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# Characterization and Purification of Bile Salt Hydrolase from *Lactobacillus* sp. Strain 100-100

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**We have characterized and purified the bile salt hydrolase from *Lactobacillus* sp. strain 100-100. Bile salt hydrolase from cells of the strain was purified with column and high-performance liquid chromatography. The activity was assayed in whole cells and cell-free extracts with either a radiochemical assay involving [$^{14}$C]taurocholic acid or a nonradioactive assay involving trinitrobenzene sulfonate. The activity was detectable only in stationary-phase cells. Within 20 min after conjugated bile acids were added to stationary-phase cultures of strain 100-100, the activity in whole cells increased to levels three- to fivefold higher than in cells from cultures grown in medium free of bile salts. In cell-free extracts, however, the activity was about equal, 1.41 and 1.53 μmol/min per mg of protein, respectively, whether or not the cells have been grown with bile salts present. When supernatant solutions from cultures grown in medium containing taurocholic acid were used to suspend cells grown in medium free of the bile salt, the bile salt hydrolase activity detected in whole cells increased two- to threefold. Two forms of the hydrolase were purified from the cells and designated hydrolases A and B. They eluted from anion-exchange high-performance liquid chromatography in two sets of fractions, A at 0.15 M NaCl and B at 0.18 M NaCl. Their apparent molecular weights in nondenaturing polyacrylamide gel electrophoresis were 115,000 and 105,000, respectively. However, discrepancies existed in the apparent molecular weights and number of peptides detected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the two forms. Both had similar substrate specificities, highest on taurodeoxycholic and glycocholic acid, and pH optima between 3.8 and 4.5. The kinetic properties were also similar, with $V_{max}$s of 17 and 53 μmol/min per mg of protein and $K_m$s of 0.76 and 0.95 mM taurocholic acid for A and B, respectively. Therefore, whether the enzyme exists in two forms in the cells remains to be determined.**

Bile acids conjugated via an amide bond to either taurine or glycine in the liver are released into the small intestine in those salt forms (11). The salts are modified by numerous microbial biotransformations in the intestines (11). In the first of such reactions, the amide bond is hydrolyzed to release free amino acid and the primary bile acid. This deconjugation reaction is catalyzed by the enzyme bile salt hydrolase (BSH). BSH activity has been detected in many members of the autochthonous microflora of the gastrointestinal tract, including species of the genera *Lactobacillus* (7), *Bacteroides* (16, 18), *Clostridium* (16, 18), *Streptococcus* (13), *Bifidobacterium* (6, 21), *Fusobacterium* (21), and others (4, 17). The activity has been examined in cells and crude cell extracts from strains of several of these genera (1, 13, 16). The enzyme has been purified and characterized, however, only from *Bacteroides fragilis* (12, 22) and *Clostridium perfringens* (8). These purified enzymes differ with respect to physical, kinetic, and genetic properties, including subunit molecular weight, pH optimum, $V_{max}$, and genetic regulation.

*Lactobacillus* species colonize the gastrointestinal tract of mammals and birds to high population levels (20). In rodents, lactobacilli colonize the stomach, forming a thick layer of bacterial cells on the keratinized, nonsecreting, squamous epithelium (20). Many strains of intestinal *Lactobacillus* species deconjugate bile salts (7). Gastric lactobacilli contribute approximately 86% of the total BSH activity in the ileum and 74% in the cecum in mice (23). However, the enzymes catalyzing the reaction in these bacteria have not been extensively studied.

To initiate such a study, we have characterized the BSH activity of *Lactobacillus* sp. strain 100-100, a strain able to colonize the gastric epithelium in mice, and purified two forms of the enzyme by using column chromatography and high-performance liquid chromatography (HPLC). We report here on the findings from that effort and compare the lactobacillus enzymes with the hydrolases in *B. fragilis* (12, 22) and *C. perfringens* (8).

## MATERIALS AND METHODS

**Strains and growth conditions.** *Lactobacillus* sp. strain 100-100 was isolated from the keratinized, nonsecreting, squamous epithelium of a rat stomach (G. W. Tannock and D. C. Savage, unpublished data), identified as a *Lactobacillus* isolate, and given the strain designation 100-100 (J. Hansley and D. C. Savage, unpublished data). This strain has the capacity, as was found for other lactobacilli isolated from the stomach epithelium of rodents (15), to colonize the nonsecreting epithelium of the mouse stomach (Hansley and Savage, unpublished data). *Lactobacillus* sp. strain 100-100 and other *Lactobacillus* strains used were maintained at −80°C in 15% glycerol. They were cultured for use in MRS broth (Difco Laboratories, Detroit, Mich.) under the experimental conditions outlined in the Results: in either screw-cap tubes in an atmosphere of 92% $N_2$–5% $CO_2$–3% $H_2$ or bottles with an environment of 92% $N_2$–8% $CO_2$. In both cases the cultures were grown without shaking and mixed prior to use. The BSH was purified from strain 100-100 grown to stationary phase with constant agitation (100 rpm) in MRS broth containing taurocholic acid (TCA) in a Braun UE20G fermentor (B. Braun Biotech Inc., Allentown, Pa.). The culture was maintained under anaerobic conditions by bubbling 90% $N_2$ and 10% $CO_2$ through the culture. The cells were concentrated with a tangential-flow filtration system

