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New insight into the catalytic properties of bile salt hydrolase

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ABSTRACT

Bile salt hydrolase (BSH), the enzyme deconjugating bile potentially plays an important role in reduction of blood cholesterol level. BSH enzymes from various sources differ in characteristics, substrates preference and specific catalytic activity. In this study, two BSH enzymes (BSH1 and BSH2) from *Lactobacillus salivarius* were heterologously expressed and purified. Both of them were characterized as homotetramer according to their molecular weight from size exclusion chromatograph. BSH1 showed a broad pH optimum over the range from 5.5 to 7.0, while a narrower range of pH optimum from 5.5 to 6.0 for BSH2 was detected. The enzymatic kinetics of the purified BSH1 and BSH2 have demonstrated BSH enzymes from bacteria were allosteric enzymes, and have also revealed their striking differences in positive cooperativity, catalytic efficiency and substrate preference for the first time. In contrast to the enzymatic reactions of BSH in the absence of dithiothreitol, the kinetics curves of BSH1 and BSH2 were similar to hyperbolic forms of Michaelis–Menten kinetics in the presence of dithiothreitol.

1. Introduction

Cardiovascular diseases caused by hypercholesterolemia have now become one of major factors that seriously influence people's health across the world [1]. Recent reports revealed that a reduction in the level of serum cholesterol was associated with the presence of bile salt hydrolase (BSH). This has led to an increasing attention to the possible application of BSH enzymes to treat hypercholesterolemia in humans [2–4]. Bile salt hydrolase is an important isoenzyme in mammal gastrointestinal (GI) tract that helps microorganism resisting bile acid or bile salt during their transit or colonization in the GI tract. It was found that several microbial species, including *Clostridium* [5], *Bifidobacterium* [6–8], *Lactobacillus* [9–15], *Enterococcus* [16–18], *Listeria monocytogenes* [19], *Brevibacillus* [20], *Bacteroides* [21,22], *Brucella abortus* [23] and *Streptococcus* [24], can either produce bile salt hydrolase or have genes encoding for BSH in their genomes. *Lactobacillus salivarius* is a flexible and versatile microorganism species that could inhabit a wide variety of environmental niches, including the human gastrointestinal (GI) tract [25–27]. Previous work has revealed that the ability to catalyze the deconjugation of bile salts is a common characteristic among members of the *L. salivarius* species [13].

Bile is a mixture of bile salts and bile acids. Independent of its function in dietary fat emulsification, bile is a key signaling molecule regulating its own biosynthesis, lipid absorption, cholesterol homeostasis, and local mucosal defenses in the intestine [28–30]. Bile salt hydrolase (BSH) catalyses the hydrolysis of glyco-conjugated bile salts (G-CBAs) or tauro-conjugated bile salts (T-CBAs) into amino acid residues and unconjugated bile acids [31–33]. The unconjugated bile acids may precipitate at low pH and be excreted in the feces [4]. Because of the less efficiency at solubilization of lipids and intestinal reabsorption, generation of unconjugated bile acids leading to an increase in demand for cholesterol for the synthesis of new bile salts [34,35] and, resulting in a decrease in the serum cholesterol level of the host. Hence, investigation and comparison of biochemical and biological properties for bile salt hydrolases from different resources is of great importance for revealing the gene functionality during bacteria and host interaction.

However, the catalytic mechanism as well as substrate preference of BSH remains unclarified. The aim of the present study was to over-express and purify two BSHs of *L. salivarius*, and comparably characterize the kinetic properties of these BSH enzymes.

Abbreviations: BSH, bile salt hydrolase; DTT, dithiothreitol; IPTG, isopropyl thio-β-D-galactopyranoside; GI, gastrointestinal; G-CBAs, glyco-conjugated bile salts; T-CBAs, tauro-conjugated bile salts; GCA, sodium glycocholate; GDCA, sodium glycodeoxycholate; GCDCA, sodium glycochenodeoxycholate; TCA, sodium taurocholate; TDCA, sodium taurodeoxycholate; TCDCA, sodium taurochenodeoxycholate.

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2. Materials and methods

2.1. Substrates and chemicals

BSH substrates sodium glycocholate (GCA), sodium glycodeoxycholate (GDCA), sodium glycochenodeoxycholate (GCDCA), sodium taurocholate (TCA), sodium taurodeoxycholate (TDCA) and sodium taurochenodeoxycholate (TCDCA) were purchased from Sigma–Aldrich Co. LLC. All chemicals used in this study were commercially available and were analytical grade.

