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Protease activities before and after germination of garlic (*Allium sativum* L.) bulbs

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Abstract. When casein was used as a substrate, garlic bulbs had lower proteinase activity levels before germination than after. The opposite was observed with hemoglobin as the substrate. Before germination, when 4-nitroanilides were used, Ala-Ala-Phe-Na was hydrolyzed most rapidly at pH 3.4, Ala-Ala-Ala-Na and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 7.0, and Gly-Na, Ala-Na, and Met-Na were hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Lys-Nap and Arg-Nap were hydrolyzed most rapidly at pH 3.4, Lys-Nap, Trp-Nap and Arg-Nap were hydrolyzed most rapidly at pH 7.0, and Ala-Nap, Trp-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 8.9. After germination, when 4-nitroanilides were used, Ala-Ala-Ala-Na, Gly-Nap, and Ben-Arg-Nap were hydrolyzed most rapidly at pH 3.4. This was not observed before germination. Gly-Na was hydrolyzed most rapidly at pH 7.0 (more than four times the rate before germination). When β-naphthylamine derivatives were used, Tyr-Nap, Ben-Phe-Nap, Ser-Nap, and Cbz-Phe-Nap were hydrolyzed most rapidly at pH 3.4. Trp-Nap, Tyr-Nap, Phe-Nap, Arg-Nap, and Pro-Nap were the five leading substrates at pH 7.0 and 8.9. Garlic enzymes hydrolyze derivatives of 4-nitroaniline and β-naphthylamine of amino acids and peptides differently at different pHs. The same was observed for those of onion.

Keywords: Garlic; Germination; Protease; Synthetic substrates.

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; Dunaevsky and Belozersky, 1989; Collier and Murray, 1977; Crump and Murray, 1979; 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 other plants (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 activiteis 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 carboxypeptidase (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, which were separated by anion-exchange chromatography (Weil 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). Although some developmental patterns occur in some seed tissues, different organs within the same seedling may display markedly different patterns of changing proteolytic enzyme activities, as evidenced by the results of studies comparing pea radicles to pea cotyledons (Crump and Murray, 1979).

There is much literature about the beneficial effects of some components of garlic (Apitz-Castro et al., 1994; Kojima et al., 1994; Lewin and Popov, 1994; Lin et al., 1994; Yeh and Yeh, 1994), but studies of garlic proteases have rarely been reported. We report proteolytic activities of garlic (*Allium sativum* L.) bulbs before and after germination.

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Materials and Methods

Chemicals

Casein, hemoglobin, and synthetic substrates were used as the substrates for proteolytic enzymes. For simplicity, the molecular configuration of an amino acid is levo unless otherwise specified, and for amide derivatives of aspartic or glutamic acid, COOH in position 1 is implied if not specified. 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, and Ben-R-Nap; and Succinyl-F-G-L-Nap.

Plant Material

Garlic (*Allium sativum* L.) was purchased in a local market. Garlic bulbs were placed in 1–2 cm water in petri dishes and germinated in an incubator at 30°C in darkness. Germinated bulbs were removed from the incubator for preparation of crude extract 30 days after imbibition, when the length of the shoots reached about 2–3 cm.

Preparation of Crude Extract

The garlic was 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 liquid was collected (crude extract) and immediately subjected to assays of protease activities.

Assays of Protease Activities

Proteinase assays using casein or hemoglobin as substrate were performed according to to the method of Bergmeyer (1984).

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 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, 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 was 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 (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 by assays of aminopeptidase activity except that reactions were carried out at only at pH 7.5.

Boiled enzyme solutions were used as 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 hydrolyze 1 μmol of substrate per hour under the assay conditions. All enzyme activities were expressed on the basis of unit per g fresh weight or unit per mg protein.

Determinations of Water-Soluble Protein

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

Results

Endopeptidase Activities

Figure 1 shows endopeptidase activities of garlic bulbs before and after germination, using casein or hemoglobin as a substrate. With casein as the substrate, garlic bulbs had lower activity levels before germination than after. The opposite was observed with hemoglobin as the substrate.

