Antioxidant peptides from goat milk protein fractions hydrolysed by two commercial proteases

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Abstract

Goat milk microfiltration fractions were hydrolysed with subtilisin or trypsin, or both enzymes in combination, and tested for 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging capacity, iron chelation capacity, and lipid peroxidation inhibitory activity in liposomes. The most active hydrolysates were further fractionated and characterized in order to identify the active compounds. The retentate treated with subtilisin was one of the most active samples regarding the radical scavenging capacity ($SC_{50} \approx 4$ μg mL^{-1}), while the permeate treated with subtilisin had the best iron chelation capacity ($IC_{50} \approx 65$ μg mL^{-1}) and lipid peroxidation inhibitory activity (33% inhibition at 25 μg mL^{-1}). In the retentate hydrolysate various short peptides active as radical scavenger as well as lipid peroxidation inhibitors were identified, both novel and already known peptides. It was not possible to assign an activity to a specific peptide, but it was clear that tyrosine had a fundamental role in the ABTS radical scavenging capacity of the peptides, and also some effect in the inhibition of lipid peroxidation, in which the amino acid phenylalanine seemed to play the most important role. The presence of non-protein low molecular weight compounds in the permeate hydrolysate seemed more important than peptides for the antioxidant activities detected. Such hydrolysates may be relevant for use in foods as natural antioxidants to increase shelf-life.

1. Introduction

Goats' milk has some nutritional benefits in comparison to cows' milk (Barrionuevo, Alferez, Lopez Aliaga, Sanz Sampelayo, & Campos, 2002; Martinez-Ferez et al., 2006; Park, 1994). Furthermore, goats' milk seems to have better *in vivo* antioxidant properties than cows' milk, since rats fed a goats' milk-based diet had lower levels of lipid peroxidation in plasma (measured as thiobarbituric acid reactive substances) than rats fed a cows' milk-based diet (Diaz-Castro et al., 2012). Kullisaar et al. (2003) showed that fermentation of goats' milk improved the antioxidant properties in humans, suggesting that such beneficial effects might be related to the release of bioactive peptides from the milk proteins during fermentation.

Milk proteins, like a number of other food proteins, contain sequences, that when liberated as peptides by fermentation or enzymatic hydrolysis, exert beneficial physiological effect. Such bioactive peptides can affect the cardiovascular system by exerting antithrombotic, antihypertensive, opioid, antioxidant, or immunomodulatory effects among others (Phelan, Aherne, FitzGerald, & O'Brien, 2009; Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007; Korhonen & Pihlanto, 2006; Silva & Malcata, 2005). Bioactive peptides have been identified mainly from cows' milk, but also goats' milk proteins include peptides with activities such as antioxidant, antibacterial, immune-modulating and ACE-inhibitory (Atanasova & Ivanova, 2010; Eriksen, Vegarud, Langsrud, Almaas, & Lea, 2008; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013; Li et al., 2013; Park, 2009; Quirós, Hernàndez-Ledesma, Ramos, Amigo, & Recio, 2005; Santillo, Kelly, Palermo, Sevi, & Albenzio, 2009;).

Antioxidant peptides are particularly interesting because they can potentially prevent or delay oxidative deterioration of foods and thus prolong shelf-life (Hernàndez-Ledesma, Davalos, Bartolome, & Amigo, 2005; Pihlanto, 2006; Suetsuna, Ukeda, & Ochi, 2000). In foods, lipid oxidation generates toxic compounds and undesirable odours and flavours (Laguerre, Lecomte, & Villeneuve, 2007). Processing

of food increases the oxidative stress by introducing oxygen (e.g. mixing), removing natural antioxidant (e.g. oil refining), increasing the amount of prooxidative compounds (e.g. light exposure) or including labile species (omega-3 fatty acids) into the food (Elias, Kellerby, & Decker, 2008; Skibsted, 2008). Antioxidant compounds can inhibit oxidation by different mechanisms, such as scavenging of free radicals, a major source of oxidation (Valko et al., 2007), or chelation of metal ions (e.g. Fe^{2+}), an initiator of oxidation (Welch, Davis, Van Eden, & Aust, 2002). For this reason there are multiple ways to measure the antioxidant activity of a compound and the use of different assays is preferable in order to confirm the activity (Huang, Ou, & Prior, 2005); it is also important, when testing potential antioxidants, to include evaluation methods with specific food related oxidation substrates (Becker, Nissen, & Skibsted, 2004).

Many peptides with antioxidant activity have been released from bovine milk proteins, either during fermentation of milk or through enzymatic hydrolysis using commercial enzymes, as reviewed by Power, Jakeman and FitzGerald (2013). These peptides have been found to protect various foods from oxidation, like omega-3 fatty acids in fortified yogurt, which are more stable than in the corresponding milk, a protection attributed to the peptides and free amino acids present in the yogurt (Sabeena Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010); furthermore, antioxidant peptides derived from milk proteins can prevent lipid oxidation in beef homogenates, both in raw meat (Rossini, Noreña, Cladera-Olivera, & Brandelli, 2009; Sakanaka, Tachibana, Ishihara, & Juneja, 2004) and after cooking and storage (Dìaz & Decker, 2004; Hogan, Zhang, Li, Wang, & Zhou, 2009).

Only a few studies on the antioxidant properties of goats' milk protein-derived peptides have been performed. Nandhini, Angayarkanni, and Palaniswamy (2012) found that goats' milk fermented with *Lactobacillus plantarum* had potent radical scavenging and lipid peroxidation inhibition activity, but the active peptides were not identified. Silva, Pihlanto and Malcata (2006) identified three antioxidant

peptides in a water extract from a goat cheese-like system made using an extract from *Cynara cardunculus* (YQEP and YQEPVLGP from β-casein, and YQKFPQY from α_{s2}-casein). In a recent study, Li et al. (2013) identified five antioxidant peptides in a goats' milk casein hydrolysate made using Alcalase and Pronase (VYPF, FPYCAP, FGGMAH, YPPYETY and YVPEPF), although the last four did not correspond to sequences in the goat caseins available in protein databases. More research is needed to identify new antioxidant peptides from goats' milk proteins to increase the exploitation of the health benefits of milk proteins from this species.

In our previous study on ACE-inhibitory activity of hydrolysed goats' milk protein fractions (Espejo-Carpio et al., 2013), we identified a number of casein-derived peptides which were liberated enzymatically by a combination of subtilisin and trypsin, among them many which contained tyrosine in their sequence and which were expected also to exert antioxidant activity. Therefore, the aim of this study is to isolate and identify the most potent antioxidant peptides (as radical scavenger, iron chelators or lipid oxidation inhibitors) formed from goats' milk protein fractions by enzymatic hydrolysis using trypsin and subtilisin individually and in combination.

2. Materials and methods

2.1. Materials

2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid (Ferrozine™) and L-α-phosphatidylcholine were purchased from Sigma-Aldrich A/S (Brøndby, Denmark).

99 *2.2. Production of hydrolysates*

100 Hydrolysates were produced and characterized as described in Espejo-Carpio et al. (2013). Briefly,
101 samples of skimmed commercial UHT goats' milk were microfiltered through a tubular ceramic
102 microfiltration membrane with pore size 0.14 μm, and the retentate and permeate fractions as well as a
103 milk sample were hydrolysed for 3 h in a stirred-tank reactor with subtilisin (Alcalase 2.4 L FG (EC.
104 3.4.21.62), trypsin (pancreatic trypsin PTN 6.0 S Saltfree (EC. 3.4.21.4), both from Novozymes A/S
105 (Bagsværd, Denmark), or a mixture of both enzymes. The pH was kept at 8.0 during hydrolysis by
106 addition of 1 M NaOH, and the degree of hydrolysis (DH) of the hydrolysates was calculated from the
107 amount of NaOH used during the reaction. The nine resulting hydrolysates are identified with two
108 letters, one for the substrate (M, R or P for milk, retentate and permeate, respectively) and the other for
109 the enzyme (S, T or ST for subtilisin, trypsin and both, respectively). For instance R-S is the retentate
110 hydrolysed with subtilisin. The hydrolysates were freeze-dried and the protein content was determined
111 using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma) (n=3).

112

113 *2.3. Determination of antioxidant activity*

114 The antioxidant activity of hydrolysates was determined by three different assays testing the ability to
115 scavenge radicals, to chelate iron and also to prevent lipid oxidation in a model system.

