Examination of Functional Properities and Bioactive Peptide Contents of Tulum Cheese

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Abstract

This study examined the presence of antioxidant, mineral binder and antimicrobial peptides in traditional Turkish goat and cow milk Tulum cheeses. Water soluble extracts (WSE) were extracted from these cheeses at ripening periods (0, 15, 30, 60, 90 and 120 days). WSEs of these cheeses were subjected to reverse-phase HPLC. The highest amounts of peptides (>500 mAU) were at 120th ripening period in both cheeses. Results of DPPH and ABTS demonstrated that the antioxidant activity increased with ripening periods for these cheeses. While no differences were observed between these cheeses in DPPH assay, the highest antioxidant activity was observed in cow milk Tulum cheese at 90th and 120th days in ABTS assay. The highest Iron(II) binding activity was determined in goat milk Tulum cheese at 60th day. WSE obtained from goat milk Tulum cheese at 90th day demonstrated inhibitory effect against *Salmonella typhimurium* ATTC 14028.

Key words: Turkish Tulum cheese, bioactive peptides, antioxidant, antimicrobial, cheese ripening

1. Introduction

Many cheese varieties that are produced only in restricted geographic areas of the world are consumed locally in large quantities. More than 150 varieties of cheese can be found in Turkey. However, 3 of them (White pickeled cheese, Kasar, and Tulum cheeses) are the most popular [1]. The annual production of Tulum cheese was 11,118 tonnes in 2012 [2]. Its production has increased greatly, and this trend has continued over recent years in a number of cheese plants. Tulum cheese has a white or cream color, a high fat content, and a crumbly and semihard texture; it is dispersible in the mouth and has a buttery and pungent flavor. The name Tulum means “goat’s or sheep’s skin bag” in Turkish, which is the bag used for packaging and ripening. Furthermore, because of increasing interest in traditional cheeses in Turkey and many other countries, new studies are needed to better understand the role of proteolysis.

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Bioactive peptides are specific protein fragments that have a positive impact on body functions and may ultimately influence health [3]. They can be from 2 to 20 amino acids in size and many have multifunctional features [4, 5]. Bioactive peptides have been isolated from many dairy products including cheese, kefir, milk, and yoghurt. Cheeses contain a high protein amount and may present as a natural source of milk protein-derived peptides, because of the diversity of the proteolytic systems in cheese ripening, and the intensity of proteolysis during ripening [6]. Furthermore, secondary proteolysis during cheese ripening may lead to the formation of other bioactive peptides, and the occurrence of bioactivity appears to be dependent on the ripening stage of the cheese [7]. Enzymes present in milk (especially plasmin), from rennet, or released by microorganisms, hydrolyze casein (α_{s1}-, α_{s2}-, β- and κ-caseins) and can enrich cheeses with bioactive peptides [8]. Milk borne bioactive peptides have been found to exhibit various physiological activities such as antihypertensive, immunomodulatory, antimicrobial, antioxidative, antithrombotic as reviewed in many recent articles [9, 10].

It was reported that bioactive peptides from caprine cheese like product had antioxidant properties [11]. Recent studies have shown that antioxidative peptides can be released from caseins in hydrolysis by digestive enzymes and in fermentation of milk with proteolytic lactic acid bacteria strains [10].

Peptides occured by *in vitro* or *in vivo* hydrolysis act as mineral holders through specific and non-specific binding sites. They may then function as carriers, chelators, of various minerals and thus support or inhibit bioavailability. The main mineral binder or chelator peptides of calcium are the caseins, α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein, but also whey proteins and lactoferrin bind specific minerals like Ca, Mg, Zn, Fe, Na and K [12]. Quantity of phosphopeptides showed an alteration in semi-hard and extra-hard cheeses. In a semi-hard cheese (Herrgård) a large of small phosphopeptides derived from α_{s1}-casein, α_{s2}-casein and β-casein was defined in a notably higher amount than in an extra-hard cheese (Parmigiano-Reggiano) [13].