Time Course of Gly-Na Hydrolyzing Activity

Figure 2 shows time course of Gly-Na hydrolyzing activity at pH 7.0 of germinated garlic bulbs. The increase of absorbance at 410 nm is linear within 150 min.

Aminopeptidase and Some Endopeptidase Activities

Figures 3 and 4 show amino peptidase and some endopeptidase activities of garlic bulbs before germination using 4-nitroaniline and β-naphthylamine derivatives of amino acids and peptides as substrates at pH 3.4, 7.0, and 8.9 on the basis of g fresh weight and mg protein. When 4-nitroanilides were used, Ala-Ala-Phe-Na was hydrolyzed

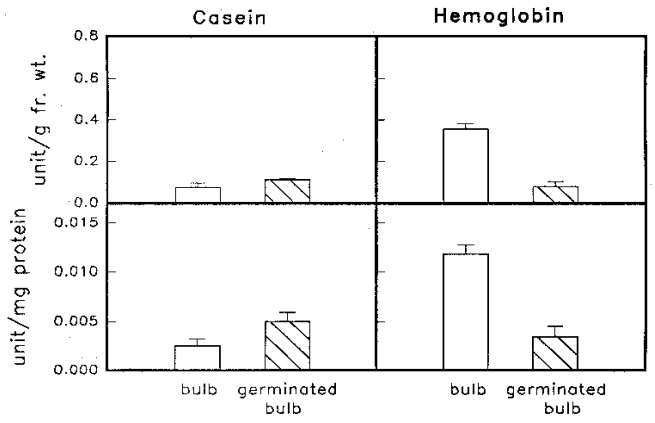

Figure 1. Proteinase activity of garlic bulbs. Garlic bulbs 30 days after imbibition were used as germinated materials. Proteinase assays using casein or hemoglobin as substrate were done according to Bergmeyer (1984).

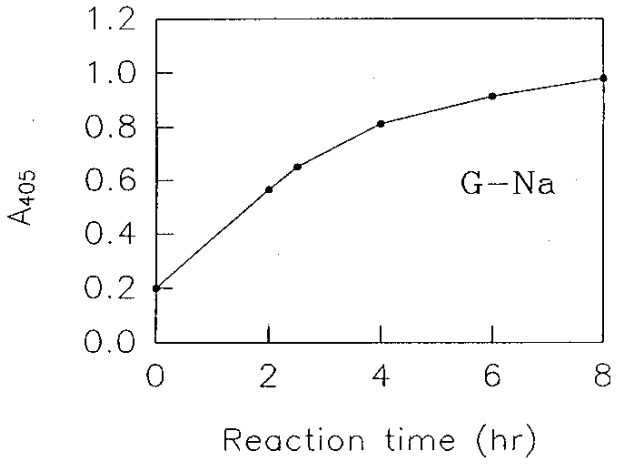

Figure 2. Time course of Gly-hydrolyzing activity of garlic bulbs. Garlic bulbs 30 days after imbibition were used as germinated materials. Protease assays for Gly-4-nitroaniline were carried out at pH 7.0 (phosphate buffer). Crude extracts were diluted with double-distilled water to about 0.25 mg protein per ml as enzyme sources. Each micro plate contained 270 µL of the reaction mixture that consisted of 20 mM buffer, 2.5mM substrate, and crude extract with about 30 µg protein. This 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 various times. Absorbance at 405 nm of zero time was used as the blank value for each corresponding assay.

