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The mobilization of storage proteins in germinating *Vicia faba*.

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ABBREVIATIONS

AEE;	Arginine ethyl ester.
BAEE;	N α- Benzoyl-L-arginine ethyl ester.
BTEE;	N α- Benzoyl-L-Tyrosine ethyl ester.
BAPNA;	N α- Benzoyl-DL-arginine-paranitroanalide.
BDH;	British Drug Houses, England.
BRL;	Bethesada Research Laboratories, Cambridge, England.
CBZ;	N-Carbobenzoxy.
DTT;	Dithiothreitol.
EDTA;	Ethylenediaminetetraacetic acid (Sodium salt)
ELISA;	Enzyme linked immunosubstrate assay.
HEPES;	N-2-Hydroxyethylpiperazine-N'-2-ethane Sulphonic acid.
HPLC;	High pressure liquid chromatography.
IEF;	Isoelectric focussing.
NaAC;	Sodium acetate.
NPE;	Nitrophenol ester
PAGE;	Polyacrylamide gel electrophoresis.
PBA;	Phenyl butyl amine.
PBS;	Phosphate buffered saline.
PMSF;	Phenylmethylsulphonylfluoride.
PNA;	Paranitroanalide.
SDS;	Sodium dodecyl sulphate.
TCA;	Trichloroacetic acid.
TEMED;	N, N, N",N'-Tetramethylethylenediamine.
TRICINE;	N-tris (Hydroxymethyl) methyl glycine; N-(2-Hydroxy-1, 1, - bis (hydroxy methyl) ethyl glycine.
ZIPE;	Zonal isoelectric precipitation electrophoresis.

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ABSTRACT

The Mobilization of storage proteins in germinating Vicia faba

Philip Leslie Richard Bonner

Proteolysis in germinating Vicia faba has been investigated in two ways. Firstly, by isolating and characterising two protease enzymes and secondly, by examining the reserve proteins present in seeds at different times during germination.

One of the enzymes was a proteinase with an apparent molecular weight of 12,000 daltons, the other an aminopeptidase with a molecular weight of 50,000 daltons, both molecular weights estimated by gel filtration. The enzymes were inhibited by N-ethylmaleimide, iodoacetamide and p-chloromercuribenzene sulphonic acid, whilst their activity was stimulated by reducing agents, indicating the presence of a thiol group at their active sites. The proteinase had a pH optima of 5.4 and was active on the storage proteins legumin and vicilin isolated from days 0, 4, 7 and 11 germinated seeds, as well as the nitrophenol esters of asparagine and glycine. The peptidase had a pH optima of 7.4 and showed no activity on the storage proteins legumin and vicilin, but was active on a variety of di- and tri-peptides, prefering glycine as the N-terminal residue next to a hydrophobic amino acid containing an aromatic residue. Antibodies raised to the peptidase in rabbits were used to show that there is an antigenically similar enzyme in cotyledons of Pisum sativum, Vigna radiata, Phaseolusvulgaris and another variety of Vicia faba, Green Windsor, but not in cereal seeds.

Legumin and vicilin were isolated from seeds at various stages of germination and checked for purity by Zonal isoelectric precipitation electrophoresis, a method developed during this study. Using PAGE, SDS-PAGE and IEF in dissociating and reducing conditions, the storage proteins were shown to alter in composition as germination proceeded. Larger subunits disappeared whilst smaller subunits accumulated. These changes were more apparent in vicilin than legumin.

INTRODUCTION

All seeds contain reserve materials, located principally in the endosperm of monocotyledons and the cotyledons of dicotyledonous plants. Germination is accompanied by marked changes in chemical composition and ultrastructure of the various, seed parts. The reserve materials, which consist mainly of lipids, carbohydrates and proteins are hydrolysed during germination, the products of which are transported to the growing axis and used for the metabolic processes which eventually leads to the seedling growth (Mayer and Marbach, 1981; Ashton, 1976).

In many seeds, proteins are the major reserve material; on average the percentage protein in cereal grains is 10-15% of dry weight, in legume seeds 20-25% of dry weight in wild species (Derbyshire et al., 1976) and up to 50% of dry weight in some cultivated species (Brohult, and Sandegren, 1954). These proteins are hydrolysed to amino acids, which can be used as substrates for the synthesis of new proteins, or after deamination or transamination the carbon skeletons may be oxidised to produce ATP and other metabolically important compounds. The hydrolysis of the proteins to amino acids progresses in an orderly fashion, the changes in the substrates brought about by one group of proteolytic enzymes allows another group of enzymes to further the degradation of the proteins. To understand this problem

information into both the changes observed in the storage proteins as germination proceeds, and the proteolytic enzymes involved in their hydrolysis is required.

In Vicia faba seeds the major storage proteins are the globulins legumin and vicilin, these comprise approximately 70% of the total seed protein (Mosse and Pernollet, 1982). These proteins are synthesized as the seed develops (Bailey et al., 1970; Millerd, 1975; Pernollet, 1978) during the phase of growth (cell expansion) which follows the phase of rapid cell division, and are stored in discrete vesicles called protein bodies.

Danielsson, (1949), showed that the globulin storage proteins were characterized in the ultracentrifuge by the presence of two peaks with sedimentation coefficients of about 11-12S and 7S. The 11-12S peak corresponded to legumin and the 7S peak to vicilin.

The structure of these proteins is still a subject of much discussion. Legumin has a molecular weight of approximately 350,000 daltons (Bailey and Boulter 1970). Comprising of acidic subunits (α) of approximately 37,000 daltons and basic subunits (β) of approximately 20,000 daltons, with equimolar amounts of di-sulphide bound α and β chains (Wright and Boulter, 1974). The subunit pairs belong to two structural types, one contains methionine the other does not, arising from two classes of gene families. The heterogeneity of the subunits, arising from mutational alterations

of a common ancestral gene (Horstmann, 1983), Both the α and β chains are heterogeneous in charge and size, with the proposed arrangement of the holoprotein being $\alpha_6\beta_6$ (Vaintraub et al., 1962; Wright and Boulter, 1974).

Croy et al., (1980 a, b), investigating in vitro proteo synthesis of the storage proteins in pea found that the legumin α and β chains are synthesized as a single 60,000 dalton polypeptide chain. This coupled with the fact that SDS-PAGE of legumin without the reducing agent 2-mercaptoethanol, reveals only three protein bands at 62,000, 60,000 and 48,000 daltons (Mori and Utsumi, 1979), has led to the proposal that these represent the real subunits.

However, after the synthesis of the 60,000 dalton chain, post translational enzymes cleave the polypeptide into two chains. It has been postulated that this event maybe the equivalent of the "signal" peptide observed in animal secretory proteins (Austen, 1979), allowing the transport of the storage proteins to eventual deposition in the protein bodies.

The structure of vicilin is still the subject of intense investigation, this maybe because the protein is the minor globulin constituent of the majority of legumes, or it maybe because of the heterogeneous nature of the protein.

The molecular weight of the holoprotein is reported as 150,000 daltons for Vicia faba (Wright and Boulter, 1973), but varies considerably from species to species, from 193,000 daltons for vicilin from Vigna sativa (Shutov and Vaintraub, 1966) to 105,000 daltons for the vicilin type protein from Glycine max (Naismith, 1955).

The subunit composition of the vicilin proteins again varies from species to species, but the major subunits range from 23,000 daltons to 56,000 daltons (Derbyshire et al. 1976) and vicilin type proteins can be classed into 5 distinct groups. These polypeptides are synthesized initially as 50,000 and 47,000 dalton subunits which are cleaved to 33,000, 19,000, 13,000 and 12,500 dalton subunits found in the holoprotein (Croy et al., 1980 (b); Gatehouse et al., 1981; Higgins and Spencer, 1981). The exact arrangements of the subunits to compose the holoprotein has yet to be suggested.

Other storage proteins have been isolated from a variety of legumes including a third storage protein, convicilin from Pisum sativum (Croy et al., 1980 (c)), also globulins with low sedimentary coefficients 2S-4 S (Blagrove and Gillespie, 1975; Catsimpoolas and Ekenstam, 1969) and storage albumins from Pisum sativum seeds (Grant et al., 1976 ; Murray, 1979).

The storage proteins of legumes are stored within the protein bodies (Weber and Neumann, 1980; Graham and Gunning, 1970), which are bounded by a single membrane which apparently originates from a vacuole during germination (Beevers, 1982). When the seed germinates and the storage proteins are metabolised the opaque contents of the protein bodies disappear and the normally small protein bodies fuse together, eventually to form a large central vacuole (Briarty et al., 1970).

The protein bodies have been proved to be the site of storage protein metabolism by Nishimura and Beevers, (1979). They isolated vacuoles from the endosperm of germinating castor beans by generating protoplasts and locating the proteinase activity in the protein bodies. Baumgartner et al. (1978), located the major proteinase of mung bean, vicilin peptidohydrolase, within the protein body by means of immunofluorescence microscopy.

Protein degradation in germinating seeds has been investigated by a number of workers. Catsimpoolas et al. (1968) working with germinating soybean seeds and Daussant et al. (1969), working with germinating peanut seeds, observed change in the electrophoretic mobility of the storage proteins isolated from cotyledons at various stages of germination. They attributed these changes to a loss in amide nitrogen, proposing that the loss in amide nitrogen was a necessary prerequisite before any proteinase could metabolize the storage

proteins. Korolyora et al.(1975) also noted these changes in electrophoretic mobility of the storage protein of vetch seeds, but attributed the changes to the depletion in lysine rich regions of the storage proteins.

Lichtenfeld et al. (1979), working on germinating Vicia faba seeds found the decrease in amide nitrogen of the storage proteins did not occur until after the sixth day from the onset of germination. This was after the storage proteins showed signs of proteolytic degradation. They concluded that the loss in amide nitrogen was unlikely to be the prelude to proteinase activity, favouring preformed proteases as the likely candidates, before de novo synthesized enzymes concluded the breakdown of the storage proteins.

The rate of breakdown of the storage proteins has been investigated by means of immunology by Lichtenfeld et al., (1979), who concluded that legumin was broken down earlier and at a faster rate than vicilin, this was in agreement with earlier work by Basha and Beevers (1975). Both of these studies observed that the larger subunits of a crude globulin extract were the first to disappear as germination proceeded.

What types of proteolytic enzymes are involved in the metabolism of storage proteins? Enzymes which cleave peptide bonds are termed "proteases" , those which cleave internal peptide bonds are termed "proteinases" and those which cleave single amino acids from either the

C- or N- terminal end of a protein are termed "peptidases" (Barret, 1980). There is also another group of enzymes which have a specificity for peptides of varying lengths, these are normally referred to as tri-or dipeptidases depending on their preference for the substrates.

There are six different sets of proteases based on their active site catalytic mechanisms (Hartley, 1960; Walsh, 1975). There are four sets of proteinase; sulphydryl, serine, metal and acid, and two sets of peptidase; serine and metal.

Proteolytic activity in plants has been the subject of intense investigation over the past twenty years and the progress has been reviewed many times. (Ryan, 1973; Ryan and Walker-Simmons, 1981; Mikola, 1983). Despite the intensity, investigation has been hampered by a number of difficulties,succinctly set out by Ashton (1976). The comparison of results between workers has been difficult because of different growth conditions used to germinate the seeds. Often, workers have used crude extracts to assay proteolytic activity, which can lead to misleading results due to the possible presence of more than one enzyme, endogeneous inhibitors, or natural substrates.

An early example of this is work done by Laufer et al.(1944).They prepared crude extracts from 1, 6 and 12 day germinated soybean seeds and found that the proteolytic activity against casein and gelatin increased four fold. The pH optima for this activity was between 6.7 and 7.0.

However, with the advent of synthetic substrates "specific" for proteolytic enzymes and the development of a quantitative assay for amino acids using ninhydrin (Yemm and Cocking, 1955), progress has been accelerated in the last twenty years. To avoid the difficulties outlined above, research has concentrated on the purification and characterization of the proteases present in plants and in particular those involved in germination.

Mainguy et al. (1972) isolated a serine peptidase from six day old peanut cotyledons, which had a pH optima of 7.4. This enzyme degraded Nα-Benzoyl-L-arginine-p-nitroanalide but would not hydrolyse complex proteins such as casein. Mikola (1976), working with the same organism found carboxypeptidase activity at pH 5.2 and three "naphthylamidases" with a pH optima of 7.2. These enzymes were active on Nα-Benzoyl amino acid naphthylamides, which maybe considered as neutral or alkaline peptidases. Elleman (1974) isolated three amino peptidases from germinating pea seeds, one was proline specific, of the other two, one was specific for hydrophobic amino acids, the other with a broader specificity showing preferential hydrolysis of non hydrophobic amino acids. These latter two proteins were purified and partially characterised, their molecular weights were 58,000 and 74,000 daltons as estimated by gel filtration, and they were inhibited to varying degrees by sulphydryl inhibitors.

The majority of other investigators have concentrated on enzymes present in monocotyledonous seeds. Visuri et al. (1969), isolated a carboxypeptidase from germinating barley seeds. Preston and Kruger (1976, 1977) isolated and characterised two carboxypeptidases from germinating wheat seeds. These enzymes exhibited a broad specificity on synthetic substrates and inhibition studies indicated the presence of a serine group at the active site. Sopanen (1976) isolated a dipeptidase from germinating barley seeds, which had a pH optima of 8.8 and was inhibited by sulphydryl reagents and metal chelators.

These results indicate that germinating seeds contain a wide variety of peptidases with differing specificities and active sites. The location of these enzymes has been investigated by Nishimura and Beevers (1978); they found carboxypeptidase activity in the central vacuole formed by the fusion of protein bodies. The aminopeptidase location is believed to be cytoplasmic because of their in vitro pH optima.

A wide range of proteinases have been purified from germinating seeds, they all appear to be small molecules approximately 25,000 daltons, with a sulphydryl group in the active centre, and a pH optima between 3.5 and 5.5.

Vavreinova and Turkova (1975), isolated a sulphydryl proteinase from germinating Phaseolus vulgaris with a molecular weight of 27,000 which was most stable at pH's between 6 and 10.

Shutov and co-workers (Korolyora et al.,1975; Shutov et al.,1976; Bul'maga and Shutov, 1977) in a series of papers investigated the action of vetch seed proteases on the storage proteins from germinating vetch seeds. They found that there were three enzymes,one from dormant seeds which was inactive on the storage proteins isolated from dry seeds, but was active on the storage proteins isolated from germinated seeds. Later, they partially purified an enzyme, which they termed "Protease A" from germinating seeds, which was also active on the storage proteins.

The only other detailed investigation into the isolation and characterization of a proteinase involved in seed germination of legumes is the work by Chrispeels and co-workers on germinating mung bean (Vigna radiata) (Chrispeels and Boulter, 1975; Chrispeels et al.,1976; Baumgartner and Chrispeels, 1976; Baumgartner and Chrispeels, 1977; Baumgartner et al.,1978; Van der Wilden et al., 1980).

They found that the protease activity had a broad pH optima between 5.0 and 9.0 and increased gradually to a maximum after four days. Carboxypeptidase activity was maximal after six days and aminopeptidase activity declined gradually as germination proceeded. The proteinase and carboxypeptidase activity was associated with the protein bodies, but the aminopeptidase activity was found within the cytoplasm.

The proteinase, vicilin peptidohydrolase had a molecular weight of 23,000 daltons, with a pH optima of 5.1. It had a broad specificity capable of digesting other proteins as well as the storage proteins. They found that vicilin peptidohydrolase accounted for 95% of all proteolytic activity of the cotyledons, five days after the onset of germination. The enzyme in conjunction with carboxy-peptidases was capable of completely digesting vicilin to amino acids.

As mentioned previously, the enzyme was located within the protein bodies, three days after the onset of germination. The enzyme first appeared in vesicles emanating from the rough endoplasmic reticulum and then later in the protein bodies. They concluded that the enzyme was synthesized de novo on the endoplasmic reticulum, then transported in vesicles which fuse with the protein bodies.

Proteolytic activity and the enzymes involved has also been investigated using other plant tissues and some details are included for comparison, for example; Drivadhl and Thimann (1977, 1978) isolated two proteases from senescing oat leaves, one a proteinase with a pH optima of 4.2 was a serine type proteinase, the other a peptidase with a pH optima of 6.6 was a sulphydryl enzyme, both had molecular weights of 76,000 daltons. Several acid proteinases have been isolated from green wheat leaves

(Frith *etal*., 1978 a,b.) which hydrolyse haemoglobin as well as an endogenous protein 1,ribulose 5-bisphosphate carboxylase. Two proteinases with an alkaline pH optima have been identified in soybean (*Glycine max*) leaves, one was a metalloenzyme the other a sulphydryl enzyme (Ragster and Chrispeels,1979).

Peptide hydrolases were isolated from maize root by Shannon and Wallace (1979),Proteinase I and Carboxypeptidase I had a serine group at the active site with molecular weights of 54,000 and 77,000 daltons respectively. Proteinase I was responsible for the majority of nitrate-reductase-inactivation, unlike proteinase II,a sulphydryl enzyme. As with the peptidases there is a wide range of proteinases in plant tissue.

In germinating seeds, because of the lack of detailed investigation into other legume species, the model for storage protein hydrolysis of Chrispeels *et al*. (1979) is the most reasonable. The acid proteinase is associated with the protein bodies and is responsible for the primary degradation of the storage proteins, to be completed by carboxypeptidases also present in the protein bodies. The acid proteinase is *de novo* synthesized as germination proceeds, and does not appear to be responsible for the immediate changes observed in extracted storage proteins. The role of the aminopeptidases and di or tri peptidases. has yet to be elucidated; this is primarily because of their high pH optimas, but it seems likely that they are

involved in the last step of amino acid generation. Their location appears to be cytoplasmic, but Waters et al.,(1982) found high concentration of aminopeptidases in the chloroplast of mesophyll tissue of wheat leaves. Van der Wilden (quoted from Mikola, 1983) has proposed that the alkaline peptidases may be located within the cell wall.

Despite the seemingly diverse information available, the actual number of plant enzyme systems investigated is still quite small. Research into the metabolism of storage proteins in economically important plants is necessary to help improve our understanding of the processes involved. Improvements in the amino acid composition of the storage proteins will improve their nutritional value and,as oil prices rise and fertilizers become more expensive, the status of legumes (capable of "fixing" atmospheric nitrogen with symbiotic bacteria) will be elevated. The seeds of Vicia faba, the field or broad bean, are already an important source of protein in many developing countries, such as Egypt (Hegazi,1975). They have also been used as a meat substitute (Flink and Christiansen, 1973).

Vicia faba was chosen for investigation because of these reasons and because it is easy to grow. Previous work carried out at Trent (Taylor, 1979), using crude extracts free of storage proteins and amino acids, showed that protease activity had several pH optima,

at pH 5.4, 6.4 and 7.2. In ungerminated seeds the pH 7.2 enzyme was most active and this corresponded to amino-peptidase activity. The pH 5.4 enzymes activity increased gradually as germination proceeded to a maximum after seven days, this corresponded to proteinase activity.

By using a novel electrophoretic assay, in which casein was immobilized in a polyacrylamide matrix, several different proteinases could be detected. Each one had maximal activity at different stages of germination, and the activity varied with each legume species and different extraction procedures.

This work still left a number of questions to be answered; what types of enzymes were involved? what were their characteristics compared to other documented enzymes? how many enzymes were involved? were they physiologically important i.e. were they active on storage proteins? and where were they located within the cell?. These questions would also help verify the model of Chrispeels *et al.* (1979).

In order to answer some of these questions during this study it was necessary to devise a procedure to isolate and purify the proteases present in germinating *Vicia faba* cotyledons. Particular reference was made to the alkaline peptidases, in order to help clarify the role of these enzymes in the cotyledons of germinating legumes.

In parallel with the above investigations, the storage protein substrates were isolated from various stages of germination, separated into legumin and vicilin and the detailed changes of the storage proteins were also investigated.

MATERIALS

(i) Biological

Seeds of Vicia faba var. Yates Fillbasket were obtained from Asmer Seeds Ltd, Leicester, England. Other seeds were obtained locally.

(ii) Chemical

Most chemicals were obtained from either BDH Chemicals Ltd, Atherstone, England, or The Sigma Chemical Company Ltd, Poole, England. and were the highest grade available.

Other specialist chemicals included Phenyl butyl amine, N-Carbobenzoxy-Citrulline-para-nitroanalide, Citrulline paranitroanalide obtained from Koch Light Laboratories Ltd, Slough, England and Pharmalytes from Pharmacia Uppsala, Sweden.

All chromatography media was obtained from Pharmacia Uppsala, Sweden.

METHODS

1. Growth of seeds

Seeds of Vicia faba (var. Yates fillbasket) were surface sterilized in 10% (v/v) 'Chloros' for one hour and then soaked overnight in running tap water. The seeds were further surface sterilized in 5% (v/v) 'Chloros' for 30 minutes before sowing the imbibed seeds in sterile, moist vermiculite in plant propagators.

The seeds were then grown at room temperature (ca. 20-23^{o}) under continuous illumination, provided by 6 'Atlas Grow Lux' fluorescent tubes, 0.5m above the vermiculite surface.

2. Harvesting the seeds

The testas and axes were removed from ungerminated seeds and the cotyledons were ground into a fine flour using an 8 inch laboratory mill. The flour was stored at 4^{o} until required.

Imbibed seeds were germinated for different time periods, after which the testas and axes were removed. The cotyledons were soaked in 70% (v/v) aqueous methanol for 5 minutes followed by repeated washing in distilled water and finally dried on tissue. The cotyledons were then stored at -20^{o} or used immediately for extraction.

3. Preparation of globulins

(i) Total globulins.

The flour from day 0 cotyledons was stirred for one hour with 0.1M sodium phosphate buffer pH7.0 (5.3g $NaH_2PO_4:H_2O$; 5.43g $Na_2HPO_4:2H_2O$ dissolved in 1.0ℓ of distilled water), the ratio of flour to buffer being 1:4 (w/v).

Cotyledons of different ages were homogenized in 0.1M sodium phosphate buffer with a Waring blender, using two thirty second bursts.

The homogenate was gently stirred for one hour at 4^{o}, the ratio of cotyledons to buffer being 1:2 (w/v) (Taylor, 1979).

Extracts of ungerminated and germinated seeds were filtered through two layers of muslin and then centrifuged at 20,000g for 30 minutes to remove cell debris. The supernatants were dialysed against 2.0ℓ distilled water, with four changes of water over a 24 hour period at 4^{o}.

The globulins that precipitated were collected by centrifugation at 20,000g for 30 minutes. The precipitate was washed with distilled water and re-centrifuged at 20,000g for 30 minutes.

The precipitate was slurried in distilled water and freeze dried overnight. The freeze-dried protein was stored at -20^{o} in airtight vials until used.

(ii) Purified globulins: vicilin and legumin.

Initial extracts of the cotyledons were prepared as described above, except that the extraction buffer used was 1M NaCl, 0.05M $Na_2HPO_4.2H_2O$ pH 7.0 (58.44g of NaCl, 7.80g $NaH_2PO_4.2H_2O$ dissolved in 900.0ml distilled water and the pH adjusted to 7.0 with dilute NaOH and made up to 1.0ℓ with distilled water.) (Wright and Boulter, 1974).

The supernatant fluid was brought to 90-100% saturation with 'Aristar' ammonium sulphate (B.D.H), stirred for 1 hour at 4^{o}, and then centrifuged at 20,000g for 30 minutes to collect the precipitated protein.

The precipitate was washed with saturated ammonium sulphate and the precipitate was recentrifuged at 20,000g for 30 minutes. The washed precipitate was then freeze dried overnight. The freeze-driedprotein was stored at -20^{o} in air tight vials until used.

For further purification of the globulins, 50.0mg of the protein material was dissolved in 10.0ml of 0.2M NaCl, 0.05M NaH_2PO_4pH 8.0, (11.69g of NaCl, 7.80g of $NaH_2PO_4.2H_2O$ was dissolved in 900ml of distilled water, the pH was adjusted with dilute NaOH and the volume made up to 1.0ℓ with distilled water) and applied to a G.50/80 Sephadex column (2.6 x 60cm) equilibrated with 0.2M

NaCl, 0.05M citric acid pH4.7 (11.69g of NaCl, 10.57g of citric acid dissolved in 900ml of distilled water, the pH was adjusted with dilute NaOH and the volume made up to 1.0ℓ with distilled water.) The column was eluted with 0.2MNaCl, 0.05M citric acid pH4.7 at 30ml $hour^{-1}$ and monitored continuously at 280nm using an LKB Uvicord SII.

The protein fractions were eluted from the column and were labelled peak I and peak II. They were dialysed separately against 2.0ℓ of distilled water, with four changes of water over a 24 hour period at 4^{o}. The precipitated protein was collected by centrifugation at 20,000g for 30 minutes.

The peak I fraction was resuspended in 0.1M NaCl, 0.05M citric acid pH4.7, (5.84g of NaCl, 10.57g of citric acid dissolved in 900ml of distilled water, the pH was adjusted with dilute NaOH and the volume made up to 1.0ℓ with distilled water) and recentrifuged at 20,000g for 30 minutes to remove contaminating legumin. The supernatant was dialysed against 2.0ℓ of distilled water, with four changes of distilled water over a 24 hour period at 4^{o}. The precipitated protein was centrifuged at 20,000g for 30 minutes.

The two precipitates were freeze dried and the protein stored in air tight vials at -20^{o} until used.

4. Electrophoretic procedures

(i) Polyacrylamide gel electrophoresis. (PAGE)

The method followed was outlined in the LKB application note 306, for the LKB Multiplier 2117 (Ornstein, (1964); Davis, (1964)). This method was also used in disc rod electrophoresis in a Shandon vertical electrophoresis chamber.

Stock solutions

(A) Tris-glycine buffer stock solution pH 8.9.

30.04g glycine

1.00g sodium azide

The glycine and azide were dissolved in 1200.0ml of distilled water, and titrated with 'Trizma' (Sigma) base solution to pH 8.9, finally made up to 2.0ℓ with distilled water.

(B) Electrode buffer.

1 volume buffer stock (A): 1 volume distilled water.

(C) Sample buffer.

5.0ml buffer stock (A) made up to 100.0ml with distilled water.

(D) Monomer Solution.

22.2g acrylamide

0.6g N-N' methylene bis acrylamide

The acrylamide and N'N' methylene-bis-acylamide were dissolved in 60.0ml of distilled

water, filtered through Whatman no 1 filter paper and made up to 100.0ml with distilled water.

(E) Ammonium persulphate solution.

150.0mg of ammonium persulphate was dissolved in 10.0ml of distilled water. This solution was freshly prepared for each experiment.

(F) Bromophenol blue solution.

25.0mg of bromophenol blue was dissolved in 10.0ml of sample buffer (C).

(G) Fixing solution.

57.0g trichloroacetic acid
17.0g sulphonosalicyclic acid.

The trichloroacetic acid and sulphonosalicyclic acid were dissolved in 350.0ml of distilled water and made up to 500.0ml with methanol.

(H) Staining solution.

1.25g PAGE blue 83 (B.D.H.) was dissolved in 227.0ml of methanol and 227.0ml of distilled water. The solution was filtered through Whatman no 1 filter paper, after which 46.0ml of glacial acetic acid was added.

(I) Destaining solution.

300.0ml of ethanol, 100.0ml of glacial acetic acid was made up to 1.0ℓ with distilled water.

Method

(a) Gel Solutions.

Solution	ml solution for 7.5% gel (T=7.7% C=2.6%)	ml solution for 10% gel (T=10.2% C=2.6%)
Distilled water	7.5	-
Buffer Stock A	33.0	33.0
Acrylamide Solution (D)	22.2	29.7
Ammonium persulphate(E) solution	3.2	3.2
TEMED	0.1	0.1
Final Volume	66.0	66.0

When using the LKB Multiplier 2117, the gel casette had a nominal volume of 66.0ml. When using the Shandon disc electrophoresis chamber, the above volumes were reduced by one third, leaving enough gel solution for 10 disc gels of 2.0ml in volume.

(b) Preparation of the gel.

All the glassware was washed in detergent, and rinsed with distilled water. After drying the gel cassette was assembled according to the manufacturers instructions.

The distilled water was mixed with the buffer stock (A) and the acrylamide solution (D) in a vacuum flask, and deaerated for 10 minutes. The ammonium persulphate and TEMED was carefully added and mixed before adding to either

the gel cassette or disc rods. Polymerization was complete after one hour.

(c) Electrophoresis.

The gel was removed from the cassette and placed on the LKB Multiplier 2117 with a circulating water supply at 8^{o}. The wicks were placed on the gel surface and the buffer wells filled with the electrode buffer (B).

Pre-electrophoresis was performed at 50 ma for 30 minutes, after which the samples were applied.

Freeze-dried protein was dissolved in the sample buffer (C), with the addition of 10µℓ of bromophenol blue solution (F) to every 250.0µℓ of sample.

10.0µℓ of sample was applied to the preformed wells, concentrated for 10 minutes at 20 ma when the field strength was increased to 40 ma. until the dye front was 1 cm from the anodic wick.

