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Mungbean (*Vigna radiata* L. Wilczek) contains some high proteolytic activities already before germination

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Abstract. When casein was used as a substrate, mungbean seeds had higher proteinase activity levels based on g fresh weight or on mg protein before germination than after germination. While hemoglobin was used, the same was observed as based on g fresh weight but the opposite was observed as based on mg protein. Carboxypeptidase activities toward Hip-His-Leu or Hip-Arg at pH 7.5 based on g fresh weight were also higher before germination than after germination, but activity toward Hip-Arg based on mg protein before germination was lower than after germination. Before germination, when 4-nitroanilides were used, Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 3.4. Gly-Na, Phe-Na, Ala-Ala-Ala-Na, and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 7.0. Gly-Na and γ-Glu-Na were hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Phe-Nap, Leu-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 3.4 while Leu-Nap and Phe-Nap were hydrolyzed most rapidly at pH 7.0. Lys-Nap, Gly-Nap, and Ben-Arg-Mx-Nap were hydrolyzed most rapidly at pH 8.9. After germination, when 4-nitroanilides were used, Gly-Na, Phe-Na, Ala-Ala-Ala-Na, and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 3.4. Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 7.0. Gly-Na was hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Phe-Nap, Leu-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 3.4 and 7.0. Lys-Nap and Gly-Nap were leading substrates at pH 8.9. Mungbean enzymes hydrolyze derivatives of 4-nitroaniline and β-naphthylamine of amino acids and peptides differently at various pH values.

Keywords: Protease; Protein substrates; Synthetic substrates; Germination; pH.

Abbreviations: Ace, acetyl; **Ben,** benzoyl; **Cbz,** benzylcarbonyl; **Cp,** N-3-(carboxy propionyl)-; **Hip,** hippuryl; **Mx,** methoxy; **Na,** nitroanilide; **Nap,** naphthylamide.

Introduction

There are many reports of plant protease activities in the literature. Most reports are on legumes (Citharel and Garreau, 1987; Couton et al., 1991; Collier and Murray, 1977; Crump and Murray, 1979; Dunaevsky and Belozersky, 1989; Elleman, 1974; Mikkonen, 1986; Mikkonen, 1992; Wong, 1991; Wynn and Murray, 1985; Yamaoka et al., 1990; Yamaoka et al., 1994; Yu and Greenwood, 1994); some are on crops (Horiguchi and Kitagishi, 1976; Kolehmainen and Mikola, 1971; Vodkin and Scandalios, 1980; Waters and Dalling, 1983); some on vegetables (Kitamura and Maruyama, 1985; Lin and Chan, 1990); and a few reports are on others (Ninomiya et al., 1981; Pallavieni et al., 1981).

Protease activities in a variety of plants are reported in two books edited by Dalling (1986). Aspartic proteinase and some amino peptidase activities are present in ungerminated seeds. Some of these enzymes have been purified and cloned (Kolehmainen and Mikola, 1971; Ninomiya et al., 1981, 1983; Runeberg-Roos et al., 1991; Salmia and Mikola, 1975; Sarkkinen et al., 1992). Cysteine proteinase (Shutov and Vaintraub, 1987) and carboxypeptidases (Dunaevsky et al., 1987) are expressed in germinating and post-germinating seeds. Amino peptidases are expressed in sprouts of sweet potato (Lin and Chan, 1992). There are two major storage proteins in soybean (*Glycine max* [L.] Merrill) seed, glycinin and β-conglycinin. During germination and early growth, these storage proteins are degraded by proteolysis. The predominant pattern is one of limited proteolytic cleavage by proteases specific to the reserve protein, followed by more rapid proteolysis by less specific proteases (Muntz et al., 1985; Shutov et al., 1982). Several soybean proteases have been described, including six proteolytic enzymes from ungerminated seeds separated by anion-exchange chromatography (Wei et al., 1966), two carboxypeptidases from germinating soybean (Kubota et al., 1976), two endopeptidases, one exhibiting an acidic pH optimum and the other a basic pH optimum (Bond and Bowles, 1983), and a trypsin-like protease (Nishikata, 1984).