116 *2.3.1. ABTS radical scavenging capacity*

117 This assay is based on the reductive decolorization of the radical form of ABTS ($ABTS^{\bullet+}$) as described
118 in previous papers (Jensen et al. 2011) with slight modifications. In order to create $ABTS^{\bullet+}$, a solution
119 of ABTS (19.4 mM) was first allowed to react overnight with a solution of potassium persulfate (6.7
120 mM) in water. The $ABTS^{\bullet+}$ solution was then diluted approximately 400 times with phosphate buffer

(10 mM, pH 7.4) until an absorbance of 0.6 – 0.7 was reached. The $ABTS^{\bullet+}$ working solution (200 µL) was mixed with 50 µL of hydrolysate (at different concentrations) and the absorbance at 405 nm was recorded for 30 min using a TECAN GENios Plus microtiter-plate multiscan fluorometer (Tecan Austria GmbH, Grödig/Salzburg, Austria). The final readings were used for calculating the ABTS radical scavenging activity (%) according to the formula:

$$\text{Radical scavenging (\%)} = 100 - (100 \times (A_{sample})/ (A_{ctrl}));$$

where A_{ctrl} is the absorbance of the control sample with water instead of hydrolysate, A_{sample} is the absorbance of the hydrolysate sample. Samples were diluted in water to various concentrations, and the dilution which resulted in a scavenging activity between 10 and 90% for all samples was chosen for comparison. Samples were assayed in triplicate. In order to calculate the SC_{50} (the protein concentration needed to scavenge 50% of the $ABTS^{\bullet+}$), a curve was plotted of the scavenging activity as function of hydrolysate concentration, and the slope of the linear part around 50% activity was used to calculate the SC_{50}.

2.3.2. Iron chelation capacity

This assay was performed as described by Wu, Huang, Lin, Ju and Ching, (2007) with some modifications. It is based on colour changes of Ferrozine™, which is an uncoloured compound that becomes purple when complexed with iron ions (Fe^{2+}). In a transparent microtiter plate, 25 µL of hydrolysate were mixed with 100 µL of a $FeSO_4$ solution (75 µM in water) and incubated for 10 min at room temperature. Then, 100 µL of Ferrozine™ solution (500 µM in water) were added and the absorbance was read at 560 nm using a Multiskan EX plate reader (Labsystems Oy, Helsinki, Finland). The inhibition of iron-Ferrozine complex formation was calculated according to the formula:

$$\text{Iron chelation (\%)} = 100 - (100 \times (A_{sample}/A_{ctrl})$$

where A_{ctrl} is the absorbance of Ferrozine complex in the control sample with water instead of hydrolysate, and A_{sample} is the absorbance of the Ferrozine complex when the hydrolysate sample is present. Before testing, samples were diluted in water to various concentrations and the concentration which gave an inhibition between 5 and 90% for all samples was chosen for comparison. Samples were assayed in triplicate. In order to calculate the IC_{50} (the protein concentration needed to inhibit 50% of the iron-Ferrozine complex formation), a curve was plotted of the iron chelation as function of hydrolysate concentration, and the slope of the linear part around 50% activity was used to calculate the IC_{50}.

2.3.3. Prevention of lipid oxidation in liposomes

This assay is based on the method developed by Tirmenstein, Pierce, Leraas and Fariss (1998). Briefly, L-α-phosphatidylcholine liposomes were prepared and kept for 1 wk at 5°C in order to increase the level of hydroperoxide groups. The assay was performed in black microtiter plates. To each well was added 10 µL of liposomes, 50 µL of diluted hydrolysate (0.1 mg mL^{-1} for samples or water as positive control) and 120 µL of glycine ascorbate buffer (GAB, 50 mM Potassium Phosphate Buffer, 100 mM glycine and 450 µM ascorbate, pH 7.4). The reaction was started by addition of 20 µL of oxidizing reagent (25 µM $FeCl_3$ with 1 mM ADP in 50 mM sodium phosphate buffer pH 7.4). The plate was then incubated at 37°C and the fluorescence was monitored for 4 h with 10 min intervals using excitation at 360 nm and emission at 460 nm in the TECAN microplate reader described above. In order to avoid differences due to a different fluorescent starting point, all the fluorescent values were subtracted the

value of the lowest reading, so that the lowest point was zero, and the area under the curve (AUC) was calculated using the formula:

$$AUC = (2 \times \sum (a_2{:}a_{n-1}) + a_1 + a_n) \times time / 2,$$

where a_1 is the first fluorescent value (in rfu) and a_n is the last fluorescent value, time is the time interval between two measurements (10 min). The % inhibition of lipid oxidation was calculated using the formula:

$$\text{Lipid peroxidation inhibition (\%)} = 100 - (100 \times AUC_{sample}/AUC_{ctrl}),$$

where AUC_{sample} is the AUC of the sample with hydrolysate and AUC_{ctrl} is the AUC of positive control (with GAB buffer added instead of hydrolysate). Before testing, samples were diluted in water to various concentrations, and the concentrations which gave inhibition values between 10 and 90% were chosen for comparison.

2.4. Fractionation by size-exclusion chromatography

Selected hydrolysates with high antioxidant activity were fractionated by size-exclusion chromatography (SEC) using an fast protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) mounted with a column packed with Superdex^{TM} 30 prep grade gel filtration resin Amersham Biosciences, Hillerød, Denmark) as described previously (Espejo-Carpio et al. 2013), with some modifications. Two millilitres of a hydrolysate solution with 5 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 214 nm and fractions of 10 mL were collected.

185 *2.5. Purification by semipreparative RP-HPLC*

186 The most active SEC fractions from the retentate and permeate samples hydrolysed by subtilisin were
187 further fractionated in order to identify the active peptides. The SEC fractions from the permeate
188 hydrolysate were fractionated on a Gilson high-performance liquid chromatography (HPLC) system
189 (Middleton, WI, USA) as described by Buskov, Olsen, Sørensen and Sørensen (2000) but modified for
190 RP-HPLC with buffers containing trifluoroacetic acid (TFA) and acetonitrile. Samples (100 µL) were
191 injected and separated on a C-18 RP column (Phenomenex Gemini, 5 µm, 250 x 10 mm, 110 Å,
192 Phenomenex, Værløse, Denmark) at 40°C with a flow rate of 1 mL min^{-1}, using a gradient consisting of
193 95% Buffer A (0.1% TFA in water) for 10 min, followed by a linear gradient from 5 to 50% buffer B
194 (0.1% TFA, 90% acetonitrile) over the next 70 min. The effluent was monitored at 214 nm and 280 nm
195 and data collected using Unipoint v. 1.71 system software (Gilson Inc. Middleton, WI, USA). Fractions
196 of 2 or 3 mL were collected, and the same fractions from 3 repeated RP-HPLC runs were pooled (a
197 total of 6 or 9 mL) and evaporated to dryness. The SEC fractions from the retentate hydrolysate were
198 fractionated using the same conditions and same column but mounted on an HPLC system composed
199 of an Agilent series 1100 Autosampler (G1313A) and binary pump (G1312A) and a Gilson DAD
200 detector (G1315A). The software used was Chemstation for LC 3D systems software version B.03.02
201 (Agilent technologies ApS, Glostrup, Denmark). Fractions of 2 mL were collected, and the same
202 fractions from 3 repeated RP-HPLC runs were pooled (a total of 6 mL) and evaporated to dryness for
203 bioactivity testing, each evaporated fraction was resuspended in 500 µL of water.

204

205 *2.6. Peptide profiles and identification by LC-MS/MS analysis*

206 Peptide profiles were revealed by liquid chromatography – tandem mass spectrometry (LC-MS/MS)
207 analysis using an Agilent 1100 LCMSD Trap as described previously by Espejo-Carpio et al. (2013).
208 From the fractions obtained after the SEC and RP-HPLC a sample volume of 25 µL was injected. On-
209 line mass spectra were recorded using settings as described previously and data were processed by
210 Bruker Daltonics Data Analysis version 3.3.

