Carbohydrate Research, 127 (1984) 109–120
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION OF A β-D-GALACTOSIDASE FROM BOVINE LIVER BY AFFINITY CHROMATOGRAPHY

RICHARD A. DICIOCCIO, JOSEPH J. BARLOW, AND KHUSHI L. MATTA
Department of Gynecologic Oncology, Roswell Park Memorial Institute, Buffalo, New York 14263 (U.S.A.)

(Received September 26th, 1983; accepted for publication, October 17th, 1983)

ABSTRACT

A β-D-galactosidase from bovine liver was purified to apparent homogeneity. The major purification step was affinity chromatography on a column of D-galactose attached to a Sepharose support activated with divinyl sulfone. Affinity media prepared by binding ligands to Sepharose activated with cyanogen bromide were unsuitable for purification of the enzyme, even though such media have been used to purify β-D-galactosidases from other sources. The molecular weight of the denatured enzyme was 67,000. The molecular weight of the native enzyme at pH 7.0 was 68,000, and at pH 4.5 or 5.0, was 141,000. These data suggest that the enzyme has a single, fundamental subunit with a molecular weight of 67,000, and that the enzyme exists as a monomer at pH 7.0, and a dimer at pH 4.5 or 5.0. The V_{max} values of the enzyme with *p*-nitrophenyl β-D-galactoside, *p*-nitrophenyl β-D-fucoside, lactose, and β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* were 10,204, 11,550, 9,479, and 8,859 nmol/min/mg of protein, respectively, and the K_m values for these substrates were 0.08, 14.9, 14.2, and 1.6mM, respectively. D-Galactose, β-D-galactosylamine, *p*-aminophenyl 1-thio-β-D-galactoside, and D-galactono-1,4-lactone were competitive inhibitors of the enzyme, with K_i values of 0.9, 0.6, 0.6, and 0.8mM, respectively. The enzyme catalyzed the transfer of the D-galactosyl group from *p*-nitrophenyl β-D-galactoside to D-glucose. The pH optimum of the enzyme was 4.5, and the pI was 4.7.

INTRODUCTION

Partially purified, enzyme preparations from bovine liver have been reported[1,2] to contain at least two distinct β-D-galactosidases (β-D-galactoside galactohydrolase, EC 3.2.1.23). One catalyzed the hydrolysis of β-galactose from the artificial substrate, *o*-nitrophenyl β-D-galactoside, but was inactive with the natural substrates lactose and asialo-α_1-acid glycoprotein. The other was reported to catalyze the hydrolysis of D-galactose from these natural substrates, but not from the artificial D-galactoside. The latter observation suggested that bovine liver may contain a novel β-D-galactosidase having a strict substrate-specificity for D-galac-

0008-6215/84/$ 03.00

tose linked to another sugar. In an effort to demonstrate such a unique β-D-galactosidase rigorously, a program for the purification to homogeneity of β-D-galactosidases from bovine liver was initiated. The present report is concerned with our experience in the purification of a β-D-galactosidase, from bovine liver, that is capable of hydrolyzing lactose. The purification of the enzyme could not be achieved by affinity chromatography with ligands attached to Sepharose supports activated with cyanogen bromide, even though this method has been widely used[3–11] to purify β-D-galactosidases from other sources. Instead, an affinity technique, with D-galactose, as the ligand bound to a Sepharose support activated with divinyl sulfone, was successfully employed.

EXPERIMENTAL

Materials. — β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* was prepared as described[12]. All other nitrophenyl glycosides, as well as lactose, *N*-acetylneuraminyl-lactose (Grade I), β-D-galactosylamine, D-galactono-1,4-lactone, *p*-aminophenyl 1-thio-β-D-galactoside, D-galactose, D-glucose, β-D-galactose dehydrogenase from *Pseudomonas fluorescens*, *N*-acetyl-β-D-glucosaminidase from Jack bean, D-glucose oxidase (type VII) from *Aspergillus niger*, peroxidase (type II) from horseradish, *o*-dianisidine dihydrochloride, bovine serum albumin, and NAD (Grade III) from yeast, were obtained from Sigma. Sephadex G-200, Sepharose 4B, and Sepharose 6B were purchased from Pharmacia. Azocoll, fetuin, and divinyl sulfone were obtained from Calbiochem, Grand Island Biological Company, and Polysciences Inc., respectively. Ultrafiltration membranes (PM 10) and apparatus were from Amicon. Bovine liver was purchased from a local slaughterhouse. All other chemicals were of the highest grades commercially available.

Enzyme assays. — Several assays were used to determine β-D-galactosidase activity. All reaction mixtures had a final volume of 200 μL, and contained 50 μL of phosphate–citrate buffer[13], pH 4.5, and 0.7–7.0 units of β-D-galactosidase.

