Germination In Pea Seeds:

Proteolytic Enzyme Activity And Electron Microscopy

A Thesis

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CHRISTOPHER P. MROZ ©

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LIST OF ABBREVIATIONS

ANA	alanine-p-nitroanilide
BAPNA	N-benzoyl-DL-arginine-p-nitroanilide
pC1-HgBzO	*p*-chloromercuribenzoate
PhMeSuF	phenylmethyl sulphonyl fluoride

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1 GENERAL INTRODUCTION

This study was undertaken with two primary objectives in mind. The first objective was to isolate several pea seed hydrolases present during early seed germination. The second objective was to perform an ultrastructural investigation of protein mobilization in normally senescing and de-shooted cotyledons. It has been demonstrated biochemically that protein mobilization is delayed in de-shooted cotyledons (Guardiola and Sutcliffe 1971; Malek 1987). Peas were chosen for this study due to their large size, availability and the wealth of literature that was available as background. Despite the large amount of study, few pea seed enzymes have been purified to homogeneity. Isolation, characterization and localization of pea seed proteases will be necessary in order to piece together the puzzle of protein mobilization during germination.

Seeds of leguminous plants synthesize and accumulate large amounts of storage protein as well as hydrolytic enzymes in the course of their development. In mature seeds the storage proteins are present in protein bodies, membrane-bound organelles measuring 0.1 to 25 um in diameter, in the storage parenchyma cells of cotyledons (Bewley and Black 1978). Protein bodies originate from the central vacuoles of storage parenchyma cells in legumes and other dicot seeds after the deposition of storage protein has started (Opik 1968; Bain and Mercer 1966a; Harris and Boulter 1976; Neuman and Weber 1978; Yoo and Chrispeels 1980; Craig *et al.* 1980). The appearance of protein bodies follows the time course of globulin synthesis during seed development (Varner and Schidlovsky 1963). Analysis of isolated

protein bodies shows that they contain not only storage proteins and lectins, but also numerous hydrolytic enzymes with an acid pH optimum, such as phosphatase, α- mannosidase and ribonuclease (Van der Wilden *et al.* 1980).

Germination of leguminous seeds is accompanied by a dramatic increase in proteolytic activity in the cotyledons owing to de novo synthesis of proteolytic enzymes (Csoma and Polgar 1984; Chrispeels 1984). During seed germination, changes take place in the fine structure of cotyledon cells which can be related to the utilization of stored reserves which supply energy and biosynthetic intermediates to the growing plant (Briarty *et al.* 1970; Wilson and Tan-Wilson 1987). After germination, protein bodies disappear gradually over a period of several days. Digestion of cotyledons of bean is practically completed in 8 days at 25°C (Opik 1966), but is delayed in deshooted cotyledons of pea (Guardiola and Sutcliffe 1971; Malek 1987). After the reserves are hydrolyzed the storage cells die. Vascular bundles and epidermal cells survive until the cotyledons abcise (Opik 1966).

Storage proteins are hydrolyzed into their constituent amino acids by proteolytic enzymes. These enzymes are classified as either proteinases or peptidases (Mikola 1983). Proteinases are endoproteases that cleave internal peptide bonds of polypeptides. There are four major groups of proteinases based on the catalytic mechanisms of their active site (Ryan and Walker-Simmons 1981). These are the serine proteinases, sulfydryl proteinases, metalloproteinases and acid proteinases. Peptidases are exoproteases that are further classified as aminopeptidases or carboxypeptidases depending on whether they cleave the amino or carboxyl terminal amino acid residue from the polypeptide chain.

Pea seed storage proteins are composed of water soluble albumins and salt soluble globulins. The globulins were classified as legumin and vicilin by Osborne and Campbell (1898), and the methodology was later improved by Danielson (1951). Since the work of Danielson (1956) on peas, the predominant view of seed enzymes was that they belonged exclusively to the albumin class and acted upon legumin and vicilin. However, subsequent evidence has revealed that enzymes are not confined to the albumin fraction and that albumins are actually utilized as reserves during and after germination (i.e. Basha and Beevers 1975; Collier and Murray 1977).

Conventional biochemical protein purification techniques were utilized to separate some proteolytic activities of pea seeds. Subsequent studies could build on this study by employing such methods as affinity chromatography. Once a homogenous prepartion is obtained, its antibody could be developed for immunocytochemical localization of the enzyme. By localization of an enzyme, its possible movement between organelles can be followed during germination. This will help to elucidate whether it is involved in the breakdown and mobilization of reserve proteins.

2 CHAPTER 1

Isolation and partial characterization of pea seed proteolytic enzymes hydrolyzing alanine-p-nitroanilide, N-benzoyl-DL-arginine-p-nitroanilide and ^{3}H-casein after two and five days of germination.

2.1 INTRODUCTION

Storage proteins of seeds serve as a store of amino acids during germination and early seedling growth. These reserve proteins which are stored in membrane-bound protein bodies in cotyledons of leguminous seeds, are synthesized on the rough endoplasmic reticulum (Baumgartner and Chrispeels 1977). Proteolytic enzymes play a central role in the biochemical mechanism of germination by hydrolysing the storage proteins. This allows for mobilization of resulting amino acids to the growing axis of the developing plant. However, protein bodies isolated from dry seeds do not contain the proteolytic enzymes necessary to digest their own reserve proteins (Harris and Chrispeels 1975). Chrispeels *et al.* (1976) concluded that storage protein hydrolysis in the cotyledons involves de novo synthesis of the required proteinase and its transfer to and accumulation in the protein bodies.

Acid, neutral and alkaline protease activities have been reported in cotyledons of germinating legume seeds (Yomo and Varner 1973; Elleman 1974; Basha and Beevers 1975; Storey and Beevers 1977; Shutov *et al.* 1984; Mikkonen and Mikola 1986; Boylan and Sussex 1987; Malek 1987). At least two main groups of proteases likely function in storage protein hydrolysis during germination and seedling growth. These include a group of neutral to alkaline peptidases that are present in dry seeds at low levels and do not increase in activity with germination and a group of acid proteinases that increase in activity during germination following a lag period of one to four days (Nielsen and Liener 1984; Mikkonen 1986; Mitsuhashi *et al.* 1986).

The acid proteinases belong to the class of sulfhydryl proteases judging by their sensitivity to thiol reactive inhibitors. They have optimum proteolytic activity in the range of pH 5.0 to 5.5 and molecular weights in the range of 20 to 30 kilodaltons (Boylan and Sussex 1987; Mitsuhashi *et al.* 1986; Beevers 1968; Feller 1979; Vavreinova and Turkova 1975; Csoma and Polgar 1984; Nielsen and Liener 1984). These enzymes have broad substrate specificity, including azocasein (Feller 1979; Caldwell and Sparrow 1976; Nielsen and Liener 1984), haemoglobin (Mitsuhashi *et al* 1986), gelatin (Mitsuhashi *et al.* 1986) and endogenous reserve proteins (Baumgartner and Chrispeels 1977). The acid protease that has been most extensively studied is mung bean vicilin peptidohydrolase which was isolated and characterized by Baumgartner and Chrispeels (1977) and later localized by Baumgartner et al. (1978). Antibodies prepared against vicilin peptidohydrolase have been utilized to determine that the enzyme is not present in leaves or in developing seeds but is synthesized de novo in the cotyledonary cytoplasm after the first two days in the cotyledonary cytoplasm of seedling growth (Chrispeels *et al.* 1976), then transported to the protein bodies (Baumgartner et al. 1978).

Neutral to alkaline peptidase enzymes have optimal activities in the range of pH 7 to 10 (Nielsen & Liener 1984; Nishikata 1984; Caldwell and Sparrow 1976). The activity is present in dry seeds and is maintained for several days following germination (Tomomatsu *et al.* 1978). The molecular weights are larger than for the acid proteinases being in the range of 58 to 360 kilodaltons (Sopanen and Mikola 1975; Elleman 1974; Mikkonen and Mikola 1986).

Caldwell and Sparrow (1976) reported the isolation from pea seeds of two serine peptidases with a requirement for a thiol group that hydrolyze N-benzoyl-DL-arginine-p-nitroanilide. The two activities were separated by ion exchange chromatography. Other alkaline peptidases that hydrolyze synthetic substrates have been reported in germinating kidney bean (Mikkonen 1986; Crump and Murray 1979), soybean (Nishikata 1984) and peas (Tomomatsu et al. 1978). As a group, these enzymes have not been well studied, probably because a suitable physiological role cannot be attributed to enzymes with such a high pH optimum.

Two other groups of peptidases are recognized as occurring in plant tissues including the acid carboxypeptidases and the napthylamidases (Mikola 1983). Naphthylamidases occur in resting seeds but their activity was found to decrease with germination in kidney beans, whereas carboxypeptidase increased during germination reaching a maximum value when the mobilization of nitrogen was highest (Mikkonen 1986).

The purpose of the present study was to isolate proteolytic enzymes that hydrolyze alanine-p-nitroanilide (ANA), N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and ^{3}H-casein to distinguish between physiologically distinct groups of enzymes and as a first step in elucidating a mechanism for the early hydrolysis of protein in pea cotyledons. Data is presented on tissue that has been imbibed for two and five days. Five distinct enzymatic activities were isolated. Some properties of the enzymes were investigated and their possible physiological role is discussed.

2.2 MATERIALS and METHODS

2.2.1 Plant Material and Growth Conditions

Pea seeds (*Pisum sativum* L. cv. Spring, Asgrow Seeds, Bradford, Ont.) treated with Captan, were sown in vermiculite, watered to field capacity and covered to prevent dehydration.

Germination took place in a Conviron growth chamber, model CMP 3023 (Controlled Environments Ltd., Winnipeg, Man.) set to 25°C for an 8-hour photoperiod provided by fluorescent lights at 70-100 $uEm^{-2}s^{-1}$ and 15°C for 16-hour nights. After 48 hours, the growing peas were uncovered and used for analysis (2-day-old seedlings) or watered and left uncovered in the growth chamber for 3 more days (5-day-old seedlings).

2.2.2 Protein Determination

Protein concentration was determined utilizing the Biorad (Richmond, Ca.) Coomasie brilliant blue dye binding method (Bradford 1976). Standard curves were produced utilizing bovine serum albumin at known concentrations. Spectrophotometric measurements were made on a Beckman model DU-50 spectrophotometer (Beckman Instruments Inc., Fullerton, Ca.) at 595 nm.

2.2.3 Enzyme Purification

2.2.3.1 Preparation of Crude Extract

Seeds of *Pisum sativum* L. that were either dry or at the desired state of germination or seedling growth, were collected. The seed coat, hypocotyl and epicotyl were removed. Forty seeds were homogenized over crushed ice with a mortar and pestle in 60 ml pH 5.5, 50 mM

sodium phosphate extraction buffer containing 1 mM mercaptoethanol and 1% sodium azide following the method of Malek (1987). 10% polyvinylpolypyrolidone (PVPP) was included here to bind the tannins. After centrifugation at 27,000 g for 10 minutes in a refrigerated Sorval RC2B centrifuge (Dupont, Wilmington, Delaware) with a SS-34 rotor, the supernatant was collected for analysis of hydrolytic activity and further purification.

2.2.3.2 **Ammonium Sulfate Precipitation**

Ammonium sulfate was added to the crude preparation to an 80% saturation over a period of 30 minutes. The preparation was stirred by a magnetic stirrer in a cold room at 2° C for an additional 30 minutes before being recentrifuged at 27,000 g for 10 minutes in the Sorval RC2B. After discarding the supernatant, the pellet was resuspended in 5 ml of the extraction buffer without PVPP.

2.2.3.3. **Dialysis**

The above preparation was loaded into dialysis tubing (Spectrapor membrane, 6,000-8,000 molecular weight cut-off, Spectrum Medical Industries, Los Angeles, Ca.) which had been soaked in distilled water for at least one hour. The enzyme preparation was dialysed for 8 hours in one litre of extraction buffer while being stirred in a cold room at 2° C. After centrifugation at 27,000 g for 10 minutes in the Sorvall RC2B, the supernatant was collected and saved for analysis or further purification.

2.2.3.4 Gel Filtration Chromatography

Gel Filtration Chromatography was performed utilizing an LKB (Fisher Scientific Co., Winnipeg. Man.) model chromatography column of dimensions 1.6 cm x 85 cm and Sephadex G150 gel (Pharmacia Chemicals, Upsala, Sweden). Five ml or about 100 mg of protein obtained from the supernatant of the centrifuged dialysed fraction was loaded on the gel. A flow rate of 15 ml/hr was maintained with an LKB (Fisher Scientific Co., Winnipeg, Man.) or Econo-Column-Pump (Bio-Rad Laboratories, Richmond, Ca.) peristaltic pump. The proteins were eluted with extraction buffer. Two hundred drops per fraction (9.5 ml per fraction) were collected with an LKB model 2111 "Multirac" fraction collector. A chart trace of the run was produced on a Linear (Can Lab, Toronto, Ont.) chart recorder by measuring UV absorbance at 280 nm in a LKB model 2238 "Uvicord SII" UV detector. The active fractions were pooled for further analysis or applied to an ion exchange column.

2.2.3.5 Ion Exchange Chromatography

A LKB chromatography column of dimensions 2.5 cm x 60 cm containing the anion exchanger Biogel A (Bio-Rad Laboratories, Richmond, Ca.) was employed to perform ion exchange chromatography. The gel was equilibrated with pH 7.5, 50 mM Tris-Cl buffer containing 1 mM mercaptoethanol and 1% sodium azide.

The three most active fractions from gel filtration chromatography were pooled, loaded on the ion exchange gel and run at 40 ml/hr. The speed was maintained as in gel filtration chromatography with a peristaltic pump. The gel was washed with the same pH 7.5 Tris-Cl buffer until absorbance at 280 nm was zero. A zero to 300 mM NaCl gradient containing 700 ml per side of pH 7.5, 50 mM Tris-Cl buffer plus 1 mM mercaptoethanol and 1% sodium azide was used to elute the proteins.

Two hundred drop (9.5 ml) fractions were collected as in gel filtration chromatography. The gel was regenerated by washing with the same buffer containing 1 M NaCl followed by 10 mM NaOH. Fractions were surveyed for enzymatic activity and active fractions were pooled.

2.2.4 **Assay for Caseinolytic Activity**

^{3}H-methyl casein substrate was prepared by reductive methylation of casein in the presence of tritiated sodium borohydride and formaldehyde by the method of Means and Feeney (1968). The tritiated casein substrate was diluted 1:1 with distilled water before use to a specific activity of 5 x 10^{6} cpm/mg protein. Each assay mix (12 x 75 mm tube) contained 10 ul of diluted ^{3}H-methyl casein substrate and between 10 ul to 70 ul of the enzyme. The volume was made up to 80 ul with the addition of pH 5.5, 50 mM sodium phosphate buffer. Mercaptoethanol (1 mM) was included in all assay mixes except for the inhibitor studies. Control assay mixes contained 10 ul dilute ^{3}H-methyl casein substrate and 70 ul pH 5.5, 50 mM sodium phosphate buffer.

Following 60 minutes of incubation at 25°C in an Orbit shaker bath (Lab-Line Instruments, Melrose Park, I11.) at 100 rpm, 20 ul of 10 mg/ml BSA was added as a carrier protein. The reaction was terminated by adding 80 ul of cold 10% (W/V) TCA and vortexing.

After 30 minutes, the assay tubes were spun at high speed in an IEC Model HN (International Equipment Co., Needham Heights, MA.) centrifuge, 80 ul of the supernatant was soaked onto 2.1 cm Whatman GF/A (Whatman Ltd., Maidstone, England) glass fibre filter paper. Following air drying overnight, the filter paper discs were counted in 5 ml toluene containing 0.5% 2,5-Diphenyloxazole in a liquid scintilation counter (LKB, model 1211 Rackbeta, Turku, Finland). Specific enzyme activity was calculated using total protein content measurements, according to Bradford (1976), using BSA as a standard.

2.2.5 **Assay for ANA and BAPNA Hydrolysing Activities**

Stock solutions of ANA and BAPNA (Sigma Chemical Co., St. Louis, Mo.) were made up to a concentration of 400 mM in dimethyl sulfoxide following the method of Malek (1987), then subsequently diluted to 4mM in water. Each assay mix (12 x 75 mm tube) contained 250 ul substrate (final concentration 1mM), 50 ul to 750 ul of enzyme and (pH 7.5), 50 mM Tris-Cl buffer to make up a total volume of 1.0 ml. Mercaptoethanol (1 mM) was included in all assay mixes except for the inhibitor studies. Controls did not contain the enzyme. Samples were agitated at 100 rpm for 30 minutes at 25° C. The reaction was stopped by the addition of 250 ul of 30% acetic acid and vortexing. Colour development was read on a spectrophotometer (Beckman DU-

50, Beckman Instruments Inc., Fullerton, Ca.) at 410 nm. For turbid samples, 1.25 ml. of ethyl acetate was added to each sample following the method of Erlanger *et al.* (1961). The assay mixes were centrifuged at full speed (JEC-HN) for 5 minutes. The supernatant was read in a spectrophotometer at 385 nm and corrected for comparison with non-turbid samples.

