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Effect of ripening time on peptide dynamics and bioactive peptide composition in Tulum cheese

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ABSTRACT

Skin bag Tulum cheeses traditionally produced in the Central Taurus region of Turkey were studied to identify peptide profiles by liquid chromatography–tandem mass spectrometry over 180 d of ripening. After mass spectrometry analysis, 203 peptides were identified: 59 from α_{S1}-casein (CN), 11 from α_{S2}-CN, 129 from β-CN, and 4 from κ-CN. Numbers of α_{S1}- and β-CN-derived peptides increased with increasing number of ripening days due to the dependence of newly formed peptides on proteolysis. However, similar increases were not observed for α_{S2}- and κ-CN-derived peptides. Most identified peptides consisted of β-CN-derived peptides, followed by α_{S1}-, α_{S2}-, and κ-CN-derived peptides. Among these, bioactive peptides were found, including antihypertensive, antibacterial, antioxidant, dipeptidyl peptidase-4 inhibitory, metal chelating, skin regenerating, glucagon-like peptide-1 secretion enhancing, opioid, cathepsin B inhibitory, prolyl endopeptidase inhibitory, immunomodulatory, brain function improving, antiamnesic, antihypercholesterolemic, anti-inflammatory, and anticarcinogenic peptides.
Key words: bioactive peptide, liquid chromatography–tandem mass spectrometry, peptide identification, proteolysis, Tulum cheese

INTRODUCTION

Tulum cheese is one of the top 3 types of cheese consumed in Turkey (Hayaloglu et al., 2007). It is produced from raw milk using traditional methods and is offered for consumption after it has ripened for a long time (Akın, 2010). Tulum cheese is manufactured by pressing cheese curd in goat or sheep skin bags and takes its name from the skin bag (tulum) in which it is pressed. In its production, generally, rennet is used as a coagulant, and sheep milk, goat milk, or a mixture of both are preferred; however, due to increasing demand in recent years, cow milk has also begun to be used in the production of Tulum cheese (Hayaloglu et al., 2007; Akın, 2010). Approximately 2 to 3% salt is added before pressing the cheese curd in the skin bag. Tulum cheeses are preripened for 5 to 7 d at 10 to 15°C to remove the remaining whey and, after this process, left to ripen for 120 to 180 d at 4 to 6°C in 85% relative humidity. Tulum cheese is characterized as a semihard cheese of white or cream color with a buttery taste and tangy flavor (Öztürk and Akın, 2017). Natural microbiota of raw milk are used to obtain a traditional flavor in Tulum cheese production without starter culture. The microbial growth is controlled by lactic acid production from lactose. Therefore, in Tulum cheese that is ripened for a long time, some of these microorganisms are eliminated and others become dominant in progressive ripening periods.

During the ripening of cheese, various biochemical processes occur, such as lipolysis, proteolysis, and glycolysis, which contribute to the development of aroma and texture in cheese (Farkye, 2004). These processes hold great importance to flavor formation in general, and proteolysis in particular is considered to be a complex and fundamental biochemical process that supports texture and flavor development during the ripening of most cheese varieties. Rennet (chymosin) remaining in cheese, endogenous proteolytic enzymes in milk (plasmin, somatic cell proteinases, cathepsins), microbial proteolytic enzymes, and exogenous proteinases or peptidases added externally to accelerate cheese maturation cause proteolysis throughout cheese ripening (Fox et al., 2017). The proteolysis reaction, which takes place due to the activity of proteinases or peptidases during ripening, first cleaves caseins into large and medium peptides. These resulting peptides induce major changes in cheese texture during the first stage of ripening. Additionally, large peptides and oligopeptides contribute to positive flavor formation in cheese, mostly functioning as substrates for the enzymatic activities of the microbiota (Ardö et al., 2017). In addition, as a result of the advanced proteolysis reaction, short

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peptides and free AA are formed, and these reaction products greatly influence the formation of aroma in cheese (Fox et al., 2017).

The enzymes that are effective in proteolysis of Tulum cheese consist of enzymes in coagulant and milk (indigenous enzymes) and microbial proteolytic enzymes (intracellular and extracellular enzymes) showing activity during ripening. Because raw milk is used without starter culture in Tulum cheese processing, the natural microbiota of raw milk more actively influence cheese properties. Thus, various proteolytic microbial enzymes can be found in Tulum cheese compared with other cheese varieties. This is thought to significantly affect the peptide profile of Tulum cheese. Moreover, production without applying heat treatment to milk also has advantages for peptide formation. Heat treatment causes various modifications in milk proteins and has negative effects on bacterial diversity and endogenous proteolytic enzymes (Gaya et al., 2005; Raynal-Ljutovac et al., 2007). Accordingly, changes in milk proteinases and substrates of these enzymes may also affect the final peptide composition in the ripened cheese.

Peptides that confer health benefits to the human body upon consumption are defined as bioactive peptides. They are encoded in structural precursor proteins and are activated by proteolysis from the inactive forms of these proteins (Korhonen and Pihlanto, 2003). Bioactive peptides are usually characterized as short-chain peptides with 2 to 20 AA residues (Hartmann and Meisel, 2007). These peptides have been reported to have various physiological functions, such as opioid action, binding of minerals, immunomodulation, antimicrobial activity, antioxidation, antithrombosis, hypocholesterolemic action, anticarcinogenesis, and so on (Korhonen and Pihlanto, 2006). It has been suggested that the great majority of bioactive peptides in milk originate from α_{S1}-CN, followed by peptides from β-CN and α_{S2}-CN (Baum et al., 2013).

Identification of peptides that arise during cheese ripening provides a better understanding of the proteolytic process and the bioactive properties of cheese. To examine proteolysis extensively, the peptides that occur during ripening in various cheese types such as Manchego (Gómez-Ruiz et al., 2004), Idiazabal (Sagardia et al., 2013), Coalho (Fontenele et al., 2017), and Old Saare (Taivosalo et al., 2018) were identified. However, no data related to peptides occurring during the ripening of Tulum cheese were found in the literature reviews. This research aimed to identify the water-soluble peptides and to describe peptide dynamics during 180 d of the ripening period of Tulum cheeses produced using traditional methods from the Karaman region (Turkey). Peptides in water-soluble extracts of Tulum cheeses were characterized by liquid chromatography–tandem MS (**LC-MS/MS**). Furthermore, the functional properties of these peptides were determined by various online databases. In addition, during the 180 d of the ripening period, physicochemical properties of Tulum cheeses were monitored.

MATERIALS AND METHODS

Cheese Sampling

Tulum cheeses were provided by local producers from the Karaman region in the Central Taurus region of Turkey. A total of 15 Tulum cheeses (5 different ripening times and 3 biological repetitions; 5 × 3) of approximately 3 to 3.5 kg were supplied from the local producers. The cheeses were supplied following production and were left to ripen under conditions similar to those used by traditional producers. Cheese samples were preripened for 5 d at 15°C to remove excess whey at the Food Processing Pilot Plant of the Food Engineering Department of Selcuk University (Konya, Turkey). Thereafter, the cheeses were allowed to ripen for 180 d under the conditions of 4 to 6°C and 85% relative humidity in the cold storage room at the pilot plant of the university. Considering the effectiveness of microbial activity and enzymatic activity, sampling times of d 7, 30, 60, 90, and 180 of the ripening period were chosen. On the designated ripening days, Tulum cheeses were taken from the cold storage room, and the cheese samples were prepared for analysis after removing the skin of the Tulum cheese and mixing samples homogeneously to represent the whole cheese. Three biological samples were used on each ripening day. All analyses were conducted over the 180 d of the ripening period.

Compositional Characteristics

Total DM or moisture, protein, fat, salt content, pH, and titratable acidity of Tulum cheeses were determined according to the methodologies of AOAC International (2000). Titratable acidity was evaluated using the alkaline titration method and calculated as percent lactic acid. Total DM content was examined using the gravimetric method. Protein content in the Tulum cheeses was measured using the Kjeldahl method. The Gerber method was used for examining fat content. A pH meter (WTW-315i, WTW GmbH, Weilheim, Germany) was used for pH determination. The salt content of Tulum cheeses was determined using the potentiometric titration (Mohr) method. All physicochemical analyses

were done in triplicate during the 180 d of the ripening period.

Preparation of Water-Soluble Extracts for Chromatographic Analysis

Water-soluble extracts (**WSE**) from Tulum cheeses were prepared by modifying the method developed by Gómez-Ruiz et al. (2002). The cheese samples were each mixed with 5 times their weight of distilled water and then homogenized with an Ultra Turrax T25 mixer (IKA, Staufen, Germany) for 3 min. Homogenates were kept in a water bath (Memmert WB22, Schwabach, Germany) at 40°C and 200 rpm for 60 min. Following this process, the pH of the homogenates was adjusted to pH 4.6 using 1 *M* HCl (Merck Millipore, Darmstadt, Germany), and then the samples were centrifuged (NF800R, Nuve, Ankara, Turkey) at 3,800 × g and 4°C for 30 min. The resulting supernatants were first filtered through Whatman no. 40 filter paper (Sigma-Aldrich, Steinheim, Germany) and then filtered through a 0.45-μm membrane filter (Merck Millipore). These extracts were used in LC-MS/MS analysis. In addition, the nitrogen ratio of these extracts was determined using the Kjeldahl method (Katsiari et al., 2001).

MS Analysis

The LC-MS/MS analysis was performed using the method previously described by Chang et al. (2013) with some modifications. Analysis of the WSE was performed with an Agilent 6530 electrospray ionization-QTOF-MS system (Agilent Technologies, Santa Clara, CA) equipped with an Agilent 1260 HPLC consisting of an autosampler, a binary pump, a degasser, and a column oven. Chromatographic separation was conducted using a C18 analytical column (Agilent Poroshell 120 EC-C18, 3 × 150 mm, 2.7 μm; Agilent Technologies). Electrospray ionization was carried out in positive ion mode. The automatic sampler was kept at 4°C to prevent sample deterioration. The WSE were used in the analysis after passing them through the 0.22-μm syringe membrane filter (Durapore, Merck Millipore), and each extract (2 μL) was injected into the LC-MS/MS system. Water:acetonitrile (98:2, vol:vol) containing 0.1% formic acid and water:acetonitrile (2:98, vol:vol) containing 0.1% formic acid were prepared as solvent A and solvent B, respectively. Samples were eluted with a linear gradient of solvent B in solvent A at 0.200 mL/min. The LC gradient was operated at 2 to 35% solvent B for 30 min, 35 to 90% solvent B for 10 min, 90% solvent B for 5 min, and 5% solvent B for 15 min. Resulting peptides were electrosprayed at an ion spray voltage of 200 V. Mass data collection was accomplished automatically with Agilent MassHunter 7.0 qualitative analysis software (Agilent Technologies). Mass spectra were recorded in full-scan ion mode (50–2,000 m/z).

