Angiotensin I-converting enzyme-inhibitory activity of the Norwegian autochthonous cheeses Gamalost and Norvegia after in vitro human gastrointestinal digestion

T. M. Qureshi, G. E. Vegarud, R. K. Abrahamsen, and S. Skeie¹

Department of Chemistry, Biotechnology and Food Sciences, Norwegian University of Life Sciences, Chr. Magnus Falsens vei 1, 1432 Ås, Norway

ABSTRACT

The angiotensin I-converting enzyme (ACE) inhibitory activity of Gamalost cheese, its pH 4.6-soluble fraction, and Norvegia cheese was monitored before and after digestion with human gastric and duodenal juices. Both Gamalost and Norvegia cheeses showed an increased ACE-inhibitory activity during gastrointestinal digestion. However, only Norvegia showed pronounced increased activity after duodenal digestion. More peptides were detected in digested Gamalost compared with digested Norvegia. Most of the peptides in Gamalost were derived from β -casein (CN), some originated from α_{s1} -CN, and only a very few originated from α_{s2} -CN and κ -CN. In general, the number of peptides increased during gastrointestinal digestion, whereas some peptides were further degraded and disappeared; however, surprisingly, a few peptides remained stable. The aromatic amino acids, such as Tyr, Phe, and Trp; the positively charged amino acids (Arg and Lys); and Leu increased after simulated gastrointestinal digestion of Gamalost and Norvegia. After in vitro gastrointestinal digestion, both Gamalost and Norvegia showed high ACE-inhibitory activity, which may contribute in lowering of mild hypertension.

Key words: Gamalost, angiotensin I-converting enzyme inhibition, human gastrointestinal digestion, peptide composition

INTRODUCTION

Gamalost, an autochthonous mold (*Mucor mucedo*)-ripened Norwegian cheese, is made by acid precipitation of fermented pasteurized skim milk and has been shown to have a high angiotensin I-converting enzyme (ACE)-inhibitory effect (Pripp et al., 2006; Qureshi et al., 2012). Gamalost differs from other mold cheeses by

having a low fat content (<0.05% fat) and by the inactivation of the starter bacteria due to intensive cooking of the cheese curd in whey for 1 to 2 h at 90 to 95°C before the mold is added to the cheese. The development of ACE inhibitory peptides in Gamalost during ripening has previously been investigated (Qureshi et al., 2012), and it was found that the ACE inhibition of the pH 4.6-soluble fraction (SF) differed during ripening due to development of potential bioactive peptides.

A large number of bioactive peptides have been identified in milk, fermented dairy products, and cheeses (Meisel, 1998; Clare and Swaisgood, 2000; FitzGerald et al., 2004; Silva and Malcata, 2005; Sieber et al., 2010). These peptides have no bioactivity in the parent protein but are released during fermentation and hydrolysis by native enzymes, rennet enzymes, and bacterial enzymes in the cheese and further by digestive enzymes (FitzGerald et al., 2004; Korhonen and Pihlanto, 2006).

For a long time, commercial enzymes such as pepsin, trypsin, and chymotrypsin from nonhuman origin have been used in simulated human digestion, and many peptides have been identified as a result of such digestion experiments. Only a few studies have been carried out using human gastrointestinal (GI) enzymes under physiological conditions to identify the peptides formed (Mullally et al., 1997; Abubakar et al., 1998; Parrot et al., 2003; Gómez Ruiz et al., 2004; Hernández-Ledesma et al., 2004; Almaas et al., 2006b; Schmelzer et al., 2007). Different peptide patterns have been observed when using commercial enzymes compared with human enzymes. The in vitro human enzyme digestion results seem to be more consistent with the in vivo digestion studies reported (Chabance et al., 1998; Almaas et al., 2006a; Eriksen et al., 2010).

Human digestion depends on many factors such as composition of the food, gastric pH, buffering capacity of the food, transit time, and concentrations and activities of the digestive enzymes and other digestive components (Dressman et al., 1990; Ekmekcioglu, 2002; Moreno, 2007). Gastric juice contains hydrochloric acid, pepsin, mucous, and gastric lipase (only in newborns), whereas pancreatic juice has high contents of bicarbon-

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¹Corresponding author: siv.skeie@umb.no

ate, bile salts, and digestive enzymes such as trypsin, chymotrypsin, amino and carboxypeptidases, amylase, lipase, and enzymes that aid in digestion of nucleic acids and phospholipids (Ulleberg et al., 2011; Campbell, 2012). Generally, gastric pH ranges from 1 to 3.5 during the fasting period. However, the gastric pH range measured in healthy adults following a meal was 3.9 to 5.5, depending on the buffering capacity of the food. The duodenal pH is normally around 6.0 to 7.8 (McCloy et al., 1984; Russell et al., 1993; Ekmekcioglu, 2002; Kalantzi et al., 2006; Campbell, 2012).

