# Ginger protease used as coagulant enhances the proteolysis and sensory quality of Peshawari cheese compared to calf rennet

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ORIGINAL PAPER

# Ginger protease used as coagulant enhances the proteolysis and sensory quality of Peshawari cheese compared to calf rennet

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**Abstract** The worldwide increase in cheese consumption combined with a scarcity of rennet as well as ethical concerns have resulted in a global interest for natural milk coagulants from plant sources. In this study, the influence of ginger protease in comparison to calf rennet on the physicochemical, microbiological, and sensory characteristics of Peshawari cheese manufactured from cow's milk was examined. For most of the physicochemical parameters (fat, protein, lactose, acidity, pH), and the main groups of microorganisms (total viable, enterobacteria, *Lactobacilli*, and molds and yeasts) investigated, no significant ($P>0.05$) differences were observed between the two cheeses made by using different coagulants. However, significantly lower ($P<0.05$) levels of moisture and higher levels of soluble nitrogen were observed in the cheese produced by ginger protease compared to that made using calf rennet. The main sensory attributes (appearance, body texture, and flavor) were significantly enhanced ($P<0.05$) in Peshawari cheese prepared with ginger protease. Importantly, no bitterness was noted by the sensory panel in the Peshawari cheese made with ginger protease. The results reveal that the ginger protease may have potential application for the manufacture of Peshawari cheese.

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**摘要- 生姜蛋白酶作为凝乳剂促进 Peshawari 干酪蛋白质水解和改善感官质量**

世界范围内奶酪消费量不断增加，而传统凝乳剂小牛皱胃酶供应相对不足以及出于宗教方面的考虑，使得一些植物来源的凝乳酶研究引起了广泛兴趣。本文以牛奶为原料，比较了生姜蛋白酶和小牛皱胃酶作为凝乳剂制备的 Peshawari 奶酪在物化特性、微生物特性以及感官特性方面的差异。结果表明，用两种凝乳酶制备的奶酪，其大部分的物化特性指标（脂肪、蛋白质、乳糖、酸度、pH）以及主要的微生物指标（活菌总数、肠道菌、乳酸菌、真菌和酵母）均无明显差异（$P > 0.05$）。但以生姜蛋白酶为凝乳剂制备的奶酪其含水量明显低于用小牛皱胃酶生产的奶酪($P < 0.05$)，相反可溶性氮（SN）的含量前者却明显高于后者($P < 0.05$)。此外，结果还显示用生姜蛋白酶为凝乳剂制备 Peshawari 奶酪可以明显改善主要的感官特性（外观、质构、风味）($P < 0.05$)，更重要的是，以这种方式生产的 Peshawari 奶酪没有苦味。这一研究表明生姜蛋白酶作为植物来源的凝乳剂在 Peshawari 奶酪生产中具有潜在应用价值。

**Keywords** Peshawari cheese · Ginger protease · Physicochemical characteristics · Microbiology · Sensory attributes

**关键词** Peshawari干酪 · 生姜蛋白酶 · 物化特性 · 微生物学 · 感官特性

## 1 Introduction

Calf rennet has been widely employed as a milk coagulating agent from antiquity. However, increasingly higher prices of calf rennet (CR) as well as ethical concerns associated with the production of such enzymes for general cheese making have led to systematic investigations on the possibility and suitability of their substitution by other enzymes of plant origin (Sousa and Malcata 1997). Apart from calf rennet, coagulation of milk can be achieved by proteolytic enzymes from various sources, such as different animal species, microbial proteases, and proteases extracted from fruits and plants such as wild cardoons. One of the most successful plant rennets, which have been used for the manufacture of traditional cheeses in Portugal and Spain, is obtained from the flowers of the thistle, *Cynara cardunculus* L (Tejada and Fernández-Salguero 2003). The use of these plant proteinases as milk coagulants is very interesting since they are natural enzymes and can also be used for producing cheeses aimed at lacto-vegetarian consumers and ecological markets (Gómez et al. 2001). They can also be used for the manufacture of Kosher and Halal products (Galán et al. 2008).

