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Priming physiology and proteinase purification from resting Jack pine seeds

A Thesis

presented to

The Office of Graduate Studies and Research

of

Lakehead University

By

Jacqueline Bourgeois

In partial fulfilment of the requirements

for the degree of Master of Science

July 1989

LAKEHEAD UNIVERSITY

The undersigned certify that they have read, and recommended to the Graduate Studies Committee for acceptance of a thesis entitled "Purification and characterization of a proteinase from resting Jack pine seeds " submitted by Jacqueline Bourgeois in partial fulfillment of the requirements for the degree of Master of Science.

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1.0 GENERAL INTRODUCTION

Pines constitute an important economic species for the lumber and paper industries in the Boreal region (Galloway, 1986). Current seedling production, dependent on the availability of high quality seed, may be considered adequate. However, better understanding of seed biochemistry and physiology may result in the development of better seed, and consequently healthier, more uniform, and perhaps stress tolerant seedlings. Based on past efforts (Ackerman and Farrar, 1965; Santon, 1970, Fraser, 1974; Fleming and Lister, 1984), we have undertaken an investigation of pine seed priming and its effect on several physiological and metabolic parameters of a single seed-lot. The technique explored may provide a method to decrease germination time and increase synchrony of those seeds germinating, resulting in more economical seedling production.

The following is an overview of literature dealing with knowledge pertinent to the study of seed priming physiology and germination. Pine seeds consist of a thin seed coat encasing a thick haploid megagametophyte surrounding a small embryo (Bewley and Black, 1978). Most gymnosperm storage seed cells contain lipid deposits in addition to protein bodies. The high energy stores needed for initial seedling growth are found, for the most part, in the parental megagametophyte tissue and the

mechanisms involved in their depletion during seedling growth have been of interest to many researchers, and is discussed below.

The metabolic shift from development i.e., the accumulation of seed reserves, to the resting state, and finally to germination is an interesting, and incompletely understood phenomenon. There are two hypotheses attempting to explain initiation of germination and control of reserve mobilization during seedling growth. First, hormonal interactions between the embryo and storage tissues are involved, which may regulate when and how quickly the reserves will be depleted (Chin et al., 1972; Bilderback, 1974; Murray and Adams, 1980; Kermode and Bewley, 1985; Kermode and Bewley, 1986). Second, less popular hypothesis suggests that an equilibrium must be achieved between the storage and embryo tissues, where by products of degradation are continually removed into the axis or their production ceases (Bain and Mercer, 1966; Vincenzini et al., 1973; Davis and Chapman, 1979; Saarelainen and Mikola, 1987; Tabor and Barret, 1987). In castor bean it has been found once the reserves are deposited in storage tissue a gradual desiccation and subsequent resting state ensues (Kermode and Bewley, 1985a). This desiccation requirement in order for germination to take place has also been well documented in cereals and has been reviewed by Michel et al. (1978). Once the seed is in a stage where germination can proceed upon hydration, the control mechanism can be investigated. It has also been noted, even during premature desiccation of desiccation-tolerant seeds, that all seed protein synthesis associated with development is halted by drying. After rehydration, only germination processes involved in protein and other reserve mobilization are allowed to proceed (Kermode et al., 1985b,c). Kermode and Bewley (1986d) suggest that the switch from development to germination may be due to altered hormonal levels or tissue responsiveness to these effectors during desiccation. Dissected embryos from desiccation-tolerant seeds will germinate whether desiccated or not. It appears that the

endosperm tissue may repress the germination process until it is desiccated (Kermode and Bewley, 1988). Chin et al. (1972), have found that in pea seeds the proteinase activity and protein breakdown is not detected when the embryo is not present. In pine, there may be a mechanism by which the embryo controls the endosperm enzymatic activities at least to a small extent. One enzyme examined in pines was isocitrate lyase. Studies by Bilderback (1974) have shown that media in which *Pinus ponderosa* embryos were incubated, stimulated this enzyme's activity in excised endosperm tissue. Furthermore, if the embryo was removed from seed early during imbibition the enzymatic activity dropped drastically in the megagametophyte while if the embryo was only removed after germination, the activity levels in the megagametophyte remained high (Bilderback, 1974). The diffusible or extractable factor but was not gibberellic acid (GA). In *Pinus sylvestris* , the ∝-amylase level in the megagametophyte was greater when the embryo was not removed (Murray and Adams, 1980). This suggests that the embryo has a positive effect on the hydrolysis of megagametophyte.

Davies and Chapman (1979) suggested a sink effect was produced by the cucumber embryonic axis. The embryo may trigger hydrolytic processes but subsequently the reserve tissue can act on its own. Drawing off the products may produce a concentration gradient allowing for the continual need for additional breakdown products. Saarelainen and Mikola (1987) used a sensitive method to show the interaction between the endosperm and embryo of barley. Specific radioactive amino acids injected into the endosperm were translocated to specific areas of the developing seedling. In pea seeds, protein mobilization decreased without the embryo (Bain and Mercer, 1966). Malek (1987) also presented evidence suggesting a sink effect is produced by the pea embryo. Isocitrate lyase levels in the megagametophyte in *Pinus pinea* can be lowered when fatty acids are added (Vincenzini et al., 1973); this indicates that there may be a feedback mechanism related to the sink effect. Tabor and

Barnett (1987) suggested that the role of the megagametophyte during growth is not only to provide reserves, but also to control seedling development. This would be based on nitrate reductase activities in both tissues and autotrophy threshold of the embryo. The studies outlined above support both hypotheses of embryo/storage tissue interaction. The regulation of the metabolic events during growth probably involves an interplay of both regulation mechanisms.

The desiccation process separating seed development and germination is an important component regulating the enzymes and hydrolytic processes which increase during priming or direct hydration. However, it is still unknown how the enzymes are triggered during imbibition (Lichtenfeld, et al. 1981). One possibility could be the removal of inhibitors from pre-existing enzymes, the second is that *de novo* synthesis of the enzymes ensues (Filner and Varner, 1967; Sze and Ashton, 1971; Royer et al., 1974; Salmia and Mikola, 1975; Chrispeels et al., 1976; Salmia and Mikola, 1976a; Salmia and Mikola, 1976b; Baumgartner and Chrispeels, 1977; Salmia et al., 1978; Koehler and Ho, 1988). The requirement for mRNA synthesis during the initial stages of germination has now been well established (Gumilevskaja et al., 1981). There is a small amount of residual mRNA conserved in the dry seed. No major role has been established for these mRNAs in germination, and they have been associated with maturation-specific rather than germination-specific proteins (Peumans et al., 1981). In pine, as in most other seeds, mRNA levels will increase in the embryo soon after imbibition but not in the megagametophyte (Sasaki and Brown, 1969). This is a reflection of protein requirement in the developing embryo and not in mobilization of storage materials. However, some pre-existing hydrolytic enzymes are present and ready to act upon imbibition (Lichtenfeld et al., 1981). One such enzyme is trypsin-like proteinase, which appears to co-exist with endogenous inhibitors in the seed (Chrispeels and Baumgartner, 1978; Leluk et al., 1983). The inhibitor is degraded

during seedling growth presumably releasing the enzyme activity (Tan-Wilson et al., 1982, Wilson and Tan-Wilson, 1983; Wilson et al., 1985). Much of the known seedling growth protein degradation events have been summarized by Lichtenfeld et al., (1981). Many enzymes in addition to proteinases are active during seedling growth; these are described in the following discussion.

Some studies in gymnosperms have only looked at well characterized enzymes in order to later determine their activities under different physiological conditions such as during storage and how their levels may be related to subsequent seedling growth capacity (Pitel and Cheliak, 1986; Pitel and Cheliak, 1988). Pine seed enzymes involved in respiration (Hatano, 1963), gene expression (Kreis et al., 1987) as well as DNA repair (Daniel and Bryant, 1988) are also active. Other researchers have been interested in pine seed protein degradation, ie. proteinases (Salmia and Mikola, 1975; Salmia and Mikola, 1976a,b; Salmia, et al. 1978; Salmia, 1980; Salmia and Mikola, 1980; Salmia, 1981a,b). These studies are discussed in subsequent sections.

Various seed treatments have been employed, aimed at increasing the rate of seed germination. One example is osmotic priming. This process involves the imbibition of seeds in solutions of negative solute water potential (Salter and Darby, 1976; Fleming and Lister, 1984). Treating seeds with these solutions prior to germination has resulted in more rapid and synchronous germination in seeds of several speices (Hansen, 1973; Heydecker et al., 1973; Darby and Salter, 1976; Salter and Darby, 1976; Coolbear et al., 1980; Bodsworth and Bewley, 1981; Donald, 1981; Georghiou et al., 1982; Fleming and Lister, 1984; and Furutani et al., 1986). Under the low water potential conditions the initial stages of germination are allowed to proceed, including such events as partial reserve hydrolysis, RNA synthesis, and translation (Coolbear et al., 1980). One can assume that these cellular events will take

place at different times in different seeds due to different hydration rates. This will presumably allow seeds of a particular species to arrive at a particular germination stage before treatment is discontinued (seeds are re-dried). Upon rehydration, such seeds continue metabolic development and germination in a more uniform manner and radicle emergence takes place in less time than if seeds were germinated without priming. The priming technique may be used in conjunction with other techniques such as inhibitor leaching (Darby and Salter, 1976) to promote more efficient germination.

A high molecular weight polyethylene glycol (PEG), a biologically inert polymer, is most commonly employed priming solution. It cannot penetrate cell walls and damage cells as easily as salts (Salter and Darby, 1976). Aeration was required due to the high viscosity of the PEG solutions, especially in large samples. Lack of O_2 in many cases impairs the germination of the seed.

Seeds require O_2 during seedling growth for respiratory use of stored energy reserves. Techniques such as seed scarification are used to allow O_2 to enter more readily. Hatano (1963) suggested that in *Pinus densiflora* and *Pinus thunbergii* the intact seed structure (eg. seed coat) may impair O_2 availability to the tissue and ultimately affect respiration. Seed respiration has been monitored in the first few hours of imbibition and these observations have been summarized by Bewley and Black (1978). The respiration in seed starts soon after imbibition. In the first phase, there is a sharp initial rise in respiration followed by a second phase characteristic of a lag period which may be due to O_2 restriction. In the third phase, a second respiration burst is seen which may be due to *de novo* synthesized enzymes and respiratory pathway constituents. Once the seed reserves are depleted, respiration drops in the storage tissue. The different phases are found in most seeds but the time scale for each event can vary from seed to seed (Moreland, et al. 1974). Correlative trends in fresh weight (Hatano, 1963; and Moreland et al., 1974), pine seed respiration (Hatano,

1963) and ATP levels (Moreland et al., 1974) have been observed as well. In particular the studies on *Pinus densiflora* and *Pinus thunbergii* by Hatano (1963) showed levels of O_2 uptake coinciding with the levels of imbibition during the early stages of germination.

Temperature and duration of solution imbibition may also alter the effect of the osmotic pretreatment on subsequent germination (Haydecker et al., 1973; Darby and Salter, 1976; and Salter and Darby, 1976). Priming of onion seeds with PEG 6000 reduced the number of seeds germinated (Furutani et al., 1986). Such reductions in germination may have been caused by decreased O_2 availability for respiration as a result of the viscosity of the PEG solution. It was also suggested that it was best to pretreat at low temperature because of decreased O_2 requirements therefore reducing the chance of anaerobic seed exposure (Ellis and Butcher, 1988). Also, it was suggested that those seeds close to death may not be able to survive the priming treatment (Ellis and Butcher, 1988). It may be that damage resulting from a lack of O_2 affects the ATP levels, retarding abnormal seeds to the point of death. Studies on *Pinus patula* by Donald (1981) have shown that when seeds were placed in a PEG solution with an osmotic potential too low to germinate, subsequent germination was lowered. In these experiments aeration was essential. In addition, the pine seeds were too sensitive to complete drying down and just surface drying was recommended.

Since PEG presents an aeration problem (Darby and Salter, 1976) priming has been performed with salts and other osmoregulators. Such regulators include: KNO_3, K_3PO_4 (Salter and Darby, 1976), and mannitol (Furutani et al., 1986). There is also the evidence as noted above, that surface drying after priming may only be required and complete drying back is not necessary or recommended. However, priming is a good pre-treatment technique since many seeds can be dried down and stored before sowing (Coolbear et al.,1980).

Biochemical investigations and physiological studies of metabolic events during germination or priming in gymnosperm seeds have been limited. This study reports the findings of research in two areas. In the first section, the effects of osmotic priming treatment of Jack pine seed on germination, protein and ∝–amino nitrogen, ATP, and a pepstatin-sensitive proteinase levels are investigated. In the second section, the aspartic (pepstatin-sensitive) proteinase found in the dry pine seeds was isolated and characterized. This characterization will provide information on the role of preexisting enzymes in pine seeds and their potential role in seedling growth.

2.0 Pine seed priming and some aspects of germination physiology

2.1 ABSTRACT

Germination acceleration by osmotic priming in Jack pine seeds was investigated using a 10% (w/v) PEG 8000 solution at 21.5°±0.5°C. Priming did not affect total, or peak rate of germination significantly, but did promote synchronous germination and increased rate of germination. A pepstatin-sensitive proteolytic activity was not affected by priming, while energy metabolism, measured as ATP levels, increased within 2 days of the priming treatment. This suggests that energy metabolism, and possibly phosphate metabolism may play an important role in early priming. Significant changes in protein hydrolysis and soluble free amino acid availability were not detected until 4 to 6 days of priming.

Key words: Osmotic priming, PEG, imbibition, germination, pepstatin, proteinase, ATP.

Abbreviations: PEG 8000, polyethylene glycol (approximate average molecular weight 8000); DTT, dithiothreitol.

2.2 INTRODUCTION

Germination of stratified, non-dormant Jack pine seeds can be uneven and complete germination of the population can take about 10 days under the most suitable conditions (20°C and 30°C) (Heit, 1958). In field seeding, this long time lag can be detrimental to the viable seeds, with the slowly germinating seeds more likely to encounter unfavourable conditions in the upper layer of the soil (Spyropoulos, 1986). The temperature and moisture extremes, as well as predation can all be factors decreasing the percentage of seeds which will successfully germinate.

Treatments which reduce the time between sowing and seedling emergence will reduce seed exposure to potential adverse field conditions and/or may save greenhouse costs in container production systems. One such technique, which often achieves more rapid and higher total germination is seed osmotic priming, which is achieved by controlled seed hydration in low water potential solutions (Salter and Darby, 1976; and Heydecker et al., 1973). Hydration of seed tissue is clearly required for early metabolic events to take place. The priming can provide controled levels of imbibition and consequently regulate the extent to which these events proceed. Ideally the level of hydration would be such that re-drying should halt the events but not reverse them completely, or even destroy already synthesized cellular compontents.

In this study, the germination of primed Jack pine seeds was examined, and complemented by the investigation of several metabolic parameters: the levels of ATP, water, readily soluble proteins, amino acids and the activity of a proteinolytic enzyme. These data will serve as a foundation for further investigations of the metabolism in primed and germinating pine seeds.

Positive effects of osmotic priming on germination are not clearly understood and may be due to increased synthesis or activity of RNA, adenosine phosphates, proteins, or hydrolytic enzymes. Detailed understanding of the metabolic and developmental benefits of osmotic priming will require deeper understanding of the molecular basis of the germination process itself and indeed, both processes are intimately linked.