Studies on ACE inhibition after digestion with commercial enzymes have been performed on different milk proteins such as β -LG, α -LA, whey protein concentrates, and cheese. In these studies, some antihypertensive peptides of β -CN, including the potent Val-Pro-Pro and Ile-Pro-Pro peptides, have been identified (Mullally et al., 1997; Parrot et al., 2003; Vermeirssen et al., 2003; Gómez Ruiz et al., 2004; Ohsawa et al., 2008). To our knowledge, no in vitro digestion studies of cheese using human gastric juice (HGJ) and human duodenal juice (HDJ) evaluating the ACE-inhibitory potential of the cheese digests have been published. Therefore, it would be interesting to follow the ACE-inhibitory activity of Gamalost, which is known to have high ACE-inhibitory activity (Qureshi et al., 2012), and Norvegia, which is representative of cheese varieties used by many consumers in Norway, during digestion with human gastric and duodenal juices. The main objective of the present study was to evaluate the ACE-inhibitory activity of 2 Norwegian cheeses (Gamalost and Norvegia) during digestion with human gastric and duodenal juices and to characterize the peptides and amino acids in the digests.

MATERIALS AND METHODS

Collection and Ripening of Cheese

Gamalost cheese, produced as described by Qureshi et al. (2012), was kindly supplied by TINE Meieriet Vik (Oslo, Norway). Five cheeses from each of 3 separate productions were selected randomly at the dairy. One cheese was frozen (-20°C) at age 0 (after cooking of the cheese in whey but before applying the mold at the surface of the cheese), the remaining 4 cheeses were frozen after 10 d of ripening. The frozen 10-d-old cheeses were thawed and one from each batch was further ripened at the Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science (Ås, Norway) at 4°C .

The cheeses were sampled after 0, 10, and 30 d of ripening, ground, and frozen until digestion and further analysis. The sampling of the cheeses was done accord-

ing to International Dairy Federation (IDF) standard 50C (IDF, 1995) and the cheese was grated with a manual grinder. From each sampling, a pH 4.6 SF was prepared according to the procedure described by Pripp et al. (2006). Three Norvegia cheeses (90 d old) were purchased from a local grocery shop, and used for comparison with Gamalost. The Gamalost cheese, its pH 4.6 SF, and Norvegia were further used in simulated digestion experiments.

Aspiration of Human Gastric and Duodenal Juices and Their Activities

Human gastric and duodenal juices were collected from healthy volunteers according to Ulleberg et al. (2011). The juices were collected from 20 fasting volunteers (7 men and 13 women; average age, 25 ± 5 yr). The collected juices were mixed together to make a batch as the juices vary in their activity from individual to individual. The main advantage of making a batch of juices was to reduce the variations in enzyme activity between the individual samples. The collected juices were centrifuged ($4,500 \times g$ for 10 min) to remove mucous and cell debris before storing at -20 or -80°C . The pepsin activity of human gastric juices was measured as described by Sánchez-Chiang et al. (1987), whereas the total proteolytic activity of the human duodenal juices was measured as described by Krogdahl and Holm (1979).

In Vitro Enzymatic Digestion

An in vitro digestion model was performed to simulate human digestion in the stomach (step 1) and the duodenum (step 2) according to Almaas et al. (2006a), with some modifications. The Gamalost (2 g; containing $\sim 50\%$ protein), Norvegia (4 g; containing $\sim 26\%$ protein), and the pH 4.6 SF (150 mg) of Gamalost were dissolved in 10 mL of physiological solution (0.9% NaCl). To mimic chewing in the mouth, the sample was incubated (37°C) for 5 to 7 min in a Stomacher (Seward stomacher 400; Seward Ltd., West Sussex, UK) with constant shaking. To simulate the gastric phase (step 1), the pH was slowly decreased to 2.5 by dropwise addition of 2 M HCl after adding HGJ (15 U/g of protein) and the samples were incubated (0.5 h for the pH 4.6 SF and 1 h for the cheeses) at 37°C in a stomacher. The following duodenal digestion (step 2) was performed by pH adjustment to 7 by 4 M NaOH and then incubated with HDJ (31.2 U/g of protein; 1 h for the pH 4.6 SF and 3 h for cheeses) at 37°C . Samples (0.3–0.5 mL) were collected before digestion, after gastric digestion (step 1) and after the subsequent duodenal digestion (step 2). To stop the enzymatic reaction, the samples

were immediately transferred to an ice bath and frozen (-20°C). The digestion of each sample was performed in duplicate.

Before further analysis, the samples were thawed and the digestive enzymes were separated from the sample by ultrafiltration through hydrophilic membranes (Amicon Ultra, cut-off molecular weight = 10 kDa; Millipore BV, Carrigtwohill, Cork, Ireland) by centrifugation ($11,148 \times g$ for 40 min at 4°C). The peptides having molecular weight lower than 10 kDa were collected in the permeate for all the samples.

Chemical Analysis of Cheese

The DM content of Gamalost was determined according to IDF standard 4/ISO 5534 (IDF, 2004). The soluble nitrogen (SN) content of the 10-kDa permeate of Gamalost cheese and of Norvegia cheese, and the pH 4.6 SF of Gamalost cheese as well as the total nitrogen (TN) content of Norvegia cheese were determined by the Kjeldahl method (IDF, 1993). A homogeneous sample for TN of Gamalost was difficult to obtain due to formation of precipitates during sample preparation. In addition, due to the presence of denatured whey proteins, foaming occurred during the digestion step in the preparation of the Gamalost sample for TN. Therefore, SN/DM of the Gamalost was used instead of SN/TN.

