Antimutagenicity and the Influence of Physical Factors in Binding *Lactobacillus gasseri* and *Bifidobacterium longum* Cells to Amino Acid Pyrolysates

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ABSTRACT

Antimutagenic and binding properties of 28 strains of *Lactobacillus gasseri* and 2 strains of *Bifidobacterium longum* on the mutagenicity of amino acid pyrolysates were investigated in vitro using a streptomycin-dependent (SD510) strain of *Salmonella typhimurium* TA 98. Four strains of *L. acidophilus* (SBT0274, SBT1703, SBT10239, and SBT10241) and 1 strain of *B. longum* (SBT 2928) exhibited the highest percentage of antimutagenicity and binding. These 5 strains were further optimized for other physical factors influencing the mechanism of binding, such as cell and mutagen concentration, pH, and incubation time. In all of the selected strains, 2 mg of cells bound with 88 to 95% of 0.2 mg of 3-amino-1,4 dimethyl-5*H*-pyrido[4,3-*b*]indole in 30 min at pH 7.0. Other amino acid pyrolysates, such as 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole, 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole, 2-amino-3-methylimidazo[4,5,*f*]quinoline, and 2-amino-3,4-dimethylimidazo[4,5,*f*]quinoline were also tested for the binding ability of these strains. We observed that the complexity of the mutagens greatly influenced the binding properties. The binding of 3-amino-1,4 dimethyl-5*H*-pyrido[4,3-*b*]indole to the purified cell walls was very high compared with that of the crude cell wall, peptidoglycan, or the cell extract. Binding was inhibited when the cell walls were subjected to treatment with metaperiodate or trichloroacetic acid but not when they were subjected to treatment with lysozyme, trypsin, or proteinase K, reflecting the role of the carbohydrate component as a binding site.
(**Key words**: amino acid pyrolysates, antimutagenicity, binding, cell wall)

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Abbreviation key: **Glu-P-1** = 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole, **IQ** = 2-amino-3-methylimidazo[4,5,*f*]quinoline, **MeIQ** = 2-amino-3,4-dimethylimidazo[4,5,*f*]quinoline, **PCW** = purified cell wall, **Trp-P-1** = 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, **Trp-P-2** = 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

INTRODUCTION

Lactic acid bacteria and their fermented products are thought to confer a variety of important nutritional and therapeutic benefits, including antimutagenic and anticarcinogenic properties (3, 5). Hosono et al. (9, 10, 11, 12) and Hosono and Sreekumar (13) have observed antibacterial mutagenicity of fermented milk products and lactic acid bacteria against some amino acid pyrolysates, N-nitroso compounds, and fecal mutagens from various mammals. In addition, Morotomi and Mutai (18) have investigated the in vitro binding to intestinal bacteria of amino acid pyrolysates such as 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole (**Glu-P-1**), 2-amino-3-methylimidazo[4,5,*f*]quinoline (**IQ**), 2-amino-3,4-dimethylimidazo[4,5,*f*]quinoline (**MeIQ**), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (**Trp-P-1**), and 3-amino-methyl-5*H*-pyrido[4,3-*b*]indole (**Trp-P-2**).

Amino acid pyrolytic products that are produced during the broiling of meat and fish are mutagenic (17). The formation of heterocyclic amines during the cooking and processing of protein-rich foods has also been reported (28). These mutagens are encountered in meat preparation involving roasting in direct flame, which is a prevalent culinary practice of various countries. Moreover, studies (16) have shown good correlation between mutagenicity and carcinogenicity.

Lactobacillus acidophilus and *Bifidobacterium bifidum* have received great attention because they reportedly exert inhibitory action toward the common foodborne pathogens (4, 6). Oral supplements of *L. acidophilus* in humans have resulted in reduced ac-

tivities of fecal bacterial enzymes, β-glucuronidase, nitroreductase, and azoreductase involved in procarcinogen activation (6) and have reduced the excretion of mutagens in feces and urine (15). Of the six groups of *L. acidophilus*, the B1 group, *Lactobacillus gasseri*, and *Bifidobacterium longum* of human origin were selected (Snow Brand Milk Co., Tokyo, Japan). This paper attempts to identify the potent strains of *L. gasseri* and *B. longum* for the maximum binding of Trp-P-1 with optimized physical parameters and partial identification of the binding receptors.

MATERIALS AND METHODS

Strains of Lactic Acid Bacteria

Twenty-eight pure culture strains of the *L. gasseri* (strains 1 to 28) and 2 strains of *B. longum* (strains 29 and 30) were obtained from Snow Brand Milk Co. (Tokyo, Japan) The *L. gasseri* strains were cultured and maintained in MRS medium (2), and *B. longum* was cultured as described by Scardovi (19).

Preparation of Mutagen Solutions

The amino acid pyrolysates, Trp-P-1, Trp-P-2, Glu-P-1, IQ, and MeIQ were purchased from Wako Pure Chemicals (Tokyo, Japan). All mutagens were prepared to a concentration of 2 mg/ml. The Trp-P-1 was dissolved in distilled water, and the other pyrolysates were dissolved in 20% methanol.