Peshawari cheese is a semi-hard, fresh cheese made from whole or partly skimmed buffalo or cow's milk. In the traditional Peshawari cheese-making process, milk is heated to 63 °C for 30 min, cooled to 32 °C, and inoculated with 5–6% *Lactobacillus* culture, i.e. Lassi (traditional yoghourt mixed with cold water). Rennet (diluted in water) is added in the proportion of 10 mL per 500 L of milk. Coagulation is achieved at 32 °C in about 1 h. The coagulum is cut into small slices and put into a cheese cloth to drain off the whey (Fox 1999). Peshawari cheese is extensively used as an ingredient in the preparation of culinary dishes, stuffing material for various vegetable dishes, snacks, and cooked meats in the Khyber Pakhtunkhwa Province of Pakistan.

Ginger rhizome (*Zingiber officinale*) has often been used as a peptic drug or in other drugs in Chinese medicine and also in various food dishes from ancient times

(Ohtsuki et al. 1995). It has also been applied as a meat tenderizing agent, the meat tenderizing component being the ginger protease (GP) (Naveena et al. 2004). However, there is paucity of information regarding the milk coagulating ability of ginger protease and its application in cheese making. Recently, we have isolated, purified, and characterized new milk coagulating cysteine protease from the ginger rhizomes in our laboratory (Hashim et al. 2011). The molecular mass of the purified enzyme was found to be 36 kg $mol^{-1}$ and a p*I* of 4.3. It was a glycoprotein and showed optimum activity at pH 5.5. The enzyme showed higher milk clotting activity in comparison to both rennet and papain and exhibited a broad degradation ability for all the three types of caseins (i.e., $\alpha_{s1}$-, β-, and κ-casein). The N-terminal amino acid sequence of the purified protease was also elucidated by Edman's degradation.

The objectives of the present work were to study the physicochemical, microbiological, and sensory characteristics of Peshawari cheese during cold storage and to monitor the impact of different types of coagulants (ginger protease and calf rennet) on the improvement of the technological quality of Peshawari cheese.

## 2 Materials and methods

### 2.1 Cheese manufacturing and sampling

Ginger protease was extracted and purified from ginger rhizomes using a three-step purification scheme including ammonium sulfate precipitation, ion exchange chromatography, and size exclusion chromatography (Hashim et al. 2011). Briefly, diced ginger rhizomes (50 g) were homogenized with five parts cold acetone (−23 °C). After filtration, the precipitate was air-dried and then ground to a fine powder. The powder was homogenized in 20 mmol $L^{-1}$ phosphate buffer (pH 7.0), and the extract was filtered through cheesecloth. The filtrate was centrifuged (12,000×*g* for 20 min) followed by purification of crude extract with ammonium sulfate precipitation (20–65%), ion exchange chromatography (MonoQ 5/50GL), and size exclusion chromatography (Superdex 75).

Fresh cow's milk was obtained from the Bright Dairy Farm of Nanjing, China. The average composition of standardized cheese milk was fat 3.2%, protein 3.2%, lactose 4.7%, solids-non-fat 8.1%, and pH 6.60. The milk was divided in two portions, and two batches of cheese were manufactured on the same day following a slight modification of the conventional technology. The raw milk was filtered through muslin cloth to remove any extraneous material and then heated to 63 °C for 30 min. One batch was coagulated with commercial CR (0.5 g·100 $L^{-1}$ milk) from Al-Amin Biotech Co., Ltd. (Shanghai, China), and the second batch was coagulated with GP (about 4.5 g·100 $L^{-1}$ milk). The experiment was replicated three times. Consequently, three experimental batches were coagulated with ginger protease and another three with calf rennet. The coagulating temperature for milk was 34±1 °C. For batches clotted with CR, the clotting time was about 30 min, and for batches clotted with GP, the clotting time was about 50 min. The curd was strained by passing through a muslin cloth and

allowed to drain. It was dipped in chilled water for about 10 min, pressed, and packed in plastic containers which were transferred to a cold room (3±1 °C) for storage up to 28 days.

For each cheese-making trial, using different milk, two cheeses were selected from each batch after 2, 7, 14, 21, and 28 days of storage for physicochemical, microbiological and sensory analyses, making a total of 60 cheeses sampled in all. All the analyses were conducted in duplicate so that 12 analyses were performed for each type of cheese on each sampling date.

