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Purification and characterization of an α-glucosidase from germinating millet seeds

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Abstract

An α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) was isolated from germinating millet (*Panicum miliaceum* L.) seeds by a procedure that included ammonium sulfate fractionation, chromatography on CM-cellulofine/Fractogel EMD SO_3, Sephacryl S-200 HR and TSK gel Phenyl-5 PW, and preparative isoelectric focusing. The enzyme was homogenous by SDS–PAGE. The molecular weight of the enzyme was estimated to be 86,000 based on its mobility in SDS–PAGE and 80,000 based on gel filtration with TSKgel super SW 3000, which showed that it was composed of a single unit. The isoelectric point of the enzyme was 8.3. The enzyme readily hydrolyzed maltose, malto-oligosaccharides, and α-1,4-glucan, but hydrolyzed polysaccharides more rapidly than maltose. The K_m value decreased with an increase in the molecular weight of the substrate. The value for maltoheptaose was about 4-fold lower than that for maltose. The enzyme preferably hydrolyzed amylopectin in starch, but also readily hydrolyzed nigerose, which has an α-1,3-glucosidic linkage and exists as an abnormal linkage in the structure of starch. In particular, the enzyme readily hydrolyzed millet starch from germinating seeds that had been degraded to some extent.

Keywords: *Panicum miliaceum* L.; Gramineae; Millet; α-Glucosidase

1. Introduction

In a previous paper, we reported that two α-glucosidases were isolated from millet seeds and that they each showed a very small K_m for maltose. In fact, the K_m values for maltose are the smallest reported to date for any plant α-glucosidase. Moreover, the enzymes hydrolyzed millet starch to liberate glucose.; millet seeds contain abundant α-glucosidase activity which approximately doubles after 24 h when seeds are soaked in water for germination. On the other hand, α-amylase activity was detected only very weakly, but increased markedly after 48 h. This shows that α-glucosidase in seeds may play an important role during the early stage of germination. On the other hand, some authors (Swain and Dekker, 1966; Nomura et al., 1969) have suggested that α-glucosidase is part of the non-phosphorolytic pathway for the breakdown of starch, and plays a role in seed germination by hydrolyzing the oligosaccharides produced by α- and β-amylases. However, Sun and Henson (1990) reported that barley seed α-glucosidase can initiate the attack of raw starch granules and that this attack is independent of the presence of α-amylase. This has been verified by Sissons and MacGregor (1994) and Sun et al. (1995). Therefore, the in vivo significance of α-glucosidase is not yet clear.

In view of the role of α-glucosidase in germination, we investigated α-glucosidase during the early stage of germination of millet in the context of starch degradation, which exhibits little α-amylase activity but abundant α-glucosidase activity. We describe here the purification and properties of α-glucosidase from millet seeds at an early stage of germination. Moreover, we

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attempted to examine its enzymatic activity toward a starch purified from millet seeds.

2. Results and discussion

2.1. Purification of α-glucosidase

The α-glucosidase activity of seeds approximately doubled after 24 h of germination, with the highest value from days 1 to 5. Therefore, a crude enzyme solution was prepared from seeds that had germinated for 24 h, as described in Section 3 and this was dialyzed overnight against 20 mM NaOAc buffer, pH 4.5.

The enzyme solution was applied to a CM-cellulofine column (3.4 × 15 cm) that had been equilibrated with 20 mM NaOAc buffer, pH 4.5. The column was eluted with a linear gradient of 0–0.5 M NaCl in 20 mM NaOAc buffer, pH 4.5. The peak that exhibited α-glucosidase activity was collected and applied to a Fractogel EMD SO_3 column (1.7 × 17 cm) that had been equilibrated with 20 mM NaOAc buffer, pH 4.5. After the column was washed with 20 mM NaOAc buffer, pH 4.5, a linear gradient of 0–1.2 M NaCl in the buffer was applied. The eluate with α-glucosidase activity was concentrated using an Amicon ultrafiltration device (PM-10 membrane; Amicon). The concentrate was subjected to gel filtration on a Sephacryl S-200 HR (Pharmacia). The eluate was purified further on a column (0.75 cm × 7.5 cm) of TSKgel Phenyl-5PW that had been equilibrated with 0.1 M phosphate buffer, pH 7.0, containing 1.5 M ammonium sulfate, by repeatedly using an HPLC system (AKTA, Pharmacia). After the column was washed with buffer containing 1.5 M ammonium sulfate, a linear gradient of 1.5–0 M ammonium sulfate was applied. The peak that exhibited α-glucosidase activity was subjected to preparative isoelectric focusing (IEF) (pH range, 6.5–9.0) using an HSI GT Tube Gel Electrophoresis Unit (Hoefer Scientific Instruments, CA). 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 5.3. The purification procedure is summarized in Table 1. The purified enzyme was homogeneous by SDS–PAGE (Fig. 1). The enzyme produced glucose as the sole product from amylose EX-I, which shows that the purified enzyme did not contain any α,β-amylases (Fig. 3).

