Pergamon 0031-9422(95)00711-3 Phytochemistry, Vol. 41, No. 3, pp. 703–705, 1996

0031-9422/96 $15.00 + 0.00

PURIFICATION AND PROPERTIES OF α-GLUCOSIDASE FROM MILLET SEEDS

YOSHIKI YAMASAKI, HARUYOSHI KONNO and HIDEYUKI MASIMA

Research Institute for Bioresources, Okayama University, Kurashiki-shi, Okayama 710, Japan

(*Received in revised form* 30 *August* 1995)

Key Word Index—*Panicum miliaceum* L.; Gramineae; millet; α-glucosidase.

Abstract—Two forms of α-glucosidase (EC 3.2.1.20), designated as I and II, have been isolated from millet (*Panicum miliaceum* L.) seeds by a procedure including fractionation with ammonium sulphate, CM-cellulofine column chromatography, Sephadex G-100 column chromatography, preparative isoelectric focusing and preparative disc gel electrophoresis. The two enzymes showed identical M_r, calculated to be 85000 on SDS-PAGE and 93000 on gel filtration. The two enzymes readily hydrolysed maltose and malto-oligosaccharides, and native starch weakly. The two enzymes hydrolysed amylose liberating α-glucose.

INTRODUCTION

The enzyme α-glucosidase (EC 3.2.1.20) is ubiquitous in higher plants. It has been suggested that α-glucosidase forms a part of the non-phosphorolytic pathway for the breakdown of starch, and functions in seed germination by hydrolysing the oligosaccharides produced by α- and β-amylases [1, 2]. However, plants seeds which do not contain α-amylase, contain much α-glucosidase. In addition, the enzymes in higher plants readily hydrolyse soluble starch, liberating glucose [3–6]. These facts suggest that α-glucosidase hydrolyses native starch slowly by itself and plays a role for the respiration of seeds. Sun and Henson [7] have reported that barley seed α-glucosidases can hydrolyse native starch granules isolated from barley kernels. There are few reports on α-glucosidases, which can hydrolyse native starch. On the other hand, we have found that several α-glucosidases exist in millet seeds, and we now deal with the purification and properties of these.

RESULTS AND DISCUSSION

Isolation of two α-glucosidase

Crude enzyme solution was prepared as described in Experimental. The enzyme solution was dialysed overnight against 20 mM sodium acetate buffer, pH 4.5. The dialysate was applied to a CM-cellulofine column (3.2 × 17.5 cm) equilibrated with 20 mM sodium acetate buffer, pH 4.5. The column was first washed with the same buffer to remove the unadsorbed protein, and then with a linear gradient of NaCl (0–600 mM) in the same buffer (Fig. 1). The eluate with α-glucosidase activity was concentrated using an Amicon ultrafiltration device (PM-10 membrane). The concentrate was subjected to gel filtration on a Sephadex G-100 column (Pharmacia) (Fig. 2). The eluate with α-glucosidase activity was concentrated using an Amicon ultrafiltration device and dialysed overnight against 20 mM sodium acetate buffer, pH 4.5. The dialysate was subjected to preparative isoelectric focusing (IEF) (pH 6.5–9.0). A Rotfor TM Preparative IEF Cell (Bio-Rad, California) was used. After focusing at 12 W for 4 hr, the active fractions were combined and applied to a CM-cellulofine column (1.8 × 17.5 cm) as described above. After washing off the unadsorbed materials with 20 mM sodium acetate buffer, pH 4.5, the column was eluted with the same buffer containing 1 M NaCl. The eluate was concentrated using an Amicon ultrafiltration device and dialysed overnight against 20 mM sodium acetate buffer, pH 4.5. The dialysate was subjected to preparative disc gel electrophoresis, pH 4, using a HSI GT Tube Gel Electrophoresis Unit (Hoefer Scientific Instruments, California) and divided into two fractions (I and II). The active fractions were pooled separately. After removing insoluble materials by filtration, the two enzyme solutions were concentrated using an Amicon ultrafiltration device and dialysed overnight against 20 mM sodium acetate buffer, pH 4.5, separately. The two preparations were used for further studies. This procedure led to a 111-fold purification with 0.2% recovery (I) and 125-fold purification with 1.3% recovery (II) (Table 1). The purified enzymes were shown to be homogeneous by PAGE (Fig. 3). The M_r of the two enzymes were calculated to be 85000 from SDS-PAGE and 93000 from gel filtration with Superdex 200 HR 10/30.

General properties

The pH optimum of the two α-glucosidases was found to be 3.5. After 20 hr pre-incubation at 30° with 50 mM McIlvaine's buffer, the two enzymes were stable in the

pH ranges 3.5–6.0 (I) and 3.5–5.5 (II). The temperature optimum of the two α-glucosidases was found to be 60° after 30 min incubation. After 15 min pre-incubation with 50 mM sodium acetate buffer, pH 4.5, at various temperatures, the two enzymes were stable at temperatures up to 35°.