2.2.6 **Protein Ultrafiltration**

Prior to electrophoresis, pooled fractions were concentrated by ultrafiltration in a 50 ml Amicon ultrafiltration cell (Amicon Corporation, Danvers, MA) utilizing Diaflow YM5 ultrafiltration membranes (Amicon Corporation, Danvers, MA) with molecular weight cut-off of 5000 daltons. The concentrated fractions were taken up in 2 ml of the ion exchange buffer.

2.2.7 **Electrophoresis**

Polyacrylamide Gel Electrophoresis (PAGE) was performed utilizing a Hoeffer (Hoeffer Scientific Instruments, San Francisco, CA) Slab Gel electrophoresis unit and following the method for the second dimension of the O'Farrell process (O'Farrell, 1975).

Native gels were run in a cold room at 2° C for about 3.5 hours at 40 mA. The front of the gel was tracked visually utilizing methylene blue dye. The gels were stained and destained following the method outlined in the Hoeffer Scientific Instruments Catalogue (1983), then photographed and dried onto blotting paper, or used for zymograms.

2.2.8 Zymograms

A 0.5% agarose solution was prepared by adding 0.1 g agarose (Bio-Rad Laboratories, Richmond, Ca.) to 20 ml pH 7.5, 50 mM Tris-Cl buffer plus 1 mM mercaptoethanol and bringing the solution to a boil. During cooling and before the agarose gelled, ANA or BAPNA was added to the solution to a final concentration of 4 mM. The solution was poured out into a flat rectangular dish and allowed to gel.

The native gel was placed over top of the agarose slab and colour development was noted after 1 hour incubation at room temperature. The R_f values where colour development occurred were compared with R_f values of stained BSA standards for molecular weight determination.

2.2.9 Proteinase Inhibitors

Proteinase inhibitors were dissolved in pH 5.5, 50 mM sodium phosphate buffer except for PhMeSuF which was dissolved in 95% ethanol and diluted with buffer. Control treatments containing either buffer or the appropriate buffer-solvent mixture without inhibitor, were included in each experiment. The inhibitors were added to the assay tubes just before the addition of the enzyme. Casein assays were incubated for 60 minutes, ANA and BAPNA assays were incubated for 30 minutes at 25°C.

Inhibition was expressed as a percentage of total activity in a control sample. The activity of the inhibited sample was divided by the activity of the control and multiplied by 100 to give the "% activity". BSA was included in the analysis to test for non-specific inhibition.

2.3 RESULTS

2.3.1 Caseinolytic Assay

The effect of enzyme concentration on the caseinolytic assay is demonstrated in Figures 1 and 2. An optimum specific activity (Figure 2) was obtained when at least 0.004 mg of protein was employed in the assay mix. Changing the protein concentration significantly affected the specific activity.

Figure 3 demonstrates that the assay was affected by added protein. Low specific activities were recorded with a high level of exogenous protein in the assay mix.

Caseinase was also inhibited by NaCl as shown in Figure 4. The effect is partially reversible (Figure 5). This may have consequence to the analysis of the $(NH_4)_2SO_4$ fractionation and in anion exchange chromatography, especially in evaluating the activity in the 1 N NaCl wash.

2.3.2 Freezing Tolerance

Enzyme preparation purified from 5-day-old cotyledons past the gel filtration stage showed no significant loss of caseinolytic activity following short-term (7 days) storage at -20° C. Long-term (75 days) storage caused a reduction in caseinolytic activity of about 30% (data not shown).

2.3.3 Purification

Figures 6 and 7 illustrate typical scans of gel filtration chromatography runs. All three enzyme activites overlap. Tubes 11, 12 and 13 were routinely collected and combined for loading onto the anion exchange chromatography column.

Figure 6 shows that the protein in 2-day-old cotyledons was primarily comprised of large molecular weight proteins. By 5 days of germination, much of the protein was degraded to smaller proteins and peptides (Figure 7).

Figures 8 to 11 show typical scans of anion exchange chromatography runs. The numbering of proteinases corresponds to their respective elution on the column. BAPNA hydrolase (BAPNAase) I and II are clearly separated from each other (Figure 8 and 9) and ANA hydrolase (ANAase) I and II are also well separated (Figure 8 and 9). ANAase I overlaps with BAPNAase II (see Figures 8 and 9) but only the shoulders of each peak were collected, minimizing cross contamination. ANAase II and BAPNAase I eluted free of contaminating enzyme activity.

Typical purification results are listed in tables 1 through 6. ANAase I and II were purified 1237 and 668 times respectively from 2-day-old cotyledons (Table 1) and 864 and 172 times respectively from 5-day-old cotyledons (Table 2). The yield of ANAase I was from four-to-six times as great as for ANAase II.

BAPNAase I and II were purified 52 and 252 times respectively from 2-day-old cotyledons (Table 3) and 24 and 125 times respectively from 5-day-old cotyledons (Table 4). The yields were comparatively low for BAPNAase compared to ANAase I. The rate of purification for BAPNAase I was very low because it eluted with the major protein peak wash on the ion exchange column (see Figures 8 & 9).

Caseinolytic activity spread throughout the ion exchange run (figures 10 and 11). Significant caseinolytic peaks appeared in the same position as BAPNA activities. The same fraction labelled for BAPNAase II was also used as the basis for purification Tables 5 and 6. Caseinase was purified over 190 times for both 2 and 5-day-old cotyledons (Tables 5 and 6).

2.3.4 pH Optima of Enzymes

The optimal pH for ANAase and BAPNAase was found to be pH 7.5. The optimal pH for Caseinase was determined to be pH 5.5. Variations of activity with pH for all 5 enzymes is given in Figures 12 through 16.

2.3.5 Molecular Weights

The positions of ANAase and BAPNAase activities were determined by comparing the Rf values from enzyme activity on zymograms with the Rf value of standard marker proteins. The less purified gel filtration fraction produced the same molecular weights for both ANA and BAPNA activities (Table 7). Once these enzyme activities were separated by ion exchange chromatography, the ANAases were found to be smaller than the BAPNAase (see Table 7). BAPNAase I and II were identical in molecular weight. ANAase II was found to be much smaller than ANAase I. Both BAPNAase activities and ANAase I were found to be smaller in the 5-day-old cotyledons (Table 7). The molecular weight of ANAase II could not be determined by the zymogram method for 5-day-old cotyledons due to the small yield and low activity of this enzyme.

2.3.6 Inhibitors

The effect of various proteinase and peptidase inhibitors on the activity of the partially purified enzyme preparation is summarized in Table 8. Dithiothreitol and mercaptoethanol stimulated the activity of caseinase but mercaptoethanol inhibited ANA and BAPNA activities. Ovomucoid and soybean trypsin inhibitors inhibited caseinase at high concentrations but the effect was similar to that obtained with BSA. Inhibition by pCl-HgB_2O was reversible by the addition of 10mM mer-

captoethanol for all enzymes. PhMeSuF was slightly inhibitory to all 3 types of enzymes. Leupeptin, however, had varying effects with each substrate.

2.3.7 Polyacrylamide Gel Electrophoresis

The composition of the purified extracts of both ANAases and both BAPNAases as well as the preparation purified past the gel filtration stage are illustrated in Figure 17. Bovine serum albumin (molecular weight 66,000 daltons) and its dimer (molecular weight 132,000 daltons) were used as marker proteins. The data presented is for 5-day-old cotyledons but enzymes from 2-day-old colyledons produced the same pattern. PAGE of the dialysed fraction and pooled fractions following gel filtration chromatography demonstrated that all the proteins smaller than 66,000 daltons were removed at the gel filtration stage by pooling fractions 10, 11 and 12 (data not shown).

FIGURE 1

Effect of protein concentration on caseinolytic activity. Enzyme was purified from 2-day-old cotyledons past the gel filtration stage.

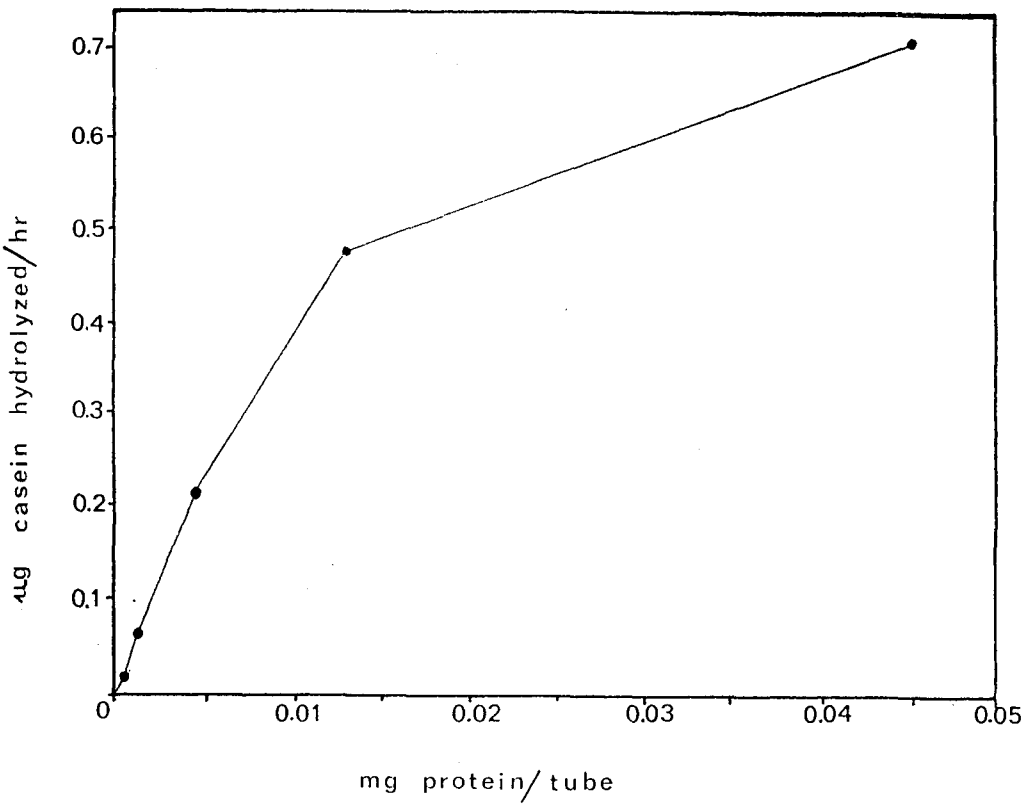

FIGURE 2

Specific activity recalculated from Figure 1.

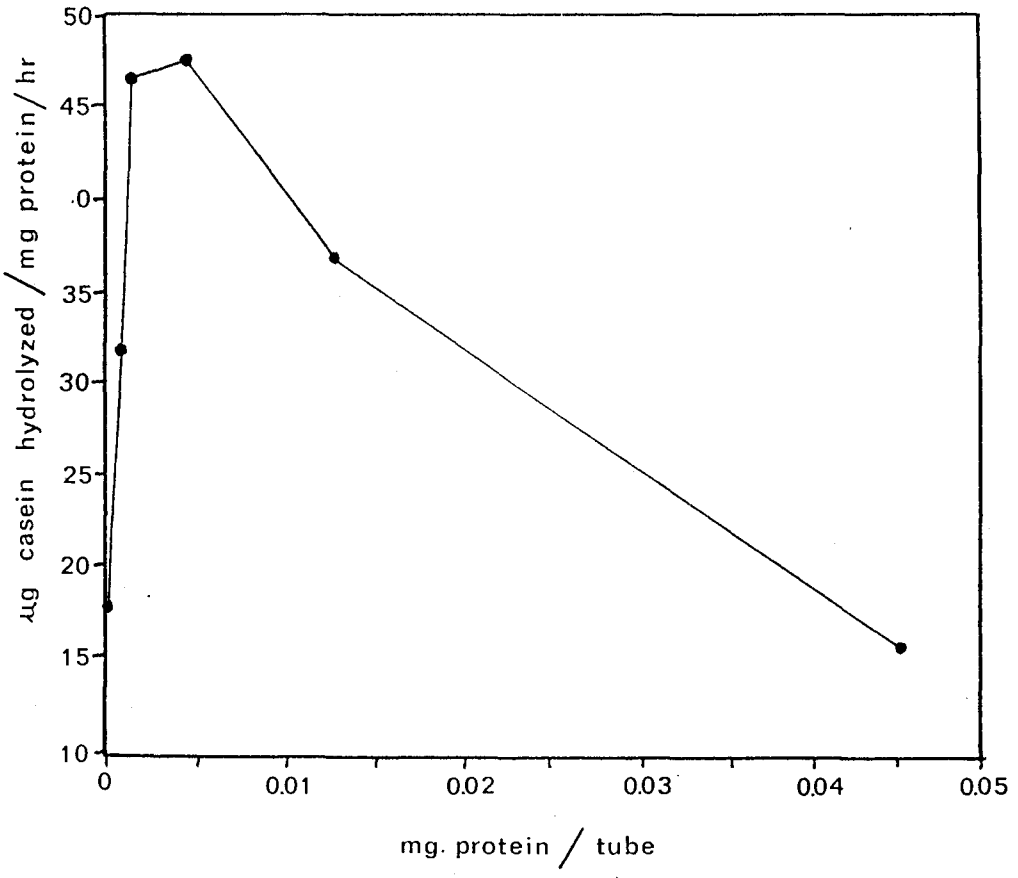

FIGURE 3

Effect of added BSA on caseinolytic activity. Enzyme was purified from 2-day-old cotyledons past the gel filtration stage; 0.05 mg enzyme preparation added per tube.

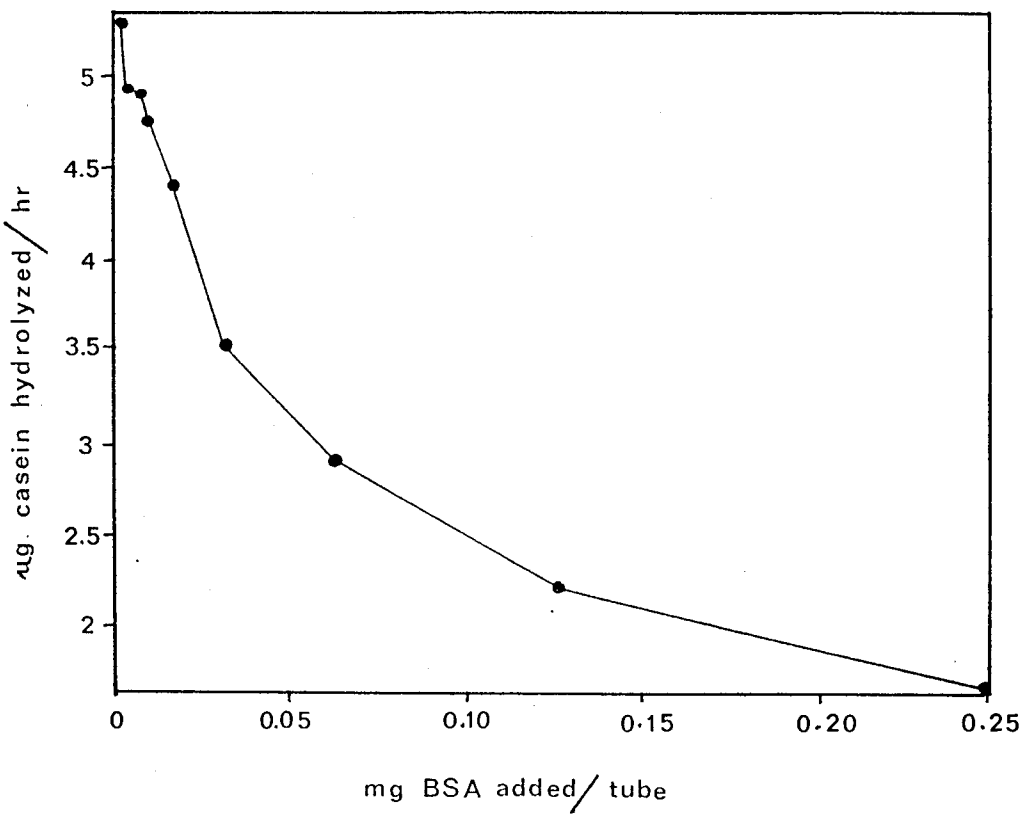

FIGURE 4

Inhibition of caseinolytic activity by NaCl. Enzyme was purified from 5-day-old cotyledons past the gel filtration stage.

