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β-Amylase in germinating millet seeds

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Abstract

β-Amylase (EC 3.2.1.2) was isolated from germinating millet (*Panicum miliaceum* L.) seeds by a procedure that included ammonium sulfate fractionation, chromatography on DEAE-cellulofine and CM-cellulofine, and preparative isoelectric focusing. The enzyme was homogeneous by SDS–PAGE. The M_r of the enzyme was estimated to be 58,000 based on its mobility on SDS–PAGE and gel filtration with TSKgel G4000SW$_{XL}$, which showed that it is composed of a single unit. The isoelectric point of the enzyme was 4.62. The enzyme hydrolyzed malto-oligosaccharides more readily as their degree of polymerization increased, this being strongest for malto-oligosaccharides larger than 13 glucose residues and very weakly for maltotriose. Amylose, amylopectin and soluble starch were the most suitable substrates for the enzyme. While the enzyme showed some activity against native starch by itself, starch digestion was accelerated 2.5-fold using α-amylase, pullulanase and α-glucosidase. This enzyme appears to be very important for the germination of millet seeds.

Keywords: *Panicum miliaceum L.*; Gramineae; Millet; β-Amylase

1. Introduction

β-Amylases (EC 3.2.1.2) are exo-hydrolases that release β-maltose from the non-reducing end of α-1,4-linked poly- and oligoglucans until the first α-1,6-branching point along the substrate molecule is encountered. The enzyme is distributed in many higher plants and some bacteria. In cereal seeds, the enzyme is present in free and bound forms (Ziegler, 1999; Shinke et al., 1988). During germination, however, a bound form is activated either by a disulfide reductase or by a proteolytic enzyme, and the enzyme activity increases markedly (Shinke et al., 1988; Sopanen and Lauriere, 1989). It has also been reported that β-amylase is synthesized de novo during the early germination of rice (Okamoto and Akazawa, 1980).

On the other hand, starch is the major component of most of the world's crop yield, and the degradation of starch is essential in the germination of these plants. This degradation is thought to occur only hydrolytically. In germinating pea seeds, starch degradation is initiated by α-amylase (Juliano and Varner, 1969). α-Amylase produces soluble oligosaccharides from starch, and these are then hydrolyzed by β-amylase to liberate maltose. Finally, α-glucosidase breaks down maltose into glucose (Swain and Dekker, 1966; Nomura et al., 1969). Therefore, β-amylase is an essential enzyme for germination. Moreover, Nandi et al. (1995) suggested that β-amylase activity is a reliable indicator of the germination ability of rice seed stocks and of their vigor during germination. Das and Sen-Mandi (1992) have also demonstrated that β-amylase is more important than α-amylase during the early hours of germination in wheat scutella. However, it has also been shown that the seeds of some mutants of rye and barley that germinate well exhibit only very low levels of β-amylase (Daussant et al., 1981; Kreis et al., 1987). Gibson and co-workers (2001) suggested that little or no β-amylase activity is required to maintain normal starch levels, rates of phloem exudation and overall plant growth . Moreover, it has also been reported that β-amylase may merely be a storage protein (Giese and Hejgaard, 1984). If so, β-amylase may be considered to be insignificant for germination.

Considering these previous findings, it is important to clarify the physiological role of β-amylase in starch degradation.

In millet seeds, β-amylase activity significantly increases during days 1–4 of germination. In accordance

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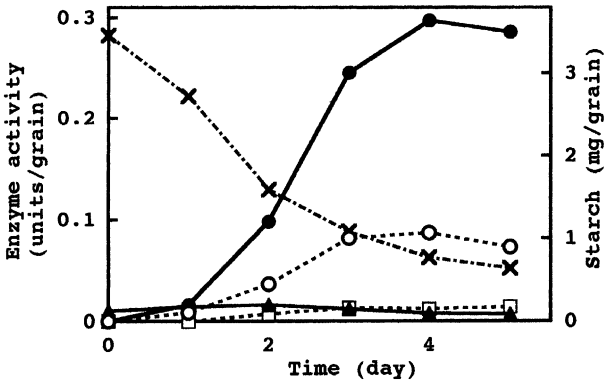

Fig. 1. Changes in some enzyme activities and the starch content in millet seeds. (○), α-amylase; (●), β-amylase; (□), pullulanase; (▲), α-glucosidase; (×), starch content.

