Short communication: A sensitive method for qualitative screening of bile salt hydrolase-active lactobacilli based on thin-layer chromatography

C. F. Guo,* L. W. Zhang,*¹ X. Han,* J. Y. Li,* M. Du,* H. X. Yi,* Z. Feng,† Y. C. Zhang,* and X. R. Xu*

*School of Food Science and Engineering, Harbin Institute of Technology, Harbin, Heilongjiang 150090, China

†College of Food Science, Northeast Agricultural University, Harbin, Heilongjiang 150030, China

ABSTRACT

A sensitive protocol based on thin-layer chromatography (TLC) was developed to screen qualitatively bile salt hydrolase (BSH)-active lactobacilli. The sodium salts of glycocholic acid and taurocholic acid were used as substrates, and bacterial BSH activity was confirmed by detecting cholic acid as a product of the bile conjugates using a TLC assay with direct visual observation. Forty-five lactobacilli isolated from human fecal samples were tested for BSH activity by the TLC assay, a conventional plate assay, and a quantitative colorimetric assay. With the TLC and quantitative colorimetric assays, the same 24 BSH-positive strains were detected. No false-positive or false-negative results were detected by the TLC assay. However, only 20 BSH-positive strains were detected with the conventional plate assay. Compared with the conventional plate assay, the TLC assay is more sensitive for the detection of BSH activity of lactobacilli and, thus, more suitable for screening of BSH-active lactobacilli of human origin.

Key words: bile salt hydrolase, thin-layer chromatography, plate assay, lactobacilli

Short Communication

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 taurine-conjugated bile acids into AA residues and free bile acids (Kim et al., 2004). Bile salt hydrolase acts upon a wide range of bile acid conjugates, including 6 major human conjugated bile acids [taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), and glychenodeoxycholic acid (GCDCA)]. To date, BSH activity has been detected only in bacteria, and is expressed primarily by several bacterial species of the gastrointestinal tract, such as

Bacteroides spp., *Clostridium* spp., *Enterococcus* spp., *Bifidobacterium* spp., and *Lactobacillus* spp. (Begley et al., 2006).

Lactobacilli are normal inhabitants of the human intestine, and have been often used as probiotic agents due to the wide range of consumer benefits associated with their consumption, such as the treatment and prevention of enteric infections, prevention of colorectal cancer, and treatment of irritable bowel syndrome (Lebeer et al., 2008). Certain strains of lactobacilli that possess BSH activity also have the capacity to decrease serum cholesterol levels in animals (De Smet et al., 1998; Nguyen et al., 2007). Thus, the ability to hydrolyze bile salts has often been included among the selection criteria for probiotic strains with cholesterol-lowering properties (Pereira et al., 2003).

Bile salt hydrolase activity has been quantified by measuring bile acid conjugates using HPLC (Corzo and Gilliland, 1999b) or free acids using spectrophotometry (Walker and Gilliland, 1993). The activity also can be measured quantitatively by measuring the amount of taurine and glycine that has been released from the bile acid conjugates using colorimetric assay (Liong and Shah, 2005a; Noriega et al., 2006). Although quantitative assays are reliable, these remain complicated and time-consuming. Therefore, a plate assay as a qualitative detection method has been widely used to mitigate labor intensity. The plate assay was developed by Dashkevich and Feighner (1989) and TDCA was used as a substrate with this method. However, Certain *Lactobacillus* strains with BSH activity lack the ability to deconjugate TDCA (Christiaens et al., 1992; Moser and Savage, 2001), with the result that false-negative outcomes would occur when using the plate assay for detection of BSH activity in such strains.

Cole and Fuller (1984) reported that certain strains of lactobacilli were able to hydrolyze glycine-conjugated, but not taurine-conjugated bile acids. In contrast, Fang et al. (2009) found that certain strains of lactobacilli were able to deconjugate taurine-conjugated, but not glycine-conjugated bile acids. However, to our knowledge, no BSH-active strain of lactobacilli exists that cannot deconjugate either GCA or TCA. On the basis

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¹Corresponding author: lanweizhang@yahoo.com.cn

of this finding, we hypothesized that use of a mixture of GCA and TCA as substrates might provide a more sensitive method to screen BSH-active lactobacilli. The objectives of this study were to develop a sensitive method for qualitative screening of BSH-active lactobacilli using a mixture of GCA and TCA as substrates based on thin-layer chromatography (TLC) assay.

The 45 strains of lactobacilli used in this study were isolated from 27 human fecal samples using LAMVAB agar (Hartemink et al., 1997) and were identified on the basis of Gram staining, catalase reaction, and carbohydrate fermentation patterns using Biolog System (Biolog, Hayward, CA). *Lactobacillus acidophilus* ATCC 43121 was used as a positive control (Brashears et al., 1998) and *Escherichia coli* ATCC 25922 as a negative control (Begley et al., 2006). The strains were cultured in de Man, Rogosa, and Sharpe (MRS) broth and stored at 4°C. Prior to testing, all strains were subcultured twice at 37°C for 18 h.