Analysis of Peptide Sequences

To identify peptides, all data from the LC-MS/MS analysis were submitted to the BioConfirm program (BioConfirm for MassHunter Acquisition Data, B.09.00, 2017; Skrzypczak et al., 2017). The search was performed against databases of major milk proteins from the NCBI (www.ncbi.nlm.nih.gov). Because the Tulum cheese was produced from mixtures of cow, sheep, and goat milks, protein data of these 3 species were used. The NCBI accession numbers of the used ovine, bovine, and caprine protein data were as follows: NP_001009795, NP_851372, and NP_001272624 for α_{S1}-CN; NP_001009363, NP_776953, and NP_001272514 for α_{S2}-CN; CAA34502, XP_010804480, and XP_005681778 for β-CN; NP_001009378, NP_776719, and NP_001272516 for κ-CN; NP_001009797, NP_776803, and NP_001272564 for α-LA; NP_001009366, NP_776354, and NP_001272468 for β-LG; and NP_001009376, 4F5S_B, and XP_005681801 for serum albumin. Peptides were sequenced by adding these databases to the BioConfirm program as fasta files. No specific enzyme cleavages or variable modifications were selected. The peptide mass tolerance was set to 10 ppm for MS and 50 ppm for MS/MS. Peptides possessing identity score higher than ≥80% were considered in this study.

Determination of Bioactivity

The functional properties of the obtained peptide sequences were evaluated using online databases proposed by Giacometti and Buretić-Tomljanović (2017). For this purpose, BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), EROP-Moscow (http://erop.inbi.ras.ru/), PepBank (http://pepbank.mgh.harvard.edu/), PeptideDB (http://www.peptides.be/), Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/), and Google Scholar (https://scholar.google.com.tr/) online databases were used.

Statistical Analysis

The data obtained from compositional analyses were analyzed using 1-way ANOVA in Minitab software (Minitab Inc., 2010). The results are presented as the mean ± standard deviation of the 3 replicates. To identify significant differences between the means of ripening periods, the Tukey multiple range test with a confidence interval set at 95% was performed.

RESULTS AND DISCUSSION

Physicochemical Characterization of Tulum Cheeses

The physicochemical properties of Tulum cheeses depending on ripening period are presented in Table 1. During the ripening period of Tulum cheeses, the total DM content was determined to be between 52.22 and 63.52% and was found to increase due to the moisture loss that occurred during ripening ($P < 0.05$). The occurrence of water loss from Tulum cheeses can be explained by increasing titratable acidity over the course of ripening, thereby reducing the water-holding capacity (Pastorino et al., 2003). Protein and fat contents of Tulum cheeses were found to increase from 22.72 to 30.87% and 24.77 to 27.34%, respectively, during ripening. Although these contents showed partial increases due to the increasing DM ratio during the ripening period, the differences were statistically insignificant ($P > 0.05$). Similarly, the salt content also increased with increasing DM ratio during the ripening period, and this increment was found to be statistically significant ($P < 0.05$). The salt content of Tulum cheeses was determined to change from 2.29 to 2.64% over the ripening period. The titratable acidity of Tulum cheeses increased from 2.50 to 2.93% with the progress of ripening based on lactic acid formation ($P < 0.05$). Despite the elevation of the titratable acidity during the ripening period, no significant decrease was observed in pH values of Tulum cheeses due to the enhancement in the buffer capacity depending on the increased DM content, and the pH values of Tulum cheeses were determined to vary between 4.74 and 5.12. Similar observations of pH during ripening were also encountered in Tulum cheeses by Öztürk and Akın (2017) and in Idiazabal cheeses by Sadat-Mekmene et al. (2013). The water-soluble nitrogen content was determined to vary between 0.30 and 0.58%, and a significant increase in the water-soluble nitrogen content of Tulum cheeses was detected due to proteolysis during ripening ($P < 0.05$).

Peptide Identification by MS

Peptides Derived from α_{S1}-CN. The α_{S1}-CN-derived peptides identified by LC-MS/MS in WSE of Tulum cheeses over the ripening period are presented in Table 2. A total of 59 α_{S1}-CN-derived peptides were identified in Tulum cheeses throughout the 180 d of the ripening period. It has been reported that α_{S1}-CN is first broken down by the activity of the coagulant remaining in cheese and hydrolyzed with microbial proteinases or peptidases and, to a lesser extent, plasmin enzyme during later ripening days (Ardö et al., 2017).

Gómez-Ruiz et al. (2004) determined 43 α_{S1}-CN-derived peptides during 240 d of ripening of Manchego cheese produced from pasteurized sheep milk using starter cultures (*Lactococcus lactis* ssp. *lactis* and *Leuconostoc mesenteroides* ssp. *dextranicum*) and rennet. In another study, Pisanu et al. (2015) detected 62 α_{S1}-CN-derived peptides during 270 d of ripening of cheeses produced from raw sheep milk. For Coalho cheeses produced with raw cow milk, 25 α_{S1}-CN-derived peptides have been identified (Fontenele et al., 2017). More peptides were observed in Tulum cheeses ripened for 180 d than in Manchego or Coalho cheeses. It is speculated that the differences in numbers of α_{S1}-CN-derived peptides among cheese varieties are due to differences in production method and the microbiota of cheeses. The number of peptides has been reported to be lower in cheeses produced using pasteurized milk than in those produced from raw milk (Gómez-Ruiz et al., 2004; Pisanu et al., 2015). In addition, Losito et al. (2006) observed that different peptides were formed in WSE as a result of using different milk types in the production of various Italian cheeses. Therefore, the formation of peptides in higher numbers in Tulum cheeses produced from a mixture of raw sheep, goat, and cow milks is not surprising.

In general, it was observed that most of the peptides were formed by hydrolysis of the N-terminal region of α_{S1}-CN. Other peptides, excluding α_{S1}-CN

Table 1. Compositional characteristics of Tulum cheeses during ripening period

Characteristic	Ripening period (d)				
	7	30	60	90	180
DM (%)	52.22 ± 0.40^{b}	56.00 ± 3.37^{ab}	57.31 ± 1.95^{ab}	61.41 ± 4.44^{ab}	63.52 ± 5.25^{a}
Protein (%)	22.72 ± 0.99^{b}	24.08 ± 0.93^{b}	25.30 ± 1.87^{b}	25.86 ± 1.67^{b}	30.87 ± 0.98^{a}
Fat (%)	24.77 ± 0.40^{a}	26.49 ± 2.71^{a}	25.84 ± 2.46^{a}	28.74 ± 3.19^{a}	27.34 ± 4.66^{a}
Salt (%)	2.29 ± 0.09^{b}	2.38 ± 0.13^{ab}	2.41 ± 0.13^{ab}	2.40 ± 0.16^{ab}	2.64 ± 0.07^{a}
pH	4.79 ± 0.06^{b}	4.74 ± 0.07^{b}	4.85 ± 0.09^{ab}	4.87 ± 0.11^{ab}	5.12 ± 0.16^{a}
Titratable acidity (lactic acid %)	2.50 ± 0.02^{b}	2.43 ± 0.03^{b}	2.37 ± 0.05^{b}	3.08 ± 0.07^{a}	2.93 ± 0.09^{a}
Water-soluble nitrogen (%)	0.30 ± 0.04^{d}	0.38 ± 0.04^{cd}	0.46 ± 0.03^{bc}	0.52 ± 0.01^{ab}	0.58 ± 0.02^{a}

$^{a–d}$Different superscripts in the same row denote significant differences ($P < 0.05$) between ripening days.