2.2. Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. *L. salivarius* LMG14476 was cultured under a microphilicanaerobic conditions at 37 °C in de Man Rogosa and Sharpe (MRS) medium. *Escherichia coli* strains were aerobically grown at 37 °C in Luria–Bertani (LB) broth with shaking at 200 rpm. When needed, ampicillin was added at 50 μg/ml for *E. coli* JM109 and 100 μg/ml for *E. coli* BL21(DE3), respectively.

2.3. Gene cloning, expression and protein purification

The genomic DNA of *L. salivarius* was isolated and purified by using the bacterial genomic DNA rapid extraction kit (Sangon, Shanghai, PRC) according to the manufacturer's instructions. *Ex Taq* polymerase, restriction enzymes, DNA ligation kit, MutanBEST Kit and PCR purification kits were purchased from TaKaRa (Dalian, PRC) and used according to the manufacturer's instructions. Plasmids were introduced into *E. coli* by standard heat-shock transformation. PCR primers used in this work were shown in Table S2. Primer pairs pETb1u-pETx1d and pETn2u-pETx2d were used to PCR amplify *bsh1* and *bsh2* from genomic DNA of *L. salivarius* LMG14476, respectively. The corresponding PCR product of *bsh1* was restricted with *BamH* I and *Xho* I, and ligated to similarly digested pET-20b(+), the construct (pET21) was then mutated to remove genes between *Nde* I and *BamH* I sites, yielding the pETM21. While the amplicon of *bsh2* was cloned into the *Nde* I and *Xho* I sites, yielding the pET22. *E. coli* JM109 was used as the cloning host for these constructs. The recombinants pETM21 and pET22 plasmids for over-expression of *bsh1* and *bsh2* were transformed into *E. coli* BL21(DE3).

E. coli pET-20b constructs were grown at 37 °C with shaking at 200 rpm until the OD_{600} reached 0.5 to 0.6, 0.1 mM or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) was then added to induce the over-expression of *bsh1*or *bsh2*. The production of BSH1 and BSH2 was performed at 20 °C for 24 h in TB medium induced with 0.1 mM IPTG and in LB medium induced with 1 mM IPTG, respectively. Cells were then harvested by centrifugation at 8000 × *g* for 15 min at 4 °C.

Cell pellets from batch fermentation were washed and resuspended in binding buffer (20 mM Na_2HPO_4–NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4), then disrupted by sonication for 20 min. The clarified supernatant was subjected to immobilized metal affinity chromatography (IMAC) using a 1.0 ml HisTrap FF crude column (GE Life Sciences) pre-equilibrated with binding buffer at 4 °C. The unbound proteins were washed off with 10 column volumes of binding buffer, and the loaded column was then eluted with elution buffer (20–500 mM imidazole, 20 mM Na_2HPO_4–NaH_2PO_4, 500 mM NaCl, pH 7.4) using a linear gradient elution method. Fractions containing BSH activity were pooled and concentrated by ultrafiltration using Amicon Ultra −15 ml (Millipore10 kDa molecular mass cutoff) at 4000 rpm to a final volume of 2 ml. The concentrated samples were purified by gel exclusion chromatography in 20 mM sodium phosphate (pH 6.0), using a superdex G-200 column (GE Life Sciences) at a flow rate of 0.5 ml/min at 4 °C.

2.4. Detection of BSH activity in E. coli

BSH activity was detected using a plate assay method [36]. For detecting BSH activity of *E. coli* BL21 derivatives, an optimized LB medium (for 1 liter, 15 g agar, 10 g tryptone, 5 g yeast extract, 5 g NaCl, 0.35 g $CaCl_2{\cdot}2H_2O$, 10 g glucose, 1 mM IPTG, 0.1 g ampicillin, pH 6.5) containing 5 g/l TDCA or 2 mM GDCA was used.

2.5. BSH molecular mass estimation

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was carried out by the method of Laemmli [37] with the molecular mass standards (Fermentas). The molecular weight of native protein was calculated according to Andrews [38]. Size elution chromatograph was carried out using a Tsk gel G3000 column (Sigma–Aldrich) at a flow rate of 0.5 ml/min with 20 mM Na_2HPO_4–NaH_2PO_4 buffer at pH 6.8. The native molecular weight (M_w) was determined by using protein standards under similar conditions (Sigma MW-GF-200 Kit) which includes β-amylase, alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase.