Assay 1 had 0.5 μmol of *o*- or *p*-nitrophenyl β-D-galactoside as the substrate. Assay 2 contained 0.06 unit of *N*-acetyl-β-D-glucosaminidase as the reagent, and 0.5 μmol of β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* as the substrate[14]. Reaction mixtures for assays 1 and 2 were incubated for various lengths of time at 37°. The reactions were terminated by chilling in an ice bath and adding 0.2M Na_2CO_3 (800 μL). Hydrolysis of substrates was determined by measurement of the absorbance of free nitrophenol at 400 nm.

Assay 3 contained 50 μmol of lactose as the substrate. After incubation for various lengths of time at 37°, the mixtures were chilled in ice, H_2O (200 μL) and 0.4M potassium phosphate, pH 6.9 (300 μL) were added, and the mixtures were heated for 5 min at 98° and then placed in an ice bath. If turbidity developed, it was removed by centrifugation at 1,200*g* for 10 min at 4°. Hydrolysis of lactose was measured by determination, by a modification of the D-glucose oxidase–peroxidase

method[15], of the amount of D-glucose released. To each heat-treated mixture were added, in 100-μL aliquots, 1.65 units of peroxidase, 12.5 units of D-glucose oxidase, and 100 μg of *o*-dianisidine dihydrochloride. Peroxidase and D-glucose oxidase solutions were made up in 0.4M potassium phosphate, pH 6.9. Mixtures were incubated for 15 min at 37°, and 4M HCl (100 μL) was added. D-Glucose was measured by the A_{400} value, and by comparing the absorbance of reaction mixtures to a calibration curve of D-glucose standards.

Assay 4 was employed in order to measure the cleavage of D-galactose from the substrates. The reaction mixtures were incubated for various lengths of time at 37°, and the reactions were terminated by chilling in an ice bath. Liberation of D-galactose was measured by the D-galactose-dehydrogenase method, using 0.1 unit of D-galactose dehydrogenase and 0.7 μmol of NAD per reaction mixture[16].

Other glycosidases, except neuraminidase, were measured by assay 1 with appropriate *p*-nitrophenyl glycosides (0.5 μmol) as the substrates. Neuraminidase was determined either with *N*-acetylneuraminyl-lactose (0.5 μmol) or fetuin (0.5 μmol of sialic acid groups) as the substrate. Cleavage of sialic acid was measured by the thiobarbituric acid method[17]. Protease, with Azocoll as the substrate, was assayed according to the directions of the supplier of the substrate.

Controls lacking either the substrate or the enzyme were routinely employed for all assays. All assays were conducted under conditions that were linear with respect to time and to concentration of protein. A unit of β-D-galactosidase is 1 nmol of β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* hydrolyzed per min. The units of reagent enzymes employed in assays 2–4 were defined by the supplier.

Preparation of affinity media. — β-D-Galactosylamine was directly coupled to Sepharose 4B activated with cyanogen bromide[18]. The content of ligand was 5.8 μmol/mL of gel. This ligand was also attached to succinylaminoethyl–Sepharose 4B, as described for coupling of β-L-fucosylamine to this support[19]. The content of ligand was 1.2 μmol/mL of gel. 1,8-Octanediamine–Sepharose 4B was prepared, and coupled to D-galactono-1,4-lactone[4,18]. The content of ligand was 0.4 μmol/mL of gel. *p*-Aminophenyl lactoside–Sepharose 4B (1.7 μmol/mL of gel) was prepared by the general method of Bloch and Burger[20]. All of the foregoing methods use cyanogen bromide to activate Sepharose.

D-Galactose was attached to Sepharose 6B activated with a divinyl sulfone spacer-arm as described[21] for the coupling of D-mannose to this support. *p*-Aminophenyl 1-thio-β-D-galactoside was similarly coupled, except that the concentration of reactant ligand was 10mM, and the coupling buffer was 0.5M $NaHCO_3$, pH 8.3. Contents of ligand were 9.6 μmol of D-galactose/mL of gel, and 5.3 μmol of *p*-aminophenyl 1-thio-β-D-galactoside/mL of gel. Cyanogen bromide is not used in the preparation of affinity media with divinyl sulfone.

For each affinity gel, a control gel was prepared in an identical manner, except for omission of the ligand. Ligand contents of gels were determined according to Bloch and Burger[20] for *p*-aminophenyl glycosides, and according to Chipowsky *et al.*[22] for the other ligands.