### 2.2 Physicochemical analyses

The moisture content, protein, fat, lactose, and acidity (expressed as lactic acid) of Peshawari cheese samples were determined by recommended methods of AOAC (1980). The pH was measured by probing the cheese directly with the glass electrode of a Schott digital pH meter (model Lab 850, Schott Instruments, Mainz, Germany). Cheese proteolysis was followed by determining the soluble nitrogen (SN) as previously described (Katsiari and Voutsinas 1994). The concentration of SN was expressed as gram per 100 g of total nitrogen (TN).

### 2.3 Microbiological analyses

Microbiological analyses were performed according to APHA procedures (APHA 1992) as follows: total viable counts were determined on plate count agar and incubated at 30 °C for 72 h; total enterobacteria on violet red bile glucose agar and incubated at 37 °C for 24–48 h; coliforms on violet red bile agar and incubated at 37 °C for 24 h; *Lactobacilli* on MRS agar and incubated at 37 °C for 24 h; and molds and yeasts on potato dextrose agar and incubated at 26 °C for 96 h. All determinations were performed in duplicate and expressed as log colony forming units (cfu) per gram of cheese sample.

### 2.4 Sensory evaluation

The cheeses were evaluated organoleptically at 2, 7, 14, 21, and 28 days after production by a nine-member panel, following the recommendations of IDF (1997). The members evaluated cheese for appearance, body and texture, and flavor (odor and taste) using a nine-point hedonic scale, with 1 being the worst and 9 the best quality. Samples were placed in white plastic cups coded with standard random numbers. The sensory evaluation was conducted at room temperature under normal laboratory light conditions and the panelists were free to judge any sample two or three times. Water was provided for mouth rinsing between samples. Panel members were also instructed to report any defects in the appearance, body and texture or flavor of the cheeses.

### 2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SPSS 13.0 (SPSS Inc., Chicago, IL). In this study, a two-way ANOVA was used to find statistical

differences between the physicochemical parameter values, microbiological counts and sensory analysis scores of Peshawari cheeses according to coagulant type, storage period, and the interaction between the two factors. Two factors (type of coagulant and storage period) were taken into consideration for ANOVA analysis in order to establish whether the significant differences due to the coagulants used depended on the storage time or remained constant independent of the storage time. Comparison of means was performed by Fisher's least significant difference test. Differences were considered significant at $P<0.05$.

## 3 Results and discussion

### 3.1 Physicochemical characteristics

Overall mean values and standard deviations for moisture, protein, fat, and pH of Peshawari cheese made with GP or CR during storage are given in Table 1. No significant differences ($P>0.05$) were found for protein, fat, lactose, acidity, and pH in cheeses made with both types of coagulants. However, the moisture level of Peshawari cheese obtained with GP was significantly lower ($P<0.05$) than that made with CR. It might be inferred that the structure of the coagulum made using GP retained less liquid during the dewheying stage of the cheese (Sanjuán et al. 2002). Significantly lower moisture levels in cheeses manufactured using vegetable coagulants than those produced with calf rennet have been reported in the literature (Galán et al. 2008; Sanjuán et al. 2002). Similarly, it has previously been found that type of coagulant (animal or plant) had no significant effect on the compositional characteristics (protein, fat, lactic acid, and pH) of different cheeses manufactured with animal or plant rennet (Fernández-Salguero et al. 2002; Pino et al. 2009; Tejada and Fernández-Salguero 2003).

The lactose content of Peshawari cheese was significantly ($P$ value of 0.0001) affected by the storage period. A decreasing trend was observed in the lactose content of Peshawari cheeses during storage. This decrease is due to the glycolytic fermentation by the microorganisms producing mainly lactic acid, which determines the cheese acidity and favors rennet action, syneresis, and dewheying, giving the

**Table 1** Overall mean values of physicochemical components[c] of Peshawari cheese obtained with calf rennet and ginger protease during storage

| Component | Coagulant | |
|---|---|---|
| | Calf rennet | Ginger protease |
| Moisture (%) | 52.19±1.31[a] | 50.76±1.48[b] |
| Fat (%) | 23.08±1.16[a] | 23.05±1.29[a] |
| Protein (%) | 19.50±0.84[a] | 19.53±0.89[a] |
| pH | 5.10±0.15[a] | 5.07±0.15[a] |

[a,b] Means in the same row sharing a common letter did not differ significantly ($P>0.05$)

[c] Values are averages±standard deviations of three cheese-making trials from different batches of milk

curd certain physical characteristics that influence the final flavor and texture (Sanjuán et al. 2002).

