Purification and Characterization of Three Different Types of Bile Salt Hydrolases from *Bifidobacterium* Strains

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ABSTRACT

Bile salt hydrolases were purified to electrophoretic homogeneity from *Bifidobacterium bifidum* ATCC 11863, *Bifidobacterium infantis* KL412, *Bifidobacterium longum* ATCC 15708, *Bifidobacterium longum* KL507, and *Bifidobacterium longum* KL515. Three different types (A, B, and C) of bile salt hydrolase (BSH) were revealed during the purification study, exhibiting the type-specific characteristics in their electrophoretic migration and elution profiles from anion exchange and hydrophobic interaction chromatographic columns. The subunit molecular mass estimated by sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was around 35 kDa, and the native molecular mass in all five *Bifidobacterium* strains was estimated to be between 130 and 150 kDa by gel filtration chromatography, indicating that all BSH enzymes have tetrameric structure. From the isoelectric focusing, an isoelectric point value of 4.45 was obtained with BSH (type B) from *B. bifidum* ATCC 11863 and the other BSH (types A and C) showed the similar pI values around 4.65. N-Terminal amino acid sequencing for the proteins of types A and C revealed that 6 out of 20 amino acid residues were different, and highly conserved residues were identified in both N-terminal sequences of types A and C. All BSH enzymes from five strains hydrolyzed six major human bile salts, and they showed a better deconjugation rate on glycine-conjugated bile salts than on taurine-conjugated forms.

(Key words: bile salt hydrolase, purification, *Bifidobacterium*)

Abbreviation key: BSH = bile salt hydrolase, FPLC = fast protein liquid chromatography, HIC = hydrophobic interaction chromatography, IEC = ion-exchange chro-

matography, IEF = isoelectric focusing, LAB = lactic acid bacteria, pI = isoelectric point, SEC = size exclusion chromatography.

INTRODUCTION

Bifidobacteria are one of the most predominant microflora in the gastrointestinal tracts of humans, and many bifidobacteria-containing dairy and pharmaceutical products have been developed and consumed for several decades due to their reported health-promoting effects. In the gastrointestinal tract, many endogenous microflora and exogenous bacteria continuously encounter a significant amount of bile salts that possess some detergent-like antimicrobial properties (Gunn, 2000). As a consequence, many enteric bacteria have developed mechanisms to resist the detergent action of bile salts and evolved the ability to transform bile salts biochemically. One important enzymatic activity of intestinal bacteria, including bifidobacteria, is the deconjugation of bile salts, and this occurs naturally in the intestinal tract of human and animal. Bile salt hydrolase (BSH, EC 3.5.1.24), which is also called cholyglycine hydrolase, is an enzyme that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and free bile acids. Even though it is well known that this enzymatic activity is widely spread among many enteric bacteria, the functions of this enzyme in producing bacterium (as well as) in the mammal host and the precise physiological impact on which the enzymatic products exert their effect are not clearly understood. From a positive viewpoint, it was proposed that high BSH-activity in the probiotic cultures could be beneficial because they have the potential to reduce serum cholesterol (Anderson and Gilliland, 1999; Pereira and Gibson, 2002). Removal of the amino acid moiety from bile salts by deconjugation generates less water-soluble compounds and is easily excreted via feces (De Smet et al., 1998). This drain on the bile salt pool could result in a loss of feedback inhibition on the de novo synthesis of bile salts from

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cholesterol. It has been observed that oral administration of a BSH-active *Lactobacillus* strain lowered the serum cholesterol levels in animal trials (De Rodas et al., 1996; De Smet et al., 1998; du Toit et al., 1998). Although the potentially positive aspects of probiotic BSH activity have been discussed, other possible negative concerns on BSH activity have become evident recently. Deconjugated bile salts are thought to be the cause of gallstones (Thomas et al., 2000). They can be responsible for depression of growth in chickens due to the poor lipid uptake in the small intestine (Knarreborg et al., 2002) and may cause colorectal cancer because some of the bile salts generated by microbial transformation have been incriminated in colonic carcinogenesis (Singh et al., 1997). Furthermore, it has been proposed that the BSH activity from the virulent strains of *Listeria monocytogenes* contribute to the virulence factor (Dussurget et al., 2002).

