

Antimicrobial activity of bioactive peptides derived from fermentation of soy milk by *Lactobacillus plantarum* C₂ against common foodborne pathogens

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Abstract

This study presents, antimicrobial activity of bioactive peptides derived from fermentation of soy milk along with their production by *Lactobacillus plantarum* C₂ strain. Bioactive peptides are specific fragment of protein, can be released by fermentation, upon release they may act as antimicrobial, antioxidant, antihypertensive, immunomodulatory and hypocholesterolemic activities. LP C₂ showed very good growth in soy milk by increasing their count and acidity significantly, consequently pH dropped. Ultrafiltration was used for the separation of peptides and their peptide contents analyzed by OPA assay. 10kDa fraction was found high in peptide (655.128±2.95 µg/ml). Antimicrobial activity of bioactive peptide fractions was checked by agar well diffusion method and found that 5 kDa showed highest activity against all the pathogens with highest inhibition against *E.coli* (12±0.57) followed by *S. dysenteriae* (11±0.57), *L. monocytogenes* (10±0.57) and *B. cereus* (10±0.57 mm). However it was observed that unfractionated sample high in antimicrobial activity, may be due to combined effect of all the fractions.

Keywords: Bioactive peptides, antimicrobial, lactobacillus, fermentation, food pathogens

Bioactive peptides are fragments that are nascent in the primary sequences of proteins and confer functions beyond basic nutritional benefits. Bioactive peptides are considered to promote diverse activities, including, antimicrobial, antioxidant, antihypertensive, immunomodulatory, hypocholesterolemic, opiate-like, mineral

binding and antithrombotic actions. They can be released during gastrointestinal digestion or food processing or fermentation from plant and animal proteins, such as milk, soy or fish proteins. Bioactive peptides usually contain 2–20 amino acid residues per molecule, but in some cases may consist of more than 20 amino acids. During digestion, the bioactive peptides can be absorbed through the intestine to enter the blood circulation and exert systemic effects. Several of the known bioactive peptides are multifunctional and can exert more than one of the effects (Saadi *et al.*, 2015; Singh *et al.*, 2014).

Although milk and milk products are greatly studied as source of bioactive peptides, many bioactive peptides are also found in other animal and plant sources. Soybean (*Glycine max*) (L) Merr. is economically the most important bean in the world, providing vegetable protein for millions of people and ingredients for hundreds of chemical products and a potential source of bioactive peptides. Glycinin and β -conglycinin, accounting for 65–80% of total soy proteins, are the precursor of most of the isolated peptides (Wynstra *et al.* 1986). Soy milk, which is an aqueous extract of soybeans, provides adequate proteins, iron, unsaturated fatty acids and other nutrients but contains low fat, carbohydrates and calcium. It naturally has the same amount of protein as cow's milk. Several bioactive peptides have been isolated, purified and characterized from soy milk with ACE-inhibitory, hypocholesteremic, immunomodulatory and anticancer activities. Fermentation with proteolytic lactic acid bacteria is efficient way to produce bioactive peptides and food grade hydrolyzed proteins. During fermentation, proteins are degraded into simpler form like oligopeptides, di-peptides and tri-peptides and serve as a precursor of bioactive peptides (Singh *et al.* 2014; Vij *et al.* 2011).

Antimicrobial peptides are known to play important roles in the innate host defense mechanisms of most living organisms, including plants, insects, amphibians and mammals (Kim *et al.* 2009). They are also known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses. Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. Once in a target microbial membrane, the peptide kills target cells through diverse mechanisms (Izadpanah and Gallo, 2005). Antimicrobial peptides are generally between 12 and 50 amino acids. These peptides include two or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion of hydrophobic residues. Several studies reveals the antimicrobial activity of soy fermented products such as soy yoghurts, mainly depends on bioactive peptides produced during fermentation. This paper presents the antimicrobial activity of bioactive peptides derived from fermentation of soy milk against food pathogens.

Materials and Methods

Materials and microorganisms

MRS (de Man, Rogosa, Sharpe) broth, BHI (Brain Heart Infusion) broth, Nutrient broth and Agar powder used in the study were purchased from HiMedia Pvt. Ltd. (Mumbai, India). OPA (Ortho-phthaldialdehyde) and Serine for peptides quantifications were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India) and HiMedia (Mumbai, India), respectively. Soybean (Glycine max) was purchased from the local market in Karnal (Haryana), India. Pathogenic microbial strains *Escherichia coli* O157 25922 and *Listeria monocytogenes* 15313 were obtained from American Type Culture Collection (ATCC), Manassas, USA. *Staphylococcus aureus* 1144 and *Shigella dysenteriae* 107 were procured from National Collection of Dairy Cultures (NCDC), ICAR-National Dairy Research Institute, Karnal, India and Microbial Type Culture Collection (MTCC), Chandigarh, India, respectively.