TABLE I

PURIFICATION OF β-D-GALACTOSIDASE FROM BOVINE LIVER

Step	*Total protein (mg)*	*Total activity (units[a])*	*Specific activity (unit/mg of protein)*	*Purification (-fold)*	*Yield (%)*
1 Crude homogenate	90,300	63,000	0.7	1.0	100
2 Extraction at pH 4 8	33,984	31,860	0.9	1.3	50.6
3 Fractionation with $(NH_4)_2SO_4$	8050	8183	1.0	1.4	13.0
4 Affinity chromatography	7.80	3120	400	571	5.0
5 Gel filtration chromatography					
Peak A	0.28	1400	5000	7142	2.2
Peak B	3.51	1296	369	527	2.1

[a]One unit of β-D-galactosidase is one nmol of β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* hydrolyzed/min at a substrate concentration of 2.5mM. This is less than a saturating amount of substrate (K_i 1.6mM).

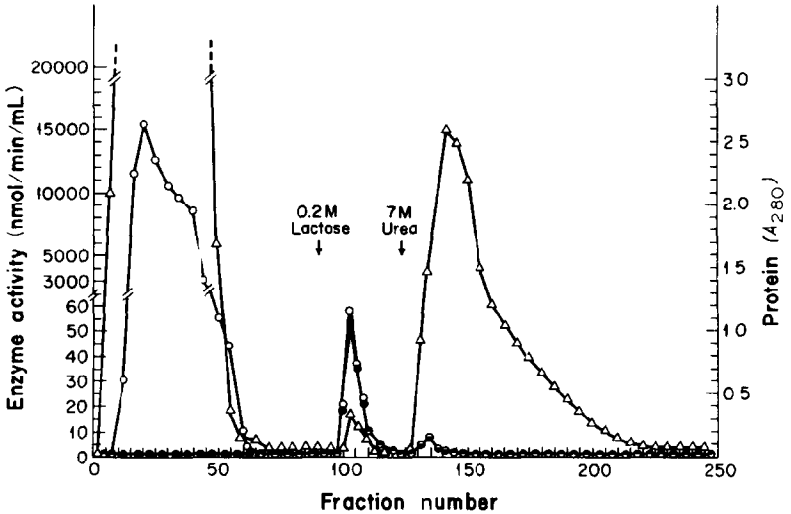

Fig. 1. Affinity chromatography of β-D-galactosidase from bovine liver. [Enzyme from step 3 was loaded onto a D-galactose–Sepharose 6B column. The column was washed with 0.05M citrate, pH 5, containing 0.1M NaCl, and then treated, as indicated, with this buffer containing either 0.2M lactose or 7M urea (see Experimental section). O——O, enzyme activity with *p*-nitrophenyl β-D-galactoside; ●——●, enzyme activity with lactose; and △——△, A_{280}.

Purification of β-D-galactosidase. —This is summarized in Table I. All operations were conducted at 4°. Fresh bovine liver (1.5 kg) in H_2O (3.0 L) was homogenized with a blender, extracted at pH 4.8, and fractionated with $(NH_4)_2SO_4$ as described[2,23]. The $(NH_4)_2SO_4$ fraction was dialyzed *versus* three changes of 0.05M citrate, pH 5, containing 0.1M NaCl. The dialyzate was centrifuged at 13,000*g* for 50 min, and the supernatant liquor collected, and applied to a column (2.7 × 54 cm) of D-galactose–Sepharose 6B which was pre-equilibrated

with 0.05M citrate, pH 5, containing 0.1M NaCl (see Fig. 1). The flow rate of the column was 35 mL/h, 22-mL fractions were collected, and alternate fractions were measured for β-D-galactosidase activity by assay methods 1 and 3, and for protein (A_{280}). The column was washed with binding buffer until A_{280} and the β-D-galactosidase activity (with *p*-nitrophenyl β-D-galactoside as the substrate) returned to the baseline. No β-D-galactosidase activity with lactose as the substrate was detected in these fractions. The column was washed with binding buffer containing 0.2M lactose, which co-eluted the β-D-galactosidase activity with either *p*-nitrophenyl β-D-galactoside or lactose as the substrate. Subsequently, the column was regenerated for future use by washing with binding buffer containing 7M urea.

Large amounts of A_{280} material and a small amount of β-D-galactosidase were eluted in these fractions. Fractions 100–115 were pooled, concentrated by ultrafiltration, and applied to a column (5 × 91 cm) of Sephadex G-200 which was pre-equilibrated with 0.05M citrate, pH 5. The flow rate of the column was 18 mL/h, 22-mL fractions were collected, and alternate fractions were measured for β-D-galactosidase activity by assay methods 1 and 3, and for protein (A_{280}) (see Fig. 2). Two peaks of β-D-galactosidase activity were eluted from the column. Fractions eluted between 720 and 860 mL (peak A) and between 940 and 1,250 mL (peak B) were separately pooled, concentrated by ultrafiltration, and stored at 4°. After each purification step, the enzyme activity was monitored, using β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* as the substrate, and protein was determined by the method of Bradford, using Bio-Rad reagent[24]. β-Gal-(1→4)-β-GlcNAc-1 →