Table 2. α_{S1}-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
1	2.554	1,196.6938	α_{S1}-CN (16–25)	Bovine	RPKHPIKHQG	ACE inhibitory	Saito et al. (2000)					×
2	2.557	929.4845	α_{S1}-CN (18–25)	Caprine	KHPINHQG	—	—				×	×
3	2.707	1,139.6692	α_{S1}-CN (16–24)	Bovine	RPKHPIKHQ	ACE inhibitory	Saito et al. (2000)	×	×	×	×	
4	4.002	1,369.7495	α_{S1}-CN (18–29)	Ovine	KHPIKHQGLSPE	—	—			×		
5	4.081	584.3519	α_{S1}-CN (125–129)	Ovine	EIVPK	ACE inhibitory	Gómez-Ruiz et al. (2006)	×	×	×	×	×
6	4.306	570.3022	α_{S1}-CN (85–89)	Bovine	EIVPN	Metal chelating	Timón et al. (2019)			×		
7	8.801	1,622.9026	α_{S1}-CN (16–29)	Ovine	RPKHPIKHQGLSPE	ACE inhibitory	Georgalaki et al. (2017)	×	×	×	×	×
8	13.540	1,014.5419	α_{S1}-CN (48–55)	Caprine	RKENINEL	—	—		×			
9	15.369	643.3639	α_{S1}-CN (33–37)	Bovine	ENLLR	—	—	×	×			
10	15.673	757.4073	α_{S1}-CN (32–37)	Bovine	NENLLR	—	—		×			
11	16.499	601.3074	α_{S1}-CN (31–35)	Bovine	LNENL	—	—		×	×	×	×
12	16.664	754.3851	α_{S1}-CN (26–32)	Caprine	LSPEVPN	—	—		×			
13	16.728	811.4082	α_{S1}-CN (25–32)	Caprine	GLSPEVPN	—	—		×	×	×	×
14	18.020	559.2625	α_{S1}-CN (41–45)	Bovine	APFPE	DPP-4 inhibitory, GLP-1 secretion enhancing, ACE inhibitory	Boots (2012, 2013); Abdel-Hamid et al. (2017)	×	×	×	×	×
15	18.099	988.5702	α_{S1}-CN (116–123)	Caprine	KKYNVPQL	ACE inhibitory	Ruiz et al. (2004)	×	×	×	×	
16	18.480	601.3050	α_{S1}-CN (51–55)	Caprine	NINEL	—	—				×	×
17	18.492	1,034.5041	α_{S1}-CN (100–108)	Bovine	DVPSERYLG	ACE inhibitory	Gómez-Ruiz et al. (2002)		×			
18	18.709	700.3743	α_{S1}-CN (30–35)	Bovine	VLNENL	ACE inhibitory	Benavente et al. (2018)	×	×	×	×	×
19	18.937	870.4925	α_{S1}-CN (31–37)	Bovine	LNENLLR	DPP-4 inhibitory	Nongonierma et al. (2017b)	×	×	×	×	×
20	19.316	730.3500	α_{S1}-CN (50–55)	Caprine	ENINEL	—	—		×	×		
21	19.776	977.4813	α_{S1}-CN (100–107)	Bovine	DVPSERYL	—	—			×	×	×
22	20.003	1,106.5237	α_{S1}-CN (99–107)	Bovine	EDVPSERYL	—	—		×	×	×	
23	20.008	1,370.8407	α_{S1}-CN (113–123)	Caprine	LRLKKYNVPQL	—	—	×				
24	20.080	569.3157	α_{S1}-CN (119–123)	Caprine	NVPQL	—	—	×	×	×	×	×
25	20.313	721.3631	α_{S1}-CN (107–112)	Bovine	LGYLEQ	—	—	×				
26	20.461	969.5614	α_{S1}-CN (30–37)	Bovine	VLNENLLR	Antibacterial	Hayes et al. (2006)	×	×	×	×	×
27	21.147	658.3328	α_{S1}-CN (40–45)	Bovine	VAPFPE	—	—	×	×	×	×	×
28	21.222	601.3069	α_{S1}-CN (32–36)	Bovine	NENLL	Antioxidant	Bezerra et al. (2013)			×	×	×
29	21.525	531.3072	α_{S1}-CN (38–42)	Caprine	FVVAP	—	—		×	×	×	×
30	22.061	664.3413	α_{S1}-CN (109–113)	Bovine	YLEQL	—	—	×	×	×	×	×
31	22.671	529.2879	α_{S1}-CN (40–44)	Bovine	VAPFP	—	—	×	×	×	×	×
32	22.899	757.4084	α_{S1}-CN (39–45)	Caprine	VVAPFPE	ACE inhibitory	Gómez-Ruiz et al. (2002)	×	×	×	×	×
33	23.813	714.3907	α_{S1}-CN (31–36)	Bovine	LNENLL	—	—	×	×	×	×	×
34	23.963	732.3813	α_{S1}-CN (118–123)	Caprine	YNVPQL	—	—	×	×	×	×	×
35	23.965	531.3034	α_{S1}-CN (39–43)	Caprine	VVAPF	—	—		×	×	×	×
36	24.192	713.3965	α_{S1}-CN (25–31)	Ovine	GLSPEVL	—	—		×	×	×	×
37	24.271	721.3626	α_{S1}-CN (108–113)	Bovine	GYLEQL	—	—	×	×	×	×	×
38	24.347	658.3308	α_{S1}-CN (41–46)	Bovine	APFPEV	—	—	×	×			
39	24.497	1,287.7276	α_{S1}-CN (39–49)	Caprine	VVAPFPEVFRK	—	—	×	×	×		
40	24.652	628.3566	α_{S1}-CN (39–44)	Caprine	VVAPFP	—	—	×	×	×	×	×
41	25.106	904.4794	α_{S1}-CN (32–38)	Bovine	NENLLRF	GLP-1 secretion enhancing	O'Halloran et al. (2018)	×	×	×	×	×
42	25.109	813.4614	α_{S1}-CN (30–36)	Bovine	VLNENLL	ACE inhibitory, antioxidant	Hernández-Ledesma et al. (2005)	×	×	×	×	×
43	25.337	790.4340	α_{S1}-CN (33–38)	Bovine	ENLLRF	ACE inhibitory	Quirós et al. (2005)	×	×	×	×	×
44	25.794	1,060.5695	α_{S1}-CN (37–45)	Caprine	RFVVAPFPE	Antibacterial	Rizzello et al. (2005)	×	×	×	×	

Continued

Table 2 (Continued). α_{S1}-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
45	26.175	757.3989	α_{S1}-CN (40–46)	Bovine	VAPFPEV	ACE inhibitory	Quirós et al. (2005)	×	×	×	×	×
46	26.402	805.4017	α_{S1}-CN (39–45)	Bovine	FVAPFPE	ACE inhibitory, antioxidant	Hernández-Ledesma et al. (2005)	×	×	×	×	×
47	26.941	1,017.5587	α_{S1}-CN (31–38)	Bovine	LNENLLRF	GLP-1 secretion enhancing	O'Halloran et al. (2018)	×				
48	27.315	834.4484	α_{S1}-CN (107–113)	Bovine	LGYLEQL	—	—		×	×	×	×
49	27.547	856.4646	α_{S1}-CN (39–46)	Caprine	VVAPFPEV	Antibacterial	Rizzello et al. (2005)	×	×	×	×	×
50	27.775	1,116.6302	α_{S1}-CN (30–38)	Bovine	VLNENLLRF	ACE inhibitory	Georgalaki et al. (2017)	×	×	×	×	×
51	27.849	579.3052	α_{S1}-CN (39–43)	Bovine	FVAPF	—	—			×		
52	27.925	1,159.6384	α_{S1}-CN (39–48)	Caprine	VVAPFPEVFR	—	—	×	×	×	×	×
53	28.230	676.3577	α_{S1}-CN (39–44)	Bovine	FVAPFP	Skin regenerating	Gorouhi and Maibach (2009)	×	×	×	×	×
54	28.306	904.4663	α_{S1}-CN (38–45)	Caprine	FVVAPFPE	ACE inhibitory	Bounouala et al. (2017)	×	×	×	×	×
55	29.753	678.3716	α_{S1}-CN (38–43)	Caprine	FVVAPF	—	—					×
56	30.058	775.4277	α_{S1}-CN (38–44)	Caprine	FVVAPFP	ACE inhibitory	Bounouala et al. (2017)	×	×	×	×	×
57	30.446	1,680.8338	α_{S1}-CN (100–113)	Bovine	DVPSERYLGYLEQL	—	—	×				
58	31.508	904.4699	α_{S1}-CN (40–47)	Bovine	VAPFPEVF	ACE inhibitory	del Mar Contreras et al. (2013)				×	×
59	32.574	1,003.5378	α_{S1}-CN (39–47)	Bovine	VVAPFPEVF	ACE inhibitory	Espejo-Carpio et al. (2013)	×	×	×	×	
Total								37	45	44	42	39

[1]Protein sequences (NCBI accession IDs: NP_851372, NP_001272624, and NP_001009795).

[2]Bioactive characteristics obtained by BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), EROP-Moscow (http://erop.inbi.ras.ru/), PepBank (http://pepbank.mgh.harvard.edu/), PeptideDB (http://www.peptides.be/), Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/), and Google Scholar (https://scholar.google.com.tr/) online databases. ACE inhibitory = lowering blood pressure (ACE = angiotensin-converting enzyme); GLP-1 secretion enhancing = increasing insulin secretion and decreasing appetite by enhancing glucagon-like peptide-1 secretion; DPP-4 inhibitory = treating type II diabetes by inhibiting dipeptidyl peptidase-4.

[3]× = the identified peptides are present on the relevant ripening day.

(85EIVPN89), originated from the middle part of α_{S1}-CN. This is consistent with previous studies of cheese, which have shown that α_{S1}-CN-derived peptides occur from the amino part of α_{S1}-CN (Sagardia et al., 2013; Fontenele et al., 2017). It has been suggested that there are 3 different hydrophobic regions on α_{S1}-CN, which consist of 1 to 44, 90 to 113, and 132 to 199 fractions (Swaisgood, 2003). α_{S1}-Casein in Tulum cheese was observed to mostly hydrolyze from its hydrophobic regions. This indicates that the hydrophobic region is more sensitive to proteolytic enzymes in Tulum cheese. Miclo et al. (2012) and Chang et al. (2013) revealed that hydrophobic regions of α_{S1}-CN are more sensitive to microbial proteolytic enzymes than are hydrophilic regions.

During the 180 d of the ripening period, the number of α_{S1}-derived peptides varied. In this ripening period, some α_{S1}-CN-derived peptides have been found to be further degraded to AA, some peptides have accumulated in cheese, and others have been hydrolyzed to form new peptides. α_{S1}-Casein-derived peptides generally appeared to occur due to carboxypeptidase and aminopeptidase activity (Table 2). It was determined that 26 of 59 α_{S1}-CN-derived peptides identified in Tulum cheese were constantly present for 180 d of ripening. These peptides included the following sequences: 85EIVPK89, 16RPKHPIKHQGLSPE29, 30VLNENLLRF38, 30VLNENLLR37, 30VLNENLL36, 30VLNENL35, 32NENLLRF38, 33ENLLRF38, 31LNENLLR37, 31LNENLL36, 39VVAPFPEVFR48, 39VVAPFPEV46, 39VVAPFPE45, 40VAPFPEV46, 39VVAPFP44, 38FVVAPFPE45, 38FVVAPFP44, 39FVAPFPE45, 39FVAPFP44, 40VAPFPE45, 40VAPFP44, 41APFPE45, 118YNVPQL123, 119NVPQL123, 108GYLEQL113, and 109YLEQL113. Continuous presence of these peptides during ripening may have resulted from their remarkably high occurrence early in the ripening period, and the cleavage products of some of the preserved peptides at different days of ripening have been identified. All of these accumulated peptides, except 125EIVPK129, were portions of the N-terminal region of α_{S1}-CN. It has been observed that 36% of these accumulated peptides consist of the cleavage products of isracidin, and 64% of them are formed by breakdown of the region following the isracidin component in α_{S1}-CN. These peptides also contained protected sequences, including 32NENL35, 119NVPQL123, 41APFP44, and 109YLEQL113. Gagnaire et al. (2011) also reported that 16RPKHPIKHQ24, 29EVLNENLLRF38, 30VLNENLLRF38, 32NENLLRF38, and 39FVAPFPEVFGKEKV52 peptides, which originate from isracidin and the region following isracidin, accumulate during the ripening of Ragusano cheese for 210 d.

α_{S1}-Casein-derived peptides in Tulum cheeses were found to be 5 to 14 AA residues in length. Among these peptides, pentapeptides and hexapeptides were predominant, followed by heptapeptides and octapeptides. Considering that bioactive peptides are also composed of short peptides containing 2 to 20 AA residues, the potential for these identified peptides to exhibit bioactive properties is very high (Hartmann and Meisel, 2007). Indeed, 7 different biological activities, including angiotensin-converting enzyme (**ACE**) inhibition, antibacterial action, antioxidation, glucagon-like peptide-1 (**GLP-1**) secretion enhancement, dipeptidyl peptidase 4 (**DPP-4**) inhibition, metal chelation, and skin regeneration, were determined for some of the identified α_{S1}-CN-derived peptides using online databases (Table 2). The numbers of ACE inhibitory peptides were higher among bioactive peptides, and 18 peptides derived from α_{S1}-CN showed antihypertensive properties. Additionally, different ACE inhibitor peptides have been found to occur with the advancement of ripening.

