Characterisation of a calcium dependent transglutaminase in *Pisum sativum* leaf and root tissue.

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Declaration

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Abstract.

Two colorimetric assays for transglutaminase activity involving protein cross-linking were developed. The assays were used to screen protein extracted from root and leaf tissue of the dicotyledons *Pisum sativum* and *Vicia faba* and the monocotyledons *Triticum aestivum* and *Hordeum vulgare* for transglutaminase activity. In one assay, biotin labelled casein was cross-linked to chemically modified casein bound to a microtiter plate via ε-(γ-glutamyl) lysine isodipeptide bonds and the biotin labelled reaction product was detected by conjugation to extravidin peroxidase. In a second assay, microtiter plate bound *N',N'*-dimethylcasein was enzymically modified using commercially available purified guinea pig liver transglutaminase to incorporate polyamines into glutamine residues. Biotin labelled casein was then cross-linked into the immobilized polyamines by the transglutaminase under assay resulting in the formation of *N',N'*-bis (γ-glutamyl) polyamine linkages. The crude plant protein preparations were also screened for the ability to catalyse the production of *N*-(γ-glutamyl) polyamine bonds using a biotin labelled cadaverine incorporation assay and a radiolabelled putrescine incorporation assay. Crude plant extracts were shown to catalyse the cross-linking of biotin labelled casein to microtiter plate bound chemically modified casein and the incorporation of biotin labelled cadaverine into microtiter plate bound *N',N'*-dimethylcasein in a calcium dependent manner. The cross-linking of casein and the incorporation of biotin labelled cadaverine into *N',N'*-dimethylcasein were time dependent reactions with a pH optimum of 7.9. Transglutaminase activity was shown to increase over a 2 week growth period in both the roots and leaves of *Pisum sativum*. A partially purified transglutaminase from the root tissue of *Pisum sativum* had an estimated molecular mass of 36 kDa and a K_m of 190μM and 0.2μg ml^{-1} for biotin labelled cadaverine and biotin labelled casein respectively. Calcium dependent transglutaminase activity was also detected in detergent treated *Pisum sativum* membrane preparations, implying that a membrane bound form of transglutaminase is also present in this tissue. The ε-(γ-glutamyl) lysine isodipeptide product of the transglutaminase reaction was detected in the root and leaf protein of *Pisum sativum* at a level of 510 pmol mg^{-1} and 210 pmol mg^{-1} respectively.

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Contents.

Publications and presentations.

Publications.

1. **Lilley, G. R.**, Griffin, M., and Bonner, P. L. R (1995). A survey of plant tissue for the presence of transglutaminase. J. Ex. Bot. (supplement) **46:** 50.
2. **Lilley, G. R.**, Griffin, M., and Bonner, P. L. R (1996). Transglutaminase in plants. J. Ex. Bot. (supplement) **47:** 74.
3. **Lilley, G. R.,** Skill, J., Griffin, M and Bonner, P. L. R (1997). The detection of ε (γ-glutamyl) lysine isodipeptide cross-links in *Pisum sativum* root and leaf tissue. J. Ex. Bot. (supplement) **48:** 30.
4. **Lilley, G. R.**, Griffin, M and Bonner, P. L. R (1997). Assays for the measurement of tissue transglutaminase (type II) mediated protein cross-linking via ε-(γ-glutamyl) lysine and *N',N'*-bis(γ-glutamyl) polyamine linkages using biotin labelled casein. J. Biochem. Biophys. Methods **34** 31-43.
5. **Lilley, G. R.,** Skill, J., Griffin, M and Bonner, P. L. R (1998). Detection of calcium dependent transglutaminase activity in root and leaf tissue of monocotyledonous and dicotyledonous plants. Plant Physiol. **117:** 1115-1123.

Presentations.

1. **A survey of plant tissue for the presence of transglutaminase (1995).** Poster presentation at the Society for Experimental Biology annual meeting, University of St. Andrews, U. K.
2. **Transglutaminase in plants (1996).** Oral presentation at the Society for Experimental Biology annual meeting, Lancaster University, U. K.
3. **The detection of ε-(γ-glutamyl) lysine isodipeptide cross-links in *Pisum sativum* root and leaf tissue (1997).** Poster presentation at the Society for Experimental Biology annual meeting, Canterbury, U. K.

Abbreviations.

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CTAB	Cetyltrimethylammonium bromide
DEAE	Diethylaminoethyl
DIECA	Diethyldithiocarbamic acid
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulfoxide
DPM	Disintegrations per minute
DTT	Dithiothreitol
EDC	Ethyldimethylaminopropyl carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FFQ	Fast flow quaternary ammonium
FW	Fresh weight
GTP	Guanosine triphosphate
HPLC	High pressure liquid chromatography
kDa	KiloDaltons
2-ME	2-mercaptoethanol
MES	(2-[N-morpholino] ethane sulphonic acid)
M_r	Relative molecular mass
NEM	N-ethylmaleimide
OPA	Orthophthaldialdehyde

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PFS	Particle free supernatant
PMSF	Phenylmethylsulfonylfluoride
PVPP	Polyvinylpolypyrrolidone
QAE	Quaternary aminoethyl
RuBisCo L	Large sub-unit of ribulose 1, 5-bisphosphate carboxylase / oxygenase
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SWR	Standard working reagent
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
Tween 80	Polyoxyethylenesorbitan

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Chapter 1- Introduction.

1.1 Introduction to the transglutaminases.

1.1.1 Definition.

Transglutaminases (E.C 2.3.2.13, systematic nomenclature: R-glutamyl-peptide:-amine-γ-glutamyl transferases) are defined as a family of calcium dependent enzymes with an active site thiol responsible for catalysis of an *in vivo* acyl transfer reaction between the γ-carboxamide group of protein bound glutamine residues and primary amino groups (Sarkar *et al.* 1957). Reaction with the ε-amino group of protein bound lysine leads to protein cross-linking via ε-(γ-glutamyl) lysine isodipeptide bond formation. Alternatively, when the acyl acceptor substrate is a polyamine, both protein cross-linking via *N'*,*N'*-bis(γ-glutamyl) polyamine cross-bridge formation and post translational modification via *N'*-(γ-glutamyl) polyamine formation may occur (Folk 1980; Griffin and Smethurst 1994; Aeschlimann and Paulsson 1994). In addition, transglutaminases are able to carry out a variety of *in vitro* reactions involving either aminolysis or hydrolysis, none of which have been shown to be of physiological significance (Folk and Finlayson 1977; Folk *et al.* 1967).

1.1.2 Biochemistry of the transglutaminases.

1.1.2.1 Specificity.

Transglutaminases are specific for protein bound glutamine as their only acyl donor substrate. Free glutamine is not utilised and this distinguishes transglutaminases from other enzymes of glutamine metabolism (Lorand and Conrad 1984). Differences in the reactivity of glutamine residues of different proteins and within the same protein has been reported (Folk and Finlayson 1977; Gorman and Folk 1981 and 1984; Aeschlimann *et al.* 1992). The observed differences in reactivity are due to charge, primary structure and conformation of the protein in the vaccinity of the glutamine residue. Different transglutaminases have also been shown to exhibit different specificity for the same protein as illustrated by the structural differences in the polymers formed by the action of factor XIII and tissue transglutaminase on fibrin. This structural difference is due to different glutamine residues within the same protein being utilised by each enzyme (Shainoff *et al.* 1991).

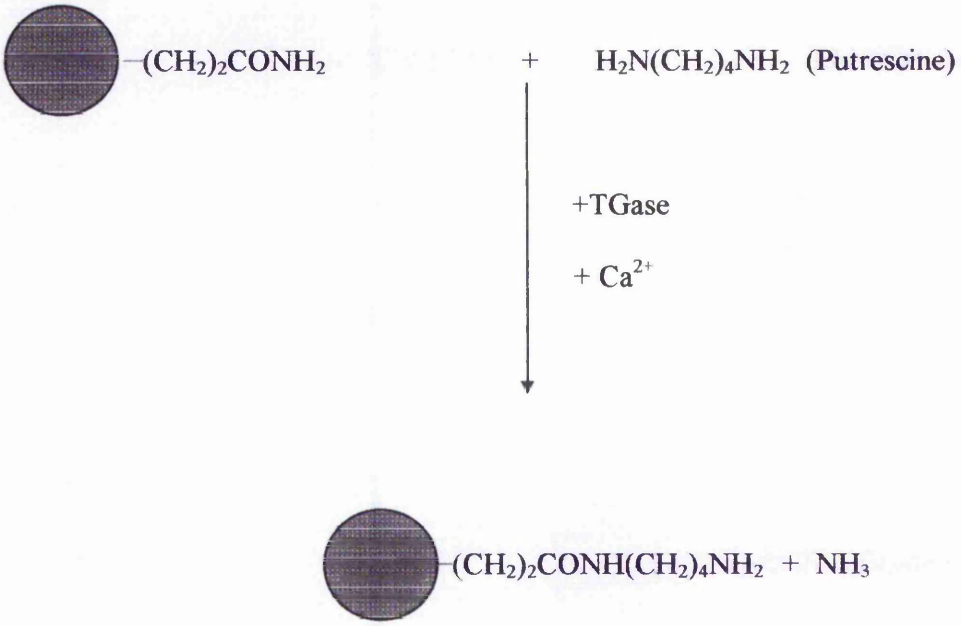
In contrast to the narrow acyl donor substrate specificity of transglutaminases, a broad specificity for amine donor substrates is exhibited. Use of the polyamines putrescine, spermine, spermidine and cadaverine results in the *in vivo* formation of either *N'*,*N'*-bis(γ-glutamyl) polyamine cross-bridges or *N'*-(γ-glutamyl) polyamine conjugates (For reviews see Folk and Finlayson 1977; Folk 1980 and 1983). Also of biological importance is the *in vivo* reaction between the ε-amino group of peptide bound lysine and peptide bound glutamine which results in ε-(γ-glutamyl) lysine formation. This bond is present in many tissues and provides mechanical and chemical stability (Griffin and

Smethurst 1994). Unlike glutamine donor proteins, a large number of lysine containing proteins are suitable substrates for transglutaminases because amino acid residues adjacent to lysine do not influence substrate potential (Aeschlimann and Paulsson 1994).

1.1.2.2 Reactions of the transglutaminases.

1.1.2.2.1 Polyamine incorporation.

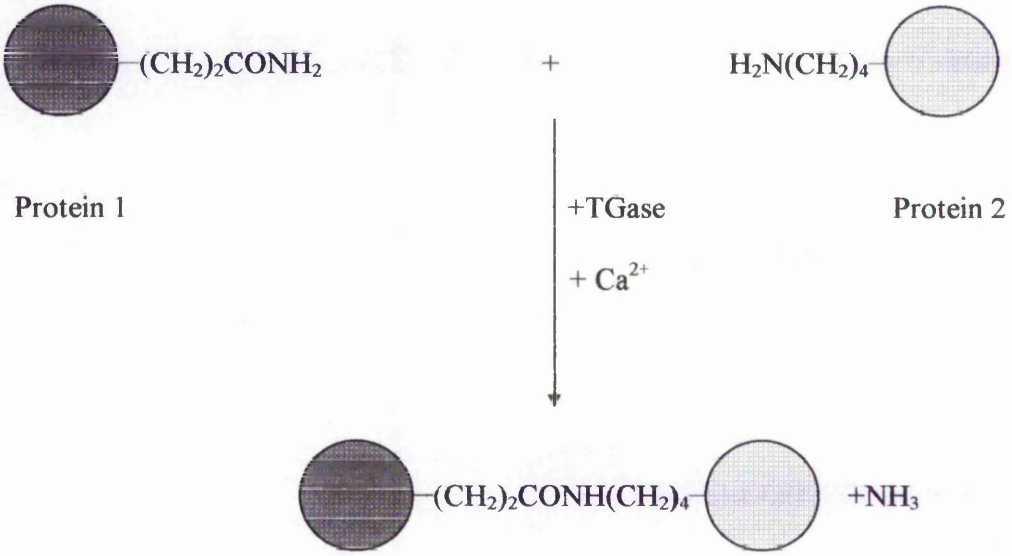
The polyamine incorporation reaction results in the formation of *N'*-(γ-glutamyl) polyamine bonds. This reaction has also been utilised in several transglutaminase assays involving the incorporation of either radiolabelled, fluorescently labelled or biotin labelled polyamines into such substrates as *N',N'*-dimethylcasein and benzyloxycarbonyl-L-glutaminylglycine (Lorand *et al.* 1972; Fink *et al.* 1992; Slaughter *et al.* 1992). When proteins are used as glutamine donors in such assays, they are dimethylated in order modify the lysine and hence prevent intramolecular cross-linking via ε-(γ-glutamyl) lysine which would compromise assay sensitivity by reducing the signal.



1.1.2.2.2 Protein cross-linking.

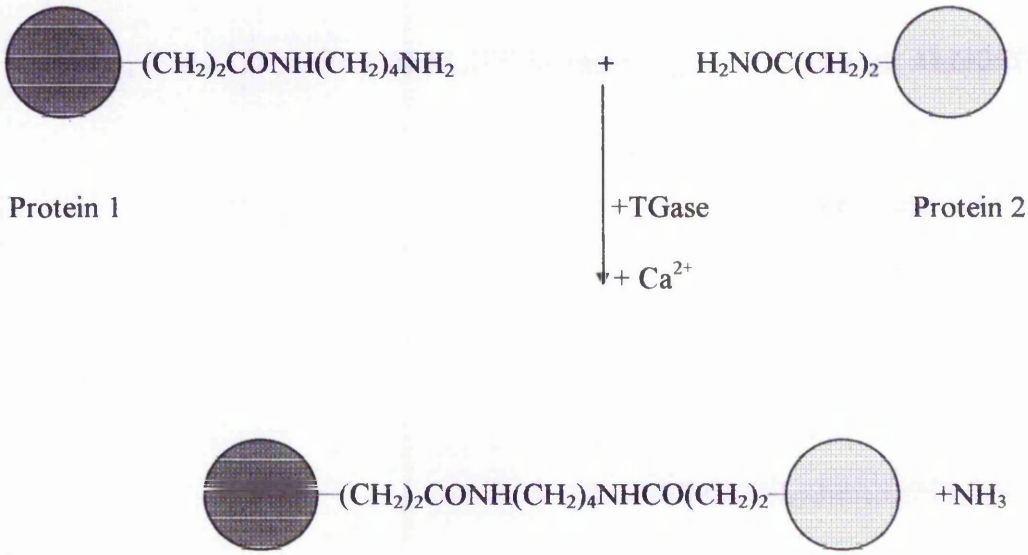
The cross-linking of two proteins via lysine and glutamine results in an ε-(γ-glutamyl) lysine isodipeptide bond. Calcium ions bind to all mammalian transglutaminase enzymes resulting in a conformational change, which exposes the active site (Folk 1983). Specificity for calcium is high and tissue transglutaminase has been shown to bind 3-4 calcium ions per molecule but it should be noted that other divalent cations including strontium and manganese have been demonstrated to activate tissue transglutaminase (Folk *et al.* 1967).

Sequencing has revealed no typical binding sequence such as the EF hand structure seen in calmodulin and other calcium binding proteins and it has been suggested that negatively charged regions of the molecule are probable binding sites for calcium (Aeschlimann and Paulsson 1994).



The ε-(γ-glutamyl) lysine cross-link is resistant to proteolysis but the bond may be hydrolysed in some specialist tissues. For example, hydrolysis of the free isodipeptide occurs in the kidney of rabbit by the action of a γ-glutamyl cyclotransferase (Fink *et al.* 1980). Furthermore, the medicinal leech, *Hirudo medicinalis* has been reported to posses enzymes capable of digesting the protein bound isodipeptide (Zavalova *et al.* 1996). The protein cross-linking reaction has been utilised in several transglutaminase assays involving the cross-linking of immobilised casein to biotin labelled casein (Seiving *et al.* 1991; Choi *et al.* 1992; Lilley *et al.* 1997a).

1.1.2.2.3 Protein-polyamine-protein cross-bridge formation.



During this reaction, the second primary amine group of an already covalently attached polyamine is able to react with a second protein resulting in the formation of an *N'*,*N'*-bis (γ-glutamyl) polyamine protein cross-link. Recently an assay has been developed at The Nottingham Trent University to detect the ability of transglutaminase enzymes to carry out this reaction *in vitro* (Lilley *et al.* 1997a).

1.1.2.2.4 Hydrolysis of peptide bound glutamine and other amides.

$$R_1CONH_2 + H_2O \longrightarrow R_1CO_2H + NH_3$$

This reaction utilises water as the attacking nucleophile instead of a primary amine and results in the de-amidation of amides to carboxylic acids. The reaction has been demonstrated *in vitro* but is thought to have no physiological relevance (Folk *et al.* 1967).

1.1.2.2.5 Hydrolysis of esters.

$$R_1CO_2R_2 + H_20 \longrightarrow R_1CO_2H + R_2OH$$

1.1.2.2.6 Aminolysis of esters.

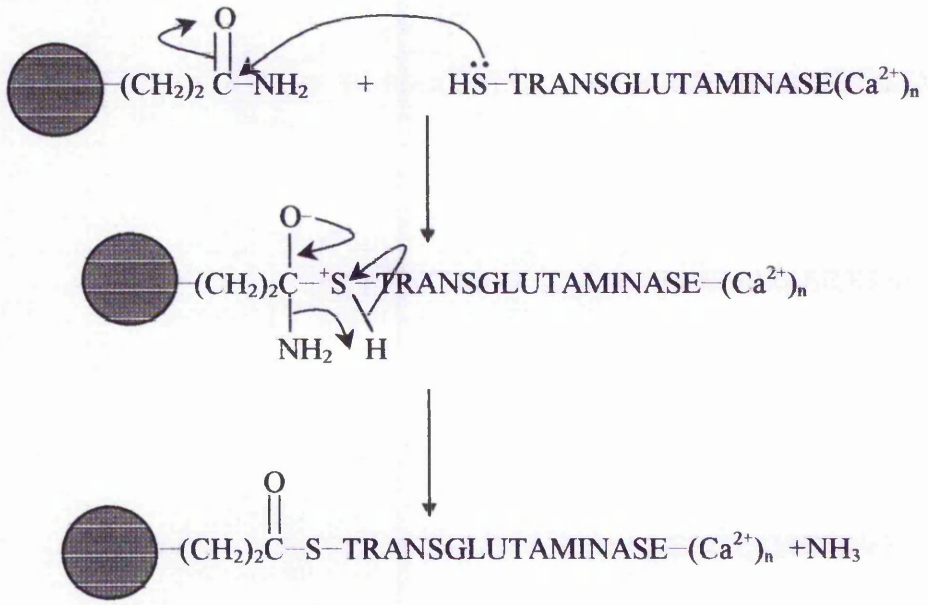
$$R_1CO_2R_2 + R_3NH_2 \longrightarrow R_1CONHR_3 + R_2OH$$

In both reaction 1.1.2.2.5 and 1.1.2.2.6, protein bound glutamine is replaced by an ester. The reaction of primary amines and water with esters occurs *in vitro* by the same double displacement reaction as the other transglutaminase reactions (see section 1.1.2.3) but has no physiological relevance and has not been shown to occur *in vivo* (Folk and Finlayson 1977; Folk *et al.* 1967).

1.1.2.3 The catalytic mechanism of the transglutaminases.

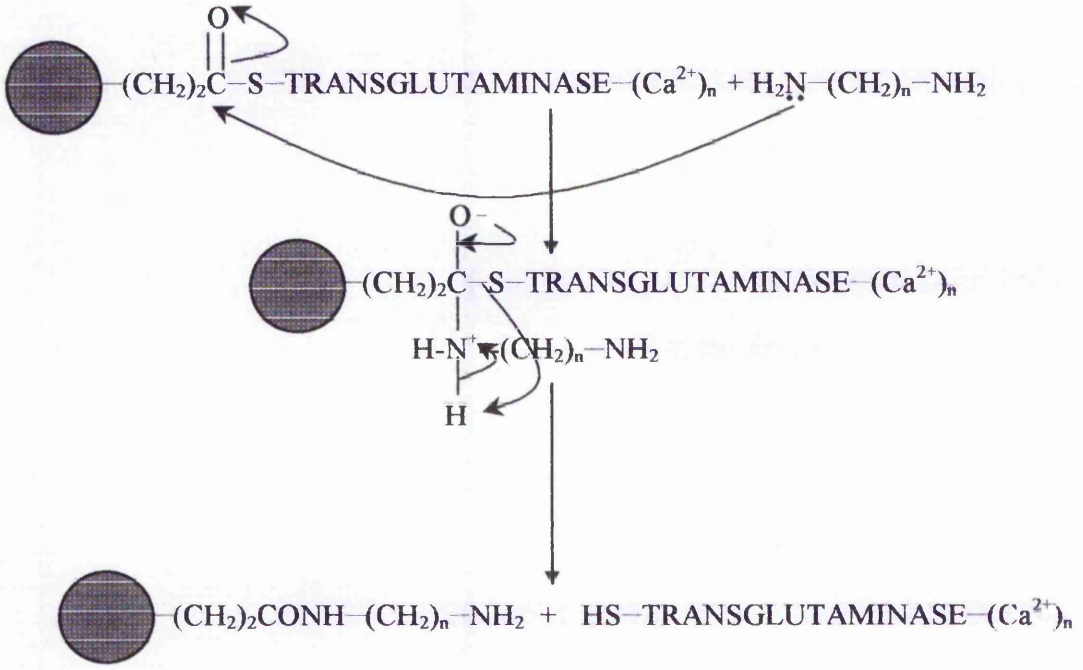
The transglutaminase reaction mechanism consists of a two step process, the initial step involving calcium ions binding to the enzyme which induces a conformational change resulting in exposure of the active site (Folk 1983). Nucleophilic attack on the electron deficient γ-carbon atom of the glutamine residue by the active site cysteine residue thiol group then occurs (Figure 1). This step was verified by the isolation of a thioester linked acyl-enzyme intermediate (for review see Folk 1983).

Figure 1. (Step 1): Nucleophilic attack on the protein bound glutamine residue by the active site thiol group.



The second step of the reaction mechanism involves the nucleophilic displacement of the protein from the active site of transglutaminase by the primary amine group (Figure 2).

Figure 2. (Step 2): Nucleophilic attack on the acyl-enzyme intermediate by the primary amine.



1.2 Varieties and biological roles of the transglutaminases.

1.2.1 Mammalian transglutaminases.

1.2.1.1 Plasma factor XIII.

Plasma transglutaminase (Factor XIII) is a heterotetramer ($\alpha_2\beta_2$) with a molecular mass of 320kDa. It consists of two catalytic (α) sub-units of molecular mass 75kDa and two non-catalytic (β) sub-units of molecular mass 80kDa (Aeschlimann and Paulsson 1994). Factor XIII is present in human blood plasma as a pro-enzyme and is activated by the calcium dependent protease thrombin during the terminal step of the blood clotting cascade. In the presence of fibrinogen, thrombin is activated by plasma levels of calcium to cleave the catalytic (α) sub-units from the (β) sub-units (Credo *et al.* 1978) and this yields an active dimer which is referred to as factor XIIIa.

Fibrin monomers produced by the thrombolytic cleavage of fibrinogen are cross-linked by factor XIIIa resulting in an insoluble fibrin clot which is mechanically stable and resistant to both chemical and enzymic degradation. The clot is also protected from the major clot degrading enzyme plasmin due to the factor XIIIa mediated incorporation of an α_2-plasmin inhibitor into the cross-linked fibrin (Tamaki and Aoki 1982). The cross-linking of fibrin and the incorporation of α_2-plasmin inhibitor occur at a faster rate than the cross-linking of other substrate proteins found in plasma and this ensures that these are the first reactions to occur (Hornyak and Shafer 1992). The cross-linked fibrin

polymer then increases the rate at which factor XIII is cleaved by thrombin by providing both factor XIII and thrombin binding sites (Greenberg *et al.* 1987).

Individuals with factor XIIIa deficiency suffer from a number of difficulties including bleeding disorders (Lorand *et al.* 1980). Wound healing problems are also common such as those experienced by sufferers of the chronic inflammatory bowel disorder, Crohn's disease which is often further complicated by the appearance of needle shaped ulcers known as refracted fistulas (Oshitani *et al.* 1995). As a consequence of the clinical importance of factor XIIIa, a number of assays have been developed to screen human blood for deficiency of this enzyme (Song *et al.* 1994, Seiving *et al.* 1991).

1.2.1.2 Prostate transglutaminase.

Prostate transglutaminase is a further example of an extracellularly active transglutaminase with a fully elucidated biological role. Guinea pig prostate transglutaminase is a calcium dependent monomer of molecular mass 70kDa which accounts for up to 25% (w/w) of total anterior prostate gland intracellular protein (Wilson and French 1980; Wing *et al.* 1974).

Prostate transglutaminase is secreted into the semen to cross-link proteins and form a stable, clotted post ejaculatory vaginal plug which aids rodent fertility by providing mechanical stability to the clotted semen. The ε-(γ-glutamyl) lysine isodipeptide bonds have been isolated *in vivo* in clotted rodent semen and if polyamines are exogenously added during clotting, both *N'*,*N'*-bis(γ-glutamyl) polyamine and *N'*-(γ-glutamyl) polyamine conjugates are formed (Folk 1980). In certain species, the reaction to cross-

link seminal proteins is inhibited by spermine and spermidine which are produced in excess by the ventral prostate. This reaction is thought to prevent premature semen coagulation, which could block the passage of sperm through the urethra (Williams-Ashman 1984). More recently, a transglutaminase has been identified in the human prostate gland. The molecular mass was determined at 77kDa and the gene encoding the enzyme is located on chromosome 3 (Dubbink *et al.* 1996).

1.2.1.3 Keratinocyte transglutaminase (Type I).

Keratinocyte transglutaminase is a calcium dependent monomer of molecular mass 90kDa (Chakravarty and Rice 1989). The presence of calcium also appears to up-regulate synthesis of this enzyme (Floyd and Jetten 1989). It is often referred to as particulate transglutaminase as it is anchored to the plasma membrane of terminally differentiating keratinocyte cells of the stratified squamous epithelia by a post translational acylation reaction with palmitate and myristate. This anchorage is cleaved by calcium dependent proteolysis to release a transglutaminase of reduced molecular mass (80kDa) which dimerises in the cytoplasm (Rice *et al.* 1990) where the function of the activated enzyme is proposed to be the cross-linking of the cell envelope proteins loricrin (Hohl *et al.* 1991) and cornifin (Marvin *et al.* 1992). Cross-linking of these proteins produces an insoluble, cornified cell envelope that serves to stabilise the outermost layer of the epidermis forming an effective barrier to the environment.

Deficiency of keratinocyte transglutaminase is thought to lead to the severe congenital skin disease lamellar ichthyosis that is characterised by large skin scales and variable redness (Huber *et al.* 1995). The skin disease psoriasis vulgaris which is characterised by

the appearance of red inflamed areas known as psoriatic plaques has been linked to premature expression of keratinocyte transglutaminase in the supra-basal spinous layer of the epidermis rather than the granular layer (Griffin and Smethurst 1994). Retinoids which are known to down regulate the expression of genes involved in cell proliferation have been shown to be therapeutically useful in the treatment of psoriasis possibly by decreasing keratinocyte transglutaminase expression in the psoriatic plaque (Rosenthal *et al.* 1992). Topical addition of the vitamin D_3 analogue calcipotriol to psoriatic skin is also an effective treatment although the mechanism of action of this compound is not fully understood (Oranje *et al.* 1997).

1.2.1.4 Tissue transglutaminase (Type II).

Tissue transglutaminase is the most widely distributed form of transglutaminase in mammals (Folk and Finlayson 1977) and has been detected in many tissues including liver, arterial muscle and lung (Folk 1980; Griffin and Smethurst 1994; Birkbichler *et al.* 1978b). Tissue transglutaminase has been purified to homogeneity from a variety of sources including guinea pig liver (Folk and Cole 1966), Rabbit liver (Abe *et al.* 1977), rat liver (Knight *et al.* 1990) and human erythrocytes (Brenner and Wold 1978). The tissue enzyme from all sources is monomeric with a molecular mass of 70-90kDa and there is 80% homology between the amino acid sequences of the guinea pig and human tissue transglutaminase enzymes (Greenberg *et al.* 1991). Tissue transglutaminase has been found to co-exist alongside other forms of the enzyme in both hair follicles and the epidermis (Lichti *et al.* 1985; Chung and Folk 1972). Although the biological role of tissue transglutaminase has not yet been defined it has been suggested to be involved in a

number of important cellular functions and these processes will be discussed later in the text.

Tissue transglutaminase is activated in the presence of calcium ions (For reviews see Folk and Finlayson 1977; Folk 1980 and 1983). At sub-optimal levels of calcium, the enzyme exhibits GTP binding resulting in inhibition of activity (Takeuchi *et al.* 1992; Bergamini and Signorini 1993; Mian *et al.* 1995; Smethurst and Griffin 1996). Tissue transglutaminase thus exhibits a weak GTPase activity (Lee *et al.* 1989) and it has recently been suggested that tissue transglutaminase may be involved in transmembrane signal mediation, acting as a G-protein (Im *et al.* 1997). Since calcium ions and GTP have been shown to have opposite effects on enzyme activity *in vitro* it is also possible that the *in vivo* activity of tissue transglutaminase may be controlled by local levels of these factors (Achyuthan and Greenberg 1987). Tissue transglutaminase is also regulated by a number of agents at the gene expression level including retinoids, sodium butyrate and transforming growth factor beta (Fukuda *et al.* 1993; Griffin and Smethurst 1994).

It has been proposed that tissue transglutaminase may have an involvement in apoptosis (programmed cell death). First described by Kerr (1971), apoptosis is a process by which cells die in an ordered fashion without the leakage of cellular contents (For review see Schwartzman and Cidlowski 1993). During apoptosis, cells become more spherical and the nuclei separate into discrete masses of condensed chromatin which then fragment along with condensed cell organelles into a number of membrane bound vesicles which are described as apoptotic bodies. Apoptotic bodies prevent the leakage of cellular components and promote the phagocytosis of dying cells by the surrounding cells due to

alterations in the chemistry of surface carbohydrates and lectins of the plasma membrane (Duvall *et al.* 1985). It has been proposed that tissue transglutaminase is involved in the formation of the insoluble, cross-linked apoptotic envelope because both transglutaminase activity and the level of ε-(γ-glutamyl) lysine are observed to increase in certain tissues undergoing programmed cell death (Fesus *et al.* 1987 and 1989). Analysis of apoptosing mouse and hamster cell lines showed that the majority of transglutaminase activity and ε-(γ-glutamyl) lysine was associated with the apoptotic bodies and not the remainder of the cell (Knight *et al.* 1991). Further evidence to link tissue transglutaminase to apoptosis is the observed increase in the intracellular calcium concentration during late stages which is known to activate tissue transglutaminase (Fesus *et al.* 1987).

A further process in which tissue transglutaminase has been implicated in is the stabilisation of the extracellular matrix. This is thought to take place via the cross-linking of such proteins as fibronectin, collagen, laminin and nidogen (Aeschlimann and Paulsson 1991). It has also been suggested that the cross-linking of extracellular matrix proteins by tissue transglutaminase may assist in the process of cell adhesion (Juprelle-Soret *et al.* 1988; Gentile *et al.* 1992). The extracellular space is an ideal environment for optimal tissue transglutaminase activity due to a high calcium ion concentration and low levels of such inhibitory factors as GTP and zinc ions (Aeschlimann and Paulsson 1994). Tissue transglutaminase has been shown to be involved in extracellular matrix cross-linking prior to calcification during the process of bone formation (Aeschlimann *et al.* 1993) but it is not yet clear how tissue transglutaminase is secreted as the enzyme has no secretory sequence (Ikura *et al.* 1988). Certain disease states of the extracellular matrix

have been coupled to elevated levels of tissue transglutaminase such as pulmonary fibrosis and eye lens cataract formation (Griffin *et al.* 1979; Griffin and Smethurst 1994). A recent paper reports a possible therapeutic role for tissue transglutaminase as an extracellular cartilage adhesive for use in orthopaedic surgery (Jurgensen *et al.* 1997).

Elevated transglutaminase activity was first related to the metastatic potential of tumours in 1966 by Laki *et al.* They observed that the administration of transglutaminase inhibitors to mouse YPC-1 tumours greatly increased mouse survival time. Birkbichler *et al.* (1976 and 1978a) observed decreases in tissue transglutaminase activity in Hovikoff hepatoma cells compared to healthy cells. They demonstrated that the remaining transglutaminase became more particulate and that levels of ε-(γ-glutamyl) lysine isodipeptide were lower in metastasising tumours compared to normal tissue, indicating that transglutaminase was required for cells to remain in a non-proliferating state.

Haughland *et al.* (1982) showed that a decrease in ε-(γ-glutamyl) lysine resulted in an increase in membrane fluidity and hence they proposed that tissue transglutaminase may be involved in membrane architecture. Using 6-P-dimethylaminophenylazobenzathiazole and diethylnitrosamine as carcinogens, Barnes *et al.* (1984 and 1985) were able to show that there is a decrease in transglutaminase activity in tumour containing liver compared to healthy liver. Indeed, other workers have noted a decrease in transglutaminase activity from healthy tissue > benign tumour > metastasising tumour (Delcros *et al.* 1986). Conversely, in certain types of cancer, expression of tissue transglutaminase has been shown to increase. In azoxymethane induced rat colon cancer cells, tissue

transglutaminase activity increased compared to healthy tissue and elevated levels of transglutaminase were found to be associated with the extracellular matrix (Dargenio *et al.* 1995). Hettasch *et al.* (1996) also demonstrated an increase in extracellular matrix levels of tissue transglutaminase by immunoblot analysis in human breast carcinoma. The alteration of tissue transglutaminase levels during tumourigenesis could mean that this enzyme is of possible prognostic value for the assessment of human cancers.

Various workers have shown that receptor mediated endocytosis may be impeded by transglutaminase inhibitors such as primary amine competitive substrates (Yarden *et al.* 1981; Leu *et al.* 1982; Teshigawara *et al.* 1985; Hucho and Bandini 1986). Yarden *et al.* (1981) showed that one round of internalisation of α-macroglobulin could be achieved in the presence of primary amines but further internalisation did not occur and this led to the proposal that α-macroglobulin receptors could not return to the cell surface without the participation of active tissue transglutaminase. A criticism of the work on transglutaminase inhibition was presented by Ahmed and Niswender (1981) who proposed that the added primary amines were accumulating in lysosomes and that the resultant increase in cellular pH was responsible for the inhibition of endocytosis. Whether or not tissue transglutaminase is involved in this process remains unclear.

Further proposed roles for tissue transglutaminase include the secretion of insulin by pancreatic β cells (Bungay *et al.* 1982 and 1984) and stiffening of the erythrocyte membrane during the ageing process (Lorand and Conrad 1984). The ubiquitous nature of this enzyme suggests that it is involved in important biological processes and much

research is therefore still being directed towards the full understanding of the biological function of tissue transglutaminase.

1.2.1.5 Epidermal transglutaminase (Type III) and hair follicle transglutaminase.

Epidermal transglutaminase is a 72kDa monomer, which is activated by proteolysis to a 50-54kDa protein (Negi *et al.* 1985; Greenberg *et al.* 1991) and has been identified in bovine snout epidermis (Buxman and Wuepper 1975) and human callus (Folk 1980). Upon activation, the cross-linking of keratinocyte proteins results in the deposition of a rigid, insoluble protein matrix beneath the epidermal cell membrane (Thacher and Rice 1985) containing both ε-(γ-glutamyl) lysine and *N'*,*N'*-bis (γ-glutamyl) polyamine cross-bridges. These covalent cross-links are thought to provide the protein matrix with the stability required to maintain skin integrity (Piacentini *et al.* 1988). The epidermal enzyme is not found in cultured keratinocytes and is immunologically distinct from factor XIII and tissue transglutaminase (Ogwa and Goldsmith 1977). Epidermal transglutaminase has the same molecular mass as hair follicle transglutaminase (50-54kDa) but hair follicle transglutaminase has been shown to be a dimer consisting of two 27kDa sub-units (Chung and Folk 1972) and the two enzymes are immunologically distinct (Buxman and Wuepper 1976 and 1978). The detection of ε-(γ-glutamyl) lysine isodipeptide in citrulline rich proteins of hair follicles has led to the suggestion that the hair follicle enzyme may be involved in hair fibre formation and stabilisation (Harding and Rogers 1976; Peterson and Wuepper 1984).

1.2.2 Non-mammalian transglutaminases.

1.2.2.1 Nematode transglutaminase.

Mehta *et al.* (1992) detected ε-(γ-glutamyl) lysine isodipeptide in the nematode *Brugia malayi* and postulated that transglutaminase plays an important role during the development of the embryonic worm. Purification of *Brugia malayi* transglutaminase yielded a calcium dependent 56kDa protein with a pH optimum of 8.5. The transglutaminase from *Brugia malayi* showed similar characteristics to mammalian tissue transglutaminase including inhibition by EDTA, primary amines and millimolar levels of GTP. Thiol blocking reagents also inhibit activity suggesting that there is a thiol group present at the active site (Singh and Mehta 1994). Further research showed that embryonic development of *Brugia malayi* was enhanced by the transglutaminase mediated cross-linking of host proteins (Mehta *et al.* 1996).

Transglutaminase has also been detected in the canine filarial parasite *Dirofilaria immitis*. Purification of this enzyme yielded a 56kDa protein which cross reacted with an antibody raised in rabbit against the N-terminal sequence of *Brugia malayi* transglutaminase suggesting sequence similarity (Singh *et al.* 1995). Further evidence for the involvement of transglutaminase in nematode development was presented by Lustigman *et al.* (1995). The transglutaminase inhibitors monodansylcadaverine, cystamine and N-benzyloxycarbonyl-D, L-beta-(3-bromo-4, 5 dihydroisoxazol-5-yl)-alaninebezylamide were shown to prevent moulting of stage 3 *Onchocerca volvulus* larvae to stage 4 which suggests that transglutaminase in nematodes may be a possible target for an anti-parasitic chemotherapeutic agent.

1.2.2.2 Bacterial transglutaminase.

Transglutaminase from a variety of bacteria including *Streptomyces lydicus* and *Streptoverticillium morbaraense* has recently been shown to improve the functional properties of some foods by the cross-linking of certain proteins to improve the processing, flavour, appearance and texture characteristics (For review see Zhu *et al.* 1995). For example, the treatment of soy protein hydrolysates by microbial transglutaminase improves their solubility properties by greatly reducing hydophobicity. Cross-linking of the protein hydrolysate serves to bury hydrophobic amino acids within the resultant polymer, which results in an improvement in the taste of soy protein (Babiker *et al* 1996). Transglutaminase from *Streptomyces morbaraense* has been shown to improve the functional properties of a variety of food proteins including casein, soybean globulins, gluten, actin and myosins. It is a monomer of 38kDa (SDS-PAGE) and differs from mammalian transglutaminases in that it is calcium independent (Zhu *et al.* 1995). Similarly *Streptomyces lydicus* transglutaminase is calcium independent (Faergemand *et al.* 1997) but in common with mammalian transglutaminase both bacterial enzymes are thiol dependent implying active site similarities. Use of the bacterial enzyme over mammalian transglutaminase for food protein modification has certain advantages including the fact that purification from bacteria is easier and large quantities may be prepared using fermentation technology.

1.2.2.3 Fungal transglutaminase.

It has recently been suggested that transglutaminase may be responsible for the cross-linking of structural proteins during cell wall formation of the yeast *Candida albicans* as cell wall protein incorporation was inhibited by the transglutaminase inhibitor cystamine

(Sentandreu *et al.* 1995; Ruizherrera *et al.* 1995). Immunoreactivity between a factor XIIIa antibody and a cell wall protein has also been demonstrated suggesting the involvement of transglutaminase in fungal development (Arrese and Pierard 1995). This research implies that inhibition of *Candida albicans* transglutaminase by a chemotherapeutic agent may reduce the pathogenicity of this fungi and result in a treatment for Candidiasis (thrush).

1.2.3 Plant transglutaminase.

1.2.3.1 Introductory statement.

The first evidence for enzyme mediated covalent incorporation of polyamines into protein by a crude plant extract, was presented in 1987 by Icekson and Apelbaum. They showed the incorporation of [^{3}H]-labelled putrescine into a number of protein substrates by an extract prepared from the 7 day old apical meristematic region of etiolated *Pisum sativum* seedlings. There has been an increasing body of evidence to support the existence of a catalytically active transglutaminase in plant tissue and the sections that follow review the observations that have been made and the conclusions drawn from them.

1.2.3.2 Sources of plant transglutaminase and methods of detection.

Detection of transglutaminase activity in 7 day old apical meristematic tissue of etiolated *Pisum sativum* seedlings was achieved using a modification of a method first described by Lorand *et al.* in 1972. Crude plant extract was incubated in the presence of *N',N'*-dimethylcasein and [^{3}H]-labelled putrescine. Following incubation at 35°C for 30 minutes, aliquots of the reaction mixture were spotted onto filter paper and the protein was precipitated by treatment with cold 10% (w/v) TCA. The filter papers were placed into liquid scintillation cocktail and the covalently incorporated [^{3}H]-labelled putrescine was quantified by liquid scintillation counting. The activity was shown to be present either in the cytosol or the extracellular fluid as only 3% of the activity was associated with a 27000×g pellet. This data was supported by the more recent observation that the [^{14}C]-labelled polyamine conjugation activity of 10 day old apical meristematic hook

regions of etiolated *Pisum sativum* seedlings was also shown to be soluble. Treatment of a membrane pellet with various detergents including Triton X-100, Tween, Brij 35 and deoxycholate yielded no transglutaminase activity (Chiarello *et al.* 1996a). It should be noted that many modifications of the original assay described by Lorand *et al.* in 1972 have been carried out in order to study plant transglutaminases. For example, recently a calcium independent transglutaminase-like activity has been purified from *Glycine max* leaves using a method that involves [^{14}C]-labelled putrescine incorporation into *N',N'*-dimethylcasein bound to nitro-cellulose discs (Kang and Cho 1996).

Membrane associated transglutaminase activities have also been detected in a number of plant tissues. Detergent treatment of membrane pellets prepared from the leaves of *Spinacia oleracea* and *Beta vulgaris* and the floral buds of *Brassica oleracea* yielded a radiolabelled amine incorporation activity (Signorini *et al.* 1991). Evidence for transglutaminase activity in intact chloroplasts of *Medicago sativa* was presented by Margosiak *et al.* (1990) using a monodansylcadaverine conjugation reaction, which was visualised by fluorescence detection in polyacrylamide gels. Furthermore, they demonstrated that the transglutaminase activity co-eluted with the large sub-unit of RuBisCo (E. C 4.1.1.39) when crude extracts were subjected to size exclusion chromatography indicating an estimated molecular mass of 58kDa for plant transglutaminase. However, further purification of the activity from *Medicago sativa* using DEAE-cellulose chromatography, isoelectric focusing and reversed phase HPLC yielded a protein of molecular mass 37-39kDa (Kuehn *et al.* 1991).

Transglutaminase activity was also observed in a Triton X-100 treated 22000×g *Helianthus tuberosus* leaf membrane pellet (Falcone *et al.* 1993). Further investigation showed the transglutaminase activity to be associated with the thylakoid membrane where a 58kDa protein band cross reacted with antibodies raised against both rat prostate and human erythrocyte transglutaminases (Del Duca *et al.* 1994). In the same laboratory, transglutaminase activity has been detected in floral buds, tuber explants and the apical meristematic region of sprouting tubers of *Helianthus tuberosus* using a radiolabelled amine incorporation assay. The authors also suggest the possibility of different isoforms of transglutaminase within the same organ (Serafini-Fracassini *et al.*1988; Del Duca *et al* 1993; Falcone *et al.* 1993). Labelled *Helianthus tuberosus* endogenous substrate proteins were demonstrated by running the reaction products on SDS-PAGE gels. Entire lanes of the gels were cut into coomassie blue positive or negative bands and following gel dissolution the bands were counted for radioactivity. Endogenous proteins with various molecular masses ranging from 30-92kDa were shown to be labelled.

Other methods used to detect plant transglutaminase activity include the cross-linking of proteins by plant extracts followed by analysis on SDS-PAGE gels (Siepaio and Meunier 1995). A Triton X-100 treated 41400×g *Lupinus albus* membrane pellet was incubated with bovine casein or spinach RuBisCo and the resultant reaction mixture was shown to contain high molecular mass protein polymers when analysed by SDS-PAGE electrophoresis. Furthermore, the extract was able to incorporate labelled polyamines into *N',N'*-dimethylcasein and the authors concluded that this was good evidence for the presence of a transglutaminase in *Lupinus albus* seedlings because both polyamine

incorporation and protein cross-linking could be carried out by the membrane extract. Covalent attachment of both [^{3}H] and [^{14}C]-labelled polyamines to oat and petunia protoplast homogenates bound to filter paper discs has been demonstrated. The homogenates were incubated with labelled polyamines and subsequently washed with either 1.0M or 5.0M NaCl to remove any label bound by an ion exchange effect (Mizrahi *et al.* 1989). They concluded that the labelled polyamine was bound to protein because washing with proteases was the only method found to reduce the number of counts on each filter paper disc.