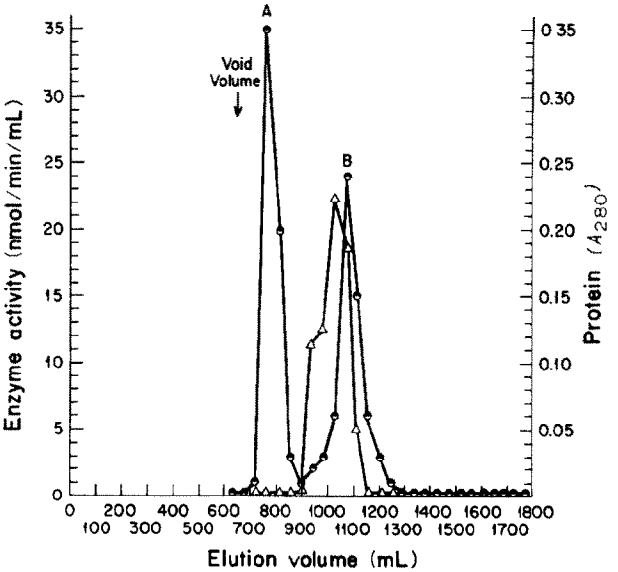

Fig. 2. Gel-filtration chromatography of β-D-galactosidase (step 4) on Sephadex G-200. [○——○, enzyme activity with *p*-nitrophenyl β-D-galactoside; ●——●, enzyme activity with lactose; and △——△, A_{280}.]

$OC_6H_4NO_2$-*p* was employed for determinations of specific activity, because crude preparations of enzyme (steps 1 and 2) contained material that interfered with the estimation of free β-glucose derived from the hydrolysis of lactose.

Electrophoresis and isoelectric focusing. — Poly(acrylamide) gel electrophoresis under denaturing or non-denaturing conditions, and analytical, isoelectric focusing, were conducted as described[19,25,26].

Sedimentation velocity ultracentrifugation. — Ultracentrifugation was conducted with 5–20% linear, density gradients of sucrose (5 mL). Separate gradients of pH 4.5, 5.0, or 7.0 were made up in the phosphate–citrate buffer system of McIlvaine[13]. Samples were layered onto gradients, and the gradients were centrifuged at 40,000 r.p.m. in a Beckman SW 50 rotor for 19 h at 4°. Gradient fractions (0.25 mL each) were collected after puncturing the bottoms of the centrifuge tubes, and fractions were assayed for enzyme activity by assay 1. Sedimentation coefficients were estimated according to Fritsch[27], and molecular weights according to Martin and Ames[28].

Kinetic and pH studies. — K_m and V_{max} were graphically determined by the method of Lineweaver and Burk[29], and K_i according to Cleland[30]. Transglycosylation (*i.e.*, the transfer of D-galactosyl group from *p*-nitrophenyl β-D-galactoside to D-glucose, catalyzed by β-D-galactosidase) was demonstrated by paper chromatography[31]. The pH optimum of β-D-galactosidase was determined in a phosphate–citrate buffer-system[13].

RESULTS

Purification. — A β-D-galactosidase activity from bovine liver was purified 571-fold by affinity chromatography on a D-galactose–Sepharose 6B column activated with divinyl sulfone (see Table I and Fig. 1). This enzyme used lactose, *o*- and *p*-nitrophenyl β-D-galactoside, and β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* as substrates. The affinity ligand, namely, D-galactose, was a competitive inhibitor of the enzyme (K_i 0.9mM) and the eluant lactose was a substrate. This suggested that both the binding and elution of the enzyme were biospecific.

Fractions 8–65 of the chromatographic profile contained a β-D-galactosidase activity that did not bind to the column. This enzyme used *p*-nitrophenyl β-D-galactoside as substrate. However, neither lactose nor β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* was a substrate, and D-galactose (125mM) did not inhibit this activity. This enzyme corresponded to the major β-D-galactosidase activity described in bovine liver[1,2,31,32], and was distinct from the enzyme eluted with lactose. In crude homogenates, β-D-galactosidase activity with β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* as the substrate was 0.5% of that with *p*-nitrophenyl β-D-galactoside. Thus, the affinity column had extraordinary resolving-power.

