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Angiotensin I-converting enzyme inhibitory activity of enzymatic hydrolysates of goat milk protein fractions

F. Javier Espejo-Carpio[a,b], Cristian De Gobba[b], Antonio Guadix[a], Emilia M. Guadix[a], Jeanette Otte[b]*

[a] *Department of Chemical Engineering, University of Granada, 18071 Granada, Spain*

[b] *Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark*

Corresponding author. Tel.: +45 35333189

E-mail address: jo@life.ku.dk (J. Otte)

Abstract

Casein and whey protein fractions from goat milk were hydrolysed by subtilisin, and trypsin, individually and in combination, to release angiotensin converting enzyme (ACE)-inhibitory peptides. Selected hydrolysates were fractionated by size-exclusion chromatography (SEC) and further characterised. The highest ACE-inhibitory activity was obtained from the casein fraction hydrolysed by the combination of enzymes. SEC presented 4 fractions with fraction F2 containing the highest concentration of peptides (< 2.3 kDa) and the highest activity. F2 contained a number of peptides not previously identified from caprine caseins but with structural similarity to other ACE-inhibitory peptides. The most active fraction in relation to protein content was F4 with IC_{50} between 9.3 and 5.1 µg mL^{-1}. This fraction contained a compound tentatively identified as WY, an active dipeptide not previously reported from caseins. The high inhibitory capacity of these fractions points towards the advantage of implementing a membrane process to concentrate the most active peptides.

1. Introduction

Bovine milk proteins have been extensively studied as a source of bioactive peptides (Korhonen 2009; Meisel, 1998; Nagpal et al., 2011), and in the last years proteins from sheep and goat milk also have attracted interest as sources of encrypted bioactive peptides (Bernacka, 2011; Hernández-Ledesma, Recio, Ramos, & Amigo, 2002). The protein content of goat milk is quite similar to that of cow milk, although the caseins content in goat milk is slightly higher, and there is great homology between the major proteins. However, β-casein is the major protein in goat milk (50% of total caseins), which is in contrast to in cow milk where β-casein and α_{S1}-casein are almost equally abundant, 37% and 30%, respectively (Park, Juárez, Ramos, & Haenlein, 2007).

Within bioactive peptides, antihypertensive peptides are among the most well studied. Hypertension is a key factor in cardiovascular diseases that are among the largest health problems in the world (Whitworth, 2003). As a first step to identify antihypertensive peptides, the angiotensin converting enzyme (ACE; EC 3.4.15.1) inhibitory activity can be assessed in vitro. ACE is a zinc metallopeptidase acting in the rennin-angiotensin-aldosterone system transforming angiotensin I into angiotensin II, which is a potent vasoconstrictor. Moreover, ACE catalyses the degradation of bradykinin, a blood pressure lowering nonapeptide in the kallikrein-kinin system. Therefore, the inhibition of this enzyme would cause a decrease in blood pressure (FitzGerald, Murray, & Walsh, 2004).

A number of peptides derived from goat milk proteins have shown the ability to inhibit ACE in vitro (Fitzgerald & Meisel, 2000; Saito, 2008). Some of these peptides

have also shown antihypertensive activity in vivo, both in animals and humans (Geerlings et al., 2006).

ACE-inhibitory peptides have been released from goat milk by fermentation, e.g., in products such as cheeses and Kefir (Gómez-Ruiz, Taborda, Amigo, Recio, & Ramos, 2006; Quirós, Hernández-Ledesma, Ramos, Amigo, & Recio, 2005), or by enzymatic hydrolysis of goat milk protein fractions (Geerlings et al., 2006; Hernández-Ledesma et al., 2002; Lee, Kim, Ryu, Shin, & Lim, 2005; Manso & López-Fandiño, 2003; Minervini, Algaron, & Rizzello, 2003). The enzymatic hydrolysis approach makes it possible to choose both the substrate and the specific enzyme and the reaction conditions, allowing optimisation of the yield of bioactive peptides, and also to subsequently enrich for them.

Many studies have used enzymes of different origin (animal, vegetal or microbial) to hydrolyse milk protein fractions (Jiang, Chen, Ren, Luo & Zeng, 2007; López-Fandiño, Otte, & van Camp, 2006; Otte, Shalaby, Zakora, Pripp, & El-Shabrawy, 2007b; Saito, 2008), but only a few have addressed the effect of a combination of enzymes (Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000; Wang, Mao, Cheng, Xiong, & Ren, 2010). Trypsin has frequently been employed in milk protein hydrolysis yielding products with good ACE-inhibitory activity (Pan, Cao, Guo, & Zhao, 2012; Pintado & Malcata, 2000; Tauzin, Miclo, & Gaillard, 2002). In contrast, subtilisin has not been employed much to hydrolyse milk proteins (Geerlings et al., 2006; Jiang et al., 2007; Mao, Ni, Sun, Hao, & Fan, 2007). Subtilisin shows low specificity and preferentially cleaves at the C-terminal of hydrophobic residues.

Hydrophobicity is desirable in the C-terminal of peptides, since ACE inhibitors generally have hydrophobic amino acids in the last three positions of the C-terminal. Moreover, it has been suggested that positively charged amino acids (e.g., basic amino

acids as released by trypsin) in the C-terminal contribute to the inhibitory activity (Li, Le, & Shi, 2004; Pripp, Isaksson, Stepaniak, & Sørhaug, 2004). Therefore, the combined effect of these enzymes on the release of ACE-inhibitory peptides should be investigated.

ACE-inhibitory sequences have been released from both whey proteins and caseins from goat milk; however, most have been identified in the casein fraction (Geerlings et al., 2006; Lee et al., 2005; Manso & López-Fandiño, 2003; Minervini et al., 2003; Quirós et al., 2005). Membrane filtration is one of the procedures most used for separation of milk protein fractions (Pouliot Pouliot, & Britten, 1996; Punidadas & Rizvi, 1998). In contrast to fractionation using precipitation, filtration does not require a second stage to dissolve the precipitated caseins; moreover an increase in the salt content is avoided. Ceramic membranes have been used extensively for separating caseins (retentate fraction) from whey proteins (permeate fraction) using cut-off values between 0.05 and 0.2 µm (Pouliot et al., 1996; Punidadas & Rizvi, 1998; Samuelsson, Dejmek, Trägårdh, & Paulsson, 1997). On the other hand, when information on the size range of ACE-inhibitory peptides is available, membrane technology could also be employed to concentrate the active peptides in the resulting hydrolysates. Indeed, an increasing number of studies are dealing with developing methods for concentrating or purifying such peptides (Bazinet & Firdaous, 2009).