Confirmation of Identity of Cultures

All cultures were tested for Gram stain reaction, catalase production, and the ability to grow at 15 and 45°C. Biochemical tests and various tests for sugar utilization were carried out to determine the identity of lactobacilli and bifidobacteria (19).

Preparation of Freeze-Dried Cells

The 24-h-old cultures of *L. gasseri* and *B. longum* were anaerobically incubated in MRS medium at 37°C for 24 h. After incubation, the cells were harvested by centrifugation at 3000 × *g* for 20 min at 4°C and washed in PBS (pH 7.0) twice, followed by a wash in distilled water. The cells were lyophilized and kept at 4°C until used. The lyophilized cells were serially diluted and tested for viability and purity on MRS agar. All lyophilized cultures were found to be pure cultures. The cultures were viable in the range of 1.85×10^5 to 1.55×10^6 cells/mg.

Preparation of Purified Cell Walls

The cell walls were purified as described by Gopal and Reilly (7). The cells grown in MRS medium were pelleted by centrifugation (10,000 × *g* for 10 min at 4°C), were washed in 20 ml of cold saline solution, and were centrifuged again. The packed cells were suspended homogeneously in 20 ml of cold distilled water, and the cells were disintegrated by ultrasonic vibrations using a sonifier (USP 300; Shimadzu Co., Kyoto, Japan). The whole cells and debris were removed by centrifugation at 1000 × *g* for 10 min at 4°C. Cell walls were sedimented by centrifugation at 15,000 × *g* for 25 min at 4°C, and the supernatant was used as the cell extract. The crude cell-wall fractions were checked microscopically, and a streak of the suspension that was incubated anaerobically at 37°C on MRS agar plates did not show growth. This result indicated the complete disruption of the cells and the sterility of the suspension. The cell walls were further washed two times in 15 m*M* NaCl, followed by a wash in 50 m*M* Tris·HCl, and were diluted to twice the volume with 10 m*M* potassium phosphate buffer (pH 7.0); RNase and DNase each were added to a final concentration of 50 μg/ml. The mixture was incubated at 37°C for 90 min and then centrifuged at 15,000 × *g* for 25 min. The washed cell walls were treated with 20 g/L of SDS and heated at 70°C for 2 h to remove the membrane. The cell walls were washed extensively with distilled water to remove the SDS and residue collected by centrifugation, were lyophilized, and were used as the purified cell-wall (**PCW**) preparation.

Isolation of Peptidoglycan

Peptidoglycans were isolated as described by Tanabe et al. (23). Briefly, 24 mg of the lyophilized cells were suspended in 4 ml of cold 250 g/L of TCA, and the volume was made up to 20 ml by the addition of cold distilled water. After incubation for 20 min at 4°C, the suspension was centrifuged at 4000 × *g* for 5 min. The residue was resuspended in 20 ml of 75% ethanol in water. The mixture was kept at room temperature for 10 min and centrifuged at 4000 × *g* for 10 min. The pellets were resuspended in 20 ml of 50 g/L of TCA, heated to 90°C, maintained for 6 min, and centrifuged again; the supernatant was discarded. To the residue, 7.6 ml of 65 m*M* PBS (pH 6.98) were added and mixed well with 0.4 ml of trypsin solution (containing 1 mg of crystalline trypsin). The mixture was incubated at 37°C for 2 h and then centrifuged, and the residue was lyophilized and used as isolated peptidoglycan.

HPLC

The amino acid pyrolysates were quantified using HPLC (model LC 6A; Shimadzu Co., Kyoto, Japan) equipped with a UV monitor (SPD-6A, Shimadzu Co.) and a reverse-phase column (Shimpack CLC-ODS 6 mm × 15 cm; Shimadzu Co.). A mobile phase of 0.1 *M* citrate-0.2 *M* Na_2HPO_4 (pH 3.0), acetonitrile, and triethylamine (50:50:0.05, vol/vol/vol) was employed for Trp-P-1 and Trp-P-2. For Glu-P-1, IQ, and MeIQ, the mobile phase ratio was 65:35:0.05 (vol/vol/vol) at a flow rate of 1 ml/min. The absorbance was measured at 254 nm and was recorded using an integrator (model C-R6A, Chromatopack; Shimadzu Co.).

In Vitro Binding Assay

During the preliminary screening, 3 mg of lyophilized cells were suspended in 0.95 ml of distilled water, and 0.05 ml of Trp-P-1 (100 μg) was added. Simultaneously, the positive controls were run without lactic acid bacteria cells. After thorough mixing, the mixtures were incubated at 37°C in a shaking water bath for 30 min, then centrifuged at 6000 × *g* for 10 min, and filtered using a microfilter (pore size 0.45 μm; Nacali Tesque, Tokyo, Japan). The filtrate was mixed with an equal volume of acetonitrile, and 20 μl of the mixture was injected into the HPLC for the detection of the unbound mutagen. All assays were repeated three times; triplicate and mean values are reported.

