Key role of peptidoglycan on acrylamide binding by lactic acid bacteria

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Abstract The primary purpose of this study was to analyze the ability of four peptidoglycan (PGN) from different lactic acid bacteria to bind acrylamide (AA) and to identify the binding mechanism. In this study, to clarify the possible binding interactions among AA and components of PGN, chemical components, surface structure, amino acids component, and functional groups of peptidoglycans were studied. It was found that PGN from *Lactobacillus plantarum* 1.0065 had the highest ability to bind AA with 87%. Furthermore, a significant positive relation was found between the carbohydrate content of PGN and percentage of bind AA, and the content of four specific amino acids of PGN and AA binding ability were also positive correlated. Thereinto, alanine of PGN had a significant impact on AA binding among four amino acids. Additionally, the C–O (carboxyl, polysaccharides, and arene), C=O amide, and N–H amines groups of PGN were involved in AA binding.

Keywords: acrylamide, lactic acid bacteria, peptidoglycan, component, binding

Introduction

Food safety issues have become a global major concern due to the great impact on the people's health and social development. In October 2006, scientists in Norway discovered that acrylamide (AA) were present in many carbohydrate-rich foods after being roasted, baked, or fried at high temperatures (1). The presence of AA in foodstuffs has resulted in wide interests because it is known to be a neurotoxic, reproductive-toxic, and potentially carcinogenic compound (1-3). Therefore, approaches to lower acrylamide levels in foodstuffs should be vigorously sought.

Some physical and chemical methods have been developed to reduce AA present in food. However, these methods are not generally practical because of high energy or reagent requirements, costly equipment, and loss of nutrition (4). A safe and efficient method using various microorganisms has been implemented for reducing AA, such as lactic acid bacteria and *Bifidobacterium* (5).

Lactic acid bacteria, due to their GRAS (Generally Recognized as Safe) status and use as probiotics, is nonpathogenic and without generating toxic residues. Therefore, it is feasible to bind toxins using lactic acid bacteria (5-7). Several scientists have demonstrated the capacity of lactic acid bacteria to bind some carcinogens, including patulin, acrylamide, and aflatoxins (M₁, M₂, and B₁) (8-12), but relevant research for binding AA is rare. In July 2015, however,

scientists in Mexico discovered that *Lactobacillus* strains were able to bind acrylamide (13). Moreover, our previous studies have shown that some strains of lactic acid bacteria have the ability to bind acrylamide and that this bind was stable and irreversible. *Lactobacillus plantarum* 1.0065 (*L. plantarum* 1.0065) and *Lactobacillus casei* ATCC393 (*L. casei* ATCC393) were the strains of lactic acid bacteria with the higher ability for binding AA (67% and 61%, respectively).

Some authors have summarized that cell wall carbohydrates, primarily peptidoglycan (PGN), were primary components responsible for binding of the toxin by lactic acid bacteria cell (14-16). PGN is a major component from the Gram-positive bacterial cell wall and is an indispensable molecule of lactic acid bacteria, accounting for approximately 90% of cell wall mass. The PGN network includes glycan chains of N-acetylglucosaminyl (GlcNAc) and N-acetylmuramoyl (MurNAc) cross-linked by peptide stems of D- and L-amino acids connected to lactyl moiety of MurNAc. The crosslinking peptides vary in amino acid residues and in the linkage of stem peptides in different bacterial species (17-19); thus, the PGN structure and properties had differences among various lactic acid bacteria strains. Till date, the mechanism of AA binding by PGN is not well understood.

Therefore, in the present study, we chose four strains of lactic acid bacteria as our research object to explore the ability of PGNs from these strains to bind AA. Further, to clarify the underlying mechanism,

we studied the chemical composition, surface structure, amino acids composition, and functional groups of PGNs.

Materials and Methods

Chemicals and media Four strains were used in this study. The lactic acid bacteria were cultured in the Mann Rogosa Sharpe (MRS) broth and agar (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA). Standard AA and other chemicals were purchased from Chemical Reagents Company.

Microorganisms and culture propagation Four strains were used in this study: *L. plantarum* 1.0065, *L. casei* ATCC393, *Lactobacillus acidophilus* KLDS1.0307 (*L. acidophilus* KLDS1.0307), and *Streptococcus thermophilus* KLDS1.0316 (*S. thermophilus* KLDS1.0316).

