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Purification and Characterization of Bile Salt Hydrolase from *Lactobacillus plantarum* CK 102

HA, CHUL-GYU[1], JIN-KOOK CHO[2], YOUNG-GYU CHAI[1], YOUNG-AE HA[3]*, AND SHANG-HUN SHIN[4]

[1]*Department of Biochemistry and Molecular Biology, Hanyang University, Ansan, 425-791, Korea*
[2]*Department of Dairy Science and Gyonggi-do Regional Research Center, Hankyong National University, Anseong 450-749, Korea*
[3]*Department of Occupational Medicine, Dong-Kang General Hospital, Ulsan 681-711, Korea*
[4]*Department of Diagnostic Radiology, University of Ulsan College of Medicine, Ulsan University Hospital, Ulsan 682-714, Korea*

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Abstract A bile salt hydrolase (BSH) was purified from *Lactobacillus plantarum* CK 102 and its enzymatic properties were characterized. This enzyme was successfully purified using ion-exchange chromatography with Q-Excellose and hydrophobic interaction chromatography with Butyl-Excellose. The purified enzyme showed a single protein band of 37 kDa by SDS-polyacrylamide gel electrophoresis, which was similar to the molecular weight of known BSHs. The amino acid sequence of *GLGLPGDLSSMSR*, determined by MALDI-TOF, was identical to that of BSH of *L. plantarum* WCFS1. Although this BSH hydrolyzed all of the six major human bile salts, glycine-conjugated bile acid was the best substrate, based on its specificity and K_m value. Among the various substrates, the purified enzyme maximally hydrolyzed glycocholate with apparent K_m and V_{max} values of 0.5 mM and 94 nmol/min/mg, respectively. The optimal pH of the enzyme ranged from 5.8 to 6.3. This enzyme was strongly inhibited by thiol enzyme inhibitors such as iodoacetate and periodic acid.

Key words: Bile salt hydrolase, *Lactobacillus plantarum* CK 102

Many lactobacilli-containing dairy and pharmaceutical products have been developed and consumed for several decades for their health-promoting effects. Lactobacilli are one of the most common microflora in the gastrointestinal tracts of humans. In the gastrointestinal tract, many endogenous microflora and exogenous bacteria continuously encounter a significant amount of bile salts that possess detergent-like antimicrobial properties [9, 11, 20].

One of those mechanisms of enteric intestinal bacteria including Lactobacilli is the deconjugation of bile salts by bile salt hydrolase (BSH), which occurs in the intestinal tract of humans and animals [16]. BSH catalyzes the deconjugation reaction by hydrolysis of glycine- or taurine-conjugated bile acids into the amino acid and the bile acid. This kind of BSH activity has been detected in many members of the genera *Clostridium, Fusobacterium, Bacteroides, Streptococcus* [1, 4, 23], *Lactobacillus* [3, 26], and *Bifidobacterium* [7, 16].

In a previous paper, we reported a number of lactic acid bacteria that produced BSH [10]. Initially, 120 strains of bacteria showing BSH activities and resistance against acidic pH were isolated from human feces. The strain with the highest BSH activity was identified as *Lactobacillus* using API kit and 16S rRNA sequencing, and named as *Lactobacillus plantarum* CK 102 [10].

In this study, we further purified a BSH from this strain and characterized some of its enzymatic properties, and herein, we discuss the relevance of our findings to future works.

Materials and Methods

Materials

Phenylmethysulfonyl fluoride (PMSF), glycine conjugates of cholic acid, deoxycholic acid, chenodeoxycholic acid, acrylamide, sodium dodecyl sulfate (SDS), iodoacetate, p-chloromercuribenzoate, and trinitrophenylbenzenesulfonate (TNBS) were obtained from Sigma Chemical Co. (St. Louise, MO, U.S.A.). Q-Excellose and Butyl-Excellose were obtained from Bioprogen Co. (Daejon, Korea).

Growth Conditions of Strains

L. plantarum CK 102 isolated from human feces was grown anaerobically in MRS medium (Difco Laboratories, Detroit, U.S.A.) for 24 h. The culture was stored in 15% glycerol at −40°C.