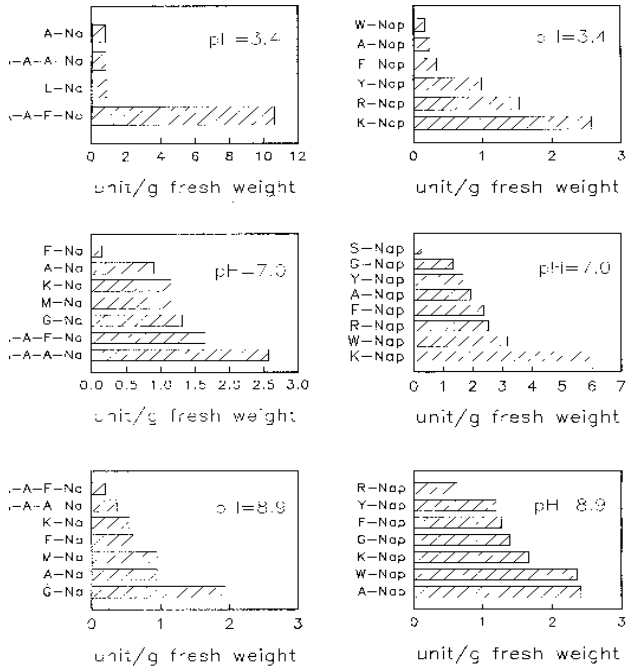

Figure 3. Amino peptidase and some endopeptidase activities of dormant garlic bulbs expressed as units per g fresh weight. The hydrolysis of aminoacyl-4-nitroanilide was measured spectrophotometrically at 410 nm by the method of Erlanger et al. (1961, 1966). The hydrolysis of aminoacyl-β-naphthylamide was measured at 540 nm (Erlanger et al., 1966). The endopeptidase activity was determined with casein (Kunitz, 1946) and hemoglobin (Bergmeyer, 1984) as substrates. The enzymatic reaction was performed and determined by assays of aminopeptidase activity, except that reactions were carried out only at pH 7.5.

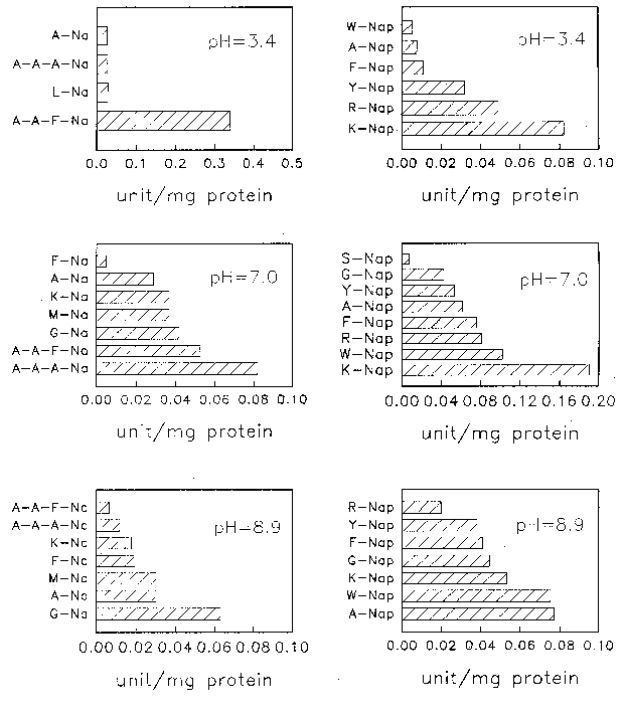

Figure 4. Amino peptidase and some endopeptidase activities of dormant garlic bulbs expressed as units per mg protein. Details are the same as in Figure 3.

most rapidly at pH 3.4. Ala-Ala-Ala-Na and Ala-Ala-Phe-Na were hydrolyzed most rapidly at pH 7.0. Gly-Na, Ala-Na, and Met-Na were hydrolyzed most rapidly at pH 8.9. When β-naphthylamine derivatives were used, Lys-Nap and Arg-Nap were hydrolyzed most rapidly at pH 3.4, while Lys-Nap, Trp-Nap, and Arg-Nap were hydrolyzed most rapidly at pH 7.0. Ala-Nap, Trp-Nap, and Lys-Nap were hydrolyzed most rapidly at pH 8.9.

Figures 5 and 6 show amino peptidase and some endopeptidase activities of garlic bulbs after germination using 4-nitroaniline and β-naphthylamine derivatives of amino acids and peptidases as substrates at pH 3.4, 7.0, and 8.9 on the basis of g fresh weight and mg protein. When 4-nitroanilides were used, Ala-Ala-Ala-Na, Gly-Nap, and Ben-Arg-Nap were hydrolyzed most rapidly at pH 3.4. This was not observed before germination. Gly-Na was hydrolyzed most rapidly at pH 7.0—the rate was more than four times that before germination. When β-naphthylamine derivatives were used, Tyr-Nap, Ben-Phe-Nap, Ser-Nap, and Cbz-Phe-Nap were hydrolyzed most rapidly at pH 3.4. Trp-Nap, Tyr-Nap, Phe-Nap, Arg-Nap, and Pro-Nap were the five leading substrates at pH 7.0 and 8.9.