All strains were activated in MRS broth, and two subcultures in MRS broth were performed prior to each experiment. For *S. thermophilus* KLDS1.0316 and other three *lactobacillus* strains, the two subculturing steps involved 4% inoculum shaking incubated at 37°C for 24 h with pH 6.2 and 5.8, respectively. Then, an aliquot of 3% from the second subculture was transferred into 500 mL MRS broth for shaking culture under the same conditions as that of two subcultures (24 h, 37°C, 150 rpm), bacteria were collected via centrifugation (8,000×g, 5 min, 4°C), and washed with sterile deionized water (20). Finally, bacterial sediment was dissolved in 50 mL of sterile deionized water. These bacterial cells were used for extracting PGN and the binding experiments.

Extraction of PGN The bacterial cells suspended in sterile deionized water were centrifuged, and the resulting pellet was resuspended in ten volumes of 10% trichloroacetic acid (TCA) and were heated by water bath for 25 min at 80°C, after cooling at 4°C, suspensions were centrifuged (8,000×g, 10 min, 4°C), and washed with sterile deionized water. Then, the washed pellets were vibrationally processed with two volumes of trichloromethane:methanol (1:2, v/v) for 12 h. The pellets were collected by centrifugation (8,000×g, 15 min, 4°C) and washed with absolute ethanol. The collected pellets were suspended in 0.1 N Tris-HCl (pH 7.6) and shaking incubated with trypsin (3 mg/mL) and neutrase (10 mg/mL) at 37°C overnight. The sample was then centrifuged (15,000×g, 20 min, 4°C), and the supernatant was removed to provide the pellets of cell wall fraction. The pellets were washed with sterile deionized water until they were free of all enzymes (16,21). These PGNs of strains were collected and stored via freeze-drying for further study.

AA binding assay A 900-μL quantity of PGN working solution (5 mg/mL) in a 2-mL plastic tube was added to 100 μL the working solution of AA (6 μg/mL), and the AA concentration reached 0.6 μg/mL. Subsequently, the reaction mixtures were incubated at 37°C for 6 h in the shaker water bath pot with moderate rotation (150 rpm).

Once the reaction was terminated, PGN was separated by centrifugation (6,000×g, 5 min, 4°C) and supernatant fluid containing residual AA were collected and analyzed via high-performance liquid chromatography (HPLC). For each assay, a working solution of AA (0.6 μg/mL) without PGN, serving as a control assay, was analyzed in the same conditions (10,13).

AA determination via high-performance liquid chromatography

In this assay, the HPLC system (LC-20A; Shimadzu, Kyoto, Japan) and an Alltima Inertsil ODS-C18 (150×4.6 mm, 5 μm) were used to determine the peak area of AA in the supernatant fluid. All samples tested must be filtered through a filter membrane (0.22 μm) prior to the experiment. The injection amount of each sample was 20 μL, and HPLC grade methanol/water (5:95, v/v) served as the mobile phase. The flow rate was 1 mL/min at 30°C. In this assay, the detection wavelength was 205 nm (22). The percentage of AA adsorption was calculated by the following equation:

$$\%B = \left[1 - \frac{A}{A_0} \right] \times 100,$$

where B is the percentage of AA adsorption, A is the peak area of AA in the supernatant fluid, and A_0 is the peak area of AA in control.

Determination of total carbohydrates First, a solution of glucose (0.1 mg/mL) was used as a standard solution. Glucose standard solutions of 0, 2, 4, 6, 8, and 10 mL were deposited in test tubes (10 mL) in borosilicate glass, adding distilled water to 10 mL in each tube, respectively. Second, 1 mL of each standard solution was placed in a glass test tube. A 1.4 mL phenol solution (5%) was added to each tube, and the test tubes were vortexed. In each tube, 5 mL of concentrated sulfuric acid was rapidly added; these tubes were cooled down for 15 or 20 min at 25°C in a thermostated bath. Third, these mixtures were heated for 25 min in a 100°C water bath. The sample was transferred to a cuvette after being cooled to room temperature, and the absorbance of each sample was measured using an ultraviolet spectrophotometer at 490 nm (23,24). Each sample was tested in triplicate.

Determination of total proteins assay The protein analysis of PGNs was performed using a KDN-812 Kjeltac auto analyzer.

FTIR analysis Fourier transform infrared (FTIR) spectroscopy (Heilongjiang province, China) was used to identify potential functional groups of PGNs from four strains of lactic acid bacteria related to AA binding. FTIR analysis was performed with an FTIR spectrophotometer, and the infrared spectra range was 4,000–400 cm⁻¹ (10).

Amino acid analysis PGNs derived from strains of lactic acid bacteria needed to be hydrolyzed at 110°C for 24 h by 6 N HCl. The amino acids of PGN were determined with the help of an L-8800

Amino Nova amino acid analyzer (Heilongjiang Province, China).