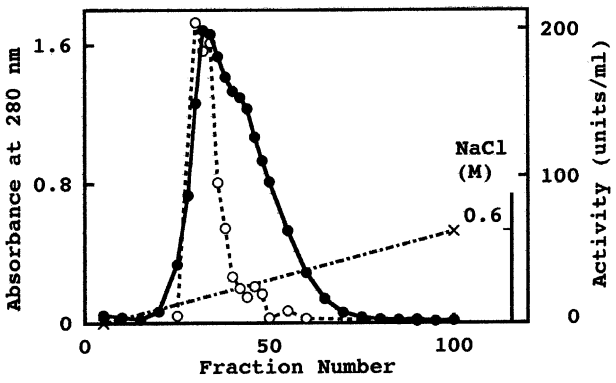

Fig. 2. Chromatography of β-amylase on CM-cellulofine. The experimental conditions are described in the text. Flow rate, 40 ml h^{-1}; fraction volume, 8.5 ml; (●), A_{280}; (○), β-amylase activity.

with this increase in enzyme activity, germination of the seeds is markedly enhanced. This shows that β-amylase in millet seeds should play an important role in germination. The purpose of this study was to elucidate the function of β-amylase in starch digestion to clarify the role of this enzyme in germination.

2. Results and discussion

2.1. Isolation of β-amylase

β-Amylase activity is significantly increased during days 1–4 of germination and shows the greatest increase after 4 days of germination (Fig. 1). Therefore, a crude enzyme solution was prepared from seeds that had germinated for 4 days, as described in the Experimental section. The enzyme solution was dialyzed overnight against 20 mM Tris–HCl buffer, pH 8.0, and then applied to a DEAE-cellulofine column (3.4×15 cm) equilibrated with 20 mM Tris–HCl buffer, pH 8.0. After the column was washed with the same buffer, a linear gradient of NaCl (0–1 M) in the same buffer was applied. The peak that exhibited β-amylase activity was collected, and $(NH_4)_2SO_4$ was added to give 0.9 satn. The precipitate was collected by centrifugation and dissolved in 20 mM NaOAc buffer, pH 4.5. After dialysis with 20 mM NaOAc buffer, pH 4.5, the dialysate was applied to a CM-cellulofine column (3.4×15 cm) equilibrated with the same buffer. After the column was washed with the same buffer, a linear gradient of NaCl (0–0.6 M) in the same buffer was applied (Fig. 2). The peak that exhibited β-amylase activity was concentrated using an Amicon ultrafiltration device (PM-10 membrane) and dialyzed overnight against 20 mM NaOAc buffer, pH 4.5. The dialyzate was subjected to preparative isoelectric focusing (pH, 3.5–5.0), using an HSI GT Tube Gel Electrophoresis Unit (Hoefer Scientific Instruments, CA, USA). The active fractions were pooled. After insoluble materials were removed by filtration, the enzyme solution was concentrated using an Amicon ultrafiltration device and dialyzed overnight against 20 mM NaOAc buffer, pH 4.5. The purification procedure is summarized in Table 1. The purified enzyme was homogeneous by SDS–PAGE. The M_r of the enzyme was estimated to be 58,000 based on its mobility on SDS–PAGE and from gel filtration with TSKgel G4000SW_{XL}, which showed that it was composed of a single unit. Most of the β-amylases in plants are composed of a single protein, although those from sweet potato and the hedge bindweed are homotetramers (Shinke et al., 1988; Van Damme et al., 2001). The isoelectric point of the purified enzyme was determined by analytical gel electrofocusing to be 4.62, which is the most acidic of the β-amylases reported thus far (Shinke et al. 1988).

2.2. General properties

The optimum pH of the enzyme was found to be 5.5–6.0. After 15 h of pre-incubation at 20° with 50 mM McIlvaine's buffer and sodium carbonate–boric acid

Table 1
Summary of purification of β-amylase from germinating millet seeds

Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg prot.)	Yield (%)
1. Ammonium-sulfate fractionation	19,200	23,410	1.22	100
2. DEAE-cellulofine column chromatography	2400	17,860	7.44	76.3
3. CM-cellulofine column chromatography	164	10,440	63.66	44.6
4. Preparative isoelectric focusing	28	2910	103.93	12.4

buffer, the enzyme was stable in a pH range of 3.5–9.0. The optimum temperature of the enzyme was found to be 55° after 30 min of incubation. After 15 min of pre-incubation with 50 mM NaOAc buffer, pH 6.0, at various temperatures, the enzyme was stable at temperatures up to 55°.

2.3. Inhibition with metal ions and PCMB

When the enzyme was preincubated with 5 mM of metal ions at 37° for 30 min, Hg^{2+}, Mn^{2+} and Cu^{2+} reduced its activity by 80% or more. PCMB completely inhibited the enzyme activity even at a low concentration of 10 μM. This result indicates that the enzyme contains sulphydryl groups, like other β-amylases.

2.4. Substrate specificity

Various substrates (180 nmol) were incubated with the enzyme under standard conditions, and the relative rates of hydrolysis are shown in Table 2. As the degree of substrate polymerization increased, it was more readily hydrolyzed by the enzyme. The enzyme hydrolyzed amylopectin and soluble starch three times faster than maltoheptaose, while it hydrolyzed maltotriose only very weakly. The enzyme hydrolyzed soluble starch and liberated only maltose.