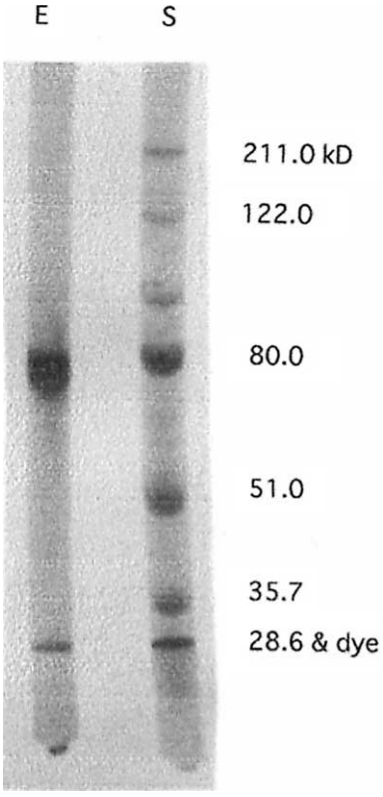

Fig. 1. SDS–PAGE of purified millet α-glucosidase. SDS–PAGE was carried out as described in Section 3. E, α-glucosiclase; S, Precision Plus Protein Standards (Dual Color, Bio-Rad).

2.2. Purification of starch

Two crude starch preparations were prepared from intact seeds and seeds that had been allowed to germinate for 24 h, as described in Section 3. The precipitates were suspended in isoamylalcohol–H_2O (1:5) to wash off proteins, and centrifuged at ca. 1940*g* for 10 min. This procedure was repeated three times. The precipitates were washed with ethyl alcohol three times and lyophilized. The starch preparations obtained from seeds and

Table 1
Purification of α-glucosidase from germinating millet seeds

Step	Total protein, mg	Total activity, units	Specific activity, U/mg prot.	Yield, %
1. Ammonium-sulfate fractionation	17,600	22,100	1.3	100
2. 1st CM-cellulofine column chromatography	3760	11,250	3.0	50.9
3. 2nd CM-cellulofine column chromatography	800	9100	11.4	41.2
4. Fractogel EMD SO3 column chromatography	290	6720	23.2	30.4
5. Sephacryl S-200 HR column chromatography	150	4800	32.0	21.7
6. TSK gel Phenyl-5 PW column chromatography	48	2330	48.5	10.5
7. Preparative isoelectric focusing	3	230	76.0	1.0

