J. Dairy Sci. 96:7490–7499
http://dx.doi.org/10.3168/jds.2013-7119

Yield, changes in proteolysis, and sensory quality of Prato cheese produced with different coagulants

L. S. Alves,* C. Merheb-Dini,*[1] E. Gomes,† R. da Silva,† and M. L. Gigante*
*Faculty of Food Engineering, University of Campinas – UNICAMP, PO Box 6121, CEP 13083-970, Campinas, SP, Brazil
†Laboratory of Biochemistry and Applied Microbiology – Instituto de Biociências, Letras e Ciências Exatas (IBILCE) – Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Rua Cristóvão Colombo 2265, CEP 15054-000, São José do Rio Preto, SP, Brazil

ABSTRACT

The objective of this research was to compare the effect of 2 fungal proteases, one that is already commercially established as a milk-clotting agent and another produced at the laboratory scale, on Prato cheese composition, protein and fat recovery, yield, and sensory characteristics. Cheeses were produced according to the traditional protocol, using protease from the fungus *Thermomucor indicae-seudaticae* N31 and commercial coagulant from *Rhizomucor* spp. as clotting agents. A 2 × 6 factorial design with 3 replications was performed: 2 levels of coagulants and 6 levels of storage time. After 5, 12, 19, 33, 43, and 53 d of refrigerated storage (12°C), cheeses were monitored for proteolysis, firmness, and casein degradation by capillary electrophoresis. Sensory acceptance was evaluated after 29 d of manufacturing. The different coagulants did not statistically affect Prato cheese composition, protein and fat recovery, and yield. Both cheeses presented good sensory acceptance. Proteolysis increased and firmness decreased for both cheeses during the storage time, as expected for Prato cheese. Caseins were well separated by capillary electrophoresis and the results showed, with good resolution, that the cheeses exhibited similar protein hydrolysis profile. Both cheeses presented good sensory acceptance. The gathered data showed that the protease from *T. indicae-seudaticae* N31 presented similar action compared with the commercial enzyme, indicating its efficiency as clotting agent for Prato cheese manufacture.

Key words: fungal enzyme, yield, sensory acceptance, capillary electrophoresis

INTRODUCTION

Enzymatic coagulation of milk for cheese manufacture involves specific modifications of the casein micelle through limited proteolysis by selected proteinases, following micelle aggregation in the presence of calcium. Rennet, made up mostly of chymosin (EC 3.4.23.4) is the most-used protease for cheese manufacture (Kloosterman, 1991). During enzymatic coagulation of milk, κ-CN is the only protein hydrolyzed by chymosin, specifically at the bond Phe_{105}-Met_{106}, destroying micelle stability (Fox et al., 2000). Afterward, the N-terminal part of the molecule, κ-CN fragment 1 to 105 (f1–105), known as para-κ-CN, remains attached to the micelle, whereas the C-terminal part, known as glycomacropeptide (f106–169), is lost to the aqueous medium (Fox et al., 2000). The physicochemical modifications of the micelles continue after the first hydrolysis and on the secondary phase of coagulation, the para-κ-CN micelles, in the presence of Ca^{2+} and at temperatures above 20°C, aggregate, forming a protein matrix referred to as coagulum or gel (Fox et al., 2000). This coagulum is then treated, and the different treatments lead to the manufacture of the various types of cheeses. Chymosin substitutes must reproduce its specific properties; that is, exhibit high milk-clotting activity, which consists of having specificity for κ-CN and low proteolytic activity at pH and temperature values normally used in cheese making (Fox et al., 2000). Currently, fermentation-produced chymosin, highly purified chymosin obtained using a host organism, and coagulants obtained from microorganisms such as *Rhizomucor miehei*, *Rhizomucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, and *Irpex lacteus*, are extensively used for cheese manufacture and among the microbial coagulants commercially available, the ones from fungal sources are the most used (Jacob et al., 2011).

The global enzyme market is expected to generate sales of $8 billion in 2015, with $1.3 billion representing the food and beverages segment and the highest sales occurring in the dairy market (Li et al., 2012). Booming of the market and its growing potential certainly stimulates research for new microbial proteases to be used as rennet substitutes, including the studies carried out with the following microorganisms: *Nocardiopsis* spp. (Cavalcanti et al., 2005), *Rhizopus oryzae* (Kumar

Received June 11, 2013.
Accepted September 2, 2013.
[1]Corresponding author: carolinamerheb@yahoo.com.br

et al., 2005), *Mucor bacilliformis* (Machalinski et al., 2006), *Bacillus subtilis* natto (Shieh et al., 2009), and *Aspergillus oryzae* MTCC 5341 (Vishwanatha et al., 2010).

With the increased search for new coagulants, a novel source is reported from time to time. Recently, Merheb-Dini et al. (2010) presented a coagulant protease obtained from the thermophilic fungus *Thermomucor indicae-seudaticae* N31 isolated in Brazil from industrial waste piles (Martin et al., 2010). The enzymatic extract was produced by solid-state fermentation using only wheat bran as substrate. In 24 h of fermentation, the microorganism secreted the extracellular enzyme, which exhibited maximum milk-clotting activity at pH 5.7 and at 70°C and stability in the pH range from 3.5 to 4.5 and from 5.0 to 6.0 and temperature range from 35 to 40–45°C. In addition, it was shown that the peptide profile formed by the enzymes from *T. indicae-seudaticae* N31 and from *R. miehei* (Hannilase; Chr. Hansen Brazil, Valinhos, SP, Brazil), a widely used milk-clotting agent for cheese manufacture, was very similar. These results encouraged laboratory-scale research using protease from *T. indicae-seudaticae* N31 for the manufacture of Prato cheese compared with a fermentation-produced chymosin, HA-LA, produced by Chr. Hansen Brazil (Merheb-Dini et al., 2012). The results suggested the possibility of obtaining a product with good technological quality.

