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PURIFICATION OF JACK BEAN MEAL β-D-GALACTOSIDASE BY A NEW AFFINITY ADSORBENT

PREMANAND V. WAGH

Connective Tissue Laboratory, Veterans Administration Hospital, Little Rock, Ark. 72206 (U.S.A.)

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Summary

A simple procedure has been developed for the purification of jack bean β-D-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) by affinity chromatography employing a new affinity adsorbent. The ligand 6-*N*-β-(4-aminophenyl)-ethylamino-3-*O*-β-D-galactopyranosyl-6-deoxy-L-gulitol was prepared by the reaction between lactose and β-(4-aminophenyl)-ethylamine and was coupled to cyanogen bromide activated Sepharose 4B via the amino groups of the 4-aminophenyl moiety. This affinity gel resulted in a 111-fold purification of β-D-galactosidase with a 64% recovery of the enzyme. With *p*-nitrophenyl-β-D-galactopyranoside as the substrate the apparent K_m and V values were 0.59 mM and 1.87 μmol/min per mg, respectively. The method for purification of β-D-galactosidase may be applicable to other glycosidases depending upon the choice of specific di- or oligosaccharides of known structures to be used in the preparation of ligands.

β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) has been purified from many sources [1—3], employing various purification techniques including affinity chromatography. Jack bean β-D-galactosidase has been used in the elucidation of structure of the carbohydrate moiety of a number of glycoconjugates in which the prosthetic group contains among other sugars β-D-galactosyl units [4—7]. Li et al. [3] have purified jack bean β-D-galactosidase using 2-[6-(6-aminohexanamido) hexanamido]-ethyl-1-thio-β-D-galactopyranoside linked to Sepharose 4B as the affinity adsorbent. The procedure resulted in a 10% yield of enzyme activity.

The present communication describes a procedure for the preparation of a new specific affinity adsorbent for β-D-galactosidase and a simple isolation procedure for obtaining purified enzyme free from other glycosidase activities in high yield.

Materials and Methods

Materials were obtained as follows: β-(4-Aminophenyl)-ethylamine and lactose, the Aldrich Chemical Company; Sepharose 4B and Sephadex G-10, Pharmacia Fine Chemicals; jack bean meal, Nutritional Biochemicals; p-nitrophenyl glycosides and 4-methyl-umbelliferyl-β-D-galactopyranoside from Pierce Chemical. All other chemicals were of reagent grade.

Preparation of the ligand. 6-*N*-β-(4-aminophenyl) ethylamino-3-*O*-β-D-galactopyranosyl-6-deoxy-L-gulitol. The procedure used was similar to that described by Jeffrey et al. [8]. Lactose (3 mmol) was reacted with 15 ml of β-(4-aminophenyl)-ethylamine for 15 h at 45°C. All subsequent steps including reduction with $NaBH_4$ and separation of the coupled sugar and amine on Sephadex G-10 were carried out essentially as described by Jeffrey et al. [8]. This gel-filtration procedure, although gave a poor separation of derivatized lactose, lactitol and β-(4-aminophenyl)-ethylamine, those fractions containing both the neutral sugars and amino groups were pooled and lyophilized. The product was used as the ligand for coupling to Sepharose 4B and will be referred to as galactosyl derivative. The ϵ_{285} value for the galactosyl derivative (mol. wt. 462.5) in 0.1 M Na_2CO_3 (pH 8.0) was 1017, which was close to the 1020 reported for aminophenyl group [8]. The molar ratio of galactose: aminophenyl group as determined by phenol/H_2SO_4 and ultraviolet absorption methods was 1.14 : 1.00. The yield of the product was 630 mg which is 45% of the theoretical value of the starting material. Thus, about one-half of the starting lactose could not be accounted for presumably due to incomplete reduction to lactitol and/or its conversion to galactosyl derivative.

Preparation of affinity adsorbent. Sepharose 4B (60 ml) was activated with 12 g CNBr at pH 10 [10]. The activated gel (462.5 mg) was reacted with galactosyl derivative as described by Jeffrey et al. [8]. The quantity of ligand coupled to Sepharose 4B was determined to be 3.7 μmol of galactosyl derivative/ml of Sepharose using ultraviolet absorption value for the aminophenyl group. The free amino groups on the gel, which would be present due to trace amounts of β-(4-aminophenyl)-ethylamine, were acetylated as described by Roseman and Daffner [11]. The gel was washed successively with 1 l each of 0.5 M NaCl, water and 0.01 M sodium phosphate (pH 7.0) and used for affinity chromatography. The gel will be referred to as galactosyl-Sepharose 4B*.

