening of a human prostate cDNA library with a probe for the active site region of tissue transglutaminase (Dubbink *et al.*, 1996).

In rodents, the enzyme is responsible for formation of the copulatory plugs in the female animal's genital tract following coitus (Williams-Ashman, 1984), and may play a role in

masking the antigenicity of the male gamete, thereby suppressing an immune response in the female genital tract against the sperm (Paonessa *et al.*, 1984; Esposito *et al.*, 1996).

1.2.6 Type V transglutaminase

Transglutaminase X (type V) has recently been discovered in cultures of human keratinocytes using a set of degenerate oligonucleotide primers specific for the conserved active site region of transglutaminases (Aeschlimann *et al.*, 1998). A full length cDNA for the protein was obtained by anchored PCR, the deduced amino acid sequence encoded a protein of 720 amino acids with a relative molecular mass of 81 000. Comparisons of transglutaminase X with other members of the transglutaminase family showed conserved sites for Ca^{2+} binding and enzymatic activity and an overall sequence identity of 35% (Aeschlimann *et al.*, 1998).

Transglutaminase X expression was found to increase in keratinocyte cultures induced to differentiate and therefore it may contribute to cornified envelope formation (Aeschlimann *et al.*, 1998). Further *in vitro* studies have suggested that transglutaminase X may have a specific role in early cornified envelope assembly due to maximal levels of expression within 24 hours of the induction of differentiation, compared to 72 hours for maximal expression of transglutaminase I and III (Candi *et al.*, 2000).

1.2.7 Erythrocyte membrane protein band 4.2

Band 4.2 is a structural component of the cytoskeletal network underlying the red blood cell membrane, but is also found in other tissues, for example kidney, platelets and brain (Aeschlimann and Paulsson, 1994). This protein represents the only catalytically inactive member of the transglutaminase family, which is due to a substitution of alanine for cysteine in the transglutaminase active site (Korsgren *et al.*, 1990). The complete amino sequence of human band 4.2 was deduced from its cDNA sequence, the protein consists of 691 amino acids with a relative molecular mass of 77 000 (Korsgren *et al.*, 1990).

Inherited band 4.2 deficiency is a rare genetic disorder causing fragility of erythrocytes. Affected individuals are primarily from Japan and suffer from severe haemolytic anaemia (Cohen *et al.*, 1993).

1.3 Non-mammalian transglutaminases

1.3.1 Fish transglutaminase

Transglutaminase has been purified from *Pagrus major* (red sea bream) and *Onchorhynchus keta* (salmon) liver and the amino acid sequences of the enzymes were deduced from the analysis of cDNA clones (Yasueda *et al.*, 1994 and 1995; Sano *et al.*, 1996). The primary structure of *Pagrus major* and *Onchorhynchus keta* liver transglutaminase showed 43% and 43.5% sequence homology with guinea pig liver transglutaminase respectively. In addition, both the enzymes contained the critical residues thought to form the catalytic-centre triad.

A transglutaminase has been found localised in the egg envelope (chorion) of *Oncorhynchus mykiss* (rainbow trout) (Ha and Iuchi, 1997). Transglutaminase catalysed formation of ε-(γ-glutamyl) lysine crosslinks between chorion subunits resulted in hardening of the chorion.

1.3.2 Invertebrate transglutaminase

Isolation and sequencing of cDNA clones coding for *Tachypleus tridentatus* (limulus) hemocyte transglutaminase (Tokunaga *et al.*, 1993a) and the *Schistocerca americana* (grasshopper) protein annulin (Singer *et al.*, 1992) revealed proteins of 764 and 772 amino acids with relative molecular masses of 86 000 and 97 000 respectively. Sequence comparison of *Tachypleus tridentatus* hemocyte transglutaminase and *Schistocerca americana* annulin with the mammalian transglutaminase family revealed significant sequence homology, particularly with the a-subunit of factor XIII and keratinocyte transglutaminase. Both *Tachypleus tridentatus* hemocyte transglutaminase and annulin protein from *Schistocerca americana* contained NH_2-terminal extensions similar to those of factor XIII a-chains and keratinocyte transglutaminase. Annulin appears to more closely resemble keratinocyte transglutaminase in that it contained possible sites of fatty acid acylation and phosphorylation, and it is intracellularly and peripherally associated with the plasma membrane in epithelial tissues (Singer *et al.*,

1992). *Schistocerca americana* hemocyte transglutaminase fulfils a function similar to that of mammalian factor XIIIa in blood coagulation (Tokunaga *et al.*, 1993b)

1.3.3 Microbial transglutaminase

Transglutaminases of microbial origin have great practical use since the enzyme can be supplied at low cost and is easily purified (Ando *et al.*, 1989). The enzyme may prove to be beneficial in the food processing industry where the crosslinking of food proteins can be used to modify the functional properties of foods (Zhu *et al.*, 1995).

1.3.4 Nematode transglutaminase

A transglutaminase-like enzyme was identified in adult female worms of *Brugia malayi* that is thought to play an important role in *in utero* development and differentiation of embryos to mature microfilariae (Mehta *et al.*, 1990). Identification of ε-(γ-glutamyl) lysine isodipeptide crosslinks further confirmed the presence of a biologically active transglutaminase in the nematode (Mehta *et al.*, 1992). Purification and characterisation of *Brugia malayi* transglutaminase strongly suggested this was a novel member of the transglutaminase family, therefore, it could serve as a good biochemical target for designing parasite-specific chemotherapeutic agents for the effective control of infections caused by these parasites (Singh and Mehta, 1994).

Cloning of transglutaminase from the filarial parasite *Dirofilaria immitis* derived a protein with a relative molecular mass of 57 000 and no sequence homology to mammalian transglutaminases (Chandrashekar *et al.*, 1998). The protein did however share significant sequence homology with protein disulphide isomerase (PDI). The recombinant protein expressed in *Escherichia coli* catalysed the isomerisation of intramolecular disulphide/sulphydryl bonds in denatured RNase as well as Ca^{2+}-dependent ε-(γ-glutamyl) lysine isodipeptide crosslink formation. Conversely, purified PDI from bovine liver was also found to catalyse Ca^{2+}-dependent protein crosslinking. These observations suggest that isodipeptide bond formation may be an integral part of several proteins (Chen and Mehta, 1999).

Transglutaminase from the nematode *Caenorhabditis elegans* was found to be involved in the molecular events occurring during programmed cell death (Madi *et al.*, 1998).

1.3.5 Fungal transglutaminase

During the differentiation of *Physarum polycephalum* microplasmodia to spherules, transglutaminase specific activity increased six-fold. Changes in organisation of the cytoskeleton of *Physarum polycephalum* that accompanies spherule formation may involve transglutaminase catalysed crosslinking processes that require actin or actin filaments (Klein *et al.*, 1992). The spherules formed have the capacity to survive starvation and desiccation.

1.4 Plant transglutaminases

1.4.1 Evidence and detection of transglutaminase in plant tissues

Based on the incorporation of radiolabelled polyamines into protein substrates there is an expanding body of evidence to support the presence of transglutaminase in plant tissue (for review see Serafini-Fracassini *et al.*, 1995). This included the recovery of mono- and bis- (γ-glutamyl) polyamine conjugates after an *in vitro* assay performed by incubating radiolabelled polyamines with cell free extracts of *Beta vulgaris* (Signorni *et al.*, 1991) and *Helianthus tuberosus* leaves (Del Duca *et al.*, 1995). In addition, the ε-(γ-glutamyl) lysine crosslink has been detected in the root and leaf tissue of *Pisum sativum*, *Vicia faba*, *Triticum aestivum* and *Hordeum vulgare* and offers unequivocal proof of transglutaminase activity in plant tissues (Lilley *et al.*, 1998a).

1.4.1.1 Incorporation of labelled polyamines

The incorporation of radiolabelled polyamines into exogenous protein substrates, such as N',N'-dimethylcasein, is a commonly used method for the detection of transglutaminase activity (Lorand *et al.*, 1972). Many modifications of this original method have been developed in order to study transglutaminase activity in plant tissues.

In 1987, Icekson and Apelbaum detected a transglutaminase-like activity in the meristematic tissue of etiolated *Pisum sativum* seedlings using a modification of the method of Lorand *et al.* (1972). The 27 000.g supernatant of crude plant extracts was incubated at 37°C for 30 minutes with N',N'-dimethylcasein and [^{3}H] labelled putrescine. Aliquots of the reaction mixture were spotted onto filter paper and protein was precipitated by treatment with ice cold 10% (w/v) TCA. Filters were washed, dried, placed in liquid scintillation cocktail and counted in a scintillation counter to quantify covalently incorporated [^{3}H] putrescine. Using a similar assay, transglutaminase-like

activity, mainly located in the 22 000.g supernatant prepared from crude extracts of *Helianthus tuberosus* sprout apices, was able to incorporate radiolabelled polyamines into endogenous proteins of different molecular masses (Serafini-Fracassini *et al.*, 1988). Kang and Cho (1996) measured transglutaminase-like activity in *Glycine max* leaf extracts using a method involving [^{14}C]-labelled putrescine incorporation into N',N'-dimethylcasein bound to nitrocellulose discs.

The incorporation of various radiolabelled polyamines into endogenous protein substrates and their separation by SDS-PAGE has also been used as a measure of transglutaminase-like activity in plant tissues (Serafini-Fracassini *et al.*, 1988 and 1989). Crude extracts prepared from *Helianthus tuberosus* sprout apices and leaves were incubated with [^{3}H] putrescine and the proteins were resolved by SDS-PAGE. SDS-polyacrylamide gel lanes were cut into either Coomassie blue positive or negative bands and following gel dissolution the bands were counted for radioactivity. Endogenous protein substrates of various relative molecular masses (30 000 – 92 000) were found to be radiolabelled. Margosiak and co-workers (1990) presented evidence of transglutaminase-like activity in meristematic floral buds of *Medicago sativa*. Monodansylcadaverine was incubated with crude plant extracts for 90 minutes and the reaction mixture was separated by SDS-PAGE. Transglutaminase catalysed conjugation of monodansylcadaverine to endogenous proteins was viewed under UV light.

A further method used for the detection of plant transglutaminase-like activity was the crosslinking of proteins by *Lupinus albus* crude extracts (Siepaio and Meunier, 1995). An enzyme present in the 41 400.g pellet of etiolated *Lupinus albus* seedlings catalysed the covalent conjugation of monodansylcadaverine and [^{14}C] putrescine to N',N'-dimethylcasein, labelled proteins were visualised by fluorescent detection or by autoradiography in SDS-polyacrylamide gels. The incubation of *Lupinus albus* extract with bovine casein, spinach Rubisco and 7S and 11S globulins produced high molecular mass polymers unable to enter the resolving gel of SDS-polyacrylamide gels.

The incorporation of biotin-labelled cadaverine into microtiter plate bound N',N'-dimethylcasein and the crosslinking of biotin-labelled casein to microtiter plate bound EDC-modified casein have been used for the detection of a calcium dependent

transglutaminase in the root and leaf tissues of *Pisum sativum*, *Vicia faba*, *Triticum aestivum* and *Hordeum vulgare* (Lilley *et al.*, 1998a).

1.4.1.2 Detection of transglutaminase products

The assays described above provide much circumstantial evidence for the binding of labelled polyamines to proteins by the action of transglutaminase. However, the only proof of the presence of a catalytically active transglutaminase is to isolate one of the products of the transglutaminase reaction (Folk and Finlayson, 1977).

N'-(γ-glutamyl) putrescine was detected after incubation of a 15 000.g pellet fraction prepared from *Beta vulgaris* leaf extract for 60 minutes with [^{14}C]-labelled putrescine (Signorini *et al.*, 1991). The conjugate was identified after exhaustive proteolytic digestion of the labelled proteins followed by ion exchange chromatography using an LKB amino acid analyser according to the method of Folk *et al.* (1980). Identification of the N'-(γ-glutamyl) putrescine conjugate was further verified by the detection of stoichiometric amounts of glutamic acid and putrescine following acid hydrolysis of the corresponding N'-(γ-glutamyl) putrescine peak after chromatographic separation. N'-(γ-glutamyl) putrescine, N^1,N^4-bis(γ-glutamyl) putrescine and N^1,N^8-bis(γ-glutamyl) spermidine have been isolated after incubation of chloroplasts from *Helianthus tuberosus* with radiolabelled putrescine and/or spermidine (Del Duca *et al.*, 1995) using a method similar to that described by Signorini *et al.* (1991).

In vivo N'-(γ-glutamyl) derivatives were recently identified in seeds *of Glycine max* (Kang *et al.*, 1998). The fixing and staining of 12 hour imbibed *Glycine max* seeds with an anti-putrescine antibody revealed that putrescine conjugates were mainly localized in the protein bodies. Probing western blots of purified glycinin isolated from *Glycine max* seeds with the anti-putrescine antibody revealed that putrescine was covalently attached to the glycinin subunits, furthermore polyamines were identified in the acid hydrolysates of the purified glycinin. In addition, polyamines were released from proteolytic digests of the purified glycinin by the action of γ-glutamylamine cyclotransferase, an enzyme specific for the disassembly of γ-glutamylamines (Fink *et al.*, 1980).

Isolation of these mono- and bis-polyamine conjugates is acknowledged as good proof of the existence of transglutaminase, however, N'-(γ-glutamyl) polyamines may be formed in tissues without the action of transglutaminase (Tack *et al.*, 1981; Beninati *et al.*, 1988) and therefore the only unequivocal proof for the existence of transglutaminase activity in plants would be the isolation of the ε-(γ-glutamyl) lysine isodipeptide crosslink (Folk and Finlayson, 1977).

Recently the ε-(γ-glutamyl) lysine isodipeptide crosslink was detected in the root and leaf tissues of two dicotyledonous and two monocotyledonous plants (Lilley *et al.*, 1998a). The leaf and root tissues of *Pisum sativum*, *Vicia faba*, *Triticum aestivum* and *Hordeum vulgare* were subject to proteolytic digestion (Griffin *et al.*, 1982) and amino acid analysis. Owing to the low levels of isodipeptide in these tissues, the isodipeptide crosslink could only be detected if an additional purification step was undertaken before amino acid analysis. To confirm the presence of the isodipeptide, the peak from the amino acid analyser was collected and subjected to reversed-phase chromatography and the isodipeptide was confirmed by co-elution with standard. In addition, the underivitized sample from the amino acid analyser was hydrolysed overnight and reversed-phase chromatography was used to confirm the presence of glutamine and lysine. The levels of ε-(γ-glutamyl) lysine isodipeptide in the plant proteins was approximately 3% of that found in clotted fibrin (Griffin and Wilson, 1984). This low level of isodipeptide and the necessity for an additional purification step could explain why Chiarello *et al.* (1996a) failed to detect isodipeptide after incubation of β-casein with protein extracted from the meristematic tissue of etiolated *Pisum sativum* seedlings.

1.4.1.3 Immunodetection of plant transglutaminases

No antibodies raised against a plant transglutaminase are currently available, however, the presence of transglutaminase in plant tissue has been assessed using polyclonal antibodies raised against purified mammalian transglutaminases.

A polyclonal antibody raised against a purified transglutaminase from rat prostatic gland cross reacted with a single protein with a relative molecular mass 58 000 in extracts prepared from *Helianthus tuberosus* chloroplasts and isolated thylakoids (Del Duca *et al.*, 1994 and 2000b). The same polyclonal antibody cross reacted with a single

protein band with a relative molecular mass of 80 000 on western blots of proteins isolated from rehydrated ungerminated pollen and germinated pollen of *Malus domestica* (Del Duca *et al.*, 1997). During the progression of the cell cycle in extracts prepared from activated *Helianthus tuberosus* tuber slices, western blots probed with the anti-prostatic gland transglutaminase polyclonal antibody revealed an increase in band density corresponding to two polypeptides with relative molecular masses of 58 000 and 90 000 (Del Duca *et al.*, 2000a). A protein with a relative molecular mass of 58 000 was immunodetected with the same antibody in callus tissue and isolated chloroplasts of cultured *Zea mays* callus (Bernet *et al.*, 1999).

Western blot analysis revealed that a polyclonal antibody raised against human erythrocyte transglutaminase cross reacted with several proteins in crude extracts prepared from *Medicago sativa* floral buds, the most prominent of these had a relative molecular mass of 80 000 (Kopecki-Fjetland *et al.*, 1996).

Western blots of proteins probed with a polyclonal antibody to guinea pig liver tissue transglutaminase cross reacted with a protein with a relative molecular mass of 72 000 in extracts prepared from the unicellular green alga *Chlamydomonas reinhardtii.* Furthermore, probing the western blot with antibody that had been pre-incubated with purified guinea pig liver transglutaminase showed that the signal was competed away in a concentration dependent manner (Waffenschmidt *et al.*, 1999). Western blots of proteins isolated from the chloroplasts of the unicellular green alga *Dunaliella salina,* probed with an anti-rat prostatic gland transglutaminase antibody cross reacted with two protein bands with relative molecular masses of 25 000 and 50 000 (Dondini *et al.*, 2000).

1.4.1.4 Purification of plant transglutaminase

Purification of transglutaminase from *Medicago sativa* floral buds using ammonium sulphate precipitation, ion exchange chromatography, isoelectric focusing and reversed-phase HPLC revealed a protein with a molecular mass of approximately 39 000 (Kuehn *et al.*, 1991). However, the same laboratory reported that a polyclonal antibody directed against human erythrocyte transglutaminase cross reacted with several proteins, the most prominent having a relative molecular mass of 80 000 in crude extracts prepared

from the same tissue (Kopecki-Fjetland *et al.*, 1996). The possibility arises that more than one form of transglutaminase is present in floral buds of *Medicago sativa*.

A transglutaminase-like activity was recently purified from *Glycine max* leaf tissue by ammonium sulphate precipitation, ion exchange chromatography and affinity chromatography. The purified enzyme had a molecular mass of 80 000 and properties similar to those of mammalian tissue transglutaminase (Kang and Cho, 1996).

1.4.2 Plant transglutaminase biochemistry

1.4.2.1 Calcium ion requirement

Mammalian transglutaminases are calcium dependent enzymes (Aeschlimann and Paulsson, 1994). In contrast, transglutaminase activity in plant tissues has been found to have no absolute requirement for the cation (for review see Serafini-Fracassini *et al.*, 1995) and transglutaminases isolated from bacteria have been found to be calcium-independent enzymes (Ando *et al.*, 1989; Kanaji *et al.*, 1993; Kobayashi *et al.*, 1998).

Icekson and Apelbaum (1987) found that calcium ions stimulated etiolated *Pisum sativum* meristematic transglutaminase activity when measured in the presence of the substrate N',N'-dimethylcasein, but they found no absolute requirement for Ca^{2+} when only endogenous protein substrates were present. The Ca^{2+} chelating compounds EDTA and EGTA produced only a small inhibition of the [^{3}H] putrescine incorporating activity.

Transglutaminase-like activity in crude extracts of *Helianthus tuberosus* sprout apices was not stimulated by the addition of exogenous Ca^{2+} and above 5mM it caused severe inhibition, EDTA and EGTA produced a 16% and 17% inhibition respectively at 10mM (Serafini-Fracassini *et al.*, 1988). In a 22 000.g pellet extract prepared from *Helianthus tuberosus* leaf tissue the addition of exogenous calcium caused a clear inhibition of [^{14}C] putrescine incorporation into endogenous proteins, 5mM EDTA provoked a 25% inhibition of the incorporation activity (Falcone *et al.*, 1993). However, transglutaminase-like activity in isolated chloroplasts of *Helianthus tuberosus* leaves was stimulated 145% by the addition of 1 – 3mM $CaCl_2$ (Del Duca *et al.*, 1994). The [^{14}C] putrescine-binding activity of *Chrysanthemum morifolium* explants cultivated in a medium promoting bud formation was inhibited above 2mM $CaCl_2$ (Aribaud *et al.*,

1995). Using partially purified Rubisco as a substrate, transglutaminase activity in *Medicago sativa* floral buds was inhibited 40% by 2.4mM $CaCl_2$ and 10% by 1mM EDTA (Margosiak *et al.*, 1990).

Transglutaminase purified from *Glycine max* leaf tissue incorporated [^{14}C] putrescine into N',N'-dimethylcasein in the absence of Ca^{2+}, the addition of up to 10mM $CaCl_2$ did not affect the [^{14}C] putrescine incorporating activity (Kang and Cho, 1996). However, in a 15 000.g pellet extract prepared from *Beta vulgaris* leaves, Signorini *et al.* (1991) reported that polyamine incorporating activity took place in the absence of calcium ions, but the addition of calcium chloride up to 5mM stimulated the activity and chelating agents reduced the activity.

Assays involving the incorporation of radiolabelled polyamines into protein substrates such as N',N'-dimethylcasein and Rubisco may not be appropriate for screening crude plant extracts for transglutaminase activity because of the possible interference by diamine oxidases (Siepaio and Meunier, 1995; Chiarello *et al.*, 1996a and 1996b; Lilley *et al.*, 1998a). Diamine oxidases catalyse the oxidative deamidation of diamines to amino-aldehyde intermediates with the release of ammonia and hydrogen peroxide (Hill, 1971), these aldehydes could be further oxidised or they could react spontaneously with protein amino groups giving rise to a Schiff base formation (Hill, 1971; Lorand and Conrad, 1984).

Measuring [^{14}C] putrescine incorporation into N',N'-dimethylcasein using crude plant extracts revealed activity in only three out of the eight crude plant extracts screened. The Ca^{2+}-chelating agents EDTA and EGTA at 5mM were unable to effect more than a 35% inhibition (Lilley *et al.*, 1998a). However screening the same leaf and root tissues of *Pisum sativum*, *Vicia faba*, *Triticum aestivum* and *Hordeum vulgare* using a biotin cadaverine incorporation assay (Slaughter *et al.*, 1992) and a casein-crosslinking assay (Lilley *et al.*, 1997), revealed activity was present in all the tissues. Furthermore, chelation of Ca^{2+} by 1mM EDTA or EGTA resulted in more than 80% inhibition of *Pisum sativum* root and leaf transglutaminase activity and 100% inhibition of all other extracts screened (Lilley *et al.*, 1998a). These data suggested that both the microtiter plate assays are more suitable for measuring transglutaminase activity in crude plant

extracts, as the activity is not masked by diamine oxidases. The assays also confirm that these plant tissues contain a calcium dependent transglutaminase.

1.4.2.2 GTP regulation

As previously stated, type II transglutaminase from mammals is inhibited by GTP at sub-optimal calcium ion concentrations (Smethurst and Griffin, 1996) and exhibits weak GTPase activity (Lee *et al.*, 1989). Using crude extracts prepared from *Pisum sativum* root tissue, the biotin cadaverine incorporation activity was not inhibited by 1mM GTP at 1μM free Ca^{2+} (Lilley *et al.*, 1998a). The use of crude extracts in GTP inhibition studies may be misleading due to the possible quenching of GTP by other GTP binding proteins. The only report to date of the inhibition of a plant transglutaminase by GTP has been shown using a purified enzyme from *Glycine max* leaf tissue (Kang and Cho, 1996).

1.4.2.3 Proteolytic regulation

The addition of the protease inhibitors PMSF and leupeptin to an extract of *Helianthus tuberosus* tuber caused a reduction in radiolabelled polyamine binding activity (Grandi *et al.*, 1992). Similarly, the extraction of chloroplasts from *Helianthus tuberosus* leaf tissue in the presence of the protease inhibitors PMSF, ε-amino-n-caproic acid, pepstatin and leupeptin caused a reduction in the labelling of endogenous proteins with [^{14}C] spermidine. Therefore *Helianthus tuberosus* tuber and chloroplast transglutaminase could be activated by proteolytic processing, as reported for transglutaminase in mammalian cells (Kim *et al.*, 1990) and micro-organisms (Pasternack *et al.*, 1998), or the protease inhibitors may directly affect transglutaminase by inactivation of an essential amino acid (Del Duca *et al.*, 2000b).

1.4.2.4 Light stimulation and regulation

Margosiak *et al.* (1990), using isolated chloroplasts from *Medicago sativa*, showed that [^{14}C] putrescine was conjugated to the large subunit of Rubisco and the reaction was almost doubled in the light. This stimulation was suggested to be due to an increase in putrescine transport into chloroplasts, enhanced accessibility of Rubisco subunits to transglutaminase catalytic action or a direct stimulation of the transglutaminase catalysed reaction (Margosiak *et al.*, 1990). Previously, Cohen *et al.* (1982) demonstrated a light dependent uptake of polyamines into isolated chloroplasts of

Brassica pekinesis and their incorporation into a TCA insoluble fraction. Similarly in isolated chloroplasts and thylakoids of *Helianthus tuberosus*, [^{14}C] putrescine incorporation into endogenous protein was increased 120% and 150%, respectively, in the light with respect to the dark (Del Duca *et al.*, 1994). In another system, light markedly inhibited the uptake of radiolabelled polyamine and consequently polyamine binding to TCA insoluble material in *Avena sativa* protoplasts (Mizrahi *et al.*, 1989).

Transglutaminase activity was detected in cultured *Zea mays* callus and their isolated chloroplasts during a 16 hour light /8 hour dark phase of culture by measuring the radioactivity due to labelled putrescine incorporation into TCA precipitated proteins (Bernet *et al.*, 1999). Calluses presented a maximum of transglutaminase activity at 3 hours into the light phase, higher activity was detected in differentiating calluses compared to growing calluses. At 16 hours when the dark phase began, transglutaminase activity presented a second maximum, but only in differentiating calluses and thereafter it slowly decreased. The trend was shown to be similar in the chloroplasts isolated from the callus. Therefore in differentiating *Zea mays* calluses and their chloroplasts' transglutaminase activity was not strictly light-dependent.

1.4.2.5 Thiol dependence

The active site region of the mammalian transglutaminases is highly conserved and contains an essential cysteine residue, and the activity was shown to be affected by thiol reactive reagents (Folk and Cole, 1966). Reducing agents such as DTT have been shown to exhibit varying effects on plant transglutaminase-like activity depending on the plant and tissue of origin. The addition of 1 - 5mM DTT induced a 40 - 80% reduction in *Pisum sativum* polyamine binding activity (Icekson and Apelbaum, 1987; Chiarello *et al.*, 1996a) and the presence of 10mM DTT reduced the rate of polymerisation of β-casein by *Lupinus albus* seedling transglutaminase (Siepaio and Meunier, 1995). The presence of 2mM 2-mercaptoethanol had no effect on polyamine incorporation activity in partially purified *Medicago sativa* floral bud extracts (Margosiak *et al.*, 1990).

Conversely, the presence of 30mM DTT was essential for the preservation of polyamine binding activity of *Helianthus tuberosus* sprout apices extracts (Serafini-Fracassini *et al.*, 1988). Kang and Cho (1996) reported that the activity of purified transglutaminase

from *Glycine max* leaves was almost doubled by the presence of 10mM DTT and was inhibited 60% by 0.1mM N-ethylmaleimide. Soluble *Pisum sativum* root and leaf transglutaminase were inhibited 32% and 24%, respectively, by 10mM iodoacetamide (Lilley *et al.*, 1998a) and the thiol reagents N-ethylmaleimide and p-chloromercuribenzoate completely blocked transglutaminase activity in both vegetative and zygotic cells of *Chlomydomonas reinhardtii* (Waffenschmidt *et al.*, 1999).

Conflictingly, the polyamine incorporating activity in isolated chloroplasts of *Helianthus tuberosus* was inhibited 43% by 10mM N-ethylmaleimide and 95% by 5mM DTT (Del Duca *et al.*, 2000b). Ten millimolar N-ethylmaleimide partially inhibited the incorporation of [^{14}C] putrescine into *Phaseolus aureus* mitochondria, however, the incorporation of [^{14}C] spermine and spermidine was increased 2.5- to 3-fold by the presence of 10mM N-ethylmaleimide (Votyakova *et al.*, 1999). The variations in thiol requirement may reflect problems with interpreting data obtained from crude plant extracts.

1.4.2.6 Substrate specificity

In contrast to their wide specificity for acyl acceptors, transglutaminases possess a limited specificity for acyl donors (Gorman and Folk, 1980) and therefore only limited numbers of endogenous proteins serve as substrates for transglutaminases. Conjugates formed by the activity of plant transglutaminases have frequently been separated by SDS-polyacrylamide gel electrophoresis and the molecular mass and identity of the modified proteins determined (Margosiak *et al.*, 1990; Falcone *et al.*, 1993; Del Duca *et al.*, 1994 and 1997).

Transglutaminase in etiolated *Pisum sativum* meristematic extracts was capable of recognising specific sites in several animal transglutaminase substrate proteins including, N',N'-dimethylcasein, insulin, pepsin, fibrinogen and thrombin, as well as cellulase and creatin kinase which have not previously been shown to be substrates for animal transglutaminases. Native BSA and catalase did not serve as amine acceptors in the [^{3}H] putrescine incorporation assay (Icekson and Apelbaum, 1987). N',N'-dimethylcasein was not recognised as a substrate for transglutaminase in *Helianthus tuberosus* extracts, however, [^{3}H] putrescine was incorporated into endogenous proteins of different molecular masses and produced high molecular mass polymers unable to

enter 10% (w/v) SDS-polyacrylamide resolving gels (Serafini-Fracassini *et al.*, 1988 and 1989; Falcone *et al.*, 1993). A 41 400.g pellet extracted from *Lupinus albus* seedlings incorporated [^{14}C] putrescine into a number of exogenous amine acceptor substrates, 7S and 11S globulins were the best amine acceptors. N',N'-dimethylcasein and spinach Rubisco also served as amine acceptors (Siepaio and Meunier, 1995).

Rubisco was shown to be a substrate for transglutaminase in *Medicago sativa* floral bud extracts by the incorporation of both [^{14}C] putrescine and monodansylcadaverine into the large subunit (Margosiak *et al.*, 1990). Analysis of the primary structure of Rubisco revealed several glutamine and lysine residues that have the potential to serve as crosslinking and conjugation sites for transglutaminase. After a 30 minutes incubation of isolated chloroplasts of *Helianthus tuberosus* with [^{14}C] putrescine or spermidine, high molecular mass polymers were not detected in SDS-polyacrylamide gels. Radiolabelled polyamines were found conjugated to apoproteins of the chlorophyll a/b antenna complex namely, the major light harvesting complex (LCHII), CP24, CP26 and CP29 and the large subunit of Rubisco (Del Duca *et al.*, 1994).

[^{14}C] putrescine supplied to the growth media of cultured parenchyma cells and activated tuber slices of *Helianthus tuberosus* was incorporated into low molecular mass polypeptides (17 500 – 19 000) in G_1 phase, with further progression of the cell cycle very high molecular mass protein polymers were formed (Mossetti *et al.*, 1987). [^{14}C] spermidine supplied to *Nicotiana tabacum* and *Oryza sativa* cell suspensions also brought about the specific labelling of a polypeptide with a relative molecular mass of 18 000 (Apelbaum *et al.*, 1988; Mehta *et al.*, 1991).

1.4.3 Roles for transglutaminase in plant tissues

Transglutaminase activity was rapidly induced in wounded stem and leaf tissue of *Nicotiana tabacum* and *Medicago sativa*, there was a 10- to 15-fold increase in transglutaminase activity within 12 to 24 hours of wounding. The response was systemic since wounding at one site resulted in new enzymatic activity in proximate leaves, therefore, transglutaminase may have a central role in wound repair in plants (Margosiak *et al.*, 1987).

Conjugated polyamines have been found in different phases of the cell cycle in activated tuber slices of *Helianthus tuberosus* (Mossetti *et al.*, 1987; Serafini-Fracassini *et al.*, 1989). This suggests that polyamines, or their derivatives, may be preferentially utilised in the bound form during certain phases of the cell cycle. Furthermore, transglutaminase activity increased 7- to 8-fold after mid G_1 phase and up to the beginning of cell division suggesting the enzyme's products may be necessary to the S phase or cell division, during which the activity remained high. At the time of cell division transglutaminase may be involved in cytoskeletal modifications, nuclear and organelle division and cell wall division which are known to occur at this stage (Favali *et al.*, 1984).

In *Medicago sativa,* Rubisco was identified as a major substrate of transglutaminase (Margosiak *et al.*, 1990). Transglutaminase may be responsible for generating the covalently crosslinked dimer of large subunits that has been discovered and characterised in isolated chloroplasts of *Medicago sativa* (Kuehn *et al.*, 1991). Formation of a dimer of large Rubisco subunits has been suggested to be the initial stage in chaperone-mediated assembly of the catalytically active L_8S_8 Rubisco structure (Roy *et al.*, 1988). The labelling of specific apoproteins of the chlorophyll a/b antenna complex was detected after incubation of isolated chloroplasts of *Helianthus tuberosus* with [^{14}C] putrescine or spermidine. The fact that the oligomeric form of the major light-harvesting complex (LCHII) was more heavily labelled by polyamines than the monomeric form suggests a role for polyamines in the stabilising of oligomers (Del Duca *et al.*, 1994). The nature of the substrates, together with the light stimulated conjugation of polyamines, suggests transglutaminase may be involved in the regulation of the light harvesting function (Del Duca *et al.*, 1994).

Considerably higher transglutaminase activity was found in non-photosynthetic medullary explants of *Helianthus tuberosus* induced to green compared to their non-green growing controls (Del Duca *et al.*, 1993). The morphological characteristics of the tissues were also quite different; the non-green growing tissue consisted of friable tissue whose cells had thin cell walls and empty intracellular spaces, and resembled tumour tissue. The green callus however had well developed chloroplasts, a compact consistency and dense material in the intracellular spaces. These authors suggested a relationship exists between these morphological features and the increased

transglutaminase activity in the green callus tissue. They proposed that transglutaminase activity in plants, as it is in animal systems, is generally higher in differentiated tissue and lower in rapidly growing tumours where the organisation of intra- and extra-cellular structural proteins is poor (Folk, 1980; Lorand and Conrad, 1984; Aeschlimann and Paulsson, 1994).

Malus domestica pollen transglutaminase catalysed the incorporation of labelled polyamine into proteins having relative molecular masses of 43 000 and 52 000 – 58 000. These bands were identified as actin and tubulin using monoclonal antibodies against the cytoskeletal proteins. *Malus domestica* pollen transglutaminase may be involved in the rapid cytoskeletal rearrangements that take place during rehydration of ungerminated pollen and the organisation and growth of pollen tubes in germinating pollen (Del Duca *et al.*, 1997). Actin has also been identified as a substrate for transglutaminase in animals (Derrick and Laki, 1966) and in the slime mould *Physarum polycephalum* actin was also recognised as a substrate for transglutaminase where it is thought to be involved in the cytoskeletal rearrangements that occur during spherule formation (Klein *et al.*, 1992).

In *Chrysanthemum morifolium* explants cultured on a medium promoting bud or root formation, levels of transglutaminase activity increased during the first few days of culture when cell manipulation was rapid then the levels declined as the rate of cell division decreased and differentiation occurred. Undifferentiated explants exhibited low transglutaminase activity. In the roots and buds initiated from the explants, transglutaminase activity increased during growth and decreased during maturity (Aribaud *et al.*, 1995).

Transglutaminase activity in *Pisum sativum* root tissue, measured using a biotin cadaverine incorporation assay and a casein-crosslinking assay, increased during the first 18 days of growth and decreased during day 22 to 32, indicating transglutaminase may be involved in early root growth and development (Lilley *et al.*, 1998a) as suggested by Aribaud *et al.* (1995). In *Pisum sativum* leaf tissue, transglutaminase activity reached peaks at day 15 and 25, the activity was present in mature and developing leaves (Lilley *et al.*, 1998a) and supports other reports suggesting a role for transglutaminase in photosynthesis (Margosiak *et al.*, 1990; Del Duca *et al.*, 1994).

Glycinin, the major storage protein of *Glycine max* seeds, was found to be modified by transglutaminase *in vivo* (Kang *et al.*, 1998). Transglutaminase may be responsible for polymerising glycinin subunits thus stabilising the protein or, the post-translational modifications brought about by the action of transglutaminase may be a signal for rapid degradation, since post-translationally modified proteins and those with abnormal structures are often degraded rapidly within cells (Goldberg and St. John, 1976). Such modifications could lead to the necessary rapid breakdown of storage proteins in imbibed seeds (Kang *et al.*, 1998).

Increased extracellular transglutaminase activity was detected during the early stages of cell wall formation in vegetative and zygotic seeds of *Chlamydomonas reinhardtii*. This transglutaminase was thought to be responsible for early cell wall protein crosslinking, which precedes the oxidative crosslinking mediated by extracellular peroxidases (Waffenschmidt *et al.*, 1999).

1.5 The aims of this project

At the present time there is conflicting evidence on the activity, characteristics and roles of plant transglutaminases. The main aim of this project is to provide detailed work on transglutaminase activity in the green leaf tissue of *Pisum sativum*, looking at features such as subcellular localisation, calcium ion requirement and thiol dependence, which may help determine a role for the enzyme within plant tissue. Transglutaminase will be purified from a suitable plant material to provide the tools to further study the enzyme.

Chapter 2 - Materials and methods

Chapter 2 - Materials and methods

2.1 Materials

2.1.1 Chemicals

Biotin cadaverine was obtained from Molecular Probes, Eugene (OR), USA. Enhanced chemoluminesence (ECL) development kit was ordered from Amersham Life Sciences Ltd, Little Chalfont, UK. Mouse anti-tissue transglutaminase 1° antibody (CUB 7402) was purchased from Stratech Scientific Ltd, Bedfordshire, UK. Goat anti-mouse HRP conjugated 2° antibody was obtained from Dako A/S, Glostrop, Denmark. Macro-Prep High Q anion exchange support was purchased from Bio-Rad, Hertfordshire, UK. Marvel non-fat dried milk was purchased from Asda Stores Ltd, Nottingham, UK. All other chemicals were purchased from Sigma Chemical Company, Dorset, UK.

2.1.2 Seeds

Seeds of *Pisum sativum* (var. Feltham First) and *Vicia faba* (var. Sutton Dwarf) were purchased from Stewarts, Nottinghamshire, UK.

2.1.3 Other materials

Nunc maxisorp 96 well assay plates were obtained from Life Technologies, Renfrewshire, UK. Muslin was purchased from Jessops department store, Nottinghamshire, UK. Dialysis tubing was purchased from Medicell International, London, UK. Kodak films were purchased from Sigma Chemical Company, Dorset, U.K. Nitrocellulose membranes were ordered from Micron Separation Inc., Westborough (MA), USA. Whatman electroblotting 3MM filter papers were obtained from Whatman International Ltd, Maidstone, UK.

2.1.4 Equipment

Beckman Instruments Limited, High Wycombe, UK.

L8-70 ultra centrifuge fitted with 70Ti and SW28 rotors.

Avanti J-S01 centrifuge fitted with JA 25-50 and JA-14 rotors.

Optima TLX Ultracentrifuge, TLA120.2 rotor.

DU 70 spectrophotometer.

Bio-Rad, Hertfordshire, UK.

Biologic HR chromatography system, fitted with model 2128 fraction collector, SV5-4 buffer selection valve, AV7-3 sample injection valve and UV and conductivity flow cells.

Protean II minigel vertical electrophoresis apparatus.

Model 3000Xi electrophoresis power supply.

Cecil Instruments, Cambridge, UK.

CE1011 spectrophotometer.

Grant Instruments Ltd, Cambridge, Hertfordshire, UK.

Water baths.

Labsystems, Finland.

Titertek multiskan MCC/340 MK II ELISA spectrophotometer.

Luckham Ltd, Sussex, UK.

R100 rotary shaker.

Mettler-Toledo AG, Switzerland.

Balances: AC100, AE240 and BD1201.

Millipore (U.K.) Ltd, Watford, Hertfordshire, UK.

Milli-Q water purification system.

Pharmacia Biotech, Cambridge, UK.

LKB 2117 multiphor II electrophoresis unit.

LKB 2002 power supply.

Philip Harris Scientific, Leicestershire, UK.

Jenway 3320 pH meter.

2.2 Methods

2.2.1 Preparation of dialysis tubing

Dialysis tubing was boiled for 5 minutes in 200.0ml of Milli Q distilled water containing 2.0g EDTA and 2.0g sodium hydrogen carbonate. The tubing was then rinsed and boiled in milli Q distilled water for 5 minutes. Tubing was stored at 4°C in a solution of 0.02% (w/v) sodium azide.

2.2.2 Preparation of plant material

2.2.2.1 Seedling growth conditions

Seeds of *Pisum sativum* (var. Feltham First) and *Vicia faba* (var. Sutton Dwarf) were soaked overnight in running water and germinated in moist vermiculite in a greenhouse at 20°C. A 16 hours photoperiod was provided by natural daylight supplemented with high-pressure sodium lamps providing a photosynthetic flux of 300 – 400 μmol m^{-2} s^{-1}. Leaf tissue of *Pisum sativum* and cotyledons of *Vicia faba* were harvested after a growth period of 14 days, unless otherwise stated, and stored frozen, at -20°C, until required.

2.2.2.2 Treatment of *Pisum sativum* leaf tissue

Stock solutions

(A) Extraction buffer (1) – 50mM Tris-HCl pH 7.4 containing 250mM sucrose, 3mM EDTA and 10mM 2-ME.

3.03g Tris, 42.8g sucrose, 0.558g EDTA and 391.0μl 2-ME were mixed with 400.0ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume was adjusted to 500.0ml with distilled water.

(B) Membrane resuspension buffer – 5mM Tris-MES pH 7.3 containing 250mM sucrose and 1mM DTT.

0.061g Tris, 8.60g sucrose and 0.015g of DTT were dissolved in 70.0ml of distilled water. The pH was adjusted to 7.3 with solid MES and the volume was adjusted to 100.0ml with distilled water.

(C) Membrane washing buffer – 5mM Tris-MES pH 7.3 containing 250mM sucrose, 1.0 M potassium chloride and 1mM DTT.

0.061g Tris, 8.60g sucrose, 7.50g potassium chloride and 0.015g of DTT were dissolved in 70.0ml of distilled water. The pH was adjusted to 7.3 with solid MES and the volume was adjusted to 100.0ml with distilled water.

Method

Fourteen day old *Pisum sativum* leaf tissue was homogenised in ice cold extraction buffer (A) containing; 5% (w/v) PVPP, 5μM leupeptin, 1μM pepstatin A, 1mM PMSF and 1μM E_{64} in a ratio of 1: 2 (w/v) using a Philips model HR 1375/A blender (Nottinghamshire, UK). The homogenate was filtered through 2 layers of muslin and the pH re-adjusted to 7.4 using solid Tris. The extract was clarified by centrifuging at 13 000.g for 20 minutes at 4°C in a Beckman Avanti J-S01 centrifuge fitted with a JA 25-50 rotor. The supernatant was further clarified by centrifuging at 100 000.g for 1 hour at 4°C in a Beckman L8-70 ultracentrifuge, 70Ti rotor. The supernatant was collected and stored in aliquots at -20°C.

The membrane pellet was washed twice with membrane washing buffer (C), washed membranes were collected by centrifugation at 100 000.g for 1 hour at 4°C. The final washed membrane pellet was resuspended in 5mM Tris-MES pH 7.3 containing 250mM sucrose and 1mM DTT (B) and stored in aliquots at -20°C.

2.2.2.3 Treatment of *Vicia faba* cotyledon tissue

Stock solutions

(A) Extraction buffer (2) – 100mM Tris-HCl pH 8.0 containing 2mM EDTA and 5mM 2-ME.

12.11g Tris, 0.744g EDTA and 391.0μl 2-ME were dissolved in 900.0ml of distilled water. The pH was adjusted to 8.0 with HCl and the volume was made up to 1.0 litre with distilled water.

(B) Dialysis buffer (1) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME.

7.266g Tris and 234.0μl 2-ME were dissolved in 2.9 litres of distilled water and the pH was adjusted to 8.5 with HCl. The final volume was made up to 3.0 litres with distilled water.

Method

Fourteen day old *Vicia faba* cotyledons were homogenised in ice cold extraction buffer (A) containing; 5μM leupeptin, 1μM pepstatin, 1mM PMSF and 1μM E_{64} in a ratio of 1: 2 (w/v) using a Philips model HR 1375/A blender (Nottinghamshire, UK). The homogenate was filtered through 2 layers of muslin and clarified by centrifugation at 10 000.g for 30 minutes at 4°C in a Beckman Avanti J-S01 centrifuge fitted with a JA 25-50 rotor. The supernatant fraction was collected and dialysed overnight at 4°C against 2 changes of 3.0 litres of dialysis buffer (B).

2.2.2.4 Ammonium sulphate precipitation

Stock solutions

(A) Protein re-dissolving / dialysis buffer – 20mM Tris-HCl pH 8.0 containing 1mM 2-ME.

7.27g Tris and 234.0μl of 2-ME were mixed with 2.9 litres of distilled water. The pH adjusted to 8.0 with HCl and the volume was adjusted to 3.0 litres with distilled water.

Method

Protein was precipitated by the addition of ammonium sulphate to 90% saturation at 4°C. After stirring for 1 hour at 4°C precipitated protein was collected by centrifugation at 10 000.g for 15 minutes at 4°C. The pellet was dissolved in 20mM Tris-HCl pH 8.0 containing 1mM 2-ME (A) and dialysed overnight against 2 changes of 3.0 litres of the same buffer at 4°C. Aliquots of dialysed protein were stored at -20°C.

2.2.3 Treatment of crude *Pisum sativum* membrane fractions

2.2.3.1 Detergent extraction of membrane proteins

Stock solutions

(A) Membrane resuspension buffer – 5mM Tris-MES pH 7.3 containing 250mM sucrose and 1mM DTT.

Prepared as described in section 2.2.2.2 (B).

(B) 2% (w/v) sodium deoxycholate.

1.0g sodium deoxycholate was dissolved in membrane resuspension buffer (2.2.3.1 (A)) to a final volume of 50.0ml.

Method

Washed membranes were prepared according to section 2.2.2.2. Sodium deoxycholate (B) was added to the membranes to yield a final detergent concentration of 0.23% (w/v). Preparations were incubated on ice for 1 hour and then centrifuged at 100 000.g for 1 hour at 4°C in a Beckman Optima TLX Ultracentrifuge, TLA120.2 rotor, to remove insoluble material. The supernatant was collected and stored in aliquots at -20°C.

2.2.3.2 Proteolytic extraction of membrane proteins

Stock solutions

(A) 1.0 mg ml^{-1} trypsin.

10.0mg trypsin were dissolved in 10.0ml of membrane resuspension buffer 2.2.3.1 (A).

(B) 100mM PMSF.

85.0mg PMSF were dissolved to a final volume of 5.0ml in ethanol.

Method

Washed membranes were prepared according to section 2.2.2.2. Trypsin was added to the washed membranes to yield a final concentration of 50 μg ml^{-1}. Membranes were incubated at 20°C for 1 minute to 60 minutes before the reaction was terminated with the addition of PMSF (B) to a final concentration of 5mM. The membranes were centrifuged at 100 000.g for 1 hour at 4°C. The supernatant was collected and stored in aliquots at -20°C. The pellet was resuspended in membrane resuspension buffer (2.2.3.1 (A)) and subjected to detergent extraction as described in section 2.2.3.1.

2.3.3.3 Triton X-114 phase partition of membranes

Stock solutions

(A) Precondensing buffer – 10mM Tris-HCl pH 7.4 containing 150mM sodium chloride and 0.004% (w/v) butylated hydroxytoluene.

1.21g Tris, 8.77g sodium chloride and 0.04g butylated hydroxytoluene were dissolved in 950.0ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume made up to 1.0 litre with distilled water.

(B) Phase separation buffer - 10mM Tris-HCl pH 7.4 containing 150mM sodium chloride.

0.61g Tris and 4.38g sodium chloride were dissolved in 450.0ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume made up to 500.0ml with distilled water.

(C) Dialysis buffer (2) - 10mM Tris-HCl pH 7.4 containing 0.23% (w/v) sodium deoxycholate and 1mM 2-ME.

1.21g Tris, 2.3g sodium deoxycholate and 78.0µl of 2-ME were dissolved in 950.0ml of distilled water. The pH was adjusted to 7.4 with HCl and the final volume was made up to 1.0 litre with distilled water.

Method

The method was essentially that of Bordier (1981), with the modifications of Brusca and Radolf (1994). Twenty millilitres of Triton X-114 were added to 980.0ml of precondensing buffer (A). The detergent solution was mixed at 0°C until clear and heated to 30°C until cloudy. The mixture was allowed to separate into an aqueous and detergent phase by incubation at 30°C. The upper aqueous phase was discarded and replaced with an equal volume of preconsensing buffer (A). The condensation step was repeated a further two times. The purified detergent was stored at room temperature.

Washed membranes were prepared according to section 2.2.2.2. Membranes were pelleted by centrifugation at 100 000.g for 1 hour at 4°C and resuspended in 10mM Tris-HCl pH 7.4 containing 150mM sodium chloride (B). Purified detergent was added to the membranes to a yield a final concentration of 2% (v/v) Triton X-114 and 1 – 3 mg ml^{-1} membrane protein. Membranes were incubated on ice for 1 hour and then centrifuged at 100 000.g for 1 hour at 4°C to remove insoluble material. The soluble material was transferred to a microfuge tube and warmed to 30°C for 5 minutes then centrifuged at 300.g for 5 minutes at 20°C in an MSE micro centrifuge.

The upper aqueous phase was transferred to a new tube and Triton X-114 was added to a final concentration of 2% (v/v). The phases were separated as before and the detergent phase was discarded.

The first detergent phase was mixed with 1.0ml of phase separation buffer (B), warmed to 37°C for 10 minutes and centrifuged at 300.g for 5 minutes at 20°C. The aqueous phase was discarded. The detergent and aqueous phases were separated a further three times to improve the purity of the two phases. The final purified aqueous phase was stored in aliquots at -20°C.

The input samples (Triton X-114 solubilized proteins) and the final detergent phases were dialysed against four changes of dialysis buffer (C) at 4°C. Aliquots of dialysed protein were stored at -20°C.

2.2.3.4 Sucrose density gradient fractionation of membranes

Stock solutions

(A) Gradient buffer – 5mM Tris-MES pH 7.3 containing 1mM DTT and 1mM EDTA.

0.153g Tris, 0.038g DTT and 0.093g of EDTA were mixed with 200.0ml of distilled water. The pH was adjusted to 7.3 with MES and the final volume made up to 250.0ml with distilled water.

(B) 20% (w/w) sucrose.

6.0g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

(C) 25% (w/w) sucrose.

7.5g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

(D) 30% (w/w) sucrose.

9.0g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

(E) 34% (w/w) sucrose.

10.2g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

(F) 38% (w/w) sucrose.

11.4g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

(G) 45% (w/w) sucrose.

13.5g of sucrose were dissolved to a final mass of 30.0g in gradient buffer (A).

Method

The method was essentially that of Hodges and Leonard (1974). Discontinuous sucrose gradients were prepared in 38.0ml Beckman tubes by carefully layering in succession 4.0ml of 45% (w/w) sucrose and 6.0ml each of 38, 34, 30, 25 and 20% (w/w) sucrose solutions. Three millilitres of washed membranes (prepared according to section 2.2.2.2) were carefully layered on to the gradient and the tubes were centrifuged at 95 000.g for 2 hours at 4°C, in a Beckman L8-70 ultracentrifuge fitted with a SW 28 rotor.

Readily visible bands of membranes at the sucrose interfaces were removed with a Pasteur pipette that was bent slightly over 90° at the tip. The membranes were diluted in gradient buffer (A) and sedimented at 100 000.g for 1 hour at 4°C. Pellets were resuspended in a minimum volume of 5mM Tris-MES pH 7.3 containing 250mM sucrose and 1mM DTT (2.2.2.2 (B)).