In view of the previous results regarding the discovery of an enzyme produced rapidly by using a low-cost substrate, with promising biochemical properties for use in cheese manufacture, the objective of this study was to produce pilot-scale Prato cheese with the new enzyme and compare it with a well-established commercial coagulant of fungal source, commonly used for Prato cheese manufacture. For the most complete characterization, cheese and whey composition were evaluated, protein and fat recovery and adjusted cheese yield were determined, sensory analysis was carried out, and proteolysis during the storage time was investigated by capillary electrophoresis, a modern technique more efficient for casein separation than conventional gel electrophoresis and HPLC. Prato is a semihard (moisture content 36–45.9%), low-scald (≈42°C) cheese manufactured by enzymatic coagulation of milk and ripened for at least 25 d (Ministério da Agricultura do Brasil, 1997), with soft texture and smooth taste, and therefore a good tool to evaluate enzyme action as both milk-clotting agent and proteolytic agent during ripening. This research should be of great contribution to the fields of biotechnology and dairy technology, as it deals with the application of a new microbial enzyme for cheese making.

MATERIALS AND METHODS

Enzyme Production

The fungus *T. indicae-seudaticae* N31 was obtained from the collection of the Laboratory of Biochemistry and Applied Microbiology, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista Júlio de Mesquita Filho (IBILCE-UNESP, São José do Rio Preto, SP, Brazil). The steps of fungal growth, inoculum preparation and protease production followed the methodology described by Merheb-Dini et al. (2010), with modifications to achieve maximum enzyme yield and concentration under laboratory conditions. Culture medium containing 20 g of wheat bran was prepared and sterilized (120°C/20 min) in 500-mL Erlenmeyer flasks, inoculated with 27 mL mycelial suspension to give a 60% initial moisture, and incubated at 45°C for 24 h under stationary conditions. For enzyme extraction, 160 mL of distilled water was added to the medium, the flasks were shaken at 100 rpm for 30 min, and the mixture was filtered and centrifuged at 30,996 × *g* for 20 min at 5°C. The resulting solution, denominated crude enzyme extract, was filtered on Whatman grade no. 1 filter paper, and concentrated by UF (Quix-Stand System; GE Healthcare Life Sciences, São Paulo, SP, Brazil).

Cheese Manufacture

The cheeses were produced in vats with heating-cooling jackets, stirrers, and speed control by the traditional manufacturing method as described by Mazal et al. (2007). For each process, 100 L of heat-treated whole milk (68°C for 2 min) were divided into 2 equal parts to be used for Prato cheese manufacture. The following treatments were carried out: (1) control Prato cheese using commercial microbial coagulant produced by *Rhizomucor* spp. (Bela Vista Produtos Enzimáticos, Alto Bela Vista, SP, Brazil) and (2) *Thermomucor* Prato cheese produced with the enzyme from *T. indicae-seudaticae* N31. For both treatments, 50% calcium chloride solution (250 mg/kg) was added to the milk at 35°C, which was followed by the addition of 1% mesophilic culture (*Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris*; R704; Chr. Hansen Brazil), annatto dye (80 mg/kg), and coagulant. Control Prato cheese was produced using 35 min of clotting time, according to the manufacturer's instructions. *Thermomucor* Prato cheese was produced using 55 min of clotting time after standardizing the firmness at cutting of the curd to that of the commercial coagulant from *Rhizomucor* spp. (Lucey and Kelly, 1994). The curd firmness was evalu-

ated by inserting a sanitized spatula into the coagulum at a 45° angle, gently lifting the spatula straight up, and observing the curd as it split open (Mazal et al., 2007). After coagulation, the curd was cut into 1.0-cm cubes and submitted to slow continuous mixing for 15 min, which was followed by removal of part (30%) of the whey and further heating of the curd to 42°C by adding hot water (80°C) to increase the temperature by 1°C every 3 min. After cooking, all the whey was drained off, and the curd was placed in rectangular plastic molds (0.5 kg) and pressed at room temperature with successive turns (0.1 MPa/15 min; 0.1 MPa/15 min; 0.24 MPa/30 min; and 0.31 MPa/90 min). Cheeses were fermented for 5 h at room temperature and salted in brine (20%) for 10 h at 5°C. Then, the cheeses were dried at 12°C for 48 h and vacuum-packed into heat-shrinkable plastic bags and stored at 12°C for 53 d. The experiment was repeated 3 times on different days and *Thermomucor* and control Prato cheeses were produced in side-by-side vats.

Sampling and Analysis

Heat-treated milk was analyzed for the activity of alkaline phosphatase (EC 3.1.3.1) to check the efficiency of the heat treatment as described by Marshall (1992). The milk was evaluated for pH, fat by the Gerber method (British Standards Institution, 1989), TS (AOAC International, 2006; methods 33.2.44 and 990.20), ash content (AOAC International, 2006; methods 33.2.10 and 945.46), total nitrogen (**TN**; AOAC International, 2006; methods 33.2.11 and 991.21), pH 4.6-soluble nitrogen (**pH 4.6 SN**; AOAC International, 2006; methods 33.2.64 and 998.05), and 12% TCA-soluble nitrogen (**12% TCA SN**; AOAC International, 2006; methods 33.2.12 and 991.21). Total protein was calculated by multiplying TN by 6.38.

For each vat, all the whey was collected, mixed, and sampled. The whey was analyzed for fat content by the Mojonnier method (AOAC International, 1995; methods 33.2.26 and 989.05), TS, ash, TN, pH 4.6 SN, and 12% TCA SN, using the same methodologies previously mentioned.

Five days after manufacture, the pH of the cheeses was determined by introducing the electrode directly into the triturated samples. The samples were analyzed for moisture content (AOAC International, 2006; methods 33.7.03 and 926.08), fat by the Gerber method (British Standards Institution, 1989), ash (AOAC International, 2006; methods 33.7.07 and 935.42), salt by the Volhard method (Richardson, 1985), TN (AOAC International, 2006; methods 33.7.12A and 2001.14), pH 4.6 SN, and 12% TCA SN by the macro-Kjeldahl method according to Bynum and Barbano (1985).