Many of the metabolic events taking place during germination such as, enzyme activation and *de novo* synthesis of hydrolases for reserve mobilization require metabolic energy. ATP is not stored in seeds in large quantities; therefore its rapid production early during imbibition is essential. Indeed, stratification increased the total adenosine phosphate levels to a point of readiness, so that when germination temperature was raised, the seeds were more prepared for rapid germination (Ching and Ching, 1972). Similar events may ensue during osmotic priming. The ATP synthetic pathways of seeds, however, have not been completely characterized, and are still debated (Moreland et al., 1974; Morohashi and Sugimoto, 1988; Hourmant and Pradet, 1981; Morohashi, 1986, Perl, 1986; Salon et al., 1988). Morohashi (1986), in agreement with observations in Ponderosa pine by Ching (1970), suggests that the development of the mitochondrial system is influenced by the type of reserve storage material present. He suggests that rapid breakdown of lipid in lipid storing seeds is by glyoxysomes and mitochondria which are synthesized *de novo*, while starch storing seeds may activate and/or repair pre-existing mitochondria. However, Morohashi and

Sugimoto (1988) have studied the levels of ATP during the first few hours of cucumber (oily seed) and mung bean (starch seed) imbibition and concluded that both the oil and starch storing seeds produced ATP by the same mitochondrial oxidative phosphorylation system. This is also in agreement with Hourmant and Pradet (1981) and Salon et al. (1988) who have found that O_2 was required to maintain a high level of adenosine phosphates and demonstrated that TCA cycle activity and oxidative phosphorylation was the main mechanism of ATP production in lettuce seeds. Finally, Perl (1986) has suggested a novel, as yet undetermined metabolic source of ATP.

In addition to increased energy metabolism, germinating seeds must develop or activate hydrolytic enzymes which will provide respiratory substrates. The enzymes involved in the reserve mobilization in gymnosperms have not been well characterized. There have been some attempts at isolation and characterization of endopeptidase in Scots pine (Salmia et al., 1978; Salmia, 1980; Salmia and Mikola, 1980; Salmia, 1981a; Salmia, 1981b). Among the activities which may be involved in megagametophyte or embryo protein reserve mobilization is a pepstatin-sensitive acid proteinase (Salmia et al., 1978). It was identified primarily in the embryo of resting seeds and may be found in the protein bodies. Its precise function during seedling growth has not been resolved.

The work in this report is focused on the potential use of osmotic priming as pre-treatment to accelerate germination in Jack pine. Data on the levels of soluble protein, amino acids, pepstatin-sensitive proteinase and ATP in germinating and primed seeds is also presented to provide baseline information for further studies of the role of energy metabolism and proteolysis in early pine seed germination.

2.3 MATERIALS AND METHODS

2.3.1 Preparation of plant material

The Jack pine *Pinus banksiana* Lamb. seeds, were all from the same seed lot Pj 4400 site region, 1981 collection with a 99% germination (8/1986). The seeds were stored at -20°C. When required, the seeds were warmed to room temperature, sterilized with 6% Na hypochlorite for 30 seconds and rinsed with sterile double distilled water. The control seeds were then dried at 4°C overnight on silica gel while the seeds to be primed were submerged in 10% w/v polyethylene glycol (PEG 8000) solution (water potential approximately -170 kPa based on Michel & Kaufman, 1973) aerated with a Neptune Dyna Pump model no.2 air pump (Fisher Scientific, Ont.). After priming for 2, 4, and 6 days respectively, the seeds were removed, rinsed with deionized water, and desiccated overnight over silica gel at 4°C. Three replicates of 50 seeds were used per trial while each experminent was repeated 3 times.

2.3.2 Germination conditions and criteria

The 50 seeds were germinated in sterile Petri dishes on a qualitative grade 1 Whatman filter paper moistened with 2 ml sterile double distilled water. These were incubated under fluorescent lights (0.088 W/m^2 night due to safety light and 0.332

W/m^2 for 8hrs) at 22.5 ± 0.5°C. The germination of the seeds was monitored daily for 10-14 days. A seed was considered germinated when the seed coat split and the white radicle was showing approximately 1mm in length.

The following germination parameters were quantified: germinative capacity, germinative speed (T50), uniformity of germination (T90). According to Fleming and Lister (1984), germinative capacity is defined as the percentage of sound seeds that germinated, and T50 is time for 50% of viable seed to germinate ie. those that did germinate. The germination uniformity (T90) is the time taken from 10% to 90% of the viable seeds to germinate. Germinative vigour (Czabator, 1962) is a measure of completeness of seed germination calculated as germinated seed/unit time. This represents the maximum number of germinated seeds in a period of time. All values were subjected to one way variance analysis and considered as completely randomized experiments. Fisher PLSD test was used to find significance differences between the means.

2.3.3 Water content

The percent water content was calculated using the following equation (where surface drying, after washing with deionized water, was for 10 minutes on top of Whatman paper and oven drying was for 48 hours at 60°C):

$$\frac{\text{Surface dried weight - Oven dried weight}}{\text{Surface dried weight}} \times 100\% = \%\ \text{Water Content}$$

2.3.4 Protein determination

The quantity of readily solubilized seed protein from each stage of priming was determined. One hundred seeds were washed with deionized water and blotted dry with Whatman paper and then ground in a Braun coffee grinder (Type DSM2). The powder was transferred to a beaker with 5-10 ml of 50 mM Na phosphate buffer, pH 7.2, 1mM DTT, 0.2% (w/v) Triton X-100 and 0.02% NaN_3 (Extraction Buffer). The slurry was homogenized for 5 min with a Tissumizer (model no.SDT 1810 Tekmar, Cincinnati, OH). The extract was centriguged at 15000 rpm for 10 minutes in a Sorvall centrifuge (Newton, CO. Model RC2-B, SS34 rotor, DuPont, Delaware). The supernatant was collected and kept on ice. The pellet was resuspended with the same volume of buffer and recentrifuged. The protein concentrations in pooled supernatants was determined (against BSA) using the Bio-Rad (Richmond CA.) method based on Bradford (1976) using a DU-50 spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

2.3.5 Amino acid determination

One hundred seeds aliquot were washed with deionized water, blotted dry with no. 1 Whatman filter paper, and ground in a Braun coffee grinder type DSM2. The powder was transferred to a beaker containing 5 ml of 80% (v/v) hot ethanol and homogenized for 5 minutes with a Tissumizer (model no.SDT 1810 Tekmar, Cincinnati OH). The extract was centrifuged at 4000 rpm for 10 minutes in a Sorvall centrifuge (Newton, CO. Model RC2-B,SS34 rotor, DuPont, Delaware). The supernatant was cooled on ice and the pellet was reextracted and centrifuged with 5 ml of 80% hot ethanol. The supernatants were combined, dried, resuspended in water then passed over a Dowex H^+(50X8-400)(Sigma Chem. Co., St Louis, MO) equilibrated with

water according to Cooper (1977). The samples were eluted in 6N HCl collected then flash evaporated (Büchi Rotavapor R110 Type KRvr 65/45, Brinkmann Inst., Rexdale Ont.) to remove the hydrochloric acid. The samples were resuspended in extraction buffer (see 2.3.4) and assayed for amino nitrogen groups using ninhydrin (Yemm and Cocking 1955). The samples were read at 570 nm using a DU-50 spectrophotometer (Beckman Instruments Inc. Fullerton, CA) against a standard comprised of a mixture glycine, threonine, and methionine.

2.3.6 Crude protease extraction

Aliquots of 0.350 grams (approximately 100 seeds) were washed with deionized water and homogenized at 4°C in a Braun KSB coffee grinder for 30 seconds. The powder was transferred to a chilled beaker with 5 mls of 50 mM sodium phosphate buffer, pH 6.5, containing 1mM DTT, 0.2% (w/v) Triton X-100, and 0.02% NaN_3 and homogenized with a Tissumizer (model no.SDT 1810 Tekmar, Cincinnati OH) for 5 minutes. To the slurry, 10% (w/v) polyvinyl polypyrrolidone (PVPP) was added prior to centrifugation at 10000 rpm for 10 minutes at 4°C in a Sorvall SS34 rotor. Supernatant was collected and placed on ice while the pellet was resuspended in 5 mls of buffer and recentrifugated under the same conditions. The supernatants were pooled and kept on ice until required for enzymatic activity analysis.

2.3.7 Enzyme activity assay

The enzyme activity of the seed extract was based on the hydrolysis of hemoglobin at pH 3.7 according to Salmia et al. (1978) adopted from Anson (1938). The hemoglobin substrate solution was prepared on a daily basis by mixing one part 2% aqueous hemoglobin (Sigma chemical Co. bovine, type II) and two parts of 0.2 M sodium lactate buffer of pH 3.7. This solution was equilibrated for 30 minutes at 30°C. Concurrently, 0.2 mls of enzyme was incubated with and without the inhibitor

pepstatin A at 10 μM (Sigma Chemical Co.) at 0-4°C for 30 minutes. The reactions were started by adding 0.6 ml of the substrate to the pre-incubated enzyme and incubated further at 30°C for 30 minutes. The reaction was stopped by adding 0.4 ml of cold 9% TCA . The tubes were cooled for 30 minutes at 0°C then centrifuged at maximum speed for 10 minutes in a benchtop centrifuge.

TCA-soluble reaction products were determined according to Lowry et al. (1951) with the Folin phenol reagent, against a BSA standard. Fifty μl of the supernatant removed from the centrifuged samples was neutralized with 50 μl of 0.184 M NaOH before assay. The absorbance was measured at 750 nm and the proteinase activity was expressed in units (one unit = TCA soluble colour produced after 1 minute at 30°C, equivalent to absorbance produced by 1 μg BSA). The pepstatin-sensitive activity was determined as follows:

Total activity - Pepstatin insensitive activity = Pepstatin-sensitive activity

2.3.8 ATP determination

The levels of ATP were determined according to Lemasters and Hackenbrock (1978). Each batch of 50 seeds was extracted with 1.5 ml of 5% (w/v) trichloroacetic acid (TCA) using a tissue grinder (Tissumizer). The extract was centrifuged for 10 minutes at 15000 rpm in a Sorvall SS34 rotor. The supernatant was collected and kept on ice. The pellet was reextracted and centrifuged with an additional 1.5 mls of 5% TCA. A 10 μl aliquot from combined supernatants was rapidly mixed with 0.5 ml of luciferin-luciferase (Sigma Chemical Co., St. Louis, MO.) in the cuvette of a M600 photometer (Oriel Optics Corp., CO), set at appropriate sensitivity level. Light

2.4 RESULTS

2.4.1 Osmotic priming treatment

More than 25% of cold-stored, dry Jack pine seeds placed on water were germinated by day 3 (Fig. 1). In contrast, during priming with the 10% PEG solution germination was delayed at least for 6 days (not shown). Priming was not continued beyond this point since some seeds were starting to germinate by day 7 (<1%). Such primed seeds, when dried and subsequently placed in water in Petri dishes, germinatied more rapidly (Fig. 1, Table 1)

As the priming time increased, less time was required to achieve 50% germination. After only two days priming it took only 76% of the time required by the dry seed to germinate to T50 and after 6 days of priming 46% of the original time. The germination uniformity was also affected by the priming process. Even after only 2 days of priming, the germination uniformity was increased. Priming increased significantly the peak value parameter. This indicates that the seeds that did germinate, germinated more quickly. However, the peak value though similar, did arrive at different times; for dry seed, it was after 4 days, for 2 and 4 days primed seeds it was after 3 days and for the 6 day primed seed it was reached after only 2 days. The overall

high germination capacity was not affected by priming (Table 1). These high quality seeds had a 99% germination capacity in 1986, which has not decreased significantly over the storage time since the total % germination is still high at 94-96% (Table 1).

2.4.2 Seed hydration during priming: protein and amino acid levels

As noted earlier, priming in 10% PEG 8000 did not prevent germination. After 6 days in the PEG solution, fewer than 1% of the seeds had radicles protruding. It took less than 2 days of priming to hydrate the seeds (Fig. 2). Protein and amino acid levels remained nearly constant for the first 4 days of priming. During the subsequent two days, as osmotically delayed germination reaches completion (radicle protrusion on day 6) there was a dramatic decrease in soluble seed protein content (about 40% of initial, Fig. 3) accompanied by an increase in amino nitrogen content (about 180% of initial, Fig. 4).

2.4.3 Pepstatin sensitive activity and ATP levels during priming

The activity of pepstatin-sensitive proteinase (nearly linear for 30 minutes, Fig. 5) was monitored at different physiological times. The enzyme activity was assayed when the seeds were dry, either following priming or not, (designated physiological time I); when the seeds were completing germination (<5%, physiological time II), and when the seeds had completely germinated (>95%, physiological time III) (Table 2). The proteinase activity did not appear to change appreciably at any of the physiological stages after 4 days of priming, or during normal germination (Table 2). In contrast, the ATP levels were higher in 4 day-primed seeds than in the resting dry seeds. By the time germination was reaching completion (physiological stage II), the unprimed seed ATP levels were nearly comparable to the primed seeds, while by the time germination was complete (physiological stage III) the levels of ATP were not significantly different in both types of seeds (Table 2).

2.4.4 The effect of pepstatin on germination

Pepstatin was found to retard germination (Fig. 6). After 7 days, 94% of the control seeds had germinated while only 78% had germinated in the presence of 25 μM pepstatin. The seeds which germinated in pepstatin did not exhibit a normal rate radicle elongation.

Figure 1. Germination of untreated and 10% PEG 8000 primed *Pinus banksiana* seeds Each point represents the average percent germinated ± std dev (Out of 50 seed trials done in triplicate from three different blocks). Dry (—), 4-day-primed (—♦—) , and 6-day-primed (—▲—) seeds.

