Short communication: Assessing antihypertensive activity in native and model Queso Fresco cheeses¹

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ABSTRACT

Hispanic-style cheeses are one of the fastest growing varieties in the United States, making up approximately 2% of the total cheese production in this country. Queso Fresco is one of most popular Hispanic-style cheeses. Protein extracts from several varieties of Mexican Queso Fresco and model Queso Fresco were analyzed for potential antihypertensive activity. Many Quesos Frescos obtained from Mexico are made from raw milk and therefore the native microflora is included in the cheese-making process. Model Queso Fresco samples were made from pasteurized milk and did not utilize starter cultures. Water-soluble protein extracts from 6 Mexican Quesos Frescos and 12 model cheeses were obtained and assayed for their ability to inhibit angiotensin-converting enzyme, implying potential as foods that can help to lower blood pressure. All model cheeses displayed antihypertensive activity, but mainly after 8 wk of aging when they were no longer consumable, whereas the Mexican samples did display some angiotensin-converting enzyme inhibitory action after minimal aging.

Key words: Hispanic cheese, bioactive peptide, angiotensin-converting enzyme inhibition, antihypertensive activity

Short Communication

Hypertension, also known as high blood pressure, is a chronic condition that can lead to more severe and deadly illnesses such as stroke and coronary heart disease. Hypertension affects up to 30% of the adult population in most countries and often goes undiagnosed (Chen et al., 2009). One of the many biological pathways that regulate blood pressure in the human body is the rennin-angiotensin-aldosterone system.

Angiotensin-converting enzyme (**ACE**) is a key component in this pathway that catalyzes the conversion of an inactive decapeptide, angiotensin I, to a potent vasoconstrictor, angiotensin II. The enzyme has an additional role in the hydrolysis of bradykinin and kallidin, both compounds with vasodilative activities (Pihlanto et al., 2010). Through these mechanisms, ACE causes blood pressure to increase. Therefore, inhibiting the action of the ACE enzyme can lead to a decrease in blood pressure (Chen et al., 2009).

Some studies have shown a correlation between dairy consumption and lowering of blood pressure (Lopez-Fandino et al., 2006). Subsequent work has targeted and characterized specific milk protein-derived compounds from various dairy products with ACE-inhibitory activity (Korhonen et al., 2006). Many of these have been sequenced and shown to be derived from the enzymatic degradation of milk proteins, mostly caseins, by bacterial proteinases during milk fermentation (Pihlanto et al., 2010). Hence, many ripened cheeses, such as Cheddar (Singh et al., 1997) or aged Gouda (Saito et al., 2000), are sources of bioactive compounds including antihypertensive peptides. Recently, milk fermented using varieties of lactic acid bacteria has been shown to provide antihypertensive peptides derived from β -casein, as well (Pihlanto et al., 2010).

Interest is increasing in the incorporation of health-promoting compounds into foods that are readily available to consumers in supermarkets. Several advantages exist to using compounds such as milk protein-derived antihypertensive peptides as food additives. First, because these peptides are milk protein derived, they are already established as safe for human consumption. Many dairy foods on the market already contain these peptides, albeit in relatively small quantities. Second, these peptides are released through proteolytic digest of the parent milk proteins. Although the proteolysis can be carried out by bacterial proteinases during cheese-making, the intact protein may make it to the gut and be hydrolyzed gastrointestinally, thereby circumventing bioavailability issues with small molecules (Lopez-Fandino et al., 2006). Well-known ACE inhibiting tripeptides VPP and IPP (Nakamura et al., 1995a,b) have also been shown to be resistant to proteolysis in vivo

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(Mizuno et al., 2004), suggesting that these compounds will have long, effective lifetimes within the body and will not be quickly degraded or inactivated.

