A.V. Schekotova et al /J. Pharm. Sci. & Res. Vol. 9(12), 2017, 2599-2603

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

The Study of the Proteolysis of Milk Proteins Obtained By Thermal Calcium Coagulation

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Abstract

The article describes the results of the studies to determine the proteolytic activity of different *Lactobacillus helveticus* strains used to produce calcium-enriched milk protein concentrates (MPC). A high level of the extra- and intracellular proteolytic activity of the crops under study has been established. It has been noted that hydrolysis by proteinases of different strains of *Lactobacillus helveticus* is almost equal in effectiveness to hydrolysis by the animal enzyme. The molecular weight distribution of the products of the hydrolysis of cow and goat milk proteins by proteolytic enzymes of lactic bacteria has been studied. It has been established that the MPC based on cow milk are split into 7 fractions, while the goat milk-based MPC - into five factions. The fractions of α_{s1}-casein and γ-casein are absent in the fermented goat milk-based MPC. It has been established that the *Lactobacillus helveticus* H_{17-18} stain is superior to the *Lactobacillus helveticus* H_9 stain by proteolytic capacity. It has been noted that the largest number of low molecular weight peptides capable of binding calcium was found in fermented protein clumps based on goat milk.

Keywords: lactic bacteria, protein concentrates, proteolysis, cow milk, goat milk.

INTRODUCTION

The state of health of the Earth's population tends to deteriorate and is characterized by an increase in the number of persons suffering from various diseases, including nutritional ones. Among the nutritional diseases caused by the lack of nutrients, the diseases caused by calcium deficiency are of the greatest practical importance. Calcium (Ca) is the second important element involved in various physiological and biochemistry processes occurring in the body. It plays an important role in regulating the permeability of cellular membranes, the electrogenesis of nervous and muscle tissues, in the molecular mechanics of muscle contraction, digestive and endocrine glands, in the activation of various enzymatic systems, including provision of blood clotting, etc. [1, 2, 3].

It is known that Ca is contained in food in the form of sparingly soluble or completely insoluble salts, mainly phosphates, carbonates, oxalate, as well as compounds with fatty acids, and proteins. Calcium, which is found in milk and dairy products, is easily digestible [4-7]. It has been established that not only cow milk is rich in calcium. Goat milk is characterized by higher content of this macronutrient [8-11], but its number is insufficient. The daily calcium intake is contained in 2l of milk and dairy products. The use of this quantity of dairy products on a daily basis is not real for most people. The process of additional introduction of calcium ions into dairy products is difficult to implement due to the physical properties of inorganic calcium salts - their insignificant solubility [12].

In solving the problems of enriching dairy products with calcium, attention should be given to milk-protein concentrates (MPC) obtained by thermal calcium coagulation (coprecipitates). The thermal calcium coagulation of milk proteins is based on the joint deposition of casein and serum proteins, with simultaneous effects of temperature and the chloride calcium on the milk [13]. This is a way to get a calcium-enriched dairy protein product.

We have developed a technology for fermented Ca-enriched MPC on the basis of goat and cow milk [14]. The MPC were obtained by thermal calcium coagulation, followed by fermentation of proteins with *Lactobacillus helveticus* lactic bacteria. The *Lactobacillus helveticus* has been selected as a culture for the fermentation of a protein concentrate due to the high proteolytic activity of microorganisms. *Lactobacillus Helveticus* produces enzymes capable of destroying milk proteins to biologically active peptides, which include antihypertensive, immunostimulating, anti-oncogenic peptides and casein phosphopeptides (CPP) [15-23]. It should be noted that the exclusive bioavailability of calcium from milk and dairy products is due to the presence of CPP, which are formed in the gastrointestinal tract at digestion of casein and provide

high Ca solubility. The CPP accumulate in the distal segment of small intestine, where they form calcium complexes that increase passive intake of Ca in the intestine independent of vitamin D [19, 24].

Biotechnology treatment of MPC using starter cultures will increase the functional properties of MPC obtained by thermal calcium deposition. Unlike the animal and vegetable-based enzymes, the proteolysis by lactic acid bacteria enzymes is performed specifically gently, through the enrichment of the product with valuable nitrogenous substances, thereby increasing its biological and tasteful value. Moreover, the use of proteolytic strains of lactic acid bacteria as starter cultures can reduce the allergenicity of milk proteins and develop hypoallergenic dairy products, as well as fermented products containing biologically active peptides [25]. Therefore, the most important task of the modern biotechnology is to find sources of the protease with a wide range of action among microbial producers, to study their properties and conditions of formation in order to replace animal proteolytic enzymes that require more time, valuable raw materials and economic costs.

