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Food Chemistry 71 (2000) 221–227

Food Chemistry

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Purification and characterization of sago starch-degrading glucoamylase from *Acremonium* sp. endophytic fungus

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Received 17 November 1999; received in revised form 24 May 2000; accepted 24 May 2000

Abstract

A novel sago starch degrading glucoamylase which had a strong amylopectin-hydrolyzing-activity was purified to homogeneity from a culture filtrate of *Acremonium* sp. isolated from forest trees. The purified enzyme was an oligomeric protein of two sub-units with molecular weights of 22 and 39 kDa. Optimum temperature and pH for enzyme activity were around 55°C and 5.5, respectively. The enzyme was stable in a pH range of 3.0–7.0 and temperatures up to 60°C. The purified enzyme was strongly inhibited by EDTA. The enzyme catalyzed hydrolysis of amylose and amylopectin, showed apparent K_m values of 10.0 and 3.8 mg/ml and V_{max} of 195 μmol/ml/min and 391 μmol/ml/min, respectively. Glucose was the sole product released by the hydrolysis, indicating that this enzyme displays an exo-action of starch-degrading activity.

1. Introduction

Fungal carbohydrases have a wide application in food industries, in addition to their role in the ecological recycling of cellulose, hemicellulosic and starchy biomass materials. Of these extracellular enzymes, starch degrading amylases represent the largest potential for industrial use. Several starch-degrading enzymes are of commercial importance, such as α-amylase in brewing and baking, and isoamylase and pullulanase, as well as α-amylase, in the production of glucose syrups.

Endophytic fungi occur within plant tissues without producing any apparent symptoms and their presence may confer certain advantages to the host plant. Endophytic fungi have also been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Schulz et al., 1995). There is no report on the extraction of starch-degrading enzymes from endophytic fungi. We report here the purification and characterization of sago starch-degrading glucoamylase from *Acremonium* sp. endophytic fungus.

2. Materials and methods

2.1. Materials

Sago starch was obtained from Gamex Factories Malaysia. Tapioca, corn, rice, potato, wheat, soluble starch, amylose, amylopectin, pullulan, Sephadex G-150 and middle molecular weight of protein standard kit were from Sigma Chem., USA. DEAE-Toyopearl-650 S ion-exchanger was obtained from Tosoh Corporation, Tokyo, Japan. Other reagents were obtained from BDH.

2.2. Organism and culture conditions

Acremonium sp., isolated from forest trees in Malaysia, was used in this investigation. The characteristics of this strain have been described by Karim et al. (1998). For the production of the enzyme, a loopful of actively growing fungal mycelium was transferred from a potato dextrose agar (Difco) plate to a 500-ml Erlenmeyer flask containing 200 ml of growth medium. The medium was

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0308-8146/00/$ - see front matter
PII: S0308-8146(00)00160-6

composed of 2% (w/v) raw sago starch, 0.5 g peptone, 3.0 g $NaNO_3$, 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl and 0.01 g $FeSO_4 \cdot 7H_2O$. The incubation was carried out at 30°C for 5 days and 120 rpm in a rotary shaker. The culture fluid was filtered and centrifuged at 15 000 *g* for 10 min and the supernatant was used as a crude enzyme extract preparation.

2.3. Purification procedure

All steps were carried out at 4°C.

2.3.1. Ultrafiltration of crude extract

One thousand millilitres of the culture supernatant were concentrated using ultrafiltration (Toyo Advantec Stirred Cell), fitted with cellulose acetate membrane (molecular weight cut-off 10 000) to a volume of 20 ml. The concentrated crude enzyme was dialyzed against four changes of distilled water and the insoluble residue was removed by centrifugation.

2.3.2. DEAE-Toyopearl 650 S

The dialyzed enzyme solution was applied to the anion-exchange column (3.0×20 cm) which was equilibrated with 0.01 M Tris–HCl buffer, pH 7.5, at a flow rate of 0.5 ml/min and fractions of 5 ml were collected. The column was washed exhaustively with the equilibration buffer. The enzyme was eluted from the column with a linear gradient of 0.01–0.2 M NaCl dissolved in 0.01 M Tris–HCl, pH 7.5.

