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Purification and characterization of beta-amylase in maize kernel

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Abstract. Five major amylolytic enzymes, Amyl-1, 2, 3, 4 and 5, were present in germinating maize kernels. Amog these five amylases, Amyl-5 was heat-labile, and mainly associated with aleurone layer. Amyl-5 was purified and identified as β-amylase based on the analysis of digestion products. The β-amylase contained one polypeptide and had an apparent molecular mass of 60 KD determined by SDS-PAGE. The pI of β-amylase was 4.20, and the enzyme had a broad pH optimum. The sensitivity of enzyme to heavy metals and p-chloromercuribenzoic acid indicated that sulfhydryl groups were essential for catalytic activity.

Key words: Beta-amylase; Maize; Purification.

Introduction

Starch is the major component of cereal grains and is the most important carbon source for the growth of germinating seedlings. Several degradative enzymes such as α- and β-amylases, are involved in the hydrolysis of starch (Beck, 1985; Kruger and Lineback, 1987; Steup, 1988) with α-amylase being the key enzyme to initiate the degradation. In barley, α-amylase is *de novo* synthesized by the scutellum and aleurone layer after the initiation of germination, and the enzyme released by aleurone layer is the major contribution to the total amylolytic activity of endosperm (MacGregor *et al.*, 1984; Mundy *et al.*, 1985). Unlike α-amylases, β-amylase in barley is synthesized during seed development and deposited in the endosperm as a latent form (Hardie, 1975; Hara-Nishimura *et al.*, 1986; Fincher, 1989). The latent form is bound to proteins such as protein Z through disulfide linkages and can be extracted with reducing agents. The bound β-amylase is activated *in vivo* by proteolytic enzymes produced during germination (Beck and Ziegler, 1989; Sopanen and Lauriere, 1989).

Early studies with maize kernels indicated that α-amylase activity was originated exclusively in the scutellum while β-amylase was formed exclusively in the endosperm (Dure, 1960). Subsequent studies on the amylolytic activity demonstrated that maize endosperm could express α-amylase activity in response to exogeneous gibberellic acid or germination (Ingle and Hageman, 1965). Goldstein and Jennings (1978) showed that three isoforms of α-amylase and one β-amylase were present in germinating maize kernels, although a more complex pattern was observed in germinating maize kernels by isoelectric focusing (MacGregor *et al.*, 1988). None of the individual maize amylase was well characterized in terms of physiological functions and localization. As the first step towards understanding the physiological role and regulated expression of starch degrading enzymes in germinating maize, maize β-amylase was purified from the endosperm of germinating seeds and its physicochemical properties were examined.

Materials and Methods

Enzyme Preparation

Seeds of maize *(Zea mays* L.) TN 351 were obtained from Taiwan Seed Service. Uniform-sized

seeds wre soaked with running water for 24 h (designated as day 1) and allowed to germinate on moist vermiculite at 28°C in the dark. Forty growing seedlings were harvested four days after seed imbibition and separated into aleurone layers, scutella, and starchy endosperms. Tissue sample was homogenized in 4 ml extraction buffer with a Polytron. The extraction buffer contained 50 mM Tris-HCl (pH 7.0), 3 mM NaCl, 4 mM $CaCl_2$, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 1 ppm leupeptin, and 10 mM β-mercaptoethanol. The homogenate was filtered through a Nitex cloth having pore size of 20×20 μm. The filtrate was centrifuged at 12,000 g for 15 min, and the supernatant was used for determining amylolytic activity.

Assay of Amylolytic Activity

The enzyme preparation was assayed directly for amylolytic activity or was treated with low pH acetate buffer (50 mM, pH 3.6) for 12 h before assaying for β-amylase activity (Dure, 1960). Amylolytic activity was measured by the method of Bernfeld (1955). The reaction mixture containing 1.5% potato starch in 50 mM sodium acetate (pH 5.4) and enzyme preparation was incubated at 37°C. At time intervals, 0.3 ml of reaction mixture was withdrawn, mixed with 0.2 ml 3,5-dinitrosalicylic acid (DNSA) reagent and then boiled for 5 min. Absorbance at 540 nm was read after adding 2.5 ml of distilled water. DNSA reagent consisted of 1% 3,5-dinitrosalicylic acid, 0.4 M NaOH, and 1.0 M potassium sodium tartrate. One unit of activity was defined as the amount of enzyme which gave one unit change in absorbance in 10 min.