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(Millipore Corp., Bedford, Mass.), after which they were centrifuged (10,000 × g for 10 min) and the cell pellet was washed twice with buffer A (10 mM $NaPO_4$, 12% glycerol, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 6.8). The cells were suspended in buffer A and frozen at −80°C. The cell density and enzymatic activity were similar in cultures grown in all the conditions used in this study.

**Radiochemical enzyme assay.** A radiochemical assay was used to assay BSH activity in whole cells and cell-free extracts (5). TCA (Sigma Chemical Co., St. Louis, Mo.) and [24-$^{14}$C]TCA (NEN Du Pont, Boston, Mass.; or Amersham Corp., Arlington Heights, Ill.) was used in the assays at a concentration of 2 mM and a specific radioactivity of 0.025 μCi/μmol (except for kinetic assays in which the concentration and specific activity varied). BSH activity was assayed in whole cells in an anaerobic atmosphere of 92% $N_2$–5% $CO_2$–3% $H_2$. Cell-free extracts were assayed aerobically. Cells to be assayed were diluted in sodium acetate buffer (5 mM; pH 5.0). Cell-free extracts were diluted in 0.2 M sodium acetate (pH 4.2). All reactions were carried out at 37°C for 10 min. pH optima were determined with purified enzymes in buffers with pHs ranging from 3.8 to 7.0. Sodium acetate (0.5 M final concentration) was used for pHs of 3.8 to 5.5. Sodium phosphate (0.5 M final concentration) was used for pHs of 6.0 to 7.0.

**Substrate specificity.** Substrate specificities were determined with an assay developed by Dashkevicz and Feighner and outlined by Gopal-Srivastava and Hylemon (8). In this assay, the amount of amino acid released from conjugated bile acids is measured in a reaction involving trinitrobenzene sulfonate (Sigma). The reactions were run for 10 min at 37°C with 5 mM conjugated bile acid, 0.2 M sodium acetate (pH 4.2), and the lactobacillus enzymes in a total volume of 1 ml. Substrates used were TCA, taurodeoxycholic acid, taurochenodeoxycholic acid, glycocholic acid, and glycodeoxycholic acid (Sigma). $A_{416}$ was measured with a Shimadzu UV160U spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.).

**Purification of BSH.** Cell-free extracts were prepared from cells passed through a French pressure cell three times at 18,000 lb/in$^2$ (SLM Aminco, Urbana, Ill.) or exposed to four 1-min pulses in a Bead Beater (Biospec Products Inc., Bartlesville, Okla.) with 0.1-mm glass beads. The suspension was centrifuged to pellet unbroken cells and debris (31,000 × g for 15 min and 105,000 × g for 2 h). BSH was purified from the soluble portion of the cell extract. The extract was fractionated by ammonium sulfate precipitation (45 to 70% saturation). This fraction was passed through a Sephadex G-200 (Sigma) gel filtration column (325-ml bed volume) equilibrated and run with buffer A (pH 6.8). Hydrophobic interaction chromatography with phenyl-Sepharose CL-4B (Sigma) followed the gel filtration. The column was developed with a 0.7 to 0.4 M ammonium sulfate gradient in buffer A (pH 6.8) with a total volume of 200 ml. The final step was anion-exchange HPLC through Spherogel TSK DEAE-3SW (Beckman Instruments, Inc., Fullerton, Calif.). The column was developed with a 0.1 to 0.2 M NaCl gradient in buffer A (pH 6.8) with a flow rate of 0.85 ml/min for 75 min. Protein concentration was determined by the Bradford assay (3). Peptide separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% slab gels (14) and in nondenaturing gels of 10% acrylamide with buffers free of SDS and 2-mercaptoethanol. Both denaturing and nondenaturing gels were stained with silver in the procedure outlined by Oakley et al. (19).