2.3.3. Sephadex G-150

The fractions containing enzyme activity were pooled and dialyzed with 0.01 M Tris–HCl buffer pH 7.5 and applied onto a Sephadex G-150 gel filtration column (2.5×90 cm), previously equilibrated with 0.1 M Tris–HCl buffer pH 7.5. The column was eluted by the same buffer solution. Elution was done at a flow rate of 0.5 ml/min and fractions of 5 ml were collected.

2.4. Enzyme activity and protein assay

Enzyme activity was assayed by measuring the reducing sugar released with sago starch as a substrate. The reaction mixture consisted of 2% (w/v) sago starch in 0.1 M acetate buffer, pH 5.5 and 0.2 ml of purified enzyme solution in a total volume of 2 ml. The reaction mixture was incubated at 55°C for 30 min. After incubation, the reaction was stopped by placing in boiling water for 5 min and the amount of reducing sugar in the supernatant was measured by the dinitrosalicylic acid method of Miller (1959). One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol reducing sugar (as glucose) per min under the standard assay conditions. Protein was assayed by Hartree's method (1972) with bovine serum albumin as the standard. The protein content of column eluates was monitored by measuring absorbance at 280 nm.

2.5. Characterization of enzyme

2.5.1. Optimum pH and stability

The optimum pH was determined by assessing the enzyme activity in the following buffer systems: 0.1 M acetate buffer, pH 3–5.5; 0.1 M phosphate buffer, pH 6.0–7.0 and 0.1 M Tris–HCl buffer, pH 8.0–9.0. Enzyme stability at different pH values was determined by measuring the remaining activity after incubating the enzyme for 22 h at 4°C at pH 3.0–9.0. The conditions of the assay were otherwise similar to that given above.

2.5.2. Optimum temperature and heat stability

The optimum temperature of enzyme activity was determined by assaying the enzyme at temperatures from 30 to 65°C. Heat stability was measured by incubating the enzyme in 0.1 M acetate buffer (pH 5.5) for 30 min at 30–65°C. After the treatment, the enzyme solution was cooled and the remaining activity was determined under standard assay conditions.

2.5.3. Effect of metal ion

The effects of various metals ions and EDTA at 1 mM on enzyme activity were determined by assaying the enzyme with the individual metal ions and EDTA under the standard assay conditions. The activity assayed in the absence of metal ions or reagents was taken as 100%.

2.5.4. Polyacrylamide gel electrophoresis

Purity of the purified enzyme and its molecular mass was determined by native-polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively, using the Laemmli system (Laemmli, 1970) on 10% polyacrylamide gel containing 1% SDS. Electrophoresis was performed in a Mini PROTEAN II dual slab cell (Bio-Rad). Gel was stained with silver staining following Morrisey (1981). Standard proteins with a molecular weight ranging from 14 400 to 97 400 (middle-molecular-weight calibration kit, Sigma) were used for calibration.

2.5.5. Substrate specificity

Several raw starches, such as sago, potato, tapioca, corn, wheat and rice, soluble starch and polysaccharides (amylose, amylopectin pullulan) at 2% (w/v) concentration were tested as substrates for the purified enzyme. The glucose released was assayed by standard procedures as described earlier.

2.5.6. Determination of kinetic parameters

The effects of amylose and amylopectin (2.5–30 mg/ml) on the reaction rate were determined at pH 5.5 and

55°C. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver–Burk plots.

2.5.7. Determination of end-product

The end-products produced were determined under optimum conditions (pH 5.5, 55°C). Reaction mixture contained 5 ml of purified enzyme solution and 5 ml of 24% substrate raw sago starch. Two millilitres samples were taken out after 6, 12, 24, 48 and 72 h and the reactions were stopped by heating in a boiling water bath for 5 min. All the reaction products were analyzed by HPLC (Waters) using the NH_2-18C column (25 cm×6.5 mm, Merck-Germany). The column was maintained at 38°C and run with 80% acetonitrile (HPLC grade) as the mobile phase at a flow rate of 1.2 ml/min. Sugar standard (glucose) was dissolved in deionized water, filtered and injected into the HPLC system. Ten microlitre samples and the standards were applied.