The binding rate was calculated as 1 – (peak area of the Trp-P-1 solution with the cells per peak area of Trp-P-1 solution without the cells) × 100.

Assay for Antimutagenicity

The streptomycin-dependent strain SD510 of *Salmonella typhimurium* TA 98 (20) from the culture collection of this laboratory was used as the tester strain. Strain SD510 was grown in Oxoid nutrient broth number 2 (Unipath Ltd., Basingstoke, United Kingdom) that had been fortified with streptomycin at a final concentration of 20 mg/ml (SM20), and this strain was maintained in SM20 agar.

The tester strain SD510 was grown in SM20 broth overnight to an optical density of 1.37 at 600 nm in a shaking water bath at 37°C. The culture was diluted 1000 times with 66 m*M* PBS, pH 6.8, and 0.1 ml of the diluted culture was used in the antimutagenicity assay.

The antimutagenic activity of the whole cell preparation of *L. gasseri* and *B. longum* was estimated by measuring the extent of decrease in mutation induced by Trp-P-1. The antimutagenicity assay was performed by the plate incorporation method (26). In this method, 0.1 ml of SD510 culture, 50 μl of Trp-P-1 solution, and 100 μl of PBS containing 3 mg of lyophilized cells were added and mixed in a sterile test tube. The contents were preincubated at 37°C in a shaking water bath for 30 min. Then, 2 ml of soft agar (25 g/L of Oxoid nutrient broth with 5 g/L of agar) kept at 45°C were added to the preincubated mixture and mixed by gentle vortexing. The soft agar was poured on Oxoid nutrient agar plates (OX broth 25 g/L with agar 15 g/L). The plate was rotated and tilted to achieve a uniform distribution on the base agar. These plates were incubated at 37°C for 48 h, and streptomycin-independent revertants were counted.

In all cases, both positive and negative controls were included. Positive controls consisted of SD510, mutagen solution, and no lactic acid bacteria cells. The negative control had SD510, bacterial cells in PBS of the same quantity, and distilled water instead of the mutagen.

The viability of tester strains was monitored by plating the diluted culture on SM20 agar on which all streptomycin-dependent and streptomycin-independent tester organisms could form colonies. The counts were tabulated after subtraction for natural revertants obtained from negative controls. The results are expressed as the number of revertants per plate. All assays were repeated three times; triplicate and mean values are reported.

Chemical and Enzymatic Treatment of the Cell Wall

Isolated PCW (1 mg/ml) was treated with 10% TCA (wt/vol) for 15 min at 100°C or with sodium metaperiodate at 4°C for 24 h. The excess periodate at the end of the reaction was destroyed by the addition of a drop of ethylene glycol. After the treatment, the cell-wall materials were centrifuged (10,000 × *g* for 10 min), washed once in distilled water, and used for the binding assay. For the enzyme treatment, 1 mg of PCW was suspended in PBS and digested with lysozyme (30,000 units/ml), trypsin (1 mg/ml), or proteinase K (1 mg/ml) at 37°C in a shaking water bath (120 rpm) until lysis occurred as monitored by measuring the decrease in optical density of the reaction mixture at 600 nm. The digested PCW were tested for binding properties.

Statistical Analyses

Statistical analyses were used to determine whether the impact of a particular physical factor was

significant or whether significant interaction with other dependent variables existed with the binding properties. The binding percentage was analyzed by one-way ANOVA and modified least significant difference (Bonferroni *t* test) was used to identify whether differences between the means were significant. The analyses were performed by using SPSS® (21) at 5% confidence. The correlation coefficient between inhibition percentage or pH and binding was also calculated.

RESULTS

All available strains of *L. gasseri* and *B. longum* were tested for their binding properties as well as their antimutagenic properties with Trp-P-1, and the most potent strains were selected. Three milligrams of cells and 100 μg of Trp-P-1 were used for binding, and antimutagenicity was determined using a streptomycin-dependent (SD510) strain of *S. typhimurium* TA98; the results are summarized in Table 1. Variations ($P < 0.05$) existed among the strains in binding properties and antimutagenicity. Nine strains of *L. gasseri* showed a high percentage of binding, and 13 strains showed a high percentage of inhibition against the mutagenicity of Trp-P-1. Of the 2 *B. longum* strains, SBT2928 showed variation ($P < 0.05$) in binding, but the difference in antimutagenicity was nonsignificant. Strain SBT2928 of *B. longum* was selected for further studies along with 4 strains (SBT0274, SBT1703, SBT10239, and SBT10241) of *L. gasseri* that were randomly selected from the

TABLE 1. In vitro binding and antimutagenicity of *Lactobacillus gasseri* and *Bifidobacterium longum* cells with 100 μg of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole.[1]