Acidity, on the other hand, increased significantly ($P$ value of 0.0001) during storage of the Peshawari cheeses. This increase in acidity is related to the conversion of the initial lactose present in the cheese into lactic acid.

Proteolysis in cheese is often measured by means of quantification of SN fraction of cheese, which consists of whey proteins, medium- and small-sized peptides from the degradation of caseins and free amino acids (Christensen et al. 1991). Figure 1 shows the SN/TN ratio plotted as a function of storage time; this ratio has been used by a number of researchers to follow aging of cheese. The values of SN/TN in cheeses manufactured with either coagulant increased throughout storage, but the levels of SN were significantly ($P<0.05$) higher in the cheese made by using the ginger protease. On day 2 of storage, the SN value for the Peshawari cheese made by using GP (7.80±0.24 g 100 $g^{-1}$ TN) was higher than that of the same cheese made by using CR (5.80±0.26 g 100 $g^{-1}$ TN). At the end of the storage period studied, at day 28, the Peshawari cheese made with GP (9.75±0.21 g 100 $g^{-1}$ TN) contained 16% more SN than that made with calf rennet (8.19±0.32 g 100 $g^{-1}$ TN). Soluble nitrogen components in cheese are produced mainly by the action of rennet but can also be produced by starter bacteria or plasmin (Visser 1977). Nevertheless, as no starter bacteria were added for the manufacture of Peshawari cheeses in our study and the levels of the indigenous plasmin is comparable between the cheeses, the higher levels of SN/TN may only be due to the action of coagulants used, with the differences being observed throughout the storage period. The higher proteolytic effect of the ginger protease in comparison to the calf rennet in the production of SN may also be contributing to increasing the proteinase activity early in the ripening of different cheese varieties. High levels of SN in cheese made with plant coagulant have been reported by several authors (O'Mahony et al. 2003; Pino et al. 2009; Prados et al. 2007; Tejada and Fernández-Salguero 2003).

### 3.2 Microbiological characteristics

Table 2 shows mean values (log $cfu^{-1}$ g cheese) and standard deviations for microbial groups: total viable, enterobacteria, coliforms, *Lactobacilli*, and molds and yeasts detected in the Peshawari cheeses made by GP and CR. The microbial profile

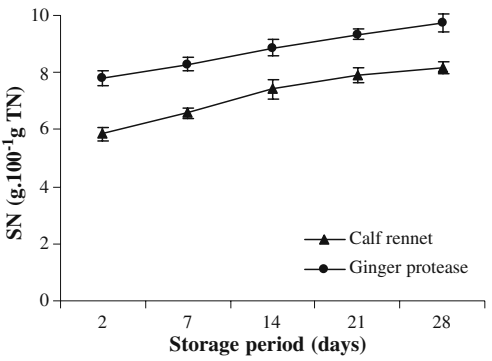


**Fig. 1** Changes in SN expressed as grams·100 $g^{-1}$ of TN of the Peshawari cheese manufactured using CR or GP during 28 days of the storage. Values are averages±standard deviations of three cheese-making trials from different batches of milk