Activity Detection on Native Gel

Slab polyacrylamide gel electrophoresis was carried out to analyze amylase pattern according to the procedure of Kakefuda and Duke (1984). The gel concentration was 8%. After electrophoresis, the gel was rinsed twice with 50 mM acetate buffer (pH 5.4), and incubated in 4% starch solution containing 50 mM sodium acetate buffer (pH 5.4) and 5 mM $CaCl_2$ for 90 min. Subsequently, the gel was stained with iodine solution containing 1 mM I_2, 10 mM KI, and 0.1 M HCl. Amylase activity appeared as a colorless band against a dark blue background.

Enzyme Purification

Whole endsperms including aleurone layers and starchy endosperms collected from 5-day-old seedlings were used for the purification of β-amylase. Endosperms were homogenized (1 g/1 ml buffer) with a Polytron in extraction buffer. The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10,000 g for 10 min. The supernatant was further centrifuged at 150,000 g for 30 min and then fractionated with ammonium sulfate. Proteins precipitated from 2.4-3.2 M ammonium sulfate was dissolved in extraction buffer and dialyzed against 50 mM Tris-HCl (pH 7.0) containing 4 mM $CaCl_2$ and 10 mM β-mercaptoethanol. The dialyzed sample was applied to Waters DEAE-5PW column (21.5 mm × 15 cm) pre-equilibrated with the dialysis buffer. The column was washed with 2 bed volumes of dialysis buffer containing 80 mM NaCl before elution with a linear gradient from 80 to 500 mM NaCl. Fractions having amylolytic activity were pooled and concentrated by ultrafiltration with a PM-10 membrane in Amicon Macrosolute Concentrator. The concentrated enzyme preparation was then subjected to size exclusion chromatography with a Waters 300SW column. The active fractions were collected and concentrated with a PM-10 membrane. Glycerol was added to give 25% final concentration, and the enzyme preparation was stored at -20°C for further studies. All extraction and purification steps were carried out at 4°C. Protein content in enzyme preparation was determined according to the Bradford method (1976) using BSA as a standard.

Determination of β-Amylase Properties

a. Products Analysis. Purified β-amylase with 0.1 unit activity was incubated with 2% starch in 50 mM acetate buffer (pH 5.4) for 2 h. Reaction mixture equivalent to the reducing power of 20 μg maltose was spotted on a TLC plate (Silica 60 F254, Merck No. 5735). The TLC plate was developed with a mixture of n-butanol: 90% ethanol:water (5:3:2, v/v/v) before drying and spraying with a coloring reagent. The coloring reagent was composed of 1 g diphenylamine, 1 ml aniline, 50 ml n-butanol, and 7.5 ml 80% phosphoric acid. The spots of reducing sugars turned brown after baking at 105°C for 10 min. Glucose, maltose, and other oligosaccharides produced by purified maize α-amylase or commercial sweet potato β-amylase (from Sigma) were co-chromatographed as reference.

b. Determination of Apparent Molecular Mass. Analytical SDS-PAGE of 10% polyacrylamide slab gel

was performed according to the procedure of Laemmli (1970). Proteins were visulalized by staining with Coomassie brilliant blue R-250. The Mr of β-amylase was determined by comparing with protein standards obtained from Bio-Rad. The Mr of native β-amylase was determined by size exclusion chromatography with a Waters 300SW column. The molecular weight protein standards used were obtained from Serva.

c. pI Determination. A 5% polyacrylamide slab gel containing 5% Serva Ampholyte (pH 3-7) was prepared and prefocused for 100 V-h with Pharmacia Multiphor-II system. Samples treated with 2% Ampholyte (pH 3-7) were applied to the gel 1.5 cm from the cathod with application filter strips. Pharmacia pI markers were co-electrofocused for pI calibration. Isoelectric focusing (IEF) was performed at 10°C for 3100 V-h. After focusing was completed, the gel was cut into two parts, one part stained with Coomassie blue for proteins, and the other stained for amylolytic activity.

