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Purification and Characterization of the Excreted α-Amylase from Germinating Water Spinach (*Ipomoea aquatica* Forsk) Seeds

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ABSTRACT

Seeds of water spinach excreted α-Amylase into soak medium from testa tissues during early germination. After 36 hours imbibition the enzymes were purified from soak medium by procedures including streptomycin sulfate precipitation, hydroxyl apatite column chromatography and fast protein purification liquid chromatography. Amylase isozymes, amy I-a and I-b purified by these procedures were classified as endoamylase. Both isozymes shared a same range of pH optimum. The molecular weight of amy I-a and I-b were 47.5 and 46.3 kDa respectively. Amy I-a was heat stable and showed a low Km value for potato starch, and high Km value for soluble starch substrates. Amy I-b was heat labile and had the similar Km value for both substrates. Those properties suggest the two isozymes may have distinct molecular conformation.

Key words: seed germination, α-Amylase, enzyme properties

Introduction

Starch is a general storage nutrient existing in different plant tissues. Amylase is recognized as the important enzyme involved in starch degradation. Its enzyme activity can be detected in almost all of starch storage organs (Greenwood and Milne, 1968; Lin *et al.*, 1988).

In the more thoroughly studied cereal grains, it is generally believed that the hydrolytic enzymes are synthesized in aleurone cells and secreated into endosperm in response to gibberellins produced by the embryo (Briggs, 1973; Varner and Ho, 1976; MacGregor and Matsuo, 1982; Beck and Ziegler, 1989). Many studies demonstrate aspects of the molecular biology of α-amylase in cereals, such as gene family and the regulation mechanism of gene expression (Nolan and Ho, 1988; Sutliff *et al.*, 1991; Ranjhan *et al.*, 1992; Yu *et al.*, 1996). The regulation of carbohydrate mobilization is less clear for dicot seeds, where the major storage organs are cotyledons (Sutcliffe and Bryant, 1977). Activity of α-amylase increased during germination and seedling growth. This activity increase was associated with a rapid loss of starch in peas and lentils (Brown and Wray, 1968; Yomo and Varner, 1973; Tarrago and Nicolas, 1976). Gibberellin tended to have no influence upon α-amylase activity in intact peas (Sprent, 1968b), whereas cytokinin was effective in promoting amylase activity (Sprent, 1968a; Gepstain and Ilan, 1970; Locker and Ilan, 1975).

It was reported that α-amylase activity was immediately induced, and following the activity increases of β-amylase and starch phosphorylase during seed germination of peas, suggesting a temporal cooperation of enzymes in carbohydrate degradation (Juliano and Varner, 1969). In dict seeds reserve carbohydrate other than starch have also been observed in the seeds of fenugreek, an endospermic legume (Reid and Meier, 1973), as well as in the endosperm cells of lettuce (Halmer *et al.*, 1975).

Water spinach is an annual herbaceous dicot plant distributed mainly in southern Asia. Seeds have rigid seed coat and contain storage organs of both cotyledon and endosperm. According to anatomical studies, endosperm in dry seeds accumulate large amount of starch granules,

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which disappear rapidly during germination (Lin, 1993). Our previous results have shown that in the endosperm both enzyme activity and mRNA of α-amylase were high 12 hours after germination. Enzyme activity decreased with time after germination and coincided with the corruption of endospermic tissue. Gibberellin administration as well as benzyladenine treatment did not affect the pattern of α-amylase activity in endosperm (Ho *et al.*, 1999). In this report the gibberellin effect on excreted α-amylase during early germination and its time course were investigated. Excreted enzymes were then purified and characterized in order to understand the possible physiological role of the enzyme.

Materials and Methods

Plant materials

Seeds of *Ipomoea aquatica* Forsk were purchased from Gau-nong seed company in Kaohsiung and sterilized by a procedure similar to that described in Ho *et al.*, (1999). After sterilization, seeds were imbibed with distilled water in a ratio of 3 ml per gram and placed in a growth chamber at 30°C in darkness.