Recent screening analyses with many lactic acid bacteria (**LAB**) strains, conducted in our laboratory as well as others, revealed that BSH activity is widely distributed in many species of lactobacilli including *Lactobacillus acidophilus* (Corzo and Gilliland, 1999; Kim et al., 1999; Ahn et al., 2003), *Lactobacillus johnsonii* (Elkins and Savage, 1998), *Lactobacillus gasseri* (Usman and Hosono, 1999), and *Lactobacillus plantarum* (De Smet et al., 1994) and almost every species of *Bifidobacterium* (Tanaka et al., 1999; Grill et al., 2000b). Interestingly, many strains of bifidobacteria possess higher BSH activity than other probiotics. In spite of this wide distribution and high activity of BSH enzyme in bifidobacteria, only 2 BSH have been purified so far from *B. longum* BB536 (Grill et al., 1995b) and *B. longum* SBT 2928 (Tanaka et al., 2000).

To investigate some biochemical characteristics of BSH in *Bifidobacterium* spp., BSH were purified from *B. bifidum* ATCC 11863, *B. infantis* KL412, *B. longum* ATCC 15708, *B. longum* KL507, and *B. longum* KL515. From the profiles of native PAGE followed by BSH activity staining and the elution profiles from an anion-exchange column, three types (A, B, and C) of BSH enzyme were identified. Some properties and purification methods of 3 different types of BSH from bifidobacterial strains are described in this study.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Among 40 strains of bifidobacteria tested for their BSH activity, 5 strains were selected for this study based on their high BSH enzyme activity and some characteristics from previous screening experiments. Authentic strains, *B. bifidum* ATCC 11863 and *B. longum* ATCC 15708, were obtained from the American

Type Culture Collection (Rockville, MD). *Bifidobacterium infantis* KL412, *B. longum* KL507, and *B. longum* KL515 were obtained from the stock culture collection of the Food R & D Centre, Agriculture and Agri-Food Canada (Ste-Hyacinthe, QC, Canada). All strains were cultured anaerobically for 24 to 36 h at 37°C in MRS broth (Difco Laboratories, Detroit, MI).

Enzyme and Protein Assays

The cell-free extracts were prepared by sonicator (Sonic Dismembrator 550, Fisher Scientific, Mississauga, ON, Canada) for 5 min at level 4 under constant cooling. The supernatant was collected by centrifugation (15,000 × g, 30 min) and stored at -20°C. The BSH activity was determined by measuring the amount of amino acids resulting from hydrolysis of the amide bond of bile salts using a ninhydrin assay (Tanaka et al., 2000). The hydrolysis rate of conjugated bile salts at 37°C was measured in a sodium phosphate buffer (0.1 M, pH 6.5). One unit of BSH activity was defined as the amount of enzyme that liberated 1 μmol of amino acid from the substrate per minute. Specific activity was defined as units/mg of protein. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Mississauga, ON, Canada) using BSA (Sigma Chemical Co., St. Louis, MO) as a standard.

Activity Staining on Acrylamide Gel

The crude enzymes were loaded and electrophoresed in a nondenaturing 10% (wt/vol) acrylamide gel with Laemmli buffer (Laemmli, 1970) omitting the SDS (Grill et al., 2000a). Partially purified CBAH (cholyglycine hydrolase) originating from *Clostridium perfringens* was purchased from Sigma Chemical Co. For comparison purposes, some BSH-positive strains of *Lactobacillus* species were selected, and their cell-free extracts were prepared. Bile salt hydrolase activity on the gel was detected by washing in a 0.4 M sodium acetate buffer (pH 4.5) and then incubating the gel at 37°C in a reaction mixture (10 mM sodium taurodeoxycholate in 50 mM sodium phosphate buffer, pH 5.5). The BSH activity band in the gel was identified by the formation of a white precipitate of deoxycholic acid at the position of the enzyme.

Enzyme Purification

Hydrophobic interaction chromatography (HIC).

The cell-free extracts were precipitated at 4°C with ammonium sulfate (40 to 80% saturation); the pellet was resuspended in a 50 mM sodium phosphate buffer (pH 7.0). Portions (1 mL) of concentrated sample were ap-

plied to HIC columns (HiTrap Phenyl FF or HiTrap Octyl FF, Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada) and eluted at a flow rate of 1 mL/min by a linear ammonium sulfate gradient (0.8 to 0 M) in 50 mM sodium phosphate buffer (pH 7.0). Fractions (2 mL) were collected and assayed for BSH activity.