Cheese Characterization During Storage

The cheeses were monitored for pH, moisture content, proteolysis, electrophoretic profile, and firmness after 5, 12, 19, 33, 43, and 53 d of refrigerated storage (12°C). The pH was measured as described above, and moisture content was determined according to AOAC International (2006; methods 33.7.03 and 926.08). For proteolysis measurements, pH 4.6 SN and 12% TCA SN were determined by the macro-Kjeldahl method according to Bynum and Barbano (1985), and expressed as a percentage of the TN of the cheese.

The electrophoretic profile was determined by capillary electrophoresis in a fused-silica capillary column of 57 cm (50-cm effective length to detector) × 75 μm, using a P/ACE MDQ system (Beckman Coulter, Santana de Parnaiba, SP, Brazil) and 32 Karat software (Beckman Coulter), according to the conditions described by Ortega et al. (2003) and Otte et al. (1997) with modifications. To dissociate the casein, 20 mg of cheese were dissolved into 1 mL of 10 m*M* sodium phosphate buffer containing 8 *M* urea and 10 m*M* dithiothreitol (DTT; pH 8.0), and left for at least 1 h at room temperature. The samples were filtered (0.45 μm) and injected for 5 s at 3.45 $\times 10^{-3}$ Pa. Separation was carried out at 18.5 kV and 23°C, and detection was performed at 214 nm for 60 min. Between runs, the capillary was conditioned by washing first with 0.5 *M* NaOH for 5 min, then with pure water for 5 min, and finally with running buffer [10 m*M* sodium phosphate containing 6 *M* urea and 0.05% hydroxypropyl methylcellulose (HPMC; pH 3.0)] for 5 min. Sigma-Aldrich Co., St. Louis, MO standards for α-CN, β-CN, κ-CN (10 mg/mL) were used for peak identification.

Firmness was measured using the texture profile analysis defined by Bourne (2002), using a TA XT2 Texture Analyzer (Stable Micro Systems Ltd., Godalming, Surrey, UK) with a 35-mm-diameter aluminum probe. Sampling and sample treatment were according to Mazal et al. (2007) as follows: 8 cylindrical samples of 2-cm diameter and 2.4-cm height were sampled from the entire length of the cheeses with the aid of an aluminum probe. The cylinders were wrapped in plastic film, packed in plastic bags, and kept in an ice-water bath (10°C) for at least 4 h for temperature stabilization. The test was performed at speed of 100 mm/min and a 40% compression of the initial height, and repeated within 5 s.

Recovery of Milk Components and Cheese Yield

The recovery of milk components and the adjusted yield were calculated as described by Mazal et al.

(2007). Protein and fat recovery (%R) were calculated according to Equation 1:

$$\%R_{ij} = \frac{(m_j \times c_{ij}) \times 100}{m_{\text{milk}} \times c_{i_{\text{milk}}}}, \quad [1]$$

where i is the milk component (fat or protein), j is the sample (cheese or whey), m is the mass of the sample (g), c_{ij} is the percentage of i in sample j, m_{milk} is the mass of milk (g), and $c_{i_{\text{milk}}}$ is the percentage of i in the milk.

The adjusted yield (Y_{adj}), which considers the desirable salt and moisture contents of the cheeses, was calculated according to Equation 2:

$$Y_{\text{adj}} = \frac{Y_{\text{act}} \times [100 - (\%\ \text{real moisture content} + \%\ \text{real salt content})] \times 100}{[100 - (\%\ \text{desirable moisture content} + \%\ \text{desirable salt content})]}, \quad [2]$$

where Y_{act} is the actual cheese yield [(g of cheese × 100)/(g of milk)]; desirable salt content for Prato cheese was considered 1.6% and desirable moisture content for Prato cheese was considered 42%.

Experimental Design and Statistical Analysis

To assess the effect of the coagulant on cheese composition, the experiments were conducted in a complete randomized block design with a 2 × 6 factorial arrangement. The factor coagulant had 2 levels (commercial coagulant and coagulant produced at a laboratory scale by *T. indicae-seudaticae* N31), and the factor storage time had 6 levels (5, 12, 19, 33, 43, and 53 d after manufacture). The blocks were the manufacture replications (processes 1, 2, and 3 for each cheese). Analysis of variance was performed to assess the effect of treatments on the composition of cheese and whey, protein and fat recovery, and cheese yield, regarding only the effect of the coagulant. To evaluate cheese characteristics during storage, the independent effects of the coagulant and storage time were assessed, as well as the interaction between these factors. The Tukey test was used for comparison between means, considering the 5% significance level using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK).

Sensory Analysis

To abide by the Brazilian legislation that specifies the ripening period for Prato cheese to be at least 25 d, the sensory acceptance test was performed 29 d after manufacturing with 100 untrained assessors, who evaluated the cheeses regarding the parameters appearance, aroma, flavor, texture, and overall impression using a 9-point hedonic scale (1 = extremely disliked, 7 = liked moderately, and 9 = liked extremely; Stone and Sidel, 1993). Results for overall impression were expressed as a percentage that represented the relationship between the number of responses regarding the acceptance (6–9), indifference (5), or rejection scores (1–4) to the total number of assessors. Purchase intent was evaluated on a 5-point hedonic scale (1 = certainly would not buy this product; 5 = certainly would buy this product) and the results were expressed as a percentage between the number of responses regarding the acceptance (5 and 4), indifference (3), or rejection scores (1 and 2) to the total number of assessors. The results were submitted to ANOVA, considering samples and assessors as variation causes at a 5% significance level.

RESULTS AND DISCUSSION

Characterization of the Raw Material

The milk used in cheese manufacture met the standards required by the Brazilian legislation (Ministério da Agricultura do Brasil, 2011) regarding the physicochemical parameters: minimum of 3% fat, acidity between 14 and 18°D, relative density (15°C) from 1.028 to 1.034 g/mL, and a minimum of 2.9% total protein. The heat treatment applied to milk was adequate, resulting in negative alkaline phosphatase activity.