d. Effect of pH on Activity. Buffers (50 mM) ranging from pH 2.0 to 10.0 were prepared with proper buffer reagents: Glycine-HCl (pH 2.0-3.5), acetic acid -NaOH (pH 3.5-6.5), Tris-HCl (pH 6.5-9.0), Glycine -NaOH (pH 9.0-10.0). Purified β-amylase was preincubated with various buffers in ice bath for 12 h before starch solution prepared in water was added for assaying the amylolytic activity.

e. Thermostability. Aliquots of purified emzyme preparation were treated at 45°, 50°, 55°, 60° or 65°C for 5 to 15 min. The remaining activity from heat treatment was determined at 37°C after centrifugation at 12,000 g for 15 min.

f. Effects of Cations and Sulfhydryl Agents. Ions in purified β-amylase preparation was removed by gel filtration. Various metal ions were added into test tube containing enzyme preparation to give 1 mM final concentration. Dithiothreitol (DTT) was included in the mixture in some cases as indicated. The mixture was pre-incubated in ice bath for 2 h before the enzyme activity was determined. Aliquots of enzyme preparation were treated with a various concentrations of pCMB (p-chloromercuribenzoate) in the presence or the absence of DTT, and the remaining activity was determined after 2 h of incubation.

Results and Discussion

Amylolytic Activity in Germinating Maize Kernels

Previous studies suggested that α-amylase was originated exclusively in scutellum and β-amylase was localized only in the maize endosperm (Dure, 1960). Our studies indicated that 4-day-old germinating maize kernels contained five bands with amylolytic activity (designated as Amyl-1, 2, 3, 4, and 5) based on separation by native polyacrylamide gel electrophoresis (Fig. 1). Analysis of hydrolytic products suggested that Amyl-1, 2, 3, and 4 were α-amylase isozymes (data not shown). Although Amyl-3 and 4 were present in starchy endosperm, scutellum, and aleurone layer, Amyl-1 and 2 were found primarily in starchy endosperm. The presence of two major α-amylases, Amyl-3 and 4, in all three kernel parts was not surprising since α-amylase was shown to be synthesized in the

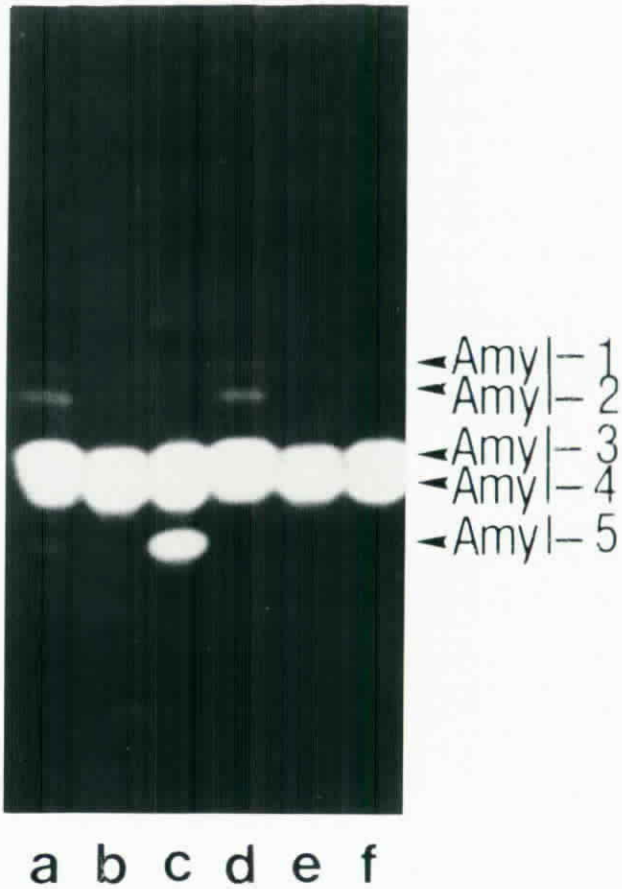

Fig. 1. Zymogram of amylolytic activity of extracts prepared from 4-day-old germinating maize kernels. Proteins extracted from starchy endosperms (lanes a and d), scutella (lanes b and e), and aleurone layers (lanes c and f) were treated with (lanes d-f) or without (lanes a-c) heating at 70°C for 15 min.

scutellum and aleurone layer before being released into starchy endosperm. Amyl-5, on the other hand, was localized only in the aleurone layer. It is interesting to note that heat treatment at 70°C for 15 min resulted in a complete loss of Amyl-5 activity, but not the others. This heat labile and aleurone layer-specific amylase was further purified, and verified as β-amylase.