2.6. Enzyme activity assay and protein assay

BSH specific activity was determined by measuring the release of amino acid from conjugated bile salts. Bile salt hydrolase assay was performed by a ninhydrin method [7,35] with minor modifications. TDCA and GDCA were used as the substrates for BSH1and BSH2, respectively. The reaction was incubated at 37 °C for 30 min in absence of DTT and terminated by trichloroacetic acid (15%, w/v). The mixture was centrifuged at 14,000 rpm for 2 min to remove the precipitate. The supernatants were mixed with ninhydrin reagent (19 ml ninhydrin solution contains 5 ml 1% ninhydrin in 0.5 M citrate buffer, pH 5.5, 12 ml glycerol, 2 ml 0.5 M citrate buffer, pH 5.5) and incubated at 100 °C for 15 min. The absorbance of the cooled sample was recorded at 570 nm. One unit of BSH activity was defined as the amount of enzyme that released 1 μmol of taurine/glycine from the substrate per minute. Protein concentration was determined by the Bradford method [39] using bovine serum albumin (BSA) as the standard.

2.7. Detection of optimum pH for enzymatic reaction

The pH dependency of BSH was examined using the 20 mM sodium phosphate buffer (pH 5–8). Enzyme activity was analyzed using 10 mM TDCA for BSH1 and 10 mM GDCA for BSH2.

2.8. Steady-state kinetics of bile salt hydrolase

BSH substrate specificity was determined using six major bile salts. Hydrolysis of conjugated bile salt was determined at 37 °C for 30 min in a 20 mM phosphate buffer (pH 6.0). The amount of amino acid released due to enzymatic reaction was determined by enzyme activity assay described previously. The cooperativities of enzymes were described by the Hill equation (Eq. (1)).

$$v = \frac{V_{max}[S]^h}{[S]^h + S_{0.5}^h} \quad (1)$$

where v is the initial velocity, h is the Hill coefficient, and $S_{0.5}$ is the substrate concentration at which v is half-maximal.

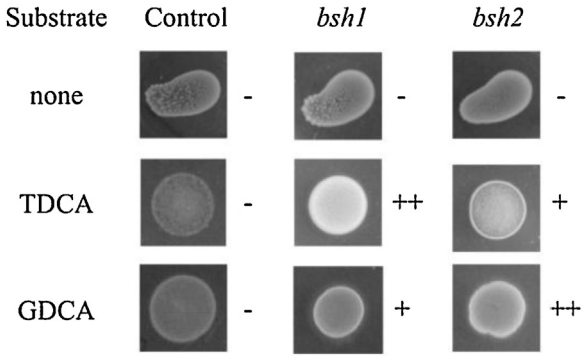

Fig. 1. Detection of BSH activity in *E. coli*. none, no bile salts were added; control, strain with empty vector; –, no BSH activity; +, BSH activity with production of opaque white colonies; ++, high BSH activity with formation of precipitation.

3. Results

3.1. Cloning and over-expression of bile salt hydrolase in E. coli

L. salivarius LMG14476 genes *bsh1* and *bsh2* were amplified by PCR using specific primers mentioned in "Materials and methods". *bsh1* was inserted at the *Bam*H I and *Xho* I sites of pET-20b(+), resulting in pET21. Then the DNA sequences between *Nde* I and *Bam*H I in pET21 was removed by mutation PCR. The resulting plasmids pETM21 for overexpression of *bsh1* was then obtained. *bsh2* was inserted at the *Nde* I and *Xho* I sites of pET-20b(+) plasmids directly, resulting in pET22. The recombinant BSH1 and BSH2 activity in *E. coli* were detected by the plate assay method and successfully demonstrated convincing expression of BSH1 and BSH2 in *E. coli* (Fig. 1). The plate assay also demonstrated that the above enzymes could hydrolyze both GDCA and TDCA.

