Influence of *Withania coagulans* Protease as a Vegetable Rennet on Proteolysis of Iranian UF White Cheese

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**ABSTRACT**

Extraction of protease from *Withania coagulans*’ fruits and the effect on proteolysis of Iranian UF white cheese in comparison with pure chymosin and fungi rennet (fromase) were investigated during ripening. The results indicated that, except for pH which was significantly (P< 0.05) lower in cheeses made with *Withania coagulans*, there was no significant difference observed among the cheeses produced with different rennet preparations as in moisture, fat and salt contents during ripening. The values of pH 4.6-SN and the Urea-polyacrylamide gel Electrophoresis (PAGE) pattern revealed severe proteolysis in cheeses produced with *Withania coagulans*’ enzyme as compared with animal and fungi rennets. The caseins \(\alpha_s1\) and \(\beta\) disappeared in cheeses made with *Withania coagulans* enzyme preparations. Furthermore, a noticeable increase of soluble nitrogen in 12% trichloroacetic acid (SNTCA) was observed during ripening of cheeses made with vegetable rennet, probably due to an unspecific proteolitic activity of *Withania coagulans* enzymes as compared to other rennets.

**Keywords:** Proteolysis, UF white cheese, Vegetable rennet, *Withania coagulans*.

**INTRODUCTION**

Rennet is a milk coagulating enzymatic preparation. The most highly active enzyme in animal rennet preparations is chymosin. The principal role of chymosin, in cheese making, is to coagulate milk by specific hydrolysis of Phe\(^{105}\)-Met\(^{106}\) bond of the \(k\)-casein (micelle-stabilising protein), causing milk coagulation (Fox *et al.*, 2000). Only 0–15% of the activity of the rennet added to the milk remains in the curd after cheese making (Guinee and Wilkinson, 1992; Guinee and Wilkinson, 1992; Fox *et al.*, 2000). Only a small number of aspartic proteinases from plant origin have been isolated and partially characterised (Sousa and Malcata, 1998; Sousa *et al.*, 2001). A unique feature shared by most of these plant proteinases is an extra segment of about 100 amino acid residues which bears no sequence similarity with proteinases of mammalian or microbial origins (Faro *et al.*, 1995). Many aspartic and other proteinases are obtained from plants with some of them having been studied as coagulants, i.e., proteinases from *Benincasa cerifera* (Sousa *et al.*, 2001), *Silybum marianum* (Cavalli *et al.*, 2008), *Dieffenbachia maculata*.

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(Padmanabhan et al., 1993), fruit parts of Sodom apple (Aworth and Muller, 1987) and Centaurea calcitrapa (Tavaria et al., 1997). It is known that, most plant coagulant preparations possess weak milk clotting activity while a high proteolytic activity, which results in low cheese yields during cheese making, and bitter peptides accumulation during cheese ripening. Cynara cardunculus coagulant, an exception among the other plant rennet proteases, is obtained from dried flowers of C. cardunculus. This coagulant possesses a milk clotting activity and it has successfully been employed, for many centuries, in the Iberian Peninsula to produce such traditional cheeses, as: Serra da Estrela, La Serena, Guia and Los Pedroches (Verissimo et al., 1995; Silva et al., 2003; Silva and Malcata, 2005; Tejada and Fernández-Salgueiro, 2003). Withania coagulans (Solanacea) grows in Pakistan, Afghanistan, India as well as in southern regions of Iran and has been employed for decades in production of traditional cheeses from raw cow’s milk (Dastur, 1948; Singh et al., 1973). Fruits of this plant possess milk clotting characteristic and are known as vegetable rennet. Dastur et al. (1948) reported that it is possible to prepare soft cheeses, using rennet preparations from Withania coagulans. Cheddar cheese manufactured, using Withania coagulans rennet preparation had a good texture and flavor but gave a perceptible bitter taste which nevertheless could be reduced to some extent, by prolonging the ripening period. The enzyme is extracted from flowers, through water extraction and successive precipitation while adding alcohol and acetone. The enzymatic precipitate is then dried at room temperature. This enzyme shows an optimum of activity between 45 to 60°C. The activity of the enzyme decreases rapidly at temperatures higher than 70°C, and it is completely inactivated at 80°C. Enzymes from Withania coagulans flowers coagulated boiled milk more slowly than raw milk but it is possible to restore its clotting capability by the addition of calcium to heated milk (Dastur, 1948). There are very few studies made regarding the proteolytic activity of Withania coagulans enzymes during cheese ripening.