Statistical analysis All assays in this study were conducted in triplicate. Data analysis was performed by variance (ANOVA) tests using the SPSS 21 statistical analysis package to identify differences between bacterial PGN and binding mechanism (SPSS Inc., Chicago, IL, USA). P -values < 0.05 were considered significantly different.

Results and Discussion

AA binding assay Several studies have reported that carbohydrate components of bacteria are primary binding sites for some carcinogens (14,25-29). Lahtinen studied different components from the cell wall of *Lact. rhamnosus* GG and summarized that PGNs are the most primary binding sites for aflatoxin B₁ (25). Zoghi reported that intact PGNs from lactic acid bacteria exhibited highest capability to remove Benzo(a)pyrene (26). There is almost no report regarding binding AA by PGN, but we speculated that PGN is the primary binding component for AA.

Our studies exhibited that PGNs from four lactic acid bacteria had definite ability to bind AA, and the ability of PGNs to bind AA ranged from 33.65% to approximately 87%. The AA binding assay of PGNs from different lactic acid bacteria was presented in Fig. 1 and showed significant differences ($p < 0.05$). The PGN of *L. plantarum* 1.0065 had the highest affinity for binding AA with 87.14%. The amount of AA bound by PGN from *L. plantarum* 1.0065 was notably more than the other PGNs examined ($p < 0.05$). PGNs from *L. casei* ATCC393 and *L. acidophilus* KLDS1.0307 showed a 75.50 and 56.75% affinity for binding AA, respectively. The PGN from *S. thermophilus* KLDS1.0316 had the lowest capacity to bind AA among four PGNs with 33.65%. Therefore, we inferred that PGN was an important binding component for AA.

Compositional analysis of PGN Some scientists have concluded that proteins and/or carbohydrates of lactic acid bacteria cell walls were primary components in the adsorption of aflatoxin B₁ (30). Furthermore, carbohydrates and proteins of lactic acid bacteria cell walls had been reported to be related to adsorption of patulin (31,32). Similar findings had been reported by Zoghi *et al.* (26) in which the adsorption of toxins by bacteria was predominantly referred to some carbohydrates and proteins in the bacteria cell wall. Carbohydrates and proteins form the major composition of PGN, and

Fig. 1. Percentage of AA bound by PGNs from different Lactic acid bacteria strains (1, *L. plantarum* 1.0065; 2, *L. casei* ATCC393; 3, *L. acidophilus* KLDS1.0307; 4, *S. thermophilus* KLDS1.0316). Treatments with different letters in each column are statistically different by each PGN ($p \leq 0.05$).

there are variations in the composition among different PGNs. Hence, it is possible that these composition variations could provide different AA binding ability; furthermore, the variations may partially explain the difference in the AA binding ability among different PGNs.

Our compositional analysis of PGNs demonstrated that the evaluated PGNs contained mainly carbohydrates and proteins. The data concerning the same are shown in Table 1. We found that the contents of carbohydrates and proteins had differences between four PGNs from different strains, the carbohydrate content ranged from 32.45 to 40.34%, and the protein content ranged from 51.49 to 60.83%. Thereinto, PGN from *L. plantarum* 1.0065, which had the highest content of carbohydrate, had the highest capacity to bind AA among the four PGNs under the same conditions. The carbohydrate content of PGN from *S. thermophilus* KLDS1.0316 was lowest, and its ability to bind AA was the lowest. This, in turn, led us to discover a conspicuous positive relation between the content of carbohydrates and the ability of PGNs to bind AA, while the protein content and the ability of PGNs to bind AA did not positively correlate. These results demonstrated that the carbohydrate content of PGN is an important factor for binding AA between four PGNs. The ability for PGN binding AA was not closely related to the content of protein of PGN, but it is probably related to the content of certain amino acids.

Table 1. Percentage of PGN components involved on AA binding

Species	Carbohydrate	Protein	Percentage of AA absorption
<i>L. plantarum</i> 1.0065	40.34±0.035 ^a	57.76±1.11 ^a	87.05±0.10 ^A
<i>L. casei</i> ATCC393	36.69±0.076 ^b	60.83±1.10 ^b	75.50±0.12 ^B
<i>L. acidophilus</i> KLDS1.0307	33.81±0.026 ^c	51.49±2.08 ^c	56.75±0.11 ^C
<i>S. thermophilus</i> KLDS1.0316	32.45±0.025 ^c	56.68±1.41 ^a	33.65±0.11 ^D

Each value is a mean±standard deviation of triplicate assays. Different letters in each column are significantly different for each PGN from different strains.

