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Onion (*Allium cepa* L.) contains some high proteolytic activities already before germination

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Abstract. Onion bulbs had higher proteinase and carboxypeptidase activity levels before germination than after, when casein or hemoglobin was used as a substrate. The substrates that were hydrolyzed most rapidly before germination, Hip-Gly-Gly and Hip-Lys, were different from those which were hydrolyzed most rapidly after germination, Hip-Gly-Lys and Hip-His-Leu. Amino peptidase and some endopeptidase activities were determined before and after germination using 4-nitroaniline or β-naphthylamine derivatives of amino acids or peptides as substrates at pH 3.4, 7.0, or 8.9. Before germination, Gly-Na and Phe-Na were hydrolyzed most rapidly at all three pHs; and Gly-Na, Phe-Na, and Ala-Na were hydrolyzed most rapidly at both pH 7.0 and 8.9. 4-nitroaniline derivatives of peptides were hydrolyzed substantially, although not most rapidly, at all pHs. Phe-Nap was hydrolyzed most rapidly at pH 3.4 and 7.0 while Ala-Nap was hydrolyzed most rapidly at pH 8.9. Phe-Nap, Lys-Nap, Ala-Nap, and Arg-Nap were among the 5 leading substrates at all pHs. After germination, Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 3.4 and 7.0 (the same as before germination), and Phe-Na, Ala-Ala-Phe-Na and Gly-Na were hydrolyzed most rapidly at pH 8.9. Ala-Ala-Ala-Na and Ala-Ala-Phe-Na were hydrolyzed rather rapidly at pH 3.4 and 8.9, respectively, which was not observed before germination. Cbz-Phe-Nap, Phe-Nap, and Trp-Nap were hydrolyzed most rapidly at pH 3.4, 7.0, and 8.9, respectively. Cbz-Phe-Nap and Ben-Phe-Nap were hydrolyzed most rapidly at pH 3.4 (characteristic of germinating onion bulbs). Variance analysis (using Gly-Na as substrate) shows that pH (P) and days after imbibition (D) were significant sources of variance. There was also interaction between P and D. Details of interaction between P and D with various substrates were analyzed by Scheffe's difference comparison.

Keywords: Germination; Onion; Protease activities; Synthetic substrates.

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

Introduction

There are many reports of 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; 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 aminopeptidase 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 proteinases (Shutov and Vaintraub, 1987) and carboxypeptidases (Dunaevsky et al., 1987) are expressed in germinating and post-germinating seeds. Aminopeptidases 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 seed separated by anion-exchange chromatography (Weil et al., 1966), two carboxypeptidases from germinating soybeans (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).

Different organs within the same seedling may display markedly different patterns of changing proteolytic enzyme activities, as evidenced by studies comparing pea radicles to pea cotyledons (Crump and Murray, 1979). We report proteolytic activities before and after germination of onion (*Allium cepa* L.) to provide data for future work on the purification of onion enzymes.

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Materials and Methods

Chemicals

We used synthetic substrates, casein, and hemoglobin as substrates for proteolytic enzymes. For simplicity, the configuration (D or L) of an amino acid is levo if not otherwise specified, and for amide derivatives of aspartic or glutamic acid, COOH in position one is implied. Both one-letter and three-letter designations of amino acids are used. N-free 4-nitro-aniline 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-), and V-NA (valine-); A-A-NA, E-F-NA, and G-P-NA; A-A-A-NA and A-A-F-NA; and 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-), and Y-Nap (tyrosine-). N-blocked hippuric acid derivatives of amino acids included Hip-F, Hip-K, and Hip-R; and Hip-G-G, Hip-G-K, and 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, and 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, and Succinyl-F-G-L-Nap. Casein and hemoglobin were purchased from E. Merck (Darmstad, Germany), and other chemicals were purchased from the Sigma Chemical Company (St. Louis, USA).

Plant Material

Onions (*Allium cepa* L.) were purchased in a local market. They were germinated in petri dishes in 1–2 cm of water at 37°C. Onions were removed 1, 6, and 14 days after imbibition, when their length reached about 0, 4, and 11 cm, respectively, for preparation of crude extract.

Preparation of Crude Extract

Onions were frozen in liquid nitrogen. Ten-millimolar phosphate extraction buffer (pH 7.8) containing 1% polyvinylpyrolidone, 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) and the mixture was ground in a mortar at 4°C. The homogenate was centrifuged at 12000 *g* for 20 min at 4°C, then the supernatant liquid was collected (crude extract) and immediately subjected to an assay of protease activities.

