Purification and characterization of bile salt hydrolase from *Clostridium perfringens*

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Abstract Bile salt hydrolase (cholylglycine hydrolase, EC 3.5.1.24) has been purified to homogeneity (792-fold) from *Clostridium perfringens* using high performance DEAE-chromatography. The purified enzyme showed a single detectable protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a relative molecular weight ca. 56,000. The intact enzyme had a relative molecular weight (M_r) of ca. 250,000 as determined by nondenaturing PAGE. The NH_2-terminal sequence of bile salt hydrolase was determined to be Met-(Ser/Cys)-Arg-Thr-Lys-Leu-Val-Ileu-Thr-Ileu-Gly-Ala-Ser. The purified enzyme was active towards both glycine and taurine conjugates of cholate. The apparent K_m and V_{max} of the enzyme for glycocholate was estimated to be 0.5 mM and 107 nmol/min · mg protein, respectively. The pH optimum was in the range of 5.8 to 6.4. The enzyme was inhibited 85%, 81%, and 83% by 2 mM iodoacetate, *p*-chloromercuribenzoate, and phenylmethanesulfonylfluoride, respectively. Rabbit polyclonal antibody was prepared and used to demonstrate a single form of the enzyme in crude cell extracts.—**Gopal-Srivastava, R., and P. B. Hylemon.** Purification and characterization of bile salt hydrolase from *Clostridium perfringens*. *J. Lipid Res.* 1988. **29:** 1079–1085.

Supplementary key words HPLC • substrate saturation kinetics

Bile salts are biotransformed into a number of metabolites by the intestinal microflora of man and animals (1). The hydrolysis of conjugated bile acids is one of the most common microbial bile salt biotransformations. Bile salt hydrolase (BSH) catalyzes the hydrolysis of the amide bond of conjugated bile acids commonly found in mammalian bile. BSH activity has been detected in members of the genera *Clostridium, Lactobacillus, Fusobacterium, Bacteroides, Bifidobacterium, Peptostreptococcus,* and *Streptococcus* (2–6). BSH has been characterized primarily from *Clostridium perfringens* (7) and *Bacteroides fragilis* (8). However, there are considerable differences in the biochemical characteristics of BSH isolated from *Clostridium perfringens* (7) and *Bacteroides fragilis* (8). Whether these differences are due to the level of purity or due to species-specific difference remains to be determined.

There is currently no standard procedure available for the purification of BSH from *Clostridium perfringens* even though this enzyme is routinely used by many laboratories to deconjugate bile salts prior to gas–liquid chromatographic analysis. In the present study, BSH has been purified to homogeneity using high performance liquid chromatography techniques and some of its properties have been determined. In addition, specific polyclonal antibody has also been prepared against the purified enzyme and used to demonstrate a single form of the enzyme in *Clostridium perfringens*.

MATERIALS AND METHODS

Organisms and growth conditions

Clostridium perfringens MCV 815 was kindly donated by Dr. H. P. Dalton of the Medical College of Virginia, Richmond, VA. Stock cultures of *Clostridium perfringens* MCV 815 were maintained in chopped meat medium under anaerobic conditions as described by Holdeman and Moore (9). *C. perfringens* was grown anaerobically in Brain Heart Infusion medium.

Materials

Glycine and taurine conjugates of cholic acid, deoxycholic acid, and chenodeoxycholic acid were obtained as the sodium salts from Sigma Chemical Co. (St. Louis, MO). Acrylamide was obtained from Bio-Rad Laboratories (Richmond, CA). Sodium dodecyl sulfate (SDS), iodoacetate, *p*-chloromercuribenzoate, and trinitrophenylbenzenesulfonate (TNBS) were obtained from Sigma Chemical Co. Diethylaminoethyl-cellulose (DE-52) was purchased from Whatman (W. & R. Balston Ltd., England).

Quantitative enzyme assay for bile salt hydrolase

Enzymatic assay of BSH was performed by measuring the release of glycine resulting from the hydrolysis of the amide bond of sodium glycocholate. The reaction mixture

Abbreviations: BSH, bile salt hydrolase; TNBS, trinitrophenylbenzenesulfonate; 2-ME, 2-mercaptoethanol.