It is known that the proteolytic activity of each strain is strictly specific and depends on many factors, including the raw materials used. Therefore, the study of the proteolytic properties of different strains of *Lactobacillus helveticus* is relevant for the purpose of their further use as starter cultures in the production of calcium-enriched MPC.

The scope of this work is to study the proteolytic activity of different strains of *Lactobacillus helveticus* used in the production of MPC obtained by thermal calcium coagulation.

MATERIALS AND METHODS

Two strains of lactic bacteria were the subjects of research: *Lactobacillus helveticus* H_9 and *Lactobacillus helveticus* $H_{17\text{-}18}$, obtained from the All-Russian Collection of Industrial Microorganisms of the FSUE GosNII Genetika (Federal State Unitary Enterprise State Research Institute of Genetics), activated by a biotechnological method developed at the ESSUTM (East-Siberian State University of Technology and Management) [26].

Milk of cows and goats was chosen as the milk raw material for milk and protein concentrate. The MPC were obtained by thermal calcium coagulation, followed by fermentation of proteins with *Lactobacillus helveticus*. Coagulation was carried out by applying a 20% solution of CaCl2 at a temperature of 950 °C. The coagulant dose for cow milk-based MPC was 1.50 g/l, and for goat milk-based MPC - 1.25 g/l. The fermentation of protein clots was carried out at (40±1) ^{o}C; the dose of the lactic bacteria ferment was 5% [14].

Proteolytic activity of intracellular enzymes of lactobacilli was determined by pricking into the gelatin column. The inoculated test tubes were incubated at 20-40^{0}C. The results were taken into account on the 1st, 10th and 20th days, noting the degree and nature of gelatin dilution. Extracellular proteolytic ability was established by its inoculation on milk agar. If the bacteria are proteolytically active, milk casein is peptonized, and light zones are formed around the colonies on a cloudy background of the medium. The 1-4 mm zone indicates a low proteolytic activity, 5-9 mm - the medium one, and 10 mm and more - a high one.

The degree of protein hydrolysis was calculated as the ratio of amine nitrogen to total nitrogen. The content of amine nitrogen in the solution was determined by titration; the concentration of protein substances - by the Kjeldahl method [27].

MPC were examined by electrophoresis in a dodecyl sulfate-sodium-polyacrylamide gel using a Mini-Protean cell [28].

All experiments were carried out in 3-5 replicates. The data obtained were processed using an Excel statistical software package with the Mann-Whitney test. Significant differences were considered if the error probability had been $p \leq 0.05$.

RESULTS AND DISCUSSION

At the first stage of the work, the proteolytic activity of the studied *Lactobacillus helveticus* strains was examined. To determine the presence of extracellular proteolytic enzymes that split casein, bacteria were grown on milk agar. The intracellular proteolytic activity in the Lactobacillus helveticus strains was studied using gelatin. The results of the study are shown in Table 1.

From the data in Table 1, it can be seen that both strains tested showed high proteolytic activity on gelatin liquefaction. Decomposition of gelatin occurred on the first day, when inoculated with a prick, the liquefaction column had a funnel shape. By the ability to lase casein, the *Lactobacillus helveticus* $H_{17\text{-}18}$ strain showed a higher level of proteolysis (the clearing zone was 11 mm), in the *Lactobacillus helveticus* $H_{9\ \text{strain}}$ - the average level of proteolytic activity (the clearing zone was 8-9 mm).

Table 1. Extracellular and intracellular proteolytic activity of different *Lactobacillus helveticus* strains

Lactic bacteria strain	Proteolytic activity (zones of clearing on medium, mm/form of liquefaction)	
	Extracellular (decomposition of casein)	Intracellular (liquefaction of gelatin)
Lactobacillus helveticus $H_{17\text{-}18}$	+++ (11±1 mm)	+++ (funnelform)
Lactobacillus helveticus H_9	++- (8±1 mm)	+++ (funnelform)
Note: proteolytic activity "+++" - strong, "++ -" - medium, "+ -" - weak, "---" - absent		

To assess the proteolytic properties of the cultures studied, the degree of hydrolysis of the protein clumps of cow and goat milk during the fermentation of *Lactobacillus helveticus* was calculated. As a control, hydrolysis using an enzyme of animal origin (pepsin) was considered at an enzyme to substrate ratio of 1:100. The results of the studies are presented in Table 2.