Fig. 2. Spectra of AA-exposed PGNs and AA-unexposed PGNs in the mid-infrared region (4,000–400 cm^{-1}). (A) *L. plantarum* 1.0065, (B) *L. casei* ATCC393, (C) *L. acidophilus* KLD51.0307, and (D) *S. thermophilus* KLD51.0316.

FTIR analysis FTIR spectra play an important role in analyzing the functional groups related to the binding process. Several authors suggested that carbonyl oxygen (C=O) atoms of AA were more

efficient than the amino group (NH_2) for the bonding at smectite using FTIR-spectroscopy and X-ray diffraction (33). Analogously, some reports have indicated that bound patulin entered the PGN

Table 2. FTIR bands observed from acrylamide-exposed peptidoglycans and acrylamide-unexposed peptidoglycans

Functional groups	Wavenumber (cm ⁻¹) ¹⁾			
	<i>L. plantarum</i> 1.0065	<i>L. casei</i> ATCC393	<i>L. acidophilus</i> KLD51.0307	<i>S. thermophilus</i> KLD51.0316
N-H amines	3406.49 (3318.48)	3418.48 (3316.53)	3432.47 (3406.49)	3432.47 (3452.47)
C-H stretching	2932.70 (2958.69)	2932.69 (2944.69)	2962.69 (2918.70)	2944.69 (2948.32)
C=O amide 1	1625.28 (1651.27)	1639.27 (1665.30)	1651.27 (1665.26)	1639.27 (1651.27)
N=O nitro compounds	1561.31 (1549.31)	1549.35 (1535.32)	1564.29 (1539.31)	* ²⁾ (1531.46)
R-C-H alkyl groups	1395.38 (1407.37)	1383.39 (1383.39)	1395.38 (1395.38)	1395.38 (1395.38)
C-O carboxyl	1241.45 (1229.45)	1267.44 (1241.45)	1267.44 (1253.44)	1267.44 (1241.45)
C-O polysaccharides	1075.52 (*)	1061.53 (*)	1075.52 (*)	1087.52 (1061.53)
C-O arene	843.63 (819.64)	857.62 (*)	847.62 (*)	843.63 (*)
C-Br alkyl halide	625.72 (*)	625.72 (625.72)	613.73 (625.72)	625.72 (625.72)
C-I alkyl halide	523.77 (575.74)	* (*)	537.76 (*)	549.76 (*)

¹⁾Wavenumbers obtained from four acrylamide-exposed peptidoglycans were shown in parentheses, while wavenumbers obtained from four acrylamide-unexposed peptidoglycans without parentheses.

²⁾*, Band not observed

layer of lactic acid bacteria cell wall because of the peptidoglycan containing mostly N-H, O-H, and C-O groups (15). In addition, hydrogen bonds probably occur between C=O groups of both AFB₁ and AA, and the hydroxyl groups of some glycerol phosphate substituents and/or glucose residues connected to the poly chain (ribitol phosphate) (13). To determine related functional groups and probable binding sites involved in AA binding, FTIR assay was performed in our study. We found that the FTIR spectra of the AA-exposed PGNs were different from AA-unexposed PGNs, and the assignment of FTIR bands and detailed wavenumber shifts were shown in Fig. 2 and Table 2, respectively. Obviously, the peak shape of each sample was basically alike. The AA-exposed PGNs did not completely lose their original structure, and the AA binding primarily caused significant changes of C-O (carboxyl, polysaccharides, and arene), C=O amide, and N-H amine groups of PGNs. These changes in the three groups could vary in different PGNs, demonstrating the participation of these three groups in AA binding.

Amino acid analysis of PGNs In October 2009, Koutsidis *et al.* (34) demonstrated that some amino acids might generate amino acid-AA adducts via Michael addition reactions to reduce the AA content. Considering the above, we further studied amino acid composition of different PGNs, and relevant data are shown in Table 3. Our results indicated that the contents of alanine, aspartic acid, glutamic acid, and lysine were clearly higher than other amino acids. These amino acids formed an important part of the amino acid component of PGN. Similar reports have been found by some authors, who discovered that alanine, glutamic acid, lysine, and aspartic acid constituted an important part of the amino acid component of peptidoglycan (19). Moreover, several authors suggested that the strength of the toxin-lactic acid bacteria interaction was influenced by the PGN constituent, more accurately, by its amino acid constituent (35). Particularly, we found a positive relation between the total content of four amino acids and the AA binding ability of PGNs. Thus, we concluded that these four amino acids were closely related to AA binding.