In contrast to the affinity support prepared with divinyl sulfone, affinity media containing ligands attached to Sepharose activated with cyanogen bromide were not useful for purification of the β-D-galactosidase, using lactose as the sub-

strate. Ligands used were β-D-galactosylamine, *p*-aminophenyl 1-thio-β-D-galactoside, D-galactono-1,4-lactone, and *p*-aminophenyl β-lactoside. The last compound was a substrate for the enzyme, but the other compounds were competitive inhibitors thereof, with K_i of values of 0.6, 0.6, and 0.8mM, respectively. The capacity for binding enzyme by these matrices was ~20% of that of the D-galactose–Sepharose 6B matrix prepared with divinyl sulfone. Moreover, β-D-galactosidase could not be eluted with lactose from matrices prepared with cyanogen bromide, but was eluted, together with large amounts of other proteins, by 7M urea. Control experiments with columns of Sepharose 4B activated with cyanogen bromide, but not containing any ligand, behaved identically to the ligand–Sepharose 4B columns prepared with cyanogen bromide. Unreacted Sepharose 4B and Sepharose 6B, as well as Sepharose 6B activated with divinyl sulfone but not containing ligand, did not bind any β-D-galactosidase. Thus, the treatment of Sepharose with cyanogen bromide is apparently responsible for the "anomalous" chromatographic behavior.

An affinity matrix containing *p*-aminophenyl 1-thio-β-D-galactoside coupled to Sepharose 6B *via* divinyl sulfone also specifically bound β-D-galactosidase activity, using lactose as the substrate. However, D-galactose was employed as the ligand for large-scale purification of the enzyme, because it was more economical.

Gel-filtration chromatography of β-D-galactosidase from step 4 resolved two peaks, A and B, of enzyme activity (see Fig. 2). The ratio of enzyme activity for the cleavage of *p*-nitrophenyl β-D-galactoside and lactose by fractions containing peak-A enzyme was constant, suggesting that a single enzyme catalyzed the hydrolysis of each substrate; the same was true for peak-B enzyme. The specific activity of enzyme purified through step 5, peak A, increased 12.5-fold over step 4, but the specific activity of step 5, peak B, was about the same as for step 4. Other properties of peak A and B enzymes will be considered separately.

Purity of peak A enzyme. — β-D-Galactosidase (peak A) migrated as a single species in poly(acrylamide)-gel electrophoresis under either nondenaturing or denaturing conditions (see Fig. 3). Enzyme activity measurements (assay 1) of replicate gels conducted under nondenaturing conditions coincided with the single species detected by staining. The enzyme preparation was devoid of several contaminating hydrolase activities possible. These included α- and β-D-glucosidase, α- and β-D-mannosidase, α- and β-L-fucosidase, α-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, *N*-acetyl α- and β-D-galactosaminidase, β-D-glucosiduronase, and β-D-xylosidase, assayed with appropriate *p*-nitrophenyl glycosides; neuraminidase assayed with either *N*-acetylneuraminyl-lactose or fetuin; and protease assayed with Azocoll. However, the enzyme preparation had β-D-fucosidase activity. At concentrations of 2.5mM, *p*-nitrophenyl β-D-fucoside and *p*-nitrophenyl β-D-galactoside were hydrolyzed at rates of 1.200 and 10.000 mmol/min/mg of protein, respectively. Enzyme activity that hydrolyzed *p*-nitrophenyl β-D-fucoside co-migrated with activity that hydrolyzed *p*-nitrophenyl β-D-galactoside in poly(acrylamide)-gel electrophoresis conducted under nondenaturing conditions. This strongly suggests that both substrates are hydrolyzed by the same en-

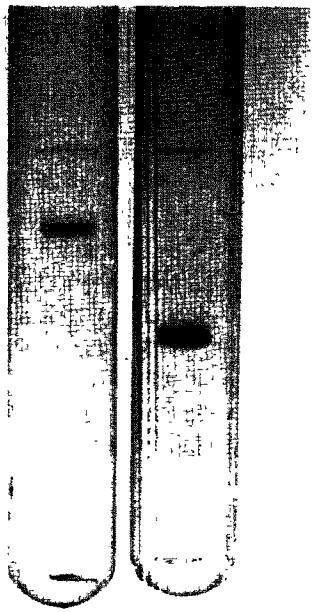

Fig. 3. Poly(acrylamide)-gel electrophoresis of β-D-galactosidase (step 5, peak A). [Left, 50 units of enzyme were electrophoresed under non-denaturing conditions at pH 7.5. Right, 50 units of enzyme were electrophoresed under denaturing conditions in the presence of sodium dodecyl sulfate.]

zyme. β-D-Galactosidases from porcine spleen and human, feline, and mouse liver have been reported to hydrolyze *p*-nitrophenyl β-D-fucoside[8,33–35].

