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Purification of Ligandin by Affinity Chromatography on Sulfobromophthalein-Agarose Gel (40408)

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In man and animals, bilirubin, sulfobromophthalein (BSP), and other organic anions, avidly bind to albumin in the vascular compartment. Their removal depends upon hepatic uptake by a process having carrier-mediated kinetic characteristics (1). These compounds accumulate within the liver cell bound to a family of soluble proteins, the GSH-transferases, of which GSH-transferase B (ligandin) is responsible for at least 70% of total organic anion binding (2, 3). Each of these GSH-transferases has been purified to homogeneity using classical protein chemistry techniques (4, 5). This report describes a rapid and simple method for purification of proteins, such as ligandin, which bind organic anions with high affinity. The method involves affinity chromatography and, in contrast with existing methods, separates proteins on the basis of organic anion binding rather than by charge or size characteristics.

Materials and methods. BSP was obtained in aqueous solution (50 mg/ml) from Hynson, Westcott, and Dunning, Inc., Baltimore, MD. Purity was confirmed by thin-layer chromatography (6). Glutathione (GSH) was obtained from Sigma, and cyanogen bromide from Eastman Organic Chemicals. Agarose (Sepharose 4B) was obtained from Pharmacia.

Synthesis of GSH-BSP. GSH-BSP was prepared by incubation of 1.2 mmoles GSH with an equimolar amount of BSP at 37° for 2 hr (7). Purity of the resulting GSH-BSP conjugate was established by thin-layer chromatography (6) and exceeded 95%.

Preparation of BSP-conjugated agarose. Cyanogen bromide activated agarose was prepared from 50 g wet wt of gel by the method of Axen *et al.* (8). The activated gel was washed with 25 vol of ice cold 0.1 *M* $NaHCO_3$ and the wet gel was placed in a plastic bottle. Immediately thereafter, approximately 200 μmoles of GSH-BSP in 10 ml of 0.1 *M* $NaHCO_3$ were added, and the mixture was kept on a stirring table for 48 hr at 4°. The resulting gel was washed sequentially with 25 vol of distilled water, 25 vol of 0.1 *M* Na_2HPO_4, 50 vol of distilled water, and 25 vol of 0.15 *M* NaCl in 0.02 *M* sodium phosphate (PBS) at pH 7.4. BSP bound to agarose was determined by increasing the pH to 12 in aliquots of the original GSH-BSP solution and collected washes of the derivatized gel. Absorption at 580 nm was determined, and bound GSH-BSP was calculated as the difference between total and unbound ligand.

Protein studies. The liver was removed from 200 to 250 g male Sprague-Dawley rats (Marland Farms) after light ether anesthesia, perfused with saline, and homogenized by a motor-driven teflon pestle with an equal volume of 0.25 *M* sucrose in 0.1 *M* sodium phosphate at pH 7.4. The crude homogenate was centrifuged at 2000*g* for 30 min, and the supernatant was centrifuged at 140,000*g* for 60 min. The supernatant was separated from the pellet and overlying lipid, and will be referred to as cytosol.

Cytosol (20 ml) was charged onto a 20 ml BSP-agarose column and washed with 20 vol of PBS at pH 7.4. Fractions were collected in 4 ml aliquots, and absorption at 280 nm was determined (Fig. 1). All fractions between cumulative vol 16 and 188 ml were combined and termed peak A. The column was eluted with 0.1 *M* Na_2HPO_4 at pH 9.2, and fractions were collected in 3 ml aliquots. Fractions between cumulative volumes 254 and 362 ml were combined and termed peak B. Flow through the column was 2 ml per min.