The gel was then removed from the apparatus, and transferred to the fixing solution (G) for 30 minutes. After which the gel was stained for 1 hour [solution(H)] and destained [solution(I)] until the background was clear of stain.

The position of the proteins in the gel were recorded by photography.

(d) Disc gel electrophoresis.

When using the Shandon vertical PAGE chamber, the gel mixtures were as previously described (Materials and Methods, 4(i) (a)). The gels were poured to a height of 8.0 cm and overlayed with 0.2ml of isopropanol to remove the meniscus. After polymerization the discs were placed in the electrophoretic chamber and the electrode buffer (B) poured into the upper and lower chambers.

The protein samples were dissolved in the sample buffer (C), with the addition of 0.025 ml glycerol and 0.01 ml Bromophenol blue solution (F) to every 0.25 ml.

After pre-electrophoresis at 6 ma per-disc tube for 30 minutes, the samples were overlayed onto the gel surface, and concentrated for 10 minutes at 3 ma per disc tube. The field strength was increased to 6 ma per disc tube until the dye front had reached 1.0 cm from the bottom of the gel.

The gels were then removed with a syringe and needle, fixed, stained and destained as previously described. (Materials and Methods, 4 (i) (a)).

(ii) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The method described was based on that of Weber and Osborn (1969) with modifications outlined in the LKB application note 306 for use with the LKB Multiplier 2117.

STOCK SOLUTIONS

(A) Imidazole buffer stock solution 0.1M pH 7.0.

13.6g Imidazole

4.0g Sodium dodecyl sulphate (SDS)

The imidazole and SDS were dissolved in 1200.0 ml of distilled water, the pH was adjusted to 7.0 with dilute orthophosphoric acid and made up to 2.0ℓ with distilled water.

(B) Electrode buffer.

1 volume buffer stock (A) : 1 volume distilled water.

(C) Sample buffer.

10.0 ml buffer stock (A), 1.0 ml 2-mercaptoethanol, 1.0g SDS, made up to 100.0ml with distilled water.

The stock solutions of; monomer (D), ammonium persulphate solution (E), bromophenol blue solution (F), fixing solution (G), staining solution (H) and destaining solution

(I) were as previously described, (Materials and Methods, 4 (i)).

METHOD

(a) Gel solutions.

The gel mixtures for SDS-PAGE were as previously described for PAGE (Materials and Methods, 4 (i) (a)).

(b) Preparation of the gel.

The preparation of the gel for SDS-PAGE were as previously described for PAGE (Materials and Methods, 4(i) (b)).

(c) Electrophoresis.

The gel was removed from the cassette and placed on the LKB Multiphor 2117 with a circulating water supply at 18°. The wicks were placed on the gel surface and the buffer wells filled with imidazole electrode buffer (B).

Pre-electrophoresis was performed at 80 ma for 30 minutes, after which the samples were applied.

Freeze-dried globulin samples were dissolved in the imidazole sample buffer (C) and incubated at 100° for 5 minutes. After cooling to room temperature 0.01 ml of bromophenol blue solution (F) and 0.01 ml of 2-mercapto ethanol were added to every 0.25 ml of sample.

0.01 ml of sample was applied to the preformed wells and concentrated for 10 minutes at 20 ma, after which the field strength was increased to a constant 100 ma, until the dye front was 1.0 cm. from the anodic wick.

The gel was removed from the apparatus and transferred to the fixing solution (G) for 1 hour. The gel was then transferred to the staining solution (H) for 2 hours, then destained in solution (I) until the background was clear of stain.

The position of the proteins in the gels were recorded by photography and by scanning spectrophotometrically sections of the gels in a PYE Unicam SP1800 spectrophotometer with an SP1802 scanning attachment.

(iii) Isoelectricfocussing (IEF) under denaturing and reducing conditions.

Isoelectric focussing is based on the method of Vesterberg (1971). The method for IEF under denaturing and reducing conditions was outlined by Matta et al. (1981) with the following modifications:

STOCK SOLUTIONS

(A) Monomer solution.

22.2g Acrylamide

0.6g N-N' methylene-bis-acrylamide

The acrylamide and N-N' methylene-bis-acrylamide were dissolved in 60.0ml of distilled and deionized water, filtered through Whatman no 1 filter paper and made up to 100.0ml with distilled and deionized water.

(B) Ammonium persulphate solution.

100.0 mg of ammonium persulphate dissolved in 10.0 ml of distilled and deionized water. This solution was made up fresh for every experiment.

(C) Electrode solutions.

(i) pH gradient 6.0-9.0

anodic solution.

1 volume Pharmalyte 4.0-6.0: 9 volumes distilled and deionized water.

cathodic solution,

1 volume Pharmalyte 8.5-10.5: 9 volumes distilled and deionized water.

(ii) pH gradient 4.0-6.0

anodic solution.

1 volume Pharmalyte 4.0-6.0 : 9 volumes distilled and deionized water.

cathodic solution,

1 volume Pharmalyte 6.0-9.0 : 9 volumes distilled and deionized water.

(D) Sample buffer.

(i) pH gradient 6.0-9.0,

75.0 ml (specially pure) formamide (B.D.H.)

5.0 ml Pharmalyte 6.0-9.0

2.0 ml 2- mercaptoethanol

12.0 ml distilled and deionzed water.

(ii) pH gradient 4.0-6.0.

75.0 ml (specially pure) formamide

5.0 ml Pharmalyte 4.0-6.0

2.0 ml 2- mercaptoethanol

12.0 ml distilled and deionized water.

(a) Gel Solutions.

Solution		Solution for pH gradient pH 6.0 - 9.0	pH 4.0 - 6.0
Monomer (A)		4.50	4.50
Specially pure formamide		14.50	14.50
Ammonium persulphate (B)		1.00	1.00
TEMED		0.03	0.03
Pharmalyte	4.0-6.0	-	2.00
	6.0-9.0	1.00	-
	8.5-10.5	1.00	-
	3.0-10.0	0.50	0.50
Total Volume		22.53	22.53

(b) Preparation of gel.

The specially pure formamide was passed through a 25.0 ml syringe packed with Amberlite MB ion exchange resin just before use. It was then mixed with the monomer Solution (A) and the Pharmalyte then degassed under vacuum.

The ammonium persulphate and TEMED were mixed, then poured into an LKB micro-thin gel casting kit with an 0.5mm gasket, and left for 1 hour to polymerize.

The gel was removed from the cassette and placed on an LKB Multiphor 2117 with a circulating water supply at 4^{o}. The electrode wicks were placed on the gel, having been soaked in the appropriate electrode solutions (C). The gel was then prefocussed for 1 hour at 8 watts before sample application.

(c) Electrophoresis.

Freeze-dried globulin samples were dissolved in the appropriate sample buffer (D) at a concentration of 2.0 mg ml^{-1}. Filter paper squares (0.5 x 1.0 cm) were placed 2.0 cm from the anode for ph gradient 6.0-9.0 and 2.0cm from the cathode for pH gradient 4.0-6.0. The samples were applied to the filter paper squares in 0.01 ml aliquots. The gel was focussed for 1 hour at 8 watts, then 1.5 hours at 12 watts.

The gel was then fixed overnight and washed in destain for 30 minutes to ensure complete removal of the ampholytes, before being placed in the stain for 2 hours. The gel was then destained until the background was clear, (The fixing, staining and destain solutions were as described previously:- Methods and Materials 4 (i)) because of the gels fragility, the protein bands were recorded by drawing.

(iv) Zonal Isoelectric precipitation electrophoresis (ZIPE).

This method was used to assess the purity of legumin and vicilin (Bonner et al., 1983).

STOCK SOLUTIONS

(A) 0.15M McIllivane buffer pH4.7 electrode stock buffer.

10.91g citric acid,

17.10g disodium hydrogen phosphate dihydrate.

The citric acid and sodium dihydrogen phosphate was dissolved in 900.0 ml distilled water, the pH was checked and made up to 1.0ℓ with distilled water.

(B) Electrode buffer.

1 volume electrode stock buffer: 2 volumes distilled water.

(C) Sample buffer, 0.15M Sodium phosphate buffer pH 7.0.

1.63g di-sodium hydrogen phosphate dihydrate,

0.81g sodium dihydrogen phosphate mono-hydrate.

The sodium phosphate salts were dissolved and made up to 100.0 ml with distilled water.

(a) Electrophoresis.

Whatman cellulose acetate membranes (5.7 x 14cm) were soaked absorbent face down for 10 minutes in electrode buffer (B). The buffer was removed and replaced with fresh electrode buffer for a further 10 minutes.

The membranes were removed, dried on tissue and placed on an LKB Multiphor 2117 with a circulating water supply at 4^{o}. The buffer wells were filled with electrode buffer (B) and filter paper wicks were placed on the absorbent surface of the membranes.

Freeze-driedglobulin samples were dissolved in sample buffer (C) at a concentration of 2.0mg ml^{-1} and applied to the membranes with either a semi-micro applicator (Whatman Lab Sales) delivering 0.005 ml or a 0.01 ml syringe.

The samples were run for 1 hour at 6 ma per membrane, then transferred to a staining solution for 10 minutes before being destained until the background was clear. (The staining and destaining solutions were as described previously:- Methods and Materials, 4 (i)).

The membranes were kept in 10% (v/v) aqueous methanol until they were photographed.

5. Preparation of Affinity Chromatography Media

(i) Preparation of globulin Sepharose 4B.

The method followed was outlined in the Pharmacia booklet "Affinity Chromatography principles and Methods" (1979).

(A) Cyanogen bromide activated Sepharose 4B (Pharmacia).

6.0g of cyanogen bromide activated sepharose was washed with 3.0ℓ of 1mM hydrochloric acid on a sintered glass filter.

(B) 0.1M Sodium bicarbonate buffer pH 8.3 containing 0.5M Sodium chloride,

8.40g Sodium bicarbonate, 29.22 g of Sodium chloride were dissolved in 900.0ml of distilled water, the pH was adjusted to 8.3 with dilute sodium hydroxide and made up to 1.0ℓ with distilled water.

(C) 0.1M Sodium acetate buffer pH 4.0 containing 0.5M Sodium Chloride,

8.20g of Sodium acetate (anhydrous), 29.22g of Sodium chloride were dissolved in 900.0ml of distilled water, the pH was adjusted to 4.0 with dilute glacial acetic acid and made up to 1.0ℓ with distilled water.

(D) 0.1M Sodium bicarbonate buffer pH 8.3 containing 0.5M Socium chloride, 0.2M glycine.

0.84g Sodium bicarbonate, 2.92g Sodium chloride, 1.50 g glycine, were dissolved in 90.0ml of distilled water, the pH was adjusted to 8.3 with dilute sodium hydroxide and made up to 100.0 ml with distilled water.

(E) Total globulins extracted from five day old cotyledons of Vicia faba.

100.0mg of globulins extracted from five day old cotyledons (as described previously) was dissolved in 40.0ml of 0.1M Sodium bicarbonate buffer pH 8.3 (B).

METHOD

Cyanogen bromide activated Sepharose 4B treated with 1mM hydrochloric acid was washed with 0.1M sodium bicarbonate buffer (B) on a sintered glass filter and mixed with the 40.0ml of total globulins (E) for 2 hours at room temperature. After which, the gel was filtered through a sintered glass filter and transferred to 0.1M Sodium bicarbonate buffer (D) containing 0.2M glycine to block the remaining cyanogen bromide groups, for 2 hours at room temperature.

The gel was then transferred to a sintered glass filter and washed alternately with 0.1M Sodium acetate buffer pH 4.0 (C) and 0.1M Sodium bicarbonate buffer pH 8.3 (B).

The gel was then stored at 4°in 0.1M Sodium bicarbonate buffer (B) containing 0.03% (w/v) Sodium azide, until used.

(ii) Phenylbutylamine (PBA) Sepharose 4B.

The method followed was that of Stevenson and Landman (1971).

The stock solutions and method of coupling the phenylbutylamine were identical to the method previously described for globulin Sepharose 4B, with the following modifications (Materials and Methods, 5(i)).

2.0g of phenylbutylamine (Koch Light) was dissolved in 5.0ml of dimethyl formamide. The activated cyanogen bromide was mixed with 7.0ml of dimethyl formamide and 28.0ml of 0.1M sodium bicarbonate buffer pH 8.3 (B) for 2 hours at room temperature. The gel was washed on a sintered glass filter with 30% (v/v) dimethyl formamide in 0.1M Sodium bicarbonate buffer pH 8.3 (B).

The gel was then mixed with buffer (D) for 2 hours at room temperature washed with buffers (C) and (B), finally stored at 4° in buffer (B) containing 0.03% (w/v) sodium azide until used.

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2.0g of phenylbutylamine (Koch Light) was dissolved in 5.0ml of dimethyl formamide. The activated cyanogen bromide was mixed with 7.0ml of dimethyl formamide and 28.0ml of 0.1M sodium bicarbonate buffer pH 8.3 (B) for 2 hours at room temperature. The gel was washed on a sintered glass filter with 30% (v/v) dimethyl formamide in 0.1M Sodium bicarbonate buffer pH 8.3 (B).

The gel was then mixed with buffer (D) for 2 hours at room temperature washed with buffers (C) and (B), finally stored at 4° in buffer (B) containing 0.03% (w/v) sodium azide until used.

6. Chromatographic procedures

(i) Preparation of Albumin extracts.

Stored frozen cotyledons were homogenized in 0.05M Sodium acetate buffer pH 4.7, in a Waring blender with two thirty second bursts.

The homogenate was stirred for one hour at 4^{o}, filtered through 2 layers of muslin and centrifuged at 20,000g for 30 minutes.

The supernatant fluid was used directly for experiments or subjected to further purification steps.

(ii) Gel filtration.

Gel filtration using Sephacryl 5-200 (Pharmacia) was used to purify a poteinase and peptidase from 5-day old Vicia faba cotyledon at 4^{o}.

Fully swollen Sephacryl S-200 was degassed under vacuum, and packed into an LKB column (2.6 x 85.0cm) using an extension attachment and a linear flow rate. The column was equilibrated with 50mM imidazole buffer pH 6.4 containing 5mM potassium chloride and 1mM EDTA. (3.4g of imidazole, 0.37g of potassium chloride, 0.37g of EDTA, were dissolved in 900.0ml of distilled water, the pH was adjusted to 6.4 with dilute hydrochloric acid and made up to 1.0l with distilled water).

Samples (10.0-20.0ml) were applied to the column in the descending mode, eluted protein was monitored continuously with a LKB Uvicord SII and the fractions collected with an LKB Redirac fraction collector.

(iii) Affinity Chromatography with globulin Sepharose 4B.

STOCK SOLUTIONS

(A) Application buffer.

50mM sodium acetate, 0.5M NaCl, 1mM EDTA, 1mM DTT pH 5.4.

4.100g sodium acetate, 0.372g EDTA, 0.154g DTT, 29.22g NaCl was dissolved in 800.0ml of distilled water, the pH was adjusted to pH 5.4 with dilute acetic acid, before making up to 1.0µl with distilled water.

(B) Elution buffer

0.1M acetic acid, 1mM EDTA, 1mM DTT pH 3.1.

600µl of glacial acetic acid, 0.037g of EDTA, 0.015g of DTT was dissolved in 80.0ml of distilled water, the pH was adjusted to pH 3.1 with 4M sodium acetate, before making up to 100.0ml with distilled water.

METHOD

The sample eluted from the Sephacryl S-200 column with proteinase and BAPNAase activity was dialysed against 50mM Sodium acetate, 1mM EDTA, 1mM DTT, 0.032% (w/v) sodium azide pH 5.4 (A) overnight at 4^{o}. The precipitated protein was centrifuged at 20,000g for 30 minutes.

The supernatant fluid was applied to a Globulin Sepharose 4B column (1.6 x 20cm) equilibrated with the application buffer (A) and eluted with the application buffer (A) until all the unbound material had been eluted from the column. Then the elution buffer (B) was applied to the column. The protein eluted was monitored continuously at 280nm with an LKB UVicord SII and fractions collected with an LKB Redirac fraction collector.

The fractions were assayed for proteinase and BAPNAase activity.

(iv) Chromatofocussing.

The sample was dialysed against 25mM piperazine buffer pH 5.5 and concentrated in a Amicon stirred cell with a PM30 membrane at 4°.

STOCK SOLUTIONS

25mM Piperazine pH 5.5.

4.85g piperazine dissolved in 800ml of distilled water, the pH was adjusted to 5.5 with dilute hydrochloric acid and made up to 1.0ℓ with distilled water.

Poly buffer (7-4)(Pharmacia) pH 4.0.
1 volume Polybuffer (7.4) : 10 volumes distilled water.

The pH was adjusted with dilute hydrochloric acid.

METHOD

Polybuffer exchanger (7-4) (Pharmacia) was supplied fully swollen. This was degassed under vacuum and packed into a Pharmacia column (1.0 x 40cm) using a linear flow rate. The column was equilibrated with 25mM piperazine pH 5.5 (A).

A small volume of Polybuffer (7-4) pH 4.0 (ca. 5.0ml) was applied to the column to prevent the sample experiencing extremes of pH. The sample was applied to the column (between 10-20 ml) and eluted with 350ml of Polybuffer (7-4) pH. 4.0 in a decending mode. Eluted protein was monitored continuously with an LKB Uvicord SII and fractions collected with an LKB Redirac fraction collector.

The pH gradient was measured with a Philips pH meter and a semi-micro electrode.

(v) Preparative gel electrophoresis.

A preparative gel electrophoresis apparatus (B.R.L) was used with the tris-glycine PAGE system previously described (Methods and Materials 4(i)). While the electrode buffer (B) and elution chamber were being degassed for 2 hours,the gel mixture was poured to a height of 3.5cm and left to polymerize.

After polymerization the gel was placed in the elution chamber and then into the lower buffer chamber already full of electrode buffer. The inlet of the elution chamber was connected to a resevoir containing electrode buffer (B) used as the elution buffer. The outlet of the elution chamber was connected to an LKB Redirac fraction collector via an LKB microperplex pump and an LKB UVicord SII constant ultra violet monitor (280 nm). The upper chamber was then filled with electrode buffer (B).

Pre-electrophoresis was carried out at a constant 5 ma for 30 minutes with a flow rate through the elution chamber of 15.0 ml $hour^{-1}$.

The BAPNAase sample was dialysed against the sample buffer (C) and concentrated to 1 ml on a B15 Amicon concentrator. 0.1 ml of glycerol and 0.025 ml of bromophenol blue solution (F) was then added before the sample was applied to the gel.

The sample was concentrated for 20 minutes at 2ma then the field strength was increased to 5ma, with a flow rate through the elution chamber of 10.0ml $hour^{-1}$.

The elution was carried out at 4^{o} overnight with 1.0ml fractions collected, which were assessed for BAPNAase activity.

(vi) Affinity Chromatography using phenylbutylamine Sepharose 4B (PBA).

Phenylbutylamine Sepharose 4B was prepared as described previously (Methods and Materials 5 (ii)) and equilibrated with 50mM TRICINE buffer pH 8.0 containing 1mM EDTA (8.96g of TRICINE, 0.37g of EDTA was dissolved in 900.0ml of distilled water, the pH was adjusted to 8.0 with dilute sodium hydroxide and made up to 1.0ℓ with distilled water). The gel was degassed under vacuum and packed into an LKB column (1.6 x 20.0cm).

Samples of BAPNAase were loaded (ca. 10.0ml) onto the column and unbound material was eluted with 50mM TRICINE buffer pH 8.0 containing 1mM EDTA. Bound material was eluted with 0.1 M acetic acid pH 3.1 containing 1mM EDTA (as previously described, Methods and Materials 6 (iii)).

Protein was continuously monitored with an LKB Uvicord SII and fractions collected with an LKB Redirac fraction collector. Fractions were assayed for BAPNAase activity as described previously (Methods and Materials, 10 (ii)) and active fractions were brought to pH 5.5 with 4M sodium acetate (32.81g of anhydrous sodium acetate was dissolved in 90.0ml of distilled water and made up to 100.0ml with distilled water.).

(vii) Ion exchange chromatography with diethylamino ethyl Sephacryl (DEAE-Sephacryl).

The anion exchanger DEAE-Sephacryl (Pharmacia) was supplied fully swollen and was equilibrated with 50mM imidazole, buffer pH 6.4 containing 5mM potassium chloride, 1mM EDTA (3.4g of imidazole, 0.37g of potassium chloride, 0.37g of EDTA, were dissolved in 900.0ml of distilled water, the pH was adjusted to 6.4 with dilute hydrochloric acid, and made up to 1.0ℓ with distilled water). The exchanger was degassed under vacuum and packed into a Pharmacia column (5.6 x 3.0cm) with a linear flow rate.

Samples were loaded (ca. 20.0ml) onto the column and washed with the equilibration buffer, until all the unbound protein had been eluted. Bound protein was eluted with a linear salt gradient 5mM-300mM potassium chloride in 50mM imidazole buffer pH6.4 containing 1mM EDTA.

Eluted protein was continuously monitored with an LKB Uvicord SII and fractions were collected with an LKB Redirac fraction collector.

7. Concentration of samples

During purification of the proteinase and peptidase the samples were concentrated in order to apply the correct volume to the various columns. An Amicon stirred cell was used with external pressure provided by nitrogen at 35 lbs $inch^{2\ -1}$ at 4^{o}. A PM10 membrane was inserted into the stirred cell, with a molecular weight exclusion of 10,000 daltons for samples of protienase. A PM30 membrane was inserted into the stirred cell, with a molecular weight exclusion of 30,000 daltons for samples of BAPNAase.

An Amicon B15 concentrator was used for volumes below 5.0 ml. This was a free standing concentrator with a molecular weight exclusion of 15,000 daltons, relying upon absorbent pads behind the membrane to concentrate samples.

8. Preparation of dialysis tubing

During purification of the proteinase,peptidase and globulins it was necessary to dialyse samples to exchange buffers.

Two metres of Visking tubing 18/32 were boiled in 500.0ml of distilled and deionized water containing 0.5g of EDTA and 0.5g of sodium carbonate for 10 minutes. The tubing was rinsed in distilled and deionized water and boiled again for 10 minutes in distilled and deionized water.

After rinsing in distilled and deionized water, the tubing was stored at 4^{o}, in distilled and deionized water until used.

9. Protein determinations

The amount of protein extracted by various procedures was assessed by the methods of Lowry et al. (1951) and Bradford (1976). Bovine serum albumin was used as the standard protein.

(i) Lowry Method.

Stock solutions

(A) Alkali reagent.

20.0g of sodium carbonate was dissolved in 1.0ℓ of 0.1M sodium hydroxide (4.0g ℓ^{-1}) to which was added 10.0ml of 1% (w/v) copper sulphate solution (1.0g ℓ^{-1}) and 10.0ml of 1% (w/v) sodium tartrate solution (1.0g ℓ^{-1}).

METHOD

0.4ml of sample or standard was incubated for 10minutes with 2.0ml of stock solution (A) at room temperature. After which 0.2ml of Folin-Ciocalteau reagent (B.D.H) (previously diluted 1 volume Folin-Ciocalteau : 2 volumes distilled water) was added and mixed vigorously. The samples were measured at 500nm after 30 minutes incubation at room temperature.

The standard protein concentrations between 0.01mg-0.50mgml^{-1} gave a linear graph and this was used to determine unknown protein concentrations.

(ii) Bradford method

Stock solutions

100.0mg coomassie brilliant blue G (Sigma) was dissolved in 50.0ml ethanol, 50.0ml 85% v/v orthophosphoric acid in distilled water was then added and the mixture made up to 1.0ℓ with distilled water.

METHOD

0.1ml of standard or unknown protein solution was mixed vigourously with 5.0ml of stock solution. The colour was left to develop for 5 minutes, after which the samples were measured at 585nm. The standard protein concentrations between 0.1-1.0mg ml^{-1} gave a linear graph and this was used to determine unknown protein concentrations.

10. Enzyme Assays

(i) Azocasein assay

The method followed was that of Taylor (1979).

Stock solutions

(A) 2% (w/v) Azocasein

2.0g Azocasein (Sigma), 0.064g sodium azide dissolved in 100.0ml of distilled water.

(B) 0.3M McIllivane buffer pH 5.4.

4.16g citric acid, 10.2g sodium dihydrogen phosphatedihydrate made up to 250.0ml with distilled water.

METHOD

0.5ml of azocasein (A) was added to 0.5ml of McIllivane buffer (B) and incubated at 40^{o} for 5 minutes. The reaction was started by the addition of 0.5ml of enzyme solution; terminated by the addition of 0.5ml of 20% (w/v) TCA.

The tubes were removed and stored at 4^{o} for 30 minutes, then centrifuged a 5,000g for 10 minutes. After which 1.0ml of the supernatant was added to 1.0ml of 2M sodium hydroxide to enhance the colour, which was measured at 440nm.

One unit of enzyme was defined as the amount of protein which caused an increase of 0.001 in absorbance per minute at 440nm.

(ii) NαBenzoyl-DL-arginine-paranitroanalide (BAPNA)

The method followed was that of Ota et al. (1964), with the following modifications.

Stock solutions

(A) 105mM BAPNA.

2.45g BAPNA dissolved in 50.0ml of dimethyl sulphoxide.

(B) 0.1M McIllivane buffer stock pH7.2.

0.64g citric acid 10.78g sodium dihydrogen phosphate dihydrate dissolved in 500.0ml of distilled water.

METHOD

0.1ml of BAPNA (A) was added to 2.0ml of McIllivane buffer (B) which was incubated for 5 minutes at 40°. The mixture was shaken gently to allow the reagent to dissolve. The reaction was started by the addition of 0.1ml of enzyme solution and terminated by the addition of 0.7ml of 20% (w/v) TCA.

The samples were removed and stored at 4° for 30 minutes before being centrifuged at 5,000g for 10 minutes. The supernatant was measured in a semi-micro cuvette at 416nm.

One unit of enzyme was defined as the amount of protein which caused an increase of 0.01 in absorbance per minute at 416 nm.

(iii) Coomassie blue dye binding assay.

The method followed was that of Saleemuddin et al. (1980).

Stock solutions

(A) Coomassie blue reagent.

100.0mg of Coomassie brilliant blue G (Sigma) was dissolved in 50.0ml of ethanol. 50.0ml of 85% (v/v) aqueous orthophosphoric acid was added and the solution made up to 1.0ℓ with distilled water.

(B) Globulin substrates.

Purified vicilin and legumin was dissolved in 0.1M McIllivane buffer pH5.4 at a concentration of either 1.0mg ml^{-1} or 2.0mg ml^{-1}.

METHOD

0.05ml or 0.10ml of globulin substrate (B) was incubated at 40^{o} in a 1.5ml disposable vial (Sterlin). The reaction was started by the addition of 0.10ml of enzyme solution. The reaction was terminated by the addition of 1.0ml of Coomassie blue reagent (A), which was mixed vigorously before the addition of another 4.0 mls of Commassie blue reagent (A).

The colour was developed for 5 minutes and the absorbance was measured at 585nm.

(iv) N-carbobenzoxy-(amino acid)- , -nitrophenol ester CBZ-NPE.

The method followed was that of Baumgartner and Chrispeels (1977).

Stock solutions

(A) 105mM N-carbobenzoxy-(amino acid)- nitrophenol ester (CBZ-NPE).

The requisite amount of (CBZ-NPE) of various aminoacids were dissolved in dimethyl sulphoxide and made up to 10ml with dimethyl sulphoxide.

(B) 0.1M McIllivane buffer pH 5.4.
0.55g of citric acid, 1.36g sodium dihydrogen phosphate dihydrate were dissolved in 50.0ml of distilled water, 10.0ml of methanol was added and the volume made up to 100.0ml with distilled water.

(C) 0.1M McIllivane buffer pH7.4.
0.13g of citric acid, 2.15g of sodium dihydrogen phosphate dihydrate were dissolved in 50.0ml of distilled water, 10.0ml of methanol was added and the volume made up to 100.0ml with distilled water.

METHOD

(i) BAPNAase activity

2.9ml of 0.1M McIllivane buffer pH 7.4(C) was incubated with 0.1ml of purified BAPNAase at 25°. The reaction was started by the addition of 50µℓ of CBZ-NPE derivatives of various amino acids and measured continuously at 410nm in a DU7 Beckman recording spectrophotometer.

(ii) Proteinase activity

2.75ml of 0.1M McIllivane buffer pH5.4 (B) was incubated with 0.25ml of purified proteinase at 25°. The reaction was started by the addition of 50µℓ of CBZ-NPE derivatives of various aminoacids and measured continuously at 410nm in a DU7 Beckman recording spectrophotometer.

(v) 100mM NαBenzoyl-L-arginine-ethyl ester (BAEE), NαBenzoyl-L-tyrosine-ethyl ester (BTEE), arginine-ethyl ester (AEE).

The method followed was that of Schwert and Takenaka (1955) with the following modifications.

Stock solutions

(A) 100mM (BAEE), (BTEE), (AEE).
0.0343g BAEE, 0.0313g BTEE, 0.0153g AEE were each dissolved in 10.0ml of 0.1M McIllivane buffer pH 5.4.

