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Use of a new milk-clotting protease from *Thermomucor indicae-seudaticae* N31 as coagulant and changes during ripening of Prato cheese

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ABSTRACT

Prato cheeses were manufactured using coagulant from *Thermomucor indicae-seudaticae* N31 or a commercial coagulant. Cheeses were characterised using the following analysis: yield; fat; acidity; moisture; ash; salt; pH; total nitrogen; total protein; NS-pH 4.6/NT∗100; NS-TCA 12%/NT∗100; casein electrophoresis; and RP-HPLC. The results were statistically analysed and revealed that the proteolytic indices were not significantly different throughout the 60 days of ripening of cheeses made with either coagulant. Even though there were some quantitative differences in the peptide profile of cheeses, the enzyme from *T. indicae-seudaticae* N31 was used in the production of good quality Prato cheese without having to change the established technological parameters of the process.

1. Introduction

Rennet and coagulants are proteolytic enzyme preparations which have been used in the cheese industry for milk clotting, being this the oldest known application of enzymes. By definition, rennet is an extract of ruminant abomasums. From the name rennet, derived the word rennin for the milk clotting enzyme, which today is called chymosin (EC 3.4.23.4) (Andrén, 2002). Rennet extracted from calf abomasum consists of chymosin, as the major component, and of another proteolytic enzyme, pepsin (EC 3.4.23.1); when rennet is extracted from adult animals this proportion is inverted, and there is predominance of pepsin (Guinee & Wilkinson, 1992; Sousa, Ardo, & McSweeney, 2001). Due to its specificity towards the bond Phe_{105}–Met_{106} of κ-casein, chymosin is more adequate to clot milk for cheese making than pepsin, which presents general proteolytic action (Visser, 1993), risking the yield and flavour of cheese. Milk-clotting enzymes other than rennet are called coagulants and are represented by fermentation produced chymosin, which is 100% calf chymosin produced by recombinant DNA technology involving *Aspergillus niger*, *Kluyveromyces lactis* or *Escherichia coli* (Andrén, 2002); by vegetable enzymes such as the aqueous extract of flowers of *Cynara cardunculus* the most popular and successful in Portugal (Sousa & Malcata, 1998); and by different microbial coagulants specially the ones from *Rhizomucor miehei*, *Rhizomucor pusillus* and *Cryphonectria parasitica* (Andrén, 2002; Nelson, 1975; Sardinas, 1968).

Approximately a third of the world's milk production is used for cheese manufacture and the use of cheese for direct consumption and as an ingredient has increased tremendously (Farkye, 2004). For example, there was a 17% worldwide increase in cheese production from 2000 to 2008, and 43% in Brazil (Embrapa, 2010). Prato cheese, a Brazilian semi-hard cow variety, is of Danish origin, similar to Gouda and Danbo, with characteristic taste and texture; it is widely distributed in Brazil and is one of the most consumed cheeses in the country (Cichoscki, Valduga, Valduga, Tornadijo, & Fresno, 2002). It is a ripened cheese made by enzymatic curdling with a smooth, thin rind and an elastic, compact consistency and rectangular in shape (Cichoscki et al., 2002; Gorostiza et al., 2004). It is clear that cheeses have a very important economical role worldwide and also that the production of cheeses obtained through enzymatic coagulation, such as Prato cheese, tends to keep rising, meaning that the demand for coagulants is growing.

Another trend in the cheese industry is that calf slaughter has decreased causing lack of calf rennet and a raise in its cost (Andrén, 2002). Therefore the dairy industry still has the challenge of overcoming the shortage of rennet with coagulants from other sources.

In previous studies carried out in our laboratory, the fungus *Thermomucor indicae-seudaticae* N31, isolated by our group, produced a protease that specifically hydrolysed κ-casein during milk clotting (Merheb-Dini, Gomes, Boscolo, & da Silva, 2010). This led

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us to develop studies with this enzyme using it as a coagulant for Prato cheese making. The properties of the resultant cheeses during ripening were compared with cheeses manufactured with a traditional commercial coagulant, since after a cheese is made, some of the coagulant remains in the cheese block and its activity contributes to the proteolysis that takes place during ripening (Guinee & Wilkinson, 1992).

2. Material and methods

2.1. Maintenance of fungal culture and enzyme production

The fungus, *T. indicae-seudaticae* N31, obtained from the Laboratory of Applied Biochemistry and Microbiology – IBILCE – UNESP, was maintained in Sabouraud dextrose agar medium (Oxoid) and prior to use it was inoculated in 250 ml Erlenmeyer flasks containing Sabouraud with 0.2% casein and incubated at 45 °C for 2 days for complete growth. Enzyme production was carried out according to Merheb-Dini et al. (2010) using wheat bran as substrate and a fermentation period of 24 h. After extraction, 1.116 ml of enzymatic extract was concentrated to 112 ml through ultrafiltration for use in cheese making.