Understanding the nature of these peptides and their structures and measuring their abilities to inhibit ACE provide information toward better, more effective antihypertensive compounds that can be incorporated into foods. Assessing the natural concentrations of these compounds within items such as ripened cheese provides information as to potential health benefits associated with consuming particular foods. Additionally, researchers have used the information obtained from these types of studies to develop new types of cheeses that contain bioactive peptides with potential health benefits (Ryhanen et al., 2001). Although significant work has been done toward characterizing antihypertensive peptides in many dairy foods, little work has been done to date on identifying them in the increasingly popular Hispanic-style cheeses such as Queso Fresco. According to the National Agricultural Statistical Service, as of 2009, Hispanic-style cheeses are one of the fastest growing cheese varieties in the United States, making up approximately 2% of total cheese production in this country. This aim of this study was to assess the antihypertensive activity in protein extracts from Queso Fresco made from raw and pasteurized milk obtained from Mexico, as well as in model cheeses developed to mimic the native flavors and taste, made with pasteurized milk without starter cultures in the Dairy and Functional Foods facility at the North Atlantic Area (NAA)–Eastern Regional Research Center (ERRC) of the Agricultural Research Service (ARS) of the USDA.

Model cheeses A, B, and C were manufactured in duplicate from raw milk obtained locally. Each batch of cheese was prepared from 180 kg of milk that had been standardized to 3.5% (wt/wt) fat, homogenized at 6.9 and 3.4 MPa and then pasteurized at 72°C for 15 s. Milk was adjusted to 32°C in a cheese vat (Kussel Equipment Co., Watertown, WI), CaCl_2 was added to the milk to a final concentration of 0.1% wt/wt, and no starter culture was used. Chymosin (130 mL per 1,000 L of milk; Chy-Max 73863, Chr. Hansen Inc., Milwaukee, WI) was added to coagulate the milk, and after 30 min the coagulated curd was cut into 1-cm cubes. The temperature was increased to 39°C and the curd was cooked for 30 min. Approximately two-thirds of the whey was drained from the vat, and dry salt (2.3 kg) was added in 3 applications and mixed into the slurry for a total of 20 min. The rest of the whey was drained and the curd trenched. When the curd reached pH 6.3, it was removed from the vat, and chilled to <21°C. Chilled curds were finely milled using a Bosch

universal 6610UC/01 FD meat grinder (Robert Bosch Hausgeräte GmbH, Dillingen, Germany) or a Waring Pro MG800 meat grinder (Waring Pro, East Windsor, NJ). Milling treatments were (a) no milling and immediately packed into molds; (b) curd was passed through the Bosch meat grinder without a blade; (c) curd was passed through the Bosch meat grinder with a small bore blade (6.3-cm-diameter blade with eighteen 0.7-cm holes); and (d) curd was passed through the Waring meat grinder with a large bore blade (6.3 cm in diameter with five 1.4-cm holes). The curd from each treatment was packed separately into molds and stored overnight at 4°C. Each cheese was then removed from the mold, divided into 3 blocks, vacuum packaged, and stored at 4°C. One block from each milling treatment was removed for testing after 1 and 8 wk of storage. Cheeses were designated A1f (i.e., cheese A aged for 1 wk at 4°C), A1t (t = aged at 10°C), A8f, A8t, B8fa, B8fb, B8fc, B8fd, B8t, C8fa, C8fb, C8fc, C8fd, and C8t, where a, b, c, and d refer to milling treatments (D. L. Van Hekken, unpublished work). Mexican Queso Fresco cheeses were obtained from different commercial sources in Sonora, Mexico, and were labeled Mr1 (Mexican raw cheese sample 1), Mr2, Mr3, Mr4, Mp1 (Mexican pasteurized cheese sample 1), and Mp2 (Renye et al., 2008).

For all samples of cheese, water-soluble proteins were extracted using a protocol adapted from multiple cheese protein studies (Gomez-Ruiz et al., 2002; Van Hekken et al., 2004; Silva et al., 2006). Milli-Q water (5 mL; Millipore, Billerica, MA) was added to a grated cheese sample (5 g) and the mixture was homogenized by sonication for 6 min. More water was added (5 mL), and the mixture was further homogenized by sonication for 6 min. The homogenate was stirred at room temperature for 30 min, and then centrifuged at $26,000 \times g$ for 60 min at 4°C. The supernatant was filtered through a Whatman no. 1 filter paper and lyophilized to yield the water-soluble protein extract used in subsequent experiments.

