

## 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 proteolytic 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<sub>105</sub>-Met<sub>106</sub> 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) and this is one of the main proteolytic factors involved in proteolysis during cheese ripening (Moatsou *et al.*, 2004). In the latter part of the last century, cheese consumption increased while the availability of calf rennet decreased, leading to rennet shortages and subsequent price increases. In addition, more restrictive ethical concerns associated

with production of such animal rennets led to a search for suitable rennet substitutes for cheese making. Several proteases from animal, microbial and plant sources were investigated (Drohse and Foltmann, 1989; 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-Salguero, 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.

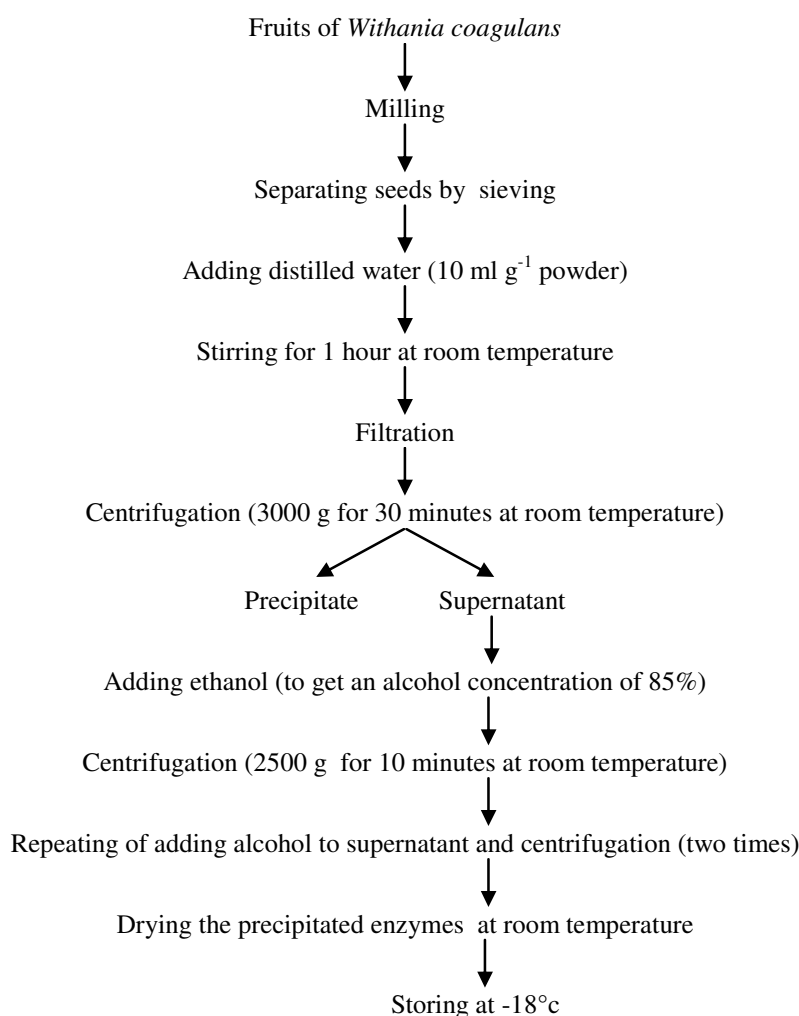
## MATERIALS AND METHODS

### 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<sup>-1</sup> retentate); 2- pure chymosin (CHR HANSEN, Denmark) (30 mg kg<sup>-1</sup> 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



**Figure 1.** Procedure of enzyme extraction from *Withania coagulans*' fruits.

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 (N° UFPH20 Invensys APV, Silkeborg, Denmark) with a nominal molecular weight cut-off of approximately 20 kg mol<sup>-1</sup> and a surface area of 16.9 m<sup>2</sup>. 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, *Lc. cremoris* and *Lc. lactis*) and thermophilic (Joghurt 709, *Str. thermophilus* and *Lb. delbrueckii* subsp. *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 (SNTCA) (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 (SNTCA) 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 slabgelunit (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 Brilliant 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 I.** Physicochemical parameters of 1-day-old experimental ultrafiltered (UF) Iranian white cheeses with three different sources of coagulant. (Results are expressed as average of three independent replicate trials ± standard deviations).

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

NS: Not significant, \* Significant at  $P < 0.01$ .

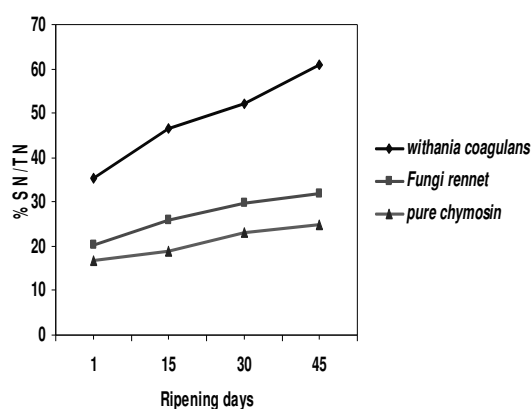
observed among cheeses in moisture, fat, protein and salt contents. The use of different rennet preparations did not have a significant effect on the main gross composition of cheeses as reported by other workers (Sousa and Malcata 1997; Wium *et al.*, 1998; Mara and Kelly, 1999).

