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Angiotensin Converting Enzyme Inhibitors and Antioxidant Peptides Release During Ripening of Mexican Cotija Hard Cheese

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Abstract

Cotija cheese is an artisanal Mexican cheese produced with raw cow´s milk. Our objective was to measure the antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of the peptides released during its ripening. For that, Cotija cheeses were ripened 6 months in a chamber at 25 ºC without humidity control. Weekly samples were taken to determine acid soluble nitrogen (ASN), non-protein nitrogen (NPN) and ethanol soluble nitrogen (EtOH-SN) indexes, by Kjeldahl method. Antioxidant and ACE inhibitory activities were measured by spectrophotometry and HPLC methods, respectively. Peptides in each nitrogen fraction were determined by HPLC. Our results showed that during ripening of Cotija cheeses peptides with antioxidant and ACE inhibitory activities were released and increased through ripening time reaching a maximum of 79.8 % of 2,2-diphenyl-1-picrylhydrazyl (DPPH) discoloration and 100 % of ACE inhibition at the end of ripening. Both activities were highly correlated with the types of peptides present in each fraction.

Keywords: Cotija cheese, ripening, bioactive peptides, antioxidant activity, ACE inhibitory activity

1. Introduction

Cotija cheese is a ripened Mexican hard cheese made from raw cow's milk. It is produced seasonally according to traditional protocol from July to October in Jalmich region, located between Jalisco and Michoacán at 700-1700 meters above sea level (Flores-Magallón et al., 2011). Cotija cheese is a 20 kg cylindrical cheese. Their distinctive characteristics are hard rind, high salt content, firm or friable texture with a strong, sharp, or pungent aroma (Hernández et al., 2009) with 35-42 % moisture, 23-30 % fat 28-31% protein and 4% salt (Hnosko et al., 2009).

Cotija cheese production and process vary among producers in the curd cutting, salting, pressing and especially in ripening time which takes 3 months minimum. The microbiological and sensorial characteristics of Cotija cheese have been described before (Chombo-Morales et al., 2016; Flores-Magallón et al., 2011; Hnosko et al., 2009), however little is known about its functional properties.

Proteolysis is the principal biochemical process during cheese ripening and it depends among other factors on the origin of milk, microbial populations, ripening time, etc. Proteolysis results in a unique peptide profile characteristic of each cheese variety. The peptides released during proteolysis may have biological activities such as antioxidants or inhibitors of angiotensin converting enzyme (ACE) (Ong et al., 2007; Vermeirssen et al., 2004). However, the biological activity depends on the state of cheese ripening (Gupta et al., 2013).

Therefore, the present study was focused on the evaluation of the biological activity as antioxidants and ACE

inhibitors of the peptides released during the ripening of Mexican Cotija cheese.

2. Method

2.1 Cheese Production and Sampling

Cotija cheeses were part of the regular production of an artisan dairy farm located in the Cotija region. Cheese elaboration was as follows: Cow's milk was standardized (3% fat) and heated to 30-35 °C for 1 h, followed by rennet addition (strength 1:10,000). Curd was cut into 1 cm^3 cubes and rested 5–10 min. Serum-free curd was salted (5 -6 %), and molded (35 kg molds) in cylindrical shape molds. After that, the curd was pressed 24 h in istle molds covered with muslin. Next day (1d), molds were open and cheeses were wrapped with a stainless steel sheet to protect them during transportation to the laboratory. Cheeses were kept wrapped during 5 days at room temperature, daily turn. On day 6, cheeses were unwrapped and placed at 25 °C in a chamber without humidity control for 6 months to be ripened. Fifty grams of cheese were sampled every week during 4 months (W1-W16), then every two weeks for the last 2 months (W18, W20, W22, W24 and W26).