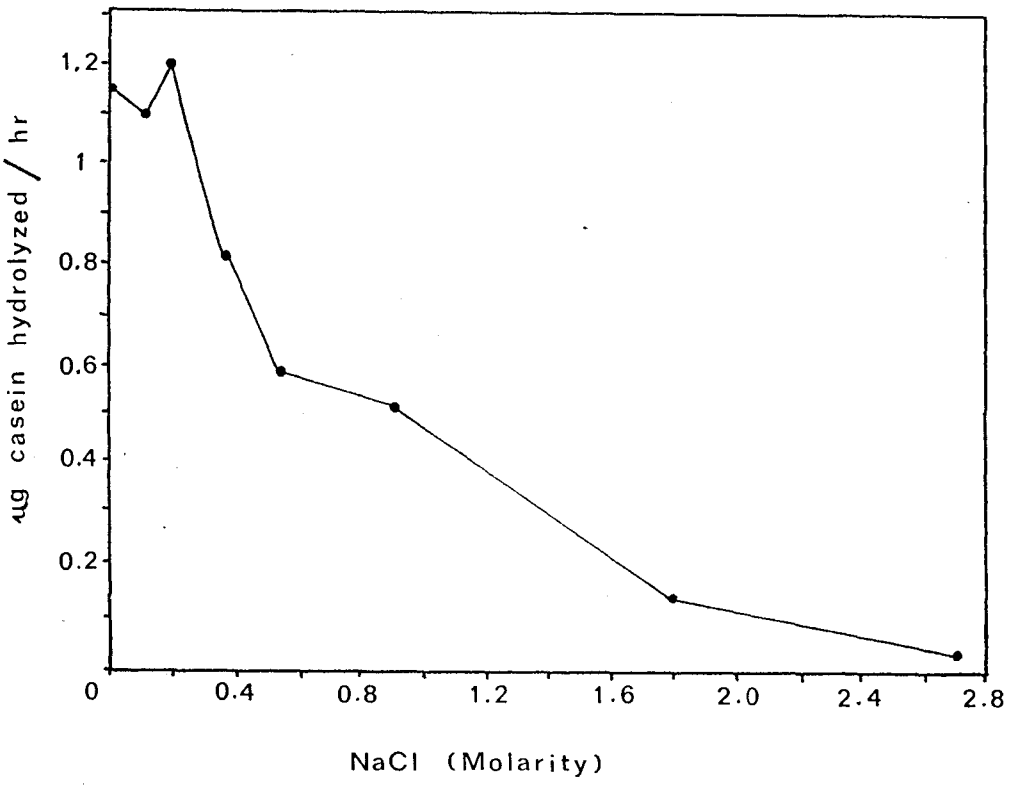

FIGURE 5

Recovery of caseinase after exposure to 2.5 N NaCl. Enzyme was purified from 5-day-old cotyledons past the gel filtration stage; 0.05 mg enzyme preparation added per tube.

(.) -Control, no NaCl added; (+) 2.5 N NaC1 added;

(○) -NaCl removed by dialysis, enzyme activity recovered.

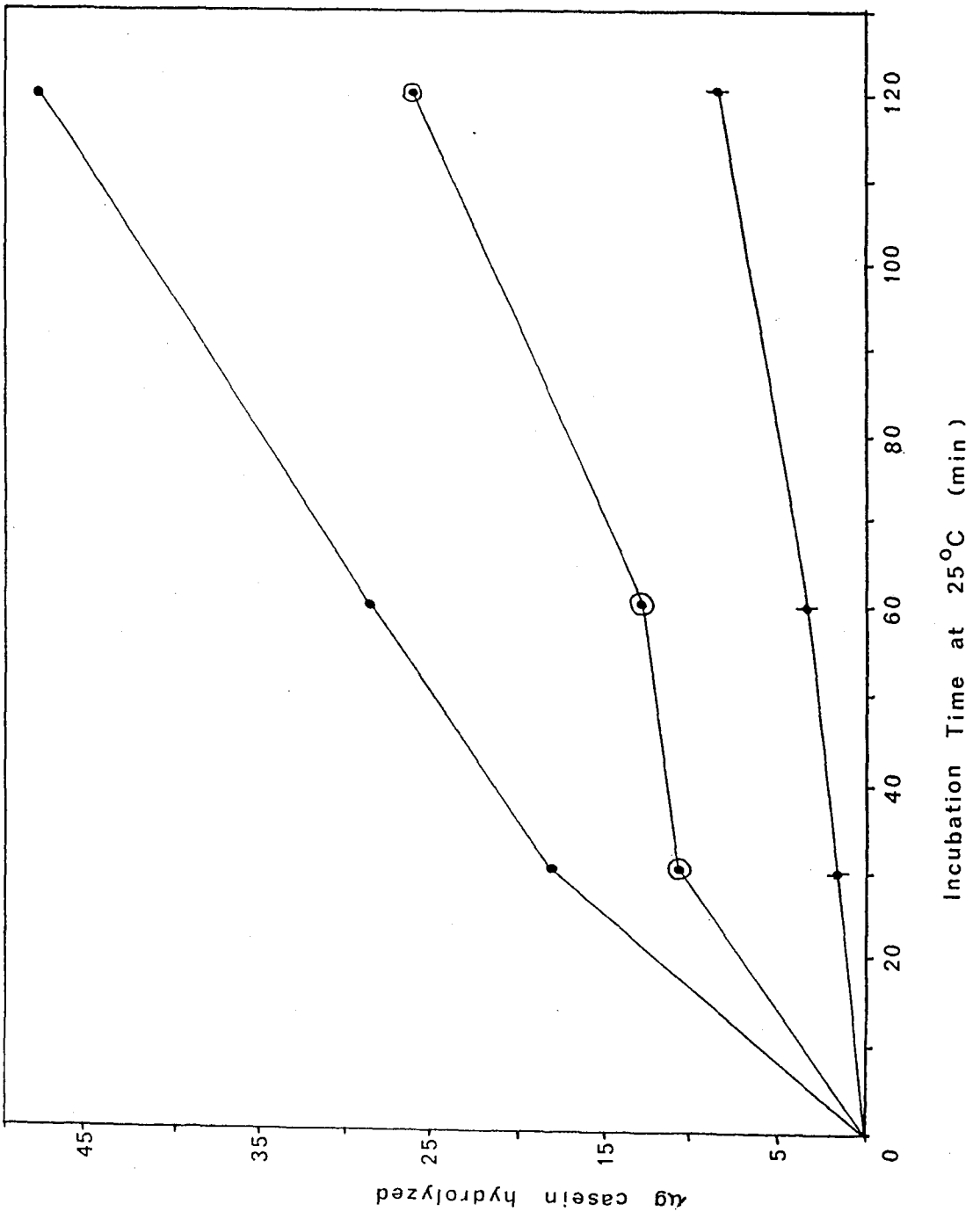

FIGURE 6

Gel filtration elution profile of a preparation from 2-day-old pea cotyledons.

(o) ANAase activity, (absorbance at 410 nm)

(..) BAPNAase activity, (absorbance at 410 nm)

(_.) ^{3}H - Caseinolytic activity.

(-) Relative A_{280}

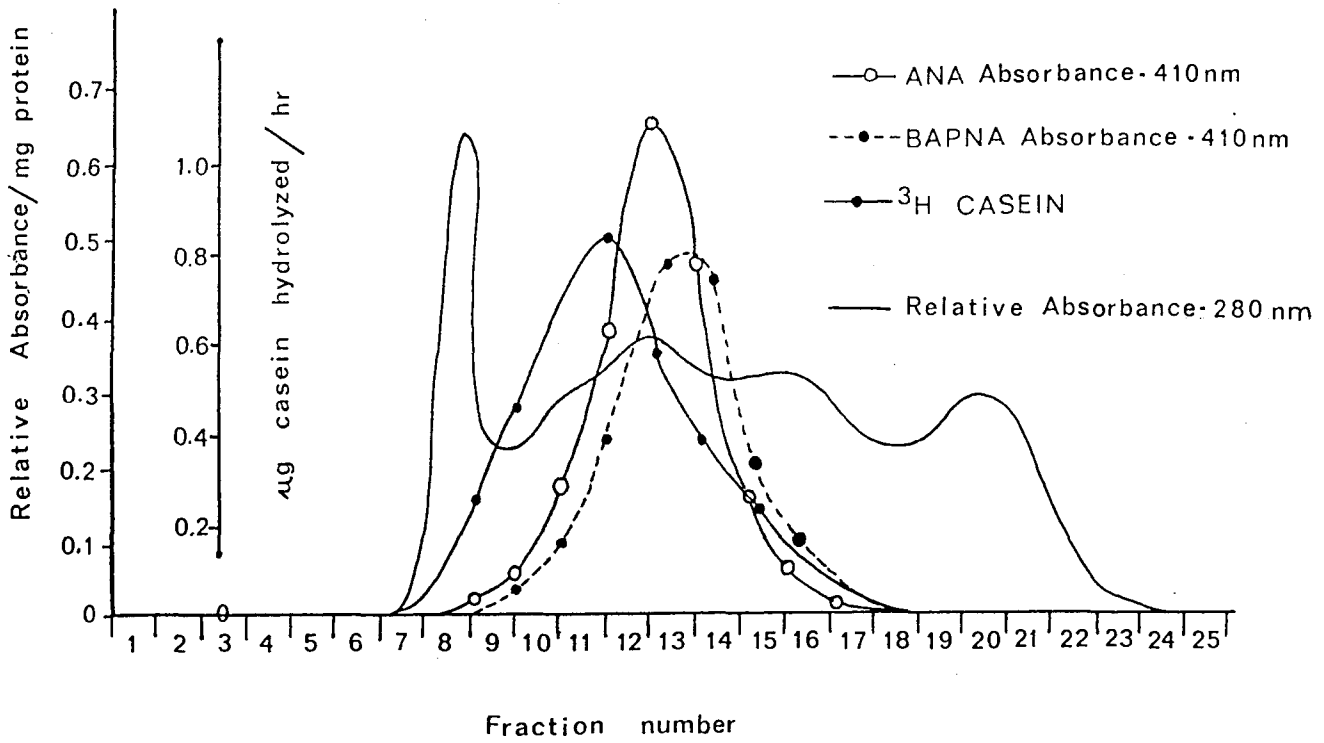

FIGURE 7

Gel filtration elution of a preparation from 5-day-old pea cotyledons.

(o) ANAase activity, (absorbance at 410 nm)

(..) BAPNAase activity, (absorbance at 410 nm)

(_.) ^{3}H - Casein activity.

(-) Relative A_{280}

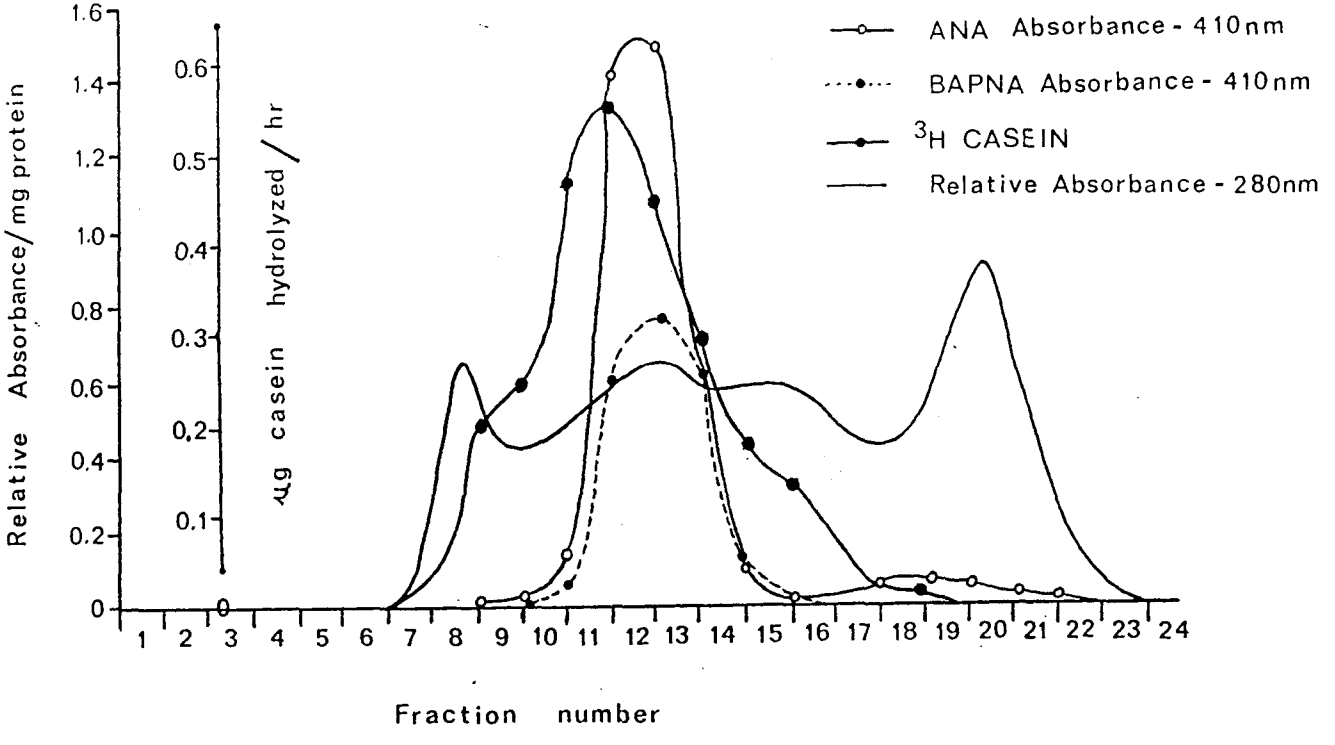

FIGURE 8

ANAase and BAPNAase activity in DEAE Sephadex ion exchange elution profile of 2-day-old pea cotyledons.

(-) relative A_{280};

(o) ANAase activity, (absorbance at 410 nm)

(.) BAPNAase activity, (absorbance at 410 nm)

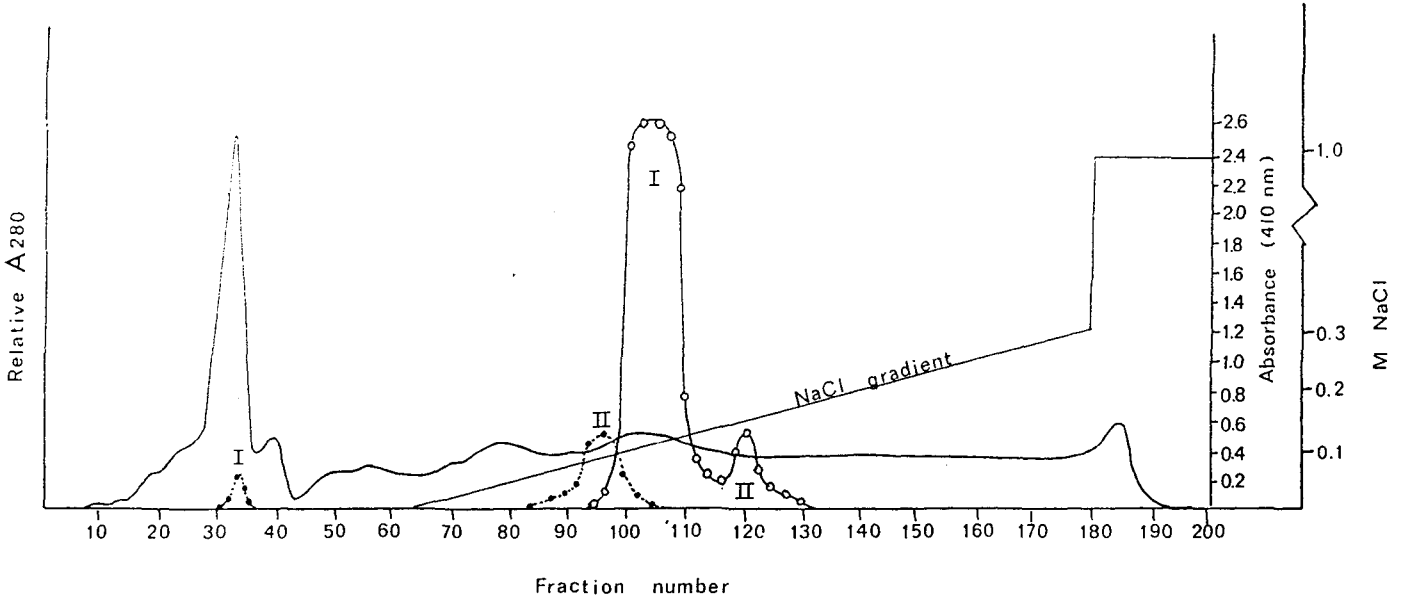

FIGURE 9

ANAase and BAPNAase activity in DEAE Sephadex ion exchange elution profile of 5-day-old pea cotyledons.