Antibacterial peptides are defined in various Italian cheeses. Antibacterial activity was found in two water-soluble extracts of nine Italian cheese varieties that showed an alteration in the types of starter strains and biotechnological applications used [14].

Proteolysis is affected by several factors, including pH of the curd, plasmin, chymosin, proteases from starter and non-starter bacteria, salt to moisture ratio, storage time and temperatures [15]. In this sense, goat and cow milk Tulum cheeses which were produced without heat treatment are likely to exhibit compounds with variable bioactives. Therefore, the aim of this investigation was to evaulate the antioxidative, mineral binding and antimicrobial properties of water soluble extracts of traditional goat and cow milk Tulum cheeses produced in Turkey during ripening periods and compared cow and goat milk Tulum cheeses about functional properties and bioactive peptides compounds.

2. Materials and Method

2.1. Turkish Tulum cheese making

Goat and cow milk was acidified by its native microflora. The homemade calf rennet was added at a level of 1.0% (vol/vol), and coagulation took place at appx. 35°C for about 90 min. The coagulum was cut into pieces (approximately 3 cm^3) and transferred into cotton bags for whey drainage. Drainage was carried out at 20°C for 12 h, and the curd was pressed by piling the cotton bags on top of each other, with regular turning. Stacking of the curd at this temperature allows curd acidity (pH drops below 6.0) to develop and increases the removal of whey. Following this step, the curd was broken into pea-size pieces by hand and salted (3.0%, wt/wt), kneaded and holded for 2-3 days. Afterward, goat skin bags (The skin bag prepared and cleaned specially) were tightly filled with salted and pea sized curd at 2 different dairies (as cow milk cheese and goat milk cheese samples), and the packaged in goat skin samples were ripened for 120 d. Cheese making was performed in duplicate, and the cheese samples were analyzed every 0,15, 30, 60, 90 and 120 d.

2.2. Sampling

The cheeses were stored in a refrigerator (working at 4 to 6°C) and were sampled from the different skin bag (4 kg) at each sampling time (0, 15, 30, 60, 90 and 120 d) after the surface section (approximately 0.5 to 1.0 cm) was discarded.

2.3. Water soluble extracts (WSE)

A water-soluble extracts were prepared according to modified the method described by Kuchroo and Fox (1982) [16]. Cheese samples (20 g) were homogenized in 40 mL of deionized water during 10 min using a Stomacher. The resulting homogenates were held at 4°C for 1 h in water bath. The insoluble material was then separated by centrifugation at 10,000× *g* for 30 min. at 4°C. The supernatants were filtered through membrane filter with 0.45 μm diameter to remove residual suspended fat.

2.4. Separation of cheese peptides by RP-HPLC

Water soluble extracts prepared from cheese samples were mixed (1:1, vol/vol) deionize water containing % 0.2 trifluoroacetic acid and filtered through a 0.45- μm cellulose acetate filter. After filtration samples are made ready for analysis taking into HPLC vials. To this end; ThermoFinnigan Spectrasystem HPLC system was used. Chromatographic separation was performed using a Phenomenex Jupiter C18 (250×4,6 mm, 5 μm, 300 A^o) (Phenomenex, Torrance, CA, USA) RP-HPLC column. Mobile phase A was deionize water containing % 0.1 trifluoroacetic acid (vol/vol) and mobile phase B was acetonitrile containing 0.1 trifluoroacetic acid (vol/vol). The measurements were made at 214 nm. Gradient elution was performed [17].