Identification of Peptide Sequences

Nano-liquid chromatography-mass spectrometry of desalted and concentrated samples was performed according to Qureshi et al. (2012) following the method described by Eriksen et al. (2010), with some modifications. Only peptides with mass above 800 and below 4,500 Da were subjected to collision-induced fragmentation and further processing.

Free Amino Acid Composition

For the analysis of amino acid composition, the samples were prepared as described by Qureshi et al. (2012), with reversed-phase HPLC using α -phthalaldehyde and fluorenylmethyloxycarbonyl chloride derivatization, following the procedure of Bütikofer and Ardö (1999), with some modifications. One hundred microliters of the 10-kDa permeate of all the samples was mixed with 100 μL of 0.1 *M* HCl containing 0.4 $\mu\text{mol/mL}$ of L-norvaline (Sigma, St. Louis, MO) and 0.4 $\mu\text{mol/mL}$ of piperidine-4-carboxylic acid (Fluka, St. Louis, MO), and used as internal standards.

Preparation of Samples for ACE-Inhibition Assay

The ACE-inhibition assays were performed using the reaction of substrate (hippuryl-histidyl-leucine; Sigma)

and enzyme (extract from rabbit lung acetone powder; Sigma), with measurement of the liberated hippuric acid (HA) by reversed-phase HPLC according to Qureshi et al. (2012), following the method described by Hyun and Shin (2000), with some modifications. Forty microliters of the 10-kDa permeate of all samples was used in the assay. Captopril ($\text{C}_9\text{H}_{15}\text{NO}_3\text{S}$; Sigma), a blood pressure-lowering pharmaceutical, was used as an inhibitory reference. The ACE inhibition (%) was calculated by using Equation 1 given below:

$$\text{ACE inhibition (\%)} = \frac{\text{HA control} - \text{HA (sample)}}{\text{HA (control)}} \times 100, \quad [1]$$

where HA (control) denotes the concentration of hippuric acid liberated after reaction of enzyme and substrate (without sample), whereas HA (sample) represents the hippuric acid released after the reaction of enzyme and substrate in the presence of the sample. The estimated volume of 10-kDa permeates extracted from 1 g of Gamalost was 5 mL, whereas the estimated volume of 10-kDa permeates extracted from 1 g of Norvegia was 2.5 mL (as described in the *in vitro* enzymatic digestion section). The results of the 10-kDa permeates were calculated as the amount of cheese. Hence, the half maximal inhibitory concentration (IC_{50}), which is the inhibitory concentration of the sample of the freeze-dried pH 4.6 SF (in mg/mL or in milligram equivalent cheese per milliliter) required to inhibit 50% of the ACE activity was determined from the linear regression equation by plotting ACE inhibition (%) versus the inhibitory concentration of each dilution of the sample. The IC_s was calculated using the following Equation 2:

$$\text{IC}_s = (\text{C}_0 \times \text{V}_s \times \text{L})/\text{V}, \quad [2]$$

where C_0 is the initial sample concentration (mg/mL), V_s is the sample volume (40 μL), L denotes the dilutions used (0.5, 0.25, and 0.125), and V is the total reaction volume (340 μL). From the IC_{50} values of the freeze-dried pH 4.6 SF, the IC_{50} values of the pH 4.6 SF were also calculated as amount of Gamalost by using the weight (g) of freeze-dried pH 4.6 SF extracted from 1 g of cheese. The ACE-inhibitory potential (IP) per unit cheese weight (mg of captopril Eq/kg of cheese) was calculated by Equation 3 given below:

$$\text{ACE (IP)} = \frac{\text{IC}_{50}(\text{captopril}) \times \text{pH 4.6 SF or cheese amount}}{\text{IC}_{50}(\text{pH 4.6 SF})}, \quad [3]$$

where IC_{50} (captopril) and IC_{50} (pH 4.6 SF) are the concentrations (mg/mL) of captopril and the freeze-

dried pH 4.6 SF (mg/mL), respectively, whereas pH 4.6 SF and cheese amount represent the amount (mg) of freeze-dried powder from 1 g of cheese and amount of cheese (mg) present in 1 mL of the physiological solution (0.9% NaCl), respectively.

Statistical Analysis

Statistical analysis was performed by Minitab statistical software version 15 (Minitab Inc., State College, PA). The Shapiro-Wilk test was used for testing the assumption of normal distribution of the data, which were satisfied for all variables. Individual cheeses from the same batch were assumed to be independent. Two-way ANOVA was performed with replicate block (random variable) and age (fixed variable) of Gamalost cheese to test the null hypothesis H_0 : that the ACE-inhibitory activity in the Gamalost and its pH 4.6 SF did not change during ripening. Similarly, 2-way ANOVA was carried out with replicate block (random variable) and digestion steps (fixed variable; including undigested samples) and Norvegia to test H_0 : that the ACE-inhibitory activity did not change during digestion of Gamalost and its pH 4.6 SF at each ripening stage and Norvegia at 90 d. The Tukey test for pairwise comparison was used to test the differences between means. For all comparisons, the level of significance was set to $P < 0.05$.