Enzyme purification

After 36 hours imbibition of 400 g seeds, crude enzyme were collected from soak medium centrifuging at 27,200g for 15 min. The supernatant was concentrated by ultrafiltration (Amicon YM 30K) to remove small molecules. The concentrated filtrate was resuspended by addition of streptomycin sulfate to a final concentration of 0.01 g/ml. The precipitate was removed by centrifugation at 27,200g for 15 min. The supernatant was then loaded on a hydroxyl apatide column (52 x 1.5 cm), pre-equilibrated by 50 mM potassium phosphate buffer (pH 6.8). The enzyme was eluted by step-concentration of same buffer from 0.2 to 0.3 M. Collection proceeded in a speed of 0.1 ml/min, generating 1.0 ml for each fraction.

Peaks of enzyme activities, named amy I and amy II, were collected separately (Figure 1). After concentration by filtration, 2.0 mM phenylmethylsulfonylfluoride was added to each filtrate and dialyzed overnight against 10 mM potassium succinate buffer (pH 6.8) containing 0.03 mM $CaCl_2$. After dialysis the major activity fraction, amy I was purified further by fast protein purification liquid chromatography (FPLC) (Shelby, 1993). A 5 x 0.5 cm Mono Q column (Pharmacia) pre-equilibrated by 10 mM potassium succinate buffer (pH 6.8) containing 0.03 mM $CaCl_2$ was used. After sample application, proteins were eluted by same buffer containing step-concentration of 0, 20, and 100% of 1.0 M NaCl. Amy I showed two peaks. A very early peak fraction of enzyme activity was pooled and designated as amy I-a. The second peak fraction , named amy I-b, was eluted with the buffer containing 0.2 M NaCl (Figure 2).

Enzyme assay and protein estimation

Activity of α-amylase was assayed according to the method of Doehlert and Duke (1983). A 0.1 ml enzyme solution was added to 0.2 ml assay mixture containing 100 mM potassium succinate buffer (pH 6.8), 3 mM calcium chloride and 2% potato starch. After incubation at 30^0 C for 30 min, a 0.25 ml reagent of dinitrosalicylic acid and 0.55 ml of distilled water was added, and heated in boiling water for 5.0 min. After cooling, the absorbance of solution was measured at 540 nm. One unit of activity release of 1 μmol of reducing power per min. Activity assay during purification was carried out by the procedures described by Rinderknecht *et al.* (1967). A 50 μl enzyme solution was added to 0.1 ml reaction mixture containing 100 mM potassium succinate buffer

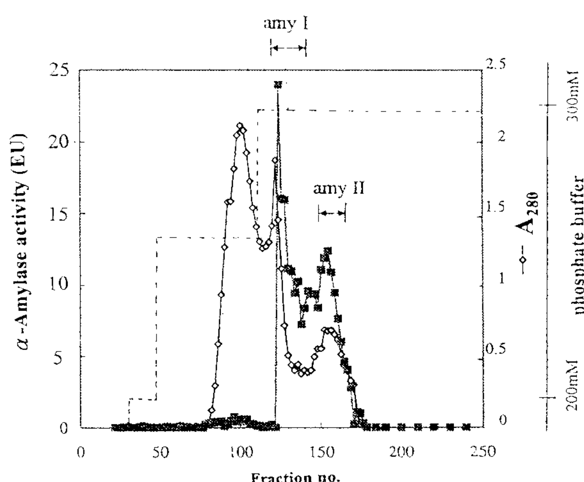

Figure 1. Profile of hydroxyl apatite column chromatography for amylolytic enzymes. Fraction volume was 1.0 ml. ◇, A_{280} absorbance; ■, enzyme activity; dashed line, concentration of phosphate buffer during elution. Arrow indicates the recovery range of amylase activity.