Enzyme Purification and Identification of Amyl-5 as β-Amylase

Germinating endosperms, including starchy endosperms and aleurone layers, were used to purify Amyl-5 because isolation of aleurone layers was very time-consuming. In the crude extract, α-amylase contributed a major amylolytic activity in germination kernels (Fig. 2, lane a). Fractionation by ammonium sulfate eliminated most of the α-amylase activity (Fig. 2, lane c); the purification was reached at the expense of a 50% loss in total β-amylase activity (Table 1). Minor α-amylase contamination was completely removed by Waters DEAE-5PW chromatography (data not shown). Fractions containing β-amylase activity were pooled and further purified by size exclusion chromatography on Waters 300SW column which resulted in one single peak of β-amylase activity. As shown in Fig. 3 (lane e), this enzyme peak contained only one single proteinc band on SDS gel.

Analysis of amylolytic products generated by the purified enzyme clearly indicated that it is a β-amylase, and the preparation was free of contamination. Besides the immobile β-limited dextrin stayed at the original spot, maltose was the only sugar produced by the Amyl-5 digestion (Fig. 4, lane c). The digestion of soluble starch by maize α-amylase produced glucose, maltose, and other oligosaccharides (Fig. 4, lane e). The reaction products of sweet potato β-amylase from Sigma showed that this commercial β-amylase was contaminated by α-amylase or some other amylolytic enzymes.

Fig. 2. β-amylase activity shown on a native gel following steps of purification. The gel was stained for amylolytic activity. Lane a, crude extract (2.0 μg); lane b, 150,000 g supernatant (2.0 μg); lane c, ammonium sulfate precipitated fraction (1.0 μg); lane d, peak from DEAE chromatography (0.1 μg); lane e, active fraction from size exclusion chromatography (0.1 μg).

Table 1. *Purification of maize endosperm β-amylase*

Purification step	Total Protein (mg)	Total Activity (unit)	Specific Activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	87.00	2075	23.85	1.00	100.0
Ultracentrifugation	78.84	2053	26.03	1.09	98.9
Ammonium sulfate precipitation	17.32	888	51.27	2.15	42.8
Ion exchange Waters DEAE-5PW	0.86	247	287.21	12.04	11.9
Size exclusion Waters 300SW	0.58	185	318.96	13.37	8.9

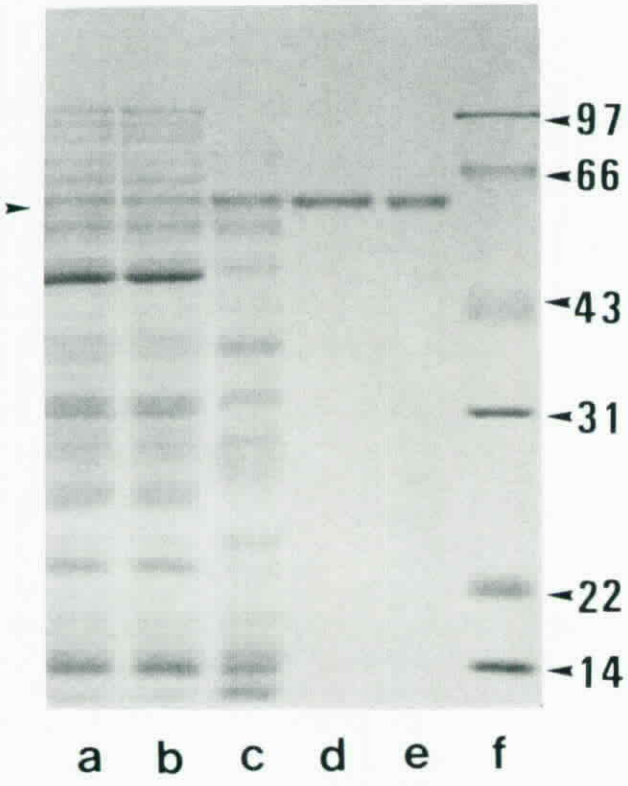

Fig. 3. SDS-PAGE of maize endosperm β-amylase prepared at different purification steps. The gel was stained with Coomassie blue for proteins. The arrow indicates β-amylase. Lane a, crude extract (100 μg); lane b, 150,000 g supernatant (100 μg); lane c, ammonium sulfate precipitated fraction (10.0 μg); lane d, peak from DEAE chromatography (0.5 μg); lane e, active fraction from size exclusion chromatography (0.5 μg).