3. Results and discussion

3.1. Purification of enzyme

An extracellular sago starch-degrading glucoamylase was purified to homogeneity from the culture filtrate of *Acremonium sp.* grown on medium containing sago starch. The Toyopearl 650 S ion-exchange column separated the enzyme into two active enzyme peaks (Fig. 1). The major portion of enzyme activity was eluted as a sharp peak at a concentration of 0.1 M NaCl, while the minor portion was eluted as a flow-through fraction with 0.01 M Tris–HCl buffer, pH 7.5. Fractions 50–70 were pooled, concentrated and subjected to Sephadex G-150 gel filtration chromatography. This step gave symmetrical peaks of enzyme activity and protein (Fig. 2). The purification of this enzyme is summarized in Table 1. The enzyme was purified 9.8 fold with a yield of 11%. The final preparation was subjected to native-PAGE polyacrylamide gel electrophoresis. A single protein band was observed indicating the enzyme homogeneity with respect to protein (Fig. 3, III).

3.2. Properties of enzyme

The molecular masses of enzyme, estimated from the relative mobility of standard proteins on SDS-PAGE, were 22 and 39 kDa (Fig. 3, II and Fig. 4). The presence of one activity band on native-PAGE with two sub-unit bands on SDS-PAGE has also been reported by Ali, Malek and Hossain (1994) for glucoamylase from a *Myrothecium* isolate and Kimura and Horikoshi (1990) for α-amylase from *Micrococcus* sp. The activity of the enzyme towards raw sago starch was determined at pH 3.0–9.0 at 55°C. The enzyme exhibited optimum activity in a range of pH 4.5–6.0 with maximum activity at pH 5.5 (Fig. 5). At below pH 4.5 and above 6.0 the activity decreases rapidly. The enzyme was stable in a broad pH range between 3.0 and 7.0 for 22 h incubation at

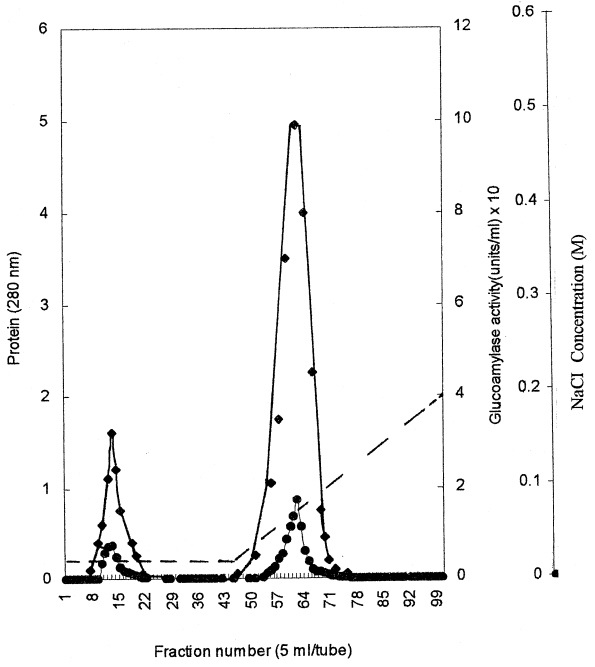

Fig. 1. Ion-exchange chromatography of sago starch-degrading glucoamylase from *Acremonium* sp. on Toyopearl-650 S (●, protein; ◆, enzyme activity; - - -, gradient elution).