(-) relative A_{280};

(o) ANAase activity, (absorbance at 410 nm)

(.) BAPNAase activity, (absorbance at 410 nm)

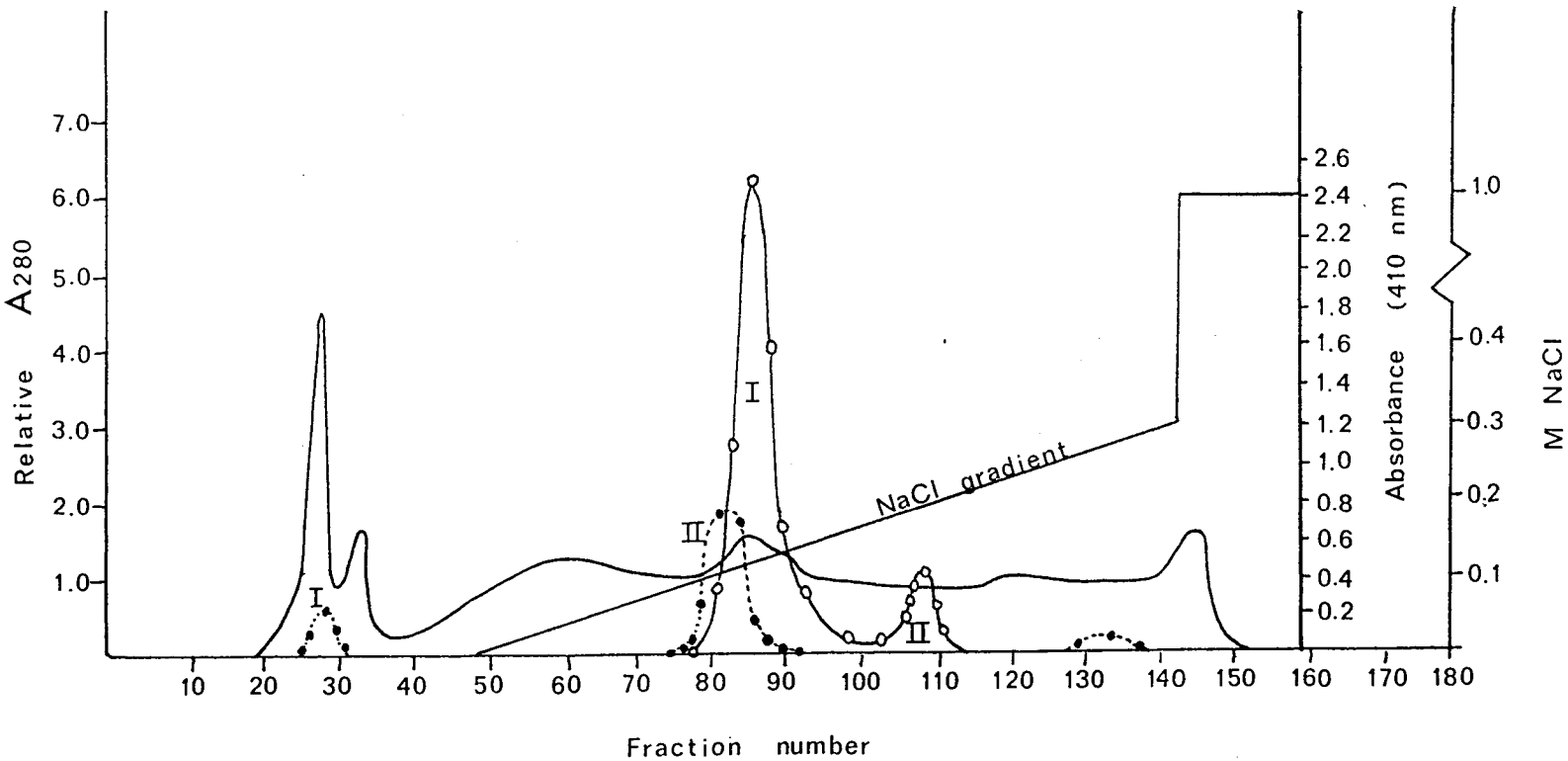

FIGURE 10

Caseinolytic activity in DEAE Sephadex ion exchange elution profile of 2-day-old pea cotyledons.

(-) relative A_{280};

(.) caseinolytic activity.

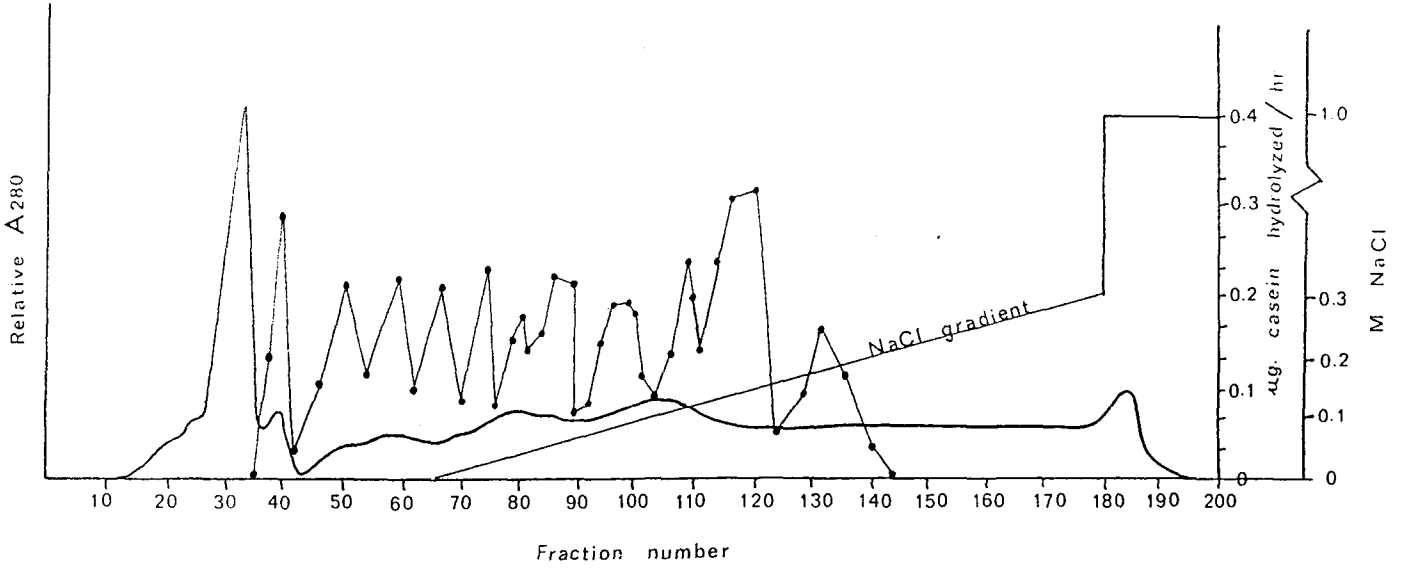

FIGURE 11

Caseinolytic activity in DEAE Sephadex ion exchange elution profile of 5-day-old pea cotyledons.

(-) relative A_{280};

(.) caseinolytic activity.

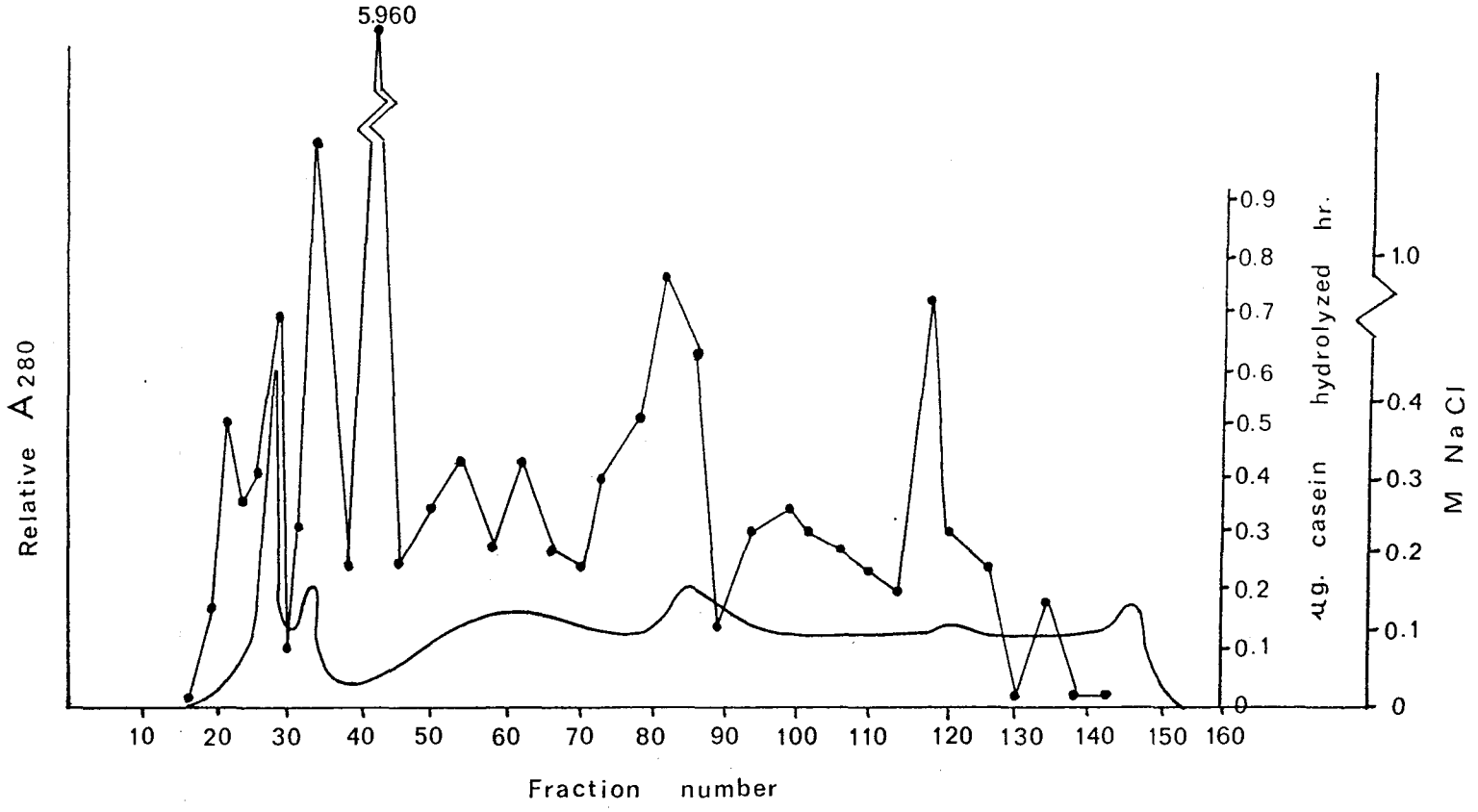

FIGURE 12

Response of ANAase I activity to pH. pH 4.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM Tris-Cl buffer. 1 mM mercaptoethanol was included in assay mix. Incubation was for 30 minutes.

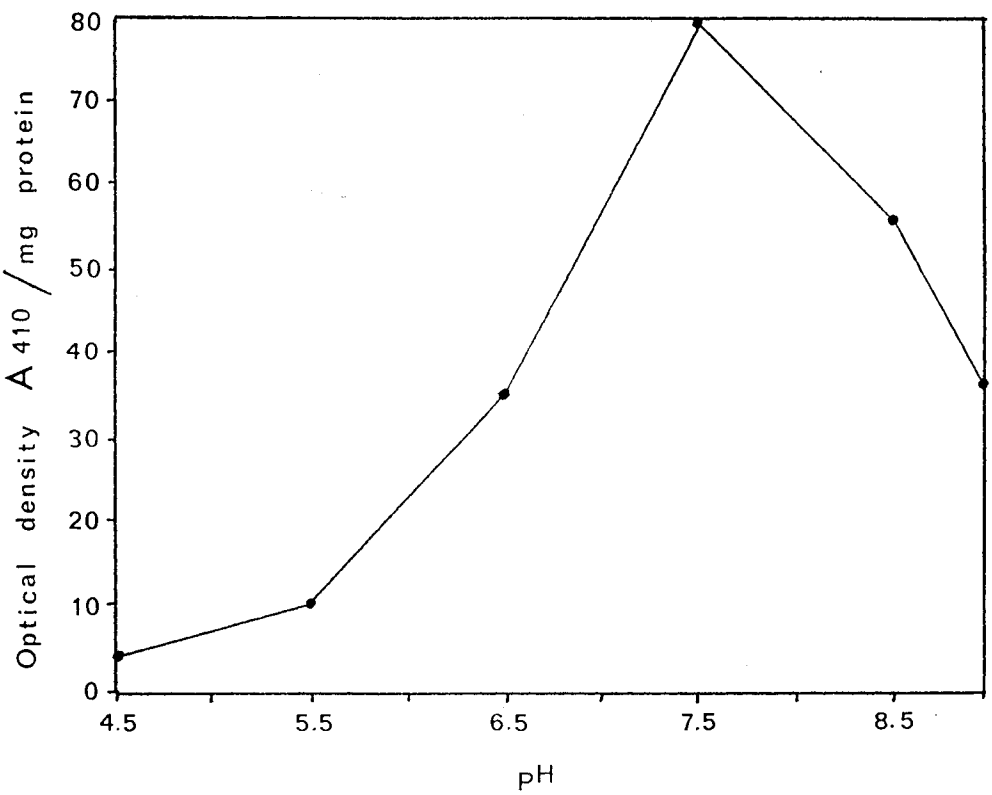

FIGURE 13

Response of ANAase II activity to pH. pH 4.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM tris-Cl buffer. 1 mM mercaptoethanol included in assay mix. Incubation was for 30 minutes.

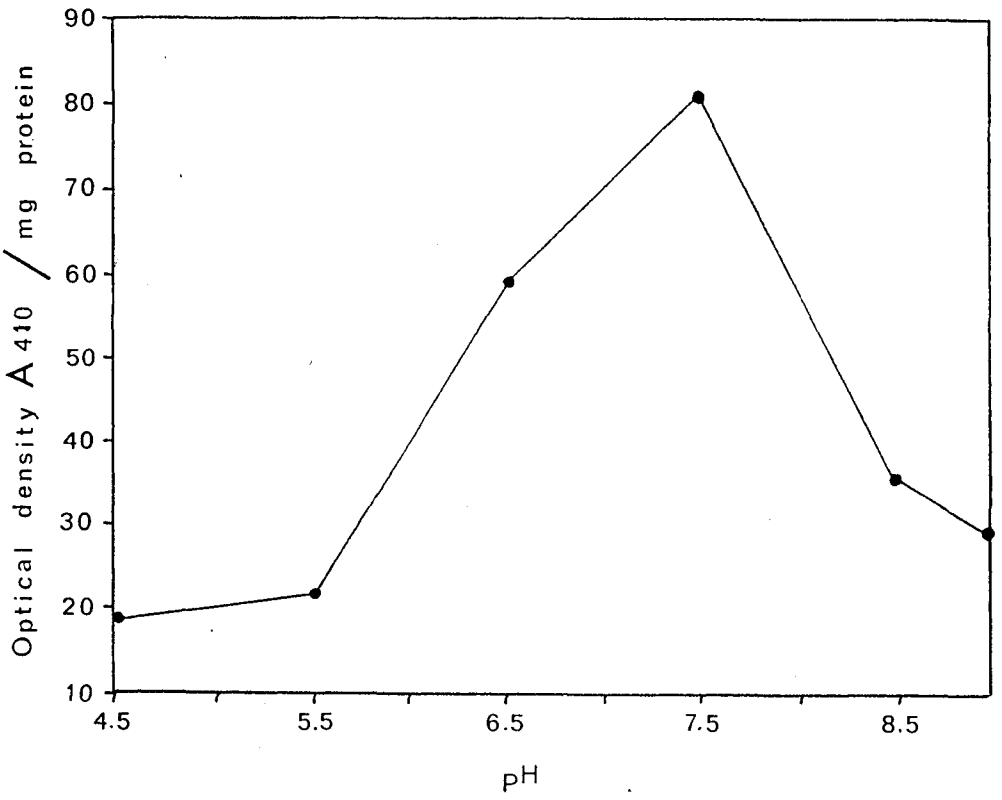

FIGURE 14

Response of BAPNAase I activity to pH. pH 4.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM tris-Cl buffer. 1 mM mercaptoethanol included in assay mix. Incubation was for 30 minutes.

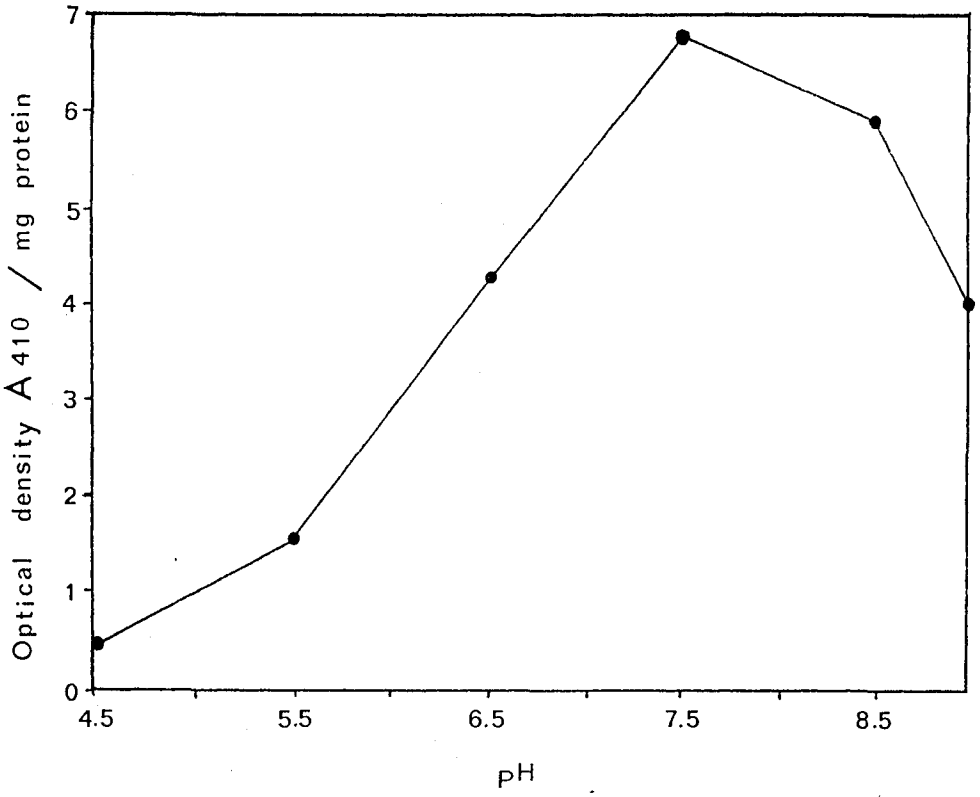

FIGURE 15

Response of BAPNAase II activity to pH. pH 4.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM tris-Cl buffer. 1 mM mercaptoethanol included in assay mix. Incubation was for 30 minutes.

