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Characterization of the Norwegian autochthonous cheese Gamalost and its angiotensin-I-converting enzyme (ACE) inhibitory activity during ripening

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Abstract

Gamalost, a mould-ripened semi-hard traditional Norwegian cheese, has previously (Pripp et al. 2006) been shown to have a very high angiotensin-I-converting enzyme (ACE) inhibition potential compared to other cheeses. In this study the development of the ACE inhibiting peptides in Gamalost, was characterized during ripening. The maximum ACE inhibitory activity of the pH 4.6 soluble fraction of Gamalost was detected after 10 and 20 days of ripening and corresponded to the initial proteolytic activity. During further ripening, a decrease of the ACE inhibitory activity was observed which corresponded to a further increase in the content of free amino acids. From the pH 4.6 soluble fraction of the cheese, 41 different peptides were identified, and were found to be derived mainly from β-casein. The results presented in this paper, confirm the ACE inhibiting activity of Gamalost which peaked between 10 and 20 days of ripening. The ACE inhibiting activities revealed, may indicate that this cheese may have an *in vitro* antihypertensive effect.

Keywords: Gamalost, cheese characteristics, cheese ripening, ACE inhibition

1. Introduction

ACE inhibitory drugs are used in the treatment of hypertension, but these drugs may have associated side effects such as cough, renal failure and a number of fetal abnormalities. Therefore, food protein derived peptides may be used in order to limit these side effects and reduce expenditure on antihypertensive drugs (Haque and Chand 2008). The ACE inhibiting peptides have previously been identified in plant and animal proteins (Li et al. 2004), milk (Haque and Chand 2008), cheese like systems from both ovine and caprine milks (Silva et al.

2006) as well as in different cheeses (Sieber et al. 2010). A number of *in vitro* and *in vivo* (blood pressure measurements on spontaneously hypertensive rats) studies have been performed on many cheese varieties to date (Sieber et al. 2010).

Gamalost, literally meaning "old cheese", is a Norwegian cheese ripened by an autochthonous mould (*Mucor mucedo*) with protected designation of origin status (PDO) (http://www.spesialitet.no). It is made from pasteurized skimmed milk and the caseins are acid precipitated by fermentation with mesophilic lactic acid bacteria (LAB). The cheese has no salt added and no other additives besides the added mould. Probably, it is one of the earliest Norwegian cheeses. It is prepared in cylindrical shapes with an average size of ca. 600 g. The normal ripening period of the cheese is up to 30 days. The fresh acidic curd has a white to yellowish colour, granular texture and a lactic acid flavour. However, a brown colour starts to dominate from the surface towards the interior of the cheese due to mould growth during the ripening and thus the ripened cheese has only a small yellowish core. The rapid growth of the mould in the cheese contributes to an extensive level of proteolysis which leads to pronounced ripening. In a study performed by Pripp et al. (2006), Gamalost showed a higher ACE inhibition potential than Brie, blue mould cheese and Gouda-type cheeses, probably due to the combination of a high initial protein content and an extensive level of proteolysis, providing a high content of ACE inhibitory peptides.

The aim of this study was to characterize the development of the ACE inhibitory activity during the ripening of Gamalost related to the levels of proteolysis of the cheese, and to identify the potential ACE-inhibitory peptides.

2. Materials and Methods

2.1. Cheese making

Gamalost cheese was made according to the following procedure: Skimmed milk was pasteurized and the LAB Starter i.e. *Lactococcus (L.) lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Chr. Hansen, Hørsholm, Denmark) were added. The fermentation was made at 20 °C until pH 4.67 (isoelectric point) was reached. The curdled milk was then heated to 60 °C and passed through a decanter centrifuge which separated the solid components in the curdled milk from the whey. The solids were then milled in an impact mill converting the cheese mass into grains which were transferred into molds and then cooked in whey (90-95 °C, 1-2 hours). The cheese was removed from the molds after cooling and placed in the mould room (18 °C and relative humidity of 92-95%) where the cheeses were sprayed with a suspension of spores from *Mucor mucedo* (a mould previously isolated from artisan Gamalost by TINE SA (Oslo, Norway) and propagated). The cheeses remained in the mould room until the next day when they were moved to the temperate store room (22 °C). After a further three days, the mould had grown sufficiently and the cheeses had a furry appearance and the mould was rubbed down on the surface of the cheese. Now the mould started to grow towards the centre of the cheese. After two days, the same rubbing process was repeated and the cheese attained a smooth golden surface after about 10 days of ripening due to the growth of mould throughout the cheese matrix. At day 10, the cheeses were packed in aluminium foil and ripened further at 4 °C (TINE Meiriet Vik, Norway, personal communication, 2009).