211 Mass spectra were exported and analysed with Biotools 3.0 software (Bruker Daltonics, Frederikssund,
212 Denmark) using the sequences of goat caseins from Swissprot (http://uniprot.org, sequences for α_{s1}-
213 casein (P18626, variants A, B and C), α_{s2}-casein (P33049, variant A), β-casein (P33048), κ-casein
214 (P02670, variants A and B), α-lactalbumin (P00712) and β-lactoglobulin (P02756)). The sequence of
215 each casein protein from the database was searched for peptides with the same masses as those
216 obtained by the LC-MS analysis, and the MS/MS fragments obtained were compared to those expected
217 from the relevant peptides. The peptide with the highest score given by the software was selected as the
218 identified one, provided that all major MS/MS peaks could be accounted for.

219

220 *2.7. Statistical analysis*

221 Differences between hydrolysate samples with respect to bioactivity was tested using one-way analysis
222 of variance (GLM procedure), and means of samples were compared using Duncan's Multiple Range
223 test (SAS 9.3 for windows, SAS Institute Inc., Cary, NC, USA.). Correlations between the various
224 parameters and activities were determined by Pearson Correlation Coefficients using the same
225 software.

226

3. Results and discussion

3.1. Antioxidant capacity of total hydrolysates

Nine goats' milk protein hydrolysates were obtained by subjecting the 3 milk protein substrates, i.e. milk (M), retentate (R), and permeate (P), to the 3 enzymatic treatments with trypsin (T), subtilisin (S) and both enzymes simultaneously (ST), respectively. The DH of these hydrolysates is given by Espejo-Carpio et al. (2013). The protein content of the lyophilized hydrolysates from milk, retentate and permeate were 20.8, 31.6 and 4.4% (w/w), respectively. The hydrolysate samples were redissolved to the same protein level and the antioxidant activity of these were compared**.**

3.1.1. ABTS radical scavenging capacity

As shown in Figure 1a, all goats' milk protein hydrolysates showed high ABTS radical scavenging activity (47 to 87%) when tested at 10 µg protein mL^{-1} in the assay. The hydrolysates prepared from milk (M-T, M-S and M-ST) and from retentate (R-T, R-S and R-ST) exhibited a radical scavenging capacity of more than 80%, which is significantly higher ($p< 0.0001$) than those exhibited by the permeate hydrolysates (P-T, P-S, and P-ST). From this it seems that the caseins, present in the milk and in the retentate but not in the permeate, play an important role in the scavenging of the radicals. This is in accordance with the results obtained by Chen, Lindmark-Månsson, Gorton and Åkesson (2003) comparing the ABTS scavenging activity of bovine milk, whey and low molecular weight (LMW) fractions. Furthermore, the enzyme used for hydrolysis had a small but significant ($p<0.05$) effect on the activity. Use of both enzymes gave rise to a slightly better radical scavenging capacity than subtilisin alone, which in turn was better than trypsin alone. This could be related to DH, since subtilisin has a broader specificity than trypsin which leads to a higher DH, and the use of both

enzymes increased the DH even more. This was confirmed by a strong positive correlation between DH (as given by Espejo-Carpio et al., 2013) and radical scavenging activity ($r = 0.72$, $p<0.0001$) for all samples, which is probably due to an increased number of peptides at higher DH available for interaction with the ABTS radical. Mao, Cheng, Wang and Wu (2011) using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a radical also found that the DH of yak milk hydrolysates had a positive effect on the radical scavenging capacity, and that Alcalase (subtilisin) gave rise to a better antioxidant effect than trypsin. .

Comparison of the concentrations needed to scavenge 50% of the ABTS radicals (SC_{50}) confirmed that the hydrolysates from milk and retentate exerted the best radical scavenging activity ($SC_{50} \approx$ 4-5 µg mL^{-1} in the final assay, Table 1). Li et al. (2013) also found high ABTS radical scavenging activity ($SC_{50} \approx 0.5$ µg mL^{-1}) in goat casein hydrolysates produced with a combination of neutral protease and Alcalase. The higher activity in their study could in part be due to the increased time of hydrolysis (6 h instead of 3 h) and the broader specificity of the two proteases used. Goats' milk hydrolysates thus seem much more active than bovine casein tryptic digest (referred as caseinphosphopeptides, CPP) ($IC_{50} \approx 350$ µg mL^{-1}) obtained by Kitts (2005) with the same method.

3.1.2. Iron chelation capacity

The iron chelation capacity values of the goats' milk protein hydrolysates were between 20 and 80% when analysed at a protein concentration of 20 µg mL^{-1} (Figure 1b). As was observed for radical scavenging activity, the nature of the substrate had a significant influence ($p< 0.0001$) on the capacity to chelate iron ions (Figure 1b). However, opposite to what was seen for the radical scavenging capacity, the permeate exerted the highest iron chelation capacity, more than 75% for all three permeate hydrolysates, against less than 40% for the retentate and milk hydrolysates. The permeate had

a low protein content (around 5%) and contained also non-protein LMW substances such as lactose, acids and salts, which are present in much lower quantity in the retentate and milk, and which might chelate iron or otherwise influence the measurement and thus be partly responsible for the high value.

Taking all hydrolysates together, the hydrolysates prepared with subtilisin exerted a slightly higher iron chelation capacity than those prepared with trypsin or both enzymes together ($p<0.05$). It thus seems that trypsin may degrade part of the active peptides formed by the action of subtilisin in the permeate hydrolysates. Subtilisin, which cleaves peptides with leucine, phenylalanine, serine, tyrosine, glutamic acid or tryptophan residues, as determined with whey proteins (Doucet, Otter, Gauthier, & Foegeding, 2003), would liberate many peptides, among them some with C-terminal tyrosine, a structural feature known to increase the antioxidant activity (Gülçin, 2007). By the action of trypsin, part of these peptides would be cleaved creating shorter and less active peptides, since free tyrosine and shorter peptides containing tyrosine are often less antioxidant than longer peptides (Guo, Kouzuma & Yonekura, 2009). However, as shown in Figure 1b, the effect of the enzymatic treatment was different for the 3 substrates. For the hydrolysates of the retentate, which contained the highest protein concentration in relation to LMW non-protein compounds, there was a positive correlation with DH, probably due to an increased number or solubility of the smaller peptides. Opposite to this, for the milk hydrolysates there was a negative correlation. We speculate that extensive hydrolysis in this case would lead to an increased number of peptides which could interact with the LMW compounds preventing them from reacting with the iron, since peptides contain more charges than the intact and less hydrolysed proteins.

Overall, the hydrolysate that was most active in iron chelation was the permeate treated with subtilisin, with an IC_{50} of 14 µg mL^{-1} (Table 1). Conway, Gauthier and Pouliot (2012) found that a whey protein concentrate hydrolysed with pepsin and trypsin had an iron chelation capacity of only 28% when tested at 4 mg mL^{-1}. The much lower capacity compared to our results highlight the importance of non-

296 protein components in the whey fraction. The IC_{50} for the retentate hydrolysates were between 57 and
297 61 μg mL^{-1} (Table 1). Li et al. (2013) also measured the chelating effect of caprine casein hydrolysates
298 and obtained a similar IC_{50} (50 μg mL^{-1}). These activities are quite high in comparison to those for
299 bovine milk protein hydrolysates, of which the best (a peptic hydrolysate of skim milk) showed an IC_{50}
300 for iron chelation between 1 and 4 mg mL^{-1} (Conway et al., 2012).
301
302 *3.1.3. Inhibition of lipid oxidation in liposomes (generation of secondary oxidation products)*
303 The goats' milk protein hydrolysates showed the ability to inhibit lipid oxidation in
304 phosphatidylcholine liposomes, ranging from 5 to 35%, when tested at a protein concentration of 25 μg
305 mL^{-1} in the final assay (Figure 1c). This seems to be in the same range or better than that found by Dìaz
306 and Decker (2004) for a bovine casein hydrolysate which inhibited the formation of TBARS in
307 phosphatidylcholine liposomes by 80% at a concentration around 260 μg mL^{-1} (estimated from 3 mM
308 nitrogen converted to protein by the factor 6.34).
309 Similar to the previous antioxidant activities, the capacity of the resulting hydrolysates to inhibit lipid
310 peroxidation in liposomes (Figure 1c) was significantly influenced by the substrate ($p<0.05$). The
311 hydrolysates from permeate showed the best activity, followed by those from retentate and milk. The
312 unexpectedly high activity of the milk sample treated with both enzymes is probably due to an unusual
313 high initial fluorescence of this sample (even when diluted), which lead to a false calculation of the %
314 inhibition. Omitting this value, the pattern for inhibition of lipid oxidation was similar to that seen for
315 iron chelation (Figure 1b). Indeed, there was a very strong correlation between iron chelation capacity
316 and inhibition of lipid peroxidation ($r = 0.88$, $p< 0.0001$), suggesting that the main mechanism for
317 inhibition of lipid peroxidation was actually the capacity to chelate ions, thus to inhibit the Fenton
318 reaction leading to formation of ·OH radicals and generation of secondary lipid oxidation products,

more than to scavenge already formed radicals. This is further supported by a strong negative correlation of ABTS radical scavenging values with values for inhibition of lipid peroxidation (r = -0.91). No significant effect of the different enzyme treatments was seen, which may be due to the relatively low lipid peroxidation inhibitory activity of the samples (maximum 33%) compared to the radical scavenging and iron chelation activities (up to 80%). The most active sample was, like for the iron chelation capacity, the permeate hydrolysed with subtilisin, reaching 33% inhibition at a protein concentration of 25 µg mL^{-1} in the assay.