In the last decades, white-brined cheese from ultra-filtered milk has been manufactured in several, well organized, dairy plants in Iran. In spite of obvious economic advantages, due to high production yields as a result of the incorporation of whey proteins in the product, UF white-brined cheese usually shows slower proteolysis and production of amino acids during ripening. Moreover it does not bear the suitable organoleptic characteristics (Hesari et al., 2006).

The aim of this study was to examine the effects of vegetable coagulant from Withania coagulans on proteolysis of Iranian UF white cheese during ripening, an attempt to use this enzyme preparation as a local new source of rennet in cheese making.

MATERIALS AND METHODS

Extraction of Enzyme

Extraction of enzyme was carried out according to the method of Dastur (1948), (Figure 1).

Cheese Making

Experimental UF white cheeses were made in three trials on separate days. Each day, from a single batch of UF milk (retentate), three types of cheeses were produced: 1-by adding fungi rennet; Rhizomucor miehei (Renco, Eltham, New Zealand) (30 mg kg⁻¹ retentate); 2- pure chymosin (CHR HANSEN, Denmark) (30 mg kg⁻¹ retentate) and 3- Withania coagulans’fruits (15 ml of 3.3% (w/v) aqueous extract from Withania coagulans’fruits was added to 450 g retentate). The enzyme activities of coagulants were adjusted to reach the rennet coagulation time of 10 minutes for the three type cheeses. The cheese making process
was repeated on two other days to obtain three replicates, on three different days, for each rennet preparation. The retentate was prepared by Iran Dairy Industry Inc., Pegah Co (Tabriz, Iran) as follows: raw cow’s milk of high microbial quality was standardized to 3.5% of fat, and after bactofugation within two steps and at 55°C, it was pasteurized at 72°C for 15 seconds and then ultrafiltered at 50°C. The applied membrane cartridges were of the spiral wound type (No UFPH20 Invensys APV, Silkeborg, Denmark) with a nominal molecular weight cut-off of approximately 20 kg mol\(^{-1}\) and a surface area of 16.9 m\(^2\). The ultrafiltration unit was operated at an inlet pressure of 5.3 and an outlet pressure of 1.7 bars. Before making of the cheese, the retentate was pasteurized at 78°C for 60 seconds and then cooled to 35°C. A mixture of mesophilic (G3 mix, \textit{Lc. cremoris} and \textit{Lc. lactis}) and thermophilic (Joghurt 709, \textit{Str. thermophilus} and \textit{Lb. delbrueckii} subsp. \textit{bulgaricus}) cultures (Laboratorium Visby, Tender Aps, Denmark) in the ratio of 7:1 was used as starter. Subsequently, following the addition of rennet preparations, the milk was poured into containers (450 g). The samples were allowed to coagulate at 30-32°C of room temperature for 20 minutes. A parchment paper was placed on top of the coagulum and dry salt (3%) added. The containers were sealed with aluminum foil. Salt gradually adsorbed moisture from curd and a
layer of brine formed around cheese samples
in the containers. Cheese packs were held at
26–28°C for 24 hours and then transferred to
a cool room (8°C). Cheeses were ripened for
45 days. One cheese sample of each trial
was sampled out at 1, 15, 30, and 45 days of
ripening.