Anion-exchange chromatography. The active BSH fractions from HIC were pooled, desalted, and further concentrated using the Ultrafree-15 centrifugal filter unit (30 MWCO, Millipore; www.millipore.com). The active fraction obtained from HIC was applied to an anionic-exchange column (Mono Q HR 5/5, Amersham Pharmacia Biotech Inc.) equilibrated with buffer A (50 mM bis-Tris propane buffer, pH 6.5). Elution was performed using a linear gradient of 1 M sodium chloride in buffer A at a flow rate of 0.5 mL/min, and 1-mL fractions were collected. Fractions exhibiting BSH activity were pooled and assayed for protein and enzymatic activity.

Enzyme Purity and Molecular Mass Estimation

Protein purity and subunit molecular mass were estimated by subjecting aliquots from each purification step to SDS-PAGE using prestained marker proteins (New England BioLabs Inc., Mississauga, Ontario, Canada). Gels were stained with Coomassie brilliant blue R-250. The native molecular mass was determined by gel filtration chromatography (Superose 12 HR 10/30, Amersham Pharmacia Biotech Inc.) using LMW and HMW gel filtration calibration kits purchased from Amersham Pharmacia Biotech Inc. The mobile phase used was 0.1 M sodium phosphate-0.15 M NaCl (pH 7.0), and the flow rate was 0.5 mL/min.

Determination of Isoelectric Point (pI)

To determine the isoelectric point of BSH enzymes, the purified enzymes were applied to an isoelectric focusing unit (IEF) (Multiphore II IEF system, Amersham Pharmacia Biotech Inc.) equipped with Immobiline DryStrip. A broad pH range strip (pH 4.0 to 7.0, 13 cm) was used for the first isoelectric focusing (IEF), and a narrow pH range strip (pH 4.0 to 5.0, 18 cm) was used for the second IEF to measure their precise isoelectric points.

Determination of the N-Terminal Amino Acid Sequence

Bile salt hydrolase was purified by consecutive HIC and Mono Q chromatography. The resulting active fractions were pooled, concentrated, and applied to a mini

electrophoresis unit (Bio-Rad). After separation, the proteins were transferred onto a polyvinylidene fluoride membrane and stained with Coomassie brilliant blue R-250, and the BSH protein was cut out and used for sequencing. N-Terminal amino acid sequencing was performed with a Procise Protein Sequencing System (Applied Biosystems, Foster City, CA).

Substrate Specificity

To determine the substrate specificity of purified enzymes, the six major human bile salts were selected (Tanaka et al., 2000). Glycocholic acid, glycodeoxycholic acid, glycocheno deoxycholic acid, taurocholic acid, taurodeoxycholic acid, and taurochenodeoxycholic acid were obtained from Sigma Chemical Co. The rate of hydrolysis of conjugated bile salts was measured at 37°C and at pH 6.5, which is similar to that of the small intestine in a healthy human (Corzo and Gilliland, 1999). The released amount of amino acid from the substrates by enzymatic reaction was measured by ninhydrin assay and compared with the standard curve prepared by using either glycine or taurine. These experiments were conducted in triplicate.

RESULTS

Electrophoretic Mobility of BSH

Native gel electrophoresis followed by BSH activity staining demonstrated that BSH electrophoretic mobility was different according to the origin of the enzymes (Figure 1). Partially purified BSH from *Clostridium perfringens* (Sigma Chemical Co.) showed the lowest migration among the enzymes tested (lane 1). The highest migration was shown from the BSH enzyme of *B. infantis* KL 412 (lane 2), and a very similar pattern was observed from the BSH of *B. longum* KL 515. Another distinct migration pattern was identified from the BSH of *B. longum* ATCC 15708 and *B. longum* KL 507 (lane 3). The BSH from *B. bifidum* ATCC 11863 showed very similar electrophoretic mobility to lane 3. Based on the electrophoretic mobility of BSH enzymes, 5 strains were separated into 2 different types (type C: *B. infantis* KL 412 and *B. longum* KL 515; type A: *B. bifidum* ATCC 11863, *B. longum* ATCC 15708, and *B. longum* KL 507).

Concerning the electrophoretic mobility of BSH from the genus *Lactobacillus*, different patterns were observed depending on the strains. Four distinct classes were identified among those tested in this experiment (lanes 4 through 7).

