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Note

A simple and rapid method for purification of the β-D-galactosidase from bovine testes

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β-D-Galactosidases from such various sources as *Aspergillus niger, Streptococcus pneumoniae, Clostridium perfringens, Escherichia coli,* jack bean, and almond either do not hydrolyze the β-Gal-(1→3)-GlcNAc linkage, or cleave it slowly[1–5]. In contrast, the β-D-galactosidase from bovine testes readily hydrolyzes this linkage[6]. Recently, we needed large amounts of the last-mentioned enzyme for structural studies of glycoconjugates prepared by the action of β-D-(1→3)-galactosyltransferase with appropriate acceptors. We now describe a rapid method for large-scale purification of the β-D-galactosidase from bovine testes.

EXPERIMENTAL

Materials. — All *p*-nitrophenyl glycosides were purchased from Sigma. Sepharose 6B was purchased from Pharmacia. Divinyl sulfone was a product of Aldrich. Bovine testes were obtained from local slaughterhouses, and used fresh, or kept frozen until used.

Enzyme assay. — Routine mixtures for the assay of β-D-galactosidase contained 0.1M citrate phosphate buffer (pH 4.3), 20mM *p*-nitrophenyl β-D-galactopyranoside, 1% of bovine serum albumin, and the enzyme in a final volume of 0.1 mL. The assay mixture was incubated for 30 min at 37°, the reaction terminated by the addition of 0.2M sodium carbonate (1 mL), and the absorbance of the *p*-nitrophenol released was measured at 400 nm. Other glycosidases were similarly assayed, using appropriate *p*-nitrophenyl glycosides as substrates. β-D-Hexosaminidase was assayed by using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside as the substrate.

Preparation of affinity media. — β-D-Galactose was attached to Sepharose

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TABLE I

PURIFICATION OF β-D-GALACTOSIDASE FROM BOVINE TESTES

Step	*Protein (mg)*	*β-D-Galactosidase (units[a])*	*β-D-Hexosaminidase (units[a])*	*β-D-Galactosidase specific activity (units/mg of protein)*
1. Acid extraction	5027	201	765	0.04
2. Ammonium sulfate fractionation	2784	142	442	0.051
3. Affinity chromatography	2.04	28.4	0.7	14.0

[a]Substrate μmol hydrolyzed per min.

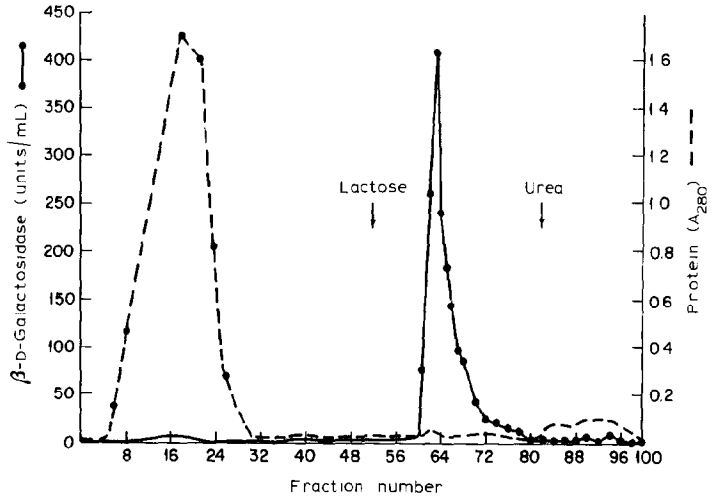

Fig. 1. Affinity chromatography of β-D-galactosidase from bovine testes. [Enzyme from step 2 was loaded onto a column of D-galactose–Sepharose 6B. The column was washed with 0.05M citrate, pH 5, containing 0.1M NaCl, and successively treated, as indicated, with this buffer containing either 0.2M lactose or 7M urea.]

6B activated with a divinyl sulfone spacer-arm, as described for the coupling of D-mannose[7].

Purification of β-D-galactosidase. — The various steps of purification are summarized in Table I. All operations were performed at 4°. The first two steps of purification were conducted according to Distler and Jourdian[6]. The precipitate obtained by ammonium sulfate fractionation was dissolved in 0.05M citrate, pH 5.0, containing 0.1M NaCl, and dialyzed against the same buffer (20 vol.) for 24 h. The dialyzate was centrifuged at 10,000*g* for 30 min. The supernatant liquor was loaded onto a column (2.5 × 90 cm) of D-galactosyl-Sepharose pre-equilibrated with 0.05M citrate, pH 5.0, containing 0.1M NaCl, and the column was washed with the same buffer. Preliminary experiments showed the binding capacity of the affi-

nity medium to be 150 units/mL of the gel, and the column was loaded accordingly. The flow rate of the column was maintained at 40 mL/h. Fractions (20 mL) were collected, and alternate fractions were assayed for β-D-galactosidase, β-D-hexosaminidase, and A_{280}-absorbing material (protein) (see Fig. 1). A major peak of protein-containing β-D-hexosaminidase was washed off the column in the first 30 fractions; these fractions contained virtually no β-D-galactosidase.