Specificity of the glycon. — The glycon specificity of β-D-galactosidase (peak A) was restricted to β-D-galacto- and β-D-fuco-syloxy among the *p*-nitrophenyl glycosides tested. The V_{max} of the enzyme with *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl β-D-fucoside were similar, 10.204 and 11.550 mmol/min/mg of protein, respectively. However, the K_m value for *p*-nitrophenyl β-D-galactoside (0.08mM) was 1/186th of that for *p*-nitrophenyl β-D-fucoside (14.9mM). This difference in K_m value explains the 8-fold difference in the rate of hydrolysis of these substrates at concentrations of 2.5mM. Furthermore, the data imply that *p*-nitrophenyl β-D-galactoside has a greater binding affinity for the enzyme than has *p*-nitrophenyl β-D-fucoside, and that the glycon of these substrates participates in their binding to the enzyme. Specifically, the 6-hydroxyl group of D-galactose must promote binding to the enzyme, because the presence or absence of this group is the sole structural difference between D-galactose and D-fucose.

The V_{max} of the enzyme with lactose and β-Gal-(1→4)-β-GlcNAc-1 → $OC_6H_4NO_2$-*p* were 9.479 and 8.859 mmol/min/mg of protein, respectively, and the K_m values were 14.2 and 1.6mM, respectively. The V_{max} values of the enzyme with these substrates were similar to that with *p*-nitrophenyl β-D-galactoside, but the K_m values were respectively 178 and 20 times that for *p*-nitrophenyl β-D-galactoside.

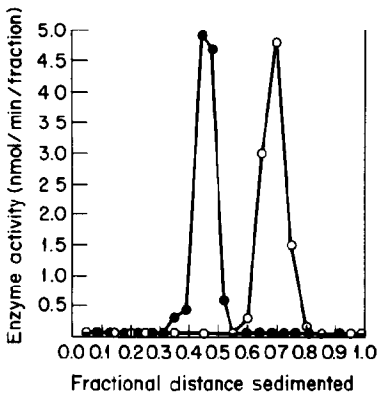

Fig. 4. Sedimentation velocity ultracentrifugation of β-D-galactosidase (step 5, peak A) on sucrose density gradients. [Aliquots of enzyme (7 units) were equilibrated at pH 4.5, pH 5.0, or pH 7.0 before layering each onto a density gradient having the same pH. ○——○, ultracentrifugation at pH 4.5; and ●——●, ultracentrifugation at pH 7.0. Ultracentrifugation at pH 5.0 gave the same results as at pH 4.5. Enzyme activity was measured with *p*-nitrophenyl β-D-galactoside.]

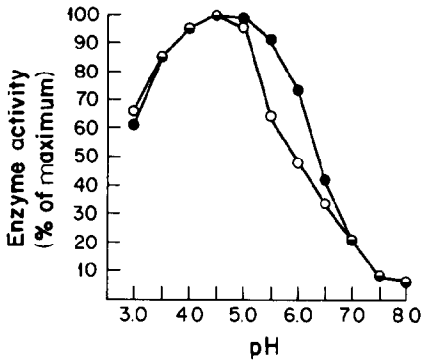

Fig. 5. pH optimum of β-D-galactosidase from bovine liver. [Enzyme (0.7 unit) was assayed at various pH values with the following substrates: ○——○, *p*-nitrophenyl β-D-galactoside, or ●——●, lactose.]

These three substrates have the same glycon, D-galactosyloxy, but different aglycons. Thus, the kinetic data for these compounds show that the aglycon can also alter the binding affinity between enzyme and substrates.

Molecular weight. — The molecular weight of β-D-galactosidase (peak A) measured under denaturing conditions by gel electrophoresis in the presence of sodium dodecyl sulfate was 67,000. The molecular weight of the native enzyme, as determined by sedimentation velocity ultracentrifugation at pH 7.0, was 68,000, and, at pH 4.5 or 5.0, 141,000 (see Fig. 4). At each pH, the enzyme sedimented as a single peak, with sedimentation coefficients of 5.3 s at pH 7.0 and 8.5 s at pH 4.5 or 5.0. Collectively, these data suggest that the enzyme has a single, fundamental subunit having a molecular weight of 67,000, and that the enzyme exists as a monomer at pH 7.0, and a dimer at pH 4.5 or 5.0.

Studies of pI, pH, salt, protein, and transglycosylation. — β-D-Galactosidase (peak A) had a pI of 4.7. The enzyme had a pH optimum of 4.5 with either lactose or *p*-nitrophenyl β-D-galactoside as the substrate (see Fig. 5). Enzyme activity with either substrate was stimulated 10% by 0.1M NaCl, but was inhibited 24% by M NaCl; also, activity with either substrate was increased 50% by bovine serum albumin (200 μg per assay).