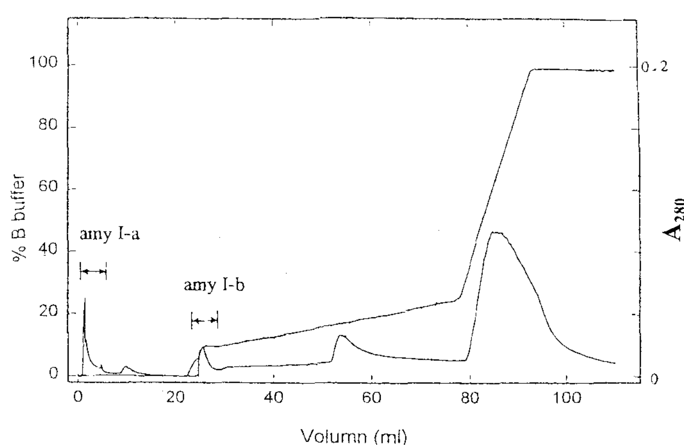

Figure 2. Profile of mono Q column chromatography for amylolytic enzymes from amy I showed in Fig. 1. Fraction volume was 1.5 ml. Arrow indicates the recovery range of amylase activity.

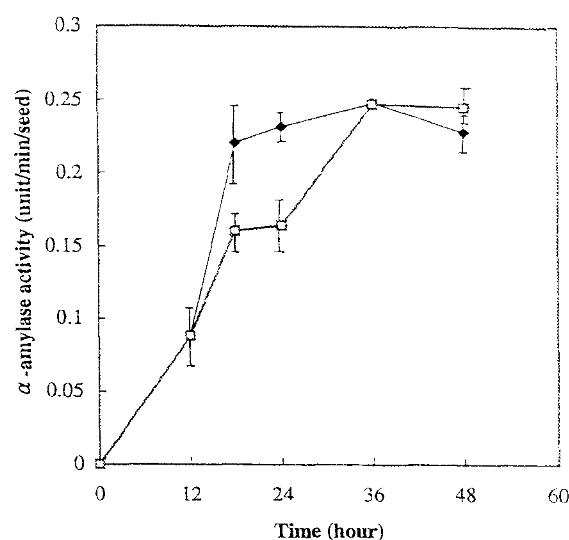

Figure 3. Time courses of α-amylase activity in soak medium of germinating water spinach seeds. ◆, with or □, without treatment of 0.1 mM gibberellic acid during imbibition.

(pH 6.8), 3 mM $CaCl_2$ and 2% starch azure. After incubation at 30^0 C for 30 min, 0.5 ml 20% TCA was added and centrifuged at 2,800 g for 10 min. The absorbance of supernatant at wave length of 595 nm was measured. One unit (EU) of α-amylase activity is defined as the amount that causes an increase of one unit of absorbance per hour.

Activity of β-amylase was assayed with the same procedures as for α-amylase except that a reaction mixture of 5 mM sodium acetate buffer (pH 3.6) containing 1 mM EDTA and 2% potato starch was used. Protein content was determined by Coomassie Brilliant Blue G-250 (Sigma) according to the method of Bradford (1976). Bovine serum albumin served as a standard.

Thermostability and Substrate specificity

Thermostability of the enzyme was assayed at various temperatures from 40 to 70^0C. In each assay, enzyme reaction was started by addition of the 100 mM potassium succinate buffer (pH 6.8) containing 3 mM $CaCl_2$ and 2% potato starch. After 60 min incubation, reaction was stopped by adding of dinitrosalicylic acid reagent. Colorimetric determination was performed at 540 nm.

Substrate specificity of the enzyme was measured using 2% of various substrates for α- and β-amylase digestion. Commercial α-amylase from *Bacillus* and β-amylase from sweet potato served as references to the reactions.

Results

Activity of α-amylase in soak medium during seed germination

As water spinach seed has a very rigid testa, isolation of seed tissues is only possible after soaking for 12 hours. Activity of α-amylase in the soak medium increased during seed germination and reaching a maximum at 36 hours. Enzyme activity was not observed to increase after 36 hours germination (Figure 3). Treatment of gibberellic acid accelerated α-amylase excretion between 18 and 24 hours of imbibition, but did not increase the maximum level of enzyme activity.

Table 1. Amylase activity and protein content in the soak medium and endosperm extract from water spinach seeds soaked for 36 hours in distilled water.