2.2.4 Enzyme assays

2.2.4.1 Biotin cadaverine incorporation assay

Stock solutions

(A) 100mM Tris-HCl pH 8.5.

12.11g of Tris were dissolved in 900.0ml of distilled water, the pH was adjusted to 8.5 with HCl and the final volume made up to 1.0 litre with distilled water.

(B) 150mM Phosphate buffered saline (PBS)-Tween 80 – 137mM sodium chloride, 2.6mM potassium chloride, 8.1mM disodium hydrogen orthophosphate, 1.47mM potassium dihydrogen orthophosphate and 0.05% (v/v) Tween 80.

8.0g sodium chloride, 0.2g potassium chloride, 1.15g disodium hydrogen orthophosphate, 0.2g potassium dihydrogen orthophosphate and 500.0µl Tween 80 were dissolved in 900.0ml of distilled water, the pH was adjusted to 7.4 with HCl and the final volume was made up to 1.0 litre with distilled water.

(C) 100mM sodium acetate pH 6.0.

8.2g of sodium acetate were dissolved in 900.0ml of distilled water, the pH was adjusted to 6.0 with acetic acid and the final volume made up to 1.0 litre with distilled water.

(D) Calcium chloride assay buffer – 100mM Tris-HCl pH 8.0 containing; 6.67mM calcium chloride, 13.3mM DTT and 225µM biotin cadaverine.

0.30g Tris, 0.025g calcium chloride, 0.051g DTT and 2.5mg biotin cadaverine were dissolved in 20.0ml of distilled water, the pH was adjusted to 8.0 with HCl and the volume made up to 25.0ml with distilled water.

(E) EDTA assay buffer – 100mM Tris-HCl pH 8.0 containing; 1.33mM EDTA, 13.3mM DTT and 225µM biotin cadaverine.

0.30g Tris, 0.012g EDTA, 0.051g DTT and 2.5mg of biotin cadaverine were dissolved in 20.0ml of distilled water, the pH was adjusted to 8.0 with HCl and the volume made up to 25.0ml with distilled water.

(F) 10 mg ml^{-1} TMB.

10.0mg of TMB were dissolved in 1.0ml of DMSO.

(G) 3% (v/v) Hydrogen peroxide

10.0µl of 30% (v/v) hydrogen peroxide were added to 90.0µl of distilled water.

(H) Developing buffer

150.0µl of 10 mg ml^{-1} TMB (F) and 25.0µl of 3% (v/v) hydrogen peroxide (G) were added to 20.0ml of 100mM sodium acetate pH 6.0 (C).

Method

This assay was used as a measure of transglutaminase activity and was performed according to the method of Slaughter *et al.* (1992) with the modifications of Lilley *et al.* (1998a). Ninety-six well NUNC Maxisorp assay plates were coated overnight at 4°C with 250.0µl per well of 100mM Tris-HCl pH 8.5 (A) containing 10 mg ml^{-1} N, N'-dimethylcasein. After discarding unbound protein, plates were washed twice with 150mM PBS-Tween 80 (B) and twice with distilled water. Plates were blocked with 250.0µl per well of 0.1M Tris-HCl pH 8.5 (A) containing 3% (w/v) BSA and shaken for 45 minutes at room temperature. Plates were washed as previously described but with an additional final wash with reagent (A).

To each well were added 150.0µl of calcium chloride assay buffer or 150.0µl EDTA assay buffer and a 50.0µl sample of plant protein. Plates were incubated for one hour at 37°C and then washed as described previously. To each well was added 200.0µl of 100mM Tris-HCl pH 8.5 containing 1% (w/v) BSA and a 1: 5 000 dilution of extravidin peroxidase. Plates were incubated at 37°C for 45 minutes and then washed as previously described but replacing the final wash with 100mM sodium acetate pH 6.0 (C). The plates were developed with 200.0µl per well of developing buffer (H). Colour development was terminated by the addition of 50.0µl per well of 5.0M sulphuric acid. The absorbance was read at 450nm using a Titertek Multiscan ELISA plate reader. One unit of transglutaminase activity was defined as a change in absorbance at 450nm of 1.0 per hour.

2.2.4.2 Azocasein endopeptidase assay

Stock solutions

(A) Assay buffer – 100mM Tris-HCl pH 8.0.

12.11g of Tris were dissolved in 900.0ml of distilled water, the pH was adjusted to 8.0 with HCl and the final volume made up to 1.0 litre with distilled water.

(B) Substrate - 2% (w/v) azocasein.

0.5g of azocasein were dissolved in 100mM Tris-HCl pH 8.0 to a final volume of 25.0ml.

(C) 10% (w/v) TCA

10.0g of TCA were dissolved in distilled water to a final volume of 100.0ml.

(D) 1.0 M Sodium hydroxide

4.0g of sodium hydroxide were dissolved in distilled water to a final volume of 100.0ml.

Method

The azocasein endopeptidase assay was carried out essentially according to the method of Sarath *et al.* (1989). Two-hundred and fifty microlitres of azocasein substrate (B) were incubated with 150.0µl of plant protein for two hours at 37°C in a microfuge tube. The reaction was terminated with the addition of 1.2ml of 10% (w/v) TCA (C), tubes

were vortex mixed and allowed to stand at room temperature for 15 minutes prior to centrifugation at 8 000.g for 5 minutes. Enzyme blanks were prepared by mixing 150.0µl enzyme, 1.2ml TCA and 250.0µl azocasein substrate (B) in that order, tubes were allowed to stand for 15 minutes at room temperature prior to centrifugation at 8 000.g for 5 minutes. One hundred and fifteen microlitres of the supernatant were transferred to an ELISA plate well containing 135.0µl of 1.0M sodium hydroxide (D). The absorbance was read at 405nm using a Titertek Multiscan ELISA Spectrophotometer. One unit of endopeptidase activity was defined as the change in absorbance at 405nm of 1.0 per hour.

2.2.4.3 Lactate dehydrogenase

Stock solutions

(A) 30mM sodium phosphate buffer pH 7.4.

0.43g of disodium hydrogen orthophosphate were dissolved to a final volume of 100.0ml in distilled water. 0.36g of sodium dihydrogen orthophosphate were dissolved to a final volume of 100.0ml in distilled water. The pH of the disodium hydrogen orthophosphate solution was adjusted to pH 7.4 by titration with the sodium dihydrogen orthophosphate solution.

(B) 10mM Sodium pyruvate.

0.011g of sodium pyruvate were dissolved in 30mM sodium phosphate buffer pH 7.4 to a final volume of 10.0ml.

(C) 2mM NADH.

0.016g of NADH were dissolved in 30mM sodium phosphate buffer pH 7.4 to a final volume of 10.0ml.

Method

The reaction mixture contained 900.0µl of 30mM phosphate buffer pH 7.4 (A), 33.0µl of 10mM sodium pyruvate (B) and 33.0µl of 2mM NADH (C); the reaction was started by the addition of 33.0µl of plant protein. The change in absorbance was followed for 3 minutes at 340nm. Rates of NADH oxidation were determined from initial linear rates and the amount of NADH oxidised was estimated using a molar extinction coefficient

for NADH of 6.22 x 10^3 M^{-1} cm^{-1}. One unit of lactate dehydrogenase activity was defined as the oxidation of 1.0 μmol of NADH per minute.

2.2.4.4 Antimycin A insensitive NADH-dependent cytochrome c reductase

Stock solutions

(A) 50mM potassium phosphate buffer pH 7.5.

3.4g of potassium dihydrogen orthophosphate were dissolved in distilled water to a final volume of 500.0ml. 4.36g of dipotassium hydrogen orthophosphate were dissolved in distilled water to a final volume of 500.0ml. The pH of the potassium dihydrogen orthophosphate solution was adjusted to 7.5 by titration with the dipotassium hydrogen orthophosphate solution.

(B) 50mM sodium cyanide.

0.061g of sodium cyanide were dissolved in 50mM phosphate buffer pH 7.5 (A) to a final volume of 25.0ml.

(C) 0.45mM cytochrome c.

0.028g of cytochrome c were dissolved in 50mM phosphate buffer pH 7.5 (A) to a final volume of 5.0ml.

(D) 5mM antimycin A.

13mg of antimycin A were dissolved in ethanol to a final volume of 5.0ml.

(E) 3mM NADH.

0.011g of NADH were dissolved in 50mM phosphate buffer pH 7.5 (A) to a final volume of 5.0ml.

Method

The method was essentially that of Hodges and Leonard (1974). The reaction mixture contained 832.0μl of 50mM phosphate buffer pH 7.5 (A), 33.0μl of 50mM sodium cyanide (B), 67.0μl of 0.45mM cytochrome c (C), 2.0μl of 5mM antimycin A (D) and 33.0μl of plant protein; the reaction was started by the addition of 33.0μl of 3mM NADH (E). The change in absorbance was followed for 3 minutes at 550nm. Rates of cytochrome c reduction were determined from initial linear rates, and the amount of

cytochrome c utilised was estimated using a molar extinction coefficient for cytochrome c of 18.5 mM^{-1} cm^{-1} at 550nm. One unit of NADH-dependent cytochrome c reductase activity was defined as the reduction of 1.0 μmol of cytochrome c per minute.

2.2.4.5 Cytochrome c oxidase

Stock solutions

(A) 50mM phosphate buffer pH 7.5.

Prepared as described in section 2.2.4.4(A).

(B) 0.3% (w/v) digitonin.

0.03g of digitonin were dissolved in 50mM phosphate buffer pH 7.5 (A) to a final volume of 10.0ml.

(C) 0.45mM cytochrome c (reduced form).

0.028g of cytochrome c were dissolved in 50mM phosphate buffer pH 7.5 (A) to a final volume of 5.0ml and chemically reduced by adding a few crystals of sodium dithionite. The excess dithionite was removed by passing air through the solution for 5 minutes.

Method

The assay was carried out essentially according to the method of Hodges and Leonard (1974). The reaction mixture contained 900.0μl of 50mM phosphate buffer pH 7.5 (A), 33.0μl of 0.3% (w/v) digitonin (B), 33.0μl of 0.45mM reduced cytochrome c (C) and 33.0μl of plant protein. The change in absorbance was followed for 3 minutes at 550nm. Rates of cytochrome c oxidation were determined from initial linear rates, and the amount of cytochrome c utilised was estimated using a molar extinction coefficient for cytochrome c of 18.5 mM^{-1} cm^{-1} at 550nm. One unit of cytochrome c oxidase activity was defined as the oxidation of 1.0 μmol of cytochrome c per minute.

2.2.4.6 Cation-stimulated vandate-inhibitable ATPase

Stock solutions

(A) 30mM ATP (Tris salt).

Sodium ATP was converted to the Tris salt by treatment with a cation exchange resin (Hodges and Leonard, 1974). 1.383g of ATP were dissolved in 50.0ml of distilled water, sufficient Dowex 50-W ion exchange resin (acid form) was added to give a pH of

between 1 and 2. The resin was removed by filtration on a Millipore filter, and the solution was brought to pH 6.0 with crystals of Tris.

(B) Assay buffer (1) – 60.5mM Tris-MES pH 6.5 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 1.6mM sodium azide and 4.8mM ATP-Tris.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 5.3mg sodium azide and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 6.5 with MES and the final volume was adjusted to 50.0ml with distilled water.

(C) Assay buffer (2) – 60.5mM Tris-MES pH 6.5 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 1.6mM sodium azide, 161μM sodium orthovanadate and 4.8mM ATP-Tris .

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 5.3mg sodium azide, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 6.5 with MES and the final volume was adjusted to 50.0ml with distilled water.

(D) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate.

5.13g sodium acetate, 1.55g ammonium molybdate and 0.27g zinc acetate were dissolved in 200.0ml of distilled water. The pH was adjusted to 4.0 with acetic acid and the final volume was made up to 250.0ml with distilled water.

Method

The assay was performed essentially according to Fischer-Schliebs *et al.* (1994). A 100.0μl reaction volume consisted of 62.0μl of reaction buffer, (B) or (C) and 38.0μl of membranes (10 – 30 μg protein). The assay was allowed to proceed for 30 minutes at 37°C.

Inorganic phosphate release was determined according to the method of Bencini *et al.* (1982). The 100.0µl reaction volume was mixed with 100.0µl of Bencini reagent (D) and the absorbance was measured at 340nm using a Titertek multiscan ELISA plate reader after incubation at room temperature for 2 - 60 minutes.

A calibration graph was prepared with 0 – 300 nmol inorganic phosphate. Vanadate-inhibitable ATPase activity was determined by subtracting the differences between assays carried out in the presence and absence of 100µM sodium orthovanadate. One unit of cation-stimulated, vanadate-inhibitable ATPase activity was defined as the release of 1.0 µmol phosphate per hour.

2.2.4.7 Anion-stimulated nitrate-inhibitable ATPase

Stock solutions

(A) 30mM ATP (Tris salt).

Prepared as described in section 2.2.4.6 (A)

(B) Assay buffer (1) – 60.5mM Tris-MES pH 8.0 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 1.6mM ammonium molybdate, 1.6mM sodium azide, 161µM sodium orthovanadate and 4.8mM ATP-Tris.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.10g ammonium molybdate, 5.3mg sodium azide, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.0 with MES and the final volume was adjusted to 50.0ml with distilled water.

(C) Assay buffer (2) – 60.5mM Tris-MES pH 8.0 containing; 4.8mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 1.6mM sodium azide, 161µM sodium orthovanadate and 4.8mM ATP-Tris.

0.731g Tris, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 5.3mg sodium azide, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.0 with MES and the final volume was adjusted to 50.0ml with distilled water.

(D) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate.

Prepared as in section 2.2.4.6 (D).

Method

The assay was carried out essentially according to the method of Fischer-Schliebs *et al.* (1994). The method was performed as described for cation-stimulated vandate-inhibitable ATPase in section 2.2.4.6. Nitrate-inhibitable ATPase activity was determined by subtracting the differences between assays carried out in the presence and absence of 50mM sodium nitrate. One unit of anion-stimulated, nitrate-inhibitable ATPase activity was defined as the release of 1.0 μmol phosphate per hour.

2.2.4.8 Azide-sensitive ATPase

Stock solutions

(A) 30mM ATP (Tris salt)

Prepared as described in section 2.2.4.6 (A)

(B) Assay buffer (1) – 60.5mM Tris-MES pH 8.5 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 161μM sodium orthovanadate and 4.8mM ATP-Tris

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.5 with MES and the final volume was made up to 50.0ml with distilled water.

(C) Assay buffer (2) – 60.5mM Tris-MES pH 8.5 containing; 80.7mM potassium chloride, 4.8 mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 1.6 mM sodium azide, 161μM sodium orthovanadate and 4.8mM ATP-Tris.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 5.3mg sodium azide, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.5 with MES and the final volume was made up to 50.0ml with distilled water.

(D) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate

Prepared as in section 2.2.4.6 (D).

Method

The assay was carried out essentially according to the method of Fischer-Schliebs *et al.* (1994). The method was performed as described for cation-stimulated vandate-inhibitable ATPase in section 2.2.4.6. Azide-sensitive ATPase activity was determined by subtracting the differences between assays carried out in the presence and absence of 1mM sodium azide. One unit of azide sensitive ATPase activity was defined as the release of 1.0 μmol phosphate per hour.

2.2.4.9 Molybdate-sensitive ATPase

Stock solutions

(A) 30mM ATP (Tris salt)

Prepared as described in section 2.2.4.6 (A)

(B) Assay buffer (1) – 60.5mM Tris-MES pH 8.5 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM sodium azide and 4.8mM ATP-Tris

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 5.3mg sodium azide and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.5 with MES and the final volume was made up to 50.0ml with distilled water.

(C) Assay buffer (2) – 60.5mM Tris-MES pH 8.5 containing; 80.7mM potassium chloride, 4.8 mM magnesium sulphate, 80.7mM sodium nitrate, 1.6mM ammonium molybdate, 1.6 mM sodium azide, 161μM sodium orthovanadate and 4.8mM ATP-Tris.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.343g sodium nitrate, 0.10g ammonium molybdate, 5.3mg sodium azide, 0.015g sodium orthovanadate and 8.05ml ATP (Tris salt) were dissolved in 30.0ml of distilled water. The pH was adjusted to 8.5 with MES and the final volume was made up to 50.0ml with distilled water.

(D) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate.

Prepared as in section 2.2.4.6 (D).

Method

The assay was carried essentially according to the method of McCarty *et al.* (1984). The method was carried out as described for cation-stimulated, vanadate-inhibitable ATPase in section 2.2.4.6. Molybdate and vanadate-sensitive ATPase activity was determined by subtracting the differences between assays carried out in the presence and absence of 1mM sodium molybdate and 100μM sodium orthovanadate. One unit of molybdate-sensitive ATPase activity was defined as the release of 1.0 μmol phosphate per hour.

2.2.4.10 Latent IDPase

Stock solutions

(A) Assay buffer - 60.5mM Tris-MES pH 7.5 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate and 4.8mM IDP.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate and 0.065g IDP were dissolved in 40.0ml of distilled water. The pH was adjusted to 7.5 with MES and the volume corrected to 50.0ml with distilled water.

(B) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate.

Prepared as detailed in section 2.2.4.6 (D).

Method

The assay was carried out essentially according to the method of Waldron and Brett (1987). A 100.0μl reaction volume consisted of 62.0μl of reaction buffer (A) and 38.0μl of membranes (10 – 30 μg protein). Following incubation at 37°C for 30 minutes the reaction was terminated by the addition of 100.0μl of Bencini reagent (B). The absorbance was measured at 340nm using a Titertek Multiscan ELISA plate reader after incubation at room temperature for 2 - 60 minutes.

Latent IDPase activity develops to a maximum after storage at 4°C for several days (Quail, 1979), therefore, latent IDPase activity was determined by subtracting the

difference between assays performed after storage of membranes at 4°C for 6 days and immediately after membrane preparation. One unit of latent IDPase activity was defined as the release of 1.0 μmol phosphate per hour.

2.2.4.11 Cation-stimulated inorganic pyrophosphatase (PPase)

Stock solutions

(A) Assay buffer (1) - 60.5mM Tris-MES pH 8.0 containing; 80.7mM potassium chloride, 4.8mM magnesium sulphate, 1.6mM ammonium molybdate and 4.8mM sodium pyrophosphate.

0.731g Tris, 0.30g potassium chloride, 0.056g magnesium sulphate, 0.10g ammonium molybdate and 0.107g sodium pyrophosphate were dissolved in 40.0ml of distilled water. The pH was adjusted to 8.0 with MES and the volume adjusted to 50.0ml with distilled water.

(B) Assay buffer (2) - 60.5mM Tris-MES pH 8.0 containing; 4.8mM magnesium sulphate, 1.6mM ammonium molybdate and 4.8mM sodium pyrophosphate.

0.731g Tris, 0.056g magnesium sulphate, 0.10g ammonium molybdate and 0.107g sodium pyrophosphate were dissolved in 40.0ml of distilled water. The pH was adjusted to 8.0 with MES and the volume adjusted to 50.0ml with distilled water.

(C) Bencini reagent – 250mM sodium acetate pH 4.0 containing 5mM ammonium molybdate and 5mM zinc acetate.

Prepared as detailed in section 2.2.4.6 (D).

Method

The method was essentially that of Walker and Leigh (1981). A 100.0μl reaction volume consisted of 62.0μl of reaction buffer (A) or (B) and 38.0μl of membranes (10 – 30 μg protein). Following incubation at 37°C for 30 minutes, the reaction was terminated with the addition of 100.0μl of Bencini reagent (C). The absorbance was measured at 340nm using a Titertek multiscan ELISA plate reader after incubation at room temperature for 2 - 60 minutes.

Cation-stimulated PPase activity was determined by subtracting the differences between assays performed in the presence and absence of 50mM potassium chloride. To

calculate PPase activity the total inorganic phosphate released was halved since hydrolysis of 1 mol of PP_i yields 2 mol of P_i. One unit of PPase activity was defined as the release of 1.0 μmol phosphate per hour.

2.2.5 Chlorophyll estimation

Method

The method for total chlorophyll estimation was that of Arnon, (1949). One hundred microlitres of plant protein were mixed with 800.0μl of acetone and 100.0μl of distilled water. Tubes were vortex mixed and allowed to stand on ice for 5 minutes. After centrifuging samples for 5 minutes at 13 000.g the absorbance of the supernatant was measured at 645 and 663nm against a blank of 80% (v/v) acetone. Total chlorophyll was calculated using the following formula:

$$\text{Total chlorophyll } (\mu g\ ml^{-1}) = \{ 20.2\ (A_{645}) + 8.02\ (A_{663}) \} \times 10$$

2.2.6 Chromatographic techniques

All column chromatography techniques were carried out using a Bio-Rad Biologic HR chromatography system.

2.2.6.1 Macro Prep Q anion exchange chromatography

Stock solutions

(A) Equilibration buffer – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME.

2.42g Tris and 78.0μl 2-ME were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 8.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

(B) Elution buffer – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 1.0M sodium chloride.

2.42g Tris, 78.0μl 2-ME and 58.44g sodium chloride were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 8.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

Method

Fifty millilitres of Macro-Prep High Q (Bio-rad) were resuspended in 100.0ml of equilibration buffer (A) and degassed by vacuum aspiration. The gel slurry was slowly poured into a 2.5 x 10 cm column. The column was equilibrated using 250.0ml of elution buffer (B) followed by 250.0ml of equilibration buffer (A). *Vicia faba* cotyledon protein, prepared as described in section 2.2.2.3, was applied to the column at a flow rate of 3.5 ml min^{-1}. Unbound material was eluted using two column volumes of equilibration buffer at 3.5 ml min^{-1}. A linear gradient between 0 and 0.3M sodium chloride was applied to the column by mixing equilibration buffer with elution buffer over 400.0ml. The gradient was held at 0.3M sodium chloride for 50.0ml before applying a linear gradient between 0.3 and 1.0M sodium chloride over 50.0ml. Five millilitre fractions were collected throughout the run and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (2.2.4.1). The active fractions were pooled and stored at -20°C.

2.2.6.2 Calcium dependent hydrophobic interaction chromatography

Stock solutions

(A) Equilibration buffer – 20mM Tris-HCl pH 7.5 containing 5mM $CaCl_2$ and 1mM 2-ME.

2.42g Tris, 0.74g $CaCl_2$ and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 7.5 with HCl. The final volume was made up to 1.0 litre with Milli Q distilled water.

(B) Elution buffer (1) – 20mM Tris-HCl pH 7.5 containing 1mM 2-ME.

2.42g Tris and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 7.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

(C) Elution buffer (2) – 20mM Tris-HCl pH 7.5 containing 5mM EDTA and 1mM 2-ME.

2.42g Tris, 1.86g EDTA and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 7.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

Method

Fifty millilitres of Phenyl Sepharose 6 fast flow (Sigma) were resuspended in 100.0ml of equilibration buffer (1) and degassed by vacuum aspiration. The gel slurry was slowly poured into a 2.5 x 10 cm column. The column was equilibrated using 250.0ml of 20mM Tris-HCl pH 8.5 containing 5mM $CaCl_2$ and 1mM 2-ME (A). Ion exchange purified *Vicia faba* cotyledon protein was brought to 5mM $CaCl_2$ by the addition of solid calcium chloride and the pH was adjusted to 7.5 with HCl. Extracts were clarified by centrifugation at 10 000.g for 15 minutes and applied to the equilibrated Phenyl Sepharose 6FF column at a flow rate of 2.0 ml min^{-1}. Unbound material was removed by washing with two column volumes of equilibration buffer (A). Calcium binding proteins were eluted by washing the column with two column volumes of elution buffer (B) followed by two column volumes of elution buffer (C), the column was finally washed with distilled and de-ionized water to remove hydrophobic proteins. Five millilitre fractions were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (2.2.4.1). The active fractions were pooled and stored at -20°C.

2.2.6.3 Q2 / Q10 anion exchange chromatography

Stock solutions

(A) Equilibration buffer – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME.

Prepared according to section 2.2.6.1 (A).

(B) Elution buffer – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 0.5 M sodium chloride.

2.42g Tris, 78µl 2-ME and 29.22g sodium chloride were dissolved in 900.0ml of Milli Q distilled water and the pH was adjusted to 8.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

Method

A 2.0ml Q2 or 10.0ml Q10 column (Bio-Rad) was equilibrated using 10 column volumes of elution buffer (B) followed by 10 column volumes of equilibration buffer (A). Phenyl Sepharose purified *Vicia faba* cotyledon protein was prepared as described in section 2.2.4.2 and applied to the Q2 or Q10 column at a flow rate of 2.0 ml min^{-1}. Unbound material was eluted using two column volumes of equilibration buffer (A) at

2.0 ml min^{-1}. A linear gradient between 0 and 0.5M sodium chloride was applied to the column by mixing equilibration buffer with elution buffer over 20 column volumes. The salt concentration was held at 0.5 M sodium chloride for 3 column volumes. Three millilitre fractions were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (2.2.4.1). Active fractions were pooled and stored at -20°C.

2.2.6.4 Q2 / Q10 calcium affinity anion exchange chromatography

Stock solutions

(A) Equilibration buffer – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME.

Prepared according to section 2.2.6.1 (A).

(B) Elution buffer (1) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 0.5M sodium chloride.

Prepared according to section 2.2.6.3 (B).

(C) Elution buffer (2) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 40mM sodium chloride.

The gradient pump on the Biologic HR system was set to run at 8% B which was equivalent to 40mM sodium chloride and 500.0 ml of buffer was collected.

(D) Elution buffer (3) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 41mM sodium chloride.

Ten microlitres of elution buffer (1) were mixed with 4990µl of elution buffer (2).

(E) Elution buffer (4) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME, 40mM sodium chloride and 1mM calcium chloride.

1.5mg of calcium chloride was mixed with elution buffer (2) to a final volume of 5.0ml.

(F) Elution buffer (5) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 42mM sodium chloride.

Twenty microlitres of elution buffer (1) were mixed with 4980µl of elution buffer (2).

(G) Elution buffer (6) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME, 40mM sodium chloride and 2mM calcium chloride.

2.9mg of calcium chloride were mixed with elution buffer (2) to a final volume of 5.0ml.

(H) Elution buffer (7) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 45mM sodium chloride.

Fifty microlitres of elution buffer (1) were mixed with 4950µl of elution buffer (2).

(I) Elution buffer (8) – 20mM Tris-HCl pH 8.5 containing 1mM 2-ME, 40mM sodium chloride and 5mM calcium chloride.

7.4mg of calcium chloride were mixed with elution buffer (2) to a final volume of 5.0ml.

Method

A 2.0ml Q2 or 10.0ml Q10 column (Bio-Rad) was equilibrated using 10 column volumes of elution buffer (B) followed by 10 column volumes of equilibration buffer (A). Q2 or Q10 anion exchange purified *Vicia faba* cotyledon protein (2.2.6.3) was diluted 20-fold with equilibration buffer and applied to the column at a flow rate of 2.0 ml min^{-1}. Unbound material was eluted using two column volumes of equilibration buffer (A) at 2.0 ml min^{-1}. The column was washed with 5 column volumes of 20mM Tris-HCl pH 8.5 containing 1mM 2-ME and 40mM sodium chloride (C) at 2.0 ml min^{-1}. 1.0ml samples of elution buffer (3) to (8) were sequentially applied to the column via a static loop, each injection was followed with 1 column volume of elution buffer 2 (C). The salt gradient was raised to 0.5 M sodium chloride over 5 column volumes. Two millilitre fractions were collected and assayed for transglutaminase using the biotin cadaverine incorporation assay (2.2.4.1). Active fractions were pooled and stored at -20°C.

2.2.6.5 Gel filtration chromatography on Sephacryl S-300 HR

Stock solutions

(A) Equilibration buffer (1) – 20mM Tris-HCl pH 7.5 containing 150mM sodium chloride and 1mM 2-ME.

2.42g Tris, 8.77g sodium chloride and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water. The pH was adjusted to 7.5 with HCl and the volume was made up to 1.0 litre with Milli Q distilled water.

(B) Equilibration buffer (2) – 20mM Tris-HCl pH 7.5 containing; 150mM sodium chloride, 0.23% (w/v) sodium deoxycholate and 1mM 2-ME.

2.42g Tris, 8.77g sodium chloride, 2.3g sodium deoxycholate and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water. The pH was adjusted to 7.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

Method

One hundred and twenty millilitres of Sephacryl S-300 HR were suspended in 120.0ml of equilibration buffer (A) and degassed under vacuum. Degassed resin was poured into a column (1.6 x 52 cm) and packed at a flow rate of 2.0 ml min^{-1}. The column was equilibrated using 500.0ml of equilibration buffer (A) or (B) at 1.0 ml $-min^{-1}$. The column was calibrated using a set of standard proteins (Blue Dextran 2000, ferritin (Mr 440 000), aldolase (Mr 158 000), albumin (Mr 67 000), ovalbumin (Mr 43 000) and ribonuclease A (13 700)). One and a half millilitre of each standard protein were applied to the column and eluted with equilibration buffer (A) or (B) at 1.0 ml min^{-1}.

One point five millilitres of plant protein were applied to the column and eluted with equilibration buffer (A) or (B) in the descending direction at a flow rate of 1.0 ml min^{-1}. One millilitre fractions were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (2.2.4.1). Active fractions were pooled and stored at -20°C.

2.2.6.6 Gel filtration chromatography on Superdex 200

Stock solution

(A) Equilibration buffer – 20mM Tris-HCl pH 8.5 containing 150mM sodium chloride and 1mM 2-ME.

2.42g Tris, 8.77g sodium chloride and 78.0µl 2-ME were dissolved in 900.0ml of Milli Q distilled water water and the pH was adjusted to 8.5 with HCl. The volume was made up to 1.0 litre with Milli Q distilled water.

Method

Thirty millilitres of fully swollen Superdex 200 (Pharmacia) were suspended in 30.0ml of equilibration buffer (A) and degassed under vacuum. Degassed resin was packed into a Pharmacia HR column (10 x 300 mm). The column was equilibrated using 300.0ml of equilibration buffer (A) at 0.5 ml min^{-1}. The column was calibrated using a set of standard proteins (Blue Dextran 2000, thyroglobulin (Mr 669 000), ferritin (Mr 440 000), catalase (Mr 232 000), aldolase (Mr 158 000), albumin (Mr 67 000), ovalbumin (Mr 43 000), chymotrypsinogen A (Mr 25 000) and ribonuclease A (13 700)). Two hundred microlitres of each standard protein were applied to the column and eluted with equilibration buffer (A) at 0.5 ml min^{-1}.

Two hundred microlitres of purified *Vicia faba* cotyledon protein were applied to the column and eluted with 20mM Tris-HCl pH 7.5 containing 150mM sodium chloride and 1mM 2-ME in the descending direction at a flow rate of 0.5 ml min^{-1}. One millilitre fractions were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (2.2.4.1).

2.2.7 Protein assays

2.2.7.1 Bicinchoninic acid protein assay

Stock solutions

(A) Bicinchoninic acid reagent A – 1% (w/v) BCA, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartrate, 0.4% (w/v) sodium hydroxide and 0.95% (w/v) sodium bicarbonate.

5.0g BCA (sodium salt), 10.0g sodium carbonate, 0.8g sodium tartrate, 2.0g sodium hydroxide and 4.75g sodium bicarbonate were dissolved in 400.0ml Milli Q distilled

water, the pH was adjusted to 11.25 with sodium hydroxide and the final volume made up to 500.0ml with Milli Q distilled water.

(B) Bicinchoninic acid reagent (B) – 4% (w/v) copper sulphate.

8.0g of copper sulphate were dissolved to a final volume of 200.0ml in distilled water.

(C) BCA standard working reagent (SWR).

The BCA SWR was prepared by mixing 50 volumes of reagent (A) with 1 volume of reagent (B).

(D) Assay buffer – 100mM Tris-HCl pH 8.5 containing 0.1% (w/v) sodium deoxycholate.

1.211g Tris and 0.1g sodium deoxycholate were dissolved in 70.0ml of distilled water, the pH is adjusted to 8.5 with HCl and the final volume was made up to 100.0ml with distilled water.

(E) 2 mg ml^{-1} BSA.

0.1g BSA were dissolved in assay buffer (D) to a final volume of 50.0ml.

Method

The assay was carried out essentially according to the method of Smith *et al.* (1985) except that the final reaction volume was 210µl instead of 2.10ml. A 10.0µl sample (standard or unknown) was mixed with 200.0µl of BCA standard working reagent (C) in a microtiter plate well. Following incubation at 37°C for 30 minutes the absorbance was read at 540nm using a titertek multliscan ELISA plate reader.

2.2.7.2 Modified bicinchoninic acid protein assay

Stock reagents

(A) Bicinchoninic acid standard working reagent (SWR).

Prepared as stated in section 2.2.7.1 (C).

(B) 0.15% (w/v) sodium deoxycholate.

0.015g sodium deoxycholate were dissolved in distilled water to a final volume of 10.0ml.

(C) 72% (w/v) TCA.

72.0g TCA were dissolved in distilled water to a final volume of 100.0ml.

(D) 5% (w/v) SDS in 0.1M sodium hydroxide.

5.0g of SDS were dissolved in 0.1M sodium hydroxide to a final volume of 100.0ml.

Method

The assay was carried out essentially according to the method of Brown *et al.* (1989). Fifty microlitres of protein were diluted to 1.0ml with distilled water in a microfuge tube. One hundred microlitres of 0.15% (w/v) sodium deoxycholate (B) were added to each tube and following a five minutes incubation period at room temperature, 100.0µl of 72% (w/v) TCA were added and tubes were mixed. Precipitated protein was collected by centrifugation at 13 000.g for 15 minutes and the supernatant was removed by vacuum aspiration. Protein was re-dissolved in 50.0µl of 5% (w/v) SDS in 0.1M sodium hydroxide (D). Ten microlitres of re-dissolved protein were mixed with 200.0µl of BCA SWR (A) in a microtiter plate well. Following incubation at 37°C for 30 minutes the absorbance was read at 540nm using a titertek multliscan ELISA plate reader.

2.2.8 Polyacrylamide gel electrophoresis (PAGE) and Western blotting

2.2.8.1 SDS-PAGE

Stock solutions

(A) Resolving gel buffer – 1.5M Tris-HCl pH 8.8 containing 0.4% (w/v) SDS.

45.41g Tris and 1.0g SDS were dissolved in 200.0ml of distilled water, the pH was adjusted to 8.8 with HCl and the final volume made up to 250.0ml with distilled water.

(B) 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide.

30.0g acrylamide and 0.8g N,N'-methylene-bisacrylamide were dissolved to a final volume of 100.0ml in distilled water.

(C) 10% (w/v) ammonium persulphate.

0.1g of ammonium persulphate were dissolved to a final volume of 1.0ml in distilled water.

(D) Stacking gel buffer – 0.5M Tris-HCl pH 6.8 containing 0.4% (w/v) SDS.

15.14g Tris and 1.0g SDS were dissolved in 200.0ml of distilled water, the pH was adjusted to 6.8 with HCl and the final volume made up to 250.0ml with distilled water.

(E) Electrode reservoir buffer – 25mM Tris, 192mM glycine and 0.1% (w/v) SDS.

3.03g Tris, 14.41g glycine and 1.0g SDS were dissolved to a final volume of 1.0 litre in distilled water. The pH of the electrode reservoir solution was 8.3.

(F) 2 x Sample buffer – 125mM Tris-HCl pH 6.8 containing; 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-ME and 0.001% (w/v) bromophenol blue.

0.76g Tris, 10.0ml glycerol, 2.0g SDS, 1.0ml 2-ME and 0.5mg bromophenol blue were dissolved in 30.0ml of distilled water. The pH was adjusted to 6.8 with HCl and the final volume made up to 50.0ml with distilled water.

(G) 5 x Sample buffer – 312mM Tris-HCl pH 6.8 containing; 50% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-ME and 0.0025% (w/v) bromophenol blue.

1.89g Tris, 25.0ml glycerol, 5.0g SDS, 2.5ml 2-ME and 1.0mg bromophenol blue were dissolved 10.0ml of distilled water. The pH was adjusted to 6.8 with HCl and the final volume made up to 50.0ml with distilled water.

Method

Bio-Rad miniprotean II gel plates were assembled according to the manufacturers instructions. Ten percent (w/v) resolving gels were prepared by mixing 5.0ml (B), 3.75ml (A), 6.25ml distilled water, 50.0µl (C) and 10.0µl TEMED. The gel solution was pipetted between the two glass plates to give a gel 80 x 60 x 1mm, gels were covered with a layer of water-saturated isobutyl alcohol and allowed to polymerise for 30 – 60 minutes at room temperature. Once polymerised, the water-saturated isobutyl alcohol was removed and the gel surface was rinsed with distilled water. Stacking gel was prepared by mixing 650.0µl (B), 1.25ml (D), 3.05ml distilled water, 25.0µl (C) and 5.0µl TEMED. The stacking gel was applied on to the polymerised resolving gel and a 10 or 12 well comb was inserted. Once polymerised, the combs were removed and the wells were rinsed with electrode reservoir buffer (E). Gels were assembled in the miniprotean II gel tanks with the correct amount of electrode reservoir buffer (E).

Protein samples were prepared by diluting protein 1: 1 with 2 x sample buffer (F) or 4:1 with 5 x sample buffer (G). Samples were boiled for 5 minutes and then cooled and centrifuged at 13 000.g for 5 minutes before applying them to the wells of the gel. Electrophoresis was conducted at 150 V for 60 – 90 minutes, or until the bromophenol blue tracking dye had reached the bottom of the gel.

2.2.8.2 Coomassie blue staining

Stock solutions

(A) 0.1% (w/v) Coomassie blue R-250, 40% (v/v) methanol and 2% (v/v) acetic acid.

0.5g of Coomassie blue R-250 were dissolved in 200.0ml methanol and 10.0ml acetic acid. The final volume was adjusted to 500.0ml with distilled water. The solution was filtered through Whatman no. 1 filter paper.

(B) Destaining solution – 30% (v/v) methanol and 10% (v/v) acetic acid.

300.0ml methanol and 100.0ml acetic acid were mixed with distilled water to a final volume of 1.0 litre.

Method

Gels were carefully removed from the glass plates and agitated slowly in 50.0ml of 0.1% (w/v) Coomassie blue solution (A) for 1 hour or overnight at room temperature. Gels were removed from the Coomassie blue solution and covered with destain solution (B) and agitated slowly for several hours. The destaining solution was changed several times, as required.

2.2.8.3 Silver staining

Stock solutions

(A) Fixer (1) - 40% (v/v) methanol and 10% (v/v) acetic acid.

400.0ml methanol and 100.0ml acetic acid were mixed with distilled water to a final volume of 1.0 litre.

(B) Fixer (2) - 10% (v/v) methanol and 5% (v/v) acetic acid.

100.0ml methanol and 50.0ml acetic acid were mixed with distilled water to a final volume of 1.0 litre.

(C) 0.01% (w/v) potassium permanganate.

0.05g of potassium permanganate were dissolved in distilled water to a final volume of 500.0ml.

(D) 0.1% (w/v) silver nitrate.

0.1g of silver nitrate were dissolved in distilled water to a final volume of 500.0ml.

(E) Developer - 3% (w/v) sodium carbonate and 0.05% (v/v) formaldehyde.

15.0g sodium carbonate and 676.0µl of 37% formaldehyde were dissolved in distilled water to a final volume of 500.0ml.

(F) Stop reagent - 10% (v/v) acetic acid.

100.0ml of acetic acid were mixed with 900.0ml of distilled water.

Method

Gels were carefully removed from the glass plates and agitated overnight in 40% (v/v) methanol, 10% (v/v) acetic acid (A). Gels were transferred into fixer 2 (B) and shaken for 20 minutes with two changes of solution. Gels were oxidised in potassium permanganate solution (C) for two minutes whilst shaking. Gels were then washed for 30 minutes with four changes of distilled water before being transferred to a solution of 0.1% (w/v) silver nitrate (D) for 20 minutes. Gels were washed quickly in distilled water and then developed with 3% (w/v) sodium carbonate containing 0.05% (v/v) formaldehyde (E). Development was terminated by transferring the gel to a solution of 10% (v/v) acetic acid (F).

2.2.8.4 Protein transfer to nitrocellulose

Stock solutions

(A) Transfer buffer – 25mM Tris, 192mM glycine, 20% (w/v) methanol and 0.1% (w/v) SDS.

3.03g Tris, 14.41g glycine, 200.0ml methanol and 10.0g SDS were dissolved in distilled to a final volume of 1.0 litre.

Method

Proteins were resolved by SDS-PAGE (as described in section 2.2.8.1) and electrophoretically transferred to nitrocellulose membranes using a semi-dry blot system (LKB 2117 Multiphor II) following the protocol of Towbin *et al.* (1979). Whatman 3MM filter paper and nitrocellulose membrane were cut into rectangles 80 x 60 mm and soaked in transfer buffer (A). Three sheets of the filter paper were placed on the anode of the electrophoresis unit, followed by a further 6 sheets. The equilibrated nitrocellulose membrane was placed on top of the filter paper stack, air bubbles were removed by rolling a glass rod over the surface of the membrane. The polyacrylamide gel was removed from the glass plates and soaked in transfer buffer before being placed on to the nitrocellulose membrane, the surface of the gel was rolled with a glass rod to expel any air. A further 3 and then 6 sheets of soaked filter paper were laid on top of the gel and rolled to remove any air bubbles. The cathode was placed on to the transfer stack and protein transfer was carried out for 1 – 2 hours at 60 mA per gel.

2.2.8.5 Antibody probing

Stock solutions

(A) Tris buffered saline (TBS) - 100mM Tris-HCl pH pH 7.5 containing 0.9% (w/v) sodium chloride.

12.11g Tris and 9.0g sodium chloride were dissolved in 900.0ml of distilled water. The pH was adjusted to 7.5 with HCl and the volume was made up to 1.0 litre with distilled water

(B) Blocking solution – TBS containing 5% (w/v) marvel.

5.0g of marvel were dissolved in TBS (A) to a final volume of 100.0ml.

(C) Washing solution – TBS containing 0.05% (v/v) Tween 20.

500.0µl of Tween 20 were mixed with TBS (A) to a final volume of 1.0 litre.

Method

Nitrocellulose membranes were blocked by incubating in 50.0ml of blocking solution (B) at room temperature on a rotary shaker for 1 hour. Membranes were incubated overnight at 4°C whilst shaking in 10.0ml of blocking solution containing a 1: 1 000 dilution of primary antibody (monoclonal anti-tissue transglutaminase antibody (CUB

7402)). Membranes were washed three times for ten minutes in washing solution (C) and then incubated with a 1: 1 000 dilution of goat-anti-mouse HRP conjugated secondary antibody in blocking solution (B), for 2 hours at room temperature whilst shaking. Nitrocellulose membranes were washed three times in washing solution (C) for ten minutes each.

Blots were developed using Enhanced Chemiluminescence (ECL) (Amersham-Pharmacia) according to the manufacturer's instructions. Reagents A and B were mixed in equal quantities (1.0ml of each per blot) and applied to the nitrocellulose membrane for one minute. The solution was then discarded and the membrane was wrapped in cling film before being placed in an autoradiograph cassette. Exposure was carried out in a dark room with Kodak X-O-Max films exposed to the nitrocellulose sheet for varying lengths of time. The film was developed and fixed using Kodak developer (IX24) and Kodak fixer (FX-40).

2.2.8.6 Protein transfer to PVDF sequencing membrane

Stock solutions

(A) Transfer buffer – 10mM CAPS pH 11.0 containing 10% (v/v) methanol.

1.107g of CAPS and 50.0ml of methanol were mixed with 400.0ml of distilled water. The pH was adjusted to 11.0 with sodium hydroxide and the volume was made up to 500.0ml with distilled water.

Method

Proteins were resolved by SDS-PAGE (as described in section 2.2.8.1) and electrophoretically transferred to PVDF sequencing membranes using a semi-dry blot system (LKB 2117 Multiphor II). Whatman 3MM filter paper and PVDF membrane were cut into rectangles 80 x 60 mm and soaked in transfer buffer (A). Three sheets of the filter paper were placed on the anode of the electrophoresis unit, followed by a further 6 sheets. The equilibrated PVDF membrane was placed on top of the filter paper stack, air bubbles were removed by rolling a glass rod over the surface of the membrane. The polyacrylamide gel was removed from the glass plates and soaked in transfer buffer before being placed on to the PVDF membrane, the surface of the gel was rolled with a glass rod to expel any air. A further 3 and then 6 sheets of equilibrated filter paper were laid on top of the gel and rolled to remove any air bubbles. The

cathode was placed on the transfer stack and protein transfer was carried out for 2 hours at 60 mA per gel.

Chapter 3 - Subcellular localisation of transglutaminase activity in *Pisum sativum* leaf tissue

Chapter 3 - Subcellular localisation of transglutaminase activity in *Pisum sativum* leaf tissue

3.1 Introduction

In mammals, transglutaminases are widely distributed amongst the tissues and body fluids and are distinguishable from each other by their physical properties and distribution within the organism (Ichinose *et al.*, 1990). Different forms of transglutaminase within a single mammal range from multi-subunit complexes that exist as protease activatable zymogens to monomeric enzymes with no observed zymogens (Folk, 1980). For example, type I transglutaminase exists in epidermal keratinocytes in several forms that are differentially partitioned between the cytosol and membranes. The partitioning of transglutaminase I is controlled by variable modifications with myristate and palmitate (Steinert *et al.*, 1996a and 1996b). Type II transglutaminase is expressed in many cell and tissue types (Folk and Finlayson, 1977). Several fractionation studies have shown that type II transglutaminase is mostly recovered in the soluble fraction of the homogenate, but a significant proportion is always present in membrane preparations (Birckbichler *et al.*, 1976; Griffin *et al.*, 1978; Chang and Chung, 1986; Slife *et al.*, 1985 and 1987). Type III and type IV transglutaminase are expressed in epidermal keratinocytes (Kim *et al.*, 1990) and rat anterior prostate gland (Seitz *et al.*, 1991), respectively.

In plants, transglutaminase has been detected in a variety of tissues and in both soluble and membrane-associated forms (for review see Serafini-Fracassini *et al.*, 1995). Differences in the subcellular distribution of the activity appear to be organ and not species specific. In etiolated apices of both *Helianthus tuberosus* (Falcone *et al.*, 1993) and *Pisum sativum* (Icekson & Apelbaum, 1987) activity was mainly localised in the soluble fraction. In *Helianthus tuberosus* leaves, as in *Beta vulgaris* and *Spinacia oleracea* leaves, the activity was mainly present in the particulate fraction (Signorini *et al.*, 1991; Falcon *et al.*, 1993). It has been proposed that chloroplasts are responsible for this activity, as first indicated by Cohen *et al.* (1982) in *Brassica pekinesis*.

The major aim of this section of the thesis was to determine the subcellular localisation of transglutaminase in *Pisum sativum* leaf tissue. To determine the extent of interaction the membrane-associated form of transglutaminase has with the membrane by using procedures directed at solubilizing membrane proteins. To ascertain if the transglutaminase activity was enriched on a microsomal membrane, membrane preparations were separated through discontinuous sucrose gradients and marker enzyme assays were used to identify the membrane types.

3.2 Results

3.2.1 The distribution of transglutaminase activity in *Pisum sativum* leaf tissue

In order to establish the subcellular location of plant transglutaminase, extracts prepared from *Pisum sativum* leaf tissue were fractionated by an initial low speed centrifugation, to remove nuclei and cell debris, followed by a high-speed centrifugation to pellet the microsomal membranes. Table 3.1 shows a typical distribution of transglutaminase activity in *Pisum sativum* leaf tissue.

Table 3.1 - Detection of transglutaminase in different fractions of *Pisum sativum* leaf extract using the biotin cadaverine incorporation assay.

	Total protein mg	Specific activity Units mg^{-1}	Total activity Units
13 000.g supernatant	1886.42	0.32 ± 0.006	607.89 ± 10.93
* 13 000.g supernatant		0.35 ± 0.024	668.76 ± 44.34
13 000.g pellet	1629.01	0.015 ± 0.006	24.33 ± 10.02
* 13 000.g pellet		0.06 ± 0.006	98.22 ± 9.65
100 000.g supernatant	1164.81	0.67 ± 0.052	780.41 ± 60.69
* 100 000.g supernatant		0.65 ± 0.047	756.32 ± 54.85
100 000.g pellet	612.32	0.071 ± 0.006	43.45 ± 3.75
* 100 000.g pellet		0.111 ± 0.013	67.86 ± 7.87

Transglutaminase activity was measured in the 13 000.g and 100 000.g supernatants and pellets of *Pisum sativum* leaf extracts using the biotin cadaverine incorporation assay (section 2.2.4.1). 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each sample. * Preparations were treated with 0.23% (w/v) sodium deoxycholate prior to the assay. Values represent the mean ± SEM of 3 replicates.

Tables 3.1 shows the distribution of the biotin cadaverine incorporation activity in *Pisum sativum* leaf extract after centrifugation at 13 000.g and 100 000.g. Approximately 9% of the total transglutaminase activity in the leaf tissue was found in the microsomal membrane fraction and 13% was localised in the low speed pellet fraction. The presence of 0.23% (w/v) sodium deoxycholate increased the total activity in the microsomal fraction from 43.5 units in the untreated to 67.9 units in the treated. In the 13 000.g pellet fraction, the presence of 0.23% (w/v) sodium deoxycholate increased the total transglutaminase activity from 24 units in the untreated to 98 units in the treated.

3.2.2 The release of *Pisum sativum* microsomal membrane transglutaminase activity from the membrane

It has previously been reported that *Pisum sativum* leaf tissue contains a soluble Ca^{2+}-dependent transglutaminase activity (Lilley *et al.*, 1998a). It was therefore necessary to thoroughly wash the *Pisum sativum* leaf microsomal fraction in order to study the activity associated with the membranes. The microsomal membrane preparation was washed with different reagents to assess their effectiveness at removing peripheral membrane proteins, whilst preventing irreversible protein denaturation. Table 3.2 shows a typical set of results, the experiment was repeated three times with similar trends on each occasion.

Table 3.2 - Biotin cadaverine incorporation activity of *Pisum sativum* microsomal membranes before and after treatment with different reagents.

Membrane Treatment	Total protein mg	Specific activity Units mg^{-1}	Total activity Units
None	1.187	1.24 ± 0.18	1.47 ± 0.21
0.1 M KCl	0.655	2.79 ± 0.09	1.83 ± 0.06
0.5 M KCl	0.501	2.87 ± 0.30	1.44 ± 0.15
1.0 M KCl	0.489	5.44 ± 0.46	2.66 ± 0.23
0.1mM EDTA	0.639	2.11 ± 0.68	1.35 ± 0.43
1mM EDTA	0.596	1.94 ± 0.32	1.16 ± 0.19
10mM EDTA	0.568	1.88 ± 0.33	1.07 ± 0.19
50mM Glycine HCl pH 3.0	0.615	0.69 ± 0.05	0.42 ± 0.03
50mM sodium carbonate pH 10.0	0.659	3.55 ± 0.22	2.34 ± 0.15

Pisum sativum leaf microsomal membranes were prepared and washed twice with the various reagents as described in section 2.2.2.2. Transglutaminase activity was measured in the crude and washed membrane preparations using the biotin cadaverine incorporation assay as described in section 2.2.4.1. Membranes were treated with 0.23% (w/v) sodium deoxycholate prior to the assay. 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each treatment. Protein concentration was determined using the modified BCA assay (2.2.7.2). Values represent the mean ± SEM of 3 replicates.