2.2. Collection of cheeses for ACE inhibition assay

In order to follow the ACE inhibitory activity, experimental cheeses were sampled at different ripening times from regular productions. Bulk milk from cows of the Norwegian red

cattle (NRF) breed from farms in the area of the dairy plant TINE Meiriet Vik was used to make the cheese. The cows were fed silage and concentrate according to the recommendations given by TINE consultancy service. Seven cheeses from each of four regular production batches (i.e. 4 different cheese making days) were selected randomly at the dairy and frozen on days 0 (after cooking of the cheese in whey and before the mould was added), 2, 5 and 10 (including cheeses supposed to be ripened for 20, 25 and 30 days). The cheeses were transported frozen to the Department of Chemistry, Biotechnology and Food Science (Ås, Norway). Those cheeses that should ripen for more than 10 days were thawed and ripened further for 20, 25 and 30 days at 4 °C. The cheeses were kept frozen from sampling until analysis. For comparison of the ACE inhibitory activity of Gamalost, four Norvegia cheeses (a Norwegian Gouda type cheese) from four different productions and ripened for 90 days were included in the study as a control. For additional analysis of fat and casein components, Gamalost (age about 1 month) produced by TINE Meieriet Vik was also purchased in a local shop.

2.3. Grating of cheese

The cylindrical cheese (ca. 600 g) was cut from the centre and then crosswise in order to get four identical sampled pieces according to the International Dairy Federation (IDF) standard 50C (IDF 1995). The cheese was grated with a manual grinder and then used for all the prescribed analyses.

2.4. Chemical analysis of cheese

The fat content was determined by the Gerber-van Gulik method using a butyrometer (Ardö and Polychroniadou 1999). The dry matter (DM) content was determined according to

IDF standard 4/ISO 5534 (IDF 2004). The pH was monitored using a PHM 92 Lab pH METER (Radiometer, Copenhagen, Denmark). The electrode (pHC 2005-7, Combined pH Electrode Red Rod, Radiometer, Villeurbanne Cedex, France) was placed in the grated cheese with a few drops of water (Ardö and Polychroniadou 1999). The pH 4.6 soluble fraction (SF) of the cheese was prepared according to the procedure described by Pripp et al. (2006). For determination of the soluble nitrogen (SN) content by the Kjeldahl method, a 10% solution of the freeze dried pH 4.6 SF (0.5 g) was prepared according to the IDF standard 20B (IDF 1993). As Gamalost was not fully soluble in any of the solvents used and therefore produced precipitates, the analysis of the total nitrogen (TN) was very difficult even by using Macro Kjeldahl, which resulted in foaming during digestion. The cheese contained < 0.5% fat and the DM of the cheese, when the ash content is subtracted from the DM, is approximately the same as the content of protein. Therefore, we decided to calculate the pH 4.6 SN/DM instead of pH 4.6 SN/TN which is normally used for cheese. But, for Norvegia cheese, we calculated pH 4.6 SN/TN as the cheese was completely soluble in the citrate solution and since this cheese contained fat, therefore, the DM of Norvegia does not reflect only the protein content.

In Gamalost ripened for around 1 month, capillary electrophoresis (CE) was performed to detect casein components i.e. any non-degraded caseins according to Recio & Olieman (1996).

2.5. Free amino acid (FAA) composition

For the analysis of free amino acids (FAA) composition of the freeze dried pH 4.6 SF, the samples were prepared according to the method of Bütikofer & Ardö (1999). 100 mg freeze dried pH 4.6 SF was mixed into 15 mL 0.1 M HCl containing 0.4 $\mu mol \cdot mL^{-1}$ L-norvalin (Sigma, St. Louis, USA) and 0.4 $\mu mol \cdot mL^{-1}$ piperidine-4-carboxylic acid (PICA) (Fluka, St. Louis, USA)

as internal standards. After sonicating the samples for 30 min (Branson, Soest, The Netherlands), centrifugation (40 min; 4 °C; 3500 rpm) (Beckman J2-MC, GMI Inc. Minnesota, USA) was carried out and 1 mL of the supernatant was added into 1 mL 4% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany). After mixing on a vortex (Gene 2, New York, USA), the samples were placed on ice for 30 min. After centrifugation (5 min; 5 °C; 13000 rpm), the samples were filtered with a 0.2 µm MFS-13 mm CA filter (Advantec, California, USA) and stored in the freezer (-20 °C) until analysis. The separation of the FAA was performed using RP-HPLC (Pump series 410, Perkin Elmer, Shelton, CT, USA), Autoinjector 1200 series (Agilent Technologies, Waldbronn, Germany), Thermostat 1200 series (Agilent), Column Oven series 200 (Perkin Elmer), Fluorescence Detector 1200 series (Agilent), Data systems: EZChrom Elite, Revision 3.3.2 (Agilent), Column (XTerra RP 150 × 4.6 mm 3.5 µm, Waters, MA, USA) with *o*-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) derivatisation and was carried out at 42 °C.

