IMMUNOADSORBENT AFFINITY PURIFICATION OF THE TWO ENZYME FORMS OF α-MANNOSIDASE FROM *PHASEOLUS VULGARIS*

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1. Introduction

The presence of α-mannosidase has been demonstrated in plants [1–5], micro-organisms [6] and animals including man [7–11]. In normal human liver there are at least 3 forms: A and B with acidic and C with neutral pH optimum [12–14]. The three enzyme forms have not been extensively purified, characterized or compared. In plant tissues 2 forms seem to exist. We have previously purified α-mannosidase from *Phaseolus vulgaris* by extensive use of column chromatography and found 2 forms, α-mannosidase I and α-mannosidase II with p*I* 5.1 and 6.1, respectively [2]. α-Mannosidase I seemed homogeneous. However, α-mannosidase II still contained some impurities.

In the present paper is described the preparation of antiserum against α-mannosidase I. There was a high degree of immunological identity between α-mannosidase I and II. This made possible the purification of both enzyme forms by specific chromatography using an immunoadsorbent column made from anti-α-mannosidase I antiserum. Once established, this is a fast and highly discriminating method. After separation by isoelectric focusing both enzyme preparations seemed homogeneous. The specific activity α-mannosidase I was about 40% higher than that obtained by extensive column chromatography [2].

2. Materials and methods

Yellow wax beans, *Phaseolus vulgaris*, were supplied by Olsens Enke, Oslo. Ampholine and Ultrogel AcA 34 were obtained from LKB. All chemicals were reagent grade.

Enzymes: α-Mannosidase (EC 3.2.1.24)

2.1. *Enzyme assay*

The assay was performed as previously described [2] using *p*-nitrophenyl-α-D-mannopyranoside as a substrate. Protein was determined by the method of Lowry et al. [15]. The protein concentration of the column effluent was estimated spectrophotometrically by absorption at 280 nm.

2.2. *Preparation of antiserum against α-mannosidase I*

Highly purified α-mannosidase I from *Phaseolus vulgaris* was obtained as described earlier [2], except for some modifications: Sephadex G 200 was substituted by Ultrogel AcA 34, which improved the separation and increased the speed. Moreover, as a last purification step after isoelectric focusing which separated the two enzyme forms, α-mannosidase was again gel filtrated through an Ultragel AcA 34 column.

Two mg per ml purified enzyme solution was mixed with an equal volume of Freunds incomplete adjuvans. Two rabbits were each given four injections of 0.1 mg enzyme. The solution was injected every second week into the thicker part of the skin above the scapula. Beginning ten days after the last injection, the rabbits were bled at about two weeks interval by earvein puncture. The sera were stored at −20°C until used.

2.3. *Immunoadsorbent column*

IgG was isolated from the rabbit antisera by ammonium sulphate precipitation, dialysis and ion exchange chromatography [16]. About 50 mg of the immuno-

globulin preparation was coupled to 5 g CNBr-activated Sepharose 4B [17], and poured into a column.

A crude extract from *Phaseolus vulgaris,* obtained after water extraction and ammonium sulphate fractionation, was dialyzed against distilled water [2]. The precipitate was removed. The supernatant, after dialysis against 0.1 M Tris–HCl buffer, pH 7.4, containing 0.3 M CaCl, was applied to an immunoadsorbent column, (2.5 × 3.5 cm), which was thoroughly washed with the same buffer before it was eluted with 0.1 M sodium bicarbonate buffer pH 10.6 containing 1 M NaCl. The fractions collected were immediately taken to pH 7.4, having 1 M Tris–HCl buffer, pH 7.4, in the collecting tubes.

2.4. *Gel diffusion*

1% Agarose in 0.05 M barbital buffer, pH 8.6, was used for double diffusion [18] and immunoelectrophoresis [19]. The plates were washed for 28 h in 0.015 M NaCl and dried before they were stained with 1% Amidoschwarz dissolved in 0.1 M sodium acetate buffer, pH 4.6, containing 10% glycerol.

2.5. *Gel electrophoresis and isoelectric focusing*

Polyacrylamide gel electrophoresis was performed at pH 8.9 [20]. Enzyme activity was localized by incubating unstained gels with *p*-nitrophenyl-α-D-mannopyranoside in the enzyme solution. An LKB 8101 column and LKB Ampholine, pH 5–7, were used for the focusing experiments. Details have been given previously [2].

