Mongolian Journal of Biological Sciences 2009 Vol. 7(1-2): 61-68

Isolation of Lactic Acid Bacteria with High Biological Activity from Local Fermented Dairy Products

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Abstract

The thirty-two strains of lactic acid bacteria were isolated from the Mongolian traditional fermented dairy products, among them 25 strains show antimicrobial activity against test microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*. Protease sensitivity assay demonstrated that the antimicrobial substances produced by isolates A23, T2 are bacteriocins as their antibacterial activities were eliminated completely after treatment with protease. Identification of bacteria is being carried out. Among the isolates 22 strains show protease enzyme producing activity. The selected strains isolated from mare's fermented milk (airag or kumis) and yoghurt (tarag) show the specific protease activity from 7.9 μg/ml to 11.9 μg/ml. The strain T2, isolated from yoghurt exhibited the highest proteolytic activity.

Key words: Lactic acid bacteria, antimicrobial activity, bacteriocins, proteolytic activity

Introduction

Fermented products are integral part of Mongolian heritage developed over a long period, which have great social, religious, cultural, economic and medicinal importance. There is a wide variety of fermented milk products in Mongolia because of variations in the raw materials, processing methods which come from the habits and customs of the different regions of country. The most common fermented milk product of Mongolia is *airag* which traditionally made from mare's milk. Another kind of fermented milk is *tarag*, which is prepared from cow, goat and sheep milk. A third indigenous dairy product is *khoormog,* which is prepared from camel milk (Baldorj & Namsrai, 1980; Damdinsuren *et al.*, 2008). Fermented products have probiotic effects as they contain live microorganisms. Lactic acid bacteria (LAB) play in vital role for fermentation of Mongolian traditional dairy products (Damdinsuren *et al.*, 2008; Batdorj *et al*., 2007).

LAB produce different antibacterial substances including organic acids (lactic acid, acetic acid), hydrogen peroxide and bacteriocins. These substances are used as bioconservants for food preservation and improve the test and quality of dairy products (Kashket, 1987; Luquet & Corrieu, 2005). Bacteriocins are ribosomally synthesized substances of proteinaceous nature during growth of lactic acid bacteria. These bacteriocins protect host organisms by killing or inhibiting the growth of other bacteria (Klaenhammer, 1993). Many bacteriocinogenic lactic acid bacteria have been found in the numerous fermented dairy products. Over the past few years, studies concerning on bacteriocins produced by LAB have received an increasing interest and many new bacteriocins were discovered recently (Nissen-Mayer & Nes, 1997; Ennahar *at al*., 1999; Luquet & Corrieu, 2005). It was revealed that bacteriocins not only used as biopreservatives, but also used as medicine during prevention of different diseases as an alternatives of antibiotics (Twomey *et al*., 2000; Ryan *et al*., 1998; Hillman, 2002). Sometimes bioactive peptides can be released in proteolysis by lactic acid bacterial enzymes.

Many industrially utilized dairy starter cultures are proteolytic to some extent. Bioactive peptides can, thus, be generated by the proteolytic activities of the strains of starter and non-starter bacteria. The single most effective way to increase the concentration of bioactive peptides in fermented dairy products is to ferment or co-ferment with highly proteolytic strains of LAB. The choice of strains influences the release of effective bioactive peptides (Gobetti *et al*., 2004)

This study is part of continuing effort to

explore the potentials of our indigenous microbial flora in developing fermented milk products with probiotic effect (Batdorj *et al*., 2006; 2007; Budragchaa, *et al*., 2009). We isolated and characterized the bioactive compound producing lactic acid bacteria from Mongolian traditional fermented dairy products.

Material and Methods

Totally 15 fermented dairy product samples (13 kumis, 1 yoghurt and 4 khoormog samples) were collected from nomads family of Umnugovi, Zavkhan, Arkhangai, Tuv and Uvurkhangai provinces in Mongolia, during the period of July 30 to August 5, 2009. The samples were collected in sterilized tubes, kept in iceboxes and transported to the laboratory. We carry out laboratory experiments at the biochemical laboratories of National University of Mongolia.