**Table 2** Log counts of different microbial groups[e] in Peshawari cheese obtained with calf rennet and ginger protease during storage

| | Coagulant | Storage days | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 7 | 14 | 21 | 28 |
| Total viable counts | CR | 5.68±0.17[b] | 5.62±0.20[b] | 6.85±0.15[a] | 6.69±0.27[a] | 6.49±0.17[a] |
| | GP | 5.70±0.30[b] | 5.80±0.19[b] | 6.77±0.18[a] | 6.71±0.21[a] | 6.62±0.27[a] |
| Enterobacteria | CR | 3.04±0.16[a] | 2.92±0.08[a] | 2.55±0.40[a] | 2.76±0.13[a] | 2.79±0.37[a] |
| | GP | 3.12±0.34[a] | 2.73±0.19[a] | 2.66±0.32[a] | 2.43±0.38[a] | 2.75±0.19[a] |
| Coliforms | CR | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| | GP | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] | 0.00±0.00[a] |
| *Lactobacilli* | CR | 5.51±0.18[d] | 6.83±0.14[c] | 7.37±0.38[bc] | 7.36±0.24[bc] | 8.27±0.28[a] |
| | GP | 5.15±0.24[d] | 6.69±0.12[c] | 7.31±0.26[bc] | 7.85±0.09[ab] | 8.25±0.38[a] |
| Molds–yeasts | CR | 3.47±0.26[ab] | 2.40±0.29[d] | 2.97±0.04[bc] | 2.83±0.11[cd] | 2.96±0.08[bc] |
| | GP | 3.52±0.28[a] | 2.50±0.19[cd] | 2.92±0.08[cd] | 2.85±0.23[cd] | 2.92±0.06[cd] |

[a–d] Means of the same microbial group in the same row sharing a common letter did not differ significantly ($P>0.05$)

[e] Values are averages ± standard deviations of three cheese-making trials from different batches of milk

*CR* calf rennet, *GP* ginger protease

of initial milk used for cheese making was: total viable microorganisms (3.60± 0.24 log cfu.mL$^{-1}$), enterobacteria (3.88±0.12 log cfu.mL$^{-1}$), coliforms (2.65± 0.28 log cfu.mL$^{-1}$), *Lactobacilli* (3.25±0.20 log cfu.mL$^{-1}$), and molds and yeasts (3.06±0.11 log cfu.mL$^{-1}$).

Mean microbial counts in the Peshawari cheese at day 2 were higher than those in the initial milk for both cheese trials. After 2 days of manufacturing, total counts in cheese were almost 2 log units higher than that in milk in terms of the total viable and *Lactobacilli*. This could be partly attributed to microbial growth during the milk coagulating stage at around 30 °C and the physical entrapment of bacteria in the curd (Tatini et al. 1971). No significant differences were found for total viable, enterobacteria, *Lactobacilli* and molds–yeast counts in Peshawari cheese made with GP and CR. Similarly, in a previous study, no differences were observed for the main groups of microorganisms (total viable, enterobacteria, coliforms, *Escherichia coli*, lactic acid bacteria, and molds and yeasts) between Los Pedroches cheeses made using animal and vegetable coagulants (Tejada and Fernández-Salguero 2003). Conversely, significantly higher counts of enterobacteria in fresh cheeses manufactured with plant rennet in comparison to the same cheeses made using animal rennet have been reported by Nuñez et al. (1991). Total viable and *Lactobacilli* counts increased significantly ($P$ value of 0.0001) over the storage period. Enterobacteria counts remained virtually unchanged during the storage period. Mold–yeast counts at 2 days of storage were almost similar to those found in initial milk. An overall reduction ($P$ value of 0.0001) was observed in counts of mold–yeasts in Peshawari cheese during storage. Comparatively higher mold and yeast counts have previously been reported in ovine cheeses manufactured using animal and plant rennet (Sousa and Malcata 1997). No coliforms were detected in Peshawari cheese manufactured with GP and CP during the storage period even though the initial

milk contained coliforms. This shows the effect of heating which completely eliminated coliforms.