Transglutaminase activity has also been demonstrated in the root tissue of a variety of higher plants. Lilley *et al.* (1996 and 1998) demonstrated the incorporation of biotin labelled cadaverine into *N',N'*-dimethylcasein and the cross-linking of chemically modified bovine casein by soluble root and leaf extracts prepared from *Pisum sativum, Vicia faba, Triticum aestivum* and *Hordeum vulgare*. Similarly, an increasing level of transglutaminase activity was detected in root explants of *Chrysanthemum morifolium* during the initial days of culture. Following the initial rapid stage of cell division, activity was shown to decrease as differentiation occurred (Aribaud *et al.* 1995). To date both soluble and membrane bound transglutaminase activity has been detected in a variety of plant root and leaf tissues indicating that this enzyme may be as widely distributed in plants as it is in animals.

1.2.3.3 Plant transglutaminase biochemistry.

1.2.3.3.1 Regulatory factors.

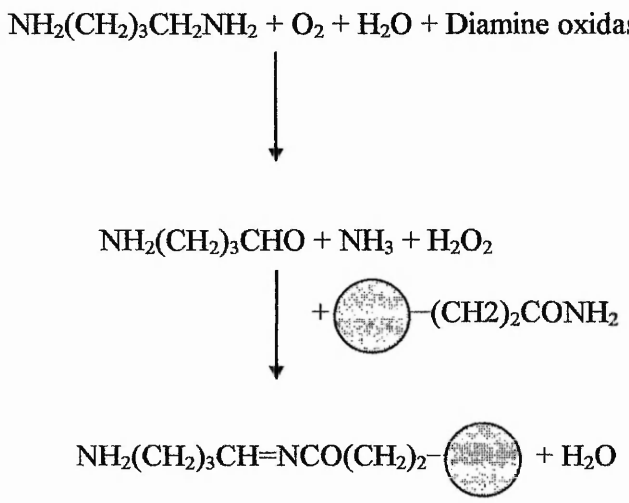
1.2.3.3.1.1 Calcium ion requirement.

As previously stated, transglutaminases are generally regarded as calcium dependent enzymes. In contrast, recent research indicates that plant transglutaminase may have no absolute requirement for this cation (for review see Serafini-Fracassini *et al.* 1995) and it should be noted that examples of calcium independent microbial transglutaminases have been also been observed (Faergemand *et al.* 1997). The pioneering plant transglutaminase research article written by Icekson and Apelbaum in 1987 considered the question of calcium ion dependence and reported that the addition of 6.6mM $CaCl_2$ to the radiolabelled amine incorporation reaction buffer stimulated transglutaminase activity by 47% but no absolute dependency upon this cation was observed. Addition of the chelation agents EDTA and EGTA produced very little inhibition of the putrescine binding activity of the *Pisum sativum* apical meristem preparation and this led to the conclusion that there were differences between the enzymes found in mammals and plants. This work was supported by the observation that the radiolabelled polyamine incorporation activity in a crude extract prepared from the apical meristem of *Helianthus tuberosus* was also not dependent upon exogenous addition of calcium (Serafini-Fracassini *et al.* 1988). Slight stimulation of activity was noted at calcium ion concentrations up to 5mM but further addition of calcium was shown to inhibit transglutaminase activity. Chelation by 15mM EDTA inhibited polyamine binding by only 16% indicating that calcium ions are not an absolute requirement for plant

transglutaminase activity. Further work in the same laboratory showed that transglutaminase activity from different organs including leaves and floral buds was stimulated by varying degrees due to addition of calcium ions to the reaction mixture but no total dependency was demonstrated (Falcone *et al.* 1993, Del Duca *et al.* 1994). Other groups have shown similar findings using radiolabelled amine incorporation assays (Margosiak *et al.* 1990; Kuehn *et al.* 1991; Signorini *et al.* 1991; Aribaud *et al.* 1995; Lilley *et al* 1995 and 1998; Kang and Cho 1996). It should however be noted that the enzymic nature of the amine binding reactions was shown in all cases by the demonstration of a time dependent increase in covalently bound radioactive label and the use of boiled extract controls.

It has recently been demonstrated that the radiolabelled polyamine incorporation assays used to detect transglutaminase activity are subject to interference by calcium independent enzymes present in crude plant extracts such as diamine oxidases (Siepaio and Meunier 1995; Chiarello *et al.* 1996a and 1996b; Martin-Tanguy *et al.* 1997). This interference would account for the observed absence of total calcium dependence since a proportion of the total activity observed is possibly due to the presence of a calcium independent diamine oxidase. Diamine oxidases are proposed to incorporate polyamines into proteins by the following mechanism (Figure 3).

Figure 3. Incorporation of polyamines into the glutamine residues of proteins by the action of diamine oxidase (according to Siepaio and Meunier 1995).



Using a *Lupinus albus* membrane extract, Siepaio and Meunier (1995) showed that both diamine oxidase and transglutaminase activities were present. Over 50% of the amine binding activity of the extract was removed by treatment with 1mM DIECA which inhibits diamine oxidase by chelation of copper ions essential for activity. The amine binding activity of a *Pisum sativum* apical meristem extract was also shown to be influenced by a diamine oxidase because addition of the copper chelator o-phenanthrolinehenanthroline inhibited the reaction by 67-96% (Icekson and Apelbaum 1987; Chiarello *et al.* 1996a). The debate concerning the relative contributions of transglutaminase and contaminating enzymes present in crude plant extracts forms a significant part of the scope of this thesis and will be discussed further in chapters 3 and 4 in terms of the development and use of alternative assay systems.

1.2.3.3.1.2 Inhibition by GTP.

Mammalian tissue transglutaminase is inhibited by GTP at sub-optimal calcium ion concentration (Takeuchi *et al.* 1992; Bergamini and Signorini 1993; Mian *et al.* 1995; Smethurst and Griffin 1996) and tissue transglutaminase exhibits a weak GTPase activity (Lee *et al.* 1989). Assaying for the inhibition of plant transglutaminase by GTP in crude extracts may result in misleading results due to the quenching of the GTP by other GTP binding proteins. This may explain why the only report to date of the inhibition of a plant polyamine binding activity by GTP has been following its purification from *Glycine max* leaves (Kang and Cho 1996). Kang and Cho estimated the relative molecular mass to be 80kDa by SDS-PAGE implying that the enzyme may be similar to mammalian tissue transglutaminase.

1.2.3.3.1.3 Proteolytic regulation.

The addition of the protease inhibitors leupeptin and PMSF to a *Helianthus tuberosus* extract was shown to inhibit radiolabelled polyamine binding implying that proteolytic cleavage may be a factor in the regulation of this type of transglutaminase (Grandi *et al.* 1992). This would suggest similarities between plant transglutaminase and both the keratinocyte and plasma mammalian transglutaminase enzymes (Credo *et al.* 1978; Rice *et al.* 1990). At present there are no other examples of proteolytic activation of plant transglutaminase in the literature.

1.2.3.3.2 The effect of inhibitors and activators.

1.2.3.3.2.1 Thiol group reagents.

A further acknowledged criteria for the identification of novel mammalian transglutaminases has been to demonstrate the presence of a thiol group at the active site of the enzyme since the active site sequence is the most conserved region of the molecule (Aeschlimann and Paulsson 1994). Thiol group activators such as the reducing agent DTT have been shown to exhibit various effects on plant transglutaminases depending on the source. 1-5mM DTT was shown to inhibit *Pisum sativum* apical meristematic transglutaminase activity by 40-80% (Icekson and Apelbaum 1987; Chiarello *et al* 1996a) and DTT was also shown to inhibit the polymerisation of bovine casein by *Lupinus albus* transglutaminase (Siepaio and Meunier 1995). 2-mercaptoethanol was shown to slightly inhibit the amine binding activity of a crude *Medicago sativa* extract (Margosiak *et al.* 1990). Conversely, 10mM DTT was found to be critical for the preservation of the amine binding activity of *Helianthus tuberosus* extracts (Serafini-Fracassini *et al.* 1988) and Kang and Cho (1996) demonstrated that exogenous addition of 10mM DTT almost doubled the amine binding activity of *Glycine max* leaf extracts. They also showed a 60% inhibition of activity due to the addition of 0.1mM NEM (a thiol group inhibitor). Furthermore, a 10mM concentration of the specific transglutaminase active site inhibitor cystamine was shown to inhibit the activity of *Chrysanthemum morifolium* transglutaminase by 100% (Aribaud *et al.* 1995) and this data suggests that there may be active site similarities between the transglutaminases present in *Chrysanthemum morifolium* and mammals.

1.2.3.3.2.2 Competitive inhibitors.

Icekson and Apelbaum (1987), working with *Pisum sativum*, demonstrated that [^{3}H]-labelled putrescine incorporation into *N',N'*-dimethylcasein could be inhibited by competition with other polyamines at a concentration of 10-100mM. Cadaverine was found to be the most effective inhibitor giving a reduction of 63% in incorporated radioactivity at the highest concentration. They also tested the capacity of a variety of amino acids to act as amine donors and inhibit the amine incorporation reaction and found that 25mM cysteine inhibited the reaction by 85% but other amino acids tested had little or no effect. The radiolabelled putrescine binding activity of *Helianthus tuberosus* apical meristematic tissue transglutaminase was also affected by competitive inhibition. 5mM histamine caused 64% inhibition of the transglutaminase reaction (Serafini-Fracassini *et al.* 1988).

1.2.3.3.3 Substrate specificity.

Some mammalian transglutaminases have been shown to catalyse the post-translational modification of more than one glutamine donor substrate (Aeschlimann *et al.* 1992). Similarly, several of the transglutaminases detected in plant tissues demonstrate the use of several glutamine donor proteins. The traditional animal transglutaminase substrate *N',N'*-dimethylcasein was shown to act as a glutamine donor for transglutaminases detected in *Pisum sativum, Lupinus albus, Chrysanthemum morifolium* and *Beta vulgaris* (Icekson and Apelbaum 1987; Signorini *et al.*1991; Siepaio and Meunier 1995; Aribaud *et al.* 1995; Lilley *et al.* 1998). Thrombin, fibrinogen, pepsin and insulin were also utilised by *Pisum sativum* transglutaminase. In addition, creatine kinase and cellulase, which had not previously been identified as animal transglutaminase substrates

were identified as substrates for the *Pisum sativum* enzyme (Icekson and Apelbaum 1987). More recent work has shown that a number of unidentified endogenous plant proteins which vary in molecular mass are modified when radiolabelled polyamines are added to crude plant extracts (Serafini-Fracassini *et al.* 1988; Margosiak *et al.* 1990; Grandi *et al.* 1992; Del Duca *et al.* 1993 and 1994).

The transglutaminase enzymes detected in plant tissue display a broad specificity for primary amine donor substrates. *Pisum sativum* transglutaminase was shown to utilise putrescine, cadaverine, spermine, spermidine and diaminopropane as amine donor substrates (Icekson and Apelbaum 1987). Using a [^{14}C]-labelled polyamine incorporation assay, *Helianthus tuberosus* transglutaminase was shown to recognise putrescine, spermidine and spermine as substrates with spermine having the lowest K_m (Serafini-Fracassini *et al.* 1988). Further evidence for the use of a number of amine substrates by *Helianthus tuberosus* transglutaminase was presented by the same laboratory upon the isolation of the transglutaminase products N^1,N^4-bis(γ-glutamyl) putrescine and N^1,N^8-bis(γ-glutamyl) spermidine from a chloroplast preparation (Del Duca *et al.* 1995). Aribaud *et al.* (1995) showed that the transglutaminase present in *Chrysanthemum morifolium* was able to incorporate both radiolabelled putrescine and spermidine into *N',N'*-dimethylcasein. This data demonstrated that the incorporation of both polyamines followed typical Michaelis-Menten kinetics and that the preferred substrate was putrescine. It should be noted that the ability of proteins to provide amine donor lysine has not been extensively tested due to the lack of suitable assay systems. Development of an assay system to test the protein cross-linking abilities of transglutaminases forms the basis for the third chapter of this thesis.

1.2.3.3.4 Plant transglutaminase product identification.

The demonstration of the calcium ion and thiol dependent incorporation of polyamines into proteins is often offered as evidence of transglutaminase activity in a tissue of interest. It has however been agreed that the only absolute proof of the presence of a catalytically active transglutaminase in an organism is the isolation of one of the products of the transglutaminase reaction (Folk and Finlayson 1977). This has been achieved in many mammalian tissues and fluids including liver, skin and blood plasma (Griffin and Smethurst 1994).

N'-(γ-glutamyl) putrescine was detected in a crude *Beta vulgaris* protein extract following a 60 minute incubation with [^{14}C]-labelled putrescine at 30°C (Signorini *et al.* 1991). Exhaustive proteolytic digestion of the labelled protein followed by ion exchange chromatography using an LKB amino acid analyser by the method of Folk *et al.* (1980) was used to identify the conjugate. The presence of this product was further verified by the detection of equimolar quantities of glutamic acid and putrescine following acid hydrolysis of the appropriate un-derivatised chromatographic fraction. Other investigations have led to the isolation of *N'*-(γ-glutamyl) putrescine, N^1,N^4-bis(γ-glutamyl) putrescine and N^1,N^8-bis(γ-glutamyl) spermidine from *Helianthus tuberosus* chloroplasts (Del Duca *et al.* 1995). This work was carried out using a similar method to that described by Signorini *et al.* (1991).

Although the isolation of these polyamine conjugates is acknowledged as good proof of the existence of a transglutaminase, it should be noted that *N'*-(γ-glutamyl) polyamines may be formed in tissues without the action of a transglutaminase (Beninati *et al.* 1988;

Tack *et al.* 1981) and as a result, the only evidence regarded as total and unequivocal proof of the presence of transglutaminase is the isolation of the ε-(γ-glutamyl) lysine isodipeptide formed by the cross-linking of proteins (Folk and Finlayson 1977). The only attempt to date to test the hypothesis that an enzyme in plant tissue is able to catalyse the formation of this product was made by Chiarello *et al.* (1996a). Following exhaustive proteolytic digestion (Griffin *et al.* 1982) of β-casein which had been exposed to protein extracted from the apical meristematic region of 10 day old etiolated *Pisum sativum* seedlings, the subsequent analysis of liberated amino acids using reversed phase HPLC yielded no detection of isodipeptide cross-link. This indicates that the concentration of transglutaminase in the extract may be too low to produce a detectable quantity of isodipeptide when β-casein is used as the substrate.

1.2.3.4 Possible biological roles of plant transglutaminase.

At present no biological role has been established for transglutaminase in plant tissue although a number of processes in which the enzyme might have an involvement have been proposed. Two research articles have related an increase in transglutaminase activity to the progression of the cell cycle of non-photosynthetically competent cells. Dinella *et al.* (1992) observed an increase in high molecular mass radiolabelled putrescine-protein conjugates when [^{14}C] or [^{3}H]-labelled putrescine was supplied to cycling *Helianthus tuberosus* tuber cells. Cycloheximide inhibited increase in transglutaminase activity before the mid-G_1 stage of the *Helianthus tuberosus* tuber cell cycle was also shown in the same laboratory (Grandi *et al.* 1992). When subjected to SDS-PAGE, the labelled conjugates were immobile but some of them could be separated by adjustment of the acrylamide concentration of the gel or by treatment with papain or

cellulase. This data suggested that the high molecular mass conjugates were proteins linked to or entrapped by carbohydrates. Since it is known that fibrillar material migrates from the cytoplasm to the cell wall of cycling tuber cells, it was concluded that transglutaminase may play a part in the assembly and organisation of the cell wall. This conclusion is reinforced by a similar suggestion that transglutaminase may be involved in the cross-linking of proteins during cell wall organisation of the fungus *Candida albicans* (Sentandreu *et al.* 1995; Ruizherrera *et al.* 1995).

A further non-photosynthetically competent tissue containing transglutaminase activity is the pollen of *Malus domestica* (Bregoli *et al.* 1994; Del Duca *et al.* 1997). During the early stages of pollen germination, which involves rapid cell division and synthesis of new cell walls, transglutaminase activity is high and an antibody raised against rat liver transglutaminase recognised proteins of 80kDa and 47kDa present in the pollen. Actin and tubulin are known to play a key role in pollen tube elongation and were identified as substrates for pollen transglutaminase implying a possible role for the enzyme in the early stages of pollen tube growth (Bregoli *et al.* 1994; Del Duca *et al.* 1997). This observation is supported by the evidence that actin has been demonstrated as a transglutaminase substrate in fungal tissues (Klein *et al.* 1992). Other proposed roles for plant transglutaminase in non photosynthetically competent tissue include a possible involvement in the development of *Chrysanthemum morifolium* root tissue and a possible role in the heat stress response of *Oryza sativa* callus (Aribaud *et al.* 1995; Roy and Ghosh 1996)

Biological roles for transglutaminase in photosynthetically competent cells have also been suggested. Margosiak *et al.* (1990) demonstrated that the large sub-unit of RuBisCo could be utilised as an acyl donor substrate by *Medicago sativa* transglutaminase and hence proposed a possible role for the enzyme in photosynthesis. The evidence presented included the covalent attachment of either monodansylcadaverine or [^{14}C]-labelled putrescine to endogenous proteins followed by fluorescence or autoradiographical detection in SDS-PAGE gels of a labelled protein of molecular mass corresponding to that of the large sub-unit of RuBisCo (57.6kDa). A protein band of 52.7kDa was also labelled and it was concluded that this may have been produced by partial proteolysis. The dependence of transglutaminase activity on the concentration of RuBisCo purified from either *Medicago sativa* or *Spinacia oleracea* was demonstrated using a [^{14}C]-labelled putrescine incorporation assay. The incorporation of radiolabelled amine into RuBisCo exhibited Michaelis-Menten kinetics with K_m values of 1.13 and 2.68 mg ml^{-1} for *Medicago sativa* and *Spinacia oleracea* RuBisCo respectively. Transglutaminase was demonstrated to co-elute with the large sub-unit of RuBisCo when crude protein extracts were subjected to gel filtration chromatography indicating a molecular mass of approximately 58kDa for the enzyme. This data coupled to the fact that the same laboratory also showed light stimulated incorporation of [^{14}C]-labelled putrescine into RuBisCo L in isolated chloroplasts implies a role for transglutaminase in the photosynthetic process. It has been suggested that the formation of a dimer of RuBisCo L is the first step of the chaperone-mediated assembly of the catalytically active L_8S_8 RuBisCo structure (Roy *et al.* 1988). A RuBisCo L dimer has been isolated in *Medicago sativa* and it has been suggested that transglutaminase may be responsible for the cross-link (Kuehn *et al.* 1991).

Del Duca *et al.* (1994) demonstrated a light stimulated transglutaminase activity in *Helianthus tuberosus* chloroplasts. Incorporation of [^{14}C]-labelled putrescine into endogenous proteins increased 120% in the light. The endogenous substrates were identified by immunoblotting with polyclonal antibodies as apoproteins of the chlorophyll-a/b antenna complex (LHCII, CP26, CP24 and CP29). The large sub-unit of RuBisCo was also labelled and this was identified using antibodies raised against maize RuBisCo. This data led Del Duca *et al.* (1994) to postulate that although the physiological function of polyamines binding to chloroplast proteins is not yet known, transglutaminase may have an involvement in the light harvesting process. This evidence was supported by more data from the same laboratory demonstrating the presence of both *N'*-(γ-glutamyl) polyamine and *N',N'*-bis (γ-glutamyl) polyamine conjugates in chloroplasts (Del Duca *et al.* 1995). Covalent attachment of exogenously added labelled polyamines to chloroplast proteins does not however demonstrate that this would occur physiologically and more work must be carried out to fully elucidate the biological role of plant transglutaminase in photosynthetically active tissue.

1.2.3.4 The aims of this project.

At present the question of the calcium ion dependence of plant transglutaminase remains unanswered as a direct result of the unsuitability of the radiolabelled polyamine assays frequently used in this field of research (Chiarello *et al.* 1996a and 1996b; Siepaio and Meunier 1995). The initial aim of this project was to develop a more suitable assay system for the detection of plant transglutaminase. A protein cross-linking assay which measures the ability of transglutaminase to cross-link biotin labelled casein into chemically modified casein bound to microtiter plates via ε-(γ-glutamyl) lysine bonds was

developed (Lilley *et al.* 1997a). The assay was developed to elucidate the calcium requirement of plant transglutaminase by overcoming the selectivity problems experienced by previous workers, who observed diamine oxidase activity in crude plant extracts.

The only unequivocal proof of the presence of a catalytically active transglutaminase is provided by the detection of the ε-(γ-glutamyl) lysine product of the transglutaminase reaction (Folk and Finlayson 1977). Hence, a second aim of the project was to detect this conjugate *in vitro* and *in vivo*.

Chapter 2- Materials and methods.

2.1 Materials.

2.1.1 Biological materials.

Seeds of *Pisum sativum* (var. Feltham first), *Vicia faba* (var. Aquadulce), *Triticum aestivum* (var. Apollo) and *Hordeum vulgare* (var. Pipkin) were purchased from Stewarts Seeds Ltd, Nottinghamshire, U.K.

2.1.2 Other materials.

Nunc maxisorp microtiter assay plates were obtained through Life technologies (Renfrewshire, U.K). Muslin was purchased from Jessops department store (Nottinghamshire, U.K). Liquid scintillation fluid was supplied by Packard (Berkshire, U.K). TLC plates were manufactured by Merck (Darmstadt, Germany) and dialysis tubing was purchased from Medicell International (London, U.K).

2.1.3 Chemicals.

[1,4-^{14}C]-labelled putrescine was obtained from Amersham Ltd (Buckinghamshire, U.K). *N',N'*-dimethylcasein was purchased from Calbiochem (Nottinghamshire, U.K). Biotin-X-cadaverine was supplied by Cambridge Bioscience (Cambridgeshire, U.K). Bovine casein was obtained through ICN (Cleveland, Ohio, U.S.A). EDC, formaldehyde and sodium borohydride were purchased from Aldrich Ltd (Dorset, U.K). Ammonium carbonate, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate,

urea, sodium hydroxide and methylamine were purchased from BDH (Dorset, U.K). All other chemicals were purchased from Sigma chemical company (Dorset, U.K).

2.2 Methods.

2.2.1 Treatment of biological material.

2.2.1.1 Preparation of dialysis tubing.

Dialysis tubing was boiled for 5 minutes in 100.0 ml of distilled and de-ionised water containing 1g of EDTA and 1g of sodium hydrogen carbonate. The tubing was then rinsed and boiled in 100.0 ml distilled water. Unused tubing was stored at 4°C in a solution of 0.02% (w/v) sodium azide (0.02g sodium azide dissolved in distilled water to a final volume of 100.0 ml).

2.2.1.2 Extraction of transglutaminase activity from plant tissue.

Stock solutions.

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

85.6g sucrose, 6.1g Tris, 651µl of 2-ME and 1.1g disodium EDTA (dihydrate) were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated HCl and the volume was adjusted to 1.0 litre with distilled water.

(When extracting transglutaminase from *Pisum sativum* root tissue for the purpose of casein cross-linking followed by isodipeptide analysis the concentration of EDTA was increased to 10mM and protease inhibitors were added to the extraction buffer at the following final concentrations: 5µM Leupeptin, 1µM pepstatin and 1mM PMSF).

2.2.1.2.2. Membrane washing buffer- 5mM Tris pH 7.2 containing: 1mM DTT, 250mM sucrose and 1.0M potassium chloride.

85.6g sucrose, 0.6g Tris, 0.15g DTT and 75g potassium chloride were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.2 using solid MES and the volume was adjusted to 1.0 litre with distilled water.

2.2.1.2.3. Membrane re-suspension buffer with detergent- 5mM Tris pH 7.2 containing: 1mM DTT, 250mM sucrose and various concentrations of detergents.

85.6g sucrose, 0.6g Tris, 0.15g DTT and various concentrations of detergent were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.2 using solid MES and the volume was adjusted to 1.0 litre with distilled water.

2.2.1.2.4. Membrane pellet re-suspension buffer- 5mM Tris pH 7.2 containing: 1mM DTT and 250mM sucrose.

85.6g sucrose, 0.6g Tris and 0.15g DTT were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.2 using solid MES and the volume was adjusted to 1.0 litre with distilled water.

2.2.1.2.5. Protein re-dissolving / dialysis buffer- 50mM Tris-HCl pH 7.4 containing: 1mM 2-ME.

18.2g Tris and 195µl of 2-ME were dissolved in 2.5 litres of distilled water. The pH was adjusted to 7.4 using concentrated HCl and the volume was adjusted to 3.0 litres with distilled water.

Method.

Seeds of *Vicia faba* (var. Aquadulce), *Pisum sativum* (var. Feltham First), *Hordeum vulgare* (var. Pipkin) and *Triticum aestivum* (var. Apollo) were soaked overnight in running water and germinated in damp vermiculite for 14 days in a greenhouse at 20°C. Over a 16 hour photoperiod a photosynthetic flux of 300-400 μmol m^{-2} s^{-1} was provided by natural daylight supplemented with high pressure sodium lamps. Root and leaf tissue was harvested after an appropriate growth period (14 days unless otherwise stated). Solid PVPP was added at a concentration of 5% (w/v) to the extraction buffer (1) (2.2.1.2.1). Plant tissue was homogenised in ice cold extraction buffer in a ratio of 1 : 2 (w/v) using a Phillips model HR1375/A blender (Nottinghamshire, U.K). The homogenate was strained through two layers of muslin and the pH was re-adjusted to 7.4 using solid Tris (Corning pH meter model 130, Essex, U.K). The extract was then centrifuged at 13000×g for 20 minutes at 4°C using an MSE model 24M centrifuge (Sussex, U.K) fitted with an 8×50.0 ml pre chilled rotor, part number 43114-143. The supernatant was further clarified by centrifugation at 80000×g for 45 minutes at 4°C to sediment the membrane fraction using a Beckman Ultra model L8 70 centrifuge fitted with a pre chilled 70 Ti rotor (Buckinghamshire, U.K). The membrane fraction was washed with membrane washing buffer (2.2.1.2.2), centrifuged at 80000×g for 45 minutes and re-suspended in membrane re-suspension buffer with detergent (2.2.1.2.3). The membrane preparations were then centrifuged at 80000×g for 45 minutes and the supernatant was stored in aliquots at -20°C. The resultant pellet was re-dissolved in membrane re-suspension buffer (2.2.1.2.4) and stored at-20°C. The initial 80000 × g

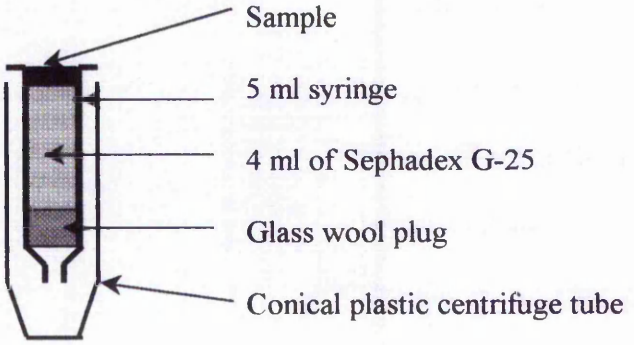
supernatant protein was precipitated by the addition of solid ammonium sulphate to 90% saturation at 4°C (0.6g ammonium sulphate per ml of supernatant).

De-salting of extracted protein.

Method a. -Protein used for activity assays. Precipitated protein was collected by centrifugation at 13000×g for 20 minutes at 4°C, re-dissolved in protein re-dissolving buffer (2.2.1.2.5) and dialysed against 2.5 litres of the same buffer at 4°C. Aliquots of dialysed protein were stored at -20°C.

Method b – after Christopherson (1983). -*Pisum sativum* root protein used for cross-linking casein followed by isodipeptide analysis. A more rapid de-salting technique was employed for these experiments to ensure that the extracted protein retained as much activity as possible: Four millilitres of pre-swollen Sephadex grade G-25 was placed into a 5.0 ml plastic syringe using a glass wool plug to stopper the end as shown in figure 4. The syringe was placed into a conical plastic centrifuge tube and distilled water was removed from the G-25 by centrifugation at 3000 rpm for 5 minutes at 4°C using a Beckman GPKR refrigerated centrifuge (Buckinghamshire, U.K). A 0.5 ml aliquot of the *Pisum sativum* root extract was then desalted by centrifugation at 3000 rpm for 5 minutes at 4°C. 25µl of 2.0M Tris-HCl pH 7.4 containing 20mM 2-ME (2.42g Tris, 14µl 2-ME dissolved in 10.0 ml of distilled water) was added to restore lost buffer.

Figure 4. Apparatus used to rapidly de-salt *Pisum sativum* root protein extracts.



2.2.1.3 Extraction of *Vicia faba* storage proteins.

Stock solution.

2.2.1.3.1. Extraction buffer (2)- 50mM Tris-HCl pH 7.5 containing: 0.5M sodium chloride and 5mM EDTA.

3.03g Tris, 14.6g sodium chloride and 0.93g disodium EDTA (dihydrate) were dissolved in 450.0 ml of distilled water. The pH was adjusted to 7.5 using concentrated HCl and the volume was made up to 500.0 ml with distilled water.

Method.

Cotyledon tissue was homogenised in ice cold extraction buffer (2) (2.2.1.3.1) in a ratio of 1 : 2 (w/v) using a Phillips model HR1375/A blender (Nottinghamshire, U.K). The homogenate was strained through two layers of muslin and the pH was re-adjusted to 7.5 using solid Tris (Corning pH meter model 130, Essex, U.K). The extract was then centrifuged at 10000×g for 30 minutes at 4°C using an MSE model 24M centrifuge (Sussex, U.K) fitted with an 8×50.0 ml pre chilled rotor, part number 43114-143. The

resultant supernatant was dialysed repeatedly against 5.0 litres of distilled water until a precipitate of globulins was observed. The extract was then centrifuged as before to pellet the globulins which were re-suspended in distilled water, freeze dried using a B. O. C model 5321 freeze drier (Sussex, U.K) and stored at -20°C.

2.2.1.4 Proteolytic digestion of plant proteins (Griffin *et al.* 1982).

Stock solutions.

2.2.1.4.1. - 72% (w/v) TCA.

72g of solid TCA was dissolved in distilled water to a final volume of 100.0 ml.

2.2.1.4.2. - 10% (w/v) TCA.

10g of solid TCA was dissolved in distilled water to a final volume of 100.0ml.

2.2.1.4.3 - 50:50 ethanol:diethylether.

25.0 ml diethylether was added to 25.0 ml ethanol and stored on ice.

2.2.1.4.4. 100mM ammonium carbonate pH 10.0.

1.57g ammonium carbonate was dissolved in 90.0 ml of distilled water and the pH was adjusted to 10.0 using concentrated sodium hydroxide. The volume was made up to 100.0 ml with distilled water.

2.2.1.4.5. Leucine amino peptidase activating solution.

This solution consisted of the following components:

250µl leucine amino peptidase (250 units)

225µl 10mM Tris-HCl pH 8.0 (0.12g Tris/100.0 ml distilled water/pH conc. HCl)

25µl 50mM manganese chloride (0.1g manganese chloride (tetrahydrate) /10.0 ml distilled water).

The solution was incubated at 37°C for 2 hours to activate the leucine amino peptidase.

2.2.1.4.6. Prolidase activating solution.

This solution consisted of the following components:

50µl prolidase (100 units)

200µl distilled water

200µl 10mM Tris-HCl pH 8.0 (made as described in section 2.2.1.4.5)

50µl manganese chloride (made as described in section 2.2.1.4.5)

The solution was incubated at 37°C for 2 hours to activate the prolidase.

2.2.1.4.7. - 0.54M magnesium chloride.

1.09g magnesium chloride (hexahydrate) was dissolved in 10.0 ml of distilled water.

2.2.1.4.8. Chloroform:methanol:HCl (200:100:2).

100.0 ml chloroform was added to 50.0 ml methanol. The solvents were mixed thoroughly and 1.0 ml of concentrated HCl was added. The mixture was used immediately.

Method.

Plant material was homogenised as described in section 2.2.1.2 and protein was precipitated from the 80000×g supernatant by the addition of an appropriate volume of 72% (w/v) TCA (2.2.1.4.1) to give a final concentration of 10% (w/v). The protein was pelleted by centrifugation at 13000×g for 20 minutes at 4°C using an MSE model 24M centrifuge (Sussex, U.K) fitted with an 8×50.0 ml pre-chilled rotor, part number 43114-143. The pellets were washed once in 10% (w/v) TCA (2.2.1.4.2), three times in 50:50 ethanol:diethylether (2.2.1.4.3) and 3 times in diethylether (Each washing was followed by centrifugation at 13000 rpm for 3 minutes using a Jouan model A-14 mini bench centrifuge (St. Nazaire, France)). The resultant pellets were dried by evaporation on a non heat cycle (Jouan model RC.10.22 centrifugal evaporator, St Nazaire, France) and re-dissolved in 1.0 ml of 100mM ammonium carbonate pH 10.0 (2.2.1.4.4). A crystal of thymol was added to each pellet to inhibit microbial growth. The pH of each tube was checked and adjusted to 10.0 by the addition of sodium hydroxide if necessary. One hundred micrograms of subtilisin was added to each tube as 10µl of a 10 mg ml^{-1} solution and the tubes were placed in an incubator at 37°C (Analytical Supplies, Derbyshire, U.K). All subsequent proteolytic enzyme incubations were carried out at this temperature. The addition of subtilisin was repeated twice more over a total subtilisin incubation period of 48 hours. One hundred and fifty micrograms of pronase was then added to each tube as 10µl of a 15 mg ml^{-1} solution. Following a further 24 hour incubation, each extract was boiled for 15 minutes and upon cooling, 10µl of both activated leucine amino peptidase solution (2.2.1.4.5) and activated prolidase solution (2.2.1.4.6) were added along with a 10µl aliquot of 0.54M magnesium chloride

(2.2.1.4.7) (5mM final concentration of magnesium chloride). The tubes were vortexed (Fisons model WM/250/SC/P) and incubated for 24 hours. The pH of each digestion was then adjusted to 7.0 with 1.0N HCl and 0.1 mg of carboxypeptidase Y was added as 10µl of a 10 mg ml^{-1} solution. Following a further 24 hour incubation the digested protein solutions were placed into conical glass centrifuge tubes and added to chloroform:methanol:HCl (200:100:2) (2.2.1.4.8) at the level of 3.6 ml to 1.0 ml of protein digestion. The tubes were centrifuged at 2500 rpm for 5 minutes at 4°C using a Beckman GPKR refrigerated centrifuge and both the upper aqueous layer containing the liberated amino acids and the central interface containing undigested protein were carefully removed and dried by centrifugal evaporation. The aqueous phase was re-dissolved in an appropriate volume of loading buffer (2.2.4.4.1) and stored at -20°C prior to subsequent amino acid analysis. The undigested protein was retained and quantified as described in section 2.2.1.6 to determine the efficiency of the hydrolysis. Hydrolysis levels were typically between 96 and 99%.

2.2.1.5 Preparation of human endothelial cell homogenate.

Stock solutions.

2.2.1.5.1. Growth medium- 10% (v/v) foetal calf serum, 1% (w/v) penicillin/streptomycin.

100g foetal calf serum and 10g penicillin / streptomycin were dissolved in 1.0 litre DMEM.

2.2.1.5.2. Trypsinising solution- 0.05% (w/v) trypsin.

500 mg trypsin was dissolved in DMEM to a final volume of 1.0 litre.

2.2.1.5.3. Re-suspension buffer- 50mM Tris-HCl pH 7.4 containing: 250mM sucrose and 1mM EDTA.

0.6g Tris, 8.6g Sucrose and 0.037g disodium EDTA (dihydrate) were dissolved in 90.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated HCl and the volume was made up to 100.0 ml with distilled water.

Method.

Human endothelial cells (ECV 304) were provided and grown in this laboratory by Dr. P. A. Smethurst at 37°C with 5% (v/v) CO_2 / 95% (v/v) air in growth medium (2.2.1.5.1). Cells were removed from the bottles by treatment with trypsinising solution (2.2.1.5.2). Following centrifugation at 2000×g for 2 minutes (Beckman GPKR), the trypsin was decanted off and the cells were re-suspended in ice cold re-suspension buffer (2.2.1.5.3). The cell suspension was sonicated on ice using an MSE Soniprep model 150 fitted with a pre-chilled narrow probe (Sussex, U.K) for three pulses of 5 seconds (6 microns amplitude) with 15 second pauses between each pulse. The un-clarified homogenate was immediately assayed for transglutaminase activity.

2.2.1.6 Bicinchoninic acid protein assay (Brown *et al.* 1989).

Stock solutions

2.2.1.6.1. - 1 mg ml^{-1} BSA.

100 mg BSA was dissolved in distilled water to a final volume of 100.0 ml.

2.2.1.6.2.- 0.15% (w/v) sodium deoxycholate.

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

2.2.1.6.3.- 72% (w/v) TCA.

7.2g TCA was dissolved in distilled water to a final volume of 10.0 ml.

2.2.1.6.4.- 5% (w/v) SDS in 0.1N sodium hydroxide.

5g SDS was dissolved in 0.1N sodium hydroxide to a final volume of 100.0 ml.

2.2.1.6.5.- BCA reagent A- 1% (w/v) BCA (sodium salt), 2% (w/v) sodium carbonate, 0.16% (w/v) sodium tartrate, 0.4% (w/v) sodium hydroxide and 0.95% (w/v) sodium hydrogen carbonate pH 11.25.

5g BCA (sodium salt), 10g sodium carbonate, 0.8g sodium tartrate, 2g sodium hydroxide and 4.8g sodium hydrogen carbonate were dissolved in 450.0 ml of distilled water. The pH was adjusted to 11.25 by the addition of either 50% (w/v) sodium hydroxide or solid sodium hydrogen carbonate and the volume was made up to 500.0 ml with distilled water.

2.2.1.6.6. BCA reagent B- 4% (w/v) copper sulphate.

4g $CuSO_4.5H_2O$ was dissolved in distilled water to a final volume of 100.0 ml.

2.2.1.6.7. BCA Standard working reagent (SWR).

This was prepared by the addition of 50 parts (2.2.1.6.5) to 1 part (2.2.1.6.6).

Method.

Standard protein solutions in the range 0-1.0 mg ml^{-1} were prepared using 1 mg ml^{-1} BSA stock solution (2.2.1.6.1). One hundred microlitres of protein to be assayed was

diluted to 1.0 ml with distilled water in an microfuge tube. One hundred microlitres of 0.15% (w/v) sodium deoxycholate (2.2.1.6.2) was added to each tube. Following a 5 minute incubation at room temperature, protein was precipitated by the addition of 100µl of 72% (w/v) TCA (2.2.1.6.3) and each tube was thoroughly vortex mixed (Fisons model WM/250/SC/P, Leicestershire, U.K). Precipitated protein was collected by centrifugation at 13500×g for 15 minutes using an MSE microcentaur (Sussex, U.K) and the supernatant was removed by vacuum aspiration. Protein was re-dissolved in 50µl of 0.1N sodium hydroxide containing 5% (w/v) SDS (2.2.1.6.4). Following the addition of 1.0 ml BCA SWR (2.2.1.6.7), each tube was vortex mixed and incubated at 37°C for 30 minutes (Grant model JB-1, Cambridgeshire, U.K). The absorbance of samples was read at 562nm (Pye unicam model SP6-400, Cambridgeshire, U.K).

2.2.2 Protein modification methods.

2.2.2.1 Biotinylation of bovine casein (Harlow and Lane 1988).

Stock solutions.

2.2.2.1.1. Reaction buffer- 100mM sodium borate pH 8.8 containing: 3 mg ml^{-1} casein.

300 mg casein and 0.62g boric acid were dissolved in 90.0 ml of distilled water. The pH was adjusted to 8.8 using concentrated sodium hydroxide and the volume was adjusted to 100.0 ml with distilled water.

2.2.2.1.2. -10 mg ml^{-1} biotin ester solution.

25 mg N-hydroxysuccinimidobiotin or biotin amidocaproate N-hydroxysuccinimide ester was dissolved in DMSO to a final volume of 2.5 ml.

2.2.2.1.3.- 1.0M ammonium chloride.

5.3g ammonium chloride was dissolved in distilled water to a final volume of 100.0 ml.

2.2.2.1.4. Dialysis buffer- 150mM PBS pH 7.4.

8.0g sodium chloride, 0.2g potassium chloride, 1.15g disodium hydrogen orthophosphate and 0.2g potassium dihydrogen phosphate were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated sodium hydroxide and the volume was made up to 1.0 litre with distilled water.

Method.

To 10.0 ml of reaction buffer (2.2.2.1.1) was added 750µl of 10 mg ml^{-1} of biotin ester solution (2.2.2.1.2). Following stirring at 4°C for 4-8 hours (Corning model PC351, Essex, U.K), 600µl of 1.0M ammonium chloride (2.2.2.1.3) was added and the reaction was left to proceed for a further 30 minutes at 4°C. The modified protein was then dialysed overnight at 4°C against 3 changes of 5.0 litres of dialysis buffer (2.2.2.1.4) and stored at -20°C.

2.2.2.2 Carbodiimide modification of bovine casein (Carraway and Koshland, 1972).

2.2.2.2.1 EDC-modification of casein in solution.

Stock solutions.

2.2.2.2.1.1. Reaction solution- 7.5M urea containing; 10 mg ml^{-1} casein and 1.0M nucleophile (glycine methyl ester hydrochloride, lysine methyl ester dihydrochloride or arginine methyl ester dihydrochloride) pH 4.75.

11.26g urea, 250 mg casein, 3.14g glycine methyl ester hydrochloride, 5.83g lysine methyl ester dihydrochloride or 6.53g arginine methyl ester dihydrochloride were dissolved in 15.0 ml of distilled water. The pH was adjusted to 4.75 using concentrated HCl and the volume was made up to 25.0 ml with distilled water.

2.2.2.2.1.2. Stop buffer- 1.0M sodium acetate pH 4.75.

2.05g sodium acetate was dissolved in 15.0 ml of distilled water. The pH was adjusted to 4.75 using concentrated acetic acid and the volume was made up to 25.0 ml with distilled water

Method.

Four hundred and seventy nine milligrams of EDC (final concentration 100mM) was added to 25.0 ml of reaction solution (2.2.2.2.1.1) and the pH was maintained at 4.75 using 0.1N HCl over a 60 minute incubation period at room temperature. Two point five millilitres of stop buffer (2.2.2.2.1.2) was then added and the modified casein was dialysed overnight at 4°C against 3 changes of 5.0 litres of distilled water. The modified protein was stored at -20°C.

2.2.2.2.2 EDC-modification of assay plate bound casein.

Stock solutions.

2.2.2.2.2.1. Coating buffer- 50mM sodium carbonate pH 9.8 containing 1 mg ml^{-1} casein.

Prepared as in section 2.2.2.3.2 but using casein and not *N',N'*-dimethylcasein.

2.2.2.2.2.2. Blocking buffer.

As section 2.2.2.3.3.

2.2.2.2.2.3. Reaction solution- 7.5M urea containing: 1.0M glycine methyl ester hydrochloride, 10 mg ml^{-1} casein and 100mM EDC pH 4.75.

11.26g urea, 250 mg casein, 3.14g glycine methyl ester hydrochloride and 0.479g EDC were dissolved in 15.0 ml of distilled water). The pH was adjusted to 4.75 using concentrated HCl and the volume was made up to 25.0 ml with distilled water.

Method.

Assay plates were coated and blocked as described in section 2.2.2.3. Two hundred and fifty microlitres of reaction solution (2.2.2.2.2.3) was then added to each well and the plates were incubated at room temperature for 30 minutes. Following addition of 1.5µl of 0.01N HCl to each well, the plate was incubated for a further 30 minutes. The assay was then carried out as described in section 2.2.3.2.

2.2.2.3 Enzymic modification of *N',N'*-dimethylcasein (Lilley *et al.* 1997a).

Stock solutions.

2.2.2.3.1.- 50mM sodium carbonate pH 9.8.

5.3g sodium carbonate was dissolved in 1.0 litre of distilled water. 4.2g of sodium hydrogen carbonate was dissolved in 1.0 litre of distilled water in a separate flask. The pH of the sodium carbonate solution was adjusted to 9.8 by titration with the sodium hydrogen carbonate solution.

2.2.2.3.2. Coating buffer- 50mM sodium carbonate pH 9.8 containing: 1 mg ml^{-1} *N',N'*-dimethylcasein.

100 mg *N',N'*-dimethylcasein was dissolved in 100.0 ml of 50mM sodium carbonate pH 9.8 (2.2.2.3.1).

2.2.2.3.3. Blocking buffer- 50mM sodium carbonate pH 9.8 containing: 1 mg ml^{-1} BSA.

100 mg BSA was dissolved in 100.0 ml of 50mM sodium carbonate pH 9.8 (2.2.2.3.1).

2.2.2.3.4. Reaction buffer- 100mM Tris-HCl pH 8.5 containing: 5mM calcium chloride, 10mM DTT, 37.5mM polyamine (spermine, spermidine, cadaverine or putrescine) and 2μg ml^{-1} guinea pig liver transglutaminase.

