J Ind Microbiol Biotechnol (2005) 32: 449–454
DOI 10.1007/s10295-005-0009-8

ORIGINAL PAPER

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Proteolytic activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in frozen-stored Kashkaval cheese

Received: 17 April 2005 / Accepted: 17 June 2005 / Published online: 10 August 2005

Abstract Proteolytic activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in Kashkaval cheeses of varying aging times, stored at −10 to −12°C for 12 months, was studied. It was established that the proteolysis of Kashkaval cheese induced by the starter culture was significantly delayed by freezing. The noncasein nitrogen (NCN/TN) and nonprotein nitrogen (NPN/TN) as a percentage of total nitrogen increased slightly during frozen storage of Kashkaval. It was found that NCN/TN and NPN/TN values increased to a larger extent in frozen-stored Kashkaval samples with shorter aging time. Enhanced proteolysis was observed during ripening of thawed Kashkaval cheese. There was greater accumulation of noncasein nitrogen in thawed Kashkaval samples compared to the control samples. The enhanced proteolysis during ripening of thawed Kashkaval cheese resulted in larger amounts of high and medium molecular weight peptides and lower amounts of low molecular weight peptides and free amino acids as compared to controls.

Keywords Proteolytic activity · Lactobacillus delbrueckii ssp bulgaricus · Streptococcus thermophilus · Frozen storage · Kashkaval cheese

Introduction

The biochemical processes that occur during storage of cheeses cause certain changes in their composition and properties and lead eventually to inferior quality [18]. Hence, the necessity of developing methods that can extend the durability and shelf life of cheeses. Freezing has been found to be an effective method of extending cheddared cheese shelf life [11, 14, 19]. It has been established that the maintenance of quality of cheeses during frozen storage depends on a number of factors, e.g. refrigeration and storage conditions [7, 8, 17, 22], composition and aging time of the product [5, 13, 25], etc. The proteolysis during ripening and frozen storage is of primary importance for cheese quality. The basic changes in cheese protein structures during ripening happen under the influence of the proteolytic enzymes produced by the starter micro organisms [18]. Therefore, the factors with an impact on cheese proteolysis by the starter culture could play a crucial role for the course of the ripening process. Kasprzak et al. [12] established a combined effect of the fat, moisture, salt content, and the freezing process on the proteolysis in Cheddar cheese, expressed through the levels of nonprotein nitrogen. That indicator increases during frozen storage as well as during subsequent ripening after thawing. According to Bertola et al. [2], the aging time, ripening after thawing, freezing rate and frozen storage time all affect the changes in nonprotein nitrogen in Mozzarella cheese. The authors found that the proteolysis was more intensive in slow-frozen cheeses and in post-thaw ripened cheeses.

The purpose of the present study was to study the proteolytic activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in frozen-stored Kashkaval cheese.

Materials and methods

Cheese making

The Kashkaval samples were obtained from four batches, consisting of 5,000 l of milk each, and produced commercially at a local dairy plant (Filippopolis-RK Ltd-Plovdiv) according to the following procedure. Cow's milk of 3.9% fat content was heat-treated at a temperature (T) of 64 ± 1°C and cooled down to $T = 33 \pm 1$°C, at which temperature curding was carried

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out. Calcium dichloride, 1% starter culture consisting of *Str. thermophilus* and *Lb. delbrueckii ssp. bulgaricus* (Genesis-laboratories, Sofia, Bulgaria), and commercial animal rennet were added. After setting, the curd was sliced and crumbled into curd grains. Curd grains were next heated at $T = 39 \pm 1$°C, drained and pressed. The pressed curd was left to cheddar for 1.5 – 2 h. At the end of the process it reached pH = 5.2–5.3 at which stage it had the desired plastic texture. The cheddared curd was then cut and treated with 13% NaCl solution at 72–74°C, milled and packed into 0.250 kg moulds. Young Kashkaval remained in the moulds for 15 h, and was then taken out, dried and packed in polyamide/polyethylene foil under vacuum at 90–99.8 Pa. Ripening took place in these packages at $T = 7 \pm 1$°C and relative humidity (ϕ) of 75–80% for 45 days. For the purposes of the experiment, the ripening process was divided into three stages, each with a duration of 5, 25 and 45 days. Thus three different Kashkaval varieties, according to aging times, were obtained: young (aged for 5 days, with a noncasein nitrogen content of 5.38 ± 0.21% of the total nitrogen); semi-ripened (aged for 25 days, with a noncasein nitrogen content of 11.22 ± 0.36% of the total nitrogen); ripened (aged for 45 days, with a noncasein nitrogen content of 16.83 ± 0.20% of the total nitrogen). Cheese samples were frozen after each of the above stages of ripening.

