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α-D-MANNOSIDASE FROM THE ALEURONE LAYERS OF RESTING WHEAT GRAINS: SUGAR-DEPLETED FORMS

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; α-D-mannosidase: aleurone layers; Con A-Sepharose; glycoprotein.

Abstract—α-D-Mannosidase from the aleurone layers of resting wheat grains has been purified to homogeneity by a procedure involving Con A-Sepharose chromatography. The enzyme has been shown to be a glycoprotein containing D-glucose, D-mannose, D-glactose and *N*-acetyl-D-glucosamine. A minor component, showing the characteristics of sialic acid has also been detected by gas chromatography. Studies dealing with the effect of Endo-H treatment on the affinity of the enzyme for Con A-Sepharose, indicate that the sugar moiety contains both high-mannose chain(s), accessible to Endo-H and interacting strongly with Con A-Sepharose, and oligosaccharide chain(s) resistent to Endo-H and interacting with Con-A-Sepharose to a lesser extent. Removal of sugar by Endo-H and periodate treatments affects the enzyme stability to heat and protease degradation.

INTRODUCTION

It is well known that the aleurone layers of resting cereal grains contain a variety of hydrolases which are secreted into the starchy endosperm during early phases of germination [1]. Although extensively studied as far as the effect of gibberellic acid is concerned [2, 3], these enzymes have been poorly characterized and little is known about their molecular properties, such as the glycoprotein nature and the composition and the structure of the oligosaccharide moiety. However, it is of interest to elucidate such molecular aspects, since the oligosaccharide moiety could play a role in the function of these enzymes. Among the roles postulated are secretion and transport, resistance to proteolysis and maintenance of conformation [4].

We have previously reported [5] that a variety of glycosidases from the aleurone layers of resting wheat grain were able to interact *in vitro* with lectins, giving evidence for their glycoprotein nature. In this paper we describe the purification and the properties of α-D-mannosidase previously reported to interact with Con A-Sepharose [5] and provide some insight into the structure of the oligosaccharide moiety of the enzyme molecule. We have also checked for a possible implication of the sugar moiety in the stability exhibited by the enzyme to heat and proteolytic degradation.

RESULTS AND DISCUSSION

Purification

The results of the purification scheme are reported in Table 1. The step involving affinity chromatography on Con A-Sepharose was found to be most valuable, giving the greatest increase in enzyme specific activity. The DEAE-chromatography step after Sephadex G-200 was required to separate α-D-mannosidase from carboxypeptidase activity co-purifying with it.

The purified enzyme preparation showed a single protein band (R_f 0.20) on polyacrylamide electrophoresis, that was coincident with the α-D-mannosidase activity. This band stained with the periodic acid–Schiff method. The enzyme preparation was devoid of the following glycosidase activities: *N*-acetyl-β-D-glucosaminidase, α- and β-D-glactosidase, β-D-xylosidase, α-L-arabinofuranosidase, α-L-fucosidase and β-D-mannosidase.

Table 1. Enzyme purification

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)
Crude extract	11200	838	0.07	—
40–60% Ammonium sulphate ppt.	4950	1660	0.33	4.7
Con A-Sepharose	77	1230	16	229
DEAE-chromatography I	33	980	29.7	424
Sephadex G-200	2.3	780	338	4830
DEAE-chromatography II	1.5	751	500	7140

Properties of the enzyme

M_r was determined by both Sephadex G-200 filtration and sucrose gradient centrifugation and results of 197 000 and 188 000, respectively, were obtained. The pH optimum was at 4.6–4.8; half maximal activity was observed at pH 5.6. The temperature optimum was at 48°; half maximal activity was observed at 35 and 55°.

The effect of EDTA and metal ions on the enzyme activity was studied. At 1 mM concentration, Ag^{2+} and Co^{2+} were inhibitory (57% and 41% inhibition, respectively), whereas Cu^{2+} and Mg^{2+} were ineffective; Zn^{2+} was slightly stimulatory (25%). When incubated in the presence of 1 mM EDTA (1 hr at 4°), the enzyme was inactivated. Inactivation could be prevented by addition of 5 mM Zn^{2+}. The kinetic parameters of the enzyme are very similar to those reported for other α-D-mannosidase isolated from higher plants [6]. As has been reported for lysosomal and jack bean α-D-mannosidase [7], Swainsonine inhibited the enzyme in a competitive manner.