Many reports also deal with mungbean, especially with cotyledons of mungbean. Results have suggested that the appearance of a sulfhydryl-type endopeptidase activity is a necessary prerequisite for the rapid metabolism of the reserve proteins which accompanies the germination of mungbean (Chrispeels and Boulter, 1975). Results have also suggested that new enzymatic activities not present

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in the protein bodies isolated from dry seed must either be activated or synthesized and possibly added to the protein bodies before storage protein breakdown can begin (Harris and Chrispeels, 1975). Further ultrastructural studies have shown that the rough endoplasmic reticulum proliferates and may give rise to visicles which fuse with the protein bodies prior to reserve protein digestion of cotyledons (Chrispeels et al., 1976). The two reserve proteins called vicilin and legumin are metabolized during the growth of mungbean seedling. Metabolism of the reserve proteins involves their hydrolysis within the membrane surrounding the protein body and is followed by the transport of the resulting amino acids to the growing parts of the seedling.

Vicilin peptidohydrolase, the major endopeptidase in the cotyledons, has been purified and characterized (Baumgartner and Chrispeels, 1977). Wong (1991) reported the separation of three neutral amino acid arylamidases (EC 3.4.11.2.) from mungbean seedlings and purified one of them (AP1).

Different organs within the same seedling may also display marked differences in patterns of changing proteolytic enzyme activities, as evidenced by studies of the pea radicle compared to pea cotyledons (Crump and Murray, 1979).

We report protease activities of mungbean (*Vigna radiata* L. Wilczek) seeds before and after germination to provide data for future work on the purification of mungbean enzymes.

Materials and Methods

Chemicals

Synthetic substrates, casein, and hemoglobin were used as substrates of proteolytic enzymes. For simplicity, if no configuration (D or L) of an amino acid is indicated, it stands for the L-form. For amide derivatives of aspartic or glutamic acid, COOH of position 1 is implied if not specified. Both one-letter and three-letter designations of amino acids were used. N-free 4-nitroaniline derivatives of L-amino acids contained A-NA (alanine-4-nitroanilide), C-bis-NA (cysteine-), E-NA (glutamic acid-), γ-E-NA (gamma-glutamic acid-), F-NA (phenylalanine-), G-NA (Glycine-), K-NA (lysine-), L-NA (leucine-), M-NA (methionine-), P-NA (proline-), R-NA (arginine-), V-NA (valine-); A-A-NA, E-F-NA, G-P-NA; A-A-A-NA, A-A-F-NA; A-A-V-A-NA. N-free β-naphthylamine derivatives of amino acids contained A-Nap (alanine beta-naphthylamide), A-4-methoxy-Nap, D-Nap (aspartic acid-), E-(4-methoxy)-Nap, F-Nap, G-Nap, H-Nap (histidine-), I-Nap (isoleucine-), K-Nap, L-Nap, DL-M-Nap, N-Nap (asparagine-), P-Nap, Pyr-Nap (pyruvic acid-), R-Nap, S-Nap (serine-), V-Nap, W-Nap (tryptophan-), Y-Nap (tyrosine-). N-blocked hippuric acid derivatives of amino acids included Hip-F, Hip-K, Hip-R; Hip-G-G, Hip-G-K, Hip-H-L. N-blocked 4-nitro-aniline derivatives of L-amino acids contained Ace-A-NA, CBz-F-NA, CP-F-NA, Ace-L-NA, Ben-R-NA, Ace-Y-NA. N-blocked β-naphthylamine derivatives of L-amino acids contained Ben-C-Nap, Ben-F-Nap, CBz-F-Nap, Formyl-M-Nap, CBz-P-Nap, Ben-R-Nap; Succinyl-F-G-L-Nap.

Plant Material

Mungbean (*Vigna radiata* L. Wilczek) seeds were purchased in a local market. Weight of seed was 0.06355± 0.02018 g (results obtained from 10 determinations, each contained 20 seeds). Seeds were placed in petri dishes with water underneath in an incubator at 30°C for germination in darkness. Seedlings were removed 4 days after imbibition when length reached about 4–5 cm for preparation of crude extract.