Enzyme activity, measured with *p*-nitrophenyl β-D-galactoside as the substrate in the presence of 0.25M D-glucose, was stimulated 69% if assay 1 was used, but was inhibited 23% if assay 4 was employed. Thus, the amount of free *p*-nitrophenol increased, but the amount of free D-galactose decreased; this suggested that the enzyme catalyzed the transfer of the D-galactosyl group from *p*-nitrophenyl β-D-galactoside to D-glucose. Evidence for the synthesis of new glycosides by such a transglycosylation was obtained by paper chromatography[31]. Transglycosylation has been demonstrated with other mammalian β-D-galactosidases[8,31,34,36]

β-D-Galactosidase (peak B). — Rechromatography of β-D-galactosidase (Step 5, peak B) on columns of D-galactose–Sepharose 6B and Sephadex-G200 yielded the same general elution-profiles as those shown in Figs. 1 and 2. The enzyme bound to the D-galactose–Sepharose 6B column was eluted with lactose, and this eluate was resolved into two peaks, A and B, by preparative, gel-filtration chromatography. The specific activity and properties of this peak A material were virtually identical to those already discussed for β-galactosidase purified through step 5, peak A. The approximate molecular weights of peak A and B material eluted from the Sephadex-G200 column were estimated to be 150,000 and 70,000, respectively. As evidence has already been presented indicating that β-D-galactosidase has a single subunit with a molecular weight of 67,000, and that the enzyme can exist as a monomer or dimer, peaks A and B are most probably monomeric and dimeric forms, respectively, of the same enzyme.

DISCUSSION

Affinity media prepared by coupling ligands to Sepharose that had been activated with cyanogen bromide have been successfully employed for the purification of β-D-galactosidases from many sources[3–11]. However, affinity media prepared by this method were found unsuitable for purification of a β-D-galactosidase from bovine liver. The results indicated that treatment of Sepharose with cyanogen bromide was responsible for a non-biospecific binding of proteins from extracts of bovine liver to Sepharose, and that biospecific interactions between β-D-galactosidase and ligands did not occur. In contrast to the cyanogen bromide method, affinity media that were prepared by coupling ligands to Sepharose with divinyl sulfone biospecifically bound a β-D-galactosidase from bovine liver. An additional feature of the divinyl sulfone method was that a plentiful and inexpensive ligand, D-galactose, could be coupled to Sepharose. This made feasible the preparation of

large amounts of affinity matrix for the scaled-up purification of β-D-galactosidase. These features of the divinyl sulfone method suggest that the D-galactose–Sepharose 6B matrix may be a valuable tool for purification of β-D-galactosidases, and of other D-galactose-binding proteins in general. Moreover, the coupling of other sugars to Sepharose with divinyl sulfone may also provide useful and inexpensive reagents for the purification of other carbohydrate-binding proteins.

The molecular weight of β-D-galactosidase from bovine liver determined under enzyme-denaturing conditions was 67,000. This was similar to molecular weights previously reported for β-D-galactosidases from feline, mouse, and human liver, porcine spleen, and human placenta and urine[8,9,11,33,35,37]. Under conditions of sedimentation velocity ultracentrifugation, the native enzyme from bovine liver apparently exists as a monomer at pH 7.0, and a dimer at pH 4.5 and 5.0. The molecular weights of β-D-galactosidases from mouse and human liver, porcine spleen, and human urine were also reported to be dependent upon the pH[11,33,35,37,38].

A partially purified preparation of the enzyme from bovine liver was reported to contain a β-D-galactosidase activity that used lactose and α_1-acid glycoprotein as substrates, but not *o*-nitrophenyl β-D-galactoside[1,2]. This suggested that bovine liver might contain a novel β-D-galactosidase having a strict specificity for D-galactose linked to another sugar, but we found no evidence for such a β-D-galactosidase. The enzyme we purified had a specificity for aryl monogalactosides and for glycosides having D-galactose linked to other sugars. In preliminary experiments, Distler and Jourdian found that bovine liver possesses a β-D-galactosidase activity that employs *p*-nitrophenyl β-D-galactoside and lactose as substrates[31], but they reported no evidence for a β-D-galactosidase that strictly requires substrates having D-galactose linked to another sugar.

Several properties of β-D-galactosidase from bovine liver were found similar to properties reported for β-D-galactosidase from bovine testes[31]. These are pH optimum, transglycosylation activity, stimulation of activity by bovine serum albumin, K_m for lactose and *p*-nitrophenyl β-D-galactoside, and the molecular weight of the native enzyme at pH 7.0. This suggests that these β-D-galactosidases may actually be the same enzyme.

ACKNOWLEDGMENTS

This research was supported by Grant CA-24051 from the National Cancer Institute, U.S.P.H.S., and by Grant GM 31425 from the National Institute of General Medical Sciences, U.S.P.H.S.

REFERENCES

1 T. J. LANGLEY, *Arch. Biochem. Biophys.*, 128 (1968) 304–311.
2 T. J. LANGLEY AND F. R. JEVONS, *Arch. Biochem. Biophys.*, 128 (1968) 312–318.