Enzyme assays. The assays for glycosidases were carried out in 0.05 M sodium citrate using 2.5 μmol of appropriate *p*-nitrophenylglycosides at the following pH: α-D-galactosidase at pH 6.0; β-D-galactosidase at pH 4.0; α-D- and β-D-glucosidases, α-D-mannosidase, β-D-*N*-acetylglucosaminidase and α-L-fucosidase at pH 5.0. The liberation of *p*-nitrophenol was followed according to the procedure of Bahl and Agrawal [12]. A unit of enzyme was defined as the amount that would liberate 1 μmol *p*-nitrophenol/min at 37°C. The protein content was determined using the method of Lowry et al. [13] with crystalline bovine serum albumin as the standard.

Purification of β-D-galactosidase. Unless otherwise indicated, all operations

* Galactosyl-Sepharose 4B: 6-*N*-β-(4-aminophenyl)-ethylamino-3-*O*-β-D-galactopyranosyl-6-deoxy-L-gulitol linked to Sepharose 4B via the amino group of the 4-aminophenyl moiety.

for the enzyme purification were carried out at 4°C. All centrifugations were performed at 23 300 × *g* for 30 min.

Step 1. The first step of extraction was according to the procedure of Li and Li [14]. A (200 g) portion of jack bean meal was stirred in (1.2 l) distilled water for 30 min. The mixture was filtered through cheesecloth and adjusted to pH 4.5 by addition of 1.5 M sodium citrate (pH 2.7) under continuous stirring. After 30 min, the extract was centrifuged and the supernatant fluid was designated "crude extract".

Step 2. The pH of the crude extract was adjusted to 6.2 by addition of solid Na_2HPO_4. To this extract, 56.1 g of solid $(NH_4)_2SO_4$/100 ml extract was added under continuous stirring, to bring the mixture to 80% $(NH_4)_2SO_4$ saturation. The solution was stirred for 16 h and was centrifuged. The precipitate was solubilized in 100 ml of 0.01 M sodium phosphate (pH 7.0) and the resulting turbid solution was dialyzed against 10 times the volume of the same buffer for 27 h with 4 changes. The dialyzed solution was centrifuged and the supernatant fluid was designated "$(NH_4)_2SO_4$ precipitate".

Step 3. A slurry of galactosyl Sepharose was poured into a column to yield 1.4 × 15 cm bed volume and the gel was extensively washed with 0.01 M sodium phosphate (pH 7.0). The $(NH_4)_2SO_4$ precipitate fraction was applied to the column. The elution of the column was initiated with 0.01 M sodium phosphate (pH 7.0) followed by 0.1 M sodium borate (pH 9.2). Effluents from the column were monitored for protein content by measuring absorbance at 280 nm. Aliquots from each fraction were assayed for glycosidase activities using the assay system described above. The column was regenerated by consecutive elution at 25°C with 6 M guanidine · HCl, deionized water and 0.01 M sodium phosphate (pH 7.0).

Fractions containing the purified β-D-galactosidase (fractions 14—16, Fig. 1) were pooled and dialyzed against 100% saturated $(NH_4)_2SO_4$ solution for 24 h. The precipitate in the dialysis bag was collected by centrifugation at 25 900 × *g* for 30 min, dissolved in 0.01 M sodium phosphate (pH 7.0) and dialyzed against the same buffer. This concentrated enzyme solution was used for the determination of kinetic constants and for electrophoresis on polyacrylamide gel [15]. The activity of the enzyme in the gel was localized by immersing the post-electrophoresis gel-column in 0.25 mM 4-methyl-umbelliferyl-β-D-galactopyranoside in 0.05 M sodium citrate (pH 4.0) essentially as described by Salafsky and Nadler [16].

Results

The elution profile of β-D-galactosidase activity from galactosyl-Sepharose 4B column is shown in Fig. 1. Eleven percent of the β-D-galactosidase activity and 97.5% of the applied protein did not bind the affinity adsorbent. It was observed in a separate experiment that the activity bound to the column could not be eluted with 0.01 M sodium phosphate (pH 7.0) containing 100 mM D-galactono-γ-lactone. However, elution of the column with 0.1 M sodium borate (pH 9.2) resulted in a 64% recovery of the applied β-D-galactosidase activity. This simple procedure resulted in a 111-fold purification from the crude extract (Table I).