Strain	Culture no.	Revertants per plate		Inhibition	Binding	
		(no.)		(%)		
		$\overline{X}$	SD	$\overline{X}$	$\overline{X}$	SD
L. gasseri						
1	SBT0267	105	20	86.5^{abc}	89.1^{ef}	0.56
2	SBT0270	305	36	$60.8^{fghjklm}$	88.7^{f}	0.12
3	SBT0273	82	28	89.5^{ab}	89.2^{ef}	0.16
4	SBT0274	45	8	94.2^{a}	95.4^{a}	0.12
5	SBT0276	353	98	54.7^{hjklm}	56.0^{p}	0.32
6	SBT0278	443	41	43.2^{m}	48.5^{r}	0.30
7	SBT0290	417	12	46.5^{lm}	63.5^{m}	0.15
8	SBT0291	317	50	59.4^{ghjklm}	76.4^{j}	1.18
9	SBT0292	395	49	49.7^{klm}	53.4^{q}	0.94
10	SBT0293	323	15	58.9^{ghjklm}	78.9^{h}	0.94
11	SBT0299	265	49	$66.0^{defghjk}$	90.8^{bc}	0.70
12	SBT0311	210	27	$73.1^{bcdefgh}$	76.4^{j}	1.71
13	SBT0312	235	54	$69.9^{cdefghj}$	73.2^{kl}	0.70
14	SBT0315	359	65	53.9^{jklm}	52.9^{q}	0.58
15	SBT0317	189	19	$75.8^{abcdefg}$	72.0^{l}	0.32
16	SBT1702	213	37	$72.7^{bcdefghj}$	75.1^{jk}	0.14
17	SBT1703	75	13	90.4^{ab}	94.7^{ab}	0.06
18	SBT1753	86	28	89.0^{ab}	89.2^{ef}	0.20
19	SBT2054	338	61	56.7^{hjklm}	59.8^{n}	0.34
20	SBT2055	131	29	83.2^{abcd}	93.2^{abc}	0.30
21	SBT2056	132	27	83.1^{abcde}	94.2^{ab}	0.37
22	SBT2057	95	13	87.8^{abc}	93.8^{abc}	0.09
23	SBT2059	110	31	85.9^{abc}	91.5^{cde}	0.08
24	SBT2061	100	36	87.2^{abc}	90.2^{def}	0.31
25	SBT10238	264	43	$64.4^{efghjkl}$	93.9^{abc}	0.39
26	SBT10239	50	13	93.6^{a}	95.3^{a}	0.04
27	SBT10240	81	15	89.6^{ab}	93.7^{abc}	0.02
28	SBT10241	69	20	91.2^{ab}	94.9^{ab}	0.05
B. longum						
29	SBT2928	105	17	86.5^{abc}	91.7^{cd}	0.12
30	SBT2928-S	168	37	78.5^{abcdef}	82.6^{g}	0.19
Positive control		780	126			
r		0.8024				

^{a,b,c,d,e,f,g,h,j,k,l,m,n,p,q,r}Means within the same column with no common superscript letters differ ($P < 0.05$).

[1]Each value is the mean (±SD) of three determinations.

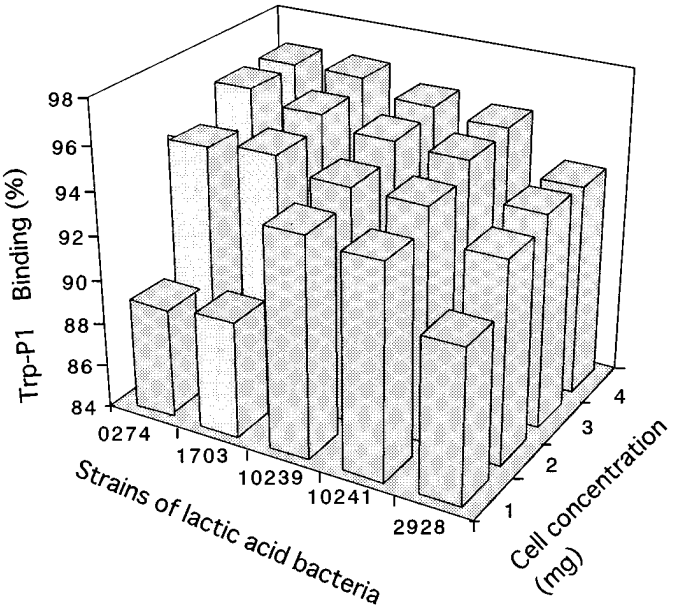

Figure 1. Effect of different cell concentrations of *Lactobacillus gasseri* (SBT0274, SBT1703, SBT10239 and SBT10241) and *Bifidobacterium longum* (strain SBT2928) on the binding of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1). The Trp-P-1 (0.1 mg/ml) was mixed with various amounts of lyophilized cells in distilled water and incubated at 37°C for 30 min. Values are the means of three determinations. Binding versus cell concentration was tested at a significance level of $P < 0.05$.