3.2. Purification and characterization of BSH1 and BSH2 enzymes

A two-step chromatographic procedure was performed for purification of the BSH enzymes from *E. coli*. A single protein band (with molecular mass around 36 kDa) indicating either the BSH1 or BSH2 was obtained after SDS-PAGE analysis of purified protein samples (Fig. 2). The molecular weight (M_w) of the pure native BSH1 and BSH2 were determined to be approximately 140 kDa and 142 kDa by gel filtration chromatography, respectively, suggesting that BSH1 and BSH2 were both homo-tetramer proteins. Detection

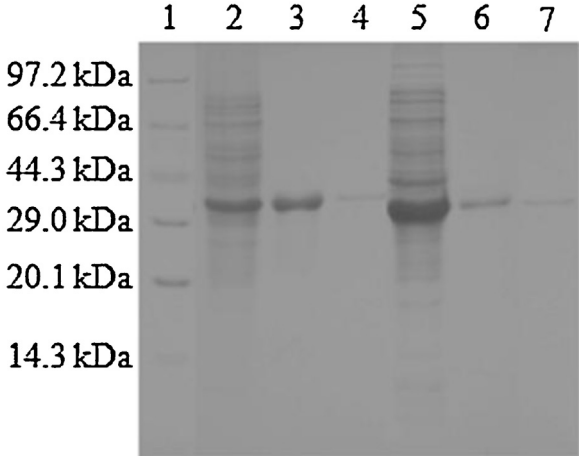

Fig. 2. Purification of BSH1 and BSH2. Lane 1, protein marker; Lane 2, crude extract of BSH1; Lane 3, IMAC-purified BSH1; Lane 4, gel filtration-purified BSH1; Lane 5, crude extract of BSH2; Lane 6, IMAC-purified BSH2; Lane 7, gel filtration-purified BSH2.

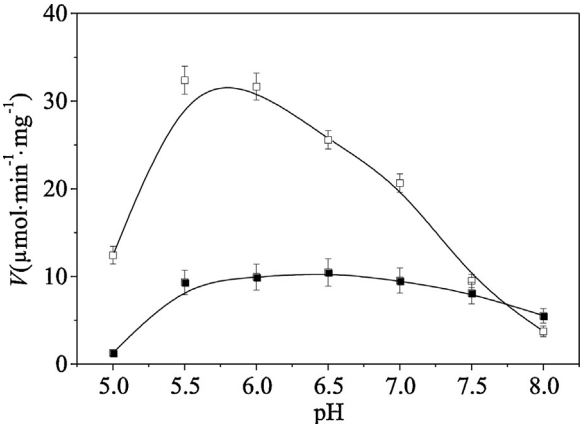

Fig. 3. Optimal reaction pH of BSH1 and BSH2. Solid symbols: BSH1 (sodium taurodeoxycholate as substrate); open symbols: BSH2 (sodium glycodeoxycholate as substrate).

of the optimum pH of enzyme catalysis (Fig. 3) showed the activity of BSH1 displayed a slight shift in a broad pH optimum over the range from pH 5.5 to 7.0, BSH2 was observed to be active in a narrower pH optimum over the range from pH 5.5 to 6.0.

3.3. Kinetic properties of BSH1 and BSH2 enzymes

BSHs from gut microbes are highly diverse in biochemical properties, especially in substrate specificity. To characterize their catalytic ability and affinity to substrate, the kinetic analysis of BSH1 and BSH2 from *L. salivarius* was performed using six conjugated bile salts as the substrates. As shown in Fig. 4, both BSH1 and BSH2 exhibited catalytic abilities against a broad range of substrates, which is similar to other reported BSHs [35,40]. Interestingly, the maximal reaction velocity catalyzed by BSH2 for all substrates was higher than that of BSH1. In addition, plots of substrate concentration against observed catalytic rate in Fig. 5 showed sigmoidal curves, indicating allosteric behavior of BSHs in the reactions.

To obtain and compare their cooperativities, the reaction velocity data were subjected to a simple kinetic analysis by fitting to the Hill equation (Eq. (1)). According to this formula, the parameters derived from the kinetics were summarized in Table 1. As shown in Table 1, values of the Hill coefficient were greater than 1.0, suggesting that the catalysis of all tested bile substrates by BSH1 and BSH2 were positively cooperative. The cooperativity of BSH1 to G-CBAs was lower than that of BSH2, whereas both enzymes share similar cooperativity to T-CBAs. Moreover, the Hill coefficiency for catalysis of GCDCA was the highest among all tested substrates despite of BSH type, indicating that more subunits of BSH enzyme were required to cooperate for catalysis of GCDCA. In contrast to the higher cooperativity of BSH1 to TDCA than that of BSH2, the cooperativities of BSH1 to CA, CDCA units and GDCA were all lower than that of BSH2. The values of k_{cat} in Table 1 demonstrated that the catalytic efficiency of BSH2 was higher than BSH1. Furthermore, catalytic efficiencies of BSH1 and BSH2 to T-CBAs were both distinctly higher than that to G-CBAs. The substrate specificities of BSH1 and BSH2 enzymes, reflected by the second-order rate constant $k_{cat}/K_{0.5}$, indicated higher preference of BSH2 to six substrates than that of BSH1. Among these tested bile salts, BSH1 exhibited the strongest preference to TCA. Asfor BSH2, much more preference was exerted to GDCA.