Assays of Protease Activities

Proteinase assays were performed according to Bergmeyer (1984), using casein or hemoglobin as the substrate. Protease assays for each synthetic substrate were carried out at three pHs: 3.4 (glycine-HCl buffer), 7.0 (phosphate buffer), and 8.9 (Tris-HCl buffer). Each synthetic substrate was dissolved either in double-distilled water or, if would not easily dissolve in water, in N,N-dimethylformamide as a stock solution, and diluted with double-distilled water before use. Crude extracts were diluted with double-distilled water to about 0.25 mg protein ml^{-1} as enzyme sources. Each micro plate contained 270 μl of reaction mixture, which consisted of 20 mM buffer, 2.5 mM substrate, and crude extract containing 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 assay.

The hydrolysis of aminoacyl-4-nitroanilide was measured spectrophotometrically at 410 nm, as described 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 the substrate.

Boiled enzyme solutions were used as controls in the 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 as units per g fresh weight and as units per mg protein.

Determinations of Water-Soluble Protein

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

Statistical Analysis

Variance analysis, Duncan's multiple range analysis, and Scheffe's difference comparison were performed using the SAS software package (SAS Institute Inc., 1988).

Results

Proteinase Activities

Proteinase activities in onion bulbs before and after germination, using casein or hemoglobin as the substrate, are shown in Figure 1. Onion bulbs have higher activity levels before germination than after germination, irrespective of the substrate used.

Carboxypeptidase Activities

Carboxypeptidase activities of onion bulbs at pH 7.5, before and after germination, are presented in Figure 2. As with endopeptidase activities, carboxypeptidase activities

before germination are higher than those after germination. The substrates that were hydrolyzed most rapidly before germination—Hip-Gly-Gly and Hip-Lys—were different than those that were hydrolyzed most rapidly after germination—Hip-Gly-Lys and Hip-His-Leu.

Aminopeptidase and some Endopeptidase Activities

Amino peptidase and some endopeptidase activities of onion bulbs before germination (using 4-nitroaniline or β-naphthylamine derivatives of amino acids or peptides as substrates, respectively) at pH 3.4, 7.0, and 8.9, are shown in Figures 3 and 4. When 4-nitroanilides were used, Gly-Na and Phe-Na were hydrolyzed most rapidly at all three pHs; Gly-Na, Phe-Na, and Ala-Na were hydrolyzed most rapidly at both pH 7.0 and 8.9. 4-Nitroaniline derivatives of peptides were hydrolyzed substantially, although not most rapidly, at all pHs. When β-naphthylamine derivatives were used, Phe-Nap was hydrolyzed most rapidly at pH 3.4 and 7.0, while Ala-Nap was hydrolyzed most rapidly at pH 8.9. Phe-Nap, Lys-Nap, Ala-Nap, and Arg-Nap were among the 5 leading substrates at all pHs.

Amino peptidase and some endopeptidase activities of onion bulbs after germination, using 4-nitroaniline or β-naphthylamine derivatives of amino acids or peptidases as substrates, respectively, at pH 3.4, 7.0, and 8.9, are shown in Figures 5 and 6. When 4-nitroanilides were used, Gly-Na and Phe-Na were hydrolyzed most rapidly at pH 3.4 and 7.0, the same as before germination; Phe-Na, Ala-

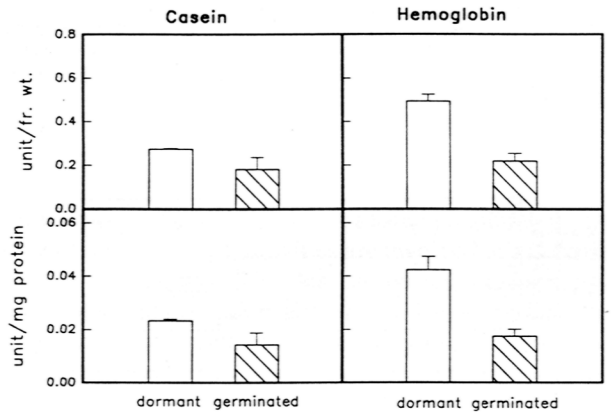

Figure 1. Proteinase activity of onion bulbs. Onion bulbs 14 days after imbibition were used as germinated materials. *See* **Materials and Methods** for details.