### 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 more 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 (Sanjuán *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



**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.



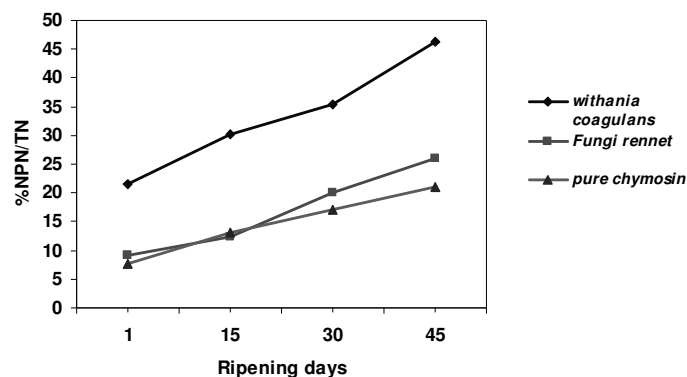
preparations are mainly responsible for the primary proteolysis of the caseins in cheese. Their action modifies the cheese texture by slowly degrading  $\alpha_1$ - and, to a lesser extent,  $\beta$ -caseins. This proteolytic process leads to the framework of cheese matrix, and produces precursors of 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 rennets than the samples made with the use of pure chymosin and fungi rennet (*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

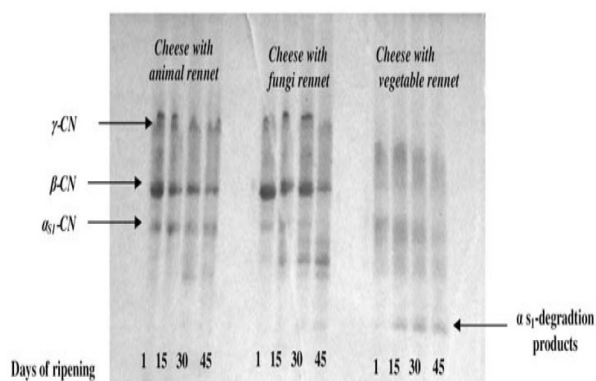
caseins in cheeses made with plant coagulants suggest that these cheeses contain more substrate (casein polypeptides) that contribute to the secondary proteolysis (Tejada et al., 2008). Hesari et al. (2006) showed that rennet had indirect but important role on the secondary proteolysis. In fact the omission 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 *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.** Evolution during ripening of non-protein nitrogen (SNTCA/TN) in UF Iranian white cheeses produced with fungi rennet, animal and vegetable rennet.



**Figure 4.** Urea polyacrylamide gel electrophoretograms of experimental UF Iranian white cheeses after 1, 15, 30 and 45 days of ripening.

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,  $\beta$ -casein degraded more slowly than  $\alpha_{s1}$ -casein, but in cheese samples produced with *Withania coagulans*' enzyme, the hydrolysis of both  $\alpha_{s1}$ - and  $\beta$ -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  $\alpha_{s1}$ -casein degradation during ripening was more intense than that in cheeses made with animal rennet, whereas  $\beta$ -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  $\beta$ -casein to hydrolysis during ripening of many cheese varieties. Furthermore, in UF type cheese, the rates of degradation of  $\alpha_{s1}$ -casein and particularly of  $\beta$ -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  $\alpha_{s1}$ - and  $\beta$ -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 پروتئاز ویتانیا کوآگولانس به عنوان مایه پنیر گیاهی روی پروتئولیز پنیر سفید فراپالایشی

ا. پزشکی، ج. حصاری، ع. احمدی زنوز و ب. قنبرزاده

### چکیده

در این تحقیق استخراج پروتئاز از میوه های گیاه ویتانیا کوآگولانس و تأثیر آن روی پروتئولیز پنیر سفید فراپالایشی در مقایسه با کیموزین خالص و مایه پنیر قارچی مورد بررسی قرار گرفت. به استثنای pH، تفاوت معنی داری بین نمونه های پنیر از لحاظ ماده خشک، چربی، پروتئین و نمک وجود نداشت. ارزیابی با اندازه گیری ازت محلول در pH برابر با ۴/۶ و روش اوره پلی آکریل آمید ژل الکتروفورز، پروتئولیز شدیدتری در نمونه های پنیر تهیه شده با آنزیم ویتانیا کوآگولانس را در مقایسه با مایه پنیر حیوانی و قارچی آشکار ساخت. پروتئینهای آلفا اس ۱ و بتا کازئین در پنیرهای تهیه شده با آنزیم ویتانیا کوآگولانس به طور کلی ناپدید شدند. علاوه بر آن سطح ازت محلول در ۱۲٪ تروکلرواستیک اسید افزایش بیشتری را در پنیرهای تهیه شده از مایه پنیر گیاهی نشان داد که احتمالاً ناشی از پروتئولیز غیر اختصاصی آنزیمهای ویتانیا کوآگولانس بود.