The purpose of this study was to investigate the release of ACE-inhibitory peptides from goat milk protein fractions by use of three different enzymatic treatments. We used milk, a milk permeate fraction and a milk retentate fraction as substrates to determine whether prior separation into whey proteins and caseins would increase the yield of bioactive peptides. Enzymatic hydrolysis was performed with subtilisin or trypsin or a mixture of both, to investigate the advantage of the latter. The hydrolysates

obtained were fractionated by size exclusion chromatography to find the size range with the highest ACE-inhibitory activity. Selected hydrolysate fractions were characterised by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with respect to peptide profile, masses of each peptide and identification of major peptides.

2. Materials and methods

2.1. Substrates and enzymes

The enzymes employed to perform the hydrolysis were subtilisin (Alcalase 2.4 L FG; EC 3.4.21.62) and pancreatic trypsin (PTN 6.0 S Saltfree; EC 3.4.21.4) both from Novozymes A/S (Bagsvaerd, Denmark). Semi-skimmed UHT goat milk (2.6% protein and 1.6% fat) purchased from a conventional supermarket was centrifuged at 4 °C and 4845 × *g* for 45 min (Sigma Laborzentrifugen 6K15 Sartorius, Osterode am Harz, Germany) to remove the fat. This skimmed milk was used as milk substrate (M).

A portion of the skimmed milk was microfiltered through a tubular ceramic microfiltration membrane with pore size 0.14 µm to obtain the retentate (R) and the permeate (F) fractions. The microfiltration device consisted of a stainless steel supply tank (2 L), a positive displacement pump (0.56 kW and Procom head, Smyrna, TN) regulated by inverter (Omron Sysdrive 3G3JV, Kyoto, Japan), and a casing containing the membrane of 1.2 m length, 3 channels and a filtration area of 0.045 m^2 (Inside Céram 120 from TAMI, Nyons, France). The temperature was maintained with a thermostated bath (Selecta, Abrera, Spain) in which the supply tank was immersed. The pressure was measured with manometers (Wika, 1-60 psi, Lawrenceville, GA) at the entrance of the membrane casing and was controlled by a membrane valve in the

retentate stream. Also in this stream, a flow meter (Badger-Meter Type Food Primo V11, Neuffen, Germany) was used to measure the flow. The permeate was collected in a tank and weighed (Mettler Toledo PB 1502-S, Greifensee Switzerland).

The process was carried out at 50 °C, with a flow of 400 L h^{-1} and a trans-membrane pressure of 1 bar until a concentration factor of 3 was reached. The microfiltration fractions were kept at 4 °C (no more than 3 days) until hydrolysis. After filtration the membrane was cleaned with a solution of 20 g L^{-1} NaOH with 2 g L^{-1} SDS at 50 ºC and 400 L h^{-1}, with the purpose of recovering the filtration characteristic of the membrane.

2.2. Production of hydrolysates

The hydrolysis reaction was carried out in a stirred-tank reactor of 250 mL equipped with a heating jacket connected to a thermostated bath to maintain the temperature in the reactor. Each substrate (M, R and P) was hydrolysed in three ways: 1) with subtilisin (S), 2) with trypsin (T) and 3) with a both enzymes simultaneously (ST) using the same operational conditions. For each batch, 200 mL of the substrate were heated in the reaction tank until the required temperature (50 °C). Then 1 M sodium hydroxide was added to set the pH to 8. These operational conditions are adequate for both enzymes (Adler-Nissen, 1986, Guadix, Guadix, Páez-Dueñas, González-Tello, & Camacho, 2000), since subtilisin would operate within its optimal conditions, and a temperature of 50 ºC would enhance the catalytic capacity of trypsin without appreciable loss of activity within a period of 3 h (Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2013; Mota et al., 2006). When the appropriate temperature and pH were reached, the enzyme was added to the medium (1.5, 5 and 0.5 g L^{-1} for M,

R and F, respectively, to reach an E/S ratio of approximately 6% for each enzyme in all reaction mixtures) and the reaction was allowed to proceed for 3 h. The pH was controlled by addition of 1 M NaOH using an automatic titrator (718 Stat Titrino, Metrohm; Herisau, Switzerland). After 3 h, the hydrolysate was immersed in boiling water for 15 min to inactivate the enzyme. Finally, the hydrolysate was lyophilised. The hydrolysates were identified with letter combinations referring to the substrate and the enzyme (i.e., R-S, R-T, and R-ST are the retentates hydrolysed with subtilisin, trypsin, and the combination of subtilisin and trypsin, respectively).

2.3. Degree of hydrolysis

The degree of hydrolysis (DH) was calculated from the amount of NaOH used during the hydrolysis reaction using to the formula proposed by Adler-Nissen (1986):

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\% \quad (1)$$

where B is base consumption (L), N_b is NaOH normality (1.0) and MP is the mass of the protein. Taking the tabulated values from the author, the parameters $1/\alpha$ (the inverse of the average degree of dissociation of the α-NH group) and h_{tot} (total number of peptide bonds in the protein substrate) were 1.13 and 8.6 meqv g^{-1} protein, respectively.

2.4. Fractionation by size-exclusion chromatography

Selected lyophilised hydrolysates were re-dissolved in 0.1 M ammonium hydrogen carbonate and fractionated by size exclusion chromatography (SEC) using an FPLC system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with a

column packed with SuperdexTM 30 prep grade gel filtration resin (2.6 × 61 cm; Amersham Biosciences, Hillerød, Denmark). Five milliliters of a hydrolysate solution (70 mg protein mL^{-1}) were injected and eluted with 0.1 M ammonium hydrogen carbonate, pH 8.0, at a flow rate of 2.5 mL min^{-1}. The effluent was monitored at 280 nm and fractions of 10 mL were collected and then pooled according to the elution profile and frozen until analysis.