Preparation of Crude Extract

Seedlings were homogenized in liquid nitrogen. Ten-millimolar phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone, 1% ascorbic acid, 1 mM potassium chloride, 10 mM magnesium chloride, and 50 mM EDTA was added in the ratio of 1/3 (gram fresh weight/ml of extraction buffer). After centrifugation at 12,000 *g* for 20 min at 4°C, the supernatant was collected as samples (crude extract). The freshly extracted sample was immediately subjected to assays of protease activities.

Assays of Protease Activities

Proteinase assays using casein or hemoglobin as a substrate were done according to the method outlined by Bergmeyer (1984).

Protease assays for each synthetic substrate were carried out at three pH values: 3.4 (glycine-HCl buffer), 7.0 (phosphate buffer), and 8.9 (Tris-HCl buffer). Each synthetic substrate was dissolved in N,N-dimethylformamide as stock solution and diluted with double-distilled water before use. Crude extracts were diluted with double-distilled water to about 0.25 mg protein per ml as enzyme sources. Each micro plate contained 270 μl of the reaction mixture that consisted of 20 mM buffer, 2.5 mM substrate, and crude extract with about 30 μg protein. The enzyme reaction was started by adding 120 μL of the crude extract to a mixture of buffer and substrate solution and carried out at 37°C for 50, 100, and 150 min. Absorbance at zero time was used as the blank value for each corresponding assay.

The hydrolysis of aminoacyl-4-nitroanilide was measured spectrophotometrically at 410 nm as reported by Erlanger et al. (1961, 1966). The hydrolysis of aminoacyl-β-naphthylamide was measured at 540 nm (Erlanger et al., 1966). The carboxypeptidase activity was determined by measuring absorbance at 254 nm using α-N-hippuryl-L-amino acid as the substrate at pH 7.5 (Folk and Schirmer, 1963). The endopeptidase activity was determined with casein (Kunitz, 1946) or hemoglobin (Bergmeyer, 1984) as a substrate. The enzymatic reaction was performed and determined as assays of amino peptidase activity except that reactions were carried out only at pH 7.5.

Boiled enzyme solutions were used in controls for the above enzymatic reactions. All enzymatic reactions were performed three times. One enzyme unit was defined as the amount of enzyme required to produce 1 μmol of product per hour under the assay conditions. All enzyme activities were expressed on the basis of either unit per g fresh weight or unit per mg protein.

Determinations of Water-Soluble Protein

Protein determinations were performed using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Proteinase Activities

Proteinase activities of mungbean seeds before and after germination using casein or hemoglobin as a substrate are shown in Figure 1. Mungbean seeds have higher activity levels before germination than after germination when casein was used. When hemoglobin was used, the same was observed based on g fresh weight, but the opposite was found based on mg protein.

Carboxypeptidase Activities

Carboxypeptidase activities at pH 7.5 of mungbean seeds before and after germination are shown in Figure 2. Carboxypeptidase activities toward Hip-His-Leu or Hip-Arg at pH 7.5 based on g fresh weight were higher before germination than after germination, but activity toward Hip-Arg based on mg protein was lower before germination than after germination.

Amino Peptidase as well as some Endopeptidase Activities

Figures 3 and 4 show amino peptidase as well as some endopeptidase activities of mungbean seeds before

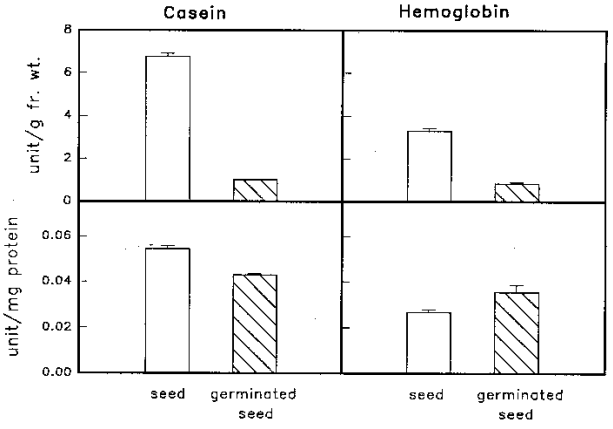

Figure 1. Proteinase activities of mungbean seeds. Seeds 4 days after imbibition were used as germinated materials. Proteinase assays using casein or hemoglobin as substrate were done according to the method outlined by Bergmeyer (1984).