3.4. Kinetic behaviors of BSHs in the presence of dithiothreitol

In previous studies, dithiothreitol (DTT) (10 mM, final concentration) was added in the enzyme reaction during the

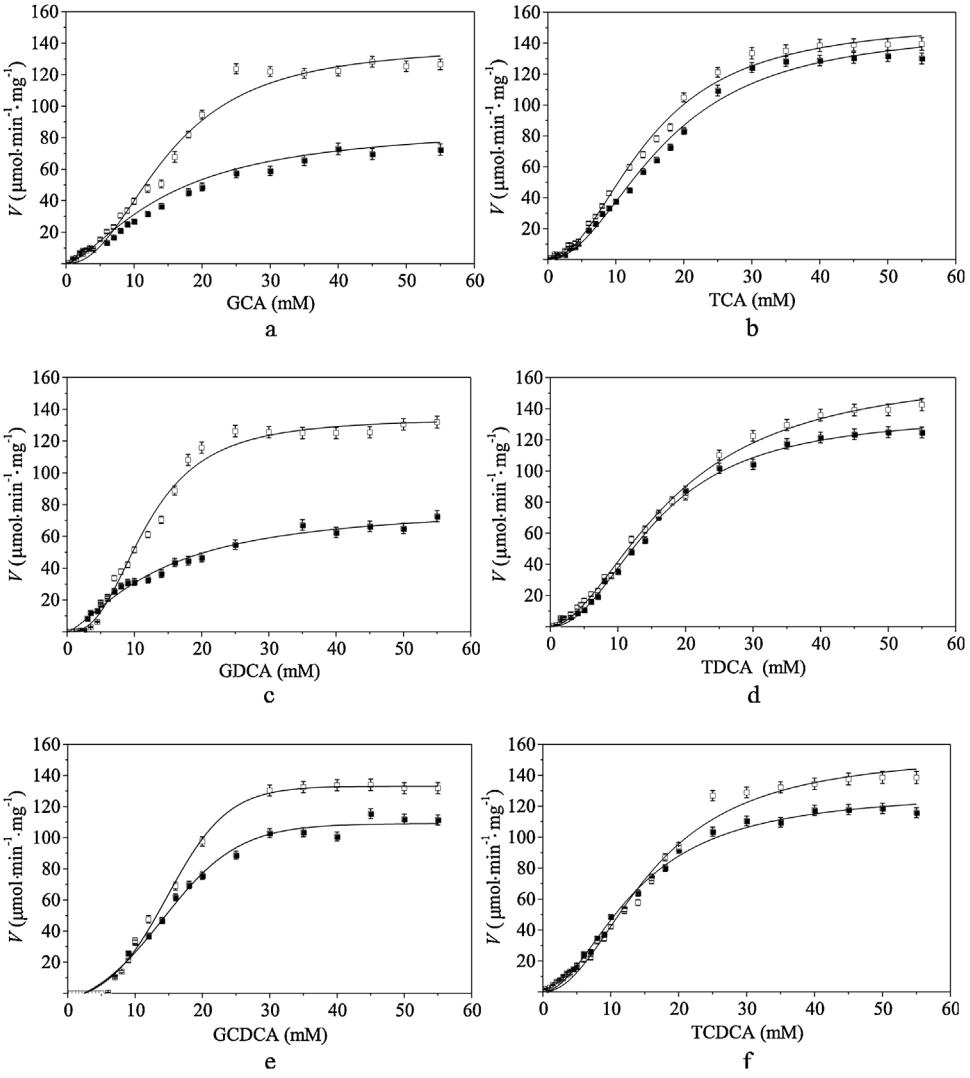

Fig. 4. Kinetic analysis of BSHs. Solid symbols, BSH1; open symbols, BSH2; (a) GCA; (b) TCA; (c) GDCA; (d) TDCA; (e) GCDCA; (f) TCDCA.