*Corresponding author
Phone: 82-52-241-1388; Fax: 82-52-241-1180;
E-mail: hya3174@netsgo.com

Purification of BSH

Preparation of Cell Extract. *L. plantarum* CK 102 cells were harvested at the late-log phase by centrifugation at 6,000 ×*g* for 15 min, and the precipitate was suspended in 10 ml of buffer A solution containing sodium phosphate (10 mM), pH 6.4, 10 mM 2-mercaptoethanol (2-ME), 1 mM ethylenediaminetetraacetic acid (EDTA), and 12% (v/v) glycerol, which contained 0.1 mg each of deoxyribonuclease and ribonuclease. The cells were ruptured by two passages through a French pressure cell (SLM Aminco, Urbana, IL, U.S.A.) at 10,000 psi. Ruptured cells were again centrifuged at 12,000 ×*g* for 30 min. The soluble supernatant of the cell extract was dialyzed against 2 l of distilled water for 1 day at 4°C, and used for the next purification steps.

Ammonium Sulfate Precipitation. Dialyzed cell extract was precipitated by slowly adding solid ammonium sulfate to 40% saturation while maintaining the pH at 7.8 using 0.1 N sodium hydroxide. After 60 min at 4°C, the precipitate was removed by centrifugation at 12,000 ×*g* for 10 min. The supernatant was brought to 70% ammonium sulfate saturation and centrifuged at 12,000 ×*g* for 10 min. The maximum enzyme activity was detected in the 40–70% ammonium sulfate fraction. The resulting pellet was suspended in 10 ml of buffer A and dialyzed against the same buffer for 24 h.

Ion-Exchange Chromatography. The supernatant fraction was loaded onto a Q-Excellose ion-exchange chromatography column (2.0×30 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 2 mM PMSF. The column was extensively washed (50 ml bed volume, 200 ml sample volume) to remove unbound materials. The adsorbed protein was eluted with a linear gradient formed from 0 to 0.5 M NaCl in equilibration buffer. BSH activity was assayed immediately, and fractions containing more than 50% of the peak activity were pooled.

Hydrophobic Interaction Chromatography. The pooled fraction from the ion-exchange chromatography column was injected onto a Butyl-Excellose hydrophobic interaction chromatography column (2.0×30 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl. The unadsorbed material was washed out with the equilibration buffer (30 ml bed volume, 100 ml sample volume). The adsorbed protein was eluted with a decreasing linear gradient formed from 1 to 0 M NaCl. Fractions were collected and immediately assayed for BSH activity. The BSH-rich fraction was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and NH_2-terminal amino acid sequence analysis.

BSH Purity and Molecular Weight Estimation. Protein purity was estimated by subjecting aliquots of pooled fractions from each step of purification to SDS-PAGE. SDS-PAGE was performed by the method of Laemmli [19], using 12% acrylamide gel (1 mm thickness) at constant current (120 V/plate) for 1 h. The protein bands were visualized by Coomassie brilliant blue R-250 [12].

Assay for BSH Activity

BSH activity was determined by measuring the release of glycine resulting from the hydrolysis of the amide bond of sodium glycocholate [6]. The reaction mixture (1 ml) contained the protein (10 to 50 μg), 5 mM sodium glycocholate, 500 mM sodium phosphate buffer (pH 6.0), and 1 mM EDTA, and was incubated at 37°C for 10 min (under the condition, the reaction approximated zero-order kinetics).

The enzymatic reaction was terminated by adding an equal volume of 20% trichloroacetic acid. The precipitated protein was removed by centrifugation at 15,000 ×*g* for 10 min, and the amount of glycine present in the supernatant was measured by quantitating the chromogenic derivative of glycine formed with trinitrophenyl-benzenesulfonate (TNBS) at 416 nm as follows: Immediately, iodoacetic acid (17.5 mM final concentration) was added to a 0.2-ml aliquot of the supernatant to acetylate 2-ME, which 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 interrupted by adding 2.0 ml of freshly prepared sodium sulfite solution (1.5 ml of 100 mM sodium sulfate 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. The BSH activity was expressed in nmol/min/mg protein.

Protein Concentration

Protein concentration was measured by the Bradford method [2], using bovine serum albumin as the standard.

Enzymatic Digestion of Protein

Protein spots were enzymatically digested in gel in a manner similar to that previously described by Shevchenko *et al.* using modified porcine trypsin (Promega modified) [25, 28]. Gel pieces were washed with 50% acetonitrile to remove SDS, salt, and stain, dried to remove solvent, rehydrated with trypsin (8–10 ng/μl), and incubated for 8–10 h at 37°C.