(1 ml) contained the protein (10 to 50 μg), 5 mM sodium glycocholate, 500 mM sodium phosphate buffer (pH 6.0), and 1 mM ethylenediaminetetraacetic acid (EDTA). Reaction mixtures were incubated at 37°C for 10 min (under these conditions the reaction approximates zero order kinetics). The enzymatic reaction was terminated by the addition of an equal volume of 20% trichloroacetic acid, and precipitated protein was removed by centrifugation at 15,000 *g* for 10 min. The amount of glycine present in the supernatant was measured by quantitating the chromogenic derivative of glycine with trinitrophenyl-benzenesulfonate (TNBS) at 416 nm using the procedure of Dashkevicz and Feighner (Dashkevicz, M. P., and S. D. Feighner, unpublished results). In brief, iodoacetic acid (17.5 mM final concentration) was added to a 0.2-ml aliquot of supernatant fluid to acetylate 2-mercaptoethanol (2-ME). This step prevents 2-ME from reacting with TNBS. The volume was brought to 1.0 ml with borate buffer (100 mM sodium borate in 100 mM sodium hydroxide, pH 9.5). After 5 min, 20 μl of 1.4 M TNBS was added and the mixture was incubated at room temperature for 5 min. Color development was stopped by the addition of 2.0 ml of freshly prepared sodium sulfite solution (1.5 ml of 100 mM sodium sulfite plus 98.5 ml of 100 mM sodium phosphate, monobasic). Glycine released from glycocholic acid was estimated by using a standard curve prepared using free glycine. Protein concentration was estimated by the method of Bradford (10), using the Bio-Rad microassay reagent.

Purification of bile salt hydrolase

Growth of bacteria and preparation of cell extract. *Clostridium perfringens* was grown anaerobically in Brain Heart Infusion medium (5 liters) and growth was monitored with a Klett Summerson colorimeter on an hourly basis. *Clostridium perfringens* cells (12 g) were harvested at the late log phase by centrifugation at 7000 *g* for 20 min and suspended in 10 ml sodium phosphate, pH 7.8, 10 mM 2-ME, 1 mM EDTA, 12% (v/v) glycerol (buffer A) containing 0.1 mg each of deoxyribonuclease and ribonuclease A. The cells were disrupted by two passages through a French pressure cell (10,000 psi). Debris and unbroken cells were removed by centrifugation at 105,000 *g* for 1.5 hr. The resulting soluble cell extract was dialyzed against 1 liter of buffer A for 20 hr at 4°C.

Ammonium sulfate precipitation. Dialyzed cell extract was precipitated by slowly adding solid ammonium sulfate to 40% saturation while maintaining the pH at 7.8 by the addition of sodium hydroxide. After 60 min at 4°C, the precipitate was removed by centrifugation at 12,000 *g* for 10 min. Supernatant fluid was brought to 70% ammonium sulfate saturation and centrifuged at 12,000 *g* for 10 min. The resulting pellet was suspended in 10 ml of buffer A and dialyzed against the same buffer for 24 hr. Maximum enzyme activity was detected in the 40–70% ammonium sulfate fraction.

DEAE-cation exchange chromatography. The dialyzed extract (ca. 800 mg protein) from 40 to 70% ammonium sulfate was applied to a DE-52 cellulose (2.6 × 25 cm) column, preequilibrated with buffer A. The column was washed extensively (5–10 times bed volume) to remove unbound materials. The protein was eluted using a linear gradient of 0–300 mM sodium chloride in buffer A; fractions (3 ml) were collected and immediately assayed for the BSH activity. Fractions containing BSH activity were pooled and concentrated by using an Amicon stirred cell with YM-10 membrane (molecular weight cutoff 10,000).

High performance gel filtration chromatography. The pooled fractions (1 ml) from the DE-52 cellulose chromatography column were injected onto a Du Pont GF-250 column fitted with a precolumn that had been preequilibrated with buffer A containing 100 mM sodium chloride. The flow rate was 0.85 ml/min. Fractions (1 ml) were collected and assayed for BSH activity.