Bacterial strains and growth media. All LAB were propagated in Non-fat skim milk (NFSM) and Man, Rogosa, Sharpe (MRS) broth (Carl Roth, Germany) and incubated at 37°C for 24 hours. The bacterial strains used as indicator micro-organisms for the screening of bacteriocin production and evaluation for antimicrobial activities are *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and they were cultivated in Nutrient Agar medium (NA). The stock cultures were maintained at -20°C in MRS broth containing 20% (v/v) glycerol. Before use, the strains were propagated twice in the appropriate broth overnight. Agar media were prepared by addition of 1.5% (w/v) agar (Carl Roth,) to the medium; overlay agar and agar well base contained 0.8% (w/v) of agar.

Isolation of the LAB strains. All samples (10%, v/v) were propagated twice in skim and sterilized milk at 37°C for 16–18 h before study. After incubation, bacteriocin-producing strains were isolated by direct plating method (Coventry *et al.,* 1997). Colonies producing zones of growth inhibition in the indicator lawn were randomly selected then inoculated in MRS broth for 24 h at 37°C.

LAB pure cultures isolated from samples were maintained as frozen stock culture stored at -20°C in MRS broth with 20% glycerol.

Antimicrobial activity assay. The antimicrobial activity of cell-free supernatant and partially purified bacteriocin was determined by well diffusion method as described by Batdorj *et al*., (2006).

Cell-free supernatant was obtained by centrifugation at 3000 rpm for 15 min, adjusted to pH 6.5 with 1 N NaOH. To investigate the antibacterial activity spectra of LAB strains by well diffusion assay, 100 µl culture of one of the test bacteria, grown to the early stationary growth phase in nutrient medium, was added to 20 ml of soft nutrient agar (0,8%, w⁄ v). Wells were made in the lawn of hardened soft agars in Petri dishes. Aliquots (100 µl) of supernatant of overnight cultures (16–18 h) were poured in the wells. The plates were left for 1 h at room temperature in sterile conditions before incubating them to the adequate temperature of growth of the test micro-organism. A clear zone of inhibition of at least 1 mm in diameter was recorded as positive.

Effects of enzyme, pH and heat treatment. The effect of various enzymes on bacteriocin activity was determined as described by Noonpakdee *et al*. (2003) with slight modification. Two hundred microlitre cell-free supernatant was incubated with 20 µl of protease K (Carl Roth, Germany) at a final concentration of 0.1 mg/ml in 20 mmol/ L phosphate buffer pH 7. After 2 h of incubation at 37°C, enzyme activity was stopped by heating at 100°C for 5 min. Untreated samples were used as controls. The residual bacteriocin activity was determined by well diffusion method against indicator strains. The pH sensitivity of the active substances was estimated by adjusting the pH of supernatant between 2 and 10 by using 5 N NaOH or 5 N HCl. After 2 h of incubation at room temperature, the pH was adjusted to 6.5, and the residual activity was tested as described earlier. To evaluate the heat stability of the active substances, neutralized cell-free supernatant (5 ml) was incubated in boiling water at 100°C for 10, 20 and 30 min, and at 121°C in an autoclave for 10, 15 and 20 min. In this latter case, time of heating was measured after a 10-min period necessary to increase the temperature up to 121°C. The residual activity was then assayed against the indicator strain.

Protease activity assay. Screening for proteolytic activity for the 33 strains of isolated lactic acid bacteria was done on MRS agar plates supplemented with 10% NFSM. After 2 days incubation at 37°C, the clear zone surrounded by colony indicates the extracellular protease.

The productivity of the colonies is measured in these plates as a ratio of the halo formed by the casein hydrolyzed to the diameter of the producer bacteria (Marcela, 2001).