2.2 Nitrogen Fractions

Cotija cheese extracts for total nitrogen (TN), acid soluble nitrogen (ASN), non-protein nitrogen (NPN) and 70 % ethanol soluble (EtOH-SN) and insoluble nitrogen (EtOH-NSN) were determined according to Guerra-Martínez et al. (2012). Where ASN is a mixture of long, medium and short size peptides, NPN is a mixture of medium and short size peptides. While EtOH-SN contains short size peptides and EtOH-NSN the long and medium size peptides from ASN. Samples were stored at -80 °C until analyses. Nitrogen content was quantified using Kjeldahl and micro-Kjeldahl methods.

2.3 Nitrogenous Fractions Analysis by RP-HPLC

Nitrogenous fractions (ASN, NPN, EtOH-SN, and EtOH-NSN) were profiled according to the method of Abadía-García et al. (2013). Resulting peaks were divided according to their retention time into hydrophilic (HI) and hydrophobic (HO) peptides following the criteria of Gonzalez de Llano et al. (1995).

2.4 Determination of Antioxidant Activity

ASN and NPN fractions antioxidant activity of each ripening point were tested using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, following the method of Abadía-García et al. (2013). Samples were tested in triplicate and the results were expressed as DPPH·discoloration percentage.

2.5 *In-vitro Determination of ACE Inhibitory Activity*

EtOH-SN and EtOH-NSN fractions ACE inhibitory activity of each ripening point were tested using angiotensin-converting enzyme from porcine kidney (0.5 UN) following the method of Wang et al. (2013). Hippuric acid (HA) was used as standard and hippuryl-histidyl-leucine (HHL) was used as equivalent of 100% of ACE activity. All reagents came from Sigma-Aldrich. Results were calculated according with the equation 1:

$$ACE\ inhibitory\ activity\ (\%) = [(\%\ HHL) - (\%HA)] \quad (1)$$

Where:

$$\%\ HHL = [HHL\ area/(HHL\ area + HA\ area)]*100 \quad (2)$$

$$\%\ HA = [HA\ area/(HHL\ area + HA\ area)]*100 \quad (3)$$

2.6 Statistical Analysis

Data analyses were carried out with Statistica v 12 (Statsoft, Inc., Tulsa, OK, USA).

Antioxidant activity in the ASN and NPN; and ACEI activity in EtOH-SN and EtOH-NSN were analyzed as a two different data sets to determinate significant differences in biological activities between fractions at each ripening point.

Antioxidant and ACE inhibitory activities were correlated with the type of peptides of the corresponding fraction.

3. Results and Discussion

3.1 Nitrogenous Fractions

Total nitrogen was stable throughout the ripening time (5.4 ± 0.4% dry base). On the other hand, during the first 5 weeks, ASN mean values through ripening, (15.5 ± 2.2 %TN), were higher than mean values of NPN during the same period (7.8 ± 4.4 %TN). These results are normal since ASN fraction is considered as an index of primary proteolysis in cheeses and in this fraction are contained all the peptides regardless the size, meanwhile

NPN contains only medium and short size peptides (McSweeney & Fox, 1997). At 5 weeks, ASN and NPN showed similar values (13.66 % and 12.41% respectively) and behavior. From this point both fractions values increased slowly reaching the highest values (41.1 %TN and 36.2 %TN, respectively) at the end of ripening (24 weeks). EtOH-SN increased from 10 %TN in week 1 to a maximum of 31.7 %TN in week 16, to later decrease reaching almost the initial value at week 24. This decrease is due to a further metabolism of the peptides in this fraction (McSweeney, 2004).

3.2 Nitrogenous Fractions Analysis by RP-HPLC

Peptide profiles showed noticeable differences between ASN, NPN, EtOH-SN and EtOH-NSN fractions. Chromatograms showed that new peaks appeared during ripening, meanwhile, peaks that existed at the beginning increased or decreased through time.

Thereby, the evolution of hydrophilic peptides (HI), hydrophobic peptides (HO) and HO/HI ratio were followed during ripening (Table 1).