Table 3.2 demonstrates that transglutaminase activity in the mixed microsomal membrane preparation was reduced after treatment with the chelating agent EDTA. Zero point four units of activity were lost from the membranes treated with 10mM EDTA with respect to the untreated microsomal membrane preparation. The most effective agent at removing protein from the membranes was the 1.0 M KCl wash since the total protein was reduced from 1.187 mg in the crude preparation to 0.489 mg in the washed preparation, treatment with potassium chloride failed to solubilize the transglutaminase activity. Washing the membranes with 50mM glycine HCl pH 3.0 reduced the recovered activity to 0.42 units compared to 1.47 units in the untreated preparation, however, treatment with this buffer brought about precipitation of proteins.

High salt washing, chelating agents and alkaline pH buffers failed to remove the remaining *Pisum sativum* microsomal transglutaminase activity, therefore, the membranes were treated with increasing concentrations of the detergent sodium deoxycholate in an attempt to solubilize the remaining transglutaminase activity. A typical solubilization profile is presented in figure 3.1.

Figure 3.1 - Transglutaminase activity following sodium deoxycholate treatment of microsomal membrane preparations from *Pisum sativum* leaf tissue.

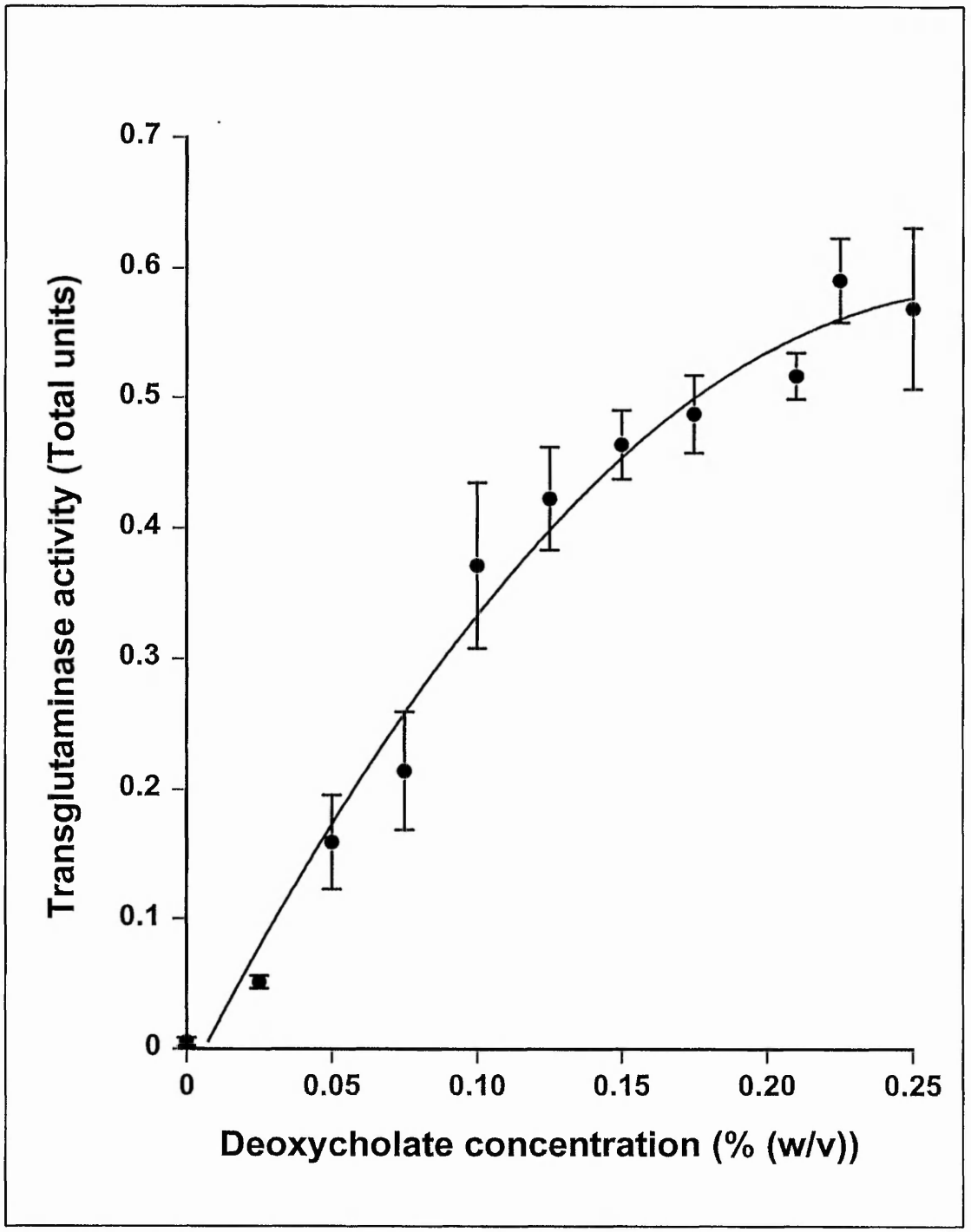


Figure 3.1 legend

Washed microsomal membranes were treated with increasing concentrations of sodium deoxycholate and allowed to stand on ice for 1 hour before being centrifuged at 100 000.g to remove insoluble material (section 2.2.3.1). The supernatants were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). 1mM EDTA replaced 5mM $CaCl_2$ as a negative control at each detergent concentration. The protein concentration was determined using the modified BCA assay as described in section 2.2.7.2. Data points represent the mean ± SEM of 3 replicates.

Figure 3.1 shows that increasing the sodium deoxycholate concentration increased the transglutaminase activity recovered in the soluble fraction. The optimum concentration of sodium deoxycholate required to extract *Pisum sativum* leaf microsomal transglutaminase was 0.23% (w/v) giving a specific activity of 0.59 units mg^{-1}.

Keratinocyte transglutaminase activity has been solubilized from the particulate fraction of cell extracts by mild trypsin treatment (Thacher and Rice, 1985). Therefore, the washed microsomal membrane preparation was subjected to trypsin treatment to determine if the activity could be solubilized from the membrane, a typical set of results are presented in table 3.3.

Table 3.3 - Solubilization of *Pisum sativum* leaf microsomal membrane transglutaminase activity by trypsin treatment.

Trypsin treatment (50 μg ml^{-1}) minutes	Trypsin Supernatant Total units	Deoxycholate supernatant Total units
0	0.032 ± 0.006 (9.0)	0.320 ± 0.044 (91.0)
1	0.116 ± 0.001 (38.4)	0.186 ± 0.027 (61.6)
2	0.113 ± 0.002 (44.5)	0.141 ± 0.010 (55.5)
5	0.110 ± 0.013 (52.6)	0.099 ± 0.006 (47.4)
10	0.079 ± 0.007 (48.8)	0.083 ± 0.012 (51.2)
30	0.063 ± 0.001 (43.8)	0.081 ± 0.001 (56.2)
60	0.052 ± 0.009 (46.8)	0.059 ± 0.005 (53.2)

Washed microsomal membrane preparations were treated with 50 μg ml^{-1} trypsin for 1 minute to 60 minutes at 20°C; proteolysis was terminated by the addition of 5mM PMSF (as described in section 2.2.3.2). Membranes were centrifugation at 100 000.g for 1 hour and the supernatant was collected. The pellet was resuspended in buffer containing 0.23% (w/v) deoxycholate and the membranes were centrifuged as before and the supernatant was retained (as described in section 2.2.3.1). Both the supernatants were assayed for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each sample. Values represent the mean ± SEM of 3 replicates. Values in brackets represent the percentage total recovery of transglutaminase activity in each of the trypsin and deoxycholate supernatants.

Table 3.3 demonstrates that treatment of the microsomal membrane preparations with 50 μg ml^{-1} trypsin for 5 minutes resulted in 53% of the total transglutaminase activity being solubilized. After 10 minutes treatment, the total transglutaminase activity recovered had been reduced from 0.352 units in the untreated to 0.162 units in the treated.

Triton X-114 phase partitioning can be used to separate hydrophilic from integral membrane proteins (Bordier, 1981). This procedure was used to investigate the solubility and partitioning of *Pisum sativum* soluble and microsomal transglutaminases in the detergent, a typical set of data is presented in table 3.4.

Table 3.4 - Triton X-114 solubilization and phase partitioning of *Pisum sativum* transglutaminase activities.

Membranes	Input sample Total units	Detergent phase Total units	Aqueous phase Total units
100 000.g supernatant	1.248 ± 0.162	0.656 ± 0.240	0.992 ± 0.112
Washed membranes (WM)	0.736 ± 0.071	0.272 ± 0.120	0.446 ± 0.016
Trypsin treated WM	0.325 ± 0.077	0.068 ± 0.004	0.228 ± 0.008
20 / 25% (w/w) sucrose gradient interface membranes	0.864 ± 0.116	0.520 ± 0.080	0.512 ± 0.216

The various membrane preparations (1 - 3 mg ml^{-1}) were mixed with Triton X-114 (final concentration 2% (v/v)) and subjected to solubilization and phase separation as described in section 2.2.3.3. The input sample (Triton X-114 solubilized proteins) and the final detergent and aqueous phases were assayed for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each sample. Values represent the mean ± SEM of 3 replicates.

Table 3.4 demonstrates that 60% of the soluble transglutaminase activity (100 000.g supernatant) separated into the aqueous phase after phase partitioning and 62% of the transglutaminase activity was found in the aqueous phase after partitioning of washed microsomal membranes. Utilising trypsin solubilized protein extracts, 77% of the total transglutaminase activity partitioned into the aqueous phase. There was an overall increase in the total transglutaminase activity recovered in the aqueous and detergent phases after phase partitioning of the soluble fraction and the washed membrane and sucrose gradient purified membranes compared with the total activity detected in the input sample.

3.2.3 The subcellular localisation of the microsomal membrane associated transglutaminase activity in *Pisum sativum* leaf tissue

To determine if the membrane associated transglutaminase activity was enriched on any one of the various membranes contained within the microsomal fraction, the washed membranes were separated by centrifugation through a discontinuous sucrose gradient (Hodges and Leonard, 1974). Fractions collected from the gradient interfaces were assayed for transglutaminase activity and the results are presented in table 3.5. Results given in table 3.5 show a typical distribution of the transglutaminase activity, the experiment was repeated 4 times with similar trends on each occasion.

Table 3.5 - Transglutaminase activity associated with fractions obtained after discontinuous sucrose gradient centrifugation of *Pisum sativum* microsomal membrane preparations.

%(w/w) sucrose interface	Total protein Mg	Specific activity Units mg^{-1}	Total activity Units
Washed membranes	260.0	0.054 ± 0.022	13.97 ± 1.59
9 / 20%	2.92	1.63 ± 0.37	4.76 ± 0.08 (18.0)
20 / 25%	1.65	4.54 ± 0.64	7.51 ± 0.34 (28.4)
25 / 30%	2.38	1.81 ± 0.06	4.29 ± 0.07 (16.2)
30 / 34%	4.69	0.61 ± 0.08	2.86 ± 0.05 (10.8)
34 / 38%	5.68	0.33 ± 0.02	1.87 ± 0.09 (7.1)
38 / 45%	8.80	0.17 ± 0.01	1.50 ± 0.17 (5.7)
Gradient pellet	135.23	0.027 ± 0.003	3.65 ± 0.41 (13.8)

Discontinuous sucrose gradient centrifugation was carried out according to section 2.2.3.4. Membranes collected at the gradient interfaces were treated with 0.23% (w/v) deoxycholate and centrifuged at 100 000.g (as described in section 2.2.3.1), the supernatants were collected and assayed for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each sample. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total transglutaminase activity detected in the fraction as a percentage of the total transglutaminase activity recovered after centrifugation.

Table 3.5 shows that an enrichment of microsomal *Pisum sativum* transglutaminase activity was located at interface 2, which corresponded to the 20 / 25% (w/w) sucrose interface and accounted for 28% of the total activity recovered from the gradient interfaces and pellet. Sixty three percent of the total transglutaminase activity recovered was located in the top three interfaces after centrifugation.

To identify the subcellular location of the microsomal transglutaminase activity, the membranes obtained after discontinuous sucrose gradient centrifugation were assayed for several enzyme activities that serve as markers for the different membrane types. Typical results from the marker enzyme assays are presented in tables 3.6 to 3.11.

Table 3.6 - Lactate dehydrogenase activity in fractions obtained after discontinuous sucrose gradient centrifugation of *Pisum sativum* microsomal membrane extracts.

% (w/w) Sucrose interface	LDH activity Total units
100 000.g supernatant	9 496.89 ± 408.56
Washed membranes	516.87 ± 95.51
9 / 20%	9.72 ± 2.82 (1.9)
20 / 25%	13.30 ± 4.05 (2.6)
25 / 30%	20.36 ± 8.31 (3.9)
30 / 34%	22.51 ± 9.27 (4.4)
34 / 38%	26.33 ± 10.99 (5.1)
38 / 45%	26.89 ± 11.15 (5.2)
Gradient pellet	257.23 ± 79.78 (49.8)

Two hundred and sixty milligrams of washed *Pisum sativum* microsomal membrane protein were fractionated by discontinuous sucrose gradient centrifugation according to section 2.2.3.4. Lactate dehydrogenase activity was measured as described in section 2.2.4.3. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total LDH activity detected in the fraction as a percentage of the total LDH activity loaded on to the gradient.

Table 3.6 demonstrates that the washed membrane preparation contained a total of 517 units of LDH activity compared to 9 497 units in the soluble supernatant fraction. Fifty percent of the total LDH activity applied on to the gradients was found in the pellet after centrifugation.

Table 3.7 - The distribution of NADH-dependent cytochrome c reductase and cytochrome c oxidase activities in *Pisum sativum* microsomal membranes fractionated by discontinuous sucrose gradient centrifugation.

% (w/w) Sucrose interface	NADH cytochrome c reductase Total units	Cytochrome c oxidase Total units
Washed membranes	1 583.57 ± 115.97	11 832.05 ± 261.87
9 / 20%	46.44 ± 5.90 (2.9)	11.12 ± 0.94 (0.09)
20 / 25%	132.43 ± 2.81 (8.4)	13.42 ± 1.89 (0.1)
25 / 30%	183.78 ± 8.83 (11.6)	38.80 ± 3.98 (0.3)
30 / 34%	257.66 ± 36.78 (16.3)	77.84 ± 3.63 (0.7)
34 / 38%	146.76 ± 9.99 (9.3)	138.64 ± 12.18 (1.2)
38 / 45%	63.47 ± 6.38 (4.0)	359.17 ± 38.01 (3.0)
Gradient pellet	226.24 ± 28.96 (14.3)	7 076.17 ± 453.94 (59.8)

Two hundred and sixty milligrams of washed *Pisum sativum* microsomal membrane protein was fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Antimycin A insensitive NADH dependent cytochrome c reductase and cytochrome c oxidase activities were determined as described in sections 2.2.4.4 and 2.2.4.5 respectively. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total activity detected in the fraction as a percentage of the total activity loaded on to the gradient.

Table 3.7 demonstrates that there was an enrichment of antimycin A insensitive NADH dependent cytochrome c reductase activity at the 25 / 30 and 30 / 34% (w/w) sucrose interfaces after centrifugation. Sixty seven percent of the total NADH dependent cytochrome c reductase activity loaded on to the gradient was recovered after centrifugation. Table 3.7 shows there was an enrichment of cytochrome c oxidase activity in the gradient pellet, which accounted for 60% of the total cytochrome c oxidase activity recovered after centrifugation.

Table 3.8 - The distribution of vanadate and azide sensitive ATPase activities in *Pisum sativum* microsomal membranes fractionated by discontinuous sucrose density gradient centrifugation.

% (w/w) Sucrose interface	Cation-stimulated, vanadate-inhibitable ATPase Total units	Azide-inhibitable ATPase Total units
Washed membranes	322.88 ± 22.94	41.03 ± 7.45
9 / 20%	12.66 ± 2.12 (3.9)	N.D
20 / 25%	16.74 ± 1.32 (5.2)	0.59 ± 0.19 (1.4)
25 / 30%	39.48 ± 3.72 (12.2)	1.22 ± 0.05 (3.0)
30 / 34%	91.44 ± 4.56 (28.3)	3.96 ± 0.06 (9.7)
34 / 38%	89.94 ± 8.94 (27.9)	3.35 ± 0.05 (8.2)
38 / 45%	15.34 ± 0.84 (4.8)	1.22 ± 0.02 (3.0)
Gradient pellet	33.47 ± 5.03 (10.4)	19.01 ± 2.36 (46.3)

Two hundred and sixty milligrams of washed *Pisum sativum* microsomal membrane protein was fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Cation-stimulated, vanadate-inhibitable ATPase and azide sensitive ATPase activities were determined as described in sections 2.2.4.6 and 2.2.4.8 respectively. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total activity detected in the fraction as a percentage of the total activity loaded on to the gradient. N.D no activity detectable.

Table 3.8 demonstrates that cation-stimulated, vanadate-inhibitable ATPase activity was enriched at the 30 / 34% and 34 / 38% (w/w) sucrose interfaces after centrifugation of *Pisum sativum* washed membranes through discontinuous sucrose gradients. The overall recovery of cation-stimulated, vanadate-inhibitable ATPase activity was 93%. Approximately 46% of the azide-inhibitable ATPase activity was detected in the gradient pellet after centrifugation. There was also an enrichment of azide-sensitive ATPase activity at the 30 / 34% and 34 / 38% (w/w) sucrose interfaces, the overall recovery of activity was 72%.

Table 3.9 - The distribution of molybdate sensitive ATPase and latent IDPase activities in *Pisum sativum* microsomal membranes fractionated by discontinuous sucrose density gradient centrifugation.

% (w/w) Sucrose interface	Molybdate sensitive ATPase Total units	Latent IDPase Total units
Washed membranes	756.23 ± 45.21	10.11 ± 1.02
9 / 20%	5.42 ± 1.68 (0.7)	N.D
20 / 25%	6.63 ± 1.32 (0.9)	0.023 ± 0.036 (0.2)
25 / 30%	24.60 ± 2.76 (3.3)	2.28 ± 1.13 (22.6)
30 / 34%	54.13 ± 6.34 (7.2)	2.34 ± 0.39 (23.1)
34 / 38%	79.84 ± 7.68 (10.6)	0.65 ± 0.06 (6.4)
38 / 45%	123.62 ± 7.68 (16.3)	N.D
Gradient pellet	364.22 ± 63.04 (48.2)	2.45 ± 0.79 (24.2)

Two hundred and sixty milligrams of washed *Pisum sativum* microsomal membrane protein was fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Molybdate sensitive ATPase and latent IDPase activities were determined as described in sections 2.2.4.9 and 2.2.4.10 respectively. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total activity detected in the fraction as a percentage of the total activity loaded on to the gradient. N.D no activity detectable.

Table 3.9 demonstrates that approximately 50% of the molybdate-inhibitable ATPase activity was recovered in the gradient pellet after centrifugation. There was also an enrichment of molybdate-inhibitable ATPase activity at the 34 / 38% and 38 / 45% (w/w) sucrose interfaces, the overall recovery of activity was 87%. Latent IDPase activity was enriched on the 25 / 30% and 30 / 34% (w/w) sucrose interfaces after centrifugation of washed *Pisum sativum* microsomal membranes through discontinuous sucrose gradients. Twenty four percent of the total latent IDPase activity loaded on to the gradients was detected in the gradient pellet after centrifugation.

Table 3.10 - The distribution of nitrate-sensitive ATPase and PPase activities in *Pisum sativum* microsomal membranes fractionated by discontinuous sucrose gradient centrifugation.

% (w/w) Sucrose interface	Anion-stimulated, nitrate-inhibitable ATPase Total units	Cation-stimulated, inorganic pyrophosphatase (PPase) Total units
Washed membranes	42.56 ± 4.77	425.23 ± 57.19
9 / 20%	9.54 ± 2.22 (22.4)	100.8 ± 12.2 (23.7)
20 / 25%	9.90 ± 1.62 (23.3)	124.8 ± 20.4 (29.3)
25 / 30%	7.62 ± 0.36 (17.9)	63.11 ± 1.83 (14.8)
30 / 34%	3.64 ± 0.46 (8.6)	50.42 ± 3.02 (11.9)
34 / 38%	N.D	13.86 ± 2.35 (3.3)
38 / 45%	N.D	N.D
Gradient pellet	3.12 ± 0.31 (7.3)	54.66 ± 9.62 (12.9)

Two hundred and sixty milligrams of washed *Pisum sativum* microsomal membrane protein was fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Anion-stimulated, nitrate-inhibitable ATPase and cation-stimulated PPase activities were determined as described in sections 2.2.4.7 and 2.2.4.11 respectively. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total activity detected in the fraction as a percentage of the total activity loaded on to the gradient. N.D no activity detectable.

Table 3.10 demonstrates that anion-stimulated, nitrate-inhibitable ATPase activity was enriched at the 9 / 20%, 20 / 25% and the 25 / 30% (w/w) sucrose interfaces after centrifugation of discontinuous sucrose gradients. No nitrate-inhibitable ATPase activity was detectable at the 34 / 38% and 38 / 45% (w/w) sucrose interfaces. Cation-stimulated inorganic pyrophosphatase activity was enriched at the 9 / 20%, 20 / 25% and the 25 / 30% (w/w) sucrose interfaces and accounted for 68% of the total PPase activity recovered after centrifugation.

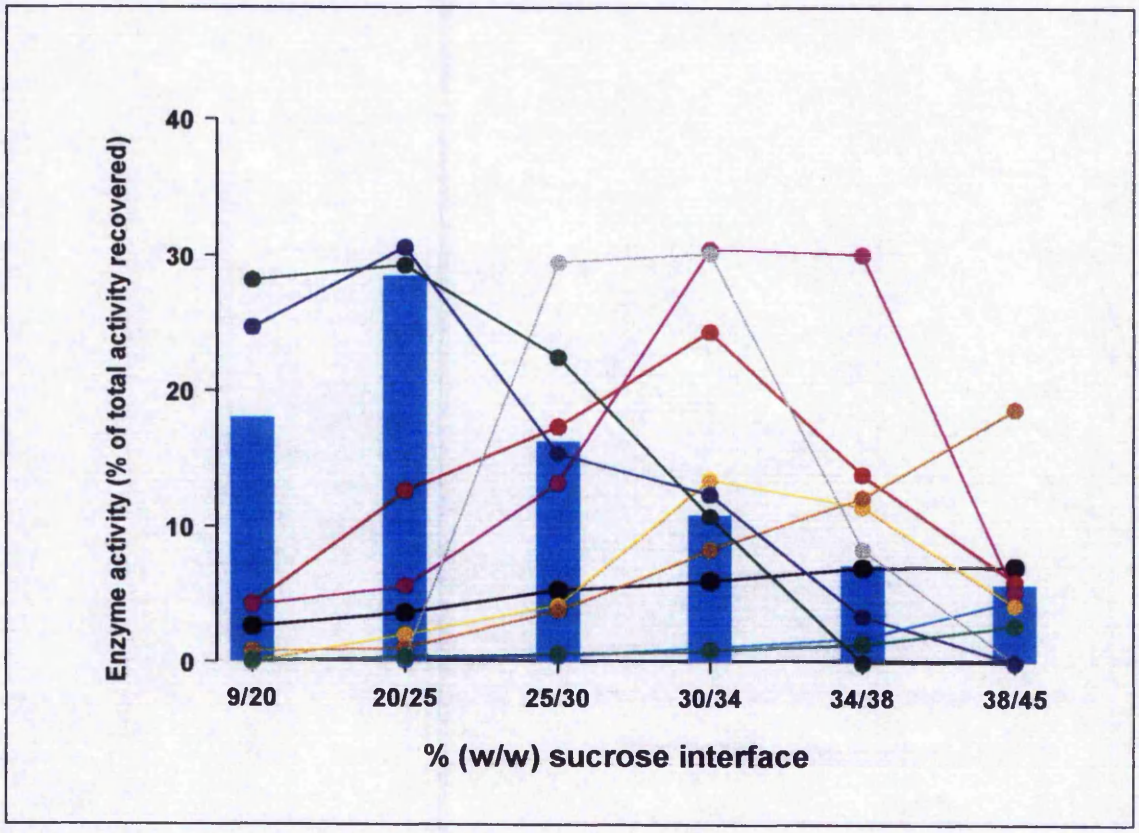
Table 3.11 - The distribution chlorophyll in *Pisum sativum* microsomal membranes fractionated by discontinuous sucrose gradient centrifugation.

% (w/w) Sucrose interface	Chlorophyll mg fraction^{-1}
Washed membranes	23.820 ± 0.769
9 / 20%	0.017 ± 0.001 (0.07)
20 / 25%	0.060 ± 0.006 (0.3)
25 / 30%	0.109 ± 0.016 (0.5)
30 / 34%	0.159 ± 0.008 (0.7)
34 / 38%	0.262 ± 0.015 (1.1)
38 / 45%	0.515 ± 0.026 (2.2)
Gradient pellet	18.521 ± 0.564 (77.7)

Two hundred and fifty milligrams of washed *Pisum sativum* microsomal membrane protein were fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Total chlorophyll concentration was determined as described in section 2.2.5. Values represent the mean ± SEM of 3 replicates. The values in brackets represent the total chlorophyll detected in the fraction as a percentage of the total chlorophyll loaded on to the gradient.

Table 3.11 shows the distribution of chlorophyll in the fractions obtained after discontinuous sucrose density gradient centrifugation of *Pisum sativum* microsomal membranes. Approximately 78% of the total chlorophyll loaded on to the gradients was detected in the gradient pellet after centrifugation. Very little chlorophyll was detected at the interfaces collected from the top of the gradients. Figure 3.2 shows a summary of the transglutaminase and marker enzyme activities presented in tables 3.5 to 3.11.

Figure 3.2 - A summary of the distribution of enzyme activities in *Pisum sativum* microsomal membranes fractionated by sucrose gradient centrifugation.



Two hundred and fifty milligrams of washed *Pisum sativum* microsomal membrane protein were fractionated by discontinuous sucrose density gradient centrifugation according to section 2.2.3.4. Enzyme activities were determined as described in tables 3.5 to 3.11. Enzyme activities are expressed as a percentage of the total activity recovered after sucrose density gradient centrifugation.

Key

■ Biotin cadaverine incorporation activity; ●— LDH activity; ●— NADH dependent cytochrome C reductase activity; ●— Cytochrome C oxidase activity; ●— Vanadate-inhibitable ATPase; ●— Azide-inhibitable ATPase; ●— Molybdate sensitive ATPase; ●— Latent IDPase; ●— Nitrate-inhibitable ATPase; ●— PPase; ●— Chlorophyll.

3.3 Discussion

3.3.1 Detection of transglutaminase activity in *Pisum sativum* leaf tissue

The biotin cadaverine incorporation assay (Slaughter *et al.*, 1992) was used to detect transglutaminase activity in crude *Pisum sativum* leaf extracts. This assay is preferred to the conventional [^{14}C] putrescine incorporation assay (Lorand *et al.*, 1972) because diamine oxidase activity present in crude plant extracts (Hill, 1971; Chiarello *et al.*, 1996a and 1996b) interferes with the [^{14}C] putrescine incorporation assay (Lilley *et al.*, 1998a).

Pisum sativum leaf homogenate was centrifuged at 13 000.g for 30 minutes to pellet unbroken cells, nuclei, chloroplasts and mitochondria, and 100 000.g for 1 hour to sediment the microsomal fraction (Morre *et al.*, 1987). Table 3.1 shows that approximately 13% of the total transglutaminase activity was pelleted by low speed centrifugation and a further 9% was sedimented with the microsomal fraction. The presence of 0.23% (w/v) sodium deoxycholate provoked a 4-fold increase in the total activity detected in the low speed pellet and a 1.5-fold increase in the total activity detected in the microsomal fraction. This increase in total transglutaminase activity maybe due to an increased accessibility of the enzyme to the assay substrates.

The transglutaminase activity detected in the 13 000.g pellet could originate from a mitochondrial form of the enzyme as described previously by Votyakova *et al.* (1999) in *Solanum tubersum* and *Phaseolus aureus*. However, the transglutaminase activity present in the 13 000.g pellet was not studied further due to the heterogeneous nature of this fraction.

In other plants, transglutaminase activity has been found in both the soluble and particulate fractions prepared from one tissue. When transglutaminase activity was studied in different organs of *Helianthus tuberosus* the activity was located within different subcellular fractions. In the leaves, 78% of the total transglutaminase activity was localised in the 22 000.g pellet; a different distribution was observed in the etiolated sprout apices, where 65% of the total activity was localised in the 22 000.g supernatant (Falcone *et al.*, 1993). Signorini *et al.* (1991) also found a transglutaminase activity in *Brassica oleracea* floral buds mainly localised in the 15 000.g pellet. In

addition, Signorini *et al.* (1991) found that in leaf extracts from *Beta vulgaris* and *Spinacia oleracea*, a transglutaminase activity was restricted to the 15 000.g pellet.

3.3.2 Association of *Pisum sativum* leaf transglutaminase with the microsomal membranes

The presence of transglutaminase in the microsomal membrane preparation does not exclude the possibility that microsomal transglutaminase originates from binding, *in vitro,* of the enzyme to the membranes taking into account the large proportion of transglutaminase activity that was recovered in the soluble fraction (see table 3.1). Therefore, the microsomal membrane preparation was washed to remove peripheral membrane proteins and any loosely associated soluble proteins. The solubilization of membrane proteins by sodium or potassium chloride (> 0.15 M), acidic or basic pH buffers or EGTA and EDTA is commonly used to distinguish between peripheral and integral membrane proteins (Thomas and McNamee, 1990). Peripheral membrane proteins are held in place by weak electrostatic and hydrogen bonding and are therefore readily solubilized by these methods

Table 3.2 demonstrates that treatment of *Pisum sativum* microsomal preparations with potassium chloride buffers (0.1 – 1.0 M) or 50mM sodium carbonate pH 10.0 failed to remove the microsomal transglutaminase activity, but did remove up to 50% of the total membrane protein. Treatment of the membranes with 50mM glycine-HCl pH 3.0 caused a reduction in recovered transglutaminase activity, but this could be due to the precipitation of proteins caused by this treatment. EDTA did effect a 27% reduction in microsomal transglutaminase activity at 10mM, this concentration of EDTA should have been sufficient to sequester membrane-bound Mg^{2+} and Ca^{2+} involved in maintenance of membrane structure (Butler, 1989). It is possible that EDTA remained in the membrane preparations after washing and was responsible for chelation of free Ca^{2+}, and hence a reduction in transglutaminase activity. Failure of these treatments to effectively solubilize transglutaminase activity suggests that the enzyme is not peripherally associated with the membrane.

The possibility that the transglutaminase activity present in *Pisum sativum* leaf microsomal fractions is an intrinsic membrane protein was further suggested by the release of the enzyme from the membranes by detergent treatment (see figure 3.1). The

transglutaminase activity recovered in the soluble fraction increased to a peak of 0.59 units at 0.23% (w/v) sodium deoxycholate. Further solubilization did not occur when the detergent concentrations were increased up to 0.5% (w/v) (data not shown). Sodium deoxycholate was chosen to solubilize the transglutaminase activity since previous work, using *Pisum sativum* root microsomal membrane preparations, showed that this detergent effectively solubilized the activity whilst other detergents lead to low recoveries of transglutaminase activity (Lilley, 1998).

The presence of an integral membrane form of transglutaminase has been reported in other plant tissues. Transglutaminase activity in the 22 000.g supernatant fraction prepared from *Helianthus tuberosus* leaf tissue was increased from 22% in the untreated to 92 – 95% after the addition of 1% (v/v) Triton X-100 to the extraction buffer (Falcone *et al.*, 1993). In pollen of *Malus domestica*, the addition of 0.5% (v/v) Triton X-100 in the extraction buffer caused a 220% increase in radiolabelled protein in the TCA pellet after a [^{3}H] putrescine incorporation assay (Del Duca *et al.*, 1997). These authors suggested transglutaminase is membrane associated and possibly an intrinsic membrane protein. However, in these instances the membranes were not subjected to rigorous washing procedures to remove peripheral membrane proteins prior to detergent solubilization.

Membrane-associated forms of mammalian transglutaminases have also been shown to exist (Thacher and Rice, 1985; Slife *et al.*, 1985 and 1987). The keratinocyte (type I) form of the enzyme can be released from the plasma membrane of cultured human epidermal keratinocytes by mild trypsin treatment (Thacher and Rice, 1985). *Pisum sativum* leaf microsomal transglutaminase activity was also released from the membrane by trypsin digestion. Table 3.3 demonstrates that treatment of microsomal membrane preparations with 50 μg ml^{-1} trypsin for 2 minutes at 20°C solubilized 45% of the total microsomal transglutaminase activity. When the incubation time was extended there was no further increase in solubilized activity and there was an overall decrease in the total transglutaminase activity recovered in the trypsin and deoxycholate supernatants, possibly due to irreversible proteolysis of the enzyme.

The release of transglutaminase by trypsin treatment would suggest that the enzyme is anchored to the membrane by 1 or 2 transmembrane protein segments or by covalent

attachment to fatty acids or lipids. Proteins of this type are often liberated from the membrane by the use of proteases or phosopholipases without destroying their native activity (Findlay, 1990). In both these forms of membrane attachment, the bulk of the protein is found in the aqueous phase and in general their activity does not involve the lipid bilayer, the bilayer simply acts as a structural support and means of localisation of the activity (Findlay, 1990).

Lipid anchors may play a role in the attachment of *Pisum sativum* microsomal transglutaminase to the membrane in a similar way to the anchorage of mammalian keratinocyte transglutaminase to the plasma membrane by fatty acid modifications, which allow the enzyme to be removed from the membrane by trypsin treatment (Thacher and Rice, 1985). The partitioning of keratinocyte transglutaminase between the cytoplasm and the membrane is controlled by variable modifications by myristate and palmitate (Steinert *et al.*, 1996b). The association of mammalian tissue transglutaminase, band 4.2 and prostate transglutaminase with membranes has also been shown to be due to interaction with lipids or attachment of a fatty acid anchor (Harsfalvi *et al.*, 1987; Cohen *et al.*, 1993; Esposito *et al.*, 1996).

The signals and sequences necessary for a protein to bind to a membrane need not be present in the primary structure of the protein and can instead be acquired through co-translational and post-translational modification (Sefton & Buss, 1987). Proteins can be covalently modified by the binding of glycosylated phospholipid, myristic acid or palmitic acid. Proteins modified by myristate may be found in cytosolic as well as membrane compartments (Olson *et al.*, 1985).

In contrast to water soluble and hydrophilic proteins, which display little or no hydrophobic interaction with non-ionic detergents, integral membrane proteins have an amphiphilic structure and upon solubilization the non-ionic detergent replaces most of the lipid molecules in contact with the hydrophobic domain of the integral membrane protein (Bordier, 1981). Table 3.4 demonstrates the distribution of soluble and microsomal transglutaminase after Triton X-114 solubilization and phase partitioning. The cytosolic *Pisum sativum* transglutaminase activity was detected in both the aqueous and detergent phase after Triton X-114 phase partitioning, with 40% of the transglutaminase recovered in the detergent phase and 60% in the aqueous phase. This

distribution was also observed with the transglutaminase activity present in the washed microsomal membrane preparation. When utilising sucrose density gradient purified microsomal membranes, 50% of the transglutaminase activity was recovered in the detergent phase. These data would suggest that the soluble and microsomal transglutaminases contain similar amounts of hydrophobic amino acids. Hydropathy analysis of human factor XIIIa and human guinea pig liver transglutaminase revealed the active sites in these enzymes are located at a transition region between hydrophilic and hydrophobic stretches of amino acids (Ichinose *et al.*, 1990). Furthermore, the binding of a hydrophobic probe, 1-anilino-8-naphthalenesulfonic acid (ANS), to tissue transglutaminase confirmed the presence of a hydrophobic pocket in the active site of the protein (Di Venere *et al.*, 2000). If the soluble and microsomal *Pisum sativum* transglutaminases contain similar sequence identity in the active site region, the hydrophobic stretch of amino acids could be responsible for detergent binding and hence partitioning of a proportion of the transglutaminase activity into the detergent phase.

When trypsin solubilized protein was subjected to Triton X-114 phase partitioning (table 3.4), 77% of the transglutaminase activity was recovered in the aqueous phase. This increased recovery in the aqueous phase could be due to the removal of hydrophobic amino acids involved in membrane anchorage. Integral membrane proteins with pronounced hydrophilic character, for example those with a single transmembrane helix and/or substantial carbohydrate content may sometimes be found in the aqueous phase after Triton X-114 phase partitioning (Findlay, 1990).

In order to measure transglutaminase activity in the input sample and the detergent phase after Triton X-114 phase partitioning, they had to be dialysed to remove the Triton X-114 (section 2.2.3.3). Activity was recovered after this detergent had been exchanged for sodium deoxycholate. The low transglutaminase activity detected in the presence of Triton X-114 could have been due to association of the hydrophobic tail portion of this detergent with a hydrophobic section of transglutaminase that was essential for its enzymatic activity (Thomas and McNamee, 1990). The recovery of transglutaminase activity after the replacement of Triton X-114 for sodium deoxycholate was probably due to differences in the hydrophobic portions of these detergents (Jones *et al.*, 1987).

3.3.3 The subcellular localisation of *Pisum sativum* microsomal transglutaminase activity

In order to establish if the microsomal *Pisum sativum* leaf transglutaminase activity was enriched on any one of the microsomal membranes, washed membrane preparations were separated by centrifugation through discontinuous sucrose gradients. Table 3.5 demonstrates that 28% of the total transglutaminase activity recovered was contained within the 20 / 25% (w/w) sucrose interface and represented an 84-fold increase in specific activity. A further 18% and 16% of the total transglutaminase activity were recovered within the 9 / 20% and 25 / 30% (w/w) sucrose interfaces respectively. The total activity recovered at these three interfaces was 16.56 units compared to only 13.97 units detected in the washed membrane preparation applied onto the gradients. This overall increase in recovered transglutaminase activity could be due to the removal and/or separation of inhibitory or regulatory substances, which could include preferred amine acceptor or donor substrates.

The membranes collected at the gradient interfaces were assayed for several enzyme activities that serve as markers of the membrane types. The activity of a soluble, cytoplasmic enzyme was also determined to estimate the extent to which soluble proteins have become associated with membrane surfaces, or trapped within membrane vesicles (Widell and Larsson, 1990).

Lactate dehydrogenase activity was used as a marker for the contamination of the membrane preparations by soluble proteins. Table 3.6 shows that the 100 000.g supernatant fraction contained 9 497 units of LDH activity compared to 517 units in the washed membrane preparation. This represents an approximate 5% contamination of the microsomal preparation by soluble proteins; therefore contamination of the washed membrane preparation by soluble proteins was low. After discontinuous sucrose gradient centrifugation of the washed membranes, 50% of the lactate dehydrogenase activity was found associated with membranes or within membrane vesicles located in the gradient pellet. Therefore the transglutaminase activity located at the top of the gradient is unlikely to be due to contamination by the soluble cytoplasmic form of transglutaminase.

Antimycin A-insensitive NADH dependent cytochrome c reductase activity was used as a marker for the endoplasmic reticulum (Quail, 1979). This enzyme activity is also found in the plasma membrane, the tonoplast and the outer mitochondrial membrane in smaller amounts (Widell and Larsson, 1990). Generally, the inclusion of millimolar levels of Mg^{2+} in the extraction and gradient buffers results in the retention of ribosomes on the endoplasmic reticulum, the inclusion of 3 to 5mM EDTA in extraction and gradient buffers usually ensures the endoplasmic reticulum is present as the smooth form in plant cell extracts (Robinson *et al.*, 1994). Table 3.7 demonstrates that NADH dependent cytochrome c reductase activity had a broad distribution over the middle four sucrose gradient interfaces, with the 30 / 34% (w/w) sucrose interface containing 16% of total activity recovered. It has been reported that smooth endoplasmic reticulum has an equilibration density in sucrose of 21 – 27% (w/w) and rough ER 30 – 40% (w/w) sucrose (Robinson *et al.*, 1994). Therefore despite the presence of 3mM EDTA in the extraction buffer and 1mM EDTA in the gradient buffer, most of the endoplasmic reticulum appeared to be present in the rough form. Since the peak of NADH dependent cytochrome c reductase activity was present at the 30 / 34% (w/w) sucrose interface and that of transglutaminase activity at the 20 / 25% (w/w) interface it is unlikely that transglutaminase is associated with the ER membranes.

Cytochrome c oxidase is localised on the inner mitochondrial membrane (Widell and Larsson, 1990) and was therefore used as a marker for the mitochondria. Most of the mitochondria would have been pelleted during the initial low speed centrifugation step since 6 000.g for 15 minutes is sufficient to pellet this organelle (Morre *et al.*, 1987). Table 3.7 shows that approximately 60% of the cytochrome c oxidase activity was found in the gradient pellet with little cytochrome c oxidase activity present at the gradient interfaces. Intact mitochondria have an equilibration density in sucrose of 40 – 44% (w/w), the mitoplast (inner membrane and matrix) 42% (w/w) and the inner membrane approximately 32% (w/w) (Quail, 1979). In this fractionation study the majority of the mitochondria appear to be present in either intact or mitoplasts forms. The microsomal transglutaminase activity is unlikely to be associated with the mitochondria as they are present at much higher sucrose densities compared to the transglutaminase activity.

The cation-stimulated, vanadate-inhibitable ATPase activity was used as a marker for the plasma membrane in the fractionation study (Widell and Larsson, 1990). Table 3.8 demonstrates that vanadate-inhibitable ATPase activity was enriched on the 30 / 34% and 34 / 38% (w/w) sucrose interfaces. This corresponds to the reported equilibration density of 30 – 40% (w/w) sucrose for the plasma membrane from plant cells (Robinson *et al.*, 1994).

The inhibition of ATPase activity by azide is a characteristic feature of the mitochondrial ATPase (Gallagher and Leonard, 1982). The distribution of azide sensitive ATPase activity (table 3.8) is similar to that observed for cytochrome c oxidase activity (table 3.7), with almost 50% of the activity being recovered in the gradient pellet. Azide sensitive ATPase activity was also found at the 30 / 34% and 34 / 38% (w/w) sucrose interfaces, at an equilibration density corresponding to that of the mitochondrial inner membrane (Quail, 1979).

A Mg^{2+}-dependent ATPase activity that is vanadate and molybdate sensitive has been found on the inner chloroplast membrane in *Pisum sativum* seedlings (McCarty *et al.*, 1984). Table 3.9 demonstrates that the molybdate sensitive ATPase was localised predominantly in the gradient pellet and at the 38 / 45% (w/w) sucrose interface. Most of the intact chloroplasts would have sedimented by the initial low speed centrifugation since 2 000.g for 2 minutes is sufficient to sediment whole chloroplasts, however, separations from total homogenates are rarely complete (Morre *et al.*, 1987).

Chlorophyll content was used as a marker for intact chloroplasts and thylakoid membranes (Quail, 1979). Table 3.11 demonstrates that approximately 78% of the recovered chlorophyll was localised in the gradient pellet after centrifugation. Thylakoid membranes have an equilibration density in sucrose of 36 – 40% (w/w), compared to 40 – 44% (w/w) for intact chloroplasts (Quail, 1979). Therefore, the presence of 78% of the chlorophyll and 48% of the molybdate sensitive ATPase in the gradient pellet suggests that chloroplasts are present in an intact form.

IDPase activity, used as a marker for the Golgi apparatus, is latent and develops to a maximum if stored at 4°C for several days or if treated with detergent (Ray *et al.*, 1969; Waldron and Brett, 1987). Table 3.9 shows the peak of latent IDPase activity to be

located at the 25 / 30% and 30 / 34% (w/w) sucrose interfaces. This distribution represents the position of the Golgi apparatus as this density corresponds to that shown for the Golgi apparatus in *Pisum sativum* and other systems of 28 – 34% (w/w) sucrose (Quail, 1979).

The inhibition of ATPase activity by nitrate is a characteristic feature of the higher plant tonoplast ATPase (Fischer-Schliebs *et al.*, 1994). The presence of cation-stimulated inorganic pyrophosphatase activity is also a feature of plant tonoplast membranes (Walker and Leigh, 1981). Table 3.10 demonstrates that the 9 / 20%, 20 / 25% and 25 / 30% (w/w) sucrose interfaces contained approximately 64% and 68% of the recovered nitrate inhibitable ATPase and PPase activities respectively, and are therefore enriched in membrane vesicles derived from the tonoplast membrane. This is consistent with the reported equilibrium densities for tonoplast vesicles in sucrose of 18 – 22% (w/w) (Robinson *et al.*, 1994).

The presence of 50% of the soluble enzyme activity, LDH, and 24% of the latent IDPase activity, 13% of the PPase activity, 11% of the vanadate-inhibitable ATPase activity and 14% of the NADH dependent cytochrome c reductase activity in the gradient pellet could be due to the formation multiorganelle complexes. Multiorganelle complexes are often formed during homogenisation of leaf tissue, these complexes consist of one or two chloroplasts surrounded by cytoplasm, mitochondria, and golgi apparatus, all enclosed by some plasma membrane (Larsson, 1994).

The transglutaminase activity enriched at the 9 / 20%, 20 / 25% and 25 / 30% (w/w) sucrose interfaces coincides with the enrichment of nitrate sensitive ATPase and inorganic pyrophosphatase activity at these three sucrose interfaces. Since the microsomal transglutaminase activity appeared to be enriched on the tonoplast membrane only, it is unlikely that the transglutaminase activity was due to non-specific binding of the soluble cytosolic form of transglutaminase to the membrane during homogenisation since the activity would be a feature of all the membranes contained within the microsomal preparation.

The microsomal transglutaminase described here appears to be different from other reported membrane associated plant transglutaminases which have been found

associated with chloroplast and mitochondrial membranes (Margosiak *et al.*, 1990; Del Duca *et al.*, 1994; Votyakova *et al.*, 1999). Intact isolated chloroplasts from meristematic tissue of *Medicago sativa* exhibited transglutaminase activity responsible for 30% of the total activity in the meristematic tissue (Margosiak *et al.*, 1990). Similarly transglutaminase activity in the 15 000.g pellet fraction prepared from *Beta vulgaris* leaves was localised with fractions containing intact and broken chloroplasts after Percoll gradient separation (Signorini *et al.*, 1991). Furthermore, Del Duca *et al.* (1994) detected transglutaminase activity in isolated thylakoids from *Helianthus tuberosus* leaves and reported the cross-reactivity of a 58 000 M_r thylakoid protein to antibodies raised against rat prostate gland transglutaminase. However, all these activities were measured using radioactive polyamine incorporation assays, which are subject to interference by diamine oxidases (Siepaio and Meunier, 1995a and 1995b; Lilley *et al.*, 1998a).

The membrane fractions of isolated *Phaseolus aureus* mitochondria were responsible for the incorporation of radiolabelled putrescine into mitochondrial proteins. The presence of 0.2% (w/v) Triton X-100 decreased putrescine incorporation by 50% suggesting membrane integrity was important for transglutaminase activity (Votyakova *et al.*, 1999). The mitochondrial enzyme was suggested to be involved in stabilising membrane protein complexes or modifying the rigidity of the membrane by the formation of crosslinks established through polyamines (Votyakova *et al.*, 1999).

At the present time it is unclear whether the soluble and microsomal transglutaminase activities of *Pisum sativum* leaf tissue are distinct and discrete enzymes. Since the microsomal transglutaminase activity was enriched on the tonoplast membrane the enzyme could be a function of the vacuole. If the activity is a function of the vacuole it would be probable that the soluble and microsomal activities are due to different isoforms of transglutaminase in *Pisum sativum* leaf tissue.

Chapter 4 - Characterisation of soluble and microsomal transglutaminase activity in *Pisum sativum* leaf tissue

Chapter 4 - Characterisation of soluble and microsomal transglutaminase activity in *Pisum sativum* leaf tissue

4.1 Introduction

The presence of transglutaminase in both the soluble and particulate fraction has been reported for mammalian tissue transglutaminase extracted from rat liver (Birckbichler *et al.*, 1976; Slife *et al.*, 1985 and 1987) lung (Griffin *et al.*, 1978) and kidney (Juprelle-Soret *et al.*, 1988).

It was deemed necessary to ascertain the relationship between the soluble and microsomal forms of transglutaminase in *Pisum sativum* leaf tissue since, at the present time, it is unclear whether they are distinct and discrete enzymes. The principal aim of this section of the thesis was to investigate the biochemical and physical properties of the soluble and microsomal *Pisum sativum* transglutaminases. The properties of the two enzymes were compared in order to assess the similarities these enzymes have with each and with other plant and mammalian forms of transglutaminase.

4.2 Results

4.2.1 *Pisum sativum* transglutaminase biochemistry

For purposes of comparison, the activities of *Pisum sativum* soluble and microsomal transglutaminase were studied in the presence of 0.23% (w/v) sodium deoxycholate.

Mammalian transglutaminases are calcium activated enzymes (Aeschlimann and Paulsson, 1994), therefore the calcium ion concentration required to activate the biotin cadaverine incorporation activities of *Pisum sativum* soluble and microsomal transglutaminases were established. Typical calcium activation curves are presented in figures 4.1 and 4.2.

Figure 4.1 - The biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

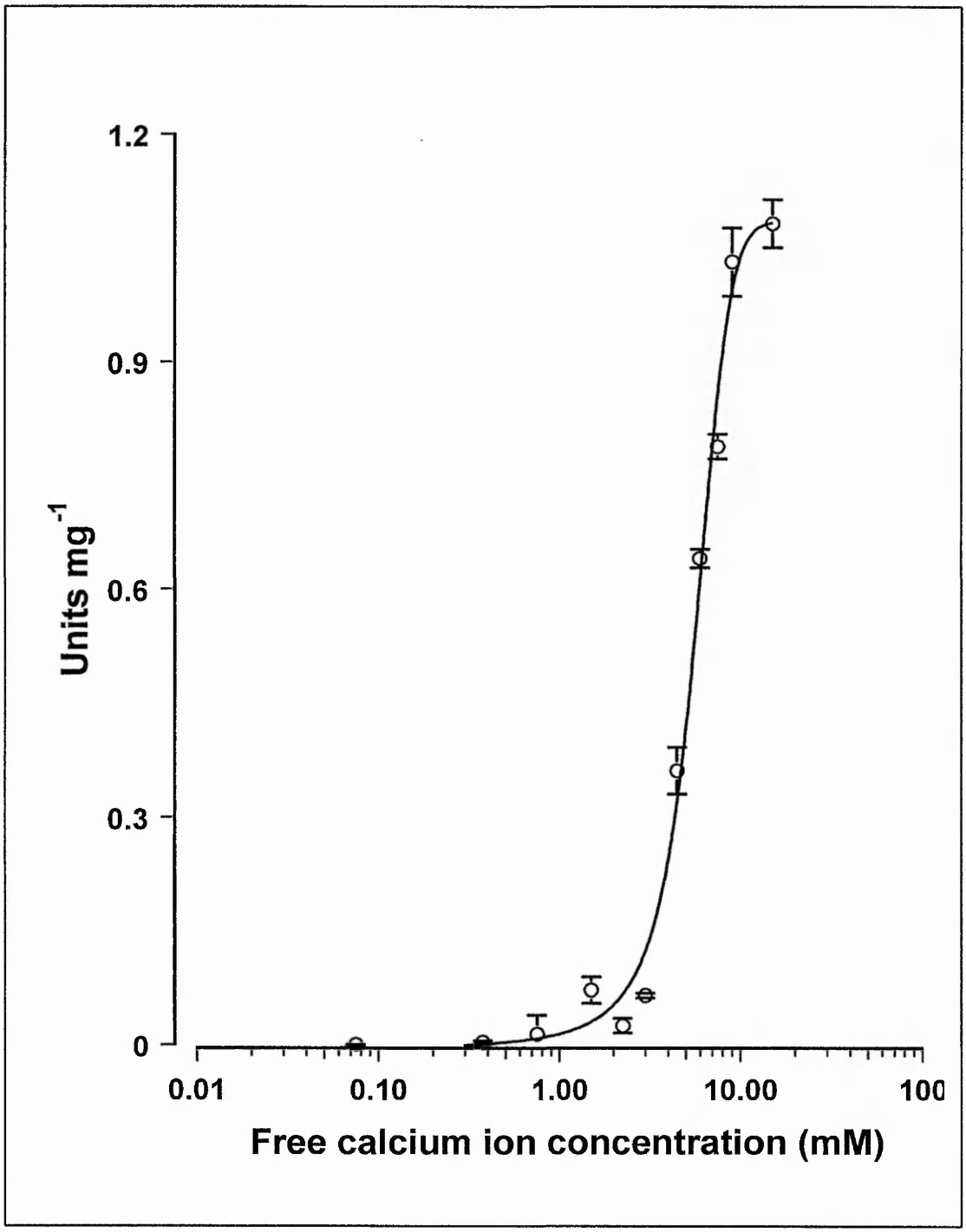


Figure 4.1 legend

Pisum sativum soluble leaf extract was incubated at 37°C for 60 minutes in the presence of various free calcium ion concentrations using the biotin cadaverine incorporation assay as described in section 2.2.4.1. Zero free calcium was achieved by the addition of 1mM EGTA. Increasing concentrations of calcium chloride were added to the reaction buffer and the equivalent free calcium ion concentration was calculated using the computer program EqCal (Biosoft, UK). The calculation of free calcium ion concentration can be found in appendix 1 (page 233). The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Data points represent the mean ± SEM of 3 replicates.