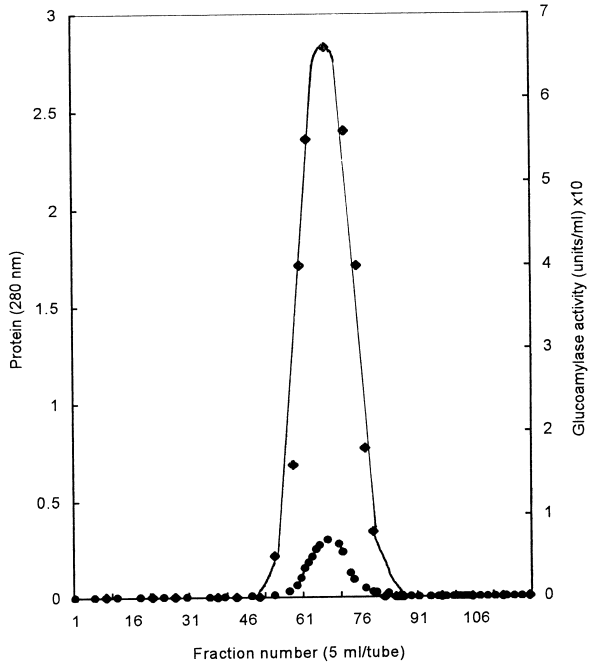

Fig. 2. Gel filtration chromatography of sago starch-degrading glucoamylase from *Acremonium* sp. on Sephadex G-150 (●, protein; ◆, enzyme activity).

Table 1
Purification of sago starch-degrading glucoamylase from *Acremonium* sp. endophytic fungus

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	1000	88	8800	100	100	1.0
Ultrafiltrate	20	17.5	2356	135	27	1.4
DEAE-Toyopearl 650 S	10	1.5	856	570	10	5.7
Sephadex G-150 gel filtration	8.5	1.0	978	978	11	9.8

[a] One unit of enzyme (U) = 1 μmol glucose produced from raw sago starch per min at pH 5.5, 55°C.

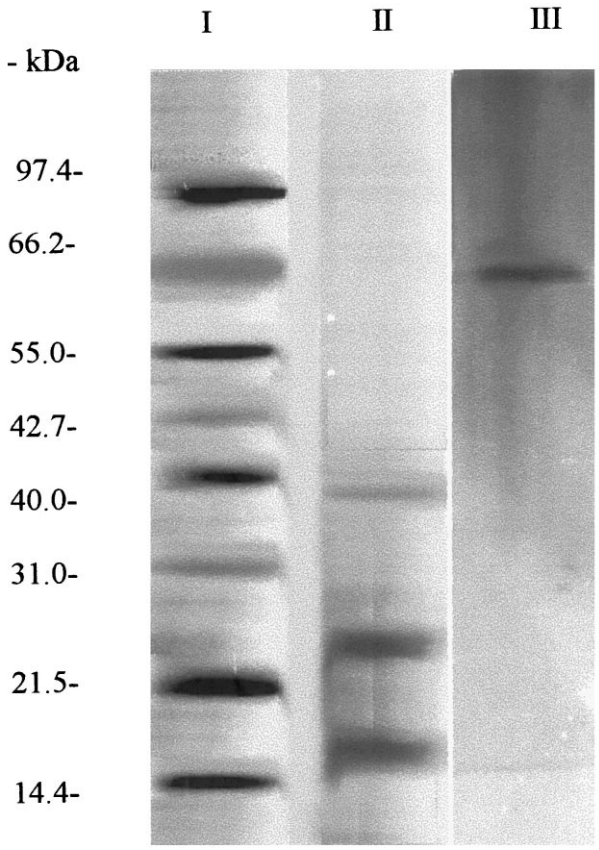

Fig. 3. Native- and SDS-PAGE of sago starch-degrading glucoamylase from *Acremonium* sp. Electrophoresis was carried out on 10% SDS polyacrylamide gel; lane I: standard phosphorylase B (97 400), bovine serum albumin (66 200), glutamate dehydrogenase (55 000), ovalbumin (42 700), aldolase (40 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400); lane II: enzyme on SDS-PAGE; lane III: enzyme on Native-PAGE.

4°C, the remaining activities at pH 8.0 and 9.0 were 38 and 28%, respectively (Fig. 5). Most fungal starch-degrading enzymes have optimum pH values of 5.0–7.0 (Okolo, Ezeogu & Ebisike, 1996; Ali et al., 1994). As shown in Fig. 6, the purified enzyme has optimum temperature of 55°C and is stable at temperatures up to 60°C for 30 min incubation. Rapid inactivation occurred above that temperature. Similar findings have been reported by Itkor, Shida and Tsukagoshi (1989) for raw starch degrading enzyme from *Bacillus* sp. B 1018.