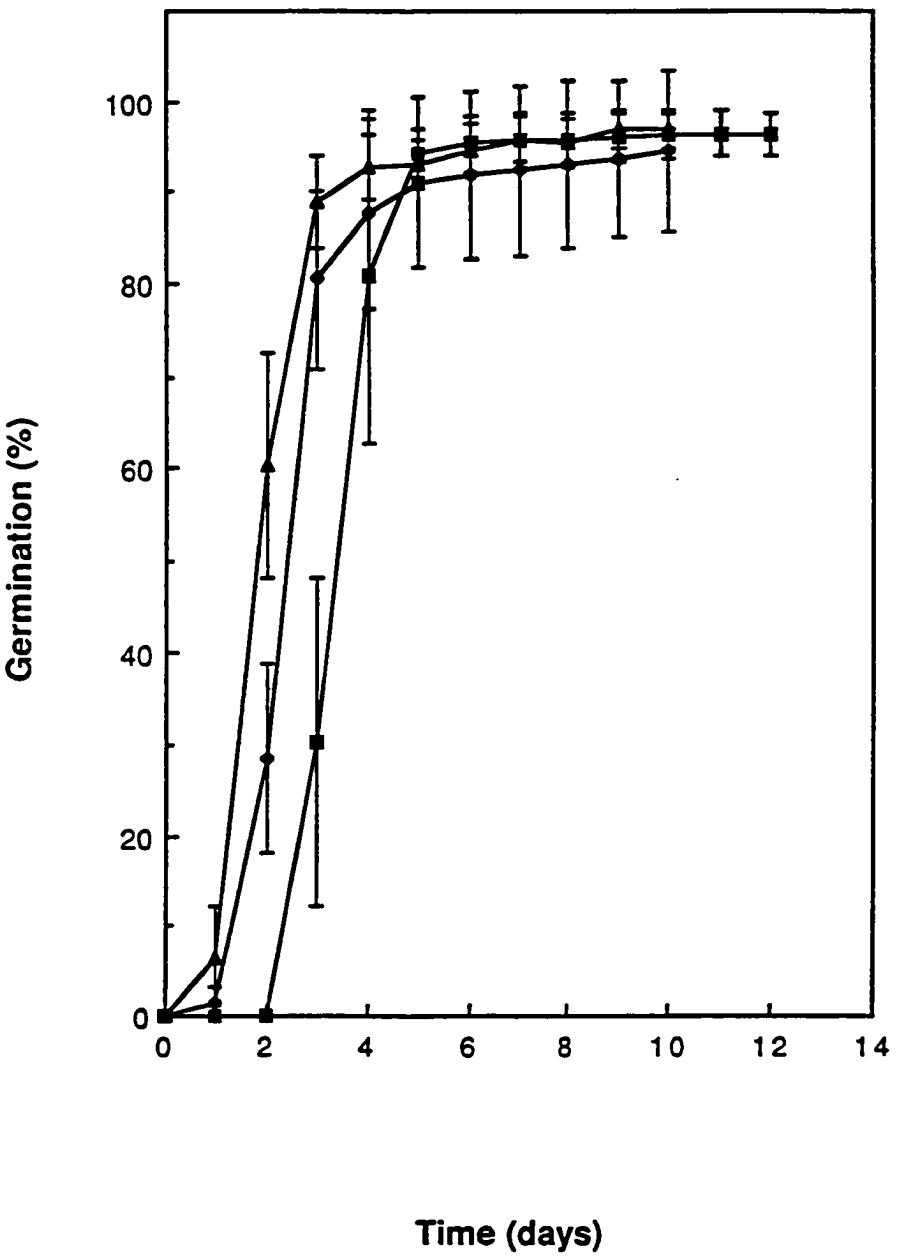


Table 1. Germination parameters of unprimed and primed *Pinus banksiana* seeds

Treatment (priming days)	Maximum Germination (germ. capacity) %	Time From 10% to 90% Viable (germ. uniformity) days	Time to 50% Germination (germ. speed) days	Peak Value (germ.rate) seeds/day
0	96.2a	2.63b	3.54d	20.22g
2	97.8a	1.77c	2.64e	26.37h
4	94.4a	2.11c	2.30e	26.82h
6	96.9a	2.00c	1.64f	30.22h

Significant differences (P=0.05) between values are shown by different lower case letters for each column.

Figure 2. Water content of dry seeds and seeds primed for 2, 4, and 6 days. The water content of three aliquots of 50 seeds was determined for each priming time.

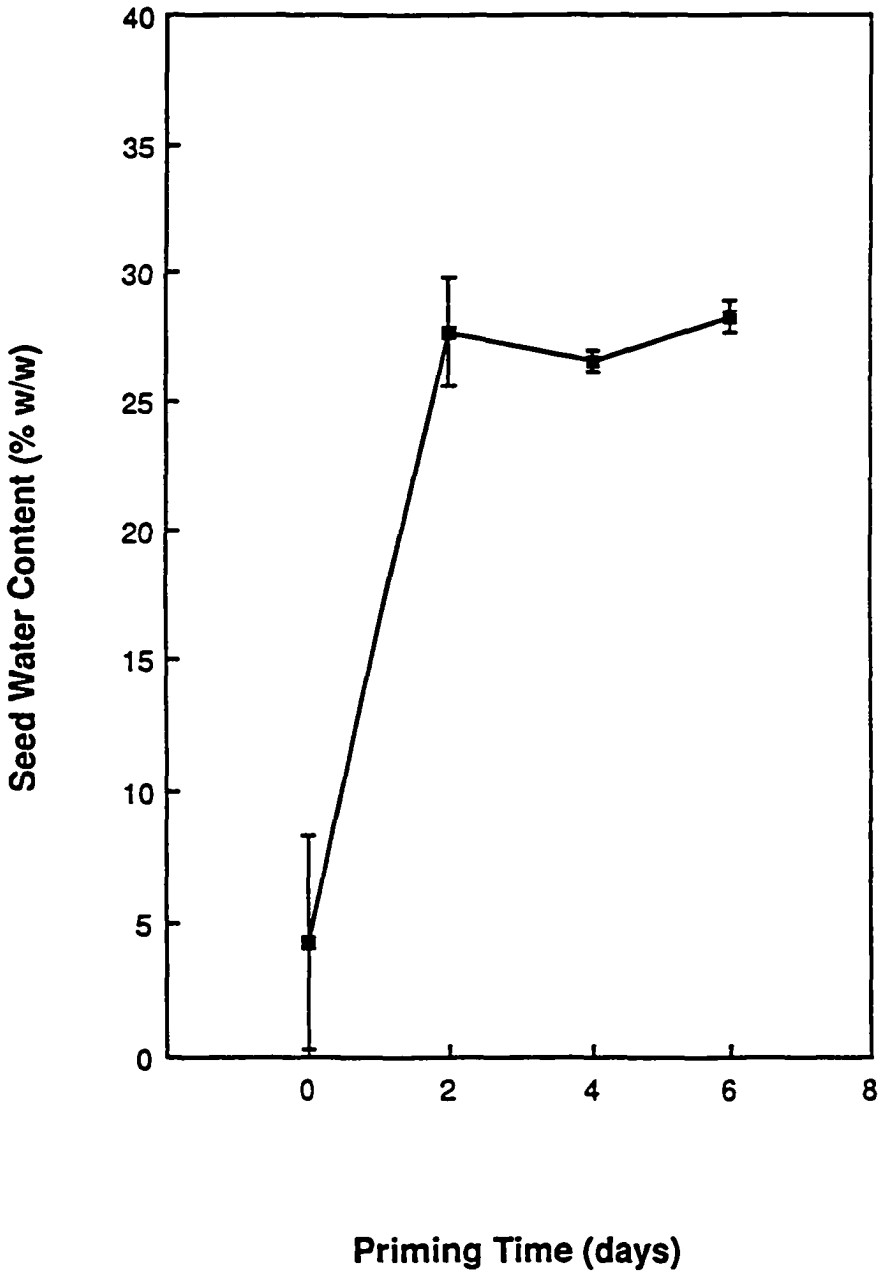


Figure 3. Readily solubilized protein content of seeds at different priming times. An aliquot of 100 seeds was extracted in aqueous buffer after 0, 2, 4, and 6 days of priming in 10% PEG 8000.

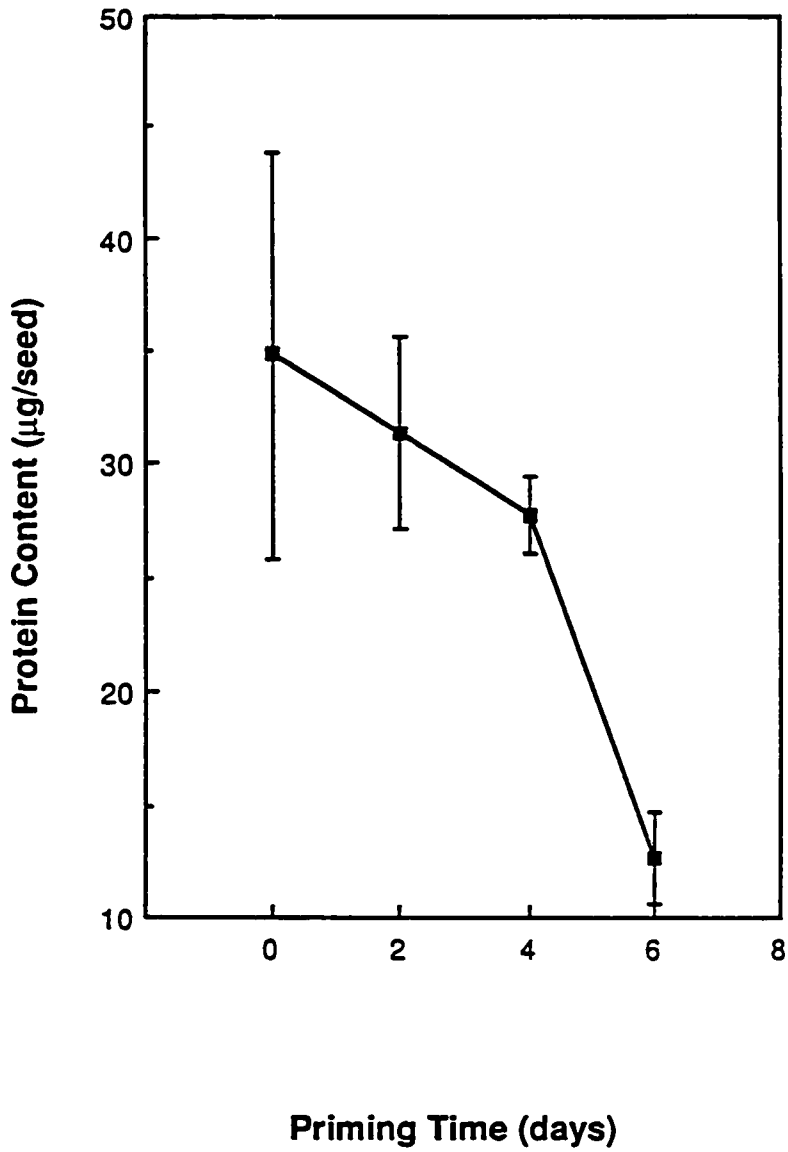


Figure 4. Seed free amino nitrogen concentration at different priming times. An aliquot of 100 seeds was extracted in 80% hot ethanol after day 0, 2, 4, and 6 of priming in 10% PEG 8000.

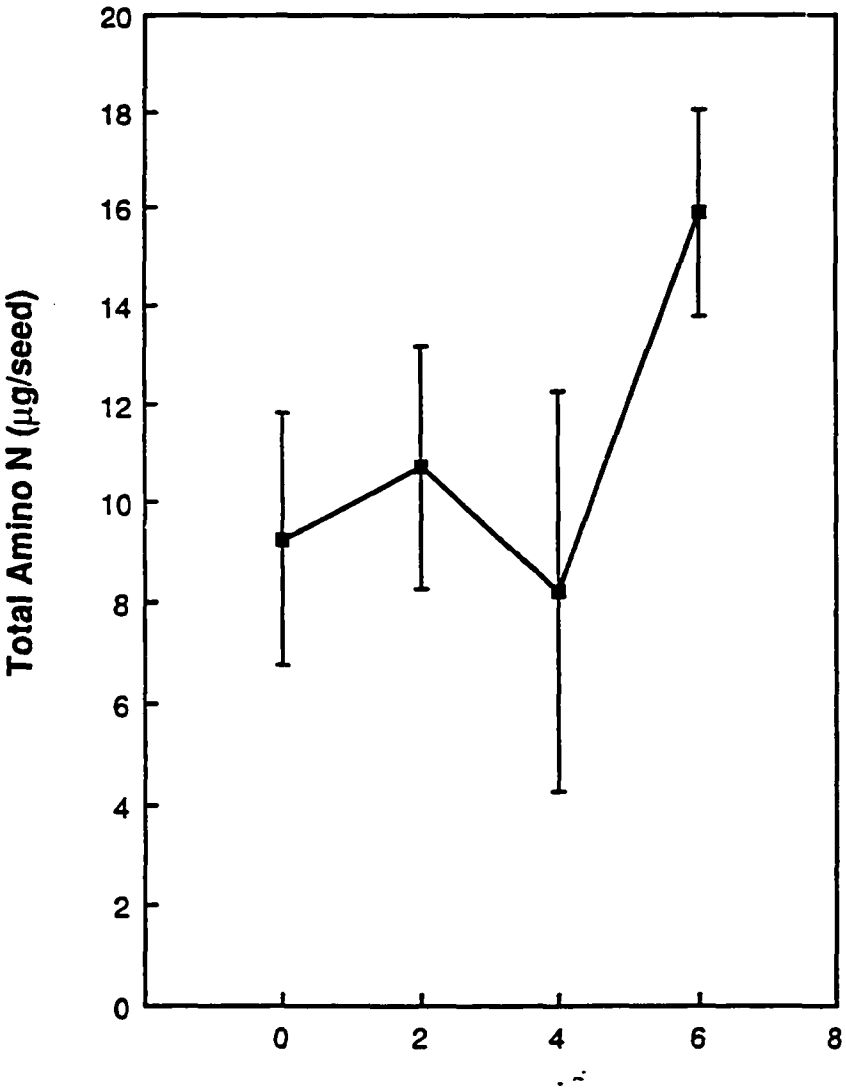


Figure 5. Kinetics of pepstatin-sensitive proteinase activity from dry seeds. Crude seed extract was assayed at 30°C.