Figure 4.1 shows that the biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf transglutaminase was activated after 0.5mM free calcium with a maximum activity at 15mM free calcium.

Figure 4.2 - The biotin cadaverine incorporation activity of microsomal *Pisum sativum* leaf transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

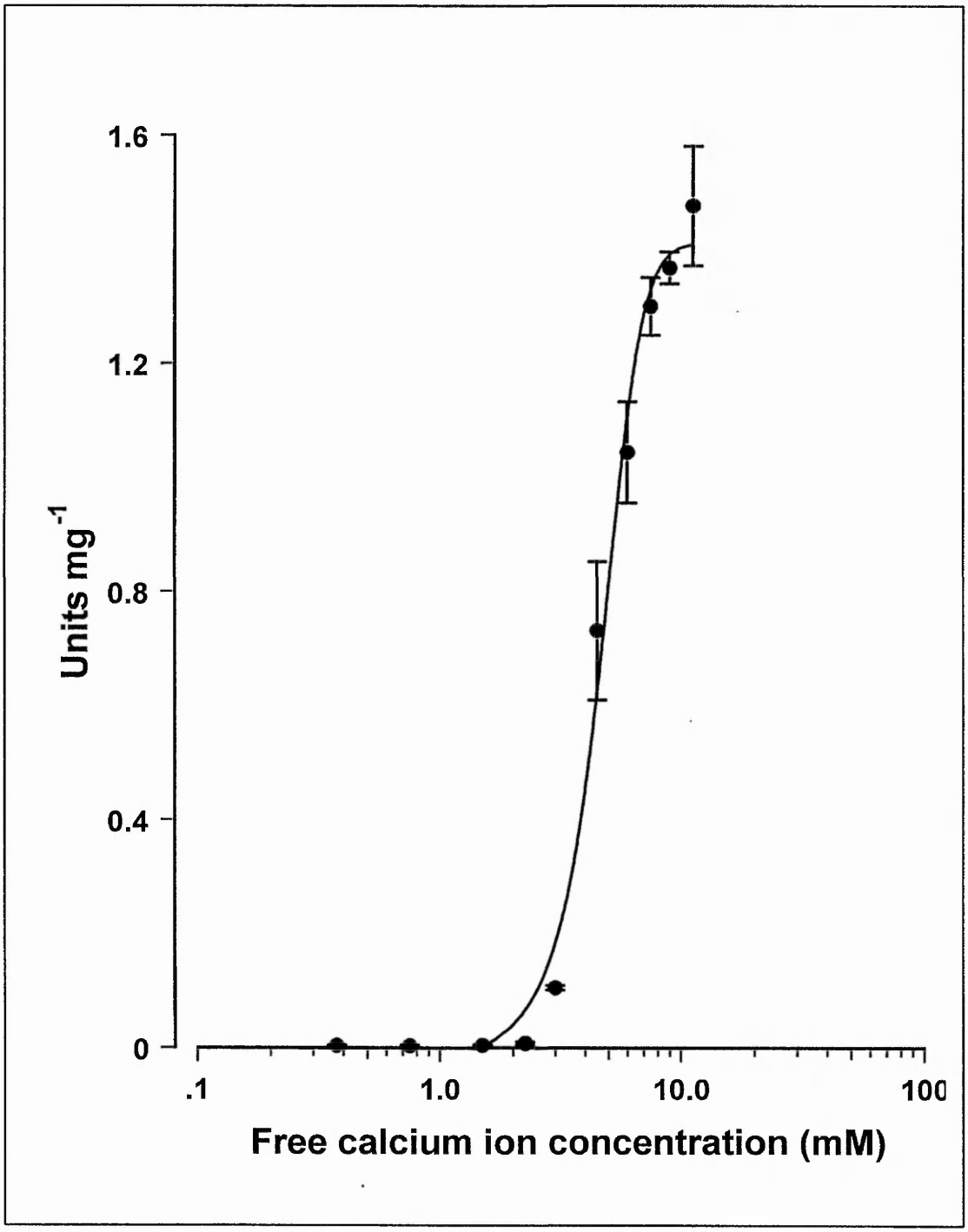


Figure 4.2 legend

Pisum sativum washed microsomal membrane protein was extracted with 0.23% (w/v) sodium deoxycholate as described in section 2.2.3.1. Detergent solubilized proteins were incubated at 37°C for 60 minutes in the presence of various free calcium ion concentrations as described in section 2.2.4.1. The free calcium ion concentration of the buffer was calculated as described for figure 4.1. Data points represent the mean ± SEM of 3 replicates.

Figure 4.2 shows that no biotin cadaverine incorporation activity occurred between 1 and 3mM free calcium. Activation of the biotin cadaverine incorporating activity of *Pisum sativum* leaf microsomal membrane transglutaminase occurred after 4mM free calcium, maximum activity was detected at 9mM free calcium.

Mammalian transglutaminases contain an active site cysteine residue and are sensitive to thiol reactive reagents (Folk & Cole, 1966). The thiol reactive reagents, iodoacetamide and N-ethylmaleimide, were used to measure the inhibition of transglutaminase activity present in *Pisum sativum* soluble and microsomal membrane preparations and typical sets of results are presented in tables 4.1 and 4.2.

Table 4.1 - The effect of thiol reactive reagents on soluble *Pisum sativum* leaf transglutaminase activity.

Inhibitor and concentration	Specific activity Units mg^{-1}	% Inhibition
Uninhibited	0.120 ± 0.003	
0.1mM iodoacetamide	0.104 ± 0.004	13.6
1mM iodoacetamide	0.074 ± 0.005	38.1
10mM iodoacetamide	0.040 ± 0.003	66.9
0.1mM N-ethylmaleimide	0.105 ± 0.002	12.3
1mM N-ethylmaleimide	0.085 ± 0.005	28.9
10mM N-ethylmaleimide	0.066 ± 0.003	45.1

Soluble *Pisum sativum* leaf extract was incubated in the presence of 5mM $CaCl_2$ and 0.1mM, 1mM or 10mM iodoacetamide or N-ethylmaleimide (NEM) and 0.23% (w/v) sodium deoxycholate for 30 minutes at 37°C. The pre-treated extracts were then assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay, but without the addition of DTT (section 2.2.4.1). Extract boiled for 10 minutes was used as a negative control at each inhibitor concentration. Values represent the mean ± SEM of 3 replicates.

Table 4.1 shows that the biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf extract was inhibited 67% and 45%, by 10mM iodoacetamide and 10mM N-ethylmaleimide, respectively.

Table 4.2 - The effect of thiol reactive reagents on microsomal membrane *Pisum sativum* leaf transglutaminase activity.

Inhibitor and concentration	Specific activity Units mg^{-1}	% Inhibition
Uninhibited	0.093 ± 0.011	
0.1mM iodoacetamide	0.059 ± 0.003	36.6
1mM iodoacetamide	0.057 ± 0.003	38.7
10mM iodoacetamide	0.018 ± 0.001	80.3
0.1mM N-ethylmaleimide	0.101 ± 0.011	0
1mM N-ethylmaleimide	0.068 ± 0.012	26.9
10mM N-ethylmaleimide	0.034 ± 0.002	63.1

Protein was solubilized from washed microsomal membrane preparations of *Pisum sativum* leaf extracts with 0.23% (w/v) sodium deoxycholate as described in section 2.2.3.1. Solubilized protein was incubated in the presence of 5mM $CaCl_2$ and 0.1mM, 1mM or 10mM iodoacetamide or N-ethylmaleimide (NEM) for 30 minutes at 37°C. The pre-treated extracts were assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay in the absence of DTT (section 2.2.4.1). Extract boiled for 10 minutes were used as a negative control at each inhibitor concentration. Values represent the mean ± SEM of 3 replicates.

Table 4.2 shows that the microsomal membrane transglutaminase activity of *Pisum sativum* leaf extract was inhibited 80% by 10mM iodoacetamide, and 27% and 63% by 1mM and 10mM N-ethylmaleimide respectively.

Legume diamine oxidases require copper ions for their activity (Hill, 1971) and are active in *Pisum sativum* leaf tissue (Hill, 1971; Siepaio and Meunier, 1995a and 1995b).

Therefore the copper ion chelators DIECA and o-phenanthroline were used to determine the extent of any contribution to the biotin cadaverine incorporation activity made by these copper dependent enzymes. Typical sets of data are presented in tables 4.3 and 4.4.

Table 4.3 - The effect of the diamine oxidase inhibitors, DIECA and o-phenanthroline, on the biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf extract.

Inhibitor and concentration	Specific activity Units mg^{-1}	% Inhibition
Uninhibited	0.131 ± 0.010	
0.1mM DIECA	0.127 ± 0.027	3.3
1mM DIECA	0.129 ± 0.025	1.3
10mM DIECA	0.096 ± 0.005	26.5
0.1mM o-phenanthroline	0.165 ± 0.022	0
1mM o-phenanthroline	0.077 ± 0.009	41.5
10mM o-phenanthroline	0.08 ± 0.007	39.2

Soluble *Pisum sativum* leaf extract was incubated in the presence of 5mM $CaCl_2$ and 0.1mM, 1mM or 10mM DIECA or o-phenanthroline and 0.23% (w/v) sodium deoxycholate for 30 minutes at 37°C. The pre-treated extracts were then assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay (section 2.2.4.1). Extract boiled for 10 minutes was used as a negative control at each inhibitor concentration. Values represent the mean ± SEM of 3 replicates.

Table 4.3 demonstrates that the biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf extract was inhibited 27% by 10mM DIECA and 42% and 39% by 1mM and 10mM o-phenanthroline respectively.

Table 4.4 - The effect of the diamine oxidase inhibitors, DIECA and o-phenanthroline, on the biotin cadaverine incorporation activity of microsomal membrane *Pisum sativum* leaf extract.

Inhibitor and concentration	Specific activity Units mg^{-1}	% Inhibition
Uninhibited	0.137 ± 0.013	
0.1mM DIECA	0.144 ± 0.009	0
1mM DIECA	0.140 ± 0.007	0
10mM DIECA	0.179 ± 0.025	0
0.1mM o-phenanthroline	0.151 ± 0.003	0
1mM o-phenanthroline	0.122 ± 0.010	11.0
10mM o-phenanthroline	0.086 ± 0.001	37.1

Protein was solubilized from washed microsomal membrane preparations of *Pisum sativum* leaf extracts with 0.23% (w/v) sodium deoxycholate as described in section 2.2.3.1. Soluble protein was incubated in the presence of 5mM $CaCl_2$ and 0.1mM, 1mM or 10mM DIECA or o-phenanthroline for 30 minutes at 37°C. The pre-treated extracts were assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay (section 2.2.4.1). Extract boiled for 10 minutes was used as a negative control at each inhibitor concentration. Values represent the mean ± SEM of 3 replicates.

Table 4.4 demonstrates that the biotin cadaverine incorporation activity of microsomal membrane *Pisum sativum* leaf extract was not inhibited by DIECA at any of the concentrations used, o-phenanthroline produced an 11% and 37% reduction in the biotin cadaverine activity at 1mM and 10mM respectively.

The effect of increasing concentrations of biotin cadaverine on transglutaminase activity was measured during a 60 minutes substrate binding experiment. The activities were found to follow Michaelis-Menten kinetics, and typical plots of the data are presented in figures 4.3 and 4.4.

Figure 4.3 - The rate of biotin cadaverine incorporation as a function of the concentration of biotin cadaverine using soluble *Pisum sativum* leaf extract.

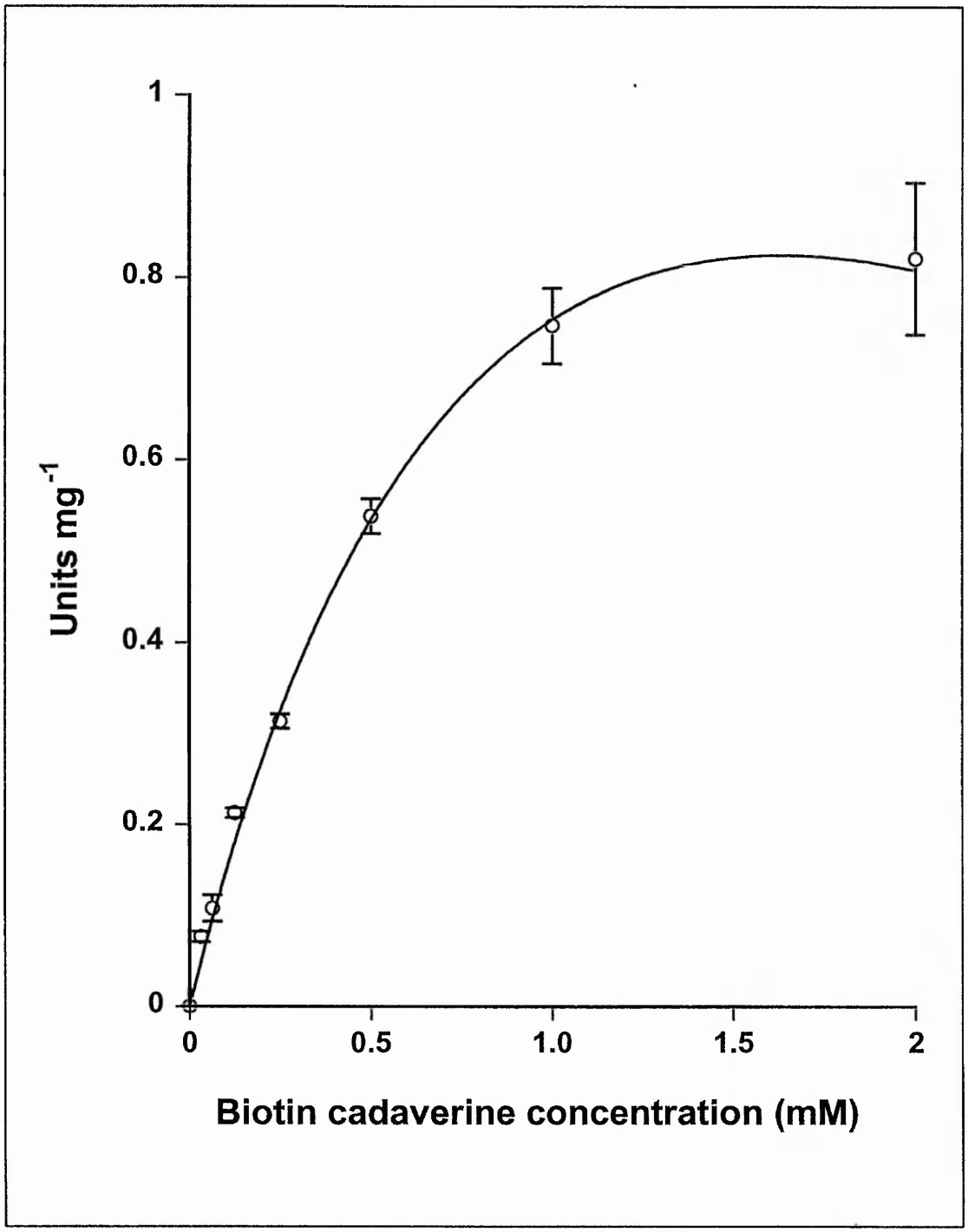


Figure 4.3 legend

Soluble *Pisum sativum* leaf extract was assayed at 37°C for 60 minutes in the presence of various concentrations of biotin cadaverine and 0.23% (w/v) sodium deoxycholate as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control at each substrate concentration. Data points represent the mean ± SEM of 3 replicates.

Figure 4.3 demonstrates the concentration dependence of biotin cadaverine incorporation into N',N'-dimethylcasein by soluble *Pisum sativum* leaf transglutaminase. The K_m for biotin cadaverine was calculated to be 472.6μM and the V_{max} 1.05 units mg^{-1} using Enzfitter software (Biosoft, U.K).

Figure 4.4 - The rate of biotin cadaverine incorporation as a function of the biotin cadaverine concentration using *Pisum sativum* microsomal membrane preparations.

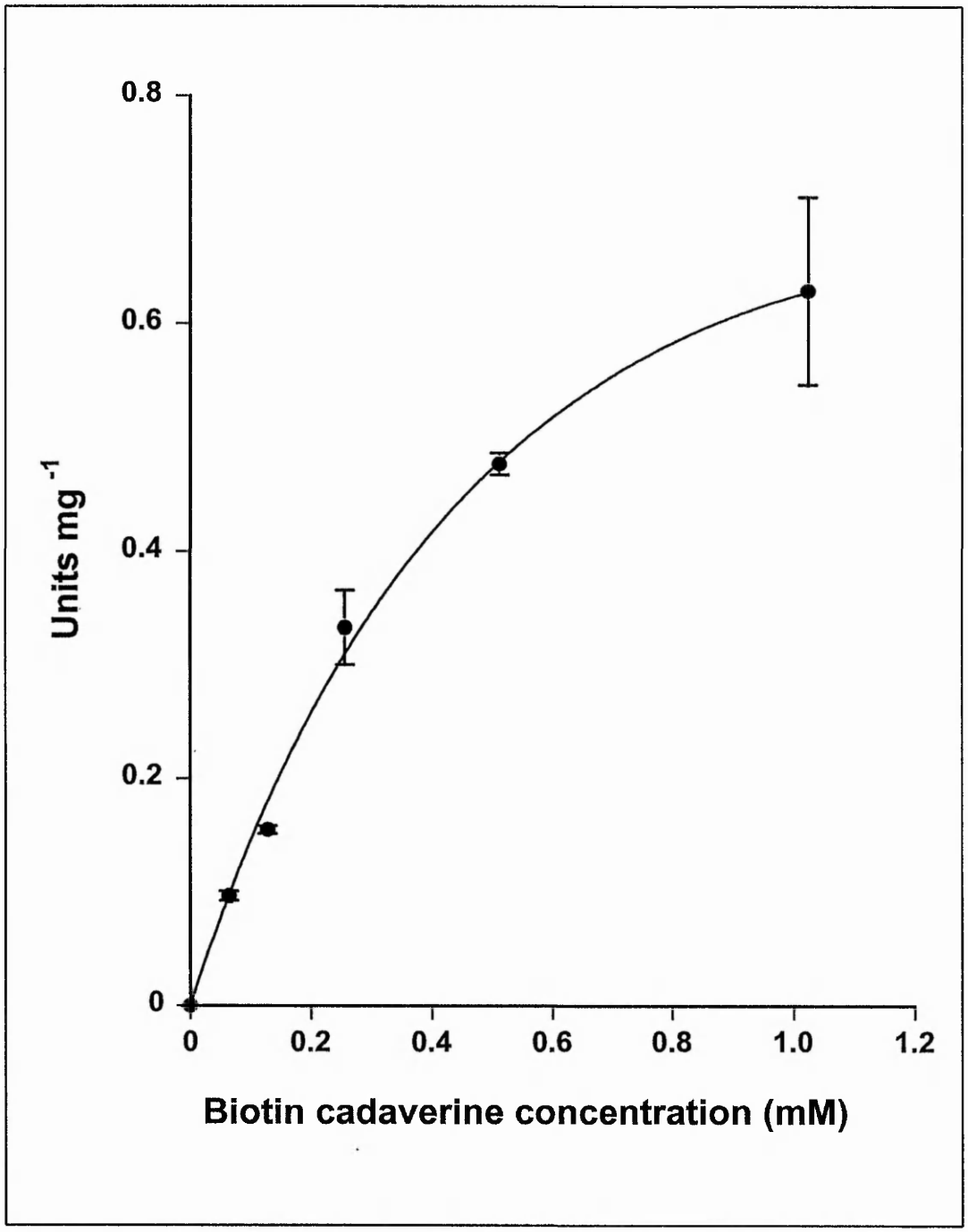


Figure 4.4 legend

Microsomal membrane associated *Pisum sativum* transglutaminase was solubilized from the membrane by treatment with 0.23% (w/v) sodium deoxycholate as described in section 2.2.3.1. The solubilized protein was assayed at 37°C for 60 minutes in the presence of various concentrations of biotin cadaverine as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control at each substrate concentration. Data points represent the mean ± SEM of 3 replicates.

Figure 4.4 demonstrates the concentration dependence of biotin cadaverine incorporation into N',N'-dimethylcasein by microsomal *Pisum sativum* leaf transglutaminase. The K_m for biotin cadaverine was calculated to be 618μM and V_{max} was estimated to be 1.01 units mg^{-1} using Enzfitter software (Biosoft, U.K).

The pH optima of the soluble and microsomal activities were investigated over the pH range 6.0 to 9.5 and a typical set of results are presented in figure 4.5.

Figure 4.5 - The variation in biotin cadaverine incorporation activity of soluble and microsomal *Pisum sativum* transglutaminase as a function of the reaction pH.

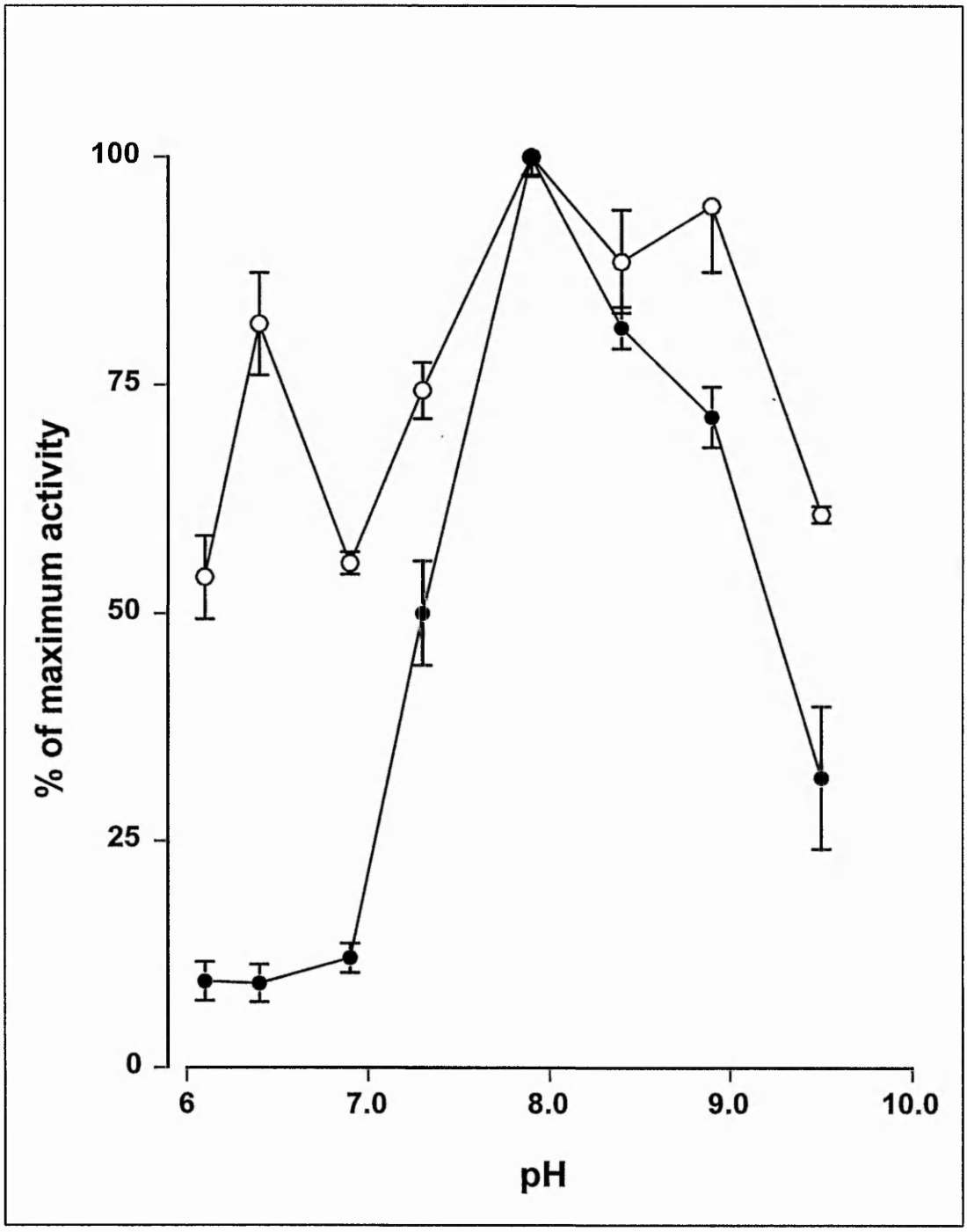


Figure 4.5 legend

Soluble *Pisum sativum* protein and sodium deoxycholate solubilized microsomal membrane protein (prepared as described in section 2.2.3.1) were assayed for 60 minutes at 37°C over a 6.0 to 9.5 pH range using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control at each pH and 0.23% (w/v) deoxycholate was present in all samples. Data points represent the mean ± SEM of 3 replicates.

Key

Soluble transglutaminase activity

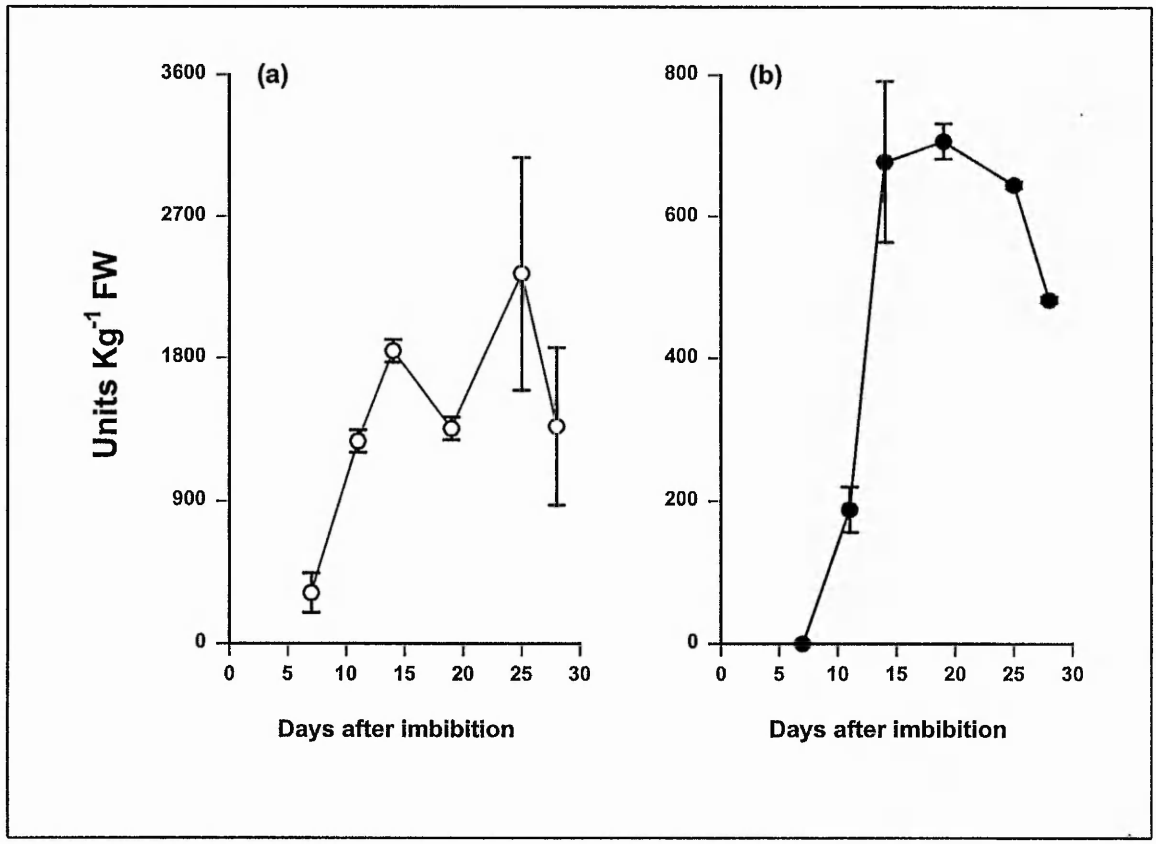
- Microsomal transglutaminase activity

Figure 4.5 shows the biotin cadaverine incorporation activities of the soluble and microsomal transglutaminase in *Pisum sativum* leaf tissue as a function of the reaction pH. The microsomal transglutaminase activity increased with increasing pH until an optimum was reached at pH 7.9. The activity of the soluble enzyme showed peaks at pH 7.9 and 8.9 and the activity remained greater than 50% of the maximum over the whole pH range tested.

4.2.2 The distribution of transglutaminase activity in developing *Pisum sativum* leaf tissue

The levels of transglutaminase activity in the soluble and microsomal fractions of leaf tissue from developing *Pisum sativum* seedlings was investigated in order to determine any change in the distribution of the soluble and microsomal activities with age of the plants. The results are presented in figure 4.6 and table 4.5.

Figure 4.6 - The variation in soluble and microsomal *Pisum sativum* leaf transglutaminase activity with plant age.



The soluble and washed microsomal membrane preparations from 7- to 29-day old *Pisum sativum* seedling leaf tissue extracts were assayed for transglutaminase activity using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each sample. Data points represent the mean ± SEM of 3 replicates.

Key

Soluble transglutaminase activity

- Microsomal transglutaminase activity

Figure 4.6 demonstrates that the soluble transglutaminase activity in *Pisum sativum* leaf tissue increased with the age of the seedlings and reached peaks at day 14 and 25, activity then fell to a lower level at day 28. Activity in the microsomal membrane preparations was not detectable in 7-day old seedlings; activity was found to increase during 7 to 14 day old plants and remained at this level until day 25. The overall activity was higher in the soluble fractions.

Table 4.5 - The variation in total transglutaminase activity with the age of *Pisum sativum* seedlings.

Seedling Age	Total soluble and microsomal transglutaminase activity Units kg^{-1} FW	% of the total activity associated with the microsomal membrane preparation
Day 7	19.97 ± 124.18	N.D
Day 11	1463.34 ± 98.79	21.8
Day 14	2522.11 ± 183.09	26.9
Day 19	2062.73 ± 56.09	34.3
Day 25	2981.36 ± 743.04	21.6
Day 29	1852.50 ± 500.42	26.0

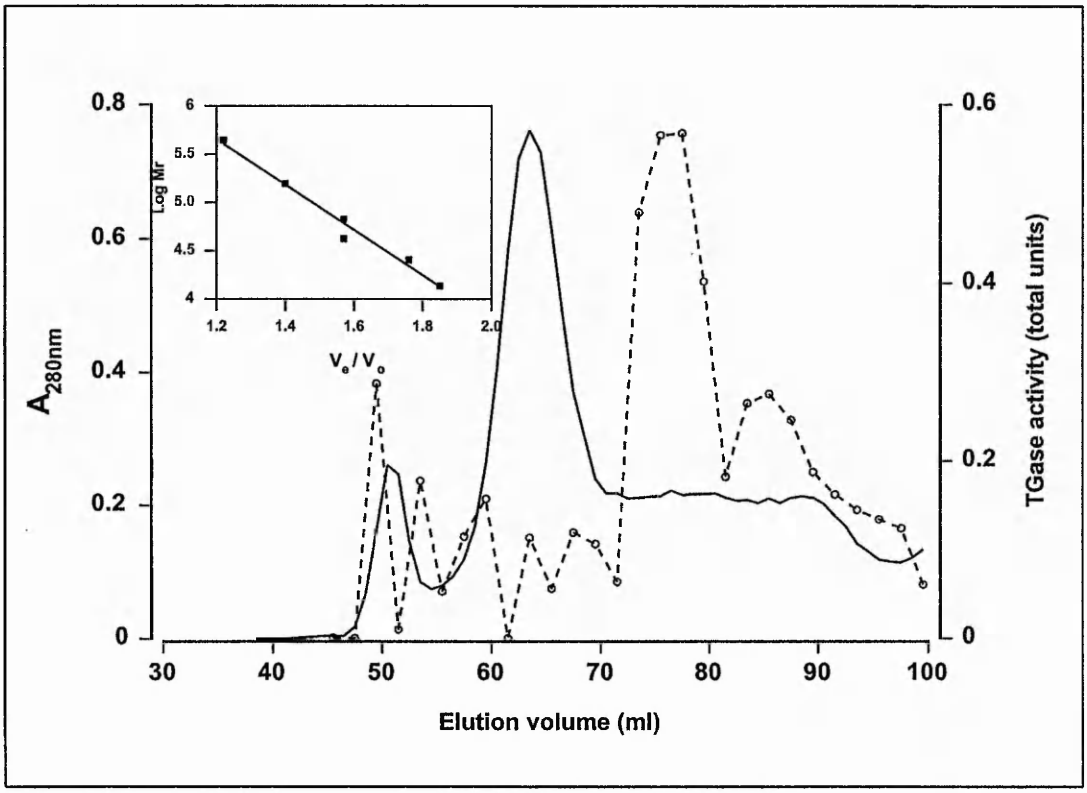
Transglutaminase activity was measured in the soluble and the washed microsomal membrane preparations after sodium deoxycholate extraction (section 2.2.3.1) using the biotin cadaverine incorporation assay (section 2.2.4.1). 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each sample. Values represent the mean ± SEM of 3 replicates. N.D no activity detectable.

Table 4.5 demonstrates that during the development of *Pisum sativum* seedlings the transglutaminase activity associated with the leaf microsomal membrane preparations remained at a level between 22% and 34%, except at day 7 when no activity was detectable in the microsomal membrane preparation.

4.2.3 Gel filtration chromatography of soluble and microsomal *Pisum sativum* transglutaminase activity

To further compare the soluble and microsomal transglutaminase activities in *Pisum sativum* leaf extracts, the relative molecular masses of the enzymes were estimated by gel filtration. Protein samples were applied to a column of Sephacryl S300 HR and fractions collected from the column were assayed for transglutaminase activity. Typical elution profiles are presented in figures 4.7 to 4.9.

Figure 4.7 - Gel filtration chromatography of soluble *Pisum sativum* leaf transglutaminase.



One point five millilitres of soluble *Pisum sativum* leaf protein were applied to a Sephacryl S300 HR column (1.6 x 50 cm) and eluted at 1.0 ml min^{-1} with 20mM Tris-HCl pH 8.5 containing 150mM NaCl and 1mM 2-ME, as described in section 2.2.6.5. The absorbance at 280nm was measured in 1.0 ml fractions using a Beckman DU 70 spectrophotometer. Transglutaminase activity was measured using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each fraction. The relative molecular mass of the active enzyme was estimated using the calibration graph (inset) prepared with standard proteins as described in section 2.2.6.5.

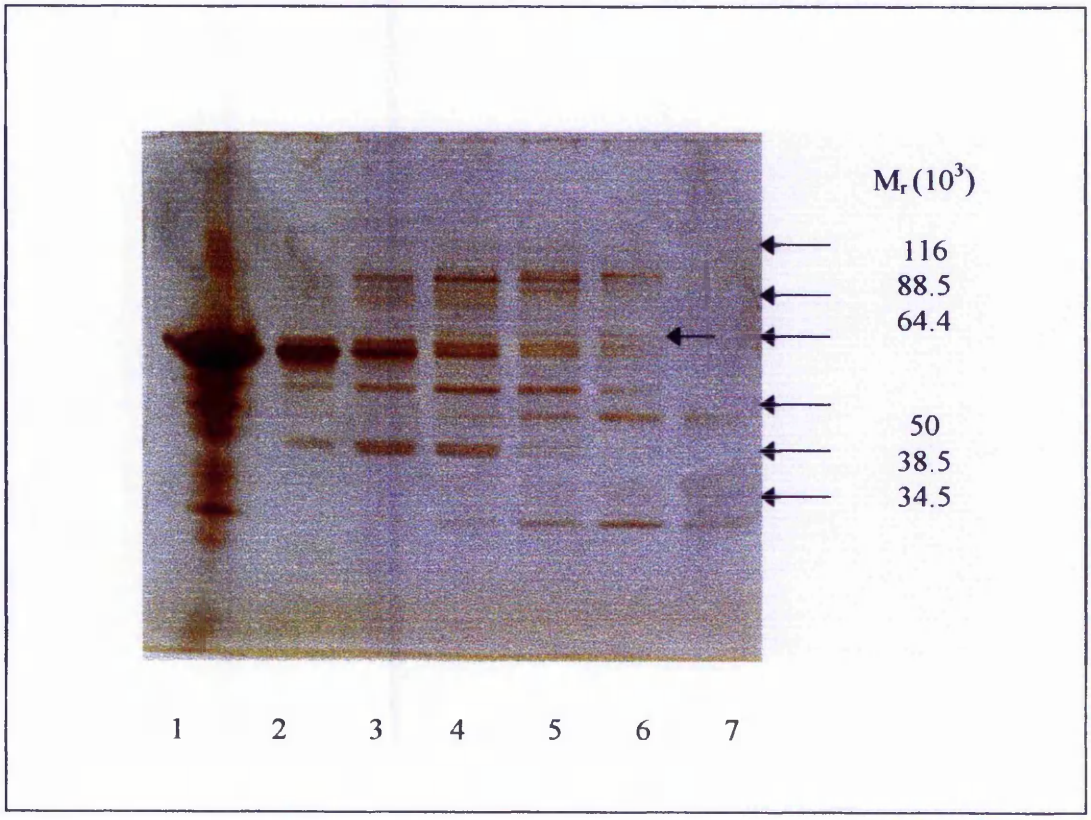
Key

—— Absorbance at 280nm

---- Biotin cadaverine incorporation activity

Figure 4.7 demonstrates that soluble *Pisum sativum* leaf transglutaminase eluted at a volume of 77.5 ml. The relative molecular mass of the active peak was estimated to be 69 000 using the calibration graph (figure 4.7 inset). Fractions across the peak of transglutaminase activity (71.5 – 81.5ml) were separated by SDS-PAGE and a silver stained gel is presented in plate 4.1.

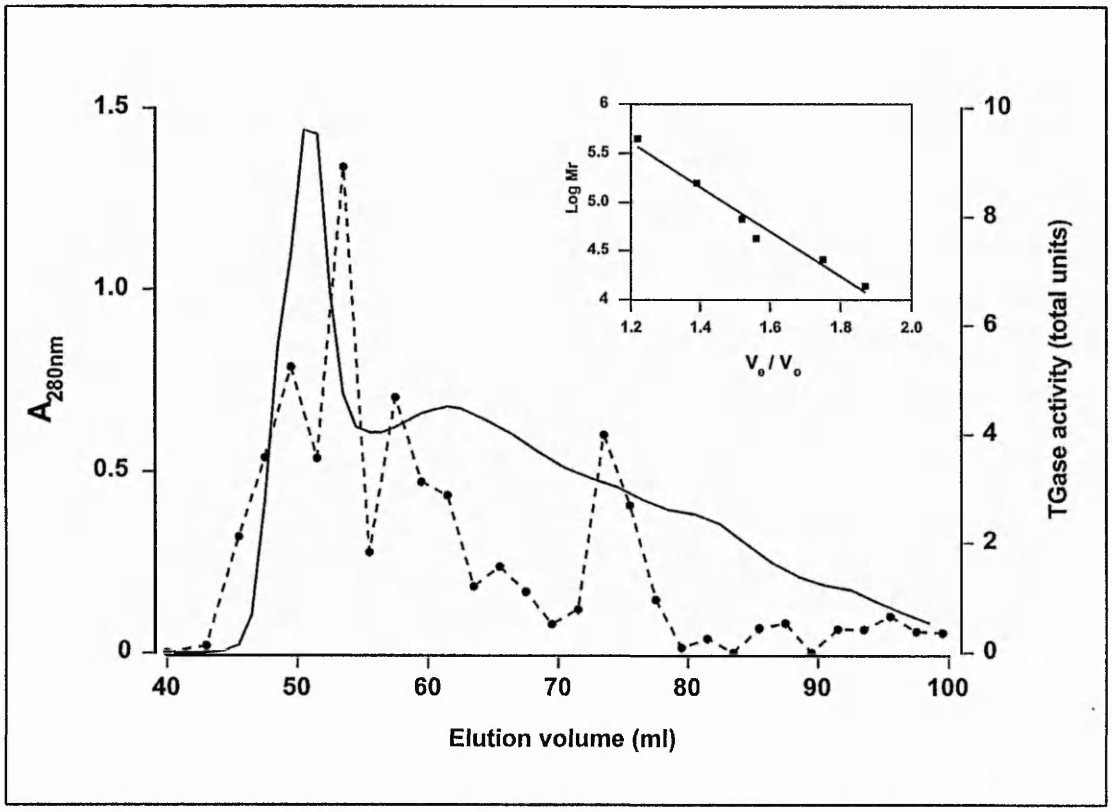
Plate 4.1 - The analysis of proteins after gel filtration chromatography of soluble *Pisum sativum* leaf transglutaminase by SDS-PAGE with silver staining.



One and a half millilitres of soluble *Pisum sativum* leaf protein were separated on a Sephacryl S300 HR column as described for figure 4.7. Thirty microlitres of protein in gel filtration fractions eluted between 71.5 and 81.5 ml were separated under denaturing conditions in a 10% (w/v) SDS polyacrylamide gel and silver stained as described in sections 2.2.8.1 and 2.2.8.3. *Lane 1*, crude soluble protein; *lane 2*, protein eluted at 71.5ml; *lane 3*, protein eluted at 73.5ml; *lane 4*, 75.5ml; *lane 5*, 77.5ml; *lane 6*, 79.5ml and *lane 7*, protein eluted at 81.5ml.

Plate 4.1 demonstrates that there was an increase in band density corresponding to a polypeptide with a relative molecular mass of 65 000 (indicated with an arrow), which increases and decreases in intensity across the peak of transglutaminase activity shown in figure 4.7 and has a relative molecular mass similar to that estimated for *Pisum sativum* leaf soluble transglutaminase activity by gel filtration chromatography (figure 4.7).

Figure 4.8 - Gel filtration chromatography of detergent solubilized microsomal *Pisum sativum* transglutaminase.



One point five millilitres of sodium deoxycholate solubilized *Pisum sativum* leaf microsomal membrane protein (prepared as described in section 2.2.3.1) were applied to a Sephacryl S300 HR column (1.6 x 52 cm). Protein was eluted at 1.0 ml min^{-1} with 20mM Tris-HCl pH 8.5 containing 150mM NaCl, 0.23% (w/v) sodium deoxycholate and 1mM 2-ME, as described in section 2.2.6.5. The absorbance at 280nm was measured in 1.0 ml fractions using a Beckman DU 70 spectrophotometer. Transglutaminase activity in the eluted fractions was measured using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each fraction. The relative molecular mass of the active enzyme was estimated using the calibration graph (figure 4.8 inset) prepared with standard proteins as described in section 2.2.6.5.

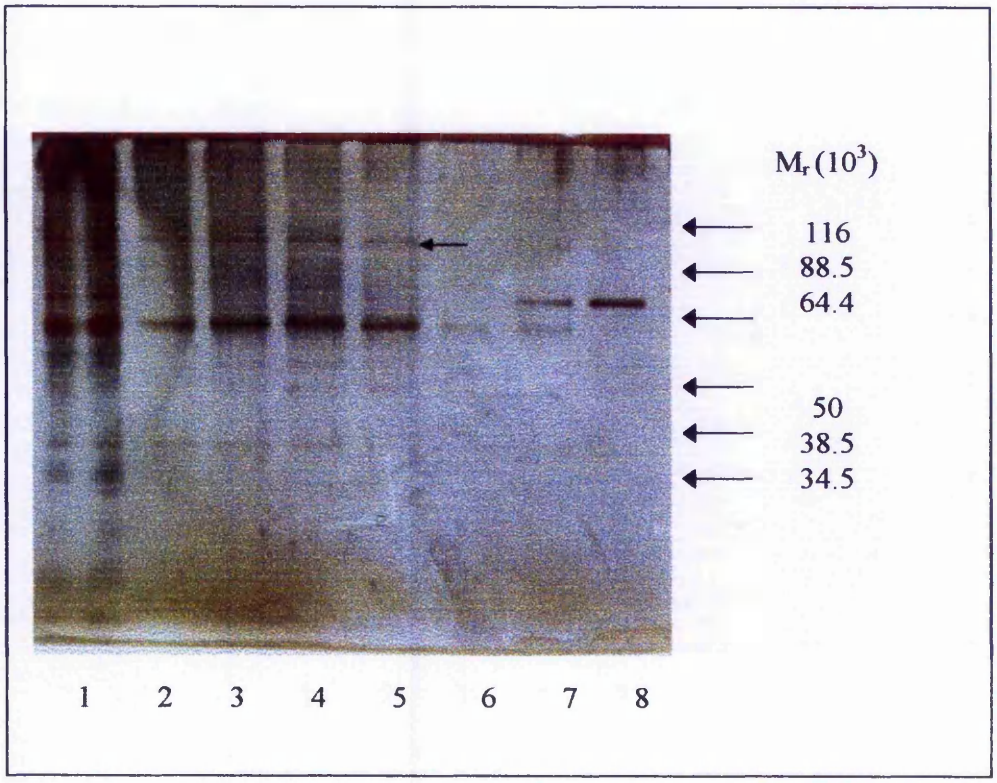
Key

—— Absorbance at 280nm

•----• Biotin cadaverine incorporation activity

Figure 4.8 demonstrates that a large proportion of the sodium deoxycholate solubilized *Pisum sativum* microsomal membrane transglutaminase eluted at a volume of 50 to 54.0 ml, a smaller peak of activity was detected at an elution volume of 73.5 ml. The relative molecular mass of the smaller active peak was estimated to be 91 000 using the calibration graph (figure 4.8 inset). The larger peak of activity eluted with the column void volume. Fractions across the smaller peak of transglutaminase activity (69.5 – 81.5ml) were separated by SDS-PAGE and a silver stained gel is presented in plate 4.2.

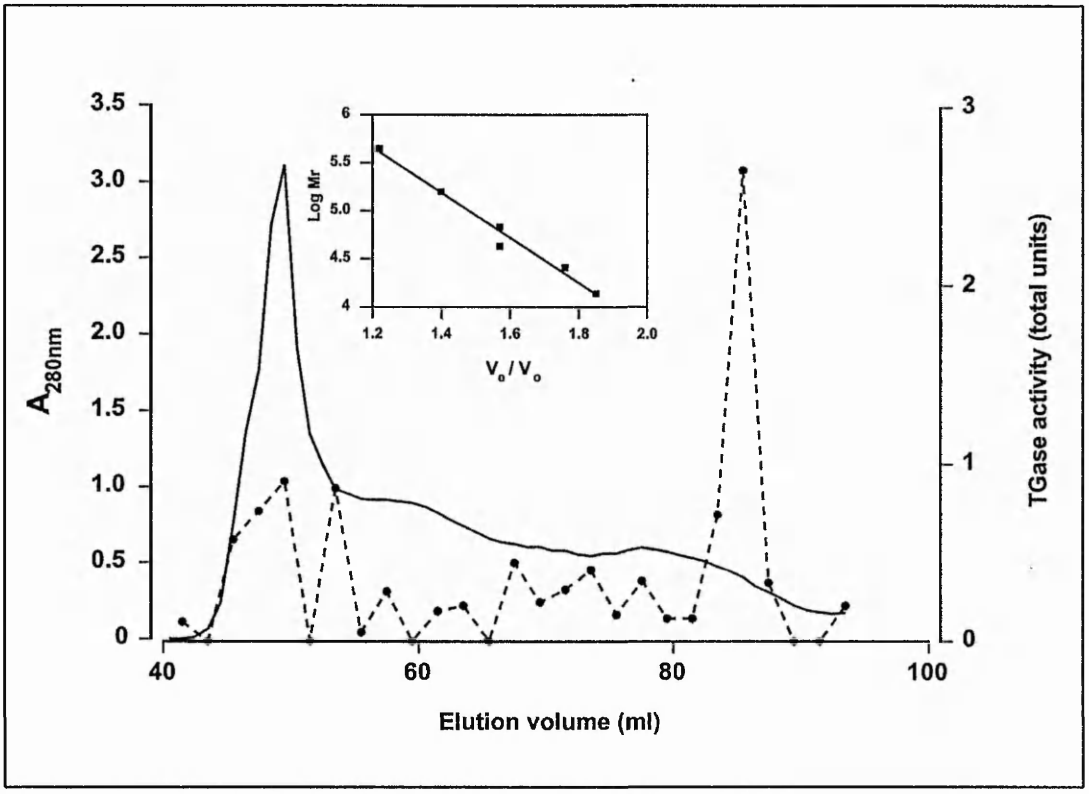
Plate 4.2 - The analysis of proteins after gel filtration chromatography of sodium deoxycholate solubilized *Pisum sativum* microsomal membrane transglutaminase by SDS-PAGE with silver staining.



One and a half millilitres of sodium deoxycholate solubilized *Pisum sativum* microsomal protein were separated on a Sephacryl S300 HR column as described for figure 4.8. Thirty microlitres of protein in gel filtration fractions eluted between 69.5 and 81.5 ml were separated under denaturing conditions in a 10% (w/v) SDS polyacrylamide gel and silver stained as described in sections 2.2.8.1 and 2.2.8.3. *Lane 1*, crude solubilized microsomal protein; *lane 2*, protein eluted at 69.5ml; *lane 3*, protein eluted at 71.5ml; *lane 4*, 73.5ml; *lane 5*, 75.5ml; *lane 6*, 77.5ml; *lane 7*, protein eluted at 79.5ml and *lane 8*, protein eluted at 81.5ml.

Plate 4.2 demonstrates that there is a protein band present with a relative molecular mass of 90 000 (indicated with an arrow), which has a relative molecular mass similar to the *Pisum sativum* deoxycholate solubilized microsomal membrane transglutaminase activity estimated by gel filtration (figure 4.8).