β-D-Galactosidase activity was eluted with buffer containing 0.2M β-lactose. Subsequently, the column was regenerated for future use by washing with binding buffer containing 7M urea, and re-equilibrated with the binding buffer prior to use. Thus, the column was successfully used thrice. Fractions (60–68) containing β-D-galactosidase were pooled, and concentrated by ultrafiltration, using an Amicon apparatus and PM 10 membrane. The concentrated enzyme solution was dialyzed against 1mM citrate buffer, pH 5.0, to remove the lactose. The final preparation of the enzyme, as well as aliquots from each purification step, was assayed for enzyme activity as described earlier. The protein content at each step was determined by the method of Bradford[8], using Bio-Rad reagent. Poly(acrylamide)-gel electrophoresis was conducted at pH 7.5, as described previously[9].

RESULTS AND DISCUSSION

Affinity column-chromatography has been widely employed for the purification of several glycosidases, including β-D-galactosidase from different sources. In this technique, a specific substrate or inhibitor of the enzyme is linked to a solid support for enzyme purification. These affinity adsorbents have frequently been prepared by coupling ligands having a spacer arm to a solid support activated with cyanogen bromide, and were expected to be highly specific for the retardation of the needed enzyme. However, such adsorbents may provide potential sites for nonspecific adsorption of unwanted enzymes and other (inert) proteins[10]. Even a matrix prepared without cyanogen bromide, such as Biogel A 15 M, may bind unwanted protein. For example, in the purification of bovine-testes β-D-galactosidase by affinity chromatography with the ligand *p*-aminophenyl 1-thio-β-D-galactoside, the retardation of some β-D-hexosaminidase was observed[6]. Furthermore, β-D-galactosidase was also bound to unsubstituted agarose.

As a result, it has been suggested that caution should be exercised in the use of affinity columns with agarose matrices. Previously, DiCioccio *et al.*[10] successfully employed affinity chromatography with a ligand D-galactose, bound to Sepharose activated with divinyl sulfone, for the purification of a β-D-galactosidase from bovine liver. For the purification of this enzyme, this affinity medium was superior to media activated with cyanogen bromide. We have now extended the use of this affinity medium for purification of the β-D-galactosidase from bovine testes.

The scheme for the purification of bovine-testes β-D-galactosidase is shown in Table I. The final preparation had a specific activity of 14 units/mg of protein. By

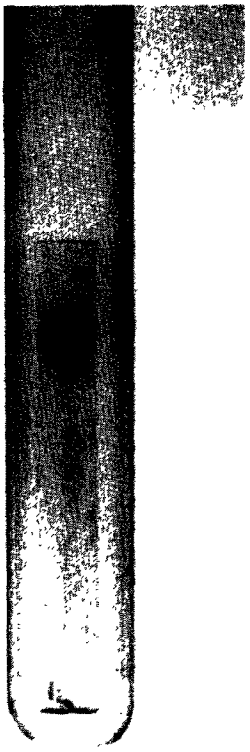

Fig. 2. Poly(acrylamide)-gel electrophoresis of β-D-galactosidase purified by affinity chromatography. [One unit of enzyme was electrophoresed, under nondenaturing conditions, at pH 7.5.]

a single, affinity-chromatography step, a 350-fold purification was achieved. The final enzyme preparation was devoid of α-L-fucosidase and β-D-mannosidase. The proportion of β-D-hexosaminidase in the final preparation was 0.025 unit per unit of β-D-galactosidase, in contrast to the 0.34 unit per unit of β-D-galactosidase reported by Distler and Jourdian[6]. These investigators also stated that β-D-hexosaminidase contamination was decreased in their procedure by an acetone fractionation-step prior to affinity chromatography. However, we obtained equally low levels of contamination (by β-D-hexosaminidase) with, or without, an acetone fractionation-step before affinity chromatography. Thus, we consider that it is not essential to use an acetone-precipitation step before chromatography with our medium.

Poly(acrylamide)-gel electrophoresis of the purified enzyme showed only one major band (see Fig. 2). This band corresponded with enzyme activity detected in slices of a duplicate gel run simultaneously (data not shown).

The major improvements in our method of purification of bovine-testes β-D-galactosidase are (*a*) fewer preparation steps, (*b*) diminished β-D-hexosaminidase

contamination, and (*c*) the use of a less expensive ligand. Thus, this method provides a simpler and faster procedure for large-scale preparation of the enzyme. However, it should be noted that this affinity medium may not be suitable for purification of β-D-galactosidases from other sources. For example, β-D-galactosidase from jack bean did not bind to matrices containing either D-galactose or lactose linked to Sepharose 6B activated with divinyl sulfone.

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