Glycoprotein nature of the enzyme

The glycoprotein nature of the enzyme was indicated by its ability to interact with Con A-Sepharose, as well as by its positive periodate–Schiff reaction on polyacrylamide gel. This was confirmed by gas liquid chromatography of the sugars that gave (% w/w): glucose 20.1, mannose 3.09, galactose 1.7 and *N*-acetyl-D-glucosamine < 1. In addition, a minor component (< 1%) was detected by gas liquid chromatography which had the same R_t as sialic acid. Since sialic acid has not so far been detected in plant glycoproteins [8], a study is in progress to determine the exact nature of this compound by GLC/MS and to exclude that possibility that it is an impurity. To give some insight into the structure of the oligosaccharide moiety, the interaction of the enzyme with Con A-Sepharose and the effect of Endo-H digestion on this interaction were studied. It is known that Endo-H cleaves specifically high mannose oligosaccharides of *N*-glycosyl proteins between GlcNAc residues of the core, while modified complex oligosaccharides are resistant [9, 10]. Native enzyme strongly interacted with Con A-Sepharose, since 200 mM methyl-α-D-glucoside was required for elution and no enzyme was eluted with 15 mM methyl-α-D-glucoside. It has been reported that neutral mannose-rich glycopeptides elute from Con A-Sepharose with 200 mM methyl α-D-glucoside, whereas complex glycopeptides with biantennary structure elute with only 15 mM methyl α-D-glucoside, and that complex glycopeptides with triantennary structure do not interact with Con A-Sepharose at all [11]. The elution pattern of the enzyme suggested that high mannose chain(s) might be present in the molecule. This was confirmed by the study with Endo-H. After treatment with this enzyme, α-D-mannosidase still interacted with Con A-Sepharose, but only 15 mM methyl α-D-glucoside was required to elute it from the affinity column. The Endo-H treatment removed only 33% of the sugar content. These results suggest that, in addition to high-mannose oligosaccharide(s), probably attached covalently to asparagine, the enzyme molecule contains oligosaccharide(s) differing from the high-mannose oligosaccharide in that it is not cleaved by Endo-H and has a lower affinity for Con A-Sepharose.

Stability of native and sugar-depleted enzyme

We examined the enzyme stability to heat treatment and proteolysis. As Fig. 1 shows, the enzyme was stable to heat treatment up to about 60°. A slight heat activation

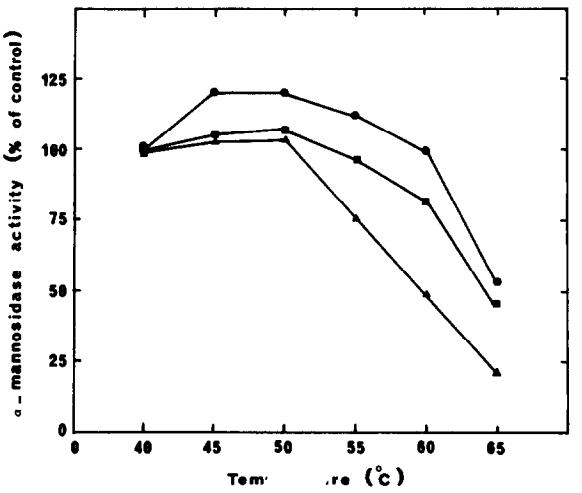

Fig. 1. Heat-stability of native and sugar-depleted enzyme. The enzyme preparations, containing 1 μg protein in 100 μl of 20 mM citrate buffer (pH 5.9), were incubated at the indicated temperatures for 15 min before measuring the activity. The activity of the enzyme preparations treated at 40° were taken as controls: —●—●—, native enzyme. —■—■—, Endo-H treated enzyme. —Δ—Δ—, Endo-H treated enzyme subjected to periodate oxidation.