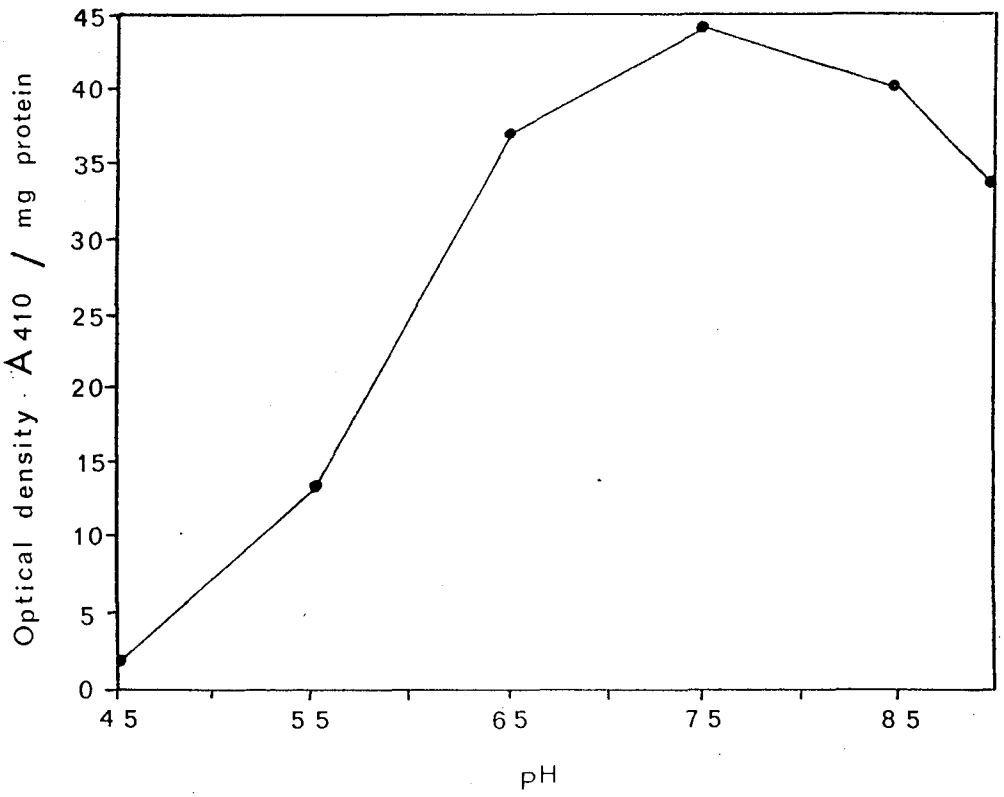

FIGURE 16

Response of Caseinase activity to pH. pH 4.5, 100 mM citrate buffer; pH 5.5 to 6.5, 100 mM sodium phosphate buffer; pH 7.5 to 9.0, 100 mM tris-Cl buffer. 1 mM mercaptoethanol included in assay mix. Incubation was for 30 minutes.

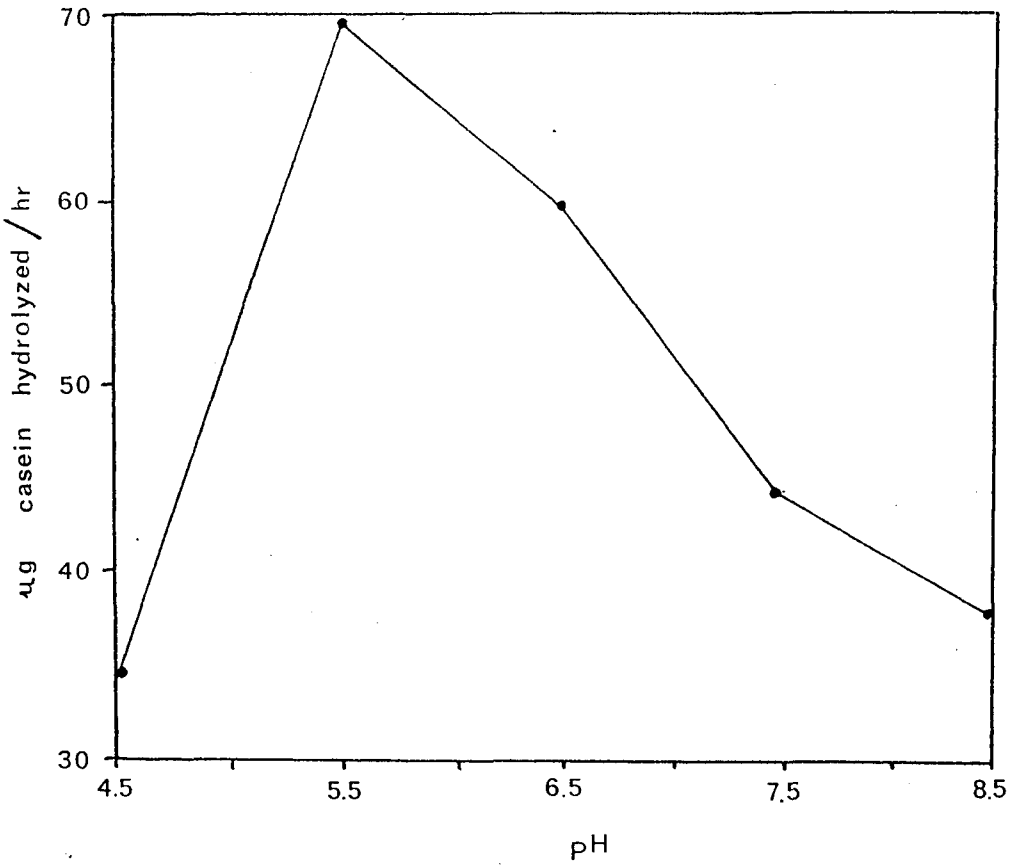

Figure 17

Non-denaturing, native polyacrylaminde gel electrophoresis of pea seed enzymes purified from 5-day-old cotyledons past the gel filtration and ion exchange stage.

Lane 1: Bovine Serum Albumin (Molecular weight 66,000) and its dimer (molecular weight 132,000) used as standard marker protein.

Lane 2: BAPNAaseII peak, after ion exchange (B).

Lane 3: BAPNAase I peak, after ion exchange (B).

Lane 4: ANAase II peak, after ion exchange.

Lane 5: ANAase I peak, after ion exchange (A).

Lane 6: fractions 11, 12, 13 pooled from gel (B) filtration stage.

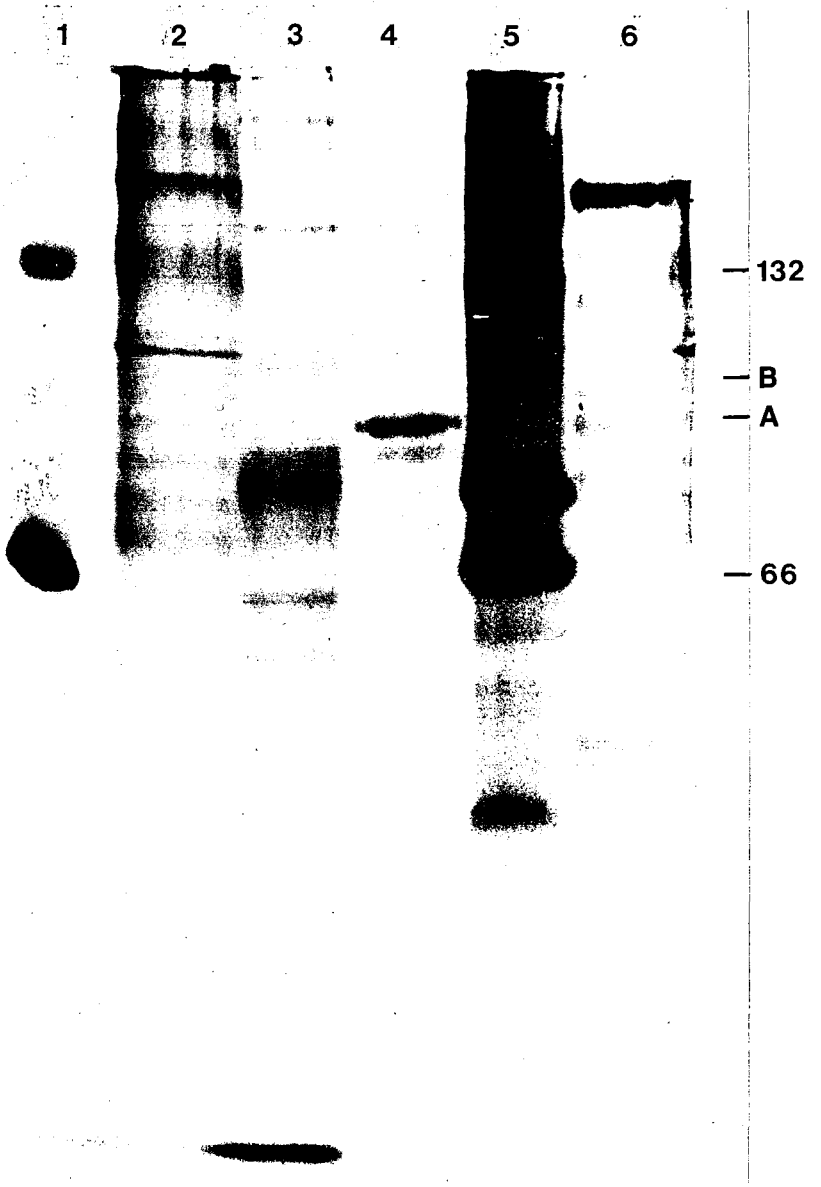

TABLE 1. Purification of ANAase from 2-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (A_{385})	Total Activity (A_{410})	Specific Activity (A_{385}/mg)	Specific Activity (A_{410}/mg)	Yield (%)	Purification
Crude	42	1776	183.12		0.103		100	1.0
$(NH_4)_2SO_4$	7	457	60.20		0.132		33	1.3
Dialysis	9	404	58.86		0.146		32	1.5
Gel Filtration (fractions 11 to 13)	19	21	43.75		2.084		24	20
Ion Exchange: ANAase I (fractions 89 to 94)	9.5	0.225		26.68		127.45	16	1237
ANAase II (fractions 109-116)	9.5	0.100		6.88		68.78	5	668

TABLE 2. Purification of ANAase from 5-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (A_{385})	Total Activity (A_{410})	Specific Activity (A_{385}/mg)	Specific Activity (A_{410}/mg)	Yield (%)	Purification
Crude	45	484	144		0.298		100	1.0
$(NH_4)_2SO_4$	7	219	69		0.315		48	1.1
Dialysis	9	157	68		0.433		47	1.5
Gel Filtration (fractions 11 to 13)	19	21	41		1.94		28	7
Ion Exchange:	9.5	0.101		26		257	18	864
ANAase I (fractions 89 to 94)								
ANAase II (fractions 109-116)	9.5	0.0973		5		51	4	172

TABLE 3. Purification of BAPNAase from 2-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (A_{385})	Total Activity (A_{410})	Specific Activity (A_{385}/mg)	Specific Activity (A_{410}/mg)	Yield (%)	Purification
Crude	42	1776	214.2		0.121		100	1.0
$(NH_4)_2SO_4$	7	457	62.58		0.137		29	1.1
Dialysis	9	404	59.04		0.146		28	1.2
Gel Filtration (fractions 11 to 13)	19	21	28.99		1.38		14	11
Ion Exchange: BAPNAase I (fractions 31 to 34)	9.5	1.146		7.45		6.49	3	53
BAPNAase II (fractions 81 to 86)	9.5	0.210		6.40		30.48	3	252

TABLE 4. Purification of BAPNAase from 5-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (A_{385})	Total Activity (A_{410})	Specific Activity (A_{385}/mg)	Specific Activity (A_{410}/mg)	Yield (%)	Purification
Crude	45	484	113		0.233		100	1.0
$(NH_4)_2SO_4$	7	219	82		0.374		73	1.6
Dialysis	9	157	70		0.446		62	1.9
Gel Filtration (fractions 11 to 13)	19	21	24		1.156		21	5
Ion Exchange: (fractions 31-34)	9.5	1.091		6		5.50	5	24
BAPNAase II (fractions 81-86)	9.5	0.171		5		29.3	4	125

TABLE 5. Purification of Caseinase from 2-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (CPM)	Specific Activity (CPM/mg)	Yield (%)	Purification
Crude	42	1776	24,414,600	13,747	100	1.0
$(NH_4)_2SO_4$	7	457	6,908,300	15,117	28	1.1
Dialysis	9	404	6,402,600	15,848	26	1.2
Gel Filtration (fracttioins 11 to 13)	19	21	3,738,657	178,031	15	13
Ion Exchange (fractions 85 to 88)	9.5	.100	269,393	2,693,930	1	196

TABLE 6. Purification of Caseinase from 5-day-old Pisum sativum L. cotyledons.

	Vol. (ml)	Total Protein (mg)	Total Activity (CPM)	Specific Activity (CPM/mg)	Yield (%)	Purification
Crude	45	484	12,601,800	26,037	100	1.0
$(NH_4)_2SO_4$	7	219	6,191,360	28,271	49	1.1
Dialysis	9	157	4,714,200	30,027	37	1.2
Gel Filtration (fractions 11 to 13)	19	21.0	2,565,543	122,169	20	5
Ion Exchange (fractions 85 to 88)	9.5	0.171	850,297	4,972,498	7	191

TABLE 7. R_f values and apparent molecular weights of Native ANAase and BAPNAase from zymograms of 2- and 5-day-old pea cotyledons.

		2-Day-Old		5-Day-Old	
		R_f	M.W.	R_f	M.W.
Gel Filtration	ANAase	.33	115,500	.28	107,000
	BAPNAase	.33	115,500	.28	107,000
Ion Exchange	ANAase I	.36	110,000	.31	107,000
	ANAase II	.61	64,000	ND*	ND*
	BAPNAase I	.34	114,000	.28	107,000
	BAPNAase II	.34	114,000	.28	107,000
	BSA**	.60	66,000	.46	66,000
	BSA Dimer	.24	132,000	.17	132,000

* ND (Not Detectable)

** 2-day-old gel run at room temperature
5-day-old gel run at 2°C.

TABLE 8. Effect of Inhibitors on Enzyme Preparations Purified Past the Gel Filtration Stage.

Inhibitor	Conc.	Age of seeds (days)	Substate	Activity (%)
Dithiothreitol	1 mM	5	3H-Casein	231
	10 mM	5	3H-Casein	225
Mercaptoethanol	1 mM	5	3H-Casein	149
	10 mM	5	3H-Casein	152
	1 mM	2	ANA	76
	10 mM	2	ANA	66
	1 mM	2	BAPNA	91
	10 mM	2	BAPNA	94
Ovomucoid	.1 mg/ml	5	3H-Casein	108
Trypsin	.5 mg/ml	5	3H-Casein	89
Inhibitor	5.0 mg/ml	5	3H-Casein	84
	50.0 mg/ml	5	3H-Casein	63
Soybean	.1 mg/ml	5	3H-Casein	103
Trypsin	.5 mg/ml	5	3H-Casein	97
Inhibitor	5.0 mg/ml	5	3H-Casein	89
	50.0 mg/ml	5	3H-Casein	69
BSA	.5 mg/ml	2	3H-Casein	93
	5.0 mg/ml	2	3H-Casein	70
	50.0 mg/ml	2	3H-Casein	32
PCl-HgB_20	1 mM	5	3H-Casein	41
	1 mM	Dry	3H-Casein	53
	1 mM	Dry	ANA	61
	1 mM	Dry	BAPNA	58
	1 mM	2	BAPNA	41
PCl-HgB_20	1 mM	5	3H-Casein	113
+ Mercaptoethanol	10 mM	Dry	3H-Casein	79
		Dry	ANA	130
		Dry	BAPNA	118
PhMeSuF	1 mM	Dry	3H-Casein	92
	1 mM	Dry	ANA	80
	1 mM	Dry	BAPNA	87
Leupeptin	.1 mM	Dry	3H-Casein	1
	.1 mM	Dry	ANA	130
	.1 mM	Dry	BAPNA	34

2.4 DISCUSSION

Two physiologically distinct groups of enzymes function in the germinating seed and their roles have been considered in the mobilization of stored protein reserves. The first group is present in dry seeds and they are either already active or are activated during the course of germination as suggested by Hobday *et al.* (1973) and Ryan (1973). These enzymes are located in the cytosol away from the stored protein reserves in protein bodies and have typically neutral or slightly alkaline pH optima. Included here are the BAPNAases and ANAases. Nishikata (1984) and Tomomatsu *et al.* (1978) concluded that similar enzymes did not participate in the initial breakdown of reserve proteins. The second group is synthesized *de novo* during the first three to six days of seedling growth and accumulates in the protein bodies (Baumgartner and Chrispeels 1977). The group includes caseinase which increases in activity following germination and has an acidic pH optimum which corresponds to the pH within protein bodies. The bulk hydrolysis of storage proteins within protein bodies corresponds with the increase in activity of acid sulfhydryl proteases (Basha and Beevers 1975). It is unlikely, however, that these enzymes are involved in the initial protein modification. One may speculate that the BAPNAases and/or ANAases may be involved in early germination events.