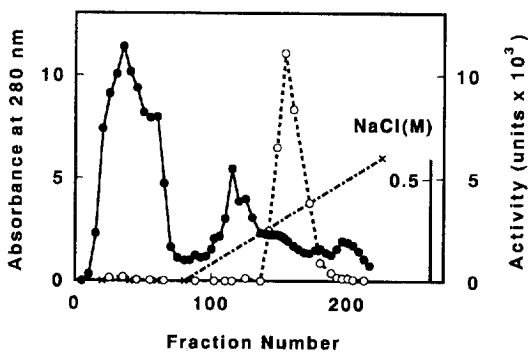

Fig. 1. Chromatography of α-glucosidase on CM-cellulofine. The experimental conditions are described in the text. Flow rate, 40 ml hr^{-1}; fraction volume, 8.5 ml; (●), A_{280}; (○), α-glucosidase activity.

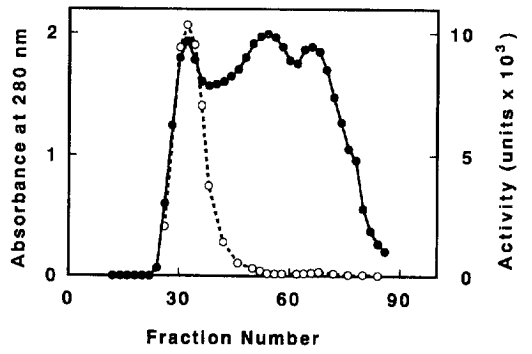

Fig. 2. Gel chromatography on Sephadex G-100. Flow rate, 40 ml hr^{-1}; fraction volume, 5.7 ml; (●), A_{280}; (○), α-glucosidase activity.

Substrate specificity

The activity of the two enzymes on malto-oligosaccharides was examined and the K_m values were calculated from Lineweaver–Burk plots (Table 2). The reaction was carried out under standard assay conditions by varying the substrate concentration. The two enzymes hydrolysed malto-oligosaccharides at a similar rate as maltose. α-Glucosidase II hydrolysed native starch from millets seeds weakly, suggesting that the enzyme may play a role for the respiration of seeds (Table 2).

Mode of enzyme action

To be able to understand the mode of action of the two α-glucosidases, the enzymes were incubated with amylose under standard conditions, and the products formed

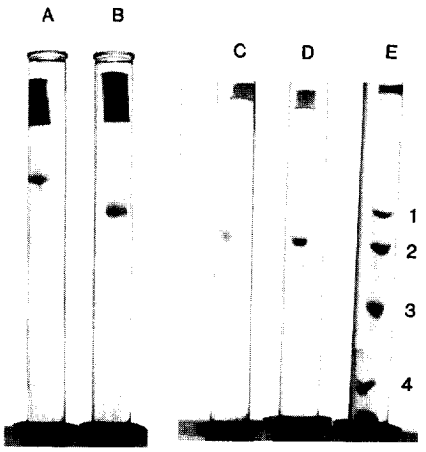

Fig. 3. Gel electrophoresis of the purified α-glucosidases. Non-denaturing disc gel electrophoresis (A, B) and SDS-PAGE (C, D, E) were carried out as described in Experimental. A and C, α-glucosidase I; B and D, α-glucosidase II; E, molecular mass standard; 1, phosphorylase b (M_r 94000); 2, bovine serum albumin (M_r 67000); 3, ovalbumin (M_r 43000); 4, carbonic anhydrase (M_r 30000).

Table 1. Summary of purification of two α-glucosidases from millet seeds

Purification step	Total activity (U)	Yield (%)	Specific activity (U mg^{-1} of protein)	Purification
Ammonium sulphate ppt.	341000	100	14	1
CM-cellulofine column chromatography	232000	68.1	520	37.1
Sephadex G-100 column chromatography	188000	55.1	1180	83.9
Preparative IEF	19100	5.6	2510	179
Preparative Page I	620	0.2	1550	111
II	4390	1.3	1760	125

Table 2. The kinetic parameters of two α-glucosidases for various substrates

Substrates	Relative rate of hydrolysis (%)		K_m (mM)	
	I*	II†	I*	II†
Maltose	100	100	0.653	0.617
Maltotetraose	41.9	41.2	0.714	0.500
Maltohexaose	46.9	48.7	0.878	0.577
Native starch		0.029		

*α-Glucosidase I.
†α-Glucosidase II.

were determined by GC. The two enzymes hydrolysed amylose liberating α-glucose, as for other α-glucosidases [8]. On the other hand, maltose was not detected at all, although α-amylase hydrolysed amylose, liberating several times greater amounts of maltose than glucose [9, 10]. The enzyme preparations, therefore, were not contaminated by the other amylases. The fact supports the above mentioned role of α-glucosidases from millet seeds, in which α-glucosidases hydrolyse native starch, playing a role in respiration.