Given the results for all the activities, the hydrolysates made with subtilisin, i.e. R-S and P-S, presenting the highest antioxidant activity, were expected to contain highly active peptides and were further characterized.

3.2. Fractionation of the R-S and P-S hydrolysates by size-exclusion chromatography and characterization of fractions

The UV traces (214 nm) of R-S and P-S samples when fractionated by SEC are shown in Figure 2. In contrast to what was seen by Espejo-Carpio et al. (2013) monitoring elution at 280 nm, in this study only one big peak with several shoulders eluted from the R-S sample (Figure 2a). The peak maximum was located at an elution volume of 220 mL, corresponding to a molecular weight of 1-2 kDa (Figure 2a). A similar picture was obtained for the P-S sample, although this contained several peak maxima and a few separate peaks at elution volumes above 300 mL (Figure 2b). Figure 2 also shows the conductivity of the samples (light grey lines). While in the R-S sample (Figure 2a) only one conductivity peak was present around 260 mL (SEC fractions 26 and 27, abbreviated SEC-26 and SEC-27), in the permeate sample (Figure 2b), various conductivity peaks were present between 230 and 290

341 mL (SEC-24 to SEC-29), indicative of elution of minerals and other non-peptide compounds occurring
342 in milk, such as lactose and uric acid (Bernacka, 2011).
343 All SEC fractions collected from both samples (R-S and P-S) were tested for radical scavenging
344 capacity and lipid peroxidation inhibitory activity. Unfortunately, the iron chelation capacity assay
345 could not be performed because the buffer used for SEC (0.1 M ammonium bicarbonate) interfered
346 with the assay by blocking the formation of the coloured Fe-Ferrozine complex, even after
347 lyophilisation and redissolution in water.
348
349 *3.2.1. Antioxidant capacity of size-exclusion chromatography fractions from the R-S hydrolysate*
350 The ABTS radical scavenging capacity of the fractions from the R-S hydrolysate seemed to be size
351 dependent, since smaller peptides ($\leq$ 1kDa) had higher activity (Figure 2a, dark grey bars). SEC-24 to
352 SEC-26 showed the highest radical scavenging capacity. Furthermore, considering the apparent lower
353 concentrations (estimated by the absorbance at 214 nm) and yet relatively high activity, also the
354 following fractions (SEC-27 to SEC -29) can be considered active. Since the activity of SEC-26 and
355 SEC-27 are affected by the presence of salts and other compounds from the original sample, SEC-24, -
356 25 and -29 were expected to contain the most interesting peptides with respect to radical scavenging
357 activity, and these were further characterized (see below).
358 Although the R-S sample was not one of the hydrolysates with the highest lipid oxidation inhibitory
359 capacity (Figure 1c), the fractions were tested also for this activity, for the possibility to find
360 particularly active peptides that were masked due to their low concentration in the total hydrolysate. As
361 can be seen in Figure 2a (light grey bars), SEC-14, -15 and -16, corresponding to the size range 6-10
362 kDa, exhibited a quite high inhibition of lipid oxidation. This correlates with partial hydrolysis leading

363 to higher activity (Corrêa et al., 2011). But also SEC-23, -24, -28, and -29 showed a relatively high
364 activity compared to their apparent concentration and should be taken into consideration.
365 Due to the high number of interesting samples, a selection based on their peptide profile was
366 performed. SEC-14, SEC-15 and SEC-16 showed similar peptide profiles (not shown), therefore only
367 SEC-16 was chosen due to its higher peptide concentration. SEC-24 included peptides from both SEC-
368 23 and SEC-25 and was chosen for further fractionation, and finally SEC-28 and SEC-29, due to low
369 protein concentration (214 nm-absorbance), were pooled. Thus, SEC-16, SEC-24 and SEC-28+29 from
370 the R-S hydrolysate were further fractionated in order to identify the most antioxidant peptides
371 contained in these fractions (section 3.3.1).
372
373 *3.2.2. Antioxidant capacity of size-exclusion chromatography fractions from the P-S hydrolysate*
374 None of the fractions from the P-S hydrolysate reached an ABTS radical scavenging activity as high as
375 the R-S fractions (more than 90%), even though the fractions were less diluted in order to have a
376 comparable concentration (according to the $UV_{214\ nm}$ profile). Like for the R-S sample, there was a
377 clear size effect on the ABTS radical scavenging activity of the SEC fractions from the P-S sample
378 (Figure 2b, dark grey bars), with SEC-24 to SEC-26 (0.5 – 1 kDa) showing the highest activity (65 to
379 75%). However, due to turbidity in these fractions, from the presence of non-protein LMW compounds
380 (see decreased conductivity in Fig. 2b) that could not be removed by centrifugation, the activity in
381 these was expected to stem mainly from non-peptidic material and they were not further characterised.
382 SEC-33 to SEC-36 also contained relatively high radical scavenging activity in relation to the low
383 apparent concentration (absorbance at 214 nm). Furthermore, their long retention times suggest that
384 they contain aromatic amino acids (Espejo-Carpio et al., 2013; Specht & Frimmel, 2000), and thus
385 would be interesting with respect to antioxidant activity.

All the SEC fractions from the P-S hydrolysate also showed a certain degree of lipid oxidation inhibition spanning from 30 to 80% with no apparent effect of peptide size (Figure 2b, light grey bars). SEC-27 was the most active fraction and the salts present in this fraction could be precipitated by centrifugation. However, also, SEC-29 to SEC-36 showed a good lipid oxidation inhibitory activity, especially in relation to the low peptide concentration (absorbance at 214 nm). Therefore, SEC-27, SEC-32, SEC-33+34 (pooled together) and SEC-35+36 (pooled together) from the P-S hydrolysate were selected for further characterisation of the active compounds therein (section 3.3.2).

3.3. Isolation and identification of antioxidant peptides

3.3.1. Antioxidant peptides in the R-S hydrolysate

All selected SEC fractions from the R-S hydrolysate (SEC-16, SEC-24 and SEC-28+29), after RP-HPLC fractionation (RPC), showed one or more fractions with good ABTS radical scavenging capacity (Figure 3, dark grey bars). RPC fractionation of SEC-16, which exerted only lipid oxidation inhibitory activity, resulted in mainly one RPC peak (in f21), which showed relatively high radical scavenging activity (40%, Figure 3a), but, surprisingly, it did not exhibit lipid peroxidation inhibitory activity. This could be due to the low concentration of peptides after RPC, even though it was concentrated 12 times before analysis. Higher radical scavenging activity (between 50 and 81%) was obtained from SEC-24 in RPC f22, f23, f24, f30 and f31 (Figure 3b) and from SEC-28+29 in RPC f9 and f22 (Figure 3c).
The highest lipid oxidation inhibitory activity (43-45%) was seen in f21, f25 and f29 from SEC-24. Also the fractions with ABTS radical scavenging activity mentioned above (except RPC f22 from SEC-28+29) were able to inhibit lipid oxidation by 20-40%.