It is difficult to interpret the results of casein assays utilizing crude or only partially purified enzyme preparations. The changes in apparent "specific activity" may not be due to the changing levels or activities of the enzyme, but a result of the presence of competitive protein substrates or unknown inhibitors (Malek 1987). Figures 1 to 3 demonstrate that potential ex-

ogenous substrates reduce the efficiency of the assay. Caseinolytic surveys of fractions from ion exchange runs were nearly impossible to interpret due to a spreading out of the activity combined with an uneven distribution of potentially competing substrates (see figures 11 and 12). A better assay, based on the analysis of casein endoproteolytic fragments may have to be developed. It is expected that casein is acted on by many enzymes and as each one is purified it is less efficient at producing 5% TCA soluble fragments. The increase in purification of a caseinase may represent a decrease in competitive inhibition.

The purification of many proteinases from seeds is complicated by their tendency to form comparitively strong complexes with other seed proteins (Shutov *et al.* 1985). It is possible that the two BAPNAases are the same enzyme that eluted in two different positions from the ion exchange column due to differential binding and isolation as a complex. This idea is supported by the molecular weights which were found to be the same for both BAPNAase I and BAPNAase II. Caldwell and Sparrow (1976) reported a similar situation with two BAPNAases being separated from pea seeds by ion exchange chromatography, but otherwise were virtually identical. Others reported only one BAPNAase in legume seeds (Tomomatsu et al. 1978; Nishikata 1984).

Two ANAases were separated using ion exchange chromatography. The level of purification was considerably higher for the ANAases than for the other enzymes studied probably because the ANAases eluted in regions of low contaminating protein. Although the enzyme preparation was not homogenous, the exoprotease activity was detected in only one main

electrophoretic band. The pH optima of the two enzymes were the same, but their molecular weights were different. ANAase I was collected in greater quantities, had a higher activity and is the major ANAase activity in germinating pea seeds.

The apparent molecular weights of ANAase and BAPNAase were smaller in the 5-day-old cotyledons than in the 2-day-old peas. The relatively small change may indicate action by amino or carboxypeptidases on the enzymes. The decrease of BAPNAase activity during germination may be due to protein degradation. The molecular weights of the BAPNAases were found to be considerably higher than what was previously published (59,000 to 65,000 daltons, Nishikata 1984; Caldwell and Sparrow 1976). The enzyme presently isolated at 107,000 to 114,000 daltons may represent a new species of BAPNAase in pea cotyledons. Fractions of molecular weight greater than 66,000 daltons were collected following gel filtration chromatography and further purified. Therefore, the enzyme species isolated are larger than BAPNAases previously isolated. These enzymes identified may be dimers. Further elaboration of the technique should include cutting out of the active band from native gels, equilibriation with SDS, then rerunning using SDS-PAGE.

It is expected that the ANAases and BAPNAases isolated may be similar to the alkaline dipeptidase isolated by Mikkonen and Mikola (1986) from kidney beans with a molecular weight of 105,000 daltons. The alkaline dipeptidase isolated by Sopanen (1976) from barley with a molecular weight of 130,000 to 175,000 daltons may also be a similar species of enzyme.

Various proteinase and peptidase inhibitors were used to identify each enzyme class. The caseinolytic activity was inhibited by sulfhydryl antagonists and was stimulated by reducing agents, indicating the presence of an essential cysteine residue. Further evidence for the presence of a cysteine residue is provided by the reversal of pCl-HgB_2O inhibition by the addition of mercaptoethanol. Thus, this enzyme belongs to the class of acid sufhydryl proteases also known as cysteine proteinases (EC 3.4.22) (Storey and Wagner 1986). The nearly complete inhibition of caseinase by leupeptin, a non-specific inhibitor of seryl and cysteinyl proteases, may indicate a serine-proteinase component in the partially purified enzyme preparation. This could not be substantiated further, since the serine proteinase inhibitor PhMeSuF had minimal effect. Moreover, the proteinaceous ovomucoid and soybean trypsin inhibitors produced results similar to BSA indicating non-specific or competitive inhibition (see Table 8).

BAPNAase exhibits characteristics of both seryl and cysteine type enzymes. It is inhibited by pC1-HgB_2O, leupeptin and PhMeSuF indicating a serine-type enzyme with a requirement for a thiol group. Similar patterns of inhibition for BAPNAase have been reported in peas by Caldwell and Sparrow (1976), in soybeans by Nishikata (1984), in kidney beans by Nielsen and Liener (1984) and in peanut seeds by Mainguy *et al.* (1972).

Further purification work should concentrate on affinity chromatography methods and the careful selection of a ligand molecule. Certain inhibitors show promise as a ligand, especially pC1-HgB_20. This inhibition was reversible by thiol compounds. Ultimately, monoclonal antibodies could be developed for immunocytochemical localization of pea seed enzymes during

several stages of germination. Tracing the sequence of appearance and location of pea seed enzymes during germination would help elucidate a mechanism for germination.

3 CHAPTER 2

An ultrastructural study of protein mobilization from pea seeds: Comparison of de-shooted with normally senescing cotyledons during early seedling growth.

INTRODUCTION

Developing legume seeds accumulate storage proteins in protein bodies. Isolated protein bodies of pea are approximately 3 um in diameter and are oval or spherical in shape (Gabara and Konopska 1974). Two types of protein bodies were distinguished by Gabara and Konopska (1974). The predominant type was composed of amorphous protein. Another protein body type contained many electron transparent areas, with various shapes and dimensions, embedded in amorphous protein. The electron transparent areas probably resulted from the loss of globoid crystals during preparation of the tissue for electron microscopy. Lott and Buttrose (1977) were successful in demonstrating the presence of globoid crystals in pea utilizing a freeze-fracture technique. They determined that phytin was the major component of globoid crystals by employing energy dispersive x-ray analysis.

During seed formation four developmental phases are recognized by Bain and Mercer (1966); cell formation, cell expansion, systhesis of storage reserves, and maturation and dormancy. Reserve protein is deposited in the central vacuoles of parenchyma cells. New protein bodies arise from the central vacuole by pinching off small masses of reserve protein surrounded by a portion of the tonoplast (Yoo and Chrispeels 1980). The protein bodies are surrounded by a single membrane (Newman and Weber 1978).

According to Greenwood and Chrispeels (1985a,b), phaseolin, which accounts for approximately 50% of the total protein in mature bean seeds, is synthesized during seed development and sequestered within protein bodies during cotyledon development. During the stage of rapid synthesis of reserve

proteins in pea, Savelbergh and Van Parijs (1971) observed a distinct increase in the amount of granular endoplasmic reticulum and polysomes. This observation is in contrast with the findings of Bain and Mercer (1966) who observed more smooth endoplasmic reticulum with free ribosomes in developing pea cotyledons.

Drying of the mature seed followed by re-imbition during germination represents the end of reserve accumulation and the beginning of reserve mobilization respectively. Degradation and mobilization of reserve proteins in legume cotyledons is expected to involve the combined activity of endopeptidases, exopeptidases and peptide hydrolases (Newman and Weber 1978). Smith and Flinn (1967) described histological changes which occur during germination, in particular, the differentiation of the vascular system. The cotyledon is apparently preparing its structures for the task of reserve mobilization. This is followed by fusion of protein bodies to form a central vacuole before all the protein is hydrolysed (Gifford *et al.* 1983).

Several seed protein localization studies have been completed. Baumgartner *et al.* (1978) succeeded in locating vicilin peptidohydrolase in the cytoplasm of cotyledons of mung bean, three days after initiation of seedling growth, using immunofluorescence microscopy. Immunohistochemical techniques have been more widely applied to the localization of storage proteins like pea seed vicilin (Craig *et al.* 1979, 1980; Craig and Millerd 1981; Craig and Goodchild 1984; Craig and Miller 1984), bean phaseolin (Baumgartner *et al.* 1980) and bean legumin (Newman 1982).

Details of the preparation of seed tissue for electron microscopy are rarely given in the literature. Therefore, it was necessary to develop a procedure that produced acceptable images of the dense cotyledon tissue. The special problems of fixation, embedding, penetration of reagents and staining, had to be taken into consideration. Procedures needed usually differ markedly from those employed in animal tissue. Although the techniques used in electron microscopy of plant tissue were originally derived from animal histology, the rigid cell wall in plant cells may prevent infiltration of fixatives and plastics. This characteristic of plant cells, as well as the presence of large amounts of reserve protein, starch and lipid in seeds, makes seed tissue particularly difficult to prepare for electron microscopy. Part of this section outlines a useful method for the preparation of pea cotyledon tissue at various stages of imbibition and senesence for observation in a transmission electron microscopy.

Protein bodies begin to portray signs of degradation after about two to three days following imbibition and are almost completely devoid of protein five to twelve days later (Opik 1966; Crump and Murray 1979; Murray 1979; Boylan and Sussex 1987). A wave of endopeptidase activity moves progressively through the cotyledons, towards the vascular bundles, leaving behind areas devoid of stored reserves and low in endopeptidase activity (Harris and Chrispeels 1975). Observations on the morphology of protein bodies during germination indicate that the membrance surrounding them remains intact, while the reserves disappear.

Guardiola and Sutcliffe (1971) compared the rates of protein hydrolysis in de-shooted and normally senescing cotyledons. They found that the rate of protein hydrolysis in normally senescing cotyledons was faster than in de-shooted cotyledons. These results are in agreement with the data of Malek (1987). This study was undertaken to extend those observations utilizing electron microscopy. Electron micrographs are presented that qualitatively illustrate delayed protein mobilization during early seedling growth in de-shooted seedlings.

3.2 MATERIALS and METHODS

3.2.1 Plant Material and Growth Conditions

Pisum sativum cv. Spring (Asgrow Seeds, Bradford, Canada) were germinated at 27 C +/- 3 C in autoclaved vermiculite moistened to field capacity. After 3 days of low light conditions with 100% humidity, the seedlings were grown under fluorescent lights at 16.1 W/m^2. Seedlings were first watered with 1/4-strength Hoaglands solution, 7 days after planting and then every 2 or 3 days thereafter, as required. De-shooting was first carried out on day 5, then every 2 or 3 days thereafter. Imbibed seeds were rinsed in distilled water to remove fungicide which could affect cell ultrastructure at early stages of development, then soaked for 24 hours at room temperature.

3.2.2 Electron Microscopy: Fixation

Cotyledons from 24-hours imbibed, 7, 14 and 21 day-old pea seedlings, or from 14 and 21-day old de-shooted seedlings, were dissected to about 1 mm^3 over crushed ice in 25 mM pH 7.1 sodium phosphate buffer containing 3% gluturaldehyde. Most of the tissue fixed was parenchyma. Occasional samples were obtained from the epidermal or vascular regions. After 1.5 hours, the tissue was washed in 5 changes of 25 mM sodium phosphate buffer for 6 minutes each. The tissue was postfixed for 3 hours in 1% OsO_4 (v/v) in 100 mM sodium phosphate

buffer, pH 7.3, then washed as above. At this point, tissue was occasionally stored in 25 mM sodium phosphate buffer at 4° C for future dehydration and embedding.

3.2.3 Dehydration

Cotyledonary tissue was dehydrated in a graded series of 25, 50, 70, and 95% acetone for one hour per step. The tissue was then exposed to at least two changes of 100% acetone for a total of three hours. Dehydration proceeded at 4°C up to and including the 70% step, then continued at room temperature.

3.2.4 Embedding

Several variations of embedding were attempted. The most successful method was adapted from O'Brien and McCully (1981). Spurr's epoxy resin was added dropwise to the dehydrated tissue in acetone. The concentration of Spurr's was brought from 0% up to 10% over a period of two hours. Acetone was allowed to evaporate in a fume hood leaving the tissue in pure resin after about 24 hours. The resin was exchanged for pure Spurr's and allowed to infiltrate for an additional hour to remove any residual acetone. Tissue was transferred to Beem Capsules (Marivac Ltd., Halifax, Can.) and the resin was polymerized at 70°C for 24 hours.

3.2.5 Sectioning and Staining

Tissue was sectioned in a Porter and Blum model MT2-B ultramicrotome with diamond or glass knives and placed on glass slides for light microscopy or on 300 mesh grids for electron microscopy. Thick sections (1 to 2 um) for light microscopy were routinely stained for 30 seconds with toluidine blue at 50° C or with amido black (10% w/v with acetic acid for 20 minutes at 50° C; Fisher 1968). Tissue for electron microscopy was stained for 10 minutes in 1% lead citrate at room temperature. Samples were viewed in a Philips EM300 transmission electron microscope.

3.3 RESULTS

A series of electron micrographs is presented in Figures 18 to 28. These Figures illustrate the senescence of pea cotyledons remaining on a normally developing seedling for imbibed, 7- and 14-day-old tissue, and also for those cotyledons, taken from 14- and 21-day-old de-shooted seedlings.

Tissue imbibed for 24 hours (Fig. 18, 19) shows storage cells that are very dense with ovoid-shaped protein bodies, mitochondria, starch grains and endoplasmic reticulum. By day 7 (Fig. 20), much of the reserves in protein bodies have been mobilized. Protein bodies are being replaced by larger vacuoles. Endoplasmic reticulum is breaking up into vesicles. By day 14, almost all the cellular contents have degraded and only remnants of cellular material remain (Fig. 21). It should be noted that Figure 21 represents a larger amount of material in the cell than was typical. The majority of cells were almost completely devoid of discernible organelles at this stage. Although reserves have been mobilized in figures 21 and 22, a thin layer of cytoplasm remains in close association to the cell wall in the storage parenchyma cells. The nucleus is also surrounded by cytoplasm. Plasmalemma of the storage parenchyma cells appears intact at these stages. Figures 22, 23 and 24 show healthy vascular cells in 14-day-old material. Vascular tissue retained its integrity until well after the senescence of storage tissue.

De-shooted tissue showed similarities to the controls but were delayed 7 to 10 days in senescence. Protein bodies of 14-day-old de-shooted tissue (Fig. 25), are in a similar state of degradation as the 7 day-old control (Fig. 21).

In 14-day-old de-shooted tissue (Fig. 25, 26) electron dense lipid bodies can be seen to be in close association with endoplasmic reticulum. Endoplasmic reticulum was often observed in an unusual concentric ring structure in this tissue (Fig. 26). The contrast of Figure 25 with Figure 27 illustrates continued degradation between 14 and 21 days.

In Figure 28, a chloroplast is evident in the epidermal layer close to the outer cell wall. This development of chloroplasts corresponds to the macroscopically visible greening of cotyledons that occurs after de-shooting.

Figure 18

Imbibed pea cotyledon storage tissue. The cells at this stage are densely packed with protein bodies (PB) and starch grains (SG). Magnification = 8,000. Bar = 5 um.