(B) 0.1M McIllivane buffer pH 5.4.
0.55g citric acid, 1.36g sodium dihydrogen phosphate dihydrate dissolved and made up to

100.0ml with distilled water.

METHOD

2.75ml of 0.1M McIllivane buffer pH 5.4 (B) 0.05ml of one of the ethyl esters (A) were incubated at 25^{o} for 5 minutes. The reaction was started by the addition of 0.25ml of purified proteinase and liberated ethyl ester was measured continuously at 253nm in a Beckman DU7 recording spectrophotometer.

(iv) Ninhydrin.

The ninhydrin method followed was outlined in the Technicon research bulletin no. 20.

It was used in the estimation of the amide content of purified legumin and vicilin according to the method of Wilcox (1968) and to estimate liberated α-amino acids as a result of peptidase activity on tri-and di-peptides, according to the method of Patterson (1976), with the following modifications.

Stock solutions

(A) 4M sodium acetate buffer.

250.0g sodium acetate trihydrate was dissolved in distilled water to a total volume of 400.0ml with heating. When the

solution had cooled 50.0ml of glacial acetic acid was added and the volume made up to 500.0ml.

(B) Ninhydrin reagent.

3.3g ninhydrin, 250.0ml methyl cellusolve, 50.0ml 4M sodium acetate buffer (A), 15.0ml glacial acetic acid made up to 500.0ml with distilled water.

(C) Hydrazine reagent.

0.13g hydrazine sulphate dissolved in 500.0ml of distilled water to which was added one drop of concentrated sulphuric acid.

(a) General method.

1.0ml of sample was taken and mixed with 1.2ml of ninhydrin reagent (B) and 0.8ml of hydrazine solution (C). The tube was heated in a boiling water bath for 15 minutes, then cooled to room temperature before the addition of 3.0ml of 50% (v/v) aqueous ethanol. This was left to stand for 10 minutes, after vigorous mixing, and the absorbance measured at 570nm.

(b) Estimation of liberated α-amino acids by ninhydrin.

Tri and di-peptides were dissolved in 0.1M McIllivane buffer pH 7.4 to a concentration of 10mM; 2.0ml was incubated at 40^{o}. The reaction was started by addition of 0.1ml of purified BAPNAase and terminated by the addition of 0.7ml 20% (w/v) TCA.

After centrifugation at 5,000g for 10 minutes, the pH of the supernatant was adjusted to pH 5.2 with 4M sodium acetate before being subjected to the above ninhydrin assay.

(c) Estimation of the amide content of purified legumin and vicilin by ninhydrin.

The method was based on that of Wilcox (1968).

METHOD

Purified samples of legumin and vicilin were dissolved in concentrated hydrochloric acid at a concentration of 2.0mg ml^{-1} contained in a glass stoppered test tube. The tubes were incubated for 10 days at 37^{o}.

0.5ml aliquots were then placed in the outer chamber of a Conway micro diffusion unit. The solution was evaporated to dryness in a vacuum desiccator over pellets of sodium hydroxide.

1.0ml of 0.04M sulphuric acid was placed in the centre well, and 1.0ml of saturated sodium tetraborate adjusted to pH 10.5 with 2M sodium hydroxide.

The micro diffusion was allowed to proceed overnight, the contents of the centre well were removed and 0.5ml aliquots were taken for estimation of ammonia by the ninhydrin method outlined previously.

II IMMUNOLOGY

(i) Immunization protocol.

Stock solutions

(A) Phosphate buffered saline (PBS).

8.00g sodium chloride, 0.02g potassium chloride, 1.15g disodium hydrogen phosphate, 0.02g potassium dihydrogen phosphate were dissolved and made up to 1.0ℓ with distilled water.

METHOD

Purified BAPNAase was passed down a G-25 Sephadex column equilibrated with phosphate buffered saline, fractions were collected and assayed for BAPNA active material. These fractions were pooled and mixed vigorously with an equal volume of Freunds' complete adjuvant prior to injection.

Two Dutch rabbits were each injected with 0.09mg of purified BAPNAase in Freunds' complete adjuvant by eight subcutaneous injections down the back of the rabbit ($0.5ml\ site^{-1}$).

After 14 days the rabbits were given another set of subcutaneous injections with purified BAPNAase in Freunds' incomplete adjuvant. The rabbits were each injected with 0.045mg of protein by eight subcutaneous injections down the back ($0.25ml\ site^{-1}$).

(ii) Bleeding.

At intervals between injections small samples of blood were obtained from an incision in a vein in the rabbits ears.

When the antibody showed no increase in antibody titre a larger volume of blood was obtained from an incision in a vein in the rabbits ears.

(iii) Isolation of the serum from blood.

The blood collected was stored at 4^{o} overnight to allow the red blood cells to clot and contract.

The serum was poured from the clot, the clot spun at 2,000g for 30 minutes at 4^{o} and free liquid pooled with the above serum.

The pooled serum was centrifuged for 20 minutes at 1,500g at 4^{o} and the supernatant stored in 1.0ml aliquots at $-20^{o}C$.

(iv) Testing thetitre of the antibody by enzyme linked immunoabsorbent assay (ELISA).

Stock solutions

(A) 0.05M sodium carbonate, containing 0.02% w/v sodium azide.

1.32g of sodium carbonate, 0.05g of sodium azide were dissolved in 200.0ml of distilled water, the pH was adjusted to pH 9.6 with dilute sodium hydroxide. The volume was made up to 250.0ml with distilled water.

(B) Phosphate buffered saline (PBS) pH 7.2.

8.00g of sodium chloride, 0.02g of potassium chloride, 1.15g of di-sodium hydrogen phosphate: $2H_2O$, 0.02g potassium di-hydrogen phosphate, was dissolved in 900.0ml of distilled water and made up to 1.0ℓ with distilled water.

(C) Phosphate buffered saline containing 1% (w/v) bovine serum albumin (PBS-BSA).

10.0g of Bovine serum albumin was dissolved in 1.0ℓ of PBS solution (B).

(D) Phosphate buffered saline containing 1% (w/v) BSA and 0.5% (w/v) Brij 35 (PBS-BSA-Brij).

0.5g of Brij 35 was dissolved in 100.0ml of PBS-BSA solution (C).

(E) Phosphate buffered saline containing 0.05% (w/v) Brij 35 (PBS-Brij).

0.05g of Brij 35 was dissolved in 100.0ml of PBS solution (B).

(F) Peroxidase substrate solution.

0.01% (w/v) o-phenylene diamine, 0.005% (v/v) Hydrogen peroxide was dissolved in 100.0ml of PBS solution (B). This solution was made up fresh at every assay and stored in the dark.

METHOD

0.2ml of purified BAPNAase (3.5µg; ml^{-1}) in carbonate buffer (A) was placed into the wells of a micro ELISA plate and incubated overnight at 4^{o}.

The BAPNAase was removed and the wells washed twice with PBS-BSA-Brij 35 solution (D) (0.25ml $well^{-1}$) for three minutes at room temperature. The remaining sites in the wells were blocked by the addition of 0.25ml $well^{-1}$ of PBS-BSA solution (C), for 1 hour at room temperature. The wells were again washed twice with PBS-BSA-Brij 35 solution (D) as above.

Doubling dilutions of test anti-BAPNAase serum were prepared in PBS-BSA-Brij 35 solution (D), 0.2ml was added to each well and incubated for 2 hours at room temperature, before being washed twice in PBS-BSA-Brij 35 solution (D) as above.

A $\frac{1}{500}$ dilution of peroxidase conjugated to anti-rabbit immunoglobulin raised in goat (Sigma) was prepared in PBS-Brij 35 (E), 0.2ml was added to each well and incubated for 2 hours at room temperature. The wells

were again washed twice with (0.25ml $well^{-1}$) PBS-BSA-Brij 35 solution (D), before 0.2ml of peroxidase substrate solution (F) was added to each well and incubated at room temperature for 30 minutes. The reaction was stopped by the addition of 0.05ml of 3M sodium hydroxide, mixed and the absorbance measured at 410nm in a micro ELISA spectrophotometer.

(V) Agarose and agarose gel electrophoretic techniques.

(a) Precoating glass plates with agarose.

METHOD

Glass plates (8.4 x 9.4 x 0.1 cm) were washed in detergent and rinsed in distilled water and then dried in an oven. 0.5g of agarose (B.D.H) was boiled in 100.0ml of distilled water for 15 minutes. The solution was cooled to 50^{o} and then 15.0ml was pipetted into the glass plates placed on a level surface. When the agarose had gelled, the plates were placed in a 37^{o} incubator until they were completely dry and ready for use.

(b) Double immunodiffusion.

This method was based on that of Ouchterlony and Nilsson (1978) with modifications outlined by Johnstone and Thorpe (1982).

Solutions

(A) Phosphate buffered saline (PBS) containing 0.02% (w/v) sodium azide.

PBS as described before (Methods and Materials, 11 (iv) with 0.2gℓ^{-1} sodium azide.

METHOD

1.0g of agarose was dissolved in 100.0ml of PBS (A) by boiling for 15 minutes. The solution was cooled to 50^{o} and 22.0ml were pipetted onto precoated glass plates (Methods and materials 11 v(a)) and allowed to set. The wells were cut 7.0 mm apart by using an LKB template for Ouchterlony double immunodiffusion.

Anti sera to BAPNAase raised in rabbits was placed in the centre well, . extracts from one day old cotyledons from various legumes were placed in the outside wells and diffusion allowed to proceed for 48 hours at 4^{o}.

Unprecipitated protein was removed from the gels by washing in 200.0ml of PBS solution (A) which was changed several times over a 48 hour period at 4^{o}.

The gels were stained in Coomassie blue overnight and destained until the background was clear of stain.

(c) Immunoelectrophoresis:

This method was based on that of Graber and Williams (1953) with modifications outlined by Johnstone and Thorpe (1982).

Solutions

(A) Barbitone buffer pH 8.2.

8.8g 5'5-diethylbarbituric acid was dissolved in 300.0ml of water at 95°. This was made up to 1800.0ml with cold distilled water. Then 24.0g of sodium 5'5-diethyl barbiturate was added and the pH was adjusted to pH 8.2 with dilute sodium hydroxide before being made up to 2.0ℓ with distilled water.

METHOD

1.0g of agarose was dissolved in 100.0ml of barbitone buffer (A) by boiling for 15 minutes. The solution was cooled to 50° and 22.0ml were pipetted onto precoated glass plates (Methods and Materials, 11 v(a)) and allowed to set.

The wells and troughs were cut by using an LKB template for immunoelectrophoresis, but only the wells were removed.

Extracts from various legumes were placed in the wells and electrophoresis carried out on an LKB Multiphor 2117

at 8^{o} for 1 hour at 60 volts, using the barbitone buffer (A) as the running buffer.

The troughs were then removed from the gel and antisera to BAPBAase raised in rabbits was placed in the troughs and diffusion allowed to proceed for 48 hours at 4^{o}.

Unprecipitated protein was washed from the gels with 200.0ml of PBS Solution A changed several times over a 48 hour period at 4^{o}.

The gels were stained in Coomassie blue overnight and destained until the background was clear of stain.

RESULTS

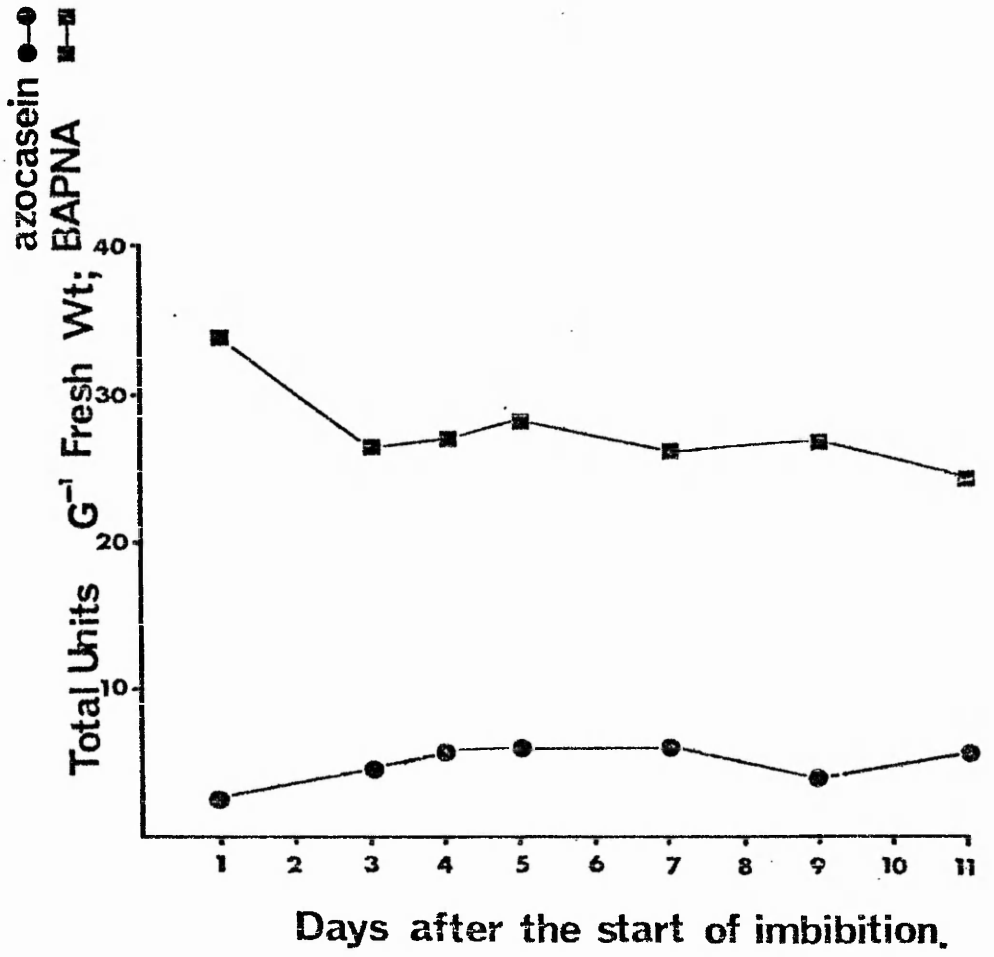
1. Enzyme isolation.

(a) Preliminary experiments

The proteolytic activities of crude extracts from cotyledons germinated for different periods of time was examined (Fig.1). BAPNAase activity altered relatively little, whereas azocasein activity reached a maximum after 5 days. Table 1 showed that different amounts of protein were extracted by different extraction buffers, but that the total amount of enzyme extracted was similar. However, extraction with sodium acetate buffer at pH 4.7 provided extracts with the highest specific activity and were therefore used for further purification.

To provide sufficient material to purify the proteolytic enzymes, ammonium sulphate precipitation was used to concentrate the protein. Table 2 shows that the majority of both azocasein and BAPNAase activity was precipitated between 30 and 60% ammonium sulphate saturation.

Fig 1. Proteinase and BAPNAase activity extracted from germinating Vicia faba cotyledons.



7.5gm of cotyledons of germinating Vicia faba were homogenized in 15.0ml of 0.1M phosphate buffer PH 7.0, strained through muslin and centrifuged at 20,000g for 30 mins.

0.5ml of the supernatant was incubated in 0.1M McIllivane buffer PH 5.4 at 40°. The reaction was started by the addition of 0.5ml of 2% (w/v) Azocasein, and terminated by the addition of 0.5ml of 20% (w/v) TCA. After centrifugation at 5,000g for 10 minutes, 1.0ml of the supernatant was added to 1.0ml of 2M NaOH and the absorbance measured at 440nm.

0.1ml of the supernatant was incubated in 2.0ml of 0.1M McIllivane buffer PH 7.4 at 40°. The reaction was started by the addition of 0.1ml of 105mM BAPNA and terminated by the addition of 0.7ml of 20% (w/v) TCA. After centrifugation at 5,000g for 10 minutes the increase in absorbance of the supernatant was measured at 416 nm.

Table 1. Proteolytic activity of 5 day old cotyledon extracts made with different buffers.

Buffer	Vol ml	Protein mg ml^{-1}	Azocasein units mg^{-1} (S.E.M)	Total units
H_2O	15	10.50	0.35 ±0.06	55.12
10mM EDTA	15	16.75	0.32 ±0.07	80.4
50mM NaAc pH 4.7	14.5	6.45	0.69 ±0.38	64.5
50mM NaAc 10mM EDTA pH4.7	15.0	9.6	0.43 ±0.28	61.9
0.1M Sodium Phosphate PH7.0	15.0	19.5	0.21 ±0.15	61.4
0.1M Sodium Phosphate 10mM EDTA PH7.0	14.0	17.35	0.26 ±0.06	63.2

7.5gm of five day old cotyledons were homogenized in 15.0ml of buffer, after centrifugation at 20,000g for 30 minutes, the supernatant was assayed for proteinase activity using azocasein, at 40^{o} for 2 hours (n=6).

Table 2. Distribution of proteolytic activity after ammonium sulphate precipitation.

% Saturation $(NH_4)_2SO4$	Volume mls	Protein content mg ml^{-1}	Azocasein units mg^{-1} (S.E.M)	Total units	BAPNA units mg^{-1} (S.E.M)	Total units
0 (Crude Extract)	35	3.50	1.04 ± 0.02	127.40	4.45 ± 0.03	545.12
0 - 30	10	0.13	1.66 ± 0.06	2.16	1.62 ± 0.66	2.11
30 - 60	20	1.57	2.45 ± 0.04	76.93	8.03 ± 0.12	252.14
60 - 100	10	2.54	0.48 ± 0.01	12.18	0.48 ± 0.02	12.20

20 gm of five day old cotyledons were homogenized in 40ml of 50mM sodium acetate buffer pH4.7, after centrifugation at 20,000g for 30 minutes, the supernatant was brought to various ammonium sulphate saturations by the addition of solid ammonium sulphate.

The precipitates were dissolved in 50 mM Imidazole pH6.4 and assayed for proteinase activity and BAPNAase activity. (n=3).

(b) Purification

Initial purification of the proteases using DEAE-Sephacryl (Table 3) and Phenyl butyl amine (PBA) sepharose Chromatography (Table 4) showed that activity was lost using these methods and the percentage yield was low; they also failed to separate the two enzymes activities and were not incorporated into the final purification procedure.

Other methods tried and abandoned for similar reasons included; Thiol-sepharose Chromatography, hydrophobic chromatography using (PBA)-sepharose, and hydroxy apatite Chromatography.

Table 3. Ion exchange chromatography of a proteinase and a BAPNAase from 5 day old Vicia faba cotyledons.

	Vol ml	Protein mgml^{-1}	Total	Azocasein Activity units mg^{-1}	Total units	BAPNA activity units mg^{-1}	Total units
Initial extract	385	3.6	1386.0	0.83	1150.40	2.89	4005.5
30-70% saturated Ammonium Sulphate	40	14.6	584.0	1.43	835.12	6.37	3720.1
Ion exchange Chromatography with DEAE-Sephacryl	10	1.2	12.0	0.94	11.28	21.66	259.9

170g of 5 day old cotyledons were homogenized in 400.0ml of 50mM Sodium acetate buffer PH4.7, stirred for 1 hour at 4^{o}, strained through muslin to remove cell debris and centrifuged at 20,000g for 30 minutes. The supernatant fluid represented the initial extract.

This was brought to 30-70% saturation with solid ammonium sulphate, stirred for 1 hour at 4^{o} and centrifuged at 20,000g for 30 minutes. The precipitate was dissolved in 50mM imidazole buffer containing 5mM potassium chloride and 1mM EDTA and passed down a G-25 sephadex column, equilibrated with the same buffer, to remove the ammonium sulphate.

The extract was then loaded onto a DEAE-Sephacryl column (3.0 x 5.6 cm) equilibrated with the same buffer. Bound protein was eluted with a linear gradient of potassium chloride from 5 mM - 300mM. Fractions were collected and assayed for proteinase and BAPNAase activity.

Table 4. Phenyl butylamine sepharose (PBA - sepharose) chromatography of a proteinase and BAPNAase from 5 day old Vicia faba cotyledons.

	Vol	Protein content		Azocasein activity		BAPNA activity	
	ml	mg ml^{-1}	Total	units mg^{-1}	Total units	units mg^{-1}	Total units
Initial extract.	70	14.60	1022.0	0.288	294.34	0.75	766.50
Dialysis against distilled water.	79	9.00	711.0	0.427	303.60	0.88	625.68
30-70% Ammonium sulphate precipitation.	10	14.20	142.0	0.435	61.79	2.90	411.80
Phenylbutyl-amine Sepharose Chromatography.	10	0.34	3.4	2.980	10.13	48.00	163.20

50g of 5 day old Vicia faba cotyledons were homogenized in 100.0ml of 50mM Sodium acetate buffer pH 4.7, stirred for 1 hour at 4^{o}, strained through muslin to remove cell debris and centrifuged at 20,000g for 30 minutes.

The supernatant was dialysed against 2.0ℓ of distilled water overnight at 4^{o} with several changes of distilled water. Precipitated material was removed by centrifugation at 20,000g for 30 minutes. The supernatant was brought to 30%-70% saturation with solid ammonium sulphate stirred for 1 hour at 4^{o} and centrifuged at 20,000g for 30 minutes.

The precipitate was dissolved in 50mM TRICINE buffer pH 8.0 containing 1mM EDTA and applied to a phenyl butyl amine column equilibrated with 50mM TRICINE buffer pH 8.0 containing 1mM EDTA. Bound protein was eluted with 0.1M acetic acid pH 3.1 containing 1mM EDTA. Fractions were collected and assayed for proteinase BAPNAase activity.

(c) Final purification

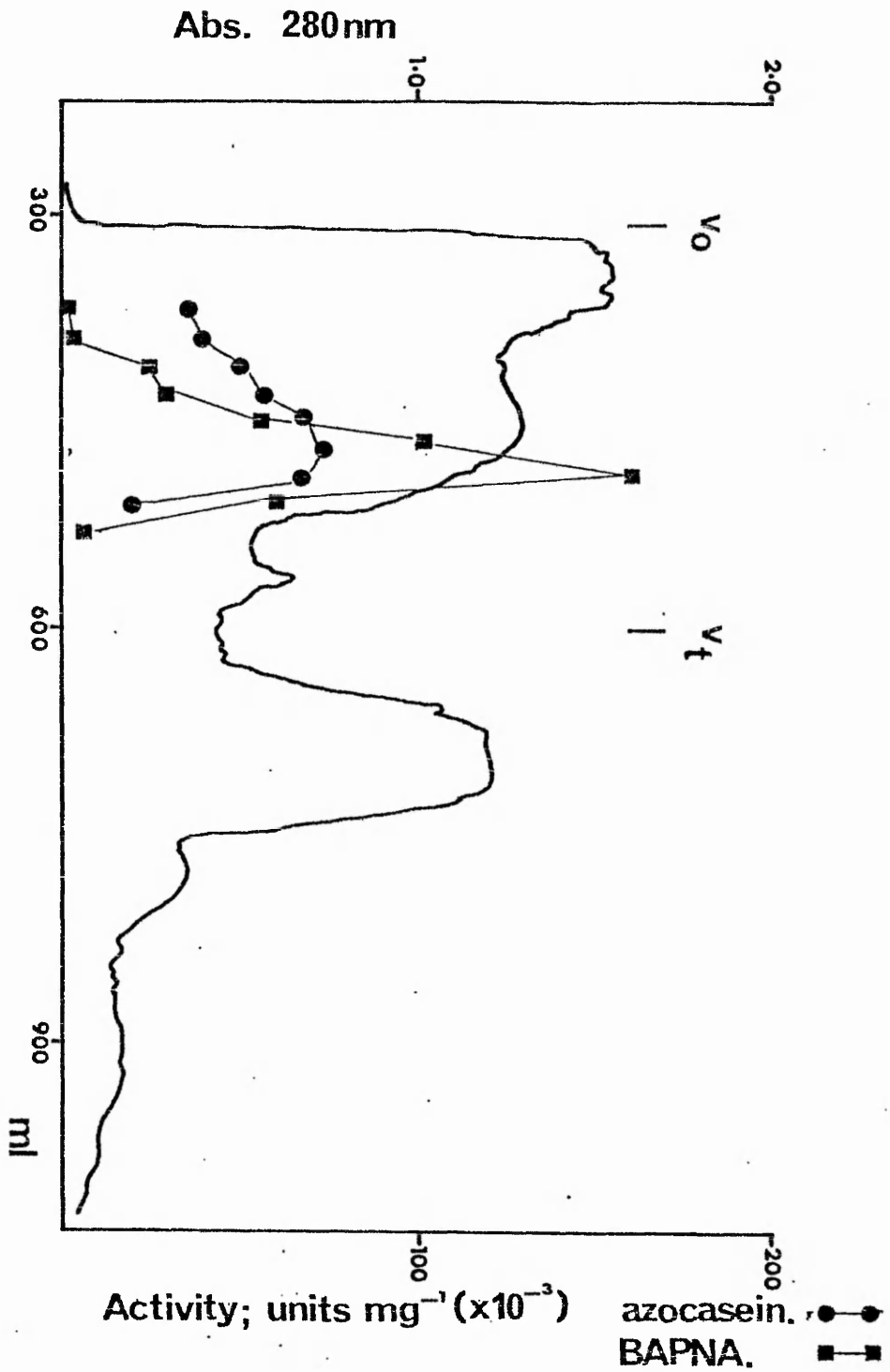
This purification procedure was evolved after many techniques of purifying enzymes were tried in a number of different sequences. At each stage it was necessary to assess the efficacy of a method.

After ammonium sulphate fractionation the enzyme extract was placed on a gel filtration medium, Sephacryl S-200 (Fig.2), which had a fractionation range ca. 100,000 - 10, 000 daltons. This gave good yields of the enzymes without globulin contamination.

After gel filtration the extracts were dialysed against the start buffer for affinity chromatography, a large amount of protein precipitated with little loss of enzyme yield.

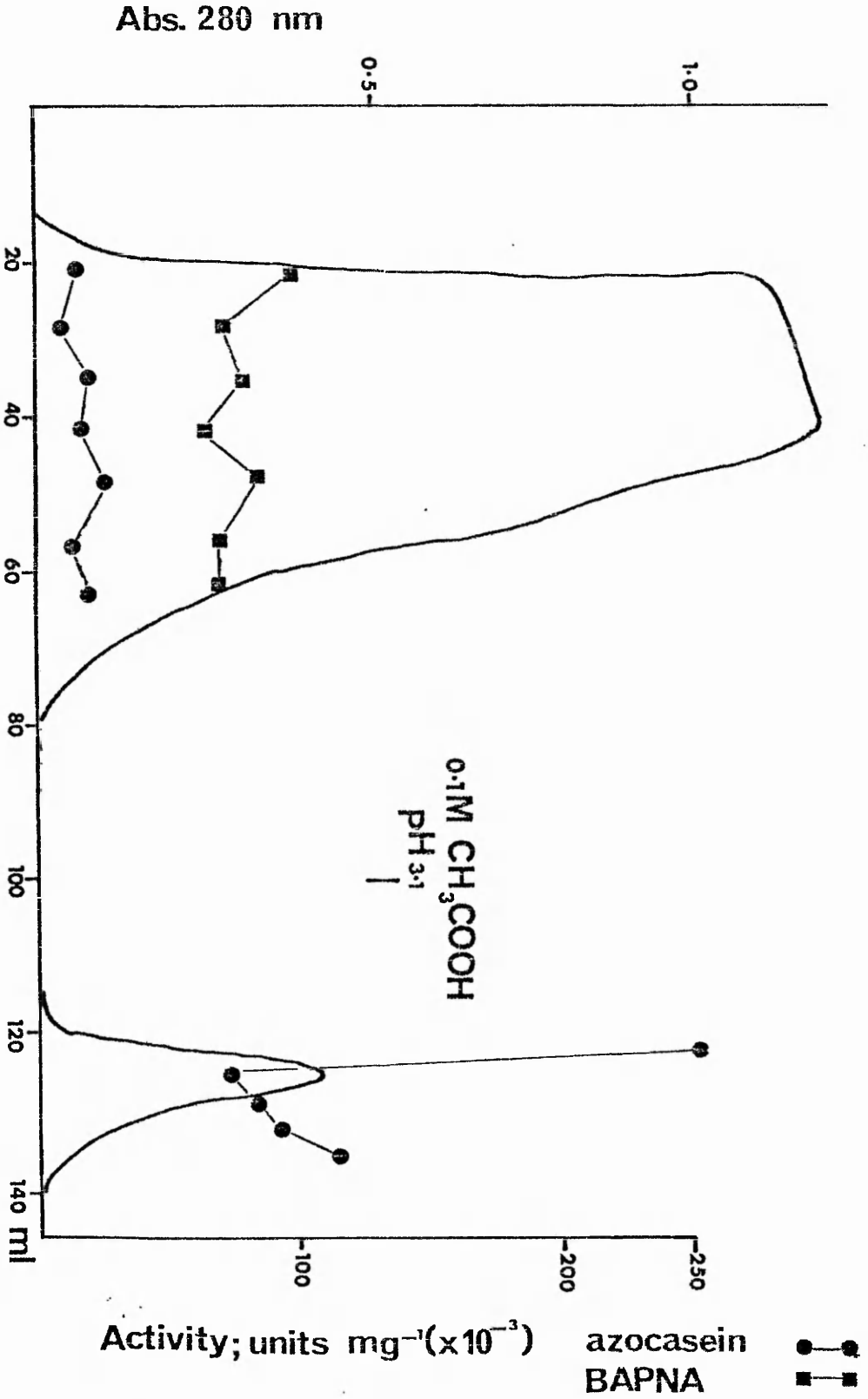
The extract was then applied to the affinity chromatography column. (Fig.3). The pH chosen for application was the pH optimum of the proteinase, to ensure maximum proteinase binding and the addition of 0.5M sodium chloride reduced non-specific protein binding. Unbound material contained both azocasein and BAPNAase activity, the bound fraction contained only azocasein activity. The majority of the azocasein activity in the unbound fraction could be recovered by repeated application to the affinity column, but the resulting yield was negligable. After elution, bound material from the affinity chromatography step was brought to pH 5.5, by the addition of 4M sodium acetate and used in assays to characterize the proteinase.

