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Use of an Exogenous Carboxypeptidase to Accelerate Proteolysis in Reggianito Cheese

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Abstract

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The effect of an exogenous commercial carboxypeptidase on the proteolysis of Reggianito cheese was evaluated. Cheeses were manufactured using 4 concentrations of enzyme: 0, 5, 10, and 20 g/100 l milk. Cheese samples were analysed at 0, 60, 90, 120, 150, 180, and 210 days of ripening. Nitrogen content values increased during ripening, but no clear effect due to the enzyme addition was observed. A profound degradation of β-casein was observed during the first 2 months of ripening in cheeses with the highest rates of enzyme addition. An increase of amino acid concentrations was clearly observed in some cheeses manufactured with exogenous enzymes compared with control cheeses at the same ripening time. However, the principal component analysis showed that experimental cheeses had a slight increase in the rate of formation and/or degradation of proteolysis products. Our results represent an important contribution to select new alternatives for enzyme addition during the manufacture of hard cheese.

Keywords: Reggianito; cheese; proteolysis; ripening; exogenous enzymes

Proteolysis is the most important and complex biochemical event that occurs during cheese maturation (McSweeney & Fox 1997). The pattern of proteolysis is very variable and it is essentially unique to a particular cheese variety. The observed differences are principally due to different moisture content, levels of NaCl, pH, cheese microflora, among many important factors. Therefore, differences in enzyme activities may be responsible for the modification of traditional characteristics, appearance, texture, and flavour of the product.

Most rennet-coagulated cheeses are ripened after manufacture for periods ranging from a couple of weeks to more than two years. Consequently, as the process can be slow, it can be expensive due to the cost associated with holding a large amount of cheese in adequate ripening facilities (Upadhyay & McSweeney 2003). Various approaches have been used to accelerate cheese ripening, including the use of elevated ripening temperatures, addition of exogenous enzymes or attenuated starters, use of adjunct cultures, genetic modification of starter bacteria, and high-pressure treatments (McSweeney 2004; Azarnia *et al*. 2006; El Soda & Awad 2011).

Particularly, addition of exogenous enzymes to cheese increases the enzyme pool accelerating the rate of certain reactions in cheese in contrast to elevated temperature which results in an increase of the rate of all reactions (Upadhyay & McSweeney 2003). In addition, the use of exogenous enzymes is at present an interesting way to accelerate ripening in different cheese varieties. For example, commercial proteinase preparations are used to accelerate ripening in Cheddar and Dutch cheeses (Wilkinson & Kilcawley 2005).

There are four main points for the addition of exogenous enzyme in cheese manufacture: with milk before cheese manufacture, with the starter

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culture or coagulant, at dry salting, and directly into a cheese block (Wilkinson & Kilcawley 2005). Addition to cheese milk appears to be the best stage for enzyme incorporation due to the homogeneous mixing of the enzyme with the milk and its subsequent transfer to cheese curd. However, most of the enzyme added to the milk is lost in the whey and proteolytic enzymes degrade caseins to peptides that are lost in the whey, which results in a reduction of cheese yield (Upadhyay & McSweeney 2003). Some commercial products are available for accelerating cheese ripening, however little data exist in relation to their partitioning or retention within cheese curd during ripening (Wilkinson & Kilcawley 2005; Doolan *et al*. 2014).

Various researchers have focused their work on accelerating lipolysis in Cheddar cheese by the use of exogenous lipases or enzyme preparations containing lipases (Upadhyay & McSweeney 2003). On the other hand, considering that proteolysis is the major biochemical process occurring during the ripening of some important cheese varieties such as Cheddar, Gouda, and Italian-type cheeses, most enzyme preparations used to accelerate ripening contain proteinases and peptidases (Upadhyay & McSweeney 2003). Most peptidases used to date are aminopeptidases, but few carboxypeptidases have been assessed in cheese ripening which cleave amino acids from the C-terminus of peptides (Kilcawley *et al*. 2002).

Reggianito is the most important hard cheese produced in Argentina and is one of the most frequently exported cheeses to different countries such as USA, Brazil, Chile, and Russia (M.A.G. y P. 2010; http://www.minagri.gob.ar/site/index.php). Italian immigrants in the late 19th and early 20th centuries developed a distinctive product inspired by Italian hard cheeses. Reggianito cheese has higher moisture and fat contents, and a shorter ripening period than Italian hard cheeses (Zalazar *et al*. 1999), and it is manufactured with pasteurised cow's milk and natural whey starter mainly composed by *Lactobacillus helveticus* (66%) and *Lactobacillus delbrueckii* subsp. *lactis* (33%) (Reinheimer *et al*. 1996). It is a cheese generally ripened at 11–13°C at 82–85% relative humidity for six months. Studies have been carried out to accelerate Reggianito cheese ripening, principally by elevating the storage temperature (Sihufe *et al*. 2007, 2010a, b, c). These studies identified that the optimal time for Reggianito Argentino cheese at 18°C ranged between 2 and 3 months. However, information about the use of exogenous enzymes to accelerate the ripening of Argentinean hard cheeses is limited. The use of carboxypeptidase may offer an interesting alternative to favour the pathways for the amino acid formation, and therefore to accelerate flavour development.

The objective of this study was to evaluate the effect of the addition of an exogenous carboxypeptidase on Reggianito cheese proteolysis.

MATERIAL AND METHODS

Cheesemaking. Four different cheese batches were prepared in a pilot plant of INTI (National Institute of Industrial Technology, Rafaela, Argentina) by experienced technicians and following the procedure proposed by Gallino (1994) using pasteurised cow's milk (fat 21.7 ± 1.2 g/l; protein 31.9 ± 0.0 g/l; lactose 46.8 ± 0.0 g/l; pH 6.60 ± 0.01). Commercial chymosin was used as a rennet (13 g/1000 l of milk). The starter medium is whey-based and is mainly composed of *Lactobacillus helveticus* and *L. delbrueckii* subsp. *lactis* (Reinheimer *et al*. 1996). The general procedure to obtain this natural whey starter and the volume used to inoculate milk were detailed by Sihufe *et al*. (2012). Free enzyme preparations (Accelerzyme® CPG, purified enzyme preparation derived from *Aspergillus niger*, > CPGU/g; DSM Food Specialties, Heerlen, The Netherlands) were added to cheese milk with the starter culture. The following enzyme treatments were used: control cheeses without enzyme addition (C), cheeses with 5 g enzyme/100 l milk (E1), cheeses with 10 g enzyme/100 l milk (E2), and cheeses with 20 g enzyme/100 l milk (E3). Cheeses (cylindrical shape and approximately 8 kg weight, 24 cm diameter, and 12 cm height) were salted by immersion in a saturated NaCl solution at 12°C for 8 days and ripened at 13°C and 85% RH. Samples were obtained at 0, 60, 90, 120, 150, 180, and 210 days of ripening.

Initial composition. After manufacturing (day 0), cheeses were analysed to determine their initial composition: moisture (ISO 2004), NaCl (ISO 2006), protein (ISO 2001), fat (IRAM 1988), and pH (ISO 2004).