0.3g Tris, 0.018g calcium chloride (dihydrate), 0.039g DTT, 0.19g spermine (or 0.14g spermidine/0.083g putrescine (dihydrochloride)/0.096g cadaverine) and 5×10^{-5}g guinea pig liver transglutaminase were dissolved in 20.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

2.2.2.3.5. Washing buffer- 150mM PBS pH 7.4 containing: 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 500μl Tween 80 were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated sodium hydroxide and the volume was made up to 1.0 litre with distilled water.

2.2.2.3.6. Washing buffer- 100mM Tris-HCl pH 8.5.

12.1g Tris was dissolved in 900.0 ml of distilled water. The pH was adjusted to 8.5 using concentrated HCl and the volume was made up to 1.0 litre with distilled water.

Method.

Microtiter plates were coated for one hour at 37°C (Analytical supplies, Derbyshire, U.K) with 250µl per well of coating buffer (2.2.2.3.2). After discarding the unbound protein, plates were washed twice with 300µl per well of washing buffer (2.2.2.3.5) and twice with 300µl per well of distilled water. Two hundred and fifty microlitres per well of blocking buffer (2.2.2.3.3) was added and the plates were shaken for 30 minutes at room temperature (Luckham model R-100, Sussex, U.K). Plates were emptied and washed as previously described but with an additional wash using 300µl per well of washing buffer (2.2.2.3.6). Two hundred microlitres of reaction buffer (2.2.2.3.4) was then added to each well. Plates were incubated overnight at 37°C and washed as before to remove guinea pig liver transglutaminase. Plates were used immediately.

2.2.2.4 De-phosphorylation of bovine casein (Cooke and Holbrook 1974).

Stock solutions.

2.2.2.4.1. Protein modification buffer- 150mM Tris-HCl pH 7.4 containing: 20 mg ml^{-1} casein.

0.45g Tris and 500 mg casein were dissolved in 20.0 ml of distilled water. The pH was adjusted to 7.5 using concentrated HCl and the volume was made up to 25.0 ml with distilled water.

2.2.2.4.2.- 20 mg ml^{-1} Alkaline phosphatase solution.

20 mg calf thymus alkaline phosphatase was dissolved in distilled water to a final volume of 1.0 ml.

2.2.2.4.3.- 10mM zinc sulphate.

0.016g zinc sulphate was dissolved in 10.0 ml of distilled water.

2.2.2.4.4. Dialysis buffer- 50mM sodium carbonate pH 9.8.

This was prepared as described in section 2.2.2.3.1.

Method.

One hundred microlitres of 10mM zinc sulphate solution (2.2.2.4.3) and 25µl of 20 mg ml^{-1} alkaline phosphatase solution (2.2.2.4.2) were added to 25.0 ml of protein modification buffer (2.2.2.4.1) and the resultant reaction mixture was stirred for 2.5 hours at room temperature. The modified protein was dialysed twice for 60 minutes

against 2.5 litres of dialysis buffer (2.2.2.4.4). The protein concentration was then adjusted to 1.0 mg ml^{-1} and aliquots of the modified protein were either used immediately or stored at -20°C.

2.2.2.5 *N',N'*-dimethylation of *Vicia faba* cotyledon storage proteins (Means and Feeney 1968).

Stock solutions.

2.2.2.5.1.- 150mM PBS pH 9.0.

0.8g sodium chloride, 0.02g potassium chloride, 0.12g disodium hydrogen orthophosphate and 0.02g potassium dihydrogen orthophosphate were dissolved in 90.0 ml of distilled water. The pH was adjusted to 9.0 with dilute sodium hydroxide and the volume was made up to 100.0 ml with distilled water.

2.2.2.5.2. Dialysis buffer- 100mM Tris-HCl pH 8.5.

24.2g Tris was dissolved in 1.9 litres of distilled water. The pH was adjusted to 8.5 using concentrated HCl and the volume was made up to 2.0 litres with distilled water.

Method.

Vicia faba storage proteins were dissolved in 10.0 ml of 150mM PBS pH 9.0 (2.2.2.5.1) to a final concentration of 10 mg ml^{-1}. The solution was placed on ice at 0°C and 0.005g sodium borohydride was slowly stirred in. Twenty five microlitres of reagent grade formaldehyde was then added slowly as five 5µl aliquots. The pH of the reaction mixture was adjusted to 5.0 for one minute using concentrated HCl and then readjusted

to 8.5 with concentrated sodium hydroxide. The modified storage proteins were then dialysed twice for 60 minutes against dialysis buffer (2.2.2.5.2). The modified protein was stored at -20°C.

2.2.2.6 The de-amidation of casein (Wilcox 1967).

Stock solutions.

2.2.2.6.1. Glassware washing solution- 0.5% (w/v) potassium dichromate in 4.0N su:phuric acid.

0.5g of potassium dichromate was added to 100.0 ml 4.0N H_2SO_4.

Method.

All glassware was thoroughly cleaned by soaking overnight in washing solution (2.2.2.6.1.) and thoroughly rinsing in 2.0N HCl. Fifty milligrams of casein was added to 25.0 ml of 2.0N HCl and the resultant mixture was placed in a boiling tube and heated in a water bath at 100°C for 2 hours. Solid sodium hydroxide was then added to neutralise the acid and the protein was dialysed against 2.5 litres of dialysis buffer (2.2.2.4.4). The final concentration of casein was adjusted to 1 mg ml^{-1} using dialysis buffer (2.2.2.4.4) and aliquots of the modified protein were stored at -20°C.

2.2.3 Transglutaminase activity assays.

2.2.3.1 Biotin cadaverine incorporation assay (after Slaughter *et al.* 1992).

Stock solutions.

2.2.3.1.1. Coating buffer- 100mM Tris-HCl pH 8.5 containing: 10 mg ml^{-1} *N',N'*-dimethylcasein.

1g *N',N'*-dimethylcasein and 1.2g Tris were dissolved in 90.0 ml of distilled water. The pH was adjusted to 8.5 using concentrated HCl and the volume was made up to 100.0 ml using distilled water.

2.2.3.1.2. Blocking buffer- 100mM Tris-HCl pH 8.5 containing: 3% (w/v) BSA.

3g BSA and 1.2g Tris were dissolved in 90.0 ml of distilled water. The pH was adjusted to 8.5 using concentrated HCl and the volume was made up to 100.0 ml with distilled water.

2.2.3.1.3. Reaction buffer- 100mM Tris-HCl pH 8.5 containing: 13.3mM DTT, 6.7mM calcium chloride and 0.67mM biotin-X-cadaverine.

0.3g Tris, 0.051g DTT, 0.025g calcium chloride (dihydrate) and 0.007g biotin-X-cadaverine were dissolved in 15.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

2.2.3.1.4. Probing buffer- 100mM Tris-HCl pH 8.5 containing: 1% (w/v) BSA and 1:5000 extravidin peroxidase.

0.3g Tris, 250 mg BSA and 5µl extravidin peroxidase (2 mg ml^{-1} stock) were dissolved in 15.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

2.2.3.1.5. Developing buffer- 100mM sodium acetate pH 6.0 containing: 0.312mM TMB and 0.004% (v/v) hydrogen peroxide.

0.16g sodium acetate, 150µl of 10 mg ml^{-1} TMB in DMSO and 25µl of 3% (v/v) hydrogen peroxide were dissolved in 15.0 ml of distilled water. The pH was adjusted to 6.0 using dilute acetic acid and the volume was made up to 20.0 ml with distilled water.

2.2.3.1.6. Washing buffer- 150mM PBS pH 7.4 containing: 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 500µl Tween 80 were dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated sodium hydroxide and the volume was made up to 1.0 litre with distilled water.

2.2.3.1.7. Washing buffer- 100mM Tris-HCl pH 8.5.

12.1g Tris was dissolved in 900.0 ml of distilled water. The pH was adjusted to 8.5 with concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.3.1.8. Washing buffer- 100mM sodium acetate pH 6.0.

4.1g sodium acetate was dissolved in 450.0 ml of distilled water. The pH was adjusted to 6.0 using concentrated acetic acid and the volume was adjusted to 1.0 litre with distilled water.

Method.

Microtiter plates were coated for 60 minutes at 37°C with 250µl per well of coating buffer (2.2.3.1.1). Following two washes with 300µl per well of washing buffer (2.2.3.1.6) and two washes with 300µl per well of distilled water, 250µl of blocking buffer (2.2.3.1.2) was added and the plates were shaken at room temperature for 30 minutes. The plates were emptied and washed as before but with an additional wash with 300µl per well of washing buffer (2.2.3.1.7). To each well was then added 150µl of reaction buffer (2.2.3.1.3) and 50µl of sample to be assayed for transglutaminase activity. After a 60 minute incubation at 37°C, plates were emptied and washed as before. Two hundred microlitres of probing buffer (2.2.3.1.4) was then added to each well and the plates were incubated at 37°C for 45 minutes. Plates were emptied and washed twice with 300µl per well of washing buffer (2.2.3.1.6), twice with 300µl per well of distilled water and once with 300µl per well of washing buffer (2.2.3.1.8). Plates were then treated with 200µl per well of developing buffer (2.2.3.1.5) and colour development was terminated by the addition of 50µl per well of 10.0N sulphuric acid. The absorbance was read at 450nm using a Titertek Multiscan ELISA spectrophotometer model MCC/340 (Laboratory systems, Hampshire, U.K). In some experiments, the 5mM final concentration of calcium chloride was replaced with various concentrations of EDTA or

EGTA in the reaction buffer. In several experiments, other reagents were added to the reaction buffer in order to investigate their effect on plant transglutaminase activity. One unit of transglutaminase activity was defined as a change of absorbance at 450nm of 1.0 per hour.

2.2.3.2 Casein cross-linking assay. Measurement of ε-(γ-glutamyl) lysine formation (Lilley *et al.* 1997a).

Stock solutions.

2.2.3.2.1.- 67.5mM sodium carbonate pH 9.8.

3.6g sodium carbonate was dissolved in distilled water to a final volume of 500.0 ml. The pH was adjusted to 9.8 by the addition of 67.5mM sodium hydrogen carbonate (2.8g sodium hydrogen carbonate dissolved in distilled water to a final volume of 500.0 ml).

2.2.3.2.2 .- 50mM sodium carbonate pH 9.8.

Prepared as stated in section (2.2.2.3.1)

2.2.3.2.3. Coating buffer- 50mM sodium carbonate pH 9.8 containing: 1 mg ml^{-1} EDC-modified casein.

20.0 ml 67.5mM sodium carbonate pH 9.8 was added to 5.0 ml EDC-modified casein (5 mg ml^{-1} stock in distilled water).

2.2.3.2.4. Blocking buffer- 50mM sodium carbonate pH 9.8 containing: 1 mg ml^{-1} BSA.

25 mg BSA was dissolved in 50mM sodium carbonate pH 9.8 to a final volume of 25.0 ml.

2.2.3.2.5. Reaction buffer- 100mM Tris-HCl pH 8.5 containing: 13.3mM DTT, 6.7mM calcium chloride and 1×10^{-3} mg ml^{-1} biotin labelled casein.

0.3g Tris, 0.051g DTT, 0.025g calcium chloride (dihydrate) and 12.5µl of 2 mg ml^{-1} stock biotin labelled casein were dissolved in 15.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

2.2.3.2.6. Probing buffer- 100mM Tris-HCl pH 8.5 containing: 1% (w/v) BSA and 1:10000 extravidin peroxidase.

0.3g Tris, 250 mg BSA and 2.5µl extravidin peroxidase (2 mg ml^{-1} stock) were dissolved in 15.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

2.2.3.2.7. Developing buffer- 100mM sodium acetate pH 6.0 containing: 0.312mM TMB and 0.004% (v/v) hydrogen peroxide.

0.16g sodium acetate, 150µl of 10mg ml^{-1} TMB in DMSO and 25µl of 3% (v/v) hydrogen peroxide were dissolved in 15.0 ml of distilled water. The pH was adjusted to 6.0 using dilute acetic acid and the volume was made up to 20.0 ml with distilled water.

2.2.3.2.8. Washing buffer- 150mM PBS pH 7.4 containing: 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 500µl Tween 80 were

dissolved in 900.0 ml of distilled water. The pH was adjusted to 7.4 using concentrated sodium hydroxide and the volume was made up to 1.0 litre with distilled water.

2.2.3.2.9. Washing buffer- 100mM Tris-HCl pH 8.5.

12.1g Tris was dissolved in 900.0 ml of distilled water. The pH was adjusted to 8.5 with concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.3.2.10. Washing buffer- 100mM sodium acetate pH 6.0.

4.1g sodium acetate was dissolved in 450.0 ml of distilled water. The pH was adjusted to 6.0 using concentrated acetic acid and the volume was adjusted to 1.0 litre with distilled water.

Method.

Microtiter plates were coated for 60 minutes at 37°C with 250µl per well of coating buffer (2.2.3.2.3). Following two washes with 300µl per well of washing buffer (2.2.3.2.8) and two washes with 300µl per well of distilled water, 250µl per well of blocking buffer (2.2.3.2.4) was added and the plates were shaken at room temperature for 30 minutes. The plates were emptied and washed as before but with an additional wash with 300µl per well of washing buffer (2.2.3.2.9). To each well was then added 150µl of Reaction buffer (2.2.3.2.5) and 50µl of sample to be assayed for transglutaminase activity. After a 60 minute incubation at 37°C, plates were emptied and washed as before. Two hundred microlitres per well of probing buffer (2.2.3.2.6) was then added and the plates were incubated at 37°C for 45 minutes. Plates were emptied

and washed twice with 300µl per well of washing buffer (2.2.3.2.8), twice with 300µl per well of distilled water and once with 300µl per well of washing buffer (2.2.3.2.10). Plates were then treated with 200µl per well of developing buffer (2.2.3.2.7) and colour development was terminated by the addition of 50µl per well of 10.0N sulphuric acid. The absorbance was read at 450nm using a Titertek Multiscan ELISA spectrophotometer. In some experiments, the 5mM final concentration of calcium chloride was replaced with various concentrations of EDTA or EGTA in the reaction buffer. In several experiments, other reagents were added to the reaction buffer in order to investigate their effect on transglutaminase activity. One unit of transglutaminase activity was defined as a change of absorbance at 450nm of 1.0 per hour.

2.2.3.3 Casein cross-linking assay. Measurement of *N',N'*-bis (γ-glutamyl) polyamine formation (Lilley *et al.* 1997a).

Stock solution.

2.2.3.3.1. Reaction buffer- 100mM Tris-HCl pH 8.5 containing; 13.3mM DTT, 6.7mM calcium chloride (dihydrate) and 1×10^{-3} mg ml^{-1} biotin labelled casein.

0.3g Tris, 0.051g DTT, 0.025g calcium chloride and 12.5µl of 2 mg ml^{-1} stock biotin labelled casein were dissolved in 15.0 ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 25.0 ml with distilled water.

Method.

To previously prepared plates coated with enzymically modified *N',N'*-dimethylcasein (section 2.2.2.3) was added 150µl per well of reaction buffer (2.2.3.3.1) and 50µl per well of sample to be assayed for transglutaminase activity. The assay was then carried out as described for the measurement of ε-(γ-glutamyl) lysine formation (section 2.2.3.2). One unit of transglutaminase activity was defined as a change of absorbance at 450nm of 1.0 per hour.

2.2.3.4 Radiometric filter paper transglutaminase assay (Lorand *et al.* 1972).

Stock solutions.

2.2.3.4.1. Reaction buffer-100mM Tris-HCl pH 8.5 containing; 9.1mM calcium chloride, 18mM DTT, 9.1mg ml^{-1} *N'*,*N'*-dimethylcasein and 2.2mM [1,4-^{14}C]-labelled putrescine.

55 µl of reaction buffer consisted of the following components:-

a) 10µl of 100mM Tris-HCl pH 8.5: (0.12g Tris was dissolved in 7.0 ml distilled water). The pH was adjusted to 8.5 with dilute HCl and the volume was adjusted to 10.0 ml with distilled water.

b) 10µl of 100mM Tris-HCl pH 8.5 containing; 100mM DTT: 0.12g Tris and 0.15g DTT were dissolved in 7.0 ml of distilled water. The pH was adjusted to 8.5 with dilute HCl and the volume was adjusted to 10.0 ml with distilled water.

c) 5µl of 100mM Tris-HCl containing; 100mM calcium chloride: 0.12g Tris and 0.15g calcium chloride (dihydrate) were dissolved in 7.0 ml of distilled water. The pH was adjusted to 8.5 with dilute HCl and the volume was made up to 10.0 ml with distilled water.

d) 10µl of 100mM Tris-HCl pH 8.5. containing; 12mM putrescine (final specific activity 3.97µCi/µmol: (1.0 ml of 0.459mM putrescine (specific activity 109 µCi/µmol) was added to 50µl of 2.1M Tris HCl pH 8.5 containing; 243mM cold putrescine).

e) 20µl of 100mM Tris-HCl pH 8.5 containing; 25 mg ml^{-1} *N',N'*-dimethylcasein: 0.12g Tris and 250 mg *N',N'*-dimethylcasein were dissolved in 7.0 ml of distilled water. The pH was adjusted to 8.5 with dilute HCl and the volume was made up to 10.0 ml with distilled water.

2.2.3.4.2. Blocking solution- 1% (w/v) methylamine containing; 100mM EDTA.

10g methylamine and 37.2g disodium EDTA (dihydrate) were dissolved in distilled water to a final volume of 1.0 litre.

2.2.3.4.3.- 10% (w/v) TCA.

100g TCA was dissolved in distilled water to final volume of 1.0 litre.

2.2.3.4.4.- 5% (w/v) TCA.

50g TCA was dissolved in distilled water to a final volume of 1.0 litre.

Method.

To 55µl of reaction buffer (2.2.3.4.1) was added 45µl of the sample to be assayed for transglutaminase activity. The pH of the reaction measured at 37°C was 7.8 when assaying crude plant extracts. The reaction was terminated after 60 minutes by pipetting

10µl aliquots of the reaction mixture onto 1cm^2 Whatman N°1 filter paper squares pre-soaked in blocking solution (2.2.3.4.2). The protein was precipitated by washing the filter papers once in ice cold 10% (w/v) TCA (2.2.3.4.3), three times in ice cold 5% (w/v) TCA (2.2.3.4.4), once in acetone:ethanol (1:1) and once in acetone prior to being dried and placed into 2.0 ml of liquid scintillant and counted for 5 minutes in a Packard model LSC 300C liquid scintillation counter (Berkshire, U.K). In some experiments, the 5mM final concentration of calcium chloride was replaced with various concentrations of EDTA or EGTA in the reaction buffer. In several experiments, other reagents were added to the reaction buffer in order to investigate their effect on plant transglutaminase activity. One unit of transglutaminase activity was defined as one nanomole putrescine incorporated into *N',N'*-dimethylcasein per hour.

2.2.4 Chromatographic techniques.

All column chromatography techniques were carried out using a Biorad Econosystem model ES-1 chromatography apparatus (Hertfordshire, U.K).

2.2.4.1. Pre-purification of ε-(γ-glutamyl) lysine isodipeptide by Dowex anion exchange chromatography (Lilley *et al.* 1998).

Stock solutions.

2.2.4.1.1. Equilibration buffer- distilled water pH 12.6.

The pH of 1.0 litre of Millipore Q (Herefordshire, U.K) grade distilled water was adjusted to 12.6 by the addition of solid sodium hydroxide.

2.2.4.1.2. Leucine elution buffer- 300mM ammonium bicarbonate.

2.4g ammonium bicarbonate was dissolved to a final volume of 100.0 ml of distilled water and the pH was measured and recorded at 7.6.

Method.

The pH of 0.3 ml of each protein digest was adjusted to 12.6 by the addition of 1.7 ml equilibration buffer (2.2.4.1.1) The samples were applied to a Dowex (2x8-200 chloride form) anion exchange column (1.5 x 1.1cm) pre-equilibrated with equilibration buffer. The column was then washed with a mixture of equilibration buffer and leucine elution buffer (2.2.4.1.2) at a flow rate of 4.0 ml min^{-1} (final concentration of ammonium bicarbonate 25mM). After 70.0 ml of the mixture had passed through the column to

elute leucine, the column was washed with 10.0 ml equilibration buffer and the isodipeptide was eluted by washing the column with 0.1N HCl. 4.0 ml fractions were collected and the pH monitored. When the pH dropped below pH 7.0 the next 20.0 ml of eluate was pooled. The pH was adjusted to 7.0 and the eluate was freeze dried. After freeze drying the samples were re-dissolved in 0.3 ml Millipore Q-grade water and stored at -20°C.

2.2.4.2 Sepharose FFQ anion exchange chromatography of *Pisum sativum* root transglutaminase activity.

Stock solutions.

2.2.4.2.1. Equilibration buffer- 50mM Tris-HCl pH 7.4 containing: 1mM 2-ME.

6.1g Tris and 70µl 2-ME were dissolved in 900.0 ml of distilled water and the pH was adjusted to 7.4 using concentrated HCl. The volume was made up to 1.0 litre with distilled water.

2.2.4.2.2. Elution buffer A- 50mM Tris-HCl pH 7.4 containing: 1mM 2-ME and 1.0M sodium chloride.

6.1g Tris, 70µl 2-ME and 58.4g sodium chloride were dissolved in 900.0 ml of distilled water and the pH was adjusted to 7.4 using concentrated HCl. The volume was made up to 1.0 litre with distilled water.

2.2.4.2.3. Elution buffer B- 50mM Tris-HCl pH 7.4 containing: 1mM 2-ME and 2.0M sodium chloride.

6.1g Tris, 70µl 2-ME and 116.9g sodium chloride were dissolved in 900.0 ml of distilled water and the pH was adjusted to 7.4 using concentrated HCl. The volume was made up to 1.0 litre with distilled water.

Method.

Pisum sativum root protein prepared as described in section 2.2.1.2 was applied to a 6 ml FFQ Sepharose anion exchange column (Pharmacia, Milton Keynes U.K) pre-equilibrated with 10 column volumes of equilibration buffer (2.2.4.2.1) at 3.0 ml min^{-1} flow rate. The column was inverted and the unbound material was then eluted by the addition of 3 column volumes of the same buffer at the same flow rate. A 3.0 ml min^{-1} linear gradient between 0 - 1.0M sodium chloride was then applied to the column by mixing equilibration buffer with elution buffer A (2.2.4.2.2) over a 25 minute period. Strongly bound material was then eluted from the resin by the addition of 45.0 ml of elution buffer B (2.2.4.2.3). Three millilitre fractions were collected and assayed immediately for transglutaminase activity. The active aliquots were pooled, concentrated to a volume of 0.5 ml against solid polyethyleneglycol (average molecular mass 3350) at 4°C and stored at -20°C.

2.2.4.3 Gel filtration chromatography of *Pisum sativum* root transglutaminase activity.

Stock solutions.

2.2.4.3.1. Elution buffer- 50mM Tris-HCl pH 7.4 containing; 1mM 2-ME and 1μM calcium chloride.

6.1g Tris, 70μl 2-ME and 10μl of 100mM calcium chloride (dihydrate) were dissolved in 900.0 ml of distilled water and the pH was adjusted to 7.4 using concentrated HCl. The volume was made up to 1.0 litre with distilled water.

2.2.4.3.2. Protein standard solution- 50Mm Tris-HCl pH 7.4 containing; 1Mm 2-ME, 1μM calcium chloride and 5 mg ml^{-1} protein of interest.

0.6g Tris, 7μl 2-ME, 1μl of 100mM calcium chloride (dihydrate) and 500mg protein of interest were dissolved in 90.0 ml of distilled water and the pH was adjusted to 7.4 using concentrated HCl. The volume was made up to 100.0 ml with distilled water.

Method.

Approximately fifty millilitres of Sephacryl 100-HR (Pharmacia, Milton Keynes U. K) was de-gassed by vacuum aspiration and slowly poured into a 17.5×2cm column. Following overnight equilibration using elution buffer (2.2.4.3.1) at 0.1 ml min^{-1} flow rate, the column was calibrated using a set of protein standards (cytochrome c (M_r = 13000), ovalbumin (M_r = 45000), casein (M_r = 100000) and BSA (M_r = 66000)). Five hundred microlitres of each 5 mg ml^{-1} protein standard (2.2.4.3.2) was applied to the column and eluted with elution buffer at 2.5 ml min^{-1} flow rate. The void volume

(15.0 ml) and total volume (35.0 ml) were determined using blue dextran and vitamin B_{12} respectively. A pre-concentrated 0.5 ml sample of ion exchange purified *Pisum sativum* root protein was then applied to the column and eluted using the same conditions. The protein was collected as 1.25 ml fractions and assayed immediately for transglutaminase activity. The active fractions were pooled and stored at -20°C.

2.2.4.4 Amino acid analysis of protein digests (Griffin *et al.* 1982).

Stock solutions.

2.2.4.4.1. Loading buffer- 200mM lithium hydroxide pH 2.2 containing: 50mM citric acid and10mM phenol.

8.4g lithium hydroxide (monohydrate), 9.6g citric acid and 1g phenol were dissolved in 900.0 ml of distilled water. The pH was adjusted to 2.2 using approximately 16.0 ml of concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.4.4.2. Elution buffer A- 200mM lithium hydroxide pH 2.8 containing: 50mM citric acid, 10mM phenol and 7.5% (v/v) isopropanol.

8.4g lithium hydroxide (monohydrate), 9.6g citric acid, 1g phenol and 75.0 ml isopropanol were dissolved in 800.0 ml of distilled water. The pH was adjusted to 2.8 using approximately 15.4 ml of concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.4.4.3. Elution buffer B- 200mM lithium hydroxide pH 3.0 containing: 50mM citric acid, 100mM lithium chloride, 10mM phenol and 7.5% (v/v) isopropanol.

8.4g lithium hydroxide (monohydrate), 9.6g citric acid, 4.3g lithium chloride, 1g phenol and 75.0 ml isopropanol were dissolved in 800.0 ml of distilled water. The pH was adjusted to 3.0 using approximately 14.8 ml of concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.4.4.4. Elution buffer C- 200mM lithium hydroxide pH 2.9 containing: 50mM citric acid, 400mM lithium chloride and 10mM phenol.

8.4g lithium hydroxide (monohydrate), 9.6g citric acid, 17.0g lithium chloride and 1g phenol were dissolved in 900.0 ml of distilled water. The pH was adjusted to 2.9 using approximately 14.8 ml of concentrated HCl and the volume was made up to 1.0 litre with distilled water.

2.2.4.4.5. Column regeneration buffer- 300mM lithium hydroxide.

12.6g lithium hydroxide (monohydrate) was dissolved in distilled water to a final volume of 1.0 litre.

2.2.4.4.6. Derivatisation buffer- 800mM boric acid (pH greater than 10.0) containing: 770mM potassium hydroxide, 0.74% (v/v) methanol, 0.34% (v/v) Brij 35, 70mM 2-ME and 4.4mM OPA.

50.3g boric acid, 43.9g potassium hydroxide, 7.5 ml methanol, 3.5 ml Brij 35, 5.0 ml 2-ME and 0.6g OPA were dissolved in distilled water to a final volume of 1016.0 ml. The pH was checked and was always between 10.1 and 11.0.

Method.

The digested protein was diluted appropriately using loading buffer (2.2.4.4.1) and injected onto a 5×250mm Ultrapac ion exchange column (lithium form / particle size 8μm ± 0.5μm) fitted to a LKB 4151 Alpha plus amino acid analyser (Cambridgeshire,U.K). 3.6 ml of elution buffer A (2.2.4.4.2) was applied to the column at a flow rate of 0.4 ml min^{-1} and column temperature of 30°C. The column temperature was then decreased to 21°C and 9.2 ml of elution buffer B (2.2.4.4.3) was applied to the column at the same flow rate. Thirty six millilitres of elution buffer C (2.2.4.4.4) was then applied at the same flow rate and column temperature. The amino acids and isodipeptide eluting from the ion exchange column due to the addition of elution buffer C were then detected using post column derivatisation with derivatisation buffer (2.2.4.4.6) flowing at 0.33 ml min^{-1} through a 2.0 ml post column reaction loop. The derivatised amino acids were then passed through a Perkin Elmer LS1 fluorescence detector (Cheshire, U.K) set to 360nm excitation, 450nm emission. Fluorescence data was recorded by a Viglen SL1 computer (Middlesex, U.K) using a Nelson Analytical 900 series interface (Lancashire, U.K) sampling at a rate of 1 point every 9.9 seconds (model 2600 V5 chromatography software). The ion exchange column was cleaned prior to re-use by the addition of 4.0 ml of column regeneration buffer (2.2.4.4.5) at 0.4 ml min^{-1} flow rate and a column temperature of 99°C. Re-equilibration was achieved by application of 12.4 ml of elution buffer A (2.2.4.4.2) at 0.4 ml min^{-1} flow rate with a stepwise decrease in column temperature to 30°C. Primary amine contamination was removed from all buffers to decrease the fluorescence base line to acceptable levels. This

was carried out by injection onto a pre-column 40mm×6mm cation exchange column ammonia trap.

2.2.4.5 Cross-link analysis of guinea pig liver transglutaminase treated EDC-modified and biotin labelled casein.

Stock solutions.

2.2.4.5.1. Reaction buffer- 0.1M Tris-HCl pH 8.5 containing: 20mM DTT, 10mM calcium chloride, 0.4mg ml^{-1} EDC-modified casein and 0.4 mg ml^{-1} biotin labelled casein.

0.12g Tris, 0.031g DTT, 0.015g calcium chloride (dihydrate), 4.0mg biotin labelled casein and 4.0mg EDC-modified casein were dissolved in 7.0ml of distilled water. The pH of the buffer was adjusted to 8.5 using dilute HCl and the volume was made up to 10.0 ml with distilled water.

2.2.4.5.2. Guinea pig liver transglutaminase solution- 0.1M Tris-HCl pH 8.5 containing: 1µg ml^{-1} guinea pig liver transglutaminase.

0.12g Tris and 10µg of guinea pig liver transglutaminase were dissolved in 7.0ml of distilled water. The pH was adjusted to 8.5 using dilute HCl and the volume was made up to 10.0 ml with distilled water.

Method.

Five hundred microlitres of reaction buffer (2.2.4.5.1) was added to 500µl of guinea pig liver transglutaminase solution (2.2.4.5.2). Boiled solution was used as control. The resultant reaction mixture was incubated at 37°C for 60 minutes. The reaction was

terminated by the addition of 165µl of 72% (w/v) TCA (2.2.1.6.3) to the reacting solution. The proteolytic digestion and cross-link analysis were then carried out as described in sections 2.2.1.4 and 2.2.4.4 respectively.

2.2.4.6 The cross-linking of casein by crude *Pisum sativum* extract.

Stock solution.

2.2.4.6.1. Reaction buffer- 0.1M Tris-HCl pH 8.0 containing: 20mM DTT, 10mM calcium chloride (or 10mM EDTA) and 20 mg ml^{-1} casein.

0.3g Tris, 0.08g DTT, 0.04g calcium chloride (or 0.09g EDTA) and 500mg casein were dissolved in 15.0ml of distilled water. The pH was adjusted to 8.0 using dilute HCl and the volume was made up to 25.0ml with distilled water.

Method.

To 500µl of reaction buffer (2.2.4.6.1) was added 500µl of crude *Pisum sativum* root protein, which had been extracted and rapidly de-salted as described in section 2.2.1.2. The resultant mixture was incubated for 0-16 hours at 37°C and the reaction was then stopped by the addition of 165µl of 72% (w/v) TCA (2.2.1.6.3). The proteolytic digestion and cross-link analysis were then carried out as described in sections 2.2.1.4 and 2.2.4.4 respectively.

2.2.5 SDS-PAGE electrophoresis (Laemmli 1970).

Stock solutions.

2.2.5.1.- 30% (w/v) Acrylamide / bis.

87.6g acrylamide and 2.4g N',N'-bis-methylene acrylamide were dissolved in distilled water to a final volume of 300.0 ml. The resultant solution was stored in the dark at 4°C.

2.2.5.2.- 1.5M Tris-HCl pH 8.8.

27.23g Tris was dissolved in 100.0 ml of distilled water. The pH was adjusted to 8.8 with concentrated hydrochloric acid and the volume was made up to 150.0 ml with distilled water and the solution was stored at 4°C.

2.2.5.3.- 0.5M Tris-HCl pH 6.8.

6.0g Tris was dissolved in 80.0 ml of distilled water and the pH was adjusted to 6.8 using concentrated hydrochloric acid. The volume was made up to 100.0 ml using distilled water and the solution was stored at 4°C.

2.2.5.4.- 10% (w/v) SDS.

10.0g SDS was dissolved in distilled water to a total volume of 100.0 ml.

2.2.5.5. Sample buffer.

1.0 ml of 0.5M Tris-HCl pH 6.8 (2.2.5.3), 0.8 ml glycerol, 1.6 ml of 10% (w/v) SDS (2.2.5.4), 0.4 ml of 2-ME and 0.2 ml 0.05% (w/v) bromophenol blue were dissolved in 4.0 ml of distilled water.

2.2.5.6.- 5× electrode running buffer- 124mM Tris-HCl pH 8.3 containing: 0.96M glycine, 0.5% (w/v) SDS.

9.0g Tris, 43.2g glycine and 3.0g SDS were dissolved in 500.0 ml of distilled water. The pH was adjusted to 8.3 using concentrated hydrochloric acid and the volume was then made up to 600.0 ml with distilled water. 60.0 ml of stock was diluted by addition of 240.0 ml of distilled water for one electrophoretic run.

2.2.5.7.- 10% (w/v) ammonium persulphate.

100 mg of ammonium persulphate was added to distilled water to give a final volume of 1.0 ml.

2.2.5.8.- 12% (w/v) Separating gel.

2.5 ml of 1.5M Tris-HCl pH 8.8 (2.2.5.2), 0.1 ml of 10% (w/v) SDS (2.2.5.4), 4.0 ml of 30% (w/v) acrylamide/bis stock (2.2.5.1), 0.05 ml of 10% (w/v) ammonium persulphate (2.2.5.7) and 0.005 ml TEMED were added to 3.35 ml of distilled water. The gel was mixed thoroughly and poured immediately.

2.2.5.9.- 4% (w/v) Stacking gel.

2.5 ml of 0.5M Tris-HCl pH 6.8 (2.2.5.3), 0.1 ml of 10% (w/v) SDS (2.2.5.4), 1.3 ml of 30% (w/v) acrylamide / bis stock (2.2.5.1), 0.05 ml of 10% (w/v) ammonium persulphate (2.2.5.7) and 0.01 ml TEMED were added to 6.1 ml of distilled water. The gel was poured immediately following thorough mixing.

2.2.5.10.- Fixing solution- 40% (v/v) methanol containing; 10% (w/v) acetic acid.

10g of acetic acid was dissolved in 40.0 ml of distilled water. 40.0 ml of methanol was then stirred in and the volume was adjusted to 100.0 ml with distilled water.

2.2.5.11.- 0.01% (w/v) potassium manganate VII.

0.1g of potassium manganate VII was dissolved in distilled water to a final volume of 1.0 litre.

2.2.5.12.- 0.1% (w/v) silver nitrate solution.

0.1g of $AgNO_3$ was dissolved in distilled water to a final volume of 100.0 ml.

2.2.5.13. Developing solution- 0.05% (v/v) formaldehyde, 3% (w/v) sodium carbonate.

0.05 ml of formaldehyde and 3g of Na_2CO_3 were dissolved in distilled water to a final volume of 100.0 ml.

2.2.5.14. Stopping solution- 5% (w/v) acetic acid containing; 10% (v/v) ethanol.

5g of acetic acid and 10.0 ml of ethanol were dissolved in distilled water to a final volume of 100.0 ml

Method.

Five point six millilitres of 12% (w/v) separating gel (2.2.5.8) was carefully poured between 1.0mm spaced polished Biorad glass electrophoresis plates using a 1.0 ml pipette. Four percent stacking gel (2.2.5.9) was then layered on top of the sample gel and a plastic sample comb was carefully inserted into the gel avoiding trapping of air bubbles between the teeth of the comb and the gel. The gel was allowed to set and it was then placed into a Biorad mini-Protean II cell (Hertfordshire U. K). Three hundred millilitres of electrode running buffer was prepared as described in section 2.2.5.6. One hundred and fifteen millilitres of electrode running buffer was poured into the upper buffer chamber and the remainder was poured into the lower buffer chamber to cover the bottom 1.0cm of the gel. Samples were diluted 1 in 4 using sample buffer (2.2.5.5) and heated to 95°C for 4 minutes. Twenty five microgram samples were then loaded into the wells of the gel using a 25µl SGE syringe (Ringwood, Australia). The gel was then connected to a Biorad powerpac 300 (Hertfordshire, U. K) at 200 volts constant voltage for approximately 45 minutes. The gel was then fixed overnight in fixing solution (2.2.5.10). The gel was placed into 0.01% (w/v) potassium permanganate VII (2.2.5.11) for 5 minutes prior to being rinsed three times in distilled water. The gel was then stained in 0.1% (w/v) silver nitrate solution (2.2.5.12) for 20 minutes and rinsed in distilled water prior to being placed in developing solution (2.2.5.13). The development was then halted by immersion of the gel in stopping solution (2.2.5.14). The gel was placed on a light box (Hulco copilite, Tunbridge Wells, U. K) and photographed using a Mitsubishi model P68b image processing system (Tokyo, Japan). The resultant image was annotated using Adobe Photoshop version 4.0 for Windows 95 (Edinburgh, U. K).

Chapter 3- Development of two transglutaminase casein cross-linking assays.

3.1 Introduction.

Many transglutaminase assays have been developed which utilise the polyamine incorporation reaction described in section 1.1.2.2.1 (for a review, see Wilhelm *et al.* 1996). The first of these was reported by Clarke *et al.* (1959) and involved the incorporation of radiolabelled amines such as putrescine and histamine into the protein substrates casein or β-lactoglobulin. Following covalent modification, the protein was precipitated using TCA and repeatedly washed and centrifuged to remove loosely bound radiolabel prior to the covalently incorporated polyamine being quantified by liquid scintillation counting. Many modifications have been made to this original method including the precipitation of protein directly onto filter paper squares or nitro-cellulose discs to increase the speed of the assay and the number of samples which may be simultaneously processed (Lorand *et al.* 1972; Kang and Cho 1996). Reductive methylation of the proteins used in the assay is a further common modification used in order to block lysine residues which would react with glutamine to form intra- and inter-molecular ε-(γ-glutamyl) lysine isodipeptide bonds resulting in a loss of signal due to a reduction in potential polyamine incorporation sites. Further modifications to the method include the substitution of the amine and protein substrates. [^{14}C]-labelled putrescine and histamine have often been replaced with a variety of [^{3}H] and [^{14}C]-labelled primary amine donor substrates including spermine, spermidine and cadaverine (Serafini-Fracassini *et al.* 1988; Aribaud *et al.* 1995). A variety of glutamine donor proteins have

also been utilised to replace casein and β-lactoglobulin in the assay including RuBisCo, thrombin, fibrinogen, pepsin, insulin, creatine kinase and cellulase (Icekson and Apelbaum 1987; Margosiak *et al.* 1990).

An alternative transglutaminase assay was developed by Jeon *et al.* (1989) which removed the need for radioisotopes and instead involved the incorporation of the biotin labelled amine donor substrate 5-(biotinamido)pentylamine into *N',N'*-dimethylcasein by tissue transglutaminase. The resultant biotin labelled product was bound to polyvinylidine difluoride membranes and quantified by reaction with streptavidin-β-galactosidase followed by the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside and subsequent measurement of the absorbance at 405nm.

Further highly sensitive amine incorporation assays have been reported (Ando *et al.* 1987; Fink *et al.* 1992). These assays involve the incorporation of monodansylcadaverine into either casein or the small synthetic peptide benzyloxycarbonyl-L-glutaminylglycine respectively. The transglutaminase reaction product was separated from reactants by HPLC and detection limits of 31 pmol and 40 pmol of product were reported for the small synthetic peptide bound product and the casein bound product respectively. Ando *et al.* (1987) used their assay to aid the purification of human erythrocyte transglutaminase and platelet factor XIII.

The amine incorporation assays described so far are carried out in the solution phase and despite the modifications described involve the use of equipment and procedures, which do not lend themselves to processing large sample numbers quickly. As a result a number

of more convenient polyamine incorporation assays have been developed which are carried out in the solid phase using 96 well microtiter plates. These assays utilise commercially available biotin labelled amine substrates such as biotinylpentylamine which are incorporated into the glutamine residues of immobilised microtiter plate bound proteins such as *N',N'*-dimethylcasein and fibrinogen. The incorporated biotin label is then detected by reaction with streptavidin conjugated to one of several reporter enzymes such as alkaline phosphatase, horse radish peroxidase or β-galactosidase (Song *et al.* 1994; Slaughter *et al.* 1992). The plate assays have several advantages over the solution phase amine incorporation assays described previously including the fact that no radiochemicals or expensive machinery are required to carry them out and up to 96 samples can be processed at once with relative ease and little processing time. Assays of this type have been used to detect both factor XIIIa and tissue transglutaminase activities (Song *et al.* 1994; Slaughter *et al.* 1992).

Assays which measure the ability of transglutaminases to cross-link proteins by the reaction scheme described in section 1.1.2.2.2 have also been developed and involve the cross-linking of immobilised 96 well microtiter plate bound casein to biotin labelled casein present in solution (Seiving *et al.* 1991; Choi *et al.* 1992). The cross-linked reaction product is quantified by reaction with avidin or streptavidin-alkaline phosphatase followed by incubation with p-nitrophenyl phosphate and subsequent measurement of the absorbance at 405nm. Both assays were found to be of use with crude and purified transglutaminase samples. Seiving *et al.* (1991) used their assay to both test the activity of pure guinea pig liver transglutaminase and then further applied the assay to the

screening of human plasma for factor XIIIa deficiency and Choi *et al.* (1992) used their assay to purify transglutaminase C from human erythrocytes.

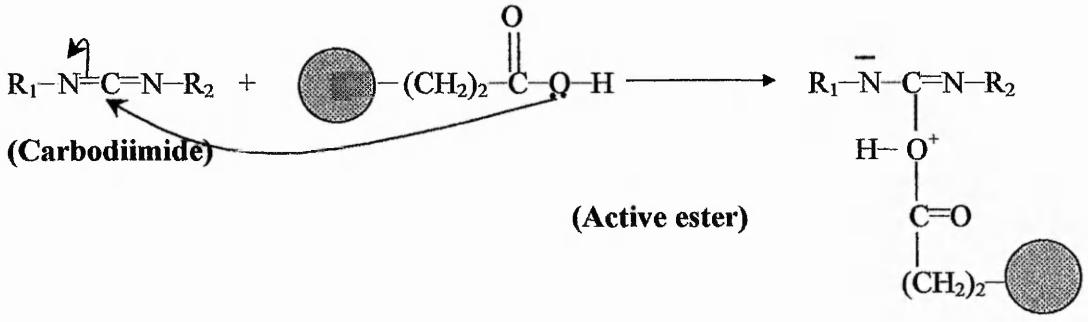
The development of two transglutaminase assays based on the protein cross-linking reaction was an initial aim of this project. There are several reasons for developing such assays, not least to answer the question of the calcium dependence of transglutaminases derived from plant sources. Because calcium independent enzymes such as diamine oxidases present in crude plant extracts have been shown to interfere with the conventional radiolabelled amine incorporation assays (Siepaio and Meunier 1995; Chiarello *et al.* 1996a and 1996b see section 1.2.3.3.1.1), we sought a new approach to measure the transglutaminase activity of crude plant extracts, which would not involve the use of radiolabelled amine substrates. Two casein cross-linking assays were developed in order to attempt to answer the calcium dependence question and also to overcome the selectivity problems experienced by other workers when using radiolabelled amine incorporation assays to screen crude plant extracts. Furthermore, to date the majority of plant transglutaminase research has demonstrated the polyamine incorporation reaction. As it is likely that plant transglutaminase may carry out the same collection of reactions as the animal transglutaminases the use of the two protein cross-linking assays will help to further characterise the plant enzyme.

The major drawback of previously developed protein cross-linking assays is that they have an inherently high background absorbance due to the presence of calcium ions in the reaction buffer resulting in a transglutaminase independent ionic interaction between the immobilised plate bound casein and the biotin labelled casein in solution. This adduct is thought to form between acidic amino acid side chains (Seiving *et al.* 1991). In the

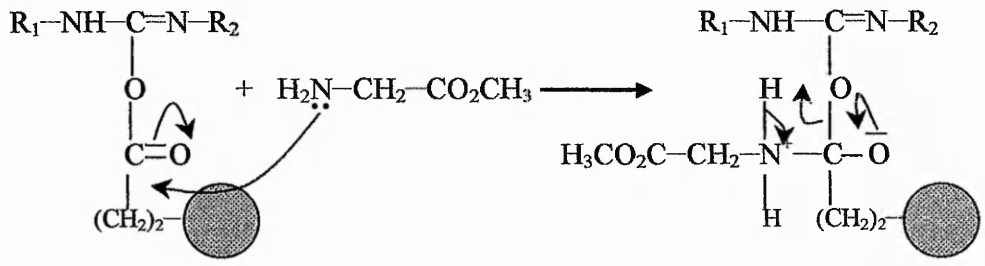
development of the first of the two protein cross-linking assays, we sought to overcome the problem caused by ionic adduct formation by blocking the glutamate and aspartate residues of the plate bound casein by the incorporation of the nucleophile glycine methyl ester using a water soluble carbodiimide coupling reaction as shown in figure 5 (Carraway and Koshland 1972).