Quantification of the proteolytic activity. Proteolytic activity was quantified to cell free supernatants. All activity measurements were done in triplicate. The method using casein as substrate was performed according to Marcela (2001). The method relies on the release of tyrosine- and tryptophan-containing peptides that react with Folin reagent. The enzyme activity was defined as the amount of tyrosine released per min per ml of the assay solution.

Morphological, physiological and biochemical tests of LAB strains. Bacteriocin and protease-producing strains were characterized and identified on the basis of their morphological and biochemical properties. Catalase reaction, oxidase test and gram staining were determined after 24 h of incubation on MRS medium. Growth at 10, 45°C, pH 9.6 and in the presence of 4% and 6.5% (w/v) sodium chloride was tested by incubating in MRS (Carl Roth) liquid medium. Acid production was tested in casein hydrolisate supplemented with different carbohydrates.

Results

Isolation of bacteriocin-producing strain. We have isolated thirty-three LAB strains from 13 samples of airag, 1 samples of yoghurt and 1 samples of hoormog and tested for their inhibitory activities against indicator bacteria by well diffusion method as described before.

All isolated strains were gram positive and catalase negative, long or short chained rods and coccus. Some physiological and biochemical characteristics confirm that these bacteria belong to Lactic Acid Bacteria (data not shown).

Thirteen isolates showed a significant growth inhibition against indicator strains on NA medium (Table 1). Four of the isolates inhibited the growth of *P. aeruginosa,* seven isolates inhibited *Ent. faecalis*, 10 isolates were active against *E. coli*, and 5 isolates inhibited the growth of *S. aureus* after neutralization of pH. We have chosen the following LAB isolates, A2, A5, A8, A13, A18, A21, A23, A28, A31 and T2 for further investigation.

Effects of enzymes, pH and heat treatment. The effects of protease on the antibacterial activity of cell free supernatants produced by isolates A2, A5, A8, A13, A18, A21, A23, A28, A31 and T2 are presented in Table 2. Protease sensitivity assay demonstrated that the antimicrobial substances produced by isolates A23 and T2 are bacteriocins as their antibacterial activity was completely eliminated after treatment with protease.

Inhibitory activity of isolate T2 did not decrease by heating at 100°C and 121°C for 10 and 20 min. However, the inhibitory activity was not completely inactivated during subsequent heat. Contrary, isolate A23 lost total activity at 100°C and 121°C for 30 min.

Screening for proteolytic activity. Thirty-two strains of lactic acid bacteria isolated from fermented milk products were screened for their proteolytic activities using MRS agar medium supplemented with 10% NFSM. Isolated colonies were cultivated on this medium in spot, and cultivated at 37°C for two days. Proteolytic strains were easily recognized by their clear halo in the medium plate, and the proteolytic activity was measured by clear halo radius (Table 3).

It was observed that 22 strains of LAB from total isolated strains show some proteolytic activity. Titrable acidity was measured after 24 h cultivation in NFSM medium. Strains A12, 16, 17, 19, 20, 26 and 28 could not coagulate skimmed milk, and their acidities were very low. For the manufacturing of yoghurt and other fermented milk products titrable acidity is one of the important characteristics of a start culture.

Among the strains that showed the proteolytic activity and sufficient titrable acidity, five strains with highest activity were chosen for the quantification of the proteases.

Specific protease activity of selected LAB strains was determined by a method, which relies on the release of tyrosine- and tryptophan-containing peptides that react with Folin reagent. Proteolytic activity was studied in cell free supernatant. Results of the measurement of protease activity are shown in Table 4. It was observed that strain T2, isolated from tarag exhibited the highest activity which equal to 11.9 μg/ml. The selected strains isolated from airag show specific activity form 7.9 μg/ml to 10.5 μg/ml.

The proteolytic behavior of LAB on casein varies according to the bacterial species and environmental conditions (Giori *et al*., 1985).