Preparation of soy milk

Soymilk was prepared according to the standard method given by Nelson *et al.* (1976). 250g of soybeans were soaked in distilled water for 14-16 hours at room temperature. The soak water was drained from the soybeans and the beans thereafter were blanched in boiling distilled water for 30 min. The drained beans were hand washed thoroughly to remove their testa. They were then placed in a mixer grinder (Bajaj electricals, India) and blended for 10 min with 600 mL of distilled water. The resulted slurry was then filtered through two layers of muslin cloth and final volume made upto 1000 mL with distilled water. Finally, obtained soy milk was then sterilized by autoclaving.

Fermentation

Lactobacillus plantarum C2 was grown in MRS broth, for preparation of the inoculum, cultures transferred twice into sterilized soymilk and incubated at 37°C. Fermentation was conducted in 1000 mL capacity flasks. Inoculum of active cultures were added in 300 mL soymilk at the rate of 1% and incubated at 37°C. Samples were collected at regular intervals of the 6h.

Growth characteristics

MRS agar was used for the enumeration of total viable count in fermented soy milk. 1.0 mL of appropriate serial dilutions of each sample were pour-plated to the appropriate media. After 24h of incubation at 37°C, the colonies that appeared on the plates were counted and the cfu/mL were calculated. The titratable acidity was determined by titration with 0.1 N NaOH solution, and expressed as percent

lactic acid. The pH values of the samples were measured using a pH meter (Orion, Thermo Scientific).

Preparation of bioactive peptide fractions

Soy bioactive peptides were fractionated into 10, 5 and 3kDa using molecular weight cut-off (MWCO) membranes (Puchalska *et al.* 2014). Firstly, supernatant of fermented soy milk was obtained by centrifugation at $12000 \times g$ for 20 min (Thermo Scientific, Heraeus, multifuge X1R Centrifuge), then filtered through 0.22 micron filter device (Millipore) and keep at 4°C. For fractionation fermented supernatant was transfer to ultrafiltration devices of different MWCO and centrifuged at 5000rpm for 30 min, then permeate and retentate were collected. 10kDa, 5kDa and 3kDa peptides fractions obtained from ultrafiltration were then frizzed dried and kept at deep freezer (-20°C).

Protein and peptide quantification

Protein content of ultrafiltered fractions was estimated by Bradford's method using Merck Protein Estimation Kit. Diluted BSA 1mg/ml to 0 to 80µg and final volume was made 200µl with distilled water. 2 ml of Bradford reagent was added to each tube and kept at room temperature for 10 min. Absorbance was taken at 595nm with blank. For unknown 200µl of sample was added instead of BSA. Standard curve was prepared by taken absorbance at Y axis and concentration at X axis. Result was expressed as µg/ml.

The determination of peptide concentration in ultrafiltered fractions was performed by the OPA (Ortho-phthaldialdehyde) assay with some modifications (Wang *et al.*, 2008). 100 µL sample was added to 2 mL of OPA reagent which is kept on ice and the absorbance of the solution was measured spectrophotometrically (Shimazu, UV-1800) at 340 nm after 5 min. at room temperature (20°C). The peptide concentration was expressed as serine equivalents, according to the standard curve using serine in a concentration range of 25-250 µg/mL.

Determination of antimicrobial activity of soy bioactive peptides

Antimicrobial activity was evaluated against four common food pathogens using agar well diffusion assay as per method of Schillinger (1989) with some modifications. The method is based on the principle that involves the ability of one microorganism to inhibit the growth of another, as exhibited by clear zone of inhibition. To check the antimicrobial activity, nutrient agar plates (15-20mL) were made and allowed to solidify. Then the nutrient agar plates were overlaid with 7 mL of soft agar (0.7% agar) inoculated with 100 µL of overnight active culture of indicator strains (Pathogen). The soft agar was allowed to solidify. The plates were refrigerated at 4°C for 1h before several wells were punched out of the

agar with sterile glass borer. The wells were then filled with 100 μ L of supernatant obtained by centrifuging the fermented soy milk at 10000 \times g for 15 min. The plates were once again refrigerated at 4°C for 3-4 h to facilitate the diffusion of supernatant and were incubated at 37°C for 24-48 h. The diameter of zone of inhibition extending laterally around the well was measured and a clear zone of 1 mm or more was considered positive inhibition.