Peptides Derived from α_{S2}-CN. α_{S2}-Casein-derived peptides in WSE of Tulum cheeses are shown in Table 3. During the 180 d of the ripening period, 11 α_{S2}-CN-derived peptides were identified by LC-MS/MS. These peptides originated from the middle and C-terminal regions of α_{S2}-CN. This result is consistent with other studies on cheese (Gagnaire et al., 2011; Sagardia et al., 2013; Pisanu et al., 2015; Fontenele et al., 2017). α_{S2}-Casein has been reported to be more resistant to chymosin enzyme than α_{S1}-CN but sensitive to plasmin enzyme (Ardö et al., 2017). In that sense, plasmin enzyme, which is the natural enzyme of milk, has been suggested to be active on α_{S2}-CN, especially hydrolyzing peptide bonds of lysine and to a lesser extent those of arginine (Fox et al., 2017). Dalsgaard et al. (2008) revealed that peptides are formed mostly from the C-terminal and middle regions of α_{S2}-CN. In addition, Larsen et al. (1996) reported that cathepsin D, another enzyme naturally found in milk, is effective with leucine and tyrosine bonds located in the middle and C-terminal regions of α_{S2}-CN. Peptides identified from the middle and C-terminal region of α_{S2}-CN in Tulum cheeses support the activity of these enzymes. Additionally, microbial proteolytic enzymes may play a role in the advanced hydrolysis of α_{S2}-CN-derived peptides during ripening (Taivosalo et al., 2018).

In Emmental cheeses ripened for 50 d (Gagnaire et al., 2001), Idiazabal cheeses ripened for 120 d (Sagardia et al., 2013), Ragusano cheese ripened for 210 d (Gagnaire et al., 2011), and Manchego cheeses ripened for 240 d (Gómez-Ruiz et al., 2004), a total of 9, 11, 17, and 12 α_{S2}-CN-derived peptides were identified,

Table 3. α_{s2}-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
1	4.382	755.3975	α_{S2}-CN (207–212)	Caprine	KPWTQP	—	—					×
2	18.176	689.3381	α_{S2}-CN (132–138)	Caprine	AGPFTPT	—	—		×	×	×	×
3	18.484	831.4574	α_{S2}-CN (215–221)	Caprine	NAIPYVR	—	—	×				
4	18.941	972.4714	α_{S2}-CN (104–110)	Bovine	YQKFPQY	Antioxidant, ACE inhibitory	Silva et al. (2006); del Mar Contreras et al. (2013)	×				
5	21.832	1,799.0249	α_{S2}-CN (204–218)	Bovine	KAMKPWIQPKTKVIP	Antibacterial	Alvarez-Ordóñez et al. (2013)		×			
6	23.885	1,744.8836	α_{S2}-CN (205–219)	Caprine	AMKPWTQPKTNAIPY	—	—	×				
7	24.499	1,648.9044	α_{S2}-CN (116–129)	Bovine	QGPIVLNPWDQVKR	—	—		×	×	×	×
8	25.487	1,811.9625	α_{S2}-CN (115–129)	Bovine	YQGPIVLNPWDQVKR	ACE inhibitory	Meira et al. (2012)	×	×	×	×	
9	25.565	1,813.9680	α_{S2}-CN (175–188)	Caprine	NRLNFLKKISQYYQ	—	—		×			
10	26.785	1,492.8081	α_{S2}-CN (116–128)	Bovine	QGPIVLNPWDQVK	—	—					×
11	30.134	894.4950	α_{S2}-CN (117–124)	Bovine	GPIVLNPW	—	—					×
Total								4	5	3	3	5

[1]Protein sequences (NCBI Accession IDs: NP_776953, NP_001272514, and NP_001009363).

[2]Bioactive characteristics obtained by BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), EROP-Moscow (http://erop.inbi.ras.ru/), PepBank (http://pepbank.mgh.harvard.edu/), PeptideDB (http://www.peptides.be/), Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/), and Google Scholar (https://scholar.google.com.tr/) online databases. ACE inhibitory = lowering blood pressure (ACE = angiotensin-converting enzyme).

[3]× = the identified peptides are present on the relevant ripening day.

respectively. The 115YQGPIVLNPWDQVKR129 peptide determined in Tulum cheeses was also observed in Ragusano cheeses (Gagnaire et al., 2011). In addition, the NAIPYVR peptide identified from Tulum cheeses was also found in Idiazabal cheeses (Sagardia et al., 2013). However, in general, α_{S2}-CN-derived peptides identified in Tulum cheeses differ from these cheeses. It is thought that the differences observed in the numbers and variety of α_{S2}-CN-derived peptides in different kinds of cheeses depend on the type of milk used in production, the activity of natural enzymes in milk, the type and amount of coagulant, the usage of starter cultures, and the activity of proteolytic enzymes belonging to microbiota.

Swaisgood (2003) reported that there are 2 hydrophobic areas on the α_{S2}-CN, consisting of 90 to 120 and 160 to 207 regions. In Tulum cheeses, 2 peptides [α_{S2}-CN (104 YQKFPQY110) and α_{S2}-CN (175NRLNFLKKISQYYQ188)] derive from the hydrophobic region and 3 peptides [α_{S2}-CN (132AGPFTPT138), α_{S2}-CN (207KPWTQP212), and α_{S2}-CN (215NAIPYVR221)] originate from the hydrophilic region. In addition, 6 peptides [α_{S2}-CN (115YQGPIVLNPWDQVKR129), α_{S2}-CN (116QGPIVLNPWDQVK128), α_{S2}-CN (116 QGPIVLNPWDQVKR129), α_{S2}-CN (117GPIVLNPW124), α_{S2}-CN (204KAMKPWIQPKTKVIP218), and α_{S2}-CN (205AMKPWTQPKTNAIPY219)] have been found to consist of sequences containing both hydrophobic and hydrophilic regions. Thus, a wide variety of proteolytic enzyme activities have been observed on α_{S2}-CN in Tulum cheeses.

During the 180 d of the ripening period of Tulum cheeses, the number of α_{S2}-CN-derived peptides showed a fluctuating course, and the peptide counts on all ripening days were found to be close to each other in value. However, within this ripening period, it was observed that some peptides disappeared due to advanced hydrolysis, and some of them newly formed. Over the ripening period of Tulum cheeses, the number of α_{S2}-CN-derived peptides did not increase as in α_{S1}- and β-CN-derived peptides. This result may be due to the fact that α_{S2}-CN is more resistant to proteolytic enzymes, such as chymosin and microbial enzymes found in Tulum cheeses, than α_{S1}- and β-CN. Bhat et al. (2016) reported that disulfide bonds in α_{S2}-CN provide a high level of stability against proteolytic enzymes. Additionally, endogenous plasmin and cathepsin D enzymes involved in the hydrolysis of α_{S2}-CN show optimum activity at pH 4.0 and 5.0 to 5.2, respectively (Larsen et al., 1996; Larsson et al., 2006; Fox et al., 2017). On this basis, the pH value between 4.74 and 5.12 of Tulum cheeses during the ripening period may have limited the activity of plasmin and cathepsin D on α_{S2}-CN.

It has been determined that α_{S2}-CN-derived peptides defined in WSE of Tulum cheese during the 180 d of the ripening period are 6 to 15 AA in length. Among these peptides, heptapeptides and pentadecapeptides were found to be predominant. Some of these peptides exhibited antibacterial, ACE inhibitory, and antioxidant activities (Table 3). Compared with α_{S1}- and β-CN-derived peptides, less variety of biological activity was observed with α_{S2}-CN-derived peptides.

Peptides Derived from β-CN

It has been reported that hydrolysis of β-CN with chymosin is very limited in cheeses, and β-CN is generally broken down in cheeses due to the activity of the endogenous milk enzymes (Ardö et al., 2017; Taivosalo et al., 2018). With respect to this limited effect of chymosin on β-CN, Fox et al. (2017) asserted that β-CN is highly hydrophobic compared with other caseins and that intermolecular hydrophobic interactions may prevent chymosin from accessing bonds in β-CN. For plasmin enzyme, mostly peptides with long AA chains have been reported to form from β-CN with the activity of this enzyme (Dalsgaard et al., 2008). In addition to the endogenous enzymes in milk such as plasmin and cathepsin D, proteolytic enzymes of the microbiota in the cheese during ripening may play a role in the advanced hydrolysis of β-CN (Sadat-Mekmene et al., 2013; Solieri et al., 2018). Long or medium peptides, which are formed by the activity of enzymes in coagulants or endogenous enzymes in milk, are broken down into oligopeptides by microbial proteinases, and oligopeptides are further hydrolyzed by microbial peptidases (Ardö et al., 2017).

β-Casein-derived peptides identified by LC-MS/MS in WSE of Tulum cheeses across the ripening period are shown in Table 4. A total of 129 types of β-CN-derived peptides have been identified during the 180 d of the ripening period. The count of β-CN-derived peptides was rather higher than that of α_{S1}-, α_{S2}-, and κ-CN-derived peptides identified in Tulum cheeses. The proteolytic system of Tulum cheeses appears to be more effective on β-CN. This can be related to proteolytic enzymes of cheese microbiota. Indeed, most studies have claimed that microbial enzymes are more active on β-CN than on other types of caseins (Requena et al., 1993; Igoumenidou et al., 2005; Veljovic et al., 2009; Miclo et al., 2012). According to Table 4, most of the β-CN-derived peptides identified in Tulum cheeses occurred as a result of carboxypeptidase, aminopeptidase, dipeptidase, and endopeptidase activities of cheese microbiota.

Gagnaire et al. (2001) identified 28 β-CN-derived peptides during the 50 d of ripening of Emmental cheeses produced using coagulant of fungal origin, thermophilic lactic acid bacteria, and propionic acid bacteria. In addition, Sadat-Mekmene et al. (2013) detected 215 kinds of β-CN-derived peptides over 41 d of ripening of Emmental cheeses produced using liquid calf rennet and starter cultures consisting of *Lactobacillus helveticus*, *Streptococcus thermophilus,* and propionic acid bacteria. Moreover, 17 β-CN-derived peptides were observed in Manchego cheeses produced using pasteurized sheep milk, and 65 β-CN-derived peptides were identified in Idiazabal cheese produced using raw sheep milk (Gómez-Ruiz et al., 2004; Sagardia et al., 2013). From these studies, it is clear that choices of coagulant, starter culture, and heat treatment affect the number of β-CN-derived peptides. Thus, the β-CN-derived peptides identified in Tulum cheeses can serve as markers to determine the specificity of this cheese depending on the details of its production method.