Characteristics of isolated strains. Biochemical and physiological characteristics

Table 1. Antimicrobial activity of isolated bacteria

Isolate	Antimicrobial activity (Inhibition zone, mm)										
	Cell free supernatant						Neutralized cell free supernatant				
	pH	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Escherichia coli*	*Micrococcus luteus*	pH	*Pseudomonas aeruginosa*	*Enterococcus faecalis*	*Escherichia coli*	*Staphylococcus aureus*
A1.	5	-	-	-	-	-	6.5	-	-	-	-
A2.	5	2	1	-	2	-	6.5	2	-	1	-
A3.	4	1	1	-	1	-	6.5	-	-	-	-
A4.	4.5	1	1	2	3	-	6.5	-	-	1	1
A5.	4.5	3	4	2	5	-	6.5	1	-	1	1
A6.	4	-	4	3	3	-	6.5	-	-	-	-
A7.	5	-	1	-	2	-	6.5	-	-	1	-
A8.	4.5	2	4	2	4	-	6.5	-	3	1	-
A9.	5	2	4	2	2	-	6.5	-	-	-	-
A10.	5.5	-	1	-	2	-	6.5	-	-	-	-
A11.	5	-	-	-	-	-	6.5	-	-	-	-
A12.	5	-	2	-	1	-	6.5	-	1	-	-
A13.	4.5	3	4	3	3	-	6.5	-	3	2	2
A14.	4.5	3	4	2	2	1	6.5	-	-	1	-
A15.	4.5	2	-	2	1	1	6.5	-	-	-	-
A16.	5	-	-	-	-	-	6.5	-	-	-	-
A17.	4.5	1	1	1	-	-	6.5	-	-	-	-
A18.	4	-	3	1	3	-	6.5	-	4	-	-
A19.	5	-	1	-	-	-	6.5	-	-	1	-
A20.	5	-	-	-	-	-	6.5	-	-	-	-
A21.	4.5	2	4	3	1	-	6.5	-	3	-	1
A22.	4.5	3	3	2	1	5	6.5	1	-	-	-
A23.	4.5	1	3	-	2	-	6.5	-	4	-	-
A24.	4.5	3	5	3	2	-	6.5	-	-	-	-
A25.	5	2	3	1	3	2	6.5	-	-	-	-
A26.	5.5	1	2	3	3	-	6.5	-	-	1	-
A27.	4.5	-	3	4	2	1	6.5	-	-	-	-
A28.	4	3	1	3	4	1	6.5	-	-	-	-
A29.	4	-	-	4	1	1	6.5	-	-	-	-
A30.	4.5	1	-	2	2	1	6.5	-	-	-	-
T1	5.5	3	2	2	4	-	6.5	-	-	2	2
T2.	4	2	5	2	3	5	6.5	2	3	-	-

Table 2. Antimicrobial activity of selected isolates after treating with protease

Test organism	Antimicrobial activity (Inhibition zone, mm)			
	E. faecalis		*E.coli*	
Isolate	Neutralized cell free supernatant	After treatment with protease	Neutralized cell free supernatant	After treatment with protease
A2	1	-	1	-
A5	2	2	2	2
A8	1	1	-	-
A13	-	-	-	-
A18	2	2	2	2
A21	3	3	3	3
A23	3	-	3	-
A28	1	2	2	2
A31	1	-	-	-
T2	3	-	2	-

Table 3. Screening for the proteolytic activity and production of general acidity