Mass Spectrometry

All mass spectrometry (MS) and power spectral density (PSD) spectra were acquired in the positive ion mode using a Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometer (Amersham Biosciences) equipped with a pulsed extraction source, a 337-nm pulsed nitrogen laser, and a curved-field reflectron. The acceleration voltage was 20 kV. The matrix α-cyano-4-hydroxycinnamic acid (CHCA) was prepared in a 1:1

acetonitrile/water mixture. A thin layer of the matrix was applied onto the sample plate first, followed by the addition of 0.5 μl each of sample and matrix and allowing to dry at room temperature [32].

Protein Identification Using CAF-MALDI Data

Chemically Assisted Fragmentation (CAF™)-MALDI was used for protein identification. Fragment masses obtained from CAF-MALDI can be submitted to either Sonar in the Ettan MALDI-TOF software or similar protein identification search engines such as PepFrag. The mass of native (non-derivatives) peptides as well as five or more fragment masses (depending on the protein) are needed for protein identification. The amino acid sequence of the peptide was determined by measuring the distances between consecutive peaks (y-ions) in the PSD spectrum. By submitting the amino acid sequence, the protein was identified by a homology search such as ProteinInfo™ (http://www.proteometrics.com) or by BLAST search such as ExPASy Molecular Biology Server (http://www.ncbi.nlm.gov/blast).

Substrate and Inhibitor of the Enzyme

The effects of substrates and inhibitors on the enzymatic activity were studied using the following compounds: glycocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid, iodoacetate, periodic acid, phenylmethysulfonyl fluoride (dissolved in 70% ethanol), $HgCl_2$, $CuCl_2$, $CaCl_2$, $MgCl_2$, and EDTA. Before substrates were added, the enzyme was incubated for 30 min at 37°C with each compound mentioned above. Then, the standard enzyme assay described previously was carried out in order to determine the residual enzyme activity.

RESULTS AND DISCUSSION

Purification of BSH

BSH from *Lactobacillus plantarum* CK 102 was purified by the procedure detailed in Materials and Methods. Each purification step was followed by measurement of enzyme activity using glycocholic acid (sodium salt) as a substrate. Approximately 92.9% of total activity was recovered from the ammonium sulfate precipitation, and the specific activity increased 1.9-fold.

When the BSH-rich fraction was fractionated by ion-exchange chromatography using a Q-Excellose column, most of the proteins were eluted in two peaks, and BSH was eluted as a sharp single peak at 120 mM NaCl. Ion-exchange chromatography using a Q-Excellose column (Fig. 1A) resulted in 71.2% recovery of protein. At this stage of purification, the BSH preparation was found to be contaminated with other proteins (Fig. 2, lane 4).

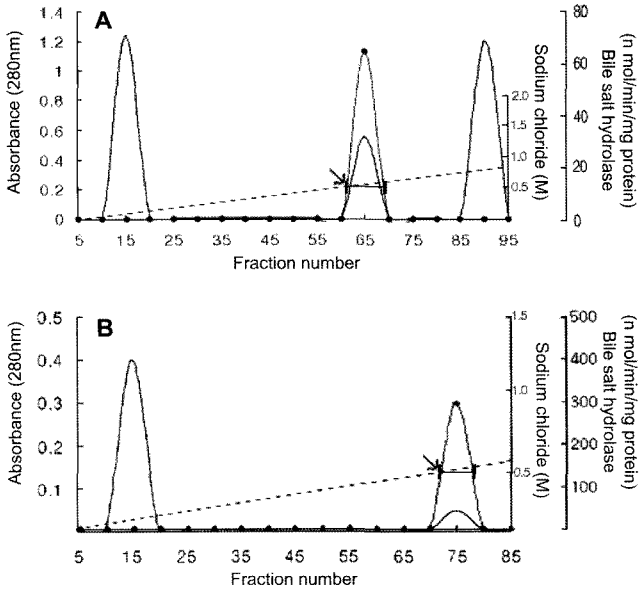

Fig. 1. Elution profiles for BSH from Q-Excellose chromatography (**A**) and Butyl-Excellose chromatography (**B**).