3. Results

3.1. *Immunological studies*

The α-mannosidase I preparation obtained by conventional column chromatography and isoelectric focusing seemed homogeneous according to gel electrophoresis and analytical ultracentrifugation [2]. α-Mannosidase II was completely separated from α-mannosidase I, but this preparation still contained impurities.

Antisera to α-mannosidase I were prepared in rabbits. One single precipitin line was seen between α-mannosidase I and this antiserum, as well as between α-mannosidase II and anti-α-mannosidase I antiserum (fig.1a). Double diffusion demonstrated considerable antigenic identity between the two enzyme forms. No spurs could be detected (fig.1b). On immunoelectrophoresis, α-mannosidase I had a higher anodic migration than α-mannosidase II.

3.2. *Purification by immunoadsorbent column*

The enzyme was adsorbed to the immunoadsorbent column when applied in 0.1 M Tris–HCl buffer, pH 7.4, containing 0.3 M NaCl. A range of buffers

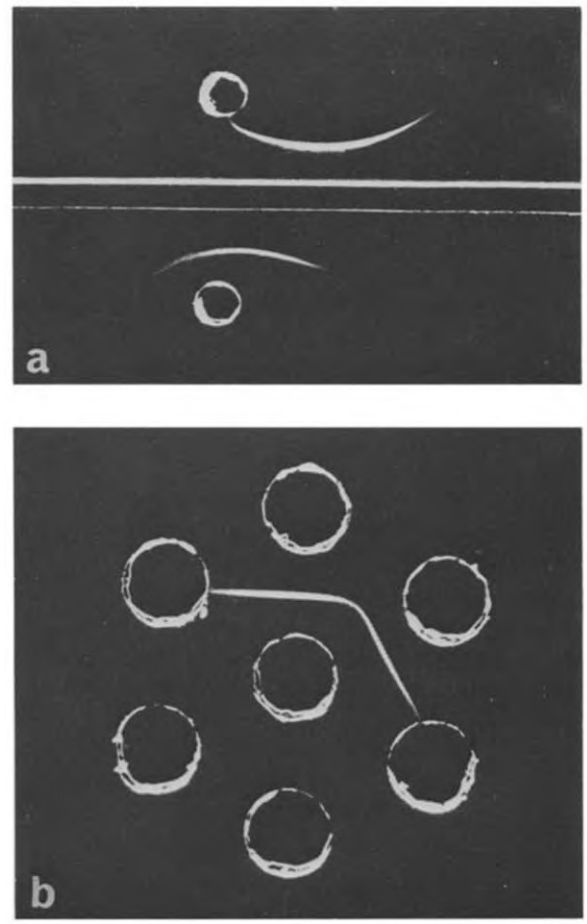

Fig.1. (a) Immunoelectrophoresis in agarose gel. Antiserum to α-mannosidase I diluted 1:1 (trough), against purified α-mannosidase I, 0.8 mg/ml (upper well), and partly purified α-mannosidase II, 0.5 mg/ml (lower well). (b) Double immunodiffusion (Ouchterlony) plate of anti-α-mannosidase I antiserum (center well), against purified α-mannosidase I, 0.6 mg/ml (top well), and partly purified α-mannosidase II, 0.5 mg/ml (right well).

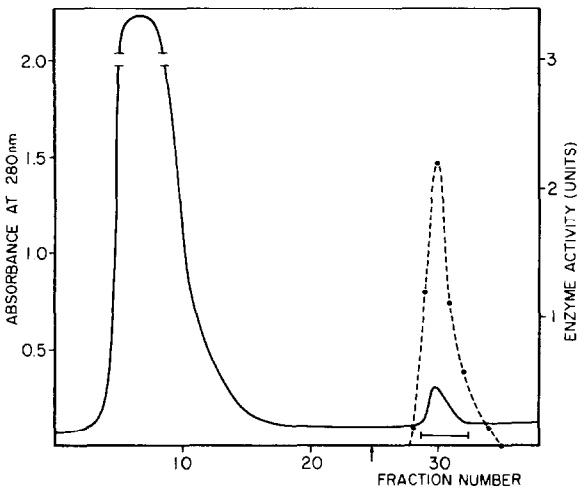

Fig.2. Chromatography of a crude α-mannosidase preparation on an anti-α-mannosidase I immunoadsorbent column. 60 mg protein from the ammonium sulphate fractionation step was applied to the column (2.5 × 3.5 cm), equilibrated and washed with 0.1 M Tris–HCl buffer, pH 7.4, containing 0.3 NaCl before elution with 0.1 M sodium bicarbonate buffer, pH 10.6, containing 1 M NaCl (arrow). (——) Absorbance at 280 nm. (•- - - - -•) α-Mannosidase activity. (├——┤) Pooled fractions.