Effect of Coagulants on the Manufacture of Prato Cheese

Figure 1 shows the protein profile of milk used in the cheese manufacture, which exhibits the casein fractions α_{s2}-CN, α_{s1}-CN 8P, α_{s1}-CN 9P, κ-CN, and β-CN with its genetic variants A^1 and A^2 (Ortega et al. 2003). These protein fractions were also observed in the electrophoretic profile of the control and *Thermomucor* Prato cheese after manufacture, with the exception of κ-CN, which was hydrolyzed, resulting in the formation of para-κ-CN, due to the action of the coagulants during the manufacturing process. After 5 d of manufacture (Table 1), both cheeses met the standards required by the Brazilian legislation, concerning fat in DM (45–59.9%) and moisture content (36–45.9%; Ministério da Agricultura do Brasil, 1997), and presented a composition similar to the results reported by Mazal et al. (2007). The coagulants did not significantly affect cheese composition, except for total protein content, which was higher in *Thermomucor* Prato cheese ($P < 0.05$). However, when expressed on a dry basis, the protein content did not differ between treatments, indi-

Table 1. Effect of treatments[1] on the composition, firmness, protein and fat recovery, and adjusted yield of control and *Thermomucor* Prato cheese after 5 d of manufacture (means ± SD; n = 3)

Item	Control Prato cheese	*Thermomucor* Prato cheese	*P*-value
pH	5.13 ± 0.11	5.08 ± 0.09	0.60
Moisture (%)	44.0 ± 1.54	41.36 ± 1.47	0.098
Fat (%)	27.83 ± 2.60	29.33 ± 2.43	0.50
FDB[2] (%)	49.67 ± 3.80	49.98 ± 2.87	0.92
Total protein (total N × 6.38) (%)	22.97 ± 0.32	24.62 ± 0.41	0.0051
PDB[3] (%)	41.03 ± 0.70	42 ± 1.02	0.24
Ash (%)	4.03 ± 0.38	4.08 ± 0.12	0.83
Salt (%)	1.93 ± 0.24	1.90 ± 0.04	0.86
S/M[4] (%)	4.38 ± 0.38	4.63 ± 0.29	0.43
Firmness (g)	1,928 ± 390.10	2,496 ± 333.52	0.13
Protein recovery (%)	76.21 ± 5.66	77.02 ± 5.53	0.87
Fat recovery (%)	81.09 ± 6.63	80.62 ± 6.02	0.93
Adjusted yield (%)	9.57 ± 0.34	9.48 ± 0.40	0.52

[1]Treatments were Prato cheese made with coagulant from *Thermomucor indicae-seudaticae* N31 and with commercial coagulant.

[2]Fat on dry basis.

[3]Protein on dry basis.

[4]Salt in moisture.

cating that the difference found in the protein contents was due to the nonsignificant difference of 3% moisture between the control Prato cheese (44.0 ± 1.54%) and *Thermomucor* Prato cheese (41.36 ± 1.47%). Despite not being statistically significant, the 3% difference in moisture content between *Thermomucor* and control Prato cheeses have technological implications being very important from the standpoint of cheese yield and quality, as discussed further on.

Cheese manufacture may be considered as a dehydration process in which the casein and fat of milk are concentrated (Lucey and Kelly, 1994) and, therefore, pro-

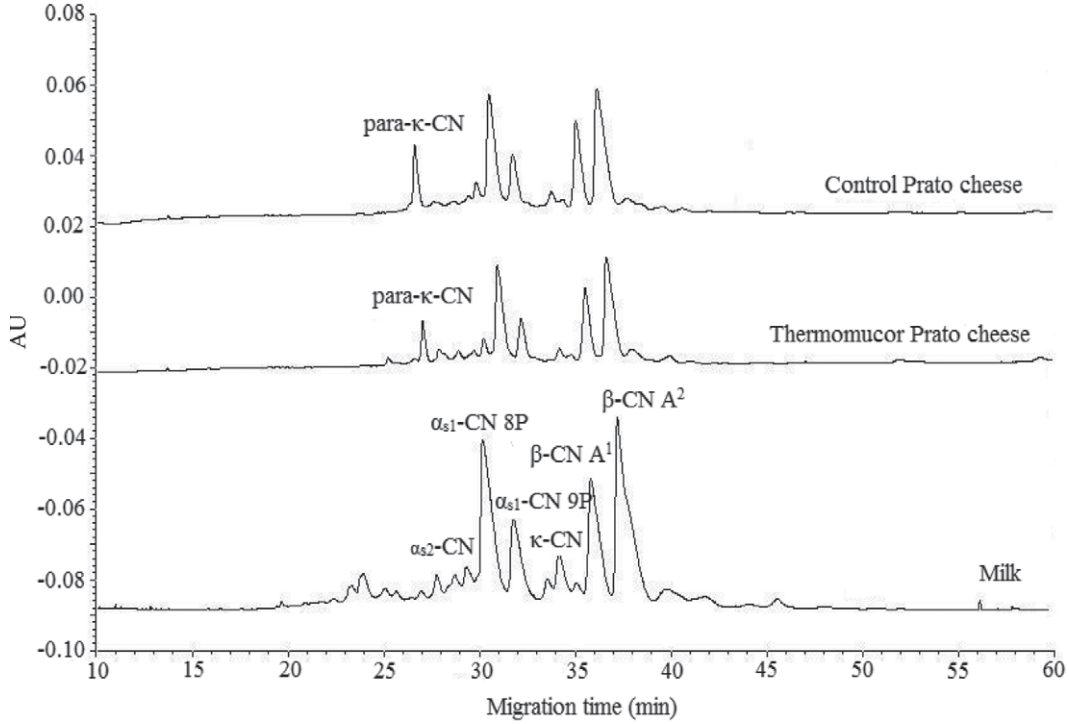

Figure 1. Capillary electrophoresis curves of proteins present in milk and in Prato cheese after manufacture. Labeling of peaks is according to Ortega et al. (2003) and Otte et al. (1997). AU = arbitrary units.