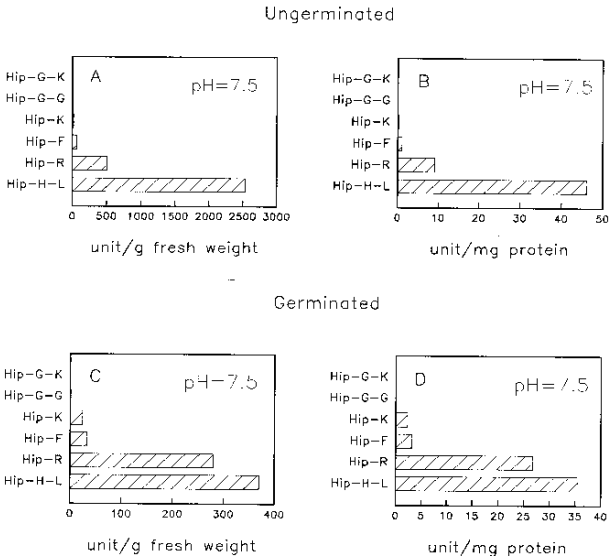

Figure 2. Carboxypeptidase activities of mungbean seeds. Seeds 4 days after imbibition were used as germinated materials. The carboxypeptidase activity was determined by measuring absorbance at 254 nm using α-N-hippuryl-L-amino acid as the substrate at pH 7.5 (Folk and Schirmer, 1963).

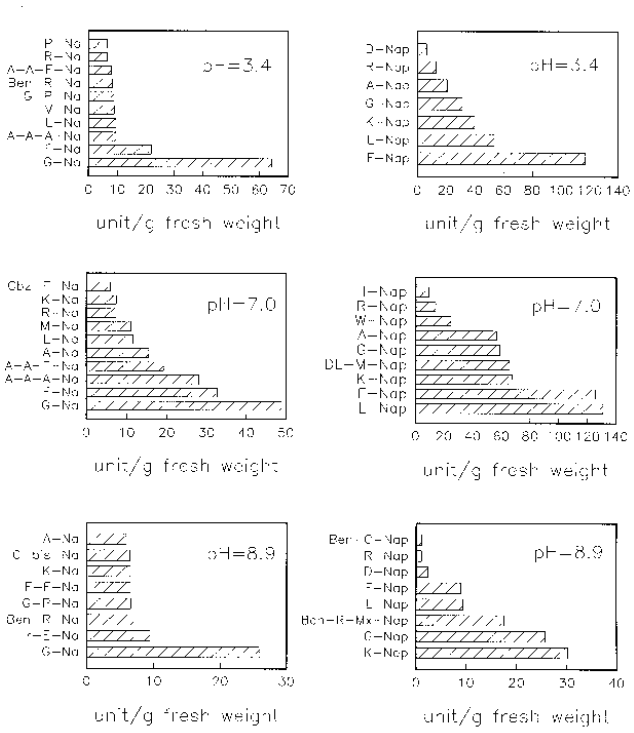

Figure 3. Amino peptidase as well as some endopeptidase activities of dry mungbean seeds expressed as enzyme unit per g fresh weight. The hydrolysis of aminoacyl-4-nitroanilide was measured spectrophotometrically at 410 nm as reported by Erlanger et al. (1961, 1966). The hydrolysis of aminoacyl-β-naphthylamide was measured at 540 nm (Erlanger et al., 1966). The endopeptidase activity was determined with casein (Kunitz, 1946) or hemoglobin (Bergmeyer, 1984) as substrate. The enzymatic reaction was performed and determined as assays of aminopeptidase activity except that reactions were carried out only at pH 7.5.