Fraction	Total activity (Unit/min/seed)	Specific activity (Unit/min/ug)	Protein content (μg/seed)
Soak medium			26
α-amylase	0.24	9.2×10^{-3}	
β-amylase	0.51×10^{-3}		
Endosperm extract			353
α-amylase	1.40	3.9×10^{-3}	
β-amylase	0.147		

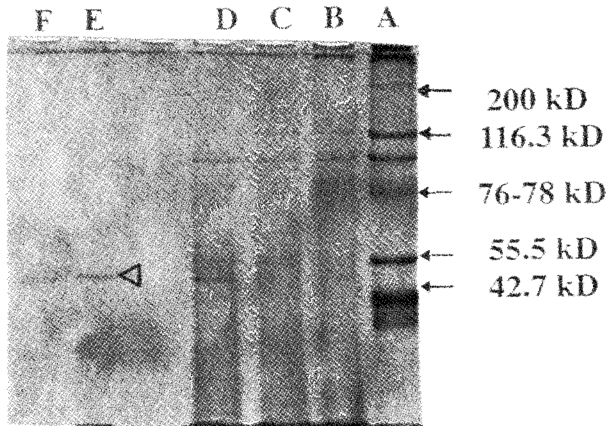

Figure 4. Silver staining of SDS-PAGE separated α-amylase at different purification steps from soak medium of water spinach seeds. Lane A, molecular weight standard; Lane B, crude soak; Lane C, 30 K membrane filtrate; Lane D, amy I collected from hydroxyl apatide chromatography; Lane E and F, amy I-a and I-b from mono Q chromatography respectively. Proteins (μg) were loaded on the gel as following: for Lane B (4); C (4); D (5); E (1.5); F (1.4).

Since α-amylase excretion reached maximum, the 36 hours germinating seeds were used to determine enzyme activity and protein content. Soluble protein content in the soak medium and the endosperm extract were 26 and 353 μg/seed respectively. Activity of α-amylase showed 0.24 unit/min/seed in soak medium which was only 17% of that in the endosperm extract (Table 1). Very low β-amylase activity was detected in the soak medium, while a significant level of activity was present in the endosperm extract. there was more than two-fold difference in specific activity of α-amylase in the medium fraction and in the endosperm extract. Since very low protein content was detected in soak medium, the high specific enzyme activity in the medium reflected a minor fraction of the proteins excreted from seed during germination.

Properties of purified α-amylase

The purification procedure was summarized in Table 2. Overall purification achievement were 32- and 15-fold increased for amy I-a and amy I-b respectively. The purified enzymes were checked by electrophoresis for both activity and size of enzyme protein. Complexity of proteins from each processes were shown on SDS-polyacrylamide gel after silver stain (Figure 4). Molecular weight, optimal pH, optimal temperature and Km values of the two isozymes, amy I-a and amy I-b are listed in Table 3.

Thermostability of the enzymes was tested by pre-incubation at temperatures from 40 to 70°C. Amy I-a was stable within one hour of incubation at 60°C. No significant activity loss was measured. When temperature was set at 70°C, activity of amy I-a decreased with time, and with only 35% activity remaining after one hour incubation (Figure 5A). In contrast with amy I-a, amy I-b was more sensitive to high temperatures. At 60°C, about 45% enzyme activity was lost in 5 min. No activity could be detected when incubated at 70°C (Figure 5B).

Table 2. Purification of α-amylase from soak medium of water spinach seeds after 36 hours imbibition.

Purification Step		Total Protein (mg)	Specific Activity (Unit/min/mg)	Total Activity (Unit/min)	Purification -fold	Recovery (%)
Crude extract		29.91	46.28	1384.47	1	100
Streptomycin Sulfate		17.90	48.72	872.11	1.05	63
Hydroxyl Apatite column	(I)	7.02	73.82	518.15	1.6	59.2
	(II)	8.05	37.41	301.20	0.8	
Mono Q Column	(Ia)	0.09	1494.4	134.5	32.3	22.2
	(Ib)	0.25	688.8	172.2	14.9	(35)*

*Recovery calculated from amylase I.

Table 3. Characteristics of α-amylase I.