DEAE-high performance chromatography. The pooled fractions (ca. 4 mg protein) from gel filtration chromatography were applied to a DEAE-HPLC column (Altex DEAE 3SW, 0.75 × 7.5 cm), preequilibrated with buffer A (pH 6.8) at a flow rate of 0.85 ml/min. Protein was eluted with a linear gradient (0–300 mM) of sodium chloride (in buffer A, pH 6.8) over 75 min. Fractions (1 ml) containing BSH activity were pooled and dialyzed against 2 liters of buffer A.

BSH purity and molecular weight estimation

Estimation of protein purity was accomplished by subjecting aliquots of pooled fractions from each step of puri-

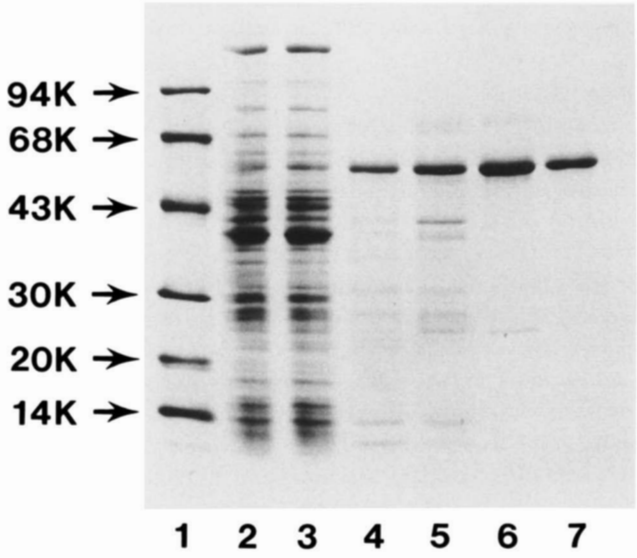

Fig. 1. Purification of bile salt hydrolase from *Clostridium perfringens* MCV 815: lane 1, molecular weight standards; lane 2, soluble extract (10 μg); lane 3, 40–70% ammonium sulfate extract (10 μg); lane 4, pooled fraction from DEAE-cellulose column (pH 7.8); lane 5, pooled fraction from HPLC GF-250 gel filtration column (pH 7.8); lane 6, pooled fraction from HPLC-DEAE column (pH 6.8); lane 7, pooled fraction from reinjected HPLC-DEAE column (pH 6.8).

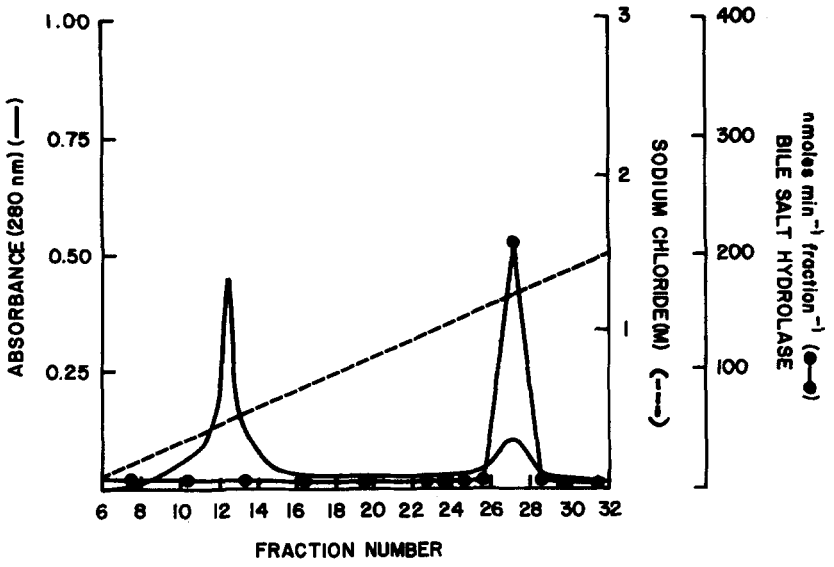

Fig. 2. HPLC-DEAE (Altex DEAE 3SW, 0.75 × 7.5 cm) elution profile of BSH from *Clostridium perfringens* MCV 815. The solid line represents absorbance (280 nm), dashed line represents the concentration of sodium chloride, and the dotted line represents BSH activity. Conditions of protein elution are described in Materials and Methods.