The elution times of nine standards with different molecular weights (BSA, Cytochrome C, Insulin, Insulin B chain oxidised, angiotensin I, Leu-Enkephalin, Gly-Tyr, and L-Trp; approximately 0.25 mg mL^{-1} of each) were used to make a calibration curve to determine the size range of the peptides in each fraction.

2.5. Protein determination

The Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the protein content of substrates (Milk, retentate and filtrate) and of the nine freeze-dried hydrolysates. The determination was carried out directly with the hydrolysates re-dissolved in Milli-Q water at protein concentrations within the assay range. The analysis was done in triplicate as indicated in the kit instructions, and the absorbance was measured at 650 nm (Varian Cary 110 Bio, Palo Alto, CA, USA).

The protein content of SEC fractions was determined in microtiter plates (611F96, Sterilin Ltd., Newport, UK), using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the kit instructions. The samples were analysed in triplicate, and the absorbance was read at 562 nm using a Multiskan EX reader (Labsystems OY, Helsinki, Finland).

215

216 *2.6. Determination of ACE-inhibitory activity*

217

218 A variation of the method optimised by Sentandreu & Toldra (2006) was used. It
219 is based on the internally quenched fluorescent substrate *o*-amino-benzoylglycyl-*p*-
220 nitro-L-phenylalanyl-proline (ABZ-Gly-Phe(NO_2)-Pro; Bachem, Bubendorf,
221 Switzerland), which upon cleavage by ACE releases the fluorescent group (ABZ-Gly)
222 resulting in an increased fluorescence. The assay was performed in 96-well black
223 microtiter plates (Microfluor 96-Well Black, Thermo Fisher Scientific OY, Vantaa,
224 Finland) using a TECAN GENios Plus microtiter-plate multiscan fluorometer (Tecan
225 Austria GmbH, Grödig/Salzburg, Austria). In each well were added 50 µL of ACE
226 (purified from rabbit lung, Sigma A6778) dissolved at 6 mU mL^{-1} in 150 mM Tris-Base
227 buffer, pH 8.3, and 50 µL of the sample solution. The control was prepared in the same
228 way, but with Tris buffer instead of sample solution. Blank samples (without ACE)
229 containing only substrate and substrate with sample, respectively, were also prepared.
230 The microplate was shaken and pre-incubated for 10 min at 37 °C. The reaction was
231 initiated by addition of 200 µL of 0.45 mM ABZ-Gly-Phe(NO_2)-Pro in 150 mM Tris-
232 base buffer (pH 8.3) containing 1.125 M NaCl. The generated fluorescence was
233 measured every minute for 30 min. Excitation and emission wavelengths were 360 and
234 415 nm, respectively. Samples were analysed in triplicate. Each of the freeze-dried
235 hydrolysates was re-dissolved in buffer (150 mM Tris-base buffer, pH 8.3) at a
236 concentration of 0.8 mg protein mL^{-1}. SEC-Fractions were analysed without pre-
237 treatment, since it was shown that the elution buffer (0.1 M ammonium carbonate, pH
238 8.0) did not influence the assay.

The inhibition of ACE was calculated using the slope of the control as 100% enzyme activity using this equation:

$$\%_{Inhibition} = 100 - \frac{100 \times [(Slope]_{inhib} - Slope_{ABZ+inhib})}{Slope_{control} - Slope_{ABZ}}, \quad (2)$$

where $Slope_{inhib}$ is the slope of the curve for the sample and $Slope_{control}$ is the slope without sample. $Slope_{ABZ}$ and $Slope_{ABZ+inih}$ are the slopes of the blank samples containing only substrate and substrate with sample, respectively.

The concentration of peptides that reduced the ACE activity to 50% (IC_{50}) was determined for fractions that showed ACE inhibitory percentages above 70%, by diluting the original fractions with the same buffer (0.1 M ammonium hydrogen carbonate, pH 8.0). The IC_{50} value was determined with the equation obtained by linear regression of the linear zone around 50% from the graph representing inhibition versus the logarithm of the protein/peptide concentration. For selected fractions with inhibition percentages lower than 50%, 1 mL of the fraction was concentrated in a vacuum centrifuge until dryness and then re-dissolved in 500 µL of water before determination of the IC_{50} value.

2.7. Peptide profiles and LC-MS analysis

Peptide profiles were revealed by LC-MS/MS analysis using an Agilent 1100 LCMSD Trap as described by Otte, Shalaby, Zakora, and Nielsen (2007a), except that the gradient consisted of 100% A for 5 min, followed by a linear increase to 50% B in 75 min. Buffer A was 0.1% trifluoroacetic acid (TFA) in water, and buffer B was 0.1%

TFA in 90% acetonitrile. On-line AutoMS(2) spectra were recorded using the standard range from 50 to 2200 *m/z* at the normal scan resolution and the target mass set to 1521 *m/z*.

The freeze dried hydrolysates were redissolved at 20 mg mL^{-1} in water and 5 μL were injected. From the fractions obtained after SEC a volume of 25 μL was injected.

Identification of the main peptides of each fraction was performed using Bruker Daltonics BioTools software 3.0. Individual MS/MS spectra were compared with theoretical fragment spectra from possible peptides with the same mass that could be found in the primary sequences of the major caprine milk proteins from the Swissprot database (α_{S2}-casein P33049, α_{S1}-casein P18626, κ-casein P02670, and β-casein P33048).

2.8. Statistical analysis

Analysis of variance was carried out (Statgraphics Centurion XV.II, Statistical Graphics Corp., Rockville, MD) to determine the effect of the treatments and the substrates on the ACE inhibitory activity. If significant influence was obtained (p-value < 0.05) the experimental data were further analysed by Fisher's least significant difference (LSD) procedure, which consists of pairwise comparisons of the means, allowing to determine if two means are statistically different at the 95% confidence level.