Protein was determined by the method of Lowry (9) and GSH transferase activity was determined using 1-chloro-2,4-dinitrobenzene (Eastman Organic Chemicals) as substrate (10). Ligandin was determined by radial immunodiffusion using a monospecific

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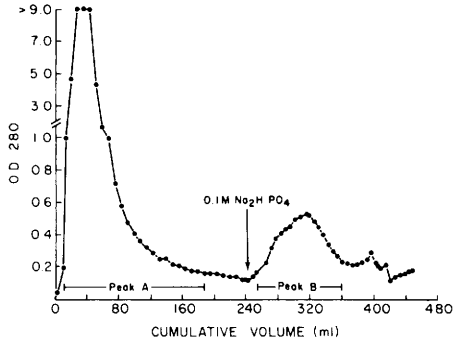

FIG. 1. Protein elution pattern, as determined by absorption at 280 nm (OD280), of 20 ml of cytosol after BSP-agarose affinity chromatography. Initially, the column was washed with PBS at pH 7.4: bound protein was eluted with 0.1 *M* Na_2HPO_4. Peaks A and B were analyzed as in the text.

antibody (11). Albumin was determined by radioimmunoassay using a monospecific antibody. In order to determine whether the conditions used resulted in loss of binding of bilirubin to ligandin, binding of bilirubin was studied by circular dichroism using a Cary model 60 spectropolarimeter with a 6001 CD attachment (12). The temperature of the cell compartment was 25–27° and a cell of 1 cm path length was used. Slit widths were programmed for a spectral band width of 15Å or less and absorption was always less than 2.0. Stock bilirubin solutions were 10^{-2} *M* in 0.02 *N* NaOH and were stored for no more than 3 hr in the dark at 4° prior to use. Negligible changes were observed in absorption of bilirubin during these conditions. Bilirubin was added in 1–30 μl aliquots to protein solutions (3–35 mg/ml) to give a final concentration of 20–200 μ*M*.

Further protein analysis was performed by slab gel electrophoresis using a spacer gel of 3% polyacrylamide and 0.1% sodium dodecyl sulfate (SDS) overlying a 10% polyacrylamide gel containing 0.1% SDS. Samples were solubilized in 0.4% SDS with 1% β-mercaptoethanol; electrophoresis was performed in Tris–glycine buffer containing 0.04% SDS, and protein was visualized after staining with 0.005% Coomassie brilliant blue (13).

Results. Covalent binding of GSH-BSP to agarose. Seventy to 80% of the 200 μmoles of GSH-BSP remained bound to agarose after washing. When GSH-BSP was incubated with agarose which had not been activated, none remained on the gel after washing.

Affinity chromatography of cytosol. Of the protein applied to the column, 45% was recovered in peak A and 13% in peak B. Only 16% of albumin was recovered in Peak B, and there was little or no enrichment over cytosol (3.73 μg/mg protein vs. 3.12 μg/mg protein, respectively). Recovery of ligandin in peak B was 55% and there was fourfold enrichment as compared to cytosol (283 μg/mg protein vs. 68.7 μg/ml protein, respectively). GSH-transferase specific activity was threefold enriched in peak B when compared to activity in cytosol (18.4 μmoles/min/mg protein vs. 5.71 μmoles/min/mg protein) (Table I).

SDS gel electrophoresis of cytosol and peaks A and B (Fig. 2) confirmed relative enrichment of ligandin in peak B. Ligandin migrated with its characteristic two subunits of molecular weights 22,000 and 25,000 (14). Protein which did not bind to the column, and cytosol, had similar SDS gel patterns. The column eluate had relatively few bands, two of which migrated identically to the two ligandin subunits. The nature of the non-ligandin protein bands in peak B is unknown, but other glutathione transferases and Z protein are presumably present.

When bilirubin was added in excess (200 μ*M*) to cytosol or to peak A, the characteristic circular dichroism ellipticity pattern of bilirubin–albumin was seen with positive extrema at 465 nm and negative at 415 nm (15, 16). When bilirubin (20 μ*M*) was added to peak B, the characteristic ellipticity pattern of bilirubin–ligandin was generated, with negative extrema at 465 nm and positive at 415 nm and 515 nm (4, 12).