Freezing and thawing

Kashkaval samples of various aging times were frozen in the laboratory freezer (natural air circulation, $T = -16$°C) until $T = -10$°C at the centre was reached. Frozen samples were stored at $T = -10$ to -12°C and relative air humidity $\phi = 75$–80% for 1, 3, 6, 9 and 12 months. After each of the above storage periods some of the samples were air-thawed at $T = +8$°C and $\phi = 75$–80%. Thawed samples of young and semi-ripened Kashkaval were then stored at $T = 6$ to 8°C and $\phi = 75$–80% for 40 and 20 days, respectively, until fully aged.

Physicochemical analyses

The Kashkaval samples were analysed for moisture content (heat at 105°C to constant weight), pH, fat content [3] and salt in moisture according to Mohr [16]. Noncasein nitrogen, nonprotein nitrogen and total nitrogen content were determined by the Vakaleris and Price method [23] modified to suit the specific conditions of the analysis. For noncasein nitrogen (NCN) determination, approximately 5 g of Kashkaval cheese was extracted in 100 ml sodium acetate buffer (pH 4.6), the homogenate was agitated at ambient temperature for 2 h and filtered. The nitrogen fraction soluble in 12% trichloracetic acid (TCA) was considered the nonprotein nitrogen (NPN). To determine the NPN content, approximately 5 g of Kashkaval cheese were homogenized in 40 ml sodium acetate buffer (pH = 4.6), the homogenate was agitated at ambient temperature for 2 h, then 10 ml of 60% TCA was added and the homogenate was filtered through Watman No 42 paper. Nitrogen determination was performed in duplicate by the Kjeldahl method using a Kjeltec Auto 1030 Analyzer (Tecator Sweden) combined with the Digestion System 20. Total protein (TP) was calculated as total nitrogen×6.38. Free amino acids determination was performed by the Mansour method [15] using automatic amino acid analyzer Hd-1200 E.

Microbiological analyses

The total numbers of viable cells of *Str. thermophilus* and *Lb. delbrueckii subsp. bulgaricus* in the studied Kashkaval samples were determined by the cultivation on synthetic culture media M17 and MRS (Merck, Darmstadt, Germany). The methodology described in IDF Standard 149 A:1997 [10] was followed. The samples were prepared according to IDF Standard 122 C:1996 [9]. Ten grams of the test Kashkaval sample were transferred into the container of a peristaltic-type blender. Ninety milliliters of diluent (20% sodium citrate solution) was added and the mixture was blended until the Kashkaval was thoroughly dispersed. Appropriate dilutions were mixed with the molten and cooled (47 ± 1°C) medium (M17 for *Str. thermophilus* and MRS for *Lb. delbrueckii ssp. bulgaricus*). After solidification the Petri dishes were inverted and incubated at 30 ± 1°C for 48 h under aerobic conditions for *Str. thermophilus* and at 37 ± 1°C for 72 h under anaerobic conditions for *Lb. delbrueckii subsp. bulgaricus*. After incubation all colonies were counted.

Statistical analysis

Statistical analyses were carried out on the averages of the triplicate results. Two-way multivariate analysis of variance (MANOVA) and multiple comparison tests were carried out to study the effect of both freezing procedures and aging time on the physicochemical characteristics and the counts of *Str. thermophilus* and *Lb. delbrueckii ssp. bulgaricus* in Kashkaval samples [4]. Differences in the averages and F tests were considered significant when the computed probabilities were less than 0.05. All statistical procedures were computed using the Microsoft Excel and Sigma Plot 2001 software.