3. Results and discussion

3.1. Production of milk protein fractions

The filtration process was followed for 2 h, at which point a concentration factor of 3 was reached. The permeate flow decreased by almost 70%, but no stationary permeate flow was reached within the operation time. Despite this relatively high flux decrease, the filtration capacity of the membrane was recovered completely after the alkali cleaning. Experimental data were fitted to the fouling models proposed for fouling in cross-flow filtrations by Field, Wu, Howell, and Gupta (1995). Complete pore blocking (Fig. 1) seemed to be the most likely fouling mechanism ($R^2 = 0.9966$). As a result of the microfiltration process the protein concentration in the retentate was increased from 26 up to 74.1 g L^{-1}, while the protein concentration in the final permeate was decreased to 2.2 g L^{-1}.

3.2. Characterisation of hydrolysates from milk and MF fractions

When the protein fractions obtained as well as the original milk sample were treated with the two enzymes, separately and in combination, nine hydrolysates were produced as shown in Table 1. For all substrates, as expected, trypsin gave the lowest DH, since it has a narrower specificity and can break fewer bonds (only Lys-X and Arg-X) than subtilisin (broad specificity with preference for large, uncharged residues). When a combination of both enzymes was used, the DH was slightly increased due to a higher number of bonds susceptible to cleavage. The ACE-inhibition percentages of the resulting hydrolysates were between 30 and 65 % (Table 1).

In accordance with a lower DH, the permeate gave rise to significantly lower ACE-inhibition percentages than the other substrates ($p < 0.0015$ for all the treatment). The lowest ACE-inhibition was obtained with trypsin hydrolysates, whereas the highest

inhibition was exerted by the hydrolysates made with the combination of subtilisin and trypsin. This tendency was seen for all substrates; however, for the retentate and permeate there was a statistically significant difference between the three enzymatic treatments ($p = 0.004$ and 0.006, respectively). With the combination of enzymes, the three substrates resulted in a percentage of inhibition of 50-65% and the highest ACE-inhibitory activity was obtained with retentate as substrate, showing the usefulness of the membrane process.

The peptide profiles of the 9 hydrolysates are shown in Fig. 2. The permeate samples had very low protein concentrations and precipitation problems appeared when a solution with adequate protein concentration was prepared. Therefore, the focus will be on the hydrolysates made from milk and the retentate fraction. Both of these substrates gave rise to different peptide profiles when hydrolysed with different enzymes. However, when the same enzyme was used, the chromatograms of both substrates were quite similar (compare adjacent chromatograms in Fig. 2A and 2B). This is in agreement with the similar ACE-inhibitory activity of these substrates hydrolysed with the same enzyme (Table 1). Also, it can be noted that when subtilisin and trypsin were used in combination, peptides with long elution times present in hydrolysates made with single enzymes, had been degraded. This is in accordance with the slightly higher DH and ACE-inhibitory activity obtained with the combination of enzymes.

The similarity between the retentate and milk hydrolysates indicates that the presence of whey proteins in the substrate (present in milk but not in the retentate, since they occurred in the filtrate after microfiltration of the milk), did not significantly affect the activity of the hydrolysate. This implies that the active peptides were derived mainly from the caseins, and is in accordance previous results showing higher activity from

casein-derived than from whey-derived peptides (FitzGerald & Meisel, 2000; Pihlanto-Leppälä et al., 1998; Saito, 2008). The retentate was formed basically by caseins and therefore was a simpler and more concentrated substrate than milk. Consequently, further fractionation of the three retentate hydrolysates was performed.

3.3. SEC Fractionation of retentate hydrolysates and ACE-inhibition of fractions

The elution profiles obtained by SEC fractionation of the three retentate hydrolysates were similar consisting basically of four peaks in each (denoted F1 to F4; Fig. 3) of which F2 was subdivided in some profiles. As indicated by DH the two hydrolysates made with single enzymes contained more large peptides (F2a and b) and less small peptides (F3 and F4) than the hydrolysate made with both enzymes. Four fractions were collected from the R-S hydrolysate, eight from the R-T, and five from R-ST as shown by the vertical lines in Fig. 3.

Using a calibration curve (not shown), it was possible to determine the approximate size ranges of peptides in each fraction. The F1 fraction contained peptides of between 70 and 22 kDa, F2 contained peptides smaller than 4 kDa in the case of R-T or R-S and smaller than 1.7 kDa for R-ST. The elution volume for F3 and F4 was higher than the total column volume suggesting that these contained peptides that interacted with the stationary phase.

The ACE-inhibitory activities of the collected fractions are shown in Fig. 4. In all hydrolysates, the highest ACE-inhibitory activity (> 80% of inhibition) was obtained with the F2 fractions (or some of the subfractions from this). For the R-ST hydrolysate this corresponded to range 1700 to 55 Da according to the calibration curve. However, in the F2a fraction from the R-S hydrolysate a very low ACE-inhibition was detected,

364 so all inhibitory peptides eluted in F2b (1300 to 75 Da). For the R-T hydrolysate, the
365 ACE-inhibitory peptides were concentrated in F2b, F2c, F2d and F2e (2300 to 55 Da).
366 The fractions F3 and F4 had lower percentages of inhibition in all the cases, however,
367 they also contained less protein. Especially F4 had very good values for ACE-inhibition
368 with a protein concentration much lower than that in F3 and all other fractions. The best
369 F4 fractions were those from the R-ST and the R-T hydrolysates which inhibited ACE
370 above 40% with a protein content of only 4% of that in the F2 fraction.