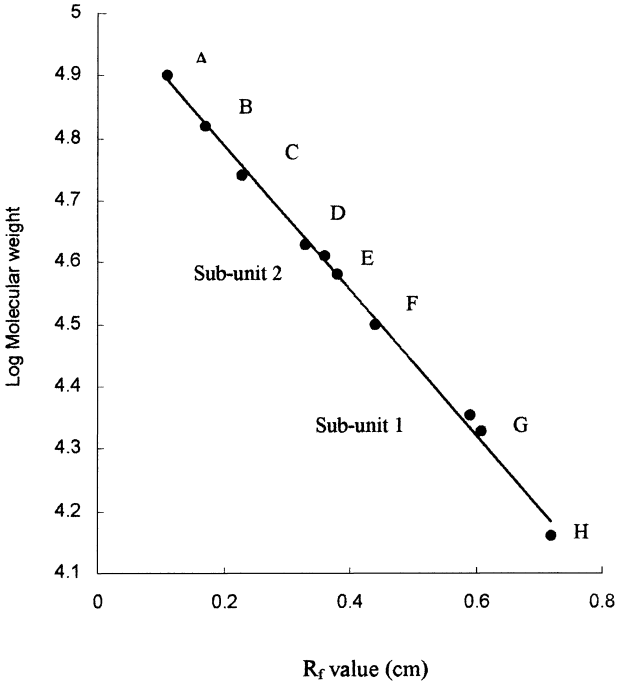

Fig. 4. Standard curves showing relationship between relative mobilities on polyacrylamide gel and relative molecular masses of enzyme subunits (●, M, standard phosphorylase B 97 400 (A), bovine serum albumin 66 200 (B), glutamate dehydrogenase 55 000 (C), ovalbumin 42 700 (D), aldolase 40 000 (E), carbonic anhydrase 31 000 (F), soybean trypsin inhibitor 21 500 (G) and lysozyme 14 400 (H); ◆, sub-unit enzyme 1; ▲, sub-unit enzyme 2).

3.3. Inhibitors and activators

As shown in Table 2, various metal ions and EDTA affected the enzyme activity. Cu^{2+}, Ca^{2+} and Mn^{2+} stimulated the enzyme activity from 7 to 57%. Al^{3+}, Co^{2+}, Zn^{2+}, Mg^{2+}, and Fe^{2+} did not significantly influence the enzymatic activity. Fe^{3+} moderately inhibited the activity while EDTA, Sr^{2+}, Hg^{2+}, Ni^{2+} and Ba^{2+} inhibited the activity more strongly (56–98%). Similar findings were also reported by Shin and Byun (1996) and Yu and Hang (1991).

3.4. Substrate specificity

The relative hydrolysis rates of various substrates by the purified enzyme are presented in Table 3. The

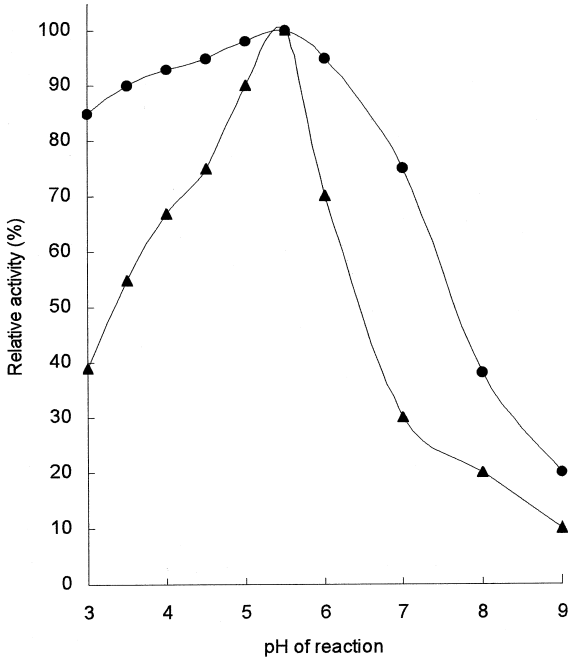

Fig. 5. Optimum pH and pH stability of purified sago starch-degrading glucoamylase from *Acremonium* sp. (▲, optimum pH; ●, pH stability).