The major peptides present in the active RPC fractions mentioned were identified by LC-MS/MS with the results shown in Table 2. Peptides originating from all 4 caseins were identified. Due to the low masses of most of the peptides, the score given by the software was not high, but the identification could be confirmed by some specific features. For example, the two dipeptides with the same mass I/LY and YI/L could be differentiated by the presence, in the MS/MS spectra, of the immonium ion of (iso)leucine (*m/z* 86) and the *y* ion of tyrosine for the first dipeptide, and by the presence of the immonium ion of tyrosine (*m/z* 136) and the *y* ion of (iso)leucine for the second peptide. The identity of the proline containing peptides could be verified by the high abundance of either the *b*-ion or the *y*-ion resulting from cleavage at the amino side of proline, a known effect in MS/MS fractionation (Contreras, Carron, Montero, Ramos, & Recio, 2009). Finally, the peptides YIPI and YLPL, which have the same mass (505 Da) and both occur in caprine caseins, could be identified due to differences in their retention times. The first peptide contained only isoleucine, an amino acid that usually elutes earlier than leucine in the RP-HPLC (Spicer et al., 2007), and eluted in RPC f30, whereas the latter eluted in RPC f31. The different elution times are also revealed in the LC-MS profiles (Figure 4a and 4b).

To our knowledge, only three of the peptides identified in this study have previously been shown to exert antioxidant activity. The dipeptides IY and LY (from SEC-24 RPC f22) and VYPF (from SEC-24 RPC f30) have been identified as radical scavengers in soybean hydrolysates (Beermann, Euler, Herzberg & Stahl, 2009), and caprine casein hydrolysates (Li et al., 2013), respectively. The tripeptide RYL (identified from RPC f23, Figure 4c) forms part of the antioxidant peptide PYVRYL, derived from ovine casein hydrolysate (Lòpez-Expòsito, Quiròs, Amigo, & Recio, 2007). These peptides are therefore expected to be the primary contributors to the antioxidant activity of SEC-24 RPC f22, f30 and f23, respectively.

Other peptides identified in this study are identical to peptides with other bioactivities. The dipeptides AF (in SEC-24 RPC f21) and VF (the second most abundant peak in SEC-24 RPC f25) and the tripeptide YGL (the main peptide in RPC f25) are known to exert ACE-inhibitory activity (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Matsufuji et al., 1994; Pihlanto-Leppälä, Koskinen, Piilola, Tupasela, & Korhonen, 2000). The peptide SRYPSY (from SEC-24 RPC f22) has been found to act as an opioid antagonist (Yoshikawa, Tani, Ashikaga, Toshimura, & Chiba, 1986) and the dipeptide YL (in SEC-24 RPC f23) was found to be a potent anxiolytic peptide in mice (Kanegawa, Suzuki & Ohinata, 2010).

In addition to the above mentioned known peptides, a number of novel peptides were identified in fractions exerting high ABTS radical scavenging activity. From SEC-24, the peptides FPKY (the most abundant peptide in RPC f23, Figure 4c), AWPQ (the second main peak in RPC f24), YIPI (in RPC f30) and three peptides from f31 (YLPL, VYPF and QPPQP) can be considered as new antioxidant peptides due to the presence of tryptophan, tyrosine and proline, three known antioxidant amino acids (Gülçin, 2007; Ma, Xiong, Zhai, Zhu, & Dziubla, 2010; Pihlanto, 2006). Tyrosine, in particular, was present in all the active fractions (and 56% of identified sequences). The abundant occurrence of this amino acid, confirms its importance for the radical scavenging activity of a peptide.

In the fractions with the best lipid oxidation inhibitory activity (SEC-24 RPC f21, f25, f29), two novel peptides were identified (ENSK and FLL from SEC-24 RPC f21 and f29, respectively). FLL could act as an antioxidant due to the presence of phenylalanine. In fact, phenylalanine was present in all the fractions inhibiting lipid oxidation (and in 50% of the identified peptides). The high inhibitory secondary lipid peroxidation activity of these phenylalanine-containing peptides could be due to the capacity of phenylalanine to interact with the hydroxyl radical (·OH), which is generated by iron-induced oxidation, turning phenylalanine into *p*-, *o*- or *m*-tyrosine (Sun, Kaur, Halliwell, Li, & Bolli,

1993), thus reducing the generation of novel lipid peroxides. Tyrosine in turn can react with lipid (peroxyl or alcoxyl) radicals, inhibiting the formation of secondary lipid oxidation products (Shchepin et al., 2010). This could also explain why RPC f9 from SEC-28+29, which contained mainly tyrosine, also prevented lipid oxidation in liposomes, and other fractions with peptides rich in tyrosine also showed some inhibitory activity in this assay/ inhibition of iron-induced generation of secondary lipid oxidation products.

3.3.2. Antioxidant compounds in the P-S hydrolysate

Even though SEC-27 showed the highest lipid oxidation inhibitory activity among the SEC fractions from the P-S hydrolysate (Figure 2b), after fractionation with RPC, the activity was distributed into several fractions with low activity (< 40%). Slightly higher activity was found in RPC fractions from SEC-32 (f5, 7, 9, and 21; Figure 5a), SEC-33+34 (RPC f3, 4 and 5; Figure 5b) and SEC-35+36 (f4; Figure 5c). The latter fraction was the only one among all fractions analysed from the P-S hydrolysate with a noticeable radical scavenging capacity (more than 50%) and it was characterized by LC-MS/MS analysis together with the fractions from SEC-32 and SEC-33+34.

The antioxidant RPC fractions from SEC-32, however, gave a low signal and no peptides could be identified. Only in RPC f5 from SEC-33+34 and RPC f4 from SEC-35+36 was it possible to identify some peptides. Both fractions contained a peptide with retention time 5 min and the mass 268 Da, which was identified as the dipeptide YS (Figure 6a and 6b). Although a similar peptide (WYS) is known to exert radical scavenging activity (Hernández-Ledesma, Amigo, Recio, & Bartolomé, 2007), it was not possible to consider YS as the main contributor to the antioxidant activity due to occurrence of other compounds in the same fractions. RPC f5 from SEC-33+34 contained a major peak (retention time ≈ 6 min) with the mass 284 Da and high 280 nm absorbance (Figure 6a), which based on its major

476 MS/MS fragment at 152 Da, was identified as guanosine (http://www.massbank.jp). RPC f4 from SEC-
477 35+36 contained another major compound with a mass of 169 Da and high 280 nm absorbance (Figure
478 6b), which based on its MS/MS fragments (Wishart et al., 2009) was identified as uric acid. Since uric
479 acid, which occurs in milk and other foods, is known to exert antioxidant behaviour (Clausen, Huvaere,
480 Skibsted, & Stagsted, 2009; Di Bernardini et al., 2011), it might have contributed to the antioxidant
481 activity of this fraction in particular and of the P-S hydrolysate in general.
482
483 **Conclusions**
484 The results of this paper showed that enzymatic hydrolysis is a way to increase the antioxidant
485 properties of goats' milk protein. Microfiltration of goats' milk before enzymatic treatment of the
486 fractions resulted in hydrolysates with significant differences in antioxidant properties.
487 The retentate fraction resulted in hydrolysates with high radical scavenging activity. In particular, the
488 retentate fraction treated with subtilisin showed a high radical scavenging activity, which was attributed
489 to the presence of short peptides. Known antioxidant peptides and novel casein-derived peptides with a
490 high abundance of tyrosine were identified in the most active fractions of this retentate hydrolysate,
491 confirming that this amino acid plays an important role in the radical scavenging capacity of the
492 peptides. Peptide fractions with a high abundance of phenylalanine also showed good lipid oxidation
493 inhibitory activity, indicating that this amino acid has a strong effect in inhibition of lipid peroxidation.
494 In contrast, the permeate fraction, particularly after treatment with subtilisin, showed the best capacity
495 to inhibit secondary oxidation products in a lipid model system, which was attributed to its capacity to
496 chelate iron ions. LMW substances other than peptides seemed to be mainly responsible for the lipid
497 peroxidation inhibition in this hydrolysate. This information can help to design a process that will
498 increase particular antioxidant characteristics of goats' milk, according to the activity needed.