A spectrophotometric assay based on the hydrolysis of the substrate peptide *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (**FAPGG**) to form the degradation product peptides furylacryloylphenylalanine (**FAP**) and glycylglycine by ACE was used to assess the ACE inhibitory activity of the water-soluble cheese protein extracts (van der Ven et al., 2002; Vermeirssen et al., 2002; Hou et al., 2003). Assays were performed in duplicate and averages were used for calculations. Protein extract mixtures were used at concentrations of 10 mg/mL. Angiotensin-converting enzyme from rabbit lung and FAPGG were obtained from Sigma-Aldrich (St. Louis, MO). Reaction buffer used for assays was 50

Figure 1. Reversed phase-HPLC C18 chromatograph of *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG; substrate) and furylacryloylphenylalanine (FAP; released by hydrolysis of FAPGG) separation as used to assess angiotensin converting enzyme (ACE) inhibitory activity, where ACE inhibition (%) = $[(FAP_{\text{control}} - FAP_{\text{sample}}) / FAP_{\text{control}}] \times 100$. mAU = milli-arbitrary units.

mM Tris-HCl, 300 mM NaCl, pH 8.3; ACE was used at a concentration of 0.0565 U/mL and FAPGG was used at a concentration of 100 μ M.

Reaction mixtures containing 2.5 μ L of ACE enzyme, 12.5 μ L of cheese protein extract, and 20 μ L of reaction buffer were mixed and preincubated before sampling at 37°C for 30 min. To this mixture, 15 μ L of FAPGG was added to give a final reaction volume of 50 μ L. Samples remained at 37°C and aliquots (10 μ L) were removed at 5-min intervals; 5 μ L of 10% trifluoroacetic acid (TFA) was added to stop the reaction. Liberated FAP was separated from FAPGG by reversed phase-HPLC (Agilent 1200 series HPLC, Agilent Technologies, Santa Clara, CA), monitoring absorbance at 300 nm, using a Vydac C18 column (5 μ m, 4.6 mm i.d. $\times$ 250 mm), eluting isocratically at 27% of 0.1% TFA in water and at 73% of 0.08% TFA in acetonitrile at a flow rate of 1 mL/min over 25 min, and subsequently quantified (Figure 1). An Agilent photodiode array detector was used as part of the HPLC instrument, and concentration of liberated FAP was calculated based on a calibration curve obtained by plotting various known concentrations of FAP versus absorbance at 300 nm. The ACE inhibitory activity was calculated according to the following formula (where FAP is liberated FAP from hydrolyzed FAPGG):

$$\text{ACE inhibition (\%)} = [(FAP_{\text{control}} - FAP_{\text{sample}}) / FAP_{\text{control}}] \times 100.$$

Protein extracts from 6 different Queso Fresco varieties obtained from Mexico as well as 12 different

Table 1. Model Queso Fresco inhibition data

Sample ¹	Inhibition activity (%)
No inhibitor	0
A1f	22.3
A8f	95.7
A1t	43.7
A8t	85.5
B8fa	72.2
B8fb	68.0
B8fc	80.1
B8fd	76.5
B8t	79.7
C8fa	18.9
C8fb	46.6
C8fc	76.0
C8fd	67.6
C8t	67.2

¹A, B, C = model Queso Fresco cheeses; 1 and 8 = aged for 1 and 8 wk, respectively; f and t = aged at 4°C and 10°C, respectively; a, b, c, and d = milling treatments: a = no milling and immediately packed into molds; b = curd was passed through a Bosch meat grinder without a blade; c = curd was passed through the Bosch meat grinder with a small-bore blade (6.3-cm-diameter blade with eighteen 0.7-cm holes); and d = curd was passed through the Waring meat grinder with a large-bore blade (6.3 cm in diameter with five 1.4-cm holes).

model cheeses made without using starter cultures were assayed for antihypertensive activity. Results for the model cheeses are presented in Table 1. Protein extracts from A1f (cheese sample A stored at 4°C for 1 wk) showed 22% inhibition of the ACE activity, whereas the same sample after 8 wk of aging (A8f) showed 95.7% inhibition. Sample A stored at 10°C showed a similar increase in inhibition activity, from 43.7% after 1 wk of aging (A1t) to 85.5% inhibition after 8 wk (A8t). Because these cheeses were not made using starter cultures, the observed increase in inhibition activity can be attributed to spoilage bacteria that could provide the enzymes needed to proteolyze the proteins to generate the ACE inhibitory compounds. This effect was universally observed with all of the B and C model cheeses as well, with 1-wk samples showing some slight ACE inhibition (data not shown) and 8-wk samples showing a significant increase in inhibition, regardless of storage temperature. In all cases (Table 1), with the exception of the C8b sample, the model cheeses displayed at least 50% inhibition after 8 wk of aging, presumably due to protein degradation by spoilage bacteria.