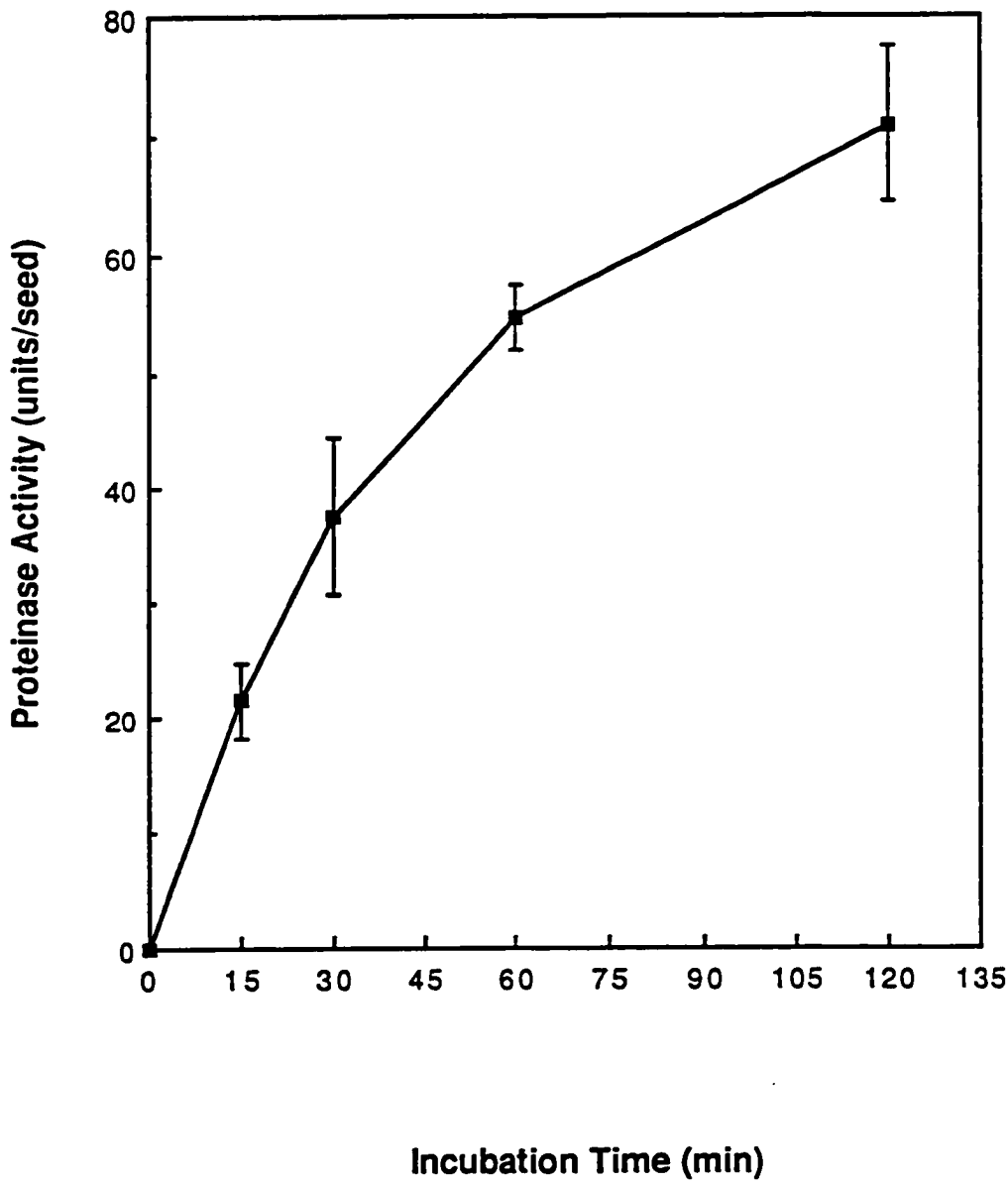
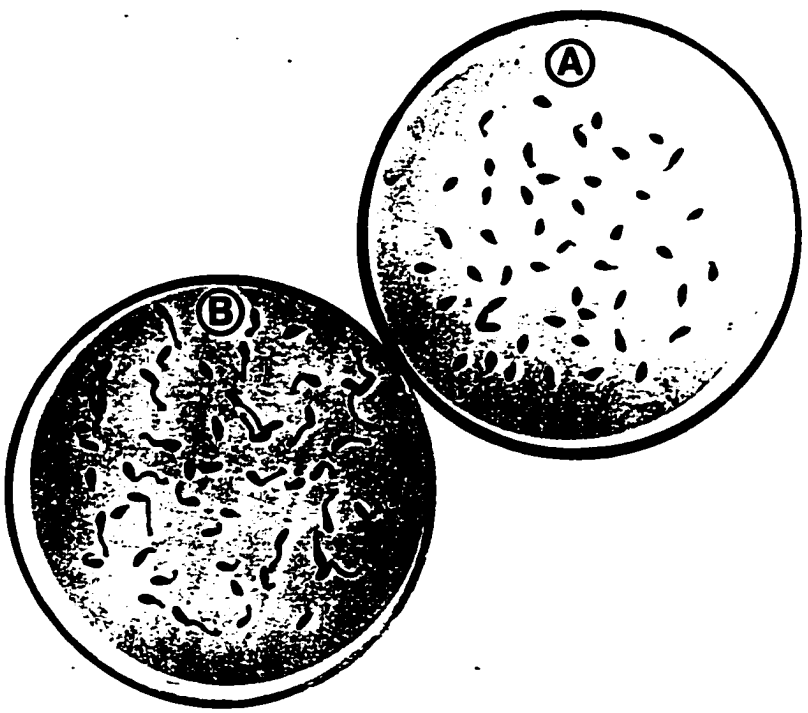


Table 2. Activity of pepstatin-sensitive proteinase and ATP levels at different physiological times in *Pinus banksiana*.. Physiological time is an arbitrary definition of the stages which the seed had attained; stage I is a dried state, stage II is < 5% germination and stage III is > 95% germination

Germination Time Starting (Days)	Seed State	Specific Activity (Proteinase units/min/seed)	[ATP] (pmoles/seed)	Physiological Time
0	Dry	1.38±0.09	5.4±1.9	I
0	4 Days Primed	1.30±0.24	23.9±7.2	I
2	Dry	0.73±0.18	17.2±2.9	II
1	4 Days Primed	0.85±0.17	24.9±3.7	II
5	Dry	0.97±0.36	41.8±7.7	III
3	4 Days Primed	1.11±0.20	53.5±7.2	III

Figure 6. *Effects* of pepstatin on germination of *Pinus banksiana* seeds after 7 days. A. Germination in 25μM pepstatin (78% germinated- radicles protruding), B. Germination in water 94%- radicles >5mm).



2.5 DISCUSSION

Positive effects of priming were noted after only two days of incubation in 10% (w/v) PEG 8000 (Table 1). Longer exposure to PEG further improved germination parameters, namely, decreased germination time, and increased the synchrony of germination. No detrimental effects on total germinative capacity were observed after post-treatment drying, as seen in some horticultural crops and *Pinus patula* seeds (Bodsworth and Bewley, 1981; Donald, 1981). The damage seen in these cases has been attributed to anaerobic conditions arising due to PEG solution viscosity (Furutani et al., 1986) and cells being unable to repair subcellular damage (Ellis and Butcher, 1986). The lack of damage found in this study may have been due to the low PEG concentration, and adequate aeration during a relatively short priming period.

Germination uniformity was also improved by priming, as were germination speed and peak value parameters (Table 1). This may indicate that the slow hydration in an osmoticum allows metabolic events in each seed to attain a comparable metabolic stage, from which all seeds start after rehydration. The acceleration of germination is most likely a result of initiations of certain metabolic events during priming, which confer on the seeds a metabolic status characteristic of nearly germinated seeds.

ATP levels indicative of active energy metabolism, were found to increase following only 2 days of priming (Table 2). Energy-related metabolic processes have been noted to proceed quickly in the initial stages of imbibition (Ching and Ching, 1972; Moreland et al., 1974; Hourmant and Pradet, 1981; Salon et al., 1988). Furthermore, ATP levels in the primed seed did not decrease during the brief re-drying. The ATP levels at later stages of germination remained high, whether the seeds were previously primed or not. Our results do not take into account the levels of ADP or AMP in the primed or untreated seed. Further evaluation of ADP and AMP levels, as well as the "energy charge" (Atkinson, 1969) may be required. The high levels of ATP during early priming may indicate either that the cell's requirements are low (low turnover) or that ATP production as well as utilization are high (high turnover). This may also require further evaluation.

The early changes in ATP level during priming suggest that the ATP requirements of the seed cells are likely linked to enzyme synthesis on activation required for storage protein degradation and the subsequent mobilization of amino acids. A decrease in protein and increase of amino acids was only measured following the last two days of priming on day 6 (Figs. 3, 4). The protein mobilization correlated well with an increase in free amino acids. This suggests that the time required for detectable protein breakdown and amino acid appearance occurs within full 4 days following complete hydration (completed during the first two days of priming, Fig. 2). Protein and amino acid concentration shifts of such magnitude may be more expected after germination, during the period of active growth of the axis. Clearly, mobilization of reserves has proceeded to an appreciable level in the absence of extensive axis growth under osmotically restricted conditions. However, such dramatic shifts in protein and amino acid levels observed in seeds primed for 6 days were not likely

required to accelerate post-priming germination, as seeds primed for only 2 or 4 days germinated nearly as rapidly as 6 day-primed seeds (Table 1).

A pepstatin-sensitive enzyme described in Scots pine seeds by Salmia et al. (1978) and Salmia (1981) may play a role in seed storage protein mobilization. The acid proteinase in resting seeds is localized mainly in the embryo (Salmia, 1981) and probably in the protein bodies. Similar activity was established in dry Jack pine seeds and the activity was monitored during the different periods of priming and germination. The enzyme activity was initially investigated in dry Jack pine seed, using a modified assay adapted from Salmia et al. (1978). Linear activity was observed for 30 minutes (Figs. 5) and not 120 minutes as found in the Scots pine seeds. This loss of activity may due to the instability of the enzyme during long periods of incubation at 30°C. This instability may be attributed to it being a complex (see Section 3.0) which may dissociate at this temperature or is autohydrolyzing at this pH.

The specific activity of pepstatin-sensitive proteinase activity showed no significant differences between dry seeds and 4 day primed seeds (either dried overnight or extracted after only surface drying), during germination, and initial stages of seedling development (Table 2), i.e. the pattern of enzymatic activity during germination was not affected by priming although our approach does not distinguish the possibility that continual turnover of the enzyme may be taking place. The pepstatin-sensitive activity does not appear to be synthesized *de novo* after imbibition as other protein reserve degrading seed peptidases (Vincenzini et al., 1973; Sze and Ashton, 1971; Chrispeels et al., 1976), since it was found in dry seed. The effect of protein synthesis inhibitors such as cycloheximide, and puromycin needs to be tested to support this hypothesis. In order to determine if the pepstatin sensitive proteinase may be a required for seed germination and growth, dry seeds were germinated on Petri dishes with and without pepstatin (Fig. 6). The enzyme inhibitor at 25 μM

concentration did not completely inhibit the germination of the seeds, but did retard the process suggesting that proteolysis is required. There is however, not enough known about the inhibitor's action on plant cell metabolism to make definite conclusions. Studies in barley by Mikola et al. (Pers. Comm., 1988) on endogenous protein degradation, have shown that the aspartic proteinase in these seeds did not initiate reserve globulin protein degradation. It was concluded that the enzyme must be important to seedling development by some other mechanism. Salmia (1981) suggests that the role of the enzyme in *Pinus sylvestris* may be linked to mobilization of protein bodies in the embryo. Further studies on the enzyme levels in the embryo and megagametophyte tissue during priming and germination may help support or refute this hypothesis.

These pilot studies were completed to confirm that priming is potentially beneficial to Jack pine seed germination. Additional studies are required where the gametophyte and embryo tissue are analyzed separately for protein, ∝–amino nitrogen, ATP, ADP, AMP, and pepstatin-sensitive proteinase under optimal priming conditions. Priming did provide a metabolic advantage compared to on unprimed seeds by decreasing germination time and resulting in more synchronous germination. This metabolic enhancement may be due to the energy metabolism being triggered early during priming. Protein hydrolysis into amino acid did not appear to be active early during priming and may not play a primary role in the germination enhancement effect. The pepstatin-sensitive proteinase levels were not affected by priming and the potential role of the enzyme in germination and in seedling development needs further analysis.

3.0 Purification and characterization of a pine seed acid protease

3.1 ABSTRACT

Pepstatin-sensitive, hemoglobin digesting enzyme was purified from dry Jack pine seeds via anion exchange, gel filtration and affinity chromatography. The enzyme has an activity optimum at pH 3.5. $HgCl_2$ (2 mM) inhibited nearly 50% of the activity while pepstatin (2 mM) and leupeptin (2 mM) inhibited the activity completely. A single band detected on non-denaturing PAGE, dissociated into several subunits in the presence of 10% SDS. The enzyme was stable for several weeks at 4°C at pH 5.0 in 0.5 M NaCl. Hemoglobin degradation products were analysed by gel filtration and urea/SDS PAGE. The potential role of the enzyme in germination is discussed.

Key words: Proteinase, seed germination, proteolysis, inhibition, hemoglobin, pepstatin A.

Abbreviations: DTT, dithiothreitol; IAA, iodoacetamide; PMSF, phenylmethane sulfonyl fluoride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; pCMBS, para-chloro mercuro benzoyl sulfonate; STI, soyabean trypsin inhibitor; ETI, egg trypsin inhibitor; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetracetic acid.

3.2 INTRODUCTION

Mobilization of seeds reserves during germination has been described in gymnosperms at the ultrastructural level (Simola, 1974) and physiological level (Goo and Furusawa, 1955; Katsuta, 1961; Ching, 1966; and Gifford, 1988) but complete understanding of the biochemical events taking place in the germinating seed is lacking. Therefore, it is essential to determine which dry seed enzymes are present and potentially active during early imbibition, and to define their role in the events taking place during germination. The immediate aims of this work were: a) to develop a method of extracting a pure and stable aspartic proteinase from resting Jack pine seeds; b) to biochemically characterize the enzyme; c) to characterize the hemoglobin substrate degradation products.

Protein degradation is an important aspect of seed germination which is a precise, genetically regulated event. Once these regulatory mechanisms are understood, they may ultimately contribute to the development of higher quality seeds. In contrast to animal proteases, those found in seeds have not been as intensively investigated. Most of the studies have concentrated on cereals and legumes (Storey, 1986), while little work has been performed with gymnosperm seeds. Mikola et al. (Salmia and Mikola, 1975; Salmia and Mikola, 1976; Salmia et al., 1978; Salmia and Mikola, 1980;

Salmia, 1980; and Salmia, 1981) have concentrated on Scots pine (*Pinus silvestris*). Two alkaline peptidase activities towards artificial substrates were described (Salmia and Mikola, 1975). The alkaline peptidases were found both in resting and germinating embryo and endosperm, but the authors could not conclude whether or not alkaline peptidases were involved in reserve protein mobilization. An acid carboxypeptidase activity was also described, which increased progressively from resting to germinating embryos and endosperm (Salmia and Mikola, 1976). The maximum activity in the endosperm came later than the stage of maximum mobilization of reserves. Therefore, the authors concluded that this activity probably did not play a significant role in the initiation of reserve protein mobilization, but may be important for seed senescence (Salmia and Mikola, 1976). They also noted that some naphthylamidase (aminopepidase) activities contributed to some extent to protein reserve depletion (Salmia and Mikola. 1976). Subsequently, Mikola's group developed a system to detect general proteinases using hemoglobin and casein as substrates (Salmia et al., 1978). These activities detected were much higher in the endosperm indicating they were probably involved in endosperm strorage protein mobilization. They also noted a pepstatin sensitive activity in resting seeds, present mostly in the embryo/seedling with only some in the endosperm.

Later studies by these authors focused on endogenous proteinase inhibitors (Salmia and Mikola, 1980; and Salmia, 1980). These inhibitors were heat tolerant and did not have any effect on the pepstatin sensitive (pepsin-like) activity or on the previously studied acid carboxypeptidase, trypsin-like or chymotrypsin-like activities. The authors noted that these inhibitors are unlike those from mung bean and barley, which do contain classical proteinaceous trypsin and chymotrypsin inhibitors (Salmia and Mikola, 1980). The pine seed inhibitors were suggested to be present for cellular protection (Salmia,1980) and not to inhibit preexisting enzymes until required for

protein degradation during germination in analogy to the findings in mung bean by Baumgartner and Chrispeels (1976).

Upon characterization of the hemoglobin and casein digesting activities in the endosperm storage tissue these two main acid-SH proteinases were concluded to be sensitive to the endogenous inhibitors. These enzymes had very little activity in the resting seeds (Salmia, 1981) and their activities only appeared later, following germination. Pepstatin sensitive activity however was active in resting seeds, especially in the embryo. Salmia (1981) suggested that the activity is found in the protein bodies where it initiates insoluble protein degradation. This pepstatin sensitive activity was chosen as the enzyme to be purified and further characterized in *Pinus banksiana* seeds. Preliminary evidence suggested this enzyme to be an aspartate protease and a brief introduction of the group of protease follows.