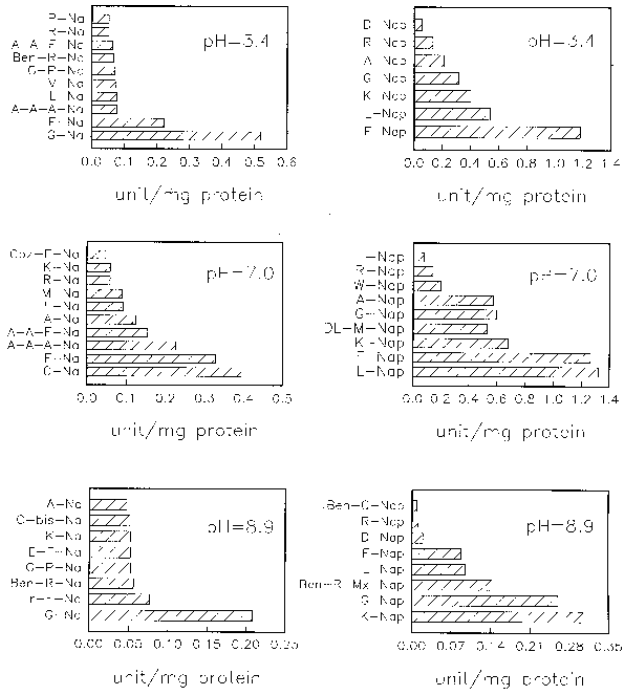

Figure 4. Amino peptidase as well as some endopeptidase activities of dry mungbean seeds expressed as enzyme unit per mg protein. Details were the same as Figure 3.

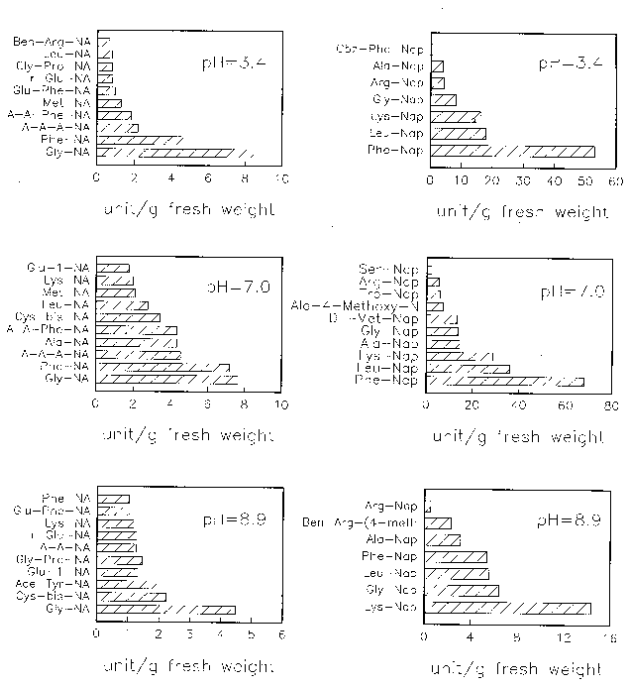

Figure 5. Amino peptidase as well as some endopeptidase activities of mungbean seedlings (4 days after imbibition) expressed as enzyme unit per g fresh weight. Details were the same as Figure 3.

germination using 4-nitroaniline or β-naphthylamine derivatives of amino acids or peptides as substrates at pH 3.4, 7.0, or 8.9 on the basis of g fresh weight or mg protein, respectively. When 4-nitroanilides were used, Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 3.4. Gly-Na, Phe-Na, Ala-Ala-Ala-Na, and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 7.0. Gly-Na and γ-Glu-Na were hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Phe-Nap, Leu-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 3.4 while Leu-Nap and Phe-Nap were hydrolyzed most rapidly at pH 7.0. Lys-Nap, Gly-Nap, and Ben-Arg-Mx-Nap were hydrolyzed most rapidly at pH 8.9.