tein and fat recoveries and cheese yield are important tools to evaluate the potential use of a new coagulating enzyme, as the one used in this study. The coagulants did not significantly affect either milk protein and fat recoveries in the cheese or the adjusted cheese yield (Table 1). Control and *Thermomucor* Prato cheeses exhibited 76.21 ± 5.66 and 77.02 ± 5.53% protein recovery, respectively, and 81.09 ± 6.63 and 80.62 ± 6.02% fat recovery, respectively. These values are slightly higher than the ones for Prato cheese made with chymosin, with 73% protein recovery and 78% fat recovery, as reported by Mazal et al. (2007). The adjusted yields for control and *Thermomucor* Prato cheeses were 9.57 ± 0.34 and 9.48 ± 0.40%, respectively, and were very similar to the 9.3% adjusted cheese yield of the Prato cheese studied by Mazal et al. (2007). The different coagulants did not influence the protein and fat loss to the whey (Table 2), indicating a similar action during Prato cheese manufacture. Values for protein and fat recoveries in whey (Table 2) complement the ones obtained in cheese (Table 1). These results are of great economic importance for the protease from *T. indicae-seudaticae* N31.

Effect of Coagulant on Ripening of Prato Cheese

Table 3 shows the effects of treatment, storage time, as well as the interaction of these factors on pH, moisture content, proteolysis, and firmness of the cheeses. The treatments, storage time, and the interaction of these factors did not significantly affect the pH of the samples, which was, on average, 5.09 ± 0.03 and 5.10 ± 0.02 for control and *Thermomucor* Prato cheeses, respectively. The moisture content was affected by the treatments, being lower for *Thermomucor* Prato cheese (41.07 ± 0.47%) than for the control Prato cheese (43.26 ± 0.55%). This difference in moisture content is probably due to the pH decrease during cheese manufacture, which was higher for *Thermomucor* Prato cheese (0.59) than for control Prato cheese (0.26). It is known that the pH behavior during cheese manufacture influences the characteristics of the final product once it favors protein contraction and, consequently, syneresis (Fox et al., 2000). Therefore, the greater pH decrease during *Thermomucor* Prato cheese production may have contributed to the lower moisture content of this cheese, which in turn possibly contributed to its proteolysis being lower and firmness being higher during the storage time (Table 3).

No significant interaction was observed between treatments and storage time for any of the evaluated parameters (Table 3) and both cheeses presented the same behavior during storage, with increased protein fractions [pH 4.6 SN and 12% TCA SN (%TN)] and reduced firmness (Table 3 and Figure 2). Although in the model system with sodium caseinate the pattern of peptide fragments formed by the action of protease from *T. indicae-seudaticae* N31 and from *R. miehei* (Hannilase) showed similarities (Merheb-Dini et al., 2010), the control Prato cheese produced with protease from *Rhizomucor* spp. in the current study presented higher values of pH 4.6 SN (%TN; 9.75 ± 3.81%) than the *Thermomucor* Prato cheese (7.81 ± 3.34%), suggesting higher proteolytic activity during the storage time. Proteolysis assessed by pH 4.6 SN (%TN) is primarily related to natural proteinases and milk-clotting agents, which degrade protein into higher-molecular-weight peptides (Fox, 1989). The higher proteolytic activity of the commercial coagulant, besides being related to the higher moisture content, may also be due to higher amount of coagulant retained in the curd. Protease from *R. miehei* is characterized by its relatively high heat stability (up to 60°C; Walsh and Li, 2000), which may have contributed to its higher residual activity after the heat treatment of the curd (42°C), leading to an increased cleavage of caseins during storage. Protease from *T. indicae-seudaticae* N31 exhibits lower heat stability, up to 40 to 45°C, (Merheb-Dini et al., 2010) and probably underwent higher denaturation during the heat treatment of the curd. The observed differences in

Table 2. Effect of treatments[1] on the composition and protein and fat recovery of control and *Thermomucor* Prato cheese whey (means ± SD; n = 3)

Item	Control Prato cheese whey	*Thermomucor* Prato cheese whey	*P*-value
Fat (%)	0.54 ± 0.06	0.63 ± 0.11	0.29
Total protein (total N × 6.38) (%)	0.78 ± 0.03	0.85 ± 0.03	0.056
pH 4.6-soluble N (%)	0.13 ± 0.01	0.14 ± 0.01	0.48
12% TCA-soluble N (%)	0.04 ± 0.003	0.05 ± 0.01	0.057
TS (%)	7.04 ± 0.09	7.16 ± 0.2	0.20
Ash (%)	0.52 ± 0.01	0.53 ± 0.03	0.67
Protein recovery (%)	22.42 ± 1.68	25.18 ± 2.81	0.22
Fat recovery (%)	13.51 ± 1.47	16.15 ± 2.48	0.19

[1]Treatments were Prato cheese made with coagulant from *Thermomucor indicae-seudaticae* N31 and with commercial coagulant.

Table 3. Summary of the ANOVA: effect of treatment, storage time, and treatment × storage time interaction on pH, moisture, pH 4.6-soluble nitrogen [pH 4.6 SN; % total nitrogen (TN)], 12% TCA-soluble nitrogen (12% TCA SN; %TN), and firmness of Prato cheeses

		P-value				
Factor	df	pH	Moisture	pH 4.6 SN (%TN)	12% TCA SN (%TN)	Firmness (g)
Treatment (T)[1]	2	0.68	<0.0001	<0.0001	0.38	<0.0001
Storage time (ST)[2]	5	0.55	0.27	<0.0001	<0.0001	<0.0001
T × ST	5	0.86	0.73	0.59	0.84	0.98

[1]Treatments were Prato cheese made with coagulant from *Thermomucor indicae-seudaticae* N31 and with commercial coagulant.