The majority of peptides (66) in Tulum cheeses occurred by hydrolysis of the hydrophobic C-terminal region of β-CN, followed by peptides from the middle (46) and N-terminal (17) regions of β-CN. Microbial proteolytic enzymes may have been involved in the formation of peptides from the N-terminal, middle, and C-terminal regions of β-CN in Tulum cheeses. Taivosalo et al. (2018) have reported that proteolytic enzymes of various lactic acid bacteria hydrolyze β-CN in Old Saare cheese from several regions including N-terminal, middle, and C-terminal areas. In other studies, the N-terminal region of β-CN has been reported to be resistant to hydrolysis by microbial proteolytic enzymes (Miclo et al., 2012; Chang et al., 2013; Sadat-Mekmene et al., 2013). It is speculated that endogenous enzymes in milk are also involved in the formation of these peptides in Tulum cheese. The plasmin enzyme has been reported to hydrolyze β-CN generally from the N-terminal region (Egito et al., 2003). Thus, peptides that originate from the N-terminal region of β-CN in Tulum cheeses may also have been formed by the activity of the plasmin enzyme. Moreover, given that cathepsin D, another natural enzyme of milk, mostly hydrolyzes β-CN from its C terminal region (Larsen et al., 1996), peptides originating from the C-terminal region of β-CN in Tulum cheeses may have arisen by the activity of the cathepsin D enzyme.

Swaisgood (2003) reported that the region containing the first 21 AA residues of β-CN is hydrophilic and that the part following this region is hydrophobic. In Tulum cheeses, all β-CN-derived peptides, except β-CN (17EQEEL21), were from the hydrophobic region.

Table 4. β-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
1	3.928	608.3279	β-CN (62–66)	Ovine	DKIHP	ACE inhibitory	Gómez-Ruiz et al. (2006)					×
2	4.309	597.3116	β-CN (78–83)	Ovine	TGPIPN	ACE inhibitory	Geerlings et al. (2006)				×	×
3	4.459	755.3967	β-CN (62–67)	Ovine	DKIHPF	ACE inhibitory	Gobbetti et al. (2000)	×	×	×	×	×
4	4.837	552.3232	β-CN (186–190)	Ovine	LPVPQ	DPP-4 inhibitory, ACE inhibitory	Cadee and Mallee (2010); Nongonierma and FitzGerald (2016)	×				
5	4.995	646.2810	β-CN (17–21)	Ovine	EQEEL	—	—		×	×	×	×
6	5.144	509.3167	β-CN (97–101)	Ovine	VVVPP	ACE inhibitory	Gómez-Ruiz et al. (2006); Quirós et al. (2009)					×
7	14.302	1,100.5611	β-CN (195–204)	Caprine	HQPPQPLSPT	—	—		×			
8	14.683	978.5120	β-CN (59–66)	Ovine	ELQDKIHP	ACE inhibitory	van Elswijk et al. (2003)		×	×	×	×
9	15.204	1,231.5981	β-CN (194–204)	Caprine	MHQPPQPLSPT	—	—	×	×	×	×	
10	15.436	1,309.7149	β-CN (226–236)	Caprine	KVVPQRDMPIQ	—	—	×				
11	16.194	602.2720	β-CN (197–201)	Ovine	DMPIQ	Antioxidant	Di Pierro et al. (2014)		×	×	×	
12	16.808	1,093.5411	β-CN (58–66)	Ovine	DELQDKIHP	—	—	×				
13	17.349	1,175.5792	β-CN (121–129)	Ovine	HKEMPFPKY	—	—	×	×			
14	18.173	1,091.6334	β-CN (181–190)	Ovine	SQPKVLPVPQ	—	—			×	×	×
15	18.630	651.3947	β-CN (185–190)	Ovine	VLPVPQ	—	—			×	×	×
16	18.706	996.5398	β-CN (60–67)	Ovine	LQDKIHPF	—	—		×	×		
17	19.546	1,642.9743	β-CN (216–230)	Caprine	SQPKVLPVPQKVVPQ	—	—	×	×	×	×	
18	19.620	707.4191	β-CN (95–101)	Ovine	TPVVVPP	DPP-4 inhibitory	Nongonierma and FitzGerald (2016)					×
19	19.697	1,815.0666	β-CN (179–195)	Ovine	SLSQPKVLPVPQKAVPQ	—	—	×	×	×		
20	19.864	1,132.5995	β-CN (138–147)	Ovine	QSLTLTDVEK	—	—		×	×	×	×
21	19.928	744.3813	β-CN (77–83)	Ovine	FTGPIPN	ACE inhibitory	Bounouala et al. (2017)	×	×	×	×	×
22	20.233	1,125.5800	β-CN (59–67)	Ovine	ELQDKIHPF	Antibacterial	Losito et al. (2006)	×	×	×	×	×
23	20.230	825.4517	β-CN (212–219)	Ovine	GPVRGPFP	ACE inhibitory	Hong et al. (2008)			×	×	×
24	20.458	917.5062	β-CN (141–148)	Ovine	TLTDVEKL	ACE inhibitory	Aslam et al. (2018)					×
25	20.534	1,204.7184	β-CN (180–190)	Ovine	LSQPKVLPVPQ	—	—	×				
26	20.839	575.3427	β-CN (149–153)	Ovine	HLPLP	ACE inhibitory	Sánchez-Rivera et al. (2016)	×	×	×	×	×
27	21.151	1,843.0893	β-CN (214–230)	Caprine	SLSQPKVLPVPQKVVPQ	—	—	×				
28	21.220	1,291.7443	β-CN (179–190)	Ovine	SLSQPKVLPVPQ	—	—	×	×	×	×	×
29	21.299	963.5753	β-CN (181–189)	Ovine	SQPKVLPVP	—	—					×

Continued

Table 4 (Continued). β-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
30	21.601	577.3119	β-CN (72–76)	Ovine	SLVYP	ACE inhibitory	Kohmura et al. (1990)	×	×	×	×	×
31	21.680	841.4336	β-CN (76–83)	Ovine	PFTGPIPN	—	—		×	×	×	×
32	21.982	1,240.6131	β-CN (58–67)	Ovine	DELQDKIHPF	ACE inhibitory	Aslam et al. (2019)	×	×	×	×	×
33	22.135	1,040.5078	β-CN (110–118)	Bovine	YPFPGPIHN	Opioid	Jinsmaa and Yoshikawa (1999)			×		
34	22.287	587.2952	β-CN (130–134)	Ovine	PVEPF	—	—		×	×	×	×
35	22.823	523.3384	β-CN (185–189)	Ovine	VLPVP	ACE inhibitory	Huang et al. (2017)			×	×	×
36	23.128	630.3370	β-CN (77–82)	Ovine	FTGPIP	—	—				×	×
37	23.433	1,163.6924	β-CN (179–189)	Ovine	SLSQPKVLPVP	—	—		×	×	×	×
38	23.585	938.5335	β-CN (211–219)	Ovine	LGPVRGPFP	ACE inhibitory	Villegas et al. (2014)			×	×	×
39	23.887	1,744.8688	β-CN (121–134)	Ovine	HKEMPFPKYPVEPF	—	—	×	×			
40	23.890	940.5490	β-CN (210–218)	Ovine	VLGPVRGPF	—	—		×	×	×	×
41	23.963	1,380.7032	β-CN (76–88)	Ovine	PFTGPIPNSLPQN	—	—					×
42	23.966	1,404.8321	β-CN (178–190)	Ovine	LSLSQPKVLPVPQ	—	—					×
43	24.118	1,465.7373	β-CN (110–122)	Bovine	YPFPGPIHNSLPQ	Opioid	Jinsmaa and Yoshikawa (1999)		×			
44	24.344	1,025.5380	β-CN (109–117)	Bovine	VYPFPGPIH	Antiamnesic, prolyl endopeptidase inhibitory	Asano et al. (1992)			×	×	×
45	24.575	1,037.6008	β-CN (210–219)	Ovine	VLGPVRGPFP	ACE inhibitory	Miguel et al. (2006); Quirós et al. (2007)		×	×	×	×
46	25.414	938.5334	β-CN (212–220)	Ovine	GPVRGPFPI	Antibacterial	Losito et al. (2006)		×	×	×	×
47	25.414	1,002.5867	β-CN (149–157)	Ovine	HLPLPLVQS	—	—					×
48	25.639	1,294.7033	β-CN (207–218)	Ovine	QEPVLGPVRGPF	Immunomodulatory	Pisanu et al. (2015)					×
49	25.715	1,166.6441	β-CN (208–218)	Ovine	EPVLGPVRGPF	—	—		×	×	×	×
50	25.642	783.4487	β-CN (84–90)	Ovine	SLPQNIL	—	—					×
51	25.794	1,245.6812	β-CN (138–148)	Ovine	QSLTLTDVEKL	—	—			×	×	×
52	25.868	890.5229	β-CN (120–127)	Bovine	LPQNIPPL	DPP-4 inhibitory	Uenishi et al. (2012)				×	
53	25.869	793.4698	β-CN (86–92)	Ovine	PQNILPL	—	—			×	×	
54	25.947	915.5538	β-CN (149–156)	Ovine	HLPLPLVQ	—	—				×	×
55	26.096	1,391.7559	β-CN (207–219)	Ovine	QEPVLGPVRGPFP	ACE inhibitory	Villegas et al. (2014)	×	×	×	×	×
56	26.249	1,263.7001	β-CN (208–219)	Ovine	EPVLGPVRGPFP	ACE inhibitory	Hayes et al. (2007)	×	×	×	×	×
57	26.404	688.4269	β-CN (148–153)	Ovine	LHLPLP	ACE inhibitory	Quirós et al. (2007)	×	×	×	×	×
58	26.633	1,457.7681	β-CN (206–218)	Ovine	YQEPVLGPVRGPF	ACE inhibitory	Torres-Llanez et al. (2011)		×	×	×	×
59	26.782	1,138.6165	β-CN (108–117)	Bovine	LVYPFPGPIH	ACE inhibitory	Pihlanto et al. (2010)	×	×	×	×	×