Discussion. This study utilizes a rapid and

TABLE I. CONTENT OF PROTEIN, ALBUMIN, LIGANDIN, AND GSH TRANSFERASE ACTIVITY IN CYTOSOL AND IN PEAKS A AND B AFTER BSP-AGAROSE AFFINITY CHROMATOGRAPHY. RECOVERY AS A PERCENTAGE OF AMOUNT CHROMATOGRAPHED IS GIVEN IN PARENTHESIS.

	Protein (mg)	Albumin (mg)	Ligandin (mg)	GSH transferase (μmoles/min)
Cytosol	354	1.11	24.3	2020
Peak A	160	0.81 (73%)	7.9 (33%)	867 (43%)
Peak B	47	0.18 (16%)	13.3 (55%)	801 (40%)

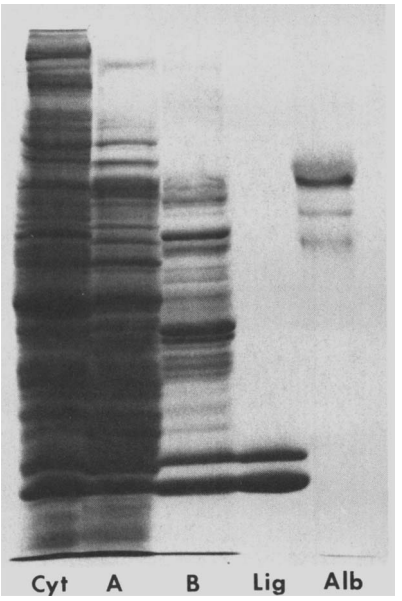

FIG. 2. SDS polyacrylamide (10%) slab gel of (left to right) cytosol, peak A, peak B, ligandin, and albumin. Peak B is enriched in ligandin and contains little albumin.

simple method for preparation of an agarose affinity column using BSP as a coupled ligand. Unlike other methods described for coupling BSP to agarose (17, 18), this procedure results in linkage of BSP to the gel through a peptide chain, ie, glutathione. The BSP-agarose gel preferentially binds ligandin when rat liver cytosol is applied. Elution of bound protein is effected by increasing pH to 9.2 with 0.1 *M* Na_2HPO_4. This is in contrast to conditions necessary for elution of glutathione transferase derived from galleria mellonella larvae (19). After elution, ligandin retains its immunologic, enzymatic, and organic anion binding properties. Significant denaturation of ligandin does not occur during affinity chromatography as evidenced by recovery of immunoreactive ligandin (88% recovery in peaks A and B) and GSH transferase activity (84% recovery in peaks A and B). Ligandin in peak B generated a typical ellipticity pattern when bound to bilirubin.

Albumin also binds BSP with high affinity, but was not enriched in peak B when compared to cytosol as determined by radioimmunoassay. By circular dichroism, peak A generated typical albumin–bilirubin complexes whereas peak B generated typical ligandin-bilirubin complexes. These results are in accord with recent studies by Listowsky *et al.* (15) which demonstrated reduced affinity for bilirubin of rat albumin when added to liver cytosol. This difference in binding is postulated to result from inhibition by other ligands within cytosol.

This method does not purify ligandin to homogeneity, which has been accomplished using classical techniques (11, 20). The fourfold purification of ligandin is similar to that obtained by gel filtration or anion exchange chromatography of cytosol; if used following one of these initial steps, a higher degree of purification of ligandin should result. However, the method rapidly separates organic anion binding proteins, such as ligandin and presumably other GSH transferases, from other proteins in liver cytosol. This technique should be useful in the study of organic anion binding to liver cell components other than cytosol, and may permit characterization of their potential role in hepatic uptake and excretory mechanisms.

Summary. Affinity chromatography of rat liver cytosol on sulfobromophthalein coupled to agarose via a glutathione bridge is described. Protein bound to the gel is eluted with 0.1 *M* Na_2HPO_4 at pH 9.2, resulting in a fourfold enrichment of ligandin as compared to cytosol. This technique should be useful in the study of organic anion binding to rat liver components other than cytosol, and may permit characterization of their potential role in hepatic uptake and excretory mechanisms.

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