2.5. Determination of antioxidant activity

2.5.1. Scavenging activity of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical

The antioxidant activities of either water soluble peptides were assayed according to the method described by Re et al. (1999) [18]. 2,2'-Azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid was dissolved in water to adjust 7 mM concentration. The ABTS radical cation ($ABTS^{\bullet+}$) solution was prepared by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate (K_2SO_4) solution and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the antioxidant analysis, the $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffered saline (PBS) pH 7.4 to an absorbance of 0.7 (±0.02) at 734 nm. 10 µL, 20 µL and 30 µL sample were mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution and absorbance (734 nm) were measured. 10 µL quantity of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (5 µM, 10 µM, 15 µM and 20 µM) as positive control was added to 1 mL of diluted $ABTS^{\bullet+}$ solution and absorbance (734 nm) were calculated. Scavenging of the $ABTS^{\bullet+}$ radical was followed by monitoring the fall in absorbance at 734 nm. A reading was taken 1 min and then periodically up to 6 min. A solvent blank was run in each analysis (negative control). According to the Trolox inhibition rates, standard curve was developed. Percentage inhibitions of absorbance at 734 nm were calculated and graphed as a function of the concentration. To calculate the Trolox equivalent antioxidant capacity (TEAC), the gradient of the plot of the percentage inhibition of sample concentration was divided by the gradient of the plot for Trolox. Results were explicated in terms of Trolox equivalent antioxidant capacity (TEAC) value.

2.5.2. Scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was used according to modified the method of [19] to determine the free radical scavenging ability of water soluble peptides. 0.1 mL of a sample was added to 0,9 mL tris hydrochloride (HCl) buffer and two millilitre of 0.1 mM DPPH dissolved in ethanol respectively in test tubes and dissolved solution was shaken vigorously. The tubes were then incubated in the dark at room temperature for 30 minute. Blank value was determined by using distilled water (free extract) and ethanol was used for the baseline correction. After 30 min., the scavenging activity was measured spectrophotometrically by the reduction in absorbance at 517 nm. All experiments were carried out in triplicate. The results were calculated as: Scavenging rate (%)=[1−(A⁄A0)]×100, where A is the absorbance of the sample and A0 is the absorbance of the blank or control.

2.6. Iron (II) chelating activity

The ferrous ion chelating ability of water soluble extract was determined using the ferrozine method [20] with some modifications. A volume of 2 mL of WSE was mixed with 0.1 mL of 2 mM iron/ferrous (II) chloride ($FeCl_2$) and 0.2 mL of 5mM ferrozine (3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4- triazine). After 10 min, the absorbance of the sample mixture was read at 562 nm. All experiments were carried out in triplicate. Likewise, 1 mL of distilled water, instead of sample, was used as a blank. Chelating activity of sample mixture was calculated as follows: Chelating activity (%)=[1−(Absorbance of sample/Absorbance of control)]×100.

2.7. Antimicrobial activity

A disk-diffusion assay was used with modifying to detect the antimicrobial activity of the water-soluble extracts [21]. *Esherichia coli* ATTC 25922, *Bacillus cereus*, *Listeria monocytogenes* ATTC 7644, *Staphylococcus auerus* ATTC 25923, *Clostridium perfringens* ATTC 13124 *and Salmonella typhimurium* ATTC 14028 on Brain-Heart agar medium were used to characterize antimicrobial activity. All cultures belong to Provincial Control Laboratory in Konya, Turkey. Water soluble extracts (5, 10 and 20 μL) were transferred into seperate 6 mm diameter paper disks (Whatman No. 1) and left to dry at room temperature under laminar flow cabin. Deionize water was used as a blank. Cultures of the different strains were activated using Brain-Heart broth. Two hundred microliters of cultures were added into 10 mL of Brain-Hearth broths and were incubated overnight at 37 ^{o}C. Then they were applied at the surface of a petri dish containing the Brain-Heart agar medium and dishes were left to dry under laminar flow cabin for 5 min. Paper disks containing water soluble extracts were put at the certain surface of inoculated petri dishes. Finally, petri dishes were incubated at 37 ^{o}C for 24 or 48 h to verify possible inhibition zones. Antimicrobial activity was determined by measuring diameter of inhibition zones.

2.8 Statistical analysis

The means of results were subjected to repeated measures and means were compared through the Duncan's multiple range test with a confidence interval set a 95%. Linear regression analysis was applied to verify correlation between water soluble protein content and ABTS assay [22].