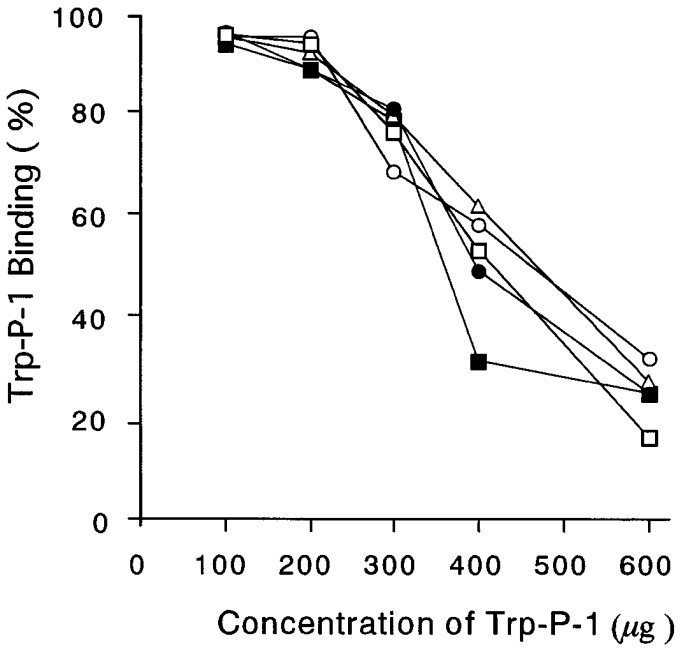

Figure 2. A dose-response curve of the binding of *Lactobacillus gasseri* SBT0274 (□), SBT1703 (●), SBT10239 (○), and SBT10241 (△) and *Bifidobacterium longum* SBT2928 (■) with 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1). Two milligrams of cells were treated with different concentrations of Trp-P-1 (final volume made up to 1 ml with distilled water) at 37°C for 30 min. Values are the means of three determinations. Binding versus mutagen concentration was tested at a significance level of $P < 0.05$.

highest binding group. There was high correlation (r = 0.804) between binding and antimutagenicity. Thus, 4 *L. gasseri* strains and one *B. longum* strain were subjected to analysis to elucidate the role of physical factors influencing the binding mechanism.

The dose-response binding of Trp-P-1 to the lyophilized cells of *L. gasseri* and *B. longum* were investigated by treating various amounts of cells with Trp-P-1 (100 μg), and differences ($P < 0.05$) were observed between the strains (Figure 1). Strains SBT0274, SBT1703, and SBT2928 showed a different ($P < 0.05$) binding properties at 1 mg of cells. The difference was nonsignificant ($P > 0.05$) for all *L. gasseri* strains at 2 mg of cells. Within the strains, the increase of more than 2 mg of cells had no significant effect with strains SBT10239, SBT0274, and SBT2928; but a difference ($P < 0.05$) was observed with strains SBT10241 and SBT1703.

A dose-response curve was drawn to show the relationships of different concentrations of Trp-P-1 with 2 mg of cells. Figure 2 shows that the binding percentage decreased as the concentration of the mutagen increased, and the quantity of the mutagen bound per milligram of cells increased to a mean of 120 μg of Trp-P-1. At lower concentrations of Trp-P-1, the binding was not significant ($P > 0.05$) within the strains, but, at higher concentrations, (300 μg) the difference was significant. Two hundred micrograms of Trp-P-1 and 2 mg of cells were used as the optimum for further studies.

The effect of incubation time on the binding of Trp-P-1 to the lyophilized cells of *L. gasseri* and *B. longum* are represented in Figure 3. Most of the mutagens were bound immediately after the addition of the cells. Strains SBT1703 and SBT0274 showed a different ($P < 0.05$) binding properties with an increase in incubation time up to 30 min, and a difference ($P < 0.05$) was observed with strains SBT10239 and SBT10241 up to 20 min incubation. The *B. longum* strain showed an increase in binding properties with an increase in incubation time up to 30 min. Only strain SBT1703 showed significantly high binding at 40 min of incubation.

The binding properties and pH in the *L. gasseri* and *B. longum* strains were highly correlated (r = 0.9341). Of the pH levels tested, the highest percentage of binding occurred at pH 7.0 (Figure 4). The increase in pH (from 3 to 7) showed variation ($P <$