Continued

Table 4 (Continued). β-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
60	26.937	529.2899	β-CN (216–220)	Ovine	GPFPI	Cathepsin B inhibitory	Lee and Lee (2000)			×	×	×
61	27.013	1,554.8143	β-CN (206–219)	Ovine	YQEPVLGPVRGPFP	—	—	×	×	×	×	×
62	27.010	796.4489	β-CN (99–105)	Ovine	VPPFLQP	ACE inhibitory	Ueno et al. (2004)					×
63	27.087	820.3792	β-CN (197–203)	Ovine	DMPIQAF	Anti-inflammatory	Altmann et al. (2016)				×	
64	27.161	1,156.7304	β-CN (147–156)	Ovine	KLHLPLPLVQ	—	—	×				
65	27.392	1,225.6480	β-CN (107–117)	Bovine	SLVYPFPGPIH	—	—		×	×	×	
66	27.544	656.3890	β-CN (97–102)	Ovine	VVVPPF	ACE inhibitory	Quirós et al. (2009)			×	×	×
67	27.775	1,051.6185	β-CN (211–220)	Ovine	LGPVRGPFPI	DPP-4 inhibitory	Nongonierma et al. (2017a)		×	×	×	×
68	28.080	1,667.8976	β-CN (205–219)	Ovine	LYQEPVLGPVRGPFP	DPP-4 inhibitory	Nongonierma et al. (2017a)		×	×	×	×
69	28.385	1,150.6847	β-CN (210–220)	Ovine	VLGPVRGPFPI	—	—	×	×	×	×	×
70	28.610	1,350.6642	β-CN (124–134)	Ovine	MPFPKYPVEPF	—	—			×	×	×
71	28.690	568.3583	β-CN (88–92)	Ovine	NILPL	Antibacterial	Losito et al. (2006)				×	×
72	28.842	1,083.5970	β-CN (93–102)	Ovine	TQTPVVVPPF	Antioxidant	Vavrusova et al. (2015)	×	×	×	×	×
73	28.991	854.4900	β-CN (95–102)	Ovine	TPVVVPPF	Antioxidant	Vavrusova et al. (2015)			×	×	×
74	29.147	1,115.6722	β-CN (148–157)	Ovine	LHLPLPLVQS	—	—			×	×	×
75	29.299	1,504.8336	β-CN (207–220)	Ovine	QEPVLGPVRGPFPI	—	—	×	×	×	×	×
76	29.375	637.3468	β-CN (73–77)	Ovine	LVYPF	—	—			×	×	×
77	29.601	1,755.9210	β-CN (73–88)	Ovine	LVYPFTGPIPNSLPQN	—	—					×
78	29.604	1,376.7719	β-CN (208–220)	Ovine	EPVLGPVRGPFPI	Immunomodulatory	Kruzel (2011)	×	×	×	×	×
79	29.756	1,780.9879	β-CN (204–219)	Ovine	LLYQEPVLGPVRGPFP	ACE inhibitory	Bounouala et al. (2017)	×	×	×	×	×
80	29.829	551.3693	β-CN (150–154)	Ovine	LPLPL	DPP-4 inhibitory	Nongonierma and FitzGerald (2016)		×	×	×	×
81	29.830	1,028.6371	β-CN (148–156)	Ovine	LHLPLPLVQ	—	—	×	×	×		
82	29.908	724.3817	β-CN (72–77)	Ovine	SLVYPF	ACE inhibitory	Kohmura et al. (1990)	×	×	×	×	×
83	30.061	1,667.9000	β-CN (206–220)	Ovine	YQEPVLGPVRGPFPI	Antibacterial	Birkemo et al. (2009)	×	×	×	×	×
84	30.210	1,123.6271	β-CN (97–106)	Ovine	VVVPPFLQPE	Brain function improving, antiamnesic	Ohsawa et al. (2013)		×	×	×	×
85	30.363	801.5106	β-CN (148–154)	Ovine	LHLPLPL	ACE inhibitory	Miguel et al. (2006)	×	×	×	×	
86	30.366	897.5346	β-CN (97–104)	Ovine	VVVPPFLQ	—	—					×
87	30.591	2,403.3674	β-CN (93–114)	Ovine	TQTPVVVPPFLQPEIMGVPKVK	—	—					×
88	30.592	1,164.6950	β-CN (211–221)	Ovine	LGPVRGPFPIL	—	—				×	×
89	30.670	1,188.6617	β-CN (149–158)	Ovine	HLPLPLVQSW	ACE inhibitory	Aslam et al. (2019)	×	×	×	×	×
90	30.744	2,073.2097	β-CN (96–114)	Ovine	PVVVPPFLQPEIMGVPKVK	—	—					×

Continued

Table 4 (Continued). β-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
91	30.746	915.5506	β-CN (182–189)	Bovine	NLHLPLPL	Anticarcinogenic, ACE inhibitory	Robert et al. (2004); Juillerat-Jeanneretet et al. (2010)	×	×	×	×	
92	30.899	1,550.8302	β-CN (93–106)	Ovine	TQTPVVVPPFLQPE	Antioxidant	Farvin et al. (2010)	×	×	×	×	×
93	30.973	1,324.7440	β-CN (93–104)	Ovine	TQTPVVVPPFLQ	—	—			×		
94	30.974	1,780.9885	β-CN (205–220)	Ovine	LYQEPVLGPVRGPFPI	Antioxidant	Ha et al. (2015)	×	×	×	×	
95	31.048	1,321.7320	β-CN (95–106)	Ovine	TPVVVPPFLQPE	Anti-inflammatory, antioxidant	Bamdad et al. (2017)					×
96	31.049	994.5891	β-CN (97–105)	Ovine	VVVPPFLQP	Antibacterial	Rana et al. (2018)				×	×
97	31.201	1,220.6816	β-CN (96–106)	Ovine	PVVVPPFLQPE	Antibacterial	Almaas et al. (2011)		×	×	×	×
98	31.284	2,516.4505	β-CN (92–114)	Ovine	LTQTPVVVPPFLQPEIMGVPKVK	—	—	×				
99	31.432	1,263.7700	β-CN (210–221)	Ovine	VLGPVRGPFPIL	—	—	×	×	×	×	×
100	31.661	1,421.7936	β-CN (93–105)	Ovine	TQTPVVVPPFLQP	—	—	×	×	×	×	×
101	31.737	1,663.9188	β-CN (92–106)	Ovine	LTQTPVVVPPFLQPE	Brain function improving, antiamnesic	Ohsawa et al. (2013)					×
102	31.887	1,192.6860	β-CN (95–105)	Ovine	TPVVVPPFLQP	ACE inhibitory	Abubakar et al. (1998)					×
103	31.966	1,617.9264	β-CN (207–221)	Ovine	QEPVLGPVRGPFPIL	—	—	×	×	×	×	×
104	32.191	1,091.6383	β-CN (96–105)	Ovine	PVVVPPFLQP	—	—			×	×	×
105	32.266	1,489.8661	β-CN (208–221)	Ovine	EPVLGPVRGPFPIL	ACE inhibitory	Miguel et al. (2006)	×	×	×	×	×
106	32.344	1,760.9722	β-CN (91–106)	Ovine	PLTQTPVVVPPFLQPE	Brain function improving, antiamnesic	Uchida et al. (2012)				×	×
107	32.347	1,894.0730	β-CN (204–220)	Ovine	LLYQEPVLGPVRGPFPI	ACE inhibitory	Bounouala et al. (2017)	×	×	×	×	×
108	32.496	1,534.8757	β-CN (92–105)	Ovine	LTQTPVVVPPFLQP	—	—					×
109	32.651	1,780.9867	β-CN (206–221)	Ovine	YQEPVLGPVRGPFPIL	Antihypercholesterolemic, antioxidant	Mahdi et al. (2017, 2018)	×	×	×	×	×
110	32.801	1,406.8172	β-CN (90–102)	Ovine	LPLTQTPVVVPPF	—	—					×
111	33.106	1,631.9235	β-CN (91–105)	Ovine	PLTQTPVVVPPFLQP	—	—					×
112	33.108	1,196.6829	β-CN (93–103)	Ovine	TQTPVVVPPFL	—	—		×	×	×	×
113	33.337	1,301.7536	β-CN (148–158)	Ovine	LHLPLPLVQSW	—	—	×	×	×	×	×
114	33.716	1,028.6390	β-CN (182–190)	Bovine	NLHLPLPLL	—	—	×				
115	33.718	1,663.9210	β-CN (93–107)	Ovine	TQTPVVVPPFLQPEI	—	—	×	×	×	×	×
116	33.792	2,085.1526	β-CN (123–141)	Bovine	NIPPLTQTPVVVPPFLQPE	Brain function improving	Ohsawa et al. (2018)		×	×		
117	34.325	1,333.7560	β-CN (96–107)	Ovine	PVVVPPFLQPEI	—	—			×	×	×
118	34.404	1,776.9948	β-CN (92–107)	Ovine	LTQTPVVVPPFLQPEI	—	—				×	×
119	34.477	1,519.8956	β-CN (89–102)	Ovine	ILPLTQTPVVVPPF	—	—	×	×	×	×	×
120	34.636	2,007.1600	β-CN (204–221)	Ovine	LLYQEPVLGPVRGPFPIL	—	—	×				
121	34.785	1,794.9573	β-CN (93–108)	Ovine	TQTPVVVPPFLQPEIM	ACE inhibitory	Pisanu et al. (2015)	×	×	×	×	×
122	35.163	1,987.1498	β-CN (89–106)	Ovine	ILPLTQTPVVVPPFLQPE	—	—	×	×	×	×	×
123	35.392	1,464.8084	β-CN (96–108)	Ovine	PVVVPPFLQPEIM	—	—			×	×	×
124	35.546	1,908.0477	β-CN (92–108)	Ovine	LTQTPVVVPPFLQPEIM	—	—				×	×

Continued

Table 4 (Continued). β-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)			
								7	30	60	90	180
125	35.711	1,761.0407	β-CN (89–104)	Ovine	ILPLTQTPVVVPPFLQ	—	—		×	×	×	×
126	35.925	1,858.0974	β-CN (89–105)	Ovine	ILPLTQTPVVVPPFLQP	—	—		×	×	×	×
127	36.842	2,288.2804	β-CN (89–109)	Ovine	ILPLTQTPVVVPPFLQPEIMG	—	—	×				
128	36.916	1,632.9815	β-CN (89–103)	Ovine	ILPLTQTPVVVPPFL	—	—			×	×	×
129	36.994	2,231.2656	β-CN (89–108)	Ovine	ILPLTQTPVVVPPFLQPEIM	—	—			×	×	×
Total								51	66	83	88	99

[1]Protein sequences (NCBI accession IDs: XP_010804480, XP_005681778, and CAA34502).