2.2. Prato cheese making

Cheeses were made from 15 l of pasteurised cow's milk (Laticínio Saboroso, São José do Rio Preto-SP): the milk was warmed to 32 °C before adding 7.5 ml of 50% calcium chloride, 12 ml of starter (LL50 A, composed of strains *Lactococcus lactis* ssp *lactis* and *Lactococcus lactis* ssp *cremoris*), 1.05 ml urucum colourant, sorbic acid (1.8 g in 90 ml of distilled water), and finally coagulant Ha-la (Chr. Hansen) – process H or coagulant from *T. indicae-seudaticae* N31 – process T (the amount of coagulant added was standardised to equal milk-clotting activity of approximately 45 min). After coagulation (45 min for both treatments), the curd was cut into 0.3–0.5 cm^3 cubes which were then submitted to slow continuous mixing for 15 min (1st mixing), followed by removal of part of the whey (30%) and further heating of the curd to 38 °C with the addition of 80 °C water (17%). The curd was mixed again for another 15 min (2nd mixing) followed by complete whey removal then placed in plastic moulds and pressed. The cheeses were turned upside down after the first 30 min and then pressed for 24 h in a vertical press, with stainless steel weights. Cheeses were then removed from the press and from the moulds and were placed in 18% (NaCl) brine solution for 5 h at 4 °C. Finally, they were dried at 9 °C/24 h, weighed, sealed under vacuum in heat-shrinkable plastic bags and stored at 9 °C/80% relative humidity for 60 days.

2.3. Cheese sampling

Two processes were carried out, one using the commercial coagulant (control) and the other substituting the commercial coagulant for the protease from *T. indicae-seudaticae* N31. Two replicates were made, A and B, with different lots of whole milk and one cheese produced with each coagulant was taken randomly for analysis with 1, 15, 30, 45 and 60 days, totalling 20 cheeses. Cheeses were triturated and homogenised for the physical chemical assays.

2.4. Chemical analysis

To characterise cheese samples the following analysis were carried out, in triplicate: fat by the method of Gerber-Van Gulik (Instituto Adolfo Lutz., 1985); titratable acidity (Instituto Adolfo Lutz, 1985); moisture (Case, Bradley, & Williams, 1985); ash (Instituto Adolfo Lutz, 1985); salt (Serres, Amariglio, & Petransxiene, 1973) and to determine pH, 10 g of grated cheese were transferred to a 100 ml beaker, 10 ml of distilled water was added and after homogenising 30 ml of distilled water was added; the mixture was left to rest for 5 min and was then filtered through hydrophilic cotton into 250 ml Erlenmeyer flask, cotton was rinsed with 10 ml of distilled water, squeezed and the clear filtrate was used for pH determination. As a common practise in our laboratory, analysis for fat, moisture, ash and salt were only determined on the first day of ripening.

To evaluate proteolysis, total nitrogen (TN) was determined by the micro-Kjeldahl method according to AOAC (1997) using a factor of 6.38 to determine total protein. For soluble nitrogen evaluation, the procedure for preparing the cheese extract was adapted from Vakaleris and Price (1959). Fifty grammes of grated cheese was homogenised with 100 ml of distilled water at 40–45 °C and 50 ml of 0.5 M sodium citrate in a mixer for 7 min. The homogenous milky solution was transferred, using distilled water, into a 250 ml volumetric flask, cooled to room temperature, brought up to volume and thoroughly mixed. This was referred to as the homogenate. To obtain the fraction of nitrogen soluble at pH 4.6, a 100 ml aliquot of the homogenate was transferred to a 250 ml beaker, 20 ml of 1.41 M HCl was added and after 5 min 15 ml of distilled water was added. The solution was filtered through a Whatman No. 1 filter paper and the clear filtrate was used for subsequent measurement of total nitrogen content. To obtain the fraction of nitrogen soluble in TCA 12%, a 50 ml aliquot of the homogenate was transferred to a 250 ml beaker, 50 ml of 24% TCA solution was added and after 15 min 15 ml of distilled water was added. The solution was filtered through a Whatman No. 1 filter paper and the clear filtrate was used for subsequent measurement of total nitrogen content. Ripening extension and depth indices were expressed as percentage of total nitrogen: NS-pH 4.6/TN∗100 and NS-TCA 12%/TN∗100, respectively.

2.5. Urea–polyacrylamide gel electrophoresis (urea–PAGE)

Proteolysis was monitored as described by Shalabi and Fox (1987). For this, 20 mg of cheese samples were incubated at 37 °C, in Eppendorf flasks, with 0.4 ml of 0.062 M tris–HCl buffer, pH 6.7, containing 42% (w/v) urea for 1 h. Afterwards, 10 μl of β-mercaptoethanol were added and the mixture again kept at 37 °C for 45 min. Finally, a drop of bromophenol blue was added. Electrophoresis was performed, according to Shalabi and Fox (1987), to these treated samples, using a Mini Protean 3 Cell vertical slabgel unit (Bio Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547). Urea–PAGE was performed at a constant voltage of 80 V, using 0.046 M tris–glycine, pH 6.7, as running buffer. Gels were stained overnight with Coomassie Brilliant Blue R-250 and destained with ethanol/acetic acid/water 3:1:6 (v/v/v) solution.