Sample analysis. Samples were analysed to determine pH and moisture content (ISO 2004). Maturation was evaluated by the determination of water-soluble nitrogen at pH 4.6 (WSN) and nitrogen soluble in 2.5 g/100 ml phosphotungstic acid (PTA-N) (Gripon *et al*. 1975). All results obtained from nitrogen frac-

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Table 1. Initial composition (Day 0) of Reggianito cheeses (average values ± SD)

Parameter	Cheese C	Cheese E1	Cheese E2	Cheese E3
Moisture (g/100 g cheese)	37.31 ± 0.28	37.92 ± 0.21	37.79 ± 0.09	37.57 ± 0.21
NaCl (g/100 g cheese)	2.35 ± 0.13	2.33 ± 0.06	2.33 ± 0.01	2.42 ± 0.01
Protein (g/100 g cheese)	30.71 ± 0.05	31.22 ± 0.06	32.28 ± 0.12	31.95 ± 0.17
Fat (g/100 g cheese)	25.00 ± 0.00	24.75 ± 0.35	23.25 ± 0.35	24.25 ± 0.35
pH	5.32 ± 0.03	5.43 ± 0.02	5.31 ± 0.01	5.34 ± 0.01

tions were expressed as a percentage of total nitrogen (TN). All determinations were carried out at least in duplicate.

Additionally, proteolysis was studied by electrophoretic, peptide, and amino acid analysis as described by Sihufe *et al.* (2010a). Some chromatographic conditions for the amino acid analysis were modified as follows. Soluble fractions in 2.5 g/100 ml sulfosalicylic acid (SSA-SF) were obtained from the water-soluble fraction at pH 4.6. Free amino acids were determined in SSA-SF using the derivatising procedure with *o*-phthaldialdehyde. The resulting solution was filtered through a disposable 0.2 µm filter before 10 µl were injected. An ISCO chromatography system (Isco, Inc., Lincoln, USA) was used, which consisted of model 2350 HPLC pump, model 2360/2361 gradient programmer, FL-2 fluorescence detector, and Chem Research 150 chromatographic data management/system controller. A VARIAN (250 × 4.6 mm) C_{18}, 100 Å column (Varian, Inc., Palo Alto, USA) at 40°C was used for chromatographic separations. Separations were carried out at a flow rate of 1.3 ml min using solvent A/tetrahydrofuran/methanol/0.05 mol/l sodium acetate(1 : 19 : 80), pH 5.9 , and solvent B: methanol/0.05 mol/l sodium acetate (80 : 20), pH 5.9 (Jones *et al.* 1981). The gradient program was: initial composition 0% B, isocratic step at 0% B for 1 min, linear step to 14% B in 5 min, isocratic step at 14% B for 5 min, linear step to 50% B in 5 min, isocratic step at 50% B for 4 min, linear step to 75% B in 6 min, isocratic step at 75% B for 4 min, linear step to 100% B in 6 min, isocratic step at 100% B for 4 minutes. Amino acids were identified according to their retention times by comparison with a standard solution chromatogram.

Statistical analysis. ANOVA was based on the replicates of determinations. The enzyme treatment and the ripening time were selected as main factors for ANOVA. When differences between treatment effects were significant ($P < 0.05$), a multiple comparison of means was performed using the least significant difference (*LSD*) test. Principal component analysis (PCA) was used to reduce the dimensionality of the data obtained. Statistical analysis was carried out using the Minitab software (Minitab Inc., State College, USA).

RESULTS AND DISCUSSION

Gross composition. Table 1 shows the initial composition of the 4 cheese enzyme treatments studied. The moisture content decreased with ripening time as expected for cheeses ripened without wrapping (Simal *et al.* 2001; Sihufe *et al.* 2007). The direct addition of proteinases may increase the syneresis of curd, therefore it may decrease cheese yield (Mohedano *et al.* 1998). However, in this case, although the level of the added enzyme significantly affected

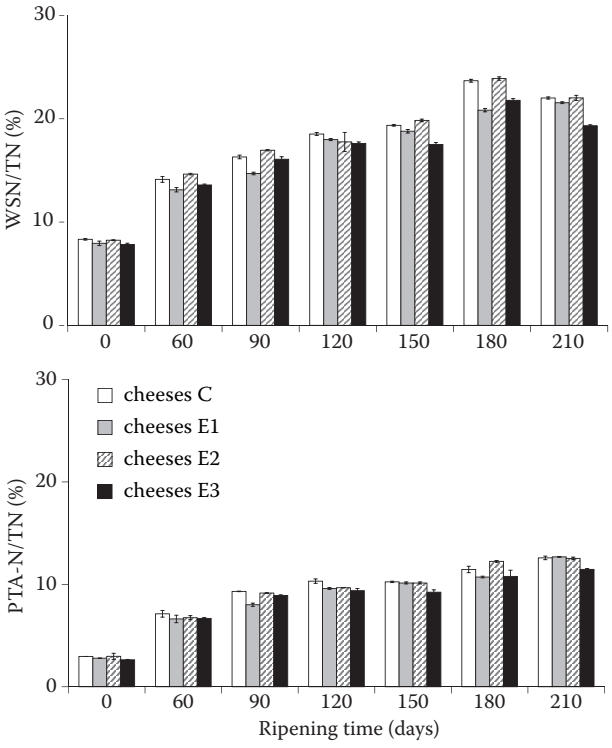

Figure 1. Profiles of (A) WSN/TN and (B) PTA-N/TN during the ripening of Reggianito cheese (results are shown as mean value with standard deviation)

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Table 2. Average values and standard deviations of moisture and pH corresponding to the studied Reggianito cheese samples

Cheese	Ripening time (days)	Moisture (g/100 g cheese)	pH
C	0	37.31 ± 0.28^{m}	5.32 ± 0.03
	60	34.31 ± 0.38^{ijkl}	5.31 ± 0.04
	90	34.15 ±0.06^{hijk}	5.32 ± 0.00
	120	33.75 ± 0.04^{efgh}	5.33 ± 0.01
	150	32.58 ± 0.01^{d}	5.43 ± 0.06
	180	31.99 ± 0.16^{bc}	5.40 ± 0.11
	210	31.52 ± 0.06^{ab}	5.44 ± 0.06
E1	0	37.92 ± 0.21^{n}	5.43 ± 0.02
	60	34.55 ± 0.38^{kl}	5.47 ± 0.06
	90	33.75 ± 0.18^{efgh}	5.44 ± 0.08
	120	33.30 ± 0.02^{e}	5.50 ± 0.07
	150	32.59 ± 0.20^{d}	5.50 ± 0.03
	180	33.31 ± 0.29^{e}	5.29 ± 0.01
	210	32.50 ± 0.13^{d}	5.29 ± 0.01
E2	0	37.79 ± 0.09^{mn}	5.31 ± 0.01
	60	34.58 ± 0.31^{kl}	5.29 ± 0.01
	90	34.02 ± 0.08^{ghij}	5.31 ± 0.02
	120	34.11 ± 0.07^{ghijk}	5.30 ± 0.00
	150	33.82 ± 0.11^{fgh}	5.33 ± 0.03
	180	34.34 ± 0.04^{jkl}	5.31 ± 0.03
	210	31.99 ± 0.06^{bc}	5.36 ± 0.01
E3	0	37.57 ± 0.21^{mn}	5.34 ± 0.01
	60	34.68 ± 0.03^{l}	5.35 ± 0.02
	90	33.62 ± 0.28^{efg}	5.35 ± 0.00
	120	33.37 ± 0.01^{ef}	5.34 ± 0.01
	150	32.27 ± 0.14^{cd}	5.33 ± 0.00
	180	33.83 ± 0.79^{fghi}	5.35 ± 0.00
	210	31.44 ± 0.25^{a}	5.35 ± 0.01
Enzyme level		*	ns
Ripening time		*	*
Interaction		*	ns

$^{a–n}$average values in the same column with different letters are significantly different ($P < 0.05$); last rows show the ANOVA result for the different factors analysed; *significant effect ($P < 0.05$); ns – no significant effect ($P > 0.05$)

the moisture content, no clear trend was observed. It is worth mentioning that the moisture content values for control and experimental cheeses were similar at the end of the studied ripening period (Table 2).