[2]Bioactive characteristics obtained by BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), EROP-Moscow (http://erop.inbi.ras.ru/), PepBank (http://pepbank.mgh.harvard.edu/), PeptideDB (http://www.peptides.be/), Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/), and Google Scholar (https://scholar.google.com.tr/) online databases. ACE inhibitory = lowering blood pressure (ACE = angiotensin-converting enzyme); DPP-4 inhibitory = treating type II diabetes by inhibiting dipeptidyl peptidase-4; opioid = tranquillizer; prolyl endopeptidase inhibitor = antiamnesic; immunomodulatory = modulating of immune system; antiamnesic = preventing forgetfulness; cathepsin B inhibitor = inhibiting pathological diseases; anti-inflammatory = preventing inflammation; anticarcinogenic = preventing cancer; antihypercholesterolemic = lowering cholesterol.

[3]× = the identified peptides are present on the relevant ripening day.

This indicates that the hydrophobic region of β-CN is more sensitive to proteolytic enzymes present in Tulum cheese than is the hydrophilic region. β-Casein-derived peptides from the hydrophobic region were found to be between 58 and 221 AA residues in length. Similar to our findings, the hydrophobic region of β-CN has been reported to be more sensitive to cell wall-associated proteinases from *Strep. thermophilus* and *Lb. helveticus* than the hydrophilic region (Miclo et al., 2012; Sadat-Mekmene et al., 2013).

It was determined that the β-CN-derived peptides identified during the 180 d of the ripening period of Tulum cheese had a length of 5 to 23 AA. These peptides have been observed to have longer chains compared with α_{S1}- and α_{S2}-CN-derived peptides. In particular, unlike α_{S1}- and α_{S2}-CN-derived peptides, 26 β-CN-derived peptides with AA length of 15 to 23 were identified in Tulum cheeses. It was found that long peptides generally arose from the middle region of β-CN, followed by the C-terminal region. Moreover, short-chain peptides originated from all regions of β-CN. Pentapeptides and undecapeptides were found to be dominant in β-CN-derived peptides identified in Tulum cheeses, followed by nonapeptides and decapeptides.

The number of β-CN-derived peptides determined in Tulum cheeses increased significantly with the progress of ripening. A total of 51 β-CN-derived peptides were identified at the beginning of the ripening period, and 99 β-CN-derived peptides were observed on d 180. The findings of the current study are consistent with those of Taivosalo et al. (2018), who found that the number of β-CN-derived peptides increased continuously until mo 3 of the ripening period of Old Saare cheese. On the other hand, the number of β-CN-derived peptides was found to decrease with the 210 d of the ripening period in Ragusano cheese, which is a pasta filata cheese. These differences among cheese varieties in the numbers of β-CN-derived peptides during the ripening period can be explained by the observations that some peptides accumulate in cheese, some peptides are formed as a result of hydrolysis of available peptides, and others are further degraded to AA. This illustrates the differences in the proteolytic systems of cheese varieties.

It was determined that 32 peptides from among the β-CN-derived peptides identified in Tulum cheeses were found to be constantly available throughout the ripening period (Table 4). Hydrolysis products of some of these peptides on different days of the ripening period were also determined, and it is thought that the permanent observation of these protected peptides during ripening was caused by their very high occurrence at the beginning of the ripening period. The vast majority of these preserved peptides originated from the C-terminal region of β-CN. In addition, most of

these peptide sequences was found to end with hydrophobic AA. Furthermore, these protected peptides were observed to contain protected sequences, including β-CN (62DKIHPF67), β-CN (72SLVYP76), β-CN (93TQTPVVVPPF102), β-CN (149 HLPLP153), and β-CN (210VLGPVRGPFP219). β-Casein-derived phosphopeptides have been reported to generally accumulate in Old Saare cheese (Taivosalo et al., 2018). Phosphopeptides were not additionally investigated in this study; however, some preserved peptides in Tulum cheeses were found to contain serine, tyrosine, or threonine AA that have the potential to be phosphorylated. Therefore, phosphorylation may have occurred in these peptides.

In some of the β-CN-derived peptides identified in Tulum cheeses, 13 different biological activities, including ACE inhibition, antibacterial action, antioxidation, DPP-4 inhibition, cathepsin B inhibition, immunomodulation, antihypercholesterolemic activity, opioid action, anti-inflammatory activity, anticarcinogenesis, prolyl endopeptidase inhibition, brain function improvement, and properties were determined (Table 4). Among the β-CN-derived peptides with biological activity, ACE inhibitory peptides were found to have the highest numbers, followed by antioxidant, antibacterial, and DPP-4 inhibitory peptides, respectively. Compared with the α_{S1}- and α_{S2}-CN-derived peptides, β-CN-derived peptides have been observed to show more diverse biological activity in Tulum cheeses.

Peptides Derived from κ-CN

As a result of the breakdown of κ-CN with rennet, hydrophilic glycomacropeptide from the C-terminal end and hydrophobic para-κ-CN from the N-terminal end occur in cheese production (Ardö et al., 2017). Generally, the hydrophilic glycomacropeptide moves away with whey, whereas the cheese contains para-κ-CN. It has been reported that the para-κ-CN remaining in cheese is not hydrolyzed with chymosin but may be hydrolyzed in cheese during ripening by endogenous enzymes or microbial enzymes (Fox et al., 2017).

κ-Casein-derived peptides identified by LC-MS/MS in WSE of Tulum cheeses during ripening are presented in Table 5. During the 180 d of the ripening period, a total of 4 κ-CN-derived peptides were detected. One of these peptides arose from the hydrophilic region, and 3 arose from the hydrophobic region of κ-CN. Peptides in the hydrophobic region generally originated from regions close to the C-terminal region of the para-κ-CN. On the other hand, peptides in the hydrophilic region were determined to be derived from the N-terminal region of the glycomacropeptide. Likewise, peptides originating from the hydrophobic region of κ-CN were

Table 5. κ-Casein-derived peptides identified by liquid chromatography–tandem MS in water-soluble extracts of Tulum cheeses during the ripening period

No.	Retention time (min)	Observed mass (Da)	Residues[1]	Derived species	Sequence	Bioactivity[2]	Reference	Ripening period[3] (d)				
								7	30	60	90	180
1	15.216	569.3167	κ-CN (98–102)	Caprine	QVLPN	—		×	×			
2	19.940	756.4014	κ-CN (139–145)	Ovine	EIPAINT	—			×			
3	20.916	632.3514	κ-CN (47–51)	Bovine	IPIQY	DPP-4 inhibitory, antioxidant	Nongonierma and FitzGerald (2014)	×	×	×		
4	22.592	651.3246	κ-CN (77–81)	Bovine	LPYPY	ACE inhibitory, DPP-4 inhibitory, antioxidant	Gómez-Ruiz et al. (2007); Nongonierma and FitzGerald (2014)			×		
Total								2	3	2	0	0

[1]Protein sequences (NCBI accession IDs: NP_776719, NP_001272516, and NP_001009378).
[2]Bioactive characteristics obtained by BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), EROP-Moscow (http://erop.inbi.ras.ru/), PepBank (http://pepbank.mgh.harvard.edu/), PeptideDB (http://www.peptides.be/), Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu/), and Google Scholar (https://scholar.google.com.tr/) online databases. ACE inhibitory = lowering blood pressure (ACE = angiotensin-converting enzyme); DPP-4 inhibitory = treating type II diabetes by inhibiting dipeptidyl peptidase-4.
[3]× = the identified peptides are present on the relevant ripening day.

also found in Coalho (Fontenele et al., 2017), Manchego (Gómez-Ruiz et al., 2004), and Emmental (Gagnaire et al., 2001) cheeses. In studies of cheese, it has been observed that peptides are generally formed from the hydrophobic region of κ-CN. However, in this study and with Idiazabal cheeses (Sagardia et al., 2013), it was determined that some peptides arose from the hydrophilic region in addition to the hydrophobic region of κ-CN. Observations of peptides from the hydrophilic region of κ-CN may result from failure to remove sufficient whey during cheese production.

κ-Casein-derived peptides found in the WSE of Tulum cheese were determined to be 5 to 7 AA in length. Among them, pentapeptides were found to be dominant. Two of these short-chain peptides were found to show functional properties; 47IPIQY51 and 77LPYPY81 peptides were identified as multifunctional peptides because they exhibit both antioxidant and DPP-4 inhibitor activities. In addition, 47IPIQY51 exerts antihypertensive action.

The number of κ-CN-derived peptides determined in Tulum cheese was found to be extremely low compared with ones derived from α_{S1}-, α_{S2}-, and β-CN. This can be attributed to the small amount of κ-CN in milk compared with other caseins, the limited amount of hydrophilic glycomacropeptide remaining in the cheese curd due to the loss of it with whey, and the resistance of κ-CN to proteolytic enzymes in cheese (Gómez-Ruiz et al., 2004; Baum et al., 2013; Pisanu et al., 2015). κ-Casein has also been reported to be resistant to proteolysis in other cheese varieties (Gagnaire et al., 2001, 2011; Pisanu et al., 2015; Fontenele et al., 2017). In Coalho and Manchego cheeses, 4 and 1 peptides were found from the hydrophobic region of κ-CN, respectively (Gómez-Ruiz et al., 2004; Fontenele et al., 2017). Moreover, 4 κ-CN-derived peptides from both hydrophobic and hydrophilic regions were observed in Idiazabal cheeses (Sagardia et al., 2013). Generally, few or no κ-CN-derived peptides are found in cheeses.

In Tulum cheeses, κ-CN-derived peptides were observed until d 60 of the ripening period, and the highest κ-CN-derived peptide number was determined on d 30. The lack of κ-CN-derived peptides observed after d 60 may be due to the further degradation of these peptides or the failure of new peptides to form from κ-CN, which is highly resistant to proteolytic enzymes. Similarly, Taivosalo et al. (2018) have reported that the number of κ-CN-derived peptides decreased in Old Saare cheeses produced from pasteurized cow milk using microbial rennet and starter cultures (mesophilic and thermophilic) with further proteolysis. In addition, the further hydrolysis of κ-CN-derived peptides on different ripening days in Tulum cheeses may have resulted from the intensive activity of proteolytic enzymes specific to these peptides on the relevant ripening day.