2.6. Reverse-phase high performance liquid chromatography (RP-HPLC)

Water soluble extract from cheese samples were prepared according to Kuchroo and Fox (1982). Cheese samples were freeze dried to eliminate possible interferences caused by differences in moisture content. Homogenisation of 1 part grated cheese with 2 parts distilled water was carried out for 5 min using a Stomacher. The resulting solution was kept at 40 °C for 1 h. To obtain fractions of pH 4.6-soluble nitrogen, HCl 1 N was used to adjust the pH of the solution to 4.6. Afterwards, samples were centrifuged at 3300*g* for 30 min at 4 °C. The supernatant was filtered through glass wool and afterwards through Whatman paper No. 1 and thus contained the nitrogenous portion soluble in pH 4.6. Samples were then freeze dried prior to RP-HPLC analyses.

RP-HPLC analyses were carried out according to Baldini (1998). For this, a Dionex P680 HPLC Pump was fitted with a Dionex 201SP C_{18} 5 μm reversed phase column (4.6 × 250 mm) and a Jasco UV-975 detector at wavelength of 214 nm. Solvents used were A: trifluoroacetic acid (TFA) at 0.1% (v/v) in water; B: TFA at 0.1% (v/v) in HPLC grade acetonitrile. One aliquot of 10 mg of freeze dried sample was dissolved in 1 ml of A, centrifuged at 13,000 rpm/20 min, filtered (0.22 μm) and 20 μl was injected and initially eluted with 100% A, then with a linear gradient of 0–50% of B for 55 min, followed by a linear gradient of 60% B for 4 min and finally with 60% B for 3 min. A flow rate of 0.75 ml/min was kept.

2.7. Statistical analysis

To establish statistical differences on data for the chemical analysis, according to the type of coagulant used, period of ripening and the interaction among these two factors, the results were analysed using the program ESTAT (Sistema para Análises Estatísticas, version 2.0, UNESP-Jaboticabal), by analysis of variance using *F* test and comparison of means by Tukey test ($p < 0.05$).

3. Results and discussion

3.1. Prato cheese yield and composition

Yield of cheese production was of 9.9 l of milk to manufacture 1 kg of cheese with the commercial coagulant and of 10.5 l of milk to manufacture 1 kg of cheese with coagulant from *Thermomucor*, which is very similar.

Table 1 shows the results from the chemical characterisation of cheeses during ripening. The data show a typical Prato cheese composition with very similar results for both processes regarding protein, fat, moisture and salt composition indicating that the production of Prato cheese with the coagulant form *Thermomucor* can be executed under conventional manufacturing conditions.

In spite of moisture content of cheese made with commercial coagulant (43.98%) be higher than the one made with coagulant from *Thermomucor* (42.47%) (Table 1), they were very similar, not implying in technological problems. Similar moisture values for Prato cheese were also reported by Cichoscki et al. (2002) (41.91% with 7 days of storage).

Traditional Prato cheese is classified as a high fat cheese for presenting 25–29% of fat. Fat content of cheeses from both processes were approximately 26% and were not significantly different (Table 1). Similar fat values for Prato cheese have also been reported by Spadoti, Dornellas, Petenate, and Roig (2003) (25.2% with 10 days of storage) and by Cichoscki et al. (2002) (26% with 1 day of storage).

Ash content for cheese were 4.60% when using coagulant from *Thermomucor* and 4.34% when using commercial coagulant being significantly higher than the first (Table 1). These values are a little superior than the one reported by Cichoscki et al. (2002) of 3.68% with 1 day of storage.

There was an increase of acidity for cheeses made with either coagulants during the 60 days of ripening, probably due to accumulation of lactose degradation products such as lactic acid and other volatile acids (Rao, Nand, Srikanta, Krishna-Swamy, & Murthy, 1979). The acidity evolution profile was similar for both cheeses in spite of contents being significantly higher for the ones made with coagulant from *Thermomucor*, except on the 15th day, where there is no difference between the two processes (Table 1). Continuous acidity increase during ripening was also noted by El-Tanboly, El-Hofi, and Ismail (2000) for Gouda cheeses made with commercial coagulant (Ha-la) and with microbial coagulant (*Mucor miehei* NRRL 3169) and by Cichoscki et al. (2002) when studying 60 days of ripening of Prato cheese made with animal rennet.

Decrease in pH values is related to lactose fermentation, as mentioned above, which is important to prevent pathogenic bacterial growth. Besides, pH variation during ripening also depends on the buffering capacity of the cheese, due to the amount of proteins and minerals present (Lawrence, Heap, & Gilles, 1984), to the formation of ammonium and/or catabolism of lactic acid (Fox, 1989).

3.2. Proteolysis assessment: Evolution of soluble nitrogen

For the development of texture, taste and aroma characteristics of ripened cheeses, such as Prato cheese, a balanced degradation of proteins into peptides and aminoacids is necessary (Singh, Drake, & Cadwallader, 2003) and the detection and quantification of these degradation products are used as parameters to express the ripening index of cheeses (McSweeney & Fox, 1997). Therefore we studied the formation of nitrogenous compounds during the ripening of Prato cheeses, through chemical analysis, to monitor

Table 1
Composition of cheeses made with commercial coagulant (H) and with protease from *T. indicae-seudaticae* N31 (T) during ripening.