Purification of BSH

To investigate the biochemical characteristics of the enzymes, BSH of bifidobacterial strains were purified

Figure 1. Activity staining on nondenaturing polyacrylamide gel. Lane 1, the commercial bile salt hydrolase from *Clostridium perfringens*; lane 2, *Bifidobacterium infantis* KL412; lane 3, *Bifidobacterium longum* ATCC 15708; lane 4, *Lactobacillus acidophilus* ATCC 43121; lane 5, *L. acidophilus* ATCC 53673; lane 6, *L. acidophilus* ATCC 53546; lane 7, *L. acidophilus* LAMA.

by a fast protein liquid chromatography (FPLC) system equipped with hydrophobic interaction, anion exchange, and size exclusion chromatography columns. Concentrated cell-free extracts from ammonium sulfate fractionation (40 to 80% saturation) were passed through HIC columns. Two types of BSH showed different elution profiles from HIC columns, suggesting that they might have different hydrophobic characteristics on the protein structure. Each enzyme seems to show different selectivity from other proteins depending on the HIC columns. Whereas a type A enzyme showed a better selectivity with aliphatic ligand columns (HiTrap Octyl FF or Butyl FF), a type C enzyme was more efficiently separated on aromatic ligand columns (HiTrap Phenyl FF). In each combination, the BSH were eluted near the end of the ammonium sulfate gradient with a good resolution.

The pooled fractions from the HIC column were loaded onto a Mono Q anion-exchanger column after the buffer was changed with 50 mM bis-Tris propane buffer (pH 6.5). From the Mono Q column, BSH were eluted differently depending on the strain. For the type C BSH enzyme, the activity was eluted at an NaCl

Figure 2. Elution profile of bile salt hydrolase (BSH) enzymes from an anionic-exchange column, Mono Q HR 5/5. A) Cell-free extract of *Bifidobacterium longum* ATCC 15708, and B) Cell-free extract of *Bifidobacterium infantis* KL412. Protein concentration (—), BSH activity (—), and NaCl gradient (----).

concentration between 0.12 and 0.16 M (Figure 2B). In the case of type A, the protein was eluted between 0.23 and 0.27 M NaCl (Figure 2A) with an exception of BSH from *B. bifidum* ATCC 11863, which was eluted around 0.35 M NaCl (data not shown). Based on this different elution profile from Mono Q, *B. bifidum* ATCC 11863 was reclassified as type B.

The electrophoretogram of the active fractions during purification steps and the purified BSH are shown in Figure 3. After consecutive purification steps of HIC and ion-exchange chromatography (IEC), type C BSH was the major protein in the active fraction (Figure 3A), and this fraction was further purified using size exclusion chromatography. For the type A BSH enzyme, 2 steps of chromatography (HIC and IEC) were enough to purify the enzyme to electrophoretic homogeneity (Figure 3B).

The purification steps of three different types of BSH are summarized in Table 1. The enzymes were purified

Figure 3. SDS-PAGE of cell extracts at each purification steps. Lane M, molecular weight marker; lanes 1 through 4: active fractions from *Bifidobacterium infantis* KL 412 (1, cell extract; 2, hydrophobic interaction [HIC]; 3, ion-exchange chromatography [IEC]; 4, size exclusion chromatography); lanes 5 through 8: active fractions from *Bifidobacterium longum* ATCC 15708 (5, cell extract; 6, HIC; 7, IEC; 8, concentrated sample of 7).

30-, 34-, and 22-fold with overall yields of 27, 29, and 25% for the types A, B, and C, respectively.

Approximately 35-kDa protein was evident throughout the different chromatography steps, and this protein formed a single band after size exclusion chromatography (Figure 3A and B).

pI Determination and N-Terminal Amino Acid Sequencing

By using an IEF system equipped with a broad pH range strip (pH 4.0 to 7.0, 13 cm), the approximate pI

values were found to be between pH 4.0 ~ 5.0. To measure more accurate pI values, a narrow pH range strip (pH 4.0 to 5.0, 18 cm) was used. Among 4 BSH enzymes tested, only type B BSH from *B. bifidum* ATCC 11863 had a pI of 4.45 and the other BSH (types A and C) from *B. longum* KL507, *B. longum* KL515, and *B. infantis* KL412 had similar pI values around 4.65 (Figure 4).

For the N-terminal sequencing, a type A BSH (from *B. longum* ATCC 15708) and a type C BSH (from *B. infantis* KL412) were selected. N-Terminal amino acid sequencing for the protein purified from each strain resulted in the following sequences: XTGVRFSDDEG-NTYFGRNLD and XTAVRFDGQNNMYFGRNLD, respectively. In both cases, the first N-terminal amino acid was not identified under the experimental conditions used.