Characters	α-amylase I-a	α-amylase I-b
Molecular Weight (kD)	47.5	46.3
Optimum pH	4.0 ~ 7.5	4.0 ~ 7.5
Optimum Temperature(°C)	40 ~ 60	40 ~ 50
Km value (g/ml)		
potato starch	2.1×10^{-4}	3.3×10^{-3}
solube starch	1.9×10^{-1}	7.2×10^{-3}

The effect of reducing agents on the stability of enzyme proteins were shown in Figure 6. With eight hours incubation of the enzyme at 30°C, mercaptoethanol as well as dithiothreitol provided certain degree of protection against denaturing of the enzyme protein. The enzyme molecule of amy I-a was clearly more stable than amy I-b over long periods of incubation.

Substrate specificity

To determine the substrate specificity of enzymes, naturally occurring and modified polysaccharides were used as substrates. Enzyme activities were composed to reference commercial enzymes from other organisms. Potato starch was the best substrate for both α- and β-amylase, but amy I-b only reached 90% reactivity (Figure 7). This indicates that potato starch was more suitable substrate than soluble starch as a substrate for either α- and β-amylase. Neither amy I-a nor amy I-b reacted with glycogen and dextrin. Both poorly digested with amylopectin as substrate. Surprisingly, amy I-b reacted with starch azure better than amy I-a. β-amylase did not react to starch azure.

Discussion

In the germination of cereal grains, α-amylase is produced mainly by aleurone tissue or scutellum undergoing gene expression, which synthesize enzyme proteins and secrete enzyme molecules into endosperm to mobilize starch nutrients (MacGregor and Matsuo, 1982; Miyata and Akazawa, 1982; Fincher, 1989; Ranjhan *et al.*, 1992). The mode of carbohydrate mobilization during seed germination of water spinach is quite different (Ho *et al.*, 1999). The seed contains both cotyledons and endosperm. Starch granules were detected mainly in the endosperm tissue. The pattern of germination is known as epigeal with the cotyledons escaping from the seed coat about

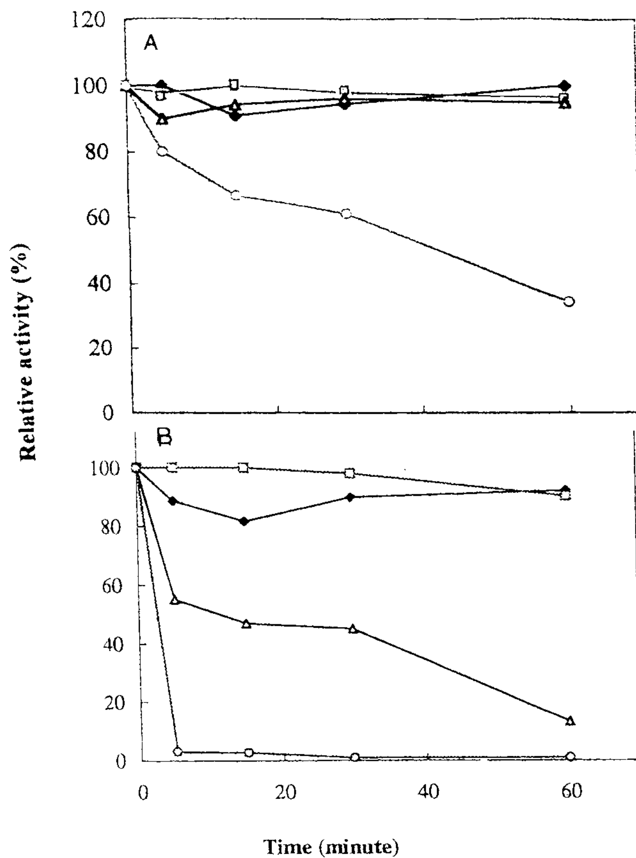

Figure 5. Thermostability of α-amylase at pH 6.8 in different temperatures. □, 40; ◆, 50; △, 60; ○, 70°C. A. amy I-a; B. amy I-b