371 Using the IC_{50} values, the peptide concentration that gives a 50% reduction of
372 the ACE-activity, it was possible to compare the inhibition capacity of the fractions
373 (Table 2). The IC_{50} values varied between 5 and 57 μg mL^{-1}. These results were similar
374 to (and slightly better than) the results obtained by Minervini et al. (2003), who, by use
375 of other artificial substrates in the ACE assay, found IC_{50} values between 16 and 100 μg
376 mL^{-1} for fractions of casein hydrolysates made with a proteinase from *Lactobacillus*
377 *helveticus* PR4. The inhibitory activity of the hydrolysate fractions produced in this
378 study, especially of F4 fractions, was only slightly lower than that of IPP, which is one
379 of the strongest ACE-inhibitory peptide identified with an IC_{50} of 1.8 μg mL^{-1}
380 (Nakamura et al., 1995) and showing antihypertensive activity in both animals and
381 humans (Jäkälä & Vapaatalo, 2010; Xu, Qin, Wang, Li, & Chang, 2008). Moreover, the
382 results shown in Table 2 show that the fractions with smaller peptide size (longer
383 elution time) have better ACE inhibitory activity. Similar results were found by Mao et
384 al. (2007) and Jiang, Tian, Brodkorb, and Huo (2010), although the lowest IC_{50} obtained
385 for a hydrolysate fraction (<3 kDa fraction) in the latter study was 461 μg mL^{-1}. The
386 lower IC_{50} of F4 in comparison to F2 might in part be due to a higher number of small
387 peptides in F4 for the same protein content, thus a higher number of the active peptides
388 available for inhibition of ACE. However, it might also stem from the presence in F2 of

389 a multitude of peptides (high protein content) of which the majority provide limited
390 contribution to the ACE-inhibitory activity.
391
392 *3.4. Characterisation of peptides in SEC fractions F2 and F4*
393
394 The peptide profiles of the F2 fractions and subfractions collected from SEC are
395 shown in Fig. 5. All F2 fractions contained many peptides; the masses of the
396 dominating ones are given above the peaks in Fig. 5. The d and e subfractions from R-
397 T-F2 contained peptides with *m/z* values of 188, 329, 345, 351, 355, 459, 496, 553,
398 515, 699, 648, 668, 799, 803, 814, and 1140, of which at least the masses 188, 496 and
399 1140 were found in both fractions. The other F2 subfractions in addition contained
400 dominating peptides with *m/z* 598, 652, 748, 751, 690, 978, 995, 1005 and 1152. All of
401 these masses represented singly charged peptides.
402 A tentative identification of these peptides was performed by processing the
403 individual fragmentation mass spectra of these peptides with BioTools software. In this
404 way peptides originating from β-casein, α_{S1}-casein and κ-casein were identified (Table
405 3). The peptide TGPIPN from β-casein has been identified as an ACE-inhibitory
406 peptide in a goat milk hydrolysate made with trypsin (Geerlings et al., 2006). Other
407 peptides identified from caprine β-casein are identical to or similar to ACE-inhibitory
408 peptides identified from bovine β-casein, e.g., LHLPLPL, HLPLPL, EMPFPK
409 (Pihlanto-Leppälä, Rokka, & Korhonen, 1998; Quirós, Contreras, Ramos, Amigo, &
410 Recio, 2009), VLPVPQ (Maeno, Yamamoto, & Takano, 1996) and YPVEPF (Robert,
411 Razaname, Mutter, & Juillerat, 2004). The activity of LHLPLPL seems to be increased
412 after ingestion (Quirós et al., 2009).

With respect to the peptides identified from κ-casein, YPSYGLNYY is very similar to the synthetic ACE-inhibitor developed by Chiba and Yoshikawa (1991). The peptide FLPYPY has not been identified as an ACE-inhibitor; however, it has been shown to stimulate neurite outgrowth in mice and could be useful to treat neurodegenerative diseases (Sakaguchi, Ishikawa, Nishimura, Sugihara, & Matsumura, 2004). Based on its hydrophobic C-terminal, this peptide might have ACE-inhibitory activity as well, in accordance with many bioactive peptides having multiple functions (Hernandez-Ledesma, Amigo, Recio, & Bartolome, 2007; Korhonen 2009; Meisel, 1998).

The peptides VVAPFPEVF and FVVAPFPEVF identified from α_{S1}-casein represent most of the sequence of the tryptic peptide FFVAPFPEVFGK from bovine α_{S1}-casein that has shown in vivo antihypertensive activity (FitzGerald et al., 2004), and may thus also exert ACE-inhibitory activity, especially considering the basic and hydrophobic residues in the C-terminal. This has been confirmed by Ong, Henriksson, and Shah (2007) who identified a similar peptide (FVAPFPEVF) in cheddar cheese as an ACE-inhibitor. Interestingly, according to their primary sequence, all of these α_{S1}-casein-derived peptides, including the peptide identified in this work, could be broken down during digestion by chymotrypsin at the carboxylic side of Phe, releasing the same peptide, PEVF, which could be the active peptide in vivo.

Several of the peptides identified could thus be primarily responsible for the ACE-inhibitory activity exerted by these goat milk protein hydrolysates and fractions. Due to the presence of C-terminal tyrosine residues in some of the identified peptides (FLPYPY and YPSYGLNYY), these are expected also to exert antioxidant activity and thus be multifunctional (Hernández-Ledesma et al. 2007). Apart from the peptides

mentioned, many other peptides were present at low concentration, some of which may also have contributed to the bioactivity of the fractions.

The F4 fractions, in contrast, contained mainly one compound with retention time 28.5 min (Fig. 6). Based on its high UV-absorbance and its long retention time in SEC and in reversed-phase high performance liquid chromatography it is expected to contain Trp and/or Tyr. Since the elution time of the compound is longer than that of Trp, which is the most hydrophobic of the free amino acids and elutes latest (Broncano, Otte, Petrón, Parra, & Timon, 2010; Parrot, Degraeve, Curia, & Martial-Gros, 2003), the compound in F4 must be a di- or tri-peptide containing at least one aromatic amino acid residue. This compound tended to fragment somewhat during the LC-MS analysis and gave rise to several masses, the major ones being 368, 351, 159 and 144. It most probably is the dipeptide Trp-Tyr corresponding to f164-165 in both caprine and bovine α_{S1}-casein, which has the mass 368.2, close to the 368.3 found. This also fits with the loss of an amino group upon fragmentation (Rogalewicz, Hoppilliard, & Ohanessian, 2000) giving the mass of 351.