Analysis	Processes	Days of ripening				
		1	15	30	45	60
Acidity (%)	H	0.60 ± 0.05^{Bc}	1.05 ± 0.15^{Ab}	1.12 ± 0.13^{Bb}	1.38 ± 0.16^{Ba}	1.55 ± 0.17^{Ba}
	T	0.70 ± 0.05^{Ae}	1.13 ± 0.04^{Ad}	1.26 ± 0.04^{Ac}	1.56 ± 0.08^{Ab}	1.78 ± 0.08^{Aa}
pH	H	5.34 ± 0.05^{Aa}	5.25 ± 0.16^{Ba}	5.33 ± 0.10^{Aa}	5.26 ± 0.05^{Ba}	5.35 ± 0.11^{Aa}
	T	5.34 ± 0.08^{Ab}	5.52 ± 0.16^{Aa}	5.42 ± 0.01^{Aab}	5.38 ± 0.02^{Aab}	5.39 ± 0.07^{Aab}
Total protein (%)	H	23.92 ± 1.56^{Aa}				
	T	24.16 ± 1.80^{Aa}				
Fat (%)	H	25.92 ± 1.20^{A}				
	T	25.83 ± 1.69^{A}				
Moisture (%)	H	43.98 ± 1.04^{A}				
	T	42.47 ± 0.82^{B}			Nd	
Ash (%)	H	4.34 ± 0.13^{B}				
	T	4.60 ± 0.14^{A}				
Salt (%)	H	1.90 ± 0.00^{B}				
	T	2.10 ± 0.00^{A}				
S/M[f] (%)	H	0.44 ± 0.01^{B}				
	T	0.50 ± 0.02^{A}				

Nd (Not determined): Analysis for fat, moisture, ash and salt were only determined on the first day of ripening.
A, B: Means within a column with equal letters, for the same analysis, are not significantly different ($p > 0.05$).
a, b, c, d, e: Means within a line with equal letters are not significantly different ($p > 0.05$).
[f] Salt in moisture.

and objectively evaluate cheese ripening when using protease from *T. indicae-seudaticae* N31 as coagulant.

Fig. 1A shows the evolution of NS-pH 4.6/TN∗100, which is represented by the presence of peptides with high/intermediate molecular mass which were produced by the action of residual coagulant, proteinases from the starter and plasmin on casein, known as primary proteolysis (Fox, 1989; Singh et al., 2003). Therefore, this is an important index to evaluate the behaviour of coagulants during cheese ripening. It can be seen that there was an increase of pH 4.6-SN for both processes, which agrees with the literature (McSweeney & Fox, 1997). Increase of NS-pH 4.6/TN∗100 during ripening of Prato cheese was also reported by Garcia, Moretti, Gomes, Casarotti, and Pena (2009) and Gorostiza et al. (2004).

Fig. 1B shows the evolution of NS-TCA 12%/TN∗100, which is represented by the presence of peptides of low molecular mass and free amino acids that were produced by the action of peptidases from the starter and non starter bacteria on peptides with high/intermediate molecular mass (Fox, 1989; Singh et al., 2003). It can be seen that there was an increase of TCA 12%-SN for both processes. Increase of NS-TCA 12%/TN∗100 during ripening of Prato cheese was also reported by Garcia et al. (2009) and Gorostiza et al. (2004).

According to the results from *F*-test of ANOVA, shown in Table 2, ripening period significantly affected ripening indices ($p < 0.01$), which was expected since for ripening to occur these indices need to increase throughout time. It can also be seen that the treatments did not significantly affect NS-pH 4.6/TN∗100 suggesting that coagulant from *T. indicae-seudaticae* N31 caused the same type of proteolysis as commercial coagulant. However, treatments affected NS-TCA 12%/TN∗100 ($p < 0.05$) but when carrying out comparison of means by Tukey test, no differences were revealed between treatments. Also, the interaction between treatments and ripening period did not significantly affect the indices, indicating that proteolysis increases throughout ripening in the same way for cheeses made with commercial coagulant and with coagulant from *T. indicae-seudaticae* N31.