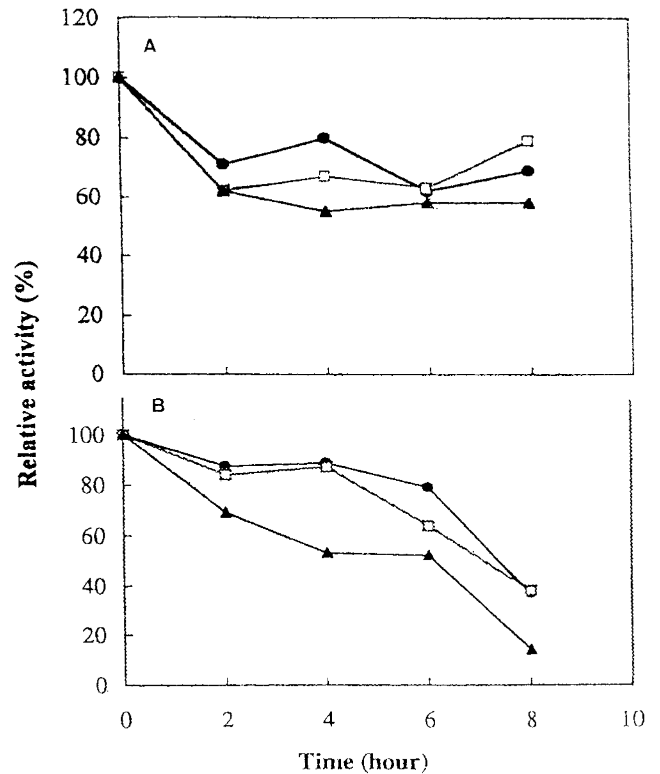

Figure 6. Change in α-amylase activity in the presence of reducing agents. Mercaptoethanol or dithiothreitol was added to the final concentration of 10 mM. Reaction mixture incubated at 30°C for various times before activity assay. A. amy I-a; B. amy I-b. ▲, control; ●, mercaptoethanol; □, dithiothreitol.

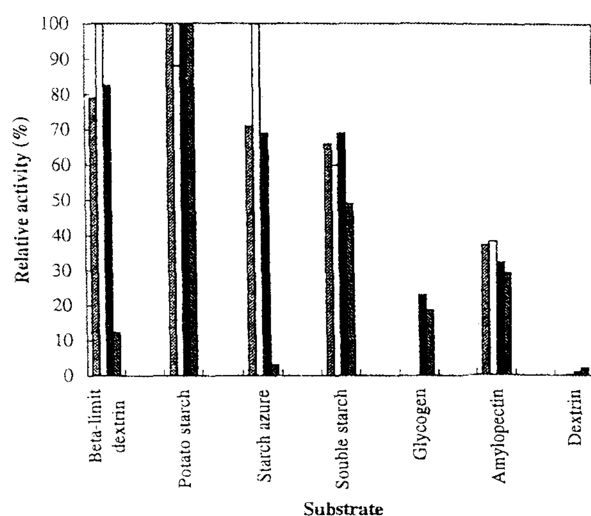

Figure 7. Relative hydrolytic activity of amylase to various glucan substrates. Amylase activities were determined by reducing power production. ▨, amy I-a; □, amy I-b; ■, α -amylase from Bacillus; ▧, β -amylase from sweet potato. Relative activities were compared with the means of three repeats and standardized to potato starch which was 100%.

the forth-day of germination in order to build photosynthetic apparatus (Lin, 1993). Both enzyme activity and mRNA of α -amylase were high in the dry seeds as well as in the endosperm of seeds soaked for 12 hours indicating that α -amylase and its mRNA should be accumulated in the endosperm during seed maturation.

During seed germination α -amylase activity decreased rapidly, correlately with the disappearance of starch granules in the endosperm. Degradation of enzyme to support the utilizable amino acids rather than inactivation or simply leakage of enzymes might be involved in the decrease of enzyme activity. Although, a fraction of α -amylase activity was detected in the soak medium during early phase of germination (Figure 3), it may not be relevant to the leakage of enzymes from endosperm. These enzymes may be excreted from the testa for the three following reasons. Treatment of gibberellic acid could accelerated the excretion of enzyme into soak medium (Figure 3), while slowing rate of decrease in enzyme activity in the endosperm (Lin, 1993). There was a six-fold difference in enzyme activity between endosperm and soak medium at 36 hours of germination (Table 1). Endosperm tissue was still intact after 36 hours of germination. Therefore, it seems unlikely that the enzymes excreted into the soak medium originated from endosperm.