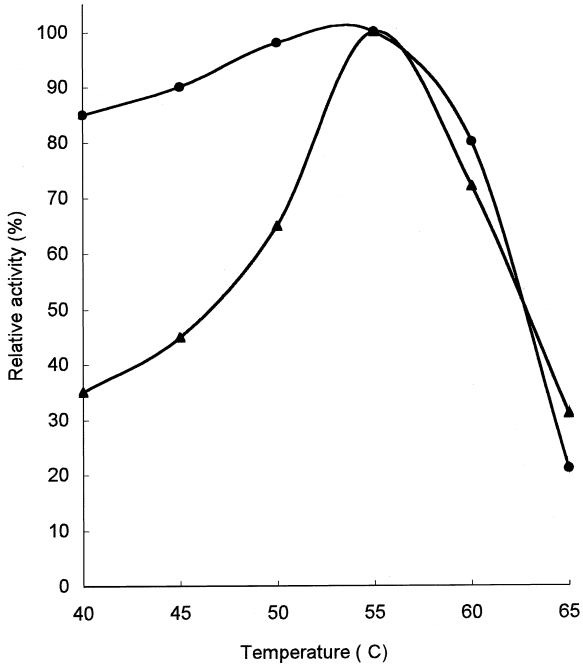

Fig. 6. Optimum temperature and stability of purified sago starch-degrading glucoamylase from *Acremonium* sp. (▲, optimum temperature; ●, temperature stability).

enzyme is not only capable of hydrolyzing polysaccharides such as amylose but also pullulan and amylopectin, both of which have α-1,6-linkages. Amylopectin was hydrolyzed 5-times faster than amylose

Table 2
Effect of various metal ions and EDTA on sago starch-degrading glucoamylase activity of *Acremonium* sp. endophytic fungus

Reagent (1 mM)	Residual activity (%)[a]
None	100
Sr^{+2}	32
Ni^{+2}	37
Cu^{+2}	107
Al^{+3}	92
Ca^{+2}	113
Co^{+2}	91
Ba^{+2}	44
Zn^{+2}	80
Mg^{+2}	84
Fe^{+2}	87
Fe^{+3}	66
Mn^{+2}	157
Hg^{+2}	30
EDTA	2

[a] The enzyme activity was assayed in the presence of 1 mM of metal ions and EDTA in a reaction mixture containing 2% of sago starch at pH 5.5, 55°C for 30 min.

Table 3
Substrate specificity of sago starch-degrading glucoamylase from *Acremonium* sp. endophytic fungus

Substrate	Relative activity (%)
Starches	
Soluble starch	100[a]
Potato starch	101
Tapioca starch	97
Corn starch	75
Rice starch	110
Wheat starch	154
Sago starch	111
Polysaccharides	
Amylose	187
Amylopectin	933
Pullulan	182

[a] Soluble starch is taken as 100%.

and pullulan. The substrate preference of the enzyme for hydrolysis can be arranged in the following order: amylopectin > amylose > pullulan. The enzyme was also able to degrade various starches, with slightly weak degrading ability towards tapioca, potato, rice and sago starch but a strong activity towards wheat starch. Corn starch was more resistant to the hydrolysis. This may be due to its composition, the starch having less amylopectin and a high amylose content. The results revealed that the rate of hydrolysis increased with the increase in amylopectin content of raw starch. In addition, Cheetham and Tao (1997) found that maize starch with higher amylose content required a longer hydrolysis time.