For the TLC assay of BSH activity, overnight cultures were harvested by centrifugation at $8,000 \times g$ for 10 min at 4°C, and the cell pellets were resuspended in a volume of Ringer's solution equal to that of the original broth. Subsequently, 1 mL of the bacterial cell suspension or distilled water (control) was added to 1 mL of a reaction mixture. The reaction mixture was prepared by dissolving MRS dehydrated media (54 g/L), sodium salt of GCA (5.6 mM), and sodium salt of TCA (2.4 mM) in sodium phosphate buffer (0.2 M, pH 7.4) and adjusting the final pH to 7.4 with 5 M NaOH. The mixtures were incubated anaerobically for 8 h at 37°C. After incubation, the mixtures were evaporated by lyophilization and the residues were redissolved in 1 mL of methanol. Following centrifugation ($8,000 \times g$, 10 min), the supernatant (3 μ L) was spotted onto TLC plates (20 cm $\times$ 20 cm Silica gel 60, Merck, Darmstadt, Germany). Standard solutions [sodium salt of GCA, TCA, and cholic acid (CA) in methanol, 5 mM each] were analyzed using an identical procedure. The TLC plates previously saturated with mobile phase vapor were developed at $18 \pm 1^\circ\text{C}$. The mobile phase consisted of isoamyl acetate–propionic acid–n-propanol–water (40:30:20:10). All solvents used were of analytical grade, purchased from Merck. When the solvent front was 1 to 2 cm from the top edges of the plates (3–4 h), the plates were removed from the jars and dried in an oven at 110°C for 3 min. Bile acids were located on the TLC plates by spraying with 10% (wt/vol) phosphomolybdic acid (Sigma-Aldrich, St. Louis, MO) in ethanol and heating at 110°C for 5 min (Hofmann, 1962). The BSH activity of a strain was regarded as positive when significantly robust CA spots appeared on the TLC plates as compared with that observed in the control.

To assess the sensitivity of the TLC assay, BSH activity was also tested with the conventional plate assay (Dashkevicz and Feighner, 1989) and the quantitative colorimetric assay (Tanaka et al., 2000). For the plate assay, overnight cultures were streaked on MRS agar plates supplemented with 0.5% (wt/vol) sodium salt of TDCA (Sigma-Aldrich). The plates were then incubated anaerobically for 72 h at 37°C. The BSH activity of strains was regarded as positive when deoxycholic acid (DCA) precipitated in the agar medium below and around a colony. For the colorimetric assay, a human bile salt mixture (9.4 mM) was used as a substrate. The mixture consisted of sodium salts of TCA, TCDCA, TDCA, GCA, GCDCA, and GDCA (Sigma-Aldrich) with molar rates of 12:12:8:23:23:16. The BSH activity of strains was expressed as the specific activity (U/mg of protein).

For the plate and TLC assays, experiments were repeated twice, and results reported on the basis of direct visual observations as described above. For the colorimetric assay, experiments were repeated 3 times. Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL). Significant differences between treatments were tested by ANOVA followed by the Fisher least significant difference (LSD) test with a level of significance of $P < 0.05$.

A TLC plate obtained with the use of this method for detection of BSH activity of strains is shown in Figure 1. For the uninoculated control sample (lane 2), a very weak CA spot appeared, which was the result of a slight impurity (CA) in the substrate sodium salts of GCA or TCA. The negative control strain (ATCC 25922, lane 8) and BSH-negative strains [F0122 (lane 3) and F0222 (lane 6)] produced a CA spot with an intensity similar to that of the uninoculated control sample. The positive control strain (ATCC 43121, lane 7) and BSH-positive strains [F0121 (lane 4) and F0221 (lane 5)] produced very robust CA spots on the plate as compared with the negative control or uninoculated control.

Forty-five lactobacilli were tested for BSH activity by the TLC, plate and quantitative colorimetric assays, and results are presented in Table 1. Results obtained from the TLC assay correlated well with that from the quantitative colorimetric assay. The same 24 strains were found to be BSH-positive with both methods. However, only 20 strains were found to be BSH-positive with the plate assay. Of the 24 BSH-positive strains as detected with the TLC assay, 4 strains (F0821, IN 3.21, IN 7.21, and F1623) failed to show BSH activity with the plate assay. Among these 4 strains, 3 (F0821, IN 3.21, IN 7.21) were found to be BSH negative with the plate assay and for the remaining strain (F1623),

BSH activity was undetectable because it was unable to grow on the MRS agar supplemented with sodium salt of TDCA.