This dipeptide has not previously been isolated from caseins, but it fits with the structural requirements for the C-terminal part of ACE-inhibitory peptides as obtained by quantitative structure-activity relationship (Wu, Aluko, & Nakai, 2006a), and the synthesised peptide WY has shown both ACE-inhibitory and antioxidant activity (Hernandez-Ledesma et al., 2007). Furthermore, the reverse peptide, YW, has also been shown to be a potent ACE-inhibitor with in vivo blood pressure lowering effect in spontaneously hypertensive rats (Marczak et al., 2003; Wu, Aluko, & Nakai, 2006b). This study is the first to demonstrate the presence of WY in a casein hydrolysate. The highest level was obtained after hydrolysis with a combination of subtilisin and trypsin (Fig. 3).

4. Conclusions

The results presented in this paper show that although a similar range of peptides was obtained from milk and the casein fraction, the active peptides were more concentrated when microfiltration was performed to isolate the caseins prior to hydrolysis. Extensive hydrolysis and production of ACE-inhibitory peptides was obtained with the two commercial enzymes, especially when used in combination. For industrial purposes, the low price and wide application field of subtilisin is especially interesting. The present results also showed that most of the active peptides were below 2 kDa and one particularly active dipeptide was very hydrophobic. This information is very useful in the design of a membrane process which can concentrate and purify the active peptides after the hydrolysis process.

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Figure legends

Fig. 1. Evolution of permeate flux with time during microfiltration of skimmed goat milk. Experimental data (○) and best fit adjusted to the pore blocking model (—) are shown.

Fig. 2. Peptide profiles of hydrolysates made from goat milk protein by use of subtilisin and trypsin. The milk substrates used were skimmed milk (column A), and retentate (column B) and permeate (column C) fractions from microfiltration of skimmed milk. Each freeze-dried hydrolysate was dissolved at 20 mg mL^{-1} before analysis.

Fig. 3. SEC Profile of goat milk protein retentate hydrolysates made with subtilisin (A), trypsin (B), and both subtilisin and trypsin (C).

Fig. 4. Protein concentration (grey) and ACE-inhibitory activity (black) of each SEC fraction from hydrolysates made from goat milk retentate with subtilisin (A), trypsin (B) and both enzymes (C). Fraction names refer to Fig. 3.

Fig. 5. Peptide profiles of F2 fractions from SEC fractionation of retentate hydrolysates. The retentate was hydrolysed with subtilisin (R-S), trypsin (R-T), and both enzymes (R-ST), respectively. Sample names refer to fractions in Fig. 3. Numbers above peaks represent the masses of major peptides ($[M+H]^+$).

Fig. 6. Peptide profiles of F4 fractions from SEC fractionation of retentate hydrolysates made with subtilisin (R-S), trypsin (R-T), and both enzymes (R-ST), respectively. Sample names refer to Fig. 3. The absorbance at 210 nm is shown as full line and the absorbance at 280 nm as dotted line.

Table 1.

Characteristics of the hydrolysates obtained by enzymatic hydrolysis (3 h, 50 °C) of caprine milk and fractions thereof; for each enzyme-substrate combination the degree of hydrolysis (DH) and the ACE-inhibitory activity are given. [a]

Hydrolysate code	Substrate	Enzyme	DH (%)	ACE-inhibition (%)
M-S	Milk	Subtilisin	28.2	60.5 ± 2.3 [a]
M-T	Milk	Trypsin	21.9	50.9 ± 0.5 [b]
M-ST	Milk	Subtilisin + trypsin	32.4	63.2 ± 2.8 [a c]
R-S	Retentate	Subtilisin	29.5	60.7 ± 1.0 [a]
R-T	Retentate	Trypsin	21.7	54.4 ± 2.3 [d]
R-ST	Retentate	Subtilisin + trypsin	32.3	64.3 ± 0.2 [c]
F-S	Filtrate	Subtilisin	21.7	41.9 ± 1.0 [$]
F-T	Filtrate	Trypsin	17.9	33.0 ± 0.6 [e]
F-ST	Filtrate	Subtilisin + trypsin	23.2	50.6 ± 2.4 [b]

[a] DH was determined from the amount of alkali added during hydrolysis. Each hydrolysate was dissolved at 0.8 mg protein mL^{-1} corresponding to 0.133 mg mL^{-1} in the assay. Values of ACE inhibition with the same superscript letter are not statistically different.

Table 2.

ACE-inhibitory activity (IC_{50}) of the most active SEC fractions from hydrolysed retentate fraction of goat milk protein.

Hydrolysate	**Fraction**	**IC50 (μg protein mL^{-1})**
R-ST	F2	13.13
R-ST	F4[b]	9.28
R-S	F2b	14.63
R-S	F4 [b]	5.53
R-T	F2b	56.96
R-T	F2c	17.74
R-T	F2d	15.37
R-T	F2e[b]	8.04
R-T	F4 [b]	5.12

[a] Hydrolysate codes are as given in Table 1; the fraction numbers refer to Fig. 3.

[b] IC_{50} were determined after a concentration of the original fraction.

Table 3.
Tentative identification of dominating peptides in the active F2 fractions of goat milk retentate hydrolysates (Sample names refer to Fig. 3); the fragment mass spectrum of each peptide was compared to theoretical fragment spectra of the proposed fragment by use of BioTools software and the score is given by this software.