Figures 5 and 6 show amino peptidase as well as some endopeptidase activities of mungbean seeds after germination using 4-nitroaniline or β-naphthylamine derivatives of amino acids or peptidases as substrates at pH 3.4, 7.0, or 8.9 on the basis of g fresh weight or mg protein, respectively. When 4-nitroanilides were used, Gly-Na, Phe-Na, Ala-Ala-Ala-Na, and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 3.4. Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 7.0. Gly-Na was hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Phe-Nap, Leu-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 3.4. Phe-Nap, Leu-Nap, and Lys-Nap were leading substrates at pH 7.0. Lys-Nap and Gly-Nap were leading substrates at pH 8.9.

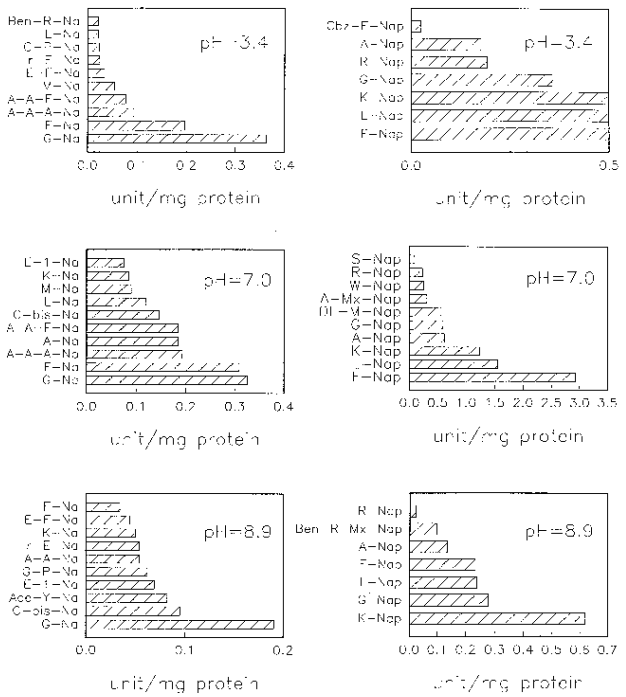

Figure 6. Amino peptidase as well as some endopeptidase activities of mungbean seedlings (4 days after imbibition) expressed as enzyme unit per mg protein. Details were the same as Figure 3.

Discussion

The casein-hydrolyzing activity of mungbean seeds is higher before germination than after germination (Figure 1) as we also observed in onion bulbs (Lin and Yao, 1995). The casein-hydrolyzing activity of mungbean seeds is about 23 and 5 times that of onion before and after germination, respectively, based on enzyme unit per g fresh weight; and about 2.3 and 3 times, respectively, based on enzyme unit per mg protein. The hemoglobin-hydrolyzing activity of mungbean seeds is higher before germination than after germination, which is also true of onion bulbs. This activity is about 6.8 and 4.3 times that of onion bulbs before and after germination, respectively, on the basis of enzyme unit per g fresh weight; and about 0.62 and 2.1 times, respectively, based on enzyme unit per mg protein.

The activity of a neutral amino peptidase hydrolyzing Leu-4-nitroanilide at pH 7.0 has been reported to decrease during germination (Feller, 1979). Collier and Murray (1977) found that the maximum activity of Leu-β-naphthylamidase in the germinating pea cotyledon was only half that found in the developing cotyledon. The activities of the naphthylamidases (hydrolyzing Leu-β-naphthylamide at pH 6.4) were high in the cotyledons of resting seeds, but decreased during germination (Mikkonen, 1986). Our results are similar in that casein-hydrolyzing activity and hemoglobin-hydrolyzing activity (only based on enzyme unit per g fresh weight) (Figure 1), carboxypeptidase (Figure 2) or some amino peptidase (Figures 3, 4, 5, and 6) activities of dry mungbean seeds are higher than those of germinated ones. In most cases, these enzyme activities remain at rather high levels after germination. It is possible that these enzymes catalyze protein turnover needed for the high rate of protein biosynthesis in developing seeds rather than a high net rate of protein breakdown during germination. A second possibility is that some of these protease activities are involved in a defense mechanism.