Substrate Specificity

Bile salt hydrolase enzymes purified from *Bifidobacterium* strains showed a broad substrate range for 6 major human bile salts. Type A and C BSH enzymes showed the highest enzyme activity with glycodeoxycholic acid (defined as 100% activity), and they exhibited a preference for glycine-conjugated bile salts over taurine-conjugated forms (Figure 5). This tendency of preference was apparent in the case of type C than type A, but no difference was observed for di- or trihydroxy-conjugated bile salts.

DISCUSSION

Wide distribution and high enzyme activity of BSH in the genus of *Bifidobacterium* has been reported by

Table 1. Purification of bile salt hydrolase from three strains of *Bifidobacterium* that are harboring different types of bile salt hydrolase enzyme.

Step	Total protein (mg)	Total activity ¹ (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
<i>Bifidobacterium longum</i> KL 515 (type A)					
Crude extract	79.7	20767	261	100.0	1.0
Ammonium sulphate	69.5	19426	280	93.5	1.1
HIC (Butyl FF) ²	5.1	16351	3206	78.7	12.3
Mono Q ³	0.7	5533	7904	26.6	30.3
<i>Bifidobacterium bifidum</i> ATCC 11863 (type B)					
Crude extract	70	12885	184	100.0	1.0
Ammonium sulphate	62.4	11914	191	92.5	1.0
HIC (Butyl FF)	4.6	10376	2256	80.6	12.3
Mono Q	0.6	3761	6168	29.2	33.5
<i>Bifidobacterium infantis</i> KL 412 (type C)					
Crude extract	43.5	10842	249	100.0	1.0
Ammonium sulphate	38.6	9749	253	89.9	1.0
HIC (Phenyl FF) ⁴	5.3	7965	1503	73.5	6.0
Mono Q	0.5	2691	5382	24.8	21.6

¹One unit of enzyme activity is defined as the amount of enzyme that liberated 1 μ mol of amino acids from sodium glycocholate per minute.

^{2,4}HIC (Butyl FF) or HIC (Phenyl FF): Hydrophobic interaction (HiTrap) columns.

³Mono Q: Anion-exchange column.

Figure 4. Isoelectric point of BSH enzymes from *Bifidobacterium bifidum* ATCC 11863 (lane 1), *Bifidobacterium infantis* KL412 (lane 2), *Bifidobacterium longum* KL507 (lane 3), and *B. longum* KL515 (lane 4). pH gradient on the strip; from pH 5.0 at the top to pH 4.0 at the bottom.

other research groups (Grill et al., 1995b, 2000b; Tanaka et al., 1999), and this fact was also confirmed from the previous experiment carried out in this laboratory. For the first time, it was demonstrated that BSH enzymes originating from the strains of the genus *Bifidobacterium* show different characteristics depending on the type of enzyme. Some type-specific characteristics of BSH enzymes were investigated in this study (Table 2).

Different electrophoretic migration of BSH was visualized at the position of the enzyme on Native-PAGE followed by the activity staining. This activity staining technique has been used to determine the electrophoretic mobility of BSH from *Bifidobacterium* strains (Grill et al., 1995b; Grill and Schneider, 1997) and a *L. amylovorus* strain (Grill et al., 2000a). However, no

Figure 5. Substrate specificity of bile salt hydrolase enzymes from *Bifidobacterium longum* ATCC 15708 (A) and *Bifidobacterium infantis* KL 412 (B). Six major human bile salts: TC, taurocholic acid; TDC, taurodeoxycholic acid; TCDC, taurochenodeoxycholic acid; GC, glycocholic acid; GDC, glycodeoxycholic acid; GCDC, glycochenodeoxycholic acid).

approach has been made to see at the intergenus level, and no picture of the activity staining has been reported so far. By using the same experimental condition, it was possible to compare the electrophoretic mobility of the BSH enzymes from a different genus of microorganism (Figure 1). The lowest migration of BSH from *C. perfringens* is not surprising, because the higher native molecular mass (250 kDa) has been reported from *C. perfringens* (Gopal-Srivastava and Hylemon, 1988).