TABLE 1. BSH activity in *Lactobacillus* species isolated from various animal sources

| Strain | Origin | Form layer on epithelium[a] | BSH activity[b] | Source |
|---|---|---|---|---|
| 100-12 | Human feces | − | 24.2 | ATCC 4963 |
| 100-16 | Mouse stomach | + | 0.9 | This lab |
| 100-33 | Pig feces | − | 12.9 | M. L. Speck |
| 100-100 | Rat stomach | + | 38.6 | This lab |
| RI | Mouse stomach | + | 4.6 | This lab |

[a] Lin and Savage (15) and Hansley and Savage (unpublished data).
[b] BSH activity expressed as nanomoles of cholic acid formed per minute per milliliter of culture.

## RESULTS

**Testing strains for BSH activity.** Five strains of lactobacilli isolated from various animal sources (Table 1) were tested for BSH activity. In addition to strain 100-100, two of these strains, 100-16 and RI, were able to colonize the mouse stomach (15). The strains were grown overnight to stationary phase; the cultures were diluted 1/10 in 5 mM acetate buffer (pH 5.0); 1 ml of each of the diluted cultures was then assayed for BSH activity. Strain 100-100 expressed the activity at levels higher than the other strains, including the two that colonized the mouse stomach (Table 1).

**Enhancement of BSH activity in cells of strain 100-100 by conjugated bile acid.** When [24-$^{14}$C]TCA was added to cells of strain 100-100 and samples were removed at intervals, the product of deconjugation, [24-$^{14}$C]cholic acid, was not detected for about 30 min. Thereafter, the free bile acid was released linearly with respect to time. This result suggested that the TCA induced or activated the hydrolase. This hypothesis was tested with cultures grown anaerobically at 37°C to stationary phase and divided into two aliquots. To one, TCA in 5 mM sodium acetate buffer (pH 5.0) was added to 0.4 mM; to the other, an equal volume of acetate buffer was added. Cells removed from both samples at 0, 10, 20, 30, and 60 min thereafter were assayed for BSH activity. Cells incubated with TCA present in the medium had reached within 20 min a three- to fivefold-higher BSH activity than those without the bile salt in the medium (Fig. 1). In addition to TCA, several other conjugated and some free bile acids were tested for the capacity to stimulate BSH activity in whole cells. Bile acids conjugated with either taurine or glycine, including taurodeoxycholic, taurochenodeoxycholic, and glycocholic acids, stimulated the activity at least threefold. However, neither the unconjugated bile acids cholic and deoxycholic acid nor the amino acid taurine increased the activity.

**BSH activity in cell-free extracts.** BSH activity was also examined in cell-free extracts prepared from cells of strain 100-100 incubated with or without TCA prior to cell lysis. Cultures (2 liters) grown to stationary phase in an atmosphere of 92% $N_2$–8% $CO_2$ were divided into two aliquots; TCA to 0.4 mM was added to one of the aliquots and no bile acid was added to the other. Both cultures were then incubated for 30 min at 37°C in the same atmosphere as above. Samples of cells removed from each were assayed for BSH activity. Once those assays had indicated that the TCA had stimulated the hydrolase activity, the cultures were centrifuged to harvest the cells. They were suspended in buffer A (pH 6.8), and extracts were prepared from the cells with a French press. The extracts were assayed for BSH activity. While the activity detected in whole cells grown in media containing TCA was threefold higher than those

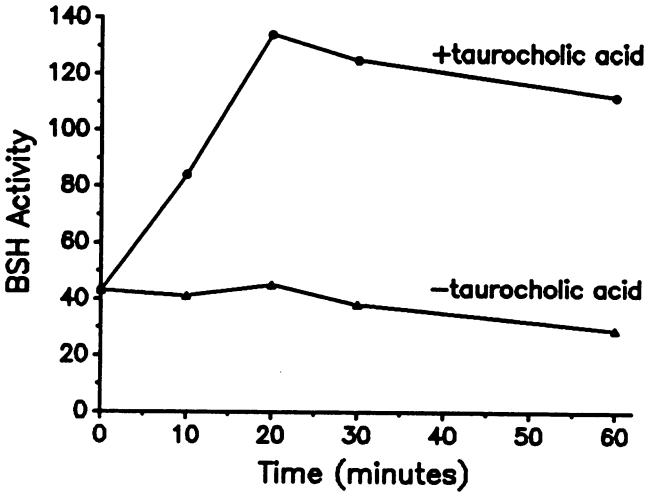


FIG. 1. Effect of TCA on BSH activity in *Lactobacillus* sp. strain 100-100. An overnight culture of strain 100-100 was divided into two aliquots; 0.4 mM TCA was added to one, but not to the other. Thereafter, cells were removed at 0, 10, 20, 30, and 60 min and assayed for BSH activity with [$^{14}$C]TCA anaerobically at 37°C for 10 min. BSH activity is expressed as nanomoles of cholic acid formed per minute per milliliter of culture.

incubated in media without TCA, 25 and 8 nmol/min per ml of culture, respectively, the activities in their cell-free extracts were about equal, 1.41 and 1.53 μmol/min per mg of protein, respectively. In addition, membrane fractions from pellets from the ultracentrifugation of crude extracts were suspended in 1% Triton X-100. No detectable levels of BSH activity were found in this fraction.