Additionally, alanine had the highest content among amino acids of PGN, and the content of alanine and AA binding ability of PGNs were positively correlated. The results demonstrated that alanine of PGN had a significant impact on AA binding and supported previous reports that suggested that the amine group of D-alanine probably reacts with AA through Michael addition (36). Moreover, Serrano-Nino *et al.* (13) suggested that D-alanine plays an important role on binding aatoxin B₁ and acrylamide. While the results in Table 3 also revealed that the aspartic acid content was higher in *L. plantarum* 1.0065 and *L. casei* ATCC393, and PGNs from these two strains had a higher capacity to bind AA among four PGNs. Thus, we inferred that aspartic acid was likely to influence the ability for binding AA of PGNs.

In conclusion, PGN from four lactic acid bacteria was the primary binding component for AA and the affinity for binding ranged from 33.65% to approximately 87%. PGNs from *L. plantarum* 1.0065 had the highest affinity for AA binding with 87%. Furthermore, we found a significant positive relation between the carbohydrate content and affinity for binding to AA. While the content of protein and AA binding ability of PGNs were not positively correlated, the content of four specific amino acids (alanine, aspartic acid, glutamic acid and lysine) and AA binding ability were positive correlated. Thereinto, alanine of PGN had a significant impact on AA binding among these four amino acids. Additionally, this study showed that C-O (carboxyl, polysaccharides, and arene), C=O amide, and N-H amines groups of PGN, which were related to carbohydrate and some amino acids components, were involved in the AA binding.

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Disclosure The authors declare no conflict of interest.

Table 3. Amino acid analysis of PGNs from different strains

	<i>L. plantarum</i> 1.0065	<i>L. casei</i> ATCC393	<i>L. acidophilus</i> KLD51.0307	<i>S. thermophilus</i> KLD51.0316
Percentage of AA absorption	87.05±0.10 ^{1A2)}	75.50±0.12 ^B	56.75±0.11 ^C	33.65±0.11 ^D
Aspartic acid	8.64±0.12^a	8.49±0.16^a	7.25±0.08^a	7.13±0.06^a
Threonine	2.42±0.08 ^b	2.30±0.06 ^b	2.13±0.11 ^b	1.71±0.02 ^b
Serine	1.62±0.07 ^b	1.53±0.04 ^b	1.40±0.08 ^b	1.25±0.05 ^b
Glutamic acid	7.95±0.06^a	7.29±0.03^a	7.22±0.04^a	6.30±0.06^a
Glycine	2.33±0.03 ^b	2.97±0.04 ^b	2.05±0.04 ^b	2.66±0.04 ^b
Alanine	11.12±0.04^c	9.82±0.04^a	8.20±0.03^a	7.51±0.03^a
Cystine	0.35±0.02 ^b	0.16±0.03 ^b	0.18±0.03 ^b	0.15±0.01 ^b
Valine	2.11±0.03 ^b	1.68±0.03 ^b	1.66±0.03 ^b	1.35±0.02 ^b
Methionine	2.26±0.05 ^b	1.23±0.04 ^b	1.12±0.04 ^b	1.08±0.04 ^b
Isoleucine	1.92±0.04 ^b	1.92±0.02 ^b	2.16±0.07 ^b	1.47±0.06 ^b
Leucine	2.94±0.03 ^b	2.99±0.04 ^b	3.11±0.03 ^b	2.37±0.03 ^b
Tyrosine	1.09±0.02 ^b	1.20±0.02 ^b	1.05±0.03 ^b	0.85±0.05 ^b
Phenylalanine	1.61±0.03 ^b	1.28±0.05 ^b	1.22±0.05 ^b	0.96±0.07 ^b
Lysine	7.36±0.02^a	7.23±0.02^a	7.08±0.03^a	6.94±0.03^a
Ammonia peak	0.95±0.03 ^b	0.61±0.03 ^b	0.66±0.03 ^b	0.51±0.03 ^b
Histidine	0.93±0.03 ^b	0.86±0.05 ^b	0.82±0.04 ^b	0.65±0.04 ^b
Arginine	2.29±0.03 ^b	1.94±0.05 ^b	1.89±0.03 ^b	1.58±0.04 ^b
Proline	1.52±0.02 ^b	1.71±0.03 ^b	1.29±0.04 ^b	1.50±0.02 ^b

¹⁾Each value is a mean±standard deviation of triplicate assays.

²⁾Different letters in each column are significantly different for each amino acid, and different letters in each row are significantly different for each PGN from different strains.

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