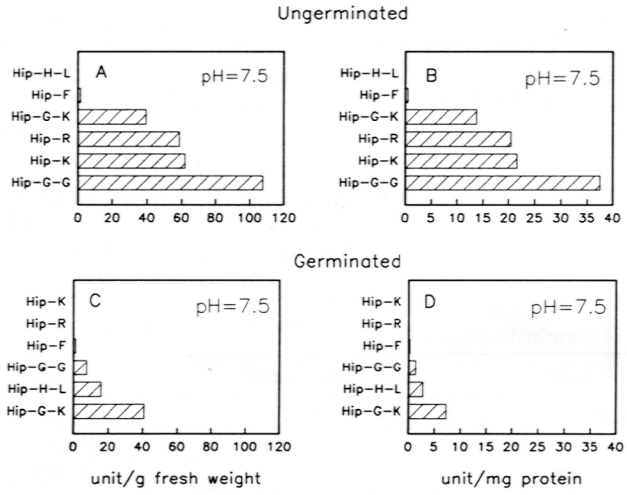

Figure 2. Carboxypeptidase activities of onion bulbs. Onion bulbs 14 days after imbibition were used as germinated materials. *See* **Materials and Methods** for details.

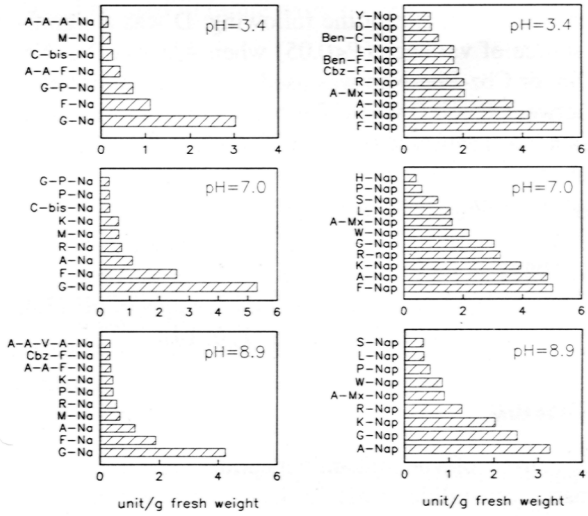

Figure 3. Amino peptidase and some endopeptidase activities of dormant onion bulbs expressed as units per g fresh weight. *See* **Materials and Methods** for details.

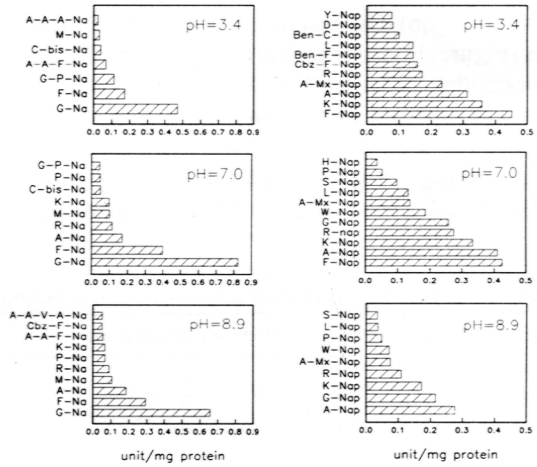

Figure 4. Amino peptidase and some endopeptidase activities of dormant onion bulbs expressed as units per mg protein. *See* **Materials and Methods** for details.

Ala-Phe-Na, and Gly-Na were hydrolyzed most rapidly at pH 8.9. Ala-Ala-Ala-Na and Ala-Ala-Phe-Na, 4-nitroaniline derivatives of peptides, were hydrolyzed rather rapidly at pH 3.4 and 8.9, respectively; this was not observed before germination. When β-naphthylamine derivatives were used, Cbz-Phe-Nap, Phe-Nap, and Trp-Nap were hydrolyzed most rapidly at pH 3.4, 7.0, and 8.9, respectively. Cbz-Phe-Nap and Ben-Phe-Nap were hydrolyzed most rapidly at pH 3.4; this is characteristic of germinating onion bulbs.

Statistical Analysis

Table 1 shows variance analysis of protease activity of germinating onion bulbs with Gly-Na as the substrate. pH (P) and days after imbibition (D) were significant sources of variance ($P<0.01$). There was also interaction between P and D. Similar results were obtained when other substrates were used, except the following: D was a significant source of variance ($P<0.05$) when Ala-Ala-Phe-Na, Phe-Na, or Cbz-Phe-Nap was used; there was no interaction between P and D when Met-Na or Gly-Nap was used; D was not a significant source of variance when Trp-Nap was used.

