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Note

Simple procedure for the isolation of jack bean α-D-mannosidase by benzidine-Sepharose chromatography

PREMANAND V. WAGH
Veterans Administration Hospital, 300 E. Roosevelt Road, Little Rock, Arkansas 72206 (U.S.A.)
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Jack bean is enriched with α-D-mannosidase (α-D-mannoside mannohydrolase, E.C. 3.2.1.24) as compared to several other glycosidases contained in it[1]. This enzyme has been purified by isoelectric focusing, by exclusion and ion-exchange chromatography[1,2]. Preliminary observations in our laboratory indicated that jack bean α-D-mannosidase was bound to Sepharose 4B when the gel contained free amino groups derived from coupling of either β-(4-aminophenyl)-ethylamine or benzidine[3]. The present study reports a rapid procedure for the isolation of jack bean α-D-mannosidase free from other glycosidase activities employing benzidine-Sepharose 4B chromatography.

EXPERIMENTAL

Materials were obtained as follows: benzidine from J. T. Baker (Phillipsburgh, N.J., U.S.A.); Sepharose 4B from Pharmacia (Uppsala, Sweden); jack bean meal, Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); *p*-nitrophenyl glycopyranosides and 4-methyl-umbelliferyl-α-D-mannopyranoside from Pierce (Rockford, Ill., U.S.A.); *p*-nitrophenyl-β-D-mannopyranoside was a kind gift of Dr. S.-C. Li. All other chemicals were of reagent grade.

Preparation of benzidine-Sepharose

Sepharose 4B (250 ml) was activated with CNBr (25 g) at pH 10[4]. The activated gel was stirred in an equal volume of 0.1 *M* Na_2CO_3 (pH 10.5) and a solution of 2 g benzidine in 250 ml dioxane was added. The contents were stirred at 25° for 16 h. The remaining active groups on the gel were eliminated by stirring for 4 h with ethanolamine (22 ml). The gel was successively washed with 2 l each of 0.1 *M* sodium carbonate buffer (pH 10.5), 0.5 *M* NaCl and 6 l of water. The quantity of benzidine bound to Sepharose gel was determined to be 15.4 μmoles/ml. This value was calculated from ultraviolet absorption of the solvent phase after 16 h of coupling reaction and using ε_{291} value of $2.75 \cdot 10^4$ for benzidine in dioxane: 0.1 *M* Na_2CO_3, pH 10.5 (1:1, v/v).

Enzyme assays

α-D-mannosidase was assayed by incubating 50 μl of properly diluted enzyme

solution with 2.5 μmoles of *p*-nitrophenyl-α-D-mannopyranoside in 450 μl of 0.05 *M* sodium citrate, pH 4.5. The assay mixture was incubated at 37° for 10 min. The reaction was stopped by the addition of 1 ml of 0.2 *M* Na_2CO_3 and the liberated *p*-nitrophenol was quantitated[5]. A unit of enzyme was defined as the amount that would release 1 μmole *p*-nitrophenol/min at 37°. The protein content was determined with crystalline bovine serum albumin as the standard[6]. The assays for other glycosidase activities were carried out similarly with appropriate *p*-nitrophenyl glycosides at pH 4.5. Additionally, α-D-galactosidase and β-D-mannosidase were also assayed at pH 6.0 and 4.0, respectively.

Isolation of α-D-mannosidase

Unless otherwise indicated, all operations were carried out at 4°.

Step 1. Jack bean meal (200 g) was fractionated with ammonium sulfate solutions essentially as described by Li[2]. The protein precipitate between 0.33–0.60 saturation was dissolved in 40 ml of 0.01 *M* sodium phosphate, pH 7.0 (standard buffer) and dialyzed exhaustively against the same buffer. The dialyzed solution was heated at 50° for 15 min and cooled to 4° in an ice bath. The precipitate was removed by centrifugation at 29,600 *g* for 30 min and the supernatant fluid was designated "crude extract". The volume of the extract was 115 ml. It contained 3306 units of α-D-mannosidase and 3.87 g protein.