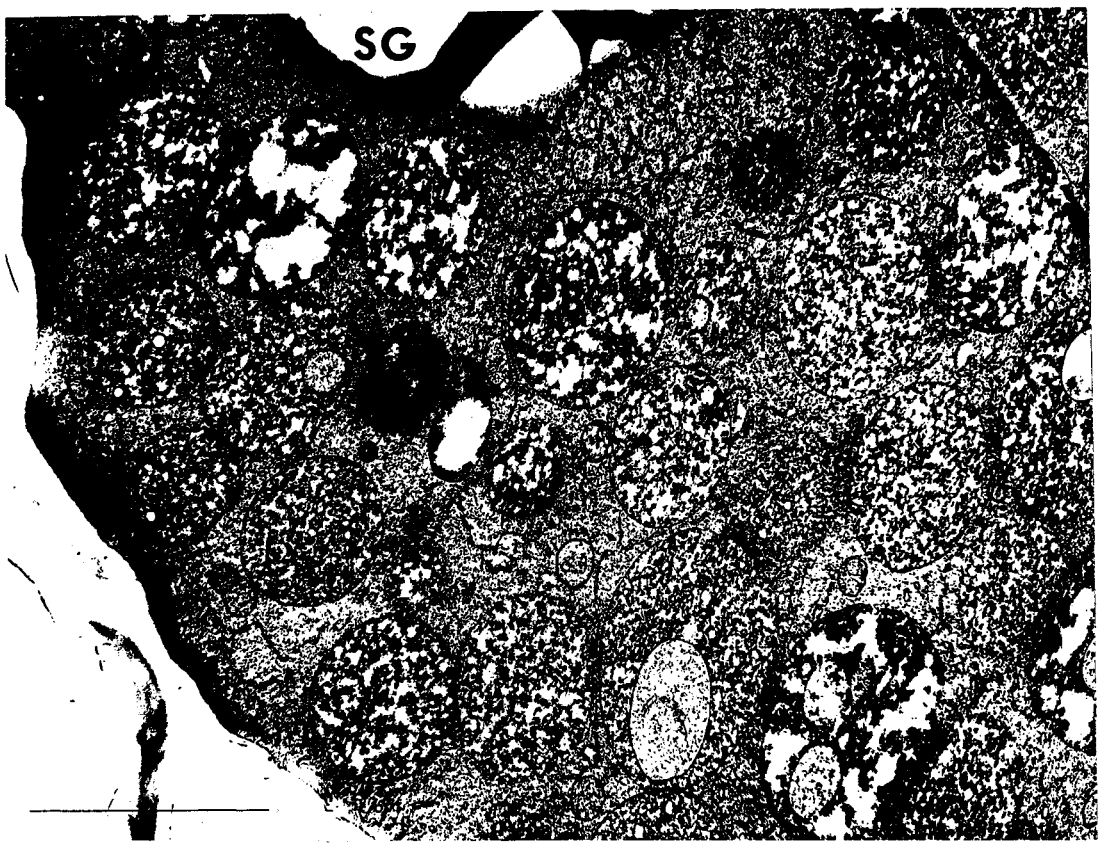

Figure 19

Same tissue as in Figure 18 showing protein bodies (PB) at high power. Endoplasmic reticulum (ER) is often in close association with the protein bodies at this stage. It is possible that synthesis of hydrolytic enzymes for transport to protein bodies is occuring. Mitochondria (M) are present. Magnification = 23,000. Bar = 1 um.

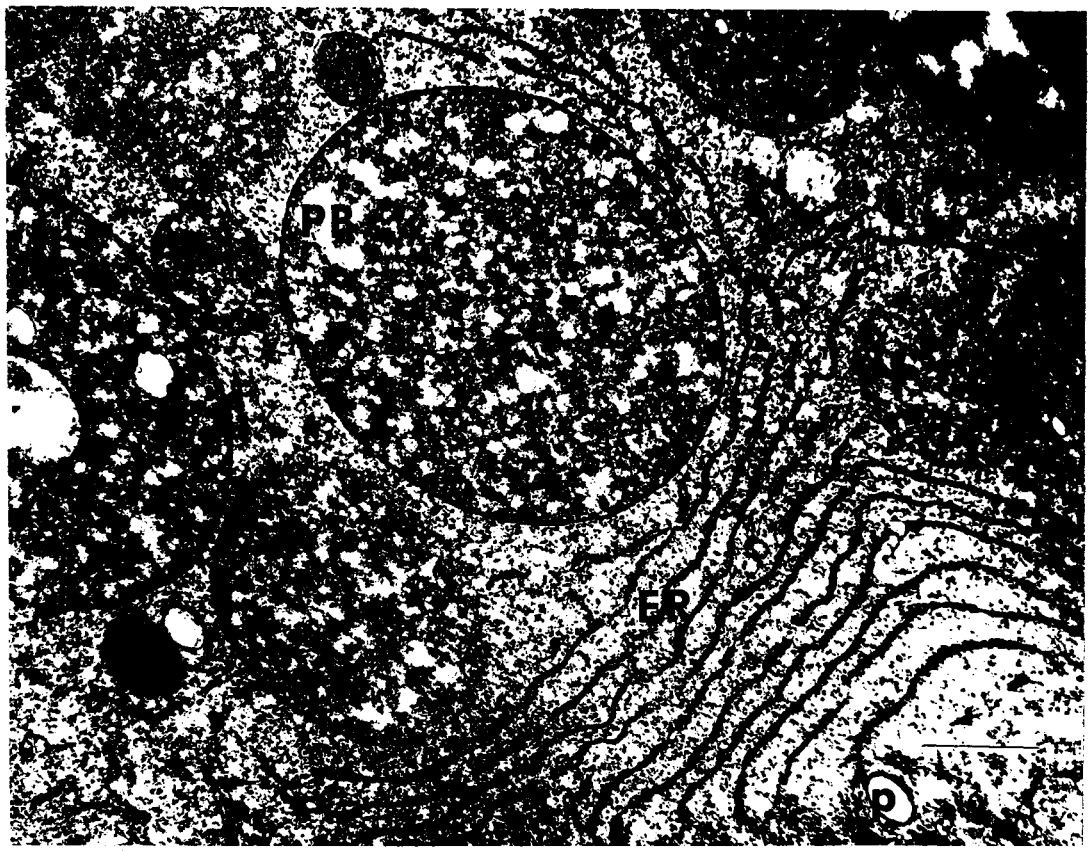

Figure 20

Seven-day-old pea cotyledon storage tissue. Most of the protein has been mobilized from protein bodies (PB), but their membrane remains intact at this stage. Endoplasmic reticulum (ER) may be breaking up into vesicles (V). Mitrochondria (M) still retain much of their internal structure. Magnification = 15,000. Bar = lum.

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Figure 21

Fourteen-day-old pea cotyledon storage tissue. At this stage, storage cells are almost completely empty. Some vesicles and nuclear material (Nu) still remains. Cell walls (CW) remain intact long after the cells have completely senesced. Magnification = 5,000. Bar = 5 um.

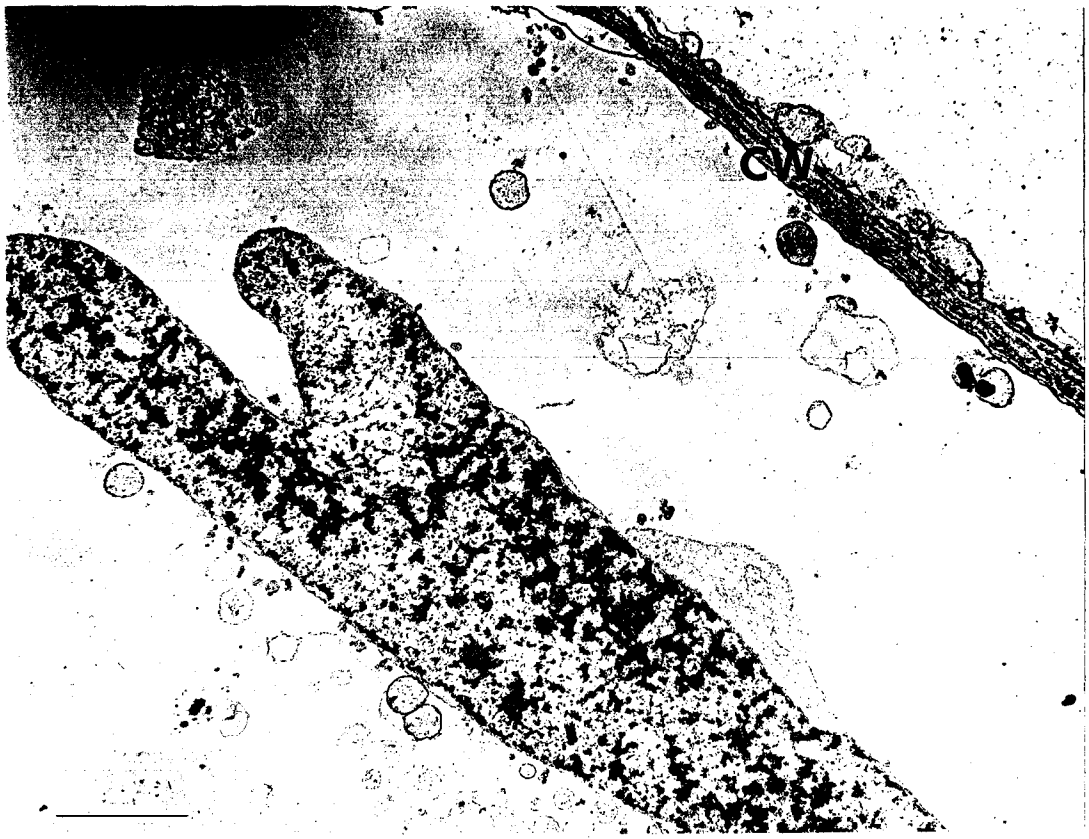

Figure 22

Xylem (Xy) from senescent 14-day-old pea cotyledon. The vascular system within pea cotyledons retains its structure until after complete senescence of the cotyledon. Magnification = 5,000. Bar = 5 um.

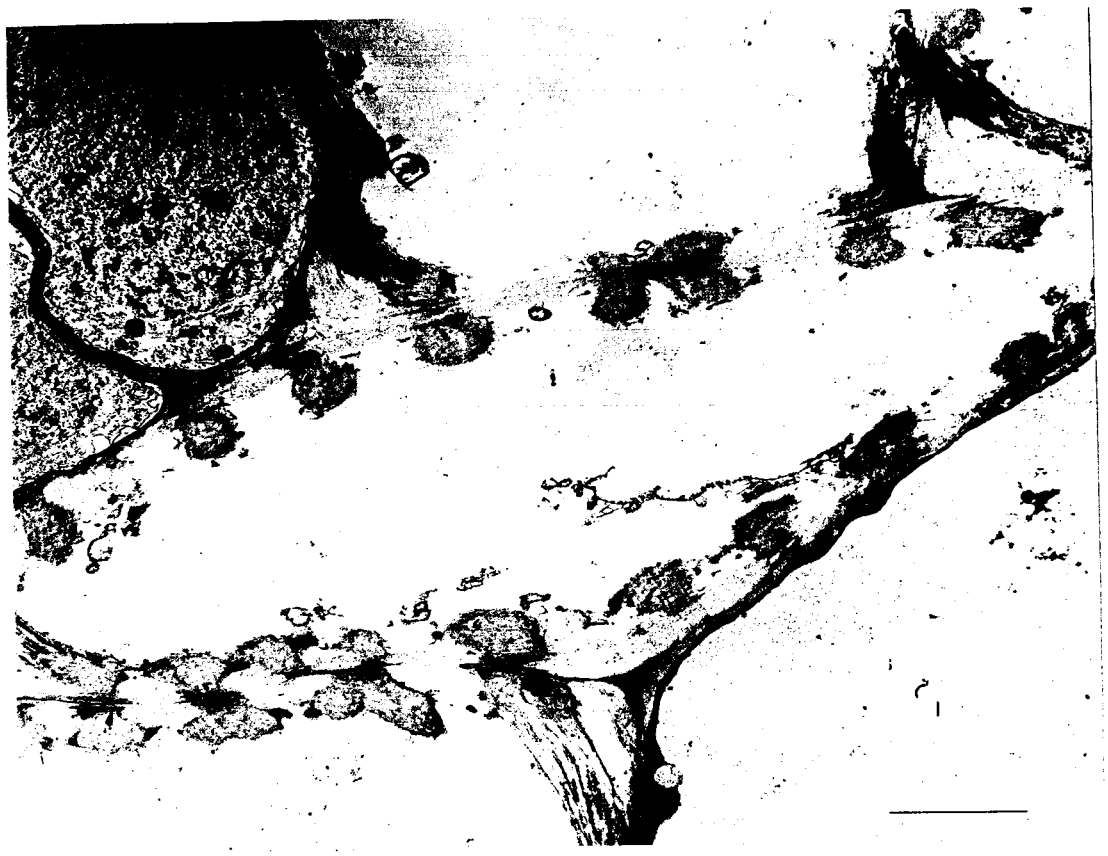

Figure 23

Tissue from vascular region of 14-day-old senescent pea cotyledon. Plasmodesmata (Pl). Magnification = 6,500. Bar = 5 um.

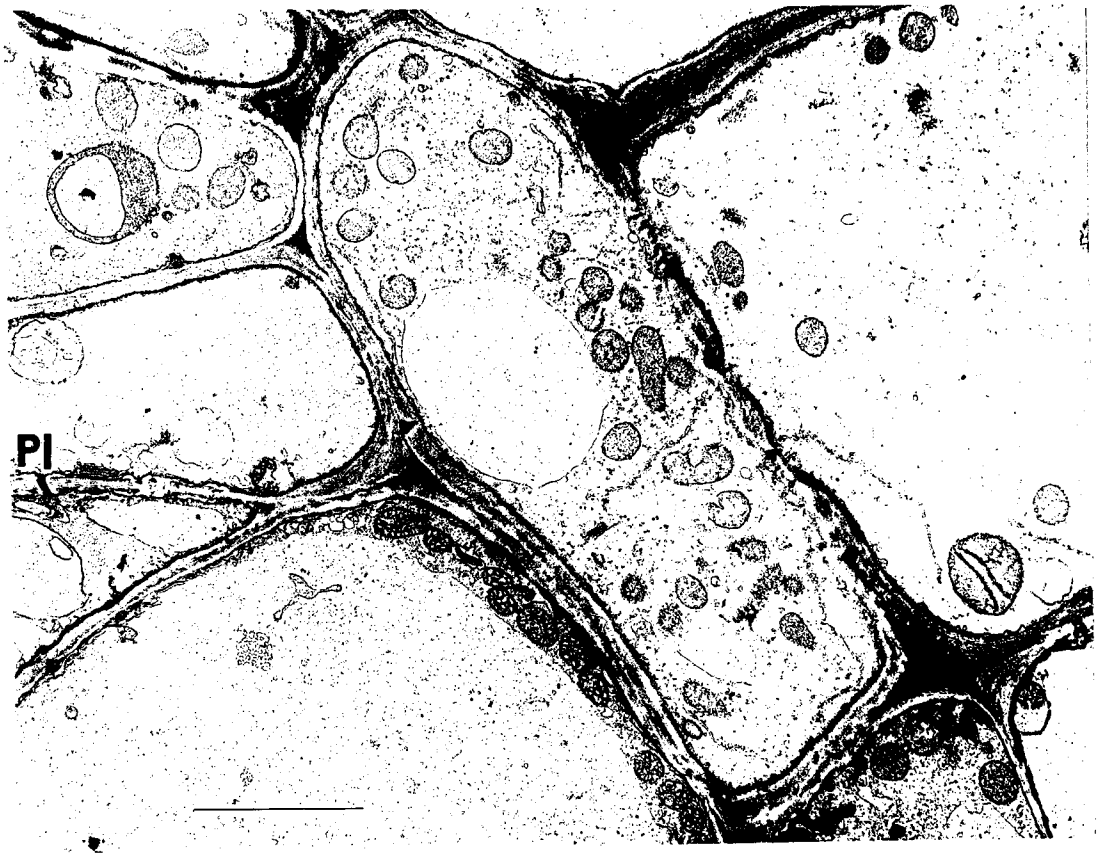

Figure 24

High power photograph of same tissue as in Figure 23. Mitochondria (M), Cell Wall (CW), Proplastid (Pr). Magnification = 18,600. Bar = 1 um.