In contrast, the Mexican cheeses showed significant ACE inhibitory activity after 1 wk of storage (Table 2). The original A and B samples, corresponding to data labeled Mr1, Mr2, Mr4, and Mp1, all exhibited 87 to 93% ACE inhibition compared with assays conducted with the enzyme alone. The Mr3 and Mp2 samples showed some inhibition activity (36 and 51%, respectively), but it was not as high as that seen in the other samples and

Table 2. Mexican Queso Fresco inhibition data

Sample ¹	Inhibition activity (%)
Mr1	89.8
Mr2	87.2
Mr3	36.3
Mr4	92.1
Mp1	93.1
Mp2	50.8

¹Mexican raw milk cheeses (Mr1, Mr2, Mr3, and Mr4) and Mexican pasteurized milk cheeses (Mp1 and Mp2) assayed without aging.

was more comparable to the activities of the protein extracts obtained from the model cheeses before aging. The overall bacterial content of these cheeses has been studied (Renye et al., 2008) and revealed that although the Mr1, Mr2, Mr3, and Mr4 samples were made from raw milk and Mp1 and Mp2 were made from pasteurized milk, the overall contamination in all samples with non-food-grade bacteria was very high. Even though the raw milk cheeses contained higher populations of bacteria, leading to the overall increased ACE inhibitory activity observed with these cheeses, the Mp1 and Mp2 cheeses were also exposed to contaminating bacteria, which would likely affect the protein breakdown in these cheeses, causing increased activity as well. Assessing the activity of protein extracts obtained from the Mexican cheese samples after aging has not yet been performed, but may show an increase in the activity of the Mr3 and Mp2 samples, as seen with the model cheeses.

A long list of cheeses, including Gouda and Cheddar, have previously been shown to exhibit some antihypertensive activity, and our results indicate that Queso Fresco could be added to the list. Numerous Spanish cheeses such as Manchego, Roncal, and Mahón have been reported to exhibit inhibitory activity, with Cabrales cheese, made without a starter culture, having the highest measured activity of 76.1% (Gomez-Ruiz et al., 2006). Studies on the ripening of Swiss cheese varieties such as Emmentaler and Le Gruyère showed that ACE inhibitory activities in these cheeses were related to elevated concentrations of 2 known ACE-inhibiting peptides, VPP and IPP, which could be associated with the presence of *Lactobacillus helveticus* strains in the starter cultures (Meyer et al., 2009). More recently, ACE inhibitory activity was identified in water-soluble extracts from the Italian cheese Asiago d'allevio (Lignitto et al., 2010).

Pooling this type of information has led to the development of novel cheeses with potential health benefits by manipulating ripening times and bacterial strains used as starter cultures (Ryhanen et al., 2001). Future work involving the isolation and characterization

of the ACE-inhibitory compounds within the Quesos Frescos in the present study will add to the current available data on cheese production. This information, along with a more comprehensive understanding of the bacterial strains, if any, responsible for the production of small bioactive peptides in the cheese matrices may eventually lead to cheeses that can lower blood pressure in American consumers.

Protein extracts from a variety of Mexican and model Queso Frescos were assayed for antihypertensive activity. Although the model cheeses displayed some ACE inhibitory activity before aging, significant increases were observed after 8 wk of storage. Presumably, this is due to the presence of proteases associated with spoilage bacteria that grow during the aging period. The Mexican Queso Frescos were made from raw and pasteurized milk, but all were contaminated to some extent by non-food-grade bacterial species. The protein extracts from these samples showed elevated levels of ACE inhibition without aging, which implies that the inhibitory compounds are either already present in the milk used for cheese-making or are generated during cheese-making, most likely as a result of milk protein breakdown leading to smaller antihypertensive peptides. Further investigations will more thoroughly examine which bacteria are needed to generate the antihypertensive peptides, as well as fully characterize those peptides responsible for this activity.

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