2.6. Identification of peptide sequences

Nano-LC-MS of desalted and concentrated samples of the peptides in the freeze dried pH 4.6 SF was done according to Eriksen et al. (2010) with some modifications. Peptide mixtures containing 1% formic acid were loaded onto a nanoACQUITY^{TM} UltraPerformance LC$^{®}$ (Waters), containing a 5 µm Symmetry$^{®}$ C18 Trap column (180 µm × 20 mm; Waters) in front of a 1.7 µm BEH130 C18 analytical column (100 µm × 100 mm; Waters). Peptides were separated with a gradient of 5-90% acetonitrile, 0.1% formic acid, with a flow of 0.4 µl·min^{-1} before identification with a Q-TOF Ultima mass spectrometer (Micromass/Waters). Peptide sequences were generated from MS/MS by the ProteinLynx Global server software (version 2.2.5; Waters)

157 and the peptides were searched against the National Center for Biotechnology Information
158 (NCBI) non-redundant protein sequence databases using an in-house Mascot server (version 2.3;
159 Matrix Sciences) (http://www.matrixscience.com). Peptide mass tolerances used in the search
160 were 100 ppm, and fragment mass tolerance was 0.1 Da.
161
162 **2.7. ACE inhibition assay**
163 The freeze dried pH 4.6 SF samples for the ACE inhibition assay were prepared
164 according to the method of Hyun & Shin (2000), a modification of the method previously
165 described by Cushman & Cheung (1971), with some modifications. Hippuryl-histidyl-leucine
166 (HHL) (Sigma) (5 mmol·L^{-1}) was dissolved in 0.1 mol·L^{-1} potassium phosphate buffer (pH 8.3)
167 containing 0.4 M NaCl. The extract from rabbit lung acetone powder (Sigma) was prepared by
168 using the method of Vermeirssen et al. (2002). A mixture of HHL solution (225 μL) and 25 μL
169 sample (0-10 mg·mL^{-1}) was incubated at 37 °C for 5 min. ACE solution (rabbit lung acetone
170 powder extract) (75 μL) was added into the mixture and again incubated for 30 min. The reaction
171 was stopped with 20 μL of 5 mol·L^{-1} HCl. After filtration with a 0.45 μm, 13 mm syringe filter
172 (Ann Arbor, MI, USA) of the samples, the liberated hippuric acid (HA) was determined by RP-
173 HPLC (Pump series 200 (Perkin Elmer), Column Oven series 200 (Perkin Elmer), Fluorescence
174 Detector series 200 (Perkin Elmer), Autosampler series 200 (Perkin Elmer) and Chromatography
175 Interface series 600 (Perkin Elmer)) on a Novapak C8 (3.9 × 150 mm, 4 μm, Waters) column.
176 The flow rate of acetonitrile (99.9%, Merck) in 0.1% trifluoroacetic acid (TFA) (99%, Sigma)
177 was 1 mL·min^{-1} with a linear gradient (1-88% in 24 min) and monitored at 228 nm. All
178 determinations were carried out in duplicate with different concentrations. ACE inhibition (%)
179 was calculated by using the formula given below (1):

$$\text{ACE inhibition (\%)} = \frac{\text{HA (control)} - \text{HA (sample)}}{\text{HA (control)}} \times 100 \qquad (1)$$

Where HA (control) denotes the concentration of hippuric acid liberated after reaction of enzyme and substrate (without sample), while HA (sample) represents the hippuric acid released after reaction of enzyme and substrate in the presence of sample. The HA (98%, Sigma) and HHL were used as standards. Captopril ($C_9H_{15}NO_3S$) (Sigma) a medical drug, was also included in the assay as an inhibitory reference. The IC_{50} which is the inhibitory concentration of the freeze dried pH 4.6 SF (mg·mL^{-1}) 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 freeze dried pH 4.6 SF (IC_s) (mg·mL^{-1}). The IC_s of the freeze dried pH 4.6 SF was calculated by the following formula (2):

$$IC_s = (C_0 \times V_s \times L) / V = 0.7692 \times L \quad (2)$$

Where C_0 is the initial sample concentration (10 mg·mL^{-1}), V_s is the sample volume (25 µL), L denotes the dilutions used (0.5, 0.25 0.125) and V is the total reaction volume (325 µL) (IC_S = 0.7692 X L). The ACE inhibitory potential (IP) per unit cheese weight (mg captopril equivalents kg^{-1} cheese) was also calculated by the formula given below (3):

$$\text{ACE (IP)} = IC_{50}\ (\text{captopril}) \times \text{pH 4.6 SF} / IC_{50}\ (\text{pH 4.6 SF}) \quad (3)$$

200

201 Where IC_{50} (captopril) and IC_{50} (pH 4.6 SF) are the concentrations ($mg \cdot mL^{-1}$) of captopril and
202 freeze dried pH 4.6 SF, respectively, and pH 4.6 SF represents mg of freeze dried pH 4.6 SF of 1
203 g of cheese.