Discussion

Although onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) belong to the same genus, their protease activity patterns are not the same. The casein-hydrolyzing activity of garlic bulbs was higher after germination than

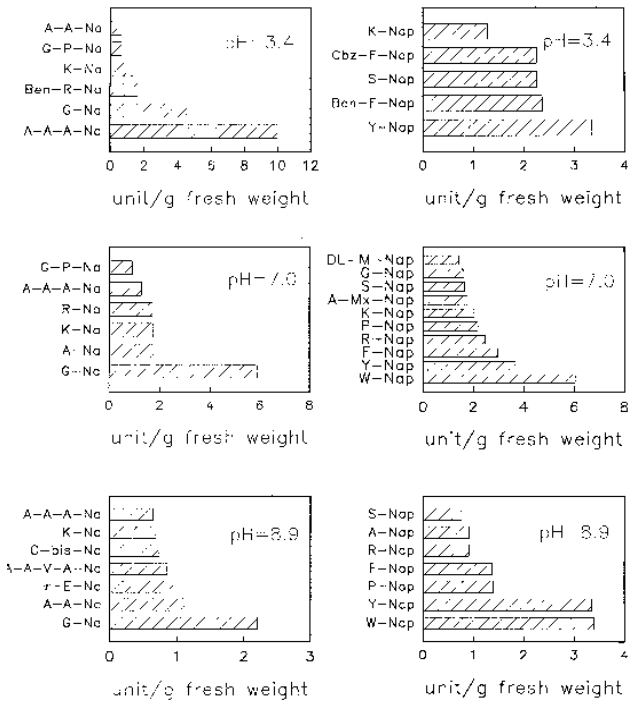

Figure 5. Amino peptidase and some endopeptidase activities of germinated garlic bulbs (30 days after imbibition) expressed as units per g fresh weight. Details are the same as in Figure 3.

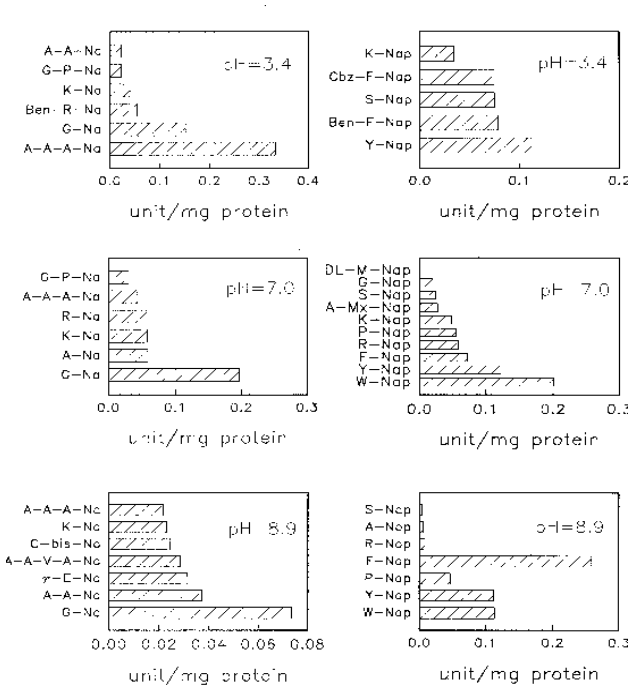

Figure 6. Amino peptidase and some endopeptidase activities of germinated garlic bulbs (30 days after imbibition) expressed as units per mg protein. Details are the same as in Figure 3.

before germination (Figure 1), while the opposite was observed in onion bulbs (Lin and Yao, 1995). The level of casein-hydrolyzing activity of garlic is only about 1/3 and 1/2 that of onion before and after germination, respectively, based on enzyme units per g fresh weight . Hemoglobin-hydrolyzing activity of garlic bulbs is lower after germination than before germination, as it is for onion. Dormant garlic has only 60% of hemoglobin-hydrolyzing activity of dormant onion, based on enzyme unit per g fresh weight.