Hydrophilic peptides (HI) are present in the all the nitrogenous fractions the first week. ASN showed the highest increase in HI mainly after 12 weeks (Table 1). On the other hand, during the first week NPN had the lowest number of peptides and then increased dramatically at week 17. In EtOH-SN, HI remained steady up to week 17 then the level almost doubled to 19736 at week 24. EtOH-NSN increased during the first 5 weeks and then remained steady until the end of ripening.

Table 1. Total area of HI, HO, and HO/HI ratio in nitrogenous fractions of Mexican Cotija cheese during ripening

Week	HYDROPHILIC (HI) 10-34 min of retention time				HYDROPHOBIC (HO) 35-100 min of retention time				HO/HI ratio			
W	NPN	ASN	EtOH- SN	EtOH-NSN	NPN	ASN	EtOH- SN	EtOH-NSN	NPN	ASN	EtOH- SN	EtOH-NSN
1	1349	1671	11662	7178	0	8194	24699	0	0	4.9	2.1	0
2	1160	3600	8787	8258	0	12923	4343	0	0	3.6	0.5	0
3	1230	8281	10520	8351	0	22886	4332	0	0	2.8	0.4	0
4	540	2493	8698	10198	0	8842	4561	0	0	3.5	0.5	0
5	241	2151	10695	11538	0	12792	5202	0	0	5.9	0.5	0
6	788	5851	10524	9815	1854	21264	22216	1927	2.4	3.6	2.1	0.2
7	555	4159	9997	9012	263	13948	8666	0	0.5	3.4	0.9	0.0
8	576	9108	10362	9403	1296	22716	15336	635	2.3	2.5	1.5	0.1
9	1551	17632	12229	9183	1235	20110	9200	1414	0.8	1.1	0.8	0.2
10	385	2658	8475	9310	0	12298	1652	0	0	4.6	0.2	0.0
11	1020	7051	9360	11322	2145	20370	21808	1966	2.1	2.9	2.3	0.2
12	ND	6339	11997	9425	ND	21139	19661	2140	ND	3.3	1.6	0.2
14	ND	29294	12661	9060	ND	24253	11305	1773	ND	0.8	0.9	0.2
17	3783	12502	11941	8504	5123	28358	13339	1786	1.4	2.3	1.1	0.2
20	4306	26206	29210	9624	3859	25055	57563	502	0.9	1.0	2.0	0.1
24	12698	38761	19736	12569	8682	14060	24137	4082	0.7	0.4	1.2	0.3

*ND= Non Determined.

**The amount of HI and HO peptides was expressed as units of chromatogram area.

Hydrophobic peptides (HO) were present only in ASN and EtOH-SN in the first week. HO in ASN increased until week six, and then remained steady up to the end of the ripening. HO in NPN and EtOH-NSN appeared at week 6 and both fractions reached their highest HO concentration at the end of the ripening. Meanwhile, HO in EtOH-SN remained steady all the time.

The evolution of HO/HI ratio was different in each nitrogenous fraction (Table 1). For ASN HO/HI ratio oscillated until week 12 to later decrease until the end of the ripening. In NPN the HO/HI ratio increased after week 6, to later decrease after week 17. HO/HI ratio in EtOH-SN fluctuated during all ripening. Meanwhile, EtOH-NSN fraction had a very low proteolysis as it showed by a constant HO/HI ratio throughout all ripening

time. The variations observed in HO and HI peptides of both ASN and NPN fractions between week 4 and 10 could be attributed to dynamic mechanisms related with changes in the microbiota of Cotija cheese. No reference were found in the bibliography to compare our results, most of the HO/HI ratio analysis in cheeses are reported only in water soluble extracts, that are only comparable with the ASN fractions, because of the size of peptides content (McSweeney & Fox, 1997). However the observed decrease in ASN HO/HI ratio is similar to the results reported by Gonzalez de Llano et al. (1995) in cheddar cheeses.

3.3 Determination of Antioxidant Activity

ASN and NPN fractions, which exhibited a content of medium and short-chain peptides, were tested for antioxidant activity (Figure 1A). Antioxidant activity showed statistically significant differences between nitrogenous fractions ($p < 0.05$) until week 23, at the end of the ripening (week 24) both fractions were similar.