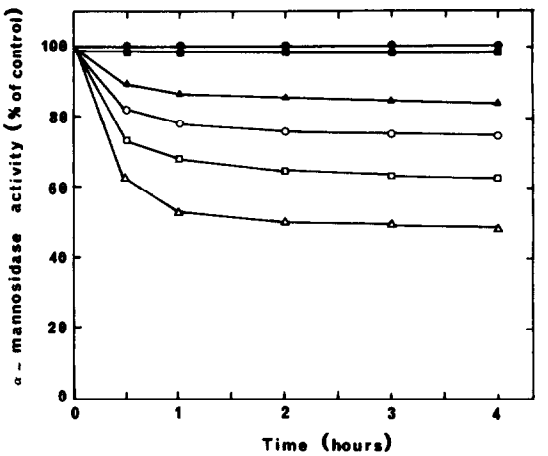

Fig. 2. Proteolytic digestion of the enzyme. The enzyme preparations, containing 10 μg protein in 0.6 ml of 50 mM phosphate buffer (pH 7), were incubated with 20 μg protease at 37°. Aliquots (20 μl) in duplicate were withdrawn for determination of residual enzyme activity. The activity of the enzyme preparations incubated with albumin instead of protease were taken as controls. —●—●—, native enzyme treated with subtilisin. —■—■—, native enzyme treated with pronase. —▲—▲—, Endo-H treated enzyme treated with pronase. —○—○—, Endo-H treated enzyme treated with subtilisin. —□—□—, Endo-H treated enzyme subjected to periodate oxidation incubated with pronase. —△—△—, Endo-H treated enzyme subjected to periodate oxidation incubated with subtilisin.

was observed in the range 45–55°. The enzyme was resistant to digestion by pronase, subtilisin (Fig. 2) and endogenous carboxypeptidase (data not shown).

To check for a possible influence of the sugar moiety on the enzyme stability, we compared the properties of three forms of α-D-mannosidase: native, Endo-H treated, and Endo-H treated enzyme subjected to periodate oxidation. Periodate oxidation of the Endo-H treated enzyme reduced the sugar content to 42% of the native enzyme and made the enzyme unable to interact with Con A-Sepharose at all. Neither Endo-H treatment or periodate oxidation affected the specific activity compared with that of native enzyme (data not shown), confirming the observation of the other authors [4, 12] that the sugar moiety is not involved in the catalytic function. Endo-H digestion and, to a greater extent, the subsequent oxidation with periodate, made the enzyme susceptible to both heat-treatment and proteolytic degradation (Figs 1 and 2).

CONCLUSION

The only data available on the sugar structure of plant acid glycosidases concern jack bean α-D-mannosidase [13]. This enzyme has been reported to be a glycoprotein whose sugar moiety contains GlcNAc and high levels of D-mannose forming mannosylated oligosaccharide(s). In the native form the oligosaccharide is sterically masked from interaction with either Endo-H or Con A. The sugar structure of the α-D-mannosidase from wheat aleurone layers appears to be different from that suggested for jack bean enzyme [13] since it contains oligosaccharide chain(s) that is (are) accessible to Endo-H and strongly interacts with Con A. Additional oligosaccharide chain(s) that is (are) resistant to Endo-H cleavage and interacts with Con A-Sepharose to a smaller extent should be present. In addition to D-mannose and D-GlcNAc, the enzyme from wheat contains D-galactose and a large amount of D-glucose. A large content of D-glucose and D-galactose have been reported to occur also in α-mannosidase from *Phaseolus vulgaris* [14].

Although there is increasing evidence that plant acid hydrolases, as lysosomal hydrolases, are glycoproteins [6, 15], the biological significance of the glycosylation is still obscure. The results here reported indicate that both the heat stability and the resistance to proteolytic attack of α-D-mannosidase from wheat aleurone are affected *in vitro* by the sugar moiety. Similar results have been obtained for invertases from neurospora crassa [16] and yeast [17], and procine pancreatic ribonuclease [18]. It has also been shown that the carbohydrate moiety of yeast invertase protects it from intracellular proteolytic inactivation [19]. It may be envisaged that the protective effect against attack by protease degradation could be important in maintaining an effective concentration of α-D-mannosidase and probably of other hydrolases in the starchy endosperm of cereal grains during early phases of germination.

EXPERIMENTAL

Materials. Chemicals and plant materials were as reported previously [5]. Aleurone layers were obtained by grinding dry de-embryonated grains and sieving the material obtained (mesh: 80). Aleurone with attached pericarps were retained, whereas most starchy material was not.