fication to SDS polyacrylamide gel electrophoresis. Gel electrophoresis was performed on slab gels (0.75 mm) (7–20% acrylamide) using the Laemmli buffer system (11). Samples were heated for 5 min at 100°C in sample buffer (50 mM Tris-HCl, 1% SDS, 1% 2-ME, 10% glycerol, pH 6.8). Electrophoresis was performed at constant current (20 mA/plate). Protein was detected using Coomassie blue stain. The molecular weight of the intact enzyme was estimated by comparing its migration with standard proteins of known molecular weight on nondenaturing polyacrylamide gels. Gel electrophoresis was carried out on the slab gels containing a gradient of polyacrylamide concentration (7–30%) according to the laboratory techniques of Pharmacia Fine Chemicals (12) except that the buffer used in gel, reservoirs, and samples was 360 mM Tris–320 mM borate–10 mM EDTA (pH 8.4). The proteins used for molecular weight calibration were as follows: chicken albumin (M_r 43,000), bovine serum albumin (M_r 67,000), *Escherichia coli* alkaline phosphatase (M_r 94,000), catalase (M_r 240,000), and jack bean urease (M_r 272,000 trimer, 575,000 hexamer). Mobility distances of the five standard proteins were plotted against molecular weight on a log scale and the molecular weight of BSH was estimated from this standard curve.

Amino acid analysis was performed on a Durrum MBF D-500 analyzer. The NH_2-terminal amino acid sequence of the purified enzyme (100 μg) was determined using a gas phase sequenator (Applied Biosystems Protein Sequenator 470 A) in the Department of Biochemistry and Molecular Biophysics, MCV-VCU, Richmond, VA 23298.

Preparation of antibody

Immunization of rabbit. Specific polyclonal antibody was produced against BSH by injecting 100 μg of purified protein emulsified in 1.0 ml of Freund's complete adjuvant into the hind leg of a white New Zealand male rabbit. It was followed by maintenance injections of 50 μg of protein

TABLE 1. Purification of bile salt hydrolase from *Clostridium perfringens*

Step	Protein	Enzyme[a,b]	Yield	Purification Factor
	mg	*U/mg protein*	*%*	*fold*
Crude cell extract	2203	1.7	100	1
Ammonium sulfate 40–70% sat.	736	3.1	60	2
DE-52	67.5	20.2	36	12
HPLC gel filtration	3.45	154	14	90
DEAE-HPLC	0.18	1346	6.4	792

[a]Unit, nmoles/min.
[b]Enzyme assay run for 10 min.

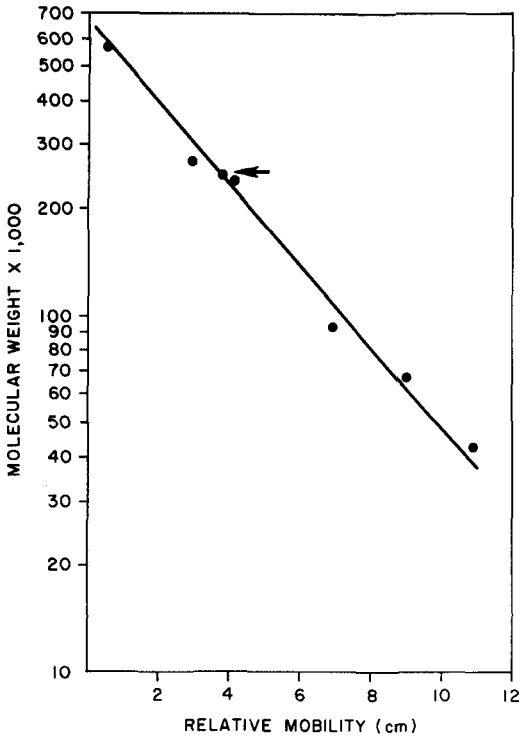

Fig. 3. Calibration curve for molecular weight estimation on nondenaturing polyacrylamide gel. The standard proteins used are chicken albumin (43,000), bovine serum albumin (67,000), *E. coli* alkaline phosphatase (94,000), catalase (240,000), and jack bean urease (272,000 trimer, 575,000 hexamer). The arrow indicates the molecular weight of BSH.