**Effects of culture supernatant solutions on BSH activity in cells of strain 100-100.** When cells of strain 100-100 grown in medium containing TCA were centrifuged, suspended in an equal volume of buffer free of the bile salt, and assayed as early as 2 min thereafter, their BSH activity as detected when they were suspended in the culture fluid dropped from 181 to 31 nmol of cholic acid formed/min per ml of culture. This finding suggested that an extracellular factor was essential for the high BSH activity detected in cells assayed in cultures containing TCA (Fig. 1). This hypothesis was tested with supernatant solutions from cultures incubated with and without TCA present in the medium.

Strain 100-100 was grown anaerobically at 37°C overnight until the cells reached stationary phase. The culture was split into two aliquots. TCA to 0.4 mM was added to one; no bile salt was added to the other. Both cultures were then incubated anaerobically for 30 min at 37°C. Thereafter, they were centrifuged (3,500 × *g* for 5 min) to pellet the cells. The supernatant solutions from cultures containing TCA were designated *SUP; those from cultures free of bile salts were designated SUP. The cell pellet from the culture incubated in medium free of TCA was then suspended with *SUP and vice versa. Samples from these cell suspensions, the cultures incubated with or without TCA, and *SUP and SUP were diluted 1/5 (vol/vol) in 0.2 M sodium acetate (pH 4.2) and assayed for BSH activity. The assays were started from 2 to no more than 5 min after the supernatant solutions were exchanged.

BSH activity increased as much as threefold when cells grown in media free of conjugated bile acids were suspended in *SUP (Table 2). The hydrolase activity was less than that of the cells grown in medium containing TCA and suspended

TABLE 2. Effect on BSH activity of supernatant solution of cultures of strain 100-100 grown with and without TCA[a]

| Cells incubated | Supernatant sol[b] | BSH activity (nmol/min per ml of culture) |
|---|---|---|
| With TCA | *SUP | 112.8 |
| With TCA | SUP | 31.6 |
| Without TCA | *SUP | 59.2 |
| Without TCA | SUP | 19.6 |
| None[c] | *SUP | 7.6 |
| None[c] | SUP | 4.5 |

[a] Experiments were repeated five times. The activity varied from one experiment to another, but the proportions remained constant.
[b] *SUP, Supernatant solution from cultures incubated with TCA; SUP, supernatant solution from cultures incubated without TCA.
[c] No cells present.

in *SUP, but still two to three times higher than cells grown in medium free of the bile salt and suspended in SUP. Moreover, when the cells grown with the bile salt in the medium were suspended with SUP, the activity dropped three to fourfold (Table 2). The supernatant solutions from both cultures, *SUP and SUP, contained low BSH activities (Table 2). These activities did not significantly differ, however, and could not account for the activities detected in the cells incubated with SUP or *SUP.

**Growth phase regulation of BSH activity.** BSH activity was examined as a function of the growth of a culture. Cells from an overnight culture of strain 100-100 were inoculated into fresh MRS broth, incubated anaerobically at 37°C, and sampled every hour thereafter. After the cell density was measured in the samples, the cells were assayed for BSH activity. The reactions were run for 30 min. The activity was detectable only in stationary-phase cells at a level increased 60-fold over that detected in exponential-phase cells (Fig. 2). Data from several experiments involving different batches of MRS broth indicate that the hydrolase activity rises at different times relative to the second phase of the biphasic growth. This suggests that the increase in BSH activity is not a result of the biphasic growth.