Fig 2. Gel filtration of enzyme extract.



Approximately 40.0ml of ammonium sulphate fractionated 5 day old cotyledon extract was applied to a Sephacryl S-200 column (2.6 x 80cm) and eluted continuously with 50mM imidazole buffer pH 6.4 containing 1mM EDTA. Protease activity on azocasein (●——●) and BAPNA (■——■) was determined on every other tube collected and A_{280nm} (———) was monitored continuously.

Fig.3. Affinity Chromatography of enzyme extract.



Approximately 30.0ml of enzyme extract after gel filtration on Sephacryl S-200 and dialysis against 50mM Sodium acetate buffer pH 5.4 containing 1mM DTT and 1mM EDTA was applied to a Vicia faba globulin Sepharose 4B column (1.6 x 20cm) equilibrated with 50mM sodium acetate buffer pH 5.4 containing 0.5M Sodium Chloride, 1mM DTT and 1mM EDTA. Unbound protein was eluted with the above buffer, bound protein was eluted with 0.1M Acetic acid pH 3.1 containing 1mM EDTA and 1mM DTT. Protease activity on azocasein (●——●), BAPNA (■——■) was determined on every other tube collected and A_{280nm} (————) was monitored continuously.

Unbound material from the affinity chromatography step was dialysed against 25mM piperazine pH5.5 and concentrated on an Amicon DM30 membrane; this prepared the extract for chromatofocussing and removed most of the remaining azocasein activity.

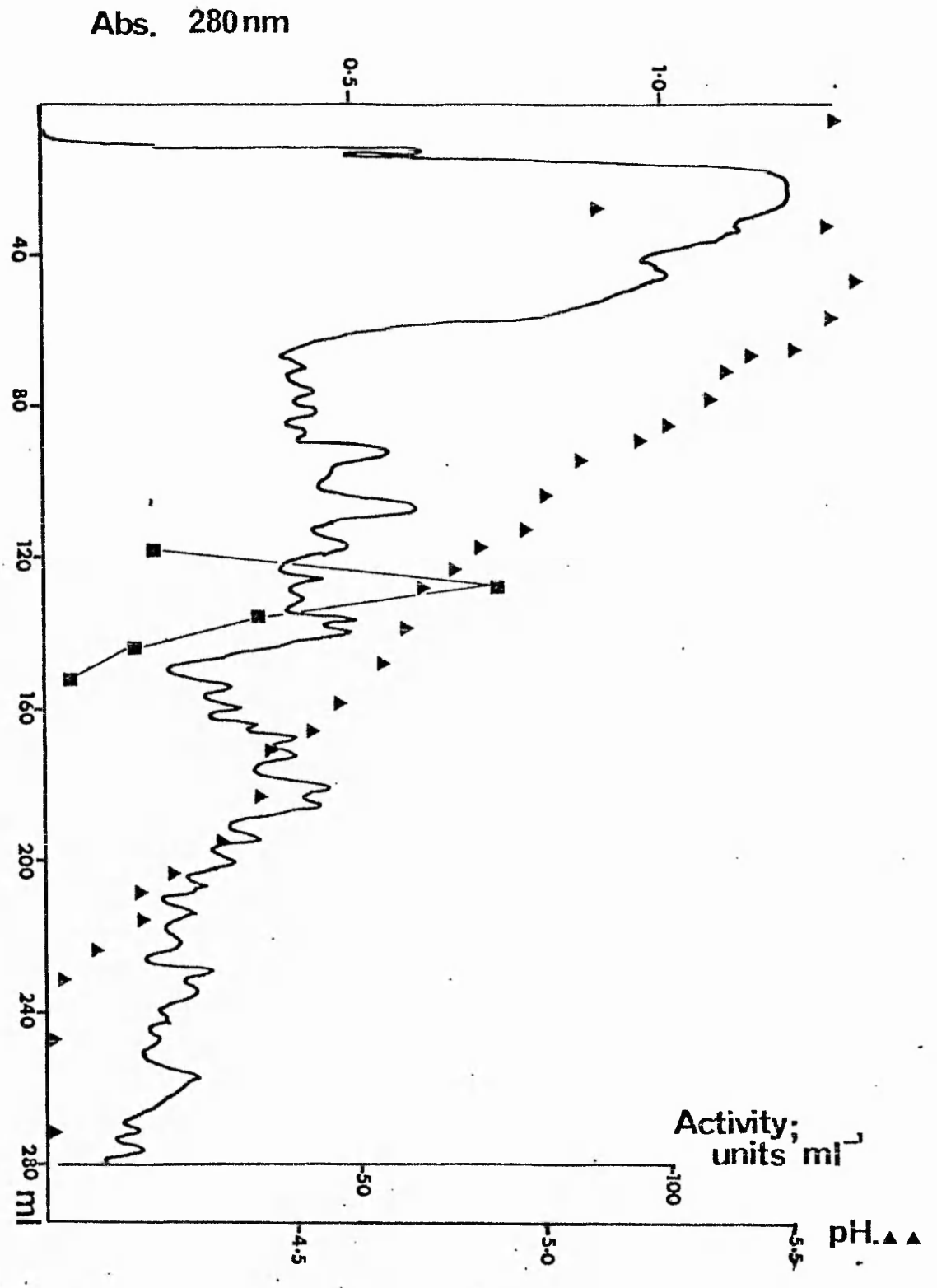
The BAPNAase eluted from the chromatofocussing column (Fig.4) at pH 4.76 ± 0.02 (the isoelectric point of the enzyme), no azocasein activity could be detected.

After concentration, the BAPNAase fractions were applied to a (B.R.L) preparative gel electrophoresis apparatus (Fig.5). The active material eluted in a narrow band and used in assays to characterise the BAPNAase.

The efficiency of these steps is shown in Table 5, which represents a typical extraction. The final purification of the azocasein active enzyme was about 200-400 fold, and the BAPNAase was 400 fold. The steps which achieved the greatest increase in specific activity were the affinity chromatography step for the proteinase and chromatofocussing for the BAPNAase.

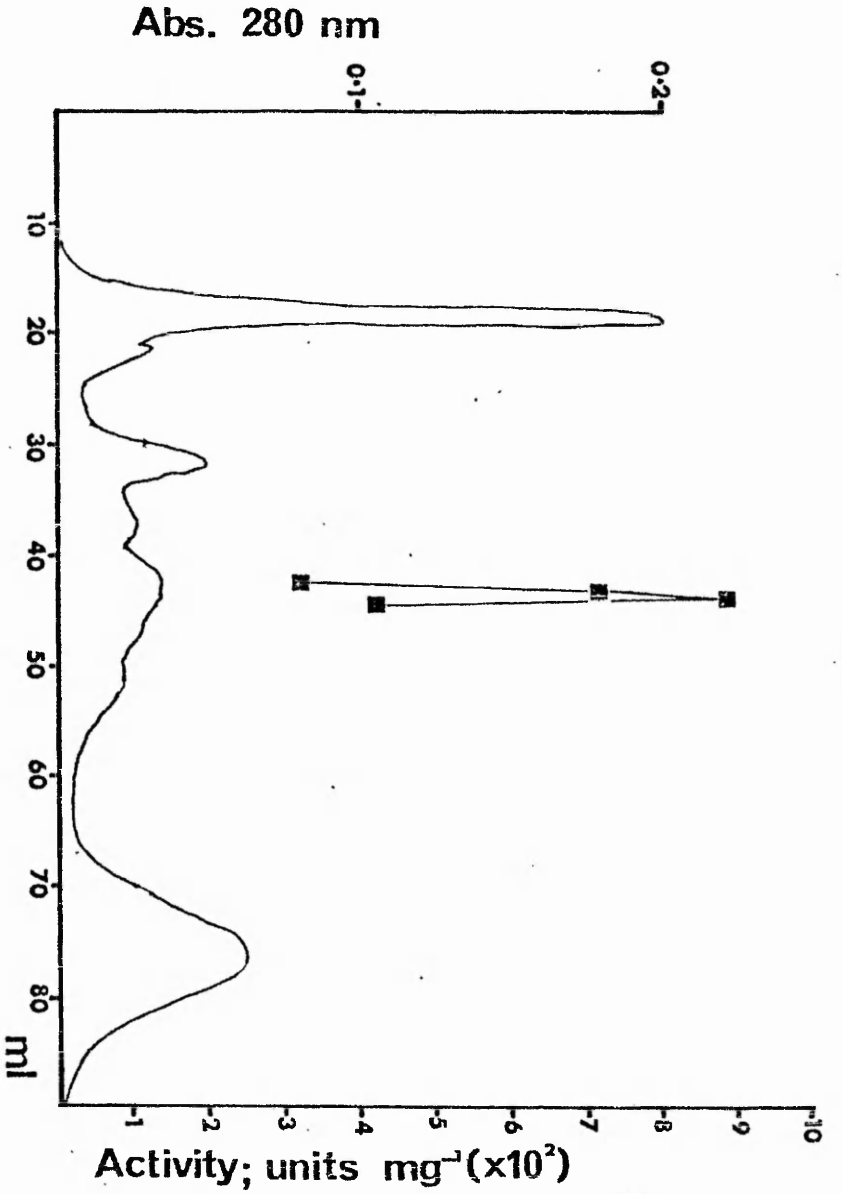
Variations in percentage yield and purity were encountered from time to time, this may have been due to the instability of the enzyme or to the different amounts of enzyme initially extracted from different batches of seeds.

Fig.4. Chromatofocussing of the enzyme extract.



Approximately 20.0ml of enzyme extract, after gel filtration on Sephacryl S-200, affinity chromatography, dialysis against 25mM piperazine pH 5.5 and concentration on an Amicon PM30 membrane was applied to a Chromatofocussing column containing polybuffer exchanger (Pharmacia) equilibrated with 25mM piperazine pH 5.5 and eluted with poly buffer (7-4) pH 4.0. BAPNAase activity (■———■) was determined on alternate tubes and A_{280nm} (▲———▲) was monitored continuously.

Fig.5. Preparative gel electrophoresis of enzyme extract.



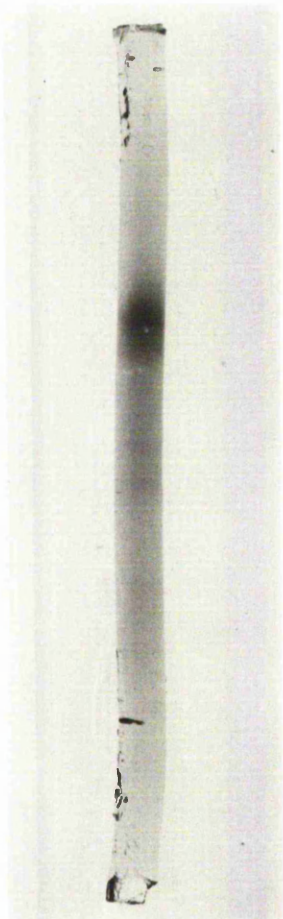
Approximately 1.0ml of enzyme extract after gel filtration, affinity chromatography, chromatofocussing and concentration on an Amicon PM30 was applied to a polyacrylamide gel column (3.5 x 1.0cm) (B.R.L) and run in a continuous Tris-glycine buffer pH 8.9 for 10 minutes at 2ma then overnight at 5ma. BAPNAase activity (■———■) was determined on each fraction and A_{280nm}(————) was monitored continuously.

TABLE 5 Purification of Enzymes

	Volume ml	Protein mg ml^{-1}	Protein Total	Azocasein Activity units mg^{-1}	Azocasein Activity Total units	Azocasein Activity % Yield	Azocasein Activity fold pure	BAPNA Activity units mg^{-1}	BAPNA Activity Total units	BAPNA Activity % Yield	BAPNA Activity fold pure
Initial Extract	520.0	6.75	3510.00	0.69	2422.00	(100.00)	(1.00)	1.30	4563.00	(100.00)	(1.00)
30% - 70% Ammonium Sulphate precipitation	38.0	36.00	1368.00	1.45	1983.60	82.00	2.10	3.00	4104.00	89.90	2.30
gel filtration S-200 Sephacryl	80.0	6.70	536.00	2.98	1597.00	65.90	4.30	5.90	3162.00	69.20	4.53
Dialysis against 50 mM Na Ac pH 5.4 1 mM EDTA, DTT	74.0	4.60	340.20	4.66	1584.40	65.40	6.75	8.00	2712.00	59.20	6.15
Affinity (a)	65.0	3.80	247.00	2.62	647.10	26.70	3.80	9.20	2272.00	49.80	7.07
Chromatography (b)	20.0	0.20	4.00	13 3.40	533.60	22.00	19 3.30	-	-	-	-
Dialysis 25 mM (a) Piperazine pH 5.5	75.0	3.08	231.00	2.38	549.78	22.70	3.40	9.15	2114.00	46.30	7.03
25mM (b) Imidazole 7.4	25.0	0.07	1.75	245.60	429.80	7.70	355.90	-	-	-	-
Concentration on Amicon PM30 membrane	20.0	9.4	18 8.	0.787	147.96	6.11	1.140	11.09	2086.	45.71	8.53
Chromato focussing 5.0 - 4.0	8.0	1.8	14.40	-	-	-	-	116.00	1670.00	36.60	89.21
preparative gel electrophoresis	2.4	0.14	0.34	-	-	-	-	523.00	175.70	3.90	402.30

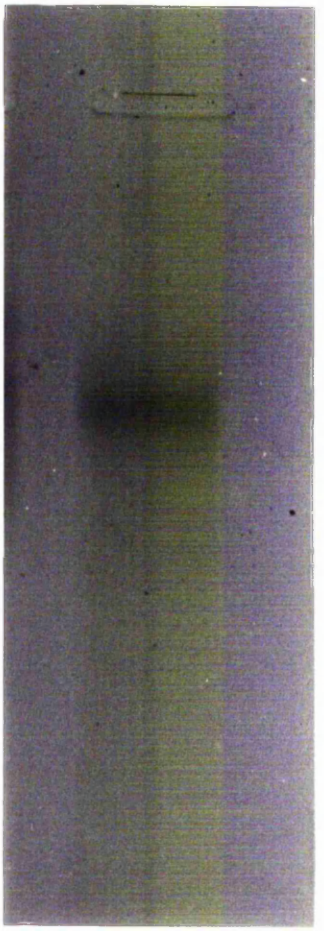
PAGE (Fig.6) of the purified proteinase showed one major band (about 90% of protein), minor bands are just visible, but these may be breakdown products of the enzyme by autolytic activity. PAGE (Fig.7) of the purified BAPNAase showed one detectable protein band, which in similar gels not stained for protein corresponded to BAPNAase activity.

Fig.6 PAGE of the purified proteinase.



0.06mg of purified proteinase was separated in a 10% (w/v) polyacrylamide gel (T=10.2% C=2.6%) with a continuous Tris-glycine buffer pH8.9 for 10 mins at 3ma, then 2.5 hrs at 6ma, using a Shandon electrophoresis chamber. The gel was stained with PAGE Blue 83 and destained with ethanol,glacial acetic acid and water (30:10:60).

Fig.7 PAGE of the BAPNAase after preparative gel electrophoresis.



0.03mg of Purified BAPNAase was separated in a 10% (w/v) polyacrylamide gel (T=10.2% C=2.6%) with a continuous Tris-glycine buffer pH 8.9 for 10 minutes at 20ma, then 2.5 hours at 40ma, using an LKB multiphor The gel was stained with PAGE Blue 83 and destained with ethanol, glacial acid and water (30:10:60).

2. Proteinase Characterisation.

(i) Molecular weight and stability.

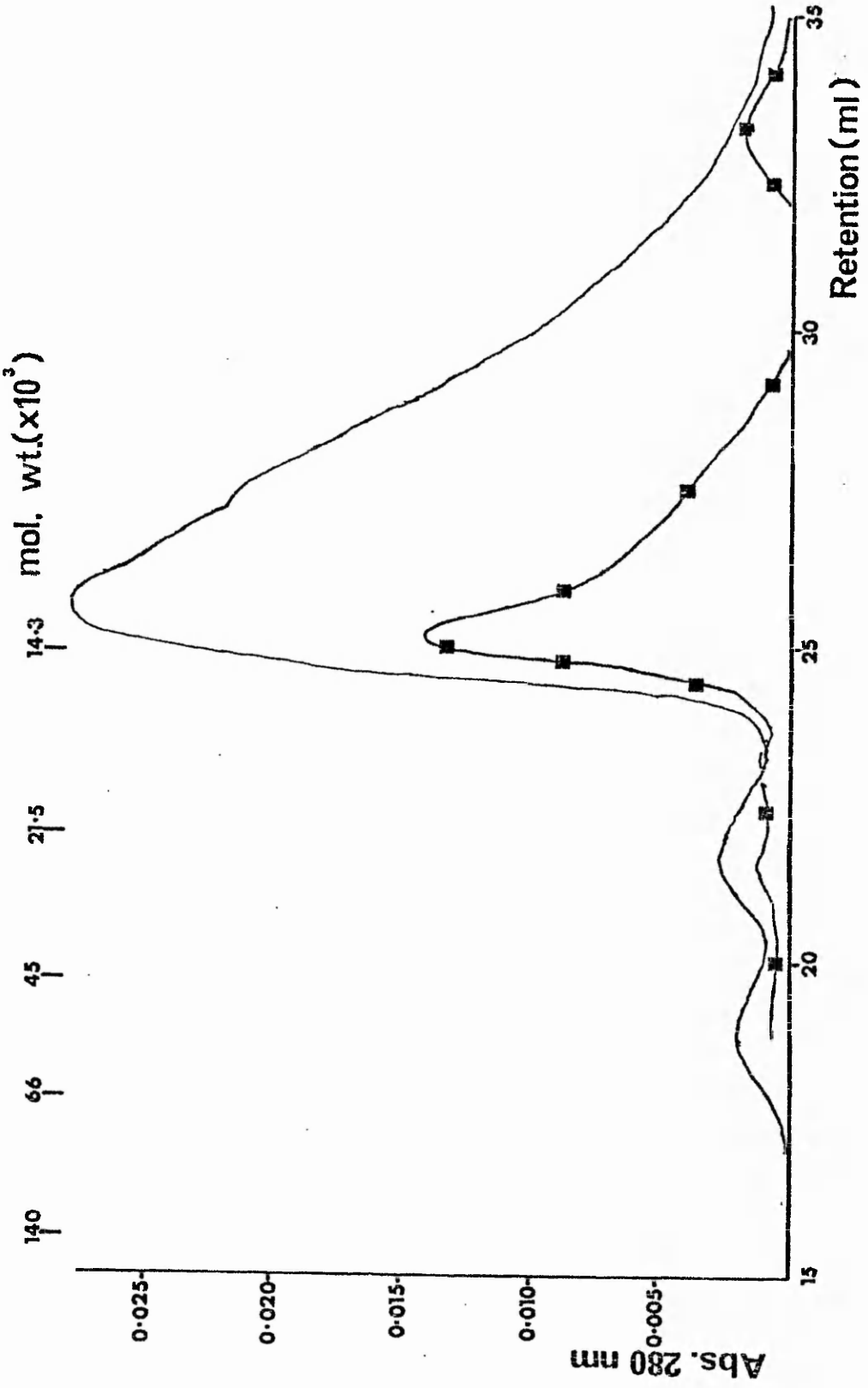
The molecular weight of the proteinase was estimated by gel filtration on a TSK 3000 HPLC Column. The column was calibrated with proteins of known molecular weight and the main peak of the proteinase sample corresponded to a molecular weight of 12,200 daltons. (Fig.8).

The other half of the sample was stored at 4^{o} for 3.5 days and reapplied to the column. There is a loss of material present (Fig.8) but the main peak remains at ca. 12,000 daltons.

In another experiment active material was applied to the column and fractions collected to try and ascertain the peak which corresponded to proteinase activity. However no active material eluted from the column.

The stability of the proteinase was assessed by storage and assay; there was a 90% loss of activity after overnight storage at 4^{o} and a similar loss at -20°. For this reason purified proteinase was used immediately in characterisation experiments.

Fig.8 The molecular weight estimation of the proteinase.



2.0ml of purified protein at 0.115 mg ml^{-1} was concentrated on an Amicon B15 concentrator to 0.2ml, this was then applied to a TSK 3000 column equilibrated with 0.1M sodium acetate buffer pH 6.0 containing 1mM EDTA, at 0.8 ml min^{-1} flow rate. (———).

After 3.5 days storage at 4^{o} a further 2.0 ml of protein was concentrated to the same volume, and applied to a TSK 3000 column, as described above. (■———■).

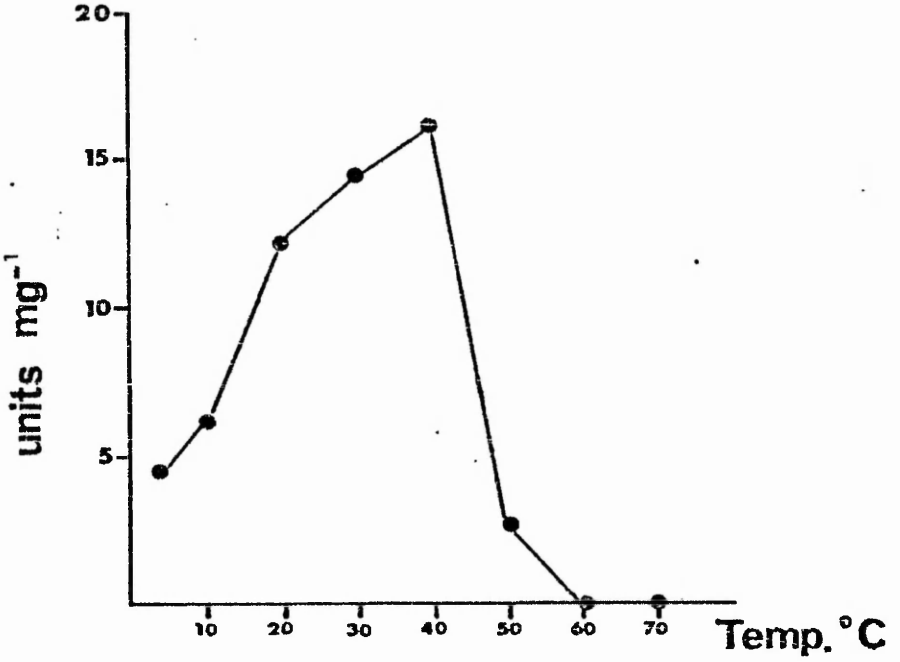
(Alkaline phosphatase (mol.wt. 140,000 daltons); Bovine serum albumin (mol.wt. 66,000 daltons); Egg albumin (mol.wt. 45,000 daltons); Chymotrypsinogen (mol.wt. 21,500 daltons); lysozyme (mol.wt. 14,300 daltons) were the standard proteins used to calibrate the column).

(ii) Factors affecting activity.

The purified proteinase was incubated and assayed at different temperatures and pH to find the optimum assay conditions for the enzyme. Figure 9 shows the enzyme was sensitive to temperature inactivation above 40^{o} when inactivation rapid. Figure 10 shows the enzyme to have a pH optimum between 5.0 to 6.0, with the peak activity at pH 5.4. There was virtually no activity above pH 7.0, therefore the proteinase did not overlap with the BAPNAase activity, when the BAPNAase was assayed at pH 7.4.

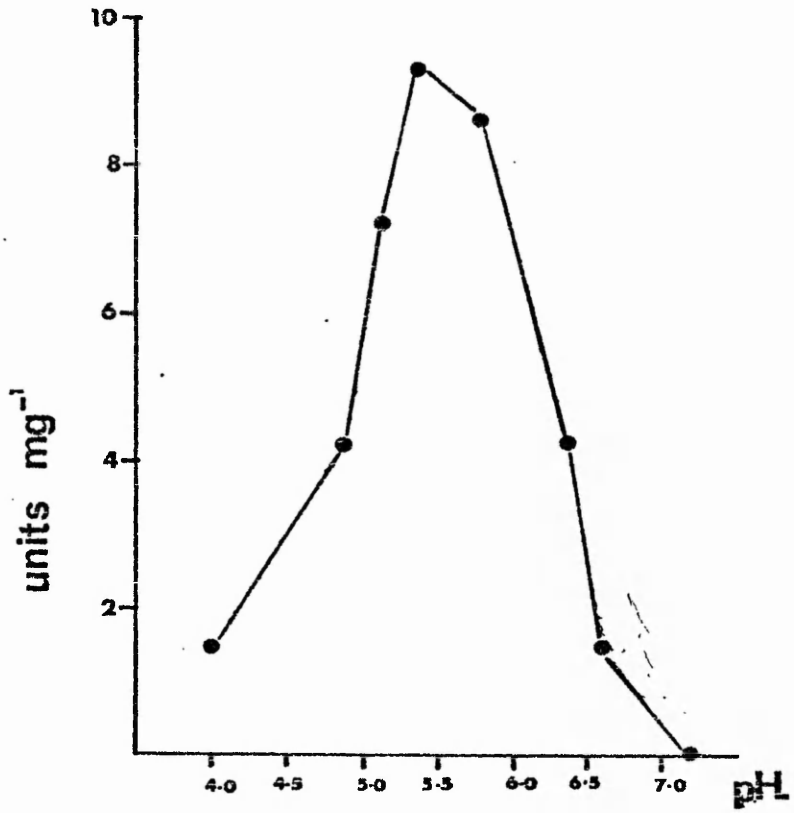
The effect of proteolytic enzyme inhibitors was investigated and Table 6 shows that significant inhibition was shown by the thiol proteinase inhibitors; mercurous ions, N-ethyl maleimide, iodo acetamide and p-chloro mercuri - benzene sulphonic acid (PCMB). This indicates that thiol groups are required for activity, a fact which should have been confirmed by activation with the reducing agent 2- mercaptoethanol, but at 3mM concentration no activation was observed. This was investigated and Table 7 shows the effect of different concentrations of 2-mercaptoethanol. At high concentrations (>1mM) the reducing agent inhibited the enzyme, greatest activation was observed at 0.1mM.

Fig.9 The effect of incubation temperature on the activity of the proteinase.



0.046 mg of purified proteinase was preincubated at various temperatures in 0.1M McIllivane buffer pH 5.4 for 20 minutes. The reaction was started by the addition of 0.5ml of 2% (w/v) Azocasein, the incubation temperature remaining the same as the pre incubation temperature. The reaction was terminated by the addition of 20% (w/v) T.C.A. After centrifugation at 5,000g for 10 minutes, 1.0ml of the supernatant was developed with 1.0ml of 2M NaOH and the increase in absorbance measured at 440 nm.

Fig. 10 The effect of pH on the activity of the proteinase



0.092mg of purified proteinase was preincubated at various pH on 0.1M McIllivane buffer for 20 minutes at 40°. The reaction was started by the addition of 0.5ml of 2% (w/v) Azocasein and terminated by the addition of 0.5ml of 20% (w/v) TCA. After centrifugation at 5,000g for 10 minutes 1.0ml of the supernatant was developed with 1.0 2M NaOH and the increase in absorbance measured at 440nm.

Table 6 shows that the serine protease inhibitor phenyl methyl sulphonyl flouride (PMSF) did not effect the enzyme activity significantly, therefore the enzyme does not possess an active serine catalytic centre. The metallo enzyme inhibitor EDTA increased activity slightly.

Table 6. The effect of proteolytic inhibitors and activators upon the activity of the proteinase.