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Figures

Figure 1. Antioxidant activity of the 9 goat milk protein hydrolysates made from milk (M) and the microfiltration fractions retentate (R) and permeate (P) by hydrolysis with trypsin (T) and/or subtilisin (S). (a) ABTS radical scavenging activity (at 0.01 mg protein mL^{-1}); (b) Iron chelation capacity (at 0.02 mg mL^{-1}); and (c) inhibition of secondary lipid peroxidation products formation (at 0.025 mg mL^{-1}). Values represent means ± std (n=3). Columns with the same letter are not significantly different ($p < 0.05$).

Figure 2. Chromatograms from size-exclusion chromatography of goat milk retentate (a) and permeate (b) hydrolysed by subtilisin for 3h. The dark grey line represents the absorbance at 214 nm, the light grey line is the conductivity. Fractions collected are shown with vertical lines. Dark grey bars represent ABTS radical scavenging activity (%) of each fraction diluted 40 times (for a) or 20 times (for b). Light grey bars represent inhibition of liposomes peroxidation (%) of each fraction diluted 5 times (for both a and b). Vertical lines on activity bars represent standard deviations (n=3). * These two fractions were pooled together.

Figure 3. Reversed-phase chromatography chromatogram (214 nm) of size-exclusion chromatography fractions 16 (a), 24 (b) and 28+29 (c) from the retentate hydrolysed with subtilisin (R-S). Black bars represent ABTS radical scavenging activity (%) and grey bars represent inhibition of lipid peroxidation (%).

Figure 4. Peptide profiles and identity of selected active reversed-phase chromatography (RPC) fractions from the R-S hydrolysate. Absorption was measured at 210 nm (black lines) and 280 nm (grey lines). a) SEC-24 RPC f30; b) SEC-24 RPC f31; c) SEC-24 RPC f23

Figure 5. Reversed-phase chromatography chromatogram (214 nm) of SEC-32 (a), -33+34 (b) and -35+36 (c) from the permeate hydrolysed by subtilisin (P-S). Black bars represent the ABTS radical scavenging activity (%), and grey bars represent inhibition of lipid peroxidation (%).

Figure 6. Peptide profiles and identity of active reversed-phase chromatography fractions from the P-S hydrolysate. Profiles are shown at 210 nm (black lines) and 280 nm (grey lines). a) SEC-33+34 RPC f5; b) SEC-35+36 RPC f4.

TABLES

Table 1. Peptides identified in the highly antioxidant reversed-phase chromatography (RPC) fractions from the retentate hydrolysed with subtilisin (R-S). The hydrolysate was first fractionated by size-exclusion chromatography (SEC), and active SEC fractions were further fractionated by RPC.

Hydrolysate fraction	RPC fractions	Observed mass ($[M+H]^+$)	Theoretical mass	Supposed Sequence	Origin [a]
SEC-24	f21	237.1	236.2	(Q) AF (L)	β-CN 187-188
		478.2	476.5	(E) ENSK (K)	αs_2-CN 134-137
		294.3	293.3	QF\KF	multiple
		295.1	294.3	IY\LY	multiple
		253.1	252.2	SF	multiple
		279.2	278.3	IF\LF	multiple
	f22	295.1	294.3	IY\LY	multiple
		237.1	236.2	(Q) AF (L)	β-CN 187-188
		772.8	771.8	(L)SRYPSY(G)	κ-CN 33-38
		685.7	684.7	(S)RYPSY(G)	κ-CN 34-38
		2313	230.2	VL	multiple
	f23	554.3	553.6	(P)FPKY(P)	β-CN 111-114
		451.5	450.5	RYL	multiple
		295.1	294.3	YL\YI	multiple
		265.1	264.3	VF	multiple
		827.7	826.9	(E)RFFDDK(I)	κ-CN 16-21
		5156	514.6	(E)NLLR(F)	αs1-CN 19-22
	f24	295.1	294.3	YL\YI	multiple
		265.1	264.3	VF	multiple
		501.6	500.3	(F)AWPQ(Y)	αs_2-CN 176-179
		313.3	312.3	(R)FF(D)	κ-CN 17-18
	f25	352.4	351.4	(S)YGL(N)	κ-CN 38-40
		295.1	294.3	YL\YI	multiple
		265.1	264.3	VF	multiple
		423.3	422.4	(S)YQL(N)	αs1-CN 154-156
		641.6	640.7	(D)KIHPF(A)	β-CN 48-52
		391.8	390.4	(N)AGPF(T)	αs_2-CN 117-120
		313.3	312.3	(R)FF(D)	κ-CN 17-18

	f29	393.4	391.5	(A)FLL(Y)	β-CN 188-190
		849.6	---	---	---
		697.8	696.8	(S)LPQNIL(P)	β-CN 70-75
		796.6	795.9	(K)YIPIQY(V)	β-CN 25-30
		756.8	755.8	(Q)WQVLPN(T)	κ-CN 76-81
	f30	525.5	524.6	(L)VYPF(T)	β-CN 59-62
		505.3	504.6	(K)YIPI(Q)	κ-CN 25-28
	f31	505.3	504.6	(Y)YLPL(G)	αs1-CN 166-169
		525.5	524.6	(L)VYPF(T)	β-CN 59-62
		566.7	565.6	(H)QPPQP(L)	β-CN 146-150
SEC-28+29	f9	181.9	181.0	Y	
	f22	205.0	204.0	W	

716 [a] CN, casein.

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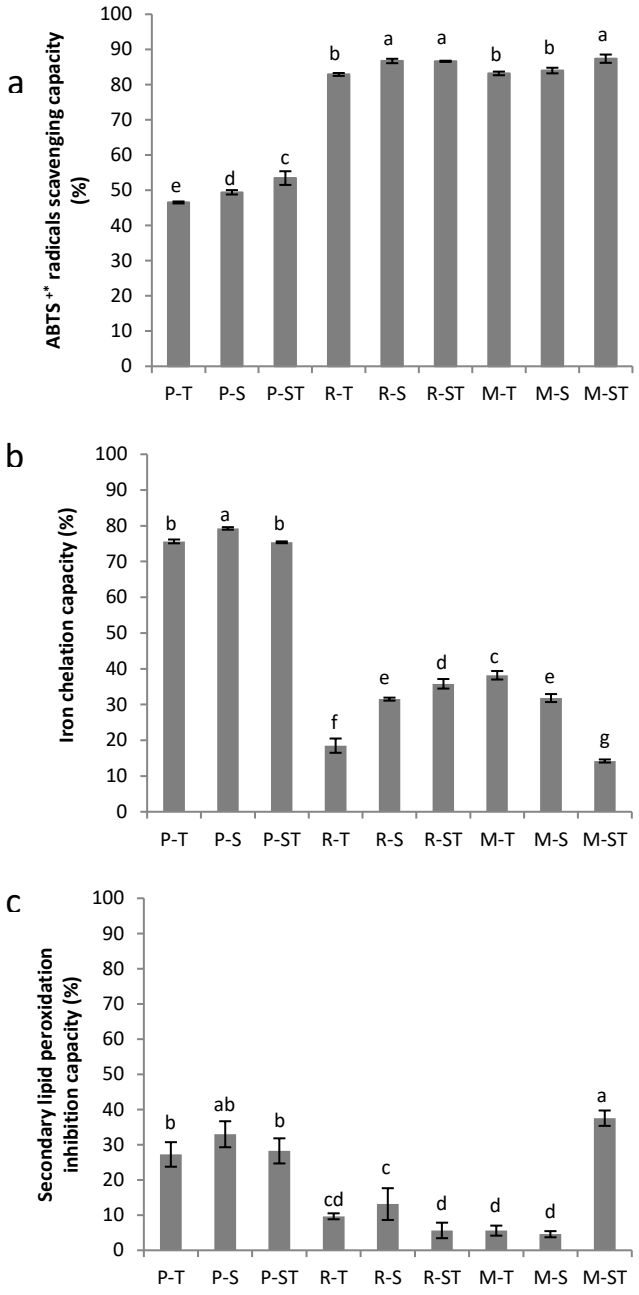
719 Figure 1.



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723 Figure 2.