Analytical Methods

Chemical Composition

Cheeses at 1, 15, 30 and 45 days of
ripening were analysed for moisture (oven
drying method at 102±2°C; IDF, 1982), salt
(potentiometric method; Fox, 1963), fat
(Gerber method; Marshal, 1992), total
nitrogen (TN), soluble nitrogen in pH 4.6
(SN) and in 12% trichloroacetic acid
(SN TCA) (macro-Kjeldahl method; IDF,
1964), total protein (NT×6.38). The pH of
the cheese was measured by direct insertion
of an electrode (PHC3031-9, Radiometer
Analytical, Copenhagen, Denmark) into
cheese (Marshal, 1992). All analyses were
performed in triplicate and results reported
as mean±standard deviation.

Assessment of Proteolysis

The nitrogen fractions of the cheese
samples, including pH 4.6-soluble nitrogen
(SN) and soluble nitrogen in 12%TCA
(SN TCA) were obtained modifying the
procedure of Kuchroo and Fox (1982) as
described by Sousa and McSweeney (2001).
Urea-polyacrylamide gel electrophoresis
(PAGE) of the pH 4.6-insoluble fraction of
the cheese was performed using a Protean II
XI vertical slab geltunit (Bio-Rad
Laboratories Ltd., Watford, UK) according
to the method of Andrews (1983) modified
by Shalabi and Fox (1987). Gels were
stained directly with Coomassie Brillant
Blue G250, as described by Blakesley and
Boezi (1977).

Statistical Analysis

Statistical analysis of the experimental
data were done using a split plot model
based on, a randomised complete block
design (CRB) (MSDATC and SPSS Version
15 for Windows 2003; SPSS Inc., Chicago,
IL, USA). The model incorporated three
treatments (cheese samples made with
Withania coagulans, cheeses made with
animal rennet and samples made with fungi
rennet) and three blocks (trials). The
significance of differences was estimated
using 1-way ANOVA.

RESULTS AND DISCUSSION

Chemical Composition

Macro nutrients and physicochemical
parameters of 1-day old experimental
Iranian UF white brined cheeses are
presented in Table 1. Except for pH that was
significantly (P< 0.05) lower in cheeses
made with Withania coagulans as coagulant
than those in cheeses with animal or fungi
rennets, there were no significant differences

<table>
<thead>
<tr>
<th>cheeses</th>
<th>pH</th>
<th>NaCl%</th>
<th>moisture%</th>
<th>Protein%</th>
<th>Fat %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Withania coagulans</td>
<td>4.69±0.03</td>
<td>3.14±0.12</td>
<td>65.78±0.8</td>
<td>11.27±0.88</td>
<td>17.85±0.35</td>
</tr>
<tr>
<td>Chymosin</td>
<td>4.48±0.02</td>
<td>3.04±0.14</td>
<td>64.73±0.36</td>
<td>12.25±0.77</td>
<td>17.92±0.41</td>
</tr>
<tr>
<td>Rhizomucor miehei</td>
<td>4.54±0.08</td>
<td>3.10±0.11</td>
<td>63.45±0.23</td>
<td>12.35±0.51</td>
<td>18.25±0.42</td>
</tr>
<tr>
<td>F value</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: Not significant. * Significant at P < 0.01.
observed among cheeses in moisture, fat, protein and salt contents. The use of different rennet preparations did not have a significant effect on the main gross composition of cheeses as reported by other workers (Sousa and Malcata 1997; Wium et al., 1998; Mara and Kelly, 1999).

**Indices of Proteolysis: pH 4.6-SN and SNTCA/TN**

Levels of pH 4.6-SN content in experimental UF white cheeses during ripening are shown in Figure 2. The levels of %pH 4.6-SN increased gradually until 45 days of ripening in all the cheeses. It is noticed that pH 4.6-SN levels differed significantly (P< 0.01) among the cheeses coagulated using the three types of rennet. Non casein nitrogen contents of cheese made with vegetable rennet were higher than those in the samples made with pure chymosin and fungi rennets (Rhizomucor miehei) during ripening. After 15 days of ripening, the content in SN compounds of cheeses made with vegetable rennet was higher than twice that in the samples produced using animal or fungi rennet. The estimation of mean SN values for the samples analysed at 1, 15, 30 and 45 days after cheese making, showed that cheese made with vegetable rennet contained about 100% more soluble nitrogen than that made with animal rennet.