The pH value may be increased in cheeses manufactured with enzyme addition due to the higher levels of proteolysis (Mohedano *et al*. 1998). In this case, the pH values were not significantly affected by the level of carboxypeptidase. The pH values for all assayed samples were in the range of 5.29–5.50 (Table 2), which were similar to those reported by other researchers for this type of cheese (Candioti *et al*. 2002; Hynes *et al*. 2003; Perotti *et al*. 2004).

Nitrogen fraction analysis. The extent of proteolysis was evaluated by determining WSN and PTA-N as a percentage of TN (Figure 1). The fraction associated with WSN contains proteins, peptides, and amino acids, while nitrogen soluble in PTA consists of free amino acids and small peptides. Values determined in control cheeses were similar to those reported by other authors for Reggianito cheese (Candioti *et al*. 2002; Hynes *et al*. 2003; Perotti *et al*. 2004).

Various authors used nitrogen indices to study the effect of enzyme addition on cheese proteolysis. Azarnia *et al*. (2010) investigated the proteolysis in enzyme-modified Cheddar cheese with added natural enzyme or recombinant aminopeptidase in the presence of a commercial proteinase. These authors did not observe any significant differences in WSN fraction between the control and experimental cheeses. However, they observed higher levels of PTA-N fraction in cheeses manufactured with exogenous enzymes. Azarnia *et al*. (2011) also studied the effect of free and encapsulated recombinant aminopeptidase on the Cheddar cheese proteolysis. They did not observe any significant differences in WSN fraction between the studied cheeses but they observed higher levels of PTA-N fraction in experimental cheeses, particularly in those with encapsulated enzymes. Kilcawley *et al*. (2012) evaluated the effect of the addition of enzyme preparations on Cheddar cheese ripening. They found that Accelerzyme®CPG

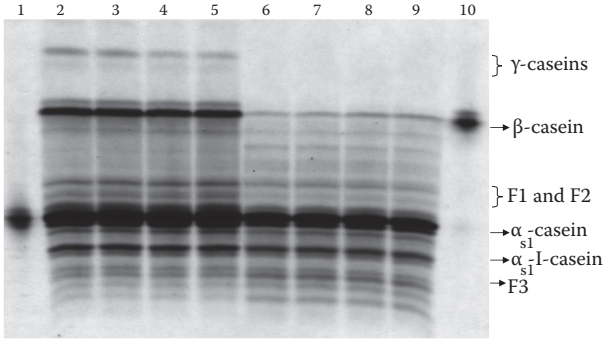

Figure 2. Urea-PAGE electrophoretogram for Reggianito cheeses manufactured with different amounts of carboxypeptidase and ripened during 60 days

1 = α_{s1}-casein standard; 2–3 = cheese C; 4–5 = cheese E1; 6–7 = cheese E2; 8–9 = cheese E3; 10 = β-casein standard

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Table 3. Average values and standard deviations corresponding to the IOD/g cheese of the electrophoretic fractions determined in Reggianito cheese

Cheese	Ripening time (days)	γ-CN	β-CN	F1	F2	a_{s1}-CN	a_{s1}-I-CN	F3
	0	13.4 ± 1.9	128.7 ± 4.2^{k}	19.7 ± 1.3	25.4 ± 1.9^{i}	151.1 ± 4.6^{m}	16.1 ± 0.2^{ij}	7.0 ± 0.7
	60	15.2 ± 2.3	72.7 ± 5.4^{h}	8.4 ± 0.8	17.4 ± 16.1^{g}	128.5 ± 2.7^{k}	22.4 ± 15.0^{n}	14.7 ± 3.4
	90	8.9 ± 4.4	18.5 ± 3.1^{e}	12.4 ± 0.7	7.7 ± 1.0^{cde}	81.4 ± 2.5^{d}	13.8 ± 1.2^{h}	5.2 ± 1.2
C	120	ND	8.9 ± 10.5^{abcd}	10.1 ± 6.3	5.8 ± 1.1^{abcde}	66.0 ± 2.1^{bc}	11.1 ± 0.8^{ef}	20.8 ± 11.7
	150	ND	14.0 ± 1.0^{de}	13.0 ± 2.1	5.5 ± 3.8^{abcde}	66.5 ± 3.6^{bc}	10.3 ± 1.0^{cde}	15.2 ± 13.7
	180	22.5 ± 3.6	11.1 ± 1.8^{cd}	15.6 ± 5.9	ND	68.5 ± 3.9^{c}	17.3 ± 1.5^{j}	9.0 ± 2.6
	210	15.3 ± 13.7	12.0 ± 0.7^{cd}	16.5 ± 11.4	2.0 ± 2.4^{abc}	92.5 ± 2.2^{fg}	13.2 ± 0.6^{gh}	9.0 ± 1.8
	0	10.3 ± 2.0	124.2 ± 4.9^{ij}	2.1 ± 1.2	19.5 ± 13.1^{ghi}	145.1 ± 2.2^{lm}	10.2 ± 1.2^{cde}	5.91 ± 1.7
	60	6.0 ± 1.1	65.5 ± 3.3^{g}	9.0 ± 0.7	10.8 ± 13.2^{ef}	124.5 ± 1.3^{k}	28.2 ± 0.9^{m}	11.7 ± 1.2
	90	12.9 ± 3.5	9.8 ± 5.6^{bcd}	11.1 ± 1.8	7.9 ± 3.9^{cde}	79.6 ± 1.0^{d}	8.3 ± 0.1^{ab}	14.4 ± 12.0
E1	120	ND	27.6 ± 2.3^{f}	12.1 ± 2.1	1.2 ± 2.5^{ab}	78.7 ± 3.9^{d}	9.7 ± 0.3^{cd}	17.14 ± 15.8
	150	ND	9.7 ± 2.6^{bcd}	9.9 ± 4.6	5.8 ± 1.4^{abcde}	64.5 ± 4.0^{bc}	7.7 ± 0.4^{a}	21.7 ± 12.3
	180	9.5 ± 4.7	1 0.5 ± 1.2^{bcd}	10.1 ± 9.0	6.0 ± 7.0^{abcde}	88.3 ± 5.9^{ef}	13.2 ± 0.4^{gh}	6.0 ± 1.2
	210	14.7 ± 7.7	12.1 ± 0.7^{cde}	19.6 ± 11.4	ND	104.1 ± 8.2^{i}	14.0 ± 1.2^{h}	7.4 ± 0.5
	0	19.5 ± 5.4	73.9 ± 3.9^{h}	14.0 ± 0.9	9.0 ± 0.5^{de}	105.5 ± 3.2^{ij}	15.3 ± 0.9^{i}	11.9 ± 2.5
	60	4.6 ± 0.9	5.6 ± 0.6^{abc}	4.8 ± 3.1	15.2 ± 1.6^{fg}	107.3 ± 1.5^{ij}	23.3 ± 1.0^{k}	21.8 ± 2.0
	90	4.2 ± 2.8	3.1 ± 2.0^{a}	11.2 ± 3.1	5.7 ± 1.9^{abcde}	69.4 ± 1.8^{c}	9.5 ± 0.2^{cd}	6.1 ± 0.4
E2	120	ND	8.2 ± 10.1^{abcd}	10.5 ± 2.8	2.9 ± 5.8^{abcd}	61.7 ± 2.4^{ab}	9.3 ± 0.6^{bc}	9.9 ± 13.0
	150	ND	12.1 ± 1.9^{de}	12.3 ± 1.6	6.7 ± 3.1^{bcde}	69.2 ± 1.29^{c}	10.7 ± 0.2^{de}	9.6 ± 14.3
	180	13.9 ± 3.2	12.3 ± 0.8^{de}	12.8 ± 15.4	0.8 ± 1.6^{ab}	83.4 ± 9.8^{de}	13.8 ± 0.3^{h}	7.7 ± 2.1
	210	18.9 ± 11.9	12.0 ± 1.1^{cd}	8.5 ± 4.2	2.4 ± 5.0^{abc}	95.3 ± 4.9^{gh}	12.4 ± 0.9^{g}	8.2 ± 1.0
	0	11.0 ± 2.2	120.6 ± 9.2^{i}	19.7 ± 2.0	24.4 ± 1.8^{hi}	143.8 ± 3.5^{l}	12.8 ± 1.0^{gh}	10.1 ± 3.1
	60	13.3 ± 12.2	8.8 ± 1.7^{abcd}	7.5 ± 0.6	18.4 ± 7.4^{gh}	111.9 ± 1.3^{j}	26.0 ± 0.7^{l}	22.3 ± 1.4
	90	5.0 ± 6.6	11.0 ± 3.7^{cd}	11.0 ± 1.9	1.2 ± 2.5^{ab}	55.2 ± 1.2^{a}	7.6 ± 0.2^{a}	6.2 ± 1.0
E3	120	ND	4.2 ± 0.8^{ab}	11.6 ± 1.6	4.5 ± 3.1^{abcd}	57.3 ± 2.0^{a}	7.8 ± 0.4^{a}	12.9 ± 11.4
	150	ND	9.7 ± 0.9^{bcd}	14.8 ± 2.4	6.8 ± 1.7^{bcde}	65.0 ± 2.4^{bc}	7.8 ± 0.4^{a}	18.2 ± 10.7
	180	16.0 ± 9.1	11.7 ± 0.6^{cd}	ND	11.2 ± 6.2^{ef}	85.1 ± 7.5^{de}	13.3 ± 0.3^{gh}	7.2 ± 1.5
	210	13.4 ± 8.8	12.1 ± 1.2^{cde}	15.8 ± 3.3	3.5 ± 4.4^{abcd}	101.9 ± 4.7^{hi}	12.1 ± 1.2^{fg}	6.6 ± 1.2
Enzyme level		ns	*	ns	*	*	*	ns
Ripening time		*	*	*	*	*	*	*
Interaction		ns	*	ns	*	*	*	ns