204

205 **2.8. Statistical analysis**

206 Statistical analysis was performed by Minitab statistical software version 15 (Minitab
207 Inc., State College, PA, USA), using the general linear model and Tukey's test for pair-wise
208 comparison in analysis of variance (ANOVA). The normal distribution of all variables was tested
209 by Shapiro-Wilk test and normality assumptions were found to be satisfied. Batches of cheese
210 (random variable), age of cheese (fixed variable) (with the assumption that the individual cheeses
211 from the same batch were independent) and interaction between age and batches were used as
212 classification factors in the statistical model.

213

214 **3. Results**

215 **3.1. Gross composition**

216 Gamalost did not contain any measurable amounts of fat. The levels of moisture and pH
217 of Gamalost were monitored up to 30 days of ripening (Table 1). The moisture content of the
218 cheese decreased significantly ($P < 0.05$) from 56.1% at day 0 to 45.6% at day 10. Later the
219 moisture content stabilized until 25 days with a further decrease thereafter to 43.9% at 30 days.
220 The moisture content was significantly influenced by the batches. The pH of the cheeses
221 increased from 4.43 at day 0 to 6.96 at 20 days but after that it stabilized up to 30 days. No
222 significant effect due to batches was observed on the pH or the SN content (%) of the pH 4.6 SF

of Gamalost. The soluble nitrogen (SN) content (%) of the pH 4.6 SF from 0 and 2 days ripened Gamalost was significantly ($P < 0.05$) lower than the content in cheese ripened for longer periods. It increased markedly up to 10 days of ripening but afterwards it did not vary remarkably up to 30 days. Norvegia contained almost half of SN (%) of pH 4.6 SF compared to ripened Gamalost (10-30 days). The pH 4.6 SN/DM of Gamalost increased significantly ($P < 0.05$) from 0.12% at day 0 to 8.17% at 10 days of ripening, but after that it did not vary significantly ($P < 0.05$). The pH 4.6 SN/TN of 90 day old Norvegia was approximately 11%.

3.2. Casein composition of purchased Gamalost

Gamalost did not show any peaks of intact casein which indicated no remaining caseins. The peaks shown in the Gamalost chromatogram were not identified, but most probably they represented small peptides and amino acids from the caseins.

3.3. Identification of peptide sequences in pH 4.6 SF

Table 2 shows the identified peptides with the amino acid sequences in pH 4.6 SF of Gamalost at different ripening times, 0-30 days. In total, 41 peptides were detected and among them, the longest peptide f (57-91) had a molecular weight of 3791 $g \cdot mol^{-1}$ and a length of 35 amino acids, whereas the shortest peptide f (134-141) had 8 amino acids with a molecular weight of 930 $g \cdot mol^{-1}$ and was identified after 10-30 days of ripening. In the unripened cheese, 17 peptides in total were detected and among them 8 were derived from β-CN, 6 from α_{s1}-CN and 3 from κ-CN. Only one peptide f (1-14) was derived from the N-terminal position of β-CN in the unripened cheese. Among the peptides detected from β-CN (at day 0), almost half of them were released from the ultimate C-terminal position of its whole sequence while all the peptides from

246 α_{s1}-CN and κ-CN were derived from their ultimate C-terminal position. In cheese ripened for 10
247 days, among the 13 peptides detected, ten were released from β-CN, two (f (10-23) and f (14-
248 23)) from α_{s1}-CN and one (f (99-115)) from α_{s2}-CN. Three peptides (f (129-139), f (132-141) and
249 f (134-141)) were derived from β-CN, appeared after 10 days and remained throughout ripening
250 i.e. until 30 days, were the degraded fragments of the peptides found in unripened cheese and
251 only one peptide (f (99-115)) from α_{s1}-CN, present after 10 and 20 days of ripening, was
252 degraded into another peptide (f (100-115)) which was identified after 25 and 30 days of
253 ripening. In the cheeses ripened longer (20, 25 and 30 days), most of the detected peptides were
254 derived from β-CN and were released from the internal positions of its sequence. It has been
255 found that most of the identified peptides in Gamalost had hydrophobic amino acids such as Ala,
256 Ile, Leu, Met, Val, Phe and Trp (A, I, L, M, V, F, W, shown as bold letters in table 2) at any of
257 the three C-terminal positions and the Pro (P, shown in italic) was also present at any of the three
258 C-terminal positions in some peptides. Moreover, a few peptides had positively charged (+)
259 amino acids such as Arg and Lys (R and K shown as highlighted) at any of the three C-terminal
260 positions of peptides. The results of the peptide sequences showed that in the ripened cheese,
261 most of the peptides detected were released from β-CN and some of the peptides identified were
262 common in the cheeses at the different stages of ripening.
263
264 **3.4. Free amino acid (FAA) of pH 4.6 SF**
265 The development of the amounts (mmol·kg^{-1}) of FAA in Gamalost during ripening is
266 shown in Fig. 1. The unripened cheeses (0 and 2 days) had negligible levels of FAA. The amino
267 acids Cit, GABA and Orn remained at low concentrations (< 1 mmol·kg^{-1} cheese) throughout
268 ripening. The content of Trp, Asn, Asp and Tyr increased during ripening but remained at a

relatively low concentration (5-19 mmol·kg^{-1} cheese) compared to Met, Gly, Arg, Phe, Thr, His, Ser and Ile (22-39 mmol·kg^{-1} cheese). At the end of ripening Glu, Gln, Ala, Val, Leu, Lys and Pro were the most abundant FAA detected and among them Pro reached the highest concentration, around 105 mmol·kg^{-1} cheese.