[2]Storage time: 5, 12, 19, 33, 43, and 53 d after manufacture.

proteolysis and moisture content between the cheeses of the present study probably reflected on firmness differences. The control Prato cheese presented higher proteolysis [pH 4.6 SN (%TN) = 9.75 ± 3.81%], higher moisture content (43.26 ± 0.55%), and less firmness (1,737.55 ± 153.19 g) than *Thermomucor* Prato cheese, whose values for 4.6 pH SN (%TN), moisture content, and firmness were 7.81 ± 3.34%, 41.07 ± 0.47%, and 2,296.23 ± 177.56 g, respectively. It is clear that cheeses with higher moisture content ripened faster and presented lower firmness, given that the hydrolysis of casein and its degradation products favors hydration and weakening of the protein matrix (Fox et al., 2000), resulting in greater softening of the cheese.

The hydrolysis profile of the different casein fractions during cheese storage can be seen in the capillary electropherogram shown in Figure 3. We observed that the hydrolysis of different protein fractions was similar for both *Thermomucor* (Figure 3A) and control Prato cheese (Figure 3B). The fractions α_{s1}-CN 8P and α_{s1}-CN 9P were degraded in the first 19 d of ripening, and the last one disappeared from d 33 in the control Prato cheese and from d 43 in the *Thermomucor* Prato cheese.

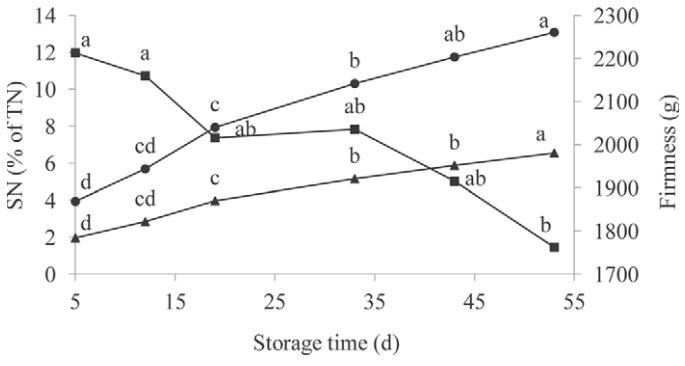

Figure 2. Effect of storage time on pH 4.6-soluble nitrogen (pH 4.6 SN; ●), 12% TCA-soluble nitrogen (12% TCA SN; ▲), and firmness (■) of Prato cheese during storage (n = 3). Values with the same letters (a–d) are not significantly different ($P < 0.05$) for each analytical determination.

The hydrolysis of α_{s1}-CN 8P formed the fraction α_{s1}-I-CN 8P that was visible on the fifth day of manufacture for both cheeses. The hydrolysis of α_{s1}-CN 9P formed the fraction α_{s1}-I-CN 9P, strongly visible after 33 d of ripening for both cheeses. The β-CN genetic variants A^1 and A^2 were also hydrolyzed, possibly by plasmin, with small peaks of γ-CN arising over time, especially γ_2-CN, whose increase was visible from d 33 in both cheeses. The hydrolysis profile of both cheeses produced with commercial microbial enzyme (obtained from *Rhizomucor* spp.) and the enzyme from *T. indicae-seudaticae* N31 was equivalent to the classic and expected hydrolysis profile of cheeses produced with chymosin [i.e., hydrolysis of α_{S1}-CN by the residual coagulant during the early stages of maturation in the bond Phe_{23}-Phe_{24}, resulting in the peptides α_{S1}-CN f24–199 (α_{S1}-I-CN) and α_{S1}-CN f1–23, and hydrolysis of β-CN by plasmin, resulting in γ-CN; Fox et al., 2000]. The set of parameters studied during ripening suggests that the enzyme from *T. indicae-seudaticae* N31 has potential as a coagulating agent for the production of Prato cheese, equivalent to the commercial enzyme used for comparison. However, to reach more conclusive results on proteolysis, as to whether the higher content of pH 4.6 SN (%TN) in the control cheeses were caused by a difference in the activity, specificity, inactivation, or retention of the coagulant or by the difference in moisture content, or by combinations of these possibilities, future work regarding the determination of residual coagulant activity in the cheeses and producing cheeses with comparable moisture content could be carried out.

Sensory Acceptance and Purchase Intent Test

The sensory evaluation was performed after approval by the Ethics in Research Committee (University of Campinas, Campinas, SP, Brazil; register no. 470/2011) and after confirming that the products met the standards required for microbiological safety established by the Brazilian legislation for Prato cheese (Agência Nacional de Vigilância Sanitária do Brasil, 2001), includ-