$^{a–n}$average values in the same column with different letters are significantly different ($P < 0.05$); ND – not detected; the last rows show the ANOVA result for different analysed factors; *significant effect ($P < 0.05$); ns – no significant effect ($P > 0.05$);

added immediately after rennet addition at a dose rate of 7.5 ml per 100 l of milk (CPG cheeses) did not impact on the levels of WSN and PTA-N in comparison with a control at any ripening time studied. In our study, it was in agreement with this data.

Electrophoretic analysis. A typical urea-PAGE electrophoretogram is shown in Figure 2. Casein fractions are labelled according to Sihufe *et al.* (2010a). Standards allowed the identification of the α_{s1}- and β-casein fractions by mobility comparison, and γ- and α_{s1}-casein f24-199 (or α_{s1}-I-casein) were identified according to Marcos *et al.* (1979) and McSweeney *et al.* (1994). Table 3 shows the average values and standard deviations of integrated optical density (IOD) for all the fractions evaluated during the ripening of Reggianito cheese. According to previous works (Sihufe *et al.* 2003, 2010a), F1 and F2 may be products of β-casein degradation, while

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Table 4A. Average concentrations and standard deviations (mg/100 g cheese) of free amino acids (aspargine, glutamic acid, asparagine, serine, histidine, glutamine, glycine, threonine, arginine) determined during ripening of Reggianito cheese