Aspartic proteinases have been found to be confined only to eukaryotes suggesting that they are relatively young evolutionarily (Kay, 1985; and Barrett, 1986). Some well known examples of aspartic proteinases are the pepsins, chymosins and gastrisins (Etherington and Taylor 1969; and Seijffers et al., 1963). These are gastric enzymes and their study has been extensive. Understanding of their action has been useful in controlling gastric ulcers, by developing inhibitory drugs for overproducing patients (Kay, 1985). Calf chymosin has traditionally been very important in milk-clotting for cheese production and much effort using recombinant DNA technology to introduce the gene into other systems has been undertaken, (Kay, 1985). Other as aspartyl proteases include cathepsin D found in spleen lysosomes (Barrett, 1981) and renin produced in the kidney and sub-maxillary gland (Inagame et al., 1985; and Kay, 1985) . The latter enzyme has been vigourously studied because of its importance in normal blood pressure maintenance. Generally, the aspartic proteinases have an acidic optimal pH, with molecular weights ranging between 35-42 kD (Kay, 1985). The

extent of the studies on this enzyme group ranges from amino acid sequencing (Takahashi and Tang, 1981) to X-ray crystallography of pure crystals (James et al., 1982).

These enzymes have but a few inhibitors. One of these is diazoacetyl-norleucine methyl ester coupled with copper ions (Barrett, 1986). The most common and powerful inhibitor used against the acid proteases is pepstatin A. Its amino acids sequence is isovaleryl-L-valyl-L-valyl-statyl-L-alanyl-statin [statin = (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid; statyl = statin residue in peptide bond] (Keesey,1987) and has been isolated from culture media of *Streptomyces* . Kinetic studies of pepstatin analogues and derivatives have found the general K_i range between 10^{-4} to 10^{-6} M for porcine pepsin (Marciniszyn et al., 1976). The inhibitor complexes in a 1:1 fashion with the enzyme's substrate active site (James et al., 1982). The competitive inhibition has been demonstrated by Corvol et al. (1973) and Marciniszyn et al. (1976) in which the latter suggest that the binding is representative of a transition state taken during bond hydrolysis. Other studies by Wilimowska-Pelc et al. (1983) and Polanowski et al. (1985) have isolated aspartic proteinases from resting squash and cucumber seeds. It was found to have a MW of 42kD, pI of 5.2, pH optimum of 3.2, was inhibited by pepstatin A, DAN, and 1,2,3,-epoxy(p-nitrophenoxy)propane and degraded seed globulins.

The isolation of the pepstatin sensitive (aspartic proteinase) activity from the Jack pine seeds will initially require classical chromatographic techniques. One of the most successful protein purification methods is affinity chromatography used in conjunction with more traditional methods such as gel filtration and ion exchange. Pepstatin A-agarose is one example of the media used. The pepstatin A inhibitor (ligand) is covalently bound to the solid agarose matrix (Corvol et al., 1973; Hackenthal et al., 1978; Kregar et al., 1977). The enzyme is allowed to bind to the

affinity media at an optimal pH while all other undesired materials are washed through a column. The enzyme of interest can then be eluted under appropriate conditions (Yamamoto et al., 1978; Takahashi and Tang, 1981; Anotov et al., 1985; Inagami et al., 1985; Kostka et al., 1985; Polanowski et al., 1985; Wilusz and Polanowski, 1985). Pepstatin A affinity chromatography step was tested and incorporated into the protocol for the purification of the pine seed enzyme.

3.3 MATERIALS AND METHODS

3.3.1 Materials

Pine (*Pinus banksiana* Lamb.) seeds from 1981 harvest site region Pj 4400 (North western Ontario) with a 99% germination when tested in August 1986 were stored frozen at -20°C until required.

3.3.2 Extraction of crude enzyme

Seeds were weighed (5 grams, 1400-1800 seeds) and ground at 4°C in a Braun coffee grinder (Type DSM2) for 30 seconds. The paste was transferred to a chilled beaker with 50 ml of 50mM sodium phosphate buffer, pH 6.5, containing 1 mM DTT and 0.1% (w/v) Triton X-100, and 0.02% NaN_3 and homogenized with a Tissumizer (Tekmar, Cincinnati, OH, Model SDT 1810) for 5 minutes. To the slurry, 10% (w/v) of polyvinyl polypyrrolidone (PVPP) was added and then centirfuged (Sorvall, Newton, CO. Model RC2-B) at 5000g at 4°C for 20 minutes.

The white opaque supernatant below the liquid spherosome layer was removed, using a Pasteur pipet and kept on ice while the pellet was resuspended in 30 ml of extraction buffer and recentrifuged at 10000 g for 10 minutes. The supernatant was collected as above and the pellet re-extracted. The three combined supernatants were

then vacuum filtered through Whatman filter paper (Qualitative grade 1, Fisher Scientific, Ont.) to remove any particulate debris.

3.3.3 DEAE agarose ion exchange and preparation for gel filtration

The extract was then loaded onto a DEAE-agarose anion exchange (Bio Gel A 100-200 mesh, Bio-Rad, Richmond, CA) 40x2.6 cm column (LKB, Bromma, Sweden) equilibrated with 50mM Tris-HCl buffer, pH 8.0, containing 0.02% NaN_3 and 0.1mM DTT. Once the unbound protein had eluted from the column, a 0-700mM NaCl gradient (500 ml total volume) was applied to remove bound proteins. Fractions of 8 ml were collected (LKB model 2111 Multirack fraction collector) at a flow rate of 30ml/hr maintained by a peristaltic pump (Econo-Column-Pump, Bio-Rad Laboratories, Richmond, CA). The UV absorbance at 280 nm was detected using a LKB model 2238 Uvicord SII and recorded (Linear, Canlab, Toronto). Fractions with the highest enzymatic activity (40-50 ml) were pooled and concentrated by ultrafiltration in a 50 ml Amicon cell through a Diaflow YM5 membrane (Amicon Co., Danvers MA) which has a molecular weight cut of 5000 Daltons.

3.3.4 Gel filtration

The concentrated extract (about 50-100 mg protein in about 5 ml) loaded on a LKB (Fisher Scientific Co., Winnipeg, Man.) Sephadex G-150 column (85x1.6 cm) (Pharmacia Chemicals, Uppsala, Sweden) equlibrated with 50 mM sodium phosphate buffer, pH 6.5, 0.1mM DTT, and 0.02% NaN_3. The flow rate was kept at 8 ml/hr. Active fractions (8 ml each) were collected, detected, pooled, and concentrated as above.

3.3.5 Affinity chromatography

About 5 ml of concentrated material was then diluted with the binding buffer, (0.1M Na acetate buffer, pH 3.5, with 1M NaCl, 0.1 mM DTT, and 0.02% NaN_3) and loaded onto a 15x1.6 cm LKB (Fisher Scientific Co., Winnipeg Man.) column of pepstatin A agarose (Sigma Chemical Co., ST. Louis Mo.) equilibrated with the same buffer. The column flow rate was maintained at 8 ml/hr. Once the sample was loaded the column was continuously washed with binding buffer until the absorbance at 280 nm had decreased to less than 0.05 A_{280}. At this point the elution buffer was used which contained 0.1 M Tris HCl, pH 8.6, 1M NaCl, 0.1 mM DTT and 0.02% NaN_3. The active fractions (about 4 ml each) were collected and concentrated to 5 ml as described above. The column was re-generated by washing with 10 x column volume of elution buffer, followed by 10 x column volume of binding buffer.

3.3.6 Protein determination

The protein concentration was determined using the Bio-Rad (Richmond, CA.) method based on Bradford (1976) against bovine serum albumin (BSA) standard using a Beckman model DU-50 (Beckman Instruments Inc., Fullerton, Ca.) or a Bausch & Lomb Spectronic 2000 (Rochester, NY) spectrophotometer at 595 nm.

3.3.7 Determination of enzyme purity and size

All electrophoretic separations were performed using the Hoefer Slab Gel Electrophoresis unit (Hoefer Scientific Instruments, San Fransisco, CA.) based on procedures of Laemmli (1970). Samples loaded on the gels were about 50 µg/ lane. The SDS and native PAGE gels were used to analyze the enzyme samples during each purification step. Both the SDS and native gels were performed according to manufactures instruction (Hoefer Scientific Instruments Catalogue 1983, San

Fransisco, CA.). The 10% SDS gels were run at 30 mA for 3 hours or until the methylene blue front reached the bottom. The standards used included BSA (66.2 kDa) , trypsin inhibitor (20.1 kDa), glyceraldehyde-3-phosphate dehydrogenase (36kDa) and ∝-lactalbumin (14 kDa). The 10% native gels were run at 30 mA for 5 hours or until the dye front reached the bottom. Standards used were: BSA (132 and 66 kDa), and ∝-lactalbumin (14kDa). The gels were fixed, stained with Coomassie blue and destained using standard methods (Hoefer Scientific Instrument Catalogue, 1983).

3.3.8 Inhibitor and effector studies

The inhibitor and effector influences were examined according to the modified method of Leluk et al. (1983). The enzyme (20-50µg/100ul) was pre-incubated with the various effectors for 30 minutes at 20 °C in 0.1 M Na acetate/HCl buffer pH 3.6 prior to substrate addition and incubation. All the divalent metal ions were at a concentration of 2 mM in the pre-incubation mixture and at 0.5mM during incubation at 30 °C. Metallic cations Ca, Cu, Mn, Co(as chloride), Mg, Zn, Hg (as acetate), Fe (as sulphate) were also tested.

The trypsin inhibitor from soybean (STT), and the bovine pancreas (Kunitz) were pre-incubated in the same manner as the divalent metal except at a weight ratio of 20:1. The remaining effectors; IAA, leupeptin, bestatin, pepstatin A, PMSF, TPCK, phenanthroline, DTT, pCMBS, and EDTA were pre-incubated as above, at a final 1mM concentration. After the pre-incubation, the samples were incubated for 30 minutes and proteolytic activity was determined as described (3.3.7).

3.3.9 Analysis of hemoglobin digestion products.

The mixture of the proteinase and hemoglobin was incubated for periods up to 12 hours. Ten µl aliquots were taken at 0.5, 2, 6, and 12 hours and prepared for SDS / urea gels. The seperation of the digestion products was done according to Koehler and Ho (1988) using a slightly modified method by Swank and Munches (1971). The Hoefer vertical slab gel unit (Hoefer Scientific Instruments, San Fransisco, CA.) was used to make 13x13 cm gels. The 20 µl samples were separated 30 mA until the dye front reached the resolving gel and then run at 60 mA for about 6 to 8 hours at 4°C or until the bromophenol blue dye front reached the bottom. The gels were stained and destained using the procedure outlined in the Hoefer Scientific Instrument Catalogue (1983) for standard SDS-PAGE gels.

The remaining portions of samples digested for 12 hours (1.4 ml) digestion mixture were kept at 4°C in the presence of 10 µM pepstatin A to inhibit further digestion and loaded onto a 16 x1.6 cm column of Bio-Gel P-2 (Bio-Rad, Richmond, CA). The column was equilibrated with 95 mM Na acetate/HCl (pH 5.0) and 0.02% NaN_3 and the sample was eluted at 3 ml/hour. The 0.75 ml fractions were collected using the Multi Rack fraction collector previously described and assays for ninhydrin reactive amino nitrogen groups (A_{570}) were performed as described Koehler and Ho (1988).

3.3.10 pH optimum

The enzyme (50µg/100ul) was incubated at different pHs with 2% hemoglobin substrate between 3.0-4.5 with 0.1 M Na Acetate/ HCl buffer 5.0-7.0, 0.1M Na phosphate buffer and 7.5-8.5 0.1 Tris/HCl buffer. Proteolyic activity was determined following 30 minutes incubation at 30°C.

3.4 RESULTS

3.4.1 Pepstatin-sensitive proteinase purification

The highly selective pepstatin A inhibited about 90 % of the hemoglobin digesting activity at pH 3.7 in the crude extract of resting seeds. The sensitivity to this inhibitor was used subsequently to distinguish this particular protease, only data for the pepstatin sensitive enzyme is presented.

The steps of purification are summarized in Table 3. After extraction, the sample was chromatographed by anion exchange on DEAE agarose at pH 8.0 where elution of the activity with a salt gradient (0-0.7 M NaCl, Fig. 8) Recovery of the pepstatin-sensitive proteinase at this point was 78.7 %. The sample was concentrated down to approximately 5 mls for gel filtration.

Gel filtration of the active ion exchange fractions at pH 6.5 resulted in at least three broad, overlapping peaks (Fig. 9). The activity was most concentrated in the area of highest optical absorbance (Fig. 9) and trailed off slowly. Fractions 9 through 17 were collected for further purification. At this point there was an 83.53 % yield of the activity. The slight unexpected increase in total enzymatic activity was found only after gel filtration regardless of whether the step was before or after DEAE separation. The acidified active fractions were loaded onto the pepstatin A-agarose. The absorbing

material recovered in pH 8.6 was collected (tubes 25 through to 32, 31.3 % recovery) and concentrated for more detailed characterization (Table 3).

3.4.2 Proteinase purity and size

During the purification, the protein was visualized on 10 % non-denaturing PAGE. One major band obtained after the pepstatin A-agarose chromatography step suggesting a large complex (Fig.10). Using denaturing 10% SDS-PAGE electrophoresis, 7 dissociated peptide bands were seen (Fig.11). Not all the bands had the same intensity. There molecular weights determined from the gel ranged from; A, 56kDa; B, 54kDa; C, 50kDa; D, 35kDa; E, 31kDa; F, 25kDa; and G, 23kDa.

3.4.3 Effect of inhibitors

At a 2mM concentration; $CaCl_2$,$CuCl_2$, $ZnSO_4 \cdot 7H_2O$ and $MgSO_4 \cdot 7H_20$ had no apparent effect on the enzyme activity, while the $CoCl_2$ and $MnCl_2$ enhanced the activity by 50 to 100 % (Table 4). $HgCl_2$ was the only divalent metal to have any inhibitory effect. Of the cysteine proteinase inhibitors (IAA, leupeptin, TCPK, pCMBS) only leupeptin showed any inhibiton (about 90%) which was not due to the solvent. The TPCK and pCMBS inhibiton was apparently due to their solvent. Among the serine proteinases inhibitors; leupeptin, PMSF, TPCK, STI and ETI, only the leupeptin exhibited any effect on the enzymatic activity. The PMSF and TPCK inhibitory action could also be accounted for by the ethanol used to dissolve the inhibitor. Bestatin, an aminopeptidase inhibitor, had no effect. The metalloprotease inhibitor o-phenanthroline's inhibition could also be accounted for by the ethanol solvent. DTT and EDTA did not seem to have any visible effect on the enzyme. The ethanol solvent at a final pre-incubation concentration of 1% inhibited up to 50% of the

enzymatic activity while a concentration of 0.7% had less than 10% inhibition, and finally the DMSO (at 1%) also inhibited the actvivity by about 10%.

3.4.4 Influence of pH on enzymatic activity

The maximum activitiy was measured at pH 3.5 (Fig.12). However, hemoglobin denatures at pH below pH 4.5 (Salmia et al., 1978) and this may not reflect the true enzymatic maximum activity, but greater accessibility of the substrate. The activity continued to decrease above pH 6.5 through to pH 8.5 (data not shown) with no indication of a second optimum.