Step 2. A column (26 × 2.2 cm I.D.) of benzidine-Sepharose was equilibrated with standard buffer until the pH of the effluent was 7.0. A portion of the crude extract (23 ml) was applied to the column. The elution of the column was initiated with standard buffer (240 ml) followed by standard buffer containing 0.2 *M* NaCl (250 ml). Effluents from the column were monitored for protein content by measuring absorbance at 280 nm. Suitable aliquots from each fraction were assayed for

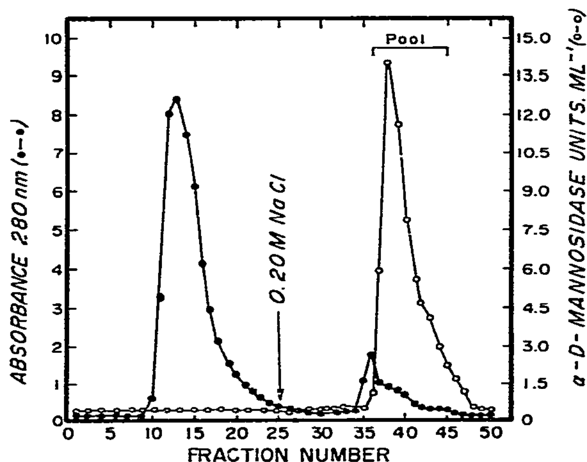

Fig. 1. Chromatography of the crude extract on benzidine-Sepharose 4B column (26 × 2.2 cm I.D.). The crude extract (23 ml, 774 mg protein and 661 units α-D-mannosidase) was applied to the column and the elution was initiated with 240 ml of standard buffer (see experimental section) followed by 250 ml of standard buffer containing 0.2 *M* NaCl. The point at which the NaCl concentration was increased from 0 to 0.2 *M* is indicated by the arrow. Fractions of 10 ml were collected at a flow-rate of 87 ml/h. The parenthesis shows the pooling of fractions.

glycosidase activities. Fractions containing the α-D-mannosidase activity (fractions 37–45, Fig. 1) were pooled and dialyzed against 1 l of 100% saturated ammonium sulfate solution for 16 h. The precipitate was collected by centrifugation at 29,600 g for 30 min, dissolved in 4 ml of standard buffer and the solution was dialyzed against the same buffer. This concentrated enzyme solution was tested for various glycosidase activities.

Aliquots from fraction number 38 (effluent with the highest specific activity) were examined by electrophoresis on polyacrylamide gel[7]. The activity of the enzyme in the gel was localized by immersing the post-electrophoresis gel column in 0.125 m*M* 4-methyl-umbelliferyl-α-D-mannopyranoside in 0.05 *M* sodium citrate, pH 4.5 for 15 min. The gel was processed according to Salafsky and Nadler[8].

RESULTS AND DISCUSSION

The elution diagram of α-D-mannosidase activity from benzidine-Sepharose 4B column is shown in Fig. 1. It was observed that virtually all of the α-D-mannosidase activity was detectable only in the 10 m*M* sodium phosphate buffer containing 0.2 *M* NaCl, pH 7.0 eluate and was absent from the unadsorbed material that was eluted with standard buffer. The pooled fraction upon concentration resulted in an 18-fold enrichment of α-D-mannosidase activity from the crude extract with a 71% recovery of the enzyme that was applied to the column (Table I). The purified material was completely free from β-D-mannosidase, α-D- and β-D-glucosidases, α-D- and β-D-galactosidases, β-D-N-acetylglucosaminidase and α-L-fucosidase activities. α-D-Mannosidase did not bind unsubstituted Sepharose 4B.

TABLE I

PURIFICATION OF JACK BEAN α-D-MANNOSIDASE

Fraction	*Specific activity (units/mg of protein)*	*Yield*	*Purification factor*
Crude extract	0.85	100	1
Benzidine-Sepharose column	15.36	71	18

The purity of α-D-mannosidase was examined by polyacrylamide gel electrophoresis at pH 9.0. The preparation was not homogenous. A major protein band and at least three minor bands were visible after staining with Coomassie Blue dye. However, when the gel column was treated with 4-methyl-umbelliferyl-α-D-mannopyranoside, the enzyme activity was localized at one place and was coincident with the major protein band, indicating that the enzyme activity is associated with a single protein species.

It is difficult to speculate on the mechanism by which α-D-mannosidase, out of several other glycosidases present in the jack bean, binds to benzidine-Sepharose. It seems that the enzyme is non-specifically bound to benzidine groups and depends upon the ionic strength. The binding is not due to interaction between the active site of the enzyme and the ligand of the adsorbent. Indeed, it has been shown that several glycosidases cannot differentiate the ligand structures in the adsorbents, notwithstand-

ing their strict enzymatic specificity[9]. Although in this study α-D-mannosidase is not purified to a homogenous degree, this enzyme can be isolated from other glycosidases employing benzidine-Sepharose in a single step. Such an enzyme preparation should be useful in structural studies of complex polysaccharides which contain α-D-mannosyl residue. Further purification may be possible by rechromatography of the enzyme employing appropriate ionic gradient.

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