Enzyme purification. All operations were carried out at 4°. Usually 50 g aleurone layers was extracted in a glass–glass homogenizer with 500 ml of 50 mM citrate buffer (pH 5.9) containing 0.2 M NaCl (Buffer A) and the homogenate was centrifuged at 20000 *g* for 30 min. The material precipitating from the supernatant between 40 and 60% saturation with $(NH_4)_2SO_4$ was recovered by centrifugation and redissolved in 20 mM citrate buffer (pH 6) containing 50 mM NaCl (Buffer B). It was desalted by Sephadex G-25 filtration. The desalted preparation (330 ml) was incubated overnight with 140 ml of Con A-Sepharose equilibrated with Buffer B under agitation and then transferred to a column to pack the gel. The column was washed with 750 ml Buffer B and then eluted with 400 mM methyl-α-D-mannoside in Buffer B. The fractions containing α-D-mannosidase were pooled, concentrated by ultrafiltration through a PM 10 membrane filter (Amicon). Methylmannoside was removed from the enzyme preparation by Sephadex G-50 filtration, equilibrated and eluted with 10 mM NaPi buffer (pH 7) (Buffer C). The material (35 ml) was applied to a 50 ml column of DEAE-cellulose that had previously been equilibrated with Buffer C. Following adsorption of the protein, the column was washed with 300 ml of Buffer C. The enzyme was eluted with 0.2 M NaCl in Buffer C. The preparation was concentrated and desalted in the Amicon apparatus and, then, chromatographed on a Sephadex G-200 column (2.1 × 96 cm) previously equilibrated with 20 mM citrate buffer (pH 5.9) (Buffer D). Elution was performed with the same buffer at an upward flow rate of *ca* 10 ml/hr. The α-D-mannosidase emerged as a single peak eluting between 177 and 220 ml. The active fractions were pooled, concd by ultrafiltration and chromatographed on a 9 ml column of DEAE-cellulose that had previously been equilibrated with Buffer D. α-D-Mannosidase was collected in the washing, whereas carboxypeptidase activity eluted with 0.2 M NaCl in Buffer D. The α-D-mannosidase preparation showing loss of activity after one year at 4°.

Enzyme assay. Enzyme activity was assayed using *p*-nitrophenyl-derivatives [5]. Unless stated otherwise, α-D-mannosidase was assayed by addition of enzyme to the reaction mixture, containing in a total vol. of 200 μl, 6.25 mM substrate and 50 mM citrate buffer (pH 4.6). After 1 hr incubation at 40°, the reaction was termined by addition of 0.7 ml of 0.2 M Na_2CO_3 and A_{420} was determined. 1 unit is defined as the amount of enzyme that hydrolyses 1 μmol of substrate per hr at 40°.

Carbohydrate analysis. This was carried out by GLC of the trimethylsilyl derivatives of the methylglycosides after methanolysis. The protein samples were dialysed against H_2O prior to analysis.

Endo-H treatment. Native α-D-mannosidase (0.4 mg of protein) was incubated with 50 mUnits of Endo-H in 500 μl of 50 mM citrate buffer (pH 5.5) for 24 hr at 37°. After incubation, the mixture was chromatographed on a Sephadex G-100 column equilibrated and eluted with 20 mM citrate buffer (pH 5.9). α-D-Mannosidase eluted with the void vol. The active fractions were pooled and concentrated by ultrafiltration. Control enzyme preparation was obtained in the same manner without Endo-H.

Periodate oxidation. An aliquot of Endo-H treated α-mannosidase (0.2 mg of protein) was exposed to 20 mM Na periodate at 4° in the dark for 3 hr. After incubation, the mixture was chromatographed on a 30 ml column of Sephadex G-25 equilibrated with 20 mM citrate buffer (pH 5.9). Enzyme activity eluted with the void vol. and was separated from periodate and sugars that were retarded. Control enzyme preparation was obtained in the same manner without periodate.

Other methods. M_r was determined by sucrose-gradient centrifugation according to ref. [20]. Polyacrylamide gel electrophoresis and the periodate–Schiff method on the gels were carried out

as previously reported [21]. Sugar content was measured by the anthrone method [22] using D-mannose as standard. Protein content was measured according to ref. [23].

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