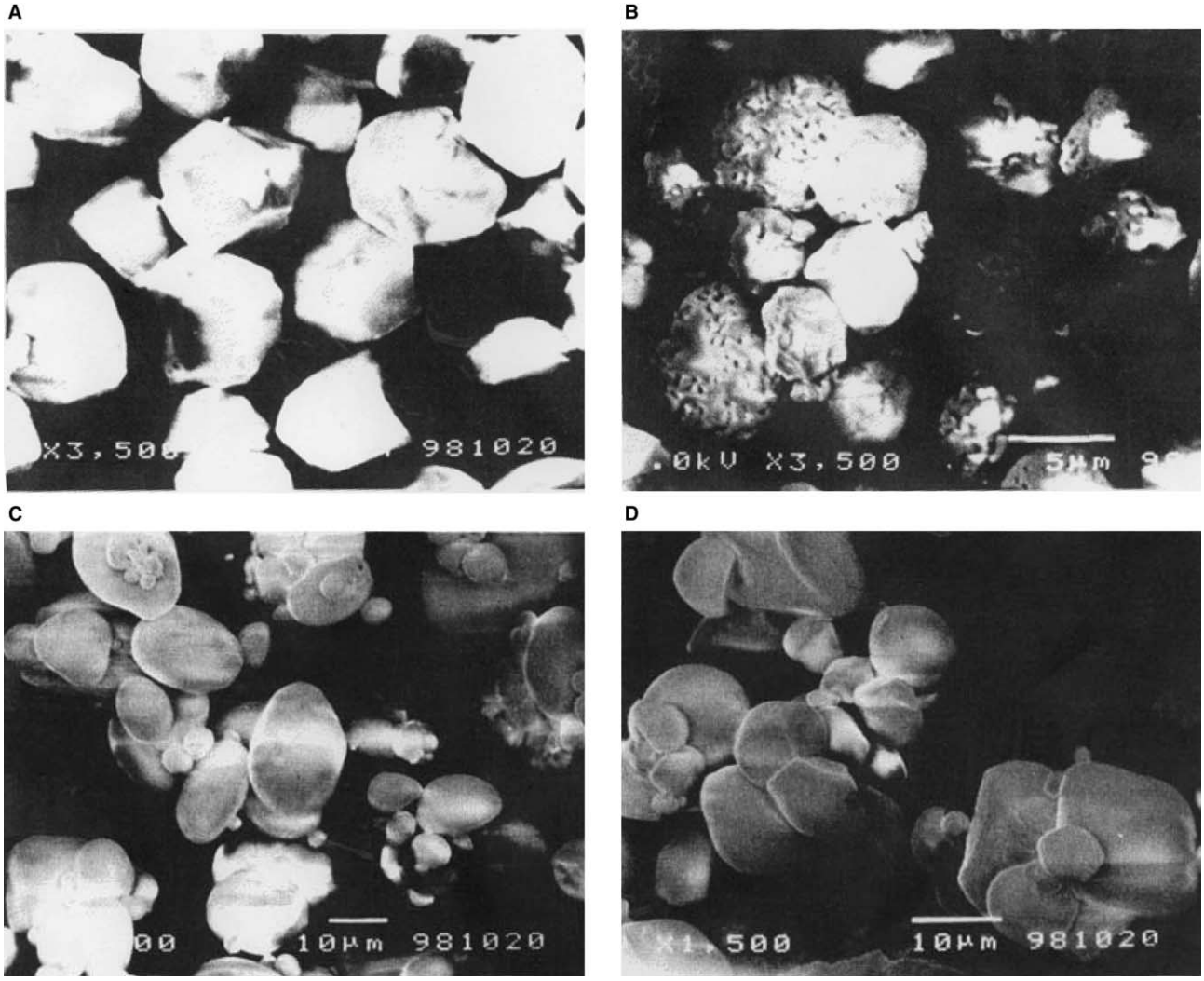

Fig. 2. SEM of starch granules. A, native starch of millet; B, starch of millet seeds germinated for 3 days; C, wheat starch; D, sweet potato starch.

germinating seeds were designated as starch I and starch II, respectively. The scanning electron micrograph clearly shows that starch II has been degraded to some extent (Fig. 2).

2.3. Chemical and physical properties of α-glucosidase

The molecular weight of purified α-glucosidase was estimated to be 86,000 based on its mobility in SDS–PAGE, and 80,000 based on gel filtration with TSKgel Super SW 3000, which showed that it is composed of a single unit. The molecular weight is similar to that of α-glucosidase from millet seeds (Yamasaki et al., 1996). The isoelectric point of the enzyme was determined to be 8.3 by analytical IEF.

The molecular weight and isoelectric point of the enzyme are similar to those of α-glucosidase from millet seeds (Yamasaki et al., 1996). Moreover, the mobility of the enzyme in native PAGE at pH 4.0 is similar to that of α-glucosidase II from millet seeds.

These results show that the enzyme is identical to α-glucosidase II protein from millet seeds.

The enzyme was again prepared from seeds after the seeds had been soaked in water for 24 h, and the activity had doubled. Therefore, it is assumed that the conformation of the enzyme protein may have changed to show higher enzyme activity than that in intact seeds.

The optimum pH of the enzyme was found to be 4.5–5.0. After 20 h of preincubation at 30 °C with 50 mM McIlvaine's buffer, the enzyme was stable in a pH range of 2.5–8.0. The optimum temperature for enzyme activity was 50–60 °C after 30 min of incubation. After 15 min of preincubation with 50 mM NaOAc buffer, pH 4.5, at various temperatures, the enzyme was found to be stable at temperatures up to 45 °C.