Figure 4.9 - Gel filtration chromatography of trypsin solubilized microsomal *Pisum sativum* transglutaminase.



One and a half millilitres of trypsin solubilized microsomal membrane protein (prepared as described in section 2.2.3.2) were applied to a Sephacryl S300 HR column (1.6 x 52 cm) and eluted at 1.0 ml min^{-1} with 20mM Tris-HCl pH 8.5 containing 150mM NaCl and 1mM 2-ME, as described in section 2.2.6.5. The absorbance at 280nm was measured in 1.0 ml fractions using a Beckman DU 70 spectrophotometer. Transglutaminase activity in the eluted fractions was measured using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each fraction. The relative molecular mass of the active enzyme was estimated using the calibration graph (figure 4.9 inset) prepared with standard proteins as described in section 2.2.6.5.

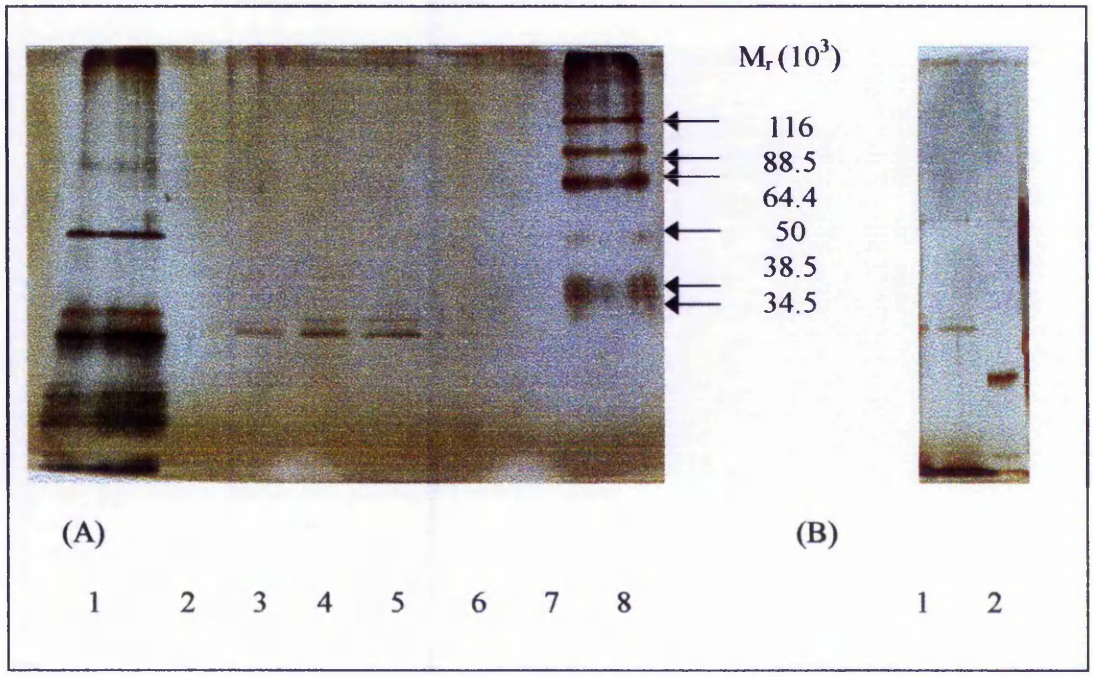
Key

——— Absorbance at 280nm

•-----• Biotin cadaverine incorporation activity

Figure 4.9 demonstrates that trypsin solubilized microsomal membrane *Pisum sativum* transglutaminase eluted at a volume of 85.5 ml. The relative molecular mass of the active peak was estimated to be 29 000 using the calibration graph (figure 4.9 inset). Fractions across the peak of transglutaminase activity (79.5 – 89.5ml) were separated by SDS-PAGE and a silver stained gel is presented in plate 4.3.

Plate 4.3 - The analysis of proteins after gel filtration chromatography of trypsin solubilized *Pisum sativum* microsomal membrane transglutaminase by SDS-PAGE with silver staining.

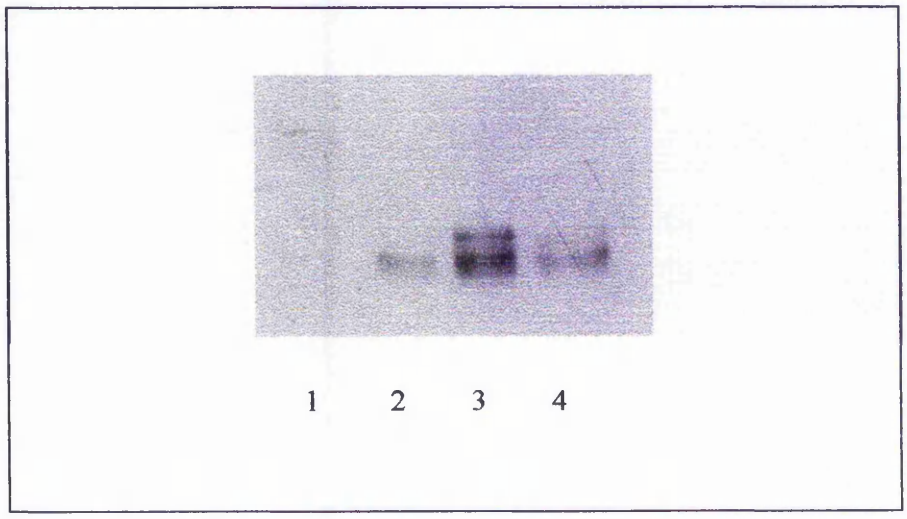


One point five millilitres of trypsin solubilized *Pisum sativum* microsomal protein were separated on a Sephacryl S300 HR column as described for figure 4.9. Thirty microlitres of protein in gel filtration fractions eluted between 79.5 and 89.5 ml were separated under denaturing conditions in a 10% (w/v) SDS polyacrylamide gel and silver stained as described in sections 2.2.8.1 and 2.2.8.3. (A) *Lane 1*, crude trypsin solubilized microsomal protein; *lane 2*, protein eluted at 79.5ml; *lane 3*, protein eluted at 81.5ml; *lane 4*, 83.5ml; *lane 5*, 85.5ml; *lane 6*, 87.5ml; *lane 7*, protein eluted at 89.5ml and *lane 8*, molecular mass markers (B) *Lane 1*, GPC purified trypsin solubilized microsomal transglutaminase; *lane 2*, trypsin.

Plate 4.3 demonstrates that SDS-PAGE analysis of proteins across the peak of transglutaminase activity revealed two major protein bands having relative molecular masses of 31 600 and 29 500.

The fractions containing the peak of transglutaminase activity after gel filtration (see figures 4.7 – 4.9) were pooled. To test for antigenic similarity between *Pisum sativum* leaf transglutaminases and mammalian tissue transglutaminase, western blots of SDS-PAGE separated proteins were probed using a monoclonal antibody against guinea pig liver tissue transglutaminase (plate 4.4).

Plate 4.4 - The analysis of proteins after gel filtration chromatography of soluble and microsomal *Pisum sativum* leaf transglutaminases by SDS-PAGE and immunoblotting with anti-tissue transglutaminase antibody.



Ten micrograms of gel filtration purified soluble and detergent solubilized protein and two micrograms of trypsin solubilized *Pisum sativum* leaf protein were separated under denaturing conditions in a 10% (w/v) polyacrylamide gel, electroblotted to nitro-cellulose and probed with anti-tissue transglutaminase antibody (CUB 7402) as described in section 2.2.8. *Lane 1*, purified guinea pig liver transglutaminase; *lane 2*, gel filtration purified soluble *Pisum sativum* leaf transglutaminase; *lane 3*, gel filtration purified detergent solubilized microsomal *Pisum sativum* leaf transglutaminase; *lane 4*, gel filtration purified trypsin solubilized microsomal *P. sativum* leaf transglutaminase.

Plate 4.4 shows that the anti-tissue transglutaminase monoclonal antibody (CUB 7402) cross reacted with proteins present in the soluble and the microsomal membrane fractions after gel filtration chromatography of *Pisum sativum* leaf extracts. The soluble fraction contained one band with a relative molecular mass of 60 000. The antibody cross reacted with two bands with relative molecular masses of 64 000 and 60 000 in the detergent solubilized microsomal fraction. The gel filtration purified trypsin solubilized fraction contained a protein with a relative molecular mass of 60 000 which cross reacted with the anti-tissue transglutaminase antibody.

The 31 000 and 60 000 M_r proteins released during trypsin digestion of the microsomal fraction and resolved by SDS-polyacrylamide gel electrophoresis were subjected to N-terminal sequence determination, after blotting on to polyvinylidene diflouride (PVDF) membranes, by automated Edman degradation. Unfortunately not enough protein was present to obtain any sequence data.

Mammalian transglutaminases are calcium activated enzymes (Aeschlimann and Paulsson, 1994), therefore the calcium ion concentration required to activate the gel filtration purified biotin cadaverine incorporation activities of *Pisum sativum* soluble and microsomal preparations were established. Typical calcium activation curves are presented in figures 4.10 to 4.12.

Figure 4.10 - The biotin cadaverine incorporation activity of gel filtration purified soluble *Pisum sativum* leaf transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

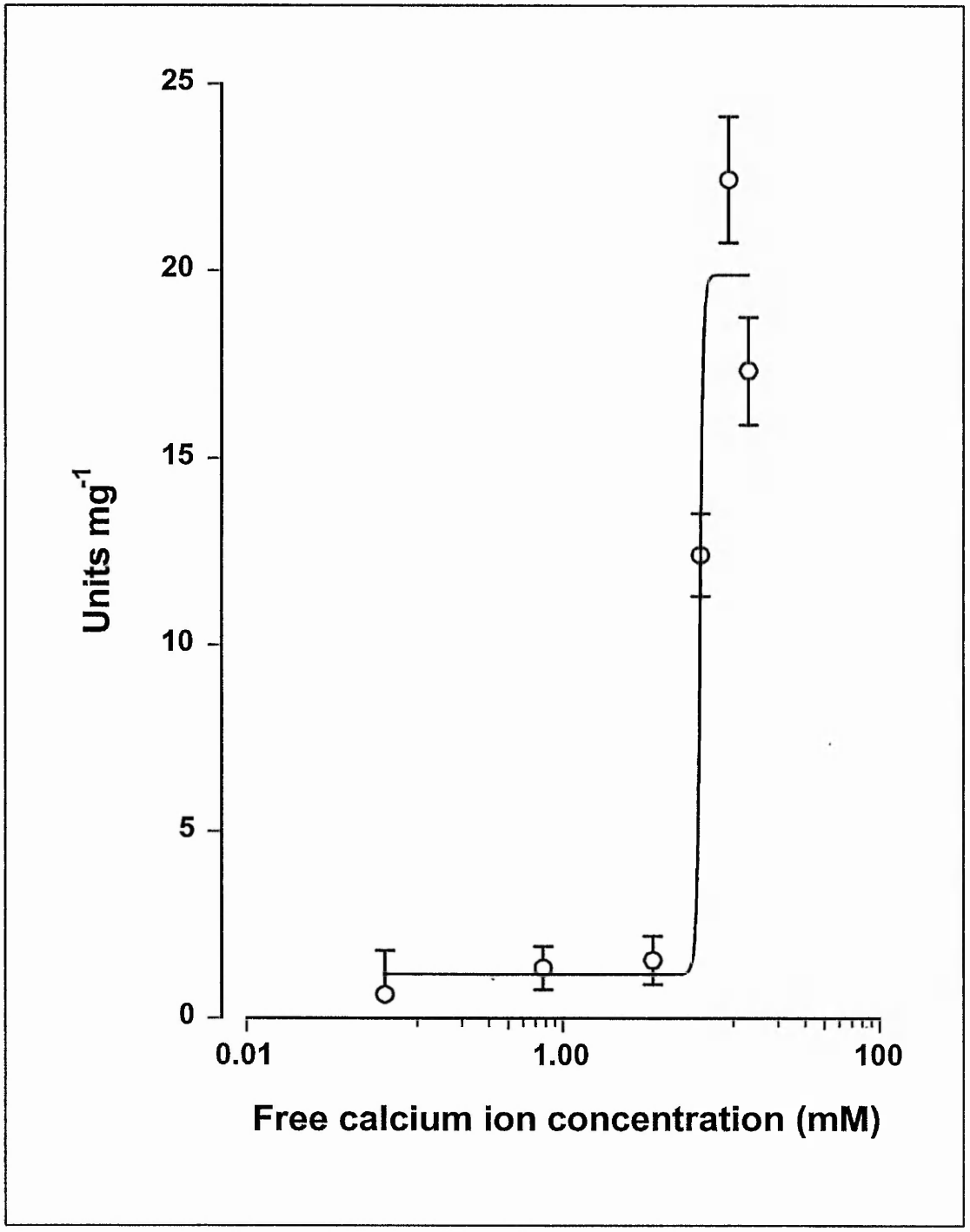


Figure 4.10 legend

Pisum sativum soluble leaf extract was separated by gel filtration chromatography as described for figure 4.7. The active peak of transglutaminase activity was pooled and incubated at 37°C for 60 minutes in the presence of various free calcium ion concentrations as described in section 2.2.4.1. Zero free calcium was achieved by the addition of 1mM EGTA. Increasing concentrations of calcium chloride were added to the reaction buffer and the equivalent free calcium ion concentration was calculated using a computer program (EqCal, Biosoft, UK) and this data can be found in the appendix (page 233). The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Data points represent the mean ± SEM of 3 replicates.

Figure 4.10 shows that very little biotin cadaverine incorporation activity was detectable between 0.1mM and 2mM free calcium. Activation of the biotin cadaverine incorporating activity of gel filtration purified *Pisum sativum* soluble leaf transglutaminase occurred after 4mM free calcium, a maximum activity was observed at 11mM free calcium.

Figure 4.11 - The biotin cadaverine incorporation activity of gel filtration purified sodium deoxycholate solubilized *Pisum sativum* leaf transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

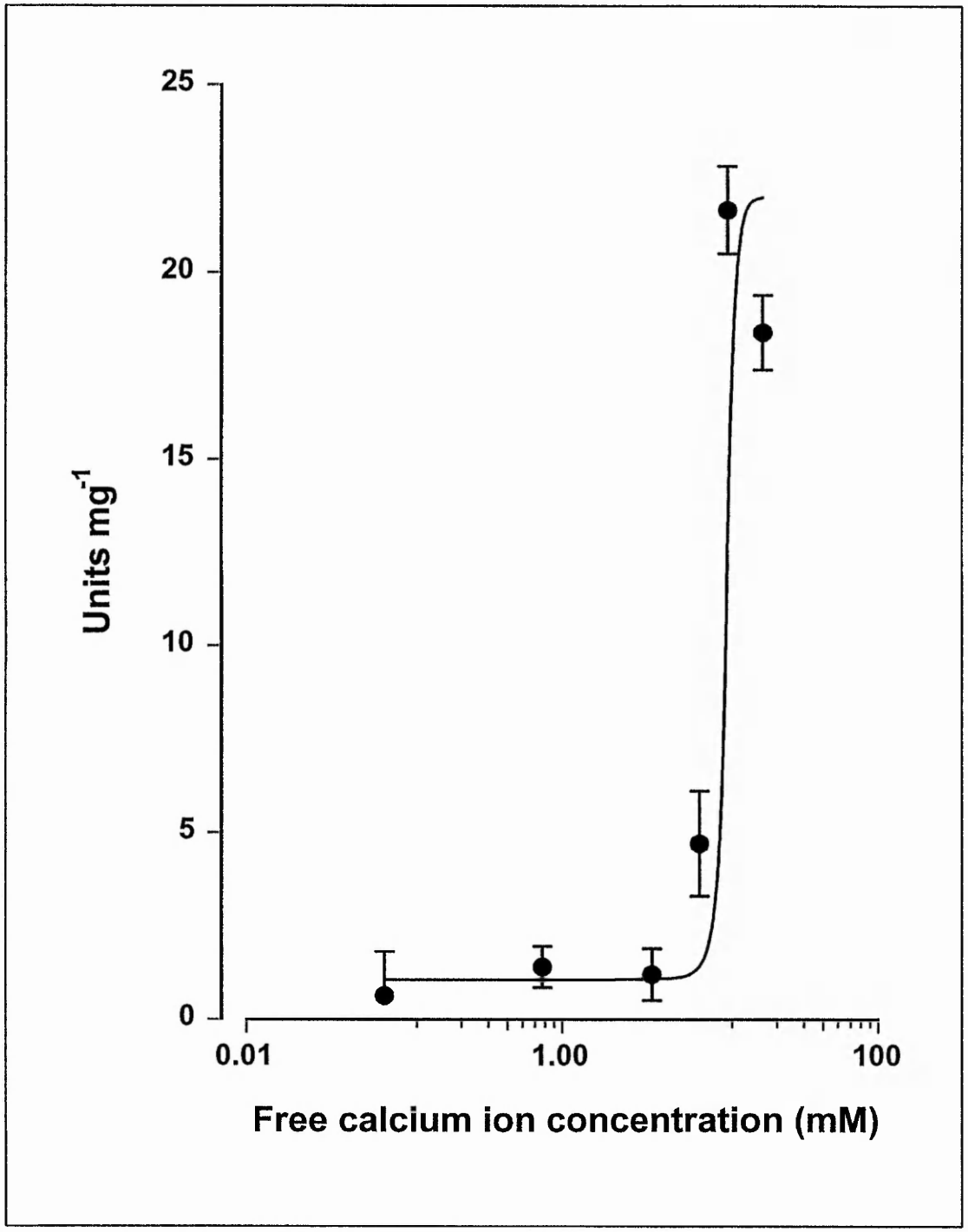


Figure 4.11 legend

Pisum sativum detergent solubilized microsomal leaf protein was separated by gel filtration chromatography as described for figure 4.8. The active peak of transglutaminase activity was incubated at 37°C for 60 minutes in the presence of various free calcium ion concentrations as described in section 2.2.4.1. Free calcium ion concentration in the buffer was calculated as described for figure 4.10. The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Data points represent the mean ± SEM of 3 replicates.

Figure 4.11 shows that little biotin cadaverine incorporation activity occurred between 0.1mM and 4mM free calcium. Activation of the biotin cadaverine incorporating activity of deoxycholate solubilized, gel filtration purified *Pisum sativum* leaf microsomal membrane transglutaminase occurred above 5mM free calcium, a maximum activity was observed at 15mM free calcium.

Figure 4.12 - The biotin cadaverine incorporation activity of gel filtration purified trypsin solubilized microsomal *Pisum sativum* leaf transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

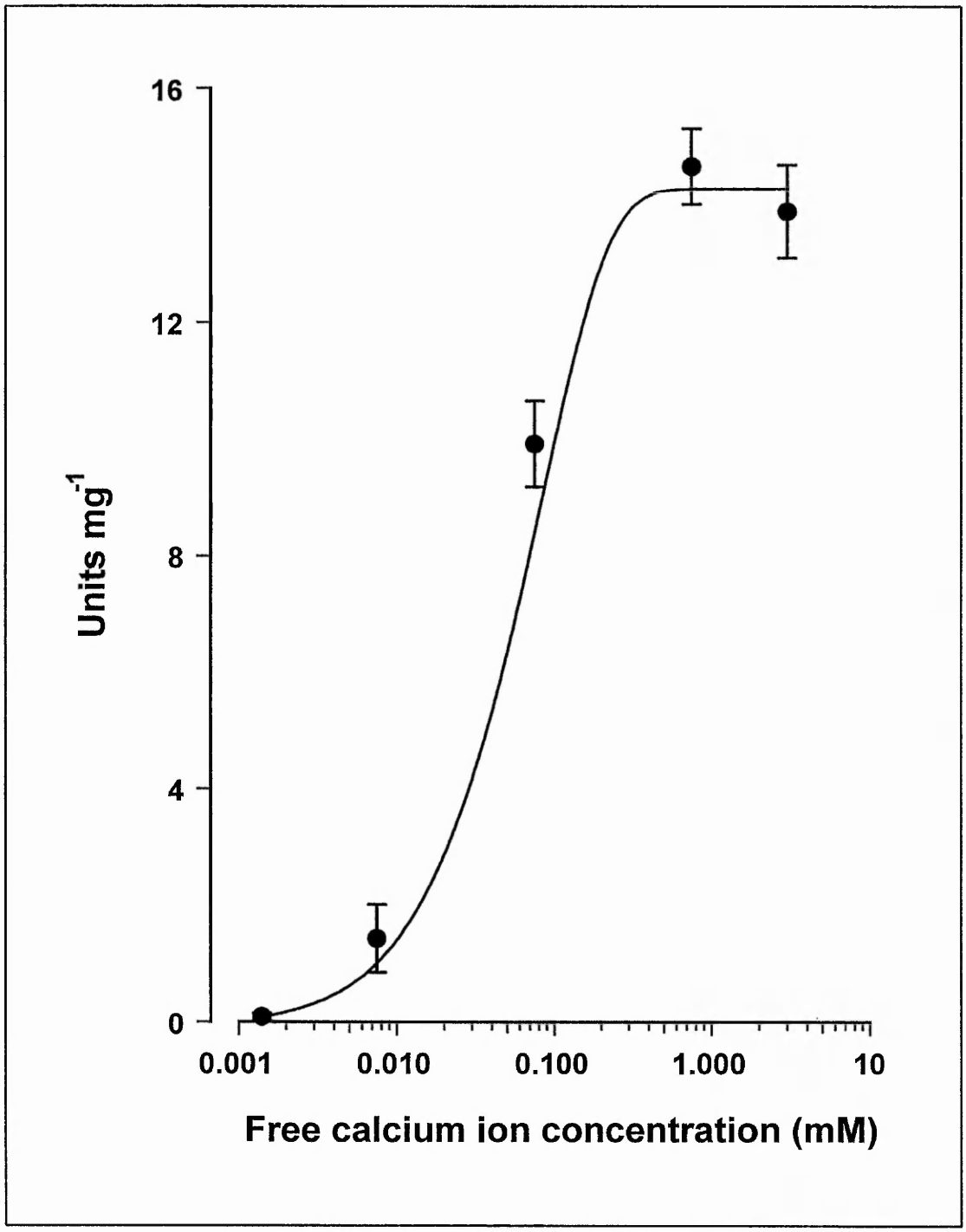


Figure 4.12 legend

Pisum sativum trypsin solubilized microsomal leaf protein was separated by gel filtration chromatography as described for figure 4.9. The active peak of transglutaminase activity was incubated at 37°C for 60 minutes in the presence of various free calcium ion concentrations as described in section 2.2.4.1. The free calcium ion concentration of the buffer was calculated as described for figure 4.10. The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Data points represent the mean ± SEM of 3 replicates.

Figure 4.12 shows that no biotin cadaverine incorporation activity occurred below 1μM free calcium. Activation of the biotin cadaverine incorporating activity of trypsin solubilized, gel filtration purified *Pisum sativum* leaf microsomal transglutaminase occurred after 5μM free calcium, a maximum activity was observed at 750μM free calcium.

It is known that mammalian type II transglutaminase activity is inhibited by GTP at sub-optimal calcium ion concentrations (Achyuthan and Greenberg, 1987; Bergamini, 1988; Smethurst and Griffin, 1996). The effect of GTP on the soluble and microsomal transglutaminase activities of *Pisum sativum* were therefore investigated using the soluble and microsomal extracts after gel filtration chromatography. The results are presented in tables 4.6 to 4.8.

Table 4.6 - The effect of 1mM GTP on the biotin cadaverine incorporation activity of gel filtration purified soluble *Pisum sativum* leaf transglutaminase.

Free calcium ion concentration mM	Specific activity Units mg^{-1} uninhibited	Specific activity Units mg^{-1} + 1mM GTP
0.75	0.97 ± 1.11	0.31 ± 0.53 (68.3)
3.75	2.59 ± 0.88	1.25 ± 0.59 (51.9)
5.6	6.23 ± 0.64	4.09 ± 0.84 (34.4)
7.5	12.61 ± 0.70	6.67 ± 0.73 (47.1)
11.25	21.73 ± 0.92	11.84 ± 0.88 (45.5)

Gel filtration purified soluble *Pisum sativum* transglutaminase was assayed for 60 minutes at 37°C in the presence and absense of 1mM GTP and varying free calcium ion concentrations (calculated as described for figure 4.10) using the biotin cadaverine incorporation assay (section 2.2.4.1). The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Values represent the mean ± SEM of 3 replicates. The values in brackets given after the specific activity represent the percentage inhibition of the biotin cadaverine incorporation activity due to the presence of 1mM GTP.

Table 4.7 - The effect of 1mM GTP on the biotin cadaverine incorporation activity of gel filtration purified deoxycholate solubilized microsomal *Pisum sativum* leaf transglutaminase.

Free calcium ion concentration mM	Specific activity Units mg^{-1} uninhibited	Specific activity Units mg^{-1} + 1mM GTP
0.75	1.40 ± 0.55	0.57 ± 0.32 (59.4)
3.75	1.20 ± 0.69	0.93 ± 0.35 (22.5)
5.6	2.73 ± 0.54	1.87 ± 0.51 (31.5)
7.5	4.71 ± 1.41	4.04 ± 0.73 (14.2)
11.25	21.67 ± 1.16	18.68 ± 1.23 (13.8)

Gel filtration purified deoxycholate solubilized microsomal *Pisum sativum* transglutaminase was assayed for 60 minutes at 37°C in the presence and absence of 1mM GTP at varying free calcium ion concentrations (calculated as described for figure 4.10) using the biotin cadaverine incorporation assay (section 2.2.4.1). Values represent the mean ± SEM of 3 replicates. The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. The values in brackets given after the specific activity represent the percentage inhibition of the biotin cadaverine incorporation activity due to the presence of 1mM GTP.

Table 4.8 - The effect of 1mM GTP on the biotin cadaverine incorporation activity of gel filtration purified trypsin solubilized microsomal *Pisum sativum* leaf transglutaminase.

Free calcium ion concentration μM	Specific activity units mg^{-1} uninhibited	Specific activity units mg^{-1} + 1mM GTP
1.4	0.09 ± 0.06	N.D (100)
7.5	1.42 ± 0.58	N.D (100)
75	9.92 ± 0.73	0.98 ± 1.01 (90.1)
750	14.67 ± 0.65	3.02 ± 0.68 (79.4)
3000	13.90 ± 0.80	8.88 ± 0.94 (36.1)

Gel filtration purified trysin solubilized microsomal *Pisum sativum* transglutaminase was assayed for 60 minutes at 37°C in the presence and absence of 1mM GTP at varying free calcium ion concentrations (calculated as described for figure 4.10) using the biotin cadaverine incorporation assay (section 2.2.4.1). The assay was carried out in the presence of 0.23% (w/v) sodium deoxycholate. Values represent the mean ± SEM of 3 replicates. N.D. no activity detectable. Values in brackets given after the specific activity represent the percentage inhibition of the biotin cadaverine incorporation activity due to the presence of 1mM GTP.

Table 4.6 shows that gel filtration purified soluble *Pisum sativum* leaf transglutainase was inhibited 68% by 750μM free calcium in the presence of 1mM GTP. Similarly, gel filtration purified detergent solubilized *Pisum sativum* leaf microsomal transglutaminase was inhibited 59% by 750μM free calcium in the presence of 1mM GTP (table 4.7), this percentage inhibition was reduced when the free calcium ion concentration was increased. Table 4.8 shows that gel filtration purified trypsin solubilized *Pisum sativum* leaf microsomal transglutaminase was completely inhibited by 1mM GTP at free calcium ion concentrations of 1.4μM and 7.5μM.

4.3 Discussion

4.3.1 Characterisation of *Pisum sativum* leaf soluble and microsomal transglutaminase activities

In resting plant cells the cytosolic free calcium ion concentration is maintained at approximately 100 to 500nM through the activity of Ca^{2+}-ATPases and Ca^{2+} / H^{+} antiporters in cell membranes (for review see Bush, 1995; Sanders *et al.*, 1999; White, 2000). However, the calcium ion content of the cytosol is far higher than this due to the high affinity of a range of binding proteins for Ca^{2+} (Sanders *et al.*, 1999), estimates from neuronal cells suggest that only 0.1 to 1% of a calcium load remains free, with the rest being absorbed (Gorman and Thomas, 1980). Calcium levels in the plant vacuole and extracellular matrix have been detected in the millimolar range (Bush, 1985). Activation of the biotin cadaverine incorporation activity of soluble *Pisum sativum* leaf transglutaminase was detected at 1mM free calcium (figure 4.1). Maximum activity was observed at 15mM free calcium suggesting the enzyme would not be active under normal physiological conditions. Figure 4.2 demonstrates that the biotin cadaverine incorporation activity of *Pisum sativum* microsomal transglutaminase was activated at 4mM free calcium with maximum activity occurring at 12mM free calcium. If the microsomal transglutaminase is orientated in the membrane such that the bulk of the protein is present in the cytosol the enzyme would not be active under normal physiological conditions, however, if the bulk of the protein is orientated inside the vacuole, calcium concentrations would be high enough to activate the enzyme. The measurement of calcium activation in crude plant extracts could be misleading due to the presence of other proteins or cell constituents that are removing calcium from the assay buffer, this could include sodium deoxycholate which is an anionic detergent and may have been soaking up calcium ions. Despite the unphysiological concentrations of calcium required for transglutaminase activation, the data presented in figures 4.1, 4.2, 4.10 and 4.11 do show that the transglutaminase activity is calcium activated.

Due to the vacuole occupying a large proportion of the plant cell, spatial gradients in cytosolic Ca^{2+} are likely to occur through localised release of Ca^{2+} from the vacuole (Bush, 1995). The tonoplast-associated transglutaminase could be inserted into the membrane close to calcium channels and these local areas of high Ca^{2+} close to the tonoplast could activate the transglutaminase if the bulk of the enzyme is present in the cytosol.

Lilley (1998) reported that activation of the biotin cadaverine incorporation activity of *Pisum sativum* root transglutaminase occurred after 20nM free Ca^{2+}, maximum activity was observed at 94nM free calcium suggesting the enzyme is able to incorporate primary amines in to proteins at resting levels of cytosolic calcium. The *Pisum sativum* root transglutaminase was extracted from 14-day old tissue in the absence of protease inhibitors and therefore the low calcium ion concentration required for enzyme activation could be due to essential proteolytic activation of the plant enzyme. Grandi *et al.* (1992) and Del Duca *et al.* (2000b) reported a higher polyamine incorporation activity in *Helianthus tuberosus* tuber and leaf tissue extracts prepared in the absence of protease inhibitors. These authors suggested that proteolytic processing of the enzyme could activate transglutaminase activity in these tissues. The activation of transglutaminases by proteolysis has also been reported necessary in mammalian cells (Kim *et al.*, 1990) and micro organisms (Pasternack *et al.*, 1998).

Mammalian transglutaminases have a cysteine residue at their active site and are therefore sensitive to thiol reactive reagents such as iodoacetamide and N-ethylmaleimide (Folk and Cole, 1966). Table 4.1 shows that soluble *Pisum sativum* leaf transglutaminase was inhibited 67% and 45% in the presence of 10mM iodoacetamide and NEM respectively. Similarly, microsomal *Pisum sativum* transglutaminase was inhibited 80% and 63% by 10mM iodoacetamide and NEM respectively. In both cases, iodoacetamide appeared to be a better inhibitor of the transglutaminase activities. The inhibition produced by the thiol reagents would suggest these transglutaminases have a cysteine residue essential for enzyme activity. Data on the thiol dependence of other plant transglutaminases is variable depending on the plant tissue used and the method used for measurement of transglutaminase activity. Previous reports have described the inhibition of plant transglutaminase-like activities by the thiol stabiliser DTT (Icekson and Apelbaum, 1987; Siepaio and Meunier, 1995; Chiarello *et al.*, 1996a). Kang and Cho (1996) however, reported a 60% inhibition of the [^{3}H] putrescine incorporation activity of purified *Glycine max* leaf transglutaminase activity in the presence of 0.1mM NEM. The data generated could be misleading due to the interference of diamine oxidase activity with the assays used to measure these plant transglutaminase activities (Lilley *et al.*, 1998a).

To confirm the biotin cadaverine incorporation activity present in *Pisum sativum* leaf tissue was due to transglutaminase activity and not diamine oxidase activity, inhibitors of diamine oxidase activity were screened. Tables 4.3 demonstrates that soluble *Pisum sativum* biotin cadaverine incorporation activity was inhibited 27% and 40% by 10mM DIECA and o-phenenthroline respectively, the microsomal activity was not inhibited by DIECA but was inhibited 37% by 10mM o-phenenthroline. This data strongly supports the findings of Lilley (1998) who reported that a similar reduction in *Pisum sativum* soluble root transglutaminase activity by DIECA and o-phenanthroline was mirrored by the purified guinea pig liver transglutaminase indicating that the biotin cadaverine incorporation assay was not affected by diamine oxidase activity.

In the presence of 0.23% (w/v) sodium deoxycholate the K_m values estimated for the incorporation of biotin cadaverine into N,N'-dimethylcasein by the soluble and microsomal *Pisum sativum* transglutaminase activities were 473μM and 618μM respectively (figures 4.3 and 4.4). The values could be somewhat misleading due to the possible presence of preferred amine donor or acceptor substrates in the crude extracts used. Thus, more meaningful data and comparisons can only be made with purified enzyme preparations.

Figure 4.5 shows the effect of reaction pH on the biotin cadaverine incorporation activities of *Pisum sativum* soluble and microsomal leaf transglutaminase. The microsomal transglutaminase activity increased with increasing pH until an optimum was reached at pH 7.9. The activity of the soluble enzyme showed peaks at pH 7.9 and pH 8.9, the activity remained greater than 50% of the maximum over the pH range from 6.5 to 9.5. A similar result was obtained with *Helianthus tuberosus* leaf extracts, the pH profiles generated by soluble and particulate fractions were different and these authors suggested that more than one enzyme or different forms of the same enzyme were present in the leaf tissue (Falcone *et al.*, 1993). Other workers have demonstrated pH optima for transglutaminase activity between 7.9 and 8.4 in other plant tissues (Serafini-Fracassini *et al.*, 1988; Falcone *et al.*, 1993; Lilley *et al.*, 1998a). The alkaline pH optimum of the *Pisum sativum* leaf microsomal transglutaminase indicates that the enzyme maybe orientated in the tonoplast membrane such that the bulk of the protein is present in the cytoplasm rather than the vacuole.

Figure 4.6 demonstrates the relationship between the age of *Pisum sativum* seedlings and the soluble and microsomal leaf transglutaminase activities. The soluble transglutaminase activity increased during day 7 to 14, the activity remained at a level comparable to that at day 14 over the remaining time points. No transglutaminase activity was detectable in the leaf microsomal fraction prepared from 7-day old *Pisum sativum* seedlings, levels of activity increased to a maximum at day 17 and remained high up to day 25 before starting to decrease. Soluble transglutaminase activity appeared to be present in both developing and mature *Pisum sativum* leaf tissue. However, microsomal transglutaminase appeared only to be present or active in mature leaf tissue. The different distribution of the soluble and microsomal transglutaminase activities in *Pisum sativum* leaf tissue further enhances a distinct role for the microsomal and soluble activities.

Young plant cells have numerous small vacuoles and during the period of cell expansion these vacuoles coalesce so that the mature plant cell contains a single large central vacuole that can occupy up to 90% of the cell volume (Hufford, 1978). The presence of tonoplast associated transglutaminase in the mature leaf tissue only, when the vacuole is fully formed, suggests the enzyme is inserted after formation of the large central vacuole since other tonoplast membrane proteins have been shown to be associated with the earliest stages of vacuole formation, the endoplasmic reticulum and provacuolar membranes (Herman *et al.*, 1994).

Table 4.5 shows that the total transglutaminase activity detected in the microsomal fraction of different aged *Pisum sativum* leaf tissue ranged from 21% to 34%. It is therefore unlikely that the microsomal activity is a consequence of non-specific binding during homogenisation, and would support other data in suggesting soluble and microsomal forms of transglutaminase are present in *Pisum sativum* leaf tissue.

4.3.2 Estimation of the relative molecular masses of *Pisum sativum* leaf transglutaminases

The relative molecular masses of *Pisum sativum* soluble and detergent and trypsin solubilized microsomal transglutaminases were estimated by gel filtration through a Sephacryl S300 HR column. Figure 4.7 demonstrates that the estimated relative molecular mass of soluble *Pisum sativum* leaf transglutaminase was 69 000. SDS-

PAGE analysis of proteins present across the peak of transglutaminase activity (plate 4.1) revealed there was a decrease in the number of protein bands, however it is difficult to predict which represents the transglutaminase enzyme at this early stage of protein purification.

Detergent solubilization of membrane proteins yields a mixture of detergent-protein micelles, detergent-lipid-protein micelles and possibly small membrane fragments (Findlay, 1990), which may account for the multiple peaks of transglutaminase activity observed after gel filtration of sodium deoxycholate solubilized microsomal membrane protein (figure 4.8). The transglutaminase activity detected at 50.0 – 54.0 ml eluted with the column void volume and was possibly due to the association of transglutaminase with small membrane fragments. The transglutaminase activity eluted at 73.5 ml had an estimated relative molecular mass of 91 000.

The transglutaminase activity released from the microsomal membrane preparation by trypsin treatment and separated by gel filtration had an estimated relative molecular mass of 29 000 (figure 4.9). SDS-PAGE analysis of proteins across the peak of transglutaminase activity revealed the presence of two major protein bands (plate 4.3A). The relative molecular masses of the two bands were estimated to be 31 600 and 29 500. The two polypeptides detected in SDS-PAGE gels could be different proteins or they both could be tryptic fragments of transglutaminase. To confirm that neither one of these bands corresponded to the trypsin used to solubilize the microsomal membrane preparation, a sample of the trypsin was run along side the gel filtration separated proteins (plate 4.3B).

Probing western-blots of SDS-PAGE separated proteins with the monoclonal anti-tissue transglutaminase antibody, CUB7402, produced rather conflicting data. Plate 4.4 shows that the antibody detected a protein with a relative molecular mass of 60 000 in the gel filtration purified soluble fraction which was in good agreement with the relative molecular mass of 69 000 estimated by gel filtration (figure 4.7). The antibody detected two bands in the detergent solubilized fraction after gel filtration chromatography corresponding to relative molecular masses of 64 000 and 60 000. The relative molecular mass of 91 000 estimated by gel filtration (figure 4.8) could be an overestimate of the actual size since the association of protein and detergent gives rise

to a complex of greater M_r than the protein alone (Findlay, 1990) and therefore the actual M_r could be close to 64 000. CUB7402 detected a protein with a relative molecular mass of 60 000 in the gel filtration purified trypsin solubilized microsomal fraction, this band is just visible on the SDS-polyacrylamide gel shown in plate 4.3A. This relative molecular mass is not in agreement with the M_r of 29 000 estimated by gel filtration of the trypsin solubilized transglutaminase (figure 4.9). These result raises two possibilities about the nature of the trypsin solubilized transglutaminase. Firstly, if the antibody is specifically detecting a plant transglutaminase, then trypsin solubilization of the microsomal activity would appear to result in the cleavage of a fragment with a relative molecular mass of 4 000 from the amino or carboxyl terminal that is responsible for membrane anchorage of the protein and for some reason this protein was slowed down in its passage through the gel filtration column. Alternatively, trypsin solubilization of the microsomal activity produces a 29 000 M_r protein which retains its transglutaminase activity. The cross-reactivity with the antibody in this instance would have to be considered to be non-specific.

The relative molecular masses of transglutaminases from other plant species have been estimated by both gel filtration chromatography and western blot analysis using polyclonal antibodies raised against mammalian transglutaminases. An enzyme with a relative molecular mass of 80 000 was reported in germinated and ungerminated pollen of *Malus domestica* (Del Duca *et al.*, 1997) and in the leaves of *Glycine max* (Kang & Cho, 1996). The purified enzyme from meristematic buds of *Medicago sativa* had a relative molecular mass of 39 000 (Kuehn *et al.*, 1991). Transglutaminase in chloroplasts of *Helianthus tuberosus* was identified immunologically to have a relative molecular mass of 58 000 (Del Duca *et al.*, 1994). From this information we can conclude that several forms of transglutaminase are present in plant tissues and as in mammals each possibly has a specific function to form within that tissue. Those having similar M_r may be closely related and perform similar functions.

The free calcium ion concentration required for activation of microsomal transglutaminase was reduced from 5mM to 7.5μM by the release of the enzyme from the membrane by trypsin and purification by gel filtration (figures 5.11 and 5.12). This results suggests that trypsin treatment resulted in a direct activation of the enzyme by the removal of a regulatory peptide. Alternatively the reduction in proteins seen in this

fraction (compare plate 4.2 and 4.3) could reinforce the necessity to carry out out these types of estimation on purified protein preparations rather than crude extracts.

GTP regulates the activity of mammalian type II transglutaminase at sub-optimal calcium ion concentrations (Smethurst and Griffin, 1996). Table 5.10 shows that the biotin cadaverine incorporation activities of Sephacryl S300 purified *Pisum sativum* leaf transglutaminase activities were also inhibited by 1mM GTP at sub-optimal free calcium ion concentrations. These results indicate that soluble and microsomal transglutaminases of *Pisum sativum* leaf tissue may contain a GTP binding site. It is uncertain at the present time whether these activities are capable of hydrolysing GTP and ultimately act as G-proteins in signal transduction, which is an important function of the type II transglutaminase in mammalian tissues.

No significant sequence homology has been found between the type II transglutaminase and the "classical" signal-transducing heterotrimeric G-proteins or low molecular weight GTP-binding proteins, suggesting that type II transglutaminase is a new class of GTP binding protein with a dual function (Nakaoka *et al.*, 1994; Iismaa *et al.*, 1997). Both heterotrimeric G-proteins and low molecular weight GTP-binding proteins with sequence homology to those of mammals and simple eukaryotes have been detected in plant tissues (for review see Ma, 1994; Bischoff *et al.*, 1999). Perroud *et al.*, (1997) reported the presence of a GTP binding activity on the tonoplast isolated form *Spinacia oleracea* leaves that cross-reacted to antibodies raised to the consensus sequence of an animal G_α protein. A GTP-binding protein was recently cloned from *Arabodopsis thaliana* that had significant sequence homology to animal and plant G_α proteins at the carboxy-terminal. The amino-terminal region contained domains homologous to the bacterial TonB-box protein, which is involved in energy transduction between the inner and outer bacterial membranes and therefore raisers the possibility that a G-protein in plant tissue could also be bifunctional (Lee and Assmann, 1999). The presence of GTP binding proteins in plant membranes with homology to those of mammalians raises the possibility that transglutaminases in plant tissues with homology to mammalian tissue transglutaminase may function as G-proteins in plants as they do in mammals.

Recombinant mouse type III transglutaminase obtained using the baculovirus expression system was shown to be inhibited approximately 80% by 100μM GTP in the

presence of 1mM $CaCl_2$. The enzyme however was not found to hydrolyse GTP (Hitomi *et al.*, 2000). Therefore the transglutaminase activities in *Pisum sativum* leaf tissue, which were shown to be inhibited by GTP (tables 4.6 to 4.8), may also be unable to hydrolyse GTP.

Additional work is required to purify transglutaminase and overcome some of the problems arising from working with crude plant extracts. In order to compare the soluble and microsomal activities further both enzymes would need to be purified to homogeneity.

Chapter 5 - Partial purification and characterisation of *Vicia faba* cotyledon transglutaminase

Chapter 5 – Partial purification and characterisation of *Vicia faba* cotyledon transglutaminase

5.1 Introduction

The purification of mammalian transglutaminases and the production of antibodies to the purified protein have enabled their role to be investigated and yielded information on enzyme structure and properties. Purification of the plant enzyme will also provide some of the tools needed to further characterise transglutaminase activity and expression in plant tissues and ultimately help establish the biological function of the enzyme(s). Although the different forms of transglutaminase in mammals share a common active site sequence, major differences in amino acid sequence occur at the amino- and carboxy-terminus (Iismaa *et al.*, 1997). These differences deem it necessary to employ different purification procedures for each enzyme. In most transglutaminase purification protocols, the techniques of ion exchange and gel filtration chromatography have been used, other chromatographic methods described include hydrophobic interaction, affinity, absorption, and metal-chelating chromatography as well as electrophoretic separation methods (for review see Wilhelm *et al.*, 1996).

Ion exchange chromatography separates proteins based on their charge at a defined pH. Heterogeneous mixtures of proteins are applied to the resin and those carrying an opposite charge to the ion exchange resin bind reversibly to it. Elution is performed by displacing the protein from the resin by changing the ionic strength or pH of the buffer. The introduction of an increasing salt concentration gradient causes the release of protein from the resin in order of their strengths of binding, the most weakly bound proteins are eluted first (for review see Rossomando, 1990).

Hydrophobic interaction chromatography (HIC) is performed by loading proteins onto a hydrophobic matrix and eluting them by changing the polarity of the eluent through modifications of salt concentration or addition of organic solvents (Kennedy, 1990). This procedure has been modified to specifically purify calcium binding proteins (Walsh *et al.*, 1984). Calcium-dependent HIC is generally performed at low salt concentration in the presence of calcium chloride, this enables the initial capture and selection of calcium dependent hydrophobic proteins since most other hydrophobic

proteins don't bind under these conditions. Elution is performed by removing the bound calcium from the protein.

In gel filtration chromatography, molecules in solution are separated according to differences in their size as they pass through a column of porous beads. A column constructed from such beads has two measurable liquid volumes, an external volume consisting of the liquid between the beads and an internal volume consisting of the liquid within the pores. A mixture of proteins applied to the column move down the column as eluent is added at the top. The small proteins, which can diffuse into the pores of the beads, are delayed in their passage through the column compared to larger proteins that cannot enter the pores and therefore elute from the column first followed by smaller molecules in order of their size (for review see Stellwagen, 1990).

Several methods have described the purification of tissue transglutaminase from guinea pig liver (Brookhart *et al.*, 1983; Leblanc *et al.*, 1999), rat liver (Croall and DeMartino, 1986), rat and bovine testes (Bergamini and Signorini, 1992) and human erythrocytes (Lee *et al.*, 1986). Tissue transglutaminase was purified from rat liver extracts by calcium dependent affinity chromatography on casein-Sepharose (Croall and DeMartino, 1986). In the presence of 5mM $CaCl_2$ the enzyme bound to the casein-Sepharose and was subsequently eluted with 5mM EDTA. Tissue transglutaminase from bovine testes was purified to homogeneity by means of anion exchange, size exclusion and hydrophobic interaction chromatography (Bergamini and Signorini, 1992). Protein was bound to a phenyl Sepharose column in the presence of 0.5 M sodium chloride and transglutaminase was specifically eluted with binding buffer containing 3mM GTP. Conformational changes induced by the binding of GTP to tissue transglutaminase caused elution of the protein. The purified transglutaminase had a relative molecular mass of 80 000 and displayed properties of type II transglutaminases.

Transglutaminases have also been purified from sources other than mammalian tissues. Ando *et al.* (1989) purified a Ca^{2+} independent transglutaminase from the microorganism *Streptoverticillium* S-8112 using ion exchange chromatography on Amberlite CG-50 and blue Sepharose, the purified enzyme had a relative molecular mass of 40 000. Klein *et al.* (1992) purified a transglutaminase from the slime mould *Physarum polycephalum* by combined methods of polyethylene glycol precipitation, DEAE-cellulose chromatography and isolelectric focusing on a pH 5 to 7 gradient, the

relative molecular mass of the purified enzyme was 77 000. Transglutaminase has also been purified from the filarial nematode, *Brugia malayi*, using thermoprecipitation, ammonium sulphate precipitation, gel filtration and ion exchange chromatography (Singh and Mehta, 1994). Tokunaga *et al.* (1993b) reported the purification of *Tachypleus tridentatus* (Japanese horseshoe crab) hemocyte transglutaminase with a relative molecular mass of 86 000.

To date, only two attempts to purify a plant transglutaminase have been reported. Kuehn *et al.* (1991) purified transglutaminase from *Medicago sativa* meristematic buds using the techniques of ammonium sulphate precipitation, DEAE-cellulose chromatography, isoelectric focusing and reversed phase HPLC. The purified protein had a relative molecular mass of 37 – 39 000 and catalysed amine exchange between [^{14}C] putrescine and glutaminyl residues of the large subunit of Rubisco, the enzyme did not require Ca^{2+} for catalytic activity. Using the leaves of *Glycine max*, Kang and Cho (1996) purified transglutaminase using ammonium sulphate precipitation, ion exchange chromatography (using DEAE Sepharose, blue Sepharose CL-4B and ω-aminohexyl-agarose) and a novel affinity elution step using α-casein agarose. After binding protein to the α-casein agarose, transglutaminase was specifically eluted by 1mM spermidine. The relative molecular mass of the enzyme, estimated by gel filtration and SDS-PAGE, was 80 000. The purified enzyme was activated by DTT and inhibited by N-ethylmaleimide and GTP suggesting some similarities with the mammalian enzymes. However, the enzyme showed no absolute requirement for calcium ions.

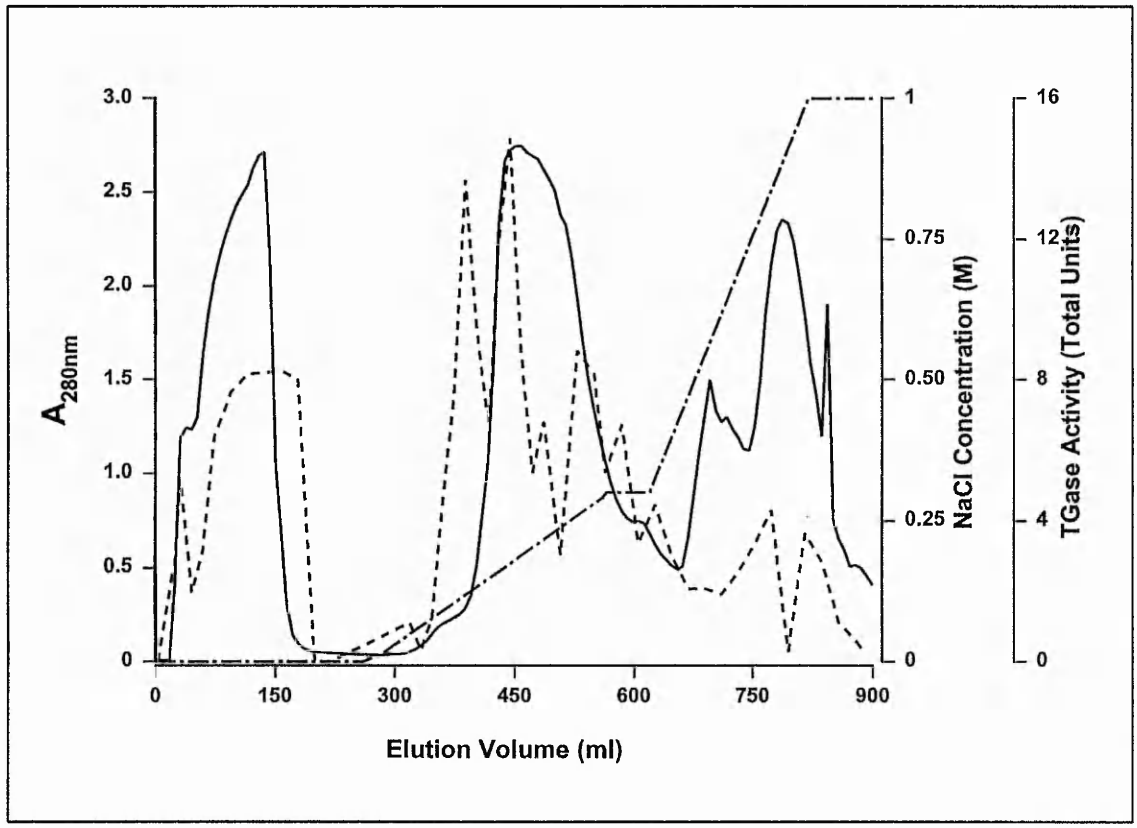
The objectives of this section of the thesis were to develop appropriate techniques to purify transglutaminase from plant tissue and compare its activity with those reported in other plant and animal tissues. Determine the molecular mass of the purified enzyme and establish its pH optimum, calcium ion requirement and thiol dependence. Finally, establish if the purified enzyme is inhibited by GTP and cross reacts with antibodies raised against mammalian type II transglutaminase.