Figure 5. Mechanism for the EDC coupling of glycine methyl ester to the acidic amino acid side chains of casein.

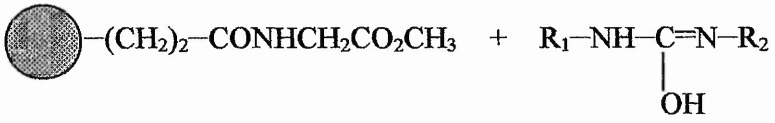
The initial step involves nucleophilic addition of the protein bound glutamate residue to the carbodiimide via its hydroxyl lone pair of electrons.



Following rapid proton transfer, the nucleophile, glycine methyl ester then attacks the carbonyl carbon of the active ester intermediate.

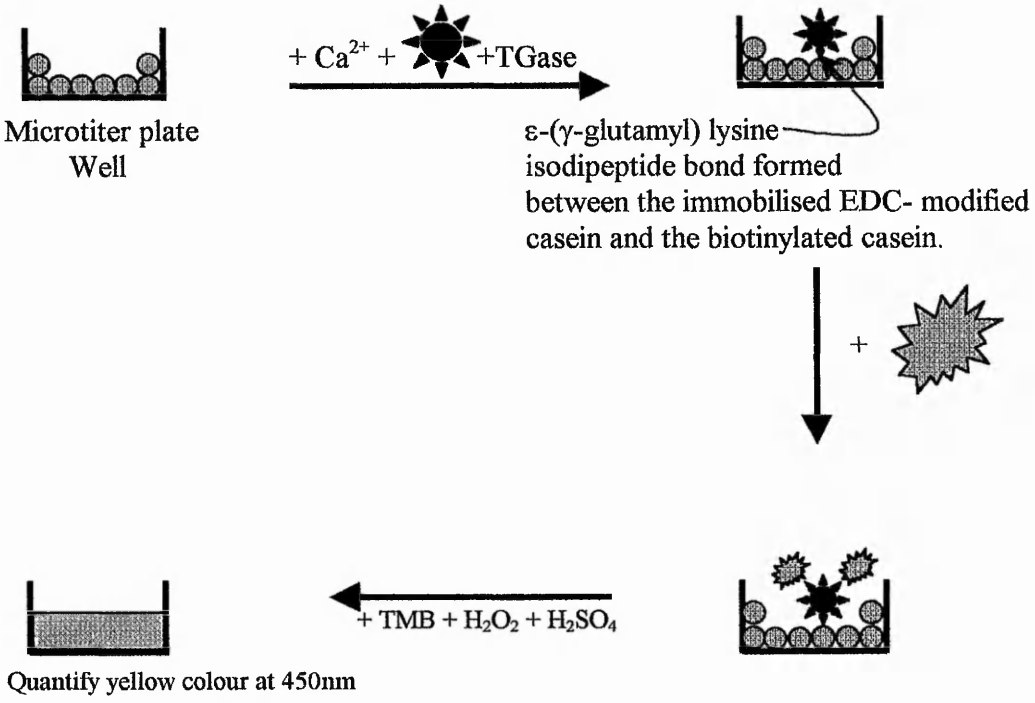


Rearrangement then yields the methyl ester blocked protein and an acyl urea bi-product.

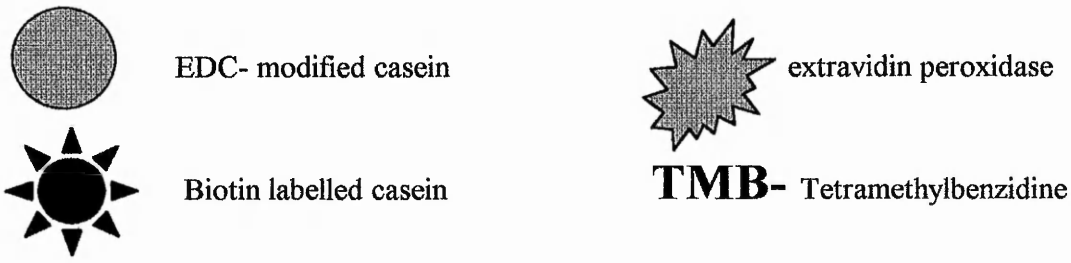


The assay was then developed using the carbodiimide modified casein and commercially available guinea pig liver transglutaminase. The methodological scheme for the assay is given in figure 6.

Figure 6. Scheme for the ε-(γ-glutamyl) lysine formation assay.



Key.

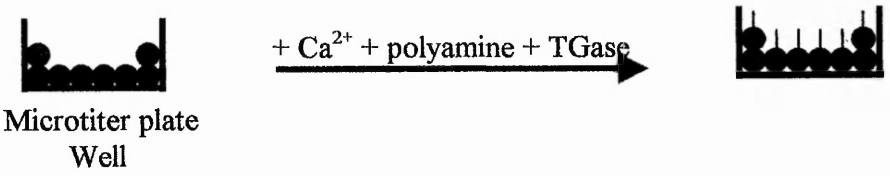


A second transglutaminase protein cross-linking assay was developed to measure the ability of transglutaminases to form *N',N'*-bis (γ-glutamyl) polyamine-protein cross-links *in vitro*. *N',N'*-dimethylcasein bound to microtiter plates was modified enzymically by the incorporation of polyamines into glutamine residues using commercially available

guinea pig liver transglutaminase. Biotin labelled casein was then incorporated via its glutamine residues into the remaining free amino group of the polyamines to form *N',N'*-bis (γ-glutamyl) polyamine bonds. This assay is a novel method for the detection of protein-polyamine-protein cross-linking which is reported to be of physiological importance in mammalian tissues such as the anterior prostate gland of rodents where its function may be to stabilise the post ejaculatory vaginal plug (Folk 1980). A methodological scheme for this second protein cross-linking assay is presented in figure 7.

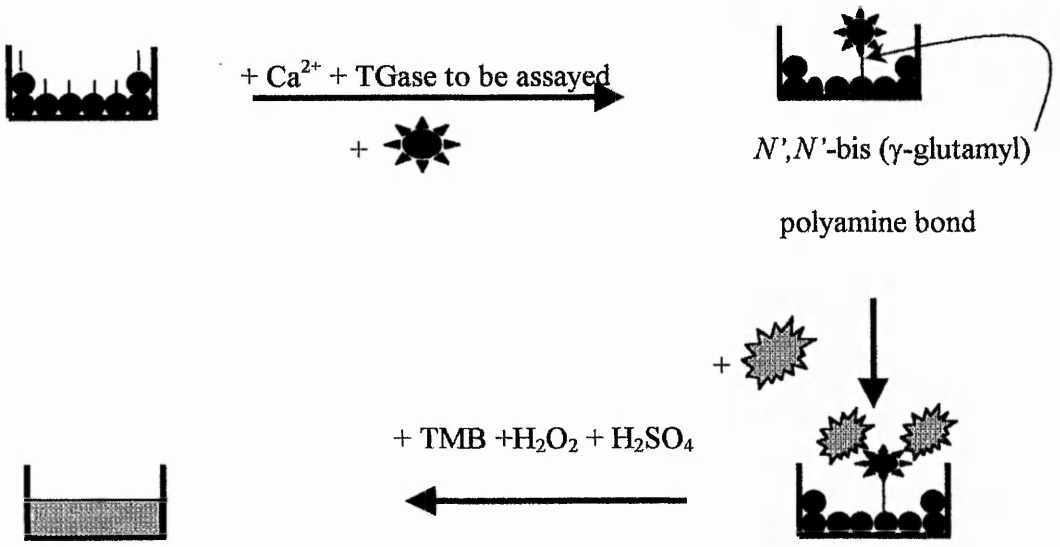
Figure 7. Scheme for the *N',N'*-bis (γ-glutamyl) polyamine formation assay.

Part 1.- Pre-treatment of *N',N'*-dimethylcasein to incorporate polyamines into glutamine residues.



Microtiter plate Well

Part 2.- Formation of *N',N'*-bis (γ-glutamyl) polyamine cross-bridges.



Quantify yellow colour at 450nm

Key.

N',N'-dimethylcasein

extravidin peroxidase

Biotin labelled casein

TMB- Tetramethylbenzidine

Enzymically modified *N',N'*-dimethylcasein.

3.2 Results.

The first method attempted to overcome the high assay background previously described by Seiving *et al.* (1991) was to de-phosphorylate the casein bound to the microtiter plate in order to prevent calcium ions binding to the protein via phosphorylated serine and threonine residues thus causing unwanted adduct formation. In a further experiment, the plate bound casein was de-amidated to reduce the quantity of available glutamine residues and hence prevent intra-molecular ε-(γ-glutamyl) lysine isodipeptide bond formation as this would result in a reduction of assay signal.

Table 1. The effect of de-amidation and de-phosphorylation on bovine casein as a substrate of tissue transglutaminase.

Type of modification	100ng/well Transglutaminase $\Delta A_{450}\ h^{-1} \pm$ SEM	Background $\Delta A_{450}\ h^{-1} \pm$ SEM	Increase in signal (%) above background
None.	0.61 ± 0.01	0.53 ± 0.02	15.1
De-phosphorylation.	0.46 ± 0.02	0.42 ± 0.01	9.5
De-amidation.	1.09 ± 0.05	1.11 ± 0.03	0

One hundred nanograms of guinea pig liver transglutaminase was incubated for 60 minutes at 37°C with de-phosphorylated or de-amidated casein bound to the microtiter plate as described in section 2.2.3.2. Casein was de-phosphorylated and de-amidated as described in sections 2.2.2.4 and 2.2.2.6 respectively. One hundred nanograms of boiled guinea pig liver transglutaminase was used to provide the background. Data points represent the mean ± SEM of 8 replicates.

Table 1 shows that the use of un-modified bovine casein in the casein cross-linking assay resulted in a signal of 15.1% above background at the 100ng per well level of guinea pig liver transglutaminase and a substantial assay background of 0.53 ΔA_{450} hr^{-1} was observed. De-amidation and de-phosphorylation of the casein substrate did not improve the overall above background signal of the assay. Furthermore, high assay background values of 1.11 ΔA_{450} h^{-1} and 0.42 ΔA_{450} h^{-1} were observed using de-amidated and de-phosphorylated casein respectively. As de-phosphorylation and de-amidation did not reduce the assay background to acceptable levels, the next step was to EDC-modify the casein to block acidic amino acid side groups as explained in figure 5. The results from this experiment are presented in table 2.

Table 2. The effect of carbodiimide modification on bovine casein as a substrate of tissue transglutaminase.

Nucleophile used in the carbodiimide reaction	100ng/well Transglutaminase $\Delta A_{450}\ h^{-1} \pm$ SEM	Background $\Delta A_{450}\ h^{-1} \pm$ SEM	Increase in signal (%) above background
None (un-modified casein).	0.59 ± 0.02	0.51 ± 0.03	15.6
Glycine methyl ester.	0.56 ± 0.01	0.16 ± 0.01	250.0
Lysine methyl ester.	0.24 ± 0.01	0.16 ± 0.02	50.0
Arginine methyl ester.	0.23 ± 0.01	0.17 ± 0.02	35.3
On plate modification with glycine methyl ester.	1.17 ± 0.04	1.16 ± 0.02	0.9

One hundred nanograms of guinea pig liver transglutaminase was incubated for 60 minutes at 37°C with EDC-modified and biotin labelled casein as described in section 2.2.3.2. The EDC-modification reactions were carried out as described in section 2.2.2.2. One hundred nanograms of boiled guinea pig liver transglutaminase was used to provide the background. Data points represent the mean ± SEM of 8 replicates.

Table 2 shows that using a range of nucleophiles in the EDC-modification reaction, the signal obtained was between 0.9 and 250.0% above background compared to 15.6% when using un-modified bovine casein. As the EDC-modification resulted in reduced assay background levels and an acceptable signal above background, further assay development was carried out using casein, which had been modified using the glycine methyl ester / EDC reaction.

Table 3. The effect of variation of biotinylation parameters on bovine casein as a substrate of tissue transglutaminase.

Biotinylation conditions	$\Delta A_{450}\ h^{-1} \pm$ SEM above background using 100ng/well transglutaminase
4 hour reaction time with d-biotin N-hydroxysuccinimide ester.	0.39 ± 0.04
8 hour reaction time with d-biotin N-hydroxysuccinimide ester.	0.38 ± 0.02
4 hour reaction time with biotin amidocaproate N-hydroxysuccinimide ester.	0.31 ± 0.03

One hundred nanograms of guinea pig liver transglutaminase was incubated with plate bound EDC-modified casein and biotin labelled casein for 60 minutes at 37°C as described in section 2.2.3.2. The biotinylation was carried out as described in section 2.2.2.1. One hundred nanograms of boiled guinea pig liver transglutaminase was used to provide the assay background. The data points represent the mean ± SEM of 8 replicates.

Table 3 demonstrates the variation of assay signal with varying methods of casein biotinylation. Values of between 0.38 ΔA_{450} h^{-1} and 0.39 ΔA_{450} h^{-1} were obtained using the N-hydroxysuccinimide ester of d-biotin whereas a value of 0.31 ΔA_{450} h^{-1} was obtained when using a similar ester with an incorporated amidocaproate spacer arm. Since the most satisfactory assay signal was obtained when casein was modified using the N-hydroxysuccinimide ester of d-biotin, this was utilised for further assay development.

Table 4. The effect of 7.5M urea on bovine casein as a substrate of tissue transglutaminase and the effect of stopping the carbodiimide reaction using sodium acetate.

Condition used	$\Delta A_{450}\ h^{-1} \pm$ SEM above background using 100ng/well transglutaminase
Treatment of casein with 7.5M urea alone.	0.11 ± 0.01
Use of 1.0M sodium acetate to stop the carbodiimide reaction.	0.40 ± 0.03
No sodium acetate used to stop the carbodiimide reaction.	0.39 ± 0.02

One hundred nanograms of guinea pig liver transglutaminase was assayed at 37°C for 60 minutes in the presence of plate bound casein, which had been subjected to urea solution without EDC and glycine methyl ester modification. Guinea pig liver transglutaminase was also incubated with EDC-modified casein, which had not been treated with sodium acetate to stop the modification reaction. One hundred nanograms of boiled guinea pig liver transglutaminase was used to provide the assay background. Data points represent the mean ± SEM of 8 replicates.

Table 4 shows that treatment of the casein with urea containing buffer alone results in a signal of 0.11 ΔA_{450} h^{-1} above background at the 100ng per well transglutaminase level. The table also demonstrates that cessation of the carbodiimide reaction using 1.0M sodium acetate has little effect on the overall assay signal as values between 0.40 ΔA_{450} h^{-1} and 0.39 ΔA_{450} h^{-1} were observed for the reaction with and without sodium acetate respectively.

In order to show that the casein cross-linking assay signal observed was due to the presence of guinea pig liver transglutaminase, several controls were carried out including the use of transglutaminase inhibitors such as iodoacetamide and EDTA. The results are presented in table 5.

Table 5. The effect of various controls on the casein cross-linking assay signal.

Control used	ΔA_{450} ± SEM using 100ng/well transglutaminase
100ng per well guinea pig liver transglutaminase (0')	0.17 ± 0.04
100ng per well guinea pig liver transglutaminase (30')	0.41 ± 0.02
100ng per well guinea pig liver transglutaminase (60')	0.57 ± 0.03
100ng per well boiled guinea pig liver transglutaminase.	0.14 ± 0.01
5mM calcium chloride removed from the reaction buffer and replaced with 0.25mM EDTA.	0.11 ± 0.01
5mM calcium chloride removed from the reaction buffer and replaced with 0.25mM EGTA.	0.12 ± 0.01
10mM iodoacetamide added to the reaction buffer.	0.16 ± 0.03
50 µl Tris-HCl pH 8.5 added to replace the guinea pig liver transglutaminase (zero enzyme control).	0.15 ± 0.02
Zero biotin labelled casein.	0.04 ± 0.00
Zero extravidin peroxidase.	0.04 ± 0.00

One hundred nanograms of guinea pig liver transglutaminase was assayed at 37°C for 60 minutes (unless otherwise stated in the table) in the presence of various controls as described in section 2.2.3.2. Data points represent the mean ± SEM of 8 replicates.

Table 5 demonstrates a variety of assay controls varying in absorbance between 0.04 ΔA_{450} h^{-1} for zero biotin labelled casein and zero extravidin peroxidase and 0.17 ΔA_{450} h^{-1} for the zero time control.

In order to further optimise the assay in terms of the concentration of substrates used, the kinetics of casein cross-linking were investigated and the results are presented in figures 8-9.

Figure 8. The variation of absorbance at 450nm as a function of the concentration of microtiter plate bound EDC-modified casein.

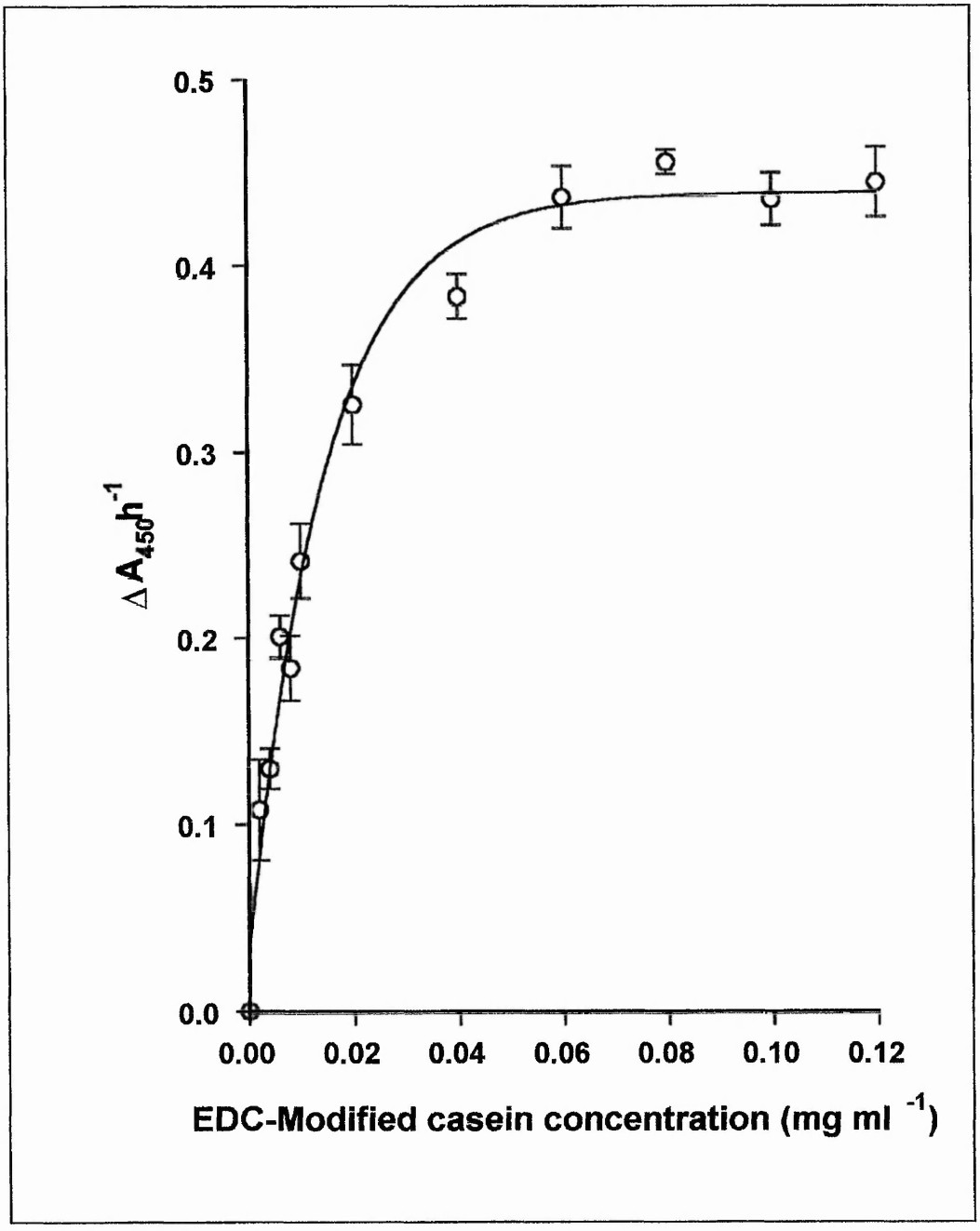


Figure 8 legend.

One hundred nanograms of guinea pig liver transglutaminase was assayed for 60 minutes at 37°C in the presence of varying concentrations of plate bound EDC-modified casein as described in section 2.2.3.2. One hundred nanograms of boiled guinea pig liver transglutaminase was used to provide the assay background of 0.12 ΔA_{450} h^{-1}. Data points represent the mean ± SEM of 8 replicates.

Figure 8 shows as the concentration of EDC modified casein bound to the microtiter plate increases, the absorbance at 450nm also increases up to a saturation point. The variation of assay signal with EDC modified casein concentration followed typical Michaelis-Menten type saturation kinetics with an optimum concentration of EDC-modified casein of 0.08 mg ml^{-1}. Subsequent experiments were carried out using microtiter plates coated with a 1 mg ml^{-1} excess of this protein to ensure that all assay plate binding sites were occupied.

Figure 9. The variation of absorbance at 450nm as a function of the concentration of the biotin labelled casein substrate.

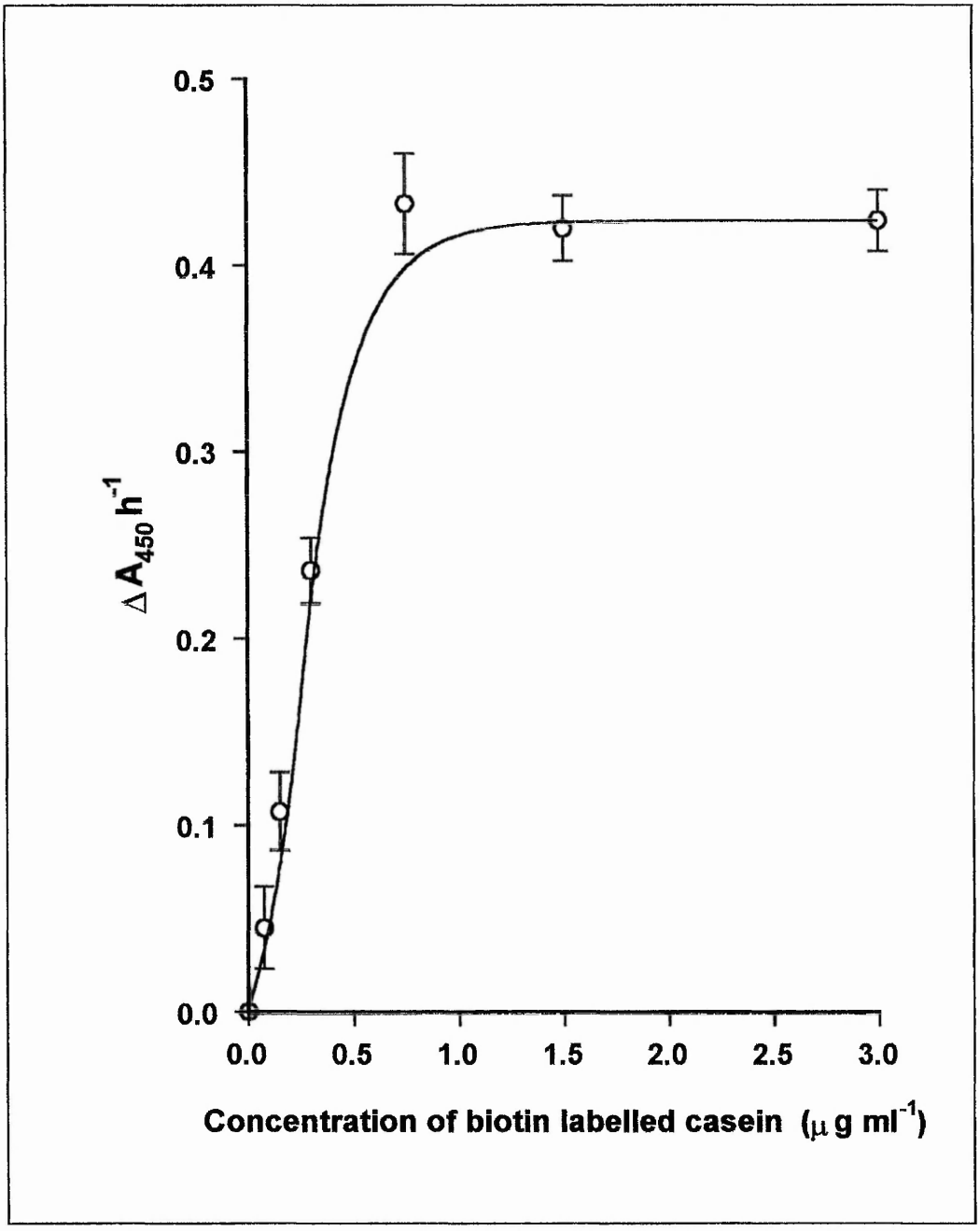


Figure 9 legend.

One hundred nanograms of guinea pig liver transglutaminase was assayed at 37°C for 60 minutes in the presence of varying concentrations of biotin labelled casein as described in section 2.2.3.2. One hundred nanograms of boiled guinea pig liver transglutaminase was used as a negative control giving an average value of 0.15 ΔA_{450} h^{-1}. Data points represent the mean ± SEM of 8 replicates.

Figure 9 demonstrates that an increase in biotin labelled casein concentration resulted in an increase in absorbance at 450nm until a saturation point was achieved at 0.75 $\mu g\ ml^{-1}$. The K_m of biotin labelled casein was calculated to be 0.36$\mu g\ ml^{-1}$ for guinea pig liver transglutaminase (Enzfitter, Biosoft U.K). A biotin labelled casein concentration of 0.75$\mu g\ ml^{-1}$ was used in all subsequent experiments.

Figure 10 (overleaf) demonstrates the detection limit of the casein cross-linking assay when using guinea pig liver transglutaminase.

Figure 10. The variation in absorbance at 450nm as a result of using different concentrations of guinea pig liver transglutaminase.

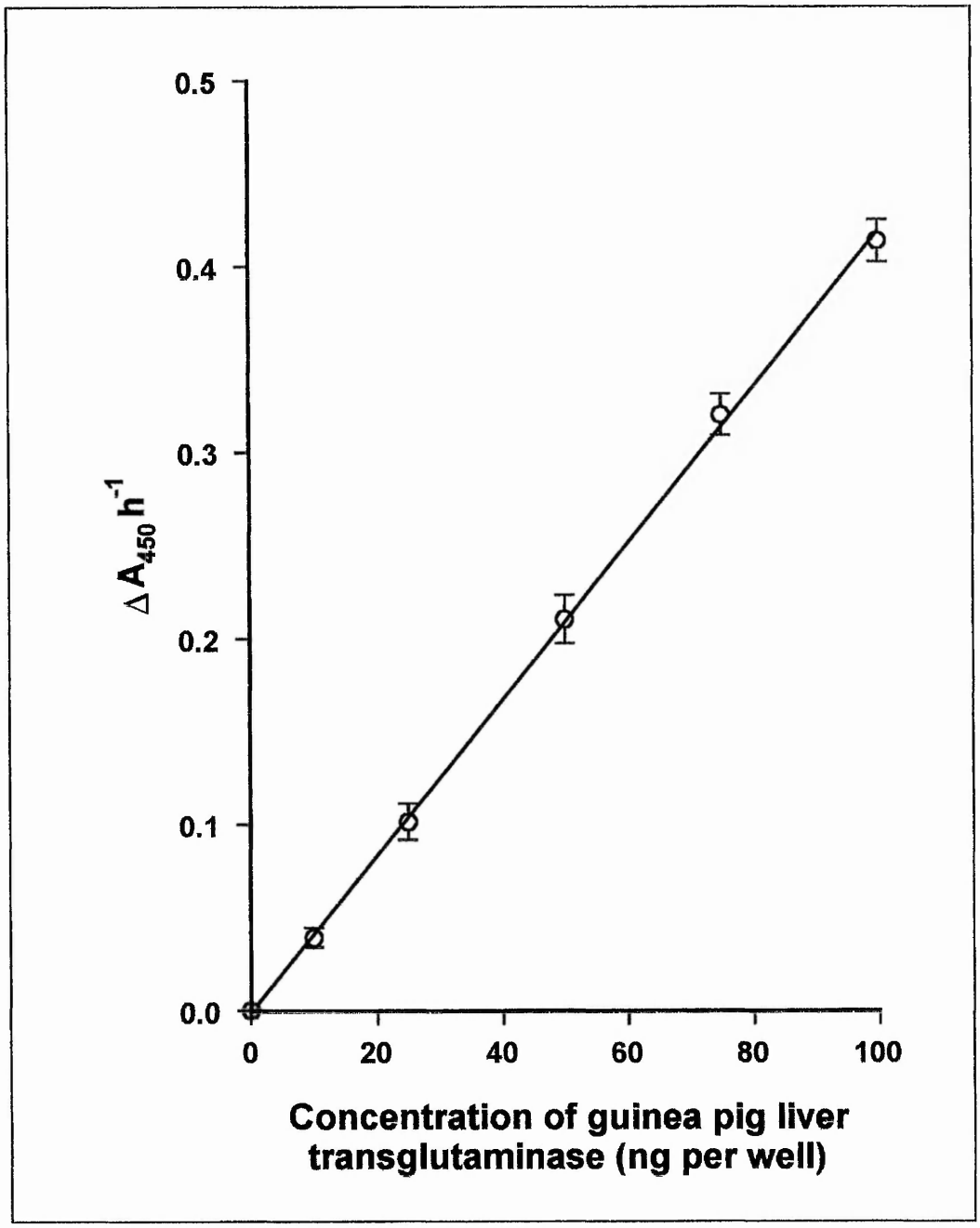


Figure 10 legend.

Guinea pig liver transglutaminase in the range 0-100ng per well was assayed at 37°C for 60 minutes as described in section 2.2.3.2. Boiled transglutaminase negative controls gave an average background of 0.14 ΔA_{450} h^{-1}. Data points represent the mean ± SEM of 8 replicates.

Figure 10 demonstrates that a linear relationship between absorbance at 450nm and transglutaminase concentration was observed over a 0-100ng per well range of transglutaminase concentrations. Since the assay was sensitive enough to detect 10ng of purified guinea pig liver transglutaminase it was decided to test the suitability of the assay for transglutaminase detection in crude extracts. Figure 11 shows the results obtained when screening human endothelial cell homogenate for casein cross-linking activity.

Figure 11. The variation in absorbance at 450nm as a function of different quantities of homogenised ECV 304 human endothelial cells.

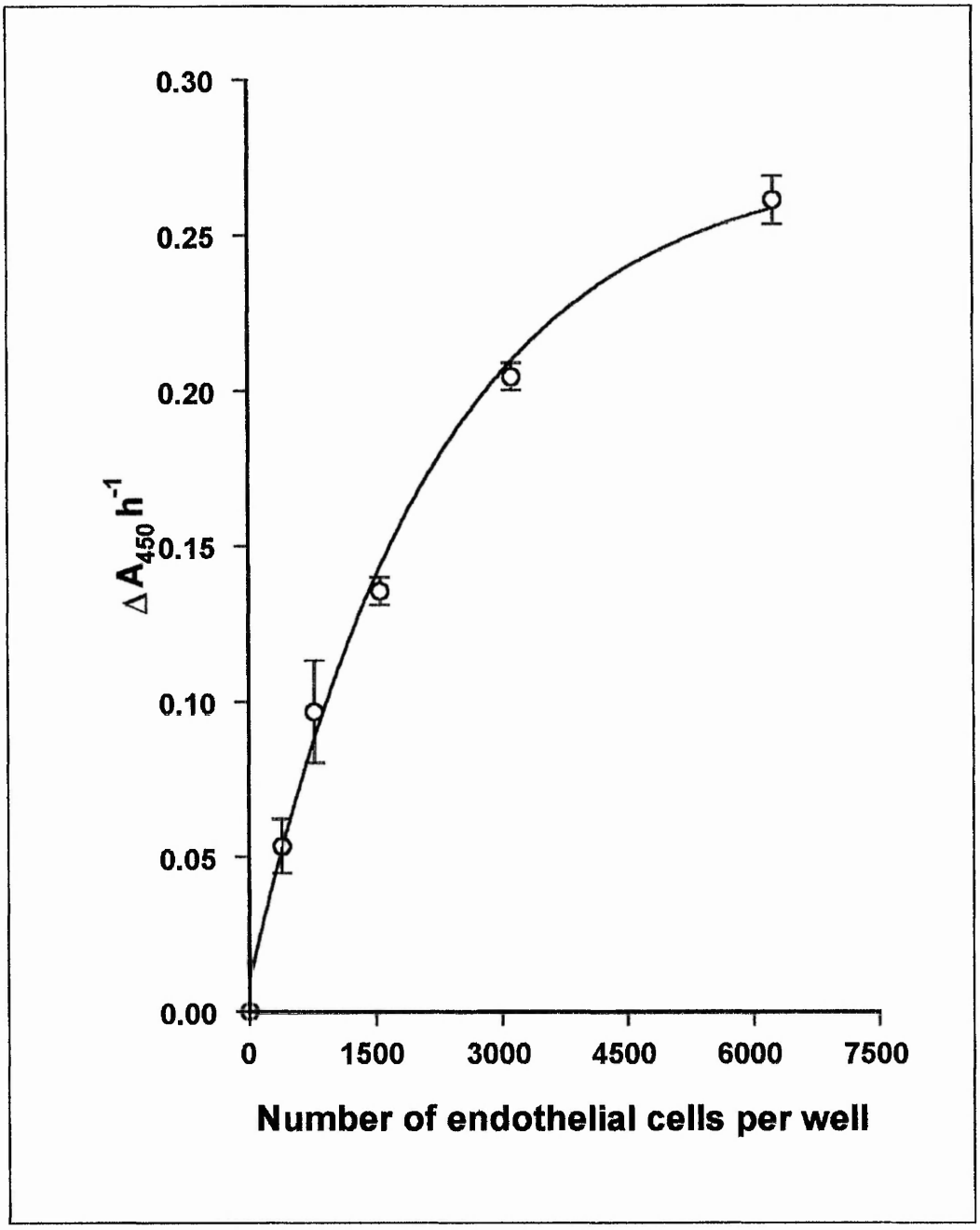


Figure 11 legend.

Human ECV 304 endothelial cells were homogenised and assayed for casein cross-linking activity over the range 0-6000 cells per well. The assay was carried out for 60 minutes at 37°C as described in section 2.2.3.2. Endothelial cells were prepared as described in section 2.2.1.5 and boiled endothelial cell homogenate was used as negative control giving an average background of 0.11 ΔA_{450} h^{-1}. Data points represent the mean ± SEM of 8 replicates.

Figure 11 demonstrates that absorbance at 450nm increases non-linearly with the number of homogenised ECV 304 cells per well. The lower detection limit was determined to be 400 homogenised cells per well which gave a transglutaminase activity of 0.05 ΔA_{450} h^{-1} above background. The assay was carried out as described in section 2.2.3.2.

A sensitivity comparison between the casein cross-linking assay and the more conventional [1.4-^{14}C]-labelled putrescine incorporation assay was now sought using guinea pig liver transglutaminase over the range 0-100ng. The results are presented in figure 12.

Figure 12. Correlation between the casein cross-linking assay and the more conventional [1,4- ^{14}C] - labelled putrescine incorporation assay.

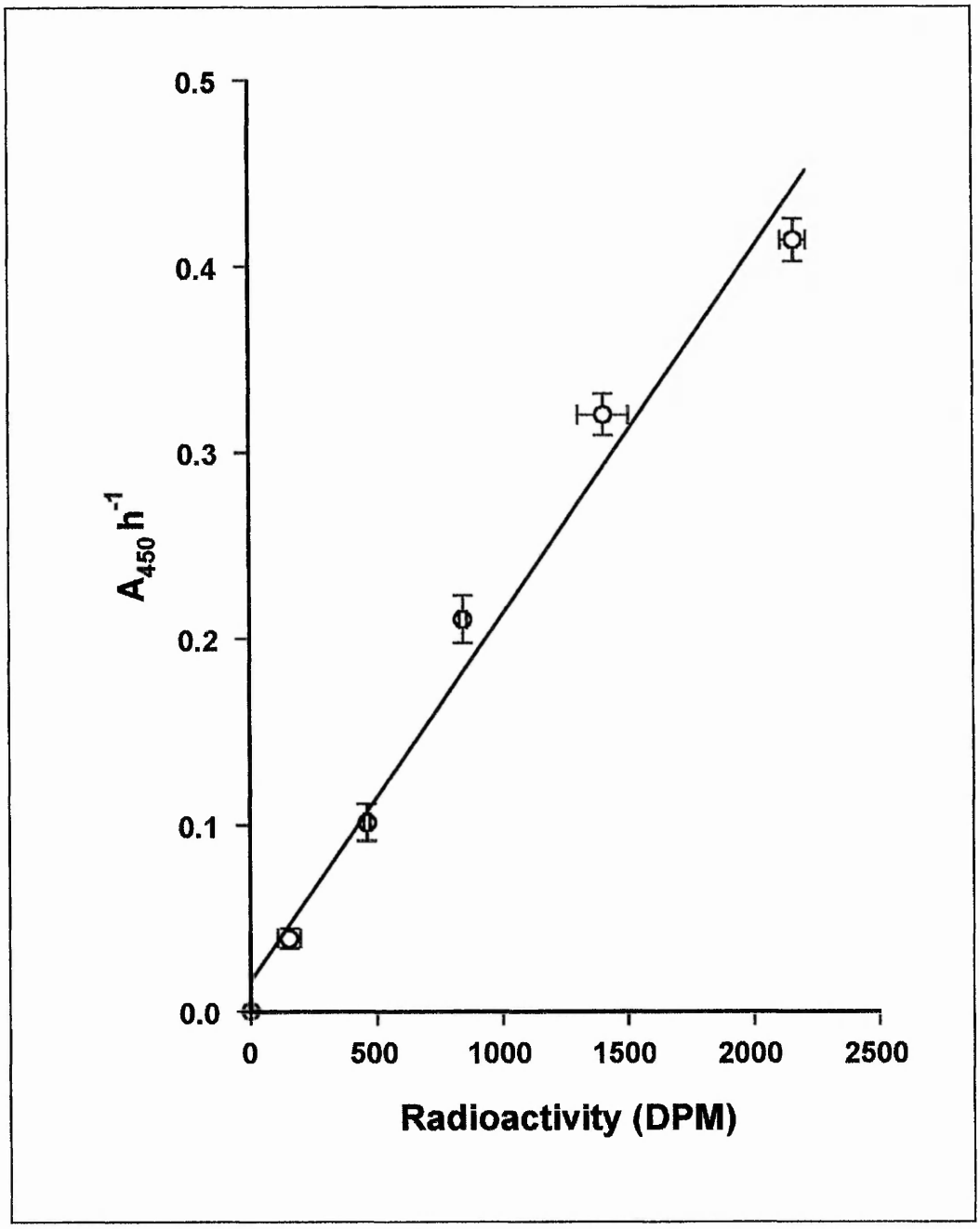


Figure 12 legend.

Guinea pig liver transglutaminase was assayed using the radiolabelled putrescine incorporation assay described in section 2.2.3.4 and the casein cross-linking assay described in section 2.2.3.2. The assays were carried out for 60 minutes at 37°C using a transglutaminase range of 0-100ng. Boiled transglutaminase was used as negative control for both assays giving background values of 0.15 ΔA_{450} h^{-1} and 101cpm for the casein cross-linking assay and the [1,4-^{14}C]-labelled putrescine incorporation assay respectively. Data points represent the mean ± SEM (n=3 for putrescine incorporation and n=8 for casein cross-linking).

Figure 12 shows the correlation between the casein cross-linking assay and the more conventional [^{14}C]-labelled putrescine incorporation assay. A linear relationship exists between absorbance at 450nm and disintegrations per minute for transglutaminase quantities between 0-100ng.

To further demonstrate that the casein cross-linking was transglutaminase-mediated, the ε-(γ-glutamyl) lysine isodipeptide product of the transglutaminase reaction was identified in a proteolytic digestion of biotin labelled and EDC- modified caseins, which had been incubated with guinea pig liver transglutaminase for 60 minutes. The results of this experiment are shown in figures 13a and 13b.

Figure 13a. Control for the ε-(γ-glutamyl) lysine cross-link analysis of EDC-modified and biotin labelled caseins which were incubated for zero time with guinea pig liver transglutaminase.

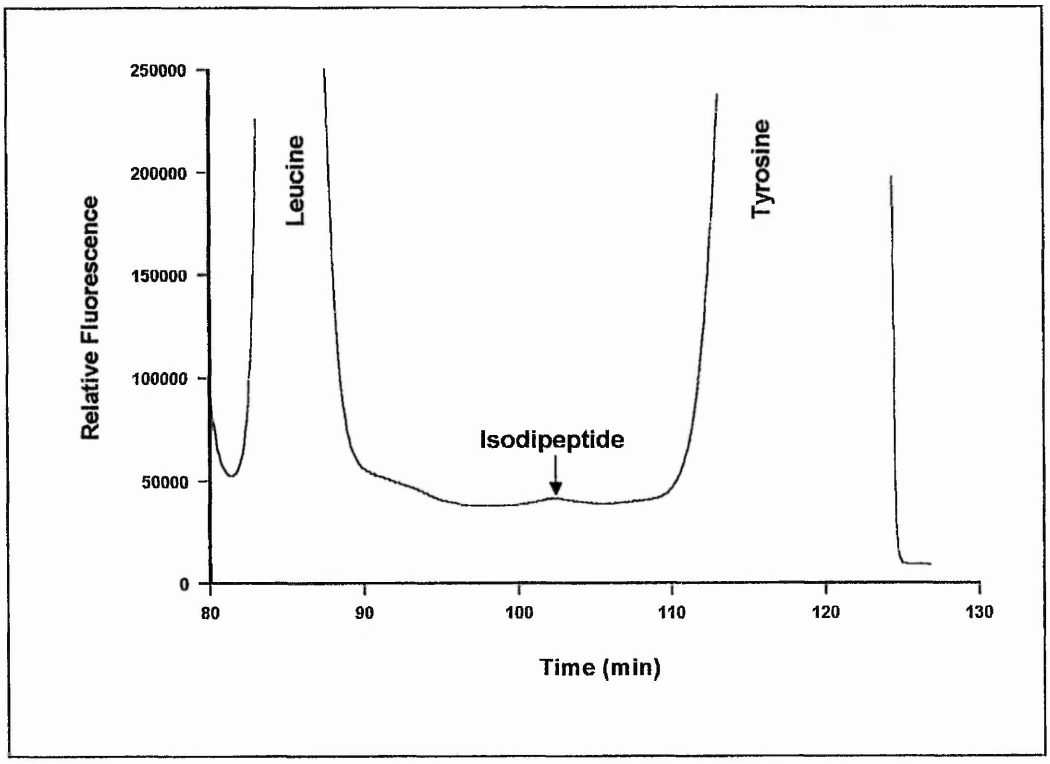


Figure 13a legend.

One hundred and twenty five nanograms of guinea pig liver transglutaminase were incubated with 0.05 mg of biotin labelled casein and 0.05 mg of EDC-modified casein for zero time at 37°C according to section 2.2.4.5. The resultant protein was proteolytically digested and analysed as described in sections 2.2.1.4 and 2.2.4.4. The plot shown is a representative trace of three runs. As a negative control in all isodipeptide analysis experiments, a tube containing proteolytic enzymes alone was carried through the procedure to eliminate any contribution made by endogenous isodipeptide. Isodipeptide levels between 25 and 48 pmol were detected in these controls.

Figure 13a shows that the endogenous level of cross-link present in the casein was 489 ± 54 pmol mg^{-1}.

Figure 13b. The ε-(γ-glutamyl) lysine isodipeptide present following cross-linking of EDC-modified and biotinylated caseins using guinea pig liver transglutaminase.