The higher levels of SN found in cheeses produced, using vegetable rennet, suggest a more intense proteolytic activity of *Withania coagulans* protease than microbial and animal proteases. Similar results were reported by other authors in relation with various cheeses. Galan et al. (2007) and Prados et al. (2006) showed that in ewe’s milk cheese the SN and SNTCA contents were significantly higher in cheeses produced with vegetable coagulant than those made, using calf rennet. During ripening, the levels of SN and SNTCA fractions of Los Pedroches cheese produced, using either *Cynara cardunculus* L. proteinases or animal rennet, were found higher in samples made with vegetable rennet and were similar to the levels of La Serena cheese (Sanjúan et al., 2002). Malcata and Freitas (1995), in a study about the influence of cheese making conditions on the characteristics of Picante da Beira Baixa cheese, concluded that vegetable rennet increased proteolysis as compared with animal rennet.

The pH 4.6-SN fraction used as an index of ripening (Sousa et al., 2001), includes peptides of medium to small molecular weight, proteoso peptones, whey proteins and free amino acids. These nitrogen compounds are the main results of rennet and plasmin proteolytic activity and/or microorganism peptidases (O’keefe et al., 1978). The proteinases of milk clotting

![Graph showing the evolution of pH 4.6-soluble nitrogen fraction (pH 4.6-SN) in UF Iranian white cheeses produced with fungi, animal and vegetable rennet.](image)

**Figure 2.** Evolution during ripening of pH 4.6-soluble nitrogen fraction (pH 4.6-SN) in UF Iranian white cheeses produced with fungi, animal and vegetable rennet.
preparations are mainly responsible for the primary proteolysis of the caseins in cheese. Their action modifies the cheese texture by slowly degrading $\alpha_s1$- and, to a lesser extent, $\beta$-caseins. This proteolytic process leads to the framework of cheese matrix, and produces precursors of sapid compounds (Hayaloglu et al., 2004) but it contributes to minority and indirectly to the formation of free amino acids (O’keefe et al., 1978).

The levels of non-protein nitrogen (SNTCA), soluble in 12% TCA, as a percentage of total nitrogen (TN), in experimental UF white cheeses during ripening are shown in Figure 3. The extent of secondary proteolysis, in terms of SNTCA or its main components (peptides, aminoacids and ammonia), were significantly higher (P< 0.01) in cheeses produced using vegetable rennet than the samples made with the use of pure chymosin and fungi rennet ($\text{Rhizomucor miehei}$) at 1, 15, 30 and 45 days of ripening. In cheeses produced using vegetable rennet, the levels of SNTCA/TN increased from 22% at the beginning of ripening to 46% at the end of ripening (45 days). On the contrary, in cheeses made with animal or fungi rennet, the SNTCA/TN content improved more slowly during ripening (Figure 3). Although lactic acid bacteria and other enzymes are the principal agents for the production of SNTCA in cheese, the higher breakdown of caseins in cheeses made with plant coagulants suggest that these cheeses contain more substrate (casein polypeptides) that contribute to the secondary proteolysis (Tejada et al., 2008). Hesari et al. (2006) showed that rennet had indirect but important role on the secondary proteolysis. In fact the omission of rennet or starter during cheese making causes an obvious reduction in the levels of peptide and free amino acids produced during ripening of Iranian UF white cheese. Prados et al. (2006) showed that % SNTCA/TN was higher in Mancheco type cheese, manufactured with powdered vegetable rennet of $\text{Cynara cardunculus}$ as compared with animal rennet. On the contrary different studies on ewe’s milk cheeses (Fernández-Salguero and Sanjuán, 1999) and Los Pedroches cheese (Sanjuán et al., 2002) showed that SNTCA values and their changes, during ripening, were closely similar in cheeses made with vegetable or animal rennet.