Strains	Proteolytic activity determined on NFM agar medium	Milk coagulation rate, after 24h	General acidity after 24h incubation in milk (°T)
A1.	++	Coagulated	76.0
A2.	+++	Coagulated	95.0
A3.	-	Coagulated	80.0
A4.	+	Coagulated	81.0
A5.	++	Coagulated	82.0
A6.	+	Coagulated	100.0
A7.	+	Coagulated	100.0
A8.	+	Coagulated	47.5
A9.	-	Coagulated	95.0
A10.	-	Coagulated	145.0
A11.	+	Coagulated	90.0
A12.	+	Not coagulated	42.5
A13.	++	Coagulated	97.5
A14.	-	Coagulated	85.0
A15.	-	Coagulated	80.2
A16.	++	Coagulated	77.0
A17.	+	Not coagulated	63.0
A18.	+++	Coagulated	72.5
A19.	+	Not coagulated	65.0
A20.	+	Not coagulated	48.0
A21.	+	Coagulated	112.0
A22.	-	Coagulated	100.0
A23.	-	Coagulated	85.0
A24.	-	Coagulated	83.0
A25.	+	Coagulated	91.0
A26.	+	Not coagulated	47.5
A27.	-	Coagulated	93.0
A28.	++	Coagulated	76.5
A29.	-	Coagulated	91.5
A30.	+	Coagulated	100.0
T1	++	Coagulated	79.0
T2.	++	Coagulated	87.0

- no halo; + halo radius is 1-3mm; ++ halo radius is 3-5mm; +++ halo radius > 5mm

were shown in Table 5. Growth of bacteria at different temperatures, salt and methylente blue tolerance, carbohydrate assimilation and NH_3 production from arginine, were determined. All of them were able to grow at 45°C, and in 2% NaCl, 4% NaCl, and produce acids from different carbohydrates. Carbohydrate fermentation profile of selected LAB was different. On the basis of biochemical and physiological characteristics, the strains A8, A3 and A21 belong to *Streptococcsus*-species. Strains A2, A5, A18, A23, A28 and T2 belong to *Lactobacillus*-species. Both these groups are very important for the production of fermented dairy products, and therefore, further studies are necessary to complete identification of bacteria.

Table 4. Specific protease activities in the culture supernatants of the strains

№	Strains	Specific protease activities (μg/ml)
1	A2	10,5
2	A5	9,2
3	A18	7,9
4	A28	10,2
5	T2	11,9

Discussion

Mongolia is relatively large country with little population, but it has rich centuries-old tradition of processing the dairy products. There are enough sources of milk in Mongolia, however the majority of milk production is taking place in

Table 5.Biochemical and physiological characteristics of selected strains

Characteristics	Starains									
	A2	A5	A8	A13	A18	A21	A23	A28	T1	T2
Cell shape	rods	rods	coccus	coccus	rods	coccus	rods	rods	rods	rods
Growth at :										
45°C	+	+	+	+	+	+	+	+	+	+
0,1% of methylene blue	+	+	-	+	+	+	+	+	-	+
0,005% of methylene blue	+	+	-	+	+	+	+	+	-	+
2% Na Cl	+	+	+	+	+	+	+	+	+	+
4% Na Cl	+	+	+	+	+	+	+	+	+	+
6,5% Na Cl	-	-	+	+	-	+	-	-	-	-
NH_3 from arginine	-	-	+	-	-	+	+	-	-	-
Acid from:										
L-Arabinose	-	-	w	-	+	-	+	-	+	w
Dultcitol			-	-	-	-	-	-	-	-
D-Fructose	+	-	-	+	-	+	+	+	-	+
Inulin	-	-	+	-	-	-	-	+	-	-
Galactose	-	-	+	-	-	-	-	+	-	-
D-Glucose	-	-	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	-	-
Mannose	-	-	-	-	-	-	-	+	-	+
Mannitol	-	-	-	-	-	-	-	+	-	-
Raffinose	+	+	+	-	+	-	+	-	+	+
D-Ramnose	+	-	-	-	-	+	-	+	-	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	-	+	+	+	+	+	+	-
Trehalose	-	-	-	-	-	-	-	+	-	+

Symbols: + positive; - negative, w - weak

the rural areas, but only small part of milk and dairy products are reach to the city consumers. Nowadays, the majority of Mongolian population is concentrated at urban areas, especially in the capital city, Ulaanbaatar, hence there is increasing need for dairy products.