Results and discussion

The mean values for pH, moisture content, fat in dry matter, total nitrogen, total protein and salt in moisture of Kashkaval samples are shown in Table 1. There were no significant ($P < 0.05$) differences in the moisture, fat

Table 1 Mean values for pH, moisture, fat in dry matter (FDM), total nitrogen (TN), total protein (TP) and salt in moisture (SM) content of Kashkaval samples

Aging time	pH	Moisture (%)	FDM (%)	TN (%)	TP[a] (%)	SM (%)
Young Kashkaval	5.40 ± 0.08^{a}	47.9 ± 1.0^{a}	51.1 ± 0.5^{b}	3.10 ± 0.07^{a}	19.78 ± 0.45^{b}	5.0 ± 0.6^{c}
Semi-ripened Kashkaval	5.29 ± 0.10^{c}	48.3 ± 0.7^{a}	50.9 ± 0.6^{b}	3.16 ± 0.09^{a}	20.16 ± 0.57^{b}	5.3 ± 0.8^{c}
Ripened Kashkaval	5.19 ± 0.09^{b}	48.1 ± 0.9^{a}	51.3 ± 0.4^{b}	3.12 ± 0.05^{a}	19.91 ± 0.32^{b}	5.1 ± 0.6^{c}

[a,b,c] Means within same column bearing a common superscript did not differ significantly ($P < 0.05$)
[a]Calculated from nitrogen ($N \times 6.38$)

in dry matter, total protein and salt in moisture contents of tested samples. It can be seen that the pH values slightly decreased when the aging time of Kashkaval cheese was increased. That was due to the lactic acid fermentation initiated by the starter micro organisms *Lb. delbrueckii* ssp. *bulgaricus* and *Str. thermophilus* during the first stages of aging. During the ripening, there was a significant ($P < 0.05$) increase in the viable cell counts of the starter culture micro organisms (Fig. 1) in the control samples. The proteolytic activity of these microorganisms caused a significant ($P < 0.05$) increase in noncasein nitrogen (NCN/TN) and non-protein nitrogen (NPN/TN) as a percentage of total nitrogen (Fig. 2). NCN/TN values increased from $5.38 \pm 0.21\%$ in young Kashkaval to $16.83 \pm 0.20\%$ in ripened Kashkaval. For NPN/TN, the increase was from $3.42 \pm 0.24\%$ in young Kashkaval to $10.18 \pm 0.20\%$ in ripened Kashkaval. The nitrogen components soluble in the pH 4.6 acetate buffer (NCN) include high and medium molecular weight peptides derived from α_s-caseins and ß-casein, as well as low molecular weight peptides and amino acids [6]. Therefore, NCN is a good indication of the extent of proteolysis of cheese. On the other hand, only low molecular weight peptides and amino acids from the cheese are soluble in the 12% TCA solution [6]. Thus, 12% TCA soluble nitrogen (NPN) is a good indication of the depth of proteolysis in cheese. The results obtained (Fig. 2) show that proteolysis of Kashkaval cheese was significantly ($P < 0.05$) delayed by freezing. That is probably due to the reduced number of the starter microorganisms (Fig. 1) and the lower activity of proteolytic enzymes in frozen stored Kashkaval cheese. Over the 12-month period of storage, there was a slight increase in the levels of noncasein nitrogen (Fig. 2a). NCN/TN values increased from 5.38 ± 0.21 to $9.94 \pm 0.41\%$ for young Kashkaval, from 11.22 ± 0.36 to $14.35 \pm 0.28\%$ for semi-ripened Kashkaval, and from 16.83 ± 0.20 to $18.82 \pm 0.22\%$ for ripened Kashkaval. Sendra et al. [21] also found a slight increase in the levels of noncasein nitrogen in frozen-stored curd. NPN/TN values increased during storage of young Kashkaval from 3.42 ± 0.24 to $5.43 \pm 0.52\%$, although they did not undergo significant ($P < 0.05$) changes during storage of semi-ripened and ripened Kashkaval (Fig. 2b). Bertola et al. [2] found a similar effect of aging time of Mozzarella cheese on the changes of nonprotein nitrogen during frozen storage. We found that noncasein and nonprotein nitrogen increased in larger extend in frozen-stored Kashkaval samples with shorter aging time (Fig. 2b). That is probably due to the high pH values of the samples subjected to shorter ripening times