3.5. ACE inhibition of pH 4.6 SF of Gamalost

Results from the measurement of the ACE inhibition of freeze dried pH 4.6 SF of Gamalost are presented in Table 3. The ACE inhibition was affected significantly ($P < 0.05$) by the ripening of the cheese. The pH 4.6 SF from the unripened (0 day) cheese showed the lowest ACE inhibition (42.5%). The ACE inhibiting effect started to develop faster early in the ripening of the cheese as the level after 2 days of ripening was significantly ($P < 0.05$) higher than after 0 days and that, after 5 days of ripening, the inhibiting effect was significantly ($P < 0.05$) higher than after 2 days of ripening. The ACE inhibition (%) was significantly ($P < 0.05$) higher in the cheeses ripened for 10 and 20 days than in the younger cheeses. Similarly, ACE inhibition was significantly ($P < 0.05$) lower in cheese after 25 and 30 days of ripening than after 10 and 20 days of ripening but no significant ($P < 0.05$) difference between the values after 25 and 30 days was observed. Gamalost (10 days) showed higher ACE inhibition (~ 74%) compared to Norvegia (~ 60%). The IC_{50} value (mg·mL^{-1}) of freeze dried pH 4.6 SF of the cheeses varied from 0.92 (0 day) to 0.34 (10 days) and was significantly ($P < 0.05$) influenced by age with the lowest values (i.e. lowest amount of cheese needed to obtain a 50% ACE inhibition) in cheese ripened for 10 and 20 days. The IC_{50} value shown by Gamalost after 10 days of ripening was almost half of the value obtained by Norvegia after 90 days of ripening. The measured IC_{50} value of captopril was 2.5×10^{-6} (mg·mL^{-1}) ± 3.5×10^{-7}. The ACE (IP) per unit cheese weight of Gamalost increased

with the progression of ripening and reached its highest level after 10 days of ripening and after 20 days it started to decrease again. Gamalost (10 days) was found to have 10 times higher ACE inhibitory potential than Norvegia. No significant effect due to batches was observed on the ACE inhibition (%) or the IC_{50} values ($mg \cdot mL^{-1}$) of freeze dried pH 4.6 SF of Gamalost or of the ACE (IP) of Gamalost.

4. Discussion

Extensive levels of proteolysis in Gamalost was shown by high levels of FAA in the ripened cheese. It has been reported that by increasing the concentration of casein, the protease activity of *Mucor mucedo* is also increased and the pH optimum for enzyme production from *Mucor mucedo* was 5 (Joel-Gnanadoss et al. 2011). It has also been reported that *Mucor mucedo* produces extracellular aspartic proteases (optimal pH 4.5) and chitinases (optimal pH 5.55-5.65) (Humphreys and Gooday 1984; Yegin et al. 2010) which may explain the increased concentrations of some amino acids such as Ala, Glu, Gln, Leu and Pro during ripening.

The high content of Glu in ripened Gamalost is interesting, as in a study conducted on Swiss (Emmental) and Cheddar cheeses, it has been found that Glu contributes to the umami taste (Drake et al. 2007). This possible influence of the high content of Glu in Gamalost on the flavour development in Gamalost should be further investigated by sensorial analysis.

Monitoring the ACE inhibitory activity of pH 4.6 SF of Gamalost during ripening was one of the objectives of this work. In unripened Gamalost, the peptides remained encrypted in the parent protein. A rapid increase in ACE inhibition during ripening was a result of the progressive liberation of the peptides from protein by the action of the fungal proteases. It has previously been reported that the ACE inhibition in general increased during the ripening of cheese but also

that it started to decrease after a attaining certain level of proteolysis (Lignitto et al. 2010) owing to further degradation of the relevant peptides which is consistent with our findings that after 20 days of ripening, the ACE inhibiting capacity of these peptides decreased slowly.