The enzyme was preincubated in 50 mM NaOAc buffer, pH 4.5, containing 5 mM metal ions at 37 °C for 30 min, Na^+, K^+, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Mg^{2+}, Sn^{2+} and Al^{3+} had no effect, but Hg^{2+} and Pb^{2+} reduced enzyme activity by 80% or more.

2.4. Substrate specificity

Various substrates (180 nmol) were incubated with the enzyme under standard conditions (Table 2). The enzyme readily hydrolyzed maltose, nigerose, kojibiose, malto-oligosaccharides, and solubilized polysaccharides, such as amylose, soluble starch and amylopectin. However, the enzyme hydrolyzed polysaccharides more rapidly than maltose, even at considerably low concentrations compared with maltose. The enzyme preparation did not contain any enzyme contaminant (Figs. 1 and 3). Moreover, inhibitors of α-amylase and β-amylase were used in the experiment. PCMB completely inhibited β-amylase of millet seeds at a low concentration (Yamasaki, 2003). α-Amylase of millet seeds was strongly inhibited by Cu^{2+} (data not shown). The same results were obtained when PCMB and Cu^{2+} were reacted with the substrates under the same conditions as in Table 2. Consequently, PCMB and Cu^{2+} gave the same results as in Table 2. Therefore, the results in Table 2 were obtained with single α-glucosidase.

α-Glucosidases in higher plants also readily hydrolyze polysaccharides (Chiba et al., 1978; Grinna and Robbins, 1979; Yamasaki and Konno, 1989; Yamasaki et al., 1996). The enzyme hydrolyzed amylose to liberate α-glucose, which is an inherent property of α-glucosidase (Yamasaki and Suzuki, 1978, 1980; Yamasaki et al., 1995). The K_m value for maltose is lower than those of α-glucosidases from other plants, such as rice seed (Eksittikul et al., 1993), buckwheat (Chiba et al., 1979), sugar beet (Matsui et al., 1978; Yamasaki and Konno, 1991), and barley (Henson and Sun, 1995). The K_m value for malto-oligosaccharide decreased with an increase in the molecular weight of the substrate. The value for maltoheptaose is about 4-fold lower than that for maltose. Therefore, α-glucosidase from germinating millet seeds has a stronger affinity for malto-oligosaccharides liberated from starch by α-amylase than for maltose produced by β-amylase. The α-glucosidase may preferably hydrolyze oligo-saccharides liberated from starch by α-amylase to glucose without the preceding action of β-amylase during the germination of millet seeds, although it has been suggested that α-glucosidase needs the preceding action of α- and β-amylases to play a role during the germination of plants (Swain and Dekker, 1966; Nomura et al., 1969). Moreover, the K_m value for amylopectin (MW, 6,600,000) was about 1000-fold lower than that for maltose. The enzyme hydrolyzed soluble starch more rapidly than amylopectin. Therefore, the enzyme is similar to glucoamylase, although glucoamylase has not yet been found in plants. Since glucoamylase readily hydrolyzes starch to glucose without the preceding action of α-amylase and β-amylase, the

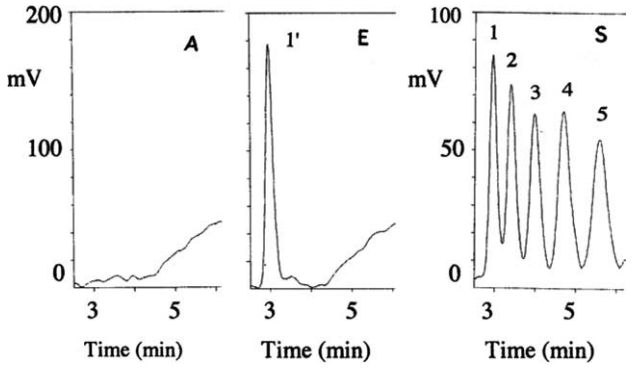

Fig. 3. HPLC of the product of the digestion of amylose by α-glucosidase. A reaction mixture (1 ml) containing amylose EX-I (10 mg) and the purified α-glucosidase was incubated at 37 °C. After 60 min, the reaction was stopped by boiling for 5 min. A 20-μl sample of the mixture (E) was assayed by eluting the column with CH_3CN–H_2O (6:4) as described in the Section 3. A, reaction mixture (20 μl) without enzyme; S, standard (each sugar, 4 μg). 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraose; 5, maltopentaose.