Inhibitors 3mM	units	(S.E.M)	(%)control
PMSF	4.39	±0.10	89.41
N-ethylmaleimide	0.64	±0.05	13.03
Iodoacetamide	1.22	±0.05	24.84
PCMB	0.51	±0.05	10.38
Hg^{2+}	0.00		Zero
EDTA	5.16	±0.05	105.09
Activator 3mM			
^{2}-Mercaptoethanol	4.32	±0.14	87.98
Control	4.91	±0.11	100.00

0.215mg of purified proteinase was preincubated for 15 minutes with 0.1M McIllivane buffer pH 5.4 containing 3mM of the above inhibitors. The reaction was started by the addition of 0.5ml of 2% (w/v) Azocasein and terminated by the addition of 0.5ml of 20% (w/v) T.C.A., after centrifugation at 5,000g for 10 minutes, the supernatant was measured at 440 nm after the addition of an equal volume of 2M NaOH. (N=3)

Table 7. The effect of different concentrations of 2-mercaptoethanol upon the activity of the proteinase.

mM 2-mercaptoethanol	units	(S.E.M)	(%)Activation
Control	24.00	±0.35	100.00
100	6.55	±0.35	27.29
10	20.79	±0.23	86.62
1	29.62	±0.46	123.41
0.1	33.04	±2.03	137.66
0.01	27.91	±0.46	116.29
0.001	28.48	±0.93	118.66

0.0325mg of purified proteinase was preincubated for 30 minutes with 0.1M McIllivane buffer pH 5.4 containing various concentrations of 2-mercaptoethanol. The reaction was started by the addition of 0.5ml of 2% (w/v) Azocasein and terminated by the addition of 0.5ml of 20% (w/v) T.C.A after 3 hours. After centrifugation at 5,000g for 10 minutes the supernatant was measured at 440 nm after the addition of an equal volume of 2M NaOH (n=3).

(iii) Substrate Specificity

The proteinase activity on purified storage proteins was measured and Table 8 shows that the proteinase was active on both legumin and vicilin isolated from various stages of germination. The amount of protein hydrolysed increased in storage proteins isolated from the latter stages of germination, this increase was more noticeable using vicilin rather than legumin. Fig 11 (a+b) shows that the storage proteins are degraded at approximately the same rate and that the enzyme alone is not capable of completely degrading the storage proteins. The different reaction rates may reflect the activity of different enzyme preparations.

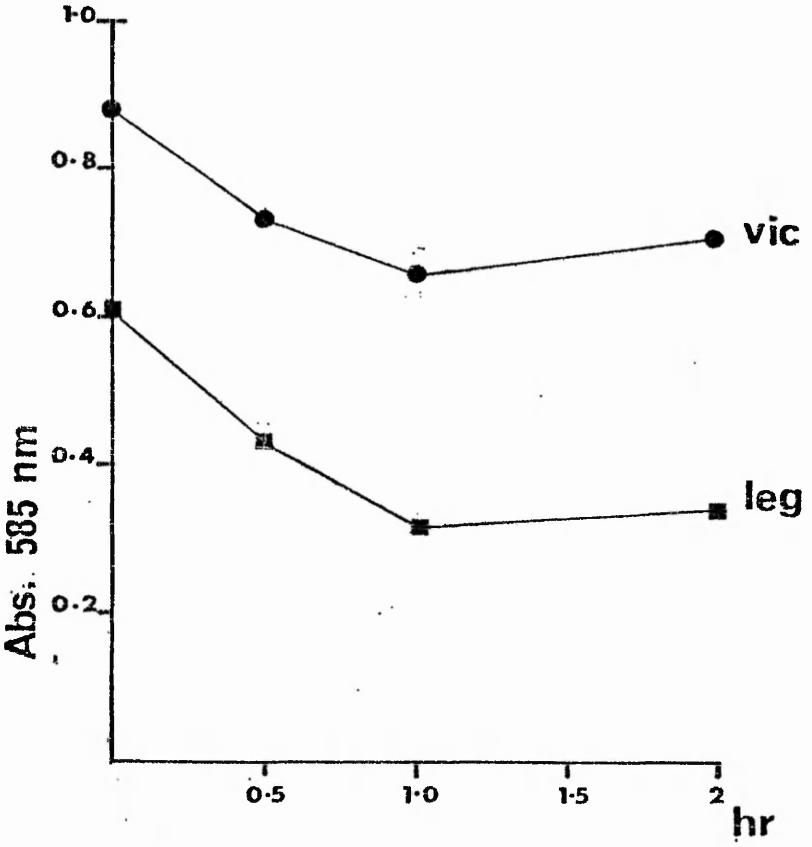
Table 9 shows that the enzyme possesses some esterase activity, only nitrophenol esters of asparagine and glycine were hydrolysed. The citrulline paranitroanalides were not hydrolysed, but all the ethyl esters were hydrolysed to varying degrees.

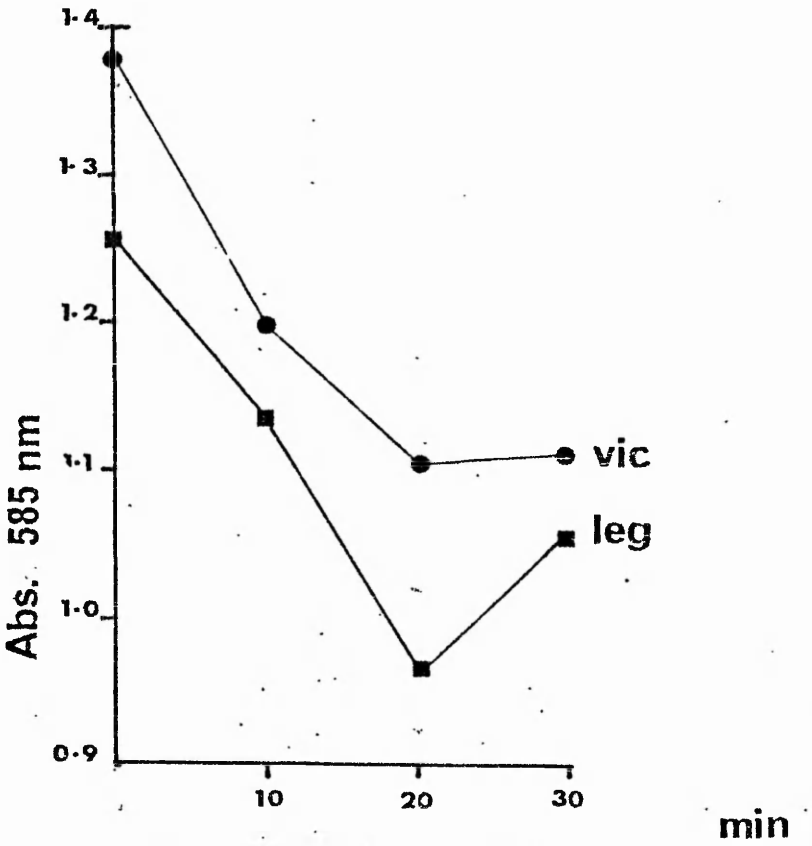
Table 8. The activity of the proteinase on purified storage proteins.

Substrate	Abs 585nm (S.E.M) Zero Time		2 hrs		Difference	µg Protein released
Day 0 vicilin	0.713	±0.020	0.526	±0.005	0.187	14.50
Day 4 vicilin	0.75		0.603	±0.015	0.147	11.75
Day 7 vicilin	0.75	±0.012	0.460	±0.004	0.290	22.25
Day 11 vicilin	0.735		0.476	±0.012	0.259	20.00
Day 0 legumin	0.800	±0.028	0.600	±0.009	0.2	15.50
Day 4 legumin	0.763	±0.009	0.496	±0.016	0.267	20.50
Day 7 legumin	0.895		0.650	±0.014	0.245	19.00
Day 11 legumin	0.725	±0.008	0.406	±0.011	0.319	24.50

0.032mg of purified protinase was incubated with approximately 0.08mg of purified storage proteins in 0.1M McIllivane buffer pH 5.4 extracted from cotyledons at different stages of germination. The reaction was terminated after 2 hours by the addition of 5.0ml of Coomassie blue reagent and the decrease in absorbance measured at 585 nm. (n=3).

Fig.11 (a,b) Activity of the proteinase on seven day old purified legumin and vicilin.





0.035mg of proteinase was added to 0.01mg of seven day old purified legumin and vicilin (Fig.11a) dissolved in 0.1M McIllivance buffer pH 5.4 at 40°. 0.037 mg of another proteinase preparation was added to 0.02mg of seven day old purified legumin and vicilin (Fig. 11 b).

After various incubation periods the reaction was terminated by the addition of 5.0ml of Coomassie blue reagent, after vigorous mixing the decrease in absorbance was measured at 585nm.

Table 9. Proteinase activity on a variety of synthetic substrates.

Substrates	Change in Absorbance 410nm min^{-1} mg^{-1} of proteinase
CBZ-asparagine NPE (1.67 mM)	0.059
CBZ-glycine NPE (1.67 mM)	0.208
CBZ-le cine NPE (1.67 mM)	not detectable
CBZ-Phenylalanine NPE (1.67 mM)	not detectable
CBZ-Tryptophan NPE (1.67 mM)	not detectable
CBZ-Tyrosine NPE (1.67 mM)	not detectable
CBZ-Valine NPE (1.67 mM)	not detectable
CBZ - Citrulline PNA (1.67mM)	not detectable
Citrulline PNA (1.67mM)	not detectable
	Change in Absorbance 253/256nm min^{-1} mg^{-1} of proteinase added.
Benzoyl arginine ethyl ester (1.67mM)	0.116
Benzoyl Tyrosine ethyl ester (1.67mM)	0.203
Arginine ethyl ester (1.67 mM)	0.293

0.062mg of purified proteinase was added to 2.75ml of 0.1M McIllivane buffer pH 5.4 at 25^{o} containing 15% (v/v) methanol and 0.05ml of 100mM carboxybenzoyl nitrophenol ester derivatives of amino acids, 0.05ml of 100mM Benzoyl ethyl ester derivatives of amino acids, 0.05ml of 100mM carboxybenzoyl citrulline paranitroanalide, or 0.05ml of 100mM Citrulline paranitroanalide. The increase in absorbance was measured directly in a Beckman DU7 recording spectrophotometer; non-enzymic spontaneous degradation of the substrates was subtracted.

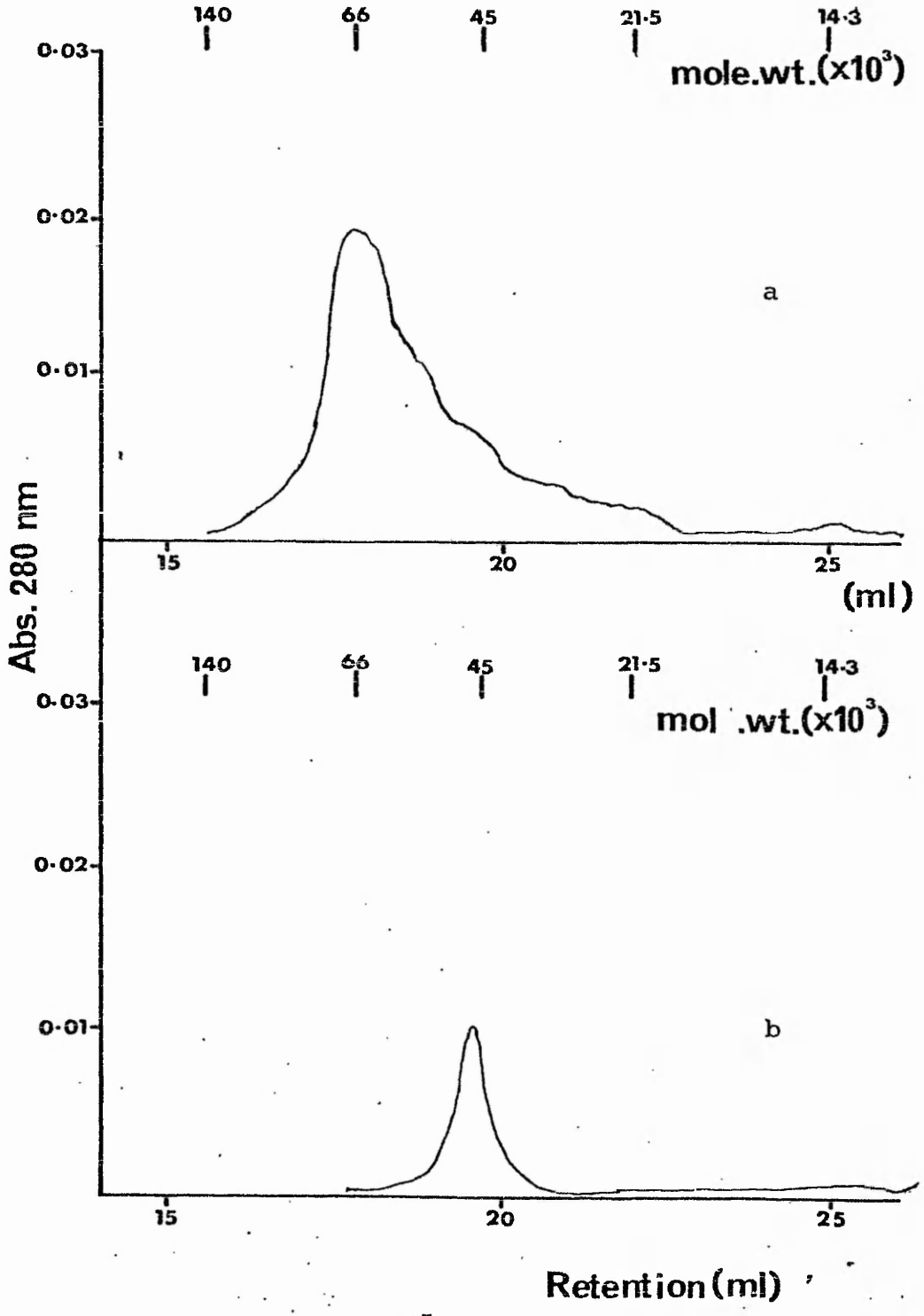
3. BAPNAase Characterization.

(i) Molecular weight and stability.

The molecular weight of the BAPNAase was estimated by gel filtration on a TSK 3000 HPLC column; the column was calibrated with proteins of known molecular weight. Fig. 12 (a) is a sample of BAPNA active material after chromatofocussing and the main peak corresponds to a molecular weight of 63,100 daltons. Figure 12 (b) is a sample of BAPNAase after preparative gel electrophoresis, and the peak corresponds to a molecular weight of 50,100 daltons.

The stability of the purified BAPNAase was assessed and Table 10 shows that the enzyme is more stable than the proteinase, At 4^{o} with no additives the activity drops by 50% during the first 65 hours and then remains at the same level over a 233 hour period. The addition of bovine serum albumin (BSA) reduces the loss of activity, and the addition of glycerol increases the loss of activity. Storage at -20^{o} with no additives results in a 25% loss in activity after 65 hours with a further 25% loss in original activity after 233 hours. The presence of BSA reduces the loss of activity after 65 hours better than glycerol, but after 233 hours the presence of an additive does not appear to affect the loss of activity. The exception was the addition of 5% B.S.A. .

Fig. 12. (a, b). The molecular weight of BAPNAase by gel filtration.



0.005mg of protein was applied to a TSK 3000 Column at a flow rate of 1.0ml min^{-1} in 50 mM HEPES buffer pH 7.0 containing 10% (v/v) glycerol and 1mM EDTA. Fig (a) is BAPNAase active material after chromatofocussing. Fig (b) is purified BAPNAase after preparative gel electrophoresis.

(Alkaline phosphatase (mol. wt. 140,000 daltons); Bovine serum albumin (mol. wt. 66,000 daltons); Egg albumin (mol. wt. 45,000 daltons); Chymotrypsinogen (mol. wt. 21,500 daltons); lysozyme (mol. wt.14,300 daltons) were the standard proteins used to calibrate the column.)

Table 10. Stability of the BAPNAase.

		Time after purification. Percentage of original units (1.23)		
		Zero Time	65 hrs	233 hrs
4°	no additions	100%	56.7%	54.0%
	5% BSA	100%	64.8%	86.5%
	10% BSA	100%	85.1%	75.6%
	20% BSA	100%	83.8%	67.5%
	5% glycerol	100%	74.3%	2.7%
	20% glycerol	100%	81.1%	21.6%
-20°	no additions	100%	78.4%	54.0%
	5% BSA	100%	75.6%	75.6%
	10% BSA	100%	91.2%	54.0%
	20% BSA	100%	87.8%	45.9%
	5% glycerol	100%	50.0%	29.7%
	10% glycerol	100%	59.4%	54.0%
	20% glycerol	100%	56.7%	35.1%

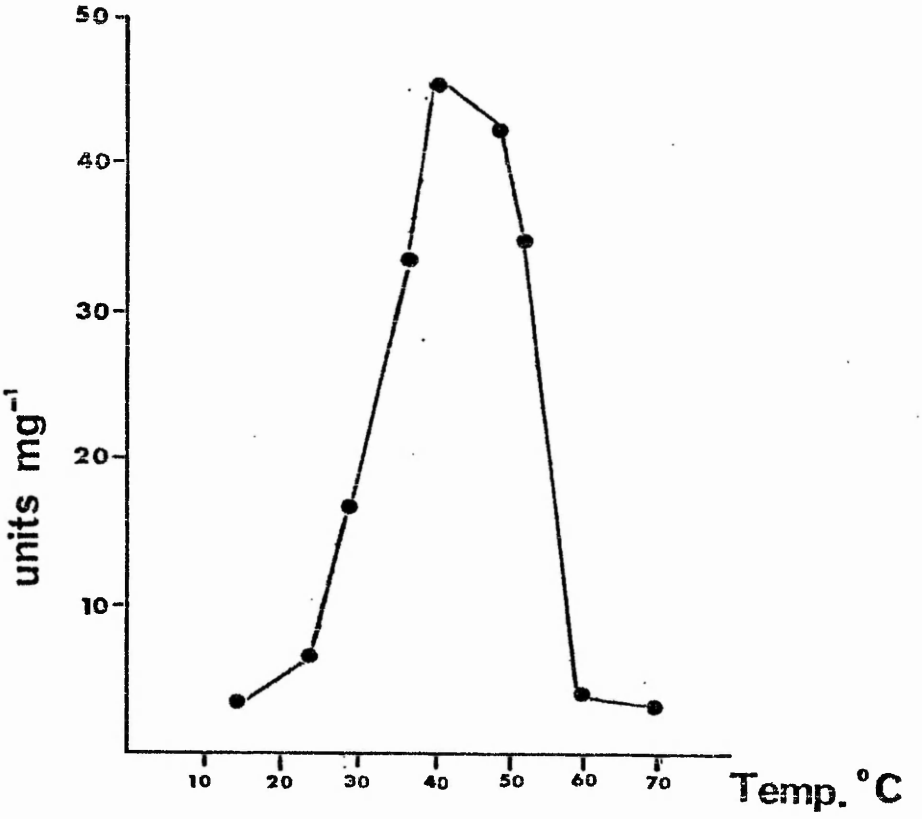
1.25µg of purified BAPNAase was stored in 0.15ml aliquots with and without the above additives, at 4° and -20° and removed to be assayed with BAPNA.

(ii) Factors affecting activity

The purified BAPNAase was incubated at different temperatures and pH to find the optimum assay conditions for the enzyme. Figure 13 shows that the enzyme is stable at higher temperatures than the proteinase; activity declining when the temperature exceeded 50^{o}. This is confirmed in Fig.15 showing the time course of the reaction; at 60^{o} the enzyme is inactivated after one minute. The pH profile of the enzyme in Fig.14 shows an optimum pH of 7.4 and the Tris/HCL buffers apparently inhibit activity.

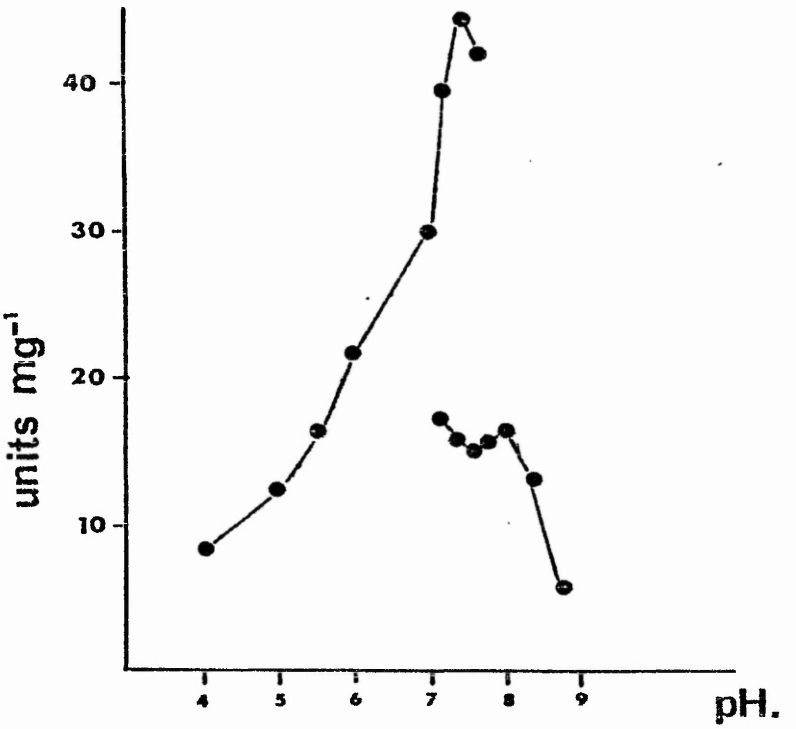
The effect of protease inhibitors on BAPNAase activity (Table 11) is similar to that of the proteinase; the thiol protease inhibitors N-ethyl maleimide, iodoacetamide and PCMB all inhibiting the enzyme, with N-ethyl maleimide being the most effective. This confirms, as with the proteinase, that thiol groups are required for activity. The enzyme was extremely sensitive to N-ethylmaleimide. Table 12 shows that 0.01 mM produced over 50% inhibition of activity, the reducing agents (Table 11) all increased enzyme activity with 2- mercaptoethanol being the most effective. In contrast to the proteinase, the BAPNAase showed greater tolerance of the presence of high concentrations of 2- mercaptoethanol (Table 13). Even at low concentrations there was activation of enzyme activity. The serine protease inhibitor PMSF and the metaloprotease inhibitor EDTA did not inhibit the enzyme, indicating that neither serine or a metal is present at the active site of the enzyme.

Fig. 13. The effect of incubation temperature on the activity of BAPNAase.



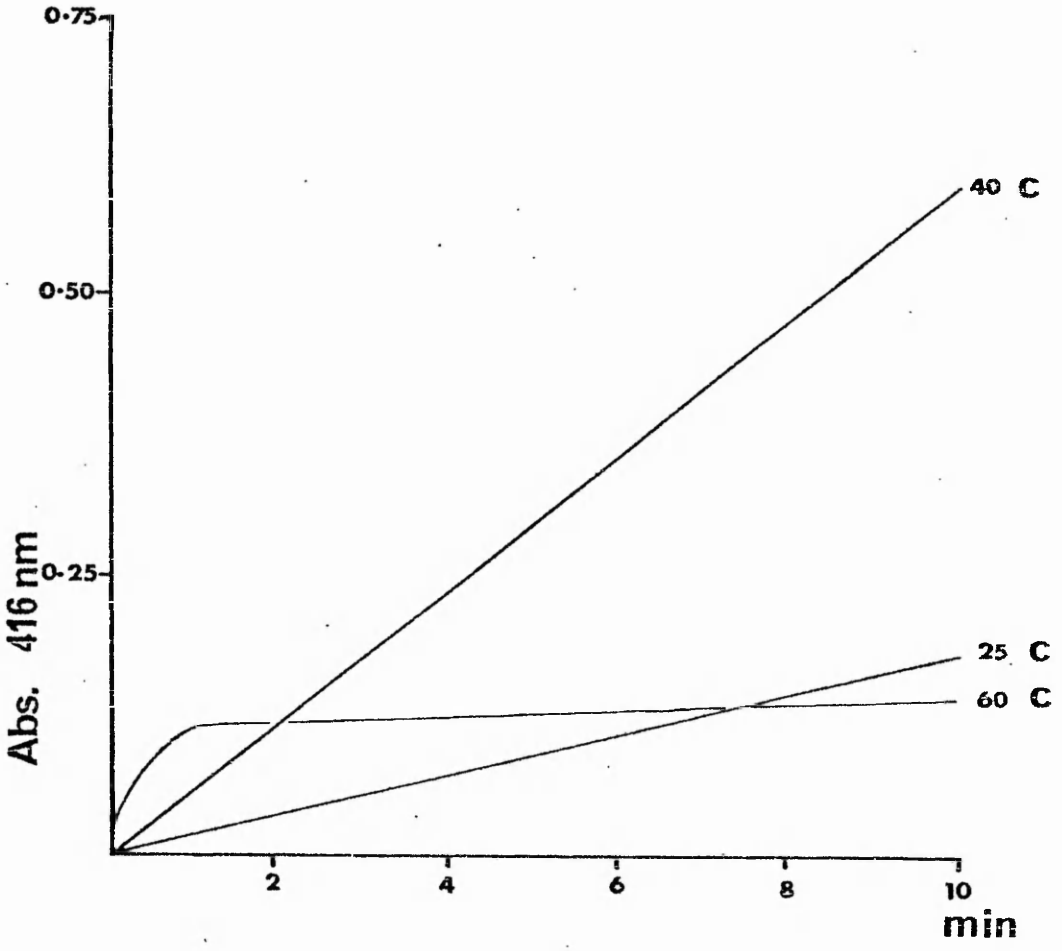
0.035mg of purified BAPNAase was preincubated at various temperatures in 2.0ml of 0.1M McIllivane buffer pH 7.4 for 15 minutes when the reaction was started by the addition of 0.1M of 105 mM BAPNA. The reaction was terminated by the addition of 0.7ml of 20% (w/v) TCA. After centrifugation at 5,000g for 10 minutes, the supernatant absorbance was measured at 416 nm.

Fig. 14. The effect of different pH on the activity of BAPNAase.



0.025mg of purified BAPNAase was preincubated in either 2.0ml of 0.1M McIllivane buffer (pH 4.0 - 7.6) or 0.1M Tris/HCL buffer (pH. 7.0 - 8.8) for 15 minutes. The reaction was started by the addition of 0.1ml of 105 mM BAPNA and terminated by the addition of 0.7ml of 20% (w/v) TCA. After centrifugation at 5,000g for 10 minutes the supernatant's absorbance was measured at 416nm.

Fig. 15 The effect of temperature on the time course of BAPNAase activity.



0.035mg of purified BAPNAase was added to 2.0ml of 0.1M McIllivane buffer pH 7.4 containing 0.1ml 105mM BAPNA preincubated at different temperatures. The increase in absorbance at 416nm over a ten minute period was measured continuously using a Beckman DU7 spectrophotometer.

Table 11. The effect of proteolytic inhibitors and activators upon the activity of BAPNAase.

Inhibitors 10mM	units	(%) control
PMSF	2.6	95.24
N-ethyl maleimide	0.233	8.53
Iodoacetamide	1.16	42.5
PCMB	1.03	37.73
EDTA	3.86	141.4
Activators 10mM		
2-Mercaptoethanol	3.73	136.63
Dithioethrietol	2.95	107.3
Glutathione (red)	3.14	114.6
control	2.73	100

0.006mg of purified BAPNAase was preincubated for 30 minutes with 0.1M McIllivane buffer pH 7.4 containing 10mM of the above inhibitors. The reaction was started by the addition of 0.1ml of 105mM BAPNA and terminated by the addition of 0.7ml of 20% (w/v) T.C.A. After centrifugation at 5,000g for 10 minutes the absorbance was measured at 416nm.

Table 12. The effect of different concentrations of N-ethyl maleimide upon the activity of BAPNAase.

mM N-ethyl maleimide	units	(%) Inhibition
100	Zero	100%
10	0.13	94.72
1	0.33	86.59
0.1	0.3	87.81
0.01	1.33	45.94
0.001	2.86	Zero
control	2.46	Zero

0.015mg of purified BAPNAase was preincubated for 30 minutes with 0.1M McIllivane buffer pH 7.4, containing various concentrations of N-ethyl maleimide. The reaction was started by the addition of 0.1ml of 105mM BAPNA and terminated by the addition of 0.7ml of 20% (w/v) T.C.A. After centrifugation at 5,000g for 10 minutes, the absorbance was measured at 416 nm.

Table 13. The effect of different concentrations of 2-mercaptoethanol upon the activity of BAPNAase.

mM 2-mercaptoethanol	units	(%) Activation.
100	2.86	116.21
10	2.66	108.11
1	2.73	110.81
0.1	2.73	110.81
0.01	2.16	87.83
0.001	2.56	104.05
control	2.46	100

0.015mg of purified BAPNAase was preincubated for 30 minutes with 0.1M McIllivane buffer pH 7.4, containing various concentrations of 2-mercaptoethanol. The reaction was started by the addition of 0.1ml of 105 mM BAPNA and terminated by the addition of 0.7ml of 20% (w/v) T.C.A., after centrifugation at 5,000g for 10 minutes, the absorbance was measured at 416nm.

(iii) Substrate specificity.

BAPNAase activity on a variety of synthetic substrates was assessed, and Table 14 shows that the BAPNAase, like the proteinase, possesses esterase activity, but unlike the proteinase the BAPNAase was active on leucine as well as asparagine and glycine nitrophenol esters. The BAPNAase was also active on benzoyl-arginine ethyl ester but unlike the proteinase had no arginine-ethyl esterase activity. The enzyme showed no activity on any of the paranitroanalides or purified storage proteins.