**Urea-PAGE of the pH 4.6: Insoluble Fraction**

Urea-PAGE electrophoretograms of the pH 4.6-insoluble nitrogen fraction of experimental UF white cheeses (samples of trial 1) at different ripening stages are shown.

![Figure 3](image-url) **Figure 3.** Evolution during ripening of non-protein nitrogen (SNTCA/TN) in UF Iranian white cheeses produced with fungi rennet, animal and vegetable rennet.
in Figure 4. Results of the other two experimental trials were similar (data not shown). Electrophoretic profile indicates that initial proteolysis of caseins in experimental Iranian UF white cheeses was carried out mainly by coagulant enzymes. There were notable differences in electrophoretic patterns among the three cheese typologies. In samples made with animal rennet, β-casein degraded more slowly than αS1-casein, but in cheese samples produced with Withania coagulans‘ enzyme, the hydrolysis of both αS1- and β-casein occurred more rapidly and intensely with the intact caseins disappeared apparently. Tejada et al. (2008) showed in Murcia al vino cheeses made with plant coagulant that αS1-casein degradation during ripening was more intense than that in cheeses made with animal rennet, whereas β-casein hydrolysis was very similar in both cases. Similar observations were reported by Moatsou et al. (2002) for Feta cheese. Sanjuán et al. (2002) in their study showed that in Los Pedroches cheese during ripening the proteolytic process was more intense in cheeses made with vegetable rennet as compared with cheeses made through animal rennet. Many authors (O’Mahony et al., 2003; Sarantinopoulos et al., 2002; Hayaloglu et al., 2004) have reported the resistance of β-casein to hydrolysis during ripening of many cheese varieties. Furthermore, in UF type cheese, the rates of degradation of αS1-casein and particularly of β-casein are lower than in conventional cheeses. The inhibition of plasmin (EC 3.4.21.7) and rennet enzymes by whey proteins has also been suggested as reason for the slow proteolytic process of UF cheeses (Hesari et al., 2006). These experimental results show the high ability of enzymes from Withania coagulans in αS1- and β-casein degradation in UF cheeses, this behavior being possible to be used in accelerating the proteolysis of UF cheese during ripening at a suitable level.

CONCLUSIONS

The experimental results indicated that rennet enzymes are the principal ripening agent in primary proteolysis of UF white cheese, but they also have notable effects on the secondary proteolysis and insoluble peptide profiles of UF white cheeses. The assessment of proteolytic process showed an intense proteolysis in cheese produced with Withania coagulans‘ enzyme compared to
animal and fungi rennets. This behavior could be foreseen in the use of *Withania coagulans*’ enzyme in cheese making to accelerate the UF white cheese ripening process.

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تأثیر پروتئز ویتامین کوآگولاس به عنوان مایه بینر گیاهی روی پروتولیز بینر سفید فرآبادی در

چکیده

در این تحقیق استخراج پروتئز از میوه‌های گیاه ویتامین کوآگولاس و تأثیر آن روی پروتولیز بینر سفید فرآبادی در مقایسه با کمربودن خالص و مایه پنیر فارقی مورد بررسی قرار گرفت. به تفاوت معنی‌داری بین نمونه‌های پنیر از لحاظ ماده خشک، جریان پروتئین و نمک وجود نداشت. ارزیابی با اندازه‌گیری اثر محوله در pH برای pH 4.6 و روش اوره بلی آکریل آمید زل الکتروفورز، پروتولیز شدیدتری در نمونه‌های پنیر تهیه شده با آنزیم ویتامین کوآگولاس را در مقایسه با مایه پنیر حیوانی و فارقی آشکار ساخت. پروتئین‌های آلفا اس 1 و پنیر کازنین در پنیرهای تهیه شده با آنزیم ویتامین کوآگولاس به طور کلی نابود شدند. علاوه بر آن سطح ازت محوله در 12/12 درصد کلر بروز است. افزایش پنیرهای تهیه شده از مایه پنیر گیاهی نشان داد که احتمالاً ناشی از پروتولیز غیر احتمالی آنزیم‌های ویتامین کوآگولاس بود.