Table 2
Substrate specificity of α-glucosidase from germinating millet seeds

	Relative rate of hydrolysis, %	K_m value, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $mM^{-1}\ s^{-1}$
Maltose[a]	100	1.46	33.5	22.9
Nigerose[a]	93	1.46	35.7	24.5
Kojibiose[a]	79	0.34	9.44	27.4
Isomaltose[a]	3	9.76	5.75	0.589
Maltotriose[a]	138	1.13	43.9	38.8
Maltotetraose[a]	154	0.86	40.7	47.4
Maltohexaose[a]	236	0.45	36.1	80.4
Maltoheptaose[a]	247	0.39	47.5	123.4
Amylose EX-I[a]	233	0.28	26.2	92.3
Amylopectin[b]	129	1.3×10^{-3}	24.1	191.3
Soluble starch[b]	175	3640[d]	49.7	
Phenyl α-glucoside[a,c]	10	3.90	3.80	0.974

A reaction mixture containing substrate, enzyme solution and 50 mM NaOAc buffer, pH 4.5, in a final volume of 0.5 ml was incubated at 37 °C for 30 min. Liberated glucose was determined by the glucose oxidase–peroxidase method as described in Section 3.

[a] Concentration = 180 nmol in 0.5 ml of reaction mixture.

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

[c] Liberated glucose was measured according to the method of Somogyi (1952).

[d] mg/1.

α-glucosidase from germinating millet seeds may also hydrolyze starch to glucose independently during the early stage of millet seed germination, when only weak α-amylase activity was detected. On the other hand, the α-glucosidase from germinating millet seeds readily hydrolyzed nigerose, which contains an α-1,3-glucosidic linkage. Since this linkage is a universal abnormal linkage in the structure of starch, nigerose-hydrolyzing activity may be vital for the digestion of starch.

On the other hand, polysaccharide powder (100 mg) was incubated with the enzyme in 0.3 ml of 50 mM acetate buffer, pH 4.5, at 28 °C for 48 h (Table 3). The enzyme hydrolyzed native starch granules, similar to α-glucosidases of barley seed (Sun and Henson, 1990), malt (Sissons and MacGregor, 1994) and pea (Sun et al., 1995). The enzyme hydrolyzed starch I (millet starch) and wheat starch at half the rate of soluble starch to liberate only glucose, but hydrolyzed sweet potato starch very weakly. The α-glucosidase may by itself initiate the attack of native starch granules during an early stage of germination. It has been reported that enzyme activity can be affected by the origin of the starch substrate. An α-amylase from poplar leaves hydrolyzes corn starch at about a 4-fold higher rate than potato starch (Witt and Sauter, 1996). The authors speculated that this difference is due to the large size and the resulting relatively small surface area of potato starch. The size of millet starch was similar to that of wheat starch, but much smaller than that of sweet potato (Fig. 2). Therefore, α-glucosidase may also exhibit stronger hydrolytic activity toward the small starches of millet and wheat than the large starch of sweet potato. The enzyme hydrolyzed starch II that had been degraded to some extent at more than twice the rate of soluble starch. This fact supports the notion that the enzyme may carry out vigorous digestion toward native starch by itself in the middle stage of germination.

Table 3
Activity of α-glucosidase for insoluble polysaccharides

	Glucose liberated, μg	Rate[a], %
Soluble starch	1116	100
Starch I	563	50.4
Starch II	2715	243.3
Wheat starch	584	52.3
Sweet potato starch	65	5.8
Amylose EX-III	628	56.3
Amylopectin	5588	500.0

A reaction mixture containing polysaccharide powder 100 mg, enzyme solution 0.669 units and 50 mM NaOAc buffer, pH 4.5, in a final volume of 0.3 ml was incubated at 28 °C. After 48 h, deionized water 5 ml was added to the reaction mixture, which was then thoroughly stirred. After insoluble materials were removed by centrifugation, glucose in the supernatant was measured by the glucose oxidase–peroxidase method (Papadopoulos and Hess, 1960; Dahlqvist, 1961).

[a] Relative rate of hydrolysis.