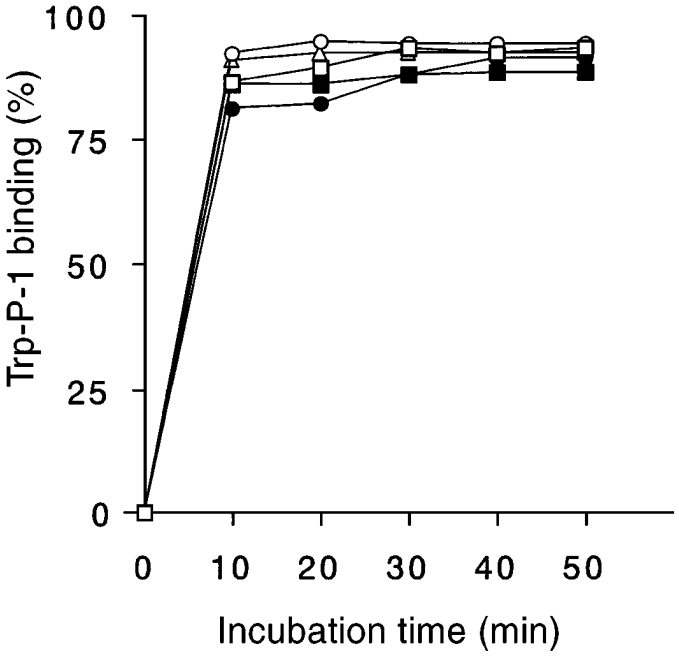

Figure 3. Effect of incubation time on the binding of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) by *Lactobacillus gasseri* SBT0274 (□), SBT1703 (●), SBT10239 (○), and SBT10241 (△) and *Bifidobacterium longum* SBT2928 (■). The 0.2 mg of Trp-P-1 was treated with 2 mg of lyophilized cells in distilled water (total volume 1.0 ml), and the mixture was incubated at 37°C for different times. Values are the means of three determinations. Binding versus incubation time was tested at a significance level of $P < 0.05$.

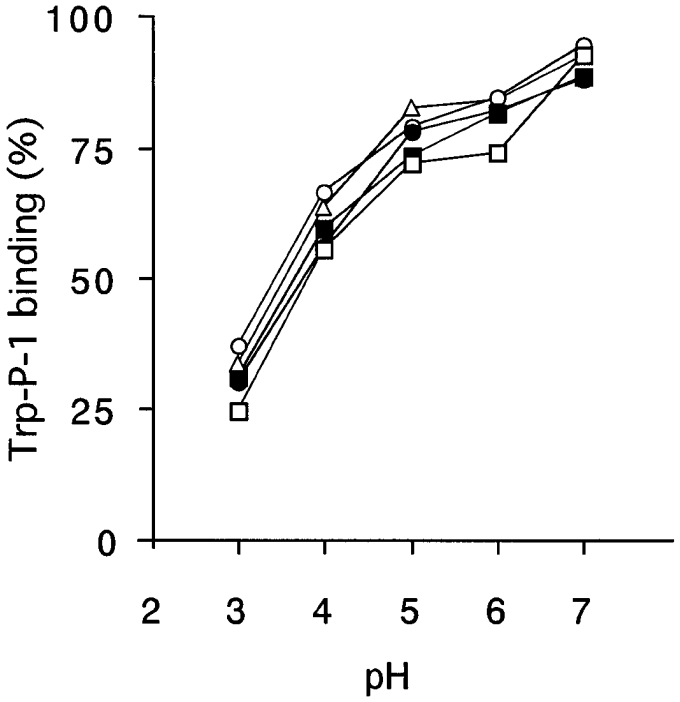

Figure 4. Effect of pH on the binding of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) to the cells of *Lactobacillus gasseri* SBT0274 (□), SBT1703 (●), SBT10239 (○), and SBT10241 (△) and *Bifidobacterium longum* SBT2928 (■). One milliliter of citric acid-Na_2HPO_4 buffer (pH 3 to 6) varying pH conditions, and distilled water was used for pH 7.0 with 2 mg of lyophilized cells and 0.2 mg of Trp-P-1 incubated at 37°C for 30 min. Values are the means of three determinations. Binding versus pH was tested at a significance level of $P < 0.05$. $r = 0.9341$; $r^2 = 0.8725$. The equation used was $y = a + bx$; binding = –2.168 + 14.084pH. Standard error was 1.122; $P < 0.001$.

0.05) in binding properties among all selected strains. Strain SBT10239 and SBT10241 were different ($P < 0.05$) from the other *L. gasseri* strains at acidic pH conditions and bound 60 to 64 and 84% Trp-P-1 at pH 4.0 and 6.0, respectively.

The binding with other amino acid pyrolysates differed with their chemical structure. A comparison of the binding of Trp-P-1, Trp-P-2, IQ, MeIQ, and Glu-P-1 with four *L. gasseri* strains and one *B. longum* strain is shown in Table 2. All strains of *L. gasseri* showed a difference ($P < 0.05$) in binding Trp-P-1. The Glu-P-1 binding with strain SBT10239 was different ($P < 0.05$) from that of other strains, but the amount of mutagen that was bound with the cells was less than that for the other mutagens. *Bifidobacterium longum* also showed a difference ($P < 0.05$) in binding Trp-P-2, IQ, and MeIQ from all the *L. gasseri* strains. The binding of Trp-P-2 was comparatively less with strains SBT1703, SBT10241, and SBT2928, but strain SBT10239 had 86% binding, and strain SBT0274 had 80% binding. Comparatively, strain SBT1703 showed better ($P < 0.05$) performance with IQ (59%) and MeIQ (61%) and was different from all other strains.

The *L. acidophilus* and *B. longum* strains were compared by varying the concentrations of Trp-P-1 and Trp-P-2. One milligram of cells of *L. gasseri* and *B. longum* bound 46 to 48 μg of Trp-P-1 and 40 to 45 μg Trp-P-2 at an initial concentration of 100 μg. At higher concentrations (200 μg) of Trp-P-1 and Trp-P-2, the binding was 88 to 95 μg/mg and 74 to 86 μg/mg, respectively. The difference was significant ($P < 0.05$) among the concentrations of mutagens but not among the *L. gasseri* strains.