Lignitto et al. (2010) observed that the water soluble extracts (WSEs) of Asiago d'allevo cheese with peptides having a molecular mass of less than 3 $kg \cdot mol^{-1}$ had a more significant contribution to ACE inhibitory activity than the WSEs containing peptides having molecular mass of higher than 3 $kg \cdot mol^{-1}$. All peptides (except β-casein f (57-91) and f (129-160)) observed in Gamalost had molecular masses lower than 3 $kg \cdot mol^{-1}$. Usually 2-12 amino acids in a peptide have been found to be active for ACE inhibition, however, the peptides with up to 27 amino acids have also shown considerable ACE inhibitory effect (López-Fandiño et al. 2006). All the identified peptides (except β-casein f (57-91) and f (129-160)) in Gamalost were found to have a number of amino acids in the range mentioned. Very few peptides from our study matched with previously reported ACE inhibiting peptides such as β-casein f (191-209) (Yamamoto et al. 1994) and f (126-143) (Otte et al. 2007). Moreover, it has been reported that the hydrophobic (either aromatic (Tyr, Phe, Trp) or branched chain aliphatic (Ala, Ile, Leu, Val)) or positive charged (+) amino acid such as Arg and Lys and Pro at any of the three C-terminal positions of the peptides show good binding of ACE (Haque and Chand 2008; López-Fandiño et al. 2006). Our findings are consistent with the above mentioned reports regarding the structure-specificity relationship as shown due to presence of hydrophobic or positive charged amino acids or Pro at the C-terminal ends of peptides detected in Gamalost which might provide a clue that the peptides detected in Gamalost were ACE inhibiting peptides. Consumers would be interested in the bioactivity per unit cheese weight, therefore further human trials should be performed to

clarify the bioavailability and *in vivo* antihypertensive activity of Gamalost cheese or its peptides.

5. Conclusions

This study showed that the ACE inhibitory effect was at its highest between 10 and 20 days of ripening of Gamalost. Hence, the optimal age for consumption of Gamalost for obtaining optimal ACE inhibition would be when the cheese has been ripened for 10-20 days. Many peptides expected to be responsible for the ACE inhibition were found to be present in the cheeses and their presence differed throughout ripening. About 41 potentially active peptides were identified and only some of them showed homology with peptides previously described in the literature, therefore, also new peptides may be considered as very important. Further studies to identify the peptides responsible for the detected ACE inhibitory activity will be performed. The pronounced rise in pH and the pH 4.6 SN during the first 10 days of ripening and therefore the increase in the ACE inhibitory activity can be attributed to the distinct levels of proteolysis caused by *Mucor mucedo*.

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428

429 **Figure caption:**

430 **Fig. 1.** Development of the free amino acids (mmol·kg^{-1} cheese) during ripening;

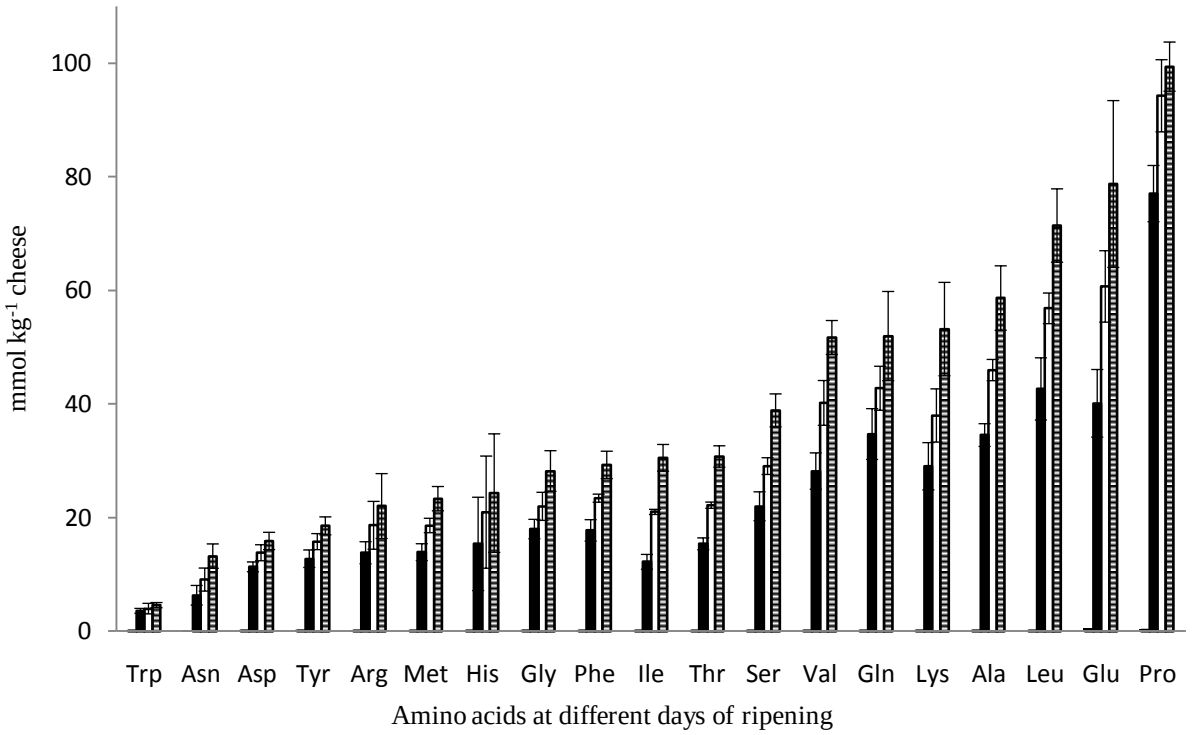
431 ▫ 0 day, ■ 10 days, □ 20 days, ▤ 30 days