To understand the basis for the discrepancy in results between the TLC and plate assays, the substrate specificity of the BSH from these 3 strains (F0821, IN 3.21, IN 7.21) was assessed with the colorimetric assay using the 6 individual conjugated bile salts at a 9.4 mM substrate concentration as described by Tanaka et al. (2000). As shown in Table 2, all 3 strains lacked the ability to hydrolyze TDCA, whereas they were able to hydrolyze GCA and TCA, with the exception of strain F0821, which was able to hydrolyze GCA, but not TCA.

In the majority of cases, lactobacilli with BSH activity are able to hydrolyze simultaneously many types of human conjugated bile acids (Lundeen and Savage, 1990; Lambert et al., 2008). However, some exceptions to this generalization exist. Moser and Savage (2001) reported that *L. buchneri* JCM 1069 expressed BSH activity against TDCA, but not against TCA. Christiaens et al. (1992) found that *L. brevis* ATCC 14869 exhibited BSH activity toward GDCA, but not toward TDCA. These findings suggest that it is possible for certain BSH to recognize bile acids on both the cholate steroid nucleus and the amino acid groups. In the present study, strains IN3.21, IN7.21 and F0821 were able to hydrolyze only bile acids with the CA, CA and CDCA, and glycin group, respectively (Table 2).

The BSH-positive strains detected by the TLC assay were the same as those detected by the quantitative colorimetric assay, indicating that all the BSH-positive strains were able to deconjugate GCA and TCA. Both GCA and TCA, as primary bile acids, predominate in the human bile as compared with TDCA, which exists as a secondary bile acid (Staggers et al., 1990). Thus, both GCA and TCA are often used as an indicator of human bile acids when studying bacterial BSH activity (Corzo and Gilliland, 1999a; Ramasamy et al., 2010). Although BSH activity is often found in intestinal lactobacilli, its exact biological significance is still unclear. Several hypotheses have been proposed suggesting that BSH activity benefits bacterium by enhancing its resistance to conjugated bile salts and increasing its survival in the gastrointestinal tract for colonization (Ridlon et al., 2006; Jones et al., 2008). The property that BSH-positive strains of lactobacilli prefer to deconjugate GCA or TCA may underlie their ability to enhance such potential benefits.

The finding that distinct spots can be yielded on TLC plates at very low levels of CA (0.5 µg), indicates that BSH activity of cultures can be detected by the TLC assay with as little as 5% of substrate hydrolyzation (data not shown). Although spots of bile

Figure 1. Typical pattern of bile salt hydrolase (BSH) activity from several strains of lactobacilli as detected by the thin-layer chromatography (TLC) assay. A solution of the samples was spotted manually on the TLC plate in a volume of 3 µL. Lane 1, mixed bile acid standard [glycocholic acid (GCA), taurocholic (TCA), and cholic acid (CA) in methanol, 5 mM each]; lane 2, uninoculated control (1 mL of sterile distilled water was added to the reaction mixture instead of the bacterial suspension); lane 3, strain F0122; lane 4, strain F0121; lane 5, strain F0221; lane 6, strain F0222; lane 7, strain ATCC 43121 (positive control strain); lane 8, strain ATCC 25922 (negative control strain).

acids can also be visualized by spraying the TLC plate with an aqueous solution of concentrated sulfuric acid (Eneroth, 1963), we found this approach to lack the sensitivity as that obtained with the ethanol solution of phosphomolybdic acid. The weakly corrosive chemical nature of phosphomolybdic acid makes it safer for use as a TLC visualization reagent, as opposed to sulfuric acid, which is a strongly corrosive chemical that can cause severe burns and tissue damage when contacting skin or mucous membranes. Given these considerations, phosphomolybdic acid was used as a visualization reagent in this study.

In humans, bile acid deconjugation mainly occurs in the ileum. The mean ileac pH is approximately 7.4 (Evans et al., 1988; Ibekwe et al., 2008), and the mean bile acid concentration in the ileum is approximately 4 mM with the ratio of glycoconjugates to tauroconjugates usually (2–3):1 (Liong and Shah, 2005b). The pH and concentration of the substrates used in the present TLC assay simulated physiological conditions of the human ileum. Accordingly, the results obtained from this in vitro test might be similar to those from the in vivo test.