The activity of a neutral aminopeptidase hydrolyzing Leu-4-nitroanilide at pH 7.0 has been reported to decrease during germination (Feller, 1979). Collier and Murray (1977) have found that the maximum activity of Leu-β-naphthylamidase in germinating pea cotyledon was only half that found in 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; we found that hemoglobin-hydrolyzing activity (Figure 1), or some aminopeptidase (Figures 3, 4, 5, and 6) activities of dormant garlic 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 ensure a high net rate of protein breakdown during germination.

Enzyme activity hydrolyzing Ben-Arg-Na at pH 3.4 was not observed before germination. Those hydrolyzing Ben-Phe-Nap and Cbz-Phe-Nap rapidly at pH 3.4 were found only after germination. Those hydrolyzing Tyr-Nap and

Ser-Nap at pH 3.4, and Trp-Nap, Tyr-Nap, and Pro-Nap at pH 7.0 and 8.9 increased after germination. This is also the characteristic of germinating garlic 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 can still be found in different organs within the same seedling or among different plant tissues, as evidenced by the results of studies comparing pea radicles to pea cotyledons (Crump and Murray, 1979) and by 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 resting seeds, but increased during germination and reached their maximum values when the mobilization of nitrogen was highest (Mikkonen, 1986). Our results with hemoglobin as a substrate show the opposite patterns (Figures 1 and 2). The activities of the naphthylamidases (hydrolyzing Leu-*b*-naphthylamide at pH 6.4) were high in the cotyledons of resting seeds, but decreased during germination (Mikkonen, 1986). Our results show that the changing patterns depend on which substrate is used (Figures 3, 4, 5, and 6).

Garlic enzymes hydrolyze derivatives of 4-nitroaniline and β-naphthylamine of amino acids and peptides differently at different pHs. The same was observed in onion.

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大蒜 (*Allium sativum* L.) 發芽前後之蛋白酶活性

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大蒜鱗莖粗抽液以酪蛋白 (casein) 爲基質測得之蛋白酶活性發芽前比發芽後低；如果以血紅素 (hemoglobin) 爲基質則發芽前比發芽後高。發芽前，以氨基酸或胜肽的 4-nitroaniline 的衍生物爲基質時，Ala-Ala-Phe-Na 在 pH 3.4 被水解最快；Ala-Ala-Ala-Na 及 Ala-Ala-Phe-Na 在 pH 7.0 被水解最快；Gly-Na 、Ala-Na 及 Met-Na 則在 pH 8.9 被水解最快。當以 β-naphthylamine 的衍生物爲基質時，Lys-Nap 及 Arg-Nap 在 pH 3.4 ；Lys-Nap 、Trp-Nap 及 Arg-Nap 在 pH 7.0 ；而 Ala-Nap 、Trp-Nap 及 Lys-Nap 則在 pH 8.9 被水解最快。在發芽後，當以 4-nitroaniline 的衍生物爲基質時，Ala-Ala-Ala-Na 、Gly-Nap 及 Ben-Arg-Nap 在 pH 3.4 被水解最快。這在發芽前未曾測到。Gly-Na 在 pH 7.0 被水解最快而且爲發芽前之四倍。當以 β-naphthylamine 的衍生物爲基質時，Tyr-Nap 、Ben-Phe-Nap 、Ser-Nap 及 Cbz-Phe-Nap 在 pH 3.4 被水解最快。Trp-Nap 、Tyr-Nap 、Phe-Nap 、Arg-Nap 及 Pro-Nap 在 pH 7.0 及 8.9 均排在被水解最快之前五名。大蒜之蛋白酶在不同 pH 顯然以不同速率水解氨基酸或胜肽之 4-nitroaniline 或 β-naphthylamine 的衍生物。這和洋蔥的情形是類似的。

關鍵詞：大蒜；發芽；蛋白酶；合成基質。