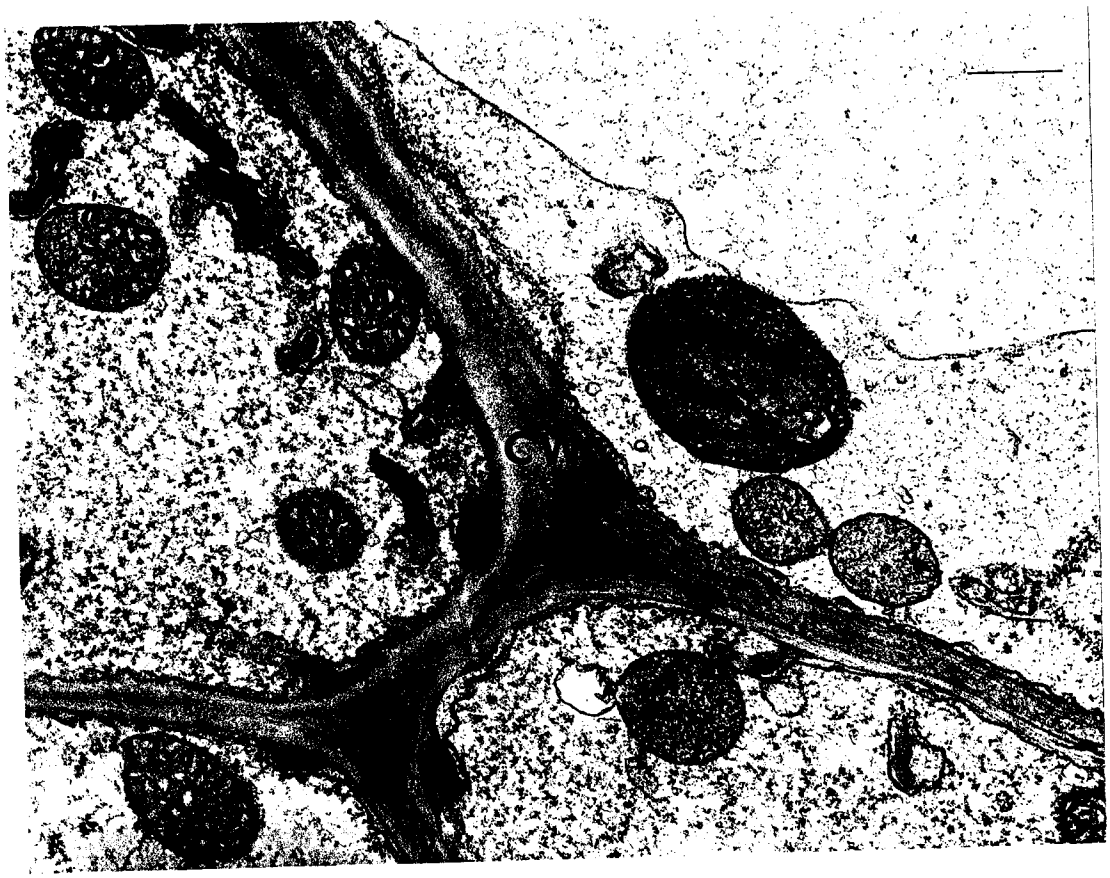

Figure 25

Fourteen-day-old de-shooted pea cotyledon tissue. Protein bodies (PB) remain intact. Their contents have not completely emptied. Lipid vesicles (L) are in close association with endoplasmic reticulum (ER). Magnification = 8,000. Bar = 5 um.

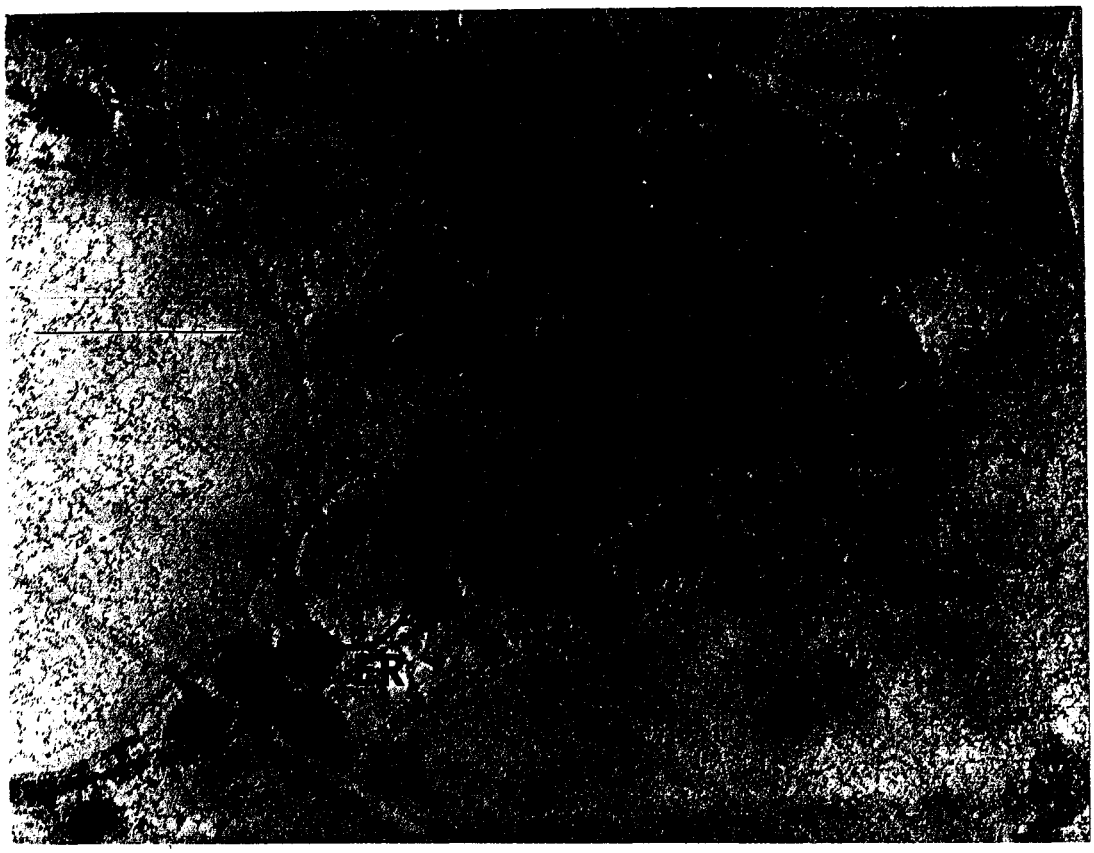

Figure 26

Same tissue as illustrated in Figure 25. A membranous ring structure has developed and seems to be continuous with the endoplasmic reticulum (ER). Lipid vesicles (L) and mitochondria (M) are in close association with these ring structures. Magnification = 7,000. Bar = 5 um.

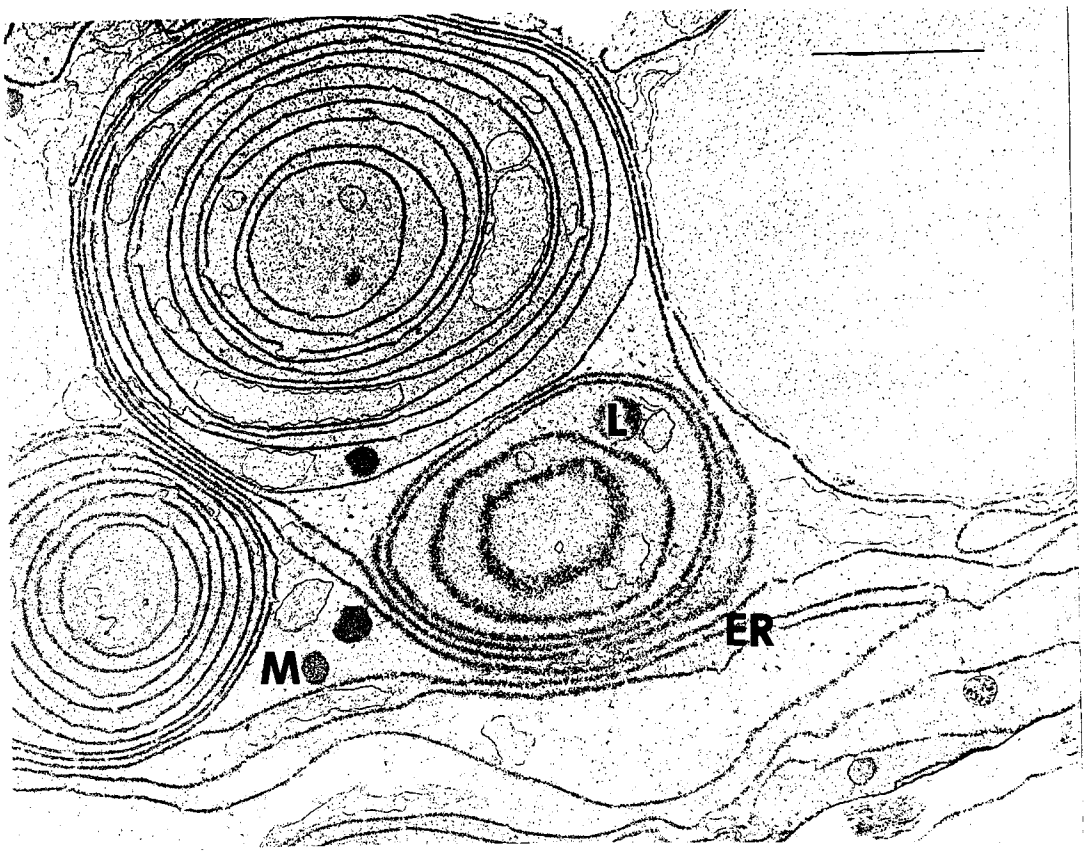

Figure 27

Twenty-one-day-old de-shooted pea cotyledon storage tissue. Cells are in a highly senescent condition by this time. Most of the membrane systems have broken down. This figure illustrates a more than average amount of cellular material compared with most cells at this stage. Magnification = 7,000. Bar = 5 um.

Figure 28

Twenty-one-day-old de-shooted cotyledon tissue. This photomicrograph was taken from a cell in the epidermal layer. The chloroplast (Ch) has developed close to the outside cell wall (CW). This development corresponds with the greening of cotyledons in de-shooted conditions. A cuticle (Cu) surrounds the epidermal cell wall. Magnification = 30,000. Bar = 1 um.

3.4 DISCUSSION

The rate and sequence of senscence in control cotyledons is very comparable to that described in the literature. For example, Murray (1979) performed quantitative protein analysis on pea cotyledons. He determined that at 14 days, more than 90% of the albumins and 83% of the globulins were mobilized at 25° C. This is in agreement with qualitative observation of 14-day-old tissue (Fig. 21). Protein mobilization, as determined by the amount of cellular contents remaining on day 14, was probably higher in this experiment than was found by Murray (1979). This would be accounted for by the increased growing temperature.

Opik (1966) found a different situation for *Phaseolus vulgaris*. She reported that digestion of starch and protein reserves was nearly complete in only 8 days at 25° C. Storage cells died once the reserves were hydrolysed. Opik (1966) and Briarty et al. (1970) demonstrated that vascular bundle and epidermal cells survive until the cotyledons abscise. This is supported here by Figures 22, 23 and 24 which illustrate intact vascular cells on day 14 despite empty storage parenchyma cells.

A close association could be seen between endoplasmic reticulum, ribosomes and protein bodies early in germination (Fig. 19). According to Opik (1966), this ribosome-endoplasmic reticulum complex could secrete the hydrolytic enzymes responsible for digestion. The cisternae could also provide a channel for the removal of the products of hydrolysis. However, Briarty *et al.* (1970) disagreed with this view based on his studies with *Vicia faba*. From the cytological evidence, it appeared to Briarty *et al.* (1970) that proteolysis occurred uniformly throughout the protein bodies. There was no

sign that breakdown was from the outside inwards as might be expected if proteases were produced external to the protein bodies. It is possible that the effect of an endoplasmic reticulum-protein body association varies between legume species. However, no direct evidence has been produced that demonstrates proteolysis of the protein body from the outside-in.

De-shooting germinating cotyledons delays, but does not stop, senescence of pea cotyledons (Malek, 1987). The mobilization of protein reserves as a function of the degradation of protein bodies was at least 7 to 10 days behind that of normally senescing cotyledons. This is in agreement with Guardiola and Sutcliffe (1971) who found decreased rates of protein mobilization in cotyledons of de-shooted seedlings.

It would be interesting to determine where the protein reserves are translocated to under de-shooted conditions. Since new shoots were allowed to form for up to 3 days before de-shooting (following the initial de-shooting 5 days after planting) a significant amount of protein reserve could have been lost to the growing shoots. It is likely that root growth accounted for the greatest drain on protein reserves. Ultimately, all protein reserve material will be exhausted and translocated either to the continually reforming shoots or to the roots. The slower pace of reserve translocation could be accounted for by a reduced demand for mobilized material in the de-shooted seedlings. Alternatively, it is speculated that a buildup of cytokinins in the cotyledons, as a result of de-shooting, could suppress protein mobilization.

Some interesting cellular developments occurred in de-shooted cotyledons that were unique to them and have not been observed in normally senescing cotyldeons. Some cotyledons eventually produce chlorophyl. This may lead to

partial autotrophic maintenance of the organ. However, senescence eventually occurs. Malek (1987) found that the de-shooted cotyledon remained turgid for an average of 58 days. It is possible that the cotyledon attempts two physiologically incompatible functions. One is to mobilize its reserves for seedling growth, the other is to conserve reserves for autotrophic growth. Its imminent death demonstrates that while it may be successful in mobilizing its reserves, it cannot sustain growth.

The greening of the de-shooted cotyledon is explained by the formation of chloroplasts in the epidermal layer (Fig. 28). However, evidence of starch production was not available in these chloroplasts. The other possible adaptation to de-shooted growth was the curious formation of concentric spherical membranes, appearing as "rings" in the cross sections of the cytoplasm of storage cells (Fig. 26). The "ring" formation was very common in 14-day-old de-shooted tissue but was not observed at earlier or later stages of senescence. Such rings appeared continuous with the endoplasmic reticulum. Tanaka *et al.* (1980) discovered a similar formation in isolated protein bodies from rice endosperm. They were described as having alternately light and dark layers and were referred to as *lamellar structure*.

The endoplasmic reticulum was observed in a net-like structure discernable at higher magnifications (Fig. 25). This structure is similar in appearance to the cisternal endoplasmic reticulum that forms 12 to 24 hours after imbibition of mung bean (Bewley and Black 1985). The cisternal endoplasmic reticulum is the site of synthesis of proteases that are then

transported to the protein bodies. The presence of this structure after 14 days of de-shooted seedling growth provides further evidence for the delay of protein mobilization in this tissue.

Lipid vesicles were usually present and in close association with endoplasmic reticulum (Fig. 25). Lipid vesicles may be contributed to by the degredation of endoplasmic reticulum. The ring and net-like structures appear to be forming in, or near, degrading protein bodies. It is speculated that there may be a high concentration of amino acids at this location that could be reprocesed into proteins in the parenchyma cells. This possible attempt at autotrophic growth is unsuccessful since there are no remnants of these structures seven days later (Fig. 27).

The methodology adopted for the photomicrographs was developed after several unsuccessful attempts to embed the dense tissue using conventional methods. This successful procedure could be utilized for other dense, non-porus material. Details of the development of the embedding protocol are included in the appendix.

3.5 APPENDIX

The cytological evidence produced for this thesis was a result of testing various embedding techniques on pea cotyledon tissue. The density of cell walls and the relatively dehydrated state of seed tissue, act as barriers to the penetration of fixatives and plastics (Mollenhauer and Totten 1971). Consequently, seed tissues are exceptionally difficult to process for electron microscopy. The problem of preparing tissue for electron microscopy could be lessened if details of dehydration and embedding were published in the primary literature. Usually, only a brief mention is made of such techniques.

Problems with embedding caused holes, knife marks and difficult sectioning. Figure 20 was produced using a fast but commonly employed embedding schedule. The tissue was transferred from 100% acetone into a 2:1 mixture of acetone:epoxy followed by 1:1, 1:2, then pure resin for one hour per step. The result was clearly inferior with knife marks and holes being common (Fig. 20). Other methods, results of which are demonstrated in the remaining figures resulted in better images.

It was difficult to obtain electronmicrographs from this material. Even shorter embedding and dehydration times were attempted, schledules commonly employed at this institution, but the tissue was so full of holes that it could hardly even be sectioned. Vacuum infiltration did not lead to an improvement. The best method was the one used in producing the remaining illustrations and involved the slow evaporation of acetone from a dilute epoxy:acetone mixture. The concentration of epoxy in the tissue was increased from 0 to 100% over 24 hours.

Despite the relative success of the technique employed, there is room for improvement, especially with the dense imbibed tissue. As can be seen in Figures 18 and 19, pinholes were common. There are several possible reasons for this. The tissue may not have been completely dehydrated, some residual acetone remained in the tissue, or air bubbles were present in the tissue.

The presence of water in the tissue would have prevented the infiltration of Spurrs into those hydrated areas. Spurrs' resin is not miscible with water. This problem could possibly be overcome by increasing the dehydration schedule from one hour per step to two hours. This is much longer than any published schedules. Care should be taken to prevent extraction of intracellular components.

If acetone was present in the tissue during the polymerization process, it would have evaporated leaving holes without resin. This may be prevented by substituting 100% propylene oxide for acetone once the tissue is completely dehydrated in acetone. Propylene oxide, when mixed with Spurr's resin produces a less viscous solution than an acetone-Spurr's solution. This would allow easier impregnation of the resin into dense tissue. In addition, whereas acetone may never completely evaporate from Spurr's resin, propylene oxide will, and in a shorter time.

Air bubbles may have been present in the tissue. This would prevent complete resin infiltration. Vacuum infiltration is a technique that is used to remove air bubbles during fixation and embedding. This technique was used in early embedding attempts, but did not show any significant improvement. However, it was not attempted with the acetone evaporation method and it is

unknown whether vacuum infiltration would improve embedding. J. Greenwood (personal communication) recommends a brief period of vacuum infiltration during the glutaraldehyde prefix.

The technique developed here provided adequate tissue preservation of pea cotyledons at various stages of senescence. It is believed that the method could be further improved by: (i) using vacuum infiltration at the prefix stage; (ii) increasing the dehydration times from 1 to 2 hours per step, and (iii) substituting propylene oxide for acetone once the tissue is completely dehydrated. This method should provide excellent thin sections of difficult seed tissue. It is expected that this procedure would be a useful embedding method for the immunocytochemical localization of pea seed proteolytic enzymes.

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