5.2 Results

5.2.1 Purification of soluble *Pisum sativum* leaf transglutaminase

Pisum sativum leaf tissue was chosen as a starting material for the purification of plant transglutaminase. The soluble fraction was initially purified by anion exchange chromatography using Macro Prep High Q anion exchange resin (Bio-Rad); a typical chromatogram is presented in figure 5.1.

Figure 5.1 - Ion exchange chromatography of soluble *Pisum sativum* leaf extract on a Macro Prep High Q column.



Soluble *Pisum sativum* leaf protein was brought to 90% saturation with ammonium sulphate; the precipitated protein was collected and dialysed overnight as described in section 2.2.2.4. Six hundred and forty milligrams of protein were applied to a 50.0ml Macro Prep Q anion exchange column (2.5 x 10 cm) and unbound proteins were eluted with two column volumes of 20mM Tris-HCl pH 8.5. Bound proteins were eluted by applying a salt gradient from 0 – 1.0 M sodium chloride over 10 column volumes (as described in section 2.2.6.1). Seven millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). The absorbance at 280nm was measured using a Beckman DU 70 spectrophotometer.

Key

——— Absorbance at 280nm

------ Biotin cadaverine incorporation activity

– · – · Sodium chloride concentration

Figure 5.1 demonstrates that the transglutaminase activity eluted as three main peaks of activity, one corresponding to the unbound material, one at a sodium chloride concentration between 0.07 and 0.19 M and one at a sodium chloride concentration between 0.23 and 0.3 M, some activity also eluted after 0.8 M sodium chloride. The unbound protein and the protein eluting between 0.07 - 0.19 M and 0.23 - 0.3 M sodium chloride were pooled separately and investigated further.

Table 5.1 - The biotin cadaverine incorporating activity of anion exchange purified soluble *Pisum sativum* leaf transglutaminase.

Fraction	Protein	Total Protein	Specific activity	Total activity
	mg ml^{-1}	mg	Units mg^{-1}	Units
Crude extract	5.31	636.67	0.44 ± 0.03	282.88 ± 18.72
Unbound	0.607	98.35	0.69 ± 0.06	67.33 ± 5.65
0.07 – 0.19 M NaCl	1.35	146.48	0.86 ± 0.06	125.96 ± 8.53
0.23 – 0.3 M NaCl	0.97	84.02	0.76 ± 0.09	63.55 ± 7.26

Six hundred and forty milligrams of soluble *Pisum sativum* leaf protein were separated by anion exchange chromatography as described for figure 5.1. The biotin cadaverine incorporation activity was measured in the pooled fractions as described in section 2.2.4.1. Protein concentration was measured using the modified BCA assay as described in section 2.2.7.2. Values represent the mean ± SEM of 3 replicates.

Table 5.1 shows that anion exchange chromatography separated transglutaminase activity into three main peaks. The unbound activity accounted for 24% of the total transglutaminase activity applied to the column and the low and high salt eluting peaks 45% and 22% respectively.

Mammalian transglutaminases contain a cysteine residue at their active site and are sensitive to thiol reactive reagents (Folk and Cole, 1966). The thiol reactive reagents, iodoacetamide and N-ethylmaleimide, were used to measure the inhibition of the biotin cadaverine incorporation activity in each of the pooled fractions.

Table 5.2 - The effect of thiol reagents on the transglutaminase activities of soluble *Pisum sativum* leaf protein separated by anion exchange chromatography.

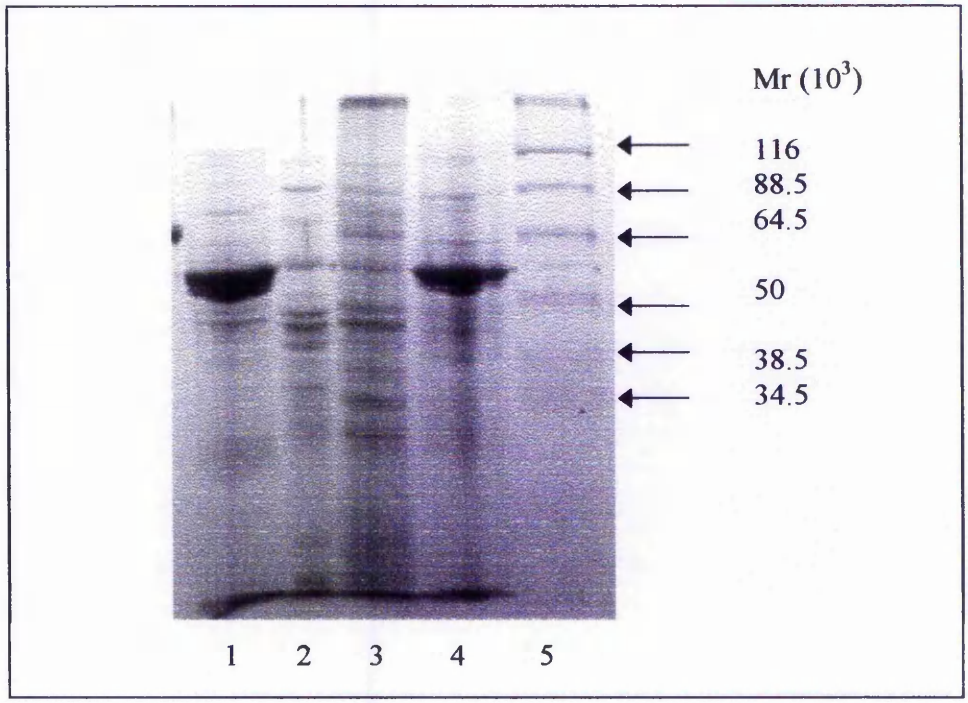
Fraction	Total transglutaminase activity Units		
	Uninhibited	+ 1mM Iodoacetamide	+ 1mM N-ethylmaleimide
Crude extract	282.88 ± 18.72	26.24 ± 1.36 (90.7)	69.20 ± 2.98 (75.5)
Unbound IE	67.33 ± 5.65	0.61 ± 0.02 (99.1)	7.95 ± 0.56 (88.2)
0.07 - 0.13 M NaCl	125.96 ± 8.53	N.D (100)	N.D (100)
0.23 - 0.3 M NaCl	63.55 ± 7.26	4.51 ± 0.09 (92.9)	3.31 ± 0.23 (94.8)

Crude and ion exchange purified *Pisum sativum* leaf transglutaminase were incubated with 5mM $CaCl_2$ and 1mM iodoacetamide or N-ethylmaleimide for 30 minutes at 37°C. The pre-treated extracts were assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay (section 2.2.4.1). Extract boiled for 10 minutes was used as a negative control at each inhibitor concentration. Data points represent the mean ± SEM of 3 replicates. N.D no activity detectable. The values in brackets represent the percentage inhibition of the biotin cadaverine incorporation activity in the presence of 1mM iodoacetamide or NEM.

Table 5.3 demonstrates that the presence of 1mM iodoacetamide or N-ethylmaleimide produced a 76% to 100% inhibition of the biotin cadaverine incorporation activity of crude and ion exchange purified *Pisum sativum* leaf extracts.

To determine if the ion exchange chromatography procedure provided a good separation of proteins, the crude extract and the pooled peaks of transglutaminase activity were separated by SDS-PAGE and a Coomassie blue stained gel is presented in plate 5.1.

Plate 5.1 - SDS-PAGE analysis of *Pisum sativum* soluble leaf protein after separation by ion exchange chromatography.



Twenty micrograms of crude *Pisum sativum* soluble protein extract and unbound, 0.07 – 0.19 M and 0.23 – 0.3 M NaCl peaks after anion exchange chromatography (prepared as described for figure 5.1) were separated under denaturing conditions on a 10% (w/v) SDS polyacrylamide gel as described in section 2.2.8.1 and stained with Coomassie blue R 250 (section 2.2.8.2). *Lane 1*, crude extract; *lane 2*, unbound proteins; *lane 3*, 0.07 – 0.19 M NaCl peak; *lane 4*, 0.23 – 0.3 M NaCl peak; *lane 5*, molecular mass markers.

Plate 5.1 demonstrates that the population of proteins present in the unbound material and in each of the pooled ion exchange peaks are different.

5.2.2 Purification of *Vicia faba* cotyledon transglutaminase

Due to the multiple peaks encountered whilst purifying soluble *Pisum sativum* leaf transglutaminase (see figure 5.1) alternative starting material was investigated. Transglutaminase activity in germinating *Vicia faba* cotyledons has been shown to be high (Lilley *et al.*, 1998b), and the low phenol oxidase activity in this tissue makes it a suitable alternative starting material. Seeds are particularly rich in proteases (Ryan and Walker-Simons, 1981) therefore the main consideration when designing a purification protocol for this starting material was to inhibit or isolate the transglutaminase activity from proteolytic activities. Endopeptidase assays were carried out to determine the types of proteases active in germinating *Vicia faba* cotyledons and the data is presented in table 5.3.

Table 5.3 - Measurement and inhibition of endopeptidase activities in *Vicia faba* cotyledon extracts.

Inhibitor	Specific activity Units mg^{-1}	% inhibition of endopeptidase activity
None	0.043 ± 0.0003	
1mM PMSF	0.046 ± 0.0006	0
2mM EDTA	0.047 ± 0.002	0
10μM E_{64}	0.021 ± 0.002	51.9
10μM leupeptin	0.023 ± 0.001	45.9
1mM N-ethylmaleimide	0.019 ± 0.001	56.0
1mM iodoacetamide	0.024 ± 0.001	43.6
10μM pepstatin A	0.05 ± 0.002	0

Ten grams of 14-day old *Vicia faba* cotyledons were homogenised and centrifuged as described in section 2.2.2.3, but without the addition of protease inhibitors. Aliquots of *Vicia faba* cotyledon protein were preincubated with protease inhibitors for 30 minutes at 37°C. Endopeptidase activity was measured using an azocasein assay as described in section 2.2.4.2. Values represent the mean ± SEM of 3 replicates.

Table 5.3 shows that the cysteine / serine protease inhibitor PMSF, the aspartate protease inhibitor pepstatin A and the metallo-protease inhibitor EDTA did not inhibit endopeptidase activity present in *Vicia faba* cotyledons. The cysteine protease inhibitors iodoacetamide, N-ethylmaleimide and E_{64} and the cysteine / serine protease inhibitor leupeptin inhibited endopeptidase activity by 44% to 56%. Combinations of these inhibitors were used to try and increase the inhibition of the endopeptidase activity further; this data is presented in table 5.4.

Table 5.4 - The effect of protease inhibitors on *Vicia faba* cotyledon endopeptidase activities.

Inhibitors	Specific activity Units mg^{-1}	% Inhibition of activity
None	0.043 ± 0.0003	
10μM E_{64} + 1mM NEM	0.018 ± 0.001	57.1
10μM E_{64} + 2mM EDTA	0.022 ± 0.004	49.6
10μM E_{64} + 10μM benzamidine	0.021 ± 0.002	52.2
10μM E_{64} + 10μM leupeptin	0.018 ± 0.001	57.8

Ten grams of 14-day old *Vicia faba* cotyledons were homogenised and centrifuged as described in section 2.2.2.3, but without the addition of protease inhibitors. Aliquots of *Vicia faba* cotyledon protein were preincubated with protease inhibitors for 30 minutes at 37°C. Endopeptidase activity was measured using the azocasein assay as described in section 2.2.42. Values represent the mean ± SEM of 3 replicates.

Table 5.3 and 5.4 show that only 50 to 60% of the endopeptidase activity present in *Vicia faba* cotyledons could be inhibited by the cysteine protease inhibitors iodoacetamide, N-ethylmaleimide, E_{64} or leupeptin. The remaining uninhibitable endopeptidase activity needs to be separated from the transglutaminase activity rapidly during the purification procedure to ensure minimal proteolytic degradation of transglutaminase. Due to the inhibition of *Vicia faba* cotyledon transglutaminase by iodoacetamide and N-ethylmaleimide (table 5.8) these were not be included in the extraction buffer.

During purification the uninhibitable endopeptidase and transglutaminase activities were assayed to measure the effectiveness of the purification technique at separating the transglutaminase activity from the uninhibitable endopeptidase activity. Ammonium sulphate precipitation was used initially to try and separate the two activities early in the purification procedure; the results are presented in table 5.5.

Table 5.5 - Transglutaminase and endopeptidase activity in ammonium sulphate precipitated proteins extracted from *Vicia faba* cotyledons.

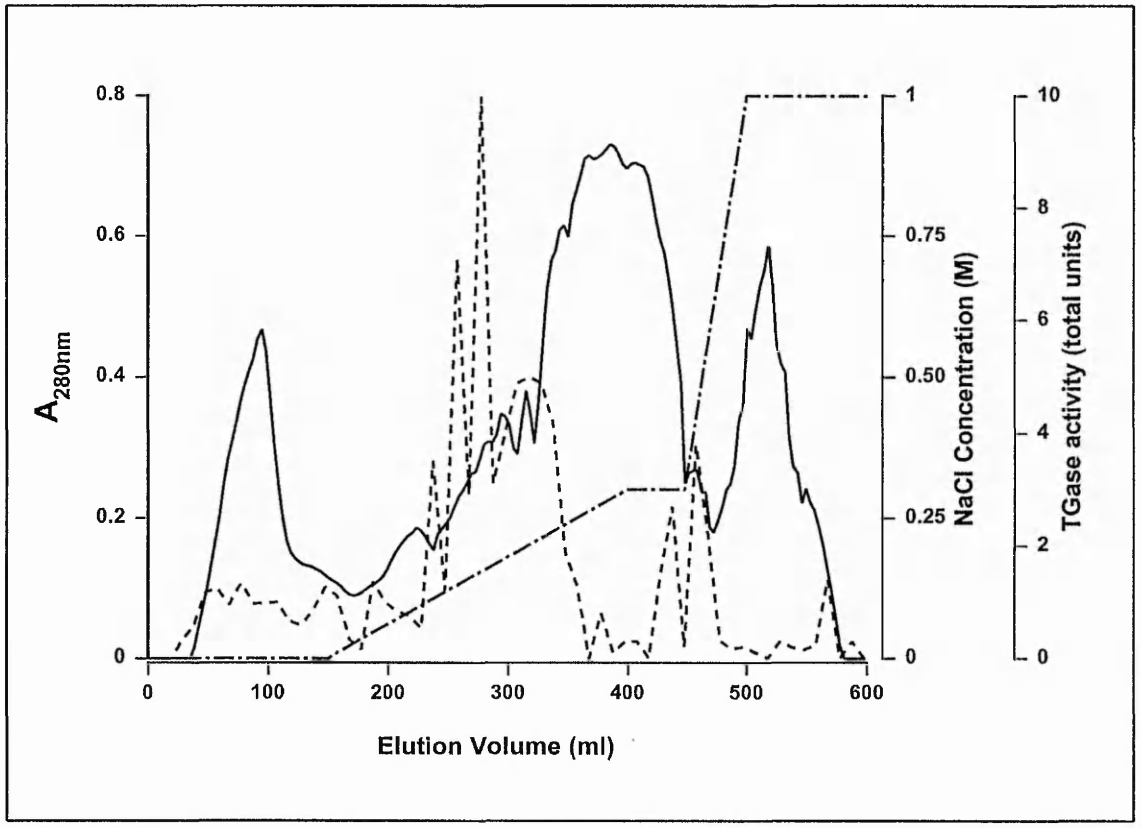
Ammonium sulphate saturation (%)	Total protein mg	Transglutaminase activity Total units	Endopeptidase activity Total units
0 – 20	8.25	2.30 ± 0.51	0.44 ± 0.08
0 – 30	21.21	4.95 ± 0.48	1.43 ± 0.22
0 – 40	51.59	18.78 ± 1.49	2.03 ± 0.36
0 – 50	75.35	33.11 ± 1.38	6.08 ± 0.36
0 – 60	80.24	41.03 ± 0.88	7.71 ± 0.03
0 – 70	86.74	45.94 ± 1.61	7.66 ± 0.19
0 – 80	101.60	27.01 ± 5.82	7.57 ± 0.53

Twenty millilitres of *Vicia faba* cotyledon protein (section 2.2.2.3) were precipitated by the addition of ammonium sulphate to 20 - 80% saturation. Protein was stirred for 1 hour at 4°C and collected by centrifugation at 10 000.g for 15 minutes, as described in section 2.2.2.4. Following dialysis, protein was assayed for transglutaminase activity using the biotin cadaverine incorporation activity (section 2.2.4.1) and endopeptidase activity using the azocasein assay (section 2.2.4.2). Values represent the mean ± SEM of 3 replicates.

Table 5.5 shows that the uninhibitable endopeptidase activity was completely precipitated by the addition of ammonium sulphate to 60% saturation. Transglutaminase activity was completely precipitated by 70% saturation of ammonium sulphate. The ammonium sulphate precipitation step was omitted from future purification procedures.

The initial method chosen for the purification of *Vicia faba* cotyledon transglutaminase was ion exchange chromatography since this technique had previously been used to successfully purify mammalian transglutaminases (for review see Wilhelm *et al.*, 1996). The resin chosen for the purification was Macro Prep High Q anion exchange support (Bio-Rad) and typical separations are presented in figures 5.2 and 5.3.

Figure 5.2 - The biotin cadaverine incorporation activity in fractions obtained after ion exchange chromatography of *Vicia faba* cotyledon protein.



Five hundred milligrams of *Vicia faba* cotyledon protein were applied to a 50.0 ml Macro Prep High Q anion exchange column (2.5 x 10 cm) and unbound protein was eluted with two column volumes of 20mM Tris-HCl pH 8.5. Bound proteins were eluted by applying a salt gradient from 0 – 1.0 M sodium chloride over 10 column volumes, as described in section 2.2.6.1. The absorbance at 280nm was monitored continuously using the online UV detector. Five millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1).

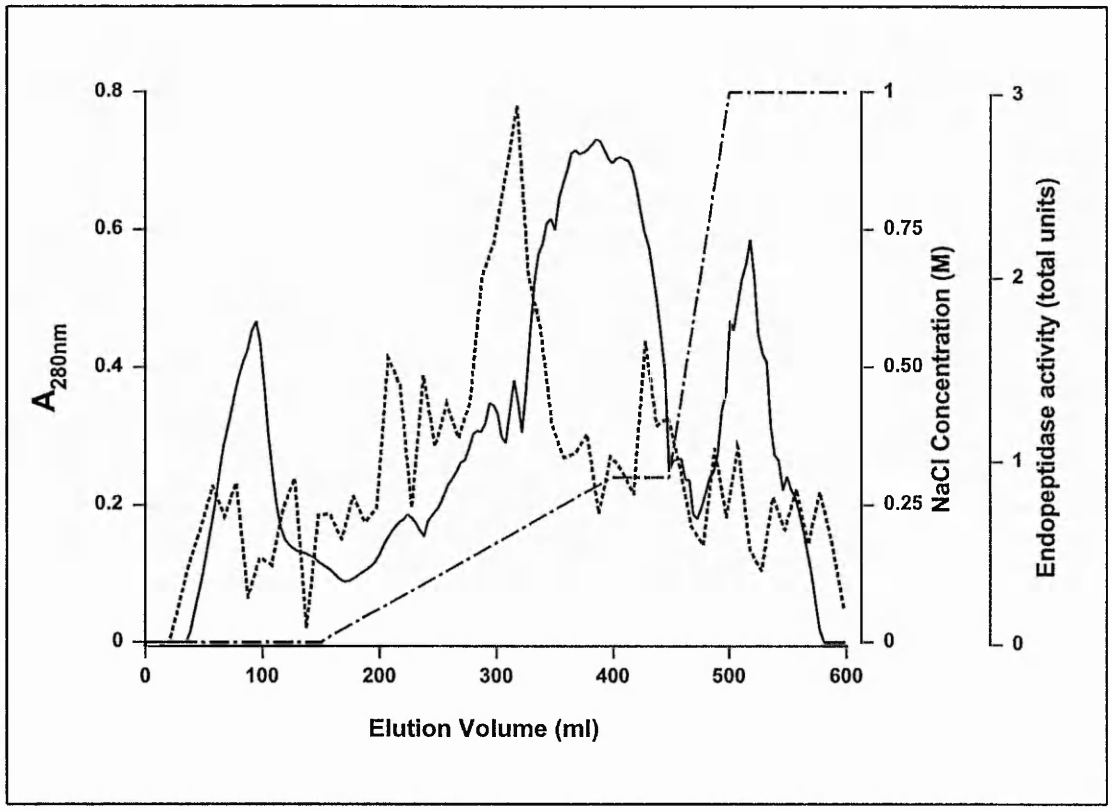
Key

——— Absorbance at 280nm ·

------ Biotin cadaverine incorporation activity

– · – · Sodium chloride concentration

Figure 5.3 - Endopeptidase activity in fractions obtained after ion exchange chromatography of *Vicia faba* cotyledon protein.



Five hundred milligrams of *Vicia faba* cotyledon protein were purified by ion exchange chromatography as described for figure 5.2. Five millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and for uninhibitable endopeptidase activity using the azocasein assay (section 2.2.4.2). The absorbance at 280nm was monitored continuously using the online UV detector.

Key

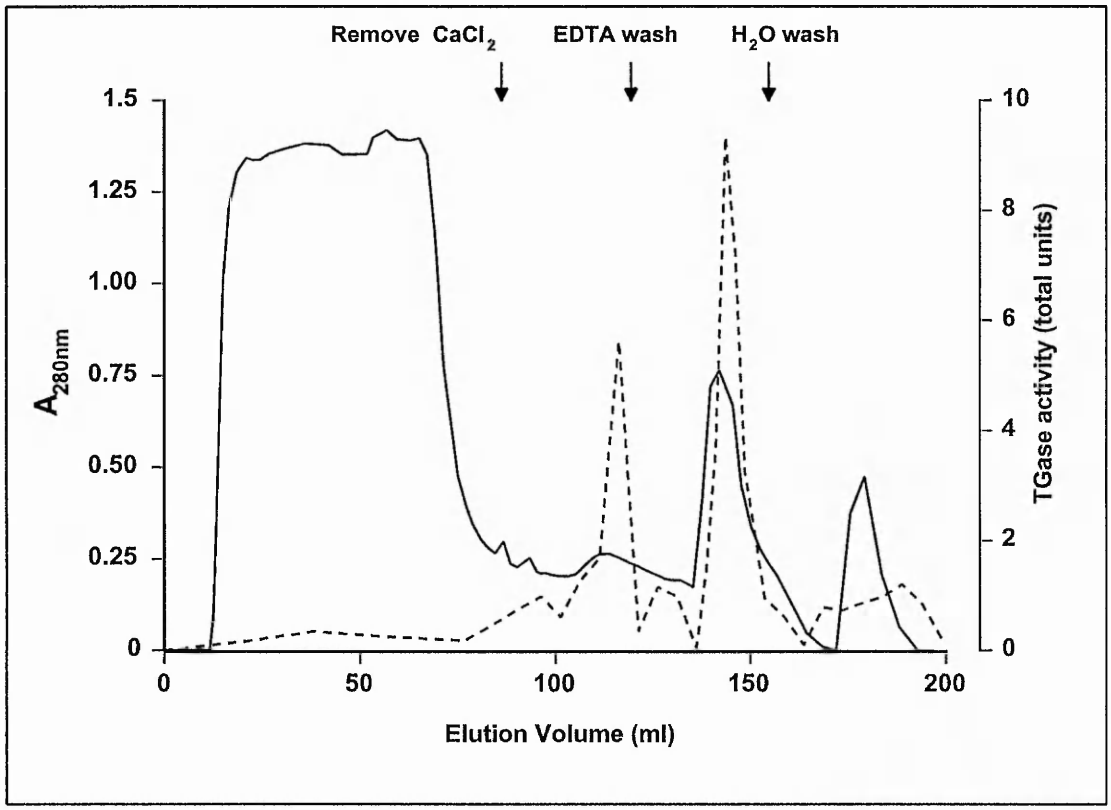
—— Absorbance at 280nm

..... Uninhibitable endopeptidase activity

– · – · Sodium chloride concentration

Figure 5.2 demonstrates that the majority of transglutaminase activity eluted at a sodium chloride concentration between 0.1 and 0.2 M. Figure 5.3 shows that the majority of the uninhibitable endopeptidase activity eluted at a sodium chloride concentration between 0.1 and 0.2 M. The ion exchange purified *Vicia faba* cotyledon transglutaminase activity was pooled and further purified by calcium dependent hydrophobic interaction chromatography using phenyl Sepharose 6FF, typical chromatograms are presented in figures 5.4 and 5.5.

Figure 5.4 - Calcium dependent hydrophobic interaction chromatogram for the purification of *Vicia faba* cotyledon transglutaminase.



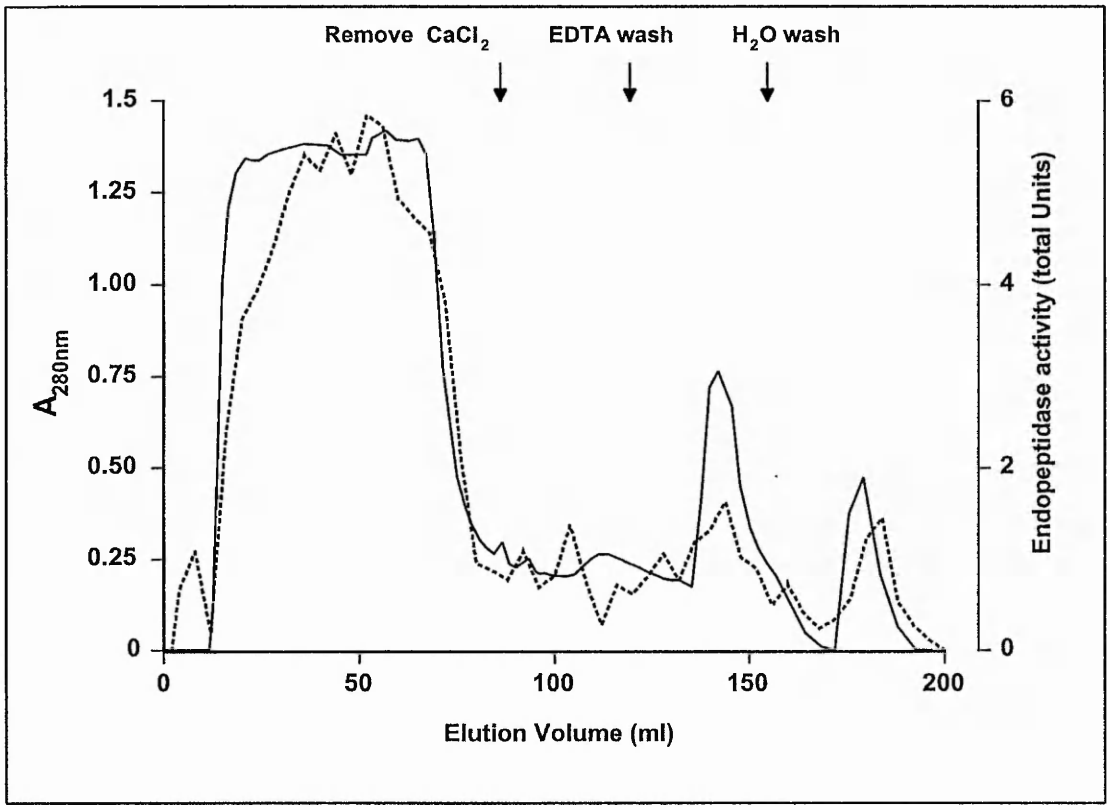
One hundred and thirty milligrams of ion exchange purified *Vicia faba* cotyledon protein were applied to a 50.0 ml phenyl Sepharose 6FF column (2.5 x 10 cm) and eluted as described in section 2.2.6.2. The absorbance at 280nm was measured continuously using the online UV detector. Four millilitre fractions were collected during the entire run and assayed for protein (section 2.2.7.1) and transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1).

Key

——— Absorbance at 280nm

------ Biotin cadaverine incorporation activity

Figure 5.5 - Endopeptidase activity in fractions obtained after calcium dependent hydrophobic interaction chromatography of ion exchange purified *Vicia faba* cotyledon extract.



One hundred and thirty milligrams of ion exchange purified *Vicia faba* cotyledon protein were applied to a 50.0 ml column of phenyl Sepharose 6FF (2.5 x 10 cm) and eluted as described in section 2.2.6.2. The absorbance at 280nm was monitored continuously using the online UV detector. Four millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and uninhibitable endopeptidase activity using the azocasein assay (section 2.2.4.2).

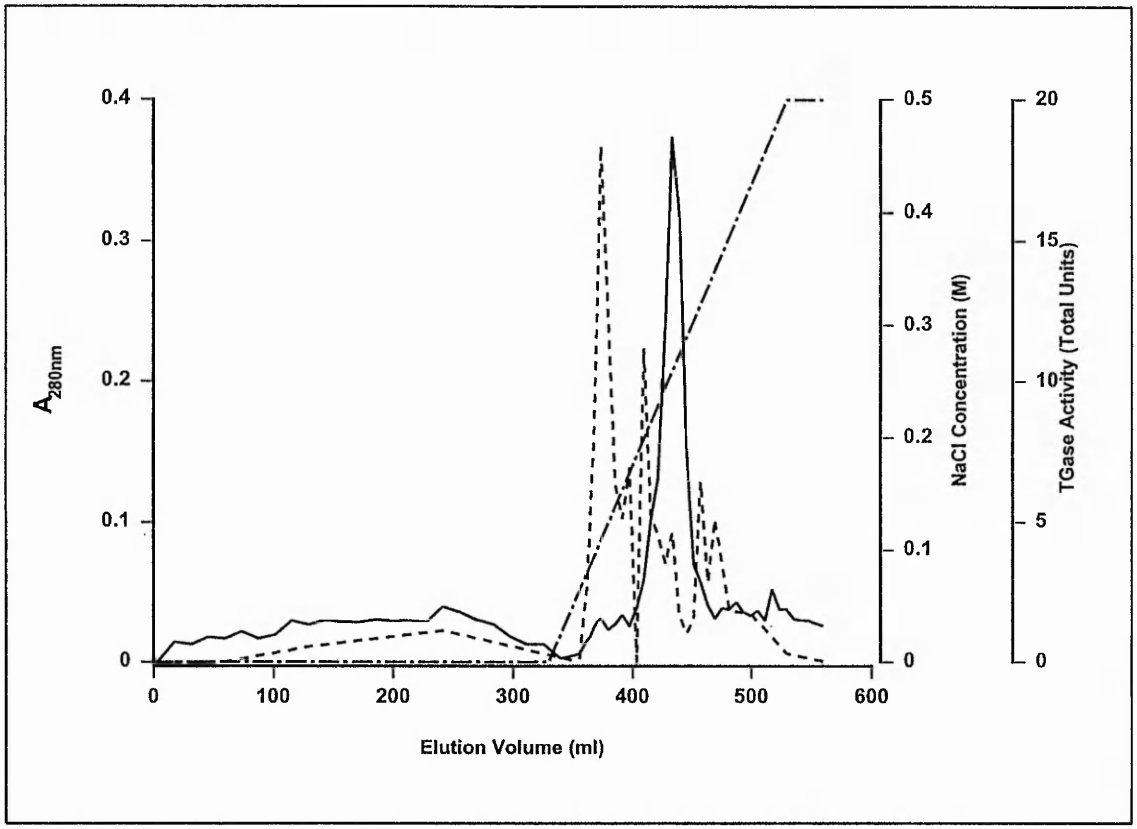
Key

—— Absorbance at 280nm

..... Uninhibitable endopeptidase activity

Figure 5.4 demonstrates that transglutaminase activity was eluted by washing the column with 20mM Tris-HCl pH 8.5 containing 5mM EDTA and 1mM 2-ME. Figure 5.5 shows that the majority of the uninhibitable endopeptidase activity did not bind to the column and eluted with the unbound material. *Vicia faba* cotyledon transglutaminase was purified further by calcium affinity ion exchange chromatography, typical chromatograms are presented in figures 5.6 and 5.7.

Figure 5.6 - Ion exchange chromatogram for the purification of *Vicia faba* cotyledon transglutaminase.



Twenty-five milligrams of phenyl Sepharose purified *Vicia faba* cotyledon protein were applied on to a 10.0 ml Q10 anion exchange column (Bio-Rad) and unbound protein was eluted with two column volumes of 20mM Tris-HCl pH 8.5. Bound proteins were eluted by applying a salt gradient from 0 – 0.5 M sodium chloride over 20 column volumes, as described in section 2.2.6.3. Three millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). The absorbance at 280nm was measured using a Beckman DU 70 spectrophotometer.

Key

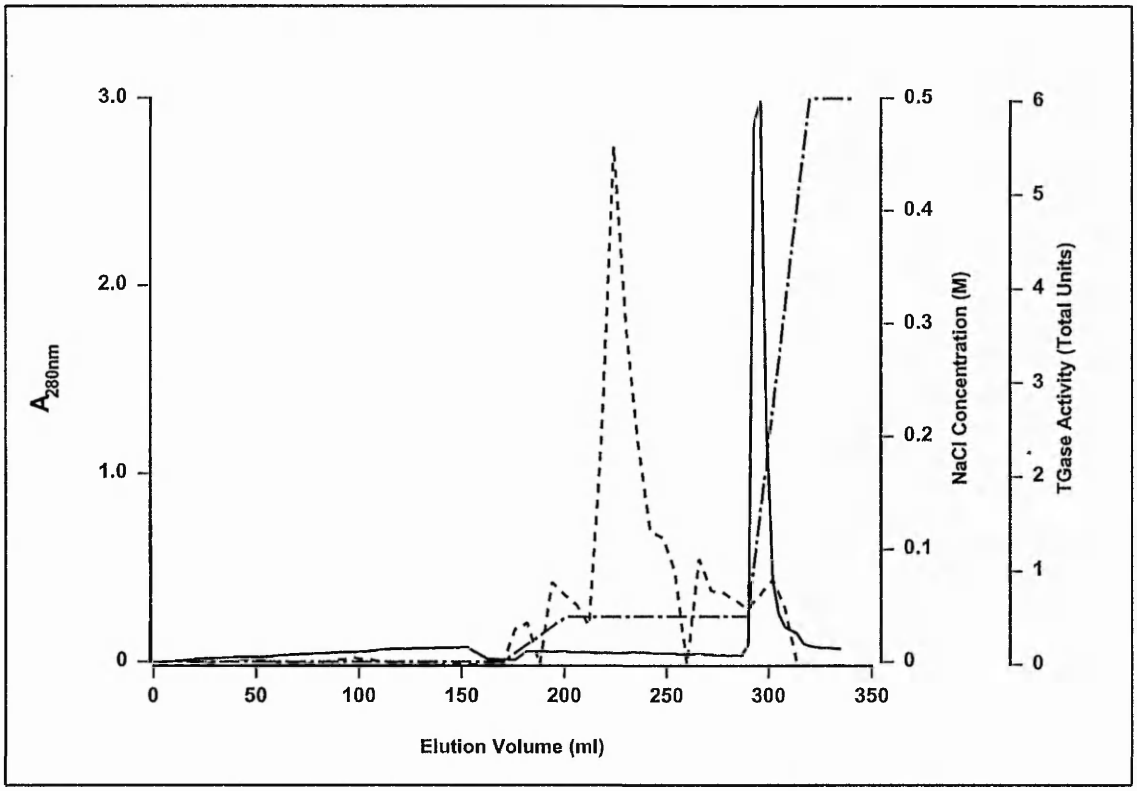
——— Absorbance at 280nm

------ Biotin cadaverine incorporation activity

– · – · Sodium chloride concentration

Figure 5.6 demonstrates that transglutaminase activity eluted at a sodium chloride concentration between 90mM and 150mM. In order to perform the calcium affinity ion exchange step the exact salt concentration at which transglutaminase begins to dissociate from the resin had to be estimated. This was done by identifying the fraction the transglutaminase activity was first detected in and knowing the volume of the column and tubing from the column to the fraction collector it was possible to determine the exact elution volume at which the enzyme would have initially dissociated from the resin. The salt concentration at this elution volume was approximately 60mM sodium chloride.

Figure 5.7 - Calcium affinity ion exchange chromatogram for the purification of *Vicia faba* cotyledon transglutaminase.



Twenty milligrams of Q10 ion exchange purified *Vicia faba* cotyledon protein were applied on to a 10.0 ml Q10 anion exchange column (Bio-Rad) and unbound protein was eluted with two column volumes of 20mM Tris-HCl pH 8.5. Bound proteins were eluted by applying a salt gradient from 0 – 40mM sodium chloride over 5 column volumes. Calcium affinity elution was performed by applying sequentially 1.0 ml injections of 41mM NaCl, 40mM NaCl plus 1mM $CaCl_2$, 42mM NaCl, 40mM NaCl plus 2mM $CaCl_2$, 45mM NaCl and 40mM NaCl plus 5mM $CaCl_2$ as described in section 2.2.6.4. Two millilitre fractions were collected throughout the run and assayed for protein (section 2.2.7.1) and for transglutaminase activity using the biotin cadaverine incorporation assay (section 2.2.4.1). The absorbance at 280nm was measured using a Beckman DU 70 spectrophotometer.

Key

——— Absorbance at 280nm

------ Biotin cadaverine incorporation activity

– · – · Sodium chloride concentration

Figure 5.7 shows that transglutaminase activity was specifically eluted by the addition of 1mM calcium chloride in 40mM sodium chloride elution buffer. Table 5.6 shows a summary of the four purification steps in terms of percentage yield and purification fold.

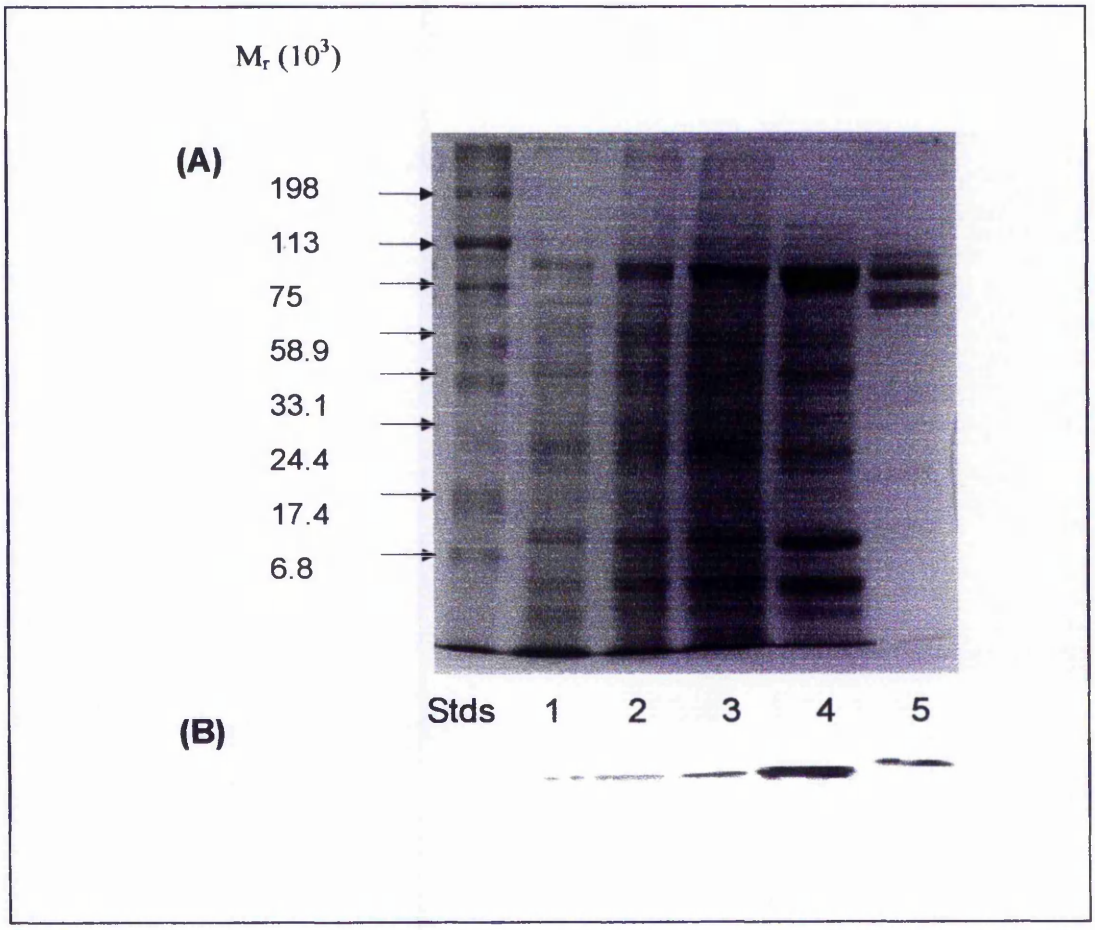
Table 5.6 - A summary of the purification of transglutaminase from *Vicia faba* cotyledons.

Fraction	Total protein mg	Specific activity Units mg^{-1}	Total activity Units	Yield %	Purification fold
Crude extract	1127.5	0.49	557.3		
IE purified	268.7	1.84	495.7	88.9	3.76
HIC purified	44.15	7.62	336.4	60.4	15.55
IE Q10 purified	18.31	16.18	296.2	53.1	33.01
AIE Q10 purified	0.59	246.78	145.6	26.1	503.6

Vicia faba cotyledon protein was purified using the techniques of anion exchange chromatography (section 2.2.6.1), hydrophobic interaction chromatography (section 2.2.6.2) and affinity ion exchange chromatography (sections 2.2.6.3 and 2.2.6.4). The biotin cadaverine incorporation assay was used to detect the transglutaminase activity present at each stage of the purification (section 2.2.4.1). Protein concentration was determined using the modified BCA assay (section 2.2.7.2).

Table 5.6 demonstrates that 600μg of purified transglutaminase were obtained from 1.1g of *Vicia faba* cotyledon protein. The transglutaminase was purified 500-fold with a final yield of 26%. The proteins present at each stage of purification were separated and visualised in 10% (w/v) SDS polyacrylamide gels (plate 5.2A). To test for immunological similarities between *Vicia faba* cotyledon transglutaminase and mammalian tissue transglutaminase, western blots of SDS-PAGE separated proteins were probed using a monoclonal antibody against guinea pig liver transglutaminase (plate 5.2B).

Plate 5.2 - Analysis of proteins during the purification of *Vicia faba* cotyledon transglutaminase.



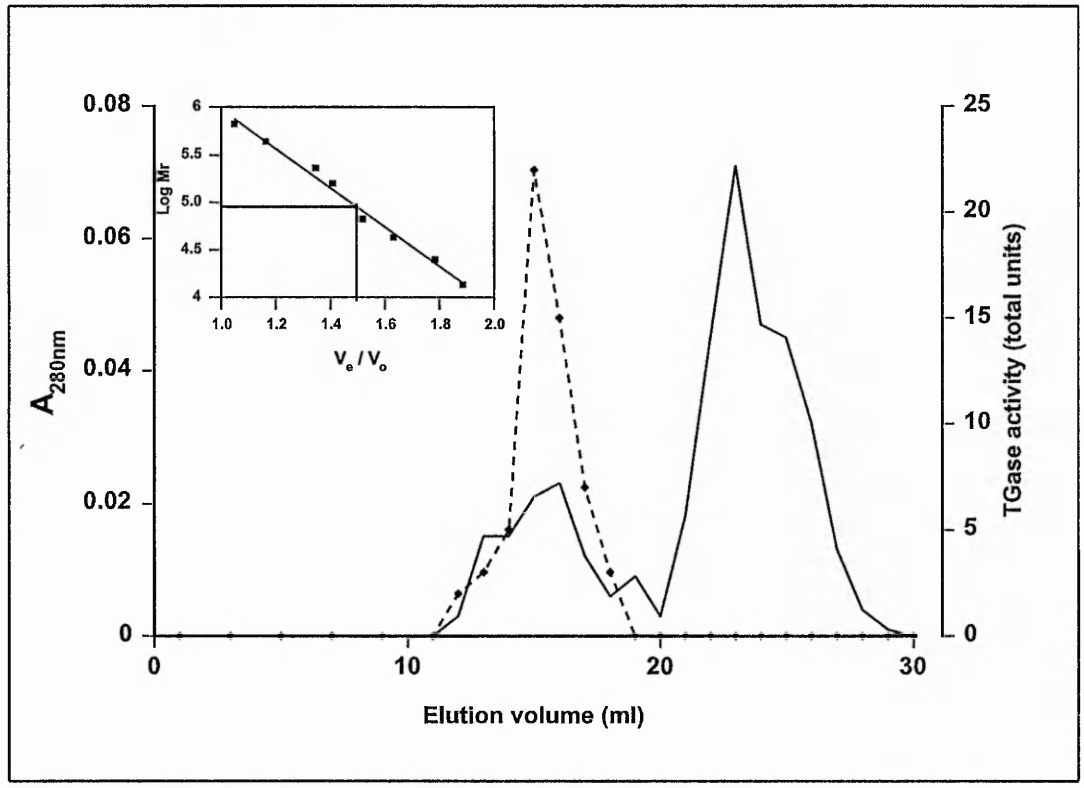
Forty micrograms of protein obtained at each stage of the purification of *Vicia faba* cotyledon transglutaminase were separated under denaturing conditions in a 10% (w/v) SDS polyacrylamide gel and stained with Coomassie blue R 250 (A), or electroblotted to nitrocellulose and probed with anti-tissue transglutaminase antibody (CUB 7402) (B), as described in section 2.2.8. *Lane 1*, crude extract; *lane 2*, IE purified protein; *lane 3*, Ca^{2+}-dependent HIC purified protein; *lane 4*, Calcium affinity IE purified protein; *lane 5*, purified guinea pig liver transglutaminase.

Plate 5.2A shows that there is an increase in band density corresponding to a polypeptide with a relative molecular mass of approximately 85 000, which is similar in molecular mass to purified tissue transglutaminase from guinea pig liver (figure 5.9A *lane 5*). Plate 5.2B shows that the anti-tissue transglutaminase antibody (CUB 7402) cross reacted with one polypeptide band with a relative molecular mass of 85 000.

5.2.3 Characterisation of partially purified *Vicia faba* cotyledon transglutaminase

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was fractionated by gel filtration chromatography on Superdex 200 to estimate the relative molecular mass of the enzyme. A typical chromatogram is presented in figure 5.8.

Figure 5.8 - Gel filtration chromatography of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase.



Two hundred microlitres of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase were applied to a Superdex 200 column (10 x 300 mm) and eluted at 0.5 ml min^{-1}, as described in section 2.2.6.6. The absorbance at 280nm was measured in 1.0 ml fractions using a Beckman DU 70 spectrophotometer. Transglutaminase activity in the eluted fractions was detected using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 1mM EDTA replaced 5mM $CaCl_2$ as a negative control for each fraction. The relative molecular mass of the protein was estimated using the calibration graph (inset) prepared with standard proteins as described in section 2.2.6.6.

Key

—— Absorbance at 280nm

------ Biotin cadaverine incorporation activity

Figure 5.8 demonstrates that *Vicia faba* cotyledon transglutaminase eluted at a volume of 15.0ml. The relative molecular mass of the active peak was estimated to be 99 000 using the calibration graph (figure 5.8 inset).

The purified *Vicia faba* cotyledon transglutaminase was further characterised in terms of calcium ion activation, substrate kinetics, pH optimum and inhibition by thiol reagents. The results are displayed in figures 5.9 to 5.11 and tables 5.7 to 5.9.

Figure 5.9 - The biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase as a function of the free calcium ion concentration of the reaction buffer.

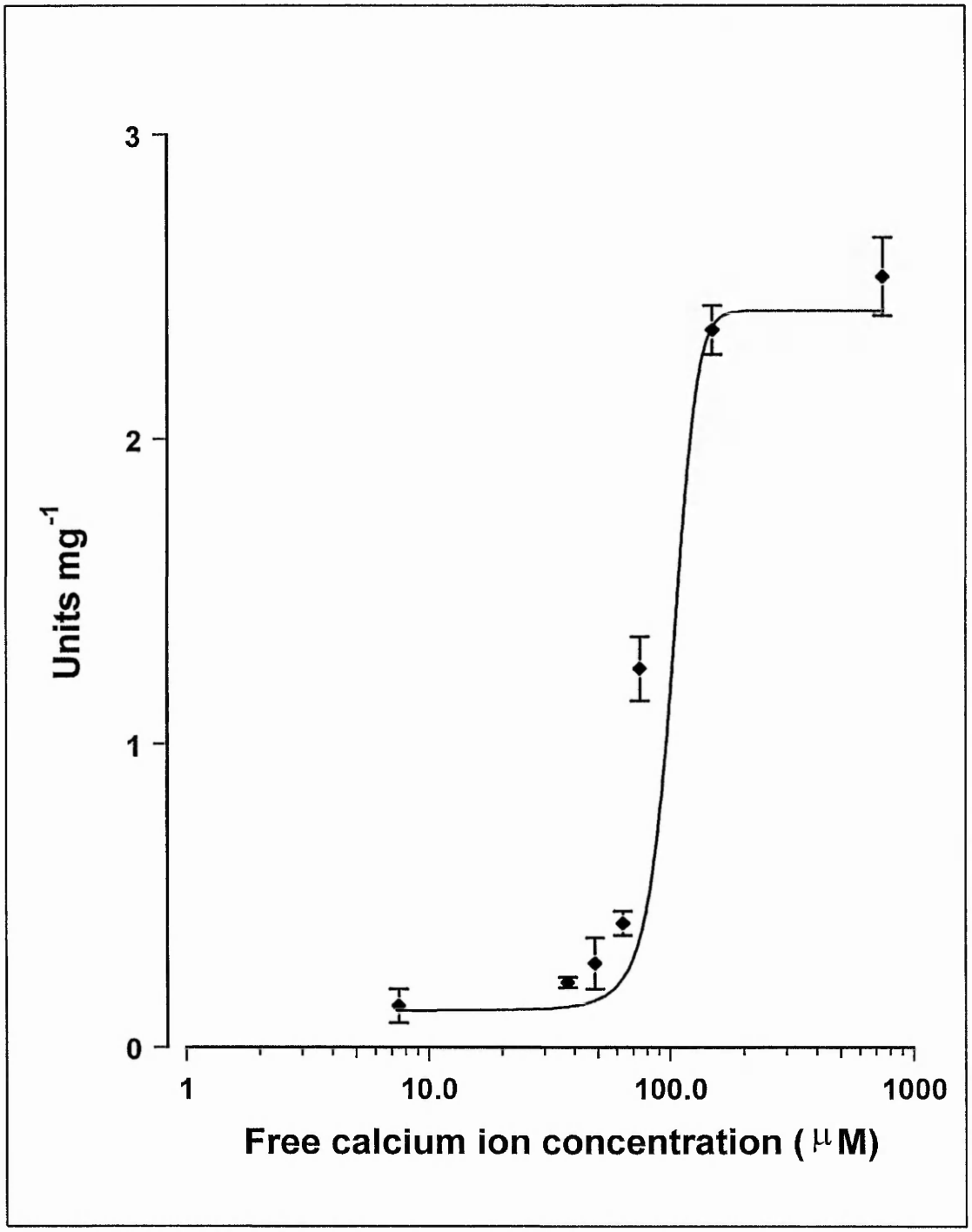


Figure 5.9 legend

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was assayed at 37°C for 60 minutes in the presence of varying free calcium ion concentrations as described in section 2.2.4.1. Zero free calcium was achieved by the addition of 1mM EGTA to the reaction buffer. Increasing amounts of calcium chloride were added to the reaction buffer and the equivalent free calcium ion concentration was calculated using the computer program EqCal (Biosoft, UK). The calculation of the free calcium ion concentration is shown in appendix 1 (page 233). Data points represent the mean ± SEM of 3 replicates.