Mass observed $[M+H]^+$	Sample name	Retention time (min)	Possible peptide	Mass calculated $[M+H]^+$	Score	Origin
459.3	R-T-F2d	23.8	n.i.[a]			
553.3	R-T-F2d	30.0	n.i.			
496.5	R-T-F2d+e	33.9	n.i.			
696.6	R-T-F2d	36.6	VPNSAE(1P)[b]	696.7	96	α_{S1}-CN f72-77
648.7	R-T-F2d	39.9	n.i.			
667.5	R-T-F2c + d	44.2	n.i.			
803.0	R-T-F2b + c	55.0	LHLPLPL	802.5	130	β-CN f133-139
1140.6	R-T-F2d+e	42.0	YPSYGLNYY[b]	1140.2	322	κ-CN f35-43
799,7	R-T-F2d	44.9	FLPYPY	799.4	64	κ-CN f55-60
814.9	R-T-F2d	50.4	n.i.			
598.6	R-ST-F2, R-S-F2b, R-T-F2b+c	25.1	TGPIPN	598.7	65	β-CN f63-68
652.7	R-ST-F2, R-S-F2b, R-T-F2b	32.6	VLPVPQ[b]	652.4	47	β-CN170-175
748,8	R-ST-F2, R-T-F2c	35.0	EMPFPK	748.9	92	β-CN f108-113
751.5	R-ST-F2, R-T-F2c	40.0	YPVEPF	751.9	65	β-CN f114-119
690.0	R-ST-F2, R-S-F2b, R-T-F2b+c	48.5	HLPLPL	689.9	63	β-CN f134-139
978.0	R-ST-F2, R-T-F2b	37.0	n.i.			
995.1	R-T-F2b	51.0	n.i.			
1005.0	R-T-F2b+c	54.1	VVAPFPEVF	1005.2	79	α_{S1}-CN24-32
1152.1	R-T-F2b+c	59.0	FVVAPFPEVF	1152.4	350	α_{S1}-CN23-32

[a] n.i., could not be identified in the sequences used.
[b] This sequence is uncertain.

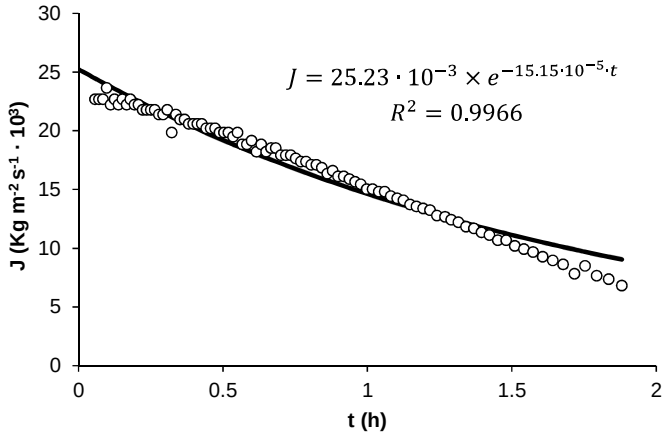

Figure 1. Espejo et al.

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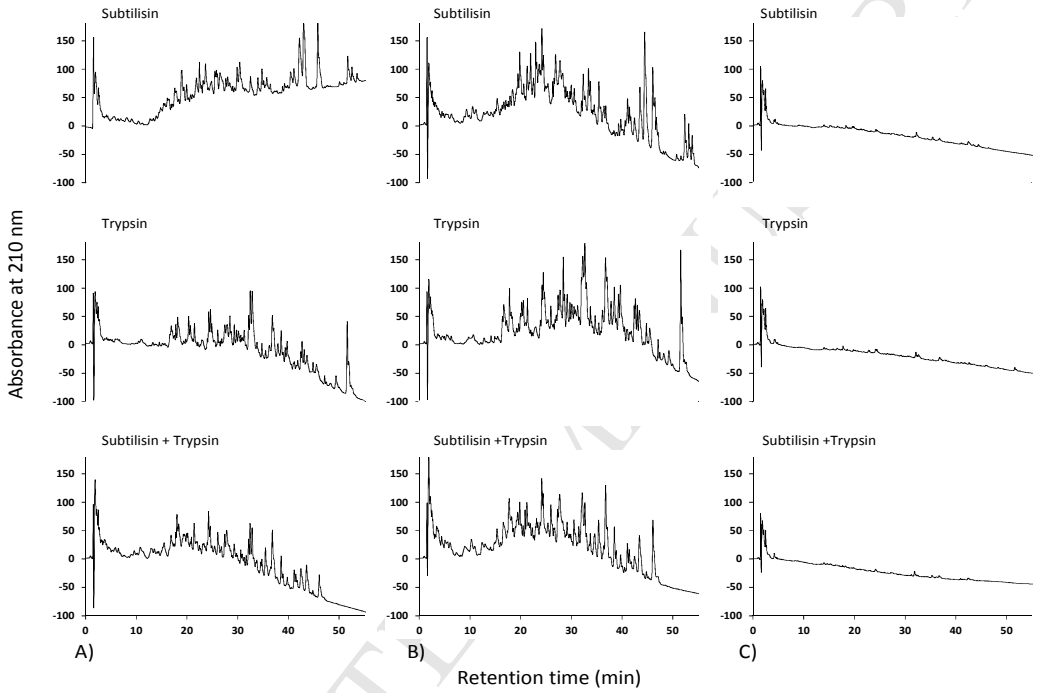
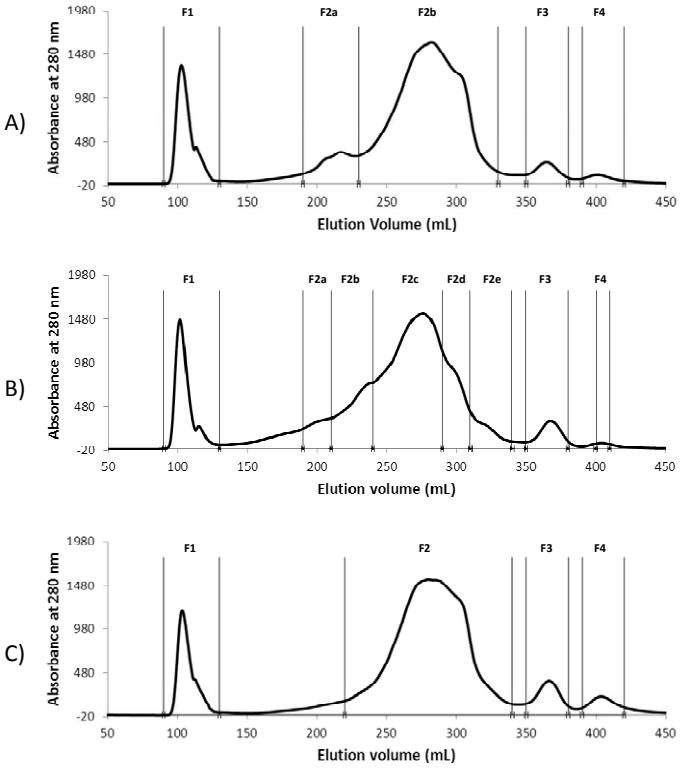
Figure 2. Espejo et al.