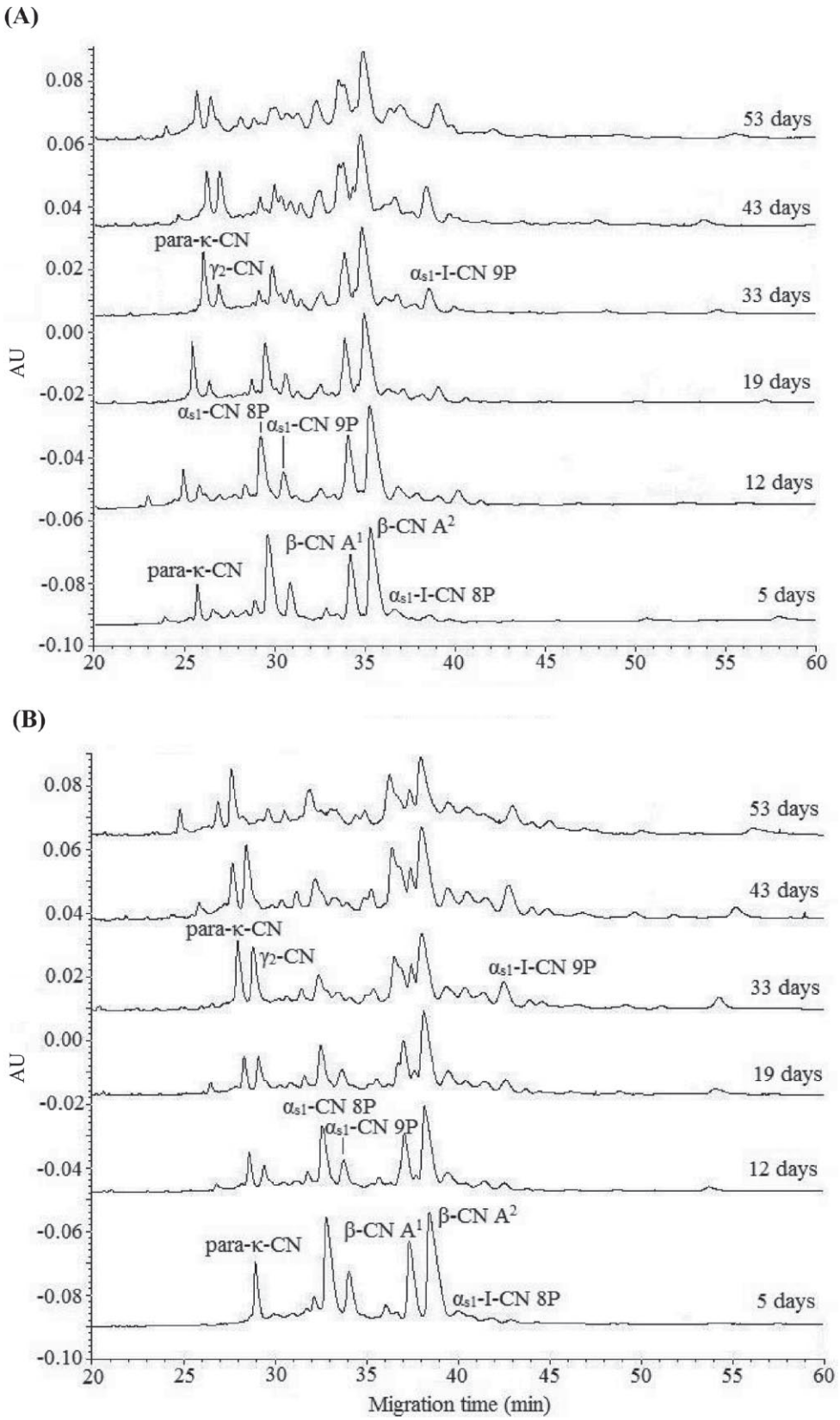

Figure 3. Capillary electrophoresis curves presenting casein breakdown during storage of *Thermomucor* Prato cheese (A) and control Prato cheese (B). Labeling of peaks is according to Otte et al. (1997) and Rehn et al. (2010). AU = arbitrary units.

Table 4. Scores for the attributes of control and *Thermomucor* Prato cheeses obtained in the sensory evaluation (means ± SD; n = 100)

Attribute	Control Prato cheese	*Thermomucor* Prato cheese	*P*-value
Appearance[1]	7.75 ± 1.00	7.55 ± 1.01	0.16
Aroma[1]	7.31 ± 1.23	7.43 ± 1.20	0.48
Flavor[1]	6.98 ± 1.40	7.07 ± 1.43	0.65
Texture[1]	7.20 ± 1.55	6.99 ± 1.41	0.32
Overall impression[1]	7.13 ± 1.31	7.06 ± 1.33	0.71
Purchase intent[2]	3.81 ± 1.09	3.80 ± 1.02	0.95

[1]Scale: 1 = extremely disliked, 7 = liked moderately, and 9 = liked extremely.

[2]Scale: 1 = certainly would not buy this product, 5 = certainly would buy this product.

ing coliform at 45°C [most probable number (MPN)/g <3], coagulase-positive S*taphylococcus* (cfu/g <10), *Salmonella* spp. (absent in 25 g of sample), and *Listeria monocytogenes* (absent in 25 g of sample). As can be seen in Table 4, the control and *Thermomucor* Prato cheeses showed no significant differences for the sensory parameters studied (appearance, aroma, flavor, texture, and overall impression). Both cheeses presented high acceptability indexes for all parameters, with scores between 7 and 8, which are equivalent to the "like moderately" and "like very much" categories of the sensory evaluation scale. With respect to the overall impression, 88% of the assessors scored in the acceptance range, 5% reported having no opinion, and 7% rejected the cheeses. With respect to purchase intention, 66% of the assessors declared that they would buy *Thermomucor* and control Prato cheeses and only 13% declared that they would not buy the cheeses.

Proteases from microbial sources generally present high proteolytic activity, which may result in bitterness in aged cheese varieties, due to their action on highly hydrophobic sequences of casein (Sousa et al., 2001). The higher proteolysis of control Prato cheese did not reflect in the sensory assessment of cheese texture and flavor and the good sensory acceptance of the *Thermomucor* and control Prato cheeses indicate that the enzyme from *T. indicae-seudaticae* N31 presented similar action to the enzyme from *R. miehei*, being able to impart typical sensory properties to the finished cheese.

CONCLUSIONS

The use of protease from *T. indicae-seudaticae* N31 in Prato cheese manufacture presented satisfactory results regarding the main requirements when choosing a clotting agent: good cheese yield and proteolysis during ripening that resulted in the development of soft texture and good sensory acceptance.

ACKNOWLEDGMENTS

The authors thank the National Council for Scientific and Technological Development (CNPq, Brasília, DF, Brazil) and Technological and Foundation for Research Support of the State of São Paulo (FAPESP), process 2011/51158-8 and 2011/50844-5, for financial support.

REFERENCES

Agência Nacional de Vigilância Sanitária do Brasil. 2001. Regulamento técnico sobre padrões microbiológicos para alimentos. Resolução RDC No. 12-02/01/2001. Accessed Jan. 3, 2013. http://portal.anvisa.gov.br/wps/wcm/connect/a47bab8047458b909541d53fbc4c6735/RDC_12_2001.pdf?MOD=AJPERES.