3. Results and Discussion

3.1. Separation of Tulum cheese peptides by RP-HPLC

The peptide profiles of the extracts obtained from cow and goat milk Tulum cheeses changed with ripening periods. Peptides peaks were identified on 500 mAU. Numbers of these peptides increased with times as shown in Figure 1.

The numbers of peaks were significantly different comparing with cow and goat milk Tulum cheeses ($P<0.01$). The highest number were observed WSE from cow and goat milk Tulum cheeses at 120th ripening period. However, in other periods water soluble extracts acquired from goat milk Tulum cheese had higher numbers of peptide peaks which increased with ripening time. Depending on ripening periods in cheeses begin by coagulant activity retained in the curd and by plasmin (or other indigenous proteolytic enzymes) to a range of large and intermediate-sized peptides from caseins, which were then hydrolyzed by proteinases and peptidases from starter or non-specific lactic bacteria, and finally secondary microbiota to shorter peptides and amino acids [23].

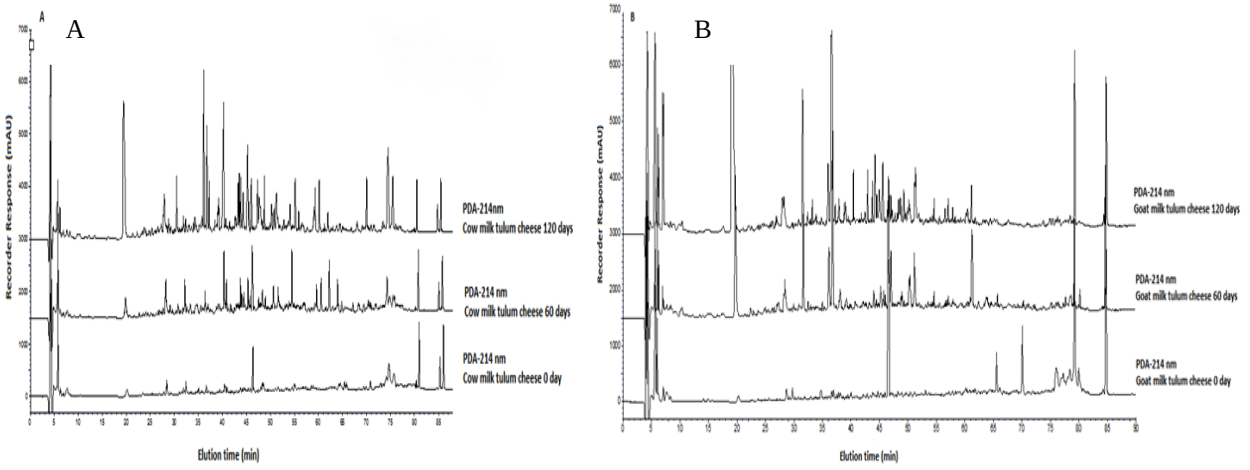

Figure 1. Peptide profiles of water-soluble extract (WSE) of cow and goat milk Tulum cheeses. Detection was at 214 nm. Profiles were determined by reversed-phase chromatography using a Phenomenex jupiter C18 column. A: WSE from cow milk Tulum cheese; B: WSE from goat milk Tulum cheese. Results are given as 3 periods. Theese periods are 0, 60 and 120 days.

3.2. Antioxidative activity

Antioxidative properties of WSE of cheese samples were evaluated by two methods, radical scavenging activity of ABTS and DPPH.

Figure 2 shows values (%) of DPPH radical inhibition for cow and goat milk Tulum cheeses. Other authors have also used the analysis of the DPPH radical scavenging activity to determine the antioxidant activity of WSE from cheese [24, 23, 25, 26]. It was reported that antioxidant capacity of the extracts was higher as the peptide concentration increased [27]. Inhibition results of DPPH demonstrated that the antioxidant activity increased with ripening periods for both Tulum cheese samples. These results caused by further proteolysis occured in ripening periods. There were no differences observed statistically ($P<0,05$) between cow and goat milk Tulum cheeses. In another study about antioxidant activity of WSE in Cottage cheese using DPPH method, it was observed that the antioxidant activity increased with storage times [24].