3 E. STEERS, P. CUATRECASAS, AND H. POLLARD, *J. Biol. Chem.*, 246 (1971) 196–200.
4 J. N. KANFER, G PETROVICH, AND R. A. MUMFORD, *Anal. Biochem.*, 55 (1973) 301–305.
5 M. SATO AND I. YAMASHINA, *J. Biochem. (Tokyo)*, 76 (1974) 1155–1163.
6 S.-C. LI, M. Y. MASSOTTA, S. F. CHIEN, AND Y.-T. LI, *J. Biol. Chem.*, 250 (1975) 6786–6791.
7 A. L. MILLER, R. G. FROST, AND J. S. O'BRIEN, *Anal. Biochem.*, 74 (1976) 537–545.
8 E. W. HOLMES AND J. S. O'BRIEN, *Biochemistry*, 18 (1979) 952–958.
9 J. LO, K. MUKERJI, Y. C. AWASTHI, E. HANADA, K. SUZUKI, AND S. K. SRIVASTAVA, *J. Biol. Chem.*, 254 (1979) 6710-6715.
10 O. P. VAN DIGGELEN, A. W. SCHRAM, M. L. SINNOTT, P. J. SMITH, D. ROBINSON, AND H. GILJAARD, *Biochem. J.*, 200 (1981) 143–151.
11 E. PASCHKE, R. NIEMANN, G. STRECKER, AND H. KRESSE, *Biochim. Biophys. Acta*, 704 (1982) 134–143.
12 S. S. RANA, J. J. BARLOW, AND K. L. MATTA, *Carbohydr. Res.*, 113 (1983) 257–271.
13 T. C. MCILVAINE, *J. Biol. Chem.*, 49 (1921) 183–186.
14 R. A. DICIOCCIO, P. J. KLOCK, J. J. BARLOW, AND K. L. MATTA, *Carbohydr. Res.*, 81 (1980) 315–322.
15 N. M. PAPADOPOULOS AND W. C. HESS, *Arch. Biochem. Biophys.*, 88 (1960) 167–171.
16 P. R. FINCH, R. YUEN, H. SCHACHTER, AND M. A. MOSCARELLO, *Anal. Biochem.*, 31 (1969) 296–305.
17 D. AMINOFF, *Biochem. J.*, 81 (1961) 384–392.
18 P. CUATRECASAS *J. Biol. Chem.*, 245 (1970) 3059–3065.
19 R. A. DICIOCCIO, J. J. BARLOW, AND K. L. MATTA, *J. Biol. Chem.*, 257 (1982) 714–718.
20 R. BLOCH AND M. M. BURGER, *FEBS Lett.*, 44 (1974) 286–289.
21 N. FORNSTEDT AND J. PORATH, *FEBS Lett.*, 57 (1975) 187–191.
22 S. CHIPOWSKY, Y. C. LEE, AND S. ROSEMAN, *Proc. Natl. Acad. Sci: U.S.A.*, 70 (1973) 2309–2312.
23 B. WEISSMAN, S. HADJIIOANNOU, AND J. TORNHEIM, *J. Biol. Chem.*, 239 (1964) 59–63.
24 M. BRADFORD, *Anal. Biochem.*, 72 (1976) 248–252.
25 I. R. BROWN, *Biochim. Biophys. Acta*, 191 (1969) 731–734.
26 K. WEBER, J. R. PRINGLE, AND M. OSBORN, *Methods Enzymol.*, 26 (1972) 3–27.
27 A. FRITSCH, *Anal. Biochem.*, 55 (1973) 57–71.
28 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372–1379.
29 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658–666.
30 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 173–187.
31 J. J. DISTLER AND G. W. JOURDIAN, *J. Biol. Chem.*, 248 (1973) 6772–6780.
32 J. A. RODRIGUES, J. A. CABEZAS, AND P. CALVO, *Int. J. Biochem.*, 14 (1982) 695–698.
33 Y. YAMAMOTO AND K. NISHIMURA, *J. Biochem. (Tokyo)*, 88 (1980) 705–713.
34 A. G. W. NORDEN, L. L. TENNANT, AND J. S. O'BRIEN, *J. Biol. Chem.*, 249 (1974) 7969–7976.
35 S. TOMINO AND M. MEISLER, *J. Biol. Chem.*, 250 (1975) 7752–7758.
36 K. WALLENFELS AND J. FISCHER, *Hoppe-Seyler's Z. Physiol. Chem.*, 321 (1960) 223–245.
37 R. G. FROST, E. W. HOLMES, A. G. W. NORDEN, AND J. S. O'BRIEN, *Biochem. J.*, 175 (1978) 181–188.
38 H. L. HOEKSEMA, O. P. VAN DIGGELEN, AND H. GALJAARD, *Biochim. Biophys. Acta*, 566 (1979) 72–79.