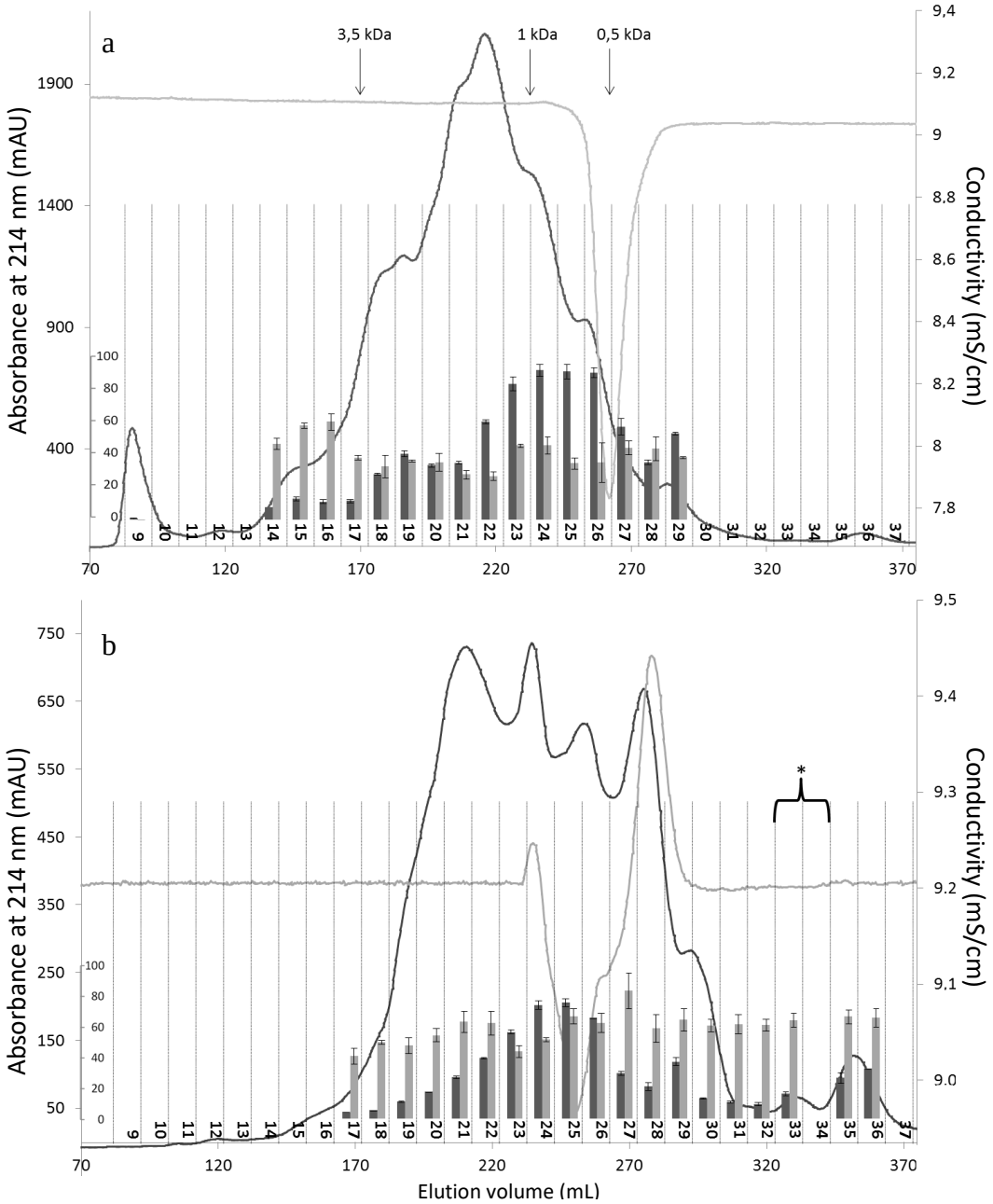

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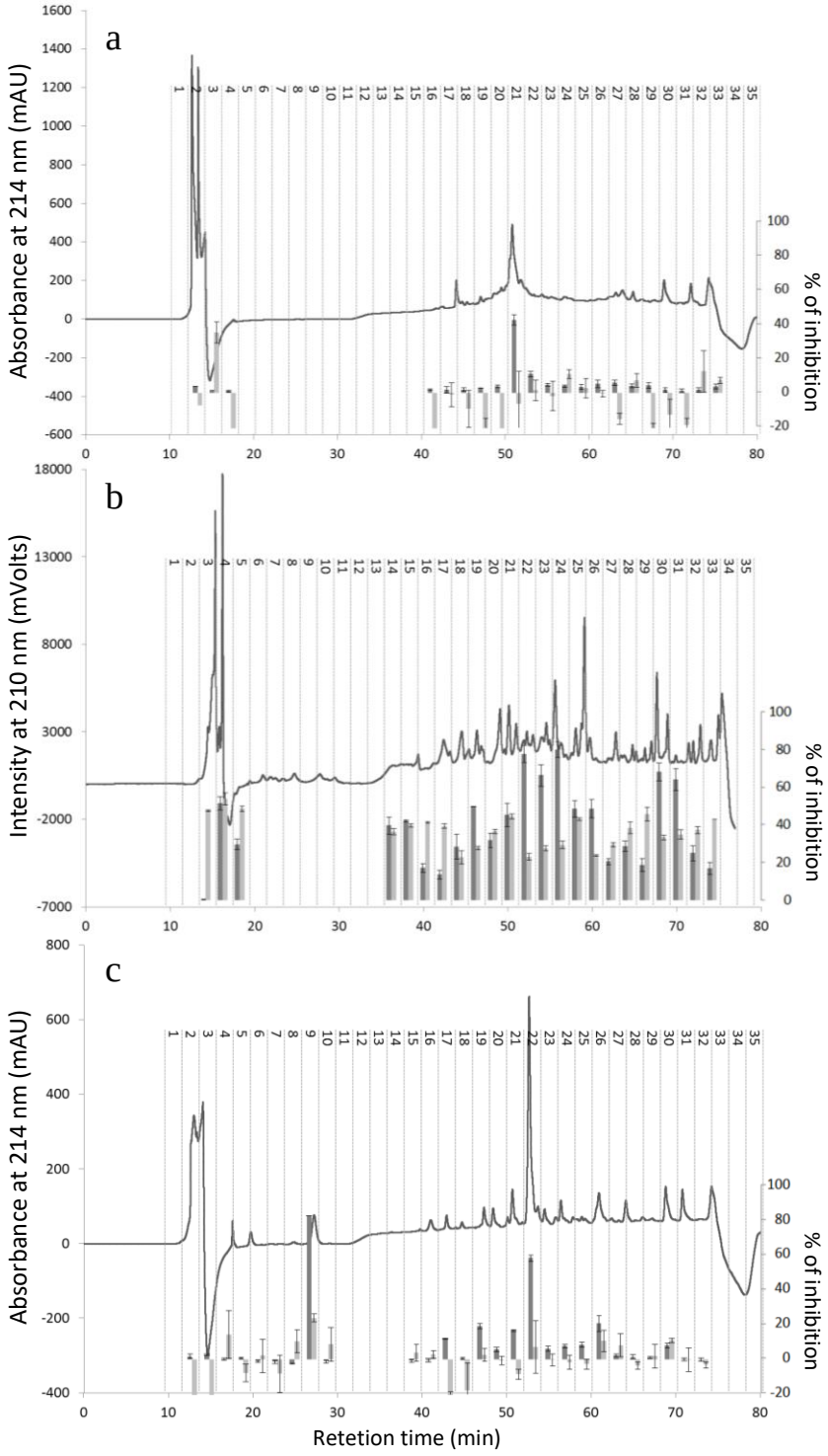
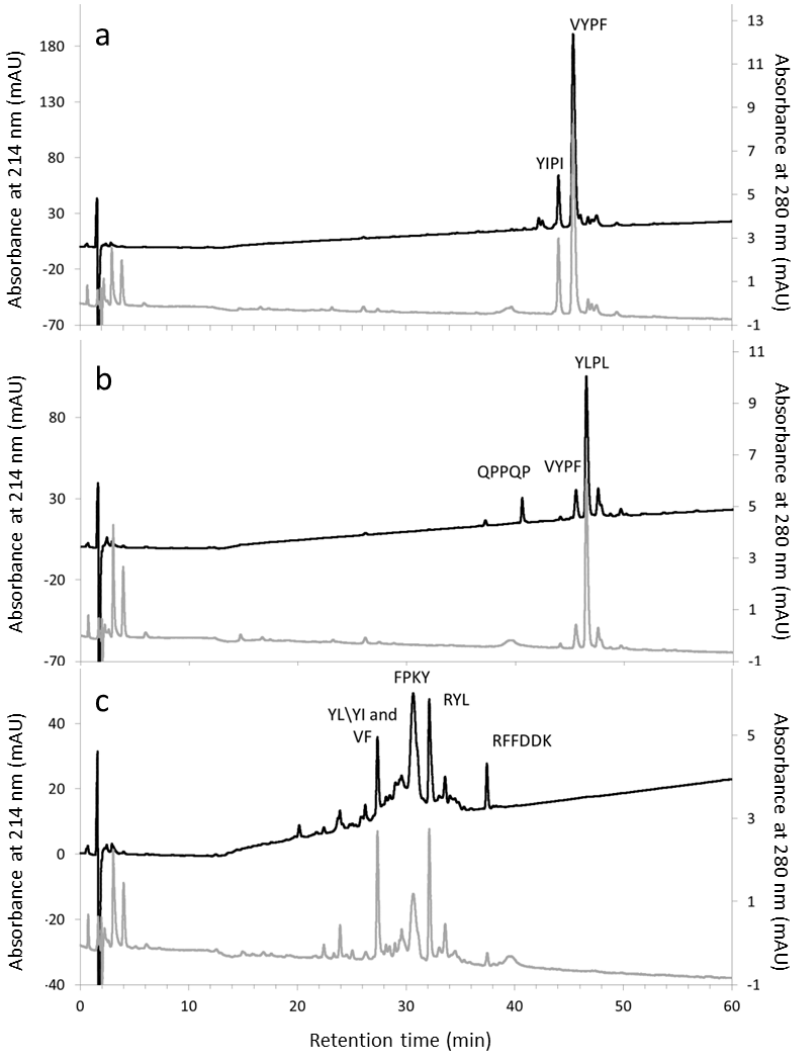
Figure 3.