### 3.3 Sensory characteristics

The results of the sensory panel's assessment of Peshawari cheese coagulated with GP and CR at 2, 7, 14, 21, and 28 days of storage are shown in Table 3. The type of rennet (GP and CR) significantly ($P<0.05$) affected all the sensory attributes of Peshawari cheeses. The scores for appearance of the cheese made with GP were significantly ($P<0.05$) higher than those made with CR at all sampling points. The former cheese had a viscous yoghurt-like appearance. It should be noted that a desirable body of Peshawari cheese to the consumers is neither too firm nor too soft. It should be sufficiently firm to hold its shape during cutting/slicing yet tender enough not to resist crushing during mastication. The scores for body and texture of the Peshawari cheese made with GP were significantly ($P<0.05$) higher than those made with CR at all sampling points. The cheeses made with GP had a more uniform and softer body and texture. In a previous study, it was observed that Murcia al Vino cheese made with animal rennet was significantly harder ($P<0.01$), more grainy ($P<0.001$), and less creamy ($P<0.001$) than the same cheese made using plant coagulant (Tejada et al. 2006). Similar findings have been reported for ewe's milk cheeses made using vegetable coagulant from *C. cardunculus*, which were softer and creamier than those made using rennet (Tejada et al. 2007). The intense level of proteolysis which occurred in cheeses made using vegetable protease hydrolyzed the casein network, creating a more homogeneous structure, thus prompting greater creaminess and softening of the cheese (Tejada et al. 2007).

Peshawari cheese made using GP received significantly higher ($P<0.05$) flavor scores than cheeses made using CR. No off-flavor or bitterness was noted by any member of the taste panel in the Peshawari cheeses made using GP during storage. This characteristic of GP is of great importance for cheese-making from cow's milk.

**Table 3** Mean values of sensory attributes[e] of Peshawari cheese obtained with calf rennet and ginger protease during storage

| | Coagulant | Storage days | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 7 | 14 | 21 | 28 |
| Appearance | CR | $7.40\pm0.07^{abc}$ | $7.31\pm0.12^{bcd}$ | $7.22\pm0.14^{bcd}$ | $7.09\pm0.06^{cd}$ | $6.98\pm0.12^{d}$ |
| | GP | $7.66\pm0.18^{a}$ | $7.53\pm0.13^{ab}$ | $7.40\pm0.11^{abc}$ | $7.25\pm0.10^{bcd}$ | $7.19\pm0.09^{bcd}$ |
| Body and Texture | CR | $6.41\pm0.10^{b}$ | $6.34\pm0.06^{b}$ | $6.28\pm0.09^{b}$ | $6.19\pm0.09^{b}$ | $6.21\pm0.18^{b}$ |
| | GP | $7.33\pm0.26^{a}$ | $7.41\pm0.10^{a}$ | $7.31\pm0.19^{a}$ | $7.47\pm0.31^{a}$ | $7.27\pm0.16^{a}$ |
| Flavour | CR | $6.75\pm0.12^{abcd}$ | $6.51\pm0.38^{bcd}$ | $6.61\pm0.21^{abcd}$ | $6.39\pm0.09^{cd}$ | $6.27\pm0.17^{d}$ |
| | GP | $7.15\pm0.26^{a}$ | $7.04\pm0.04^{ab}$ | $6.91\pm0.31^{abc}$ | $7.00\pm0.15^{ab}$ | $6.88\pm0.11^{abc}$ |

[a–d] Means of a given parameter sharing a common superscript did not differ significantly ($P>0.05$)

[e] Values are averages ± standard deviations of three cheese-making trials from different batches of milk

*CR* calf rennet, *GP* ginger protease

On the contrary, vegetable coagulant obtained from cardoon flowers, although currently successful, is regarded only as a potential clotting agent in the manufacture of cheeses from goat's milk (Tejada et al. 2008). Certain typical cheeses manufactured in Spain and Portugal from goat's and ewe's milk with cardoon-based coagulants exhibit no distinct bitter flavor, whereas cheeses made from cow's milk and extracts of *C. cardunculus* flowers tend to have an undesirable bitter taste (Barbosa et al. 1981).

## 4 Conclusion

No significant differences were detected for most of the physicochemical components and all the microbiological characteristics between Peshawari cheeses manufactured with either ginger protease or calf rennet. However, the level of proteolysis and organoleptic attributes scores of the cheeses made with the ginger protease were higher than those made with calf rennet. Softening of the cheese texture, as noticed by sensory analysis, was considerably more pronounced in ginger protease cheese which also showed significantly higher flavor quality and intensity.

This study has demonstrated the feasibility of producing Peshawari cheese using ginger protease which significantly enhanced all the organoleptic properties of this type of cheese.

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