AOAC International. 1995. Official Methods of Analysis. 16th ed. AOAC International, Arlington, VA.

AOAC International. 2006. Official Methods of Analysis. 18th ed. AOAC International, Arlington, VA.

Bourne, M. 2002. Food Texture and Viscosity: Concept and Measurement. 2nd ed. Academic Press, New York, NY.

British Standards Institution. 1989. Determination of fat content of milk and milk products (Gerber method)—Methods. British Standards Institution, London, UK.

Bynum, D. G., and D. M. Barbano. 1985. Whole milk reverse osmosis retentate for Cheddar cheese manufacture: Chemical changes during aging. J. Dairy Sci. 68:1–10.

Cavalcanti, M. T. H., C. R. Martinez, V. C. Furtado, B. B. Neto, M. F. Teixeira, J. L. Lima Filho, and A. L. F. Porto. 2005. Milk-clotting protease production by *Nocardiopsis* sp. in an inexpensive medium. World J. Microbiol. Biotechnol. 21:151–154.

Fox, P. F. 1989. Proteolysis during cheese manufacture and ripening. J. Dairy Sci. 72:1379–1400.

Fox, P. F., T. P. Guinee, T. M. Cogan, and P. L. H. McSweeney. 2000. Fundamentals of Cheese Science. Aspen Publishers Inc., Gaithersburg, MD.

Jacob, M., D. Jaros, and H. Rohm. 2011. Recent advances in milk clotting enzymes. Int. J. Dairy Technol. 64:14–33.

Kloosterman, J. 1991. The role of biotechnology in the manufacturing of wholesome natural ripened cheese. Food Biotechnol. 5:207–215.

Kumar, S., N. S. Sharma, M. R. Saharan, and R. Singh. 2005. Extracellular acid protease from *Rhizopus oryzae*: Purification and characterization. Process Biochem. 40:1701–1705.

Li, S., X. Yang, S. Yang, M. Zhu, and X. Wang. 2012. Technology prospecting on enzymes: Application, marketing and engineering. Comput. Struct. Biotechnol. J. 2:e201209017.

Lucey, J., and J. Kelly. 1994. Cheese yield. Int. J. Dairy Technol. 47:1–14.

Machalinski, C., M. L. Pirpignani, C. Marino, A. Mantegazza, and M. B. de Jiménez Bonino. 2006. Structural aspects of the *Mucor bacilliformis* proteinase, a new member of the aspartyl-proteinase family. J. Biotechnol. 123:443–452.

Marshall, R. T. 1992. Standard Methods for the Determination of Dairy Products. 16th ed. American Public Health Association, Washington, DC.

Martin, N., M. A. U. Guez, L. D. Sette, R. Da Silva, and E. Gomes. 2010. Pectinase production by a Brazilian thermophilic fungus *Thermomucor indicae-seudaticae* N31 in solid-state and submerged fermentation. Microbiology 79:306–313.

Mazal, G., P. C. B. Vianna, M. V. Santos, and M. L. Gigante. 2007. Effect of somatic cell count on Prato cheese composition. J. Dairy Sci. 90:630–636.

Merheb-Dini, C., G. A. C. Garcia, A. L. B. Penna, E. Gomes, and R. da Silva. 2012. Use of a new milk-clotting protease from *Thermomucor indicae-seudaticae* N31 as coagulant and changes during ripening of Prato cheese. Food Chem. 130:859–865.

Merheb-Dini, C., E. Gomes, M. Boscolo, and R. da Silva. 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 Chem. 120:87–93.

Ministério da Agricultura do Brasil. 1997. Regulamento técnico para fixação de identidade e qualidade do queijo Prato. Portaria 358-04/09/1997. Ministério da Agricultura, Pecuária e Abastecimento, Brasília, Brazil. Accessed Jan. 3, 2013. http://sistemasweb.agricultura.gov.br/sislegis/action/detalhaAto.do?method=consultarLegislacaoFederal.

Ministério da Agricultura do Brasil. 2011. Regulamentos Técnicos de Produção, Identidade e Qualidade do Leite tipo A, do Leite Cru Refrigerado, do Leite Pasteurizado e o Regulamento Técnico da Coleta de Leite Cru Refrigerado e seu Transporte a Granel. Instrução Normativa No. 62-29/09/2011. Accessed Jan. 3, 2013. http://sistemasweb.agricultura.gov.br/sislegis/action/detalhaAto.do?method=consultarLegislacaoFederal.

Ortega, N., S. M. Albillos, and M. D. Busto. 2003. Application of factorial design and response surface methodology to the analysis of bovine caseins by capillary zone electrophoresis. Food Contr. 14:307–315.

Otte, J., M. Zakora, K. R. Kristiansen, and K. B. Qvist. 1997. Analysis of bovine caseins and primary hydrolysis products in cheese by capillary zone electrophoresis. Lait 77:241–257.

Rehn, U., M. A. Petersen, K. Hallin Saedén, and Y. Ardö. 2010. Ripening of extra hard cheese made with mesophilic DL-starter. Int. Dairy J. 20:844–851.

Richardson, G. H. 1985. Standard Methods for Examination of Dairy Products. 15th ed. American Public Health Association, Washington, DC.

Shieh, C.-J., L.-A. P. Thi, and I.-L. Shih. 2009. Milk-clotting enzymes produced by culture of *Bacillus subtilis* natto. Biochem. Eng. J. 43:85–91.

Sousa, M. J., Y. Ardö, and P. L. H. McSweeney. 2001. Advances in the study of proteolysis during cheese ripening. Int. Dairy J. 11:327–345.

Stone, H., and J. L. Sidel. 1993. Sensory Evaluation Practices. 2nd ed. Academic Press, New York, NY.

Vishwanatha, K. S., A. G. A. Rao, and S. A. Singh. 2010. Production and characterization of a milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341. Appl. Microbiol. Biotechnol. 85:1849–1859.

Walsh, M. K., and X. Li. 2000. Thermal stability of acid proteinases. J. Dairy Res. 67:637–640.