NPN showed higher antioxidant activity during all ripening time at a constant level (67.7 ± 4.9 % discolorations). While ASN remained steady (18.8 ± 1.6 % discoloration) during the first 18 weeks of ripening. Then, it increased significantly ($p < 0.05$) until week 24 (79.8% discoloration), reaching similar values to those of NPN (Figure 1A).

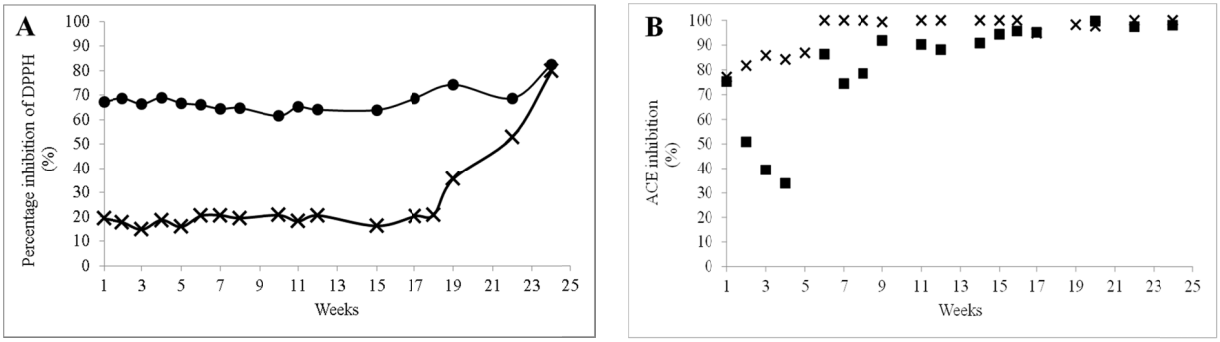

Figure 1. Biological activity evolution of nitrogenous fractions obtained during ripening of Mexican Cotija cheese. A) Antioxidant activity: ● NPN, **X** ASN; B) ACE inhibitory activity: **X** EtOH-SN, ■ EtOH-NSN

Our results indicated that Cotija cheese exhibited important antioxidant activity. Differences between ASN and NPN at the beginning of ripening can be explained by differences in the peptide content. At the beginning, NPN had shorter size peptides, which are the holders of biological activity (Pihlanto, 2006) but as the ripening goes on, the bigger peptides in ASN go through higher proteolysis, releasing smaller peptides with antioxidant activity. Similar results were observed in cheddar cheeses by Gupta et al. (2009) who observed that changes in the antioxidant activity where related to the rate of formation of soluble peptides.

Table 2. Correlation coefficients of nitrogenous fraction, peptide profile and biological activity of Mexican Cotija cheese during ripening

		ACEI[b]		Antioxidant. Activity	
		EtOH-SN	EtOH-NSN	NPN	ASN
NPN	HI			0.92*	
	HO			0.79*	
ASN	HI				0.81*
	HO				-0.18
EtOH-NSN	HI		0.33		
	HO		0.60*		
EtOH-SN	HI	0.23			
	HO	0.28			

Bold values are significant at *p*-value< 0.05 (*).
†ACEI: Angiotensin converter enzyme inhibitory activity.

Cotija cheese exhibited high antioxidant activity and ACE inhibitory activity from the beginning of ripening, in NPN, EtOH-SN and EtOH-NSN, however, it's at the end of the ripening that the highest values of activity in ASN fraction are reached. Still is after 12 weeks, that this cheese is commercialized and is after this point when its biological activity is most important and reached its maximum.

As shown in Table 2, NPN antioxidant activity exhibited a higher correlation with HI (0.92) but also significant correlation with HO peptides (0.79). ASN antioxidant activity was positive correlated with the HI peptides (0.81) but there was no significant correlation with HO peptides.