Figure 5.9 shows that activation of the biotin cadaverine incorporating activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase occurs after 40μM free calcium, maximum activity was observed at 150μM free calcium.

It is known that GTP inhibits mammalian type II transglutaminase activity at sub-optimal calcium ion concentrations (Bergamini, 1980; Achyuthan and Greenberg, 1987; Smethurst and Griffin, 1996). The effect of GTP on affinity ion exchange purified *Vicia faba* cotyledon transglutaminase is demonstrated in table 5.7.

Table 5.7 - The effect of 1mM GTP on the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase.

Free calcium ion concentration μM	Specific activity Units mg^{-1} Uninhibited	Specific activity Units mg^{-1} + 1mM GTP
49	0.275 ± 0.084	0.042 ± 0.035 (84.7)
64	0.407 ± 0.040	0.023 ± 0.014 (94.3)
75	1.250 ± 0.106	0.048 ± 0.011 (96.2)
150	2.357 ± 0.080	0.313 ± 0.073 (86.7)
750	2.533 ± 0.129	3.374 ± 0.468 (0)

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was assayed for 60 minutes at 37°C in the presence and absence of 1mM GTP at varying free calcium ion concentrations (calculated as described for figure 5.9) using the biotin cadaverine incorporation assay as described in section 2.2.4.1. Values represent the mean ± the SEM of 3 replicates. Values in brackets represent the percentage inhibition of the biotin cadaverine incorporation activity due to the presence of 1mM GTP.

Table 5.7 shows that the biotin cadaverine incorporation activity of affinity ion exchange purified transglutaminase was inhibited 85% to 96% in the presence of 1mM GTP and 49μM to 150μM free calcium. The biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was stimulated 33% by the presence of 1mM GTP at a free calcium ion concentration of 750μM.

Table 5.8 - The effect of thiol reagents on the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase.

Inhibitor and concentration	Specific activity Units mg^{-1}	% Inhibition
Uninhibited	6.696 ± 0.538	
0.1mM iodoacetamide	0.986 ±0.316	85.28 ± 4.73
1mM iodoacetamide	N.D	100
10mM iodoacetamide	0.011 ± 0.042	99.82 ± 0.62
0.1mM N-ethylmaleimide	6.429 ± 1.200	3.98 ± 17.93
1mM N-ethylmaleimide	0.493 ± 0.101	92.64 ± 1.51
10mM N-ethylmaleimide	N.D	100
0.1mM HMBA	2.852 ± 0.055	57.40 ± 0.83
1mM HMBA	0.272 ± 0.103	95.90 ± 1.53
10mM HMBA	N.D	100

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was incubated in the presence of 5mM $CaCl_2$ and 0.1, 1, or 10mM iodoacetamide, N-ethylmaleimide or HMBA for 30 minutes at 37°C. The pre-treated extracts were assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay in the absence of DTT (section 2.2.4.1). Extract boiled for 10 minutes was used as a negative control at each inhibitor concentration. Values represent the mean ± SEM of 3 replicates. N.D no activity detectable.

Table 5.8 demonstrates that the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was inhibited 100%, 93% and 96% by 1mM iodoacetamide, N-ethylmaleimide and HMBA respectively.

Table 5.9 - The effect of adding amine donor substrates to the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase.

Inhibitor and concentration	Specific activity Units mg^{-1}	% inhibition
Uninhibited	3.067 ± 0.513	
0.1mM putrescine	1.547 ± 0.782	49.53 ± 25.50
1mM putrescine	1.420 ± 0.055	53.69 ± 1.80
10mM putrescine	0.499 ± 0.046	83.75 ± 1.51
0.1mM cystamine	2.046 ± 0.159	33.27 ± 5.20
1mM cystamine	1.820 ± 0.222	40.64 ± 7.25
10mM cystamine	0.696 ±0.061	77.32 ± 1.99

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was incubated with 5mM $CaCl_2$ and 0.1, 1, or 10mM putrescine or cystamine for 30 minutes at 37°C. The pre-treated extracts were assayed for 60 minutes at 37°C using the biotin cadaverine incorporation assay (section 2.2.4.1). 1mM EDTA replaced 5mM $CaCl_2$ as a negative control at each substrate concentration. Values represent the mean ± SEM of 3 replicates.

Table 5.9 demonstrates that the amine competitors putrescine and cystamine inhibited the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase by 84% and 77% respectively at 10mM.

Figure 5.10 - The rate of biotin cadaverine incorporation as a function of the concentration of biotin cadaverine using affinity ion exchange purified *Vicia faba* cotyledon transglutaminase.

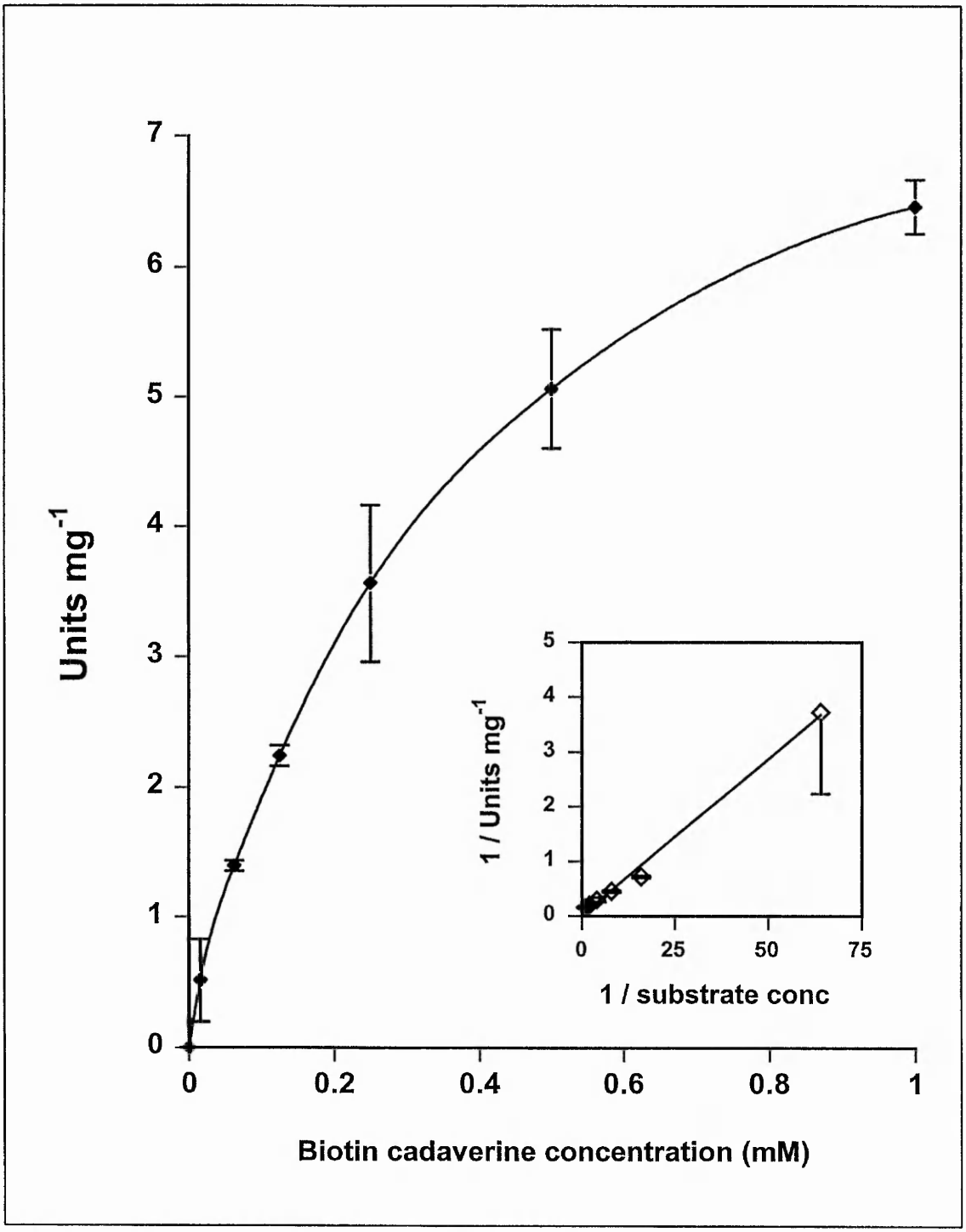


Figure 5.10 legend

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase (prepared according to section 2.2.6.4) was assayed at 37°C for 60 minutes in the presence of various concentrations of biotin cadaverine as described in section 2.2.4.1. 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control at each substrate concentration. Data points represent the mean ± SEM of 3 replicates.

Figure 5.10 shows that the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase approximates Michaelis-Menten kinetics with respect to the biotin cadaverine concentration. The K_m and V_{max} values were estimated to be 330μM and 8.51 units mg^{-1}, respectively, using Enzfitter software (Biosoft, UK).

Figure 5.11 - The variation in biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase as a function of the pH of the reaction buffer.

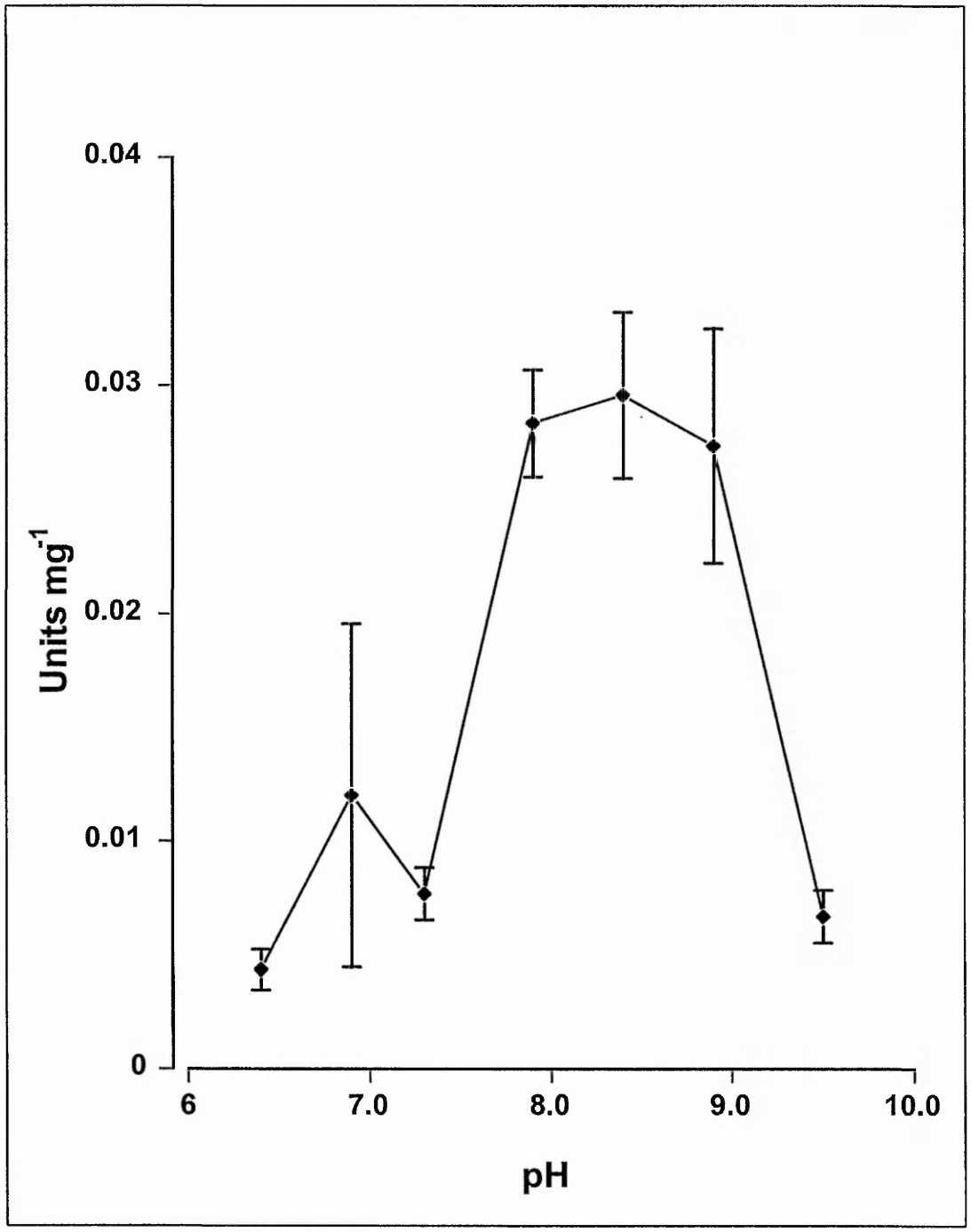


Figure 5.11 legend

Affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was assayed at 37°C for 60 minutes over the pH range 6.0 to 9.8 using the biotin cadaverine incorporation assay (section 2.2.4.1). 1mM EDTA replaced 5mM $CaCl_2$ as a negative control at each reaction pH. Data points represent the mean ± SEM of 3 replicates.

Figure 5.11 shows that purified *Vicia faba* cotyledon transglutaminase is active over a broad pH range, with an optimum at pH 8.5.

5.2.4 Activity and expression of transglutaminase during *Vicia faba* cotyledon germination

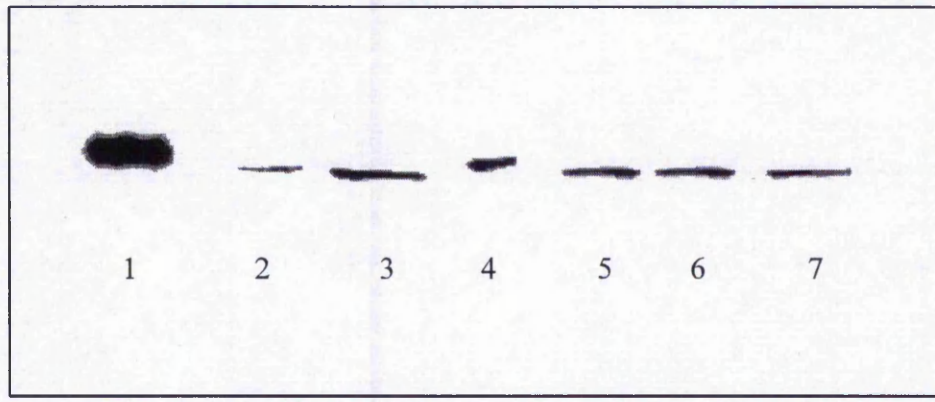
The levels of transglutaminase activity in germinating *Vicia faba* cotyledons was investigated in order to determine if the activity varied with age of the cotyledons. These results are presented in table 5.10. Proteins extracted from the cotyledons were separated by SDS-PAGE, electroblotted to nitrocellulose and probed with anti-tissue transglutaminase antibody (CUB 7402), a probed western blot is presented in plate 5.3.

Table 5.10 - The variation in transglutaminase activity with age of germinated *Vicia faba* cotyledon tissue.

Days after imbibition	Protein content mg g^{-1} FW	Total TGase activity Units g^{-1} FW
0	88.67	1.71 ± 0.36
7	55.70	2.37 ± 0.21
10	55.38	3.55 ± 0.60
14	30.34	18.41 ± 0.84
17	15.99	32.80 ± 1.67
21	13.02	33.37 ± 0.82

Protein extracted from zero to twenty one day old *Vicia faba* cotyledon tissue (as described in section 2.2.2.3) was assayed for transglutaminase activity using the biotin cadaverine incorporation assay as described in section 2.2.4.1. 5mM $CaCl_2$ was replaced with 1mM EDTA as a negative control for each sample. Protein concentration was determined using the BCA assay as described in section 2.2.7.1. Values represent the mean ± SEM of 3 replicates.

Plate 5.3 - Immunoblotting of proteins present in day 0 to day 21 *Vicia faba* cotyledon tissue with an anti-tissue transglutaminase antibody.



Forty micrograms of protein extracted from 0 to 21 day old *Vicia faba* cotyledon tissue (prepared according to section 2.2.2.3) were separated under denaturing conditions in a 10% (w/v) SDS polyacrylamide gel, electroblotted to nitrocellulose and probed with anti-tissue transglutaminase antibody (CUB 7402), as described in section 2.2.8. *Lane 1*, purified guinea pig liver transglutaminase; *lane 2*, day 0 cotyledon protein; *lane 3*, day 7 cotyledon protein; *lane 4*, day 10 cotyledon protein; *lane 5*, day 14 cotyledon protein; *lane 6*, day 17 cotyledon protein; *lane 7*, day 21 cotyledon protein.

Table 5.10 demonstrates that transglutaminase activity was low in *Vicia faba* cotyledons germinated for between 0 and 10 days. Transglutaminase activity increased substantially during day 14 and 17 and remained high in 21-day old cotyledons. Immunodetection of *Vicia faba* cotyledon transglutaminase with CUB 7402 antibody revealed the presence of a protein with a relative molecular mass of 85 000 in similar quantities at each stage of germination (plate 5.3).

5.3 Discussion

5.3.1 Purification of *Pisum sativum* soluble leaf transglutaminase

The anion exchange chromatography procedure carried out using *Pisum sativum* soluble leaf protein resulted in multiple peaks of transglutaminase activity. Figure 5.1 shows that there were three main peaks of transglutaminase activity, one corresponding to the unbound material, one eluting at a sodium chloride concentration between 0.07 and 0.19 M and one at a sodium chloride concentration between 0.23 and 0.3 M, some activity also eluted after 0.8 M sodium chloride. Table 5.1 demonstrates that 45% of the total transglutaminase activity applied to the column eluted between 0.07 M and 0.19 M sodium chloride, and represented a purification index of only 1.95 due to the removal of 55% of the transglutaminase activity. The biotin cadaverine incorporation activity of each of the pooled peaks was strongly inhibited by 1mM iodoacetamide and N-ethylmaleimide (table 5.2), indicating a cysteine residue was essential for the biotin cadaverine incorporation activity.

Lilley (1998) reported the detection of two peaks of transglutaminase activity when using anion exchange chromatography to purify *Pisum sativum* root transglutaminase, one in the unbound fraction and one in the range of sodium chloride concentrations between 0.3 and 0.5 M. The loss of large amounts of the transglutaminase activity to the unbound fraction was suggested to be due to column overloading. Plate 5.1 shows an SDS-PAGE separation of proteins present after anion exchange chromatography of *Pisum sativum* leaf extract. The proteins present in the unbound material and in each of the anion exchange peaks are different and the proteins present in the unbound material do not closely resemble those of the crude protein extract. Therefore the presence of transglutaminase activity in the three different fractions is probably not due to column overloading. Each peak represents a possible isoform or proteolytically active fragment of transglutaminase and therefore each would have to be processed separately after the initial anion exchange separation. In this case the initial loss of transglutaminase activity after one stage of purification would be approximately 55% and therefore it was decided to find a more suitable starting material for the purification of a plant transglutaminase.

5.3.2 Purification of *Vicia faba* cotyledon transglutaminase

Lilley *et al.* (1998b) showed that transglutaminase activity in germinating *Vicia faba* cotyledons increases to a peak 14-days after imbibition and the low phenol oxidase

activity in this tissue makes it a good alternative starting material. The main consideration when using cotyledons for the purification of transglutaminase is the high content of proteolytic enzymes responsible for the degradation of storage proteins in this tissue (for review see Shutov and Vaintraub, 1987).

As a measure of protease activity in *Vicia faba* cotyledons, an endopeptidase assay was used (Sarath *et al.*, 1989). Tables 5.3 and 5.4 show that 50 to 60% of *Vicia faba* cotyledon endopeptidase activity was inhibited with the cysteine protease inhibitors iodoacetamide, NEM or E_{64} and the cysteine / serine inhibitor leupeptin. The remaining endopeptidase activity was not inhibited with aspartate or metalloprotease inhibitors. Purification procedures were designed to remove this uninhibitable endopeptidase activity from the transglutaminase activity early in the purification procedure to avoid proteolysis of the purified transglutaminase. Iodoacetamide and NEM could not be included in the extraction buffer due to their inhibition of *Vicia faba* cotyledon transglutaminase activity (table 5.8).

Ammonium sulphate precipitation resulted in the *Vicia faba* cotyledon transglutaminase activity being completely precipitated by 70% saturation and the uninhibitable endopeptidase activity was completely precipitated by 60% saturation. The transglutaminase and uninhibitable endopeptidase activities could not be successfully separated by ammonium sulphate precipitation and therefore this procedure was not included in future purification protocols.

The anion exchange chromatography procedure carried out using *Vicia faba* cotyledon protein resulted in one major peak of transglutaminase and uninhibitable endopeptidase activity. Figure 5.2 shows that transglutaminase activity eluted at a sodium chloride concentration between 0.1 and 0.2 M and figure 5.3 demonstrates that the uninhibitable endopeptidase activity also eluted at a sodium chloride concentration between 0.1 and 0.2 M.

Since the binding of calcium ions to transglutaminases induces conformational changes that expose the active site cysteine residue (Chen and Mehta, 1999), it was decided to exploit the conformational changes that occur upon the binding of calcium to further purify *Vicia faba* cotyledon transglutaminase. Calcium dependent hydrophobic

interaction and affinity chromatography have been successfully used to purify transglutaminase from *Physarum polycephalum* (Mottahedeh and Marsh, 1998) and rat liver (Croall and DeMartino, 1986) respectively.

Ion exchange purified *Vicia faba* cotyledon transglutaminase was applied onto a phenyl Sepharose column in the presence of 5mM $CaCl_2$ and eluted by the chelation of calcium with 5mM EDTA (figure 5.4). In the presence of calcium *Vicia faba* transglutaminase exposes a hydrophobic surface enabling the selective binding of the enzyme. Removal of the bound calcium with 5mM EDTA, caused conformational changes, which masked the hydrophobic surface and eluted the enzyme from the resin. Figure 5.5 demonstrates that the majority of the uninhibitable endopeptidase activity was separated from the transglutaminase activity by this procedure because the endopeptidase activity failed to bind to the resin under these conditions.

Calcium dependent ion exchange chromatography has been used to purify calcium binding proteins (Maruyama *et al.*, 1985). A vitamin D-dependent calcium binding protein was purified from bovine cerebellum and kidney by binding the protein to a DEAE-cellulose column, the protein was eluted at 120 to 150 mM NaCl in the absence of Ca^{2+} (0.1mM EGTA) or with 60 to 90mM NaCl in the presence of 0.2mM $CaCl_2$. In a modification of this procedure, *Vicia faba* cotyledon transglutaminase was shown to elute from a Q10 anion exchange column with 60 to 120mM sodium chloride in the absence of calcium (figure 5.6) or with 40mM sodium chloride in the presence of 1mM calcium chloride (figure 5.7). The conformational changes induced by the binding of calcium to transglutaminase were sufficient to elute the protein from the column at a lower salt concentration. This calcium affinity ion exchange procedure represents a novel application to transglutaminase purification.

A summary of the four steps involved in the purification of *Vicia faba* cotyledon transglutaminase are presented in table 5.6. *Vicia faba* cotyledon transglutaminase was purified 500-fold with an overall recovery of 26% of the enzyme activity.

SDS-PAGE analysis of the proteins present at each stage of purification (plate 5.2A) demonstrates the reduction in the number of protein bands at each stage of purification and an increase in band density corresponding to a polypeptide with a relative molecular

mass of 85 000. Plate 5.2B shows a western blot of the SDS-PAGE separated proteins probed with a monoclonal anti-tissue transglutaminase antibody (CUB 7402) and shows the relative amounts of the enzyme at each stage of the purification. A single band with a relative molecular mass 85 000 was present. This observation closely corresponds to an estimated relative molecular mass of 80 000 for transglutaminase purified from *Glycine max* leaf tissue (Kang and Cho, 1996).

5.3.3 Characterisation of *Vicia faba* cotyledon transglutaminase

Gel filtration chromatography of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase on Superdex 200 showed a single peak of activity corresponding to a relative molecular mass of 99 000 (figure 5.8). The molecular mass estimated by gel filtration closely resembles that estimated by SDS-PAGE (plate 5.2) suggesting that transglutaminase from *Vicia faba* cotyledons functions as a monomer.

Activation of the biotin cadaverine incorporating activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase by calcium ions occurs after the addition of 40μM free calcium (figure 5.9). Maximum activity was observed at 150μM free calcium. Activation of *Vicia faba* cotyledon transglutaminase activity occurs at higher free calcium concentrations compared with *Pisum sativum* root transglutaminase, which had maximum activity at 94nM free calcium when the activity was measured using the biotin cadaverine incorporation assay and crude preparations of the enzyme (Lilley *et al.*, 1998a).

The activity of mammalian tissue transglutaminase is inhibited by GTP at sub-optimal calcium ion concentrations (Achyuthan and Greenberg, 1987; Bergamini, 1980; Smethurst and Griffin, 1996). Achyuthan and Greenberg (1987) reported a complete inhibition of purified guinea pig liver transglutaminase at 1mM $CaCl_2$ and 1mM GTP and a 50% reduction in [^{3}H] putrescine incorporation when 5mM $CaCl_2$ was present. Smethurst and Griffin (1996) reported that tissue transglutaminase activity in permeabililized ECV-304 cells was decreased virtually to zero in the presence of 10nM and 10μM free calcium when ATP and GTP were present at concentrations mimicking cytosolic levels, only at 100μM free calcium when nucleotides were low or absent was transglutaminase activity detected. Table 5.7 shows that the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon

transglutaminase was inhibited 85 to 96% by 1mM GTP at 49 to 150μM free calcium. In the presence of 1mM GTP and 750μM free calcium the biotin cadaverine incorporation activity was stimulated 32%. These results suggest that *Vicia faba* cotyledon transglutaminase is similar to mammalian tissue transglutaminase in that it contains a GTP binding site and the transglutaminase activity could be regulated by GTP at resting levels of cytosolic calcium. Very little data is available on the GTP binding properties of other plant transglutaminases, however, Kang and Cho (1996) reported that transglutaminase purified from *Glycine max* leaf tissue was inhibited by increasing concentrations of GTP, however this enzyme had no absolute requirement for calcium ions. In crude preparations from *Pisum sativum* root tissue the biotin cadaverine incorporating activity was not inhibited by 1mM GTP at 1μM free calcium (Lilley *et al.*, 1998a), indicating this plant transglutaminase activity is different from mammalian tissue transglutaminase.

Mammalian transglutaminases have a cysteine residue at their active site and are inhibited by thiol reactive reagents such as iodoacetamide, N-ethylmaleimide and HMBA (Folk and Cole, 1966). Table 5.8 shows that the biotin cadaverine incorporation activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase was inhibited 100, 93 and 96% by 1mM iodoacetamide, NEM and HMBA respectively. The data presented in table 5.8 strongly suggests there is a cysteine residue essential for the activity of *Vicia faba* cotyledon transglutaminase. Similarly, Kang and Cho (1996) reported a 60% inhibition of the [^{14}C]-labelled putrescine incorporating activity of purified *Glycine max* leaf transglutaminase in the presence of 100μM NEM.

The competitive amine donor substrates putrescine and cystamine were shown to inhibit the transglutaminase catalysed incorporation of biotin cadaverine in to N,N'-dimethylcasein by 83% and 77% respectively at 10mM (table 5.9). This data would suggest that the affinity purified *Vicia faba* cotyledon transglutaminase is able to utilise other acyl acceptor substrates in place of cadaverine and strongly supports the findings of other workers (Icekson and Apelbaum, 1987; Serafini-Fracassini *et al.*, 1988).

Figure 5.10 shows the relationship between biotin cadaverine concentration and the activity of affinity ion exchange purified *Vicia faba* cotyledon transglutaminase. The K_m and V_{max} values were estimated to be 330μM and 8.51 units mg^{-1} respectively. A K_m

value of 190μM was estimated for ion exchange purified *Pisum sativum* root transglutaminase using the biotin cadaverine incorporation assay (Lilley, 1998). The K_m value for the *Vicia faba* cotyledon transglutaminase is lower than those estimated for *Pisum sativum* leaf soluble and microsomal activities of 473μM and 618μM respectively (figures 4.3 and 4.4). These differences could represent the enzyme's differences in affinity for biotin cadaverine as the acyl acceptor substrate. Alternatively, the reduction in other preferential endogenous acyl donor and / or acceptor substrates brought about by the purification of *Vicia faba* cotyledon transglutaminase would produce a lower estimate for the K_m value for biotin cadaverine.

The pH optimum of *Vicia faba* cotyledon transglutaminase was shown to be pH 8.5. Figure 5.11 demonstrates that the activity remained at approximately 100% 0.5 pH units either side of the optimum, but fell sharply as the pH was increased above pH 9.0 or decreased below pH 8.0. Other workers have demonstrated similar pH optima, *Pisum sativum* root transglutaminase was shown to have a pH optimum of 7.9 (Lilley *et al.*, 1998a).

5.3.4 Possible roles for transglutaminase in *Vicia faba* cotyledon tissue

Transglutaminase activity was shown to be low in *Vicia faba* cotyledon tissue up to 10-days after imbibition and increased in 14- to 21-day old cotyledon tissue (table 5.10). SDS-PAGE and western blotting of proteins present in day 0 to day 21 *Vicia faba* cotyledons revealed cross reactivity with the monoclonal anti-tissue transglutaminase antibody CUB 7402 (plate 5.3). The enzyme was detected in 0-, 7- and 10-day old cotyledons in comparable amounts to that found in 14-, 17- and 21-day old cotyledons even though the activity was considerably lower. It has been reported that the appearance of transglutaminase activity in *Vicia faba* cotyledons coincides with an increase in the elecrophoretic mobility of extracted globulins (Lilley *et al.*, 1998b) and therefore transglutaminase could be responsible for the deamidation of storage proteins which has been proposed as a necessary prelude to the proteolytic digestion of reserve proteins (Daussant *et al.*, 1969). In addition, the ammonia released by deamidation could serve as an immediate nitrogen source for the developing seedling (Lilley *et al.*, 1998b). Alternatively, transglutaminase could be synthesized and stored during seed development from where it is activated during germination. In *Glycine max,* transglutaminase specific activity was shown to be 37-fold higher in the developing

seeds compared to the mature leaves (Kang *et al.*, 1998). In developing *Glycine max* seeds transglutaminase has been postulated to be involved in the polymerisation and stabilisation of glycinin subunits in the developing seeds or alternatively the transglutaminase catalysed modifications of glycinin could be a signal for rapid degradation of the storage protein during seed germination (Kang *et al.*, 1998).

Transglutaminase activity measured using the biotin cadaverine incorporation assay only measures one of the four possible reactions that transglutaminases can catalyse, the incorporation of amines in to proteins.

Transglutaminase present within developing seeds could be involved in the formation of the testa, the outer barrier of the seed that protects the embryo during desiccation. Other forms of transglutaminase have been shown to be involved in stabilizing structures such as the extracellular matrix, apoptotic bodies, fibrin clots formed in haemostasis and wound healing and the cornified envelope, by the formation of ε-(γ-glutamyl) lysine crosslinks between proteins (for review see Aeschlimann and Paulsson, 1994). In the slime mould *Physarum polycaphalum* transglutaminase was suggested to be involved in spherule formation, which have the capacity to survive starvation and desiccation (Klein *et al.*, 1992).

Chapter 6 – Concluding remarks

In *Pisum sativum* leaf tissue, transglutaminase activity was present in both the soluble and microsomal fraction (table 3.1). Solubilization of the microsomal activity by detergent (figure 3.1) or trypsin treatment (table 3.3) and not by high salt, treatment with chelating agents or alkaline pH buffer (table 3.2) suggests the enzyme is associated with the membrane via 1 or 2 transmembrane segments or through lipid anchorage. Incubation of *Pisum sativum* washed microsomal preparations with increasing concentrations of detergent and the removal of insoluble material by centrifugation, revealed that optimum transglutaminase activity was solubilized by treatment with 0.23% (w/v) sodium deoxycholate (figure 3.1). If the insoluble material was not removed by centrifugation prior to assaying for transglutaminase activity, the microsomal activity was only enhanced 1.5-fold by the presence of 0.23% (w/v) sodium deoxycholate (table 3.1), suggesting that transglutaminase is orientated in the membrane such that it is readily accessible to substrate. Alternatively, solubilization of the microsomal preparation could solubilize amine acceptor substrates which are preferentially utilised in place of N,N'-dimethylcasein.

A membrane-associated form of the mammalian tissue transglutaminase has been described in several different tissues (Birckbichler *et al.*, 1976; Slife *et al.*, 1985 and 1987; Juprelle-Soret *et al.*, 1988) and preliminary data suggests the presence of a fatty acid anchor on tissue transglutaminase (Seitz *et al.*, 1991). Several other mammalian forms of transglutaminase are membrane bound via lipid anchors, however, the nature of the anchor and the site of attachment is not conserved among transglutaminases and multiple types of modifications can occur on the same enzyme (Steinert *et al.*, 1996b; Esposito *et al.*, 1996; Cohen *et al.*, 1993).

The fractionation of *Pisum sativum* microsomal membrane preparations by sucrose density gradient centrifugation and identification of membranes using marker enzyme assays revealed that the transglutaminase activity was enriched on the tonoplast membrane (tables 3.5 – 3.11). The mechanism targeting transglutaminase to the tonoplast is presently unknown. The protein could be co- or post-translationally modified by lipids, thereby sequestering the enzyme onto the membrane. Alternatively the protein could be targeted to the tonoplast through the transport vesicle pathway,

which would require targeting information to be present within the protein (Jiang and Rogers, 1999). Without protein or gene sequence information details of protein targeting and membrane attachment can only be hypothesised.

Since the *Pisum sativum* transglutaminase activities were studied in the whole leaf tissue, it is not possible to determine which cell type(s) are responsible for the soluble and tonoplast activities or whether the two activities are expressed in several cell and tissue types.

The association of transglutaminase with the tonoplast membrane may suggest that the enzyme preferentially incorporates polyamines into tonoplast proteins or crosslinks cytoskeletal proteins to tonoplast associated proteins. The distribution of actin cytoskeleton in root statocytes of *Lens culinaris* was shown to be scattered in the cytoplasm always close to or on the nuclear and amylopast envelopes and the tonoplast (Driss-Ecole *et al.*, 2000). Actin microfilaments were also found in association with the vacuoles and the endoplasmic reticulum of *Pyrus communis* pollen tubes (Tiwari and Polito, 1988). Isodipeptide crosslinks have been detected in the cytoskeleton of *Physarum polycephalum* (Loewy and Matacic, 1981) and actin was shown to be a substrate for transglutaminase in the same organism (Klein *et al.*, 1992). Klein *et al.*, (1992) proposed that changes in organisation of the cytoskeleton that accompany spherule formation may involve *Physarum polycephalum* transglutaminase catalysed crosslinking of actin or actin filaments. Furthermore, a membrane associated transglutaminase detected in *Malus domestica* pollen was shown to use actin as a substrate, the pollen transglutaminase was suggested to be involved in the rapid cytoskeletal rearrangements that occur during the organisation and growth of pollen tubes (Del Duca *et al.*, 1997).

Characterisation of the soluble and microsomal transglutaminases of *Pisum sativum* leaf tissue revealed the enzymes had similar biochemical activities in the presence of sodium deoxycholate. For example, calcium ion requirement (figures 4.1 and 4.2), response to thiol reagents (table 4.1 and 4.2), substrate kinetics (figures 4.3 and 4.4) and inhibition by GTP (tables 4.6 and 4.7). The molecular mass estimate predicted by SDS-PAGE and immunoblotting with the monoclonal anti-tissue transglutaminase antibody (CUB7402) revealed similarities in the molecular masses of the two proteins (plate 4.4). Despite

these similarities it remains uncertain whether these two activities are closely related, the true relationship between the two transglutaminases can only be truly elucidated by comparing the two proteins after purification to homogeneity. Obtaining the protein sequences would reveal the overall degree of homology and would provide valuable information regarding the nature of the membrane association, for example, if there is a transmembrane domain or a conserved sequence characteristic of lipid modified proteins.

The presence of more than one form of transglutaminase in *Pisum sativum* leaf tissue is not unlikely since, in mammals for example, at least three forms of transglutaminase are expressed in the epidermis (Aeschlimann and Paulsson, 1994). Similarly, when transglutaminase activity was studied as a function of [^{3}H] putrescine incorporation using extracts prepared for *Helianthus tuberosus* sprout apices, a sigmoidal relationship was observed indicating the presence of two enzyme activities contributing to the assay signal (Serafini-Fracassini *et al.*, 1988). In the 22 000.g supernatant fraction prepared form *H. tuberosus* leaves and the 1 500.g supernatant fraction prepared for tuber parenchyma, pH profiles showed bimodal trends, suggesting two activities may be present in each of the organs (Falcone *et al.*, 1993). However, in these plant tissues one cannot rule out the presence of diamine oxidases, which could interfere with the assay used to detect transglutaminase activity in these tissues.

The conformational changes induced by the binding of calcium to *Vicia faba* cotyledon transglutaminase were exploited in the purification of the enzyme by the binding and elution of the enzyme from both ion exchange (figure 5.7) and hydrophobic interaction chromatography resins (figure 5.4). The enzyme was purified 500-fold with an overall yield of 26% (table 5.6) using the techniques of ion exchange (figure 5.2), calcium dependent hydrophobic interaction (figure 5.4) and calcium affinity ion exchange chromatography (figures 5.6 and 5.7). The conformational changes induced by the binding of calcium to transglutaminases are well documented. Dynamic simulations of calcium binding to type II transglutaminase confirmed that a flexible loop on the enzyme's surface between the core domain and the C-terminal domain is modified in the presence of calcium (Casadio *et al.*, 1999). This loop unwinds and looses its secondary structure and as a consequence, the two C-terminal β barrel domains move apart allowing interaction between the active site and substrate (Casadio *et al.*, 1999).

Furthermore, the binding of a hydrophobic probe, ANS, to type II transglutaminase was substantially increased in the presence of calcium confirming that changes in protein structure allow the ANS molecules to penetrate better into a hydrophobic pocket in the presence of calcium (Di Venere *et al.*, 2000).

The transglutaminase from *Vicia faba* cotyledons shares several properties with transglutaminases isolated from other sources, both mammalian and plant. Firstly, the relative molecular mass of 85 000 estimated for the enzyme from *Vicia faba* cotyledons (plate 5.2 and figure 5.8) is similar to that of 80 000 reported for the enzyme purified from *Glycine max* leaves (Kang and Cho, 1996), and that with a relative molecular mass of 79 000 reported for the enzyme purified from guinea pig liver (Ikura *et al.*, 1988). Like the mammalian enzymes, and unlike that from *Glycine max* leaf tissue, the transglutaminase purified from *Vicia faba* cotyledons is calcium dependent and activated at 50μM free calcium (figure 5.9). The transglutaminase purified from guinea pig liver (Folk and Cole, 1966), *Physarum polycephalum* (Klein *et al.*, 1992) and *Glycine max* (Kang and Cho, 1996) all have a thiol group at their active site that is essential for catalytic activity. The enzyme from *Vicia faba* cotyledon also has a presumptive free thiol group that is essential for activity, since iodoacetamide, N-ethylmaleimide and HMBA, at 1mM, caused 100, 93 and 96% inhibition of the biotin cadaverine incorporating activity, respectively (table 5.8). A conserved active site sequence Tyr-Gly-Gln-Cys-Trp may prove to be a feature of *Vicia faba* cotyledon transglutaminase as it is for other mammalian transglutaminases inhibited by thiol reagents (Ikura *et al.*, 1989; Ichinose *et al.*, 1990; Kim *et al.*, 1990).

Mammalian transglutaminases evolved from a common ancestor and show strong sequence conservation in a number of regions including that containing the acyl-accepting cysteine residue of the active site (Aeschlimann and Paulsson, 1994). This common ancestry is shared with the arthropod transglutaminases of limulus hemocytes (Tokunaga *et al.*, 1993a) and grasshopper embryo (Singer *et al.*, 1992). The calcium independent bacterial transglutaminase from *Streptoverticillium mobarage* appears to have evolved independently (Kanaji *et al.*, 1993) as have the filarial parasite transglutaminases (Mehta *et al.*, 1992). However, the similarities between the *Vicia faba* cotyledon transglutaminase and mammalian tissue transglutaminase may indicate that

this plant transglutaminase has evolved in a similar manner to the mammalian and arthropod transglutaminases.

The biotin cadaverine incorporation activity of *Pisum sativum* and *Vicia faba* extracts has been used as a measure of transglutaminase activity in these tissues. The incorporation of polyamines in to peptide-bound glutamine residues is just one of the four possible reactions which transglutaminases are able to catalyse (Folk and Chung, 1973). It is hypothesised that the transglutaminases described in this thesis may also catalyse polyamine and protein crosslinking and protein deamidation. However, the only unequivocal proof for this would be to isolate the ε-(γ-glutamyl) lysine isodipeptide crosslink from the tissues studied (Folk and Finlayson, 1977).

Mammalian transglutaminases are responsible for stabilising structures such as the extracellular matrix, apoptotic bodies formed during apoptosis, the cornified envelope in terminally differentiating keratinocytes and semen coagulation in the rat genital tract (for review see Aeschlimann and Paulsson, 1994). Similar transglutaminases in fish (Ha and Iuchi, 1997), grasshopper embryo (Singer *et al.*, 1992), limulus hemocyte (Tokunaga *et al.*, 1993b) and fungi (Klein *et al.*, 1992) have also been found to function in the stabilisation of protein structure. It is possible that transglutaminases perform similar stabilising roles to these in plant tissues. It has been reported previously that a transglutaminase activity in *Medicago sativa* is rapidly induced in leaf and stem tissue following wounding, with a 10- to 15-fold increase in activity within 12 to 24 hours (Margosiak *et al.*, 1987). Fluorescence microscopy and immunoblotting revealed that mechanical wounding of post harvest potato tubers resulted in localised increases in crosslinked actin within 12 hours of wounding (Morelli *et al.*, 1998). The crosslinking of actin in potato tubers could be carried out by transglutaminase, which has previously been detected in potato tubers (Votyakova *et al.*, 1999) and would support other data in the utilisation of actin as a substrate for transglutaminase and the induction of transglutaminase activity in wounded tissue (Margosiak *et al.*, 1987; Klein *et al.*, 1992; Del Duca *et al.*, 1997).

The ε-(γ-glutamyl) lysine isodipeptide crosslink detected in plant tissues (Lilley *et al.*, 1997a) could possibly be formed through the crosslinking of proteins in cells undergoing programmed cell death. Apoptosis in animal cells is characterised by an

identifiable set of biochemical and morphological changes including nuclear and cytoplasmic condensation and fragmentation, cleavage of DNA at specific sites, plasma membrane blebbing, apoptotic body formation and phagocytosis of these bodies by neighbouring cells and macrophages (Fesus *et al.*, 1991). The presence of a rigid cell wall in plant cells prevents the dying cell from being packaged into apoptotic bodies and being engulfed by neighbouring cells (Lam *et al.*, 1999). The self-ingestion approach, involving disruption of the vacuole and autolysis of the cell's contents, is likely to be a common strategy for the disassembly of plant cells undergoing cell death (Lam *et al.*, 1999). Indeed programmed cell death involving autolysis of the cell has been shown to occur during tracheary element differentiation (Groover and Jones, 1999; Fukuda, 2000), leaf and whole plant senescence (Matile, 1997) and death of the suspensor in developing *Phaseolus coccineus* seeds after its role is completed (Jones and Dangl, 1996). Changes in the cell morphology may differ depending on the particular inductive cell death signal. Hypersensitive cell death is a form of programmed cell death that can occur at the site of attack by an avirulent pathogen in a plant containing resistant genes (Pennell and Lamb, 1997). Cells that die during the hypersensitive response in *Arabidopsis thaliana* and *Glycine max* leaves and *Nicotiana tobacum* and *Glycine max* suspension culture cells condense, shrink and ultimately resemble apoptotic corpses (Levine *et al.*, 1996). Wang *et al.* (1996) described the appearance of distinct bodies, resembling apoptotic bodies, after treatment of tomato protoplasts with the hypersensitive response elicitor arachidonic acid. The cell corpses produced during programmed cell death are eventually pushed together mechanically by new cell growth (Jones and Dangl, 1996). Plant transglutaminase could therefore be involved in the formation of apoptotic bodies in these tissues in a similar process to that described previously in animal cells (Fesus *et al.*, 1991).

The increasing transglutaminase activity detected in 14- and 21-day old *Vicia faba* cotyledons could be involved in programmed cell death of the cotyledons after their role in germination is complete.

The work presented in this thesis establishes the presence of different forms of transglutaminase in plant tissues. In order to elucidate the role for the enzyme in plant tissues, the purification procedures described in chapter 5 require improvement or additional steps need to be included to enable the enzyme to be purified to homogeneity.

It may be possible to improve the purification techniques described in chapter 5 by altering and optimising the salt gradients and $CaCl_2$ concentrations used for the binding and elution of the transglutaminase activity. Alternatively an affinity purification step could be introduced involving the selective binding of transglutaminase to a matrix crosslinked to the CUB7402 monoclonal antibody or GTP nucleotides.

The purified enzyme may be sequenced and the sequence compared to those of known mammalian transglutaminases to determine areas of sequence homology. Nucleotide probes could be designed, used to monitor mRNA expression and to isolate the plant transglutaminase gene. Additionally, monoclonal antibodies may be raised to the purified protein and used as a probe for transglutaminase in plant tissue sections, which will help determine the subcellular localisation, the tissue distribution of the activity and subsequently elucidate possible roles for the enzyme in plant tissues.

Chapter 7 – References

Achyuthan, K.E. and Greenberg, C.S. (1987) Identification of a guanosine triphosphate-binding site on guinea pig liver transglutaminase. Role of GTP and calcium ions in modulating activity. *J. Biol. Chem.* **262**, 1 901-1 906.

Aeschlimann, D. and Paulsson, M. (1991) Cross-linking of laminin-nidogen complexes by tissue transglutaminase. *J. Biol. Chem.* **266**, 15 308-15 317

Aeschlimann, D. and Paulsson, M. (1994) Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb. Haemostasis* **71**, 402-415.

Aeschlimann, D., Koeller, M.K., Allen-Hoffman, B.L. and Moshar D.F. (1998) Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. *J. Biol. Chem.* **273**, 3 452-3 460.

Aeschlimann, D. and Thomazy, V. (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. *Conn. Tiss. Res.* **41**, 1-27.

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H. and Motoki, M. (1989) Purification and characterisation of a novel transglutaminase derived from microorganisms. *Agric. Biol. Chem.* **53**, 2 613-2 617.

Apelbaum, M., Canellakis, Z.N., Applewhite, P.B., Kaur-Sawney, R. and Galston, A.W. (1988) Binding of spermidine to a unique protein in thin layer tobacco tissue culture. *Plant Physiol.* **88**, 996-998.

Appelt, D.M. and Balin, B.J. (1997) The association of tissue transglutaminase with human recombinant tau results in the formation of insoluble filamentous structures. *Brain Res.* **745**, 21-31.

Aribaud, M., Carre, M. and Martin-Tanguy, J. (1995) Transglutaminase-like activity in chrysanthemum leaf explants cultivated *in vitro* in relation to cell growth and hormone treatment. *Plant Growth Regul.* **16**, 11-17.

Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* **24**, 1-15

Barsigian, C., Stern, A.M. and Martinez, J. (1991) Tissue (type II) transglutaminase covalently incorporates itself, fibrinogen, or fibronectin into high molecular weight complexes on the extracellular surface of isolated hepatocytes. Use of 2-[(2-oxopropyl)thio] imidazolium derivatives as cellular transglutaminase inactivators. *J. Biol. Chem.* **266**, 22 501-22 509.

Bencini, D.A., Wild, J.R. and O'Donovan, G.A. (1983) Linear one-step assay for the determination of orthophosphate. *Anal. Biochem.* **132**, 254-258.

Bendixen, E., Borth, W. and Harpel, P.C. (1993) Transglutaminases catalyse crosslinking of plasminogen to fibronectin and human endothelial cells. *J. Biol. Chem.* **268**, 21 962-21 967.

Beninati, S., Piacentini, M., Cocuzzi, E.T., Autuori, F. and Folk, J.E. (1988) Covalent incorporation of polyamines as γ-glutamyl derivatives into CHO cell protein. *Biochim. Biophys. Acta.* **952**, 325-333.

Bergamini, C.M. (1988) GTP modulates calcium binding and cation-induced conformational changes in erythrocyte transglutaminase. *FEBS. Lett.* **239**, 255-258.

Bergamini, C.M. and Signorini, M. (1992) Purification of testicular transglutaminase by hydrophobic chromatography on phenyl-Sepharose. *Biochem. Int.* **27**, 557-565.

Bernet, E., Claparols, I., Dondini, L., Santos, M.A., Serafini-Fracassini, D. and Torne, J.P. (1999) Changes in polyamine content, arginine and ornithine decarboxylases and transglutaminase activities during light/dark phases (of initial differentiation) in maize calluses and their chloroplasts. *Plant Physiol. Biochem.* **37**, 1-11.

Birckbichler, P.J., Orr, G.R. and Patterson M.K. (1976) Differential transglutaminase distribution in normal rat liver and rat hepatoma. *Cancer Res.* **36**, 2 911-2 914.

Bischoff, F., Molendijk, A., Rajendrakumar, C.S.V. and Palme, K. (1999) GTP-binding proteins in plants. *Cell. Mol. Life Sci.* **55**, 233-256.

Board, P.G., Losowsky, M.S. and Mloszewski, K.J. (1993) Factor XIII: inherited and acquired deficiency. *Blood Rev.* **7**, 229-242.

Bohn, H., Haupt, H. and Kranz, T. (1972) Molecular structure of human fibrin stabilizing factors. *Blut.* **25**, 235-248.

Boothe, R.L. and Folk, J.E. (1969) A reversible, calcium-dependent, copper-catalyzed inactivation of guinea pig liver transglutaminase. *J. Biol. Chem.* **244**, 399-405.

Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**, 1 604-1 607.

Bottenus, R.E., Ichinose, A., and Davie, E.W. (1990) Nucleotide sequence of the gene for the b subunit of human factor XIII. *Biochemistry* **29**, 11 195-11 209.

Bowness, J.M., Venditti, M., Tarr, A.H. and Taylor, J.R. (1994) Increase in ε(γ-glutamyl)lysine crosslinks in atherosclerotic aortas. *Atherosclerosis* **111**, 247-253.

Brookhart, P.P., McMahon, P.L. and Takahashi, M. (1983) Purification of guinea pig liver transglutaminase using a phenylalanine-Sepharose 4B affinity column. *Anal. Biochem.* **128**, 202-205.

Brown, R.E., Jarvis, K.L. and Hyland, K.J. (1989) Protein measurement using bicinchoninic acid: Elimination of interfering substances. *Anal. Biochem.* **180**, 136-139.