Statistical analysis

Data were analyzed by using GraphPad Prism (La Jolla, CA, USA) (version 5.01) software. Results are expressed as means \pm SEM. Differences between the means were tested for statistical significance using analysis of variance (ANOVA) followed by Bonferroni post hoc test. The significance level was set at 5% ($P < .05$) for all calculations.

Results and Discussion

Growth characteristics of *Lactobacillus plantarum* C2 during fermentation of soy milk

Fermentation of soy milk improves bioavailability of isoflavones, assists in digestion of protein, and provides more soluble calcium, reduced level of carbohydrates and increased level of bioactive isoflavones and bioactive peptides (Chien *et al.* 2006). Keeping these facts into consideration, growth patterns of *Lactobacillus plantarum* C2 was studied in soymilk, monitored in terms of alteration in viable counts, pH and acidity. LP C2 showed excellent growth in soy milk (Figure 1), by increased log count from initial 5.935 ± 0.016 to 10.406 ± 0.055 log cfu/ml after 30h of fermentation. Consequently, pH was decreased from 7.03 ± 0.05 to 3.38 ± 0.03 and acidity increased from 0.109 ± 0.004 to $0.839 \pm 0.004\%$.

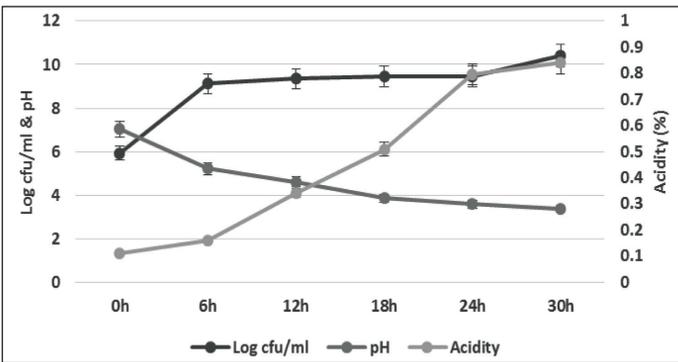


Fig. 1: Growth characteristics of *Lactobacillus plantarum* C2 during fermentation of soy milk, the level of significance was preset $\alpha = 0.05$ and significant $P < 0.0001$, \pm standard error of three replicates.

It was also observed that LP C2 increased highest log count between 0 to 6h of fermentation, it may be due to log phage and adapted culture in the soy milk medium used for inoculation as well as better availability of substrate. Several previous studies also reported such high viable count increase during soy product fermentation by *L. casei* (2.5 log after 14h), *L. rhamnosus* 6013 (2.3 log after 6h) and *L. rhamnosus* CRL981 (1.47 log after 12) (Marazza *et al.* 2009; Liu *et al.* 2011; Liu *et al.* 2006).

Protein and peptide quantification in bioactive peptides fraction of fermented soy milk

Protein and peptide content in fermented soy milk and their respective fractions of bioactive peptides were estimated by Bradford method and OPA methods, respectively (Figure 2). It was found that 10kDa fraction of peptides showed highest protein (589.26±21.24 µg/ml) content, which is higher significantly (P<0.001) from others, followed by 5kDa (299.87±8.49 µg/ml) and unfractionated (UF) sample (270.21±5.83 µg/ml). It was observed that 3kDa (211.77±15.15 µg/ml) fraction showed less protein in comparison of other.

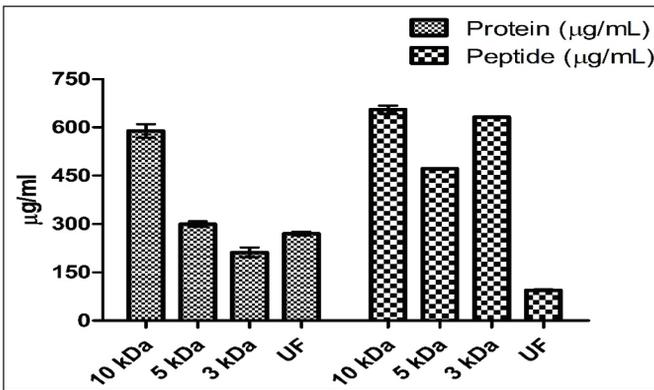


Fig. 2: Protein and peptide contents in fractions of bioactive peptides of fermented soy milk, the level of significance was preset $\alpha = 0.05$ and significant $P < 0.0001$, \pm standard error of three replicates.