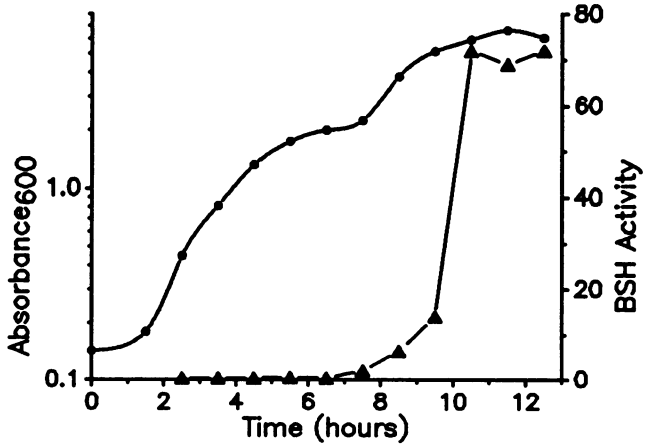


FIG. 2. BSH activity during growth of a *Lactobacillus* sp. strain 100-100 culture. Cells from an overnight culture were used to inoculate fresh MRS broth (0.5% inoculum). Cells were removed every hour thereafter. The cell density of the sample was determined by measuring $A_{600}$ (●), and BSH activity was determined with [$^{14}$C]TCA (▲). BSH activity is expressed as nanomoles of cholic acid formed per minute per milliliter of culture.

TABLE 3. Purification table for BSH from *Lactobacillus* sp. strain 100-100

| Step | Total protein (mg) | μmol of cholic acid formed/min | | Recovery (%) | Purification factor |
|---|---|---|---|---|---|
| | | Sp act (per mg of protein) | Total activity | | |
| Crude extract | 349 | 0.69[a] | 241 | 100 | 1 |
| Ammonium sulfate | 240 | 0.55[a] | 132 | 55 | 0.79 |
| G-200 gel filtration | 96 | 0.47[a] | 45 | 19 | 0.68 |
| Phenyl-Sepharose | 2.5 | 9.3[a] | 23 | 9.5 | 13.5 |
| DEAE-HPLC | | | | | |
| Hydrolase A | 0.38 | 18.5 | 7 | 3 | 27 |
| Hydrolase B | 0.065 | 30.3 | 2 | 0.8 | 44 |

[a] The specific activities in the crude extract, ammonium sulfate, and G-200 gel filtration fractions are lower than each other probably because proteases degrade the enzyme. The proteases are apparently separated from the BSH during the gel filtration chromatography.

**Purification of BSH from strain 100-100.** Proteins with BSH activity were purified from cell-free extracts of strain 100-100 as outlined in Materials and Methods (Table 3). The crude extracts were prepared from cells incubated in medium containing TCA or free of the bile salt. When the extracts were fractionated with ammonium sulfate, the activity was found in the 45 to 70% fraction. This fraction was passed over a Sephadex G-200 gel filtration column calibrated with four protein standards: ferritin (440,000), catalase (232,000), bovine serum albumin (68,000), and RNase A (13,000). Most of the activity was found in fractions eluting between those in which catalase and bovine serum albumin eluted, yielding an estimate of the apparent molecular weight of proteins with BSH activity of 140,000. At this point, the specific activity of the enzyme had declined rather than increased from the values in the crude extracts (Table 3). This result may have been due to the enzyme being degraded in the fractions. Proteins with hydrolase activity were found in fractions eluted from a phenyl-Sepharose column at about 0.55 M ammonium sulfate. However, they appeared in two sets of widely separated fractions eluted from an anion-exchange HPLC column (Fig. 3). The activity in the first set eluted at about 0.15 M NaCl, and that in the second eluted at 0.18 M NaCl. The enzymes in both sets of fractions could be isolated from cells whether or not they were grown in media containing TCA. When the proteins in the two sets of fractions were separately passed through the DEAE-HPLC, each eluted in a set of fractions at the same salt concentrations as during their initial passage through the column. Each of the two sets of fractions contained a protein migrating as a single band in nondenaturing PAGE (Fig. 4). The protein in the first set had an apparent molecular weight of approximately 115,000, while that in the second set migrated to a location indicating a molecular weight of about 105,000. The larger protein was designated hydrolase A; the smaller was designated hydrolase B. Hydrolase A separated into two