was tried for elution. 0.1 M sodium bicarbonate buffer, pH 10.6, containing 1 M NaCl was satisfactory. A typical elution profile is shown in fig.2. When the resulting enzyme preparation was assayed by gel electrophoresis using 6% gels, two bands were found (fig.3a). These corresponded to α-mannosidase I and II. The enzymes were separated by isoelectric focusing, followed by ultrafiltration. No impurities could be detected in either enzyme preparation (fig.3b,c). The purification is summarized in table 1. The specific activity of α-mannosidase I was somewhat higher than

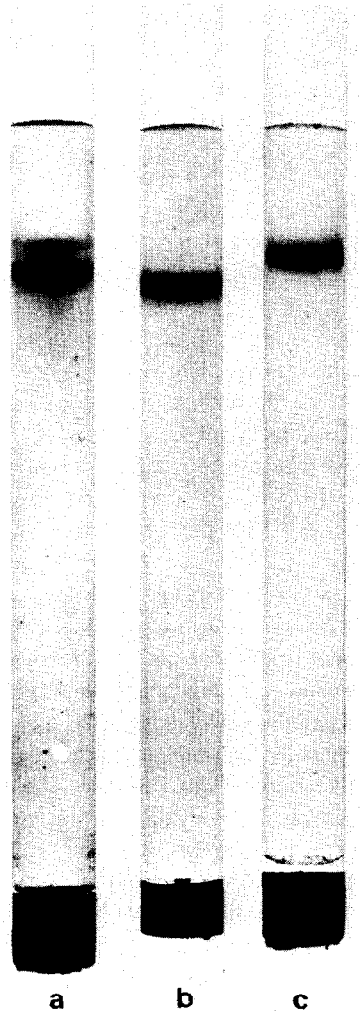

Fig.3. Polyacrylamide gel electrophoresis of α-mannosidase fractions in 6% gel at pH 8.9. (a) Immunoadsorbent eluate, (b) α-mannosidase I after isoelectric focusing, (c) α-mannosidase II after isoelectric focusing. About 20 μg protein was applied to each gel.

Table 1
Purification of α-mannosidase I and II

	Fraction	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
Step 1	Water extract	0.028		100
Step 2	$(NH_4)_2SO_4$ fractionation	0.196	6.9	69
Step 3	Immunoadsorbent column	49.6	1764	44
Step 4	Isoelectric focusing peak I	53.5	1910	26
	Isoelectric focusing peak II	51.9	1853	

that of α-mannosidase II, and some 40% higher than that obtained by our previous purification method [2].

4. Discussion

Double immunodiffusion and immunoelectrophoresis suggested considerable structural resemblance between α-mannosidase I and II. α-Mannosidase II seemed to have the same antigenic determinants as α-mannosidase I, as no spurs could be detected in the Ouchterlony assay (fig.1b). However, the enzymes have not been tested against an anti-α-mannosidase II antiserum. Conceivably, α-mannosidase II could posses antigenic determinants not present in α-mannosidase I. All the same, the antiserum against α-mannosidase I was equally suitable for the purification of both enzyme forms since only one precipitin line was produced. When an immunoadsorbent column was made from this antiserum, no α-mannosidase activity was eluted in front of the main α-mannosidase peak (fig.2). Further, the relative amounts of α-mannosidase I and II, when separated by isoelectric focusing after antibody column, were the same as when a crude extract from *Phaseolus vulgaris* was analyzed. The recovery from the immunoadsorbent column was good, and the purification considerable. Although no other proteins could be detected in the pooled fractions, impurities were probably still present, since the specific activity was increased by the subsequent isoelectric focusing. The specific activity was considerably higher than that obtained by extensive column chromatography, and the resulting enzyme preparations seemed homogenous. The availability of both α-mannosidase I and II in a separated and highly purified state will make it possible to extend the characterization and to make further comparisons of the two enzyme forms.

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