Brusca, J.S. and Radolf, J.D. (1994) Isolation of integral membrane proteins by phase partitioning with Triton X-114. *Methods Enzymol.* **228**, 182-193.

Bush, D.S. (1995) Calcium regulation in plant cells and its role in signalling. *Ann. Rev. Plant Physiol.* **46**, 95-122.

Butler, P.E. (1989) Solubilization of membrane proteins by proteolysis. In *Proteolytic enzymes A practical approach.* Eds Beynon, R.J. and Bond, J.S. IRL Press, Oxford, UK. pp 193-200.

Bylund, D.B., Regan, J.W., Faber, J.E., Hieble, J.P., Triggle, C.R. and Ruffalo, R.R. Jr. (1995) Vascular alpha-adrenoceptors: from the gene to the human. *Can. J. Physiol. Pharm.* **73**, 533-543.

Candi, E., Terrinoni, A., Oddi, S., Paradisi, A. and Melino, G. (2000) Biochemical characterization of transglutaminase X. *Proceedings of the VIth international conference on transglutaminase and protein crosslinking.*

Casasdio, R., Polverini, E., Mariani, P., Spinozzi, F., Carsughi, F., Fontana, A., Polverino de Laureto, P., Matteucci, G. and Bergamini, C.M. (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions. *Eur. J. Biochem.* **262**, 672-679.

Chakravarty, R. and Rice, R.H. (1989) Acylation of keratinocyte transglutaminase by palmitic and myristic acids in the membrane anchorage region. *J. Biol. Chem.* **264**, 625-629.

Chandrashekar, R., Tsuji, N., Morales, T., Ozols, V. and Mehta, K. (1998) An Erp60-like protein from the filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. *Proc. Natl. Acad. Sci. USA*. **95**, 531-536.

Chang, S.K. and Chung, S.I. (1986) Cellular transglutaminases the particulate-associated transglutaminase from chondrosarcoma and liver: partial purification and characterization *J. Biol. Chem.* **261**, 8 112-8 121.

Chen, J.S. and Mehta, K. (1999) Tissue transglutaminase: an enzyme with a split personality. *Int. J. Biochem. Cell Biol.* **31**, 817-836.

Chen, S., Lin, F., Iismaa, S., Lee, K.N., Birckbichler, P.J. and Graham, R.M. (1996) α1-adrenergic receptor signalling via G_h is subtype specific and independent of its transglutaminase activity. *J. Biol. Chem.* **271**, 32 385-32 391.

Chiarello, M.D., Larre, C., Kedzior, Z.M. and Gueguen, J. (1996a) Pea seedling extracts catalyze protein amine binding and protein cross-linking. 1. Evidence for the role of a diamine oxidase. *J. Agric. Food. Chem.* **44**, 3 717-3 722.

Chiarello, M.D., Larre, C., Kedzior, Z.M. and Gueguen, J. (1996b) Pea seedling extracts catalyze protein amine binding and protein cross-linking. 2. Contribution of diamine oxidase to these reactions. *J. Agric. Food. Chem.* **44**, 3 723-3 726.

Chung, S.I. (1972) Comparative studies on tissue transglutaminase and factor XIII. *Ann. N. Y. Acad. Sci.* **202**, 240-255.

Clark, D.D., Mycek, M.J., Neidle, A. and Waelsch, H. (1959) The incorporation of amines into proteins. *Arch. Biochem. Biophys.* **79**, 338-354.

Cohen, C.M., Dotimas, E. and Korsgren, C. (1993) Human erythrocyte membrane protein band 4.2. *Semin. Hematol.* **30**, 119-137.

Cohen, S.S., Marcu, D.E. and Balint, R.F. (1982) Light-dependent fixation of polyamines into chloroplasts of Chinese cabbage. *FEBS lett.* **141**, 93-97.

Cooke, R.D. and Holbrook, J.J. (1974) The calcium-induced dissociation of human plasma clotting factor XIII. *Biochem. J.* **141**, 79-84.

Cooper, A.J.L., Sheu, K.R., Burke, J.R., Onodera, O., Strittmatter, W.J., Roses, A.D. and Blass, J.P. (1997) Transglutaminase-catalyzed inactivation of glyceraldehyde 3-phosphate dehydrogenase and alpha-ketoglutarate dehydrogenase complex by polyglutamine domains of pathological length. *Proc. Natl. Acad. Sci. USA.* **94**, 12 604-12 609.

Credo, R.B., Curtis, C.G. and Lorand, L. (1978) Ca^{2+}-related regulatory function of fibrinogen. *Proc. Natl. Acad. Sci. USA.* **75**, 4 234-4 237.

Croall, D.E. and DeMartino, G.N. (1986) Calcium-dependent affinity purification of transglutaminase from rat liver cytosol. *Cell. Calcium* **7**, 29-39.

Das, T., Baek, K.J., Gray, C. and Im, M.J (1993) Evidence that the G_h protein is a signal mediator from α1-adrenoreceptor to a phospholipase C. *J. Biol. Chem.* **268**, 27 398-27 405.

Daussant, J., Nencare, N.J. and Conberton, E.J. (1969) Immunochemical studies on *Arachis hypogaea* proteins with particular reference to reserve proteins II. Protein modification during germination. *Plant Physiol.* **44**, 480-484.

Del Duca, S., Beninati, S. and Serafini-Fracassini, D. (1995) Polyamines in chloroplasts: Identification of their glutamyl and acetyl derivatives. *Biochem. J.* **305**, 233-237.

Del Duca, S., Bregoli, A.M., Bergamini, C. and Serafini-Fracassini, D. (1997) Transglutaminase-catalyzed modification of cytoskeletal proteins by polyamines during the germination of *Malus domestica* pollen. *Sex. Plant Reprod.* **10**, 89-95.

Del Duca, S., Creus, J.A., D'Orazi, D., Dondini, L., Bregoli, A.M. and Serafini-Fracassini, D. (2000a) Tuber vegetative stages and cell cycle in *Helianthus tuberosus:* Protein pattern and their modification by spermidine. *J. Plant Physiol.* **156**, 17-25.

Del Duca, S., Dondini, L., Della Mea, A., Munoz de Rueda, P. and Serafini-Fracassini, D. (2000b) Factors affecting transglutaminase activity catalysing polyamine conjugation to endogenous substrates in the entire chloroplast. *Plant Physiol. Biochem.* **38**, 429-439.

Del Duca, S., Favali, M.A., Serafini-Fracassini, D. and Pedrazzini, R. (1993) Transglutaminase activity during greening and growth of *Helianthus tuberosus* explants *in vitro*. *Protoplasma* **174**, 1-9.

Del Duca, S., Tinu, V., Bassi, R., Esposito, C. and Serafini-Fracassini, D. (1994) Identification of chlorophyll-a/b proteins as substrates of transglutaminase activity in isolated chloroplasts of *Helianthus tuberosus* L. *Planta* **193**, 283-289.

Derrick, N. and Laki, K. (1966) Enzymatic labelling of actin and tropomyosin with ^{14}C-labelled putrescine. *Biochem. Biophys. Res. Commun.* **22**, 82-88.

Dinnella, C., Serafini-Fracassini, D., Grandi, B. and Del Duca, S. (1992) The cell cycle in *Helianthus tuberosus*: Analysis of polyamine-endogenous protein conjugates by transglutaminase-like activity. *Plant Physiol. Biochem.* **30**, 531-539.

Di Verne, A., Rossi, A., De Matteis, F., Rosato, N., Finazzi Agro, A. and Mei, G. (2000) Opposite effects of Ca^{2+} and GTP binding on tissue transglutaminase tertiary structure. *J. Biol. Chem.* **275**, 3 915-3 921.

Dondini, L., Bonazzi, S.B. and Serafini-Fracassini, D. (2000) Recovery of growth capacity and of chloroplast transglutaminase activity induced by polyamines in a polyamine-deficient variant strain of *Dunaliella salina*. *J. Plant Physiol.* **157**, 473-480.

Driss-Ecole, D. Vassy, J., Guivarc'h, A., Prouteau, M., Dewitte, W. and Perbal, G. (2000) Immunolocalization of actin in root statocytes of *Lens culinaris* L. *J. Exp. Bot.* **51**, 521-528.

Dubbink, H.J., Verkaik, N.S., Faber, P.W., Trapman, J., Schroder, F.H. and Romijn, J.C. (1996) Tissue specific and androgen-regulated expression of human prostate-specific transglutaminase. *Biochem. J.* **315**, 901-908.

Duckert, F. (1972) Documentation of the plasma factor XIII deficiency in man. *Ann. N. Y. Acad. Sci.* **202**, 190-199.

Ellis, R.E., Yuan, J. and Horvitz, H.R. (1991) Mechanism and functions of cell death. *Ann. Rev. Cell Biol.* **7**, 663-698.

Esposito, C., Pucci, P., Amoresano, A., Marino, G., Cozzolino, A. and Porat, R. (1996) Transglutaminase from rat coagulating gland secreation: post-translational modifications and activation by phosphatidic acids. *J. Biol. Chem.* **271**, 26 242-26 250.

Falcone, P., Serafini-Fracassini, D. and Del Duca, S. (1993) Comparative studies of transglutaminase activity and substrates in different organs of *Helianthus tuberosus*. *J. Plant Physiol.* **142**, 265-273.

Favali, A., Serafini-Fracassini, D. and Sartorato, P. (1984) Ultrastructure and autoradiography of dormant and activated parenchyma of *Helianthus tuberosus*. *Protoplasma* **123**, 192-202.

Fesus, L., Davies, P.J.A. and Piacentini, M. (1991) Apoptosis: molecular mechanisms in programmed cell death. *Eur. J. Cell Biol.* **56**, 170-177.

Fesus, L., Metsis, M.L., Muszbek, L. and Koteliansky V.E. (1986) Transglutaminase-sensitive glutamine residues of human plasma fibronectin revealed by studying its proteolytic fragments. *Eur. J. Biochem.* **154**, 371-374.

Findlay J.B.C. (1990) Purification of membrane proteins. In *Protein purification applications A practical approach.* eds Harris, E.L.V. and Angal, S. IRL Press, Oxford, UK. pp 59-82.

Fink, M.L., Chung, S.I. and Folk, J.E. (1980) γ-Glutamylamine cyclotransferase: Specificity toward ε-(L-γ-glutamyl)-L-lysine and related compounds. *Proc. Natl. Acad. Sci. USA*. **77**, 4 564-4 568.

Fischer-Schliebs, E., Varanini, Z. and Luttage, U. (1994) Isolation of H^+-transport-competent plasma membrane vesicles from corn roots by discontinuous sucrose gradient centrifugation: Effect of membrane protectant agents. *J. Plant Physiol.* **144**, 502-512.

Folk, J.E. (1980) Transglutaminases. *Ann. Rev. Biochem.* **49**, 517-531.

Folk, J.E. (1983) Mechanism and basis for specificity of transglutaminase-catalyzed ε(γ-glutamyl) lysine bond formation. *Adv. Enzymol. Relat. Areas Mol. Biol.* **54**, 1-56.

Folk, J.E. and Cole P.W. (1966) Mechanism of action of guinea pig liver transglutaminase: Purification and properties of the enzyme; identification of a functional cysteine essential for activity. *J. Biol. Chem.* **241**, 5 518-5 525.

Folk, J.E. and Chung, S.I. (1973) Molecular and catalytic properties of transglutaminases. *Adv. Enzymol.* **38**, 109-187.

Folk, J.E. and Finlayson, J.S. (1977) The ε-(γ-glutamyl)-lysine crosslink and the catalytic role of transglutaminase. *Adv. Protein Chem.* **31**, 1-133.

Folk, J.E., Park, M.H., Chung, S.I., Schrobe, I., Lester, E.P. and Cooper, H.L. (1980) Polyamines as physiological substrates for transglutaminases. *J. Biol. Chem.* **255**, 3 695-3 700.

Fukuda, H. (2000) Programmed cell death of tracheary elements as a paradigm in plants. *Plant Mol. Biol.* **44**, 245-253.

Gallagher, S.R. and Leonard, R.T. (1982) Effect of vandate, molybdate, and azide on membrane-associated ATPase and soluble phosphatases-activities of corn roots. *Plant Physiol.* **70**, 1 335-1 340

Gaudry, C.A., Verderio, E., Aeschlimann, D., Cox, A., Smith, C. and Griffin, M. (1999) Cell surface localization of tissue transglutaminase is dependent on a fibronectin binding site in its N-terminal β-sandwich domain. *J. Biol. Chem.* **274**, 30 707-30 714.

Gentile, V., Saydak, M., Chiocca, E.A., Akande, O., Birckbichler, P.J., Lee, K.N., Stein, J.P. and Davies, P.J.A. (1991) Isolation and characterization of cDNA clones to mouse macrophage and human endothelial call tissue transglutaminase. *J. Biol. Chem.* **266**, 478-483.

Gerard, P., Bresson-Hadne, S., Vuitton, D.A. and Ricard-Blum, S. (1999) Crosslinking by transglutaminase stabilizes the extracellular matrix of fibrotic liver. *Proceedings of the XVIth meeting of the federation of the European connective tissue societies*. C10.

Goldberg, A.L. and St. John, A.C. (1976) Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annu. Rev. Biochem.* **45**, 747-803.

Gorman, J.J. and Folk, J.E. (1980) Structural features of glutamine substrates for human plasma factor XIIIa (activated blood coagulation factor XIII). *J. Biol. Chem.* **255**, 419-427.

Gorman, J.J. and Folk, J.E. (1981) Structural features of glutamine substrates for transglutaminases. Specificities of human plasma factor XIIIa and the guinea pig liver enzyme toward synthetic peptides. *J. Biol. Chem.* **256**, 2 712-2 715.

Gorman, J.J. and Folk, J.E. (1984) Structural features of glutamine substrates for transglutaminases. Role of extended interactions in the specificity of human plasma factor XIIIa and of the guinea pig liver enzyme. *J. Biol. Chem.* **259**, 9 007-9 010.

Gorman, A.L.F. and Thomas, M.V. (1980) Intracellular calcium accumulation during depolarisation in a molluscan neurone. *J. Physiol.* **308**, 259-285.

Grandi, B., Del Duca, S., Serafini-Fracassini, D. and Dinnella, C. (1992) Re-entry in cell cycle: Protein metabolism and transglutaminase-like activity in *Helianthus tuberosus*. *Plant Physiol. Biochem.* **30**, 415-424.

Green, H. (1979) Keratinocyte as differentiated cell type. *Harvey Lect.* **74**, 101-139.

Greenberg, C.S., Achyuthan, K.E. and Fenton, J.W. (1987) Factor XIIIa formation promoted by complexing of α-thrombin, fibrin and plasma factor XIII. *Blood* **69**, 867-871.

Greenberg, C.S., Birckbichler, P.J. and Rice, R.H. (1991) Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* **5**, 3 071-3 077.

Griffin, M., Barnes, R.N., Wynne, J. and Williams, C. (1978) The effects of bleomycin and copper bleomycin upon transglutaminase enzymes. *Biochem. Pharm.* **27**, 1 211-1 219.

Griffin, M. and Smethurst, P.A. (1994) Transglutaminases: Enzymes that crosslink proteins. *Retinoids Today and Tomorrow* **37**, 4-10.

Griffin, M. and Wilson, J. (1984) Detection of ε-(γ-glutamyl)lysine. *Mol. Cell Biochem.* **58**, 37-49.

Griffin, M., Wilson, J. and Lorand, L. (1982) High-pressure liquid chromatographic procedure for the determination of ε-(γ-glutamyl)lysine in proteins. *Anal. Biochem.* **124**, 406-413.

Groenen, P.J., Bloemendal, H. and de Jong, W.W. (1992) The carboxy-terminal lysine of α B-crystallin is an amine-donor substrate for tissue transglutaminase. *Eur. J. Biochem.* **205**, 671-674.

Groover, A. and Jones, A.M. (1999) Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. *Plant Physiol.* **119**, 375-384.

Grundmann, U., Amann, E., Zettlmeissl, G. and Kupper, H.A. (1986) Characterization of cDNA coding for human factor XIIIa. *Proc. Natl. Acad. Sci. USA.* **83**, 8 024-8 028.

Ha, C.R. and Iuchi, I. (1997) Purification and partial characterisation of 76kDa transglutaminase in the egg envelope (chorion) of rainbow trout, *Oncorynchus mykiss*. *J. Biochem.* **122**, 947-954.

Harsfalvi, J., Arato, G. and Fesus, L. (1987) Lipids associated with tissue transglutaminase. *Biochim. Biophys. Acta.* **923**, 42-45.

Herman, E.M., Li, X., Su, R.T., Larsen, P., Hsu, H.T. and Sze, H. (1994) Vacuolar-type H^+-ATPases are associated with the endoplasmic reticulum and provacuoles of root tip cells. *Plant Physiol.* **106**, 1 313-1 324.

Hill, J.M. (1971) Diamine oxidase (pea seedling). *Methods Enzymol.* **17B**, 730-735.

Hitomi, K., Ikura, K. and Maki, M. (2000) GTP, an inhibitor of transglutaminase, is hydrolysed by tissue-type transglutaminase (TGase 2) but not by epidermal-type transglutaminase (TGase 3). *Biosci. Biotech. Biochem.* **64**, 657-659.

Ho, K.C., Quarmby, V.E., French, F.S. and Wilson, E.M. (1992) Molecular cloning of rat prostate transglutaminase complementary DNA. The major androgen-regulated protein DP1 of rat dorsal prostate and coagulating gland. *J. Biol. Chem.* **267**, 12 660-12 667.

Hodges, T.K. and Leonard, R.T. (1974) Purification of a plasma membrane bound adenosine triphosphate from plant roots. *Methods Enzymol.* **32**, 393-406.

Huber, M., Rettler, I., Bernasconi, K., Frenk, E., Lavrijsen, S.P.M., Ponec, M., Bon, A., Lautenschlager, S., Schorderet, D.F. and Hohl, D. (1995) Mutations of keratinocyte transglutaminase in lamellar ichthyosis. *Science* **267**, 525-528.

Hufford, T.L. (1978) Plant cells and tissues. In *Botany: Basic concepts in plant biology*. Harper and Row publishers, New York. pp 29-63.

Icekson, I. and Apelbaum, A. (1987) Evidence for transglutaminase activity in plant tissue. *Plant Physiol.* **84**, 972-974.

Ichinose, A., Bottenus, R.E. and Davie, E.W. (1990) Structure of transglutaminases. *J. Biol. Chem.* **265**, 13 411-13 414.

Ichinose, A., Hendrickson, L.E., Fujikawa, K. and Davie, E.W. (1986) Amino acid sequence of the a-subunit of human factor XIII. *Biochemistry* **25**, 6 900-6 906.

Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., Sato, A., Egawa, S., Ikeuchi, T., Tanaka, H., Nakano, R., Tanaka, K., Hozumi, I., Inuzuka, T., Takahashi, H. and Tsuji, S. (1998) Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. *Nat. Genetics* **18**, 111-117.

Iismaa, S.E., Chung, L., Wu, M.J., Teller, D.C., Yee, V.C. and Graham, R.M. (1997) The core domain of tissue transglutaminase G_h hydrolyses GTP and ATP. *Biochemistry* **36**, 11 655-11 664.

Ikura, K., Nasu, T., Yokota, H., Tsuchiya, R. and Chiba, H. (1988) Amino acid sequence of guinea pig liver transglutaminase from its cDNA sequence. *Biochemistry* **27**, 2 898-2 905.

Ikura, K., Yokota, H., Sasaki, R. and Chiba, H. (1989) Determination of amino- and carboxyl-terminal sequences of guinea pig liver transglutaminase: evidence for amino-terminal processing. *Biochemistry* **28**, 2 344-2 348.

Jiang, L. and Rogers, J.C. (1999) Sorting of membrane proteins to vacuoles in plant cells. *Plant Sci.* **146**, 55-67.

Johnson, T.S., Griffin, M., Thomas, G.L., Skill, J., Cox, A., Yang, B., Nicholas, B., Birckbichler, P.J., Muchaneta-Kubara, C. and Meguid El Nahas, A. (1997) The role of transglutaminase in the rat subtotal nephrectomy model of renal fibrosis. *J. Clin. Invest.* **99**, 2 950-2 960.

Jones, A.M. and Dangl, U.L. (1996) Logjam at the styx: programmed cell death in plants. *Trends plant Sci.* **1**, 114-119.

Jones, O.T., Earnest, J.P. and McNamee, M.G. (1987) Solubilization and reconstitution of membrane proteins. In *Biological membranes A practical approach.* Eds Findlay, J.B.C. and Evans, W.H. Oxford University Press, Oxford, UK. pp 139-177.

Juprelle-Soret, M., Wattiaux-De-Coninck, S. and Wattiaux, R. (1988) Subcellular localization of transglutaminase. *Biochem. J.* **250**, 421-427.

Kahlem, P., Green, H. and Djian, P. (1998) Transglutaminase action imitates Huntington's disease: Selective polymerization of huntingtin containing poly-glutamine. *Mol. Cell* **1**, 595-601.

Kahlem, P., Terre, C., Green, H. and Djian, P. (1996) Peptides containing glutamine repeats as substrates for transglutaminase-catalyzed crossliking: Relevance to diseases of the nervous system. *Proc. Natl. Acad. Sci. USA.* **93**, 14 580-14 585.

Kanaji, T., Ozaki, H., Kawajiri, H., Ide, H., Motoki, M. and Shimonishi, Y. (1993) Primary structure of microbial transglutaminase from *Strepoverticillium* sp. strain s-8112. *J. Biol. Chem.* **268**, 11 565-11 572.

Kang, H. and Cho, Y.D. (1996) Purification and properties of transglutaminase from soybean leaves. *Biochem. Biophys. Res. Commun.* **223**, 288-292.

Kang, H., Lee, S.G. and Cho, Y.D. (1998) Identification of glycinin *in vivo* as a polyamine-conjugated protein via a γ-glutamyl linkage. *Biochem. J.* **332**, 467-473.

Kang, H., Park, S.J. and Cho, Y.D. (1997) Identification of γ-glutamylamine cyclotransferase as the preform enzyme at the dormant stage, from soybean *(Glycine max)* seeds. *J. Biochem. Mol. Biol.* **30**, 438-442.

Kennedy, R.M. (1990) Hydrophobic chromatography. *Methods Enzymol.* **182**, 309-316.

Kim, H.C., Lewis, M.S., Gorman, J.J., Park, S.C., Girard, J.E., Folk, J.E. and Chung, S.I. (1990) Protransglutaminase E from guinea pig skin. Isolation and partial characterization. *J. Biol. Chem.* **265**, 21 971-21 978.

Kim, I.G., Gorman, J.J., Park, S.C., Chung, S.I. and Steinert, P.M. (1993) The deduced sequence of the novel protransglutaminase E (TGase3) of human and mouse. *J. Biol. Chem.* **268**, 12 682-12 690.

Kim, S.Y., Chung, S.I. and Steinert, P.M. (1995a) Highly active soluble processed forms of the transglutaminase I enzyme in epidermal keratinocytes. *J. Biol. Chem.* **270**, 18 026-18 035.

Kim, S.Y., Chung, S.I., Yoneda, K. and Steinert, P.M. (1995b) Expression of transglutaminase I in human epidermis. *J. Invest. Dermatol.* **104**, 211-217.

Klein, J.D., Guzman, E. and Kuehn, G.D. (1992) Purification and partial characterization of transglutaminase from *Physarum polycephalum. J. Bacteriol.* **174**, 2 599-2 605.

Knight, C.R.L., Rees, R.C., Elliot, B.M. and Griffin, M. (1990) Immunological similarities between cytosolic and particulate tissue transglutaminase. *FEBS. Lett.* **265**, 93-96.

Kobayashi, K., Sukuki, S.I., Izawa, Y., Yokozeki, K., Miwa, K. and Yumanaka, S. (1998) Transglutaminase in sporulating cells of *Bacillus subtilitis. J. Gen. Appl. Microbiol.* **44**, 85-91.

Kopecki-Fjetland, A., Morales, T.L., Flanigan, R. and Kuehn, G.D. (1996) Isolation and characterization of a gene coding for transglutaminase in *Medicago sativa* L. (alfalfa). *FASEB J.* **10**, A1493.

Korsgren, C., Lawler, J., Lambert, S., Speicher, D. and Cohen, C.M. (1990) Complete amino acid sequence and homologies of human erythrocyte membrane protein band 4.2. *Proc. Natl. Acad. Sci. USA* **87**, 613-617.

Kuehn, G.D., Sotelo, M., Morales, T., Bruce-Carver, M.R., Guzman, E. and Margosiak, S.A. (1991) Purification and properties of transglutaminase from *Medicago sativa. FASEB. J.* **5**, A1510.

Kunico, G.S., Tsyganskaya, J., Zhu, S.L., Liu, L., Nagy, V., Thomazy, P.J., Davies, M.A. and Zern, M.A. (1998) $TNF_{-\alpha}$ modulates expression of the tissue transglutaminase gene in liver cells. *Am. J. Physiol.* G240-G245.

Lam, E., Pontier, D. and del Pozo, O. (1999) Die and let live – programmed cell death in plants. *Curr. Opin. Plant Sci.* **2**, 502-507.

Larsson, C. (1994) Isolation of highly purified intact chloroplasts and of multiorganelle complexes containing chloroplasts. *Methods Enzymol.* **228**, 419-424.

Leblanc, A., Day, N., Menard, A. and Keillor, J.W. (1999) Guinea pig liver transglutaminase: A modified purification procedure affording enzyme with superior activity in greater yield. *Protein Expres. Purif.* **17**, 89-95.

Lee, K.N., Arnold, S.A., Birckbichler, P.J., Patterson, M.K. Jr., Fraij, B.M., Takeuchi, Y. and Carter, H.A. (1993) Site-directed mutagenesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not GTPase activity. *Biochim. Biophys. Acta.* **1202**, 1-6.

Lee, K.N., Birckbichler, P.J. and Fesus, L. (1986) Purification of human-erythrocyte transglutaminase by immunoaffinity chromatography. *Prep. Biochem.* **16**, 321-335.

Lee, K.N., Birckbichler, P.J. and Patterson, M.K. Jr. (1989) GTP hydrolysis by guinea pig liver transglutaminase. *Biochem. Biophys. Res. Commun.* **162**, 1 370-1 375.

Lee, Y.R.J. and Assmann, S.M. (1999) *Arabidopsis thaliana* 'extra-large GTP-binding protein' (AtXLG1): a new class of G-protein. *Plant Mol. Biol.* **40**, 53-64.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R. and Lamb, C. (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Curr. Biol.* **6**, 427-437.

Lilley, G.R., Griffin, M. and Bonner, P.L.R. (1997) Assays for the measurement of tissue transglutaminase (Type II) mediated protein crosslinking via ε-(γ-glutamyl) lysine and N,N'-bis(γ-glutamyl) polyamine linkages using biotin labelled casein. *J. Biochem. Biophys. Methods* **34**, 31-43.

Lilley, G.R. (1998) Characterisation of a calcium dependent transglutaminase in *Pisum sativum* leaf and root tissue. Ph.D. thesis, The Nottingham Trent University, UK.

Lilley, G.R., Skill, J., Griffin, M. and Bonner, P.L.R. (1998a) Detection of Ca^{2+}-dependent transglutaminase activity in root and leaf tissue of monocotyledonous and dicotyledonous plants. *Plant Physiol.* **117**, 1 115-1 123.

Lilley, G.R., Skill, J., Griffin, M. and Bonner, P.L.R. (1998b) The detection of transglutaminase in *Vicia faba* cotyledons. In *Plant proteins from European crops. Food and non-food applications*. Eds Gueguen, J. and Popineau, Y. Springer-Verlag, Berlin.pp 99-101.

Loewy, A.G. and Matacic, S.S. (1981) Modulation of the ε-(γ-glutamyl) lysine cross-link in cellular proteins. I *In vivo* and *in vitro* studies. *Biochim. Biophys. Acta.* **668**, 167-176.

Lorand, L. (1986) Activation of blood coagulation factor XIII. *Ann. N.Y. Acad. Sci.* **285**, 144-158.

Lorand, L. (1988) Transglutaminase-mediated cross-linking of proteins and cell ageing: The erythrocyte and lens models. *Adv. Exp. Med. Biol.* **231**, 79-94.

Lorand, L. (1996) Neurodegenerative diseases and transglutaminases. *Proc. Natl. Acad. Sci. USA.* **93**, 14 310-14 313.

Lorand, L., Campbell-Wilkes, L.K. and Cooperstein L. (1972) A filter paper assay for transamidating enzymes using radioactive amine substrates. *Anal Biochem.* **50**, 623-631.

Lorand, L. and Conrad, S.M. (1984) Transglutaminases. *Mol. Cell Biochem.* **58**, 9-35.

Lorand, L., Gray, A.J., Brown, K., Credo, R.B., Curtis, C.G., Domanik, R.A. and Stenberg, P. (1974) Dissociation of the subunit structure of fibrin stabilizing factor during activation of the zymogen. *Biochem. Biophys. Res. Commun.* **56**, 914-922.

Lorand, L., Velasco, P.T., Murthy, S.N., Wilson, J. and Parameswaran, K.N. (1992) Isolation of transglutaminase-reactive sequences from complex biological systems: A prominent lysine donor sequence in bovine lens. *Proc. Natl. Acad. Sci. USA.* **89**, 11 161-11 163.

Ma, H. (1994) GTP-binding proteins in plants: new members of an old family. *Plant Mol. Biol.* **26**, 1 611-1 636.

Madi, A., Punyiczki, M., Di Rao, M., Piacentini, M. and Fesus, L. (1998) Biochemical characterisation and localisation of transglutaminase in wild-type and cell-death mutants of the nematode *Caenorhabditis elegans*. *Eur. J. Biochem.* **253**, 583-590.

Margosiak, S.A., Alvarez, M.E., Louie, D. and Kuehn, G.D. (1987) Transglutaminase and its protein substrates in plants. *Fed. Proc.* **46**, 2 114.

Margosiak, S.A., Dharma, A., Bruce-Carver, M.R., Gonzales, A.P., Louie, D. and Kuehn, G.D. (1990) Identification of the large subunit of ribulose 1,5-Biphosphate carboxylase / oxygenase as a substrate for transglutaminase in *Medicago sativa* L. *Plant Physiol.* **92**, 88-96.

Martinet, N., Kim, H.C., Girard, J.E., Nigra, T.P., Strong, D.H., Chung, S.I. and Folk, J.E. (1988) Epidermal and hair follicle transglutaminases. Partial characterization of soluble enzymes in newborn mouse skin. *J. Biol. Chem.* **263**, 4 236-4 241.

Maruyama, K., Ebisawa, K. and Nonomura, Y. (1985) Purification of vitamin D-dependent 28 000-Mr calcium-binding protein from bovine cerebellum and kidney by calcium dependent elution from DEAE-Cellulose DE-52 column chromatography. *Anal. Biochem.* **151**, 1-6.

Matile, P. (1997) The vacuole and cell senescence. In *Advances in botanical research vol. 25.* Eds Leigh, R.A. and Sanders, D. Academic press Ltd, London. pp 87-112.

Matsuki, M., Yamashita, F., Ishida-Yamamoto, A., Yamada, K., Kinoshita, C., Fushiki, S., Ueda, E., Morishima, Y., Tabata, K., Yasuno, H., Hashida, M., Iizuka, H., Ikawa, M., Okabe, M., Kondoh, G., Kinoshita, T., Takeda, J. and Yamanishi, K. (1998) Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase I (keratinocyte transglutaminase) *Proc. Natl. Acad. Sci. USA* **95**, 1 044-1 049.

McCarty, D.R., Keegstra, K. and Selman, B.R. (1984) Characterization and localization of the ATPase associated with pea chloroplast envelope membranes. *Plant Physiol.* **76**, 584-588.

Mehta, A.M., Saftner, A.R., Schaeffer, G.W. and Mattoo, A.K. (1991) Translational modification of an 18 kDa polypeptide by spermidine in rice cell suspension cultures. *Plant physiol.* **95**, 1 294-1 297.

Mehta, K., Rao, U.R., Vickery, A.C. and Birckbichler, P.J. (1990) Significance of transglutaminase-catalysed reactions in growth and development of filarial parasite, *Brugia malayi. Biochem. Biophys. Res. Comm.* **173**, 1 051-1 057.

Mehta, K., Rao, U.R., Vickery, A.C. and Fesus, L. (1992) Identification of a novel transglutaminase from the filarial parasite *Brugia malayi* and its role in growth and development. *Mol. Biochem. Parasit.* **53**, 1-15.

Melino, G. and Piacentini, M. (1998) "Tissue" transglutaminase in cell death: A downstream or a multifunctional upstream effector? *FEBS Lett.* **430**, 59-63.

Mirza, A., Liu, S.L., Venditti, E., Zhu, J., Maddukuri, S., Martinez, J., Davies, P., Schwarting, R., Norton, P. and Zern, M.A. (1997) A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by $NF_{-\kappa}B$. *Am. J. Physiol.* **272**, G281-G288.

Mizrahi, Y., Appelwhite, P.B. and Galston, A.W. (1989) Polyamine binding to protein in oats and *petunia* protoplasts. *Plant Physiol.* **91**, 738-743.

Morelli, J.K., Zhou, W., Yu, J., Lu, C. and Vayda, M.E. (1998) Actin polymerisation affects stress-induced translational activity of potato tuber tissue. *Plant Physiol.* **116**, 1227-1237.

Morre, D.M., Brightman, A.O. and Sandelius, A.S. (1987) Membrane fractions from plant cells. In *Biological membranes A practical approach.* Eds Findlay, J.B.C. and Evans, W.H. Oxford university press, Oxford, UK. pp 37-72.

Mossetti, U., Serafini-Fracassini, D. and Del Duca, S. (1987) Conjugated polyamines during dormancy and activation of tuber of Jerusalem artichoke. In *Conjugated plant hormones, structure, metabolism and function.* Eds Schreiber, K., Schutte, H.R. and Sembdner, G. Deutscher verlag der wissenschaften, Berlin. pp 369-375.

Mottahedeh, J. and Marsh, R. (1998) Characterization of 101-kDa transglutaminase from *Physarum polycephalum* and identification of LAV1-2 as substrate. *J. Biol. Chem.* **273**, 29 888-29 895.

Muesch, A., Hartmann, E., Rhode, K., Rubartelli, A., Sitia, R. and Rapoport, T.A. (1990) An alternative pathway for secretory proteins? *Trends Biochem. Sci.*. **15**, 86-88.

Nakanishi, K., Nara, K., Hagiwara, H., Aoyama, Y., Ucno, H. and Hirose, S. (1991) Cloning and sequence analysis of cDNA clones for bovine aortic-endothelial-cell transglutaminase. *Eur. J. Biochem.* **202**, 15-21.

Nakaoka, H., Perez, D.M., Baek, K.J., Das, T., Husain, A., Misono, K., Im, M.J. and Graham, R.M. (1994) G_h: A GTP-binding protein with transglutaminase activity and receptor signalling function. *Science* **264**, 1 593-1 596.

Olson, E.N., Towler, D.A. and Glaser, L. (1985) Specificity of fatty acid acylation of cellular proteins. *J. Biol. Chem.* **260**, 3 784-3 790.

Paonessa, G., Metafors, S., Tajana, G., Abrescia, P., de Santis, A., Gentile, U. and Porta, R. (1984) Transglutaminase-mediated modifications of the rat sperm surface *in vitro*. *Science* **226**, 852-855.

Parmentier, L., Blanchet-Bardon, C., Nguyen, S., Prud'homme, J.F., Dubertret, L. and Weissenbach, J. (1995) Autosomal recessive lamellar ichthyosis: Identification of a new mutation in transglutaminase I and evidence for genetic heterogeneity. *Hum. Mol. Genet.* **4**, 1 391-1 395.

Pasternack, R., Dorsch, S., Otterbach, J.T., Robenek, I.R., Wolf, S. and Fuchsbauer, H.L. (1998) Bacterial pro-transglutaminase from *Streptoverticillium mobaraense*: Purification, characterisation and sequence of the zymogen. *Eur. J. Biochem.* **257**, 570-576.

Pennell, R.I. and Lamb, C. (1997) Programmed cell death in plants. *Plant Cell* **9**, 1 157-1 168.

Perroud, P.F., Crespi, P., Chevecoeur, M., Fink, A., Tacchini, P. and Greppin, H. (1997) Detection and characterization of GTP-binding proteins on tonoplast of *Spinacia oleracea*. *Plant Sci.* **122**, 23-33.

Perutz, M.F. (1999) Glutamine repeats and neurodegenerative diseases: molecular aspects. *Trends Biochem. Sci.* **24**, 58-63.

Philips, M.A., Qin, Q., Mehrpouyan, M. and Rice, R.H. (1993) Keratinocyte transglutaminase membrane anchorage: Analysis of site-directed mutants. *Biochemistry* **32**, 11 057-11 063.

Pieredda, L., Amendola, A., Colizzi, P.J.A., Davies, M.G., Farrace, M.G., Fraziano, M., Gentile, V., Uray, I., Piacentini, M. and Fesus, L. (1997) Lack of 'tissue'transglutaminase cross-linking leads to leakage of macromolecules from dying cells: relationship to development of autoimmunity in MRL/pr/lpr mice. *Cell Death Diff.* **4**, 463-472.

Quail, P.H. (1979) Plant cell fractionation. *Ann. Rev. Plant Physiol.* **30**, 425-484.

Ray, P.M., Shininger, T.L. and Ray, M.M. (1969) Isolation of β-glucan synthase particles from plant cells and identification with Golgi membranes. *Proc. Natl. Acad. Sci. USA* **64**, 605-612.

Rice, R.H. and Green, H. (1979) Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell.* **18**, 681-694.

Rice, R.H., Rong, X. and Chakravarty, R. (1990) Proteolytic release of keratinocyte transglutaminase. *Biochem. J.* **265**, 351-357.

Robinson, D.G., Hinz, G. and Oberbeck K. (1994) Isolation of endo- and plasma membranes. In *Plant Cell Biology A practical approach.* Eds Harris, N and Oparka, K.J. Oxford university press, Oxford, UK. pp 245-272.

Rossomando, E.F. (1990) Ion-exchange chromatography. *Methods Enzymol.* **182**, 309-316.

Roy, H., Cannon, S. and Gilson, M. (1988) Assembly of RuBisCo from native sub-units. *Biochim. Biophys. Acta.* **957**, 323-334.

Russell, L.J. DiGiovanna, J.J., Rogers, G.R., Steinert, P.M., Hashem, N., Compton, J.G. and Bale, S.J. (1995) Mutations in the gene for transglutaminase I in autosomal recessive lamellar ichthyosis. *Nat. Genet.* **9**, 279-283.

Ryan, C.A. and Walker-Simons, M. (1981) Plant proteinases. In *The biochemistry of plants Volume 6 Proteins and nucleic acids.* Ed. Marcus, A. Acadamic Press Inc, New York. pp 321-250.

Sanders, D., Brownlee, C. and Harper, J.F. (1999) Communicating with calcium. *Plant Cell* **11**, 691-706.

Sano, K.I., Nakanishi, K., Nakamura, N., Motoki, M. and Yasueda, H. (1996) Cloning and sequence analysis of a cDNA encoding salmon (*Onchorhynchus keta*) liver transglutaminase. *Biosci. Biotech. Bioch.* **60**, 1 790-1 794.

Sarath, G., De La Motte, R.S. and Wagner, F.W. (1989) Protease assay methods. In *Proteolytic enzymes A practical approach.* Eds Beynon, R.J. and Bond, J.S. IRL Press, Oxford. pp 25-55

Sefton, B.M. and Buss, J.E. (1987) The covalent modification of eukaryotic proteins with lipid. *J. Cell Biol.* **104**, 1 449-1 453.

Seitz, J., Keppler, C. and Huntemann, S.B. (1991) Purification and characterization of transglutaminases from the genital tract of the male rat. *J. Chromatogr.* **587**, 55-60.

Selkoe, D.J., Abraham, C. and Ihara, Y. (1982) Brain transglutaminase: In vitro crosslinking of human neurofilament proteins into insoluble polymers. *Proc. Natl. Acad. Sci. USA* **79**, 6 070-6 074.

Serafini-Fracassini, D., Del Duca, S. and Beninati, S. (1995) Plant transglutaminases. *Phytochem.* **40**, 355-365.

Serafini-Fracassini, D., Del Duca, S. and D'Orazi, D. (1988) First evidence for polyamine conjugation mediated by an enzyme activity in plants. *Plant Physiol.* **87**, 757-761.

Serafini-Fracassini, D., Del Duca, S. and Torrigiani, P. (1989) Polyamine conjugation during cell cycle of *Helianthus tuberosus*: non enzymatic and transglutaminase-like binding activity. *Plant Physiol. Biochem.* **27**, 659-668.

Shainoff, J.R., Urbanic, D.A. and DiBello, P.M. (1991) Immunoelectrophoretic characterizations of the cross-linking of fibrinogen and fibrin by factor XIIIa and tissue transglutaminase. Identification of a rapid mode of hybrid alpha-/gamma-chain cross-linking that is promoted by the gamma-chain cross-linking. *J. Biol. Chem.* **266**, 6 429-6 437.

Shutov, A.D. and Vaintraub, I.A. (1987) Degradation of storage proteins in germinating seeds. *Pytochem.* **26**, 1 557-1 566

Siepaio, M.P. and Meunier, J.F. (1995) Diamine oxidase and transglutaminase activities in white lupine seedlings with respect to cross-linking of proteins. *J. Agric. Food Chem.* **43**, 1 151-1 156.

Signorini, M., Beninati, S. and Bergamini, C.M. (1991) Identification of transglutaminase activity in the leaves of silver beet. *J. Plant Physiol.* **137**, 547-552.

Simon, M. and Green, H. (1988) The glutamine residues reactive in transglutaminase-catalysed cross-linking of involucrin. *J. Biol. Chem.* **163**, 18 093-18 098.

Singer, M.A., Hortsch, M., Goodman, C.S. and Bentley, D. (1992) Annulin, a protein expressed at limb segment boundaries in the grasshopper embryo, is homologous to protein cross-linking transglutaminases. *Develop. Biol.* **154**, 143-159.

Singh, R.N. and Mehta, K. (1994) Purification and characterisation of a novel transglutaminase from filarial nematode *Brugia malayi*. *Eur. J. Biochem.* **225**, 625-634.

Slaughter, T.F., Achyutham, K.E., Lai, T.S. and Greenberg, C.S. (1992) A microtiter plate transglutaminase assay utilizing 5-(biotinamido)pentylamine as substrate. *Anal. Biochem.* **205**, 166-171.

Slife, C.W., Dorsett, M.D., Bouquett, G.T., Register, A., Taylor, E. and Conroy, S. (1985) Subcellular localization of a membrane-associated transglutaminase activity in rat liver. *Arch. Biochem. Biophys.* **241**, 329-336.

Slife, C.W., Morris, G.S. and Snedeker, S.W. (1987) Solubilization and properties of the liver plasma membrane transglutaminase. *Arch. Biochem. Biophys.* **257**, 39-47.

Smethurst, P.A. and Griffin, M. (1996) Measurement of tissue transglutaminase activity in a permeabilized cell system: Its regulation by Ca^{2+} and nucleotides. *Biochem. J.* **313**, 803-808.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, R.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoinic acid. *Anal. Biochem.* **150**, 76-85.

Steinert, P.M., Chung, S.I. and Kim S.Y. (1996a) Inactive zymogen and highly active proteolytically processed membrane-bound forms of the transglutaminase I enzyme in human epidermal keratinocytes. *Biochem. Biophys. Res. Commun.* **221**, 101-106

Steinert, P.M., Kim, S.Y., Chung, S.I. and Marekov, L.N. (1996b) The transglutaminase I enzyme is variably acylated by myristate and palmitate during differentiation in epidermal keratinocytes. *J. Biol. Chem.* **271**, 26 242-26 250.

Stellwagen, E. (1990) Gel filtration. *Methods Enzymol.* **182**, 309-316.

Tack, B.F., Janatova, J., Thomas, M.L., Harrison, R.A. and Hammer, C.H. (1981) The third, fourth and fifth components of human complement: Isolation and biochemical properties. *Methods Enzymol.* **80**, 64-110.

Takahashi, N., Takahashi, Y. and Putman, F.W. (1986) Primary structure of blood coagulation factor XIIIa (Fibrinoligase, transglutaminase) from human placenta. *Proc. Natl. Acad. Sci. USA.* **83**, 8 019-8 023.

Tamaki, T. and Aoki, N. (1985) Cross-linking of α2-plasmin inhibitor to fibrin catalyzed by activated fibrin-stabilizing factor. *J. Biol. Chem.* **257**, 14 767-14 772.

Tarcsa, E., Kedei, N., Thomazy, V. and Fesus, L. (1992) An involucrin-like protein in hepatocytes serves as a substrate for tissue transglutaminase during apoptosis. *J. Biol. Chem.* **267**, 25 648-25 651.

Thacher, S.M., and Rice, R.H. (1985) Keratinocyte specific transglutaminase of cultured human epidermal cells: Relation to cross-linked envelope formation and terminal differentiation. *Cell.* **40**, 685-695.

Thomas, T.M. and McNamee, M.G. (1990) Purification of membrane proteins. *Methods Enzymol.* **182**, 499-520.

Tiwari, S.C. and Polito, V.S. (1988) Organization of the cytoskeleton in pollen tubes of *Pyrus communis*: a study employing conventional and freeze-substitution electron microscopy, immunofluorescence, and rhodamine-phalloidin. *Protoplasma* **147**, 100-112.

Tokunaga, F., Muta, T., Iwanaga, S., Ichinose, A., Davie, E.W., Kuma, K.I. and Miyata, T. (1993a) Limulus hemocyte transglutaminase: cDNA cloning, amino acid sequence, and tissue localization. *J. Biol. Chem.* **268**, 262-268.

Tokunaga, F., Yamada, M., Miyata, T., Ding, Y.L., Hiranaga-Kawabata, M., Muta, T., Iwanaga, S., Ichinose, A. and Davie, E.W. (1993b) Limulus hemocyte transglutaminase: Its purification and characterisation, and identification of the intracellular substrates. *J. Biol. Chem.* **268**, 252-261.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4 350-4 354.

Upchurch, H.F., Conway, E., Patterson, M.K. Jr. and Maxwell, M.D. (1991) Localization of cellular transglutaminase on the extracellular matrix after wounding. *J. Cell Physiol.* **149**, 375-383.

Verderio, E., Nicholas, B., Gross, S. and Griffin, M. (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: Effects on the processing of fibronectin, cell attachment, and cell death. *Exp. Cell Res.* **239**, 119-138.

Votyakova, T.V., Wallace, H.M., Dunbar, B. and Wilson B. (1999) The covalent attachment of polyamines to proteins in plant mitochondria. *Eur. J. Biochem.* **260**, 250-257.

Waffenschmidt, S., Kusch, T. and Woessner, J.P. (1999) A transglutaminase immunologically related to tissue transglutaminase calalyzes cross-linking of cell wall proteins in *Chlamydomonas reinhardtii. Plant Physiol.* **121**, 1 003-1 015.

Waldron, K.W. and Brett, S.T. (1987) The subcellular localisation of a glucuronyltransferase involved in glucuronoxylan biosynthesis in pea (*Pisum sativum*) epicotyls. *Plant Sci.* **49**, 1-8.

Walker, R.R. and Leigh, R.A. (1981) Mg^{2+}-dependent, cation-stimulated inorganic pyrophosphatase associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). *Planta* **153**, 150-155.

Walsh, M.P., Valentine, K.A., Ngai, P.K., Carruthers, C.A. and Hollenberg, M.D. (1984) Ca^{2+}-dependent hydrophobic-interaction chromatography. Isolation of a novel Ca^{2+}-binding protein kinase C from bovine brain. *Biochem. J.* **224**, 117-127.

Wang, H., Li, J., Bostock, R.M. and Gilchrist, D.G. (1996) Apoptosis: A functional paradigm for programmed cell death by a host-selective phytotoxin and invoked during development. *Plant Cell* **8**, 375-391.

Weinberg, J.B., Pippen, A.M. and Greenberg, C.S. (1991) Extravascular fibrin formation and dissolution in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. *Arthritis Rheum.* **34**, 996-1 005.

Weraarchakul-Boonmark, N., Jeong, J.M., Murphy, S.N.P., Engel, J.D. and Lorand, L. (1992) Cloning and expression of chicken erythrocyte transglutaminase. *Proc. Natl. Acad. Sci. USA* **89**, 9 804-9 808.

White, P.J. (2000) Calcium channels in higher plants. *Biochim. Biophys. Acta* **1465**, 171-189.

Wilhelm, B., Meinhardt, A. and Seitz, J. (1996) Transglutaminases: Purification and activity assays. *J. Chromatogr. B* **684**, 163-177

Widell, S. and Larsson, C. (1990) A critical evaluation of markers used in plasma membrane purification. In *The plant plasma membrane. Structure, function and molecular biology.* Eds Larsson, C. and Moller, I.M. Springer-Verlag, Berlin. pp 16-43.

Williams-Ashman, H.G. (1984) Transglutaminases and the clotting of rodent seminal fluids. *Mol. Cell Biochem.* **58**, 51-61.

Yasueda, H., Kumazawa, Y. and Motoki, M. (1994) Purification and characterization of a tissue-type transglutaminase from red sea bream (*Pagrus major*). *Biosci. Biotech. Biochem.* **58**, 2 041-2 045.

Yasueda, H., Nakanishi, K., Kumazawa, Y., Nagase, K., Motoki, M. and Matsui, H. (1995) Tissue-type transglutaminase from red sea bream (*Pagrus major*) sequence analysis of the cDNA and functional expression in *Escherichia coli*. *Eur. J. Biochem.* **232**, 411-419.

Yee, V.C., Pedersen, L.C., Le Trong, I., Bishop, P.D., Stenkamp, R.E. and Teller, D.C. (1994) Three-dimensional structure of a transglutaminase: Human blood coagulation factor XIII. *Proc. Natl. Acad. Sci. USA* **91**, 7 296-7 300.

Yee, V.C., Le Trong, I., Bishop, P.D., Pedersen, L.C., Stenkamp, R.E. and Teller, D.C. (1996) Structure and function studies of factor XIIIa by X-ray crystallography. *Sem. Thromb. Haemostasis* **22**, 377-384.

Yeong, J.M., Murphy, S.N.P., Radek, J.T. and Lorand, L. (1995) The fibronectin-binding domain of transglutaminase. *J. Biol. Chem.* **270**, 5 654-5 658.

Zhu, Y., Bol, J., Rinzema, A. and Tramper, J. (1995) Microbial transglutaminase, a review of its production and application in food processing. *Appl. Microbiol. Biotech.* **44**, 277-282.

Chapter 8 – Appendix

Appendix 1 - Estimation of free calcium ion concentration

$[CaCl_2]$ of reaction buffer mM	Equivalent free $[Ca^{2+}]$ mM
1	0.0019
1.01	0.01
1.05	0.05
1.065	0.065
1.085	0.085
1.1	0.1
1.2	0.2
1.5	0.5
2	1
3	2
4	3
5	4
6	5
7	6
9	8
11	10
13	12
16	15
21	20
26	25

The free calcium ion concentration of the reaction buffer used in the biotin cadaverine incorporation assay at pH 8.0. Data was generated using the software EqCal (biosoft, UK) and used in the production of figures 4.1, 4.2, 4.11 – 4.13 and 5.9 and tables 4.6 – 4.8 and 5.7.