3.4.5 Stability of the proteinase

The enzymatic activity of extract was tested over time at different pHs (3.5,5.0, and 8.0), with or without 0.5 M NaCl (data not shown). The extract was most stable at pH 5.0, in 0.5 M NaCl. Under these condition it did not lose any activity after 6 days at 4 ^{o}C. At pH 8.0 or at pH 3.5 the sample containing salt lost only about 5% of activity, while samples without salt lost more than half of their activity. The heat stability of the enzyme, was also investigated at different pHs. The enzyme was preincubated at pH 3.5, 5.0, and 8.0 at temperatures of 15, 30, 45, and 60 ^{o}C for 30 minutes. At lower temperatures, the enzyme was most stable at pH 3.5 but at the higher temperatures, pH 5.0 resulted in the greatest stability.

3.4.6 Hemoglobin digestion product analysis

The hemoglobin digestion products were characterized, using the enzyme after the first step of purification, (DEAE-anion exchange, Fig. 13) and after the final step of purification (pepstatin A affinity chromatography, Fig.14). The hemoglobin monomer, (14.4 kDa), was quickly digested into several bands by the ion-exchange purified preparation. As for the more purified enzyme hemoglobin digestion proceeded more slowly, via two defined intermediate digestion peptides, and producing only one final band (arrow) after 12.5 hours of incubation. Further analysis of the digestion products on Bio Gel P-2 are shown in Fig. 15. A large peak eluted between fractions 17 and 24 corresponding to the non-digested hemoglobin. When the hemoglobin was incubated with the purified enzyme the peak in these fractions decreased while an additional peak appeared between fractions 35 and 42, co-eluting with labeled ^{3}H leucine. Finally, the ion-exchange purified enzyme digested hemoglobin produced a broader peak between fractions 20 and 30 and an additional peak between fractions 35 and 42. This latter peak co-eluted with ^{3}H leucine.

Table 3. Purification of the pepstatin-sensitive proteinase from dry *Pinus banksiana* seeds. The resting seeds were extracted according to the 'Materials and Methods' and the enzymatic activity was determinaed at each purification step using hemoglobin as substrate.

Purification Step	Total Activity Units [1]	Total Protein mg	Specific Activity Units /mg	Total Activity Yield %	Purification -fold
1. Crude extract	1237.0	207.0	6.0	100.0	1.0
2. DEAE-Anion Exchange	974.3	53.5	18.2	78.7	3.1
3. G-150 Gel Filtration	1033.9	22.4	46.0	83.5	7.8
4.Pepstatin A-Affinity Chromatography	387.4	0.5	700.7	31.3	117.8

[1]One unit for proteinase activity is one A_{750} equivalent to 1μg of BSA/ min

Figure 7. Separation of pepstatin -sensitive proteinase by anion exchange. The 90 ml of extract from resting seeds was chromatographed on a 40x2.6 cm DEAE-agarose anion exchanger column. The salt gradient was started once the A_{280} had decreased below 0.05 OD indicated by the arrow. A_{280} (—) and the proteolytic activity (-●-) at pH 3.5 was determined on 8 ml fractions of the eluate as described in 'Materials and Methods'. Fractions from 52 to 57 indicated by the horizontal bar were pooled for further purification.

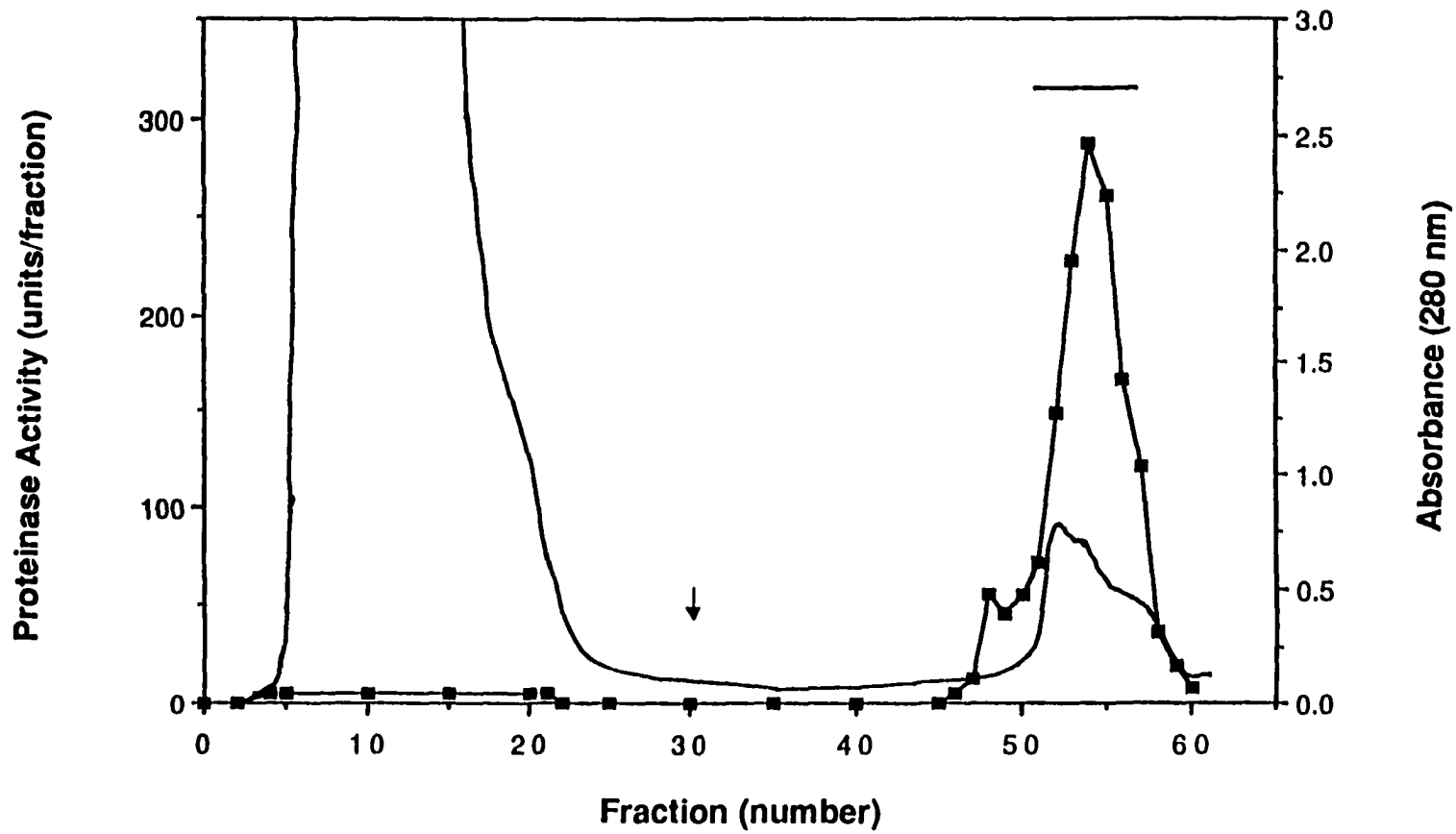


Figure 8. Separation of pepstatin-sensitive proteinase by gel filtration. The fractions collected from the anion exchange step were pooled, concentrated (5 ml) and loaded on an 8.5 X 1.6 cm G-150 Sephadex gel filtration column equilibrated with 50mM Na phosphate buffer, pH 6.5, 0.02 % NaN_3 and 0.1 mM DTT at a flow rate of 8 ml/hr. A_{280} (—) and proteolytic activity (–●–) at pH 3.5 was determined on each fraction as described in the 'Materials and Methods'. The active fractions 9 through to 17 as indicated by the bar were collected, pooled and pH adjusted for further purification.

Figure 9. Separation of pepstatin-sensitive proteinase by affinity chromatography. The fractions collected from gel filtration were pooled and adjusted with 0.1 M Na acetate/HCl buffer, pH 3.5, 1M NaCl, 0.1 mM DTT, and 0.02 % NaN_3. The solution was then chromatographed on a pepstatin A agarose affinity column equilibrated with the adjustment buffer at a flow rate of 8 ml/hr. Once the A_{280} decreased to levels below 0.05 A_{280} the eluent buffer 0.1 M Tris/HCl, pH8.6, 1M NaCl, 0.1mM DTT and 0.02 % NaN_3 was added indicated by the arrow and 4 mls fractions were collected. The proteolytically active fractions (indicated by the horizontal bar) were pooled and concentrated as described in the 'Materials and Methods'.

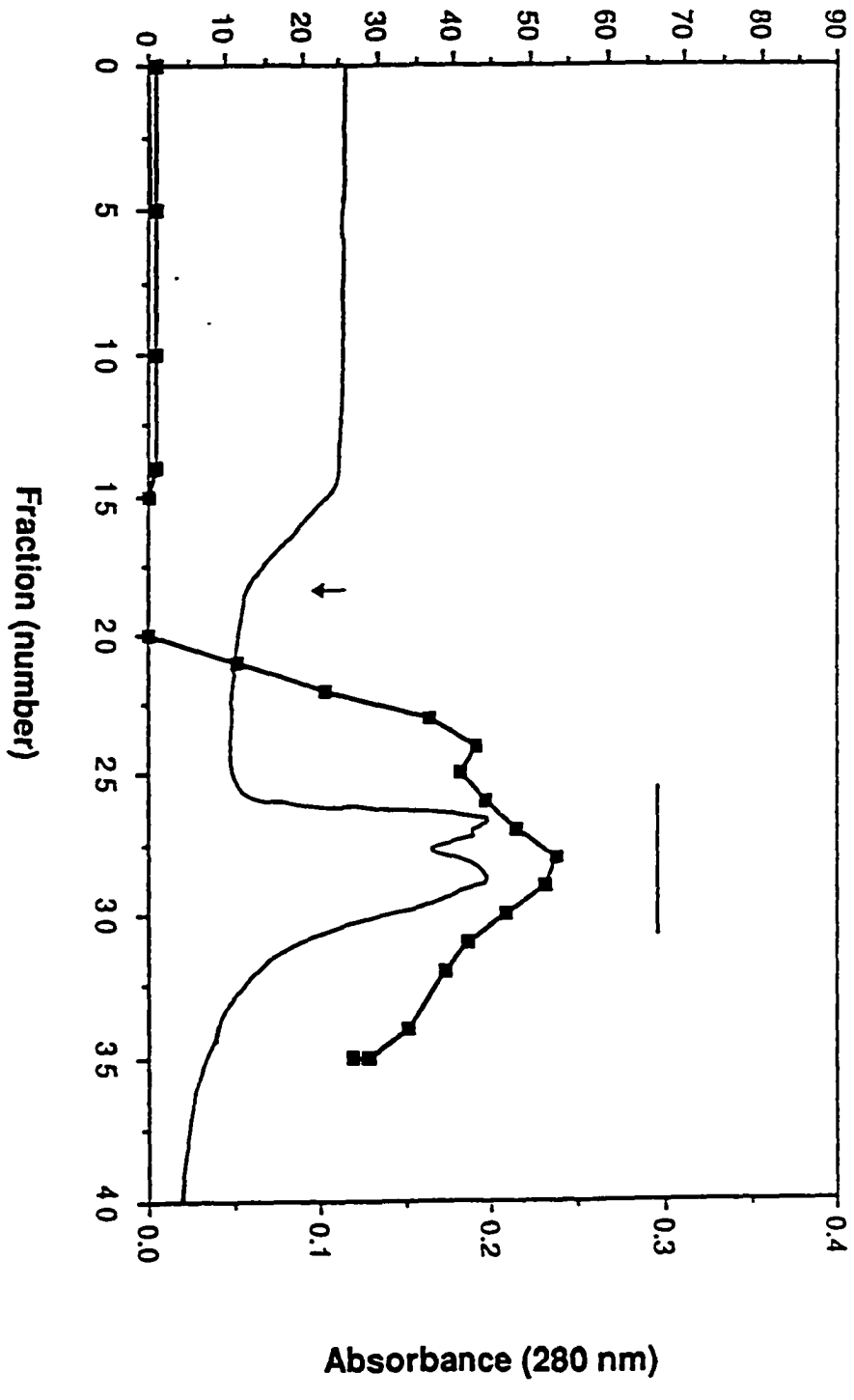


Figure 10. Electrophoretic analysis of native protein at each purification step as described in 'Materials and Methods' of the Pepstatin-sensitive proteinase. The samples were analyzed by non-denaturing 10% PAGE made visible with Coomassie brilliant blue R-256 as described in 'Materials and Methods'. Lane 1 represents the proteins from crude extract; lane 2 the pooled fractions after DEAE-agarose anion exchange, lane 3 material collected after G-150 Sephadex gel filtration; lane 4 purified proteinase from pepstatin A-agarose affinity chromatography. Lane 1 contained about 80 μg of protein lanes 2, 3, and 4 contained about 50 μg protein. The migration of the molecular weight markers; BSA (132kDa, and 66kDa), and ∝-lactalbumin (14.2kDa) are indicated to the left while the purified peptide is indicated at the right (arrow at 140 kDa).

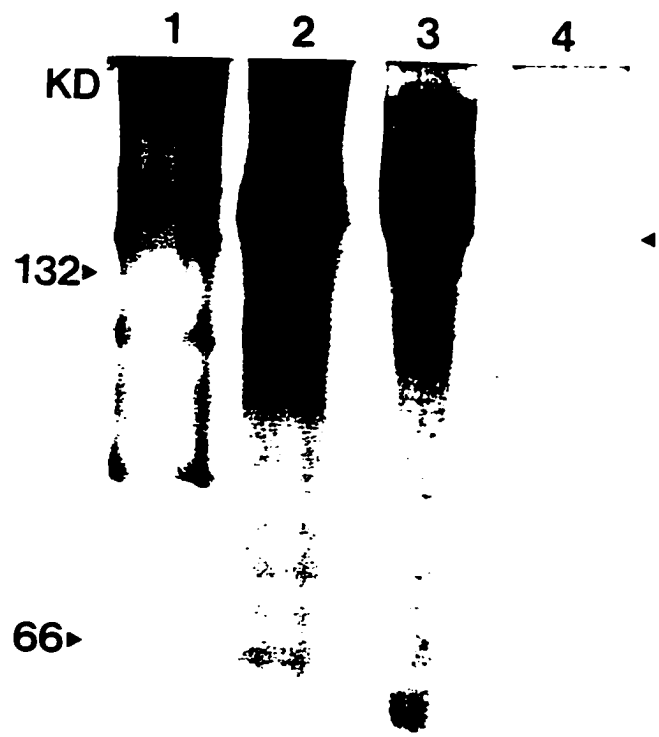


Figure 11. Electrophoretic anaiysis of SDS-denatured protein at each purification step of the pepstatin-sensitive proteinase. The samples were analyzed by SDS-PAGE on 10 % gels and made visible with Coomasie brilliant blue R-256 as described in 'Materials and Methods'. Lane 1 represent the peptides from crude extract; lane 2 the pooled fractions after DEAE-agarose ion exchange ; lane 3 material collected after G-150 Sephadex gel filtration; lane 4 purified proteinase from pepstatin A-agarose affinity chromatography. The migration of the molecular weight markers; BSA (66 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa),trypsinogen (24 kDa) and soybean trypsin inhibitor (20.1 kDa) are indicated to the left. All lanes contained about 50 μg of protein. Purified sample bands are indicated at the right; A, 56kDa; B, 54kDa; C, 50kDa; D, 35kDa; E, 31kDa; F, 25kDa; and G, 23kDa.