a
Absorbance at 214 nm (mAU)
% of inhibition
b
Intensity at 210 nm (mVolts)
% of inhibition
c
Absorbance at 214 nm (mAU)
% of inhibition
Retetion time (min)

731 Figure 4.



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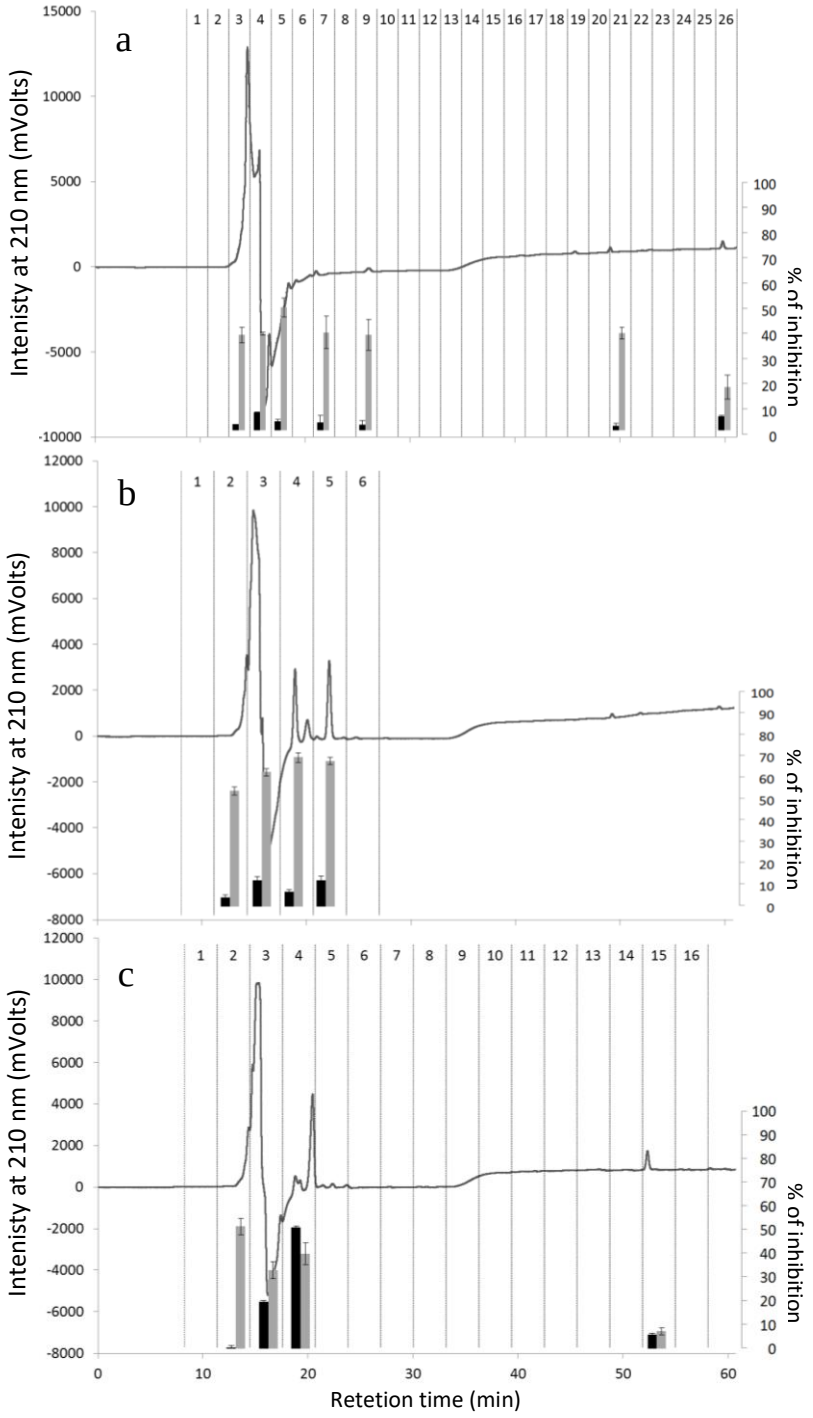
734 Figure 5.



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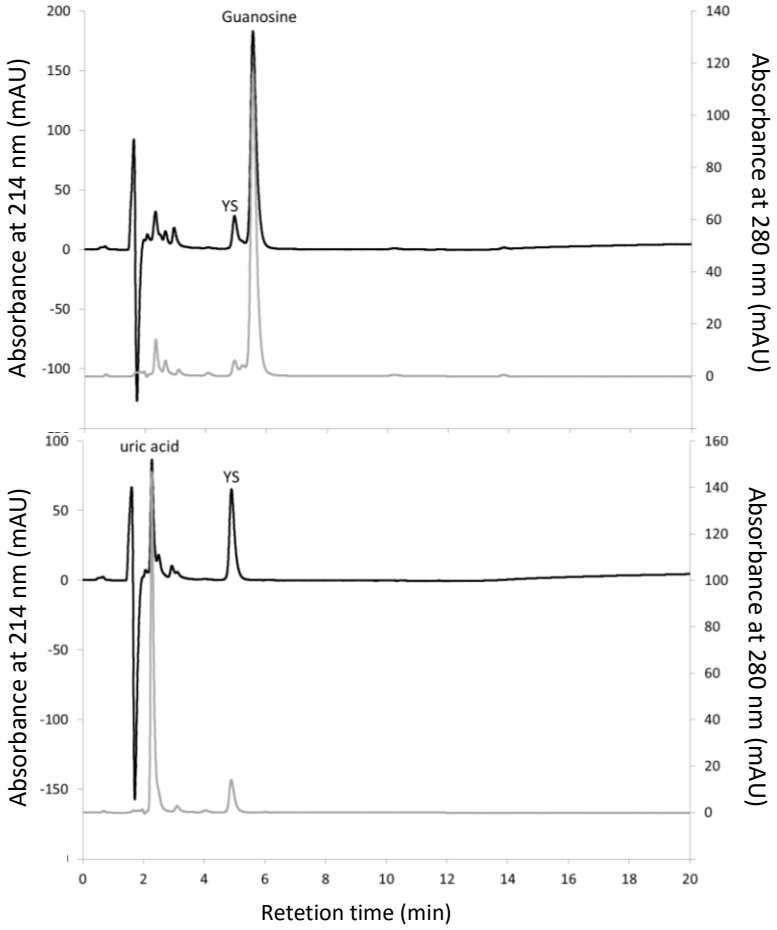
739 Figure 6.



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