kinetic detection process for protection of the sulfhydryl group in BSH enzyme [13]. BSHs in those reactions were taken as Michaelis–Menten enzyme. However, the kinetic data obtained above for BSH1 and BSH2 were evaluated in the absence of DTT. Therefore, the kinetics of BSH1 and BSH2 when catalyze GCDCA in the presence and absence of DTT were compared. Surprisingly, the kinetic sigmoidal curves for both BSH1 and BSH2 when catalyze GCDCA were disappeared and replaced by the kinetic curves similar to Michaelis–Menten enzyme in the presence of DTT. This DTT triggered changes in enzyme kinetics for allosteric enzymes was also detected for BSHs from other bacteria [7,9,13,40,41]. Further analysis on kinetics constants (Table 2) indicated that the presence of DTT increased BSH substrate affinity and reduced maximum velocity for both BSH1 and BSH2. In addition, BSH1 catalysis cooperativity was increased by addition of DTT while it is vice versa for BSH2 (showing in reduced Hill coefficiency, Table 2).

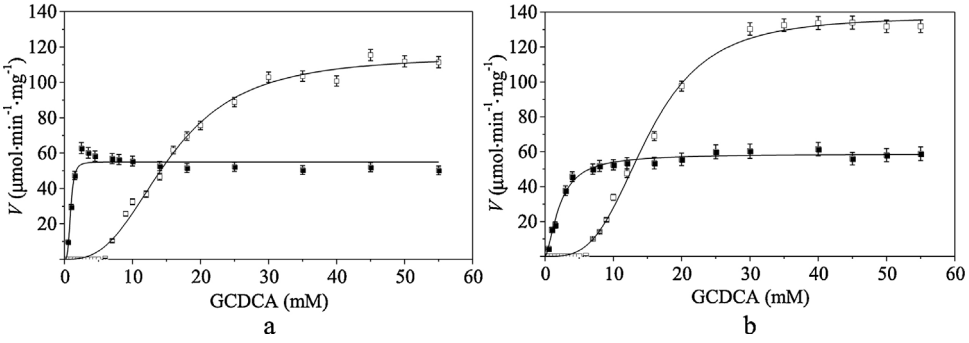

Fig. 5. Kinetic curves of BSHs in GCDCA in the presence or absence of DTT. Steady-state kinetics for BSH1 (a) and BSH2 (b), in the presence of DTT (solid symbols) and absence of DTT (open symbols).

Table 1
(a) Steady-state kinetics constants of BSH1. (b) Steady-state kinetics constants of BSH2.

Substrates	h	$S_{0.5}$ (mM)	V_{max} (μM min^{-1} mg^{-1})	k_{cat} (s^{-1})	$k_{cat}/S_{0.5}$ (mM^{-1} s^{-1})
(a)					
GCA	1.49 ± 0.08	15.02 ± 0.97	88.30 ± 3.15	53.82 ± 1.92	3.58 ± 0.10
GDCA	1.38 ± 0.12	14.12 ± 1.54	80.00 ± 4.54	48.76 ± 2.77	3.45 ± 0.18
GCDCA	2.81 ± 0.18	15.48 ± 0.43	114.96 ± 2.46	70.07 ± 1.50	4.53 ± 0.03
TCA	2.04 ± 0.13	17.16 ± 0.83	150.18 ± 5.03	91.53 ± 3.07	5.33 ± 0.08
TDCA	2.11 ± 0.09	15.77 ± 0.45	136.43 ± 2.59	83.15 ± 1.58	5.27 ± 0.05
TCDCA	1.91 ± 0.08	13.57 ± 0.47	129.66 ± 2.77	70.03 ± 1.69	5.16 ± 0.05
(b)					
GCA	2.23 ± 0.20	15.04 ± 0.84	139.19 ± 5.35	83.77 ± 3.22	5.57 ± 0.10
GDCA	2.63 ± 0.19	11.88 ± 0.39	134.37 ± 3.02	80.87 ± 1.82	6.81 ± 0.07
GCDCA	3.35 ± 0.17	15.05 ± 0.31	137.23 ± 1.88	82.59 ± 1.13	5.49 ± 0.04
TCA	2.06 ± 0.11	14.83 ± 0.54	154.70 ± 3.71	93.11 ± 2.32	6.28 ± 0.07
TDCA	1.82 ± 0.07	18.12 ± 0.72	165.20 ± 4.33	99.43 ± 2.61	5.49 ± 0.07
TCDCA	2.10 ± 0.15	15.92 ± 0.81	154.76 ± 5.41	93.14 ± 3.26	5.85 ± 0.09

Table 2
Steady-state kinetics constants of BSHs with addition of DTT.