432

433

Figure

Fig. 1


100
80
60
40
20
0
mmol kg-1 cheese
Trp
Asn
Asp
Tyr
Arg
Met
His
Gly
Phe
Ile
Thr
Ser
Val
Gln
Lys
Ala
Leu
Glu
Pro
Amino acids at different days of ripening

Table

Table 1: Moisture, pH, soluble nitrogen (SN) and pH 4.6 SN/DM (%) (Mean ± SD) of Gamalost during ripening.

Age (days)	Cheese type	Moisture (%)	pH	SN of pH 4.6 SF [1] (%)	(pH 4.6 SN/DM)[2] or (pH 4.6 SN/TN)[3] (%)
0	Gamalost	$56.14^{e} \pm 0.51$	$4.43^{a} \pm 0.05$	$2.09^{a} \pm 0.10$	$0.121^{a} \pm 0.01$
2	Gamalost	$53.87^{d} \pm 0.72$	$4.59^{b} \pm 0.03$	$2.80^{b} \pm 0.23$	$0.161^{a} \pm 0.02$
5	Gamalost	$49.77^{c} \pm 0.38$	$5.53^{c} \pm 0.02$	$13.00^{c} \pm 0.12$	$4.89^{b} \pm 0.44$
10	Gamalost	$45.64^{b} \pm 0.53$	$6.85^{d} \pm 0.02$	$14.05^{d} \pm 0.11$	$8.17^{c} \pm 0.20$
20	Gamalost	$45.76^{b} \pm 0.54$	$6.96^{e} \pm 0.03$	$13.91^{d} \pm 0.11$	$8.55^{c} \pm 0.25$
25	Gamalost	$45.38^{b} \pm 0.89$	$6.99^{e} \pm 0.02$	$13.77^{d} \pm 0.10$	$8.73^{c} \pm 0.19$
30	Gamalost	$43.86^{a} \pm 0.55$	$7.03^{e} \pm 0.02$	$13.80^{d} \pm 0.08$	$8.73^{c} \pm 0.28$
90	Norvegia	-	-	7.87 ± 0.46	10.88 ± 1.26

Data in columns with different superscript are significantly different using Tukey's pair-wise comparison test at 5% level.

[1]Percentage of SN of freeze dried pH 4.6 SF.

[2]Percentage of pH 4.6 SN on dry matter (DM) basis in case of Gamalost.

[3]Percentage of pH 4.6 SN on total nitrogen (TN) basis in case of Norvegia.

Table 2: Peptide sequences of pH 4.6 SF of Gamalost after different ripening times (0-30 days) obtained by Nano LC-MS.