The binding of Trp-P-1 was 97 to 99% with PCW of *L. gasseri* and *B. longum* (Table 3) but was nonsignificant ($P < 0.05$) among the strains. The other cell components also showed binding with Trp-P-1: cell wall, 70.1 to 78.98%; peptidoglycan, 30.4 to 40.04%; and cell extract, 11.28 to 26.71%; these components differed ($P < 0.05$). These results also show that the binding of mutagenic pyrolyzates were attributable mainly to the cell walls.

To identify the cell-wall component that was involved in the binding of heterocyclic amines, PCW

TABLE 2. Binding properties of *Lactobacillus gasseri* and *Bifidobacterium longum* cells with different amino acid pyrolysates.[1]

Strain no.	Binding[2] Trp-P-1		Trp-P-2		MeIQ		IQ		Glu-P-1	
	(%)									
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
L. gasseri										
SBT0274	92.4[b]	0.24	80.3[b]	0.16	56.6[b]	0.33	39.1[c]	0.75	11.3[c]	2.47
SBT1703	88.3[d]	0.33	78.6[c]	0.44	59.3[a]	0.48	61.1[a]	0.30	14.9[b]	0.49
SBT10239	94.7[a]	0.16	86.3[a]	0.57	41.3[c]	0.83	39.6[c]	1.10	21.7[a]	0.66
SBT10241	92.3[c]	0.09	78.2[c]	0.11	31.1[e]	0.20	35.8[d]	1.93	13.9[bc]	0.17
B. longum										
SBT2928	88.8[d]	0.11	74.2[d]	0.11	39.4[d]	0.28	43.9[b]	0.28	12.3[bc]	0.11

[a,b,c,d,e]Means within the same column with no common superscript letter differ ($P < 0.05$).

[1]Values of mutagen binding represent the mean (±SD) of three experiments.

[2]Glu-P-1 = 2-amino-6-methyldipyrido[1,2-a:3′,2′-*d*]imidazole, IQ = 2-amino-3-methylimidazo[4,5,*f*]-quinoline, MeIQ = 2-amino-3,4 dimethylimidazo[4,5,*f*]quinoline, Trp-P-1 = 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, Trp-P-2 = 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole.

was subjected to enzymatic and chemical treatments. Proteinase K, lysozyme, or trypsin failed to reduce the binding properties of PCW, but sodium metaperiodate treatment on the PCW of SBT10239 and SBT10241 reduced the binding to 15.4 and 17.4%, respectively, and TCA treatment reduced the binding properties to 7.5 and 17.6%, respectively.

DISCUSSION

The streptomycin-dependent strain SD510 of *S. typhimurium* TA 98 was used in the antimutagenicity assay because it possessed a sharp response. Moreover, the SD510 strain serves as a reliable indicator of organisms and has some additional advantages, such as simple media, fewer background colonies, and no need of metabolic activation by S-9 mix (20, 26).

The antimutagenicity and binding properties of *L. gasseri* and *B. longum* revealed a high correlation between the two processes and were mostly dependent on strain. The antimutagenicity was comparatively less than the binding in a few strains. Lactic acid bacteria have shown binding and antimutagenicity with strains isolated from traditional fermented foods (11, 27). Many reports have clearly shown that the lactic acid bacteria cells could bind with most of the heterocyclic amines (10, 16), and the binding

TABLE 3. Binding of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole with *Lactobacillus gasseri* and *Bifidobacterium longum* cells, crude cell wall, purified cell wall, peptidoglycan, and cell extract.[1]

Strains	Binding[2] Cells		Crude cell wall		Purified cell wall		Peptido -glycan		Cell extract	
	(%)									
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
L. gasseri										
SBT0274	61.4[b]	1.2	70.2[b]	0.9	97.7[a]	1.3	40.0[a]	4.3	22.8[b]	0.3
SBT1703	62.1[b]	0.1	77.8[a]	1.0	99.0[a]	0.1	31.4[b]	3.1	19.4[c]	6.3
SBT10239	67.8[a]	0.7	79.0[a]	1.0	97.0[a]	1.0	30.5[b]	2.9	26.7[a]	5.3
SBT10241	63.3[ab]	1.5	72.5[b]	5.3	99.6[a]	0.6	32.4[c]	2.1	11.3[d]	0.2
B. longum										
SBT2928	60.5[b]	2.0	70.2[b]	3.2	99.3[a]	1.0	37.2[a]	1.4	17.7[c]	0.1

[a,b,c,d]Means within the same column with no common superscript letters differ ($P < 0.05$).

[1]Value of mutagen binding represents the mean (±SD) of three determinations.

[2]One milligram of the cell, crude cell wall, purified cell wall, and peptidoglycan and 1 ml of cell extract were mixed with 200 μg of 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole and incubated at 37°C for 30 min.

seems to be stable unless some change in chemicals or in pH is applied to the cells. Recent studies (22) also revealed that bound mutagens were not released during distilled water washing, but part were released when a second amino acid pyrolysate was added to the bound cells. Kawase and Hosono (14) have also reported that the Trp-P-1 and Trp-P-2 bound with lactic acid bacteria were released in the presence of oxgall. The high correlation between antimutagenicity and binding revealed that the bound mutagens were not active because the number of revertants formed correspond to the amount of unbound Trp-P-1 that was present in the assay mixture in most of the strains.