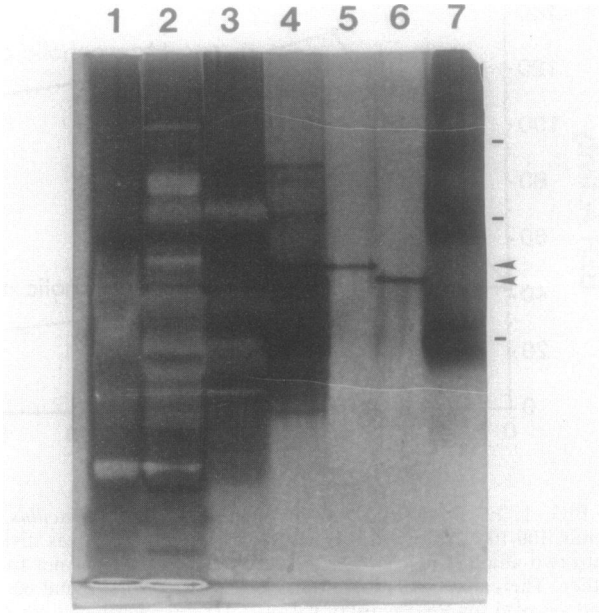


FIG. 4. Electrophoresis of cell extracts, at each step of the purification, through 10% nondenaturing polyacrylamide gel. Lanes: 1, crude cell extract; 2, 45 to 70% ammonium sulfate fraction; 3, pooled fractions from G-200 column; 4, pooled fractions from phenyl-Sepharose column; 5, pooled fractions with hydrolase A from DEAE-HPLC column (5 μg); 6, pooled fractions with hydrolase B from DEAE-HPLC column (5 μg); 7, molecular weight standards (—). Molecular weights, top to bottom: 232,000, 158,000 and 68,000. Gel is stained with silver (19). Arrows indicate both hydrolases A and B.

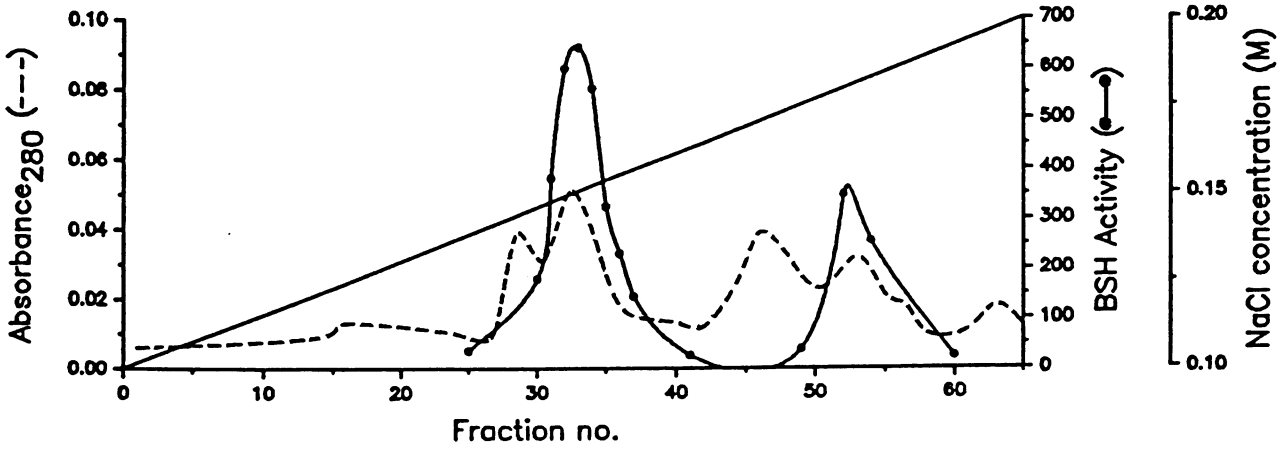


FIG. 3. Elution profile of BSH activity of *Lactobacillus* sp. strain 100-100 from a Spherogel TSK DEAE-3SW HPLC column. The column was developed with a 0.1 to 0.2 M sodium chloride gradient in buffer A. BSH activity is expressed as micromoles of cholic acid formed per minute per fraction.

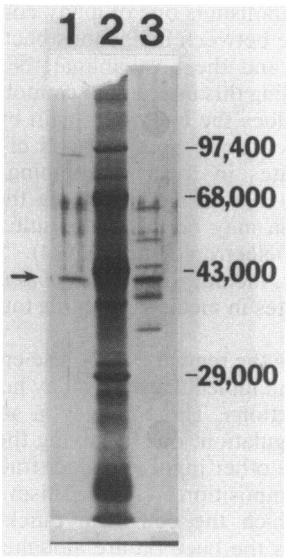


FIG. 5. Electrophoretic analysis of hydrolases A and B in SDS-PAGE (12.5%) stained with silver (19). Lanes: 1, hydrolase A (5 μg); 2, molecular weight standards; 3, hydrolase B (7 μg). (→) indicates the peptide with an apparent molecular weight of 42,000 common to both hydrolases A and B.