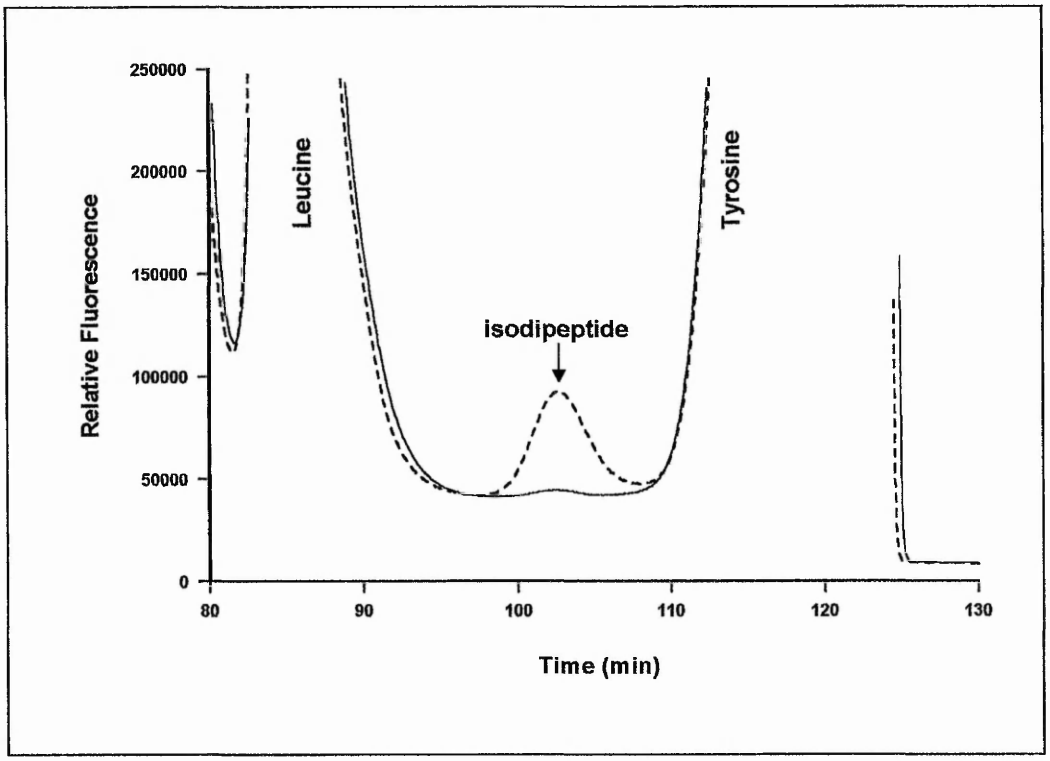


Figure 13b legend.

One hundred and twenty five nanograms of guinea pig liver transglutaminase was incubated with 0.05 mg of EDC-modified casein and 0.05 mg of biotin labelled casein for 60 minutes at 37°C as described in section 2.2.4.5. The resultant protein was proteolytically digested and analysed as described in sections 2.2.1.4 and 2.2.4.4. The dashed line indicates the sample with an added 2 nmol isodipeptide standard spike to confirm the identity of the reaction product and enable accurate quantitation of the isodipeptide formed following the action of transglutaminase on the caseins. The plot shown is a representative trace of three runs.

Figure 13b shows that the level of isodipeptide formed was 158 ± 17 pmol mg^{-1}. A method to measure the ability of transglutaminase enzymes to catalyse the cross-linking of proteins via *N'*,*N'*-bis (γ-glutamyl) polyamine bonds was also sought as described in section 3.1. Table 6 shows the results obtained when using putrescine, cadaverine, spermidine and spermine as bridging polyamines in the transglutaminase reaction.

Table 6. The effect of enzymic modification on *N',N'*-dimethylcasein as a substrate of tissue transglutaminase.

Polyamine used	Background $\Delta A_{450}\ h^{-1} \pm$ SEM	100ng/well Transglutaminase $\Delta A_{450}\ h^{-1} \pm$ SEM	Increase in signal (%) above background
No modification	0.33 ± 0.01	0.35 ± 0.01	6.1
Putrescine	0.34 ± 0.02	0.62 ± 0.01	82.3
Cadaverine	0.21 ± 0.01	0.38 ± 0.01	81.0
Spermine	0.23 ± 0.01	0.44 ± 0.01	91.3
Spermidine	0.25 ± 0.01	0.50 ± 0.01	100.0

One hundred nanograms of guinea pig liver transglutaminase was incubated with enzymically modified *N',N'*-dimethylcasein (see section 2.2.2.3) and biotin labelled casein. The assay was carried out for 60 minutes at 37°C as described in section 2.2.3.3. Boiled transglutaminase was used as negative control and gave background values of 0.21-0.34 $\Delta A_{450}\ h^{-1}$. Controls run without extravidin peroxidase or biotin labelled casein typically resulted in background absorbance values of 0.03-0.05 $\Delta A_{450}\ h^{-1}$. Buffer only controls resulted in plate background absorbance values of 0.19-0.35 $\Delta A_{450}\ h^{-1}$. Data points represent the mean ± SEM of 8 replicates.

Table 6 demonstrates that enzymic modification using spermidine gave a 100% increase in signal above background. The other polyamines when incorporated into *N',N'*-dimethylcasein resulted in increases in signal of 80-90%. Unmodified *N',N'*-dimethylcasein was used as an experimental control and gave a signal above background of only 6.1% when 100ng per well of guinea pig liver transglutaminase was assayed. As the use of spermidine as the modifying polyamine resulted in the highest specific activity this was used for further assay development. Figure 14 demonstrates the guinea pig liver transglutaminase detection limit of the assay when using spermidine.

Figure 14. Absorbance at 450nm as a function of guinea pig liver transglutaminase concentration using the *N'*,*N'*-bis(γ-glutamyl) polyamine cross-linking assay.

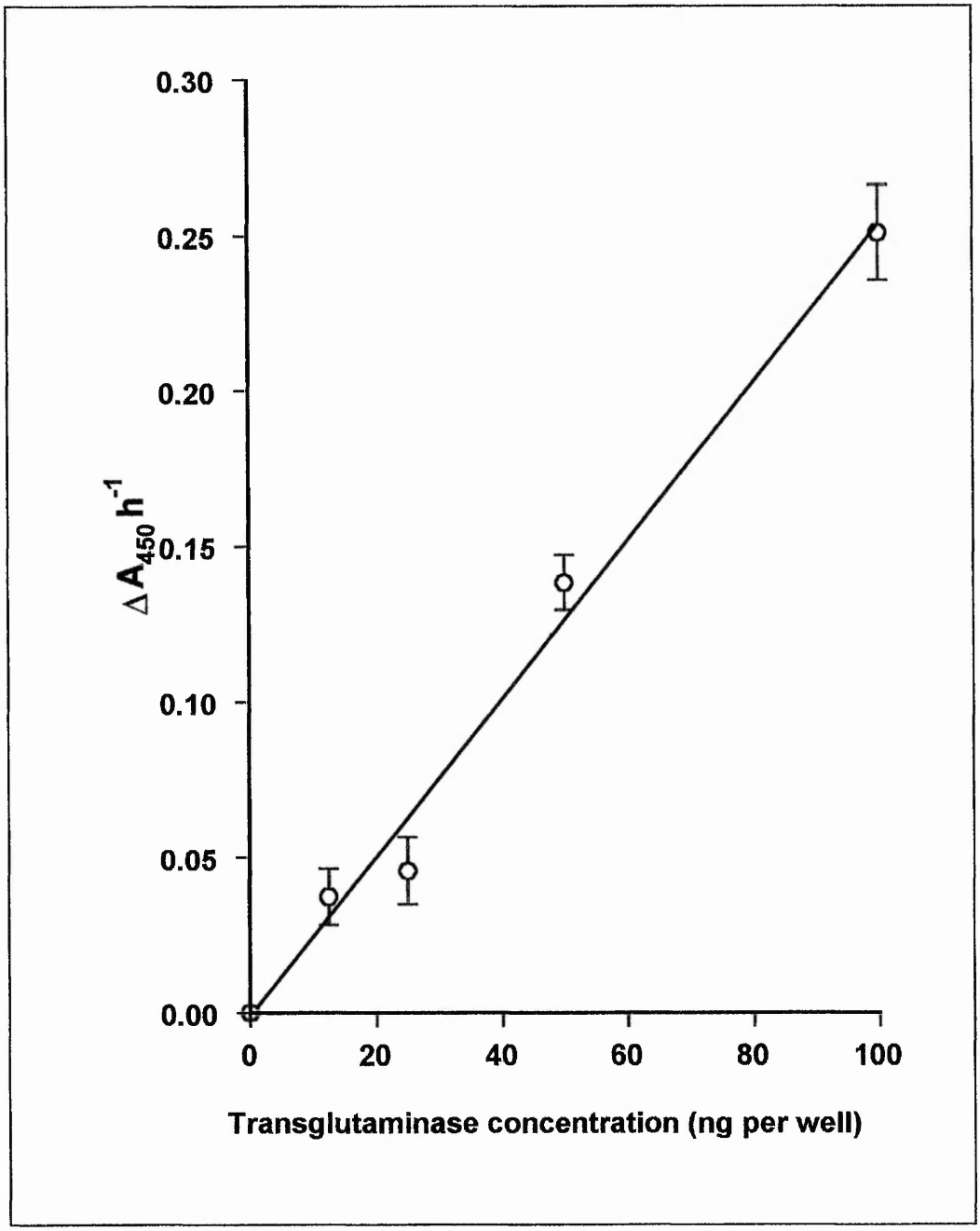


Figure 14 legend.

Guinea pig liver transglutaminase was assayed over the range 0-100ng for 60 minutes at 37°C as described in section 2.2.3.3. Boiled transglutaminase was used as the negative control giving an average background of 0.27 $\Delta A_{450}\ h^{-1}$. The assay signal obtained was time dependent with zero time and 30 minute above background absorbance values of 0.04 $\Delta A_{450}\ h^{-1}$ and 0.11 $\Delta A_{450}\ h^{-1}$ respectively at the 100ng per well level of guinea pig liver transglutaminase. Data points represent the mean ± SEM of 8 replicates.

Figure 14 shows that a linear relationship was observed between transglutaminase concentration and absorbance at 450nm over the range 0-100ng per well. A lower detection limit of 12.5ng per well was demonstrated when using spermidine as the modifying polyamine.

3.3 Discussion.

The main drawback of the casein cross-linking assay described by Seiving *et al.* (1991) was the inherently high background observed due to transglutaminase independent adduct formation between the casein bound to the microtiter plate and the biotin labelled casein in solution possibly mediated by the calcium ions present in the reaction buffer. Table 1 reinforces this observation because use of un-modified bovine casein in the assay results in an assay background of 0.53 ΔA_{450} h^{-1} and an increase in signal above background of only 0.08 ΔA_{450} h^{-1} at the 100ng per well level of guinea pig liver transglutaminase. To eliminate the possibility of phosphate groups on the protein interacting with calcium ions in the reaction buffer the casein bound to the microtiter plate was de-phosphorylated. Table 1 shows that de-phosphorylation resulted in a small reduction in assay background to 0.42 ΔA_{450} h^{-1} and an increase in signal above background of 0.04 ΔA_{450} h^{-1} at the 100ng per well level of guinea pig liver transglutaminase.

The casein bound to the microtiter plate was also de-amidated to convert glutamine residues to glutamate and hence prevent intramolecular protein cross-linking via ε-(γ-glutamyl) lysine isodipeptide bond formation. This could result in a reduction of assay signal by competing with intermolecular ε-(γ-glutamyl) lysine isodipeptide bond formation between the plate bound casein and the biotin labelled casein. Table 1 shows that this approach proved unsuccessful as an assay background of 1.11 ΔA_{450} h^{-1} was observed and no increase in signal above boiled enzyme background could be attained. The higher background observed following de-amidation could be attributed to an

increase in the number of acidic amino acid side chains in the casein bound to the plate since these side chains are thought to be involved in the formation of the transglutaminase independent adduct (Seiving *et al.* 1991).

Table 2 shows that the solution phase chemical modification of casein by the carbodiimide mediated incorporation of small nucleophiles into acidic amino acid side chains resulted in much reduced background rates of 0.16-0.17 ΔA_{450} h^{-1} compared to 0.51 ΔA_{450} h^{-1} for un-modified casein. The best increase in signal above background at the 100ng per well level of guinea pig liver transglutaminase was 250.0% which was achieved using glycine methyl ester as the modifying nucleophile. Smaller increases in signal above background of 35.3 and 50.0% were observed using the methyl esters of arginine and lysine respectively. Arginine and lysine methyl ester were possibly less effective modifiying agents due to steric hindrance caused by their 'R' groups. This results in table 2 also indicate that the calcium mediated adduct formed when using un-modified casein not only serves to increase the assay background but also compromises assay sensitivity by sterically hindering the transglutaminase reaction as an increase in signal of only 15.6% above background was observed when using un-modified casein at the 100ng per well level of guinea pig liver transglutaminase.

Calcium ions are essential for transglutaminase activity but also mediate an intermolecular interaction between aspartate and glutamate residues of biotin labelled casein and casein bound to the microtiter plate. This unwanted adduct formation was prevented by subjecting the casein to the carbodiimide modification to incorporate the nucleophile glycine methyl ester hydrochloride into acidic amino acid side groups of the

casein bound to the microtiter plate. Table 2 also shows an attempt to carbodiimide-modify the casein following binding to the microtiter plate. This approach proved un-successful as an assay background of 1.16 ΔA_{450} h^{-1} was produced and an increase in signal above background of only 0.9% was achieved using this type of modification. The high assay background experienced using this approach was possibly due to removal of the plate bound casein and the BSA blocking protein followed by non-specific binding of extravidin peroxidase due to the action of the 7.5M urea used in the reaction buffer.

Subjecting bovine casein to an 8 hour biotinylation using d-biotin N-hydroxysuccinimide ester did not improve the signal obtained when using 100ng per well of guinea pig liver transglutaminase (table 3). A 4 hour biotinylation time was therefore used for all subsequent preparations. The biotin amidocaproate succinimide ester was also investigated as a possible biotinylation reagent because it has been reported that the incorporated amidocaproate spacer arm results in a reduction of stearic hindrance when binding avidin to some biotin labelled compounds (Costello *et al.* 1979). A lower signal of 0.31 ΔA_{450} h^{-1} was however obtained using this reagent compared to the 0.39 ΔA_{450} h^{-1} observed using the d-biotin N-hydroxysuccinimide ester.

To demonstrate the involvement of the carbodiimide and the glycine methyl ester in the improvement of assay signal, the casein was incubated with 7.5M urea alone. Table 4 shows that this approach has little effect on casein as a substrate of tissue transglutaminase since a signal of only 0.11 ΔA_{450} h^{-1} above background was obtained when assaying 100ng per well of guinea pig liver transglutaminase. Table 4 also shows the effect of stopping the carbodiimide reaction with sodium acetate. This experiment

was carried out as it has been reported that protein amino groups may be spontaneously acetylated using this approach and this may reduce assay signal due to the removal of available lysine residues (Carraway and Koshland 1972). Quenching of the reaction using acetate buffer was however found not to be detrimental to assay signal at the 100ng per well level of guinea pig liver transglutaminase. A signal of 0.40 ΔA_{450} h^{-1} above background was obtained when using acetate buffer compared to 0.39 ΔA_{450} h^{-1} when no acetate buffer was used.

Table 5 demonstrates the effect of various negative controls on the activity of the guinea pig liver transglutaminase used in the assay. Boiling the transglutaminase for 20 minutes resulted in total removal of activity as did replacement of the transglutaminase in the assay with an equal volume of buffer. Mammalian transglutaminase enzymes are inhibited by iodoacetamide because they contain an active site thiol group (Aeschlimann *et al.* 1994). At the 10mM level, this inhibitor was shown to reduce assay signal by 100% when added to the reaction buffer. It should be noted that the transglutaminase was pre-incubated with calcium ions and iodoacetamide for 20 minutes prior to carrying out the assay to allow exposure of the active site and subsequent binding of the iodoacetamide to the enzyme. The calcium ion chelators EDTA and EGTA also fully inhibited 100ng per well of guinea pig liver transglutaminase and resulted in background values of 0.11 ΔA_{450} h^{-1} and 0.12 ΔA_{450} h^{-1} respectively at the 0.25mM level. Table 5 also shows that removal of biotin labelled casein from the reaction buffer resulted in total removal of signal with a background of 0.04 ΔA_{450} h^{-1} observed. This control was carried out in order to eliminate any contribution to the assay signal by endogenous biotin labelled proteins present in the tissue transglutaminase preparation. Also

eliminated was the possibility of a contribution to the assay signal by endogenous peroxidase enzymes present in the tissue transglutaminase preparation by carrying out a zero extravidin peroxidase control which resulted in a background of 0.04 ΔA_{450} h^{-1}.

Figure 10 demonstrates that the detection limit of the assay was 10ng for purified guinea pig liver transglutaminase and that a linear relationship exists between absorbance and tissue transglutaminase concentration over the range 10-100ng per well. To demonstrate the versatility of the assay, ECV 304 human endothelial cell homogenate was chosen as an example of a crude physiological extract from which to detect tissue transglutaminase activity. Figure 11 shows that the tissue transglutaminase activity present in the homogenate derived from as little as 400 human endothelial cells was detected. This implies that the assay may be of use in the purification of human tissue transglutaminase from ECV 304 endothelial cell homogenates. The assay was found to be non-linear when using ECV 304 homogenate, suggesting possible interference due to incorporation of biotin labelled casein into endogenous proteins present in the crude extract. Figure 12 shows that the biotin labelled casein cross-linking assay is as sensitive as the conventional [1,4-^{14}C]-putrescine incorporation assay (Lorand *et al.* 1972) when detecting guinea pig liver transglutaminase as a correlation of r^2=0.977 was observed.

There was a time dependent increase in the presence of ε-(γ-glutamyl) lysine isodipeptide product formed when biotin labelled casein and EDC-modified casein were incubated at 37°C in the presence of guinea pig liver transglutaminase. Figure 13a shows a typical amino acid analysis profile obtained following the digestion of 0.05 mg biotin labelled

casein and 0.05 mg EDC modified casein which had been incubated for zero minutes at 37°C with 125ng of guinea pig liver transglutaminase. The profile demonstrates that there is an endogenous level of 489 pmol mg^{-1} isodipeptide present in the casein. Figure 13b shows that after a 60 minute incubation under assay conditions the level of isodipeptide increased above the endogenous level by 158 pmol mg^{-1} of casein. The quantity of the isodipeptide was determined by addition of a 2 nmol authentic standard isodipeptide preparation which is represented by the dashed line on the chart. Detection of the isodipeptide product of the transglutaminase mediated protein cross-linking reaction is regarded as unequivocal proof of the presence of transglutaminase activity (Folk and Finlayson 1977) and is evidence that the signal obtained in the casein cross-linking assay is due to the cross-linking of EDC modified casein to biotin labelled casein via ε-(γ-glutamyl) lysine isodipeptide bonds.

To date there has been no reported method for the detection of the *in vitro* formation of *N′,N′*-bis (γ-glutamyl) polyamine bridges which may be compounds of significant physiological importance (Folk 1980). Verification of the existence of such species was originally carried out by Folk *et al.* (1980) and involved the exhaustive proteolytic digestion of samples followed by the chromatographic analysis of the resultant digests which is a procedure which may take 5 days or more to complete. In a second assay we measured the ability of tissue transglutaminase to catalyse the conjugation of polyamine modified *N′,N′*-dimethylcasein to biotin labelled casein. *N′,N′*-dimethylcasein bound to the microtiter plate was enzymically modified by the incorporation of polyamines into the free γ-glutamyl residues using commercially available guinea pig liver transglutaminase. Biotin labelled casein was then conjugated to the polyamine modified *N′,N′*-

dimethylcasein using the tissue transglutaminase sample which is to be assayed for activity. This conjugation is due to the formation of N',N'-bis (γ-glutamyl) polyamine bridges. In order to develop this assay, commercially available guinea pig liver transglutaminase was used as the test enzyme. Folk (1980) showed that when the clotting of rat seminal plasma by prostate transglutaminase was carried out in the presence of 0.2mM spermine and spermidine, N',N'-bis (γ-glutamyl) spermine and N',N'-bis (γ-glutamyl) spermidine were formed. The results in table 6 indicate that tissue transglutaminase is able to conjugate spermine and spermidine modified N',N'-dimethylcasein to biotin labelled casein but also demonstrate that putrescine and cadaverine modified N',N'-dimethylcasein are suitable substrates for tissue transglutaminase. Figure 14 shows that biotin labelled casein incorporation into enzymically modified N',N'-dimethylcasein was found to be linear over a range of tissue transglutaminase concentrations between 0 and 100ng and that the detection limit for guinea pig liver transglutaminase was found to be 12.5ng.

Chapter 4- The properties of crude plant transglutaminase.

4.1 Introduction.

As stated in chapter 1, the assays used to investigate the properties of crude plant transglutaminase preparations have involved the incorporation of radiolabelled amine substrates into proteins, either in solution or in solid phase on filter paper discs. This has led to ambiguity regarding the calcium ion requirement of plant transglutaminase due to the presence of contaminating calcium independent diamine oxidases contributing to any observed activity (Icekson and Apelbaum 1987; Serafini-Fracassini *et al.* 1988; Siepaio and Meunier 1995; Chiarello *et al.* 1996a and 1996b). Consequently, it was a principal aim of this part of the practical investigation to screen several plant extracts using a variety of transglutaminase assays to determine the most suitable system(s) for use with crude plant transglutaminase preparations.

The assays selected for the investigation were the two casein cross-linking assays (Lilley *et al.* 1997a) and an assay involving the incorporation of biotin labelled cadaverine into microtiter plate bound *N',N'*-dimethylcasein (Slaughter *et al.* 1992). These assays were chosen to provide an insight into the range of possible plant transglutaminase activities in terms of ε-(γ-glutamyl) lysine, *N',N'*-bis (γ-glutamyl) polyamine and *N'*-(γ-glutamyl) polyamine formation. The assays were also chosen to eliminate the possibility of interference by calcium independent enzyme activities such as diamine oxidases since these enzymes are not known to cross-link proteins or utilise amines with a single primary amine group such as biotin labelled cadaverine.

A further aim was to use the assays to investigate the general biochemical properties of plant transglutaminase such as pH optima and effect of known mammalian transglutaminase inhibitors, which include iodoacetamide, NEM and GTP (Aeschlimann and Paulsson 1994; Takeuchi *et al.* 1992; Bergamini and Signorini 1993). The assays were also used to identify a suitable source of plant transglutaminase from which to begin a purification.

4.2 Results.

In order to compare the conventional [1,4-^{14}C]-labelled putrescine incorporation assay (Lorand *et al.* 1972) with the casein cross-linking assay (Lilley *et al.* 1997a) and the biotin labelled cadaverine incorporation assay (Slaughter *et al.* 1992), eight plant tissues were screened for transglutaminase activity using the three assay systems. The results are presented in table 7.

Table 7. Detection of transglutaminase activity in soluble crude plant extracts using three assay systems.

	Specific activity units mg^{-1} ± SEM		
Tissue type	**Putrescine incorporation**	**Cadaverine incorporation**	**Casein cross-linking**
***Pisum sativum* root**	1.84 ± 0.95	1.21 ± 0.10	1.02 ± 0.08
***Pisum sativum* leaf**	2.57 ± 0.31	0.29 ± 0.02	0.26 ± 0.02
***Vicia faba* root**	N.D	0.20 ± 0.02	0.42 ± 0.01
***Vicia faba* leaf**	0.11 ± 0.01	0.07 ± 0.003	0.16 ± 0.03
***Triticum aestivum* root**	N.D	2.74 ± 0.15	2.18 ± 0.23
***Triticum aestivum* leaf**	N.D	0.18 ± 0.002	0.16 ± 0.01
***Hordeum vulgare* root**	N.D	1.78 ± 0.11	0.71 ± 0.12
***Hordeum vulgare* leaf**	N.D	0.29 ± 0.01	0.32 ± 0.02

One unit of casein cross-linking or cadaverine incorporation activity was defined as a change of absorbance at 450nm of 1.0 per hour. One unit of putrscine incorporation activity was defined as one nanomole of putrescine incorporated into *N',N'*-dimethylcasein per hour.

Table 7 legend.

Crude plant extracts were assayed for 60 minutes at 37°C as described in section 2.2.3. Typical boiled extract background values for casein cross-linking were between 0.05 and 0.15 units. Boiled extract controls for biotin cadaverine incorporation produced background values of 0.05-0.10 units. Removal of extravidin peroxidase or biotin labelled substrate from either plate assay resulted in background values of 0.04-0.05 units. Zero extract controls (extract replaced with buffer) resulted in a casein cross-linking assay background of 0.15 units and a biotin cadaverine incorporation assay background of 0.09 units. Boiled controls or zero extract controls resulted in a putrescine incorporation assay background of between 100 and 200cpm. Data points represent the mean ± SEM of 4 replicates.

Table 7 demonstrates that using both the biotin-cadaverine incorporation assay and the casein cross-linking assay, activity was detected in the soluble extracts of all eight plant tissues screened. Root tissue exhibited a higher specific activity in all species than leaf tissue of the same age and in both assays the greatest specific activity was observed in *Triticum aestivum* root. Use of the [1,4-^{14}C]-labelled putrescine incorporation assay resulted in activity being detected in only three of the eight tissues screened (*Pisum sativum* root and leaf and *Vicia faba* leaf).

The time dependence of each reaction was demonstrated (figures 15 and 16) and this data was used to optimise the incubation time of each assay

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Figure 15. Time dependent casein cross-linking and cadaverine incorporation by soluble *Pisum sativum* root extract.

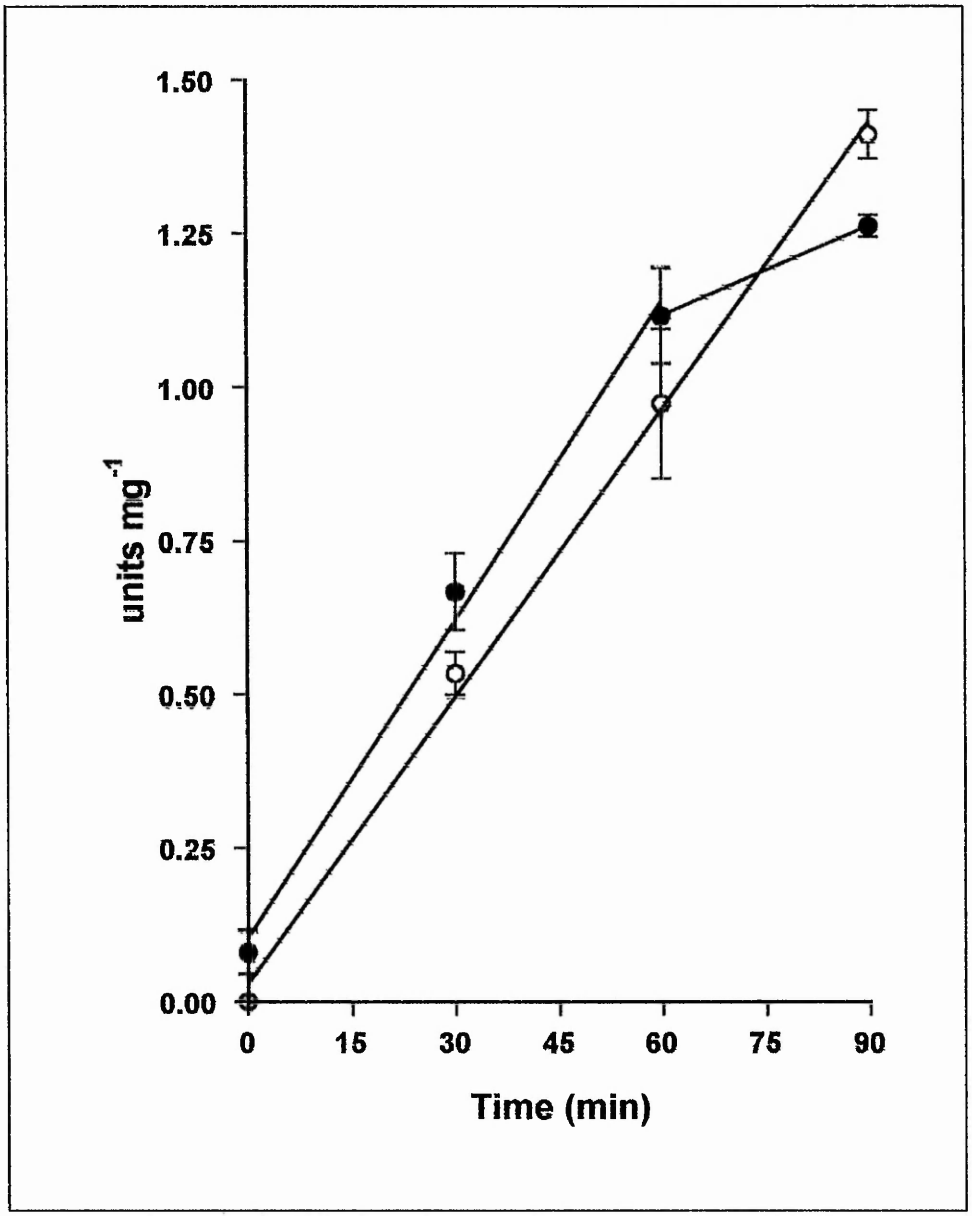

Figure 15 legend.

Pisum sativum root extract was assayed for cadaverine incorporation activity and casein cross-linking activity as described in section 2.2.3. The assays were carried out at 37°C for 0-90 minutes. Each reaction was stopped at the specified time points by removal of the reaction mixture from the wells of the microtiter plate and its replacement with distilled water. Boiled extract was used as a negative control at each time point giving background values of 0.05-0.08 units for biotin cadaverine incorporation and 0.05-0.1 units for casein cross-linking. Data points represent the mean ± SEM of 4 replicates. (○=casein cross-linking, ●=biotin cadaverine incorporation).

Figure 15 shows that the biotin-cadaverine incorporation and casein cross-linking transglutaminase reactions are time dependent and linear up to 60 minutes for soluble *Pisum sativum* root extract. Figure 16 shows the time dependence of the putrescine incorporation reaction.

Figure 16. Time dependent [1,4-^{14}Cl- labelled putrescine incorporation by soluble *Pisum sativum* root extract.

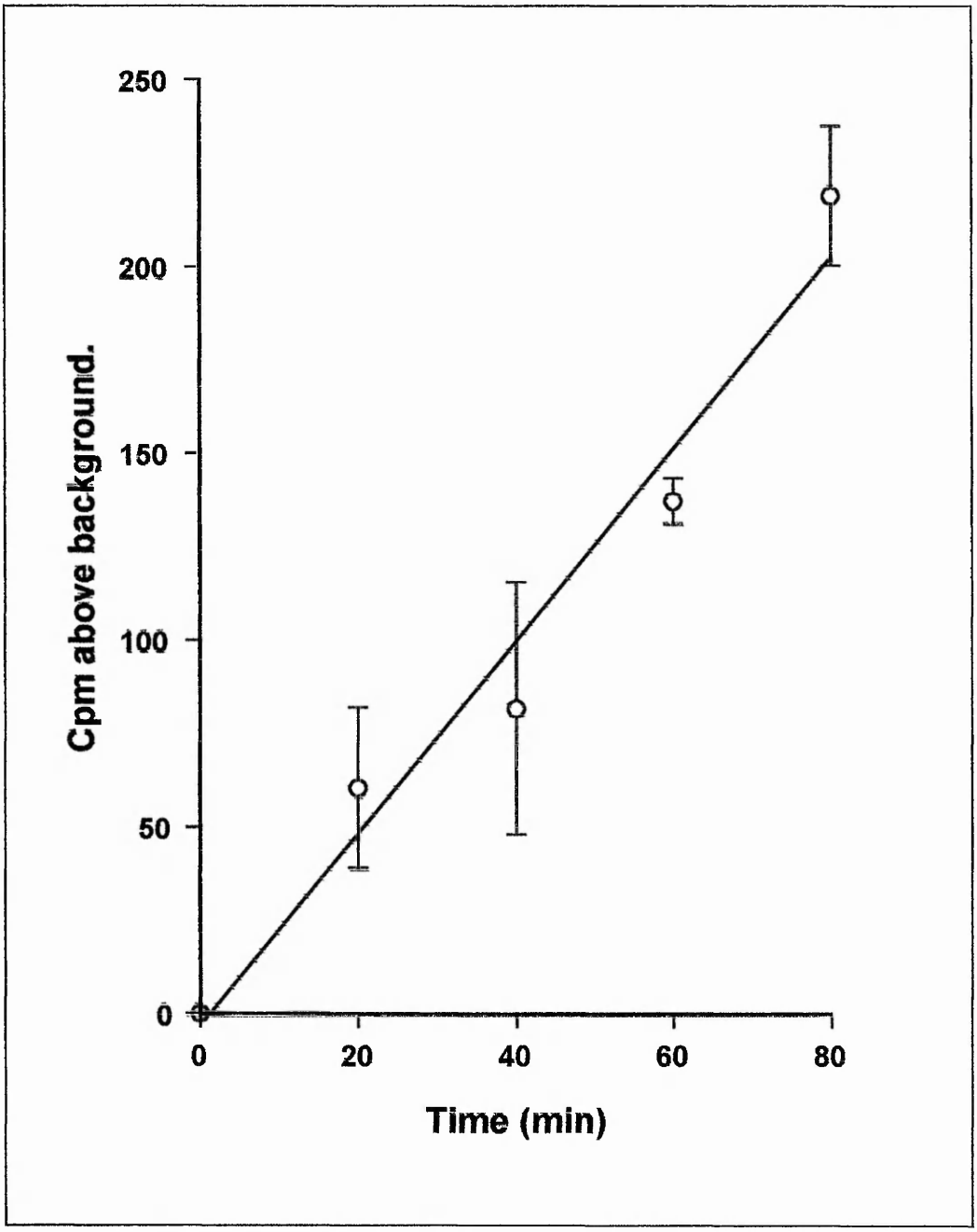

Figure 16 legend.

Pisum sativum root extract was assayed for 0-80 minutes at 37°C as described in section 2.2.3.4. The reaction was stopped at the specified time points by removal of 10µl of the reaction mixture, which was spotted onto $1cm^2$ sections of Whatman N^O1 filter paper (pre-soaked as described in section 2.2.3.4). Boiled root extract was used as a negative control at each time point and gave background values of between 100-150cpm. Data points represent the mean ± SEM of 4 replicates.

Figure 16 shows that [1,4-^{14}C]-labelled putrescine incorporation is time dependent and linear up to 80 minutes for soluble *Pisum sativum* root extract. As all three assays were shown to be linear up to 60 minutes, subsequent experiments were carried out for this time period unless stated.

The calcium dependence of the three *Pisum sativum* mediated reactions was now investigated using EDTA and EGTA to remove calcium from the reaction buffers. These results are presented in tables 8, 9 and 10.

Table 8. Inhibition of the [1,4-^{14}C]-labelled putrescine binding activity of soluble crude plant extracts by calcium ion chelation.

As [1,4-^{14}C]-labelled putrescine binding activity was observed only for *Vicia faba* leaf and *Pisum sativum* root and leaf and extracts (table 7), the inhibition of this reaction by calcium chelation was investigated using these extracts only.

	Specific activity units mg^{-1} ± SEM		
Tissue type	**+5mM $CaCl_2$**	**+5mM EDTA**	**+5mM EGTA**
***Pisum sativum* root**	0.82 ± 0.18	0.70 ± 0.04 (14.6)	0.82 ± 0.15 (0)
***Pisum sativum* leaf**	2.51 ± 0.12	2.02 ± 0.11 (19.5)	1.63 ± 0.10 (35.1)
***Vicia faba* leaf**	0.17 ± 0.03	0.19 ± 0.05 (0)	0.15 ± 0.03 (11.8)

Plant protein extracts were assayed for 60 minutes at 37°C in the presence of either 5mM $CaCl_2$, 5mM EDTA or 5mM EGTA as described in section 2.2.3.4. Data points represent the mean ± SEM of 4 replicates.

Table 8 shows that small reductions in the putrescine binding activity of two of the extracts of between 15-20% were observed due to the addition of 5mM EDTA (presented in brackets immediately after the specific activity). 5mM EGTA inhibited activity of extracts by up to 35%. Note that all inhibition experiments, percentages are calculated by assuming boiled extract absorbance to be zero activity.

Table 9. Inhibition of the biotin cadaverine incorporation activity of soluble crude plant extracts by calcium ion chelation.

	Specific activity units mg^{-1} ± SEM		
Tissue type	**+5mM $CaCl_2$**	**+1mM EDTA**	**+1mM EGTA**
***Pisum sativum* root**	1.19 ± 0.03	0.08 ± 0.02 (93.3)	0.04 ± 0.03 (96.6)
***Pisum sativum* leaf**	0.65 ± 0.03	0.06 ± 0.01 (90.8)	0.08 ± 0.01 (87.7)
***Vicia faba* root**	0.25 ± 0.01	0 (100)	0 (100)
***Vicia faba* leaf**	0.06 ± 0.01	0 (100)	0 (100)
***Triticum aestivum* root**	2.07 ± 0.07	0 (100)	0 (100)
***Triticum aestivum* leaf**	0.27 ± 0.01	0 (100)	0 (100)
***Hordeum vulgare* root**	2.28 ± 0.08	0 (100)	0 (100)
***Hordeum vulgare* leaf**	0.43 ± 0.01	0 (100)	0 (100)

Plant protein extracts were assayed for transglutaminase activity in the presence of 5mM $CaCl_2$, 1mM EDTA or 1mM EGTA as described in section 2.2.3.1. Data points represent the mean ± SEM of 4 replicates.

Table 9 shows that large reductions in the biotin cadaverine incorporation activity of the extracts of between 91-100% were observed due to the addition of 1mM EDTA (presented in brackets immediately after the specific activity). 1mM EGTA inhibited activity of extracts by 88-100%.

Table 10. Inhibition of the casein cross-linking activity of soluble crude plant extracts by calcium ion chelation.

	Specific activity units mg^{-1} ± SEM		
Tissue type	**+5mM $CaCl_2$**	**+1mM EDTA**	**+1mM EGTA**
***Pisum sativum* root**	1.15 ± 0.06	0 (100)	0 (100)
***Pisum sativum* leaf**	0.68 ± 0.02	0.09 ± 0.01 (87.5)	0.12 ± 0.01 (82.8)
***Vicia faba* root**	0.64 ± 0.04	0 (100)	0 (100)
***Vicia faba* leaf**	0.12 ± 0.02	0 (100)	0.02 ± 0.01 (81.0)
***Triticum aestivum* root**	3.41 ± 0.15	0 (100)	0 (100)
***Triticum aestivum* leaf**	0.51 ± 0.03	0 (100)	0 (100)
***Hordeum vulgare* root**	0.92 ± 0.06	0 (100)	0 (100)
***Hordeum vulgare* leaf**	0.48 ± 0.07	0 (100)	0 (100)

Plant protein extracts were assayed for transglutaminase activity in the presence of 5mM $CaCl_2$, 1mM EDTA or 1mM EGTA as described in section 2.2.3.2. Data points represent the mean ± SEM of 4 replicates.

Table 10 demonstrates that large reductions in the biotin casein cross-linking activity of the extracts of between 88-100% were observed due to the addition of 1mM EDTA (presented in brackets immediately after the specific activity). 1mM EGTA inhibited activity of extracts by 81-100%.

Since the [1,4-^{14}C]-putrescine incorporation reaction was not fully inhibited by calcium ion chelation, the effect of 5mM DIECA and o-phenanthrolinehenanthroline was investigated to determine the extent of any contribution to the assay signal made by copper dependent enzymes such as diamine oxidase. This data is shown in tables 11 and 12.

Table 11. The effect of the diamine oxidase inhibitors, DIECA and o-phenanthroline on the transglutaminase activity of soluble *Pisum sativum* root extract.

	Specific activity units mg^{-1} ± SEM		
Assay	**Uninhibited**	**+ 5mM DIECA**	**+ 5mM o-phenanthroline**
[1,4-^{14}C]-putrescine incorporation	0.96 ± 0.21	0.79 ± 0.15 (17.7)	0.68 ± 0.17 (29.2)
Casein cross-linking	1.31 ± 0.08	1.38 ± 0.12 (0)	1.02 ± 0.07 (22.1)
Cadaverine incorporation	1.08 ± 0.07	1.09 ± 0.11 (0)	0.94 ± 0.06 (13.0)

Pisum sativum root extract was assayed for transglutaminase activity in the presence of 5mM DIECA or 5mM o-phenanthroline for 60 minutes at 37°C as described in section 2.2.3. Data points represent the mean ± SEM of 4 replicates.

Table 11 demonstrates that the addition of 5mM DIECA to the assay reaction buffers resulted in inhibition of 0-18% (presented in brackets after the specific activity). o-phenanthroline at the same concentration resulted in inhibition of activity between 13% and 29%.

The data shows that in addition to inhibition of the [1,4-^{14}C]-putrescine binding reaction, the casein cross-linking and cadaverine incorporation activities of *Pisum sativum* root extract were inhibited by o-phenanthroline. As a result, a control was carried out to investigate the effect of o-phenanthroline on the activity of guinea pig liver transglutaminase. The data relating to this control is shown in table 12.

Table 12. The effect of the diamine oxidase inhibitors, DIECA and o-phenanthroline on the activity of guinea pig liver transglutaminase.

	Specific activity units mg^{-1} ± SEM		
Assay	**Uninhibited**	**+ 5mM DIECA**	**+ 5mM o-phenanthroline**
[1,4-^{14}C]-putrescine incorporation	2510 ± 333	2620 ± 281 (0)	2490 ± 305 (0.8)
Casein cross-linking	4320 ± 399	3140 ± 332 (27.3)	3510 ± 305 (18.7)
Cadaverine incorporation	4010 ± 338	3500 ± 354 (12.7)	3240 ± 289 (19.1)

One hundred nanograms of guinea pig liver transglutaminase was assayed for 60 minutes at 37°C in the presence of 5mM DIECA or 5mM o-phenanthroline as described in section 2.2.3. Data points represent the mean ± SEM of 4 replicates.

Table 12 demonstrates that the addition of 5mM DIECA results in inhibition of 0-27% (presented in brackets after the specific activity) when assaying guinea pig liver transglutaminase. o-phenanthroline at the same concentration resulted in inhibition of activity between 1 and 19%.

Information concerning the concentration of calcium required to activate the casein cross-linking and cadaverine incorporation reactions of *Pisum sativum* root transglutaminase was now sought. This is presented in figures 17 and 18.

Figure 17. The biotin cadaverine incorporation activity of soluble *Pisum sativum* root extract as a function of the free calcium ion concentration of the reaction buffer.

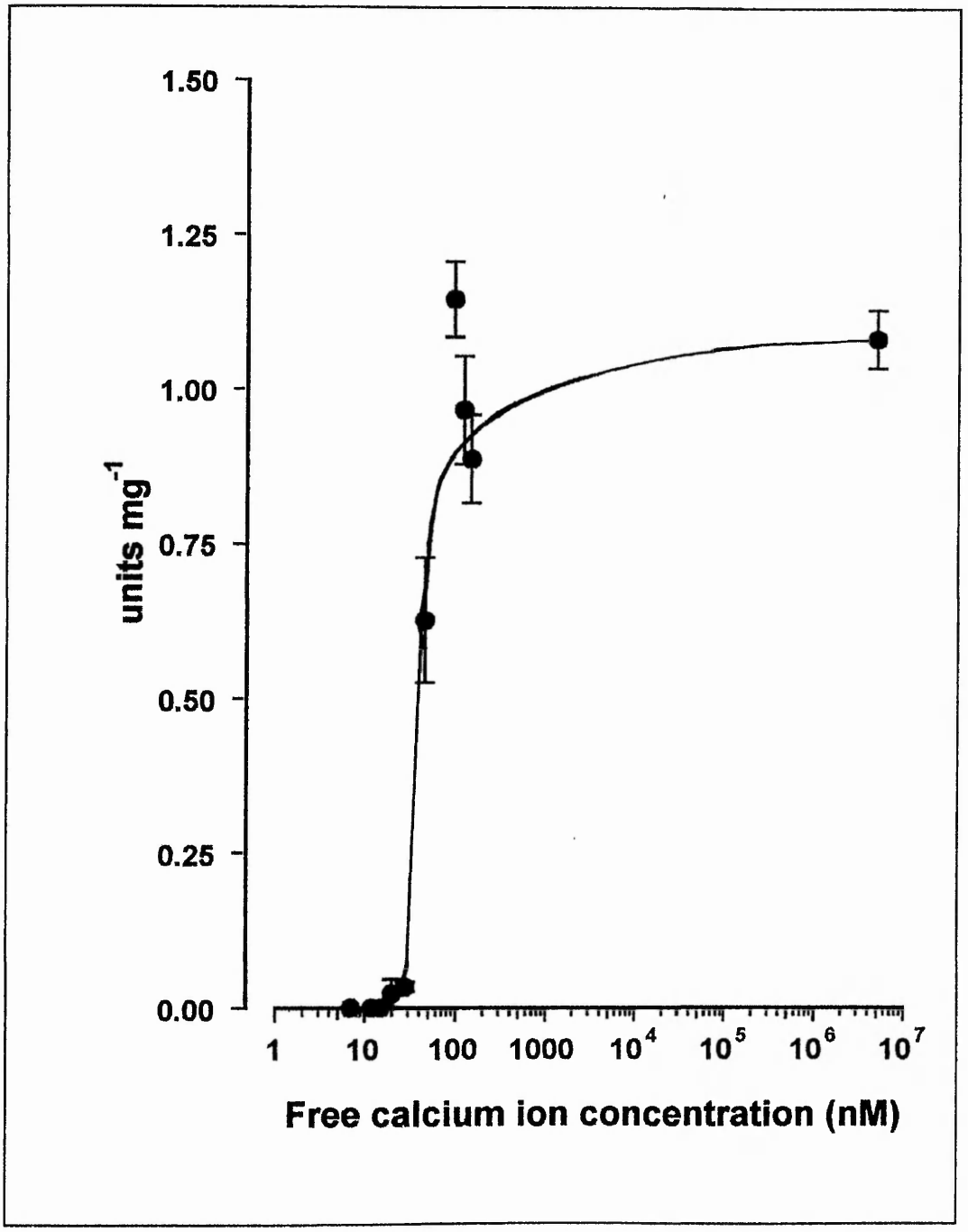

Figure 17 legend.