Inhibition with metal ions

The two enzymes were pre-incubated with 5 mM concentrations of various metal ions in 50 mM sodium acetate buffer, pH 4.5, at 37° for 30 min. Copper ions, Hg^{2+} and Zn^{2+} reduced the activity of α-glucosidase I by 20% or more. On α-glucosidase II, however, Cu^{2+} and Zn^{2+} showed no inhibition.

EXPERIMENTAL

Preparation of crude α-glucosidase. Millet (*Panicum miliaceum* L.) seeds (2.6 kg) were suspended in 5 l 25 mM NaOAc buffer, pH 4.5, containing 2 M NaCl, and homogenized in a mixer (National electric mixer MX-V 253, Matsushita Electric Industries). The homogenate was left overnight at 4° and the debris was removed by filtration. The supernatant was brought to 0.9 satn with $(NH_4)_2SO_4$. The ppt. was collected by centrifugation and dissolved in 25 mM NaOAc buffer, pH 4.5.

Preparation of native starch. Millet seeds (118 g) were suspended in 500 ml H_2O, and homogenized in a mixer. The homogenate was filtered through gauze, and centrifuged at 1940 *g* for 10 min. The ppt. was suspended in isoamyl alcohol–H_2O (1:5) and centrifuged at 1940 *g* for 10 min. The ppt. was washed twice, and lyophilized.

Assay of α-glucosidase. Maltose (2.8 μmol) was used, together with 50 mM NaOAc buffer, pH 4.5, and suitably diluted enzyme prepn in a total vol. of 0.5 ml. After 30 min at 37° for 1 hr, the reaction was stopped by boiling for 5 min. The amount of glucose formed was measured by the method of ref. [11], as modified in ref. [12]. One unit of activity was defined as the amount of enzyme which liberated 1 μmol hr^{-1} of glucose from maltose under the conditions described above.

Determination of protein. Protein was determined by the method of ref. [13]. The protein profiles in column chromatography were followed by measuring the *A* of eluates at 280 nm.

Gel electrophoresis. Non-denaturing disc-gel electrophoresis was carried out at pH 4.3 on tube gels (7.5%, w/v) in β-alanine–HOAc buffer, pH 4.5, according to ref. [14], and SDS-PAGE was performed on 7.5% gels according to ref. [15]. After the run, the gels were stained for proteins with Coomassie Brilliant Blue.

Estimation of M_r. The M_r of the purified enzyme was estimated by SDS-PAGE [15] and gel filtration with Superdex 200 HR 10/30.

Gas–liquid chromatography. The products formed from amylose with α-glucosidases were determined by GC as their TMSi derivatives. GC was carried out in glass columns (3 mm × 3 m) packed with 3% OV-1 on Chromosorb W (80–100 mesh); carrier gas, N_2; flow rate, 40 ml min^{-1}; temp., 170–300° (5° min^{-1}).

REFERENCES

1. Swain, R. R. and Dekker, E. E. (1966) *Biochim. Biophys. Acta* **122**, 87.
2. Nomura, T., Kono, Y. and Akazawa, T. (1969) *Plant Physiol.* **44**, 765.
3. Grinna, L. S. and Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 8814.
4. Yamasaki, Y. and Konno, H. (1987) *Phytochemistry* **26**, 711.
5. Murashige, T. and Skoog, F. (1962) *Physiol. Plant* **15**, 473.
6. Yamasaki, Y. and Konno, H. (1989) *Phytochemistry* **28**, 2583.
7. Sun, Z. and Henson, C. A. (1990) *Plant Physiol.* **94**, 320.
8. Yamasaki, Y. and Suzuki, Y. (1979) *Agric. Biol. Chem.* **43**, 481.
9. Yamasaki, Y. and Suzuki, Y. (1979) *Plant Cell Physiol.* **20**, 553.
10. Yamasaki, Y. and Konno, H. (1991) *Phytochemistry* **30**, 2861.
11. Papadopoulos, N. M. and Hess, W. C. (1960) *Arch. Biochem. Biophys.* **88**, 167.
12. Dahlqvist, A. (1961) *Biochem. J.* **80**, 547.
13. Warburg, O. and Christian, W. (1942) *Biochem. Z.* **310**, 384.
14. Reisfeld, R. A., Lewis, U. J. and Williams, D. E. (1962) *Nature (London)* **195**, 281.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680.