On the other hand, the enzyme preferably hydrolyzed malto-oligosaccharides larger than 13 glucose residues, when it was incubated with amylose EX-I (Hayashibara Co., Ltd., Okayama), which consists of many malto-oligosaccharides (Fig. 3). These results show that an α-1,4-glucan larger than 13 glucose residues is the best substrate for β-amylase.

2.5. Changes in the activity of β-amylase during germination of millet seeds

Fig. 1 shows the activity of β-amylase from millet seeds on different days of germination. The activity of β-amylase increased markedly from days 1–4 of germination, and the highest activity was seen after 4 days of germination. A similar trend was seen for α-amylase activity. However, the starch content of millet seeds markedly decreased during this period. Therefore, starch digestion was carried out using the same volume of α, β-amylases, pullulanase (Seikagaku Corporation) and purified α-glucosidase from millet seeds (Yamasaki et al., 1996) as contained in millet seeds after day 4 of germination (Table 3). Starch digestion was accelerated 2.5-fold when β-amylase was added to a reaction mixture using α-amylase, pullulanase and α-glucosidase. Thus, β-amylase should be a key enzyme in starch degradation during the germination of millet seeds.

On the other hand, Volenec and co-workers showed that alfalfa taproots contained high starch concentrations and high β-amylase activities, but defoliation reduced starch levels and β-amylase activities (Gana et al., 1998). They suggested that β-amylase was used as nitrogen source for the shoot regrowth and was not a key enzyme in starch degradation in the roots. Therefore, β-amylase will play a different role at each tissue

Table 2
Substrate specificity of the purified β-amylase Solubilized substrates (180 nmol) in 0.5 ml of 50 mM NaOAc buffer, pH 4.5, were incubated with the purified enzymes at 37 °C for 30 min. Maltose liberated was determined by the method of Kreis et al. (1987) as described in Experimental

	Relative rate of hydrolysis (%)
Maltotriose	Trace
Maltotetraose	7.6
Maltopentaose	13.8
Maltohexaose	23.3
Maltoheptaose	34.6
Amylose EX-I	100
Amylopectin[a]	114.6
Soluble starch[a]	106.9
Glycogen[a]	49.4

[a] Concentration = 3 mg (below 180 nmol) in 0.5 ml of reaction mixture.

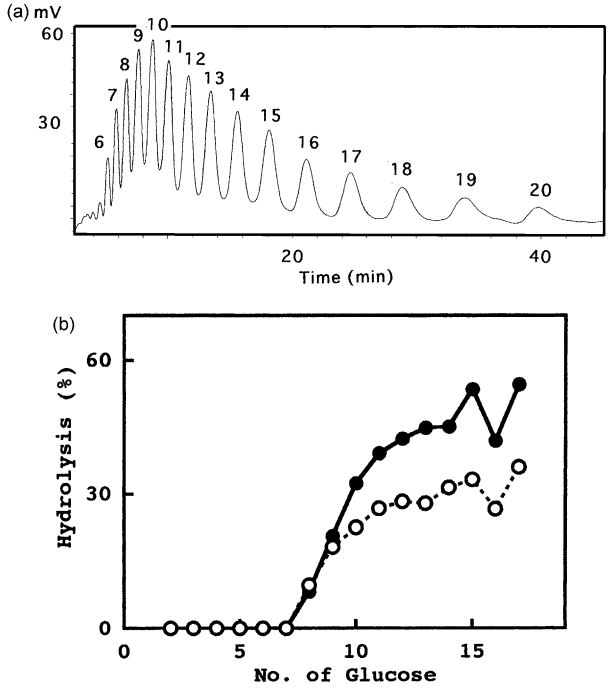

Fig. 3. HPLC of products of Amylose EX-I digestion by β-amylase. A reaction mixture (1 ml) containing amylose EX-I (20 mg) and β-amylase was incubated at 37 °C. After 30 and 60 min, the reaction was stopped by boiling for 5 min. The content of each malto-oligosaccharide in amylose EX-I was determined by HPLC as described in the Experimental section. (A) Amylose EX-I; 6-20, number of glucose residues in malto-oligosaccharides (for example, 6 means malto-hexaose); (B) degree of hydrolysis of malto-oligosaccharides in amylose EX-I by β-amylase; (○), 30 min incubation; (●), 60 min inciubation.