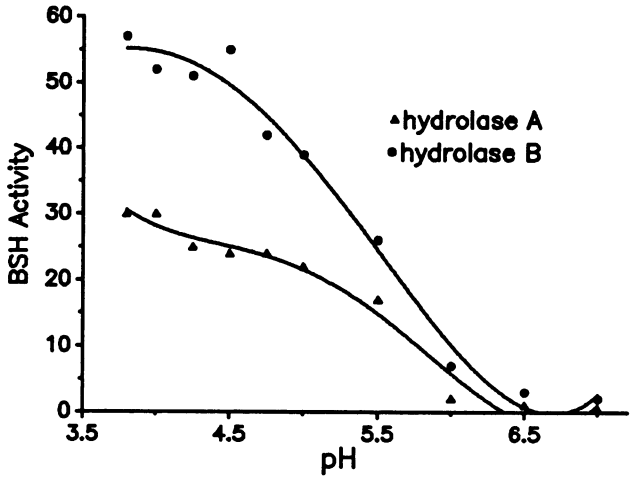


FIG. 6. pH optima of hydrolases A and B for TCA. Hydrolases A and B were added to buffers of pHs ranging from 3.8 to 7.0 and assayed with the radiochemical assay. BSH activity is expressed as micromoles of cholic acid formed per minute per milligram of protein. Sodium acetate (final concentration, 0.5 M) was used for buffers with pHs of 3.8 to 5.5, and sodium phosphate (final concentration, 0.5 M) was for buffers with pHs of 6.0 to 7.0.

peptides with apparent molecular weights of 42,000 and 90,000, while hydrolase B separated into at least six peptides with molecular weights of 33,000, 38,000, 42,000, 46,000, 55,000, and 63,000 when the same fraction used for the nondenaturing gels were analyzed in SDS-PAGE (Fig. 5).

**Substrate specificities and kinetic analysis of hydrolases A and B.** Both enzymes were active with bile acids conjugated to taurine or glycine and did not significantly differ in their substrate specificities (Table 4). Kinetic analysis of the two enzymes were carried out as follows. Hydrolases A (2.7 μg) and B (1.55 μg) were added to buffers containing TCA in increasing concentrations. The [$^{14}$C]TCA radioactivity was kept constant at 0.05 μCi for each reaction. This amount of labeled compound did not significantly increase the total concentration of TCA in the reactions. The $V_{max}$ for hydrolase A was 17, and that of hydrolase B was 53, μmol of TCA hydrolyzed/min per mg of protein. The $K_m$s were 0.76 and 0.95 mM TCA, respectively.

**pH optima of hydrolases A and B.** Buffers with pHs of 3.8 to 7.0 were used. Reactions were run with the purified enzymes at 37°C for 10 min. The pH optima for A and B were similar; both had a broad optimum from pH 3.8 to 4.5 (Fig. 6).

TABLE 4. Substrate specificities of hydrolases A and B[a]

| Substrate | Enzyme velocity (μmol/min per mg of protein) | |
|---|---|---|
| | Hydrolase A | Hydrolase B |
| TCA | 32.8 | 41.3 |
| Taurodeoxycholic acid | 49.4 | 72.7 |
| Taurochenodeoxycholic acid | 29.0 | 44.8 |
| Glycocholic acid | 46.6 | 70.3 |
| Glycodeoxycholic acid | 8.7 | 19.3 |

[a] Activity was determined with the nonisotopic assay (8).

## DISCUSSION

We have characterized the BSH activity and purified the enzymes with this activity from *Lactobacillus* sp. strain 100-100. The activity increased only when a culture of the organism entered stationary phase (Fig. 2). The BSH activity of *B. fragilis* also increases during that phase of the growth curve (12). As noted earlier, lactobacilli from the stomach produce up to 86% of the BSH activity in the small intestines of mice (23). Therefore, the finding that the activity is regulated by growth phase suggests that the lactobacillus cells passing through the small intestine may be in a physiological state similar to that of cells in stationary phase in laboratory culture media. The mechanism of this regulation remains unclear.

BSH activity is higher in cells grown in medium containing TCA than in cells grown in medium free of bile salts. However, the activity in cell-free extracts prepared from cells cultured in medium containing TCA is the same as that in extracts of cells grown in medium free of the bile salt. No activity of the enzyme can be detected in membrane fractions from these cells. Therefore, the bile salts apparently do not regulate the hydrolase, an intracellular enzyme, by conventional induction or derepression (9). Little enzymatic activity can be found in spent culture media free of lactobacilli, whether or not TCA is present. However, the BSH activity is enhanced when assayed in cells that are suspended in the supernatant solutions from cultures incubated with TCA. Therefore, an extracellular factor present in the spent medium must in some way enhance the enzymatic activity. To our knowledge, this is the first report of bile salts regulating BSH activity. That regulation appears to us to be mediated, however, by an extracellular factor functioning by other than recognized mechanisms.