The activity of the enzyme on a variety of peptides was also assessed and Table 15 shows that there was virtually no activity upon the carboxy benzoyl derivatives of amino acids, indicating the enzyme removed the amino acids from the amino end of the peptides. The enzyme prefered glycine, not alanine, at the amino acid, and this amino acid should be linked to an aromatic amino acid.

The Km of the enzyme for the substrate BAPNA was established (Fig. 16) at 0.243 mM.

Table 14. BAPNAase activity on a variety of synthetic and natural substrates.

Substrates	Change in absorbance 410nm min^{-1} mg^{-1} of BAPNAase
CBZ-Asparagine NPE (1.67 mM)	1.47
CBZ-Glycine NPE (1.67 mM)	0.93
CBZ-leucine NPE (1.67mM)	0.54
CBZ-Phenylalanine NPE (1.67mM)	not detectable
CBZ-Tyrosine NPE (1.67 mM)	not detectable
CBZ-Tryptophan NPE (1.67 mM)	not detectable
CBZ-Valine NPE (1.67 mM)	not detectable
	Change in absorbance 416nm min^{-1} mg^{-1} of BAPNAase
lysine paranitroanalide (1.75 mM)	Zero
leucine paranitroanalide (1.75 mM)	Zero
CBZ-citrulline paranitroanalide (1.75 mM)	Zero
citrulline paranitroanalide (1.75 mM)	Zero
	Change in absorbance 253nm min^{-1} mg^{-1} BAPNAase
Benzoyl-arginine ethyl ester	0.261
Arginine ethyl ester	not detectable
	mM α amino acids released min^{-1} mg^{-1} of BAPNAase
legumin Days 0, 4, 7, 11.	Zero
vicilin Days, 0, 4, 7, 11.	Zero

0.029mg of BAPNAase was incubated with 2.9ml of 0.1M McIllivane buffer pH 7.4 at 25°, containing 15% (w/v) methanol and 0.1ml of 100mM carboxybenzoyl nitrophenol ester derivatives of amino acids, or 0.1ml of 100mM paranitroanalide derivatives of amino acids. The increase in absorbance was measured directly in a Beckman DU7 recording spectrophotometer, non enzymic degradation of the substrates was subtracted.

0.029mg of BAPNAase was incubated with 2.5ml of 0.1M McIllivane buffer pH 7.4 at 40°, 0.1ml of 100mM benzoyl arginine ethyl ester or arginine ethyl ester was then added. The increase in absorbance was measured directly in a Beckman DU7 recording spectrophotometer, non enzymic degradation of the substrates was subtracted.

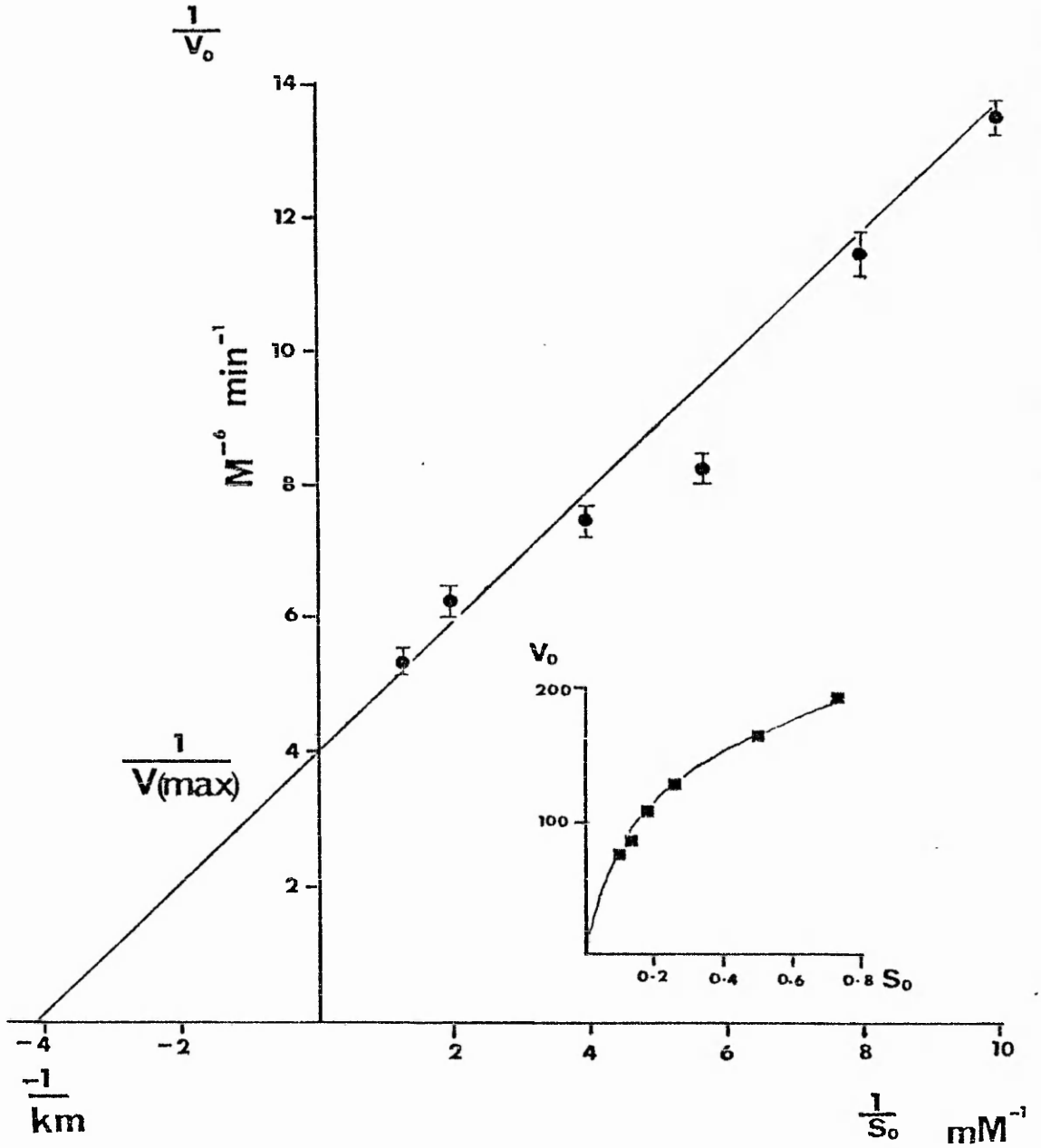
0.027mg of BAPNAase was incubated with 2.0ml of 0.1M McIllivane buffer pH 7.4 at 40°, containing 4.0mg of purified legumin and vicilin. The reaction was terminated by the addition of 0.7ml of 20% (w/v) TCA and liberated α-amino acids were estimated by ninhydrin.

Table 15. BAPNAase activity on di- and tri-peptides.

Substrate 10mM		µM α-amino acid released
DL Alanyl-glycine		Zero
DL Alanyl-phenylalanine		Zero
CBZ Alanyl-phenylalanine		Zero
CBZ Glutamyl-Tyrosine		Zero
CBZ Glycyl-phenylalanine	pH 7.4	0.070
	pH 5.4	Zero
CBZ phenylalanyl-leucine		Zero
Glycl-Glycine		0.050
Glycyl-Phenylalanine		0.505
Glycyl-Tryptophan		0.065
Glycyl-Tyrosine		0.810
Glycyl-Glycyl-phenylalanine		0.245
Glycyl-phenylalanyl-phenylalanine		0.545

0.027mg of purified BAPNA enzyme was added to 2.0ml 0.1M McIllivane buffer pH 7.4 containing 10mM of the above substrates. The reaction was terminated by the addition of 0.7ml of 20% (w/v) T.C.A., liberated α-amino acids were measured with ninhydrin.

Fig. 16. K_m and V_{max} of BAPNAase.



$y = 0{\cdot}914 + 4{\cdot}03X$

$km = 0{\cdot}243\ mM.$

$V(max.) = 0{\cdot}248 M^{-6} min.^{-1}$

0.033mg of purified BAPNAase was incubated with 0.1M McIllivane buffer pH 7.4 at 25^{o}. The reaction was started by the addition of 0.1 ml of BAPNA at various concentrations. The increase in absorbance was measured directly using a Beckman DU7 spectrophotometer. The rate of reaction, and substrate concentration were used to give a Lineweaver-Burk plot and the K_m and V_{max} established.

4. Immunology.

Preliminary experiments.

After immunization of the rabbits the level of response was measured by enzyme linked immunoabsorbent assay (ELISA). Fig. 17 (a, b) shows that the two rabbits immunized with the BAPNAase have a higher level of measurable antibody binding than the control rabbit after 38 days and 48 days. The purity of the antibodies raised was tested by immunoelectrophoresis and Fig. 18 shows that there was only one immunoprecipitation line evident in all the various legume extracts tested. This precipitation line co-incided with the location of the BAPNAase. Fig.19 (a) shows the migration of the crude extract into the gel and Fig.19 (b) shows the location of BAPNAase activity.

The antibody raised against the BAPNAase was tested against the various legume extracts by Ochterlany double immunodiffusion and Fig.20 shows that all the legume extracts tested showed a precipitation line. These lines all overlapped, indicating that there is an enzyme present in all the legume cotyledons tested, with an antigenic similarity to the enzyme in Vicia faba cotyledons. A similar experiment using extracts of various cereals (Wheat, oat, maize and barley) resulted in no visible precipitation.

Fig. 17 (a, b) ELISA of anti sera to the BAPNAase antigen.

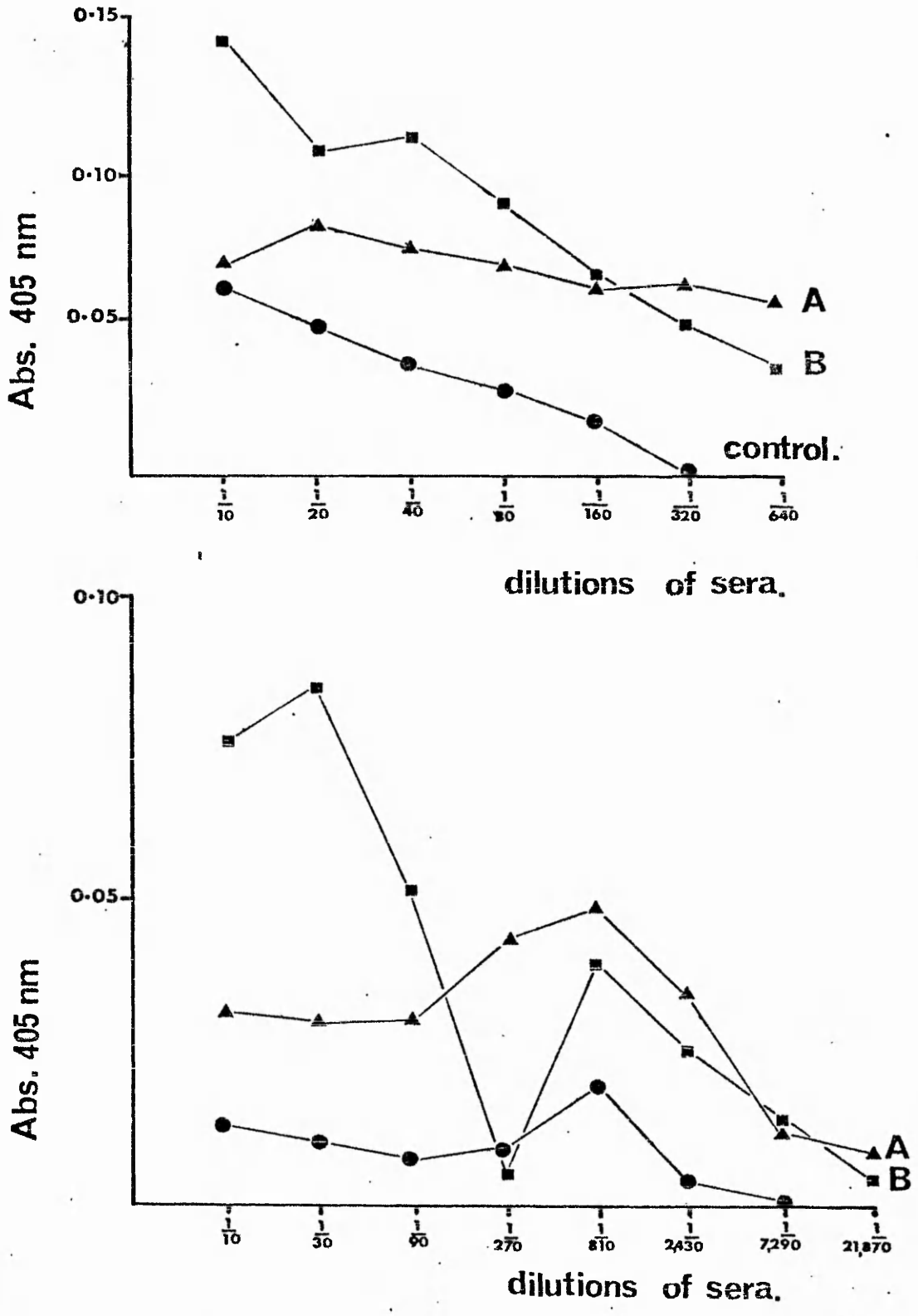
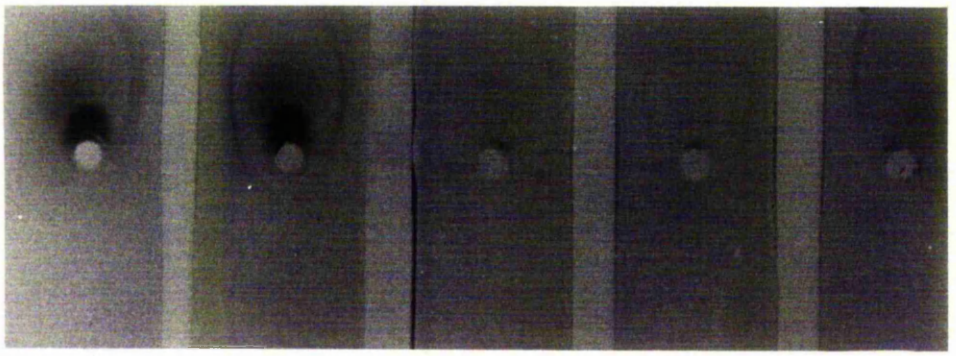


Fig.17 (a) represents the antibody titre 38 days after immunization and Fig. 17 (b) represents the antibody titre 48 days after immunization. The titre of rabbit A is represented by (▲——▲) of rabbit B by (■—■) and control (●——●) 0.2ml of BAPNAase 0.0035 mg ml^{-1} in carbonate buffer was placed in a microelisa plate overnight at 4^{o}. The antigen was removed and the wells washed twice with 0.25ml of phosphate buffered saline solution containing 1% (w/v) Bovine Serum albumin and 0.5% (w/v) Brij-35 (PBS-BSA-Brij). The remaining binding sites were blocked with PBS-BSA solution for 1 hour at room temperature before being washed twice with PBS-BSA-Brij, Doubling, Fig.17 (a) or tripling, Fig.17 (b) dilutions of antisera and control were made in PBS-BSA-Brij, 0.2ml were incubated in the wells for 2 hours at room temperature. The wells were again washed twice with 0.25ml of PBS-BSA-Brij before being incubated with 0.2ml of 1:500 dilution of anti rabbit IGg-peroxidase conjugate in PBS-BSA-Brij for 2 hours at room temperature. The wells were washed three times with 0.25ml of PBS-BSA-Brij, 0.2ml of 0.01% (w/v) O-phenylene diamine, 0.005% (v/v) H_2O_2 in PBS, was added to the wells, and incubated for 30 minutes at room temperature in the dark.

The reaction was terminated by the addition of 0.05ml of 3M sodium hydroxide. The absorbance was measured at 405nm in a micro ELISA reader.

Fig. 18. Immunoelectrophoresis of extracts of various legume cotyledons.



a) Vicia faba var Yates Fillbasket

b) Pisum saturum var Meteor

c) Phaseolus vulgaris var-Scarlet Emperor

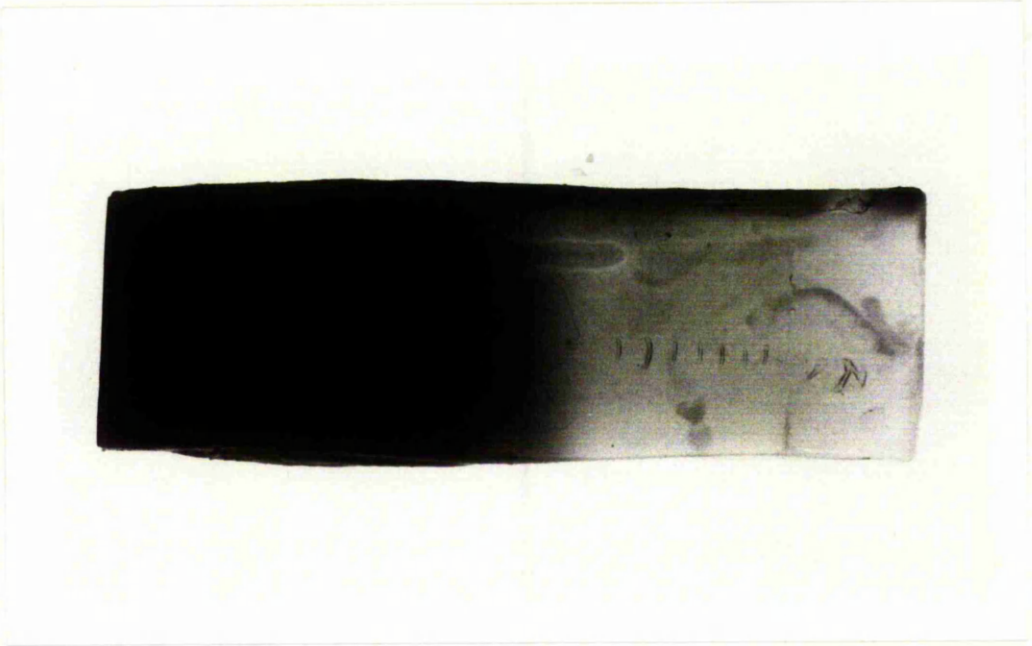
d) Vicia faba var. Windsor Green

e) Vigna radiata

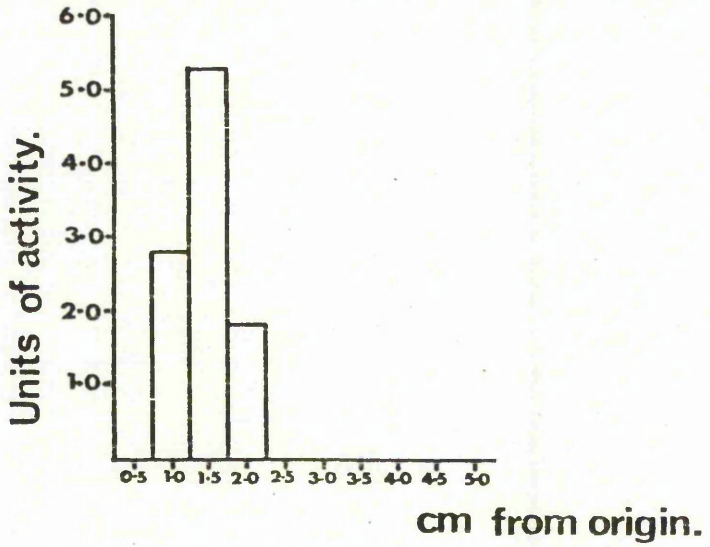
Precoated glass plates were covered with 20.0ml of 1% (w/v) agarose in barbitone buffer pH 8.2. Wells and troughs were cut into the solid agarose using the LKB Immunoelectrophoresis template. 0.02ml samples of various legume cotyledon extracts were loaded into the wells and electrophoresis was carried out for 1 hour at a constant 60 volts. When the troughs were removed and filled with anti sera. Diffusion was allowed to proceed for 48 hours and unprecipitated protein was removed from the gel by washing in phosphate buffered saline. Precipitation arcs were were visualized by staining with PAGE blue 83 and destaining with ethanol, glacial acetic acid and water. (30: 10: 60).

Fig.19. Location of BAPNAase after Immunoelectrophoresis.

a) Precipitation lines.



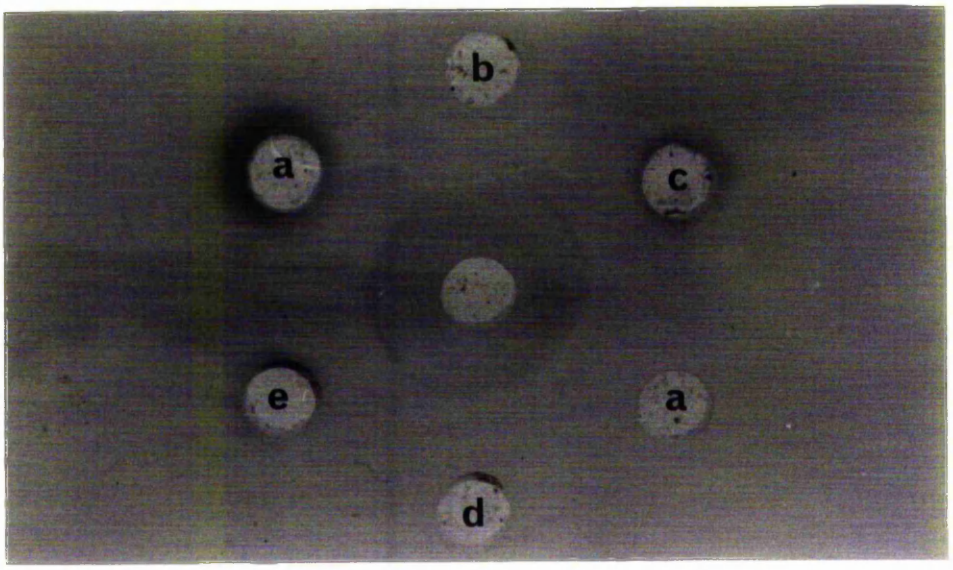
b) BAPNAase activity



Precoated glass plates were covered with 20.0ml of 1% (w/v) agarose in barbitone buffer pH 8.2.

A trough was cut in the middle of the agarose place and 1ml of dialysed albumin extract of Vicia faba cotyledons was added. Electrophoresis was carried out for 1 hour at constant 60 volts sections were cut from the gel. Some were stained for protein with Coomassie blue, others were cut into 0.5mm sections, homogenized in 2.0ml of 0.1M McIllivane pH 7.4 and assayed for BAPNAase activity.

Fig.20. Double Immunodiffusion of antisera (raised against the BAPNAase) and cotyledon extracts of various legumes.



a) Vicia faba var. Yates Fillbasket

b) Pisum satiuum var. Meteor

c) Phaseolus vulgaris var. Scarlet Emperor

d) Vicia faba var. Windsor Green

e) Vigna radiata

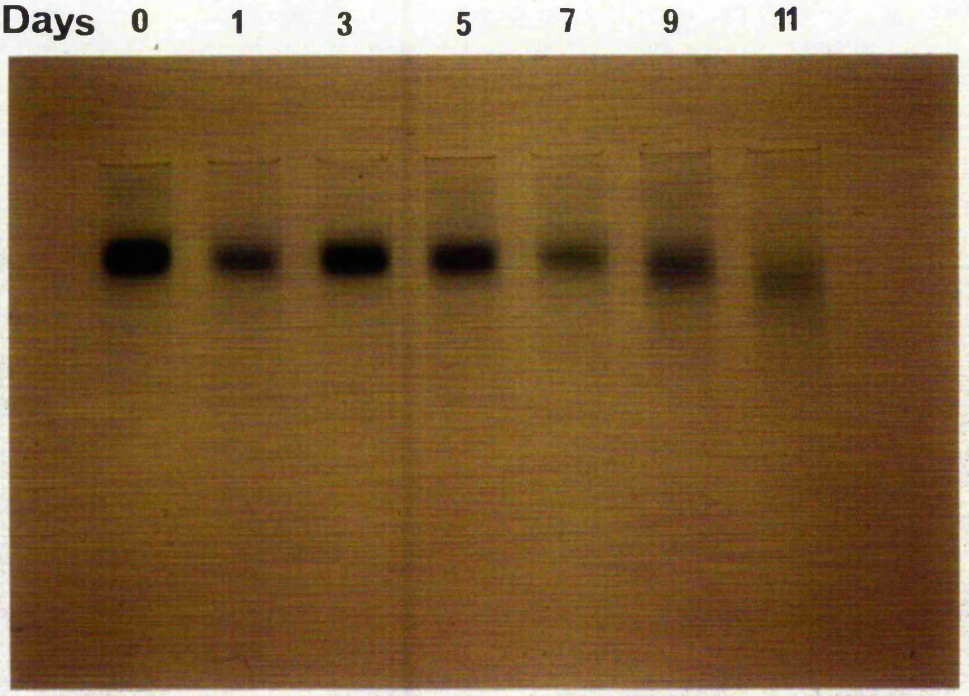
Precoated glass plates were covered with 20.0ml of 1% (w/v) agarose in Phosphate buffered saline containing 0.02% (w/v) Sodium azide. Wells were cut into the solid agarose using an LKB Double Immunodiffusion template, 0.02ml Antisera and control were added to the centre wells. 0.02ml of extracts from various legume cotyledons were added to the outside wells. Diffusion was allowed to proceed for 48 hours, unprecipitated protein was washed from the gel with excess phosphate buffered saline and precipitation visualized by staining with Coomassie blue.

5. Storage proteins.

(i) Crude globulin extract.

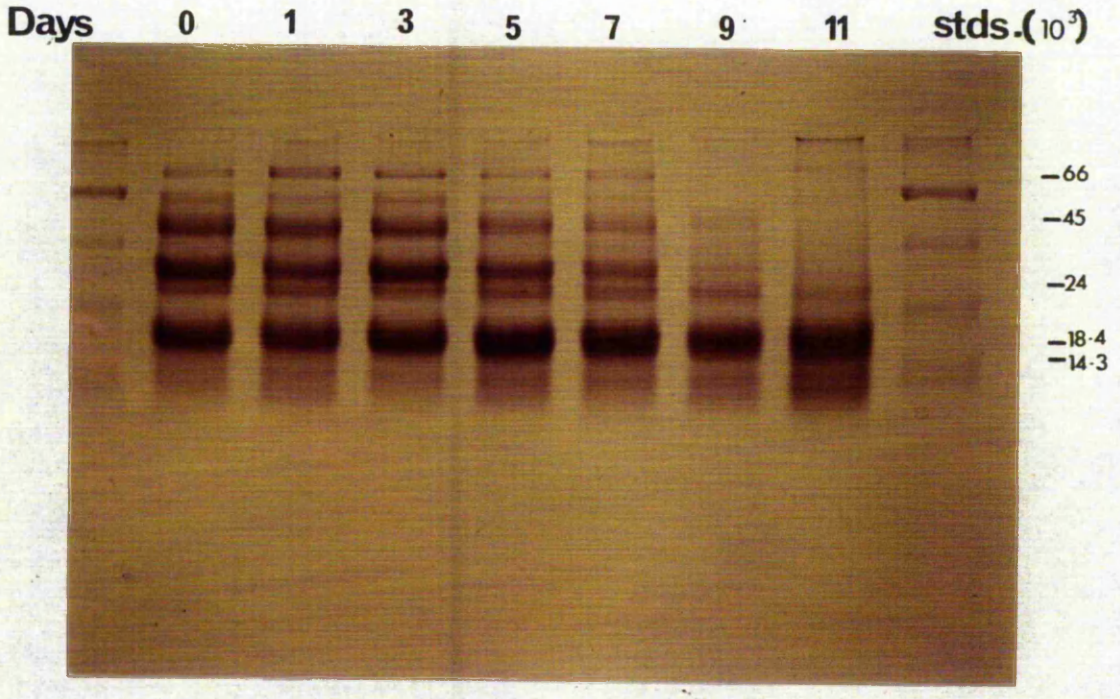
PAGE (Fig.21) showed that the globulins isolated from seeds germinated for 7 days or more became more electronegative and hence more mobile. SDS-PAGE of the same proteins (Fig.22) shows that from day 7, seeds start to lose the higher molecular weight protein subunits with a concurrent increase in lower molecular weight subunits. This is clearly evident in the globulin isolated from day 11 seeds.

Fig.21. PAGE of total globulin proteins.



Globulins were extracted from cotyledons of Vicia faba at different stages of germination, in 0.1M Sodium phosphate buffer pH 7.0. 0.03mg of protein was separated in a 7.5% (w/v) polyacrylamide gel (T= 7.7%, C= 2.6%) with a continuous Tris glycine buffer pH 8.9, for 10 minutes at 20ma, then 3 hours at 40ma. The gel was stained with PAGE Blue 83 and destained in ethanol, glacial acetic acid and water (30: 10: 60).

Fig.22. PAGE of total globulin proteins in dissociating and reducing conditions.



Globulins were extracted from cotyledons at different stages of germination; in 0.1M sodium phosphate buffer pH 7.0. 0.03mg of protein was separated in a 10% (w/v) polyacrylamide gel (T= 10.2%, C= 2.6%) with a continuous 0.05M Imidazole/SDS buffer pH 7.0, for 10 minutes at 20ma, then 2.5 hours at 100ma. The gel was stained in PAGE Blue 83 and destained in ethanol, glacial acetic acid and water. (30; 10: 60).