The flow rate was 10 ml/h (**A**) and 1 ml/min (**B**), respectively. The solid line represents absorbance (280 nm), dashed line represents the concentration of NaCl, and the connected line with black circle represents BSH activity. The condition of protein elution is described in Materials and Methods. Bars indicate fractions that contained high BSH activity from each chromatography. Arrows indicate the starting point for elution with 0.5 M NaCl. —: Absorbance (280 nm); • •: Bile salt hydrolase (nmol/min/mg protein); ---: Sodium chloride (M).

The fraction exhibiting BSH activity was applied to hydrophobic interaction chromatography to increase the purity (Fig. 1B). Typical protein concentration, enzyme activity,

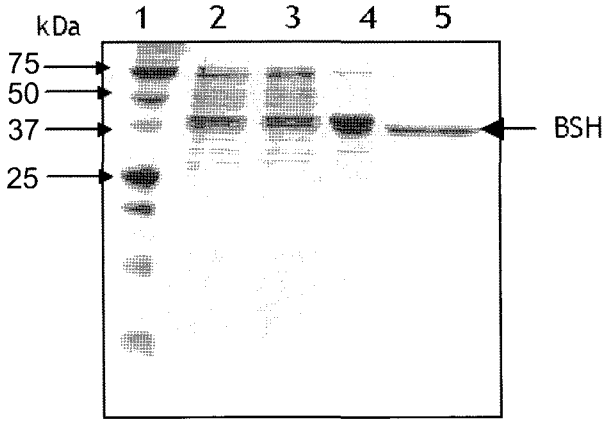

Fig. 2. SDS-PAGE patterns of the fractions obtained from each purification step of bile salt hydrolase from *Lactobacillus plantarum* CK 102.

Lane 1, molecular weight standards; lane 2, crude cell extracts (10 μg); lane 3, 40–70% ammonium sulfate precipitate (10 μg); lane 4, pooled fractions from ion-exchange chromatography; lane 5, fraction from hydrophobic interaction chromatography. Bands were visualized by Coomassie brilliant blue R-250 for protein.

Table 1. Purification of bile salt hydrolase from *Lactobacillus plantarum* CK 102.

Step	Protein		Enzyme			
	Content (mg)	Yield (%)	Specific activity (nmol/min/mg protein)	Purification fold	Total activity (nmol/min)	Yield (%)
Crude cell extract	585.2	100	2.4	1	1,413.3	100
Ammonium sulfate 40–70% saturation	287.6	49.1	4.6	1.9	1,312.3	92.9
Ion-exchange chromatography	17.2	2.9	58.5	24.2	1,006.5	71.2
Hydrophobic interaction chromatography	0.7	0.1	825.9	342.0	578.1	40.9

recovery, and yield are listed in Table 1. Approximately 0.1% of proteins and 40.9% of BSH activity were recovered by the procedure. The purity of the enzyme was confirmed by SDS-PAGE. A single band appeared at the region of an apparent molecular weight of 37 kDa (Fig. 2, lane 5), which coincided with the previous data [6].

The optimal pH of the purified BSH was measured in an assay mixture. Figure 3 shows a broad pH optimum over the range of 5.8 to 6.3.

The substrate-dependency of enzyme was determined by varying the concentration of glycocholic acid (sodium salt), and the maximum BSH activity was achieved at 4 mM glycocholic acid. The saturation curve followed Michaelis-Menten kinetics (Fig. 4): The apparent K_m and V_{max} values of BSH for glycocholic acid were determined by Lineweaver-Burk plot to be approximately 0.5 mM and 94 nmol/min/mg protein, respectively (n=3). The K_m value for the purified enzyme was identical to that of BSH in *Clostridium perfringens*, but it was lower than that of the other BSHs [5].

Mass Analysis Using MALDI-TOF

For the identification of gel-separated proteins, selected proteins were subjected to enzymatic digestion, and the resulting peptide fragments were analyzed using the complementary mass spectrometry methods MALDI-TOF-MS. The partial amino acid sequence of the purified protein obtained from MALDI-TOF mass analysis was GLGLPGDLSSMSR and, as shown in Fig. 5, this sequence of BSH protein from *Lactobacillus plantarum* CK 102 is identical to the sequence, corresponding from 211th to 223rd, of the BSH from *L. plantarum* WCFS1 [17, 21].