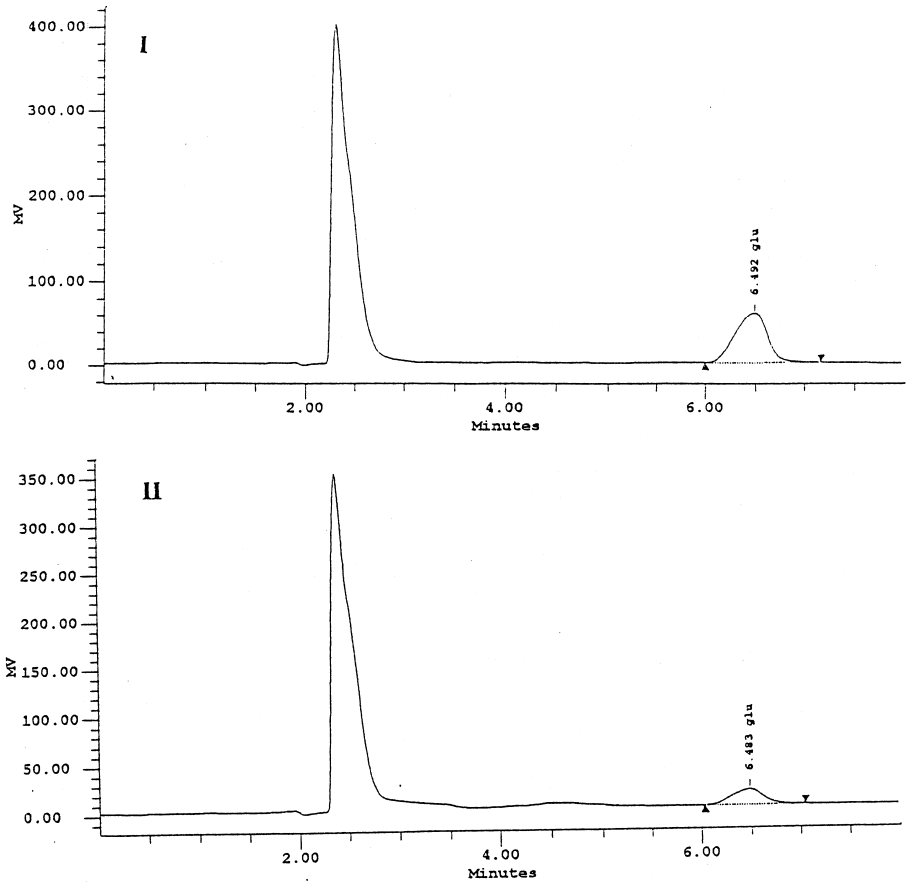

Fig. 7. HPLC chromatogram of the product of degradation of sago starch by purified sago starch-degrading glucoamylase from *Acremonium* sp. (I: standard; II: product).

3.5. Kinetic constant

The K_m and V_{max} value determined from Lineweaver–Burk plots, were 10.0 mg/ml and 195 μmol/ml/min for amylose and 3.8 mg/ml and 391 μmol/ml/min for amylopectin, respectively. The low K_m value for amylopectin possibly reflects a higher number of hydrogen or hydrophobic interactions between the substrate and active site residues of the enzyme. The activity of purified enzyme decreases with decreasing chain length of the substrate. James and Lee (1996) also reported a decrease in K_m as the substrate chain length increased.

3.6. End-product

Determination of end-product hydrolysis was carried out by incubating the purified enzyme with 24% (w/v) raw sago starch for 6, 12, 24 and 48 h. The end-product was detected by HPLC. The results showed that the enzyme released solely glucose (Fig. 7). Percentage conversion rates to glucose were 4.0, 4.5, 6.5, 8.6 and 14.0% for 6, 12, 24, 48 and 72 h hydrolysis, respectively. These results seem to indicate that hydrolysis was affected by the penetration time and was also strongly dependent on both the property of the enzyme and surface structure of sago starch.

Acknowledgements

An award from the Ohgushi Yoshika Foundation, in memory of Mrs. Yoshika Ohgushi, supported this work. We thank Professor Fusao Tomita for facilities for identification of this fungus at the Faculty of Agriculture, Hokkaido University, Japan.

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