emulsified in 1.0 ml of Freund's incomplete adjuvant, at intervals of 1 month, for 3 months. Prior to the initial injection of protein, blood (30–50 ml) was withdrawn to prepare nonimmune serum. Blood was also withdrawn prior to each maintenance injection of protein to prepare immune serum. For the preparation of serum, blood was allowed to clot at room temperature for 2 hr and then overnight at 4°C. Blood was centrifuged for 10 min at 6000 *g* (4°C) and the serum was removed and stored in 5-ml aliquots at −70°C. Specific antibody was detected by the Western blot technique (13).

Western blot analysis (immunoblotting). Proteins were electrophoresed on SDS-PAGE and then were transferred onto a nitrocellulose membrane using the Bio-Rad electroblot assembly. Electroblotting was carried out at 30 volts for 12 hr (4°C), using 25 mM Tris, 192 mM glycine, 20% methanol (v/v), pH 8.3. After the transfer, the nitrocellulose membrane was incubated in 20 mM Tris, 500 mM NaCl, pH 7.5, (TBS) containing 3% gelatin for 60 min at room temperature. The membrane was incubated with the first antibody (specific for BSH, diluted in 1% gelatin TBS) for 2 hr with gentle agitation. After washing with Tween-80-TBS to remove unbound antibody, the membrane was incubated with the second antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate, diluted 1:3000 with 1% gelatin-TBS), and washed with Tween-80-TBS. The membrane was next immersed in a color development solution containing 60 mg of 4 chloronaphthol, 20 ml of methanol, and 60 μl of 30% hydrogen peroxide in TBS. The color reaction was stopped after 30 min by immersing the membrane in distilled water for 10 min. The color development solution was prepared immediately prior to use and protected from light.

RESULTS

BSH was purified from *Clostridium perfringens* MCV 815 by the procedure detailed under Materials and Methods. The progress of purification was followed by measuring enzyme activity using glycocholic acid (sodium salt) as the substrate. The electrophoretogram of the active fractions at the various stages of purification is shown in **Fig. 1**.

When chromatographed on DEAE-HPLC at pH 6.8, the bulk of the protein passed through the column while BSH was eluted as a sharp single peak at approximately 120 mM sodium chloride (**Fig. 2**). At this stage of purification BSH was occasionally contaminated with other proteins. This problem was, however, overcome by reapplying the sample to the DEAE-HPLC column. BSH could be stored for 2 months at −20°C without marked loss in activity. **Table 1** summarizes the results of a typical purification from 12 g (wet weight) of *Clostridium perfringens*.

When subjected to SDS-PAGE, the final enzyme preparation migrated as a single polypeptide band with an apparent M_r of 56,000 (Fig. 1, lane 7). The M_r 56,000 band could be detected throughout purification as exemplified in Fig. 1, and only this band increased in intensity with the progress of purification. The molecular weight of the

TABLE 2. Amino acid composition of BSH from *Clostridium perfringens*

Amino Acid	Residue/mol Protein
Asp	7.9
Thr + Ser	3.8
Glu	6.8
Ala	15.3
Val	5.5
Met	2.0
Ile	5.5
Leu	4.5
Tyr	1.4
Phe	1.9
Lys	6.8
His	3.4
Arg	3.4
Pro	n.e.[a]
Cys	n.e.
Trp	n.e.
Gly	n.e.

[a]Not estimated.