Biochemical Characterization of the Purified BSH

The purified BSH revealed broad substrate specificity, which could hydrolyze not only human bile salts but also taurodeoxycholic acid and taurochenodeoxycholic acid (Table 2). The highest level of enzyme activity was observed with glycocholic acid. Among the six major human bile salts tested (Table 2), the enzyme exhibited higher affinity for glycine-conjugated bile acids than for taurine-conjugated bile acids. As shown in Table 3, the enzyme was strongly inhibited by substances known as sulfhydryl-enzyme inhibitors. Little inhibition was observed with either metal enzyme inhibitor (EDTA) or serine enzyme inhibitor (PMSF).

In this study, BSH was successfully purified from *Lactobacillus plantarum* CK 102 and the properties were characterized. An effective purification of BSH was achieved using a three-step process including ammonium sulfate

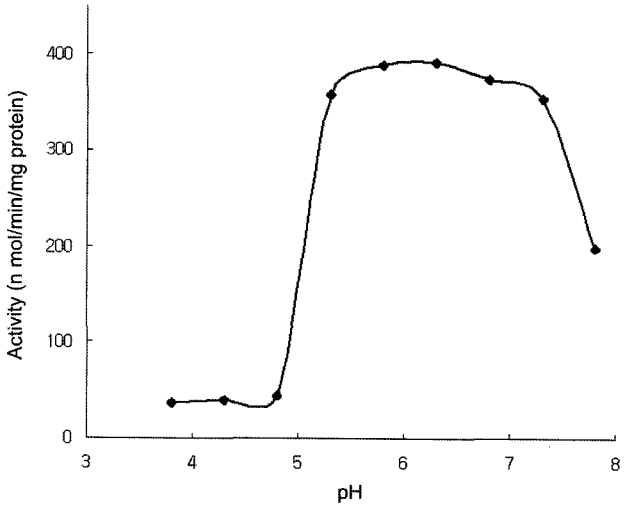

Fig. 3. Effects of pH on bile salt hydrolase activity.
The enzyme activity was measured in 50 mM sodium acetate buffer at pH 3.8–5.3, and 50 mM potassium phosphate buffer at pH 5.8–7.8.

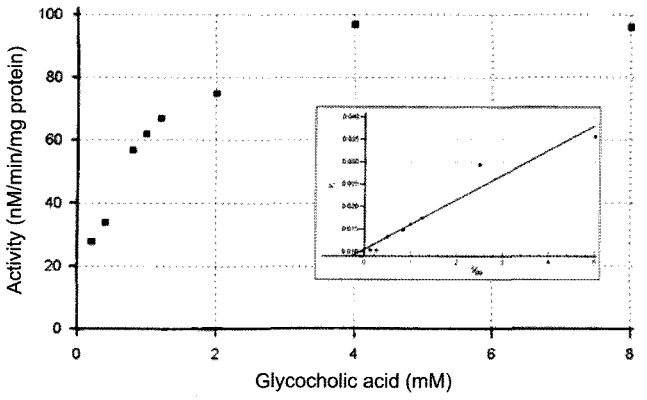

Fig. 4. Effect of glycocholic acid concentration on bile salt hydrolase activity.
Substrate (sodium salt of glycocholic acid) was added to initiate the reaction. Reaction mixtures contained 10 μg of enzyme. Initial velocities of hydrolysis were measured for 10 min. Insert represents the double reciprocal plot of the data (Curve; $y=-3.2301x^2$; line; $y=0.0055x+0.0106$)

1 MCTAITYQSYNNYFGRNFDYEISYNEMVTITPRKYPLVFRKVENLDHHYA 50
51 IIGITADVESYPLYYDAMNEKGLCIAGLNFAGYADYKKYDADKVNITPFE 100
101 LIPWLLGQFSSVREVKKNIQKLNLVNINFSEQLPLSPLHWLVADKQESIV 150
151 IESVKEGLKIYDNPVGVLTNNPNFDQLFNLNNYRALSNSTPQNSFSEKV 200
201 DLDSYSRGMGGLGLPGDLSSMSRFVRAAFTKLNSLPMQTESGSVSQFFHI 250
251 LGSVEQQKGLCEVTDGKYEYTIYSSCCDMDKGVYYYRTYDNSQINSVNLN 300
301 HEHLDTTELISYPLRSEAQYYAVN