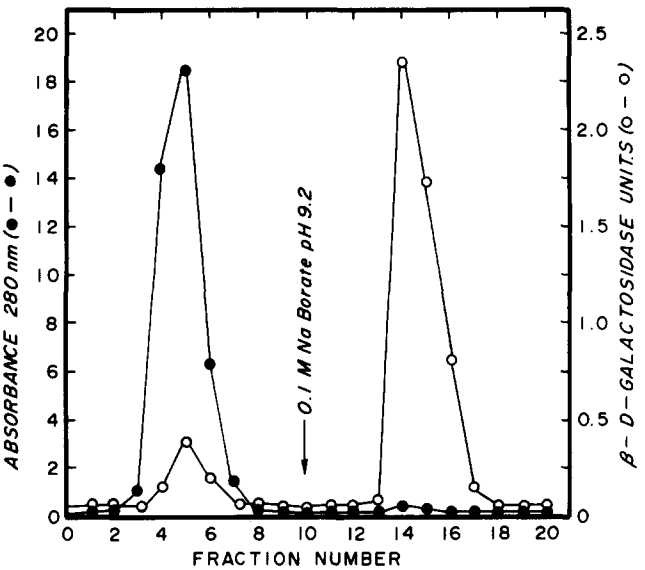

Fig. 1. Affinity chromatography of $(NH_4)_2SO_4$ precipitate fraction on galactosyl Sepharose 4B column (1.4 X 15 cm). Ammonium sulfate precipitate solution (18 ml, 342 mg protein and 8.1 units β-D-galactosidase) was applied to the column and the elution was initiated with 100 ml of 0.01 M sodium phosphate (pH 7.0) followed by 100 ml of 0.1 M sodium borate (pH 9.2). Fractions of 10 ml were collected at a flow rate of 51 ml/h. Aliquots of 50 μl were assayed for β-D-galactoside activity as described in the methods section. ●———●, Absorbance at 280 nm; ○———○, β-D-galactosidase activity.

The purified enzyme did not hydrolyze other *p*-nitrophenyl glycosides tested in these studies. All of the other glycosidase activities appeared in the unadsorbed fraction. However, a rather important observation was made in relation to α-D-mannosidase activity. If the free amino groups on the affinity gel are not blocked by the N-acetylation reaction, β-D-galactosidase together with α-D-mannosidase (70%) activities were bound to the adsorbent. After N-acetylation of the amino groups on the gel, all of the α-D-mannosidase activity occurred in the unadsorbed fraction. Preliminary studies indicate that α-D-mannosidase from jack bean meal binds benzidine Sepharose 4B. This may be probably due to ion-exchange effect.

The effect of the substrate on the velocity of purified β-D-galactosidase was

TABLE I

PURIFICATION OF JACK BEAN β-GALACTOSIDASE

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Recovery (%)
Crude extract	156	10 846	0.014	—	100
$(NH_4)_2SO_4$ precipitate	154	6 458	0.024	1.7	99
Galactosyl-Sepharose 4B *	98	63	1.555	111	64

* Corrected values presented for fractionation steps normally performed on a smaller scale. Samples containing 342 mg protein from ammonium sulfate precipitate fraction were used for affinity chromatography. Values obtained after analyses of the pooled fractions 14—16 (Fig. 1).

studied with *p*-nitrophenyl-β-D-galactoside at pH 4.0. The enzyme followed simple Michaelis and Menten kinetics as observed by Lineweaver-Burk plot with an apparent K_m value of 0.59 mM and V of 1.87 μmol/min per mg. The K_m value was similar to that obtained by Li et al. [3].

Examination of the purified β-D-galactosidase by polyacrylamide gel electrophoresis (pH 9.0) showed one major diffused protein band. A very faint minor band with faster mobility was observed indicating that the enzyme was not still homogeneous. The enzyme activity was localized at one place and was coincident with the major protein band as seen by fluorescence due to 4-methyl-umbelliferone.

Discussion

The present paper describes a procedure for the purification of jack bean β-D-galactosidase free from other glycosidase activities in high yield (64%) using a new specific affinity adsorbent. The principle used in the preparation of a specific carbohydrate ligand is based on the observation that oligosaccharides do not react with arylamines in the absence of catalyst. However, reducing oligosaccharides react with alkylamines to form *N*-alkyl glycosides [17] which are easily hydrolyzed but can be reduced to stable secondary amines. This principle has been successfully used in the purification of antibody directed against lacto-*N*-difucohexaose I [8] and now appears feasible in the purification of specific glycosidases.

Li et al. [3] using a 2-[6-(6-aminohexanamido)-hexanamido]-ethyl-1-β-thio-D-galactopyranoside as the ligand, have purified jack bean meal galactosidase. Although the specific activity of the purified enzyme obtained in this study is in one order of magnitude lower than reported by Li et al. [3], the yield of enzyme activity was 5 times greater, suggesting that there may be non-specific binding of some other protein(s) to the affinity gel and/or some inactivation of the enzyme due to borate buffer elution. β-D-galactosidase specifically bound to the galactosyl Sepharose column could not be eluted with buffer containing D-galactono-γ-lactone. The specific elution of human liver β-D-galactosidase by D-galactono-γ-lactone [18] seems to be due to the difference of specificities between human liver and jack bean enzymes. Although the purified enzyme is not homogeneous, such an enzyme preparation devoid of other glycosidase activities should be useful in structural studies of complex polysaccharides which contain β-D-galactosyl units.

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