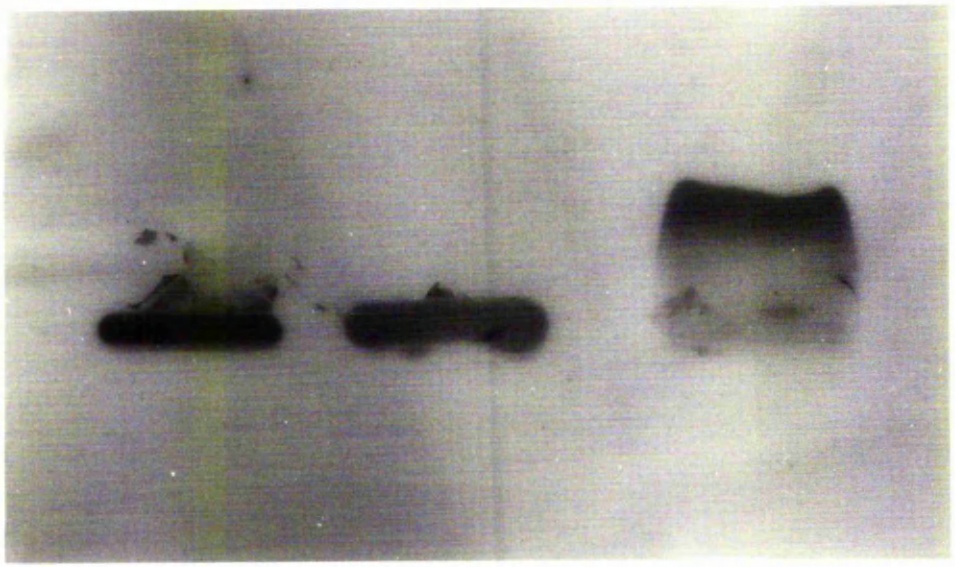
(ii) Purified Globulins

(a) Assessment of purity.

The globulins were separated into legumin and vicilin and assessed for mutual contamination using Zonal isoelectric precipitation electrophoresis (ZIPE). Fig 23 (a) shows that legumin and vicilin isolated from day 0 seeds were free from contamination Fig 23 (b) shows that a sample of vicilin isolated from day 7 seeds had legumin contamination. This fraction was then subjected to further isoelectric precipitation at pH 4.7, followed by dialysis and freeze drying, it was then retested using ZIPE. The protein was only used for further experiments when it was shown to be free of contamination.

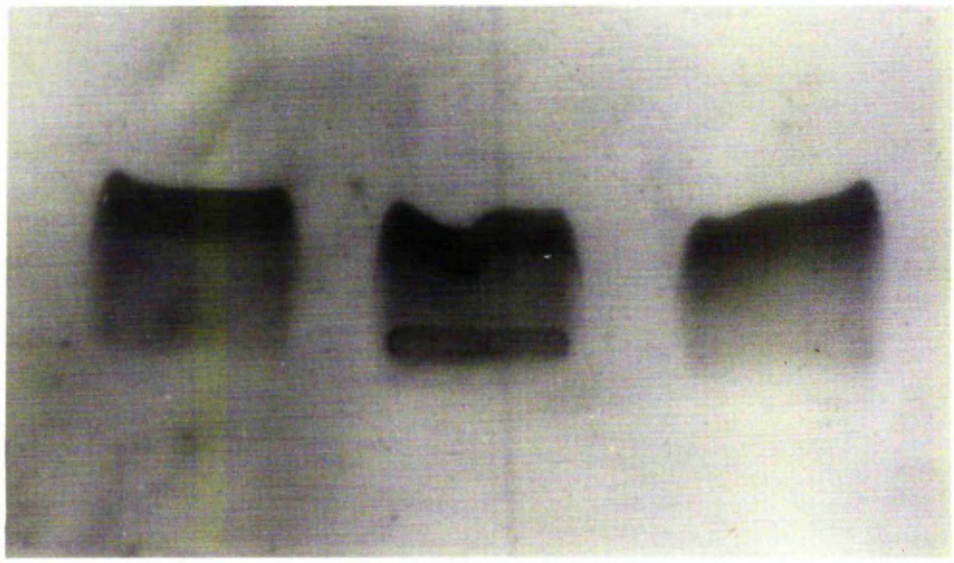
Fig.23 ZIPE of purified legumin and vicilin.

a) Day 0 globulins.



Legumin. Vicilin.

(b) Vicilins.



Days 4 7 11

Legumin and vicilin purified from cotyledons at different stages of germination was dissolved in 0.15M sodium phosphate buffer pH 7.0 (2mg ml^{-1}). 0.01 mg of protein was applied to a cellulose acetate membrane and separated in 0.05M McIllivane buffer pH 4.7 for 1 hour at 250 volts. The membranes were stained with PAGE Blue 83 and destained in ethanol, glacial acetic acid and water (30:10:60).

(b) SDS-PAGE

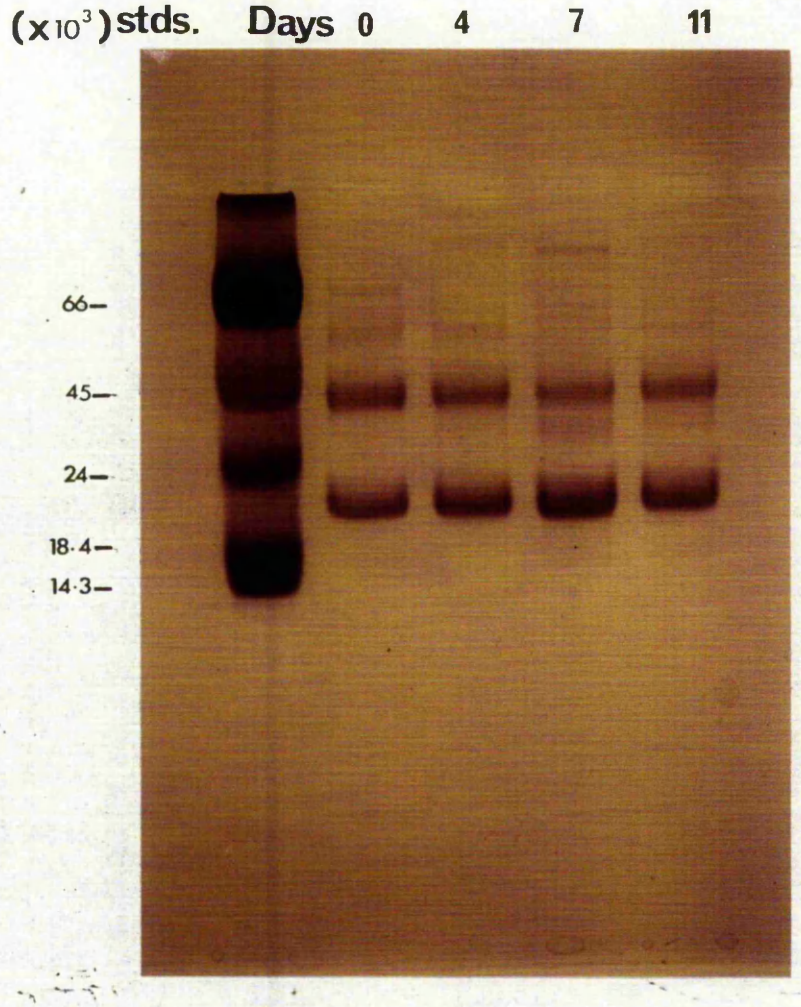
SDS-PAGE of the purified legumin (Fig.24) shows that the proteins isolated from the later stages of germination do not show the obvious changes observed by SDS-PAGE of the total globulins, although there is one new band evident. The molecular weight of the main bands were 37,300 daltons and 21,500 daltons, additional bands were noted at ca. 60,000 daltons and >100,000 daltons. The main bands remained constant throughout germination, but on day 7 there was an increase in a band with a molecular weight of 31,000 daltons. The 60,000 dalton and >100,000 dalton subunits also decrease in intensity.

SDS-PAGE of the purified vicilin (Fig.25) shows the most evident change, with lower molecular weight material accumulating in the later stages of germination. The main bands were 59,000 daltons, 32,000 daltons and 21,700 daltons, with additional bands at ca. 90,000 daltons and >100,000 daltons. In the protein isolated from day 11 seeds there are two additional bands one at 18,800 daltons another at 17,400 daltons. There is also a slight increase in the mobilities of the main bands from 59,000 to 54,000 daltons and from 32,000 to 31,000 daltons.

Changes in the storage proteins is more easily seen in the gel scans (Fig.26). There is an increase in low molecular weight subunits in the proteins isolated

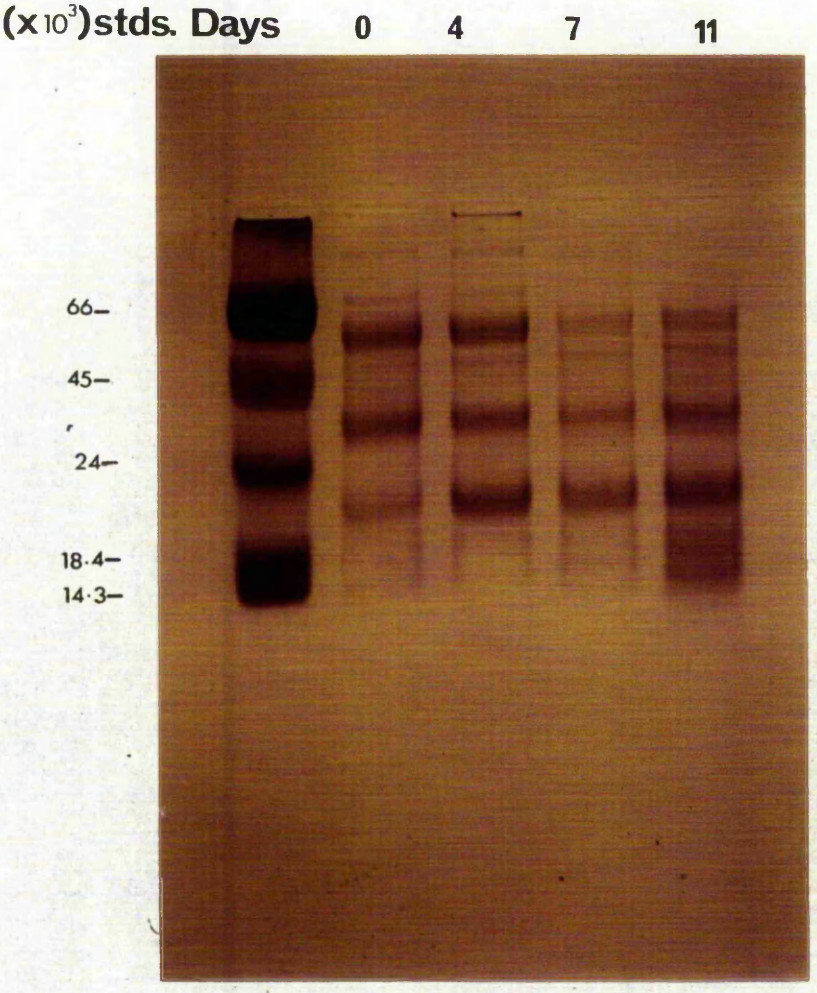
from day 11 seeds as compared with the proteins isolated from day 0 seeds. Several new smaller subunits can be seen in the vicilin isolated from day 11 seeds.

Fig. 24 PAGE of purified legumin in dissociating and reducing conditions.



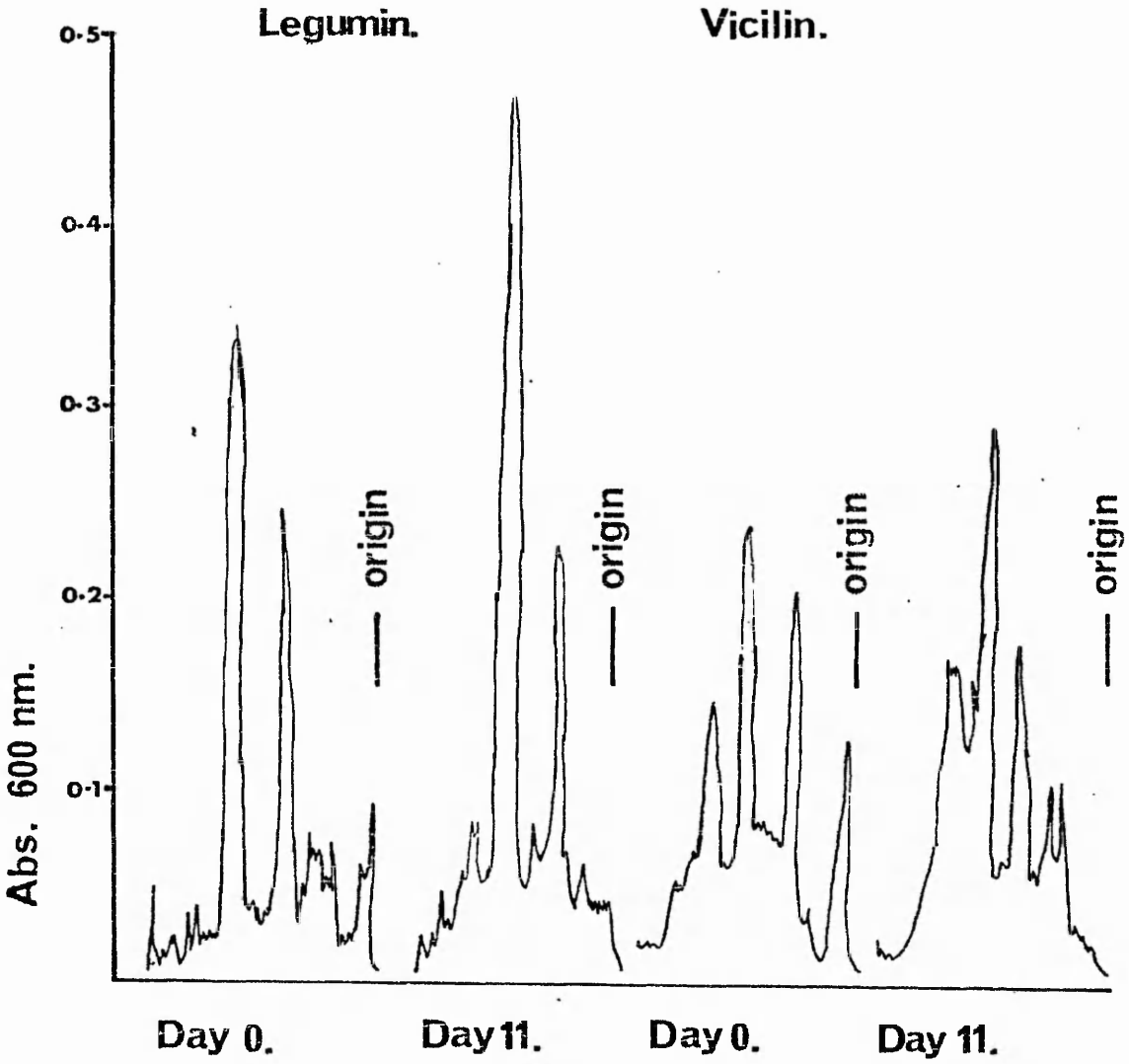
Legumin was purified from cotyledons at different stages of germination 0.04mg of protein was separated in a 10% (w/v), polyacrylamide gel (T=10.2% , C=2.6%) with a continuous 0.05M imidazole SDS buffer pH 7.0 for 10 minutes at 20 ma, then 2.5 hours at 100ma, The gel was stained with PAGE Blue 83 and destained in ethanol, glacial acetic acid and water (30:10:60).

Fig.25 PAGE of purified vicilin in dissociating and reducing conditions.



Vicilin was purified from cotyledons at different stages of germination 0.04mg of protein was separated in a 10% (w/v) polyacrylamide gel (T=10.2%, C=2.6%) with a continuous 0.05M imidazole SDS buffer pH 7.0, for 10 minutes at 20ma, then 2.5 hours at 100ma. The gel was stained in PAGE Blue 83 and destained in ethanol, glacial acetic acid and water (30:10:60).

Fig.26 Gel scans of purified legumin and vicilin from germinating seeds after SDS-PAGE.



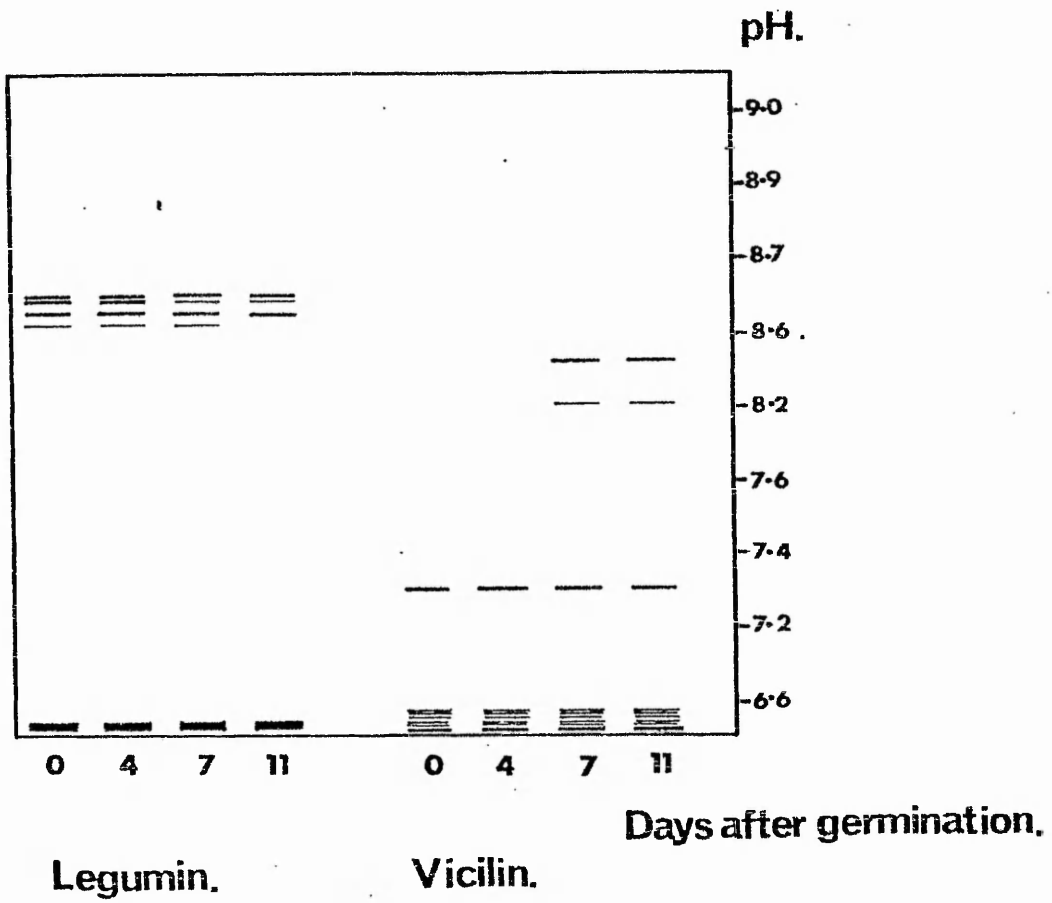
After the proteins had been subjected to SDS-PAGE stained for protein using PAGE-Blue 83 and destained using ethanol, glacial acetic acid water (30:10:60) the gels were cut mounted on a microscope slide and placed in an SP1802 scanning accessory for the Pye Uvicam SP1800 spectrophotometer. The gels were scanned at 600nm and a plotted printout was used to record the scan.

(c) IEF in dissociating and reducing conditions.

The basic subunits (Fig.27) of both proteins show little change as germination progresses. The group of legumin basic subunits do become slightly less basic as germination proceeds, (this is more easily seen in Fig.28) and two extra basic subunits are visible in vicilin isolated from day 7 and 11 seeds.

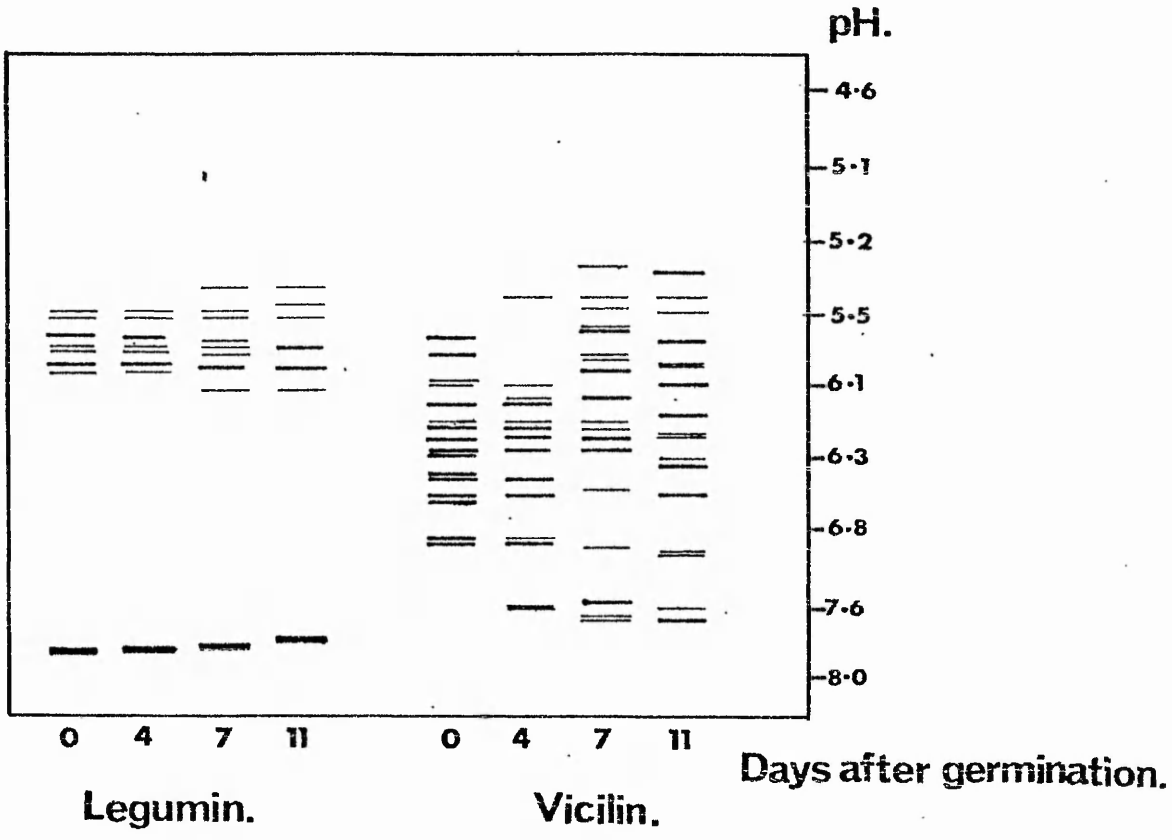
The IEF of the acidic subunits (Fig.28) of both legumin and vicilin shows the biggest change as germination proceeds. The acidic subunits of both proteins became more dispersed. Some subunits of vicilin disappear to be replaced by subunits with different isoelectric points. The subunits of legumin show some change with the appearance of subunits with both a lower and a higher isoelectric point in the proteins isolated from days 7 and 11 seeds.

Fig.27 IEF of the basic subunits of purified legumin and vicilin.



Legumin and vicilin was purified from cotyledons at various stages of germination. The protein was dissolved in formamide, Pharmalyte (6-9), 2-mercaptoethanol, and water. (75:5:2:18) 0.02mg of protein was applied to $0.5cm^2$ filter paper squares on a prefocussed polyacrylamide gel (T=4.5%, C=2.6%) and run for 1 hour at 8 watts, then 1.5 hours at 12 watts. The gel was stained with PAGE Blue 83 and destained with ethanol, glacial acetic acid and water. (30:10:60).

Fig. 28 IEF of the acidic subunits of purified legumin and vicilin.



Legumin and vicilin were purified from cotyledons at different stages of germination. The protein was dissolved in formamide, pharmalyte (4-6), 2-mercaptoethanol and water (75:5:2:18) 0.02mg of protein was applied to $0.5cm^2$ filter paper squares on a prefocussed polyacrylamide gel (T=4.5%, C=2.6%) and run for 1 hour at 8 watts, then 1.5 hours at 12 watts. The gel was stained with PAGE Blue 83 and destained with ethanol, glacial acetic acid and water (30:10:60).

(d) Amide Content.

The amount of amide nitrogen present in the purified storage proteins, legumin and vicilin (Table 16), decreases only slightly as germination progresses.

Table 16. Amide content of purified legumin and vicilin.

Sample	$\mu M\ NH_3^+\ mg^{-1}$	±S.E.M (n=3)
Day 4 vicilin	0.906	0.02
Day 7 vicilin	0.881	0.04
Day 11 vicilin	0.844	0.31
Day 0 legumin	1.013	0.14
Day 4 legumin	1.082	0.13
Day 7 legumin	0.976	0.03
Day 11 legumin	0.928	-

Legumin and vicilin was purified from cotyledons at various stages of germination and hydrolysed with concentrated HCl for 10 days at 37^{o}. 0.5ml of the hydrolysate was subjected to micro distillation in a Conway vessel. The ammonia released was measured by ninhydrin.

DISCUSSION

A number of different approaches have been used to investigate the metabolism of seed storage proteins during germination. The majority of workers have been content to measure the levels of protease activity in the seeds at different stages of germination, but some workers have isolated the proteases involved and determined some of their characteristics (Ryan, 1973; 1981).

Investigations have been hampered by a lack of specific assays for proteinases, whilst they are specific for proteins and often show a preference for a particular amino acid sequence, they will also hydrolyse a number of different amino acid sequences (Glazer and Smith, 1971). This lack of specificity makes the measurement of a single proteinase activity difficult.

A number of different assays have been used. The Kunitz assay (Kunitz, 1947) which measures the increase in absorbance at 280 nm was found to be unsuitable for crude plant extracts because of the high levels of phenolic and nucleotide compounds (Beevers, 1976).

Pusztai and Duncan (1971) used the Kunitz assay to measure proteolytic activity in *Phaseolus vulgaris*; they found great fluctuations in activity, contrasting with the work by Yomo and Varner (1973) on the same seed, but assaying with ninhydrin. They found that proteolytic activity reached a peak 10 days after the start of germination with a four fold increase in activity.

Ninhydrin reacts quantitatively with α amino groups yielding a dark blue colour which can be measuredspectrophotometrically. A number of workers have used ninhydrin to assay proteolytic activity in plant tissue (Visuri et al; 1969; Guardiola and Sutcliffe, 1971; Yomo and Varner, 1973; Drivadahl and Thimann, 1977; Shepard and Moore, 1978; Shannon and Wallace, 1979; Nishimura and Beevers, 1979; Miller and Huffaker, 1982; Waters, et al., 1982; Gifford et al., 1983; Dalling et al., 1983).

However, because ninhydrin measures α-amino nitrogen, peptides will produce a lower colour yield, and an under estimate of proteinase activity. Taylor (1979) found that compounds such as 2-mercaptoethanol enhance the colour yield of ninhydrin, whereas N-ethyl maleimide decreases the colour yield. This means that the ninhydrin assay is unsuitable for the assay of sulphydryl proteinase activity.

The use of diazotised protein substrates has also been popular (Hob day et al; 1973; Pike and Briggs, 1972; Lichten feld et al., 1979; Ragster and Chrispeels, 1979; Weckenmann and Martin, 1981). The assay is a good indication of proteinase activity, provided the diazotised groups are spread evenly along the polypeptide chain (Moore, 1969).

Recently workers have tried to use endogenous substrates to assay proteolytic enzymes in plants (Shannon and Wallace, 1979; Taylor, 1979; Miller and Huffaker, 1981; Bond and Bowles, 1983). This may give a better indication of in vivo activity.

Salumeedin et al., (1979) developed a proteinase assay which allows the use of endogenous substrates and enzymes using the dye Coomassie blue. This dye does not bind to peptides below 3,000 daltons and the volumes used in the assay are frugal. However, the assay proved suitable only for purified proteinase preparations. When assaying crude preparations the dye binding increased, this was taken to mean that the proteinase was present at a very low concentration and was hydrolysing a large number of proteins, revealing a larger binding area for the dye.

Synthetic substrates which release a chromophore or fluorescent amine have been used such as N α-Benzoyl-DL-arginine p-nitroanilide (BAPNA) (Mainguy, 1972) and Benzoyl-arginine-ethyl ester (BAEE) (Ihle and Dure, 1972). However, Beevers (1968); Ihre and Dure (1972); and Lichtenfeld et al., (1979) found that the activity on these substrates could not be correlated with proteinase activity.

All protease assays on crude enzyme extracts may not reveal the physiological levels of activity in germinating seeds, due to the presence of endogenous proteinase inhibitors, natural substrates that the enzymes may "prefer" or the presence of more than one enzyme which will act upon the added substrate. These problems occur even with a good assay and for this reason it is necessary to separate the proteolytic enzymes to establish their nature and characteristics.