Four different electrophoretic migration patterns were revealed from the genus *Lactobacillus*. Interestingly, 5 consecutive BSH activity bands were identified from *L. acidophilus* ATCC 43121 (lane 4 in Figure 2). Corzo and Gilliland (1999) reported that the intracellular BSH enzyme from this strain had higher activity than other strains tested, and the molecular mass of the enzyme was estimated as 126 kDa by gel filtration chromatography. Further study must be carried out to

Table 2. Summary of some biochemical characteristics of purified bile salt hydrolases from different strains of *Bifidobacterium*.

	R _f value ¹	Elution from mono Q ²	pI ³	Mw (kDa)		N-Terminal amino acid homology	Type ⁹
				Sub-unit ⁵	Native ⁷		
<i>B. longum</i> SBT2928	...	0.33–0.36 M	4.71 ⁴	35.024 ⁶	125~130	20	A
<i>B. longum</i> ATCC 15708	0.47	0.23–0.27 M	...	35	140~150	20/20	A
<i>B. longum</i> KL 515	0.47	0.23–0.27 M	4.65	35	140~150	20/20	A
<i>B. bifidum</i> ATCC 11863	0.50	0.35–0.38 M	4.45	35	140~150	19/20	B
<i>B. infantis</i> KL 412	0.75	0.12–0.16 M	4.63	35	130~140	14/20	C
<i>B. longum</i> KL 507	0.75	0.12–0.16 M	4.63	35	130~140	...	C

¹R_f value of major activity band on a native PAGE.²NaCl concentration (M).³pI value determined by isoelectric focusing.⁴Deduced pI value based on the nucleotide sequence.⁵Determined by SDS-PAGE.⁶Deduced molecular weight based on the nucleotide sequence.⁷Determined by gel filtration chromatography.⁸Number of identical amino acid to the BSH of *B. longum* SBT2928.⁹Typing of BSH enzymes based on the biochemical characteristics from purification study.

investigate whether those 5 consecutive activity bands are due to hetero-isoenzymes combined with two different subunits α and β as reported in *L. johnsonii* 100-100 (Lundeen and Savage, 1992; Elkins et al., 2001).

The electrophoretic migration of BSH enzymes from *Bifidobacterium* strains was higher than that of *Clostridium* and *Lactobacillus*. Based on the electrophoretic mobility from the activity staining, Grill and Schneider (1997) reported 5 classes of BSH from animal and human origin strains of *Bifidobacterium*. They proposed that the different property of BSH electrophoretic mobility might be used to differentiate bifidobacterial species, in particular, differentiation between *B. longum* and *B. animalis* was proposed. From human origin strains tested in this study, 2 major types (A and C) were revealed. The mobility of the major band of type A BSH (lane 3 in Figure 2) was lower than that of type C (lane 2 in Figure 2).

Chromatographic steps were optimized in this study using an FPLC system equipped with 3 different types of columns for the purification of three different types of BSH from bifidobacteria strains. Hydrophobic interaction chromatography was appropriate as the first step of the purification, since no buffer change was necessary after ammonium sulphate precipitation. Furthermore, HIC showed high selectivity and high resolution for the BSH enzyme at the end of ammonium sulphate, and the high purification fold was obtained after this chromatographic step (Table 1). The BSH enzyme was eluted from the HIC column at a low concentration of ammonium sulphate, suggesting that the BSH enzyme belongs to a group of hydrophobic proteins. Considering some hydrophobic characteristics in their steroid ring structure of the substrates (bile salts), it is speculated

that some hydrophobic amino acid residues on the surface of a BSH enzyme play a major role in binding via hydrophobic interactions. The similar phenomenon was reported by Alkema et al. (2002) from penicillin acylase, which has been proposed as the same Ntn-hydrolase superfamily (Suresh et al., 1999; Oinonen and Rouvinen, 2000).

Three types of BSH (A, B, and C) showed different elution profiles from anion exchanger Mono Q, suggesting that BSH enzymes of different types have some differences in their amino acid compositions, charge characteristics, as well as their tertiary and quaternary protein structures. Such a difference in the elution pattern has been reported previously. Where BSH from *B. longum* BB 536 was eluted between 0.11 and 0.15 M NaCl (Grill et al., 1995b), BSH from *B. longum* SBT 2928 was eluted between 0.33 and 0.36 M NaCl using Na-phosphate buffer system (Tanaka et al., 2000). Compared to Na-phosphate buffer (an anionic buffer with Na⁺ as counter-ions), bis-Tris propane (a cationic buffer with Cl⁻ as counter-ions), which was used in this study, eluted proteins more efficiently from the anion-exchanger column. A different elution pattern from the Mono Q column was confirmed by measuring the fructose-6-phosphate phosphoketolase (F6PPK) enzyme activity as a marker protein. Regardless of types, F6PPK enzyme, which is a unique enzyme in bifidobacteria, was eluted at NaCl concentration between 0.30 and 0.34 M. This observation is similar to that made for the F6PPK enzyme, which was reported by Grill et al. (1995a).