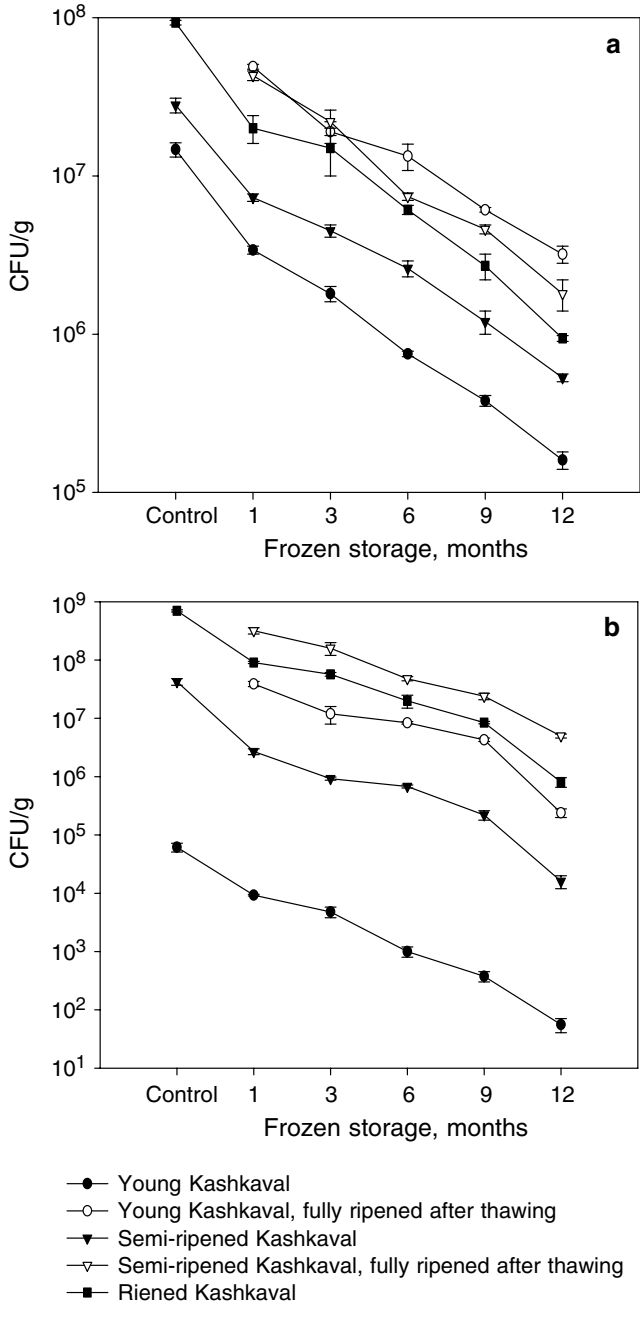

Fig. 1 Changes in the count of *Str. thermophilus* (**a**) and *Lb. delbrueckii* subsp. *bulgaricus* (**b**) in frozen stored Kashkaval cheese