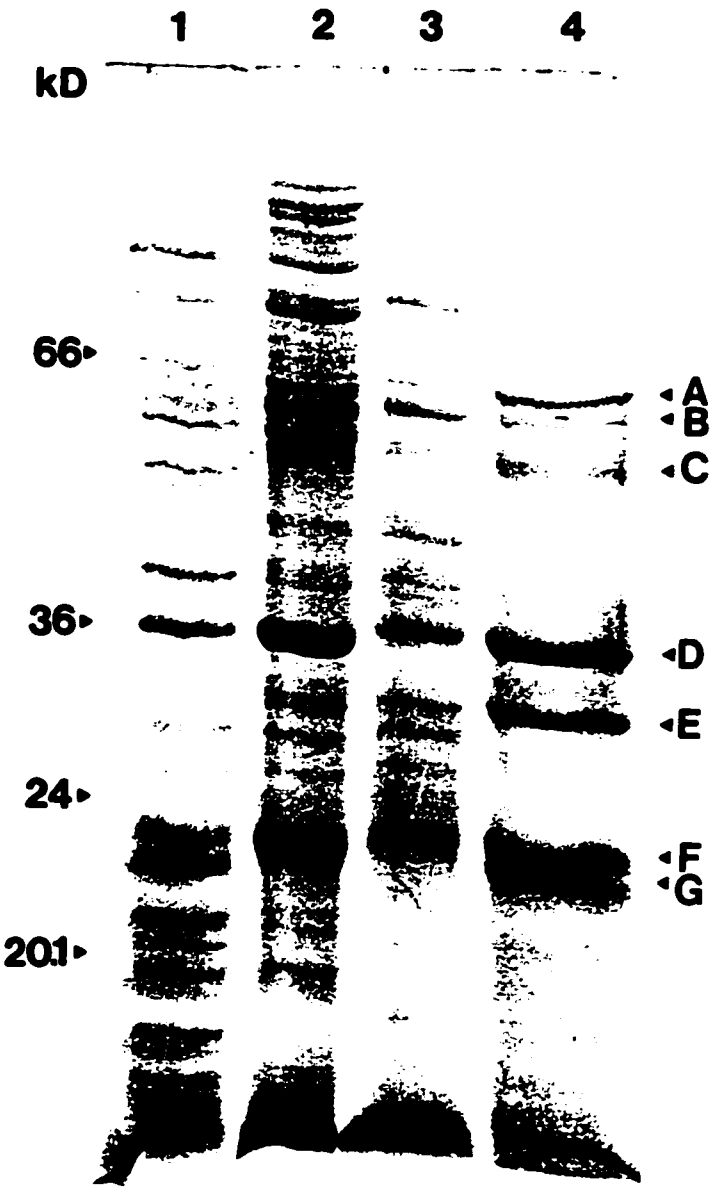


Table 4. Influence of selected potential effectors on the dry seed pepstatin-sensitive proteinase activity. The concentrated proteinase preparation, 20 μg protein/assay tube, was pre-incubated with all effectors for 30 min at room temperature. In the preincubation the divalent metal had a concentration of 2 mM, STI and ETI were in a ratio of 20:1 (400ug/tube) to the activity, while all other inhibitors were concentration of 1mM final concentration.

Effector	Activity % of control[1]
$CaCl_2$	102.7
$CuCl_2$	122.7
$CoCl_2$	186.1
$HgCl_2$	58.5
$MnCl_2$	177.7
$ZnSO_4 \cdot H_2O$	99.9
$MgSO_4 \cdot H_2O$	100.7
IAA	98.5
Leupeptin	11.5
Bestatin	108.7
Pepstatin A (0.7% ethanol)[2]	2.5
PMSF (1% ethanol)[2]	48.6
TPCK (1% ethanol)[2]	56.3
o-Phenanthroline (1% ethanol)[2]	43.3
pCMBS (1%DMSO)[2]	91.8
STI	111.5
ETI	124.9
DTT	92.2
EDTA	90.7
1% Ethanol	53.3
0.7% Ethanol	88.8
1% DMSO	93.7

[1]Each value is an average of 3 replicates of at least 2 extractions.

[2]Concentration of solvent in the pre-incubation mixture.

Figure 12. Activity of pepstatin-sensitive proteinase at varying pH. The concentrated enzyme preparation collected from the final affinity chromatography step of purification was tested at different pH levels. All buffers used were at 0.1 M concentrations. The following buffers were used: pH 3.0-4.5 Na-acetate/HCl, 5.0-7.0 Na phosphate, and 7.5-8.5 Tris/HCl.

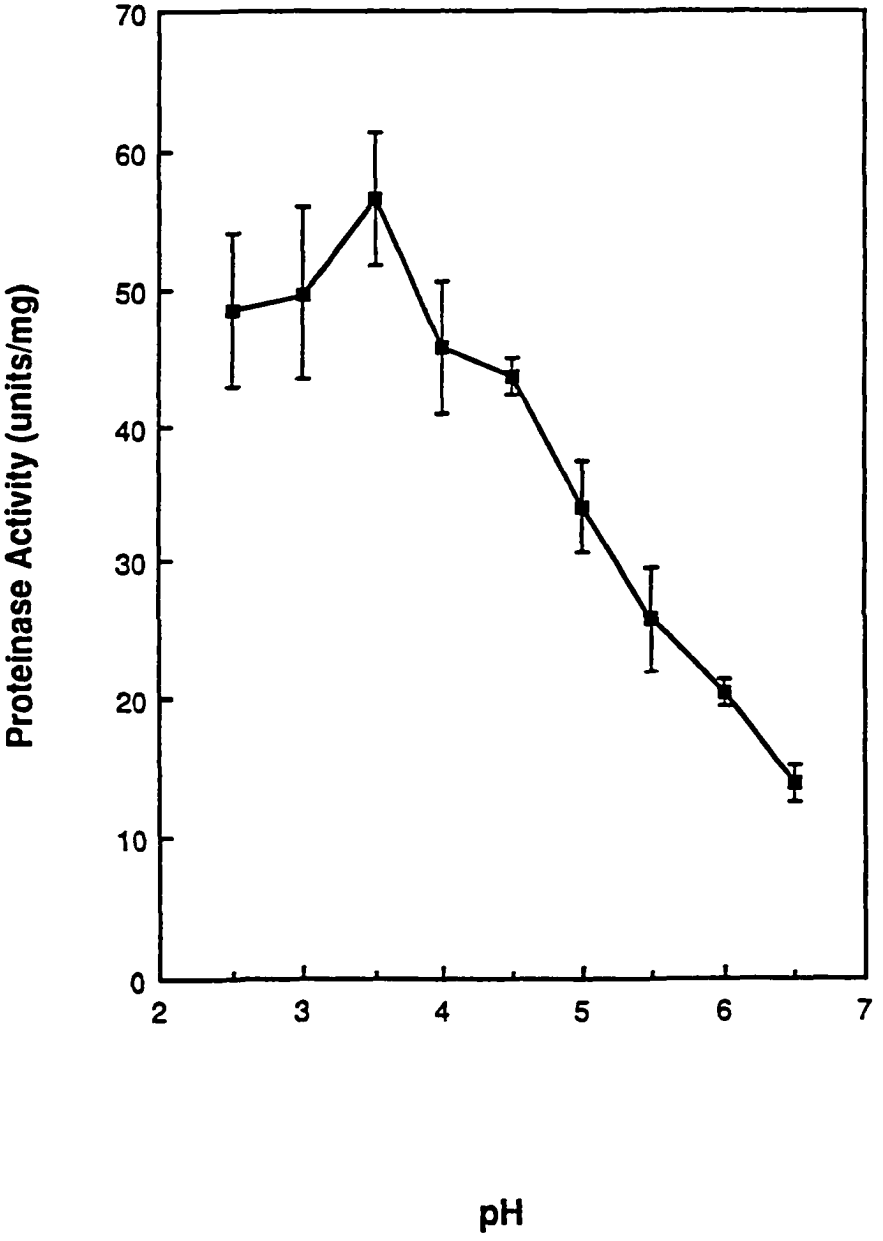


Figure 13. SDS/urea PAGE separation of hemoglobin proteolysis products by enzyme purified beyond DEAE-ion exchange stage. Hemoglobin was incubated with 20 μg of the enzyme extract as described in 'Materials and Methods'. Protein in lanes (1-5) was incubated for 0, 0.5, 2, 6, 12 hours respectively, 50 μg (10 μl) aliquots from each time was prepared for electrophoresis. The hemoglobin monomer is indicated at 14.4 kDa while smaller peptides represent digestion products.

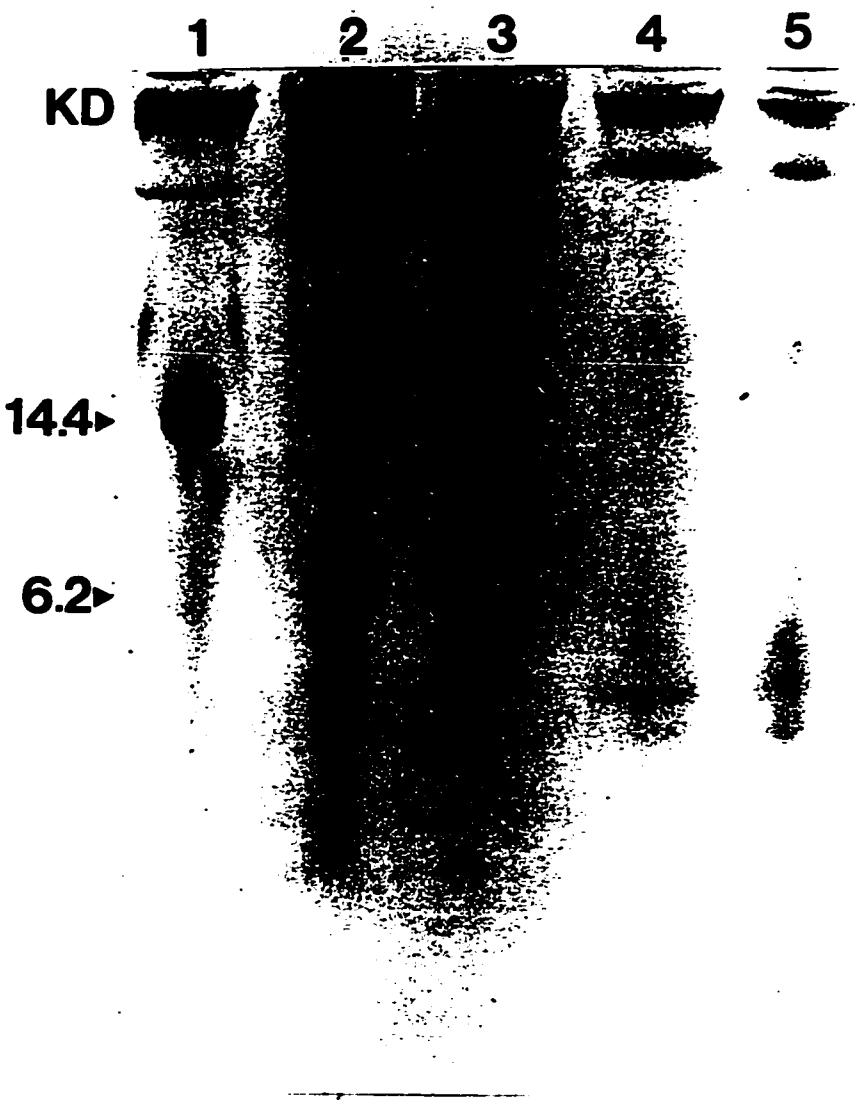


Figure 14. SDS/urea PAGE (10%) separation of proteolytic products after digestion with purified pepstatin-sensitive protease. Hemoglobin was incubated with 20 μg of the enzyme as described in 'Materials and Methods'. Lanes (1-5) were incubated for 0, 0.5, 2, 6, 12 hours respectively, 50 μg (10 μl) aliquots from each time was prepared for electrophoresis. The hemoglobin monomer is indicated at 14.4 kDa while smaller peptides represened by arrows are digestion products.

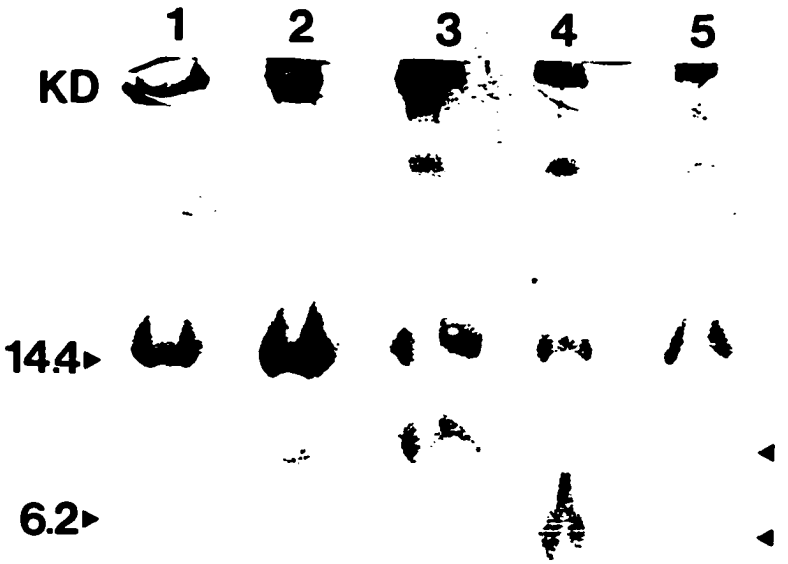
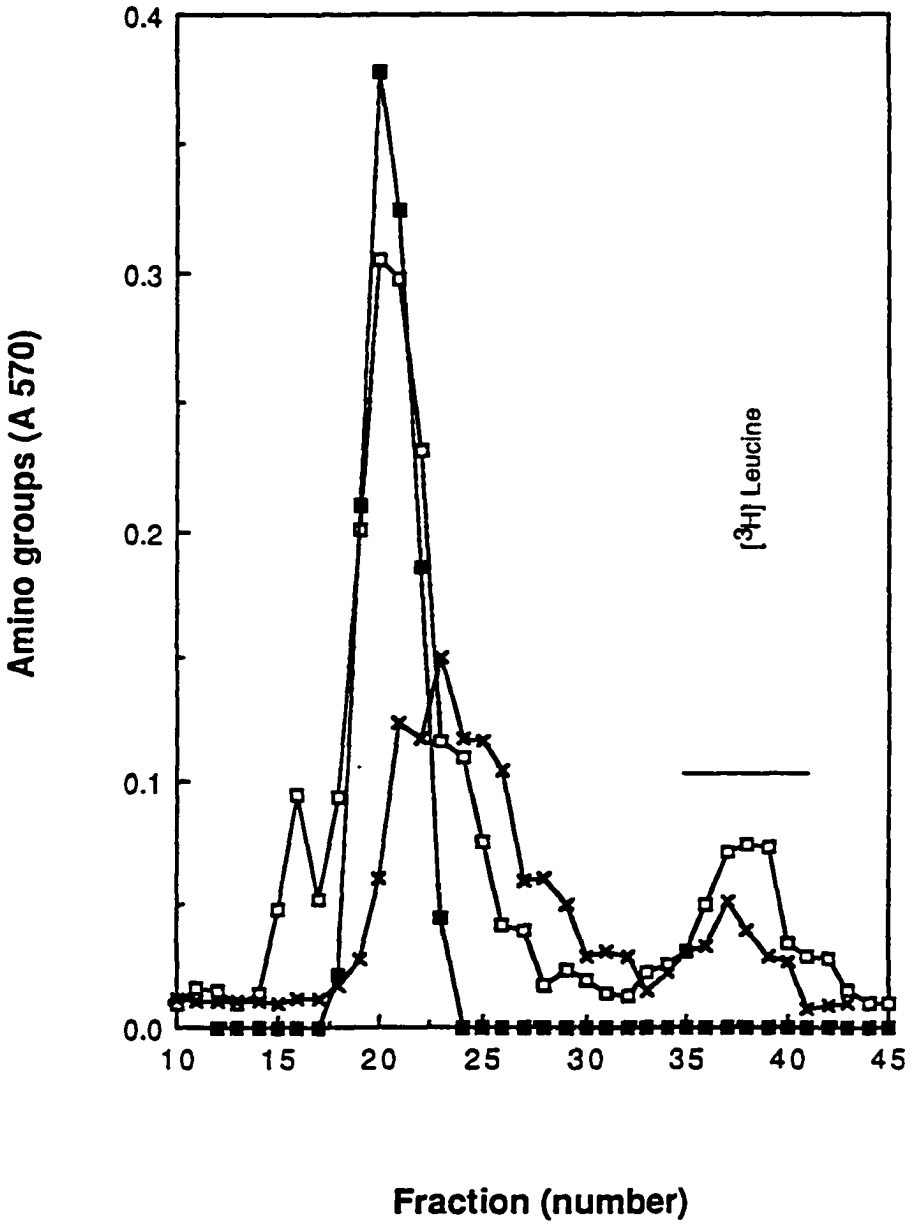