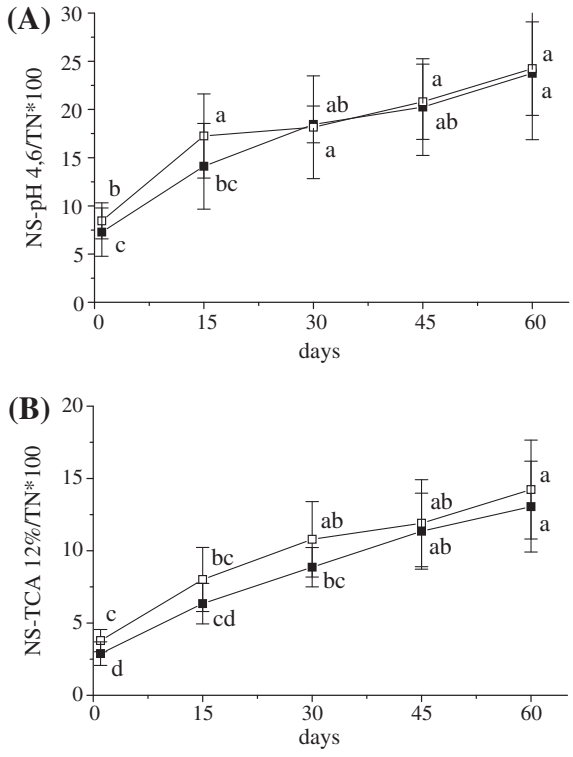

Fig. 1. Evolution of ripening indices NS-pH 4.6/TN∗100 (A) and NS-TCA 12%/TN∗100 (B), of cheeses made with commercial coagulant (□) and with coagulant from *Thermomucor indicae-seudaticae* N31 (■) during ripening. Means within a line with equal letters are not significantly different ($p > 0.05$).

Table 2
Effect of treatments and ripening period on ripening indices, NS-pH 4.6/TN∗100 and NS-TCA 12%/TN∗100.

Causes of variation	FD	F	
		NS-pH 4,6/TN∗100	NS-TCA 12%/TN∗100
Treatment[a]	1	0.7886[NS]	4.1020[*]
Period	4	22.6678[**]	34.9906[**]
Treatment × period	4	0.2570[NS]	0.1880[NS]

[a] Use of commercial coagulant and coagulant from *Thermomucor indicae-seudaticae* N31.
[NS] *F*-test was not significant at a level of 5% probability ($p > 0.05$).
[*] *F*-test was significant at a level of 5% probability ($p < 0.05$).
[**] *F*-test was significant at a level of 1% probability ($p < 0.01$).

Residual chymosin rapidly hydrolyses α_{s1}-casein at the bond Phe_{23}–Phe_{24} during initial stages of ripening, resulting in the formation of a large peptide α_{s1}-CN f24–199 (α_{s1}-I-casein) and the small one α_{s1}-CN f1–23. Hydrolysis of this bond causes a rapid change in the rubbery texture of Cheddar cheese into a more homogenous and smoother product (Lawrence, Creamer, & Gilles, 1987; Singh et al., 2003). Since the NS-pH 4.6/TN∗100 evolution was not significantly different for cheeses produced with each coagulant, a similar α_{s1}-casein hydrolysis profile was expected for these cheeses, however this was not observed as seen in Fig. 2B as explained earlier by the different action of the coagulants due to ripening pH and temperature.

Plasmin acts on β-casein resulting on the formation of three γ-caseins [γ_1-(β-CN f29–209), γ_2-(β-CN f106–209) and γ_3-(β-CN f108–209)], representing the C-terminal region and of five proteose–peptones, representing the N-terminal region (Singh et al., 2003). These proteose–peptones are soluble at pH 4.6 affecting pH 4.6-SN, although their contribution is small (McSweeney & Fox, 1997). According to Singh et al. (2003), the γ-caseins seem to accumulate in Cheddar cheese during ripening, but the proteose–peptones are extensively hydrolysed by peptidases and cell envelope-associate proteinases from starter cultures to small peptides and free amino acids, affecting TCA 12%-SN. In Cheddar cheese, the peptide α_{s1}-CN f1–23 is further hydrolysed by proteinases from *Lactococcus lactis* ssp. *cremoris* into smaller peptides, which present bitter taste (Singh et al., 2003). Since Prato cheese is also made with this starter culture, it is probable that this hydrolysis also occurs, affecting TCA 12%-SN. Thus, pH 4.6-SN and TCA 12%-SN in Prato cheese were essentially affected by residual chymosin, plasmin and by proteolytic enzymes of lactic acid bacteria.

3.3. Proteolysis assessment: Urea–PAGE and RP-HPLC

According to Sousa et al. (2001), since proteolysis is one of the main biochemical events during the ripening of cheese, it is desirable to include a general assay for proteolysis, such as the determination of soluble N as % of total N, as has been done. If the objectives of the study cover investigating the effect of one of the agents of proteolysis in cheese, such as different types of coagulants, the methodology should be chosen so as to emphasise the level of proteolysis caused by that agent. In this case, for example, proteolysis evolution could be followed by urea–polyacrylamide gel electrophoresis (urea–PAGE) and the peptide profiles of the pH 4.6-soluble fraction should be determined by reverse phase-high performance liquid chromatography (RP-HPLC). Therefore, proteolysis was assayed by the frequently used method of monitoring casein proteolytic processes: polyacrylamide gel electrophoresis using