RESULTS

SN of Gamalost, Its pH 4.6 SF, and Norvegia Cheese

The SN/DM (%) of the 10 kDa permeates of Gamalost and its pH 4.6 SF and SN/TN (%) of the 10 kDa permeates of Norvegia is presented in Table 1. The SN/DM (%) of Gamalost and its pH 4.6 SF increased during ripening as well as during subsequent digestion. A difference between the different batches of Gamalost was found during ripening on the content of SN/DM (%), whereas in the digested Gamalost, an effect of productions was found on the SN/DM (%) after 30 d of ripening (results not shown). The SN/TN (%) content of Norvegia cheese (90 d) also increased significantly during both the gastric and duodenal digestion, and the SN/TN (%) of Norvegia reached its peak after duodenal digestion.

ACE Inhibition During Ripening and After GI Digestion

The results of the ACE-inhibition (%) measurements and the IC_{50} values of Gamalost (10–30 d) and its pH 4.6 SF, and of Norvegia (90 d) are presented in Figures 1 and 2, respectively. Optimum ACE-inhibitory activity

(%) and the lowest IC_{50} values of the Gamalost and its pH 4.6 SF were observed after 10 d of ripening. Digestion affected the ACE-inhibitory activity of Gamalost and its pH 4.6 SF, as well as Norvegia. The highest ACE-inhibitory activity of Gamalost was observed after gastric digestion, and further duodenal digestion did not seem to affect the ACE-inhibitory activity further. The IC_{50} values showed similar results after gastric and duodenal digestion of Gamalost (Figure 2). By recalculation of the IC_{50} of the pH 4.6 SF to the corresponding amount of the original Gamalost (10 and 30 d), the trend was similar to what was found for the pH 4.6 SF. The IC_{50} values were higher in the Gamalost cheese before digestion (2.36 and 1.85 in Gamalost and the recalculated pH 4.6 SF, respectively, at 10 d) and lower in Gamalost after gastric digestion (0.82 and 1.65 in Gamalost and the recalculated pH 4.6 SF, respectively, at 10 d) as well as duodenal digestion (0.99 and 2.22 in Gamalost and the recalculated pH 4.6 SF, respectively, at 10 d). Unlike Gamalost, Norvegia showed a gradual increase in the ACE-inhibitory activity, with a drastic decrease in IC_{50} values after the successive gastric and duodenal digestion. However, Gamalost differed from Norvegia by showing much lower IC_{50} values at all digestion steps. The IC_{50} value of captopril measured in the assay was $4.1 \times 10^{-6} \pm 1.3 \times 10^{-7}$ mg/mL. An influence of different productions was observed on the IC_{50} values of Gamalost during ripening, whereas no influence of production was found on the ACE inhibition (%) of Gamalost cheese during ripening. An effect of different productions was observed on the ACE inhibition (%) of pH 4.6 SF at 30-d Gamalost during GI digestion. An effect of different productions was also found on the IC_{50} values in 30-d Gamalost during GI digestion (results not shown).

On the basis of the results of the IC_{50} values of Gamalost, its pH 4.6 SF, and Norvegia, the ACE IP per unit of cheese weight was calculated (Figure 3). Gamalost cheese as well as its respective pH 4.6 SF showed high ACE IP after gastric digestion, but after duodenal digestion the IP values were somewhat reduced. Norvegia, however, showed an increasing trend after subsequent digestion, but its ACE IP remained at lower levels compared with Gamalost.

Peptides Generated During Ripening and After GI Digestion

Many peptides were identified during ripening and GI digestion. A summary table (Table 2) shows the number of peptides present before and after digestion of Gamalost, its pH 4.6 SF, and Norvegia. Most peptides derived from β -CN, whereas some were released from α_{s1} -CN and only a few derived from α_{s2} -CN and κ -CN.

Table 1. Soluble nitrogen (SN) in DM (%) of the 10-kDa permeates, the pH 4.6-soluble fraction (SF; 0–30 d), and Gamalost cheese (10 and 30 d), and SN in total nitrogen (TN; % of 10-kDa permeates of Norvegia cheese (90 d) before (undigested, U) and after gastrointestinal digestion by human gastric juice (HG.J) at pH 2.5 and human duodenal juice (HD.J) at pH 7.0

Age (d)	Sample type	SN/DM (%) ¹ of pH 4.6 SF			SN/DM (%) ¹ of Gamalost or SN/TN (%) ² for Norvegia		
		U	HG.J	HD.J	U	HG.J	HD.J
0	pH 4.6 SF	0.05 ± 0.02 ^{c,B}	0.82 ± 0.34 ^{c,A}	0.87 ± 0.47 ^{c,A}	—	—	—
10	pH 4.6 SF or Gamalost	4.34 ± 0.47 ^{b,C}	8.64 ± 0.80 ^{b,B}	11.45 ± 0.67 ^{b,A}	6.09 ± 0.21 ^{b,C}	6.98 ± 0.40 ^{b,B}	10.52 ± 0.47 ^{b,A}
30	pH 4.6 SF or Gamalost	6.26 ± 0.88 ^{a,B}	12.05 ± 2.39 ^{a,A}	12.29 ± 1.57 ^{a,A}	7.59 ± 0.28 ^{a,C}	8.25 ± 0.37 ^{a,B}	12.16 ± 0.45 ^{a,A}
90	Norvegia	—	—	—	8.84 ± 1.02 ^C	14.13 ± 0.73 ^B	51.36 ± 5.22 ^A