$ABTS^{\bullet+}$ results are presented in Table 1. $ABTS^{\bullet+}$ radical scavenging values, expressed TEAC, were different among water soluble extracts of cow and goat milk Tulum cheeses. The highest antioxidant activity (TEAC) was observed in cow milk Tulum cheese at 90^{th} and 120^{th} days ($P<0,01$). The results showed that antioxidant activity of WSE from cow and goat milk Tulum cheeses increased with ripening periods. The $ABTS^{\bullet+}$ scavenging activity demonstrated a certain relation with proteolysis for cheeses; the highest scavenging rates were consistent with higher values of soluble peptides [25]. Peptides present in WSE from caprine cheese-like systems, released upon break down of the peptide bond Leu190-Tyr191 in the β-casein sequence, showed $ABTS^{\bullet+}$ scavenging activity [11]

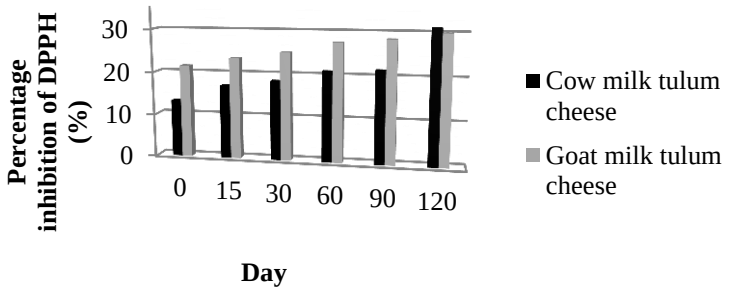

Figure 2. Average percentage inhibition of DPPH by water soluble peptides obtained from cow and goat milk Tulum cheeses. No significant differences observed between cow and goat milk Tulum cheeses (P>0,05).

These results show different scavenging patterns for DPPH and $ABTS^{\bullet+}$ radicals. The different behaviour of the same WSE with the two methods appraised could be due to the different structure of the radical that can react on a different way depending on the peptides were in the WSE. The water soluble pre-formed radical cation of $ABTS^{\bullet+}$ is occured by oxidation of $ABTS^{\bullet+}$ with potassium persulfate and could be reduced in the presence of hydrogen-donating and of chain breaking antioxidants, while DPPH is an oil soluble free radical that obtains an electron or hydrogen to become a stable diamagnetic molecule [28]. Thus, DPPH is pre-dissolved in ethanol and may not easily diffuse to target peptides that are in an aqueous medium, whereas $ABTS^{\bullet+}$ could easily reach peptides in the aqueous solution assay. It was suggested that the $ABTS^{\bullet+}$ scavenging assay was more sensitive and appropriate method for measurement of antioxidant activity of WSE. On the other hand, in a study about antioxidant activity of WSE in Cheddar cheeses, both DPPH and $ABTS^{\bullet+}$ scavenging activities of WSE were dependent on proteolysis degree and microbial flora [23].

Table 1. Antioxidant and mineral binding properties of WSE obtained from cow and goat Tulum cheeses

Tulum cheese samples	Ripening periods (day)	$ABTS^{\bullet+}$ TEAC (μM Trolox)	Chelating activity (%)
Cow milk Tulum cheese	0	$185\pm,7.1^{I}$	4.68 ± 0.04^{J}
	15	205 ± 7.1^{HI}	13.61 ± 0.05^{I}
	30	240 ± 0.0^{FG}	36.94 ± 0.02^{E}
	60	350 ± 14.1^{C}	30.19 ± 0.02^{G}
	90	375 ± 7.1^{AB}	35.31 ± 0.03^{F}
	120	390 ± 0.00^{A}	63.30 ± 0.40^{C}
Goat milk Tulum cheese	0	125 ± 7.1^{J}	22.20 ± 0.04^{H}
	15	225 ± 7.1^{GH}	56.58 ± 0.03^{D}
	30	260 ± 0.00^{EF}	64.51 ± 0.02^{B}
	60	280 ± 0.00^{DE}	65.99 ± 0.02^{A}
	90	300 ± 0.00^{D}	56.24 ± 0.02^{D}
	120	355 ± 7.1^{BC}	62.99 ± 0.02^{C}

Values are the means±SEM of observations. The same letters in a column indicate no significant difference at the 1% level of significance.