Details of interaction between P and D of various substrates were analyzed by Scheffe's difference comparison (SAS, 1988) and are summarized in Table 2. There is a total of 36 comparison items for combinations of pH (3.4, 7.0, 8.9) and days after imbibition (1, 6, 14).

Discussion

The activity of a neutral aminopeptidase that hydrolyzes Leu-para-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 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 endopeptidase (Figure 1), carboxypeptidase (Figure 2), or some aminopeptidase (Figures 3, 4, 5, and 6) activities of dormant onion bulbs are higher than those of germinated ones. It is possible that these enzymes catalyze the protein turnover needed for the high rate of protein biosynthesis in developing bulbs rather than a high net rate of protein

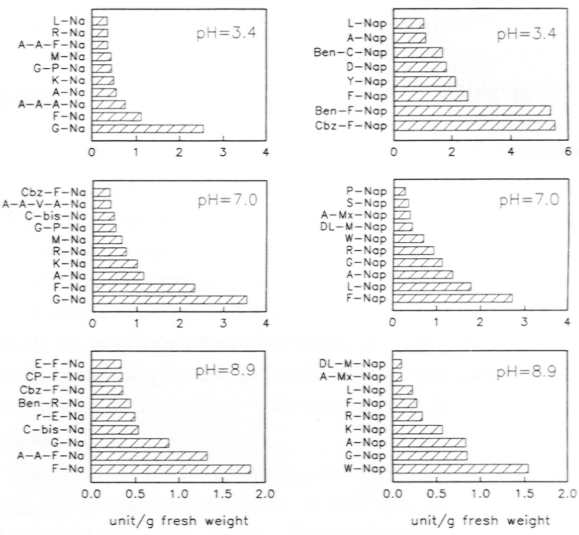

Figure 5. Amino peptidase and some endopeptidase activities of germinated onion bulbs (14 days after imbibition) expressed as units per g fresh weight. *See* **Materials and Methods** for details.

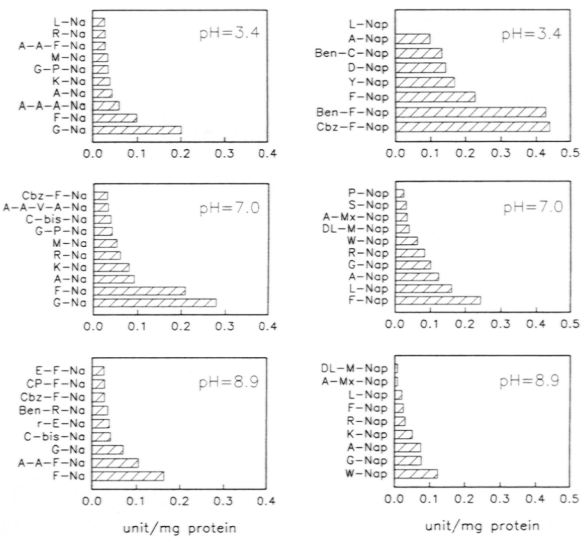

Figure 6. Amino peptidase and some endopeptidase activities of germinated onion bulbs (14 days after imbibition) expressed as units per mg protein. *See* **Materials and Methods** for details.

Table 1. Variance analysis of protease activity (units per mg protein) of germinating onion bulbs with Gly-Na as the substrate.

Source of variance	Degrees of freedom	Sum of squares	Mean square	F-value
pH (P)	2	0.2493	0.1247	686.7[a]
(D)[b]	2	0.2377	0.1188	654.6[a]
P×D	4	0.05586	0.01397	76.92[a]
Error	18	0.003268	0.0001816	
Corrected total	26	0.5462		

[a]Significant of F at $P=0.01$.
[b]Days after imbibition.

Table 2. Summary of Scheffe's difference comparison of interaction between pH and growth stage (days after imbibition) of protease activities using 18 substrates.