Cheese	Ripening time (days)	Asp	Glu	Asn	Ser	His	Gln	Gly	Thr	Arg
C	0	15.8 ± 0.1^{a}	59.2 ± 0.7^{a}	24.0 ± 0.2^{a}	17.6 ± 0.3^{a}	ND	26.6 ± 0.3^{a}	10.9 ± 0.1^{a}	24.5 ± 0.1^{b}	28.4 ± 0.6^{a}
	60	38.7 ± 0.7^{bc}	227.8 ± 2.9^{bc}	87.1 ± 1.9^{bc}	55.4 ± 1.8^{bc}	52.7 ± 7.6^{hijkl}	83.4 ± 1.9^{c}	33.8 ± 1.2^{bc}	49.5 ± 1.0^{c}	93.0 ± 1.6^{cd}
	90	52.0 ± 2.1^{ef}	298.6 ± 4.5^{efg}	113.6 ± 1.7^{e}	70.9 ± 0.7^{de}	44.9 ± 21.3^{fghij}	93.75 ± 2.2^{def}	43.7 ± 0.9^{fg}	62.3 ± 0.7^{e}	108.2 ± 12.0^{efg}
	120	65.9 ± 0.0^{hi}	368.2 ± 3.1^{hi}	140.0 ± 0.0^{gh}	91.5 ± 0.7^{ghi}	63.4 ± 1.2^{ijkl}	96.3 ± 1.2^{defg}	46.2 ± 3.1^{gh}	66.0 ± 0.3^{e}	119.8 ± 1.5^{ghi}
	150	76.5 ± 0.8^{kl}	423.0 ± 1.8^{jk}	156.4 ± 2.9^{ij}	110.8 ± 9.6^{jk}	34.3 ± 3.3^{efgh}	105.1 ± 1.3^{ghij}	66.1 ± 3.5^{lm}	92.0 ± 2.2^{gh}	122.5 ± 3.8^{hi}
	180	91.9 ± 0.4^{op}	561.6 ± 2.3^{op}	201.3 ± 0.1^{op}	139.6 ± 4.1^{no}	46.7 ± 1.1^{ghijk}	124.0 ± 0.7^{lm}	80.0 ± 0.0^{op}	116.3 ± 0.1^{n}	154.4 ± 1.0^{hl}
	210	75.5 ± 7.3^{kl}	495.4 ± 57.1^{m}	166.7 ± 16.5^{jklm}	135.5 ± 16.3^{mno}	63.4 ± 24.4^{ijkl}	105.4 ± 11.1^{hij}	83.7 ± 9.0^{p}	101.4 ± 4.0^{ijk}	157.5 ± 17.9^{l}
E1	0	15.3 ± 0.1^{a}	57.3 ± 0.4^{a}	21.9 ± 0.3^{a}	15.8 ± 0.8^{a}	ND	27.4 ± 0.2^{a}	10.9 ± 0.1^{a}	23.8 ± 0.3^{b}	26.9 ± 0.1^{a}
	60	49.7 ± 3.3^{e}	278.9 ± 12.3^{def}	105.5 ± 5.0^{de}	66.7 ± 2.0^{cde}	43.4 ± 4.4^{fghi}	100.8 ± 2.4^{fghi}	40.6 ± 1.9^{def}	61.4 ± 0.0^{e}	108.4 ± 2.6^{efg}
	90	48.1 ± 0.28^{de}	251.9 ± 1.4^{cd}	92.6 ± 0.3^{bcd}	56.7 ± 3.0^{bc}	ND	70.6 ± 0.2^{b}	37.5 ± 0.9^{cde}	59.7 ± 0.2^{de}	78.1 ± 2.0^{b}
	120	69.7 ± 0.4^{ij}	397.4 ± 4.6^{ij}	145.3 ± 1.9^{hi}	101.3 ± 1.9^{ij}	32.0 ± 1.9^{efgh}	98.0 ± 1.9^{efgh}	51.5 ± 0.3^{hi}	80.6 ± 0.0^{f}	116.1 ± 1.7^{fgh}
	150	89.3 ± 4.2^{no}	511.3 ± 28.9^{mn}	177.1 ± 8.0^{klm}	129.1 ± 9.7^{lmn}	45.3 ± 22.1^{fghij}	118.9 ± 6.1^{kl}	70.4 ± 4.2^{mn}	105.3 ± 1.8^{jk}	143.7 ± 7.4^{jk}
	180	90.8 ± 0.6^{o}	545.0 ± 7.3^{nop}	178.9 ± 2.2^{lm}	132.5 ± 3.0^{lmno}	31.0 ± 0.7^{efgh}	112.1 ± 0.9^{jk}	75.2 ± 0.1^{no}	107.9 ± 0.6^{kl}	148.7 ± 0.5^{kl}
	210	81.2 ± 5.8^{lm}	542.2 ± 26.9^{nop}	179.3 ± 12.4^{mn}	141.6 ± 6.2^{no}	71.9 ± 29.9^{lm}	108.5 ± 4.5^{ij}	80.8 ± 1.5^{p}	94.4 ± 17.3^{ghi}	174.4 ± 6.8^{m}
E2	0	13.1 ± 0.1^{a}	67.6 ± 0.1^{a}	26.3 ± 0.2^{a}	2 1.1 ± 1.0^{a}	13.4 ± 1.9^{cde}	34.2 ± 0.6^{a}	10.8 ± 0.4^{a}	16.0 ± 0.1^{a}	35.6 ± 1.3^{a}
	60	43.1 ± 0.1^{cd}	263.3 ± 4.6^{cde}	97.2 ± 1.2^{cd}	64.2 ± 0.4^{cd}	70.4 ± 5.3^{klm}	91.4 ± 1.1^{cde}	36.7 ± 0.5^{cd}	53.4 ± 0.3^{cd}	102.1 ± 1.7^{de}
	90	60.3 ± 1.6^{gh}	338.7 ± 6.9^{gh}	129.5 ± 0.9^{fg}	78.6 ± 1.9^{efg}	34.3 ± 6.3^{efgh}	97.9 ± 2.4^{efgh}	43.0 ± 0.8^{efg}	64.5 ± 1.1^{e}	103.9 ± 3.13^{def}
	120	79.5 ± 3.6^{lm}	444.9 ± 11.3^{kl}	165.8 ± 2.0^{jkl}	111.0 ± 9.6^{jk}	22.1 ± 16.5^{def}	111.3 ± 2.2^{jk}	56.6 ± 2.4^{ij}	97.8 ± 0.9^{hij}	132.6 ± 2.9^{ij}
	150	84.1 ± 6.8^{mn}	512.0 ± 56.5^{mn}	178.7 ± 18.9^{lm}	124.0 ± 15.9^{klm}	37.6 ± 16.6^{efgh}	124.7 ± 14.6^{lm}	71.4 ± 5.8^{mn}	108.2 ± 5.7^{klm}	152.8 ± 17.0^{kl}
	180	93.9 ± 0.8^{op}	583.1 ± 9.3^{pq}	192.5 ± 2.8^{no}	144.6 ± 0.9^{op}	43.7 ± 0.5^{fghi}	125.2 ± 2.2^{lm}	82.2 ± 1.1^{p}	113.1 ± 0.1^{lmn}	154.0 ± 2.0^{kl}
	210	77.4 ± 0.8^{kl}	524.0 ± 25.2^{mno}	168.6 ± 7.0^{jklm}	134.9 ± 7.0^{lmno}	90.1 ± 16.8^{m}	108.7 ± 4.7^{ij}	83.0 ± 0.0^{p}	80.6 ± 2.3^{f}	171.1 ± 7.8^{m}
E3	0	11.9 ± 0.0^{a}	51.3 ± 0.7^{a}	19.9 ± 0.0^{a}	14.8 ± 0.3^{a}	4.5 ± 1.7^{bcd}	25.4 ± 0.3^{a}	8.4 ± 0.1^{a}	14.8 ± 0.1^{a}	28.1 ± 0.9^{a}
	60	35.9 ± 0.3^{b}	203.1 ± 0.4^{b}	79.9 ± 1.6^{b}	48.7 ± 1.3^{b}	31.2 ± 3.8^{efgh}	73.1 ± 0.1^{b}	31.0 ± 0.2^{b}	47.8 ± 0.1^{c}	84.5 ± 0.6^{bc}
	90	56.3 ± 0.3^{fg}	305.4 ± 4.0^{fg}	116.1 ± 0.9^{ef}	72.9 ± 0.4^{def}	25.3 ± 5.0^{defg}	89.2 ± 0.7^{cde}	40.0 ± 0.1^{def}	62.2 ± 0.3^{e}	93.6 ± 1.0^{cd}
	120	71.7 ± 0.1^{jk}	412.3 ± 16.5^{jk}	146.5 ± 5.4^{hi}	97.1 ± 2.1^{hi}	25.2 ± 2.9^{defg}	103.3 ± 3.8^{ghij}	59.4 ± 0.9^{jk}	88.7 ± 0.8^{g}	123.4 ± 3.7^{hi}
	150	66.2 ± 2.9^{ij}	361.3 ± 21.6^{hi}	129.4 ± 4.9^{fg}	84.5 ± 4.7^{fgh}	ND	87.6 ± 3.4^{cd}	55.8 ± 1.5^{ij}	90.9 ± 0.1^{gh}	104.0 ± 4.2^{def}
	180	97.4 ± 4.1^{p}	607.9 ± 35.3^{q}	207.0 ± 10.5^{p}	156.2 ± 8.0^{p}	68.0 ± 13.0^{jklm}	132.5 ± 7.2^{m}	85.4 ± 4.9^{p}	115.8 ± 5.3^{mn}	172.6 ± 10.5^{m}
	210	88.1 ± 2.0^{no}	486.3 ± 18.0^{lm}	164.1 ± 9.0^{jk}	121.7 ± 11.6^{kl}	63.0 ± 1.9^{ijkl}	91.7 ± 4.1^{cde}	63.8 ± 3.3^{kl}	78.1 ± 0.6^{f}	149.0 ± 6.4^{kl}
Enzyme level		*	*	*	*	*	*	*	*	*
Ripening time		*	*	*	*	*	*	*	*	*
Interaction		*	*	*	*	*	*	*	*	*