^{a-c}Means within a column with different superscript lowercase letters represent differences ($P < 0.05$) between the ages of the cheeses.
^{A-C}Means within a row with different superscript capital letters show differences ($P < 0.05$) between the digestion steps (including undigested samples) in the pH 4.6 SF or cheeses at the same ripening stage.
¹Soluble nitrogen as a percentage of DM of Gamalost.
²Soluble nitrogen as a percentage of TN in Norvegia.

In short, Gamalost cheese contained almost twice as many peptides as detected in its pH 4.6 SF and Norvegia.

In general, most of the peptides that were present in Gamalost matched the peptides present in the pH 4.6 SF; however, only a few peptides were common between Norvegia and Gamalost cheese or its pH 4.6 SF (Figures 4–8). The peptide pattern of Norvegia differed from that of Gamalost by a much lower content of peptides. The peptide pattern of Gamalost (10 and 30 d) differed between undigested, gastric, and duodenal digested samples, and digestion affected the peptide profile in different ways. Figures 4 and 5 show in more detail the peptide pattern derived from β -CN during gastric and duodenal digestion of Gamalost (10 and 30 d). Many new peptides were released after gastric and further duodenal digestion. The peptides that were stable during GI digestion of the 10-d-ripened Gamalost were β -CN (126–139), β -CN (129–139), β -CN (144–160), β -CN (192–207), and β -CN (193–207), and a few peptides in the 30-d-ripened Gamalost [β -CN (78–91), β -CN (129–139), β -CN (144–160), and β -CN (193–207)] were also stable through GI digestion. In the pH 4.6 SF (10–30 d) of Gamalost, many peptides derived from β -CN were detected in the extract before digestion. Almost all of the peptides present in the undigested pH 4.6 SF of Gamalost were also detected in the undigested Gamalost cheese. Some peptides derived from β -CN were common between the gastric digested and duodenal digested pH 4.6 SF (results not shown) and Gamalost. In Norvegia cheese, some peptides [β -CN (193–206), β -CN (194–209), and β -CN (195–206)] remained stable during GI digestion; however, some new peptides were observed during digestion (Figure 6).

A lower number of peptides were derived from α_{s1} -CN than from β -CN during GI digestion of Gamalost cheese (Figure 7). In Gamalost cheeses (10 and 30 d), some new peptides derived from α_{s1} -CN were generated during GI digestion, and only 1 peptide [α_{s1} -CN (180–194)] in 30-d Gamalost remained stable during GI digestion (Figure 7a and b). The pH 4.6 SF from fresh cheese (0 d) contained a higher number of peptides derived from α_{s1} -CN compared with the pH 4.6 SF of 10- and 30-d-ripened cheese (results not shown). Figure 8a and b shows the peptides derived from α_{s2} -CN in Gamalost. Only 1 peptide [α_{s2} -CN (99–115)] remained stable in the 10-d-old Gamalost cheese during GI digestion. In addition, Gamalost contained 2 peptides [β -LG (124–136) and β -LG (124–139)] derived from β -LG (Table 2). In undigested Norvegia cheese, a few peptides such as α_{s1} -CN (1–13), α_{s1} -CN (1–14), α_{s1} -CN (1–16), α_{s1} -CN (10–23), α_{s1} -CN (15–23), and α_{s1} -CN (26–34) were derived from α_{s1} -CN. Only 1 peptide [α_{s2} -CN (151–162)] from undigested samples and 1

Figure 1. Angiotensin I-converting enzyme (ACE) inhibition (%; mean $\pm$ SD) of the <10-kDa permeates of Gamalost cheese (10 and 30 d), its pH 4.6-soluble fraction (SF; 0–30 d), and Norvegia cheese before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Different lowercase letters (a–c) over the bars (except for Norvegia) represent significant differences ($P < 0.05$) between the ages of cheeses at the same digestion step, whereas different uppercase letters (A–C) show significant differences ($P < 0.05$) during digestion of the same sample at the same ripening stage.

[α_{s2} -CN (100–114)] from HDJ digestion, derived from α_{s2} -CN, were present in Norvegia cheese (results not shown). From κ -CN, only a few peptides were observed in Gamalost (30 d), pH 4.6 SF (0 d), and Norvegia (Table 3). During GI digestion, only 1 peptide [κ -CN (155–169)] derived from κ -CN in the pH 4.6 SF from 0-d Gamalost, remained stable.