Name of substrate	Number of items[a] with significant difference (P) < 0.05
Pro-Nap	32
Met-Nap	31
Trp-Nap	30
Gly-Na	30
Leu-Nap	29
Phe-Nap	27
Ala-Na	27
Lys-Na	26
Ala-Nap	26
Ser-Nap	25
Ala-Ala-Ala-Nap	23
Lys-Nap	23
Arg-Nap	23
Gly-Nap	23
Leu-Na	22
Cbz-Phe-Nap	20
Met-Na	12
Ala-Ala-Phe-Na	12
Phe-Na	11

[a]There is a total of 36 items of comparison between combinations of pH (3.4, 7.0, 8.9) and days after imbibition (1, 6, 14).

breakdown during germination. A second possibility is that some of these protease activities are involved in a defense mechanism. Carboxypeptidase activities are normally expressed during germination, and are thought to hydrolyze storage proteins (Dunaevsky et al., 1989). The differences between the carboxypeptidase activities of legume seedlings and onion require further study.

On the other hand, enzyme activity that hydrolyzes Ala-Ala-Ala-Na and Ala-Ala-Phe-Na (para-nitroaniline derivatives of peptides) rather rapidly at pH 3.4 and 8.9, respectively, was not observed before germination. Those hydrolyzing Cbz-Phe-Nap, Phe-Nap, and Trp-Nap most rapidly at pH 3.4, 7.0, and 8.9, respectively, were found only after germination. Cbz-Phe-Nap and Ben-Phe-Nap were hydrolyzed most rapidly at pH 3.4; this is also characteristic of germinating onion bulbs. These enzyme activities may be responsible for breakdown of storage proteins during germination.

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) and in this work.

The activities of carboxypeptidase (hydrolyzing carbobenzoxy Phe-Ala at pH 5.9) and 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 and reached their maximum values when the mobilization of nitrogen was highest (Mikkonen, 1986). Our results, however, show the opposite patterns (Figures 1 and 2). 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 also show the opposite patterns (Figures 3, 4, 5, and 6). These dissimilarities require further study.

Table 2 suggests that enzyme activities that hydrolyze Phe-Na, Met-Na, and Ala-Ala-Phe-Na are affected by pH and growth stage to a lesser extent than are those that hydrolyze Pro-Nap, Met-Nap, Trp-Nap, and Gly-Na, and that enzyme activities hydrolyzing other substrates are affected to intermediate degrees.

As mentioned in the literature (Qi et al., 1992), using only exogenous or synthetic substrates may, at times, lead to the identification of proteases that are physiologically irrelevant. Hence, endogenous substrates, if available, should be used in future work on onion proteases.

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洋蔥(*Allium cepa* L.)發芽前已具有高的蛋白質水解酵素活性

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洋蔥鱗莖粗抽液以 casein 或 hemoglobin 爲基質測得之蛋白質水解酵素活性發芽前比發芽後高。Carboxypeptidase 活性也是發芽前比發芽後高。發芽前 Hip-Gly-Gly 和 Hip-Lys 被分解最快，而發芽後 Hip-Gly-Lys 和 Hip-His-Leu 被分解最快。發芽前後之 aminopeptidase 及 endopeptidaseh 的活性以氨基酸或肽的 4-nitroaniline 或 β-naphthylamineha 衍生物爲基質分別在 pH3.4, 7.0 和 8.9 測定。發芽前， Gly-Na 及 Phe-Na 在三種 pH ；而 Gly-Na, Phe-Na ，及 Ala-Na 在 pH7.0 和 8.9 被分解最快。肽之 4-nitroaniline 衍生物在三種 pH 雖然不是被分解最快者，但也相當可觀。 Phe-Nap 在 pH3.4 及 7.0 ；在 Ala-Nap 在 pH8.9 被分解最快。 Phe-Nap, Lys-Nap, Ala-Nap ，及 Arg-Nap 在三種 pH 均排名前五名。發芽後 Gly-Nap 及 Phe-Na 在 pH3.4 和 7.0 被水解最快（和發芽前相同）；而 Phe-Na, Ala-Ala-Phe-Na 及 Gly-Na 則在 pH8.9 被分解最快。 Ala-Ala-Ala-Na 及 Ala-Ala-Phe-Na 分別在 pH3.4 和 8.9 被分解最快，這在發芽前未曾測到。 Cbz-Phe-Nap, Phe-Nap ，和 Trp-Nap 分別在 pH3.4, 7.0 ，及 8.9 被分解最快。 Cbz-Phe-Nap 及 Ben-Phe-Nap 在 pH3.4 被分解最快，此爲發芽時之特性。變方分析（以 Gly-Na 爲基質）顯示 pH(P) 和浸水後日數（D）均爲極顯著變異來源。 P 和 D 之間有交感。 P 和 D 交感之詳情以 "Scheffe's difference comparison" 探討。

關鍵詞：洋蔥；蛋白質水解酶；發芽；合成基質。