Pisum sativum root extract was assayed for 60 minutes at 37°C in the presence of varying free calcium ion concentrations as described in section 2.2.3.1. Zero free calcium was achieved by the addition of 1mM EGTA to the reaction buffer. Calcium chloride was then added in increasing levels using a computer program to calculate the free calcium ion concentration increase due to each addition (Fuhr *et al.* 1993). Data points represent the mean ± SEM of 4 replicates.

Figure 17 shows that no biotin cadaverine incorporation occurs between 1 and 19nM free calcium. Activation of biotin cadaverine incorporation activity of soluble *Pisum sativum* transglutaminase occurs after 20nM free calcium with maximum activity observed at 94nM.

Figure 18. The casein cross-linking activity of soluble *Pisum sativum* root extract as a function of the free calcium ion concentration of the reaction buffer.

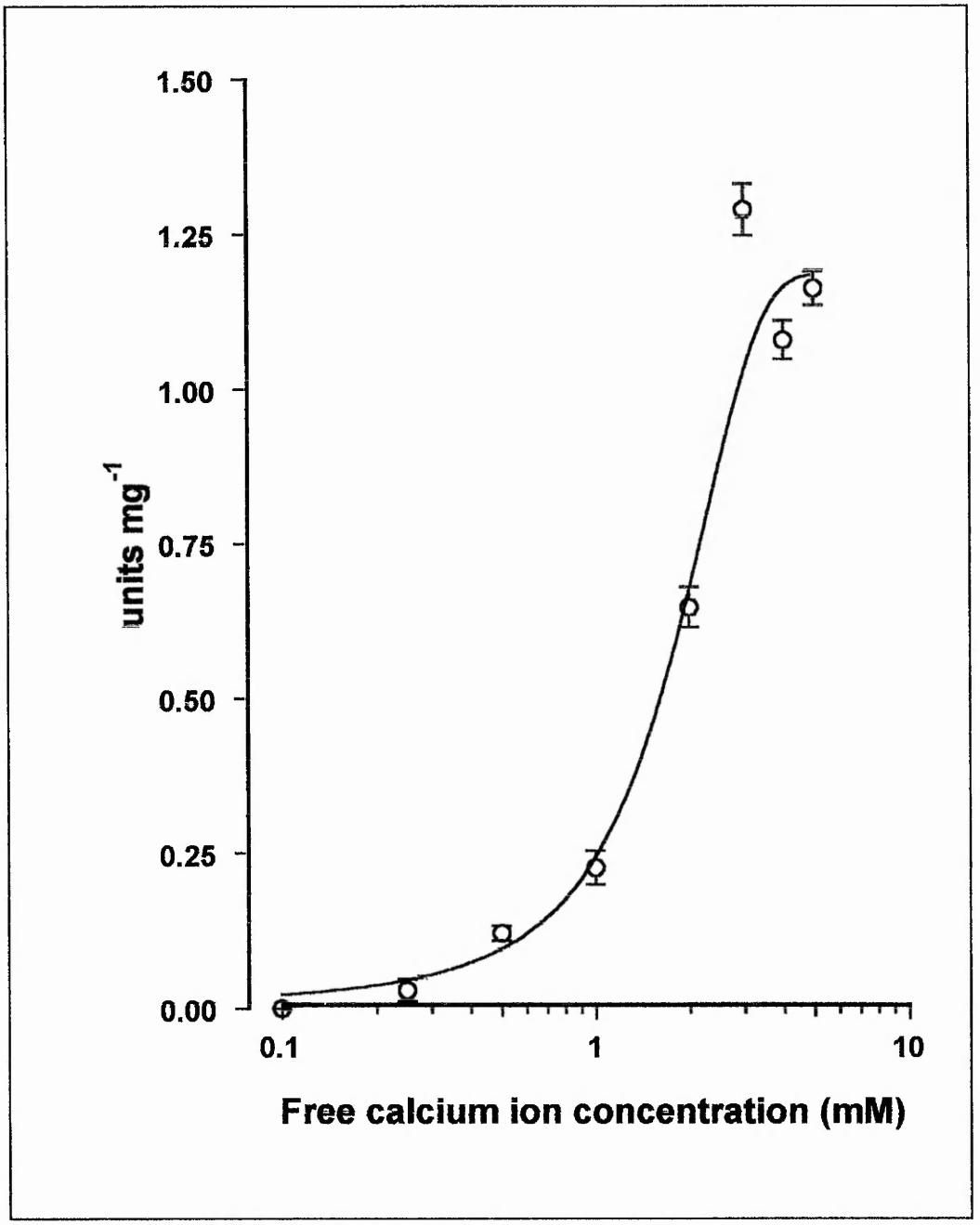

Figure 18 legend.

Pisum sativum root extract was assayed for 60 minutes at 37°C in the presence of free calcium concentrations between 0 and 5mM as described in section 2.2.3.2. The concentration of free calcium ions in the buffer was calculated as described for figure 17. Data points represent the mean ± SEM of 4 replicates.

Figure 18 shows that approximately 20% of the activity is observed between 250μM and 1mM free calcium. The remaining 80% occurs after 1mM with a peak observed at 3mM free calcium.

It is known that mammalian tissue transglutaminase is inhibited by GTP in the presence of sub-optimal calcium (Takeuchi *et al.* 1992; Bergamini and Signorini 1993; Mian *et al.* 1995; Smethurst and Griffin 1996). It was therefore decided to investigate the effect of this agent on the activity of *Pisum sativum* root transglutaminase. This is demonstrated by the results in table 13.

Table 13. The effect of 1mM GTP on the biotin labelled cadaverine incorporation and casein cross-linking activities of soluble *Pisum sativum* root transglutaminase.

	Specific activity units mg^{-1} ± SEM			
Source of TGase	**Cadaverine incorporation**		**Casein cross-linking**	
	Uninhibited	**+ 1mM GTP**	**Uninhibited**	**+ 1mM GTP**
***Pisum sativum* root**	1.24 ± 0.11	1.21 ± 0.08 (2.4)	0.88 ± 0.08	0.91 ± 0.05 (0)
Guinea pig liver	3550 ± 443	1700 ± 144 (52.1)	3030 ± 398	1960 ± 281 (35.3)

Pisum sativum root extract was assayed for 60 minutes at 37°C in the presence of 1mM GTP and sub-optimal calcium concentration (80nm for cadaverine incorporation and 2mM for casein cross-linking) as described in sections 2.2.3.1 and 2.2.3.2. As a positive control, 100ng per well of guinea pig liver transglutaminase was incubated at a calcium concentration of 2mM in the presence of 1mM GTP. Data points represent the mean ± SEM of 4 replicates.

Table 13 shows that a small cadaverine incorporation inhibition of 2.4% was observed in the presence of 1mM GTP (presented in brackets after the specific activity). Zero casein cross-linking inhibition was observed in the presence of 1mM GTP. Inhibitions of 52.1% and 35.3% were observed for cadaverine incorporation and casein cross-linking respectively.

Mammalian transglutaminases are also inhibited by thiol group reagents such as iodoacetamide and NEM due to the presence of an active site cysteine residue (Smethurst and Griffin 1996). Tables 14, 15 and 16 show the results of adding a 10mM concentration of iodoacetamide or NEM to the reaction buffers of the three assay systems.

The three tissues, which gave detectable [1,4-^{14}C]-labelled putrescine binding activity in the initial screening experiment (table 7) were now tested to investigate the effect of thiol blocking reagents NEM and iodoacetamide on transglutaminase activity. The results are presented in table 14.

Table 14. The effect of the addition of 10mM iodoacetamide or 10mM NEM to the [1,4-^{14}C]-labelled putrescine incorporation assay reaction buffer on the transglutaminase activity of soluble crude plant extracts.

	Specific activity units mg^{-1} ± SEM		
Tissue	**Uninhibited**	**+ 10mM iodoacetamide**	**+ 10mM NEM**
***Pisum sativum* root**	0.64 ± 0.19	0.48 ± 0.24 (25.0)	0.33 ± 0.02 (48.4)
***Pisum sativum* leaf**	1.37 ± 0.07	1.20 ± 0.10 (12.4)	1.54 ± 0.04 (0)
***Vicia faba* leaf**	0.39 ± 0.02	0.31 ± 0.02 (20.5)	0.42 ± 0.03 (0)

Plant protein extracts were incubated at 37°C for 30 minutes in the presence of 5mM $CaCl_2$ and 10mM iodoacetamide or NEM. The pre-treated extracts were then assayed for 60 minutes at 37°C as detailed in section 2.2.3.4. As a positive control, 100ng of guinea pig liver transglutaminase was also incubated in the presence of 10mM concentrations of the thiol group inhibitors and no activity was observed in these controls. Data points represent the mean ± SEM of 4 replicates.

Table 14 shows that reductions in activity of the extracts of between 12-25% were observed due to the addition of 10mM iodoacetamide (presented in brackets immediately after the specific activity). 10mM NEM inhibited activity of extracts by 0-48%.

Table 15. The effect of the addition of 10mM iodoacetamide or 10mM NEM to the biotin cadaverine incorporation assay reaction buffer on the transglutaminase activity of soluble crude plant extracts.

	Specific activity units $mg^{-1} \pm SEM$		
Tissue	**Uninhibited**	**+ 10mM iodoacetamide**	**+ 10mM NEM**
***Pisum sativum* root**	1.12 ± 0.07	0.82 ± 0.02 (26.8)	1.11 ± 0.09 (0.9)
***Pisum sativum* leaf**	0.34 ± 0.02	0.26 ± 0.01 (23.5)	0.34 ± 0.04 (0)
***Vicia faba* root**	0.18 ± 0.01	0.15 ± 0.01 (16.7)	0.16 ± 0.02 (11.2)
***Vicia faba* leaf**	0.05 ± 0.01	0.02 ± 0.00 (60.0)	0 (100)
***Triticum aestivum* root**	2.53 ± 0.09	1.29 ± 0.08 (49.0)	2.59 ± 0.11 (0)
***Triticum aestivum* leaf**	0.25 ± 0.01	0.14 ± 0.01 (44.0)	0.17 ± 0.02 (32.0)
***Hordeum vulgare* root**	1.58 ± 0.07	1.45 ± 0.03 (8.2)	1.47 ± 0.08 (7.0)
***Hordeum vulgare* leaf**	0.30 ± 0.02	0.30 ± 0.03 (0)	0.26 ± 0.01 (13.3)

Plant protein extracts were incubated at 37°C for 30 minutes in the presence of 5mM $CaCl_2$ and 10mM iodoacetamide or NEM. The pre-treated extracts were then assayed for 60 minutes at 37°C as detailed in section 2.2.3.1. As a positive control, 100ng of guinea pig liver transglutaminase was also incubated in the presence of 10mM concentrations of the thiol group inhibitors and no activity was observed in these controls. Data points represent the mean ± SEM of 4 replicates.

Table 15 shows that reductions in activity of the extracts of between 0-60% were observed due to the addition of 10mM iodoacetamide (presented in brackets immediately after the specific activity). 10mM NEM inhibited activity of extracts by 0-100%.

Table 16. The effect of the addition of 10mM iodoacetamide or 10mM NEM to the casein cross-linking assay reaction buffer on the transglutaminase activity of soluble crude plant extracts.

	Specific activity units mg^{-1} ± SEM		
Tissue	**Uninhibited**	**+ 10mM iodoacetamide**	**+ 10mM NEM**
***Pisum sativum* root**	1.24 ± 0.10	1.34 ± 0.12 (0)	1.42 ± 0.12 (0)
***Pisum sativum* leaf**	0.32 ± 0.04	0.41 ± 0.04 (0)	0.39 ± 0.04 (0)
***Vicia faba* root**	0.40 ± 0.02	0.41 ± 0.01 (0)	0.49 ± 0.03 (0)
***Vicia faba* leaf**	0.22 ± 0.04	0.23 ± 0.01 (0)	0.21 ± 0.01 (0.5)
***Triticum aestivum* root**	2.23 ± 0.18	2.44 ± 0.19 (0)	2.59 ± 0.11 (0)
***Triticum aestivum* leaf**	0.19 ± 0.02	0.18 ± 0.01 (0.5)	0.20 ± 0.03 (0)
***Hordeum vulgare* root**	0.88 ± 0.08	0.96 ± 0.08 (0)	0.89 ± 0.09 (0)
***Hordeum vulgare* leaf**	0.41 ± 0.02	0.46 ± 0.02 (0)	0.43 ± 0.02 (0)

Plant protein extracts were incubated at 37°C for 30 minutes in the presence of 5mM $CaCl_2$ and 10mM iodoacetamide or NEM. The pre-treated extracts were then assayed for 60 minutes at 37°C as detailed in section 2.2.3.2. As a positive control, 100ng of guinea pig liver transglutaminase was also incubated in the presence of 10mM concentrations of the thiol group inhibitors and no activity was observed in these controls. Data points represent the mean ± SEM of 4 replicates.

Table 16 demonstrates that with the exception of the 0.5% reduction of activity due to the effect of 10mM NEM on *Vicia faba* leaf, no inhibition was observed due to the addition of thiol group inhibitors.

The effect of various concentrations of the thiol group activator, DTT was also investigated (table 17).

Table 17. The effect of various concentrations of DTT on the transglutaminase activity of soluble *Pisum sativum* root extract in three assay systems.

	Specific activity units mg^{-1} ± SEM		
Assay type	**+ 10mM DTT**	**+ 1mM DTT**	**+ 0mM DTT**
Casein cross-linking	0.75 ± 0.04	0.70 ± 0.06	0.76 ± 0.03
Cadaverine incorporation	0.84 ± 0.09	0.83 ± 0.05	0.81 ± 0.05
Putrescine incorporation	1.52 ± 0.28	1.54 ± 0.31	1.46 ± 0.21

Pisum sativum root extract was incubated for 60 minutes at 37°C in the presence of DTT concentrations between 0-10mM as detailed in section 2.2.3. The data points represent the mean ± SEM of 4 replicates.

Table 17 shows that little difference was observed in specific activity values over the range of DTT concentrations tested. Further aspects of the biochemistry of *Pisum sativum* root transglutaminase activity including substrate specificity and pH optima were also investigated and the results are presented in tables 18 and 19 and figure 19.

Table 18. The effect of adding 10mM spermine to the biotin cadaverine reaction buffer on the transglutaminase activity of soluble *Pisum sativum* root extract.

	Specific activity units mg^{-1} ± SEM	
Source of transglutaminase	**Uninhibited**	**+ 10mM spermine**
***Pisum Sativum* root**	0.94 ± 0.04	0.52 ± 0.03 (44.7)
Guinea pig liver	3850 ± 402	1560 ± 215 (59.5)

Pisum sativum root extract was incubated for 60 minutes at 37°C in the presence of 10mM spermine as described in section 2.2.3.1. As a positive control, 100ng per well of guinea pig liver transglutaminase was also incubated in the presence of 10mM spermine. Data points represent the mean ± SEM of 4 replicates.

Table 18 demonstrates that 10mM spermine reduces the biotin cadaverine incorporation activity of *Pisum sativum* root extract by 44.7% (presented in brackets after the specific activity). A similar inhibition of 59.5% is observed when 10mM spermine is added to the reaction buffer containing 100ng per well of guinea pig liver transglutaminase.

Table 19. The effect of substituting *N',N'*-dimethylcasein with *N',N'*-dimethylated *Vicia faba* storage proteins on the microtiter plate in the biotin cadaverine incorporation assay.

	Specific activity units mg^{-1} ± SEM	
Source of transglutaminase	**+ *N'N'*-dimethylcasein**	**+ *N',N'*-dimethylated *Vicia faba* storage proteins**
***Pisum sativum* root**	0.86 ± 0.05	0.93 ± 0.10
Guinea pig liver	4010 ± 303	5130 ± 411

Pisum sativum root extract and 100 ng per well of guinea pig liver transglutaminase were incubated for 60 minutes at 37°C in the presence of plate bound *N',N'*-dimethylated *Vicia faba* storage proteins or *N'N'*-dimethylcasein as described in section 2.2.3.1. The storage proteins were extracted and methylated as described in sections 2.2.1.3 and 2.2.2.5 respectively. Data points represent the mean ± SEM of 4 replicates.

Table 19 shows an 8% increase in the specific activity of *Pisum sativum* root extract when *N',N'*-dimethylcasein is replaced on the microtiter plate with *N',N'*-dimethylated *Vicia faba* storage proteins. An increase in specific activity of 28% was observed when 100ng per well of guinea pig liver transglutaminase was used.

Figure 19. A graph showing the variation in casein cross-linking and cadaverine incorporation activities of soluble *Pisum sativum* root extract as a function of the pH of the reaction buffer.

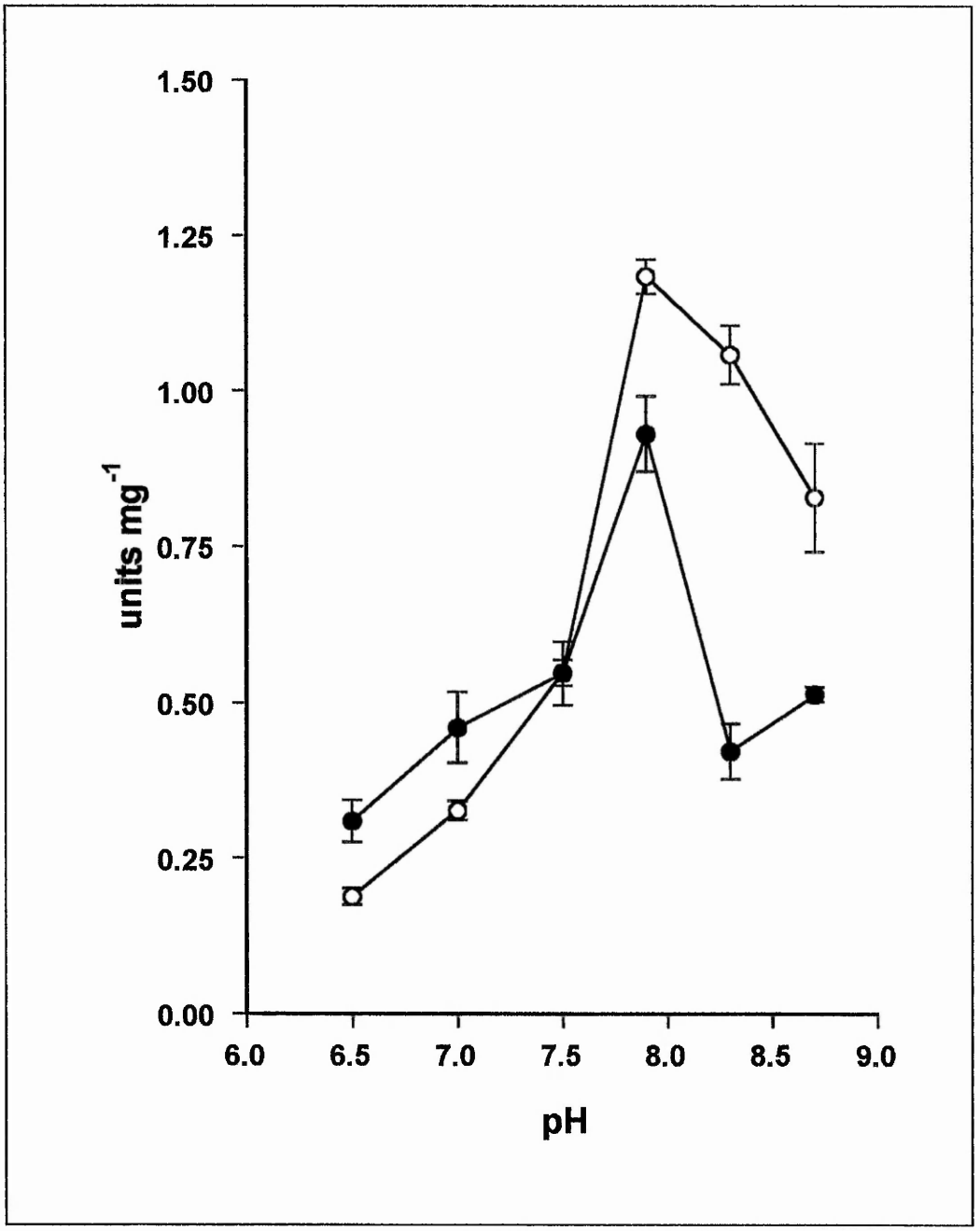

Figure 19 legend.

Pisum sativum root extract was assayed for casein cross-linking and cadaverine incorporation activities for 60 minutes at 37°C over a 6.5-9.0 pH range as described in section 2.2.3. 5mM calcium chloride was added to the casein cross-linking reaction buffer and 100μM calcium chloride was used in the biotin cadaverine incorporation reaction buffer. 250μM EDTA was used as negative control at each pH point in both assays giving an average background of 0.07 units for casein cross-linking and 0.09 units for biotin cadaverine incorporation. Data points represent the mean ± SEM of 4 replicates. (○=casein cross-linking, ●=biotin cadaverine incorporation).

Figure 19 demonstrates that both the casein cross-linking and biotin cadaverine incorporation activities of soluble *Pisum sativum* transglutaminase are influenced by the pH of the reaction buffer. Both activities increased with increasing pH until an optimum was reached at pH 7.9.

The levels of *Pisum sativum* transglutaminase activity at different stages of root and leaf development were determined in order to provide an insight into the possible involvement of the enzyme in developmental processes. The data from these experiments is shown in figures 20 and 21. Photographs illustrating the data are also presented (plates 1-4).

Figure 20. The effect of plant age on the casein cross-linking and cadaverine incorporation activities of soluble *Pisum sativum* root extract.

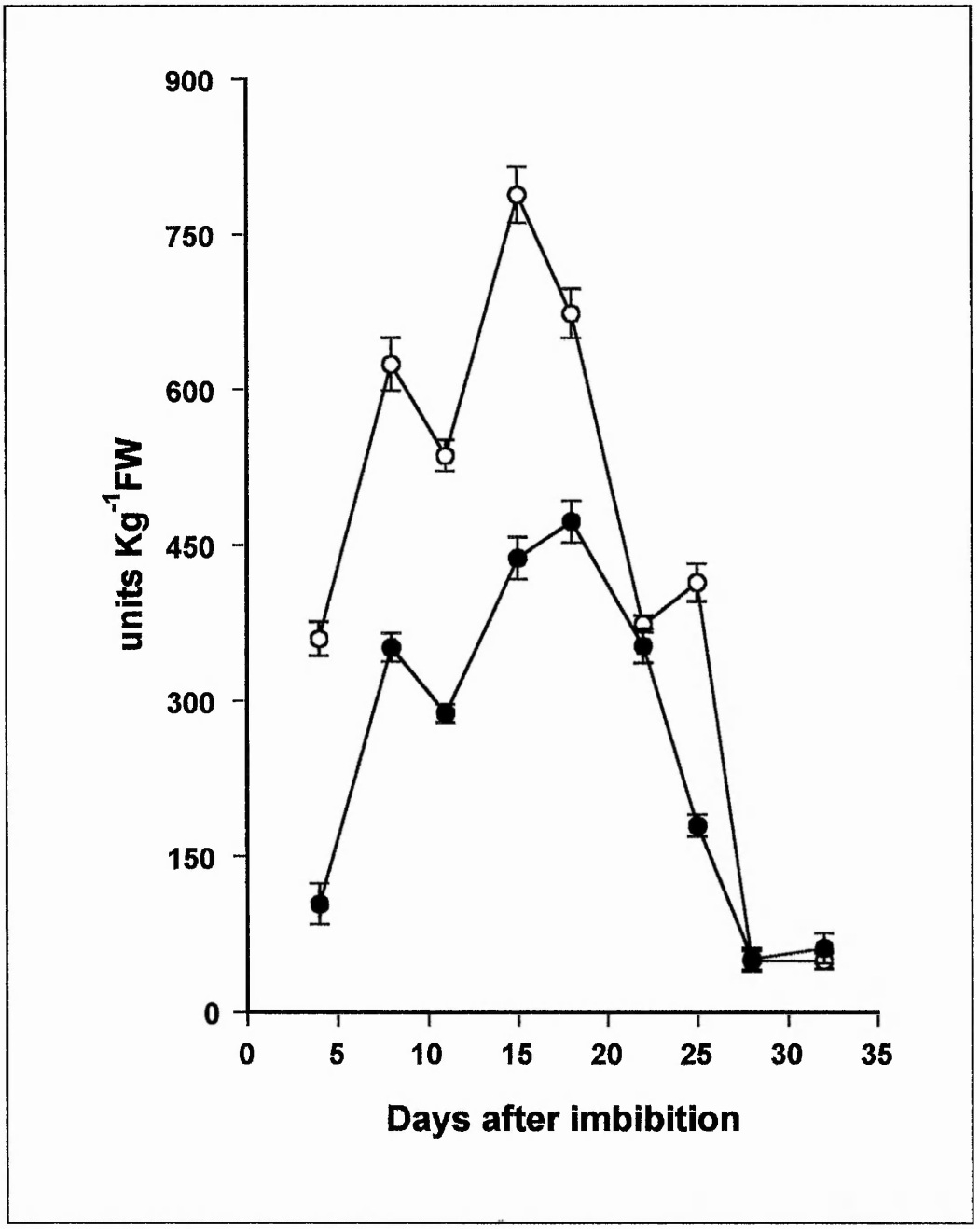

Figure 20 legend.

Four to thirty two day old *Pisum sativum* root extract was assayed for casein cross-linking and cadaverine incorporation activities for 60 minutes at 37°C as described in section 2.2.3. 250μM EDTA was used as negative control at each developmental stage giving average backgrounds of 0.08 units and 0.11 units for casein cross-linking and cadaverine incorporation respectively. Data points represent the mean ± SEM of 4 replicates. (○=casein cross-linking, ●=biotin cadaverine incorporation).

Figure 20 and plates 1 and 2 show that during root development, casein cross-linking activity of *Pisum sativum* root extract increases to a peak of 788 units Kg^{-1} FW at day 15 after imbibition and falls to 49 units Kg^{-1} FW at day 32. Biotin cadaverine incorporation activity shows a similar trend with a peak at day 18 of 473 units Kg^{-1} FW followed by a decrease to 61 units Kg^{-1} FW at day 32.

Plate 1. Photograph of a typical biotin cadaverine assay used to construct the tissue activity data shown on figure 20.

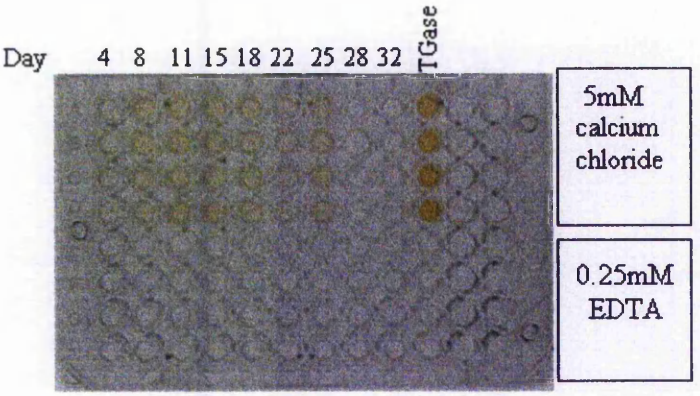

Plate 2. Photograph of a typical casein cross-linking assay used to construct the tissue activity data shown on figure 20.

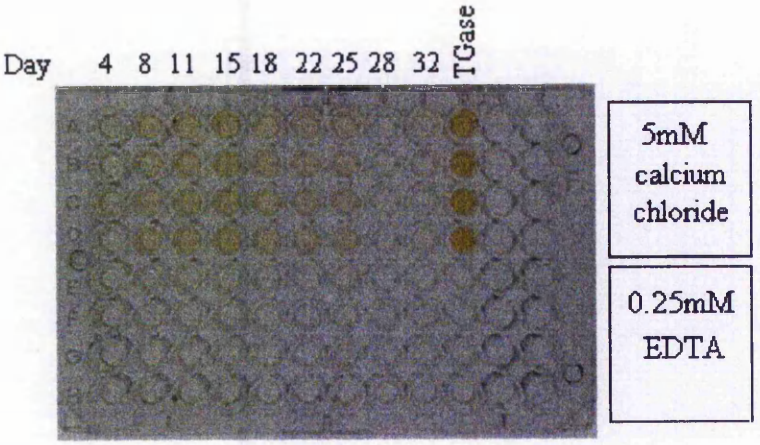

Figure 21. The effect of plant age on the casein cross-linking and cadaverine incorporation activities of soluble *Pisum sativum* leaf extract.

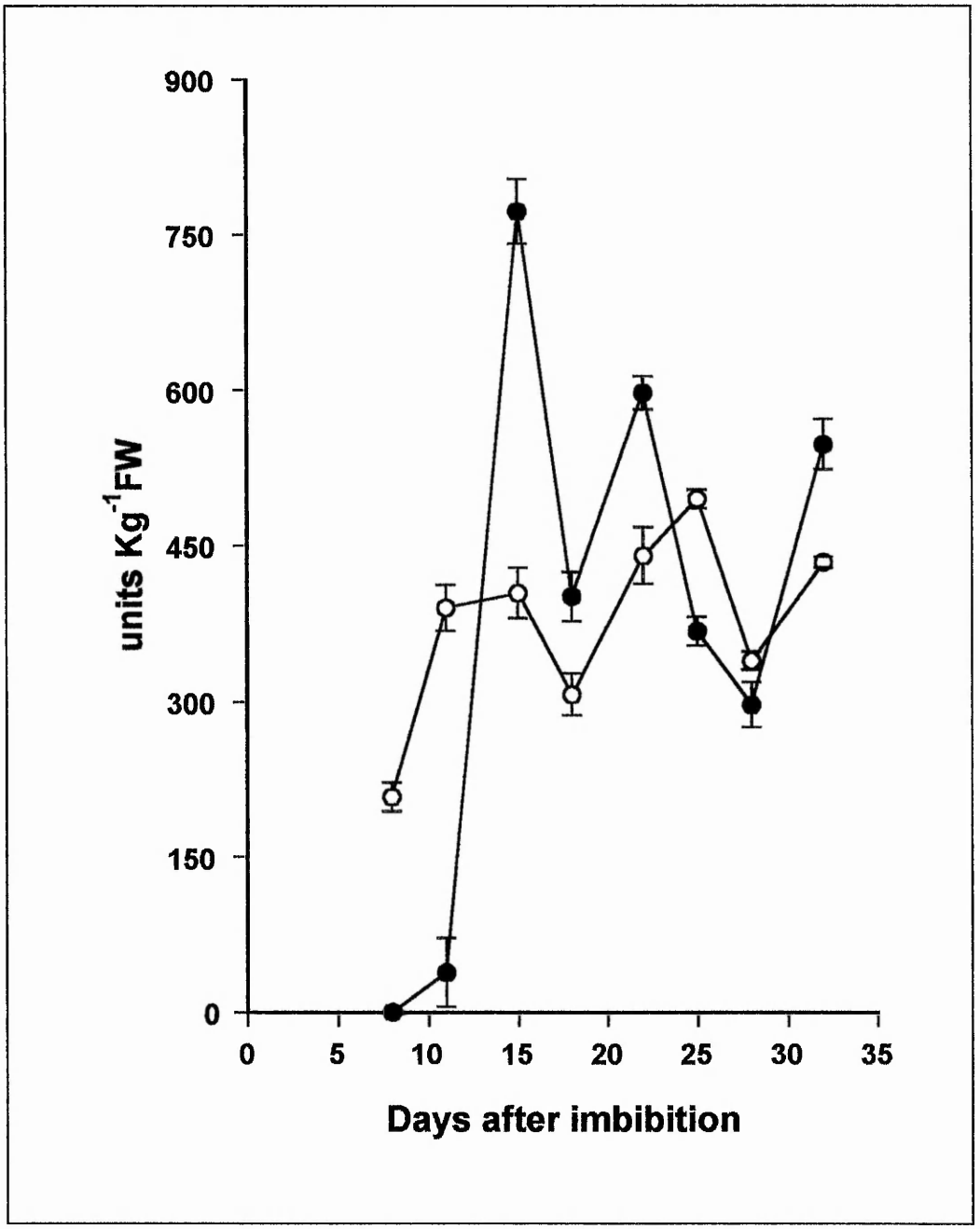

Figure 21 legend.

Eight to thirty two day old *Pisum sativum* leaf extract was assayed for casein cross-linking and cadaverine incorporation activities for 60 minutes at 37°C as described in section 2.2.3. 250μM EDTA was used as negative control at each developmental stage giving average backgrounds of 0.10 units and 0.12 units for casein cross-linking and cadaverine incorporation respectively. Data points represent the mean ± SEM of 4 replicates. (○=casein cross-linking, ●=biotin cadaverine incorporation).

Figure 21 and plates 3-4 show that during *Pisum sativum* leaf development, casein cross-linking activity increases to a peak of 500 units Kg^{-1} FW at day 25 and remains at 434 units Kg^{-1} FW at day 32. Similarly, biotin cadaverine incorporation activity peaks at 773 units Kg^{-1} FW at day 15 and remains at 548 units Kg^{-1} FW at day 32.

Membrane bound mammalian transglutaminase activity has been reported (Thacher and Rice 1985). The next experiment, illustrated by tables 20 and 21, was designed to investigate the possibility of the presence of membrane bound forms of transglutaminase in *Pisum sativum* root tissue.

Plate 3. Photograph of a typical biotin cadaverine assay used to construct the tissue activity data shown on figure 21.

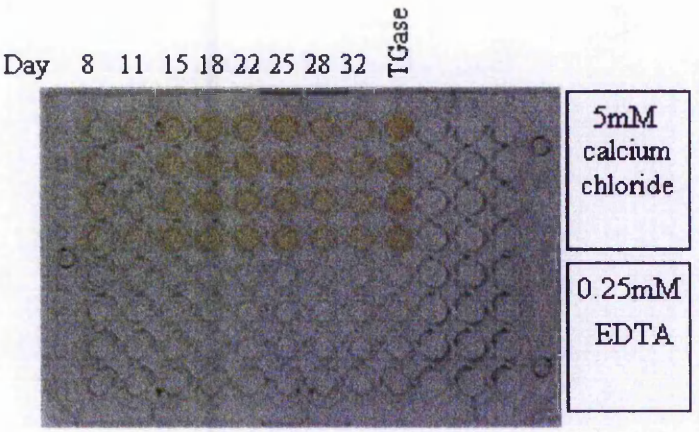

Plate 4. Photograph of a typical casein cross-linking assay used to construct the tissue activity data shown on figure 21.

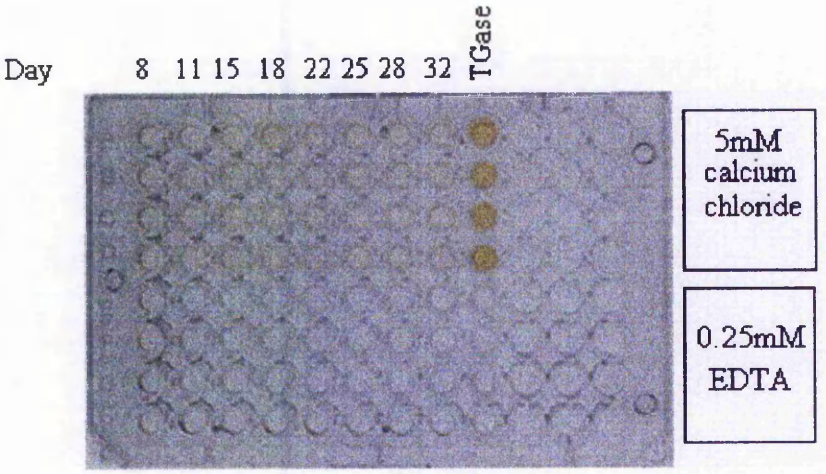

Table 20. Survey of detergents used to solubilise *Pisum sativum* root membrane transglutaminase activity.

	Specific activity units mg^{-1} ± SEM	
Conditions	**Casein cross-linking**	**Cadaverine incorporation**
1M KCl supernatant	0.39 ± 0.08	1.90 ± 0.22
0.1% (v/v) Triton X-100 PFS	6.31 ± 0.20	0.33 ± 0.10
0.1% (v/v) Triton X-100 mixed microsomal pellet	1.89 ± 0.05	0.45 ± 0.041
0.1% (w/v) Sodium deoxycholate PFS	9.32 ± 0.26	6.33 ± 0.64
0.1% (w/v) Sodium deoxycholate mixed microsomal pellet	0.76 ± 0.05	0.45 ± 0.044
0.1% (w/v) CTAB PFS	6.72 ± 0.17	8.00 ± 0.70
0.1% (w/v) CTAB mixed microsomal pellet	0.38 ± 0.04	0.80 ± 0.24

Pisum sativum root membrane extract (prepared as described in section 2.2.1.2) was incubated for 60 minutes at 37°C in the presence of different detergents as described in section 2.2.3. 250μM EDTA was used as a negative control giving average background values of 0.11 units and 0.15 units for cadaverine incorporation and casein cross-linking respectively. Data points represent the mean ± SEM of 4 replicates.

Table 20 demonstrates that some soluble transglutaminase activity was washed off the mixed microsomal membrane preparation by the 1M KCl wash since between 0.39 and 1.90 units mg^{-1} activity was found in the 80000 × g supernatant when assayed using the casein cross-linking and cadaverine incorporation assays respectively. The most effective detergent for the measurement of both types of activity, giving specific activities of 6.33-9.32 units mg^{-1} for cadaverine incorporation and casein cross-linking respectively was found to be sodium deoxycholate at a concentration of 0.1% (w/v).

The optimum concentration of sodium deoxycholate was now determined and is presented in table 21.

Table 21. Optimisation of the concentration of sodium deoxycholate used to extract the *Pisum sativum* root membrane transglutaminase activity.

Conditions	Specific activity units mg^{-1} ± SEM	
	Casein cross-linking	Cadaverine incorporation
0.01% (w/v) sodium deoxycholate PFS	0	0
0.01% (w/v) sodium deoxycholate mixed microsomal pellet	1.83 ± 0.28	0.91 ± 0.34
0.1% (w/v) sodium deoxycholate PFS	12.30 ± 1.22	7.10 ± 0.82
0.1% (w/v) sodium deoxycholate mixed microsomal pellet	1.00 ± 0.31	0.61 ± 0.10

Pisum sativum root membrane extract (prepared as described in section 2.2.1.2) was incubated at 37°C for 120 minutes in the presence of different concentrations of sodium deoxycholate as detailed in section 2.2.3. 250μM EDTA was used as a negative control at each detergent concentration and gave average background values of 0.09 units and 0.12 units for cadaverine incorporation and casein cross-linking respectively. Assays carried out in the absence of either biotin labelled substrate or extravidin peroxidase gave background values between 0.04-0.05 units for both assays. Boiled membrane supernatant and experiments carried out with buffer to replace membrane supernatant gave average plate background absorbance values of 0.10 units and 0.15 units for cadaverine incorporation and casein cross-linking respectively. Zero time controls resulted in average background absorbance values of 0.15 units and 0.18 units for cadaverine incorporation and casein cross-linking respectively. Data points represent the mean ± SEM of 4 replicates.

Table 21 demonstrates that the optimum concentration of sodium deoxycholate required to extract *Pisum sativum* root membranes was found to be 0.1% (w/v). This gave specific activities of 12.30 and 7.10 units mg^{-1} for casein cross-linking and cadaverine incorporation respectively. The relative distribution of transglutaminase activity between the soluble and membrane bound fractions of *Pisum sativum* root tissue was calculated to be as follows:

Cadaverine incorporation

Membrane- 17 ± 1 units Kg^{-1} FW (3%) Soluble- 558 ± 37 units Kg^{-1} FW (97%)

Casein cross-linking

Membrane- 12 ± 3 units Kg^{-1} FW (1.7%) Soluble- 702 ± 41 units Kg^{-1} FW (98.3%)

The relationship between *Pisum sativum* membrane associated transglutaminase activity and age of developing tissue was also investigated. The data is presented in figure 22.

Figure 22. The effect of plant age on the cadaverine incorporation and casein cross-linking activities of *Pisum sativum* root membrane extract.

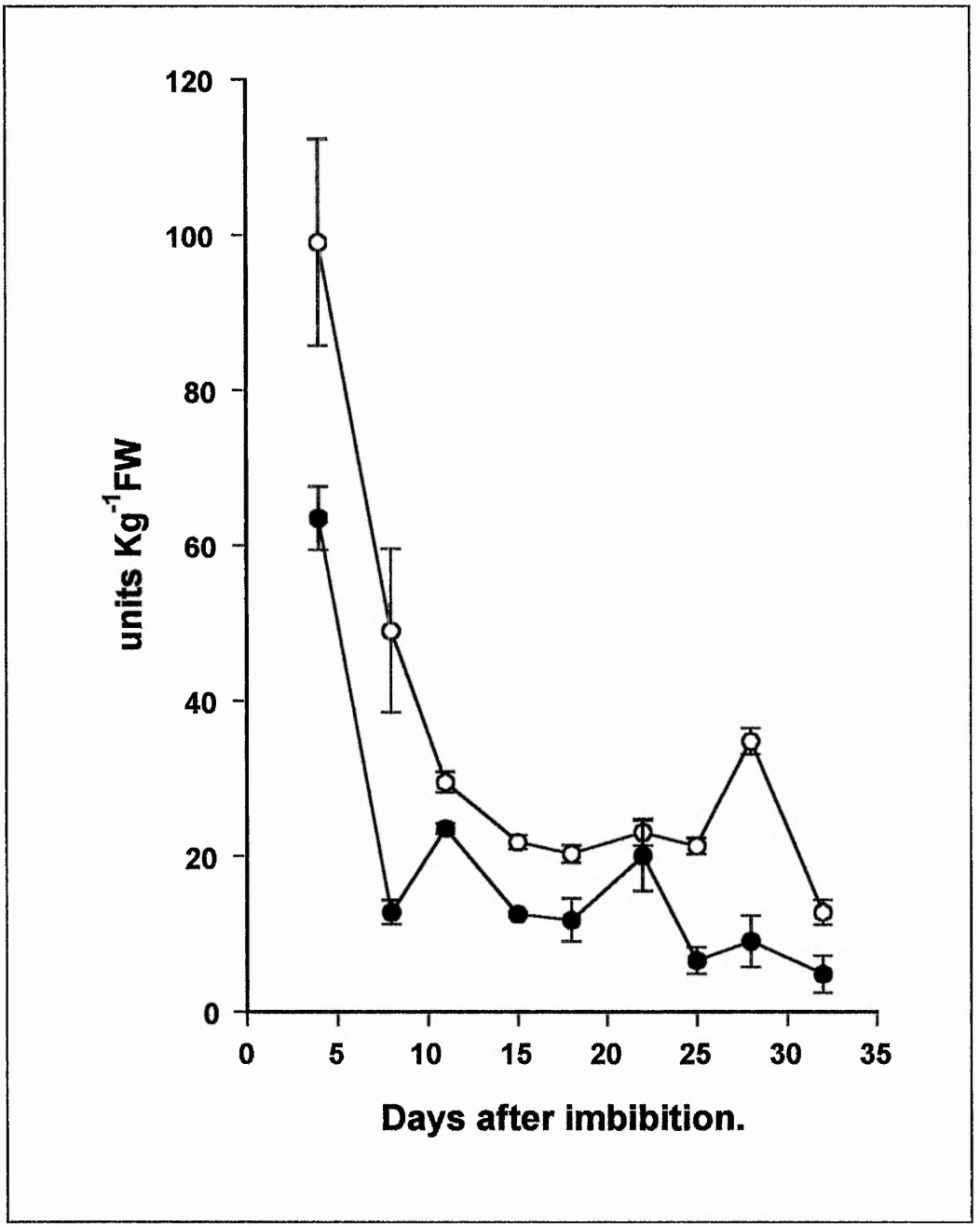

Figure 22 legend.

Four to 32 day old *Pisum sativum* root membrane extract was assayed for casein cross-linking and cadaverine incorporation activities at 37°C for 120 minutes as described in section 2.2.3. 250μM EDTA was used as negative control at each developmental stage giving average background values of 0.09 units and 0.13 units for casein cross-linking and cadaverine incorporation respectively. Data points represent the mean ± SEM of 4 replicates. (○=casein cross-linking, ●=cadaverine incorporation).

Figure 22 shows that during root development, cadaverine incorporation activity peaks at day 4 at 65 units $Kg^{-1}FW$ and falls to 5 units Kg^{-1} FW at day 32. Casein cross-linking activity also peaks at day 4 at 98 units $Kg^{-1}FW$ and falls to 14 units $Kg^{-1}FW$ at day 32.

4.3 Discussion.

The ability of crude plant extracts to incorporate radiolabelled polyamines into proteins such as *N',N'*-dimethylcasein has been demonstrated (Icekson and Apelbaum 1987; Serafini-Fracassini *et al.* 1988; Margosiak *et al.* 1990; Aribaud *et al.* 1995). In one case, this activity has in shown to be 100 fold less than that detected using animal transglutaminases (Signorini *et al.* 1991). Table 7 demonstrates that use of the conventional [1,4-^{14}C]-putrescine incorporation assay resulted in soluble transglutaminase activity being detected in only three of the eight tissues screened (*Pisum sativum* root and leaf tissue and *Vicia faba* leaf tissue). This suggests that this type of assay may be unsuitable for transglutaminase detection in some crude plant extracts. In addition, the [1,4-^{14}C]-labelled putrescine incorporation assay suffers from errors as large as ± 52% when screening *Pisum sativum* root extract for transglutaminase activity (table 7).