Most of the peptides identified in Gamalost, its pH 4.6 SF, and Norvegia had hydrophobic amino acids, such as Ala, Ile, Leu, Met, Phe, Trp, and Val, as well as Pro at any of the 3 C-terminal positions of peptides. In addition, positively charged amino acids such as Arg and Lys were detected at any of the 3 C-terminal positions of a few peptides. Moreover, most peptides in Gamalost, its respective pH 4.6 SF and in Norvegia were generated from internal as well as from the C-terminal sequences of β -, α_{s1} -, α_{s2} -, and κ -CN.

Free Amino Acid Contents Before and After GI Digestion

The free amino acid contents (mmol/kg) of Gamalost (10 and 30 d) and of Norvegia before and during diges-

tion are presented in Figure 9a, b, and c. The amino acids Cit and γ -aminobutyric acid (GABA) were detected in negligible concentrations in Gamalost and Norvegia and were, therefore, omitted from Figure 9. Digestion affected the generation of free amino acids; the content of Pro decreased significantly during gastric digestion of Gamalost whereas the content of Arg, Tyr, Phe, Leu, and Lys were not influenced considerably by gastric digestion. However, after duodenal digestion, the content of these amino acids increased significantly. Norvegia (Figure 9 c) had very low concentrations of all amino acids compared with Gamalost; however, the amino acids Arg, Tyr, Phe, Trp, and Lys increased significantly after duodenal digestion of Norvegia.

DISCUSSION

In Gamalost, the increased ACE inhibition after gastric digestion indicates a possible further release of potent peptides during digestion. After digestion of Gamalost with HGJ and HDJ, the decreased IC_{50} values might be due to the generation of new peptides that may be more active as compared with the peptides present

Figure 2. Inhibitory concentration required to inhibit 50% of the angiotensin I-converting enzyme (ACE) activity (IC_{50}) values (mean $\pm$ SD) expressed as milligram equivalent per milliliter of Gamalost (10 and 30 d) and Norvegia cheeses and pH 4.6-soluble fraction (SF; 0–30 d; mg/mL) of Gamalost before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Different lowercase letters (a and b) over the bars (except for Norvegia) represent significant differences ($P < 0.05$) between the ages of cheeses at the same digestion step, whereas different uppercase letters (A–C) show significant differences ($P < 0.05$) during digestion of the same sample at the same ripening stage.

in the undigested cheese. The considerable decreasing trend of IC_{50} of Norvegia after GI digestion revealed that the released peptides might have a very high ACE-inhibitory effect. The increased content of SN/TN (%) of Norvegia after duodenal digestion is consistent with the results of Parrot et al. (2003), who found that the SN/TN content increased drastically to almost 50% in Emmental cheese water-soluble extract (**WSE**) by the action of pepsin and trypsin. A considerable increase in Trp, Phe, and Tyr in the digests of Gamalost and Norvegia by human GI digestion is concurrent with the findings of Parrot et al. (2003) and Adt et al. (2011).

The WSE of Asiago d'allevio cheese with peptides having molecular mass less than 3 kDa were reported to have a higher ACE-inhibitory activity than the WSE containing peptides larger than 3 kDa (Lignitto et al., 2010). Most of the peptides observed in Gamalost, its pH 4.6 SF, and Norvegia had molecular masses lower than 3 kDa. López-Fandiño et al. (2006) reported that peptides with less than 27 amino acids had appreciable ACE-inhibitory activity. A slight difference existed in the IC_{50} values of the non-UF pH 4.6 SF (0 and 10 d) in our previous study (Qureshi et al., 2012) and the UF pH 4.6 SF of the present study, as some of the peptides

most probably were lost during UF of the samples in the present study. The difference in the IC_{50} values between the pH 4.6 SF of Gamalost (10 and 30 d) and Norvegia observed in the current study compared with the previously mentioned study (Qureshi et al., 2012), might be due to differences in the number of active peptides between different batches of cheese.

The presented results are consistent with previous reports regarding the structure-activity relationship between ACE inhibition and the available peptides (López-Fandiño et al., 2006; Haque and Chand, 2008). The presence of hydrophobic (Tyr, Phe, Trp, Ala, Ile, Leu, Val, and Met) or positively charged amino acids such as Arg and Lys as well as Pro at any of the 3 C-terminal positions of the peptides shows good binding of ACE (López-Fandiño et al., 2006; Haque and Chand, 2008; He et al., 2012). We observed that Gamalost also contained some of the peptides in which the 2 potent tripeptides Ile-Pro-Pro and Val-Pro-Pro were present in an encrypted form within their sequences. However, the pH 4.6 SF of Gamalost and Norvegia contained very few of those peptides. The generation of a few peptides from the hydrophobic para- κ -CN in 30-d-old Gamalost and in Norvegia might explain the suscepti-

Figure 3. Angiotensin I-converting enzyme (ACE)-inhibitory potential (IP; per unit of cheese weight, expressed as milligrams of captopril equivalents per kilogram of cheese; mean $\pm$ SD) of Gamalost cheese (10 and 30 d), its pH 4.6-soluble fraction (SF; 0–30 d), and Norvegia cheese before (undigested; black bars) and after gastrointestinal digestion by human gastric juice (HGJ; white bars) and human duodenal juice (HDJ; line pattern bars). Different lowercase letters (a and b) over the bars (except for Norvegia) represent significant differences ($P < 0.05$) between the ages of cheeses at the same digestion step, whereas different uppercase letters (A–C) show significant differences ($P < 0.05$) during digestion of the same sample at the same ripening stage.