3.3. Iron(II) chelating activity/Mineral binding properties

Table 1 presents values (%) for the chelating activity of water soluble extracts from cow and goat milk Tulum cheeses. Iron(II) chelating ability took place distinct values for WSE samples. It can be observed that the highest chelating activity of WSE was in goat milk Tulum cheese at 60th ripening day. After 60th day, iron chelating activity of WSE decreased in goat milk Tulum cheese ($P<0,01$). It was asserted that hydrolysis time affects the bioactivities of Fe^{2+} chelating ability of the WSE. Longer hydrolysis times decreased metal chelation activity [29]. Transition metals can stimulate lipid peroxidation in foods, resulting in rancidity [30]. So chelating of metal ions might contribute to the antioxidant activity of water soluble hydrolysates.

3.4. Antimicrobial activity

An antimicrobial activity against *Salmonella typhimurium* ATTC 14028 was observed at 90th ripening period in goat milk Tulum cheese. The inhibition zone was measured as 44 mm and is shown Figure 3. Zone was observed in disk used 10 µL of WSE. It was reported that hydrolyzed ovine caseinate for 3 h represent antibacterial activity against *Bacillus cereus* and *Corynebacterium fimi*. Diameters of the zones were measured, respectively, 9.3 and 11.5 mm [29]. An another study on Italian cheese varieties, antimicrobial peptides against *L. sakei* A15 were determined from Pecorino Romano, Canestrato Pugliese, Crescenza, Caprino del Piemonte, Caciocavallo and Mozzarella cheeses. Diameters of the inhibitory zones were determined 3 to 6 mm [14]. Milk protein derived antimicrobial peptides usually demonstrate a broad range of activity against microorganisms of spoilage and/or health significance [29]. But, in this study only Gram-negative bacteria was inhibited. Differences bacterial membrane compositions between Gram-positive and Gram-negative bacteria might influence action and the bacterial specificity of antimicrobial extract.

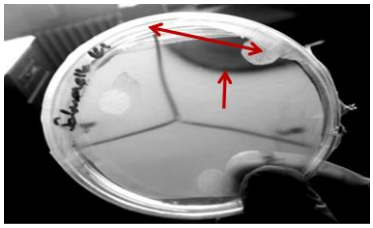

Figure 3. Growth inhibition *Salmonella typhimurium* ATTC 14028 by the WSE obtained from goat milk tulum cheese at 90th ripening period. Arrow indicates inhibition zone and two-headed arrow indicates semidiameter of zone.

Conclusions

Extracts from cow and goat milk Tulum cheeses presented antioxidant and mineral binding activities. In addition to this, WSE from goat milk Tulum cheese showed antibacterial activity against *Salmonella typhimurium* ATTC 14028. According to HPLC analysis results a peptide profile was observed at 90th day in goat Tulum chese as different from other periods. Thus, that

antibacterial effect appeared only in a certain period (90^{th} day) in goat Tulum cheese could be due to differences of peptid profiles in ripening periods. Peptide profiles of ripened goat and cow milk Tulum cheeses prepared using raw milks show statistically ($P<0,01$) differences in term of their relative numbers. These differences are likely related to exposure of the various milk components, which might act both on suitability of proteins to enzymatic cleavage, as well as on indigenous bacteria or proteolytic enzymes responsible for the formation of peptide during ripening.

Acknowledgements

This work was supported by Scientific Research Project (BAP in Konya, Turkey).

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