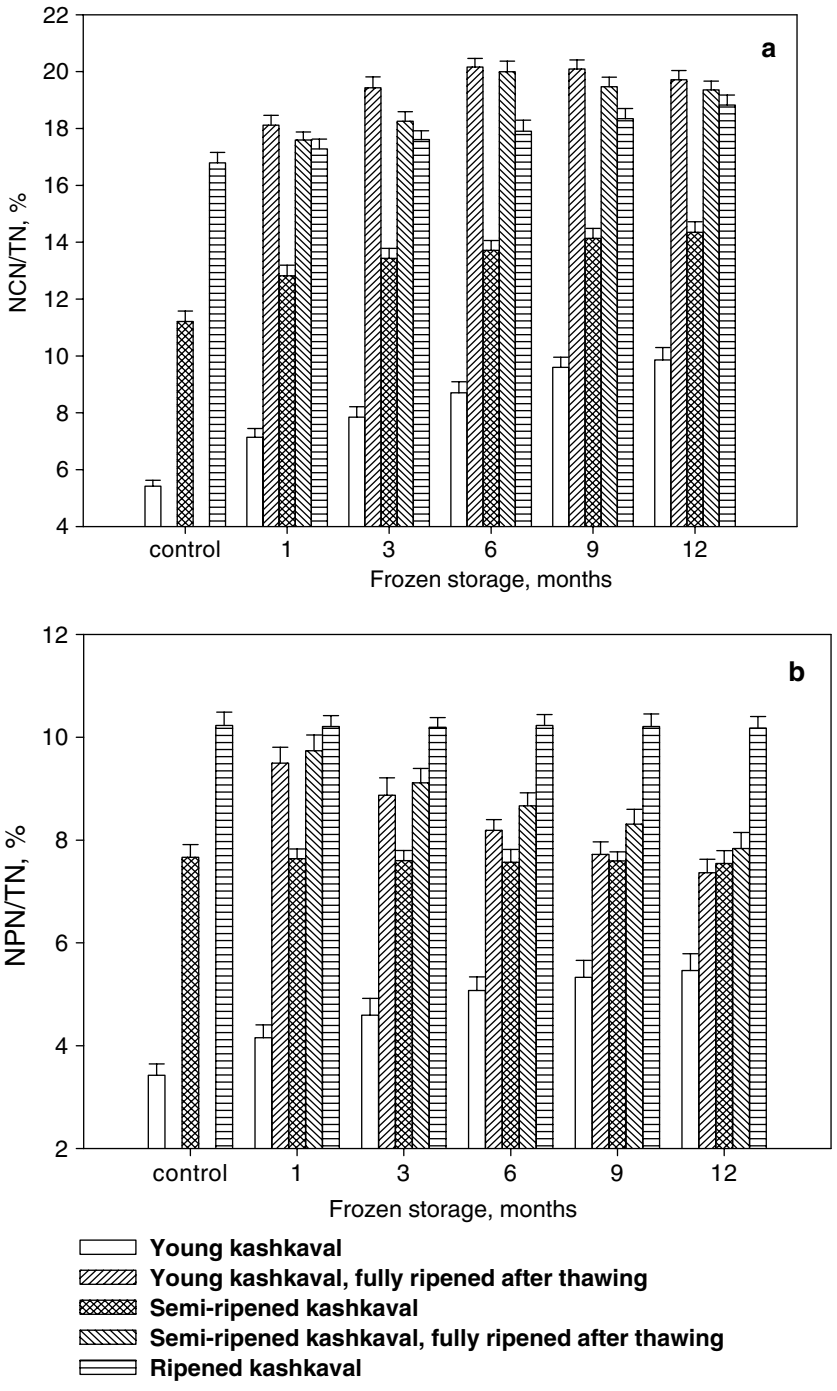

Fig. 2 Changes in the NCN/TN (**a**) and NPN/TN (**b**) during ripening and frozen storage of Kashkaval cheese

before freezing, which favours the activity of proteolytic enzymes.

It was found that during ripening of thawed Kashkaval samples, there was greater accumulation of noncasein nitrogen than during the ripening of control samples ($P < 0.05$) (Fig. 2a). This is evidence of enhanced proteolysis of thawed Kashkaval samples. During ripening of thawed Kashkaval cheese, the NPN/TN values increased ($P < 0.05$), but did not reach the levels of the control sample (Fig. 2b). That indicates that increased proteolysis during ripening of thawed Kashkaval samples leads to the formation of larger amounts of high and medium molecular weight peptides and smaller amounts of low molecular weight peptides and free amino acids as compared to the controls. It is evident also from the results obtained for the free amino acid content of the ripened control and experimental Kashkaval samples frozen stored for 12 months (Table 2). The total amount of free amino acids decreases significantly ($P < 0.05$) in frozen stored Kashkaval samples compared to the control. Enhanced proteolysis during ripening of thawed cheeses was

Table 2 Free amino acid content of the ripened control and experimental Kashkaval samples frozen stored for 12 months