However, 10kDa fraction of peptides showed highest peptide content (655.128±2.95 µg serine/ml) content, which is higher significantly (P<0.001) from others, followed by 3kDa (632.307±0.44 µg serine/ml) and 5 kDa (471.282±0.67 µg serine/ml). It was observed that unfractionated (UF) sample (94.358±0.67 µg serine/ml) showed less protein in comparison of other. Similarly Puchalska and coworkers (2014), fractionated peptide extracts by ultrafiltration through different MWCO filters to obtain peptide fractions from 5 to 10 kDa, 3 to 5 kDa, and below 3 kDa. They estimated peptide content by OPA method and found highest

peptide content in fractions containing 5–10 kDa peptides. OPA method was also used by Ghassem and coworkers (2011) in order to quantify peptides from Haruan myofibrillar protein hydrolysate and observed more peptide content with increasing MWCO.

Antimicrobial activity of bioactive peptides derived from fermentation of soy milk

The mechanism of antimicrobial activity of peptides involves interaction with membranes and may be cytotoxic as a result of disturbance of the bacterial inner or outer membranes. Alternatively, a necessary but not sufficient property of these peptides may be to be able to pass through the membrane to reach a target inside the cell. In contrast, bacterial cells have a layer rich in negatively charged phospholipids pointing toward the external environment, facilitating their interactions with peptides, most of which are positively charged (Matsuzaki, 1999). There are several antimicrobial peptides which are rich in a certain specific amino acid such as Trp or His (Epand *et al.* 1999). Antimicrobial activity of fermented soy milk and their peptide fractions was analyzed by agar well diffusion method (Fig. 3). It was observed during the study that fermented soy milk showed inhibition against all the pathogens with highest (17 ± 0.57 mm) against *E.coli*, followed by *S. dysenteriae* (16 ± 0.57) and *B. cereus* (15 ± 0.57). Between peptide fractions of fermented soy milk 5 kDa showed highest activity against all the pathogens with highest inhibition against *E.coli* (12 ± 0.57) followed by *S. dysenteriae* (11 ± 0.57), *L. monocytogenes* (10 ± 0.57) and *B. cereus* (10 ± 0.57 mm). It was observed that 10 kDa fraction showed highest inhibition against *L. monocytogenes* (12 ± 0.57 mm) and *B. cereus* (11 ± 0.57 mm), on the other hand 3 kDa fraction showed less inhibition except against *E.coli* (11 ± 0.57 mm).

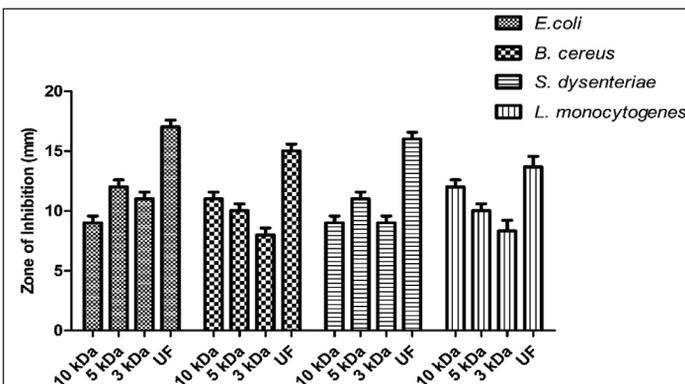


Fig. 3: Antimicrobial activity of bioactive peptides derived from fermentation of soy milk, the level of significance was preset $\alpha = 0.05$ and significant $P < 0.0001$, \pm standard error of three replicates.

Antimicrobial peptides, are the first line of defense against colonization by pathogenic microorganisms, widely distributed in nature and play a fundamental role in regulating bacterial populations on the mucosa and other epithelial surfaces (Bevins & Zasloff, 1990; Zasloff, 2002). Even great diversity in their primary structures, most antimicrobial peptides are similar in that they are short amino acid chains composed primarily of cationic and hydrophobic amino acids (Dashper *et al.* 2007). The low molecular weights of the peptide fractions facilitate, higher exposure of the amino acids and their charges, and the formation of small channels in the lipid bilayer of pathogens (Guillen *et al.* 2010; Patrzykat & Douglas, 2005).

Therefore as per the study we can conclude that lactic acid bacteria are able to grow on soy milk and release bioactive peptides during fermentation. These peptides have several biofunctional activities including antimicrobial activity. Antimicrobial activity of bioactive peptides mainly depends on their amino acid chain. Therefore, there is need to investigate sequences of amino acids those act on pathogens. There is also need to study exact mechanism of action of these peptides.