3.4 In-vitro Determination of ACE Inhibitory Activity

ACE inhibitory activity of peptides was measured in the fractions with the lowest molecular weight peptides (EtOH-SN and EtOH-NSN) since these peptides are the holders of this biological activity (Espejo-Carpio et al., 2013; Meisel, 2004). Results were expressed as percentage of ACE inhibition compared to a blank sample (Figure 1B).

ACE inhibitory activity showed significant differences ($p < 0.05$) between fractions where EtOH-SN exhibited the highest activity during the first 16 weeks (Figure 1B). %). Peptides in EtOH-SN and ETOH-NSN are small and medium peptides, respectively (McSweeney & Fox, 1997). ACE inhibitors are generally short chain peptides, which explains the differences observed between fractions (Meisel, 2004). In EtOH-SN ACE inhibitory activity increased steeply during the first 5 weeks of ripening and at week 6 reached the 100%. Whereas, in EtOH-NSN ACE inhibitory activity increased continuously after week 6, reaching a steady state after week 9 (94.1 ± 3.8). After 17 weeks ACE inhibitory activity in both fractions was similar, presumably because at this point most of the peptides in EtOH-SN and EtOH-NSN are short chain with similar activity.

ACE inhibitory activity correlations between fractions and peptide profile (HO, HI and HO/HI ratio) are shown in Table 2. In EtOH-NSN activity was highly correlated with HO and HO/HI ratio of this fraction (0.60 and 0.64, respectively). EtOH-SN activity did not show significant correlation with the peptides types in its fraction

As expected, biological activities were highly correlated with the peptide profile. ACE inhibitor activity has been always related to the hydrophobicity of the peptides (Espejo-Carpio et al., 2013; Meisel, 2004), which explains the significant correlation between HO peptides and activity in EtOH-NSN. However, the lack of correlation with EtOH-SN fraction couldn't be fully explained, and it could be only attributed to other characteristics of the peptides present like the electrostatic charge of the amino acids or the peptide conformation (Meisel, 2004).

On the other hand, antioxidant is also attributed to hydrophobic peptides, however in our results we found that this activity was mostly correlated with the HI peptide content, this could be explained by the effect that antioxidant activity can be attributed to the metal ion chelation properties of the amino acids in the peptide, or that despite the hydrophobicity or hydrophilic nature of the peptides, the antioxidant activity is furthermore attributed to chelation characteristics of the amino acids presents, or to the high amounts of cysteine in this ASN and NPN fractions that promote the synthesis of peptides that of glutathione, a potent antioxidant. Furthermore, some authors support the hypothesis that antioxidant action is most likely attributed to the cooperative effects of the mechanisms mentioned (Erdmann et al., 2008).

The increases of biological activity during ripening of Cotija cheese are in agreement with other authors (Gupta et al., 2009; Pritchard et al., 2010; Ryhänen et al., 2001). Gupta et al. (2009) reported that antioxidant activity and Pritchard et al. (2010) reported that antioxidant and ACEI activities in the water soluble extract of cheddar cheeses depended of the stage of ripening. Ryhänen et al. (2001) also showed that ACEI activity in an experimental low fat cheeses increased during ripening and after decreased after 4 months when proteolysis exceeded certain level due to catabolism of peptides.

4. Conclusions

Ripening of Mexican Cotija cheese released peptides with high antioxidant and ACE inhibitory activity which increased during ripening time. Both biological activities of the analyzed nitrogenous fractions were highly correlated with the type of peptides in the fractions. Despite the maximal antioxidant activity is reached at 24 weeks, the ACE inhibitory activity exerts its maximal levels after 13 weeks of ripening. Thus if we consider that Cotija cheese is ripened at least for 3 months (12 weeks) we can conclude that the commercialized product possess a significant content of bioactive peptides with potential health effect that makes of Cotija a functional cheese and making interesting future studies to evaluate the biological activity of peptides present in long-ripened Cotija cheese, as to evaluate other biological activities.

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