Traditionally, the preliminary steps of purification involved heating at 70^{0}C and used of ammonium sulfate, alcohol or glycogen precipitation (Sanwo and Darleen, 1992). In our preparation, we could not avoid the loss of large amounts of activity by such treatments. It was confirmed that the character of α -amylase in the soak medium was sensitively denatured by high salt or organic reagents. Heat stability has been used to eliminate starch degraded enzymes other than α -amylase (MacGregor *et al.*, 1988). Amy I-a exhibited relative heat stablility with more than 90% activity remaining after 60 min incubation at 60^{0}C. Under the same conditions 45% activity of amy I-b was lost in only 5 min incubation. Total activity was lost in 5 min when the temperature was raised to 70^{0}C (Figure 5). The different stability of amy I-a and I-b suggests variation in molecular conformation.

The patterns of digestion products from amy I-a and amy I-b after chromatography using either potato starch or soluble starch were similar to that of *Bacillus* α -amylase but much different from that of sweet potato β -amylase (data not shown). It is suggested that amy I-a and amy I-b were typical endoamylase. Molecular weight of purified enzymes of amy I-a and I-b were 47.5 and 46.3 kDa respectively. The molecular weight of α -amylase from different rice tissues are located in the range 0f 40 to 48 kDa (Okamoto and Akazawa, 1978; Chiba *et al.*, 1990). The enzyme protein of α -amylase in dicot plants was mostly monomeric (Ziegler, 1988; Morohashi *et al.*, 1989; Beers and Duke, 1990). Optimal pH of amy I-a and I-b were shown in the same range. Substrate affinity of these two isozymes was quite different; amy I-b exhibited almost same K_m values for both potato starch and soluble starch, while amy I-a showed a very low K_m value for potato starch but high K_m value for soluble starch at neutral pH (Table 3). Only under acidic conditions (pH 3.8) was soluble starch the best substrate for both amy I-a and I-b.

Amylopectin and soluble starch were shown to be better substrates for α -amylase purified from mature pea leaves (Ziegler, 1988) and poplar leaves (Witt and Sauter, 1966). Both amy I-a and I-b were able to degrade β -limit dextrins. This

substrate specificity pattern is also typical of the endoamylase. The relative rates of hydrolysis of various homoglucans by amy I-a and I-b were most similar to the α -amylase derived from pea and poplar leaves, but the reactivity was relatively lower when amylopectin was used as substrate.

Amy I-a functioned well in the hydrolysis of potato starch, whereas amy I-b reacted better to β -limit dextrins and starch azure. The difference in action pattern to substrates suggests that the isozymes can compensate each other to achieve some physiological functions in the early phase of germination. Since the time course pattern of enzyme activity in soak medium was not altered by treatment of cycloheximide, the enzyme proteins should presumably preexist in testa tissues, not synthesized during germination. This situation was also observed from testa tissues of peanut in the preliminary test. The physiological roles of the excreted α -amylase may be involved in the degradation of testa tissues, in the search for carbohydrate nutrients from the environment or in protective interaction with microbes.

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蕹菜種子萌發初期釋出性澱粉酶的純化與性質分析

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摘　要

蕹菜種子萌發初期可偵測到α澱粉酶由種皮組織釋放到浸液中。存於三十六小時浸液中之酵素則經由硫化鏈黴素沉澱、氫氧化磷酸鈣管柱層析及快速蛋白液態層析等步驟加以純化。依酶的水解產物判斷，純化的 amy Ia 及 Ib 應爲內切澱粉酶。二者具相似的最佳酸鹼作用範圍，其分子量分別爲 47.5 及 46.3kD。amy Ia 對高溫穩定，以馬鈴薯澱粉爲受質時測得的 km 值很小，而對可溶性澱粉測得的 km 值則高。相對地，amy Ib 對高溫不穩定，活性喪失很快，以馬鈴薯澱粉或可溶性澱粉爲受質所測得的 km 質相似。這些性質上的差異，顯示此二異構酶的分子結構有明顯的不同。

關鍵詞： α澱粉酶，蕹菜，種子萌發

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