bility of para- κ -CN (residues 1–105) toward hydrolysis by HGJ and HDJ. Gamalost is an acid-coagulated cheese, and the glycomacropeptide (**GMP**) is retained on the κ -CN. However, the peptides κ -CN (149–169) and κ -CN (155–169) derived from the hydrophilic GMP were found in the undigested cheese in the pH 4.6 SF from the unripened Gamalost (d 0) and further degradation occurred during digestion. These peptides were not found after 30 d of ripening, which indicated that the GMP was completely degraded. The presence of 2 peptides, derived from β -LG, following duodenal digestion revealed the presence of some whey proteins in Gamalost, which is reasonable, as the cheese was cooked in whey during manufacturing and denatured whey proteins were, therefore, retained in the cheese matrix.

Chymotrypsin, trypsin, and pepsin have specific amino acid targets during hydrolysis of proteins. It has been shown in many studies that trypsin attacks on the carboxyl side of positively charged amino acids such as Arg and Lys of the peptide sequences, as well as cleaving before Pro (Neurath, 1957; Custódio et al., 2005; Rodriguez et al., 2008). It has also been reported that chymotrypsin has a broader specificity spectrum than

trypsin and, therefore, attacks on the carboxyl side of nonpolar, hydrophobic amino acids or aromatic amino acids (Tyr, Phe, and Trp; Neurath, 1957). Pepsin has been shown to hydrolyze the amino side of the Leu residues and, similar to chymotrypsin, it also attacks on the carboxyl side of the aromatic amino acids (Neurath, 1957; Auffret and Ryle, 1979). Schmelzer et al. (2007) concluded, from an in vitro peptic digestion of β -CN, that pepsin cleaves the C-terminal region that is rich in hydrophobic residues. Our findings are mostly consistent with the aforementioned reports regarding cleavage site specificities due to the activity of enzymes present in HGJ and HDJ. During HGJ digestion of 10- or 30-d-ripened Gamalost, some peptides such as β -CN (59–93), β -CN (126–140), β -CN (129–141), β -CN (193–209), and β -CN (193–209) were generated, which have also been detected in the peptic digests of β -CN during in vitro digestion by pepsin (Schmelzer et al., 2007). Some of the peptides [β -CN (125–140), β -CN (126–141), β -CN (126–142), β -CN (129–140), β -CN (143–163), β -CN (190–209), β -CN (191–209), and β -CN (192–209)] present in undigested Gamalost in the present study were also detected by Schmelzer et al. (2007) in pepsin-digested β -CN. *Mucor mucedo* might have a

Table 2. Number of identified peptides in Gamalost cheese (ripened for 10 and 30 d), its pH 4.6-soluble fraction (SF), and Norvegia cheese (90 d) present before (undigested samples, U) and after gastrointestinal digestion by human gastric juice (HGJ) and human duodenal juice (HDJ)¹

Protein type	0 d ²				10 d				30 d				90 d ²			
	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total	U	HGJ	HDJ	Total
Gamalost cheese																
β-CN					32	47	30	72	38	33	19	68				
α _{s1} -CN					12	7	4	20	6	7	5	14				
α _{s2} -CN					1	3	3	5	5	5	1	7				
κ-CN					0	0	0	0	0	2	0	2				
β-LG					0	0	0	0	0	0	2	2				
Total					45	57	37	97	51	47	27	93				
Norvegia																
β-CN													19	14	15	36
α _{s1} -CN													6	0	0	6
α _{s2} -CN													1	0	1	2
κ-CN													1	4	0	5
β-LG													0	0	0	0
Total													27	18	16	49
pH 4.6 SF of Gamalost																
β-CN	6	15	08	22	21	12	8	33	22	15	7	33				
α _{s1} -CN	5	4	7	10	2	2	2	4	3	1	3	5				
α _{s2} -CN	0	0	0	0	1	2	2	4	3	3	2	5				
κ-CN	2	2	2	4	0	0	0	0	0	0	0	0				
Total	13	21	17	36	24	16	12	41	28	19	12	43				

¹Totals in each column correspond to the sum of the peptides derived from the different caseins in the sample. Totals in each row represent the number of different peptides (common peptides are counted only once) present before (U) and after gastrointestinal digestion by HGJ and HDJ in Gamalost, its pH 4.6 SF, and Norvegia.

²Samples were not prepared from 0-d Gamalost cheese and pH 4.6 SF from Norvegia.

Figure 4. The peptides derived from β -CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark gray lines) and human duodenal juice (HDJ; light gray lines) in the <10-kDa permeate of 10-d ripened Gamalost cheese.

Figure 5. The peptides derived from β -CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark gray lines) and human duodenal juice (HDJ; light gray lines) of the <10-kDa permeate of 30-d ripened Gamalost cheese.

Figure 6. The peptides derived from β -CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark gray lines) and human duodenal juice (HDJ; light gray lines) of the <10-kDa permeate of Norvegia cheese.