Fig. 5. The partial amino acid sequence (underline) measured by MALDI-TOF and its comparison with that of BSH of *L. plantarum* WCFS1.

fraction, ion-exchange chromatography, and hydrophobic interaction chromatography. As the BSH activity was sensitive to sonication or similar cell disruption methods as well as to oxygen [30], we used the French pressure method in order to preserve the enzymatic activity. Although there was no significant change of protein pattern in SDS-PAGE, ammonium sulfate precipitation enabled a 2-fold increase of enzyme activity.

BSH could easily be purified from *Lactobacillus plantarum* CK by taking advantage of the difference in affinity to anionic ligand and butyl groups.

The molecular mass of the purified BSH was estimated to be 37 kDa, which is similar to the value reported for BSH purified from *Bifidobacterium* sp. [16]. This result suggests that BSH in *Lactobacillus plantarum* CK 102 may be a genetic variant of BSH.

The purified enzyme showed a broad pH optimum, ranging from 5.8 to 6.3, in contrast to the pH optimum reported for *Bacteroides fragilis* (pH 4.0–5.0) [16, 22] but close to *Clostridium perfringens* (5.8–6.4) [5].

In recent years, the identification of proteins by proteomic analysis has gained a significant importance in studying cellular functions and MS has become a central element for proteomic analysis. Peptide Mass Fingerprinting (PMF) remains the simplest and most powerful of various MS techniques for high-throughput protein identification, where peptide mass fingerprints acquired by MALDI-TOF mass spectrometry are compared with theoretical peptide mass fingerprints calculated for all the proteins in a given protein sequence database [8, 13, 14, 17, 18, 27]. For bacterial strains, PMF is found to be even more reliable for species-specific protein identification than NH_2-terminal sequencing.

Table 2. Substrate specificity of BSH from *Lactobacillus plantarum* CK102.

Substrate[a]	BSH activity[b]	Relative activity (%)	K_m (mM)
GCA	252	100	0.50
GDCA	196	77.8	0.41
GCDCA	218	86.5	0.21
TCA	76	30.2	1.08
TDCA	69	27.4	0.74
TCDCA	51	20.2	0.41

[a]GCA, glycocholic acid; GDCA, glycodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycholic acid, TCDCA, taurochenodeoxycholic acid.
[b]Each substrate was assayed at 5.0 mM concentration under standard assay condition. The reaction was initiated by the addition of 10 μg of pure BSH. Activity is expressed as nmol of substrate hydrolyzed/min/mg of protein.

Table 3. Effect of various ions and inhibitors on BSH activity.

Inhibitor	Concentration (mM)	BSH activity[a] (nmol/min/mg)	% Inhibition
Control	–	252	0
Iodoacetate	2	45.4	82
Periodic acid	2	0	100
PMSF[b]	2	54	79
$HgCl_2$	0.2	40	84.1
$CuCl_2$	2	0	100
$CaCl_2$	2	12	95.2
$MgCl_2$	2	252	0
EDTA	2	232	8

[a]The enzyme was first incubated with inhibitors for 15 min at room temperature prior to the addition of the substrate sodium glycocholate, and the enzyme activity was then measured as described in Materials and Methods.
[b]PMSF, Phenylmethylsulfonyl fluoride.

Recently, interest in the possibility of using BSH from bacteria to lower the serum cholesterol level has piqued: BSH could potentially be applied in prevention and cure of hypercholesterolemia [24, 28, 29, 31]. *Lactobacillus plantarums* that contain BSH have earlier been shown to have an excellent cholesterol lowering effect in SD rats [13, 15, 17, 30]. It is not yet clearly understood whether this effect is due to the cholesterol regulatory mechanism of BSH. However, it has been hypothesized that the bile salt deconjugation plays an important role. In order to understand the biochemical mechanism and the linkage of BSH to control hypercholesterolemia, study on the physiological effects of BSH on cholesterol level *in vivo* is needed.

In this article, an efficient method to purify BSH from *Lactobacillus plantarum* CK 102 is described and some properties were characterized. Some observations in the present research are indirectly related to the understanding of the cholesterol regulation mechanism of BSH.

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