Peptide sequence	Molecular mass (exp)	Age (days)	Casein type (bovine)	Amino acid segment[a]
1-14	1624.76	0	β-CN	RELEELNVPGE**IV**E
129-160	3735.90	0	β-CN	DVENLHLPLPLLQSWMHQPHQPLPPTVMF*PP*Q
132-143	1429.76	0	β-CN	NLHLPLPLLQS**W**
185-209	2793.55	0	β-CN	MPIQAFLLYQEPVLGPVRGPFP**IIV**
195-209	1588.93	0	β-CN	EPVLGPVRGPFP**IIV**
176-199	2617.21	0	α_{s1}-CN	APSFSDIPNPIGSENSEKTTM*P***LW**
180-199	2214.90	0	α_{s1}-CN	SDIPNPIGSENSEKTTM*P***LW**
182-199	2012.87	0	α_{s1}-CN	IPNPIGSENSEKTTM*P***LW**
184-199	1802.74	0	α_{s1}-CN	NPIGSENSEKTTM*P***LW**
185-199	1688.69	0	α_{s1}-CN	PIGSENSEKTTM*P***LW**
188-199	1421.61	0	α_{s1}-CN	SENSEKTTM*P***LW**
149-169	2196.06	0	κ-CN	SPEVIESPPEINTVQVTST**AV**
151-169	2011.92	0	κ-CN	EVIESPPEINTVQVTST**AV**
155-169	1541.73	0	κ-CN	SPPEINTVQVTST**AV**
191-209	2106.02	0, 10	β-CN	LLYQEPVLGPVRGPFP**IIV**
192-209	1993.11	0, 10, 30	β-CN	LYQEPVLGPVRGPFP**IIV**
144-160	1980.96	0, 25	β-CN	MHQPHQPLPPTVMF*PP*Q
124-142	2102.09	10	β-CN	SLTLTDVENLHLPLPL**L**QS
10-23	1640.76	10	α_{s1}-CN	GLPQEVLNENL**L**R**F**
14-23	1245.67	10, 20, 30	α_{s1}-CN	EVLNENL**L**R**F**
124-143	2288.17	10, 20, 30	β-CN	SLTLTDVENLHLPLPLLQS**W**
129-139	1258.69	10, 20, 25, 30	β-CN	DVENLHLP**L***P***L**
132-141	1156.70	10, 20, 25, 30	β-CN	NLHLPLP**LL**Q
134-141	929.57	10, 20, 25, 30	β-CN	HLPLP**LL**Q
193-207	1667.90	10, 20, 25, 30	β-CN	YQEPVLGPVRGP**F***P***I**
126-139	1573.81	10, 25	β-CN	TLTDVENLHLP**L***P***L**
124-140	1887.01	10, 30	β-CN	SLTLTDVENLHLPL*P***LL**
99-115	2038.10	10, 20, 30	α_{s2}- CN	LYQGPIVLNPWDQVKRN
124-138	1660.77	20	β-CN	SLTLTDVENLHL*P***L***P*
126-143	2088.05	20	β-CN	TLTDVENLHLPLPLLQS**W**
142-164	2640.16	20	β-CN	SWMHQPHQPLPPTVMFPPQS**VL**S
129-143	1772.85	20, 25	β-CN	DVENLHLPLPLLQS**W**
57-91	3791.91	25	β-CN	SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQ*P*E
124-139	1773.85	25	β-CN	SLTLTDVENLHLP**L***P***L**
132-142	1243.63	25	β-CN	NLHLPLPL**L**QS
144-164	2367.06	25, 30	β-CN	MHQPHQPLPPTVMFPPQS**VL**S
199-208	1051.62	25, 30	β-CN	GPVRGPF*P***II**
100-115	1926.01	25, 30	α_{s2}- CN	YQGPIVLNPWDQVKRN
151-162	1473.64	25,30	α_{s2}- CN	TKLTEEEKNR**L**N
106-123	2189.94	30	β-CN	HKEMPFPKYPVEPFTESQ
128-143	1873.89	30	β-CN	TDVENLHLPLPLLQS**W**

[a]One letter amino acid codes used.

Bold letters represent hydrophobic amino acids, Ala, Ile, Leu, Val, Phe and Trp (**A, I, L, V, F, W**) at any one of the three C-terminal positions of the peptides.

Italic letters represent Pro (*P*) at any of the three C-terminal positions of the peptides.

Highlighted letters represent positive charged amino acids, Arg (R) and Lys (K) at any of the three C-terminal positions of the peptides.

Table 3: ACE inhibitory activity and IC_{50} of pH 4.6 SF (Mean ± SD) in cheese after different ripening times (0-30 days).

Age (days)	Cheese type	pH 4.6 SF[1] ($mg \cdot g^{-1}$ cheese)	ACE inhibition (%)	IC_{50}[2]	ACE (IP) per cheese unit[3]
0	Gamalost	$25.3^{a} \pm 0.86$	$42.5^{a} \pm 3.67$	$0.92^{d} \pm 0.11$	$0.07^{a} \pm 0.01$
2	Gamalost	$26.4^{a} \pm 0.93$	$51.2^{b} \pm 2.86$	$0.73^{c} \pm 0.04$	$0.09^{a} \pm 0.01$
5	Gamalost	$188.7^{b} \pm 15.82$	$60.5^{c} \pm 1.37$	$0.67^{c} \pm 0.02$	$0.71^{b} \pm 0.08$
10	Gamalost	$316.1^{c} \pm 10.09$	$74.0^{e} \pm 3.25$	$0.34^{a} \pm 0.07$	$2.40^{d} \pm 0.50$
20	Gamalost	$333.4^{cd} \pm 8.62$	$71.1^{de} \pm 2.89$	$0.39^{a} \pm 0.03$	$2.12^{d} \pm 0.16$
25	Gamalost	$346.0^{d} \pm 8.37$	$66.2^{cd} \pm 2.13$	$0.47^{ab} \pm 0.04$	$1.87^{cd} \pm 0.19$
30	Gamalost	$355.0^{d} \pm 13.59$	$64.2^{c} \pm 2.11$	$0.58^{bc} \pm 0.04$	$1.53^{c} \pm 0.14$
90	Norvegia	58.2 ± 4.35	60.2 ± 2.41	0.59 ± 0.04	0.25 ± 0.03

Data in columns with different superscript are significantly different using Tukey's pair-wise comparison test at 5% level.

[1]Weight of freeze dried powder of pH 4.6 SF ($mg \cdot g^{-1}$ cheese).

[2]IC_{50} per unit weight of freeze dried pH 4.6 SF, expressed as mg pH 4.6 SF mL^{-1}.

[3]ACE inhibitory potential (IP) per unit cheese weight, expressed as mg captopril equivalents kg^{-1} cheese.