ND – not detected; the last rows show the ANOVA result for different analysed factors; *significant effect ($P < 0.05$); $^{a–n}$average values in the same column with different letters are significantly different ($P < 0.05$)

the fraction F3 may be associated with products of α_{s1}- or α_{s1}-I-casein degradation. Fractions corresponding to the most important caseins (α_{s1}-, α_{s1}-I, or β-casein) were significantly affected by the levels of added enzyme and ripening time, the most extensive degradation being observed for β-casein during the first 2 months of ripening (Figure 2 and Table 3). These results are very interesting taking

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Table 4B. Average concentrations and standard deviations (mg/100 g cheese) of free amino acids (alanine, tyrosine, methionine, phenylalanine, isoleucine, leucine, lysine) and total amino acids determined during the ripening of Reggianito cheese

Cheese	Ripening time (days)	Ala	Tyr	Met	Val	Phe	Ile	Leu	Lys	Total
C	0	14.0 ± 0.1^{a}	14.1 ± 0.3^{a}	9.1 ± 0.1^{a}	24.9 ± 0.2^{a}	14.3 ± 0.2^{a}	16.2 ± 0.2^{a}	48.9 ± 0.7^{a}	80.2 ± 0.4^{a}	419.7 ± 5.4^{a}
	60	37.4 ± 1.3^{bcd}	41.1 ± 2.0^{bcd}	33.1 ± 0.8^{b}	92.0 ± 0.9^{b}	57.5 ± 1.4^{bc}	66.4 ± 0.6^{bc}	181.5 ± 4.9^{bc}	288.1 ± 5.6^{bc}	1518.7 ± 36.9^{bc}
	90	43.1 ± 1.5^{cde}	47.0 ± 1.5^{def}	42.1 ± 1.7^{cd}	124.4 ± 3.9^{de}	73.7 ± 4.1^{def}	92.1 ± 3.8^{ef}	222.5 ± 13.3^{d}	347.3 ± 26.2^{def}	1880.8 ± 98.0^{de}
	120	52.5 ± 0.7^{fg}	54.1 ± 0.2^{gh}	48.8 ± 0.8^{e}	150.3 ± 3.1^{fg}	87.9 ± 0.1^{ghi}	113.8 ± 3.3^{hi}	261.2 ± 5.9^{e}	348.5 ± 15.3^{ef}	2174.4 ± 37.8^{fg}
	150	63.1 ± 4.7^{hi}	66.0 ± 3.5^{j}	58.1 ± 2.3^{fgh}	172.4 ± 6.4^{hi}	108.3 ± 3.6^{jk}	128.5 ± 4.5^{jk}	304.7 ± 10.4^{fg}	465.5 ± 4.3^{gh}	2553.4 ± 68.9^{ij}
	180	74.2 ± 1.6^{jk}	79.6 ± 0.8^{klm}	72.6 ± 0.9^{lm}	225.7 ± 1.8^{l}	133.3 ± 0.4^{n}	168.1 ± 1.3^{o}	375.1 ± 7.1^{j}	578.8 ± 7.7^{jkl}	3223.4 ± 0.20^{mn}
	210	73.3 ± 11.4^{jk}	80.6 ± 10.5^{lm}	73.1 ± 6.1^{lmn}	181.8 ± 16.7^{ij}	130.3 ± 14.2^{mn}	145.5 ± 14.5^{l}	359.9 ± 39.1^{ij}	579.8 ± 71.4^{kl}	3008.8 ± 347.7^{lm}
E1	0	14.6 ± 0.0^{a}	13.9 ± 0.5^{a}	9.0 ± 0.4^{a}	22.6 ± 0.2^{a}	13.3 ± 0.2^{a}	14.5 ± 0.3^{a}	44.4 ± 0.5^{a}	82.2 ± 1.4^{a}	413.6 ± 6.3^{a}
	60	47.0 ± 1.3^{ef}	48.7 ± 0.9^{efg}	42.1 ± 1.1^{cd}	112.7 ± 2.8^{cd}	66.5 ± 0.0^{cd}	82.5 ± 1.2^{de}	212.9 ± 0.2^{d}	336.9 ± 2.4^{cde}	1804.8 ± 38.6^{de}
	90	36.2 ± 2.2^{bc}	37.7 ± 0.0^{bc}	34.4 ± 0.2^{b}	99.6 ± 0.4^{bc}	53.4 ± 0.2^{b}	74.2 ± 0.78^{cd}	165.11 ± 0.2^{b}	269.6 ± 3.5^{b}	1459.9 ± 9.50^{b}
	120	58.4 ± 1.9^{gh}	56.5 ± 1.1^{hi}	54.3 ± 0.5^{f}	161.7 ± 4.8^{gh}	90.3 ± 0.9^{hi}	118.2 ± 1.7^{ij}	260.9 ± 4.9^{e}	354.9 ± 2.5^{ef}	2247.2 ± 32.1^{gh}
	150	72.0 ± 6.7^{kl}	73.3 ± 7.4^{k}	67.0 ± 3.2^{jk}	197.4 ± 9.8^{k}	114.8 ± 11.1^{kl}	146.5 ± 5.7^{l}	325.6 ± 26.2^{gh}	514.4 ± 60.4^{hi}	2901.6 ± 223.0^{kl}
	180	71.3 ± 0.8^{jkl}	77.1 ± 0.6^{kl}	69.8 ± 0.8^{kl}	205.8 ± 1.2^{k}	121.0 ± 0.8^{lm}	158.5 ± 1.3^{mno}	337.3 ± 1.0^{hi}	547.1 ± 6.5^{ijk}	3010.0 ± 25.5^{lm}
	210	76.6 ± 1.6^{kl}	84.8 ± 2.2^{mn}	76.7 ± 2.2^{mn}	200.7 ± 8.5^{k}	134.1 ± 1.8^{n}	165.9 ± 4.8^{no}	370.5 ± 3.0^{j}	604.4 ± 5.2^{l}	3188.1 ± 67.4^{mn}
E2	0	19.2 ± 1.3^{a}	17.5 ± 1.3^{a}	11.2 ± 0.4^{a}	29.1 ± 0.8^{a}	16.9 ± 1.1^{a}	19.3 ± 0.5^{a}	58.6 ± 3.6^{a}	98.6 ± 8.0^{a}	508.6 ± 22.8^{a}
	60	44.4 ± 1.5^{de}	46.2 ± 1.9^{de}	39.4 ± 1.3^{c}	107.8 ± 2.3^{c}	65.5 ± 2.2^{cd}	76.1 ± 1.7^{cd}	205.1 ± 5.3^{cd}	334.9 ± 10.0^{cde}	1741.1 ± 41.0^{cd}
	90	48.6 ± 0.0^{ef}	49.7 ± 0.2^{efgh}	46.3 ± 0.3^{de}	138.9 ± 2.2^{ef}	78.4 ± 1.0^{efg}	99.1 ± 0.5^{fg}	231.3 ± 2.3^{d}	322.9 ± 5.8^{b}	1966.1 ± 37.0^{def}
	120	64.4 ± 4.2^{hij}	63.0 ± 2.5^{ij}	60.2 ± 2.1^{hi}	179.7 ± 5.0^{ij}	101.9 ± 5.0^{j}	131.9 ± 2.6^{k}	296.3 ± 8.4^{f}	395.3 ± 14.6^{f}	2514.3 ± 95.7^{ij}
	150	69.3 ± 8.6^{ijk}	74.6 ± 7.2^{kl}	67.2 ± 7.2^{jk}	201.6 ± 20.6^{k}	118.1 ± 13.2^{kl}	148.1 ± 15.1^{lm}	335.7 ± 35.9^{hi}	528.4 ± 58.9^{ij}	2936.3 ± 324.8^{kl}
	180	77.4 ± 0.1^{kl}	81.0 ± 0.1^{lm}	76.8 ± 0.5^{mn}	224.5 ± 1.3^{l}	135.1 ± 1.5^{n}	166.4 ± 0.05^{o}	375.8 ± 3.9^{j}	602.5 ± 7.5^{l}	3271.9 ± 11.8^{n}
	210	76.3 ± 1.6^{jk}	81.3 ± 3.5^{lm}	77.9 ± 2.8^{n}	203.1 ± 9.0^{k}	135.3 ± 4.5^{n}	162.1 ± 6.1^{no}	367.7 ± 17.1^{j}	606.4 ± 24.3^{l}	3148.6 ± 142.8^{lmn}
E3	0	13.8 ± 0.4^{a}	13.1 ± 0.2^{a}	8.8 ± 0.2^{a}	22.2 ± 0.7^{a}	12.7 ± 0.6^{a}	14.4 ± 0.4^{a}	44.8 ± 2.0^{a}	79.3 ± 5.3^{a}	388.0 ± 13.8^{a}
	60	32.5 ± 0.2^{b}	35.4 ± 0.4^{b}	30.7 ± 0.1^{b}	87.4 ± 0.8^{b}	49.2 ± 0.6^{b}	62.4 ± 0.5^{b}	157.8 ± 3.5^{b}	254.7 ± 9.5^{b}	1345.3 ± 19.6^{b}
	90	44.2 ± 0.2^{de}	44.5 ± 0.4^{cde}	42.3 ± 0.1^{cd}	123.9 ± 2.0^{d}	69.6 ± 0.8^{de}	89.2 ± 1.6^{ef}	207.2 ± 1.9^{cd}	296.6 ± 3.2^{bcd}	1778.7 ± 3.6^{d}
	120	60.9 ± 2.3^{h}	62.0 ± 2.8^{ij}	55.7 ± 3.3^{fg}	164.6 ± 7.9^{gh}	97.6 ± 7.7^{ij}	123.3 ± 5.5^{ijk}	283.5 ± 14.8^{ef}	452.2 ± 30.1^{g}	2427.5 ± 111.2^{hi}
	150	46.3 ± 2.1^{ef}	53.4 ± 2.8^{fgh}	46.6 ± 2.1^{de}	141.3 ± 7.5^{f}	80.5 ± 4.9^{fgh}	106.3 ± 4.1^{gh}	227.9 ± 12.7^{d}	374.0 ± 23.0^{ef}	2033.3 ± 118.9^{efg}
	180	84.1 ± 4.6^{l}	90.2 ± 4.0^{n}	84.2 ± 3.0^{o}	241.4 ± 11.3^{m}	149.5 ± 6.7^{o}	186.1 ± 5.4^{p}	408.2 ± 18.0^{k}	673.3 ± 30.9^{m}	3559.8 ± 182.9^{o}
	210	63.5 ± 5.3^{hi}	65.5 ± 5.0^{j}	62.7 ± 3.5^{ij}	191.1 ± 10.5^{jk}	107.7 ± 6.2^{jk}	155.6 ± 10.3^{lmn}	304.9 ± 21.0^{fg}	469.4 ± 26.7^{gh}	2726.5 ± 145.5^{jk}
Enzyme level		*	*	*	*	*	*	*	*	*
Ripening time		*	*	*	*	*	*	*	*	*
Interaction		*	*	*	*	*	*	*	*	*