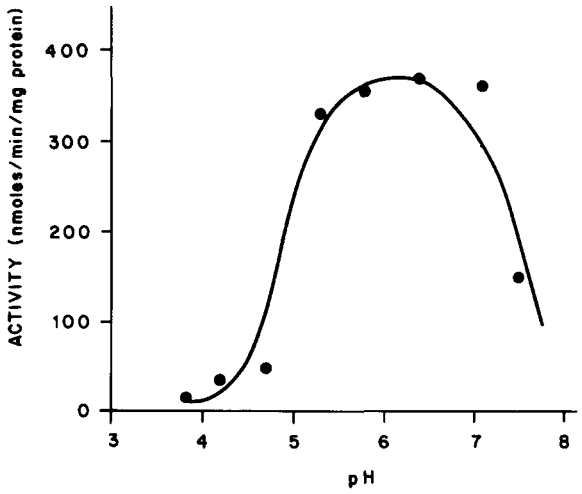

Fig. 4. Effect of pH on bile salt hydrolase activity. The pH values plotted indicate the final pH of the reaction mixture. Sodium acetate buffer (0.5 M) was used for the pH range of 3.5 to 5.5, and potassium phosphate buffer (0.5 M) was used for the pH range of 6.0 to 7.5.

intact enzyme was estimated by nondenaturing PAGE and found to be ca. 250,000 (**Fig. 3**). The NH_2 terminal sequence analysis of protein revealed methionine to be the NH_2 terminal residue (Met-Ser/Cys-Arg-Thr-Lys-Leu-Val-Ileu-Thr-Ileu-Gly-Ala-Ser-). An amino acid analysis of the purified protein (**Table 2**) showed it to be rich in alanine.

Studies on the effect of pH on enzyme catalysis (**Fig. 4**) showed a broad pH optimum over the range of 5.8 to 6.4. For pH dependency studies, a pH range of 3.8–7.5 in sodium acetate and sodium phosphate buffers was used to assay the enzymatic activity. The study was limited to this pH range because the solubility of the substrate (glycocholic acid, sodium salt) in these buffer systems decreases upon lowering the pH below 3.8.

The substrate saturation of the enzyme was determined by varying the concentration of glycocholic acid (sodium salt). The saturation curve followed Michaelis-Menten kinetics (**Fig. 5**). The apparent K_m and V_{max} were found to be approximately 0.5 mM and 107 nmol/min · mg protein, respectively, as derived from a Lineweaver-Burk plot. Substrate specificity studies indicated that glycine conjugates of di- and trihydroxy bile acids were more readily hydrolyzed as compared to the taurine conjugates of bile acids (**Table 3**).

The effect of inhibitors on enzyme activity is shown in **Table 4**. Sulfhydryl group inhibitors (iodoacetate, *p*-chloromercuribenzoate) and phenylmethanesulfonylfluoride were tested for their effects on the enzymatic activity of BSH. Following incubation of the enzyme with inhibitors for 15 min prior to the addition of substrate, enzyme activity was assayed. At a concentration of 2 mM, phenylmethanesulfonylfluoride exhibited 83% inhibition of enzyme activity. Iodoacetate (2 mM) and *p*-chloromercuribenzoate (2 mM) inhibited 85% and 81%, resepectively.

Western blot analysis was used to determine whether specific antibody had been produced against the purified enzyme and to determine whether a single form was detectable in crude cell extracts. Specific antibody detected a single polypeptide with a relative M_r of 56,000 on Western blot (**Fig. 6**, lanes 3 and 4). Nonimmune serum did not cross-react with bile salt hydrolase (Fig. 6, lanes 1 and 2). However, immune blots using nonimmune serum showed the presence of antibodies to other clostridial proteins. The reason for this is not known.