Figure 15. Separation of digestion products after 12 hr proteolysis by BioGel P-2 chromatography. The substrate, hemoglobin, was incubated as described in 'Materials and Methods' in the absence (—●—), presence of pure enzyme (—○—), and DEAE-ion exchange purified enzyme (—x—). The incubation mixture (0.8 mls) was spiked with 10 μCi of [^{3}H] leucine prior to loading as described in 'Materials and Methods'. Aliquots of 100 μl from each 0.75 ml fraction were assayed for radioactivity and for amino nitrogen by the ninhydrin reaction. The radioactive leucine elution is indicated by the bar.



3.5 DISCUSSION

Several proteolytic activities and their inhibitors have been observed in resting and germinating Scots pine seeds (Salmia and Mikola, 1975; Salmia and Mikola, 1976a; Salmia and Mikola, 1976b; Salmia and Mikola, 1980; Salmia, 1981a; and Salmia, 1981b). One particular proteinase investigated was a pepstatin-sensitive enzyme (Salmia et al., 1978; Salmia, 1980; Salmia and Mikola, 1980; Salmia, 1981a; and Salmia, 1981b). This proteinase activity has now also been detected and purified Jack pine seeds.

The related enzyme identified in *Pinus sylvestris* L. (Salmia, 1981) lost activity when dialysed and therefore this step was avoided in this work with Jack pine seeds. The enzyme did not bind to anion exchange media at pH 3.5, but did so at pH higher than 7.5 and was released using a salt gradient. The fractions with the highest enzyme activity eluting from the gel filtration came after the void volume peak and the activity trailed off into the third peak (Fig. 8) suggesting that the protein has a large molecular weight. The total activity after this step was higher than that following anion exchange (Table 3). This may be a result of some inhibitors being released from the enzyme during this separation.

At the final purification step, affinity chromatography on pepstatin A agarose, a large increase of specific activity was expected. However, the required change of pH

from 6.5 to 3.5 for matrix binding resulted in a loss in activity. This loss might have been due to two major factors. First, the loading of the protein onto the column took approximately 8-10 hours. The enzyme is most active at this pH and may have been self-hydrolysing. Secondly, the change in pH also prompted a precipitation after an hour or so. This precipitate may be accounted for by either the pH changes directly, or be due to self degradation at its optimal pH. In both cases, producing a change in K_{sp} and a subsequently change in conformational structure resulting in its coming out of solution. In order to reduce this precipitation problem prior to affinity chromatography, the pH was only brought down to 4.3 instead of 3.5. In this case, one peak came off after elution with pH 8.6 buffer, but there was very little to no activity found in these fractions. The initial eluate also contained no activity, but after decreasing its pH from 4.3 to 3.5 and reloading it on the column another non-active peak was eluted at pH 8.6. When the two peaks eluted a pH 8.6 were combined a slight rise in the almost nil activity was seen (results not shown). Both overlapping peaks seen in Fig. 10 may be needed to contribute to the activity. If considered as subunits of the same enzyme, one may induce a required conformational change on the other, therefore increasing its activity by either exposing its active site or changing the active site's shape. Additional experiments with preparative gels were understanding. The results did not contribute to purification and are described in the Appendix.

Electrophoresis of the enzyme under denaturing conditions, dissociated into 7 bands (Fig. 11 bands A to G). The partial loss of bands D, F, and G after gel filtration may account for the activity being found throughout a large number of fractions during this extraction step where a complex of proteins may be dissociating under these conditions (Fig. 9). The large number of bands seen after affinity chromatography may be due to two possibilities. Firstly, the activity may correspond to a complex of subunits or secondly the precipitation occuring when the pH is lowered prior to affinity chromatography caused non-specific binding of proteins to the protease prior to binding

to immobilized pepstatin A. If the binding was random and non-specific, the SDS pattern should not be reproducible. When the purified enzyme was electrophoresed on native PAGE, only one band was apparent (Fig. 12). This finding supports the contention that a complex was purified by affinity chromatography. If the complex was resulting from non specific binding at pH 3.5, it would be expected to dissociate at pH 8.6 as the bound protein is released from the affinity matrix and appear as separate band on a native gel. Some of the individual components of the complex may be endogenous substrate. Competitive inhibition by endogenous substrates may explain the relatively low yield of activity following affinity chromatography. The time-dependent precipitation may also be a reflection of a partial substrate hydrolysis, in analogy to chymosin attack on milk caseins (Kay, 1985).

The enzyme complex can be considered homogeneous, based on Fig 11. Immunological studies could now be performed. Production of a polyclonal mixture of IgG similar to those methods used by Kushner et al. (1964) and Jensen et al. (1982) may be appropriate. Immunoglobulins obtained could be used to isolate the enzyme more directly and efficiently by immuno adsorbent affinity (Paus, 1976). These IgG's could also be used to visualize the protein on electron micrographs of the seeds. Several probes have been used in such immunocytochemical techniques (Alexander et al., 1985).

The inhibitor sensitivities have been well characterized for enzymes of animal but not of plants origin (Barret, 1986). The enzyme purified was most sensitive to pepstatin and ethanol solvent (Table 4) suggesting that the activity is due to an aspartate proteinase. Unfortunately, ethanol was used to dissolve several of the inhibitors therefore 50% of the inhibition of the o-phenathroline, TPCK, IAA, PMSF, and TPCK, can be accounted for by the solvent alone. The only inhibitor, leupeptin, (generally considered to be cysteine or serine protease specific) nearly completely inhibited the enzyme. The lack of inhibition by the aqueous STI and ETI supports the

contention that ethanol was the effector decreasing the activity with the PMSF, and TPCK inhibitors. The enzymatic activity is also probably not a metalloprotease since EDTA had little inhibitory effect. Finally, the enzyme sensitivity to Hg^{2+} but a lack of response to IAA and pCMBS, which are all considered cysteine protease inhibitors, has no apparent explanation (Wagner, 1986). The hemoglobin digestion activity apparently consists mostly of an aspartic protease with some additional activity perhaps as a result, of a serine or cysteine.

One curious aspect of the results is the marked increase in TCA soluble products produced in the presence of $CoCl_2$ and $MnCl_2$. The enhancement of activity may be explained in two ways. First, the enzyme may be stabilized by these heavy metals. Secondly, the hemoglobin structure could be particularly affected by the presence of these metals at pH 3.7 increasing its susceptibility to the protease. The optimum hydrolysis activity of the enzyme appears to be about pH 3.5 (Fig. 12). This may not be a true catalytic pH optimum but a combination of increased activity and increased scissile bond accessibility (Salmia et al., 1978) due to the denaturation of the protein substrate. It is safe to conclude that the overall activity is optimal in an acidic range. Above pH 6.5, the activity continued to decrease (to zero at pH 8.5) suggesting that only one enzyme is present. This acidic pH optimum is similar to that found in *Pinus sylvestris* (Salmia et al., 1978), as well as *Cucumis sativus* (Wilimowska-Pelc et al., 1983).

The conditions for best long term storage of the enzyme activity was similar to that found for *Cucumis sativus* (Wilimowska-Pelc et al., 1983) except that the enzyme investigated here was more sensitive to the more alkaline solution. If the enzyme is self-hydrolyzing the ideal storage conditions would decrease its activity without destroying its conformation. Storage at low pH's may promote autohydrolysis and, therefore, destroy its own activity, while high pHs disrupting it's active configuration permanently. The activity of the enzyme is less than 60% of the maximum at pH 5.0

and salt is known to disrupt hydrostatic bonds, therefore these two factors may account for the enzymes stability without the loss of activity under these conditions.

Since inhibitor studies did not result in a clear categorization of the protease, the hydrolysis products of hemoglobin (Hb) substrate were investigated (Figs. 13, 14, and 15). Enzyme at two levels of purity was tested. A definite mixture of activities was present in more crude extract since both the electrophoresis gel and gel filtration chromatography show a series of peptide sizes ranging from smaller than hemoglobin monomer substrate to amino acid size. Pure enzyme resulted in a large peptide similar in size to hemoglobin monomer and some the size of amino acids. The BioGel P-2 molecular weight exclusion limit is 1000 daltons, the Hb tetramer, monomer and digestion products are greater than this size and will all elute in the void volume. Depending on the resolution of the gel, the "amino acid" peak co-eluting with ^{3}H leucine may also contain some small peptides comprising of a few amino acids. Therefore the digestion products of hemoglobin by the purified enzyme may be a consequence of either of two types of digestion. First, if digested by an endopeptidase, very long and very short peptides may result. Alternately, if the activity was an exopeptidase, progressively smaller peptides would be produced resulting in a smear in Fig. 14 and 15. During digestion the substrate is denatured and all bonds should be relatively accessible, therefore the only factor affecting the rate of peptide production would be the reaction rate. Further investigation of the digestion products should be using specific amino acid labelling (Glazer et al., 1975) of the hemoglobin substrate. This would produce a digestion profile which would reveal whether the digestion is happening at specific points along the chain or only at the ends.

Preliminary investigation on protein reserve mobilization by proteinases in conifers has been for the most part restricted to Scots pine (Salmia and Mikola, 1975; Salmia and Mikola, 1976; Salmia 1981; Salmia et al., 1978; Salmia and Mikola, 1976; and Salmia, 1981). The peptidases endogenous substrates and potential roles within

the seed has not been extensively reported. Methods developed by Koehler and Ho (1988) have shown substrate digestion by an endopeptidase in barley seeds. This does not explain, however, the role of the enzyme in the seed. Preliminary germination experiments have shown that pepstatin retards the radicle elongation. The isolation of different storage proteins from the seed (This et al., 1988) and their incubation with peptidases under similar conditions as performed on mung bean by Baumagartner and Chrispeels (1977), on field bean by Lichtenfeld et al. (1981), and on barley (Mikola et al. pers. comm., 1988) would possibly provide evidence to determine the identity of endogenous substrates of the pepstatin sensitive enzyme isolated.

Metabolic control mechanisms of the enzymes involving endogenous inhibitors should also be investigated. In the seed, the enzyme activity will probably be localized in the acid environment of the protein bodies or vacuoles, separated form the cytoplasm. Such studies would concentrate on identifying endogenous inhibitors which are present in developing or resting seeds. Preliminary studies carried out on conifer seeds by Salmia and Mikola have not revealed any endogenous heat tolerant inhibitors specific towards the pepstatin-sensitive activity. However, if subsequent studies on the Jack pine seeds do reveal endogenous inhibitors (possibly heat intolerant), they too could provide clues to the role of the enzyme during germination. More extensive studies of this kind have been performed on a trypsin-like activity (BAPNA hydrolyzing) and its endogenous inhibitors in mung bean and soybean (Baumgarther and Chrispeels, 1976; Chrispeels and Baumgarther, 1978; Lorensen et al., 1981; and Tan-Wilson et al., 1982; Wilson and Chen, 1983; Wilson and Tan-Wilson, 1983; Wilson et al., 1985). Other seed endogenous inhibitors have been studied to a lesser extent form other sources (Salmia, 1980; Salmia and Mikola, 1980; Leluk et al., 1983; Hayashi, 1987). The aspartate enzyme could therefore be involved in activating other proteases within the protein body by specificly cleaving areas of the protein chain or degrading bound inhibitors. If the enzyme is stricly self-hydrolyzing

it may provide amino acids for the *de novo* synthesis of other proteases and other metabolic enzymes.

In this study the isolation and purification of an acid protease activity from Jack pine seeds was accomplished. The results suggest that in fact the enzyme activity has an optimum pH in the acidic range, and is inhibited by pepstatin and leupeptin, inhibitors of both aspartate and cysteine or serine enzymes. It also degrades hemoglobin in a heterogeneous manner apparently consisting of endo- and exo-proteolytic activities. This suggests that the activity isolated from the Jack pine seeds may not fit the conventional classification rules of the enzymes isolated from animal tissues.

APPENDIX

In addition to these purification steps, trial preparative polyacrylamide gels were performed. Polyacrylamide gel electrophoretograms have been used in the detection of many types of activities. Casein and other zymograms have been used by Westergaard et al., 1980; and Every, 1981. Direct detection of proteases has been achieved by separating them on agar electrophoresis gels, immersing the gel in substrate solution and staining with a general protein dye (Uiel, 1960; and Kushner et al., 1964). Using this process active areas are seen as spots cleared of substrate protein. This particular method has subsequently been modified for several proteinases and substrates (Foltmann, et al., 1985; and Samloff, 1969). Some have carried the method even further by converting these analytical methods to a preparative method (Braatz and McIntre, 1985 and Shuster, 1971). The yields from these methods however have been limited and leading some investigators to focus their efforts on producing a soluble gel which would yield higher amounts of the enzyme upon gel extraction (Hansen, 1981; and Hansen, et al., 1980).

The purified pepstatin A sensitive protease was electophoresed on a 10% native PAGE and incubated with an acidic hemogloglobin solution since the enzyme did not

digest casein at pH 5.5. However, activity was not detected on gels used under these conditions. It was subsequently found that activity was sensitive to the ammonium persulphate the gel. In order to avoid this problem, the gels were UV polymerized. UV polymerization was not suitable for soluble gels (Bio-Rad Laboratories, Richmond, CA) and for this reason trials with these gels were discontinued. Even after the switch from chemically to UV polymerized gels, no activity was recovered from the gels. This indicates that it may actually be the electrical current which was destroying the activity. To these problems, techniques involving radial diffusion (Santarius and Ryan, 1977; and Rudolph and Tauschel, 1982) may need to be further investigated. The activity seems to be very sensitive to the molecular weight seperation methods as noted during the gel filtration step where the activity trailed off extensively.

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