To assay the proteolytic enzymes in Vicia faba cotyledons, two assays were chosen using as substrates BAPNA and azocasein. These assays were convenient to use and enabled large numbers of fractions from columns to be assayed quickly. They were also the assays used by Lichtenfeld et al., (1979) to measure the levels of proteolytic activity in germinating Vicia faba cotyledons.

Total activities gramme^{-1} fresh weight of cotyledons differ between the graph depicting the activity over an eleven day germination period, compared with the final purification table. When small amounts of cotyledons were extracted (7.5g, Fig.1) less azocasein active enzyme was extracted (ca. 40%) and more BAPNAase was extracted (ca. 40%) than when large amounts of cotyledons were extracted (250.0g, Table 5). This maybe due to different homogenization procedures; for small amounts of cotyledons a mortar and pestle was used, for larger amounts a Waring blender was used. The blender may have broken up a greater percentage of protein bodies, releasing vesicle bound proteinase into the extraction medium, at the same time the harsher homogenization may have denatured more of the cytoplasmic peptidase. Different batches of seeds and slight changes in temperature during germination may have altered the amounts of the proteases present within the seeds.

A number of techniques have been used to purify proteases from plant seeds and other plant tissue. Ion exchange chromatography was used by Mainguy, et al., (1972); and Shepard and Moore (1978). A combination of gel filtration and ion exchange chromatography was used by Visuri, et al., (1969); Pike and Briggs (1972); Bulmaga and Shutov (1977); Shutov et al., (1982); Shannon and Wallace (1979); Caldwell and Sparrow (1980); and Miller and Huffaker (1981). Adsorption chromatography on hydroxy apaptite with gel filtration and ion exchange chromatography was used by Elleman (1974) and Sopanen (1976). Preparative gel electrophoresis with gel filtration and ion exchange chromatography was used by Barth and Hermann (1974). Affinity chromatography, electrofocussing, gel filtration and ion exchange chromatography was used by Preston and Kruger (1976); and Baumgartner and Chrispeels (1977). Affinity chromatography on sepharose was used by Drivadhl and Thimann (1977). Finally, co-valent chromatography on mercury derivatives of hydroxymethylacrylate gels was used by Vavreinova and Turkova (1975) and on glutathione sepharose by Brocklehurst et al., (1973).

The majority of these methods were used in this study to purify the two proteases investigated and a number discarded; these included ion exchange chromatography adsorption chromatography, co-valent chromatography and hydrophobic chromatography. These methods failed to separate the two activities and usually had a deleterious effect on the total activities recovered.

The separation of the two activities was achieved by using affinity chromatography on globulin sepharose. Drivadhl and Thimann (1977) used haemoglobin sepharose to separate a proteinase from senescing wheat leaves, but in this work, to bind the maximum amount of a physiologically important proteinase, a natural substrate i.e. 5 day old globulins, was attached to a column. This method of specific enzyme isolation is not classic affinity chromatography, where a substrate analogue tightly binds the enzyme. This method, it is believed, retards the emergence of enzyme from the column. The proteinase will bind to the globulin, hydrolyse a peptide bond and move on down the column. Evidence to support this was provided by a decrease in binding of proteinase to the column after approximately 10-15 runs.

The proteinase that bound to the globubin sepharose column had no BAPNAase activity, similar to vicilin peptidohydrolase (Baumgartner and Chrispeels, 1977). However, whereas vicilin peptidohydrolase has a wide nitrophenol esterase activity, the proteinase from Vicia faba cotyledons could only hydrolyse the nitrophenol esters of glycine and asparagine, out of the esters tested. It is interesting to note that the storage proteins do contain a large percentage of asparagine residues (Jackson et al., 1969 and Wright and Boulter, 1974).

Hydrolysis of the native storage proteins legumin and vicilin was assessed using the decrease in Coomassie blue dye binding assay (Salumeedin et al., 1980). Unlike

protease A isolated from germinating vetch seeds (Bulmaga and Shutov, 1977), the proteinase from Vicia faba was active on all the storage proteins isolated from different ages, including the storage proteins isolated from ungerminated seeds. Lichtenfeld et al., (1979), suggested that the storage proteins needed to be altered before de novo synthesized enzymes could complete their hydrolysis, this suggestion was not corroberated. However, storage proteins isolated at an early stage of germination were hydrolysed, in vitro, less rapidly than those from a later stage. This indicates that some changes have occurred which makes the storage proteins a more attractive substrate. The proteins isolated from the latter stages of germination may have already been subjected to proteolytic attack by the proteinase within the seed; the increase in activity on the storage proteins from the latter stages of germination maybe a direct result of this prior hydrolysis. However, these changes in the storage proteins are not necessary for their degradation to start.

The molecular weight of the proteinase, as calculated by gel filtration was ca. 12,500 daltons. This is surprisingly low compared with the known molecular weights of other plant and animal proteinases, which have a molecular weight of ca. 25,000 daltons. Zwilling et al., (1981) found a low molecular proteinase (11,000 daltons) in crayfish (Astacus fluviatilis) and proposed that a new family of proteinases be formed to account for these enzymes. The low molecular weight maybe due to autolytic activity; as

the protein was concentrated the proteinase would be in closer contact with neighbouring molecules and with no other substrate to hydrolyse, autolytic activity maybe expected to be high. The molecular weight could reflect the method chosen to assess it i.e. gel filtration. Bulmaga and Shutov (1977) found that protease A had a molecular weight of ca. 8,000 daltons using gel filtration, and ca. 23,000 daltons using SDS electrophoresis. Woodrow, et al.,(1974) also noted this discrepency for molecular weight estimations of some proteases. This phenomena maybe due to a slight binding reaction between the protein and the gel matrix, this would retard the progress of the protein down the column and so record a lower molecular weight. Usually, an ionic strength of 0.1M or the addition of 10% (v/v) glycerol as used in our experiments is sufficient to reduce these interactions to a negligable level. The molecular weight needs to be determined by another method, PAGE was not possible as a preparation with suitably high enzymic activity and protein concentration could not be obtained because of enzyme instability.

A number of workers have noted the instability and autolytic activity of some plant proteinases. Drivadhl and Thimann (1977, 1978) and Pike and Briggs (1972) found that, on storage, the main enzyme peak, using gel filtration, decreased in concentration with the appearance of low molecular weight peaks suggesting that autolytic activity was occurring. This was corroberated when the proteinase

Vicia faba was stored at 4°; the main peak on gel filtration decreased. Vavreinova and Turkova (1975) showed that the proteinase from Phaseolus vulgaris was unstable below pH 4.0 and above pH 10.0. The proteinase from Vicia faba was only stable within the pH optima (4.5-6.0). Storage above pH 7.0 or below pH 3.0 completely and irreversibly inactivated the enzyme. This maybe of benefit to the cell, if the enzyme is contained within the protein bodies as was vicilin peptidohydrolase in mung bean (Baumgartner et al., 1978), accidental escape into the cytoplasm may inactivate the enzyme.

Many of the characteristics of the proteinase from Vicia faba cotyledons were similar to those of other plant proteinases from germinating seeds, e.g. pH optima, temperature deactivation and sulphydryl dependence (Mikola, 1983). The proteinase had a pH optima of 5.4, vicilin peptidohydrolase from mung bean has a pH optima of 5.1 (Baumgartner and Chrispeels, 1977), protease A and B from vetch seeds have pH optimas of 4.6 and 5.6 respectively (Bulmaga and Shutov, 1977; Shutov et al., 1982) and a protease from germinating lupin seeds had a pH optima of 4.0 (Shepard and Moore, 1978). The proteinase from Vicia faba cotyledons was deactivated above 40°, vicilin peptidohydrolase was deactivated above 50° and the protease from germinating lupin seeds was deactivated above 45°.

The sulphydryl inhibitors PCMB, N-ethyl maleimide, iodoacetamide and mercurous ions were all inhibitors of the proteinase from Vicia faba. Phenyl methyl sulphonyl flouride (PMSF) was developed as an inhibitor of hydroxyl dependent enzymes (Fahrney and Gold, 1963), but has also been shown to have some papain inhibitory activity (Whitaker and Perez-Villaseunor, 1968) which may account for the 10% inhibition of the proteinase from Vicia faba by this compound. EDTA increased the activity of the proteinase by ca. 5%, this was possibly because of chelation of heavy metal ions present in small amounts in the reagents used. The unusual finding of inactivation by 3mM 2-mercaptoethanol, revealed that concentrations above 1mM did not increase the activity of the enzyme and a concentration of 100mM substantially reduced activity. However, 2-mercaptoethanol is used in electrophoresis to disrupt any disulphide bridges involved in protein structure. The concentrations normally used are ca. 1% (v/v), 100mM is ca. 0.78% (v/v) which is approaching the necessary concentration for disruption of disulphide bridges. Thus, at high concentrations of 2-mercaptoethanol the reagent maybe disrupting a susceptible disulphide bridge within the proteinase structure, unwinding the protein and removing activity. Papain has three disulphide bridges holding the structure of the protein in the correct conformation and under suitable conditions (0.32M 2-mercaptoethanol in 8M Urea pH 8.2) one of the

disulphide bridges could be selectively reduced (Shapiro and Arnon, 1969).

The BAPNAase enzyme also showed a varying degree of susceptability to sulphydryl protease inhibitors, with N-ethyl maleimide being the most effective. The other inhibitors did not remove activity completely even at 10 mM, which maybe due to the orientation of the active site of the BAPNAase. In contrast to the proteinase from Vicia faba, all reducing agents increased activity with 2-mercaptoethanol being the most effective, even at concentrations up to 100mM. This may mean there are no easily accessible disulphide bridges in the structure of the BAPNAase.

As previously mentioned, peptidases are classified into two groups, either as serine or metalloenzymes. Recently a number of sulphydryl peptidases have been isolated from plant tissue which may required an additional group to be added to the nomenclature. Elleman (1974) isolated two aminopeptidases from pea seeds with sulphydryl dependancy and Caldwell and Sparrow (1980) isolated another sulphydryl aminopeptidase from pea seeds. Hermann and Barth (1976) found that the alanine aminopeptidase from rape seeds (Barth and Hermann, 1974) was dependent on metal ions and reduced sulphydryl groups, Sopanen (1976) found a similar dependancy with a dipeptidase from barley seeds. Gifford et al., (1983) found evidence for two sulphydryl aminopeptidases in germinating castor bean endosperm.

Mikola (1982) has arranged peptidases in plants into three groups depending on their pH optima; carboxypeptidases, neutral peptidases (napthyl amidases) and alkaline peptidases. The BAPNAase isolated from Vicia faba cotyledons would be grouped with the neutral peptidases, because of the pH optima of 7.4 and the ability to hydrolyse peptide bonds between amino acids and aromatic amines as well as hydrolysing di- and tri-peptides. This group of enzymes has few documented cases of purification and characterisation (Kolehmainen and Mikola, 1971; Elleman 1974; Caldwell and Sparrow 1976, 1980). However, the BAPNAase differs from other neutral peptidases because it cannot hydrolyse leucine or lysine paranitro-analide. Taylor (1979) used leucine paranitroanalide to assay aminopeptidase activity at pH 7.0 in crude extracts of Vicia faba cotyledons, therefore there appears to be at least one other amino peptidase in Vicia faba cotyledons.

The molecular weight of the BAPNAase was 50,000 daltons as estimated by gel filtration. In general the molecular weight of aminopeptidases vary considerably, from 54,000 daltons (Elleman, 1974) to 500,000 daltons (Caldwell and Sparrow, 1980).

Although both the BAPNAase and the proteinase from Vicia faba were sulphydryl proteases neither enzyme could hydrolyse the citrulline paranitroanalides, which are substrates for plant sulphydryl proteinases such as papain, ficin and bromelain. This means that the enzymes from

Vicia faba have a different substrate specificity despite sulphydryl dependent activity.

However, the BAPNAase did hydrolyse di- and tri-peptides. The only peptide hydrolysed with the amino end blocked was CBZ-glycylphenylalanine. This peptide is a carboxypeptidase substrate and when tested at the normal carboxypeptidase pH optima of ca. 5.5 no activity was detected ruling out carboxypeptidase contamination. The BAPNAase is a neutral peptidase with a preference for glycine at the N terminal end of the peptide chain connected to a hydrophobic amino acid. The BAPNAase bound well to the phenyl butyl amine sepharose column, this confirms a hydrophobic environment near the active site. The exact substrate specificity could be confirmed by using a wider range of di- and tri-peptides than used in this study.

The presence of small amounts of other proteins on polyacrylamide gels of purified proteinase from Vicia faba ruled out the possibility of immunological location of the proteinase. The additional bands on the gel may have been due to autolytic hydrolysis of the proteinase, but may have raised additional antibodies and confused any future experiments.

The high pH optima of the neutral and alkaline peptidases has led to the belief that they cannot be located within the protein bodies because the proteinases and carboxypeptidases located within the protein bodies have pH

optimas of ca. 5.5 (Mikola, 1983). Van der Wilden (quoted from Mikola, 1983) proposed that these peptidases maybe located within the cell wall.

The titre of antibody produced in rabbits in response to the BAPNAase was not high. This was indicated by ELISA and the need to incubate the double immunodiffussion experiments for 48 hours, rather than the usual 24 hours (Johnstone and Thorpe, 1982). The immunodifussion experiments showed that there is an antigenically similar enzyme in all the legume seeds tested, but not in the cereal grains tested. This suggests a conserved role for the enzyme in protein metabolism in the cotyledons of these legume seeds. Whether the role is in the general protein metabolism of the cell or in the metabolism of the storage proteinsis not known.

Caldwell and Sparrow (1976) reported a BAPNAase enzyme in pea seeds and Nakano and Asahi (1974) reported that the BAPNAase enzyme in pea seeds was located with the membrane associated with the Golgi vesicles. Preliminary results with fluorescence microscopy suggest that the BAPNAase is located within the cell walls or membranes and not within the protein bodies. Further investigation is needed to confirm these results.

A number of workers have noted the changes that occur in the storage proteins as germination progresses (Basha and Beevers, 1975; Konopska, 1979; Guldager, 1978; working

with Pisum sativum. Kumar and Venkataraman, 1978; working with chickpea seeds. Daussant et al., 1969; working with Arachis hypogaea. Lichtenfeld et al., 1979; working with Vicia faba.). Despite this wealth of information a detailed examination of the changes in Vicia faba has yet to be presented.

The results with the total globulins confirm earlier studies (Taylor,1979), that the changes occur about six days after the start of germination; the proteins show increased electrophoretic mobility and the larger subunits dissappear first. When the two major storage proteins were purified and analysed, vicilin showed the greatest change. Two new subunits (ca. 18,000 and 19,000 daltons) increased in intensity at eleven days after the onset of germination. This could mean that vicilin is first broken down in the protein bodies (Graham and Gunning, 1970; Craig et al., 1980) into these smaller subunits before some of these fragments are processed further (Bollini and Chrispeels, 1978; Basha and Cherry, 1978). Legumin, on the other hand, is either processed completely by the proteinase(s) ±carboxypeptidases (Baumgartner and Chrispeels, 1977), or it is broken down into fragments smaller than ca. 12,000 daltons which are not trapped by the dialysis tubing.

Another possible explanation for the differences observed in vicilin could arise from the heterogeneity of the vicilin species (Derbyshire et al., 1976; Thompson et al.,

1980). A vicilin molecule with low molecular weight polypeptides maybe present in ungerminated seeds but at low concentrations. Wright and Boulter (1972) investigating the synthesis of vicilin in developing pea seeds, found a vicilin protein with low molecular weight components. If present at low concentrations in ungerminated seeds it may not be visualized an SDS-PAGE gels initially. However, as germination proceeds and this protein is not degraded, then the concentration would increase in the latter stages of germination.

Both these proposed explanations of the appearance of low molecular weight material in vicilin isolated from the latter stages of germination, imply preferential breakdown of one or more proteins. Lichtenfeld et al., (1979) showed that legumin was broken down at a faster rate than vicilin in Vicia faba cotyledons. This study has shown that the proteinase isolated from Vicia faba cotyledons is active in vitro on both proteins at all stages of germination, Graham and Gunning (1970) found that some protein bodies in developing cotyledons of Pisum Sativum contain only vicilin and some contain neither legumin nor vicilin. Could similar protein bodies be the source of vicilins with low molecular weight components? Raising antibodies to the proteins or the low molecular weight polypeptides, and then examining their location in the early stages of germination would provide additional information on this problem.

Isoelectricfocussing of legumin confirms that it is the acidic subunits (ca. 40,000 daltons) which show the biggest change as germination proceeds, with the basic subunits losing some of their basic character, becoming more negatively charged. The proteinase showed a preference for asparagine nitrophenol esters and one may have expected a greater change in the basic subunits. The change in amide content of the storage proteins is much less than observed by Shutov and Vaintraub (1972) in vetch seeds.

Goldberg and St John (1976) proposed that the physical properties of proteins determine their rates of degradation. Many of these properties, for example size, subunit size, isoelectric point, carbohydrate content, - SH content and amide content have been correlated with _in vivo_ protein degradation (Davies, 1982). The majority of this work has been done with animal tissue, but Acton and Gupta (1979) found a similar correlation in higher plant tissue. Wolf (1982) proposed an alternative explanation of selective protein degradation; that the selectivity resides in specific proteolytic hydrolysis. Coates and Davies (1983) found some evidence to support this hypothesis in senescent barley leaves. All these studies have used enzyme half life to judge the selectivity of protein degradation, but the hypothesis may apply to all proteins, including the storage proteins in legumes. Thus the degradation of the storage proteins maybe dependent upon their own genetically determined physical properties, or upon the action of specific proteases or upon both these factors.

Catsimpoolas et al., 1968; Daussant et al., 1969; Shutov and Vaintraub, 1973 and Lichtenfeld et al.,1979 have proposed that changes in the storage proteins are necessary for their complete hydrolysis. However, this may not be so in Vicia faba; the proteinase from Vicia faba is active on the storage proteins extracted at all stages of germination and this precludes any changes necessary for hydrolysis to begin. There are no obvious differences in the rates of hydrolysis of seven day old legumin and vicilin, but further investigation on storage proteins from other stages of germination is necessary to confirm this result.

Another control mechanism could be hormonal; hormones produced in the axis controlling de novo enzymes synthesis (Guardiola and Sutcliffe, 1971). Yomo and Varner (1973) however have suggested that the accumulation of amino acids in detached cotyledons inhibits further proteolytic enzyme formation. The axis, they propose, would provide a "sink" for the amino acids.

Enzyme inhibitors present in the cytoplasm of the seeds were thought to regulate enzyme activity (Royer, et al., 1974). The majority have been found to be serine proteinase inhibitors (Ryan, 1981) and most plant proteinases are sulphydryl proteinases. Chrispeels and Baumgartner (1976, 1978) have isolated two proteinase inhibitors from mung bean, one a trypsin inhibitor, the other inhibited the major proteinase present in the seed. They concluded that the role of the endogenous proteinase

inhibitor was to protect the cytoplasm from the non specific proteinase.

The proposed role of the proteinase isolated from Vicia faba cotyledons is in the hydrolysis of the storage proteins within the protein bodies as the pH optima is in the correct range for this location. The proteinase bound to the globulin sepharose column and hydrolysed isolated storage proteins. These characteristics suggest an important physiological role. 10.3m of proteinase was extracted from 250.0g of cotyledons (fresh weight), or 410.1 µg of proteinase g^{-1} fresh weight (Table 5). The proteinase could digest ca. 0.31 µg of globulin $hour^{-1}$ (Table 8). Taylor (1979) reported that between days 5 and 7 the total globulins extracted decreased 6.0 mg g^{-1} fresh weight; Lichtenfeld et al., (1979), reported that in the same period the globulins legumin and vicilin dropped 1.48 mg g^{-1} fresh weight. The proteinase, in vitro, could hydrolyse ca. 600 µg of protein in 48 hours, so the proteinase could account for ca. 10% or 40% of the decrease in extractable globulins between days 5 and 7, However, the total amount of proteinase extracted will be underestimated due to inactivation during extraction, purification and assay. Taking this into account it seems likely that the enzyme is a major proteinase involved in the hydrolysis of storage proteins in Vicia faba.

Baumgartner and Chrispeels (1977) found that vicilin peptidohydrolase from mung bean could account for 95% of total proteinase activity, 5 days after the start of germination. Bulmaga and Shutov (1977) and Shutov , et al., (1982) found that at least two proteinases were involved in vetch seed storage protein breakdown. From this study it is evident that further investigation is needed to clarify the mechanism of storage protein breakdown in Vicia faba. It is still not clear whether there is more than one proteinase, as suggested by Taylor (1979), involved in storage protein degradation in Vicia faba. A different assay or initial extraction procedure may reveal other proteinases.

The role of the BAPNAase is likely to be in processing peptides produced from the breakdown of the storage proteins and intracellular proteins. The fact that there is an antigenically similar enzyme in other legume seeds indicates an important role for this enzyme. If, as suggested by fluorescence microscopy, the location is mainly within the cell wall or membrane, the role of the enzyme maybe in aiding the transport of amino acids from the cell to intercellular spaces. However, further investigation is needed to confirm any of these proposals.

The enzymes isolated maybe further investigated to clarify their substrate specificity and location; other analytical methods such as sucrose density gradient centrifugation to purify protein bodies should be attempted if the use of antibodies proves impossible.

There are other enzymes to isolate and characterise; Taylor (1979) reported a carboxypeptidase and a leucine aminopeptidase to be present in the cotyledons of germinating Vicia faba. Information on their location and role in the degradation of storage proteins, could only increase the present understanding of germination.

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Separation of legumin and vicilin, the storage proteins from germinating *Vicia faba* seeds, by electrophoresis on cellulose acetate membranes

Conventional electrophoretic methods of assessing the purity of a protein are both expensive and laborious. The method of zonal isoelectric precipitation electrophoresis involves precipitation of the protein under investigation at its isoelectric point after application onto cellulose acetate membranes. Contaminating proteins will be mobile and are easily visualized by conventional staining methods. Application of zonal isoelectric precipitation electrophoresis to the storage proteins of *Vicia faba* seeds (legumin and vicilin) has proved valuable in this laboratory, and, by adjustment of the buffering system, can be made to work for other protein mixtures.

In many laboratories the assessment of the purity of protein samples is routinely performed by a number of expensive and time-consuming electrophoretic techniques, such as polyacrylamide gel electrophoresis (PAGE) with [1] or without sodium dodecyl sulphate (SDS) [2, 3] and isoelectric focusing [4]. In this laboratory, the routine preparation of the storage proteins in germinating *Vicia faba* seeds [5] led to a search for a relatively inexpensive and less time-consuming method for assessing the purity of the separated proteins vicilin and legumin. Blagrove and Gillespie [6] described an electrophoretic method, using cellulose acetate to separate the storage proteins of lupin seeds: α-conglutin, β-conglutin and γ-conglutin. However, when the system was applied to the storage proteins of *Vicia faba* seeds, vicilin and legumin migrated to similar final positions forming broad overlapping bands. This method was not improved by altering the salt concentration or the buffering system (unpublished results). The method described in this paper involves the precipitation of legumin protein at its isoelectric point (p*I* 4.7) at the point of application. Thus when the electrical field is applied, only the vicilin protein is mobile. The name zonal isoelectric precipitation electrophoresis (ZIPE) is suggested for the method.

Whatman cellulose acetate membranes (5.7 × 14 cm) were transferred from storage in 30 % v/v methanol to a tray containing citric acid – Na_2HPO_4 buffer, pH 4.7 [7]. The buffer consists of 3.64 g/liter citric acid monohydrate (0.0334 M) and 5.70 g/liter di-sodium hydrogen phosphate dihydrate (0.0667 M). After 10 min the membranes were transferred to another tray, containing the same buffer for a further 10 min, to ensure the complete removal of the methanol. The membranes were then placed onto the glass plate of an LKB 2117 Multiphor, porous side uppermost, with a circulating cooling supply at 8 °C. The electrophoresis running buffer was the citrate-phosphate buffer (pH 4.7). Four sheets of Whatman chromatography paper no. 1, in each buffer compartment, provided the wicks. Protein samples were dissolved in 0.15 M sodium phosphate buffer, pH 7.0, and were applied either with a semi-micro applicator (Whatman Lab Sales) delivering approximately 5 µl or with a 10 µl syringe. After the protein samples had been applied to the membrane, electrophoresis was performed at a constant current of 6 mA per membrane for 1 h. The membranes were removed, from the apparatus, and stained in 0.125 % w/v PAGE Blue 83 (BDH Poole, Dorset) in 3:1:6 v/v ethanol: glacial acetic acid: distilled water for 15 min. Finally, the membranes were destained in 3:1:6 v/v ethanol: glacial acetic acid: distilled water until the background was clear of stain.

Fig. 1 shows that purified samples of legumin (A and B) remained at the point of application, and that the purified vicilin sample (C) moved appreciably under the influence of the electrical field. Contamination of samples is easily

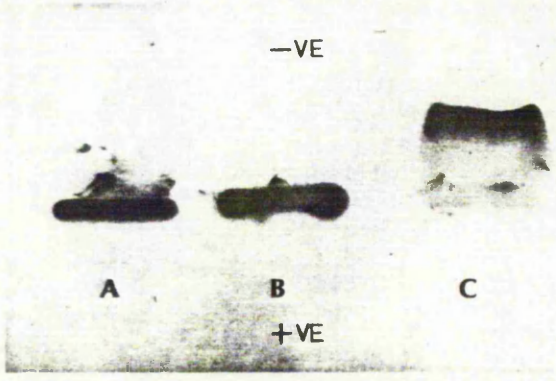

Figure 1. Lypholized samples of legumin (A and B) and vicilin (C), extracted from 0-day-old *Vicia faba* cotyledons, were dissolved in 0.15 M sodium phosphate buffer, pH 7.0, at a concentration of 2 mg/ml. Samples (each 5 µl) were applied to the midpoint of a Whatman cellulose acetate membrane for electrophoresis at pH 4.7.

detected as shown in Fig. 2, in which a purified vicilin sample from seven-day-old seeds was clearly shown to be contaminated with legumin, whereas samples from four-day and eleven-day-old seeds appear uncontaminated. Small amounts of contamination can be detected visibly (< 0.1 µg), the level of detection being limited by the sensitivity of the stain used. The method can be adapted to separate other proteins, as shown in Fig. 3. Here the electrophoresis buffer was adjusted to pH 4.3, thus precipitating the casein (BDH), and allowing the β-lactoglobulin (pI of about 6.8) to migrate. Using this buffer, contamination of casein with legumin (p*I* approximately 4.7) and alkaline phosphatase (p*I* approximately 4.5) can be detected. Both contaminants were present at a concentration of 2 mg/ml. It is envisaged that this method would benefit laboratories where characterized protein samples are routinely prepared, providing a quick visual check of the purity of the protein.

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Abbreviations: PAGE: Polyacrylamide gel electrophoresis SDS: Sodium dodecyl sulphate

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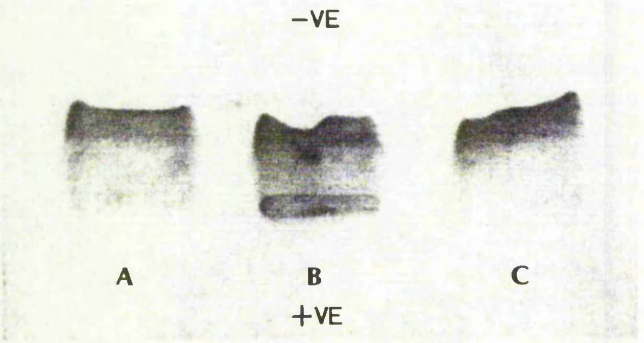

Figure 2. Lypholized samples of vicilin extracted from four-day-old (A), seven-day-old (B), and eleven-day-old (C) *Vicia faba* cotyledons were dissolved in 0.15 M sodium phosphate buffer, pH 7.0, at a concentration of 2 mg/ml. Samples (5 µl each) were applied to the midpoint of a Whatman cellulose acetate membrane for electrophoresis at pH 4.7.

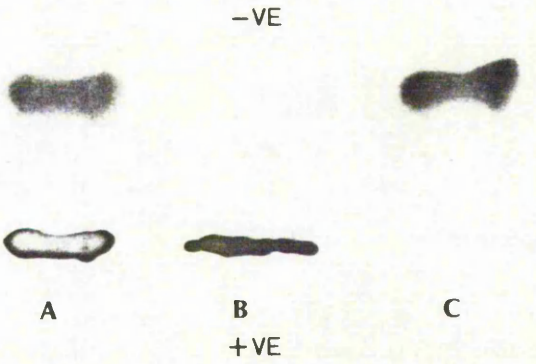

Figure 3. Casein (B), β-lactoglobulin (C) and a mixture of casein and β-lactoglobulin 1:1 (A) at 2 mg/ml were dissolved in 0.15 M sodium phosphate buffer, pH 7.0. Samples (5 µl each) were applied to the midpoint of a Whatmann cellulose acetate membrane, previously equilibrated with 0.05 M citric acid-disodium hydrogen orthophosphate buffer, pH 4.3. This buffer was also used as the running buffer.

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