As a result of the study, it has been found that 4 hours are sufficient for the fermentation of protein clumps of milk with lactic bacteria, and further there is no increase in the degree of hydrolysis (Table 2). Moreover, it has been noted that hydrolysis of goat milk proteins goes deeper, which is explained by the presence of more albumin whey protein in goat milk, due to which proteins are more easily subjected to hydrolysis and deeper decay. It has been established that the *Lactobacillus helveticus* $H_{17\text{-}18}$ strain has higher proteolytic activity from the two lactic bacteria strains under study. It should be noted that the degree of hydrolysis of cow milk proteins after 4 hours of fermentation by the *Lactobacillus helveticus* $H_{17\text{-}18}$ strain reaches 88%, and that of goat milk proteins - 91%.

From the data in Table 2 it can be seen that the hydrolysis by proteinases of different *Lactobacillus helveticus* strains is practically close to pepsin hydrolysis, which indicates a great potential for their use as sources of proteases in various areas of the food industry and biotechnology.

The electrophoregram (Fig. 1) of the test samples shows that, in contrast to cow milk proteins, the fractional composition of goat milk consists of β-casein and whey proteins: lactoglobulin (β-Lg) and lactalbumin (α-La). It is known [29, 8-11] that the goat milk belongs to a casein group, as well as cow milk. However, in the goat milk, the α_{s1}-casein protein fraction is practically absent (Figure 1 B), therefore goat milk causes less allergic reactions and digestive disorders than cow milk. The goat milk lacks the γ-casein present in cow milk, so the goat milk is better absorbed. The content of a large amount of the albumin whey protein allows goat milk to coagulate in very small and gentle flakes, which facilitates the digestion process [29, 8-11].

Table 2. The degree of hydrolysis of goat and cow milk proteins in the fermentation by different strains of lactic bacteria and pepsin

Source of proteolytic enzymes	Name of raw materials	Degree of hydrolysis of milk proteins,%		
		Time of hydrolysis, h		
		2	4	6
Lactobacillus helveticus $H_{17\text{-}18}$	Cow milk-based MPC	72±1.2	88±0.6	88±1.2
	Goat milk-based MPC	74±0.8	91±0.5	91±0.9
Lactobacillus helveticus H_9	Cow milk-based MPC	65±0.9	81±0.8	81±1.2
	Goat milk-based MPC	69±0.5	87±1.1	87±1.1
Pepsin	Cow milk-based MPC	76±0.5	92±0.8	92±0.6
	Goat milk-based MPC	79±1.2	95±0.9	95±0.7

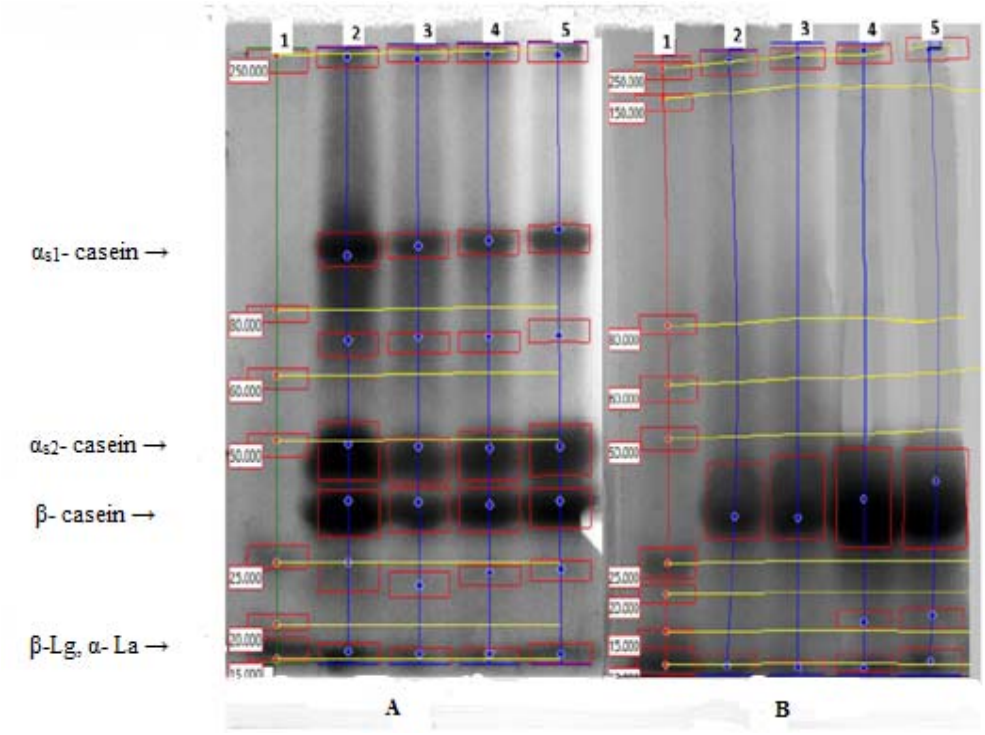

Figure 1 - Electrophoresis in polyacrylamide MPC gel:

a) Cow milk-based (row 2 and 3 - fermentation of *Lactobacillus helveticus* $H_{17\text{-}18}$, row 4 and 5 - fermentation of *Lactobacillus helveticus* H_9);

b) Goat milk-based (row 2 and 3 - fermentation of *Lactobacillus helveticus* H_9, row 4 and 5 - fermentation of *Lactobacillus helveticus* $H_{17,\,18}$);

Table 3. The molecular weight distribution of the products of the hydrolysis of cow and goat milk proteins by *Lactobacillus helveticus* proteolytic enzymes

No. of fractions	Range of molecular weights, kDa	Relative fractional distribution,% depending on the used strain of lactic bacteria and protein substrate			
		Lactobacillus helveticus H_9		*Lactobacillus helveticus* H_{17-18}	
		Cow milk-based MPC	Goat milk-based MPC	Cow milk-based MPC	Goat milk-based MPC
1	108.1-248.5	7.72	7.37	5.08	5.02
2	69.1-108.1	19.59	11.03	12.71	11.05
3	48.9-69.1	1.67	-	1.86	-
4	36.3-48.9	31.81	-	37.74	-
5	23.9-36.3	28.83	76.83	30.93	81.02
6	16.6-23.9	2.13	0.84	2.42	2.02
7	9.9-16.6	8.25	3.93	9.26	4.93

MPC based on cow's milk are split into 7 fractions, while the goat's milk-based MPC - into five factions (Table 3). In the fermented goat milk-based MPC, there are no fractions of 48.9-69.1 kDa and 36.3-48.9 kDa in size. Possibly these are the fractions of αs1- and γ-casein not contained in goat milk [10]. The fractions of 36.3-48.9 kDa and 23.9-36.3 kDa in size are the dominant fractions in MPC based on cow milk. The content of these fractions during the fermentation of milk proteins by the *Lactobacillus helveticus* H_{17-18} strain totals 68.7%, and in the fermentation with *Lactobacillus helveticus* H_9 - 60.6%. In the goat milk-based MPC, the main fraction is 23.9-36.3 kDa and at fermentation of *Lactobacillus helveticus* H_{17-18} it makes up 81%, and at that of *Lactobacillus helveticus* H_9 - 76.8%, respectively. This indicates that under equal conditions, hydrolysis of goat milk proteins with proteinases of lactic bacteria is more intensive with the formation of low molecular weight peptides than in cow milk. Moreover, the *Lactobacillus helveticus* H_{17-18} strain is characterized by higher proteolytic activity.

It should be noted that the use of the *Lactobacillus helveticus* H_{17-18} stain in the MPC production provides more low-molecular peptides capable of binding calcium and transporting it into bone tissue, bypassing regulatory functions of the body.

The results are consistent with the literature data on high Ca digestibility in milk products, fermented with *Lactobacillus helveticus,* compared to traditional fermented milk products. The long-term use of dairy products enriched by *Lactobacillus helveticus* increases the density and content of bone minerals relative to the body mass [5].

Probably, the casein phosphopeptides that provide for better fixing of calcium are formed by the protein hydrolysis with *Lactobacillus helveticus* proteolytic enzymes.

Thus, the fermentation of MPC with *Lactobacillus helveticus* lactic bacteria increases the Ca digestibility in MPC.

CONCLUSION

Collectively, it can be concluded that the *Lactobacillus Helveticus* strains under study have a high level of proteolytic activity of intracellular and extracellular enzymes. It has been noted that hydrolysis by proteinases of *Lactobacillus helveticus* is almost equal in effectiveness to hydrolysis by the animal enzyme. It has been established that the *Lactobacillus helveticus* H_{17-18} stain is superior to the *Lactobacillus helveticus* H_9 stain by proteolytic capacity. This is confirmed by data on the degree of hydrolysis and molecular weight distributions of peptides in MPC. It has also been noted that the largest number of low molecular weight peptides capable of binding calcium has been found in fermented protein clumps based on goat milk.

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