Physicochemical Properties

The purity of purified β-amylase was further verified by IEF. Only a single activity band and protein appeared at pH 4.20 on the IEF gel (Fig. 5). Previous studies indicated that the pI of partially purified maize β-amylase was 4.55 determined by tube gel IEF, and there was no isoforms (Goldstein and Jennings, 1978). However, the multiplicity of β-amylases had been reported in several other plants (Mikami *et al.*, 1982; Doehlert *et al.*, 1982). Similar to maize β-amylase, pea epicotyl β-amylase was also reported as a single form having pI of 4.35 (Lizotte *et al.*, 1990). Unlike maize and pea enzymes, β-amylases in barley (Shewry *et al.*, 1988), soybean (Mikami *et al.*, 1982), and wheat (Tkachuk and Tipples, 1966) were resolved into 5, 7 and 4 isoforms on IEF gels, respectively. Most acidic wheat isoforms had pI from 4.4 to 4.6 and were similar to that of the maize kernel β-amylase, but the maize β-esalyma is more acidic than any isoform of barley (5.0-6.0) or soybean (5.07-5.93).

The apparent molecular mass of the monomeric maize β-amylase determined by SDS-PAGE was 60 KD (Fig. 3), while determination by size exclusion exhibited a Mr of 76 KD (data not shown). Plant β-amylase may be either monomeric or multimeric in nature. Monomeric β-amylases have been found in

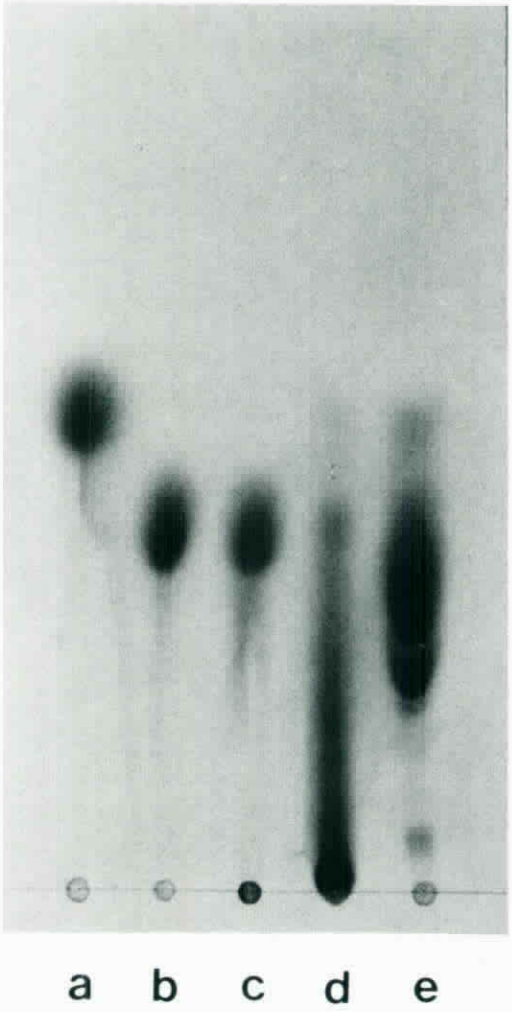

Fig. 4. Digestion products of soluble starch by amylases. Lane a, glucose standard; lane b, maltose standard; lane c, digestion products of purified maize β-amylase; lane d, digestion products of commercial sweet potato β-amylase (from Sigma); lane e, digestion products of purified maize α-amylase.