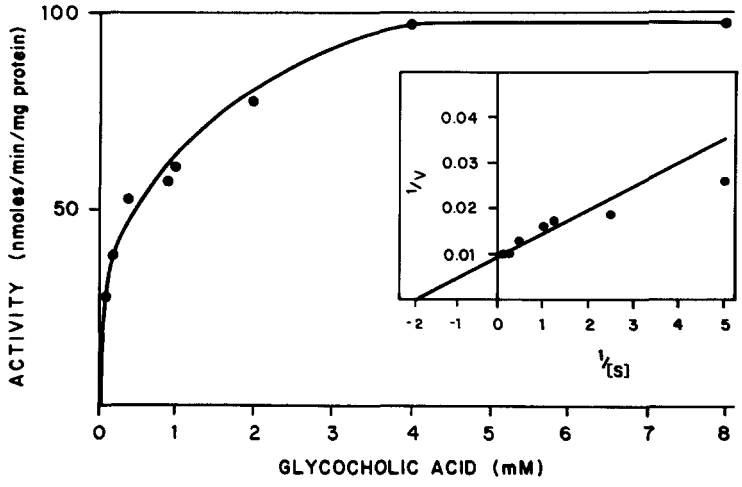

Fig. 5. Effect of glycocholic acid concentration on bile salt hydrolase activity. Substrate (sodium salt of glycocholic acid) was added to initiate the reaction. Reaction mixtures contained 10 μg of enzyme. Initial rates of hydrolysis were measured over a time course of 10 min. Inset shows the double reciprocal plot of the data.

TABLE 3. Substrate specificity of BSH from *Clostridium perfringens*

Substrate	BSH Activity[a]
Glycocholic acid	193
Glycodeoxycholic acid	155
Glycochenodeoxycholic acid	175
Taurocholic acid	80
Taurodeoxycholic acid	60

[a]Each substrate was measured at a concentration of 1.0 mM under standard assay conditions. The reaction was initiated by the addition of 8 μg of pure BSH. Activity is reported as nmol of substrate hydrolyzed/min · mg of protein.

DISCUSSION

Although certain properties of BSH have been described in whole cells of intestinal bacteria, cell extract activity has been studied in detail only from *Clostridium perfringens* (7) and *Bacteroides fragilis* (8). To our knowledge, this is the first report describing the purification of BSH to homogeneity from *Clostridium perfringens*. Crude extracts of *C. perfringens* are normally used to deconjugate bile salts samples prior to quantitation by gas–liquid chromatography. However, it is possible that a fraction of these bile salts might be modified by 3α/3β-hydroxysteroid oxidoreductases reported to be present in this organism (14, 15). A highly purified enzyme preparation of BSH should be more desirable than crude extract for bile salt deconjugation.

The molecular weight (M_r) of the intact enzyme was estimated to be 250,000 which is similar to the value reported for BSH purified from *Bacteroides fragilis* (8). However, the subunit composition of these two enzymes is different. BSH from *Bacteroides fragilis* was reported to be an octamer, whereas the intact enzyme from *Clostridium perfringens* appears to be a tetramer. BSH from *Bacteroides fragilis* is located both intracellularly and in the periplasmic space. In contrast, BSH from *Clostridium perfringens* is primarily an intracellular enzyme.

The K_m of BSH for glycocholic acid determined in this study is several fold lower than those reported for BSH from *Clostridium perfringens* (ATCC-19574, Sinai No. 43-F-4) (K_m 3.6 mM) (7). Although the K_m value appears to be closer to the value reported for BSH from *Bacteroides fragilis* (K_m 0.35 mM) (8), it is noteworthy that the substrate saturation curve of BSH purified from *Bacteroides fragilis* was biphasic for most substrates. The K_m values may vary widely among the various strains of *Clostridium perfringens*. The K_m value is different as compared to earlier studies where the enzyme was only partially purified. The apparent V_{max} value in this study was found to be 107 nmoles/min/mg protein which is much lower than the value reported for BSH from *Bacteroides fragilis* (V_{max} 80 μmol/min · mg protein) (8).