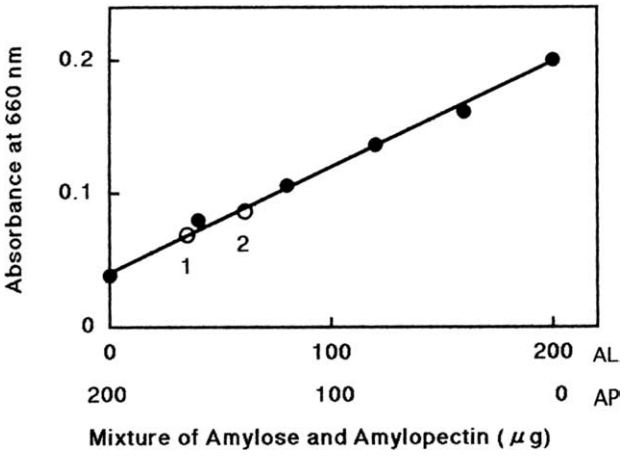

Fig. 4. Determination of amylose content in wheat starch degraded by α-glucosidase. AL, Amylose content in the mixture; AP, amylopectin content in the mixture; 1, wheat starch; 2, wheat starch degraded 25% by the purified α-glucosidase.

2.5. Mode of starch digestion by α-glucosidase

The enzyme was incubated with wheat starch under standard conditions and the remaining starch was determined by measuring the iodine color reaction. At 25% hydrolysis of wheat starch, the amylose content in the starch increased from 20% to 28% (Fig. 4). Moreover, the enzyme hydrolyzed amylopectin (mw. 6,600,000) 10-times faster than amylose EX-III (Dpn., ca. 100), when the enzyme was incubated with insoluble polysaccharides (Table 3). Therefore, the α-glucosidase from germinating millet seeds may play an important role via the preferential hydrolysis of amylopectin and hydrolysis of an α-1,3-glucosidic linkage in the structure of starch during the early stage of germination.

3. Experimental

3.1. Preparation of crude α-glucosidase

Millet (*Panicum miliaceum* L.) seeds were soaked in distilled water and grown on moist absorbent cotton at 28 °C for 24 h.

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 90% satn with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 25 mM NaOAc buffer, pH 4.5. After this was dialyzed overnight against 20 mM NaOAc buffer, pH 4.5, CM-cellulofine resin was added to the dialyzate, the resulting mixture was gently stirred for 30 min and the resin was removed by filtration. After the resin was washed with 20 mM NaOAc buffer, pH

4.5, the α-glucosidase was released by treatment with a solution, 20 mM NaOAc buffer, pH 4.5, containing 0.5 M NaCl. α-Glucosidase in the eluate was precipitated with $(NH_4)_2SO_4$ and the resulting precipitate was collected and dissolved in 25 mM NaOAc buffer, pH 4.5 as mentioned above.

3.2. Preparation of native starch

Millet seeds and germinating millet seeds were soaked in deionized water and homogenized in a homogenizer. The homogenates were filtered through gauze and the filtrates were centrifuged.

3.3. Assay of enzyme activity

α-Glucosidase activity was determined as follows. The reaction mixture containing 1 mg of maltose and enzyme solution in 0.5 ml of 50 mM acetate buffer, pH 4.5, was incubated at 37 °C for 30 min. After incubation, the reaction was stopped by boiling the mixture for 5 min. The amount of glucose formed was measured using glucose oxidase–peroxidase (Papadopoulos and Hess, 1960; Dahlqvist, 1961). One unit of α-glucosidase activity was defined as the amount of enzyme that liberated 1 μmol/min of glucose from maltose under the conditions described above.

The ratio of amylose to amylopectin was determined by measuring the iodine color of the reaction mixture and comparing it with a standard curve of the two polysaccharides mixed in various ratios.

3.4. Determination of protein

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

3.5. Electrophoresis

SDS–PAGE was performed on 7.5% gels according to the method of Laemmli (1970). After the run, the gels were stained for proteins with Phast Blue R (R-350).

3.6. Estimation of M_r

The M_r of the purified enzyme was estimated by SDS–PAGE (Laemmli, 1970) and gel filtration with TSKgel SuperSW 3000.

3.7. 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).

3.8. High-performance liquid chromatography

The product formed from amylose EX-I with α-glucosidase was 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 CH_3CN:H_2O (6:4).

3.9. Scanning electron microscopy

The digestion of starch by the enzyme was examined using a scanning electron microscope (SEM). After the reaction, the starch was washed twice with deionized water and dried. The surface was coated with gold and viewed under a Hitachi S-570 Scanning Electron Microscope operating at 5 kV.

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