This factor remains to be identified. Neither cholic acid nor taurine stimulate BSH activity when added to cultures of strain 100-100 grown in medium free of TCA. Therefore, the factor is probably not one of the products of the deconjugation reaction. Likewise, the substance is probably not TCA. TCA is deconjugated by the lactobacillus BSHs at a rate of

42 nmol of TCA hydrolyzed/min per ml of culture. Consequently, as assessed by calculation, little or no residual conjugated bile salt would be present in the medium at 60 min to stimulate the BSH activity (Fig. 1). We are now developing an assay of sufficient sensitivity directly to test for TCA in the spent medium. Finally, the activity in the cells increases to a peak level within 2 min, after they are suspended in spent medium from cultures grown with TCA present. Therefore, the substance probably does not induce a membrane transport system. Its mechanism also remains to be identified.

We have purified the BSH from strain 100-100 in two forms. Both forms, hydrolases A and B, are found in cells of the strain whether or not they have been incubated with TCA. Therefore, they are constitutively synthesized in the stationary phase of the cell growth cycle. They both appear to be homogeneous in nondenaturing PAGE, with apparent molecular weights of 115,000 and 105,000 for A and B, respectively, suggesting that they are either two separate enzymes or conformational isomers.

The proteins separate into several peptides when analyzed by SDS-PAGE. One peptide with an apparent molecular weight of 42,000 is common to and most abundant in both forms. It may represent the catalytic subunit of both. Hydrolase A appears to be a dimeric protein of heterologous subunits. By contrast, hydrolase B, which appears to be homogeneous in nondenaturing gels, separated in SDS-PAGE into six peptides, the molecular weights of which add up to 277,000, a value well over the 105,000 detected in nondenaturing gels. This discrepancy between the protein separations in nondenaturing and denaturing gels remains to be resolved. Peptides in SDS-PAGE of both enzymes may represent processed forms of the hydrolase, artifacts of the electrophoretic analysis, or peptides which copurify with the catalytic subunit and cannot be separated with the methods we have used. We are investigating this phenomenon and the relationship between the two hydrolases with genetic and antigenic techniques.

The two forms of the enzymes differ only in their behavior in anion-exchange HPLC, native molecular weight, and subunit composition. Their pH optima, kinetics, and substrate specificities are similar. They have a pH optimum of 3.8 to 4.5, but may be active at lower pHs. Because the substrate decreases in solubility, however, problems arise with the assay at pHs below 3.8. As tested, the substrate specificities of the two hydrolases were identical. Moreover, both have their highest activity on taurodeoxycholic and glycocholic acids. Therefore, since their catalytic functions are similar, we cannot rule out the possibility that the two forms are an artifact of the purification.

The BSHs from strain 100-100 have similarities to the BSH from *B. fragilis*, including pH optimum, growth phase regulation, and $V_{max}$ (12, 22). They differ from the *B. fragilis* enzyme, however, with respect to native and subunit molecular weights and $K_m$ (12, 22) and from the hydrolase from *C. perfringens* with respect to all of these properties (8). Moreover, they differ antigenically from the *C. perfringens* hydrolase (8). Polyclonal antibodies raised with the clostridial protein were used to probe Western blots (immunoblots) of crude cell extracts of strain 100-100 and *C. perfringens*. Proteins in the blot containing the clostridial extract reacted strongly with the antibodies. However, no reactions were detected with the extracts prepared from the lactobacillus cells (data not shown). Thus, the BSHs from lactobacilli represent a third type of hydrolase to be identified and characterized.

Bile acid metabolism is one of many complex interactions which take place between indigenous bacteria in the gastrointestinal canal and the host animal. Nevertheless, many questions regarding this interaction cannot be answered. For example, what does the bacterium gain by carrying out the deconjugation reaction? Some strains of *Clostridium* spp. have growth rates in media containing taurine that are increased over those in media lacking the amino acid (10, 24). The bacteria may be using the sulfate portion of this molecule as an electron acceptor (24). Not all organisms tested, however, including one *Lactobacillus* species, grew at more rapid rates in media containing taurine than in those lacking it (24).

Understanding the regulation of these enzymatic activities at the cellular and molecular levels may help to resolve their physiologic functions. The enzymes in strain 100-100 have two levels of regulation, one involving the growth phase of the cells and the other involving an extracellular effector of yet unknown composition and mechanism. The latter may be a means by which the cells can quickly adapt to their environment. As the bacteria are sloughed off the stomach epithelium, they pass through the small bowel, where they are bathed in conjugated and free bile acids. Their ability quickly to adapt to such conditions by increasing BSH activity may provide them with a nutrient source or the capacity to inhibit the growth of other bacteria by forming the free bile acid (2). Therefore, they may have a competitive advantage over bacteria of some other species for surviving in the small bowel.

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