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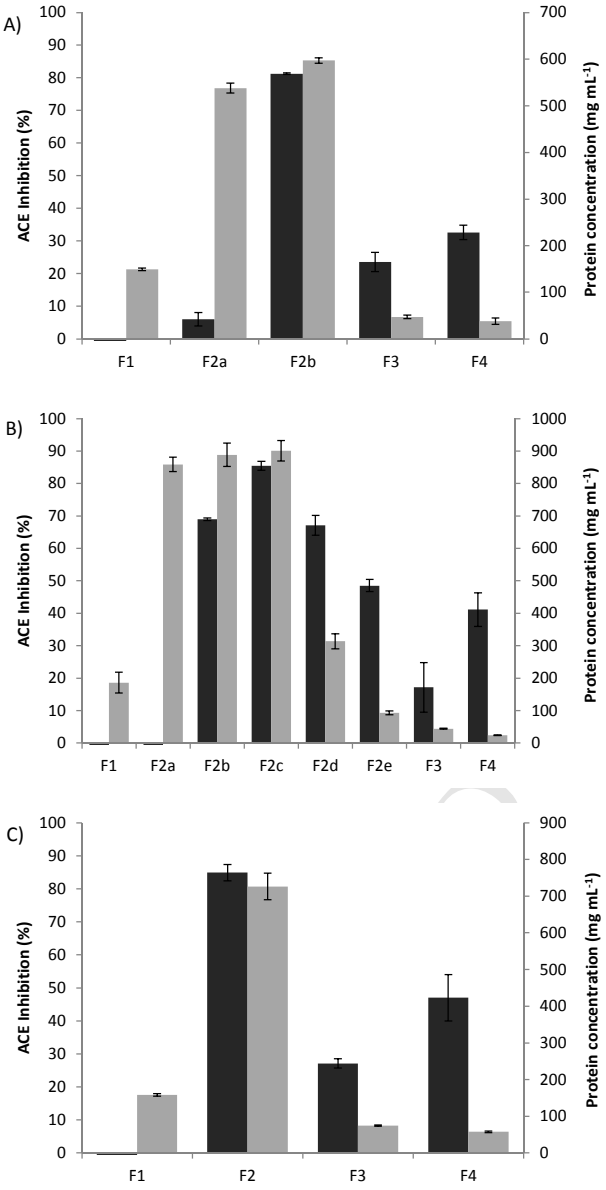

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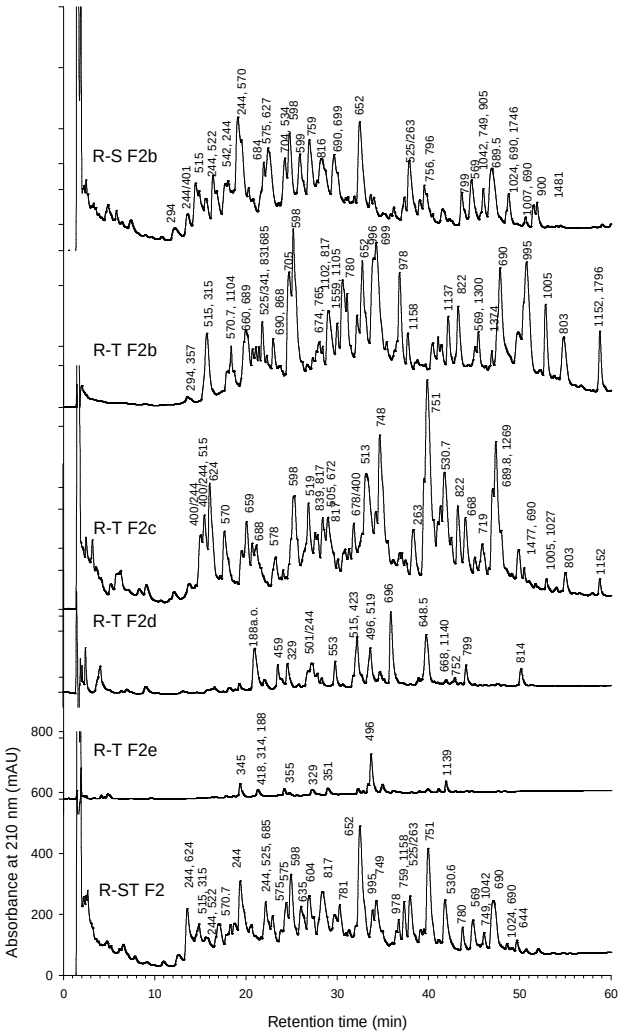

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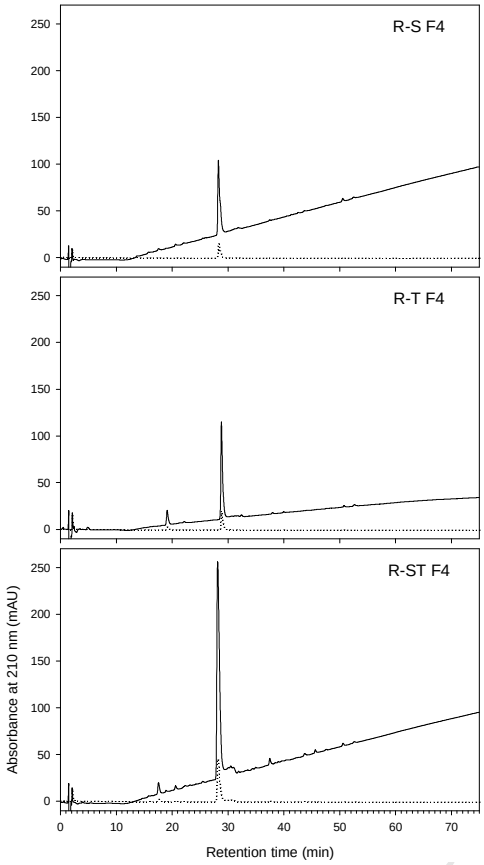

Figure 6. Espejo et al.