Figure 7. The peptides derived from α_{s1} -CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark gray lines) and human duodenal juice (HDJ; light gray lines) of the <10-kDa permeate of Gamalost cheese. (a) 10-d-ripened Gamalost; (b) 30-d-ripened Gamalost.

Figure 8. The peptides derived from α_{s2} -CN before (undigested; indicated by U and black lines) and after in vitro gastrointestinal digestion by human gastric juice (HGJ; dark gray lines) and human duodenal juice (HDJ; light gray lines) of the <10-kDa permeate of Gamalost cheese and its pH 4.6-soluble fraction (SF). (a) 10-d-ripened Gamalost; (b) 30-d-ripened Gamalost.

Table 3. Identified peptides, with molecular mass (M_r , in Da), derived from κ -CN in Gamalost cheese (30 d), its pH 4.6-soluble fraction (SF; 0 d), and Norvegia cheese (90 d) before (undigested samples, U) and after human gastric juice (HGJ) and human duodenal juice (HDJ) digestion

M_r	Fragment	Amino acid sequence ¹	pH 4.6 SF			Gamalost			Norvegia		
			0 d			30 d			90 d		
			U	HGJ	HDJ	U	HGJ	HDJ	U	HGJ	HDJ
1584.83	18–30	F.FSDKIAKYIPIQY.V					+			+	
1796.98	18–32	F.FSDKIAKYIPIQYVL.S								+	
1181.61	56–65	F.LPYPPYAKPA.A					+				
2861.53	51–75	L.INNQFLPYPPYAKPAAVRSPAQILQ.W								+	
1536.73	67–79	A.VRSPAQILQWQVL.S							+		
1197.51	96–105	M.ARHPHPLSF.M								+	
1144.56	116–137	D.KTEIPTINTIASGEPTSTPTTE.A		+							
2196.06	149–169	D.SPEVIESPPEINTVQVTSTAV	+								
1226.59	151–161	P.EVIESPPEINT.V			+						
1541.73	155–169	E.SPPEINTVQVTSTAV	+	+	+						

¹The dot (.) represents the cleavage site of the peptides.

Figure 9. The concentrations (mmol/kg of cheese; mean $\pm$ SD) of the free amino acids (FAA) in the undigested (black bars), human gastric juice- (HGJ; white bars), and human duodenal juice- (HDJ; line pattern bars) digested Gamalost and Norvegia cheeses. (a) Gamalost ripened for 10 d; (b) Gamalost ripened for 30 d; (c) Norvegia ripened for 90 d.

broader spectrum of cleavage specificities than pepsin, as it was observed in undigested Gamalost cheese that hydrophobic amino acids were present at the C-terminal position of the peptides. The presence of Lys and Arg at the C-terminal end of the peptides may be attributed to the action of plasmin (Upadhyay et al., 2004). However, as minor activity of plasmin (due to denaturation) was expected in Gamalost after cooking

of the cheese curd in whey for 1 to 2 h at 90 to 95°C, the presence of Lys and Arg at the C-terminal position of some of the peptides of Gamalost most probably were due to the activity of *M. mucedo*.

The appearance of some common peptides either after gastric digestion or duodenal digestion among pH 4.6 SF of Gamalost of different ripening times (0–30), as well as between 10- and 30-d-old Gamalost, might also

indicate the common cleavage sites of peptides cleaved by HGJ and HDJ. Most reports on identification of peptides after in vitro digestion of cheese have used commercial enzymes of nonhuman origin (Abubakar et al., 1998; Gómez Ruiz et al., 2004; del Mar Contreras et al., 2009). When comparing peptides released by commercial enzymes with peptides generated with human enzymes, very few peptides matched previously reported ACE-inhibitory peptides (Schmelzer et al., 2007). Pepsin plays an important role in the primary partial digestion of protein (10 to 15%), resulting in the production of long peptides, whereas secondary degradation of peptides was done by trypsin and chymotrypsin, resulting in oligopeptides (Goodman, 2010). More peptides were formed during gastric digestion compared with duodenal digestion, which is manifested from the number of peptides shown in Table 2.

In the present study, some peptides were not degraded and remained stable after GI digestion. If the peptides reach the cardiovascular system in an active form, they may exert a physiological (i.e., antihypertensive) effect (Segura-Campos et al., 2011). Therefore, presumable absorption of active peptides from cheese through the intestinal tract might result in a mild lowering of blood pressure. To affirm the results of our in vitro study and to clarify the bioavailability of the peptides, an epidemiological study on the effect of consumption of Gamalost on blood pressure is in progress.

CONCLUSIONS

Digestion of Gamalost with human GI enzymes increased ACE inhibition. Due to the presence of a larger amount of protein as well as a larger number of peptides (derived from β -, α_{s1} -, α_{s2} -, and κ -CN and β -LG), Gamalost showed lower IC_{50} than Norvegia cheese even though Norvegia showed an enormous decrease in the IC_{50} value during gastric and duodenal digestion. Thus, both Gamalost and Norvegia might contribute to a lowering of mild hypertension, as some of the peptides remained intact during digestion and may be absorbed through the intestine.

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