In conclusion, the TLC assay as described in this study was capable of detecting more BSH-positive

Table 1. Comparison of different methods for detection of bile salt hydrolase (BSH) activity of *Lactobacillus* strains

Strain code	BSH activity		
	Qualitative assay		Quantitative assay
	Plate assay ¹	TLC assay ²	Colorimetric assay ³ (U/mg of protein)
F1022	P(p)	P	2.00 ± 0.34*
F1421	P(p)	P	1.82 ± 0.19*
F0521	P(p)	P	1.81 ± 0.33*
F0822	P(p)	P	1.63 ± 0.36*
F1821	P(w)	P	1.47 ± 0.27*
IN2.22	P(p)	P	1.40 ± 0.21*
F0821	N	P	1.30 ± 0.24*
IN5.22	P(p)	P	1.25 ± 0.14*
F0422	P(p)	P	1.08 ± 0.17*
F0122	P(p)	P	0.93 ± 0.17*
F0121	P(p)	P	0.85 ± 0.20*
F1722	P(p)	P	0.80 ± 0.17*
IN1.21	P(w)	P	0.74 ± 0.19*
IN3.21	N	P	0.68 ± 0.07*
F1222	P(p)	P	0.59 ± 0.14*
IN2.21	P(p)	P	0.56 ± 0.14*
F0921	P(p)	P	0.52 ± 0.09*
F0621	P(w)	P	0.46 ± 0.07*
IN6.21	P(p)	P	0.39 ± 0.07*
F1623	0	P	0.39 ± 0.07*
IN7.21	N	P	0.35 ± 0.07*
F1121	P(w)	P	0.33 ± 0.06*
IN9.22	P(w)	P	0.24 ± 0.05*
F0222	P(w)	P	0.23 ± 0.04*
IN3.22	N	N	0.03 ± 0.01
F1721	N	N	0.03 ± 0.02
IN6.22	N	N	0.03 ± 0.01
F0922	N	N	0.02 ± 0.01
F1322	N	N	0.02 ± 0.02
F0421	N	N	0.02 ± 0.02
IN5.21	N	N	0.02 ± 0.03
F1522	0	N	0.02 ± 0.02
IN4.21	N	N	0.02 ± 0.03
F0622	N	N	0.02 ± 0.01
F1321	N	N	0.02 ± 0.03
F1523	N	N	0.01 ± 0.02
IN9.21	0	N	0.01 ± 0.02
F1622	N	N	0.01 ± 0.03
F0221	N	N	0.01 ± 0.03
IN1.22	0	N	0.01 ± 0.03
F1521	N	N	0.01 ± 0.03
F1021	N	N	0.00 ± 0.03
F0321	N	N	-0.01 ± 0.01
F1221	N	N	-0.01 ± 0.03
F0721	N	N	-0.01 ± 0.02
ATCC 43121	P(w)	P	1.76 ± 0.25*
ATCC 25922	0	N	0.01 ± 0.02

¹Capacity to express taurodeoxycholic acid (TDCA) hydrolase activity as detected when precipitates of deoxycholic acid appeared below bacterial colonies growing on de Man, Rogosa, and Sharpe (MRS)-TDCA. 0 = no growth; N = negative; P(p) = positive BSH activity and formation of precipitate halos around colonies; P(w) = positive BSH activity and formation of opaque granular white colonies.

²Thin-layer chromatography capacity to express glycocholic acid (GCA) and taurocholic acid (TCA) hydrolase activity as detected when visible spots of cholic acid (CA) appeared on the thin-layer plates compared with the control. P = positive; N = negative.

³Results are presented as mean ± SD of BSH activity expressed as specific activity.

*Asterisks in the same column indicate statistically significant differences ($P < 0.05$) between the tested and negative control (ATCC 25922) strains.

Table 2. Substrate specificity of 3 isolates toward the 6 individual human conjugated bile salts

Conjugated bile salt ¹	BSH activity ² (U/mg of protein)			
	Strain F0821	Strain IN3.21	Strain IN7.21	Strain ATCC 25922
GCA	0.90 ± 0.12*	0.53 ± 0.11*	0.25 ± 0.07*	0.01 ± 0.01
GCDCA	0.73 ± 0.17*	0.01 ± 0.03	0.85 ± 0.19*	0.00 ± 0.02
GDCA	0.41 ± 0.12*	−0.01 ± 0.02	0.01 ± 0.01	−0.01 ± 0.02
TCA	0.02 ± 0.01	0.26 ± 0.09*	0.22 ± 0.08*	0.02 ± 0.01
TCDCA	0.01 ± 0.02	0.02 ± 0.01	0.51 ± 0.10*	0.00 ± 0.02
TDCA	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.01	0.01 ± 0.02

¹GCA = glycocholic acid; GCDCA = glycochenodeoxycholic acid; GDCA = glycodeoxycholic acid; TCA = taurocholic acid; TCDCA = taurochenodeoxycholic acid; TDCA = taurodeoxycholic acid.

²Results are presented as mean ± SD of bile salt hydrolase (BSH) activity expressed as specific activity. The asterisk (*) in the same row indicates statistically significant differences (*P* < 0.05) between the tested and negative control (ATCC 25922) strains.

strains than that with use of the conventional plate assay. As a result, this TLC assay possesses a higher sensitivity for screening of BSH-active lactobacilli. In addition, unlike the plate assay, this TLC assay does not require that strains grow in the presence of bile salts, and, thus, has a wider application range than the plate assay.

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