Table 3
Hydrolysis of starch by α-amylase and β-amylase in millet seeds germinated for 4 days

	Relative rate of hydrolysis (%)
I + II + III + IV	100
I + III + IV	39.9
I + II + IV	62.2
I + II + III	89.5
I	29.7
II	12.9

Reaction mixture containing 25 mg of starch of millet seeds, enzyme solution (β-amylase, 6.5 units; α-amylase, 1.9 units; pullulanase, 0.27 units; α-glucosidase, 0.17 units) and 50 mM NaOAc buffer, pH 5.3, in a final volume of 1.0 ml was incubated at 35 °C for 48 h. Carbohydrates were determined by the PhOH–H_2SO_4 method. I, α-amylase; II, β-amylase; III, pullulanase; IV, α-glucosidase.

located in plants. β-Amylase also showed some activity against native starch by itself.

3. Experimental

3.1. Preparation of crude β-amylase

Millet (*Panicum miliaceum* L.) seeds (4 kg) were soaked in distilled water and grown on moist absorbent cotton at 28° for 4 days. The seeds were homogenized in a homogenizer (Nissei Excel Auto- Homogenizer; Nihonseiki Co., Tokyo) with 25 mM NaOAc buffer, pH 5.3, containing 0.5 M NaCl and 5 mM mercaptoethanol. The homogenate was left overnight at 4 °C and the debris was removed by filtration. The supernatant was brought to 0.9 satn with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 25 mM NaOAc buffer, pH 5.3.

3.2. Preparation of α-amylase

Barley malt α-amylase VIII-A (Sigma) was dissolved in 20 mM Tris–HCl buffer, pH 8.0. After dialysis with the same buffer, the dialysate was applied to a DEAE-cellulofine column (3.4×15 cm) equilibrated with the same buffer. After the column was washed with the same buffer, a linear gradient of NaCl (0–0.25 M) in the same buffer was applied. The peak that exhibited α-amylase activity was concentrated using an Amicon ultrafiltration device (PM-10 membrane) and dialyzed overnight against 20 mM NaOAc buffer, pH 4.5. The dialyzate was subjected to preparative isoelectric focusing (pH 3.5–10.0), using an HSI GT Tube Gel Electrophoresis Unit (Hoefer Scientific Instruments, CA, USA). The active fractions were pooled. After insoluble materials were removed by filtration, the enzyme solution was concentrated using an Amicon ultrafiltration device and dialyzed overnight against 20 mM NaOAc buffer, pH 4.5. The purified enzyme was homogeneous by PAGE.

3.3. Preparation of native starch

Millet seeds were soaked in distilled water and homogenized in a homogenizer. After the debris was removed by filtration, precipitate was collected from the filtrate by centrifugation. The precipitate was suspended in isoamylalcohol–H_2O (1:5) to wash off proteins, and centrifuged at ca. 1940 *g* for 10 min. The precipitate was washed four times and lyophilized.

3.4. Assay of enzyme activities

Soluble starch (1 mg) was used, together with 50 mM NaOAc buffer, pH 4.5, and suitably diluted enzyme preparations in a total volume of 0.5 ml. After 30 min at 37 °C, the reaction was stopped by boiling for 5 min. With β-amylase, the amount of maltose formed was measured according to the method of Somogyi (1952). One unit of β-amylase activity was defined as the amount of enzyme that liberated 1 μ mol min^{-1} of maltose from soluble starch under the conditions described above.

With α-amylase, the iodine color of the reaction mixture was measured. The data were converted into units of β-amylase activity using values (change in iodine color (%)/β-amylase activity (unit) = 260/1) obtained with purified α-amylase.

With pullulanase, maltotriose from pullulan was measured.

3.5. Determination of protein

Protein was determined by the method of Warburg and Christian (1942). The protein profiles in column chromatography were followed by measuring the absorbance of the eluates at 280 nm.

3.6. Assay of starch

Debris removed from homogenized seeds was treated with α-amylase (Sigma Chemical Co., pancreas type I-A). The supernatant was collected by centrifugation and hydrolyzed at 100 °C for 1 h with 2 N HCl. Liberated glucose was measured as described in the literature (Papadopoulos and Hess, 1960; Dahlqvist, 1961).

3.7. Electrophoresis

SDS–PAGE was performed on 7.5% gels according to the method of Laemmli (Laemmli, 1970). After the run, the gels were stained for proteins with Coomassie Brilliant Blue.

3.8. Estimation of M_r

The M_r of the purified enzyme was estimated by SDS–PAGE (Laemmli, 1970) and gel filtration with TSKgel G4000SW$_{XL}$.

3.9. Estimation of the isoelectric point

The isoelectric point of the purified enzyme was estimated as described in the literature (Awdeh et al., 1968; Fawcett, 1968).

3.10. High-performance liquid chromatography

The products formed from starch with α, β-amylases were determined by HPLC. Chromatography was performed on a column (0.46×15 cm) of COSMOSIL 5NH2-MS, using an HPLC System (Tosoh Co., Tokyo). The column was eluted with 60% acetonitrile.

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