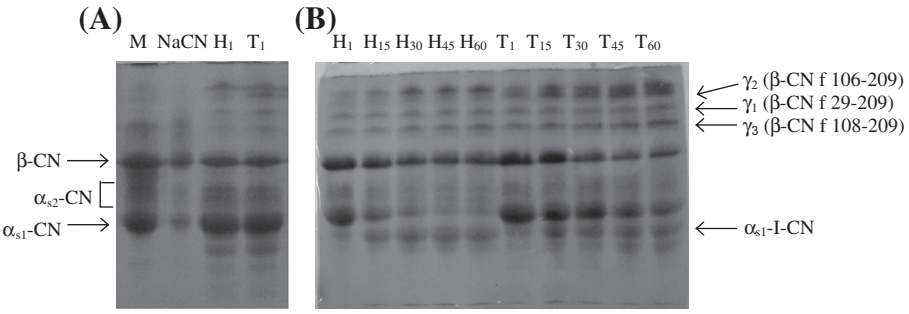

Fig. 2. Urea–PAGE of casein. (A) Electrophoretic profile of milk (M), of bovine sodium caseinate (NaCN) and of the first ripening days of Prato cheese (H_1 and T_1). (B) Casein degradation profile of Prato cheeses during the 60 days of ripening. H represents chesses made with commercial coagulant and T cheeses made with coagulant from *T. indicae-seudaticae* N31.

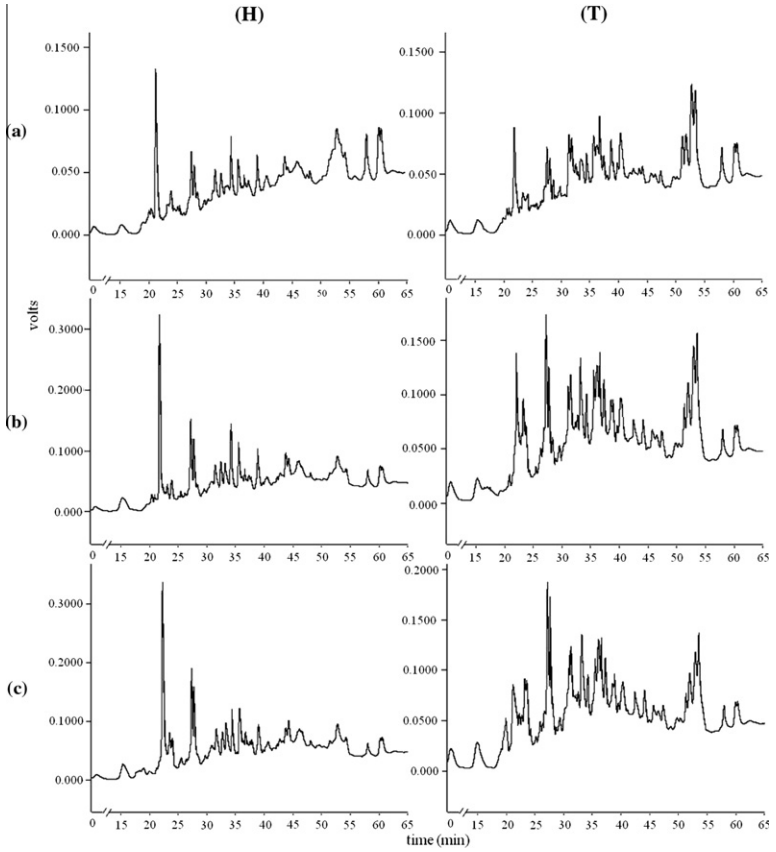

Fig. 3. RP-HPLC elution profile of the pH 4.6-soluble nitrogen fractions of cheeses made with commercial coagulant (H) and with protease from *T. indicae-seudaticae* N31 (T) with 1 (a), 30 (b) and 60 (c) days of ripening.

urea, which makes possible to visualise the integrity of casein fractions during ripening (Fig. 2). In Fig. 2A, two main casein groups were identified in the urea–PAGE: α_{s1}-casein, with higher electrophoretic mobility and β-casein, with lower mobility (Silva & Malcata, 2004). The region of family α_{s2}-casein can also be seen, whose electrophoretic mobilities is between caseins α_{s1} and β (Sgarbieri, 2005). Fig. 2B shows casein degradation in cheeses made with commercial coagulant (H_1–H_{60}) and with coagulant from *T. indicae-seudaticae* N31 (T_1–T_{60}) during 60 days of ripening. Degradation of α_{s1}-casein is seen, more pronounced in cheeses made with commercial coagulant, showing that the hydrolysis of casein molecules is specific for the type of coagulant used (Lawrence et al., 1987). Degradation of β-casein is also seen, more intense in cheeses made with coagulant from *T. indicae-seudaticae* N31, with formation of its hydrolysis products, the γ-caseins, which accumulate in cheese (Singh et al., 2003). Plasmin is one of the agents responsible for the proteolysis during cheese ripening acting especially in the initial stages along with residual coagulant, liberating peptides which will serve as substrate for proteinases from starter and non starter bacteria (Fox, 1989; Visser, 1993). Besides plasmin, chymosin also acts on β-casein, on the bond between Leu_{192} and Tyr_{193} (Visser, 1993). It can also be seen in Fig. 2B that fraction α_{s2} is also degraded, in a similar way for both processes (H and T). According to Grappin, Rank, and Olson (1985) this fraction is very resistant to chymosin and its degradation is associated to plasmin, whose preferred substrates therefore are fractions β and α_{s2}; however degradation products of α_{s2} have not yet been identified (Fox, 1989). Similar results for higher degradation of