Amino acid	Free amino acids (mg/100 g cheese)			
	Control	Young Kashkaval	Semi-ripened Kashkaval	Ripened Kashkaval
Lysine	27.81 ± 1.54	12.79 ± 1.07	24.63 ± 1.37	25.73 ± 1.05
Arginine	11.92 ± 1.15	3.12 ± 0.29	8.57 ± 0.76	12.47 ± 1.17
Aspartic acid	4.36 ± 0.53	4.16 ± 0.34	5.46 ± 0.85	4.20 ± 0.66
Threonine	3.91 ± 0.42	3.42 ± 0.27	5.15 ± 0.79	5.66 ± 0.74
Serine	5.31 ± 0.37	4.81 ± 0.35	4.92 ± 1.88	5.23 ± 0.69
Glutamic acid	16.41 ± 1.48	14.76 ± 1.34	15.24 ± 1.66	15.84 ± 1.34
Proline	14.25 ± 1.13	12.62 ± 1.22	13.73 ± 1.41	12.69 ± 1.52
Glycine	3.00 ± 0.36	1.70 ± 0.12	3.76 ± 0.52	3.99 ± 0.38
Alanine	6.60 ± 0.62	3.62 ± 0.29	5.92 ± 0.64	4.91 ± 1.26
Valine	19.53 ± 1.78	7.44 ± 0.66	16.40 ± 1.29	18.53 ± 1.98
Methionine	6.39 ± 0.73	2.49 ± 0.18	5.95 ± 0.75	6.06 ± 0.87
Isoleucine	7.58 ± 0.45	1.77 ± 0.19	4.25 ± 0.32	4.92 ± 0.66
Leucine	30.35 ± 1.88	11.34 ± 1.05	25.33 ± 1.48	29.45 ± 1.76
Tyrosine	4.53 ± 0.53	3.33 ± 0.27	4.49 ± 0.29	3.68 ± 0.64
Phenylalanine	30.11 ± 1.57	11.25 ± 1.15	25.20 ± 1.47	30.28 ± 1.53
Total	192.06 ± 14.54	98.62 ± 8.79	169.00 ± 15.48	183.64 ± 16.25

established by a number of authors [20, 22, 24]. According to Kasprzak et al. [12], the main factors contributing to such increased proteolysis are the changes in casein structure, release of endoenzymes from ruptured bacterial cells and partial destruction of the microflora that uses the amino acids released by aminopeptidases. Alichanidis et al. [1] note that the intensive proteolysis during ripening of thawed Teleme cheese results from the changes in its casein structure that occur during freezing and the increased number of proteolytic micro organisms that had more favourable growth conditions and activity in thawed curd with higher pH than the control. The greatest amounts of noncasein nitrogen were accumulated during ripening of Kashkaval samples, frozen-stored for 3–9 months ($P < 0.05$) (Fig. 2a), which indicates that in them proteolysis was at its highest. The lower proteolysis during ripening of Kashkaval samples frozen-stored for 12 months is probably due to the considerable decrease in the number of viable *Str. thermophilus* cells (Fig. 1a), and particularly *Lb. delbrueckii* ssp . *bulgaricus* (Fig. 1b) that cannot be compensated by the destructive changes in casein structure caused by freezing.

It can be concluded that the proteolysis of Kashkaval cheese induced by the starter microorganisms *Lb. delbrueckii* ssp . *bulgaricus* and *Str. thermophilus* was significantly ($P < 0.05$) delayed by freezing. Enhanced proteolysis was observed during ripening of thawed Kashkaval cheese. There was greater accumulation of noncasein nitrogen in thawed Kashkaval samples compared to the control samples. The NPN/TN values also increased significantly ($P < 0.05$), but did not reach the levels of the control sample. It was found that the enhanced proteolysis during ripening of thawed Kashkaval cheese, resulting in larger amounts of high and medium molecular weight peptides and lower amounts of low molecular weight peptides and free amino acids as compared to control. The results obtained in the present study show that the proteolysis of Kashkaval cheese can be controlled by an appropriate combination of the two factors, aging time and frozen storage period. That allows to extend the Kashkaval cheese shelf-live and to maintain its good quality.

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