On the other hand, hemoglobin-hydrolyzing activity (based on mg protein) increases after germination. Since we did not carry out inhibitor studies, we are not sure whether this proteinase activity is the same as the vicilin peptidohydrolase reported by Chrispeels and Boulter (1975); however, it is quite possible. Carboxypeptidase activity toward Hip-Arg at pH 7.5 based on mg protein also increases after germination. The observed increase in protease activities after imbibition may be responsible for the breakdown of storage proteins during germination (Chrispeels et al., 1976; Dunaevsky et al., 1989).

Although some developmental patterns occur in some seed tissues, marked differences in the changing proteolytic enzyme activities may still be found in different organs within the same seedling or among different plant tissues, as evidenced by studies comparing pea radicles to pea cotyledons (Crump and Murray, 1979), or in different young plants as comparing our onion data (Lin and Yao, 1995) with this work.

The activities of proteinases (acting on hemoglobin at pH 3.7 and on casein at pH 5.4 and 7.0) were low in the resting seeds, but increased during germination reaching their maximal values when the mobilization of nitrogen was highest (Mikkonen, 1986). However, our results with casein or hemoglobin as a substrate show the opposite patterns, except in the case of hemoglobin based on mg protein (Figures 1 and 2). Activities of the naphthylamidases (hydrolyzing Leu-β-naphthylamide at pH 6.4) were high in the cotyledons of resting seeds, but decreased during germination (Mikkonen, 1986). Our results show the same pattern (Figures 3, 4, 5, and 6).

Mungbean enzymes hydrolyze derivatives of 4-nitroaniline and β-naphthylamine of amino acids and peptides differently at various pH values. The same result was observed in onion (Lin and Yao, 1995) and in garlic (Lin and Yao, 1995).

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綠豆 (*Vigna radiata* L. Wilczek) 發芽前已具有高的蛋白質水解酵素活性

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綠豆種子粗抽液以酪蛋白 (casein) 為基質測得之蛋白酶活性發芽前比發芽後高。以血紅素 (hemoglobin) 為基質，當以 1 克鮮重為比較基準時和酪蛋白的情形相似；但以毫克蛋白質為比較基準時則和酪蛋白的情形相反。羧肽酶 (carboxypeptidase) 對 Hip-His-Leu 或 Hip-Arg 的活性，當以 1 克鮮重為比較基準時也是發芽前比發芽後高；但以 1 毫克蛋白質為比較基準時則相反。發芽前後之胺肽酶 (aminopeptidase) 及肽內切酶 (endopeptidase) 的活性以氨基酸或肽的 4-nitroaniline 或 β-naphthylamine 的衍生物為基質分別在 pH 3.4, 7.0 和 8.9 測定。發芽前， Gly-Na 及 Phe-Na 在 pH 3.4 ； Gly-Na, Phe-Na, Ala-Ala-Ala-Na 及 Ala-Ala-Phe-Na 在 pH 7.0 ；而 Gly-Na 和 γ-Glu-Na 在 pH 8.9 被水解最快。 Phe-Nap, Leu-Nap 及 Lys-Nap 在 pH 3.4 ； Leu-Nap 及 Phe-Nap 在 pH 7.0 ；而 Lys-Nap, Gly-Nap 及 Ben-Arg-Mx-Nap 在 pH 8.9 被水解最快。發芽後 Gly-Na, Phe-Na, Ala-Ala-Ala-Na 及 Ala-Ala-Phe-Na 在 pH 3.4 ； Gly-Na 及 Phe-Na 在 pH 7.0 ；而 Gly-Na 在 pH 8.9 被水解最快。 Phe-Nap, Leu-Nap 及 Lys-Nap 在 pH 3.4 和 7.0 ；而 Lys-Nap 及 Gly-Nap 在 pH 8.9 被水解最快。

關鍵詞：蛋白酶；蛋白質基質；合成基質；發芽；酸鹼度。