BSH	h	$S_{0.5}$ (mM)	V_{max} (μM min^{-1} mg^{-1})	k_{cat} (s^{-1})	$k_{cat}/S_{0.5}$ (mM^{-1} s^{-1})
BSH1	3.65 ± 0.96	0.91 ± 0.07	55.00 ± 1.26	33.52 ± 0.76	36.84 ± 2.01
BSH2	1.59 ± 0.13	2.17 ± 0.13	58.69 ± 0.94	35.32 ± 0.57	16.28 ± 0.71

Note: The substrates were sodium glycochenodeoxycholate (GCDCA) both for BSH1 and BSH2.

4. Discussion

The unique kinetic mechanism of bile salt hydrolase from *L. salivarius* was delineated by identification of their hitherto unsuspected phenomena relating to allosteric behavior as well as allosteric regulation in the reaction in this work. The results confirmed that both BSH1 and BSH2 from *L. salivarius* LMG14476 displayed allosteric behaviors in the absence of DTT, whereas kinetic curves in the presence of DTT for BSHs were consistent with the patterns proposed for other BSHs examined in previous studies [5], showing the typical hyperbolic curve similar to Michaelis–Menten enzyme. Although addition of DTT might be used for keeping the SH-group of Cys2 in BSH active site partially under reducing conditions to guarantee the native protein form [40], the allosteric features in kinetics of BSH appeared to be affected in the presence of DTT. The lost allosteric behavior indicated positive cooperativity in the presence of DTT, which may be important for bile salt hydrolase to adapt to different CBA combinations in bile, in terms of the composition of CBAs is variable at different organism stage or in different individual [28]. Notice shall be paid that BSH kinetics interpreted via Hill equation may not be sufficient due to critical micelle formation of bile salts at concentrations ranging 12.9 mM and 13.8 mM [46,47]. Those Hill coefficients are comparable in order to compare BSH1 and BSH2 cooperativities with the same substrate. Whether micelle formation will affect the enzyme reaction and how it affects the interactions between BSHs and substrates need to be further confirmed.

Previous work on probiotics and related functional genes has demonstrated diverse characteristics of different BSHs. However, the catalytic properties of BSH have not been clarified, especially their different substrates preference [20,34,42–44]. This paper has uncovered the striking differences in positive cooperativity, catalytic efficiency and substrate preference of BSH1 and BSH2 from the same *L. salivarius* strain. Both of them were characterized as homo-tetramers. BSH1 had a broader pH optimum than BSH2. BSH1 displayed stronger catalytic ability and substrates preference to T-CBAs than that to G-CBAs, whereas BSH2 showed higher catalytic ability and preference to all CBAs than that of BSH1. The best substrate for BSH1 and BSH2 are TCDCA and GDCA, respectively.

Given the fact that there are more than one type of conjugated bile salt existing in the GI tract [28], excessive deconjugation of bile in the gut may be linked with "contaminated small bowel syndrome"[4]. Thus, the different substrates preference of BSH could be employed as one of the key criteria for selecting probiotic lactobacilli, in order to provide a possible protective effect on hypercholesterolemia or obesity. The proposition of allosteric behaviors regarding to BSH provided further evidence to explain the co-exultance of both an active site and one substrate binding pocket in the enzyme crystal structure [35,40]. This discovery may give a new insight on catalytic mechanism of BSH and provide some referential significance on enzyme structure–function relationship studies. Moreover, the findings may give a new suggestion in requirement of BSH activity on selection of probiotics or development of pharmaceutical protein to maintain fitness and for wellbeing. Moreover, for better understanding of the biological functions of bile salt hydrolase in lowering cholesterol which are necessary for healthy living [32,45], the relations between BSH enzyme catalysis and protein structures will be further investigated, as well as the biophysical functions.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.06.010.

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