α_{s1}-casein and lower degradation of β-casein were also found by Gorostiza et al. (2004) when studying Prato cheese, by Irigoyen, Izco, Ibáñez and Torre (2002) when studying ovine cheese made with lamb rennet, by Bansal et al. (2009) when studying Cheddar cheese made with fermentation-produced camel or calf chymosin, and by Silva and Malcata (2004) when also studying ovine cheese made with coagulant form *C. cardunculus*. Edwards and Kosikowski (1969) also found differences in the way different coagulants acted on α_{s1}-casein; the authors saw that there was higher degradation in Cheddar cheese made with calf rennet, followed by microbial coagulants from *Mucor* and *Endothia*. Therefore we can see that even the commercial coagulants available in the market act in different ways on cheese caseins. The important thing is that this differentiated action does not technologically affect the product in such way that it can normally develop its characteristics of flavour, texture, etc.

The RP-HPLC analysis of the pH 4.6-soluble fraction (Fig. 3) was carried out, which is mainly produced by the residual coagulant since products from plasmin action such as proteose–peptones are soluble at pH 4.6 but have little contribution to pH 4.6-SN and γ-caseins are insoluble at pH 4.6 (McSweeney & Fox, 1997). The chromatograms obtained, using absorbance at 214 nm as a detection system (wavelength at which peptide bonds absorb), are very complex with many peaks and some quantitative differences between peptide profiles of both processes as ripening progressed with increase of intensity of some peaks and decrease of others. More peaks in chromatogram T may represent products of unknown hydrolysis since α_{s1}-casein was less hydrolysed in this system or it can represent β-casein hydrolysis products, which was more hydrolysed in this system, as shown in Fig. 2B. Again, the important thing is that this differentiated action does not technologically affect the product.

Despite some quantitative differences between profiles from both processes, a similar behaviour is noted as the peptides strongly increased in the first 30 days and then remained practically unchanged in the last 30 days. These results are in accordance with the determinations of NS-pH 4.6/NT∗100 discussed previously: in cheeses made with coagulant from *Thermomucor*, NS-pH 4.6/NT∗100 increased strongly from the first to the 15th day followed by a stabilisation and in cheeses made with commercial coagulant, NS-pH 4.6/NT∗100 increased from the first to the 30th day followed by stabilisation (Fig. 1A). RP-HPLC analysis of the pH 4.6-soluble fraction proved to be an important tool to complement the determinations of NS-pH 4.6/NT∗100.

4. Conclusions

Among the proteolytic agents that act during Prato cheese ripening, residual coagulant is of great importance and has a crucial role in proteolysis. The results obtained for the proteolytic indices during the 60 days of ripening for cheeses made with coagulant form *T. indicae-seudaticae* N31 did not differ statistically from the ones obtained for cheeses made with commercial coagulant. Furthermore, casein hydrolysis throughout the ripening of cheeses made with either coagulants, presented some differences as visualised by urea–PAGE and HPLC. However, making of Prato cheese with coagulant from *T. indicae-seudaticae* N31 was well executed under conventional production conditions resulting in a product with good technological quality.