One of major obstacles in sufficient supplying of high quality dairy products is the lack of a local source of start culture for fermented dairy products. Currently the majority of start cultures that are required for local dairy processing have been imported from abroad. These start cultures usually contain bacteriophages and cease to ferment milk after two transfer. However, we think that with our rich experience and long-lasting tradition of processing the fermented dairy products, it should be able to produce locally our own dairy start cultures with high biological activity by isolating good microorganisms.

Up to date most of the studies on Mongolian dairy product's microbiology have been conducted only in systematic of microorganisms (Damdinsuren *et al*., 2008; Koichi *et al*., 2008; Watanabe *et al*., 2008). There is real necessity to study the biological activity of lactic acid bacteria and to develop technology for making probiotic products.

Previously we have isolated bacteriocin producing *Entererococcus durans* from Mongolian airag, purified bacteriocins and characterized them (Batdorj *et al.,* 2006). It is tested and approved that this bacteria shows a high influence against the food-born pathogens and pathogenic bacteria.

Lactobacillus and *Streptococcus* species are widely used for the production of different types of fermented milk products (Leroy & De Vuyst, 2004; Luquet & Corrieu, 2005).

The present study revealed the inhibitory activities of some strains of *Lactobacillus* and *Streptococcus* isolated from Mongolian fermented dairy products. Totally 32 LAB strains were isolated from fermented dairy product samples and 25 strains of them show antimicrobial activity against test microorganisms. Protease sensitivity

assay demonstrated that the antimicrobial substances produced by isolates *Lactocacillus* A23 and T2 strains are bacteriocins as their antibacterial activities were completely eliminated after treatment with protease. The bacteriocin produced by T2 strain was heat resistant.

Lactobacillus A2, A5, A18, A28, T2 strains show protease enzyme producing activity on NFSM supplemented medium and show the specific protease activity from 7.9 µg/ml to 11.9 µg/ml. The strain *Lactobacillus* T2, isolated from yoghurt exhibited the highest proteolytic activity. Proteolytic activity has been found to be essential for dairy LAB to promote better growth on milk and the development of the organoleptic properties of the fermented milk products (Nieto-Arribas *et al.,* 2009). The result of this work shows that *Lactobacillus* T2 strain reveals a great potential for the production of probiotic products.

Acknowledgements

This work has been done within the framework of the project "Isolation of lactic acid bacteria with high biological activity issued from local fermented dairy products", supported by the Asia Research Center in Mongolia and Korea Foundation for Advanced Studies, Korea.

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Хураангуй

Монголын уламжлалт аргаар бэлтгэсэн эсгэлэн сүүн бүтээгдэхүүнээс сүү хүчлийн бактерийн 32 өсгөвөр ялган авснаас 25 өсгөвөр нь *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* зэрэг тест микроорганизмын өсөлтийг дарангуйлах идэвх үзүүлж байв. Протеаза ферментэд мэдрэг байдлыг шалган үзэхэд А23, Т2 өсгөврүүдээс ялгарч буй микробын эсрэг үйлчлэлтэй бодис нь бактериоцины төрлийн бодис болох нь тогтоогдлоо. Өндөр идэвх бүхий өсгөврүүдийн физиологи, биохимийн шинж чанар дээр үндэслэн ангилал зүйн тодорхойлолтыг хийв. Ялган авсан өсгөврүүдээс 22 нь протеаза ферментийг өсгөврийн шингэндээ нийлэгжүүлж байв. Айраг, тарагнаас ялган авсан өсгөврүүдийн протеазын өвөрмөц идэвх 7.9 мкг/мл-ээс 11.9 мкг/мл байна. Тарагнаас ялган авсан Т2 өсгөвөр хамгийн өндөр протеолитик идэвх үзүүлж байв.

Received: 22 January 2009
Accepted: 28 February 2010