The study of the substrate specificity of BSH from human fecal cultures and *Clostridum perfringens* by Huijghebaert and Hofmann (16) and Batta, Salen, and Shefer (17), respectively, has revealed several interesting results. For instance, the former authors have demonstrated that the position of the amide bond, changes in shape and chiral nature of the amide bond, and introduction of various amino acids at or around the amide bond influence the rate of hydrolysis. Batta et al. (17) have studied the influence of methylation of the amide bond, bile acid chain length, and the introduction of oxo groups in the bile acid nucleus on the hydrolysis of bile acid conjugate by BSH. The present studies indicated that glycine and taurine conjugates of cholic and deoxycholic acids were hydrolyzed; however, taurine conjugates were hydrolyzed less readily as compared to glycine conjugates. These studies are in agreement with previous studies (8). It is noteworthy that unlike previous studies (16, 17) where partially purified enzyme and human fecal cultures were used as the source for BSH activity, our studies used a highly purified enzyme and can be used for more detailed studies on its substrate specificity using various strategies employed by Batta et al. (17) and Huijghebaert and Hofmann (16).

The pure enzyme showed a broad pH optimum over the range of 5.8 to 6.4 in contrast to the pH optimum reported for *Bacteroides fragilis* (pH 4.2–4.5) but close to other *Clostridium perfringens* strains (pH 5.6) (7). The reason for such variances is not known.

Iodoacetate, *p*-chloromercuribenzoate, and phenylmeth-

TABLE 4. Effect of inhibitors on BSH activity

Inhibitor	BSH Activity[a]	
	nmol/min · mg Protein	Relative % Hydrolysis
Control	800	100
Iodoacetate (2 mM)	116	15
p-Hydroxymercuribenzoate (2 mM)	155	19
Phenylmethanesulfonylfluoride (2 mM)	135	17

[a]Following incubation of the enzyme with inhibitors for 15 min at room temperature prior to the addition of the substrate sodium glycocholate, enzyme activity was measured as described in Materials and Methods.

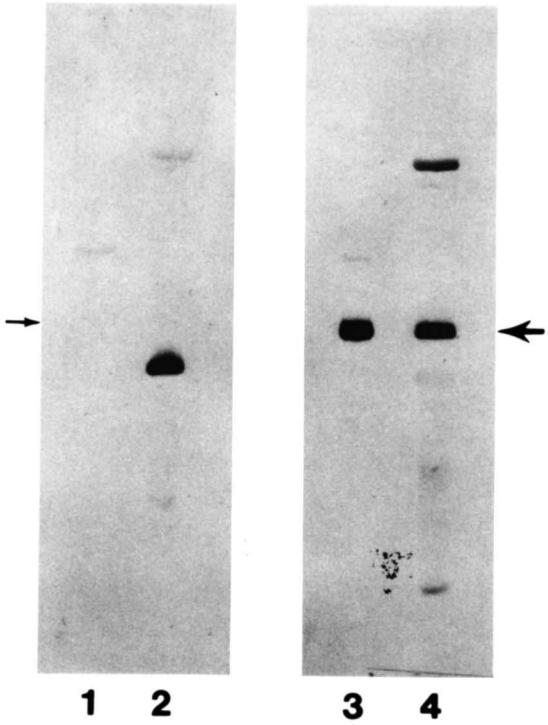

Fig. 6. Western blot analysis of SDS-PAGE separated proteins of *C. perfringens*. Numbers at the bottom of the gel indicate lane numbers: lane 1, pure bile salt hydrolase (0.5 μg); lane 2, *Clostridium perfringens* crude extract (10 μg), using nonimmune serum; lane 3, pure bile salt hydrolase (0.5 μg); lane 4, *Clostridium perfringens* crude extract (10 μg) using anti-BSH polyclonal serum.

anesulfonylfluoride significantly inhibited the BSH activity, suggesting the importance of a sulfhydryl group in the catalytic activity of BSH. Such effects of thiol group modification have been reported in earlier studies (7).

The preparation of specific polyclonal antibody to BSH and determination of the NH_2 terminal amino acid sequence is an important step in allowing the cloning of the gene coding for BSH by screening genomic clone banks of *Clostridium perfringens*.

We thank Mary Cousins for help in the preparation of the manuscript and Amy Krafft for help with column chromatography. This work was supported in part by grant CA 17747 from the National Cancer Institute and by NIH Program Project Grant P01 DK 38030.

Manuscript received 15 December 1987 and in revised form 22 February 1988.

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