The dose-dependent study using varying concentrations of the cells showed a high degree of binding of Trp-P-1 with fewer cells. The highest binding ability per milligram of cells was 0.12 to 0.13 mg of Trp-P-1 (Figure 2), which is much more than the values reported (14, 24, 25) for other strains of lactic acid bacteria. In the present study, the binding of lactic acid bacteria cells with Trp-P-1 occurred instantaneously, and a long incubation (30 to 50 min) had no significant effect. This result agreed with the result reported earlier (23, 24) using cell-wall peptidoglycan of *Leuconostoc mesenteroides* ssp. *dextranicum* and lyophilized cells of *Lactococcus lactis* spp. *lactis* T-180. A high correlation ($r = 0.9341$) between binding and pH was observed with the tested strains, and the binding properties of lactic acid bacteria decreased significantly as the pH decreased. The function of pH in binding has been studied using other strains of lactic acid bacteria, and the dissociation of Trp-P-1 and Trp-P-2 bound to *Lactobacillus casei* were shown to vary as a function of pH (14); when the pH was decreased below 7.0, a 50% dissociation of Trp-P-2 was reported for *L. casei* cells. In this context, we hypothesized that binding was not dependent on one factor, but on various factors, such as the chemical structure of the cell wall, pH, and chemical complexity of the mutagen. Other factors have not been studied so far.

The strains *L. gasseri* and *B. longum* also showed the same tendency to bind less with more complex amino acid pyrolysates such as Glu-P-1 (9, 10). A difference ($P < 0.05$) occurred in binding with different chemical mutagens among the strains, which also suggested that the chemical structure of the mutagens might also influence the binding percentage. The degree of inhibition toward different mutagens by the same organisms varied widely (Table 2) and may be attributed to the chemical nature of the cell surface. It may also be noted that, at a lower concentration, the binding percentage showed a high value (90 to 95%); in fact, the amount of bound amino acid pyrolysates was less. Moreover, the cells had more binding surfaces that were not saturated by the low concentrations of the mutagens. Strain SBT10239 was more efficient than the other strains tested, especially in binding with Trp-P-1 and Trp-P-2. Even though reports have indicated that the binding of amino acid pyrolysates with lactic acid bacteria cells is a cationic (18) or an ionic mechanism (23), no previous report has indicated the exact mechanism of binding.

From this study, we can presume that these strains of lactic acid bacteria can bind with the wide range of mutagens produced during the cooking of food (13, 28). During the consumption of food with lactic acid bacteria, a small portion of mutagens and bacteria may bind in the mouth where primary digestion starts and the pH is supposed to be favorable for the binding. As the bound components traverse through the stomach, the variation in pH may lead to the dissociation of the bound mutagen, as the pH (1 to 2) is unfavorable. But when the mutagen reaches the intestine (pH ~7), where the absorption of food is maximum, the pH favors the binding mechanism and all mutagens could potentially bind with both *L. gasseri* and *B. longum* cells.

Moreover, in this study, all components of *L. gasseri* and *B. longum* showed binding with Trp-P-1; hence, even if the lysis of some cells occurred in the intestine, the cell components would also be able to remove part of the mutagens. The cell-wall fractions of lactobacilli showed antitumor activity and inhibition of tumor growth, probably because of the immunopotentiating activities of certain cell-wall components (1). Hence, we also presume that the PCW of these strains might help to control tumor growth because of their high binding properties, but in vivo animal experiments are necessary to prove this statement.

An attempt was made to identify the probable binding receptors of the cells, and we found that surface polysaccharides have a major role; the peptidoglycan showed 70% less binding than with the purified cell walls. Moreover, the binding receptors of SBT10239 and SBT10241 were sensitive to treatment with sodium metaperiodate, which reduced the binding to 15.35 and 17.36%, respectively, and should result in oxidation of OH groups located in the *cis* position to aldehydes and carbon acid groups, suggesting that a polysaccharide component is essential for binding. The stringent TCA treatment of the PCW of SBT10239 and SBT10241 reduced the binding substantially and released most of the carbohydrates, further substantiating the role of carbohydrates in binding. Moreover, the use of TCA to extract

peptidoglycan-associated cell-wall polymers from Gram-positive bacteria is well established (8). Lysozyme, proteinase K, and trypsin treatments of PCW of both *L. gasseri* strains had no influence on the binding of Trp-P-1, ruling out the role of proteins.

CONCLUSIONS

Thus, 2 mg of cells could bind with 88 to 95% of Trp-P-1 during a 30-min incubation at pH 7.0. The binding ability was dependent on the chemical nature of the mutagen, on pH, and on strain. To remove a broad spectrum of amino acid pyrolysates, the use of more than 1 strain would be ideal. Our studies also suggest that the binding receptors of *L. gasseri* may be a complex array of polysaccharide components.

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