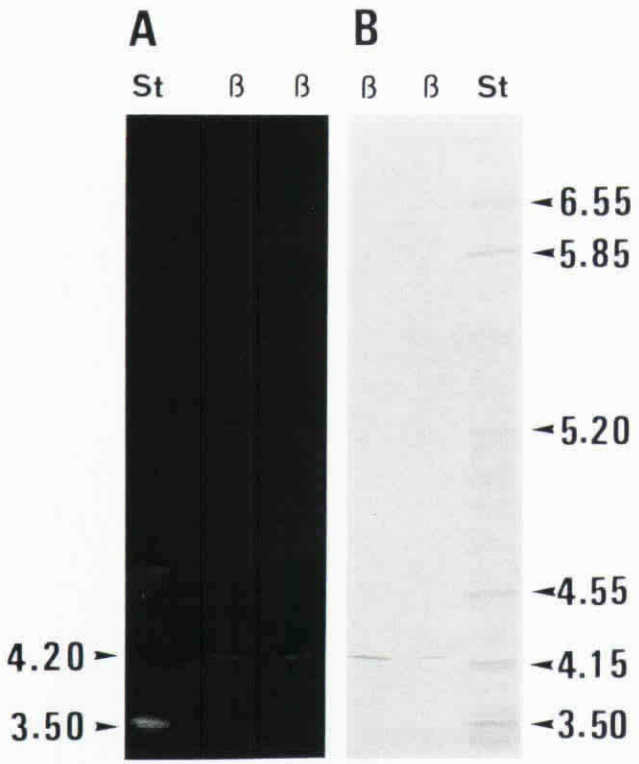

Fig. 5. Isoelectric focusing gel of purified maize endosperm β-amylase. The gel was infiltrated with starch and stained for activity (A) or stained for proteins (B). The pH gradient of gel was from 7.0 (top) to 3.0 (bottom). The Pharmacia pI markers (St) co-electrofocused contained the following proteins: amyloglucosidase (3.50), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), and human carbonic anhydrase B (6.55).

many plant species such as pea (Lizotte *et al.*, 1990), wheat (Daussant and Lauriere, 1990), and alfalfa (Doehlert *et al.*, 1982). Multimeric β-amylases have been identified in *Vicia faba* leaves with native Mr of 107 KD and subunit Mr of 26 KD (Champan *et al.*, 1972), and sweet potato with native Mr of 200 KD and subunit Mr of 50 KD (Thoma *et al.*, 1971).

In general, the pH optimum for the activity of plant β-amylase is slightly acidic, pH 4.5–6.2 (Tkachuka and Tipples, 1966; Lundgard and Svensson, 1987; Mikami *et al.*, 1982; Doehlert *et al.*, 1982; Lizotte *et al.*, 1990). However, maize β-amylase showed a broad range of pH optimum between 5 and 8 (Fig. 6). The maize β-amylase was active with slightly alkaline conditions, more than 45% of activity was retained at pH 9.0. Tkachuk and Tipples (1966) reported that fraction A and fraction E of wheat β-amylases remained only about 30% of the maximum activity at pH 8.5, and fraction B had only 12% at pH 8.0.

It is generally accepted that plant and bacterial β-amylases are not tolerant to high temperature, they are neither active nor stable at temperature above 65°C. The exception was an extracellular β-amylase of *Clostridium thermosulphurogenes* which was extremely thermostable (Shen *et al.*, 1988). The optimum temperature for the bacterial β-amylase was 75°C and some enzyme activity could be retained after incubation at 80°C for 2 h if Ca^{2+} was present. Thus, thermostability of the purified maize β-amylase was examined in the presence of 4 mM Ca^{2+} (Fig. 7). The enzyme lost about 20% of its activity after heating at 55°C for 15 min. Treatment at 60°C for five min retained about 50% activity, but 65°C caused a complete loss of activity within five min of preincubation. Unlike β-amylase, maize α-amylases are heat stable and may keep more than 80% activity after heating at 70°C for 15 min (Fig. 1). The difference in heat-stability was used as a tool to distinguish the contribution of α and β-amylase to total amylolytic activity in germinating maize (Dure, 1960).