The subunit molecular masses estimated by SDS-PAGE analyses were around 35 kDa for all BSH enzymes in this study. Numerous enteric bacteria express

BSH activity, and there are differences in the molecular structure (trimeric, tetrameric, or hexameric proteins with a molecular mass of 105,000 to 250,000) (Stellwag and Hylemon, 1976; Gopal-Srivastava and Hylemon, 1988; Coleman and Hudson, 1995; Grill et al., 1995b). The BSH of type C showed a little lower native molecular mass (130 to 140 kDa) than that of type A and B (140 to 150 kDa). This is in good agreement with those of other BSH enzymes reported previously and indicates that the native enzyme is a tetramer (Tanaka et al., 2000).

The measured pI values were close to the theoretical pI of 4.51 for the deduced protein of BSH from *B. longum* SBT 2928 (Tanaka et al., 2000). Isoelectric points of BSH enzymes from the genus *Bifidobacterium* were found to be lower than the values reported from the others; 5.29 for BSH from *L. acidophilus* KS-13 (GenBank accession number AAD03709), 4.98 from *L. gasseri* (GenBank accession number ZP_00046789), 5.05 and 4.88 from *L. johnsonii* 100-100 alpha and beta (GenBank accession numbers AAG22541 and AAC34381, respectively), and 5.12 from *L. plantarum* WCFS1 (GenBank accession number NP_786739). The lower pI value of BSH from bifidobacterial strains implies that the BSH enzymes contain a higher portion of negatively charged residues (Asp + Glu) and a lower portion of positively charged residues (Arg + Lys). In fact, the ratio of negatively charged residues to positively charged residues is 2.05 (41/20) and 1.21 (35/29) in the amino acid composition of the BSH enzymes from *B. longum* and *L. acidophilus*, respectively.

Even though the last N-terminal amino acid was not identified under the experimental condition used, the Cys residue at the N-terminal end was highly conserved in all BSH enzymes except one BSH enzyme purified from *Xanthomonas maltophilia* (Dean et al., 2002). Elkin et al. (2001) and Tanaka et al. (2000) have proposed that the Cys residue at the N-terminal end of the BSH enzyme as one of the active sites of the enzyme. From a protein homology comparison, these N-terminal sequences were homologous to those of several lactobacilli as well as *Clostridium perfringens*. In addition, an amino acid motif, XYFGRNLDX, which was highly conserved within all BSH enzymes reported in lactobacilli and bifidobacteria, was presented in both N-terminal sequences of type A and type C enzymes.

In this study, BSH from all strains of *Bifidobacterium* showed a higher deconjugation rate on glycine conjugated bile salts than on taurine conjugated forms. Considering the fact that the majority of human bile salts are glycine conjugated forms, BSH enzymes from bifidobacterial strains might be important in the deconjugation of bile salt in the human intestine.

From the foregoing discussion, one may conclude that the optimized purification conditions established with HIC and Mono Q columns were very effective in purifying three types of active BSH from bifidobacteria strains. Purified BSH enzymes from different strains showed some type-specific characteristics, and they had some different characteristics from those of other genus and species. Migration patterns on the PAGE gel and elution profiles from the anion-exchange column described in this study could be useful for the investigation of the similarities and differences among BSH enzymes from different genus and species. Because almost every strain of bifidobacteria possesses BSH enzyme activity at a significant level, and they have some genus-specific and type-specific characteristics, this phenotypic trait may be used as a means of phenotypic characterization of *Bifidobacterium* strains.

For the molecular characterization of type A, B, and C enzymes, the cloning of the BSH gene is currently being carried out. Once the nucleotide sequences for each type of *bsh* gene is obtained from the cloning experiment, it will provide some information about the phylogenetic relationship within the genus of *Bifidobacterium* as well as other genus and species of enteric bacteria that are harboring *bsh* gene. Furthermore, it will allow us to develop some genus-specific and type-specific probes based on the BSH locus and to use them as useful identification tools.

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