References

- Bevins, C. L. and Zasloff, M. 1990. Peptides from frog skin. *Annual review of biochemistry*, **59**(1): 395-414.
- Chien, H.L., Huang, H.Y. and Chou, C.C. 2006. Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria. *Food microbiology* **23**(8): 772-778.
- Dashper, S.G., Liu, S.W. and Reynolds, E.C. 2007. Antimicrobial peptides and their potential as oral therapeutic agents. *International Journal of Peptide Research and Therapeutics* **13**(4): 505-516.
- Epand, R.M. and Vogel, H.J. 1999. Diversity of antimicrobial peptides and their mechanisms of action. *Biochimica Biophysica Acta (BBA)-Biomembranes* **1462**(1): 11-28.
- Ghassem, M., Arihara, K., Babji, A.S., Said, M. and Ibrahim, S. 2011. Purification and identification of ACE inhibitory peptides from Haruan (*Channa striatus*) myofibrillar protein hydrolysate using HPLC-ESI-TOF MS/MS. *Food chemistry* **129**(4): 1770-1777.
- Guillén, G., López Caballero, M.E., Alemán, A., López de Lacey, A., Giménez, B. and Montero García, P. 2010. Antioxidant and antimicrobial peptide fractions from squid and tuna skin gelatin. *Transworld Research Network* **37/661**(2):89-115.
- Izadpanah, A. and Gallo, R.L. 2005. Antimicrobial peptides. *Journal of the American Academy of Dermatology* **52**(3): 381-390.
- Kim, S., Lim, C., Lee, C., An, G. 2009. Optimization of Growth and Storage Conditions for Lactic Acid Bacteria in Yogurt and Frozen Yogurt. *Journal of the Korean Society for Applied Biological Chemistry* **52**(1): 76-79.

- Liu, D., Wang, X., Ge, J., Liu, S. and Li, Y. 2011. Comparison of the immune responses induced by oral immunization of mice with *Lactobacillus casei*-expressing porcine parvovirus VP2 and VP2 fused to *Escherichia coli* heat-labile enterotoxin B subunit protein. *Comparative immunology, microbiology and infectious diseases* **34**(1): 73-81.
- Liu, J.R., Wang, S.Y., Chen, M.J., Yueh, P.Y. and Lin, C.W. 2006. The anti allergenic properties of milk kefir and soymilk kefir and their beneficial effects on the intestinal microflora. *Journal of the Science of Food and Agriculture* **86**(15): 2527-2533.
- Marazza, J.A., Garro, M.S. and de Giori, G.S. 2009. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. *Food microbiology* **26**(3): 333-339.
- Matsuzaki, K., Sugishita, K.I. and Miyajima, K. 1999. Interactions of an antimicrobial peptide, magainin 2, with lipopolysaccharide-containing liposomes as a model for outer membranes of gram-negative bacteria. *FEBS letters* **449**(2): 221-224.
- Nelson, A., Steinberg, M. and Wei, L. 1976. Illinois process for preparation of soymilk. *Journal of Food Science* **41**(1): 57-61.
- Patrzykat, A. and Douglas, S.E. 2005. Antimicrobial peptides: cooperative approaches to protection. *Protein and peptide letters* **12**(1): 19-25.
- Puchalska, P., Marina, M.L. and Garcia, M.C. 2014. Isolation and identification of antioxidant peptides from commercial soybean-based infant formulas. *Food chemistry* **148**: 147-154.
- Saadi, S., Saari, N., Anwar, F., Hamid, A.A. and Ghazali, H.M. 2015. Recent advances in food biopeptides: Production, biological functionalities and therapeutic applications. *Biotechnology advances* **33**(1): 80-116.
- Schillinger, U. 1989. Antimicrobial activity of *L. sake* isolated from meat. *Appl Environ Microbiol* **55**: 1901-1906.
- Singh, B.P., Vij, S. and Hati, S. 2014. Functional significance of bioactive peptides derived from soybean. *Peptides* **54**: 171-179.
- Vij, S., Hati, S. and Yadav, D., 2011. Biofunctionality of probiotic soy yoghurt. *Food and Nutrition Sciences* DOI:10.4236/fns.2011.25073.
- Wang, D., Wang, L.J., Zhu, F.X., Zhu, J.Y., Chen, X.D., Zou, L. and Saito, M. 2008. In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). *Food Chemistry* **107**(4): 1421-1428.
- Wynstra, R.J. 1986. Expanding the use of soybeans. College of Agriculture, University of Illinois at Urbana – Champaign, 20.
- Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**(6870): 389-395.