None of the cations tested was essential for maize β-amylase activity either in the presence or absence of DTT (Fig. 8). Similar to other plant β-amylases, the activity of maize β-amylase was severely inhibited by Hg^{2+} and Cu^{2+}, but DTT could resecue the enzyme

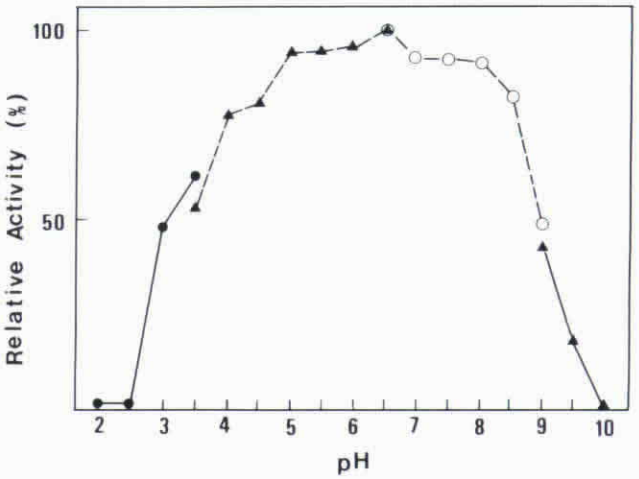

Fig. 6. Effect of pH on β-amylase activity. The enzyme activity was determined in various buffer reagents: Glycine-HCl (pH 2.0–3.5), acetic acid-NaOH (pH 3.5–6.5), Tris-HCl (pH 6.5–9.0), Glycine-NaOH (pH 9.0–10.0).

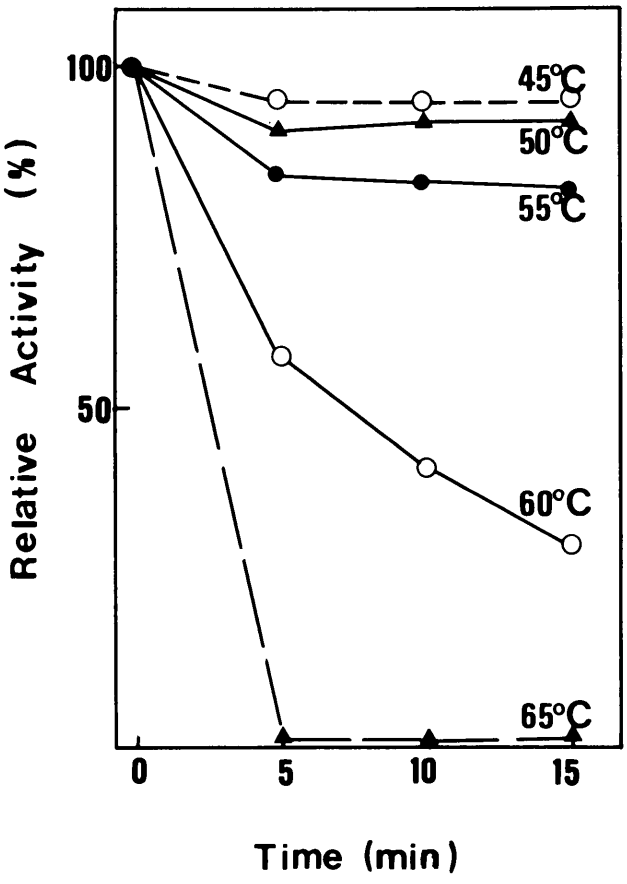

Fig. 7. Thermostability of β-amylase. Purified enzyme (0.1 μg) was treated at various temperatures for 5, 10 or 15 min before assaying the activity at 37°C.

activity completely or partly. Unlike other β-amylases, maize β-amylase activity was strongly inhibited by Fe^{3+}. The mechanism of Fe^{3+} inhibition on maize β-amylase seemed different from that of Cu^{2+} or Hg^{2+} because Fe^{3+} inhibition could not be significantly reversed by DTT. The inactivation caused by pCMB was similar to that found in other plant β-amylases (Marshall, 1975). The addition of DTT could reverse the inhibitory effect of pCMB to a great extent (Fig. 9). The results indicate that sulfhydryl groups of maize β-amylase were essential for the activity, although it is not clear whether the sulfhydryl groups were directly involved in catalysis.