Transglutaminase activity was detected in all extracts screened using both the casein cross-linking assay (Lilley *et al.* 1997a) and the biotin-labelled cadaverine incorporation assay (Slaughter *et al.* 1992). This indicates that these assays are more suitable, for the detection of transglutaminase activity in crude plant extracts possibly due in part to greater sensitivity. Errors observed using these assays were also typically as low as ± 8% for *Pisum sativum* root extract (table 7). The root tissue of all the species tested contained higher specific activity than the leaf tissue of the same species using both assays. Greatest specific activity was observed in the root tissue of *Triticum aestivum* where values of 2.74 and 2.18 units mg^{-1} were recorded for cadaverine incorporation and

casein cross-linking respectively. A decision was taken to focus the characterisation section of this research on *Pisum* sativum root transglutaminase as reasonably high specific activities of 1.21 and 1.02 units mg^{-1} were recorded for cadaverine incorporation and casein cross-linking respectively. *Pisum sativum* root tissue also produced above background plate absorbance values, which were higher than those obtained using *Triticum aestivum* root due to the low protein content of the extracts obtained from cereal root tissue. To eliminate the possibility of a contribution to assay signal by incorporation of endogenous biotinylated proteins or peroxidase enzymes, zero biotin and extravidin peroxidase controls were carried out and typically resulted in low plate background absorbances of between 0.04 and 0.05 units.

Figure 15 shows that the activity detected in *Pisum sativum* root tissue was found to be time dependent and linear for 60 minutes for both casein cross-linking and cadaverine incorporation. Further experiments carried out using *Pisum sativum* root tissue, were therefore incubated for 60 minutes. Furthermore, both the casein cross-linking reaction and the cadaverine incorporation reaction were shown to be enzymic as boiling plant root or leaf extract for 20 minutes resulted in total loss of transglutaminase activity. Figure 16 demonstrates the enzymic nature of [1,4-^{14}C]-labelled putrescine incorporation by *Pisum sativum* root extract. The reaction is time dependent and linear up to 80 minutes and subsequent experiments using this assay were carried out over a 60 minute incubation period. This figure does indicate that large errors are often observed when using this assay with *Pisum sativum* root extract. Such errors are not observed when using this assay to detect mammalian transglutaminase activity (data not presented).

Table 8 shows that the calcium chelating agent EGTA at 5mM was unable to affect more than 35% inhibition of [1,4-^{14}C]-putrescine binding activity of extracts. The same concentration of EDTA caused a maximum inhibition of 19.5% observed in *Pisum sativum* leaf extract. Similar results have been reported by other workers using comparable assays and consequently they proposed that plant transglutaminase has no absolute calcium ion requirement (Icekson and Apelbaum 1987; Serafini-Fracassini *et al.* 1988 and 1995; Signorini *et al.* 1991).

However, recent research indicates the presence of a contaminating diamine oxidase in crude plant extracts which is able to incorporate [1,4-^{14}C]-putrescine into *N',N'*-dimethylcasein in a calcium independent manner. Diamine oxidase performs this reaction via oxidation of one amine group of [^{14}C]-putrescine to its aldehyde derivative followed by spontaneous reaction with protein amine groups resulting in Schiff base formation (see figure 3) (Siepaio and Meunier 1995; Chiarello *et al.* 1996a and 1996b). These contaminating activities do not appear to interfere with biotin cadaverine incorporation as chelation of calcium by 1mM EDTA and 1mM EGTA resulted in over 80% inhibition of *Pisum sativum* root and leaf transglutaminase activity and 100% inhibition of all the other extracts screened as shown by table 9. Similarly, no interference was observed using the casein cross-linking assay. Table 10 demonstrates that the casein cross-linking activity of *Vicia faba* leaf and *Pisum sativum* leaf was inhibited by over 80% by the chelation of calcium ions. The remaining extracts were inhibited by 100%. This data suggests that both plate assays are more suitable for the study of transglutaminase from crude plant cell extracts than the conventional [1,4-^{14}C]-putrescine incorporation assay.

The contribution to [1,4-^{14}C]-putrescine incorporation by possible diamine oxidase contamination was also investigated. Table 11 shows that addition of the copper ion chelator (diamine oxidase inhibitor) DIECA at a concentration of 5mM inhibited the putrescine binding by 17.7%. Addition of the copper ion chelator o-phenanthroline at 5mM caused a more pronounced 29.2% inhibition of the putrescine binding activity of *Pisum sativum* root transglutaminase. This data broadly supports the findings of Siepaio and Meunier (1995) and Chiarello *et al.* (1996a and 1996b) and suggests that at approximately 30% of the putrescine binding activity present in *Pisum sativum* root tissue is copper dependent. As a positive control, it was found that neither of the copper chelating agents were able to inhibit the putrescine binding activity of guinea pig liver transglutaminase (table 12). A further experiment was carried out to determine the extent to which DIECA and o-phenanthroline at a 5mM concentration would inhibit the cadaverine incorporation and casein cross-linking reactions. Table 11 shows that 5mM DIECA had no effect on *Pisum sativum* root cadaverine incorporation activity but a 13.0% inhibition was observed due to the addition of 5mM o-phenanthroline. However, the 100ng per well guinea pig liver transglutaminase positive control was inhibited by 12.7-19.1% in the presence of 5mM DIECA and o-phenanthroline respectively (table 12). Similarly, table 11 shows that copper ion chelation by 5mM o-phenanthroline caused 22.1% inhibition of the casein cross-linking activity of *Pisum sativum* root transglutaminase. It should be noted that guinea pig liver transglutaminase was also inhibited by 18.7-27.3% due to 5mM concentrations of o-phenanthroline and DIECA respectively (table 12). This data suggests that the casein cross-linking assay and the cadaverine incorporation assay are not affected by interfering copper dependent enzymes since any reduction in *Pisum sativum* root transglutaminase activity caused by addition of

DIECA or o-phenanthroline is mirrored by the guinea pig liver transglutaminase positive control.

Resting levels of plant cytosolic calcium have been measured and found to be in the nanomolar range with brief rises to micromolar levels in response to appropriate stimulation. Calcium levels in the apoplast and in calcium stores have been detected in the millimolar range (Bush 1995). Activation of the biotin cadaverine incorporation activity of soluble *Pisum sativum* root transglutaminase by calcium was observed at levels of 20nM and is represented by figure 17. Maximum activity was achieved at 94nM free calcium suggesting that soluble *Pisum sativum* root transglutaminase is able to incorporate polyamines into proteins at resting levels of cytosolic calcium. Figure 18 demonstrates that activation of the protein cross-linking function of soluble *Pisum sativum* root transglutaminase occurs at 250μM but 80% of the observed activity occurs between 1 and 3mM. This observation may indicate that in order to carry out the protein cross-linking reaction, soluble *Pisum sativum* root transglutaminase must be in a high calcium environment such as the extracellular environment or in the intracellular environment during calcium release resulting from cellular damage.

Furthermore, the activity of mammalian tissue transglutaminase is regulated by GTP at sub-optimal concentrations of calcium (Takeuchi *et al.* 1992; Bergamini and Signorini 1993; Smethurst and Griffin 1996). Table 13 shows that the biotin cadaverine incorporation activity of *Pisum sativum* root transglutaminase was not significantly inhibited by 1mM GTP at a free calcium ion concentration of 80nM. The activity of guinea pig liver transglutaminase was reduced by 52.1% in the presence of 2mM free

calcium and 1mM GTP. Similarly, table 13 shows that the casein cross-linking function of soluble *Pisum sativum* root transglutaminase was found to be uninhibited by 1mM GTP at a free calcium ion concentration of 2mM whereas the guinea pig liver transglutaminase positive control was inhibited by 35.3% at this concentration. These results indicate that in this respect *Pisum sativum* root transglutaminase may differ from mammalian tissue transglutaminase in that it may not contain a GTP binding site. Alternatively, the crude extract may be removing GTP from solution due to the action of GTPase activity.

Mammalian transglutaminases have a cysteine residue at the active site and as a result are irreversibly inhibited by reagents such as iodoacetamide and NEM (Smethurst and Griffin 1996). Table 14 shows that the [1,4-^{14}C]-putrescine incorporation activity of soluble *Pisum sativum* root transglutaminase was inhibited by 25.0% in the presence of 10mM iodoacetamide. Similarly, soluble *Pisum sativum* and *Vicia faba* leaf extracts were inhibited by 12.4 and 20.5% respectively, due to addition of 10mM iodoacetamide to the reaction buffer. There is little data in the literature with which to compare this finding but recently Kang and Cho (1996) reported a 60 % inhibition of the [1,4-^{14}C]-labelled putrescine binding activity of a purified *Glycine max* leaf transglutaminase activity upon addition of 100μM NEM to the reaction buffer. Table 15 shows that the inhibition of biotin cadaverine incorporation caused by the addition of 10mM iodoacetamide varied between 0% in *Hordeum vulgare* leaf tissue and 60% in *Vicia faba* leaf tissue. The same concentration of NEM brought about a reduction of cadaverine incorporation activity of between 0% in *Triticum aestivum* root and 100% in *Vicia faba* leaf. Table 16 however, shows that the casein cross-linking activity was unaffected by a 10mM concentration of

either iodoacetamide or NEM. This data conflicts with the data presented in table 15 and one possible explanation for this is that the thiol group inhibitors are binding to thiol groups present at points other than the active site of plant transglutaminase thus sterically hindering the cadaverine incorporation reaction but not the casein cross-linking function. A further experiment was carried out to determine the effect of the thiol group stabiliser DTT (Cleland 1964) at various concentrations from 0-10mM. Table 17 shows that DTT was not required for either the casein cross-linking or cadaverine incorporation reactions. The data also shows that up to 10mM DTT did not have any inhibitory effect on *Pisum sativum* root transglutaminase. Other workers have shown DTT to slightly inhibit plant transglutaminase like activities (Icekson and Apelbaum 1987; Chiarello *et al.* 1996a; Siepaio and Meunier 1995). This data, coupled to the data presented in tables 15 and 16 suggests that there may be active site differences between mammalian transglutaminases and some transglutaminases found in plants.

Table 18 demonstrates that the biotin cadaverine incorporation activity present in *Pisum sativum* root extract can be inhibited by 45% following the addition of 10mM spermine to the reaction buffer. This data suggests that plant transglutaminase is able to utilise polyamines other than cadaverine as acyl acceptor substrates and broadly supports the findings of other workers (Icekson and Apelbaum 1987; Serafini-Fracassini *et al.* 1988). As a control 100ng per well of guinea pig liver transglutaminase was also incubated in the presence of 10mM spermine and was inhibited by almost 60%. As well as utilising other acyl acceptor substrates, table 19 demonstrates that *Pisum sativum* root transglutaminase is able to utilise a preparation of *N',N'*-dimethylated *Vicia faba* storage proteins as an amine acceptor substrate. There is however, little difference in the specific

activities observed between *N',N'*-dimethylcasein and the dimethylated storage proteins. The purified guinea pig liver transglutaminase does however show an increase in specific activity of 28% when using storage proteins as the acyl donor substrate implying a greater number of available glutamine residues. This may indicate that there is a preferential endogenous substrate present in the crude *Pisum sativum* root extract, which out-competes the glutamine residues present in the *Vicia faba* storage protein preparation.

The pH optimum of soluble *Pisum sativum* root transglutaminase was determined to be pH 7.9. Figure 19 shows that this was the optimum pH for both casein cross-linking and biotin cadaverine incorporation activity. The profiles of both pH plots are similar, suggesting that both assays are detecting the same soluble activity in *Pisum sativum* root tissue. Other workers have demonstrated basic pH optima between 7.9 and 8.4 for transglutaminase activities in different tissues of *Helianthus tuberosus* (Falcone *et al.* 1993). The high pH optima observed may reflect the biochemistry of the transglutaminase enzymes because all of the transglutaminase reactions involve the release of a molecule of ammonia which may cause a high pH in the local environment.

A relationship exists between soluble transglutaminase activity and the age of *Pisum sativum* root tissue. Figure 20 demonstrates that transglutaminase activity increased over the first 18 days of development using both the biotin cadaverine incorporation and the casein cross-linking assays. This was followed by a sharp decrease in activity in 22-32 day old root tissue indicating that transglutaminase may be involved in early root growth and development. This suggestion is supported by a similar fluctuation in

transglutaminase activity in developing roots of *Chrysanthemum morifolium* (Aribaud *et al.* 1995). Figure 21 shows that in *Pisum sativum* leaf tissue both casein cross-linking activity and biotin cadaverine incorporation activity increase to a peak at days 25 and 15 respectively. Activity does not decline rapidly as for *Pisum sativum* root but remains at a level above that detected at day 8. Martin-Tanguy *et al.* (1996) also demonstrate rising levels of transglutaminase activity in developing shoot tissue using *Chrysanthemum morifolium*. Since transglutaminase is required both in developing and mature leaf tissue of *Pisum sativum*, this observation supports other reports, which propose roles for transglutaminase in photosynthesis (Margosiak *et al.* 1990; Del Duca *et al.* 1994 and 1995).

A mixed microsomal membrane preparation prepared from *Pisum sativum* root tissue was washed in 1M KCl followed by 0.1% (w/v or v/v) concentration of either Triton X-100, sodium deoxycholate or CTAB and assayed for transglutaminase activity. The biotin cadaverine incorporation and casein cross-linking activity of the membranes was partially removed by the salt wash as shown in table 20 suggesting that a proportion of the soluble activity is still present and associated with the membrane by an ion exchange effect. The results therefore indicate that the remaining activity, which was not removed by the KCl wash but required detergent to become solubilised, is associated with the membrane. The most effective detergent for both casein cross-linking and biotin cadaverine incorporation was shown to be sodium deoxycholate which solubilised more than 90% of the activity present on *Pisum sativum* root membranes. Table 20 also shows that CTAB was an effective detergent in terms of specific activity solubilised but it should be noted that use of this detergent resulted in low absolute absorbance values

and as a result sodium deoxycholate was selected for further membrane study. The fact that the use of CTAB resulted in a low total activity but high specific activity may indicate that this detergent could be of subsequent use for membrane transglutaminase purification experiments. Membrane bound forms of animal transglutaminases are also known to exist. One such transglutaminase is found in keratinocytes, anchored to the cell membrane by a fatty acid residue (Thacher and Rice 1985).

Figure 22 shows that both cadaverine incorporation and casein cross-linking activities of *Pisum sativum* root membrane transglutaminase showed a decreasing trend over a 4-32 day development period. This data may indicate that *Pisum sativum* root membrane transglutaminase might be involved in the early stages of root development.

At this point it should be noted that each of the eight plant extracts shown in table 7 were also screened using the *N'*,*N'*-bis (γ-glutamyl) polyamine cross-linking assay described in chapter 3. No activity was observed using this assay with any of the extracts, possibly due to the lower sensitivity of this assay. As a result it was decided to re-test *Pisum sativum* root extract following partial purification and this is discussed further in chapter 6.

Chapter 5- ε-(γ-glutamyl) lysine isodipeptide analysis.

5.1 Introduction.

ε-(γ-glutamyl) lysine isodipeptide is one product of transglutaminase activity and the presence of this cross-link allows an assessment of the activity of the enzyme in a particular tissue (Griffin and Wilson 1984). Indeed, the correlation between isodipeptide levels and transglutaminase activity in human lung fibroblasts has been demonstrated by Birkbichler *et al.* (1978b). The presence of the isodipeptide in certain tissues has been an important finding as it has led to the elucidation of the role of the transglutaminase enzyme present. Pisano *et al.* (1971) showed that the mechanical and chemical stability of the clot formed during the blood coagulation cascade was directly related to a 1.6-2.0 nmol mg^{-1} level of ε-(γ-glutamyl) lysine present in the fibrin clot protein. Similarly, Williams-Ashman *et al.* (1972) demonstrated the relationship between the stability of the rodent post-ejaculatory seminal plug and the 37 nmol mg^{-1} level of isodipeptide present in the cross-linked seminal proteins. The function of both plasma and prostate transglutaminase is directly related to the formation of the ε-(γ-glutamyl) lysine isodipeptide, which stabilises the protein in which it is present.

Transglutaminase enzymes without fully understood roles do have proposed biological functions, which mostly suggest that the enzymes have a stabilising effect on the tissues in which they are found. Tissue transglutaminase is thought to be involved in the stabilisation of the extracellular matrix due to the formation of ε-(γ-glutamyl) lysine cross-links between the matrix proteins, fibronectin, collagen, laminin and nidogen

(Juprelle-Soret *et al.* 1988; Barsigian *et al.* 1988). The keratinocyte enzyme is widely believed to stabilise the outer epidermal skin layer by cross-linking proteins in the terminally differentiating keratinocyte (Simon and Green 1991; Marvin *et al.* 1992).

Proposed roles for plant transglutaminase are also related to effects which may be caused by the formation of the ε-(γ-glutamyl) lysine cross-link. These include the dimerisation of the large sub-unit of RuBisCo (Keuhn *et al.* 1991) which has been proposed as the initial step of the assembly of the L_8S_8 catalytically active structure (Roy *et al.* 1988). A further proposed role for plant transglutaminase, which is thought to involve the cross-linking of proteins, is the assembly and organisation of the cell wall (Grandi *et al.* 1992). This theory is supported by similar reports which suggest that transglutaminase may be an important factor in the assembly of the cell wall of the fungus *Candida albicans* (Sentandreu *et al.* 1995; Ruizherrera *et al.* 1995). These proposed theories regarding the cross-linking of proteins by plant transglutaminase are however unsupported since detection of the isodipeptide has not been reported in any of the listed publications.

The methods used to quantify the ε-(γ-glutamyl) lysine isodipeptide may be conveniently divided into two distinct sections, namely direct and indirect. One indirect method involves derivatisation of the un-reacted ε-lysine residues of cross-linked protein with either nitrous acid (Lorand *et al.* 1966) or acrylonitrile (Pisano *et al.* 1969) followed by total hydrolysis of the protein to liberate free lysine residues. The liberated free lysine must be derived from lysine, which was initially blocked by glutamine incorporated by transglutaminase. In a further indirect method, fluorescent amine markers have been

incorporated by transglutaminase to quantify the amine acceptor sites in fibrin (Lorand and Ong 1976; Lorand *et al.* 1968; Lorand and Chenoworth 1969).

Direct methods for ε-(γ-glutamyl) lysine quantitation involve isolation of the isodipeptide which has been released from cross-linked protein by hydrolysis (For a review of the methods see Griffin and Wilson 1984). Workers initially attempted to liberate the cross-link from guinea pig liver protein using a combination of proteolysis and acid hydrolysis (Schweet 1955 and 1956). This proved unsuccessful and was soon replaced by a method involving exhaustive proteolytic digestion alone. Korngruth *et al.* (1963) showed that the ε-(γ-glutamyl) lysine cross-link was resistant to the action of chymotrypsin, leucine amino peptidase and pronase. More recently, a variety of proteolytic enzyme cocktails have been developed which commonly include subtilisin and carboxypeptidase Y (Griffin *et al.* 1982). The cocktail of proteolytic enzymes used depends entirely on the composition of the protein to be digested and must be determined empirically.

To quantify the liberated isodipeptide, it must first be separated from the amino acids and peptides present in the proteolytic digest. This is usually carried out by one of two principle techniques, ion exchange chromatography or reversed phase HPLC (Griffin and Wilson 1984). Because the liberated isodipeptide is often present at extremely low levels (in the order of pmol mg^{-1}) a pre-purification step may be required prior to applying the digest to either method in order to prevent flanking amino acids interfering with the isodipeptide peak. Whether or not this step is carried out is dependent on the type of tissue the proteolytic digest was derived from (Griffin and Wilson 1984). The ion exchange method has been carried out using a variety of resins including Dionex DC-4

for the separation of isodipeptide from human fibroblast protein digests (Birkbichler *et al.* 1977) and Custom AA-15 for the separation of isodipeptide from clotted guinea pig vesicular protein digest (Williams-Ashman *et al.* 1972). The digested protein is subjected to either post-column derivatisation with ninhydrin or post-column derivatisation using OPA to enable post-separation amino acid analysis. A standard isodipeptide spike is usually added to allow accurate identification and quantitation of the isodipeptide present in the sample.

The reversed phase HPLC method has also been carried out using a variety of resins including Zorbax C_8 (Griffin *et al.* 1982) and Cosmosil AR C_{18} (Sato *et al.* 1992). The main advantage of this type of separation is the increased sensitivity observed (in the order of femtomoles of isodipeptide) when phenylisothiocyanate is used as the fluorescent deriviatising agent (Sato *et al.* 1992). The speed of the procedure is also improved since the HPLC technique can be carried out in 12-14 minutes compared to 140 minutes for the ion exchange method (Griffin and Wilson 1984).

The detection of the isodipeptide product of the transglutaminase reaction is regarded as the only unequivocal proof of the existence of a catalytically active transglutaminase (Folk and Finlayson 1977). Detection of *N'*,*N'*-bis(γ-glutamyl) polyamine and *N'*-(γ-glutamyl) polyamine linkages has been demonstrated in plant tissue (Signorini *et al.* 1991; Del Duca *et al.* 1995) and this does provide good evidence for the presence of a catalytically active transglutaminase but as pointed out by Tack *et al.* (1981) and Beninati *et al.* (1988), these conjugates may form without the action of transglutaminase. Chiarello *et al* (1996a) attempted to demonstrate the ε-(γ-glutamyl) lysine cross-linking

of bovine β-casein by an extract prepared from 10 day old etiolated *Pisum sativum* apical meristematic hook tissue using exhaustive proteolytic digestion followed by reversed phase HPLC analysis. This approach proved unsuccessful suggesting that concentration of transglutaminase in the extract may be too low to produce a detectable level of isodipeptide when using this physiologically irrelevant substrate. It was one aim of this section to repeat Chiarello's experiment using 14 day old *Pisum sativum* root protein as this was shown to be more active than young *Pisum sativum* leaf protein (table 7). A further aim was to analyse total soluble protein extracted from *Pisum sativum* roots and leaves for the presence of endogenous isodipeptide in order to unequivocally demonstrate the presence of transglutaminase in these tissues. The technique used was exhaustive proteolytic digestion followed by ion exchange separation and subsequent amino acid analysis of the resultant eluent.

5.2 Results.

A decision was taken to investigate the ε-(γ-glutamyl) lysine isodipeptide content of 14 day old *Pisum sativum* root tissue initially as it was shown to contain approximately four fold more transglutaminase activity than *Pisum sativum* leaf tissue of the same age (table 7). The cross-link analysis result is shown in figure 23.

Figure 23. The ε-(γ-glutamyl) lysine isodipeptide present in *Pisum sativum* root total soluble protein.

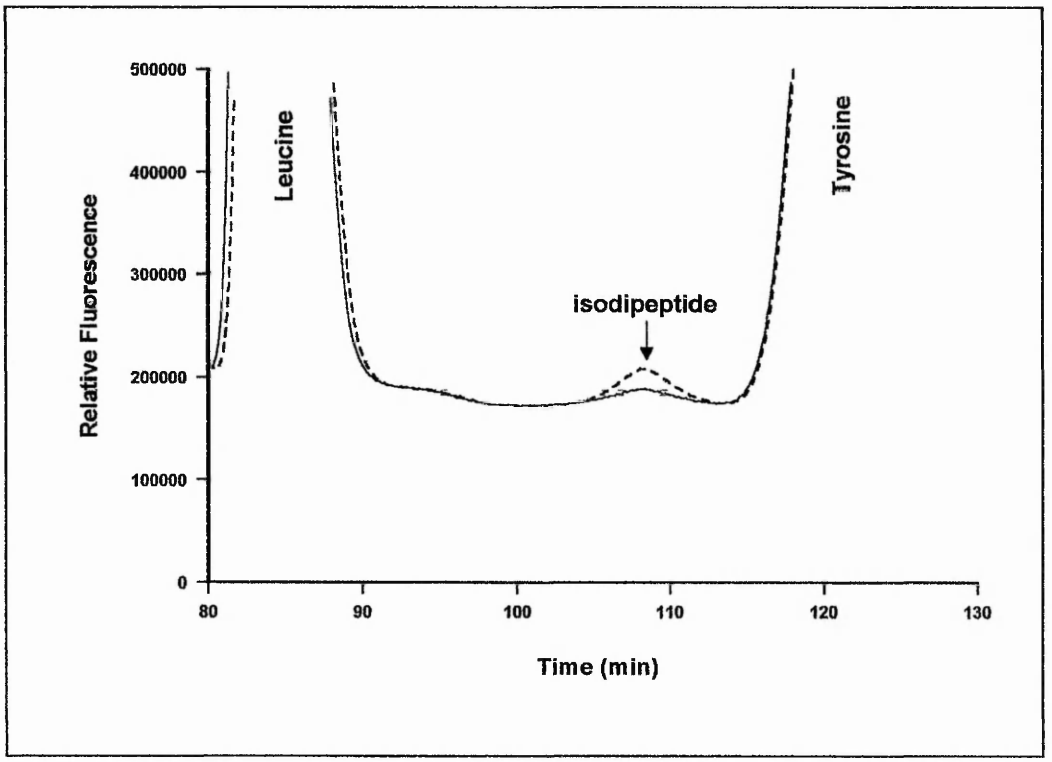

Figure 23 legend.

Four hundred micrograms of *Pisum sativum* root soluble protein was proteolytically digested as described in section 2.2.1.4. The resultant digest was then analysed according to the method detailed in section 2.2.4.4. The solid line represents the amino acid analysis profile. The dashed line indicates the same sample with an added 1 nmol isodipeptide standard spike to confirm the identity of the reaction product and enable accurate quantitation of the isodipeptide detected. Figure 23 represents one of three runs.

The level of isodipeptide present was 0.51 ± 0.04 nmol mg^{-1}. An experiment to determine the levels of isodipeptide present at different stages of root development was also carried out and the data is presented in table 22.

Table 22. The isodipeptide content of *Pisum sativum* root tissue soluble protein at various stages of root development.

Stage of root development	Isodipeptide conc. (nmol Kg^{-1} FW ± SEM)
Day 4	1200 ± 210
Day 8	160 ± 10
Day 11	240 ± 40
Day 15	160 ± 12
Day 18	240 ± 0
Day 22	310 ± 0
Day 25	140 ± 0
Day 28	80 ± 0
Day 32	60 ± 0

Four to thirty two day old *Pisum sativum* soluble root protein was proteolytically digested as described in section 2.2.1.4. Four hundred micrograms of the resultant digest was then analysed for the presence of ε-(γ-glutamyl) lysine isodipeptide as detailed in section 2.2.4.4. Data points represent the mean ± SEM of 3 replicates.

Table 22 demonstrates a relationship between the age of developing *Pisum sativum* root tissue and the endogenous level of ε-(γ-glutamyl) lysine isodipeptide present. The isodipeptide level peaks at day 4 with a value of 1200 nmol Kg^{-1} FW. There is then a decrease over the period studied to a level of 60 nmol Kg^{-1} FW at day 32.

Pisum sativum leaf total protein was also screened for the presence of ε-(γ-glutamyl) lysine isodipeptide cross-links. The result is shown in figure 24.

Figure 24. ε-(γ-glutamyl) lysine isodipeptide analysis of *Pisum sativum* leaf total soluble protein.

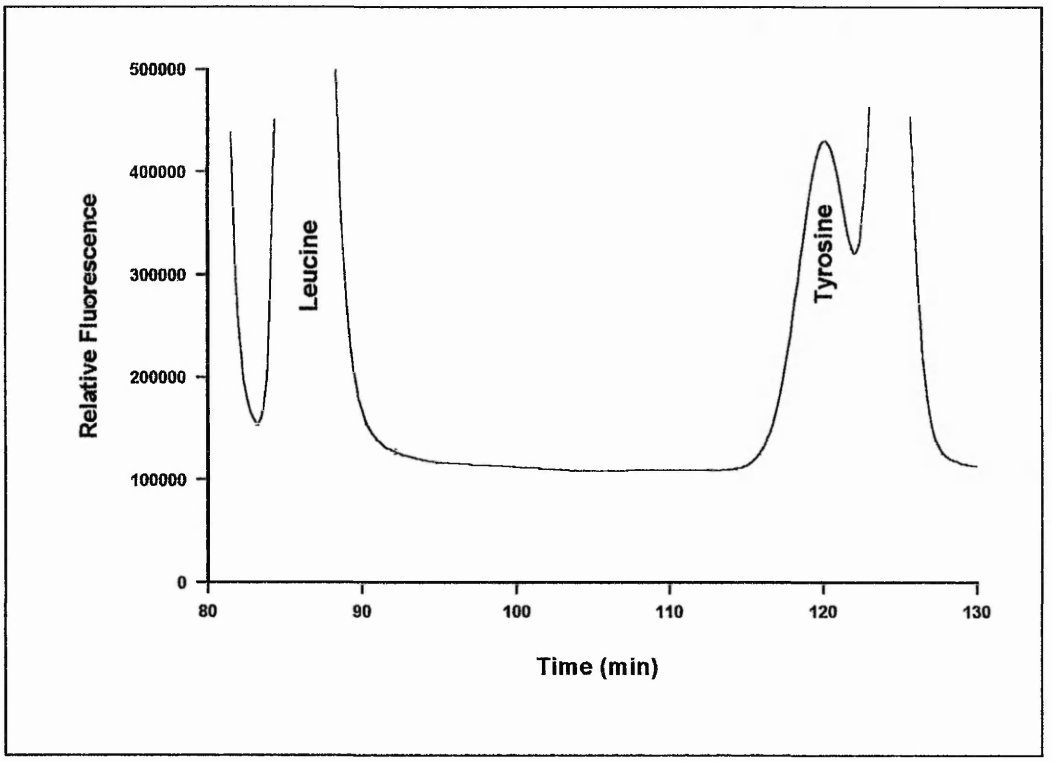

Figure 24 legend.

Four hundred micrograms of *Pisum sativum* leaf soluble protein was proteolytically digested as described in section 2.2.1.4. The resultant digest was then analysed according to the method detailed in section 2.2.4.4. Figure 24 represents the amino acid analysis profile.

The experiment was repeated 3 times but figure 24 shows that no isodipeptide was detected in *Pisum sativum* leaf protein. A pre-purification step (as detailed in section 2.2.4.1) was undertaken to attempt to remove contaminating leucine. Following pre-purification, the *Pisum sativum* extract was re-tested (figure 25).

Figure 25. ε-(γ-glutamyl) lysine cross-link analysis of anion exchange purified *Pisum sativum* leaf total soluble protein.

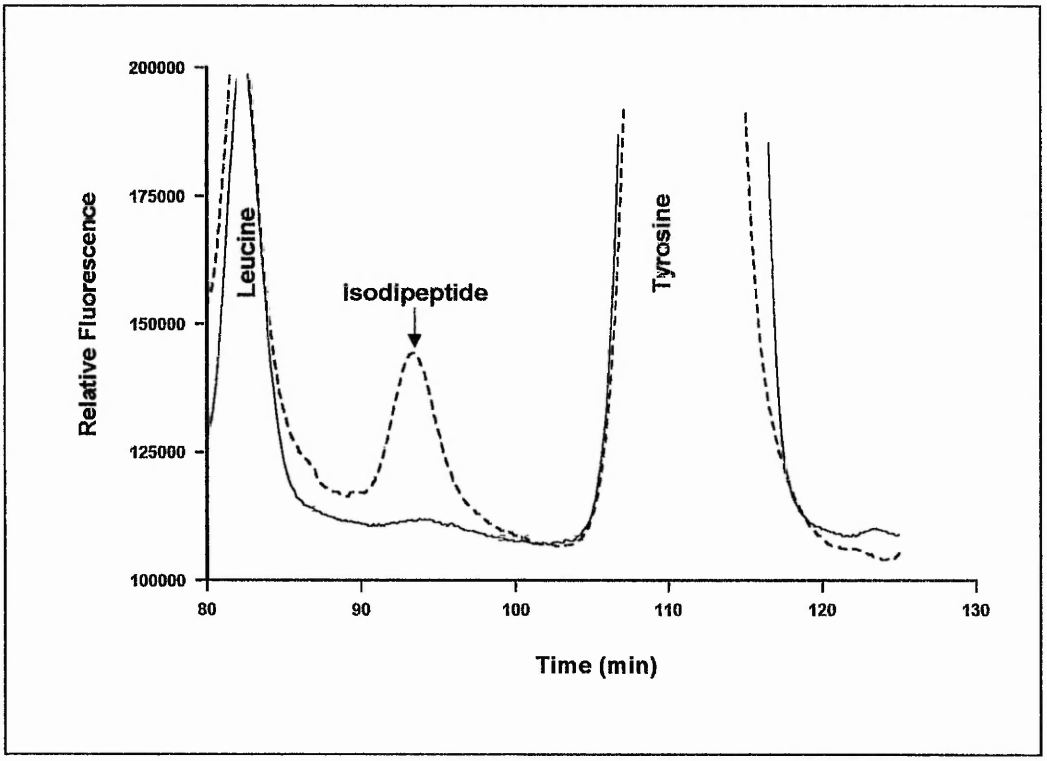

Figure 25 legend.

Four hundred micrograms of anion exchange purified *Pisum sativum* leaf soluble protein was prepared as described in section 2.2.4.1. The resultant purified digest was then analysed according to the method detailed in section 2.2.4.4. The solid line represents the amino acid analysis profile. The dashed line indicates the same sample with an added 1 nmol isodipeptide standard spike to confirm the identity of the reaction product and enable accurate quantitation of the isodipeptide detected. Figure 25 represents one of three runs.

Figure 25 shows that purification of the sample reduced the quantity of leucine considerably, allowing detection of a small ε-(γ-glutamyl) lysine isodipeptide peak of 210 ± 5 pmol mg^{-1} (allowing for 70% recovery following purification according to Lilley *et al.* 1998).

To further validate the casein cross-linking assay as a suitable method for plant transglutaminase detection, a sample of bovine casein was cross-linked in the solution phase by a protein extract prepared from *Pisum sativum* root tissue. The ε-(γ-glutamyl) lysine present following the cross-linking was detected as shown by table 23 and figure 26.

Table 23. Cross-link analysis controls and levels of isodipeptide detected due to the cross-linking of bovine casein by soluble *Pisum sativum* root extract.

Experiment	Zero time isodipeptide (pmol mg^{-1} ± SEM)	Isodipeptide after 16 hours (pmol mg^{-1} ± SEM)
Extract + casein + Ca^{2+}	320 ± 16	688 ± 32
Extract + casein + EDTA	288 ± 18	320 ± 7
Extract alone + Ca^{2+}	400 ± 24	560 ± 30
Extract alone + EDTA	384 ± 21	448 ± 41

One point five milligrams of *Pisum sativum* root protein was incubated on its own or with 10mg of bovine casein as described in section 2.2.4.6. The resultant protein was digested and analysed as described in sections 2.2.1.4 and 2.2.4.4 respectively. As a positive control, 500ng per tube of guinea pig liver transglutaminase was also incubated with 10mg of bovine casein producing an isodipeptide level of 81 pmol mg^{-1} h^{-1}. The results represent the mean ± SEM of 3 replicates.

Table 23 shows that the level of isodipeptide formed due to the action of *Pisum sativum* root transglutaminase on other root proteins was 7 pmol mg^{-1} h^{-1}. The level formed due to the action of *Pisum sativum* root transglutaminase on casein and other root proteins was 23 pmol mg^{-1} h^{-1}. By calculation, the level of casein cross-linking carried out by the *Pisum sativum* root transglutaminase was 25.3 pmol $mg^{-1}h^{-1}$. Figure 26 is one of 3 amino acid analysis profiles from which the data in table 23 was derived.

Figure 26. The ε-(γ-glutamyl) lysine isodipeptide present following the 16 hour cross-linking of bovine casein using soluble *Pisum sativum* root transglutaminase.

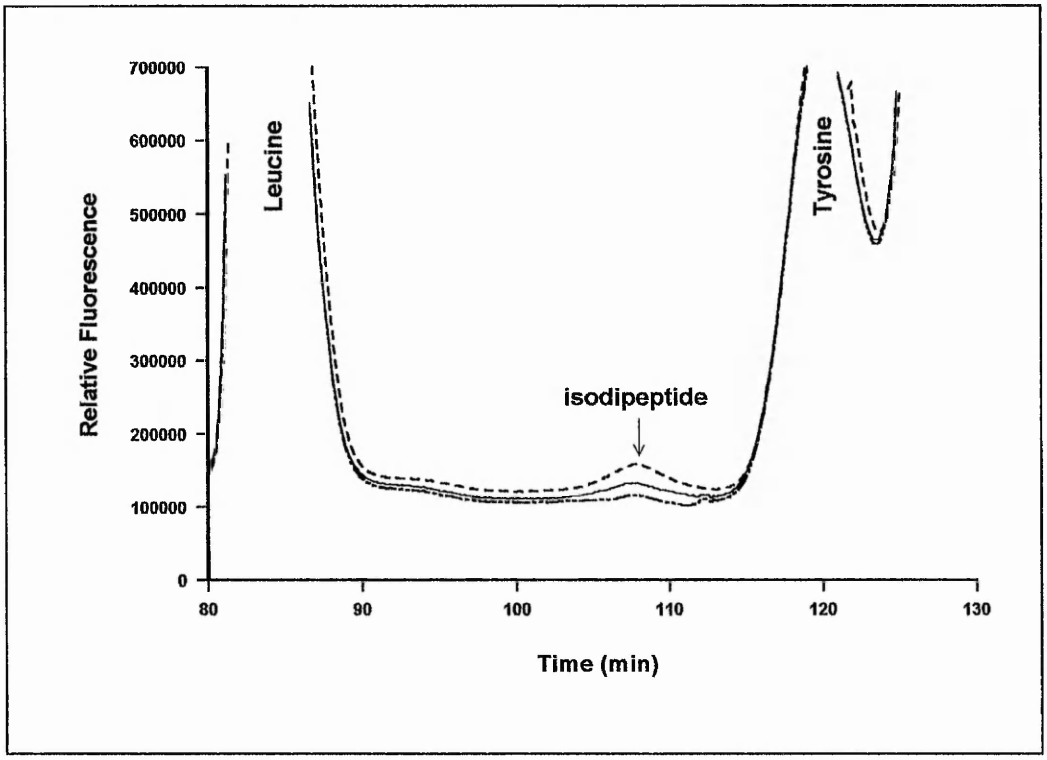

Figure 26 legend.

Ten milligrams of bovine casein was incubated for 16 hours at 37°C with 1.5mg of rapidly de-salted *Pisum sativum* root extract (see section 2.2.1.2) according to the method described in section 2.2.4.6. The resultant cross-linked protein was subjected to proteolytic digestion as described in section 2.2.1.4 and 0.4 mg was applied to the amino acid analysis column. The dashed line represents the 5mM calcium chloride activated sample plus a 1 nmol isodipeptide standard spike. The solid line shows the same sample without the isodipeptide spike. The intermittent dot/dash line shows the same sample with a final EDTA concentration of 5mM.

Figure 26 represents a level of isodipeptide of 43 ± 2 and 20 ± 0 pmol mg^{-1} h^{-1} for the calcium and EDTA controls respectively as determined by the method described in section 2.2.4.4.

5.3 Discussion.

Pisum sativum root total soluble protein was subjected to exhaustive proteolytic digestion followed by subsequent ion exchange chromatography and amino acid analysis. The level of ε-(γ-glutamyl) lysine isodipeptide present was 0.51 ± 0.04 nmol mg^{-1} (figure 23). The isodipeptide eluted with an authentic standard and this data indicates the presence of a catalytically active transglutaminase in *Pisum sativum* root tissue (Folk and Finlayson 1977; Lilley *et al.* 1997b and 1998). The data presented in figure 23 also supports the assay data in chapter 4 which shows that plant transglutaminases are able to cross-link bovine casein. The level detected is however relatively low compared to quantities present in mammalian tissues. Clotted guinea pig semen has been shown to contain over 70 times this level (Williams-Ashman *et al.* 1972) and over 3 times this level is present in the human fibrin clot (Pisano *et al.* 1971). The data concerning the level of isodipeptide present broadly concurs with assay activity data gathered by Signorini *et al.* (1991) which suggested transglutaminase activity in plant tissue was up to 100 fold less than that seen in some animal tissues.

The level of isodipeptide present in *Pisum sativum* root tissue was determined over a 32 day developmental period as shown by table 22. The concentration of isodipeptide was relatively high at day 4 with an observed quantity of 1200 nmol $Kg^{-1}FW$. This level was shown to fall sharply over the remaining 28 days of the development period to 60 nmol $Kg^{-1}FW$ at day 32. The pattern of cross-link detected over the development period does not follow the pattern of activity shown by the soluble enzyme in figure 20 but does mirror the both the casein cross-linking activity and the cadaverine incorporation activity shown by the developing root membrane enzyme in figure 22. This observation implies

that the membrane-associated transglutaminase may be actively cross-linking protein which ultimately resides in the cytosol. This may imply a role for the membrane bound enzyme in early root development. The high soluble root enzyme activity observed at day 15 (figure 20) could possibly reflect a polyamine incorporating role for the soluble enzyme at this stage of root development as no ε-(γ-glutamyl) lysine appears to be synthesised between day 4 and day 32. The observed fall in isodipeptide present over the 32 day period corresponds to a decrease in total soluble protein extracted from the tissue. This implies that the high level of isodipeptide present in the early stages of development remains in the root tissue bound to low molecular mass material derived from protein catabolism. This isodipeptide would therefore not be detected due to the method only collecting the isodipeptide present as protein cross-links.

The isodipeptide content of *Pisum sativum* leaf total soluble protein was also investigated. Figure 24 shows that by employing the same method as that used for root protein, no detectable level of isodipeptide was observed. The major peak flanking the position of isodipeptide elution is leucine and in many cases this is known to interfere with the detection of small quantities of ε-(γ-glutamyl) lysine (Griffin and Wilson 1984). Since we had already detected both cadaverine incorporation activity and casein cross-linking activity in *Pisum sativum* leaf extracts (table 7), it was decided to undertake a pre-purification step to further the attempt to detect isodipeptide in this tissue. This was carried out using the method of Lilley *et al.* (1998). When a mixture of standard isodipeptide and leucine was separated using this method. Up to 95% of the contaminating leucine was removed and on average 70% of the ε-(γ-glutamyl) lysine was recovered (Lilley *et al.* 1998). The quantity of isodipeptide present in the *Pisum sativum*

leaf soluble protein was calculated by correction for the percentage recovery. Possible explanations for losing up to 30% of the isodipeptide using this technique include the number of handling steps involved in the method or inaccurate neutralisation of samples prior to concentration by freeze drying which could facilitate hydrolysis of the isodipeptide as acid or base strength in the sample increases. Figure 25 does however show that using this method, a detectable level of 210 pmol mg^{-1} could be observed in *Pisum sativum* leaf tissue. To see and quantify the isodipeptide, the scale has been expanded by a factor of approximately 2.5 over the corresponding root tissue profile. This level is less than 50% of that seen in root tissue and this correlates with the relative casein cross-linking activities observed in day 14 *Pisum sativum* root and leaf tissue (table 7).

To attempt to validate the casein cross-linking assay, Chiarello's (1996a) casein cross-linking investigation was repeated using *Pisum sativum* root extract, which has a higher specific activity than the leaf extract of the same age (table 7). The incubation time was also increased from 4 hours up to 16 in an attempt to detect the cross-link. Table 23 and figure 26 show that the *Pisum sativum* root extract was able to catalyse the cross-linking of casein via ε-(γ-glutamyl) lysine isodipeptide cross-links. The calcium dependence of the reaction is clearly demonstrated by figure 26. The intermittent line represents the cross-linking reaction with exogenously added 5mM EDTA. This reaction has produced no significant increase in cross-links above the endogenous level of 20 pmol $mg^{-1}h^{-1}$ shown for the casein + extract zero time control in table 23. The level detected due to the addition of 5mM calcium chloride to the reaction mixture (solid line on figure 26) was 25.3 pmol mg^{-1} h^{-1} and this relates to an increase in cross-links of 1 per every 25

casein molecules over the full 16 hour period. This is a relatively low level compared to that observed due to the addition of 500ng per sample of guinea pig liver transglutaminase to the casein which resulted in an increase of 81 pmol $mg^{-1}h^{-1}$ (1 cross-link per 8 casein molecules over the 16 hours). The low level observed using the *Pisum sativum* root extract could be attributed to the low concentration of enzyme present in the extract. Nevertheless, this data does demonstrate that the *Pisum sativum* root protein extract is able to cross-link bovine casein when calcium is added to the reaction mixture thus validating the casein cross-linking assay used to gather data in chapter 4.

Chapter 6- Partial purification of soluble *Pisum sativum* root transglutaminase.