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References

Andrén, A. (2002). Rennets and coagulants. In H. Roginski, J. W. Fuquay, & P. F. Fox (Eds.), *Encyclopedia of Dairy Sciences* (pp. 281–286). London: Academic Press.
Association of Official Analytical Chemists. (1997). Dairy Products, In H. Roginski, J. W. Fuquay, & P. F. Fox (Eds), *Official Methods of Analysis* (16th ed.). Arlington.
Baldini, V. L. S. (1998). Proteolysis in Prato cheese during the maturation, (Doctorate in Food Science) – Faculdade de Ciências Farmacêuticas. São Paulo: Universidade de São Paulo (206f).
Bansal, N., Drake, M. A., Piraino, P., Broe, M. L., Harboe, M., Fox, P. F., et al. (2009). Suitability of recombinant camel (*Camelus dromedarius*) chymosin as a coagulant for Cheddar cheese. *International Dairy Journal, 19*, 510–517.
Case, R. A., Bradley, R. L., Jr., & Williams, R. R. (1985). Chemical and physical methods. In *Standard methods for the examination of dairy products* (pp. 327–404). Washington: American Public Health Association.
Cichoscki, A. J., Valduga, E., Valduga, A. T., Tornadijo, M. E., & Fresno, J. M. (2002). Characterization of Prato cheese, a Brazilian semi-hard cow variety: Evolution of physico-chemical parameters and mineral composition during ripening. *Food Control, 13*, 329–336.
Edwards, J. L., & Kosikowski, F. V. (1969). Electrophoretic proteolytic patterns in cheddar cheese by rennet and fungal rennets: Their significance to international classification of cheese varieties. *Journal of Dairy Science, 52*, 1675–1678.
El-Tanboly, E., El-Hofi, M. A., & Ismail, A. (2000). Changes of proteolytic and lipolytic activities during ripening of Gouda cheese prepared with fungal rennet substitute. *Milchwissenchaft, 55*, 624–628.
Embrapa. (2010). *Empresa Brasileira de Pesquisa Agropecuária – Embrapa Gado de Leite*. Available from www.cnpgl.embrapa.br Last consultation on 27 August 2010.
Farkye, N. (2004). Cheese Technology. *International Journal of Dairy Technology, 57*, 91–98.
Fox, P. F. (1989). Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science, 72*, 1379–1400.
Garcia, G. A. C., Moretti, B. R., Gomes, R. G., Casarotti, S. N., & Pena, A. L. B. (2009). Composition of macronutrients and evolution of ripening of low fat Prato cheese with the addition of the proteolytic enzyme fastuosain. *Brazilian Journal of Food Technology, VII BMCFB, 69–77*, 2009.
Gorostiza, A., Cichoscki, A. J., Valduga, A. T., Valduga, E., Bernardo, A., & Fresno, J. M. (2004). Changes in soluble nitrogenous compounds, caseins and free amino acids during ripening of artisanal Prato cheese; a Brazilian semi-hard cows variety. *Food Chemistry, 85*, 407–414.
Grappin, R., Rank, T. C., & Olson, N. (1985). Primary proteolysis of cheese proteins during ripening. A review.. *Journal of Dairy Science, 68*, 531–540.
Guinee, T. P., & Wilkinson, M. G. (1992). Rennet coagulation and coagulants in cheese manufacture. *Journal of the Society of Dairy Technology, 45*, 94–104.
Instituto Adolfo Lutz. (1985). *Normas analíticas do Instituto Adolfo Lutz* (3rd ed.). São Paulo.
Irigoyen, A., Izco, J. M., Ibáñez, F. C., & Torre, P. (2002). Influence of calf or lamb rennet on the physicochemical, proteolytic, and sensory characteristics of an ewe's milk cheese. *International Dairy Journal, 12*, 27–34.
Kuchroo, C. N., & Fox, P. F. (1982). Soluble nitrogen in cheddar cheese: Comparison of extraction procedures. *Milchwissinschaft, 37*, 331–335.
Lawrence, R. C., Creamer, L. K., & Gilles, J. (1987). Texture development during cheese ripening. *Journal of Dairy Science, 70*, 1748–1760.
Lawrence, R. C., Heap, H. A., & Gilles, J. (1984). A controlled approach to cheese technology. *Journal of Dairy Science, 67*, 1632–1645.
McSweeney, P. L. H., & Fox, P. F. (1997). Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait, 77*, 41–76.
Merheb-Dini, C., Gomes, E., Boscolo, M., & da Silva, R. (2010). Production and characterization of a milk clotting protease in the crude enzymatic extract from the newly isolated *Thermomucor indicae-seudaticae* N31 (Milk clotting protease from the newly isolated *Thermomucor indicae-seudaticae* N31). *Food Chemistry, 120*, 87–93.
Rao, K. S. N., Nand, K., Srikanta, S., Krishna-Swamy, M. A., & Murthy, V. S. (1979). Changes during manufacture and ripening of cheddar cheese prepared with fungal rennet substitute of *Rhizopus oligosporus*. *Die Nahrung, 23*, 621–626.
Nelson, J. H. (1975). Impact o f new milk clotting enzymes on cheese technology. *Journal of Dairy Science, 58*, 1739–1750.
Sardinas, J. L. (1968). Rennin enzyme of *Endothia parasitica*. *Applied Microbiology, 16*, 248–255.
Serres, L., Amariglio, S., & Petransxiene, D. (1973). Contrôle de la qualité des produtits laitiers. Ministère de l'Agriculture. Direction des Services Vétérinaires. Tome I. Analyse Physique et Chimiquie (Chimie VII – 6).
Sgarbieri, V. C. (2005). Review: Structural and physicochemical properties of milk proteins. *Brazilian Journal of Food Technology, 8*, 43–56.
Shalabi, S. I., & Fox, P. F. (1987). Electrophoretic analysis of cheese: Comparison of methods. *Irish Journal of Food Science Technology, 11*, 135–151.
Silva, S. V., & Malcata, F. X. (2004). Influence of the coagulant level on early proteolysis in ovine cheese-like system made with sterilized milk and *Cynara cardunculus*. *Journal of Food Science, 69*, 579–584.
Singh, T. K., Drake, M. A., & Cadwallader, K. R. (2003). Flavor of cheddar cheese: A chemical and sensory perspective. *Comprehensive Reviews in Food Science and Food Safety, 2*, 139–162.
Sousa, M. J., Ardo, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal, 11*, 327–345.

Sousa, M. J., & Malcata, F. X. (1998). Proteolysis of ovine and caprine caseins in solution by enzymatic extracts from flowers of *Cynara cardunculus*. *Enzyme and Microbial Technology, 22*, 305–314.

Spadoti, L. M., Dornellas, J. R. F., Petenate, J. A., & Roig, S. M. (2003). Evaluation of the melting capacity of Prato cheese obtained by modifications of the traditional manufacturing process. *Lait, 83*, 397–408.

Vakaleris, D. G., & Price, W. V. (1959). A rapid spectrophotometric method for measuring cheese ripening. *Journal of Dairy Science, 42*, 264–276.

Visser, S. (1993). Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. *Journal of Dairy Science, 76*, 329–350.