Although β-amylase may contribute to the amylolytic activity during germination, its physiological role remains ambiguous. Some β-amylase deficient mutants of barley, rye and rice could germinate with-

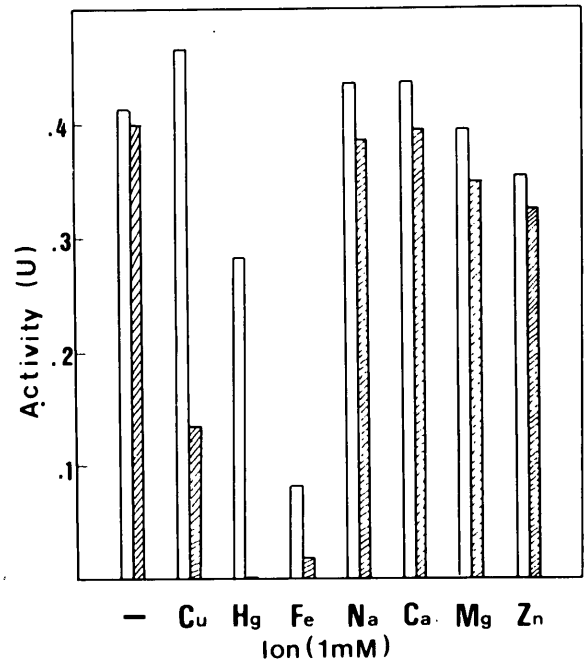

Fig. 8. Effect of cations on β-amylase activity. The enzyme (0.14 μg) was incubated with 1 mM cation in the presence (open column) or the absence (striped column) of 10 mM DTT for 2 h before the amylolytic activity was determined.

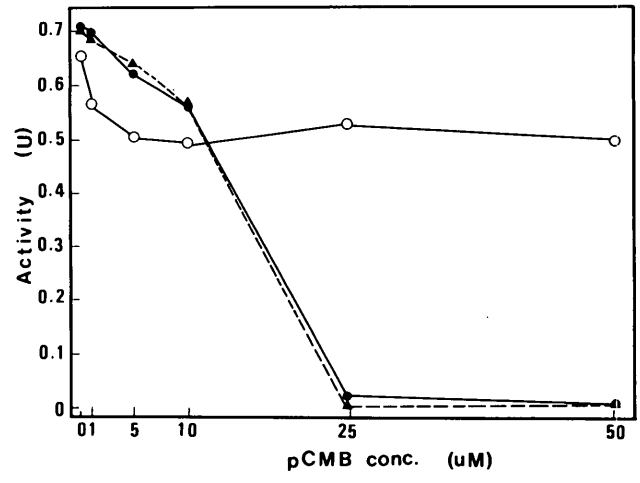

Fig. 9. Effect of pCMB on β-amylase activity. The enzyme (0.3 μg) was pre-incubated in various concentrations of pCMB for 2 h, and then dispensed equally into three tubes. The enzyme activity in first tube was assayed at once (●—●); the second and the third tubes were further incubated for additional 2 h after introducing an equal volume of 10 mM DTT (○—○) or Tris-HCl buffer, pH 7.0 (▲—▲).

out any difficulty (cited in Beck and Ziegler, 1989). Severa "nonsweet" sweet potatoes deficient in β-amylase accumulated normal amount of starch and had normal sprouting at the next generation. These findings suggest that β-amylase is not essential for either starch accumulation in developing tuberous roots or starch mobilization in the germinating cereal grains and sprouting tuberous roots (cited in Nakamura *et al.*, 1991). However, the site of synthsis and distribution of β-amylase in germinating maize kernels appeared to be different from that in barley. Further studies on the regulated expression of β-amylase in developing and germinating kernels are necessary, so that the role of β-amylase in starch metabolism in maize kernels may be established.

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玉米穀粒 β-澱粉酶的純化與定性

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在萌芽的玉米穀粒中出現五個澱粉水解酶，Amyl-1, 2, 3, 4, 5；其中 Amyl-5 的活性主要存在於糊粉層部分，且其活性在 70°C 加熱後即告消失。Amyl-5 經純化後，以色層分析法鑑定其澱粉水解的產物，可以確定其爲 β-澱粉酶。玉米 β-澱粉酶是單元體，且依 SDS-PAGE 分析結果，顯示其分子質量約爲 60 KD。以 IEF 法測得其 pI 值爲 4.20；又玉米 β-澱粉酶催化反應的適切 pH 範圍相當寬。由重金屬離子和 pCMB 的嚴重抑制結果，顯示玉米 β-澱粉酶的活性表現需要有 SH 基的存在。