Peptide Dynamics During Ripening

The changes in peptide dynamics of Tulum cheeses during the 180 d of ripening are presented in Figure 1. The occurrence ratios of α_{S1}-, α_{S2}-, β-, and κ-CN-derived peptides identified by MS were evaluated depending on the ripening period. The ratio of β-CN-derived peptides increased during the 180 d of the ripening period in Tulum cheeses, whereas the ratio of α_{S1}-CN-derived peptides decreased. That is, the ratios of these peptides in the total peptide model followed an opposite course. However, it has been found that the ratios of α_{S2}- and κ-CN-derived peptides remain almost constant during ripening. Compared with α_{S1}- and β-CN, α_{S2}- and κ-CN were found to be more resistant to proteolysis in cheese. At the beginning of the ripening period, the ratio of α_{S1}-CN-derived peptides was determined to be 40%, and in the following days of ripening, it was observed to be 38, 33, 32, and 27%, respectively, for days 30, 60, 90, and 180. On the other hand, the ratio of β-CN-derived peptides was determined to be 54% on d 7 of the ripening period, then increased to 69% on d 180 of the ripening period. κ-Casein-derived peptides were encountered until d 60 of the ripening period, and their ratios were determined to range between 2 and 3% in this period. In addition, the proportion of α_{S2}-CN-derived peptides was observed to range between 2 and 4% throughout the ripening period.

The great majority of peptides identified in Tulum cheese consisted of β-CN-derived peptides, followed by α_{S1}-, α_{S2}-, and κ-CN-derived peptides. Gagnaire et al. (2011) identified mostly β-CN-derived peptides in Ragusano cheese ripened for 210 d, followed by α_{S1}- and α_{S2}-CN-derived peptides. Likewise, it has been reported that β-CN-derived peptides are found at much higher levels in Swiss-type cheeses and Coalho cheeses, followed by α_{S1}-, α_{S2}-, and κ-CN-derived peptides. It is not surprising that the proportions of α_{S2}- and κ-CN-derived peptides are low during the ripening period. Compared with other caseins, disulfide bonds found only in α_{S2}- and κ-CN have been reported to provide a high level of stability against proteolysis (Bhat et al., 2016). It is also known that κ-CN remains present at low levels in cheese in the form of para-κ-CN, as a consequence of its hydrolysis by coagulant. Furthermore, Fontenele et al. (2017) argued that κ-CN contains rigid and flexible areas that affect access by proteolytic enzymes. Therefore, the rigid areas of κ-CN can limit hydrolysis by proteolytic enzymes found in cheese.

During the 180 d of the ripening period, the proportion of α_{S1}-CN-derived peptides decreased from 40% to

27%, whereas the proportion of β-CN-derived peptides increased from 54% to 69%. The number of α_{S1}-CN-derived peptides increased during ripening; however, because the number of β-CN-derived peptides increased at a greater rate, α_{S1}-CN-derived peptides proportionally declined. Demirci (2019) determined that *Lc. lactis* strains were predominant at the beginning of the 180 d of the ripening period of Tulum cheeses traditionally produced in the Karaman region, *Enterococcus faecium* strains were predominant in the later days of ripening period, and *Enterococcus faecalis*, *Enterococcus hirae*, *Enterococcus durans*, *Strep. thermophilus*, *Lactobacil-*

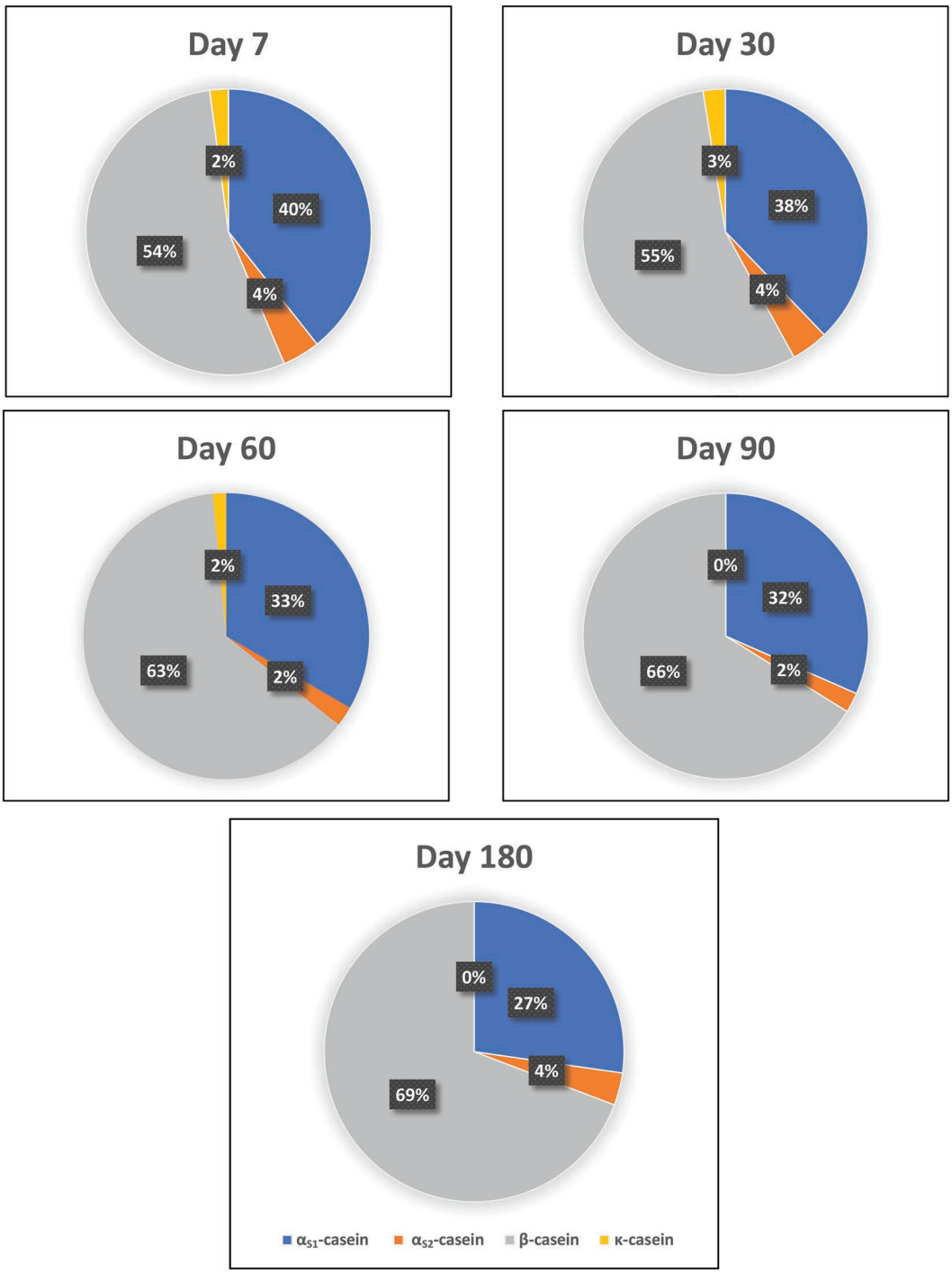

Figure 1. Changes in peptide dynamics of Tulum cheeses depending on ripening time.

lus plantarum, *Lactobacillus brevis*, and *Lactococcus garvieae* strains were also present during this time. Therefore, the proteolytic enzymes belonging to these microbiota have a significant effect on the peptide pattern in Tulum cheeses. Proteolytic enzymes of *Lc. lactis*, *Strep. thermophilus*, *E. durans*, *E. faecium*, and *E. faecalis* strains have been reported to be more effective on β-CN than on α_{S1}- and α_{S2}-CN (Requena et al., 1993; Igoumenidou et al., 2005; Veljovic et al., 2009; Miclo et al., 2012). This explains the availability of greater quantities of β-CN-derived peptides in Tulum cheeses compared with α_{S1}- and α_{S2}-CN-derived peptides.

κ-Casein-derived peptides were not observed after d 60 of ripening. They could be further broken down by microbial proteolytic enzymes to AA with progressive ripening. On the other hand, the proportion of α_{S2}-CN-derived peptides remained constant at the end of the ripening period compared with its beginning. α_{S2}-Casein has been reported to generally break down due to plasmin action (Fox et al., 2017). Considering that increased acidity values in cheese decrease the activity of plasmin (Larsson et al., 2006), it is predicted that increased acidity in Tulum cheeses during 180 d of ripening reduced the plasmin activity, thereby affecting the α_{S2}-CN-derived peptide ratio. Consequently, it has been determined that α_{S1}- and β-CN play the most important roles in peptide dynamics during the ripening of Tulum cheeses.

CONCLUSIONS

In this study, a first comprehensive description of proteolysis occurring in traditional Tulum cheeses over the 180 d of the ripening period was obtained based on peptidomic data from LC-MS/MS analysis. A total of 203 peptides were identified in Tulum cheeses during the ripening period (129 from β-CN, 59 from α_{S1}-CN, 11 from α_{S2}-CN, and 4 κ-CN). Among these peptides, β-CN-derived peptides were found to make up the largest share. Generally, α_{S1}-CN-derived peptides originated from the N-terminal portion of α_{S1}-CN, β-CN-derived peptides from the C-terminal end of β-CN, α_{S2}-CN-derived peptides from the intermediate and N-terminal areas of α_{S2}-CN, and κ-CN-derived peptides from the hydrophobic para-κ-CN region. The obtained results showed that the total number of peptides increased with the progress of ripening, especially due to aminopeptidase and carboxypeptidase activity, and the highest casein hydrolysis was found on d 180. Over the 180 d of the ripening period, the ratios of α_{S1}-CN-derived peptides and β-CN-derived peptides in total identified peptide content have shown an opposite course. On the other hand, the ratios of α_{S2}- and κ-CN-derived peptides in all defined peptides remained almost stable during ripening. A wide variety of biological characteristics have been determined in the identified peptides. In particular, ACE inhibitory peptides were found in high numbers in Tulum cheese. This was followed by antioxidant, antimicrobial, and DPP-4 inhibitor peptides. Additionally, an increase in the number of bioactive peptides was observed with the advancement of ripening days, which illustrates the health advantage of Tulum cheese ripened for a long time. In subsequent studies, the effect of ripening time longer than 180 d can be examined. Moreover, to investigate the formation kinetics of peptides during ripening in more detail, quantitative analysis of these peptides can be done in future studies.

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