ND – not detected; the last rows show the ANOVA result for different analysed factors; *significant effect ($P < 0.05$); a–qaverage values in the same column with different letters are significantly different ($P < 0.05$)

into account that carboxypeptidases are essential for promoting extensive protein hydrolysis and prevention of bitterness (Gobbetti *et al.* 1997; Forde & Fitzgerald 2000). It is worth mentioning that urea-PAGE helps detecting changes in the intact caseins and their primary proteolytic degradation products during ripening. In this case, it can be hypothesised that carboxypeptidase catalyses the hydrolysis of

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β-casein from the C-terminal and changes its charge, hydrophobicity, and size. Unfortunately, there is a lack of information about urea-PAGE to study β-casein degradation by the action of carboxypeptidase.

Peptide analysis by RP-HPLC. The chromatograms of the water soluble fraction at pH 4.6 are often referred to as 'finger prints' of the cheese proteolysis (Pripp *et al*. 1999). In our case, the regular peptide profile of Reggianito cheese was not substantially modified by the carboxypeptidase action. Twenty-eight chromatographic peaks were determined by RP-HPLC, which were present in all the analysed samples. Sixteen peaks were significantly affected by the amount of enzyme added during cheese manufacture, while all 28 peaks were significantly affected by ripening time. This result indicates the significance of the influence of the amount of added enzyme. The obtained large multivariate data set will be more easily interpreted by principal component analysis.

Free amino acid analysis. Seventeen amino acids were determined in 40 min of the chromatographic run. ANOVA highlighted that the ripening time and the amount of enzyme used during cheese manufacture significantly affected the concentrations of all the amino acids studied. Similar to Sihufe *et al*. (2010a), amino acids Lys, Glu, Leu, and Val were present at the highest concentrations and represented more than 50% of total amino acids at the end of the ripening period in all the cheeses analysed.

High amino acid concentrations were observed in some cheeses manufactured with exogenous enzymes compared with control cheeses at the same ripening time (Table 4A and B). Such behaviour can be clearly observed in cheeses E1 at 60 and 150 days of ripening, in cheeses E2 at 120 and 150 days of ripening, and in cheeses E3 at 120 and 180 days of ripening. These results are in agreement with those reported by Azarnia *et al*. (2011), who observed increased levels of free amino acids in Cheddar cheeses manufactured with free exogenous aminopeptidases. Kilcawley *et al*. (2012) concluded that the carboxypeptidase activities during ripening in cheese with added carboxypeptidase were significantly higher than in control cheese. However, the levels of total free amino acids of CPG cheeses were not significantly different from those corresponding to control cheeses at the same ripening time.

It is worth mentioning that the comparison of amino acid concentrations should be analysed taking into account that amino acids can also be degraded to catabolic products after their formation. Therefore, it may be difficult to observe higher amino acid concentrations in cheeses manufactured with exogenous enzymes at all ripening times. Thus, in the case of the cheeses manufactured with the higher enzyme level, low amino acid concentrations were also clearly observed in some cases (cheeses E3 at 150 and 210 days of ripening) compared with control cheeses at the same ripening time (Table 4).