6.1 Introduction.

Several of the mammalian transglutaminases have important physiological functions (see chapter 1) and it has therefore become desirable to purify transglutaminase enzymes in order to study and compare their structures. Purification has enabled antibodies to be raised to transglutaminase enzymes having as yet no biological function ascribed to them. This has enabled their role to be investigated extensively in such tissues as guinea pig liver where purification of the enzyme has also yielded information concerning molecular mass (Connellan *et al.* 1971), kinetics (Folk and Chung 1973) and the active site cysteine residue at position 276 (Ikura *et al.* 1988). Although the active site region of transglutaminase enzymes is extremely well conserved, there are differences in structure due to the number and arrangement of amino acid residues present (Guinea pig liver transglutaminase has 690 amino acid residues compared to factor XIIIa, which has 731 amino acid residues (Klein *et al.* 1992)). These differences make it necessary to employ different techniques to purify the enzymes but it should be noted that the core techniques of ion exchange and gel filtration chromatography are commonly used steps in many transglutaminase purifications (for review see Wilhelm *et al.* 1996).

Ion exchange chromatography involves the reversible binding of charged crude protein extracts to an immobilised, oppositely charged resin such as FFQ Sepharose. The binding reaction is based on the crude protein being applied to the resin in a pH-adjusted buffer to ensure that the protein carries an opposite charge to the resin. The

heterogeneous crude extract is then usually separated by the application of a linear sodium chloride concentration gradient to the column, which has the effect of eluting different proteins at different salt concentrations due to their charge differences. The salt ions compete with the proteins for charged sites on the resin and proteins with the lowest charge are eluted by the lowest salt concentrations. Ion exchange chromatography is capable of separating proteins with only minor charge differences and as a result it is a powerful initial purification technique (for a review see Karlsson *et al.* 1989).

Gel filtration chromatography separates protein molecules based on differences in size. The heterogeneous protein-containing buffer is passed through a column of immobilised gel filtration particles, which contain small pores, comparable in size to the protein molecules present in the heterogeneous mixture. The smallest protein molecules are able to occupy the pores in the gel filtration media and as a result they diffuse from the buffer into the pores and their progress through the column is impeded. The protein molecules with the highest molecular mass pass through the column without entering the pores of the gel filtration media and are thus eluted first resulting in separation of the higher molecular mass proteins from lower molecular mass proteins. This technique is rarely used as an initial purification step as crude protein extracts are often viscous, resulting in poor resolution. The method does, however allow for an estimation of molecular mass of the protein of interest to be made (For a review see Hagel 1989).

Tissue transglutaminase has been purified from a variety of biological sources including guinea pig liver (Folk and Cole 1966; Connellan *et al.* 1971; Brookhart *et al.* 1983) and rat liver (Chang and Chung 1986; Knight *et al.* 1990; Wong *et al.* 1990) using a variety

of different techniques. All purifications listed above did however employ an initial ion exchange step (resins used include DEAE Sepharose, MonoQ, DEAE cellulose and QAE Sephadex). A sodium chloride gradient was used in each case to elute the transglutaminase and EDTA was included in some of the elution buffers to prevent auto-aggregation of the transglutaminase via available lysine and glutamine residues. One step purifications have also been reported for tissue transglutaminase and these include an immunoaffinity purification using a monoclonal antibody to guinea pig liver transglutaminase (Ikura *et al.* 1985) and a calcium ion dependent affinity purification of rat liver transglutaminase using casein-Sepharose (Croall and DeMartino 1986).

Human platelet factor XIIIa has also been successfully purified using a combination of ion exchange chromatography on DEAE cellulose, gel filtration chromatography using Sephacryl S-300 and hydrophobic interaction chromatography using Phenyl Sepharose CL-6B (Ando *et al.* 1987). Human plasma factor XIII was purified in a similar manner using ion exchange chromatography on DEAE Sephacel and gel filtration chromatography on BioGel A-5M (Ichinose and Kaetsu 1993). Other mammalian transglutaminases, which have been purified to apparent homogeneity include haemocyte transglutaminase (Tokunaga *et al.* 1993), keratinocyte transglutaminase (Chang and Chung 1986) and epidermal transglutaminase (Ogawa and Goldsmith (1976).

Transglutaminase enzymes have also been purified from sources other than mammals. Klein *et al.* 1992 purified a 77kDa transglutaminase from the slime mould *Physarum polycephalum* using polyethylene glycol precipitation, ion exchange chromatography and isoelectric focusing. Singh and Mehta (1994) isolated a transglutaminase from the filarial

nematode *Brugia malayi* with a molecular mass of 56kDa using thermoprecipitation, ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The enzyme was found to be highly thermostable with optimum activity observed at 55°C.

To date transglutaminase-like activities have been purified from a number of plant sources including *Glycine max* leaves and *Lupinus albus* seedlings (Kang and Cho 1996; Pallavicini *et al.* 1992). The *Glycine max* crude protein extract was precipitated using 50-70% (sat.) ammonium sulphate and then subjected to a series of ion exchange purification steps (DEAE Sepharose, Blue Sepharose CL-6B and ω-aminohexyl agarose). The final purification procedure involved a unique affinity step on α-casein agarose using 1mM spermidine to elute the amine incorporating activity. The purification of the enzyme enabled Kang and Cho to estimate a molecular mass for the *Glycine max* enzyme of 80kDa by SDS-PAGE. The purified activity was inhibited by NEM and GTP and activated by DTT suggesting some similarities between this enzyme and the transglutaminase found in mammalian tissues. It should however be noted that the activity observed by Kang and Cho (1996) was calcium independent. Pallavicini *et al.* (1992) used ion exchange chromatography on QAE-Sephadex A-50 to purify *Lupinus albus* seedling transglutaminase, which they then used to polymerise milk proteins.

The principle objective of this section of the thesis was to use appropriate techniques such as ion exchange chromatography and gel filtration chromatography to purify and further characterise the transglutaminase activity present in *Pisum sativum* root protein

extract in terms of estimated molecular mass, enzyme kinetics, inhibition by iodoacetamide and NEM and the ability to cross-link casein via *N'*,*N'*-bis(γ-glutamyl) polyamine cross-bridges.

6.2. Results.

The initial method chosen to purify the crude *Pisum sativum* root extract was ion exchange chromatography since this had previously been used successfully to purify mammalian transglutaminases (for a review see Wilhelm *et al.* 1996). The resin chosen for the purification was FFQ Sepharose and the resultant profile is presented in figure 27.

Figure 27. Ion exchange chromatogram for the purification of soluble *Pisum sativum* root transglutaminase.

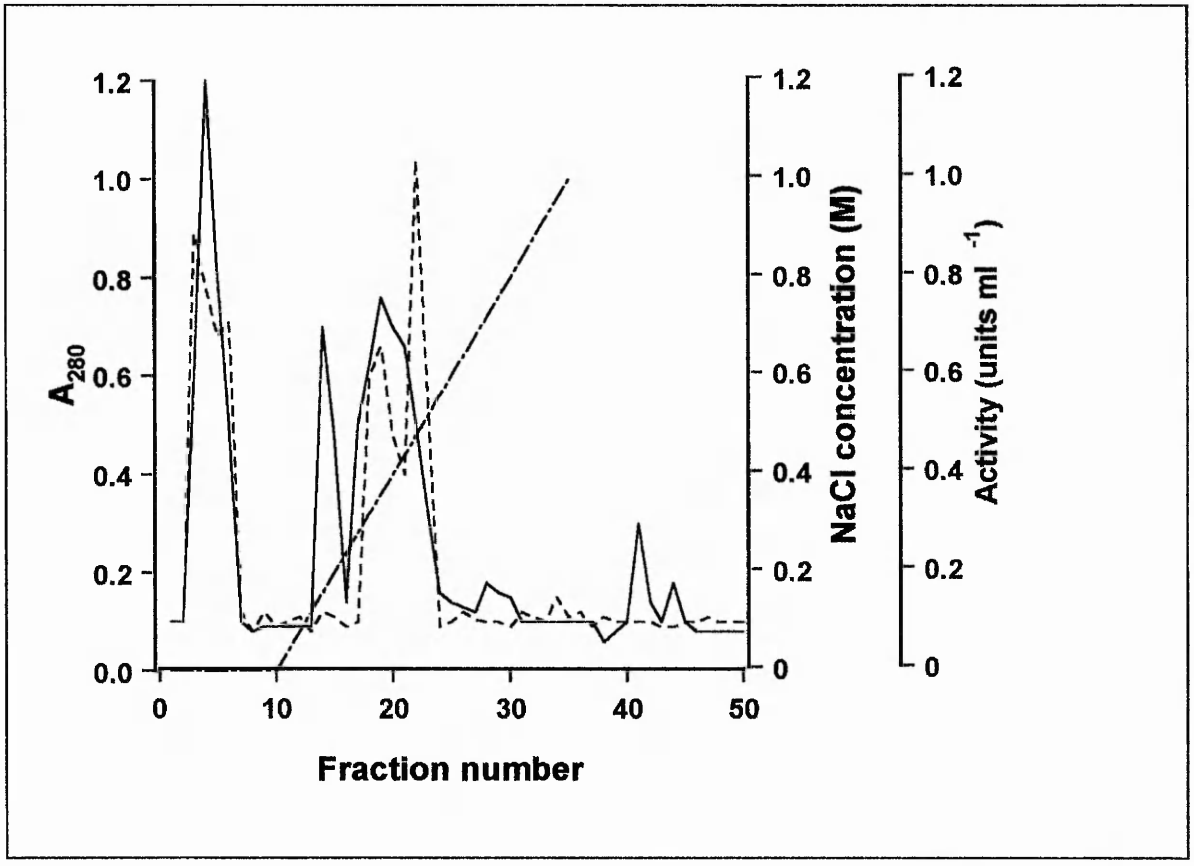

Figure 27 legend.

Eighteen point four milligrams of *Pisum sativum* root protein was applied to a 6.0ml FFQ Sepharose anion exchange column (6.5 × 1.2 cm) and eluted using a 0-1.0M sodium chloride gradient as described in section 2.2.4.2 (solid lines). The broken line shows the biotin cadaverine incorporation activity of each of the eluted fractions as determined by the method described in section 2.2.3.1. As a negative control, 250μM EDTA was added to the reaction buffer giving an average background of 0.14 units.

Figure 27 demonstrates that the transglutaminase activity eluted at a sodium chloride concentration between 0.3 and 0.5M. The ion exchange purified *Pisum sativum* salt-eluted peak was further purified by gel filtration chromatography on Sephacryl 100-HR (figure 28). **Key-** ——— Protein elution

------ Activity

_._._ Sodium chloride gradient

Figure 28. Gel filtration chromatogram for the purification of soluble *Pisum sativum* root transglutaminase.

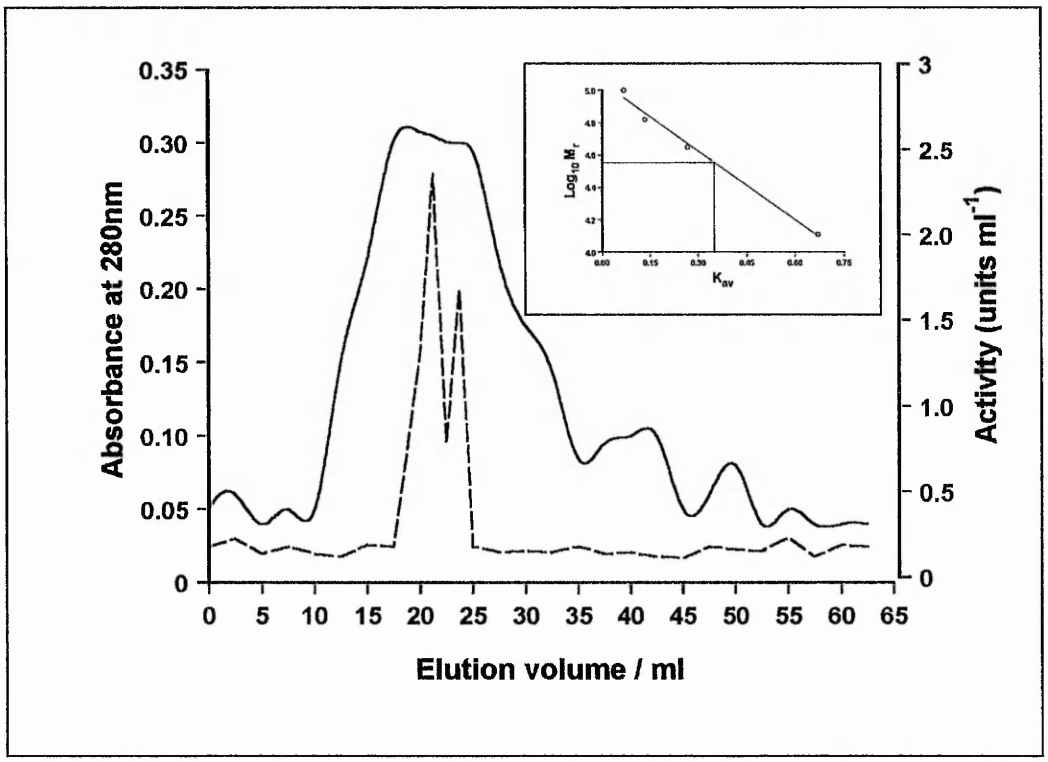

Figure 28 legend.

Seven point two milligrams of *Pisum sativum* root protein was applied to a 35.0 ml Sephacryl 100-HR column and eluted as described in section 2.2.4.3. Transglutaminase activity of the eluted fractions was detected using the cadaverine incorporation assay described in section 2.2.3.1 as shown demonstrated by the dashed line. As a negative control, 250μM EDTA was added to the reaction buffer giving an average background of 0.09. The column was calibrated (inset) as described in section 2.2.4.3.

Figure 28 demonstrates that *Pisum sativum* root transglutaminase eluted at a volume of 21.5 ml. The relative molecular mass of the active peak was estimated to be 36000. Table 24 (overleaf) gives a summary of the two purification strategies in terms of percentage yield and purification index. **Key** ▬ Protein elution

---- Activity

Table 24. The partial purification of biotin cadaverine incorporation activity from soluble *Pisum sativum* crude root extract.

Step.	Volume (ml)	Concentration (mg ml^{-1})	Total protein (mg)	Specific activity (units mg^{-1})	Total activity (units)	Yield (%)	Fold pure
Crude extract	4.0	4.6	18.4	1.30	24.0	100.0	1.0
FFQ Sepharose	18.0	0.4	7.2	1.55	11.16	46.5	1.19
Sephacryl 100-HR	5.0	0.2	1.0	8.29	8.29	34.5	6.38

A total of 18.4 mg of *Pisum sativum* root protein was purified using the techniques of anion exchange and gel filtration chromatography (sections 2.2.4.2 and 2.2.4.3 respectively). The cadaverine incorporation assay used to detect the eluted activity was carried out for 60 minutes at 37°C as described in section 2.2.3.1.

Table 24 shows that following separation, 1.0 mg of plant protein remained with an increased specific activity of 8.29 units mg^{-1}. The transglutaminase was purified 6.38-fold with a recovered yield of 34.5%. The partially purified extract was separated and visualised using SDS-PAGE electrophoresis (plate 5).

Plate 5. SDS-PAGE electrophoretogram of crude and partially purified soluble *Pisum sativum* root transglutaminase.

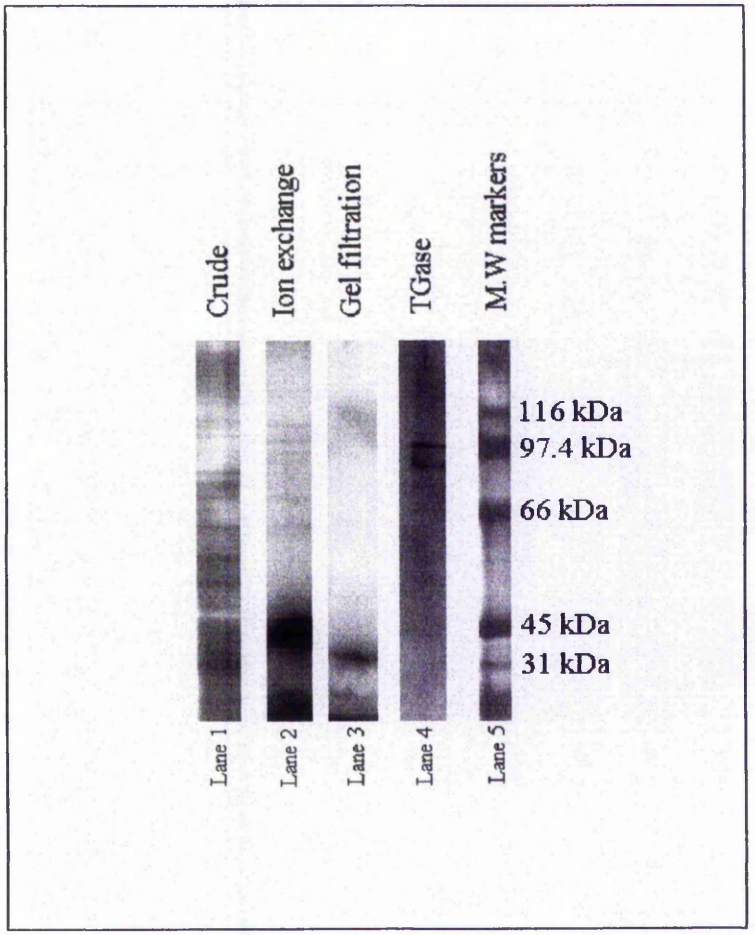

Plate 5 legend.

Twenty five micrograms of protein obtained from each stage of the purification of *Pisum sativum* root transglutaminase was applied to an SDS-PAGE gel as detailed in section 2.2.5. As a positive control, 25µg of purified guinea pig liver transglutaminase was applied to lane 4 of the gel.

Plate 5 demonstrates that there is a protein band present between 31 and 45 kDa, which is close to the molecular mass of the *Pisum sativum* root transglutaminase activity as estimated by gel filtration chromatography.

The partially purified *Pisum sativum* root transglutaminase was further characterised in terms of kinetics and the effect of transglutaminase inhibitors. The results are displayed in figures 29-30 and table 24.

Figure 29. Rate of the biotin cadaverine incorporation reaction as a function of the concentration of biotin labelled cadaverine using ion exchange purified soluble *Pisum sativum* root extract.

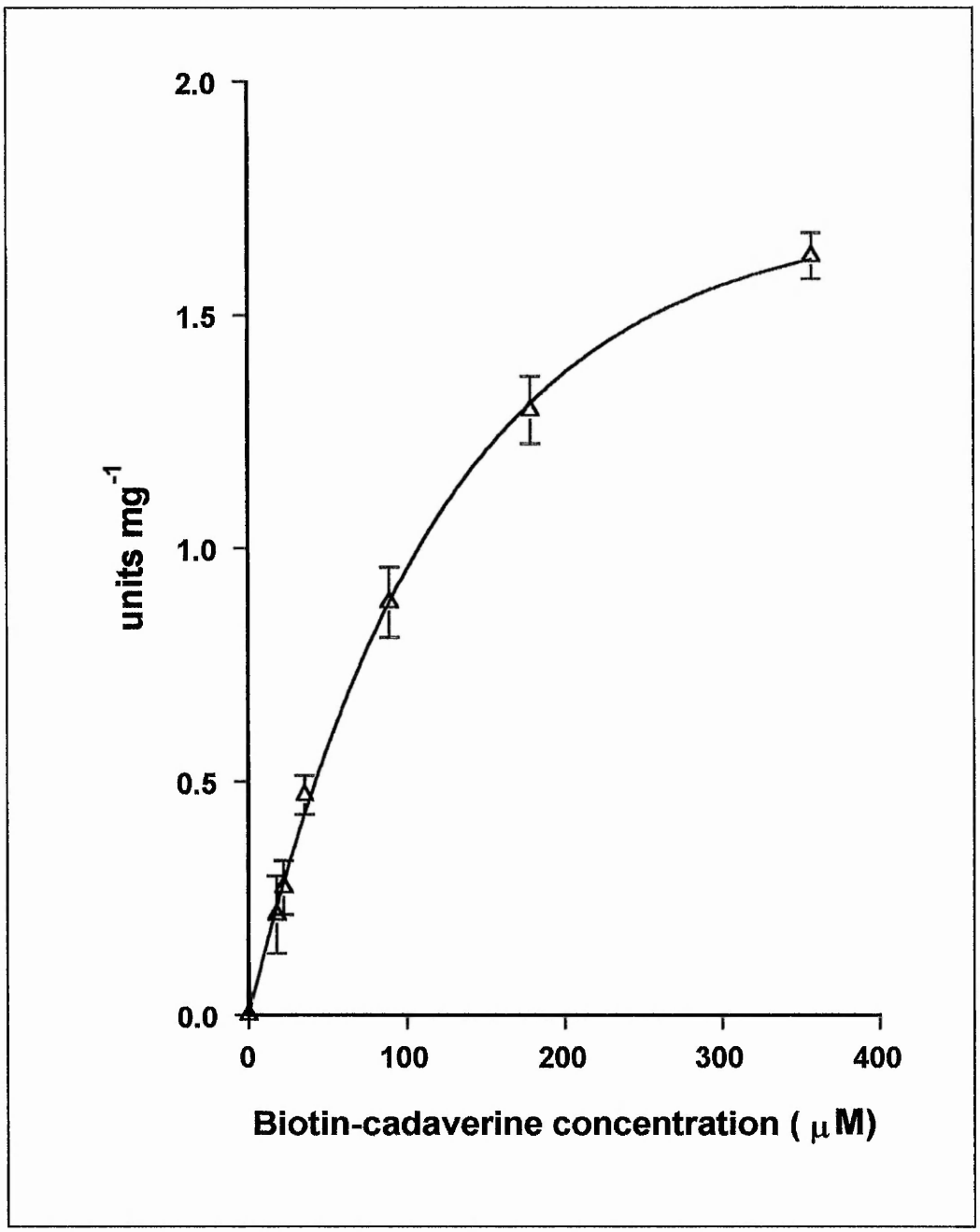

Figure 29 legend.

Ion exchange purified *Pisum sativum* root transglutaminase (prepared as described in section 2.2.4.2) was assayed at 37°C for 60 minutes in the presence of various concentrations of biotin labelled cadaverine as described in section 2.2.3.1. Negative controls containing 250μM EDTA gave an average background of 0.08 units. Boiled extract controls gave an average background of 0.11 units. A buffer only control resulted in a background of 0.10 units. Zero extravidin peroxidase and biotin labelled casein controls resulted in an average absorbance of 0.05 units. Data points represent the mean ± SEM of 4 replicates.

Figure 29 shows that an increase in biotin labelled cadaverine concentration resulted in a non-linear increase in absorbance up to a biotin cadaverine concentration of 375 μM. The K_m of biotin labelled cadaverine was calculated to be 190μM for *Pisum sativum* root transglutaminase (Enzfitter, Biosoft U.K).

Figure 30. Rate of the casein cross-linking reaction as a function of the concentration of biotin labelled casein using ion exchange purified soluble *Pisum sativum* root extract.

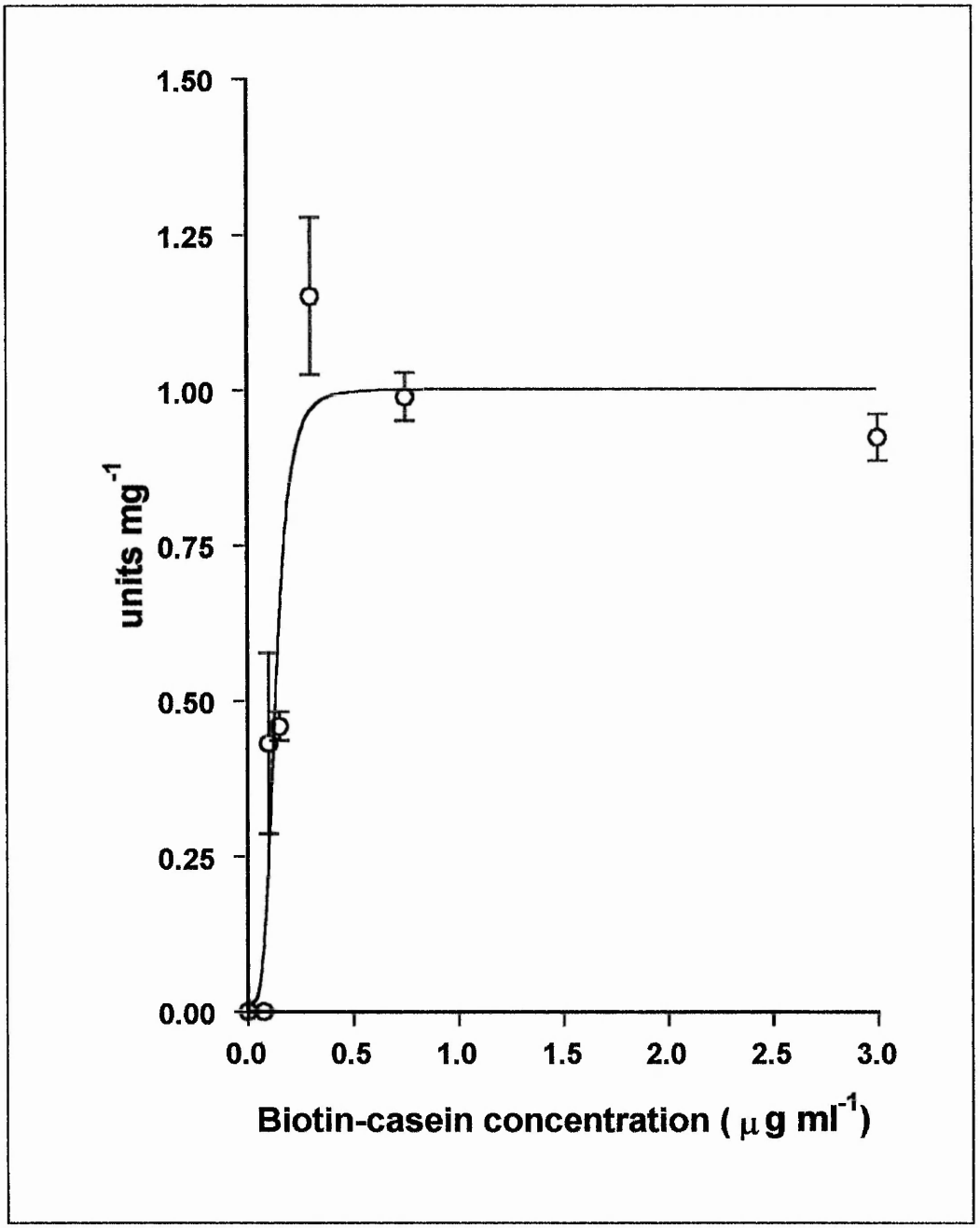

Figure 30 legend.

Ion exchange purified *Pisum sativum* root transglutaminase (prepared as described in section 2.2.4.2) was assayed at 37°C for 60 minutes in the presence of various concentrations of biotin labelled casein as described in section 2.2.3.2. Negative controls containing 250μM EDTA gave an average background absorbance of 0.13 units. Boiling the extract for 20 minutes resulted in an average background of 0.17 units. A buffer only control resulted in a background of 0.15 units. Zero extravidin peroxidase and biotin labelled casein controls resulted in an average absorbance of 0.05 units. Data points represent the mean ± SEM of 4 replicates.

Figure 30 shows that an increase in biotin labelled casein concentration resulted in a linear increase in absorbance up to a biotin casein concentration of 0.5 μg ml^{-1}. The K_m of biotin labelled casein was calculated to be 0.2 μg ml^{-1} for *Pisum sativum* root transglutaminase (Enzfitter, Biosoft U.K).

Table 25. The effect of 10mM iodoacetamide and 10mM NEM on the biotin labelled cadaverine incorporation activity of ion exchange purified soluble *Pisum sativum* root transglutaminase.

	Specific activity Units mg^{-1} ± SEM		
Type of activity	**Uninhibited**	**+ 10mM NEM**	**+ 10mM iodoacetamide**
Casein cross-linking	1.24 ± 0.11	1.29 ± 0.10 (0)	1.27 ± 0.07 (0)
Cadaverine incorporation	1.44 ± 0.09	1.31 ± 0.12 (9.0)	1.19 ± 0.08 (17.4)

Ion exchange purified *Pisum sativum* root extract was assayed for casein cross-linking and cadaverine incorporation activity in the presence of 10mM NEM or 10mM iodoacetamide. The assays were carried out at 37°C for 60 minutes as described in section 2.2.3. The thiol group inhibitors were pre-incubated with the extract in the presence of 5mM $CaCl_2$ for 30 minutes. As a positive control, 100ng per well of guinea pig liver transglutaminase was also incubated in the presence of 10mM concentrations of the thiol group inhibitors and no activity was observed. Data points represent the mean ± SEM of 4 replicates.

Table 25 shows that no inhibition of ion exchange purified *Pisum sativum* root transglutaminase was observed due to the addition of thiol group inhibitors to the casein cross-linking reaction buffer. Small inhibitions of the cadaverine incorporation reaction of 9.0 and 17.4% for NEM and iodoacetamide respectively were however observed (presented in brackets immediately after the specific activity).

6.3. Discussion.

The anion exchange procedure carried out using the crude *Pisum sativum* root extract did result in separation of the proteins. Figure 27 shows that there was a significant quantity of protein that did not bind to the FFQ Sepharose column (fractions 3-7) and a larger quantity of protein that did bind and was removed by the linear sodium chloride gradient (fractions 12-25). A further small quantity of tightly bound protein was also removed by the application of a 2.0M sodium chloride wash to the column (fractions 40-46). The cadaverine incorporation activity was detected in two peaks, one in the unbound and one in the range of sodium chloride concentration between 0.3-0.5M. Both the unbound and the salt eluted activity peaks were assayed for protein cross-linking activity giving values of 1.28 ± 0.11 and 1.41 ± 0.09 units mg^{-1} respectively suggesting that both peaks contain the same enzyme. A relatively poor purification index of 1.19 (table 24) was observed prior to gel filtration despite removal of over 60% of the protein. This was due to the loss of a large amount of the activity in the unbound fractions. If the loss of activity was due to column overloading, the ion exchange step could be improved in future development by either loading less onto the column, using a larger column or a higher capacity resin. For the purpose of the purification, the salt eluted peak of activity was applied to the gel filtration column.

The gel filtration step is represented by figure 28. The technique did successfully separate the proteins remaining after the ion exchange step with the transglutaminase activity detected at an elution volume of 21.5 ml. This step resulted in a final purity index of 6.38 since only 12% of the remaining activity was lost compared to 86% of the remaining protein. Calibration of the column using the method described in section

2.2.4.3 (calibration is inset) resulted in an estimated molecular mass of *Pisum sativum* root transglutaminase of 36kDa. This observation closely corresponds to an estimated molecular mass of 37-39kDa for an amine incorporating enzyme present in *Medicago sativa* floral buds (Keuhn *et al.* 1991). An attempt was made to store the active fractions at -20°C but no activity remained after thawing. This loss of activity may be due to proteolysis or sensitivity to a freeze / thaw cycle. In future purifications a protease inhibitor cocktail should be included to minimise the possibility of proteolytic degradation of the transglutaminase activity.

Plate 5 is an SDS-PAGE electrophoretogram demonstrating the reduction in the number of discrete protein bands present at each stage of the purification. The gel filtration lane of the electrophoretogram does contain a band of molecular mass between 31 and 45kDa. The band in question also appears to be getting stronger in intensity as one might expect following two purification steps.

Figure 29 shows the relationship between activity and the concentration of biotin labelled cadaverine for ion exchange purified *Pisum sativum* root transglutaminase. A K_m of 190μM was estimated and this is similar to the K_m of guinea pig liver transglutaminase as reported by Slaughter *et al.* (1992) using biotin cadaverine as amine donor substrate. Figure 30 demonstrates the kinetics of casein cross-linking by *Pisum sativum* root transglutaminase. A K_m of 0.2 μg ml^{-1} was estimated from the Michaelis-Menten plot using Enzfitter (Biosoft, U. K.) and this is also similar to the K_m reported by Lilley *et al.* (1997a) for guinea pig liver transglutaminase using biotin labelled casein as the glutamine donor substrate.

The inhibition of *Pisum sativum* root casein cross-linking and cadaverine incorporation by thiol group inhibitors was re-tested following ion exchange purification to ascertain whether or not the removal of contaminating proteins would affect the result reported in chapter 4. Little difference was observed using 10mM iodoacetamide in the cadaverine incorporation assay (table 25). The inhibition of *Pisum sativum* root extract by 10mM NEM was however increased by 8% following purification. The casein cross-linking inhibition was however not affected by purification as zero inhibition was observed using either reagent. This observation implies that there may be differences between the active site of mammalian transglutaminases and transglutaminase present in *Pisum sativum* root tissue. The effect of GTP on both the casein cross-linking and cadaverine incorporation activities of the *Pisum sativum* root transglutaminase was also re-tested following ion exchange purification. No inhibition was observed (data not shown) implying that the transglutaminase found in *Pisum sativum* root tissue may have a different regulatory mechanism to mammalian tissue transglutaminase.

The *N',N'*-bis (γ-glutamyl) polyamine cross-linking ability of the *Pisum sativum* root extract was also re-tested following purification by ion exchange chromatography. Despite the small increase in specific activity observed following purification, no signal was obtained using the *N',N'*-bis (γ-glutamyl) polyamine assay. This type of cross-link has been detected in plant tissue using a variation of the amino acid analysis procedure described in chapter 5 (Del Duca *et al.* 1995) and so it is likely that plant transglutaminase is able to perform the *N',N'*-bis(γ-glutamyl) polyamine cross-linking reaction. The lack of detection of this activity may be due in part to the sensitivity of the assay used. The ε-(γ-glutamyl) lysine assay did give a greater overall signal when using

guinea pig liver transglutaminase (see chapter 3). The failure of the assay to detect activity in plant extracts may also reflect the unsuitability of the casein substrate for this reaction when using plant protein.

In conclusion, the techniques of ion exchange chromatography and gel filtration chromatography are relevant methods for transglutaminase purification (Wilhelm *et al.* 1996) but in the case of the ion exchange step, further refinement will be required to allow greater yield recovery and purity of the *Pisum sativum* root enzyme. There are many other anion exchange media available including, DEAE Sephadex, QAE Sephadex, DEAE Sepharose and DEAE Sephacel, which may prove more suitable as an initial purification step for *Pisum sativum* root transglutaminase. It may also be possible to develop an affinity step, based on the transglutaminase reaction mechanism. *Pisum sativum* root transglutaminase could be bound to the glutamine residues of immobilised α-casein agarose as described for Glycine max transglutaminase (Kang and Cho 1996). It would then be possible to elute the enzyme using a polyamine such as spermidine. Using this approach, it is possible that some of the more conventional purification strategies may be bypassed.

Chapter 7- Concluding remarks.

The screening of crude plant protein preparations using the conventional [1,4 ^{14}C]-labelled putrescine incorporation assay as described in chapter 4 resulted in the detection of relatively low levels of transglutaminase activity in only three of the eight tissues tested. This observation is broadly in line with the results of other workers who report much lower levels of transglutaminase activity in plant tissue compared to mammalian tissue when using assays of this type (Serafini-Fracassini *et al.* 1988; Signorini *et al.*1991; Del Duca *et al.* 1994). The results in chapter 4 also concur with the research of the aforementioned groups, in terms of the apparent lack of calcium ion dependence of the radiolabelled amine incorporation reaction. Table 11 does however show that up to 29% of the radiolabel is incorporated in a copper dependent manner. This finding supports the conclusion of others, who propose that diamine oxidases may be responsible for the radiolabelled amine incorporation reaction of crude plant extracts (Chiarello *et al.* 1996a and 1996b; Siepaio and Meunier 1995). This growing body of evidence seriously brings into question the suitability of the radiolabel assay for plant transglutaminase research. The results in chapter 4 do show that both soluble and membrane associated plant transglutaminases are able to carry out the incorporation of biotin labelled cadaverine into *N',N'*-dimethylcasein (reaction 1.1.2.2.1) and also the cross-linking of chemically modified caseins (reaction1.1.2.2.2) in a calcium dependent manner indicating that both of the plate assays employed are more suitable for plant transglutaminase detection than the conventional radiolabelled amine method (Lilley *et al.*1998). In addition to the polyamine incorporation and protein cross-linking reactions, mammalian transglutaminases are able to carry out the formation of *N',N'*-bis(γ-glutamyl) polyamine

cross-bridges (Folk 1980- reaction 1.1.2.2.3). The crude plant extracts were unable to catalyse this reaction when using casein as the acyl donor substrate and spermidine as the modifying polyamine. This suggests either, differences in the portfolio of reactions carried out by plant transglutaminase, or more likely the unsuitability of the assay system used because protein-polyamine-protein conjugates have been isolated in plant tissue (Del Duca *et al.* 1995).

Soluble *Pisum sativum* root biotin labelled cadaverine incorporation activity is activated by nanomolar levels of calcium ions, suggesting that the polyamine incorporation reaction of the enzyme is calcium dependent. The soluble *Pisum sativum* root extract mediated casein cross-linking reaction, however, requires millimolar levels of calcium and this suggests that the protein cross-linking role of the enzyme occurs in a high calcium area such as the extracellular environment. This is consistent with proposed roles for tissue transglutaminase, which include stabilisation of the mammalian extracellular matrix due to the cross-linking of proteins (Aeschlimann and Paulsson 1991). This may also indicate that if the membrane-associated enzyme has a protein cross-linking function, its orientation within the membrane may be towards the extracellular environment.

The results in chapters 4 and 6 show that GTP has no effect on the activity of soluble *Pisum sativum* root transglutaminase when applied at sub-optimal calcium ion concentration thus implying differences between mammalian tissue transglutaminase and the enzyme present in *Pisum sativum* root tissue in terms of mechanisms of regulation. Further possible differences are apparent as shown by the effect of the thiol group

reagents, iodoacetamide and NEM. A 10mM concentration of these reagents failed to completely inhibit either the cadaverine incorporation reaction or the casein cross-linking reaction of soluble *Pisum sativum* root transglutaminase and this implies that there may be differences between the active site of mammalian transglutaminases and those present in higher plants.

The data in chapter 5 provides unequivocal proof of the presence of a catalytically active transglutaminase in *Pisum sativum* root and leaf tissue since the ε-(γ-glutamyl) lysine isodipeptide product of the transglutaminase reaction was detected (Folk and Finlayson 1977). The isodipeptide was present at significantly lower levels than that observed in mammalian fluids such as clotted semen and blood plasma (Pisano *et al.* 1971; Williams-Ashman *et al.* 1972) and this necessitated the pre-purification of the *Pisum sativum* leaf protein to reduce the level of the interfering amino acid leucine. Furthermore, the casein cross-linking assay was validated due to detection of the ε-(γ-glutamyl) lysine isodipeptide in a sample of casein, cross-linked by the soluble *Pisum sativum* root extract.

The work presented in this thesis establishes the presence of a catalytically active transglutaminase in plant tissue but in order to elucidate a role for the enzyme, the partial purification protocol described in chapter 6 should be improved and further developed in order to purify the enzyme to homogeneity. The methods chosen, namely ion exchange and gel filtration chromatography are relevant for the purification of transglutaminases (Ando *et al.* 1987; Ichinose *et al.* 1993) but require further refinement to allow greater yield recovery and purity of the *Pisum sativum* root enzyme. Following purification, the

amino acid sequence of *Pisum sativum* root transglutaminase may be determined and compared to that of known mammalian transglutaminases. Furthermore, monoclonal antibodies may be raised which will enable the localisation of plant transglutaminase and subsequent determination of possible roles for the enzyme in plant tissue.

If plant transglutaminase has a similar range of tissue stabilisation roles as the mammalian transglutaminase enzymes, one possible biological role could include root development. The data in figure 22 shows that membrane associated transglutaminase activity is highest during the first few days of *Pisum sativum* root growth. This may indicate that transglutaminase could be involved in developmental processes, which may involve the cross-linking of structural proteins at the root tip to aid the mechanical displacement of soil during root growth. It has also been suggested that transglutaminase may have a protein cross-linking role during cell wall assembly and organisation of *Helianthus tuberosus* tuber cells (Grandi *et al.* 1992). A further stabilising role for transglutaminase enzymes in plants may be to cross-link proteins required for the development of the protective outer coat (testa) of seeds.

Figure 21 shows that soluble *Pisum sativum* leaf transglutaminase activity increases during leaf development and this data is consistent with the findings of others who have speculated roles for transglutaminase in photosynthesis. If the plant enzyme has a protein cross-linking role in green tissue, it may be involved in the assembly of the catalytically active L_8S_8 RuBisCo structure, which forms via a RuBisCo L dimer (Roy *et al.* 1988).

Transglutaminase enzymes are widely distributed throughout animal tissues and body fluids and some important biological functions have been ascribed to certain members of this enzyme family. The fact that transglutaminases have important biological roles in animals implies that these enzymes may also be carrying out vital functions in plants.

Plant transglutaminase research is still in its infancy, and clearly there are interesting avenues of investigation to be followed for future work, which should eventually elucidate the role of transglutaminases in plant tissue.

Chapter 8- References.

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Chapter 9- Appendix.

Figure 31. BCA protein assay standard curve showing absorbance at 562nm as a function of BSA concentration.

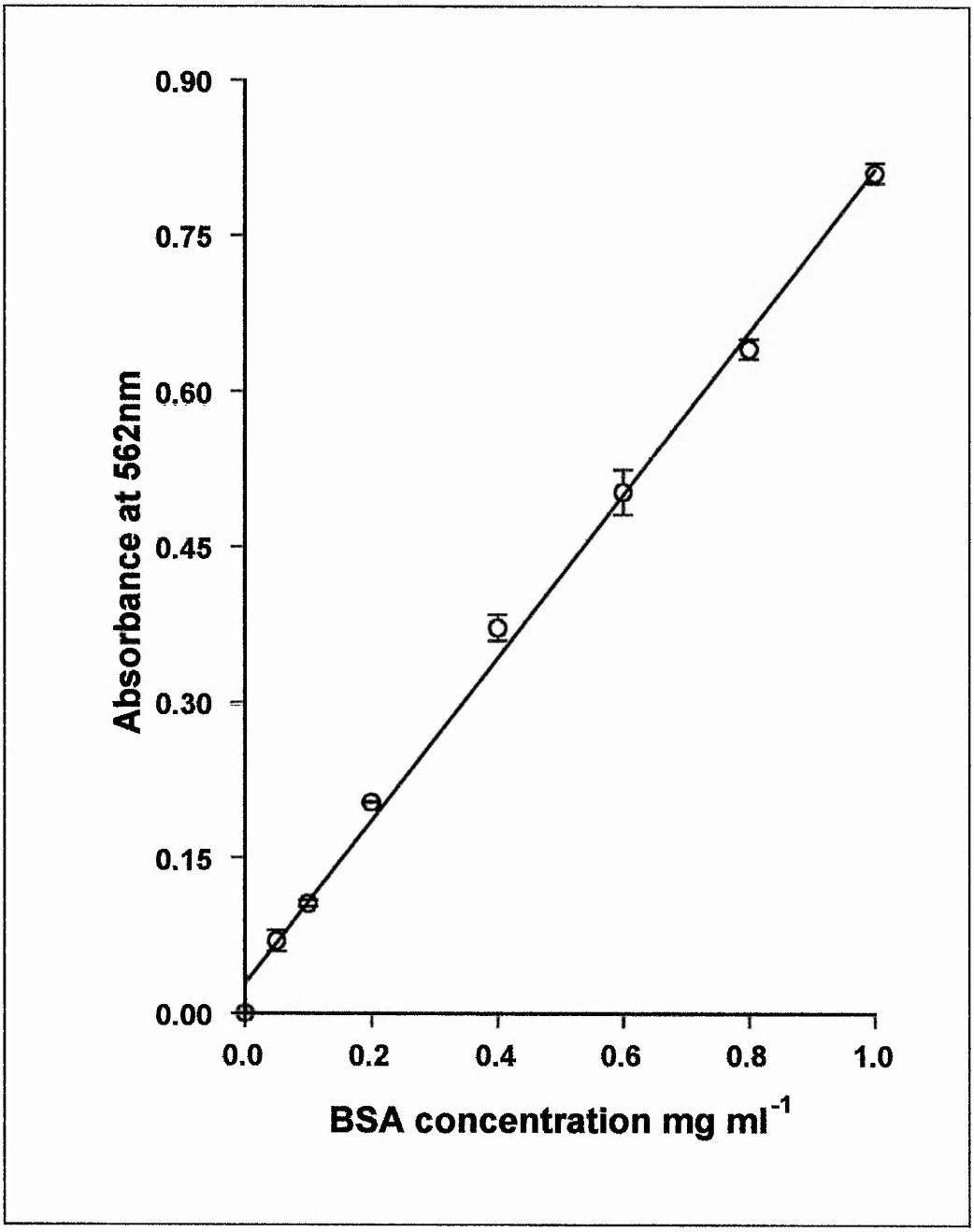

Figure 31 legend.

Figure 31 shows a typical BCA protein assay as described in section 2.2.1.6. The data points represent the mean ± SEM of duplicate experiments. Protein concentrations between 3.1 and 6.0 mg ml^{-1} were obtained for soluble *Pisum sativum* root extracts (section 2.2.1.6) giving average activities of 1.04 ± 0.06 units mg^{-1} for cadaverine incorporation and 1.06 ± 0.08 units mg^{-1} for casein cross-linking.