Principal component analysis. Principal component analysis (PCA) was used to summarise the data set and to detect possibly unanticipated patterns in the data. PCA was applied using the nitrogen content of the 2 studied fractions (WSN & PTA), 7 electrophoretic fractions analysed by urea-PAGE (γ-caseins, β-casein, F1, F2, α_{s1}-casein, α_{s1}-I-casein, and F3), 28 chromatographic peaks from the RP-HPLC analysis of the water-soluble fraction at pH 4.6, and of the 17 free amino acids studied. After using PCA with all samples, a subset of samples was selected excluding samples corresponding to 0 and 210 days of ripening to improve the analysis of the information. The first 4 principal components represented more than 70% of the total variance (73.2% VAR).

A biplot of the first 2 principal components (61.1% VAR) is shown in Figure 3. The first principal component PC1 (that explains 50.1% of the variance) can be interpreted by the behaviour of the chromatographic areas of most of the peptides and the concentration of the free amino acids which can clearly be related to the ripening process. Those variables increased with ripening time within the same treatment (in Figure 3, samples corresponding to the same treatment spread from left to right as the ripening time increases). Moreover, this effect can be observed in a cluster structure since all samples corresponding to the same ripening time but to different treatments are positioned close together.

The second principal component PC2 (that explains 11% of the variance) has a quadratic effect with respect to the PC1, having higher score values at the beginning and at the end of the process with a minimum in the middle region (Figure 3). This second component differentiates the treatments. Control cheeses at 60 days of ripening showed notably higher β-casein IOD values than those values corresponding to experimental cheeses at the same ripening time (Table 3). Similarly, but less pronounced, α_{s1}-casein IOD values were higher in control cheeses, while α_{s1}-I-casein IOD values were lower in control cheeses than in experimental ones (Table 3). However, the association of PC2 with the level of added enzyme

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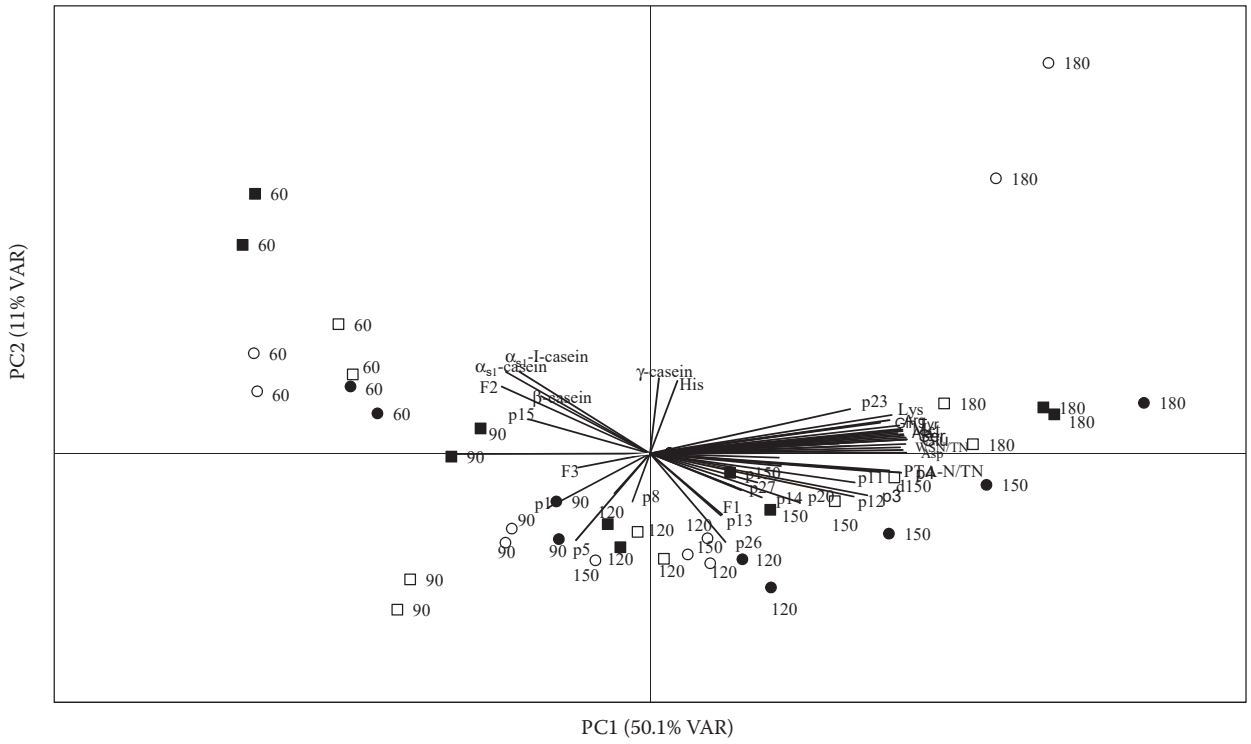

Figure 3. A biplot of scores and loadings of data obtained from different analyses used to study the proteolysis of Reggianito cheeses

(■) cheeses C; (□) cheeses E1; (●) cheeses E2; (○) cheeses E3; numbers correspond to days of ripening; labels starting with p followed by a number indicate peaks from the peptide chromatographic analysis

in the case of cheeses E3 at 180 days of ripening was not definite.

Finally, the biplot also showed that for the same ripening time, some cheeses made with carboxypeptidase are slightly on the right from the samples corresponding to control cheeses, indicating an increase in the rate of formation and/or degradation of proteolysis products. Kilcawley *et al*. (2012) studied the effect of enzyme addition on rheological properties of cheese by the texture profile analysis and found no statistical difference between control and CPG cheeses for any sensory texture attribute. They found that CPG cheeses were associated with sensory characteristics of aged Cheddar. Moreover, the authors related the greater diversity of volatile compounds in the experimental cheeses partially to the enhanced levels of secondary proteolysis that provided an additional nitrogenous substrate. Therefore, in our case no adverse impact on cheese sensory characteristics was expected because the secondary proteolysis was not extremely accelerated.

At present, little is known about the manufacture of Reggianito cheese with the addition of exogenous enzymes. Therefore, this study can be considered as the first early stage and it will allow focusing on complementary studies (sensory studies, rheological studies, etc.) and on confirmatory experiments more easily.

CONCLUSIONS

The impact of the addition of an exogenous carboxypeptidase enzyme during the manufacture of Reggianito cheese was evaluated by assessing its impact on composition and proteolysis during ripening. Fractions corresponding to the most important caseins were significantly affected by the level of added enzyme and ripening time, the most important effect being observed for β-casein in the first 2 months of ripening. The amino acids Lys, Glu, Leu, and Val were present at the highest concentrations in all analysed cheeses. An increase of amino acid concentrations was clearly observed in some cheeses manufactured with exogenous enzymes compared with control cheeses at the same ripening time. The principal component analysis summarised the information related to proteolysis determinations. The first principal component accounted for 50.1% of the total variance and it was clearly related to the

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ripening process. Useful information was obtained from the biplot of the first 2 principal components. A slight acceleration in proteolysis was observed when comparing the position of samples corresponding to control and experimental cheeses. The use of carboxypeptidase appears to be an interesting option to accelerate ripening of Reggianito cheese, but complementary studies such as sensory evaluation are necessary. This study will allow focusing on confirmatory experiments more easily regarding the enzyme addition to accelerate the ripening of hard cheeses.

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