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# Antioxidant activity of cod (*Gadus morhua*) protein hydrolysates: Fractionation and characterisation of peptide fractions

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ABSTRACT

This study aimed to characterise peptide fractions (>5 kDa, 3–5 kDa and <3 kDa) with antioxidative activity obtained from a cod protein hydrolysate. The free amino acids in all fractions were dominated by Ala, Gly, Glu and Ser. The total amino acid composition had high proportions of Lys, Ala and Glu. The 3–5 kDa and <3 kDa fractions were further fractionated by size exclusion chromatography. All sub-fractions showed high $Fe^{2+}$ chelating activity. The DPPH radical-scavenging activity of the 3–5 kDa fraction was exerted mainly by one sub-fraction dominated by peptides with masses below 600 Da. The DPPH radical-scavenging activity of the <3 kDa fraction was exerted by sub-fractions with low molecular weight. The highest reducing power was found in a sub-fraction containing peptides rich in Arg, Tyr and Phe. Both free amino acids and low molecular weight peptides thus seemed to contribute to the antioxidative activity of the peptide fractions, and Tyr seemed to play a major role in the antioxidant activity.



## 1. Introduction

The substitution of synthetic antioxidants with natural ones is gaining interest due to consumers' health concerns associated with the use of synthetic food additives. The utilisation of peptides or hydrolysates from dietary proteins for food and/or cosmetic applications has been increasing in recent years because of their low cost, safety, and high nutritional or physiological value (Hattori, Yamaji-Tsukamoto, Kumagai, Feng, & Takahashi, 1998). Fish protein hydrolysates, obtained by controlled enzymatic hydrolysis, are reported to be good in terms of nutritional properties, as they have a balanced amino acid composition and high digestibility (Kristinsson & Rasco, 2000). Also, research on fish protein hydrolysates demonstrated that they contain short-chain peptides with certain biological properties, such as angiotensin converting enzyme (ACE) inhibitory, antioxidative, anticancerous and hypocholesterolaemic activities (Farvin et al., 2014; Kim & Mendis, 2006; Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011; Wergedahl et al., 2004).

In our earlier study on cod protein hydrolysates (Farvin et al., 2014), we investigated the antioxidant activity of crude protein hydrolysates and the fractions thereof obtained by ultrafiltration (UF) (>5 kDa, 3–5 kDa and <3 kDa) both in *in vitro* assays and in 5% fish oil-in-water emulsions. When tested in 5% oil-in-water emulsions, all the fractions, including the crude protein hydrolysate, were able to protect fish oil against iron catalysed oxidation (Farvin et al., 2014). The emulsions containing peptide fractions at a concentration of 4.5 mg/mL showed lower peroxide values (PV) than those with 2 mg/mL, except in the emulsion containing the peptide fraction <3 kDa, where the PV were higher for the higher concentration of peptides than for the lower concentration. In *in vitro* assays we found that the <3 kDa fraction had very good radical-scavenging activity, $Fe^{2+}$chelating activity, and reducing power, while the fraction 3–5 kDa resulted in a higher protection against oxidation in a liposome model system (Farvin et al., 2014). The antioxidant activity of peptides is closely related to their amino acid constituents and their sequences (Chen, Muramoto, Yamaguchi, Fujimoto, & Nokihara, 1998). Therefore, the objectives of the present study were to investigate the amino acid composition of these fractions and to further characterise the peptides in the most active fractions by LC–MS/MS. This was

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done by further fractionation using size-exclusion chromatography (SEC) followed by comparison of the antioxidant activity of each sub-fraction and relating it to the composition of the fractions. Each SEC sub-fraction was characterised with respect to peptide profiles, and the nature of the antioxidant peptides and/or amino acids conferring the highest antioxidant activity. This should make it possible to produce a hydrolysate fraction highly suitable for application in foods as a natural antioxidant.

## 2. Materials and methods

### 2.1. Materials and chemicals

A commercial cod protein hydrolysate, MariPep C, made from North Atlantic cod of consumption quality with commercial proteases, was kindly donated by Marinova (Danish Fish Protein), Hoejmark, Denmark. MariPep C was supplied as a spray-dried product with the following composition: dry matter 97.60%, protein 75.18%, salt 19.92%, and fat <0.30%. The amino acid standards taurine, anserine, and carnosine, as well as L-α-phosphatidyl choline, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), thiobarbituric acid, ascorbic acid, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (Steinheim, Germany). All other chemicals used were analytical grade reagents obtained from Merck (Darmstadt, Germany).

### 2.2. Fractionation of protein hydrolysates

The sequential fractionation of protein hydrolysates was done according to our earlier study (Farvin et al., 2014) by ultrafiltration with 5-kDa and 3-kDa molecular-weight-cut-off (MWCO) membranes (polyethersulfone membrane, Sartorius Stedim Biotech, Aubagne, France), resulting in three fractions; >5 kDa, 3–5 kDa and <3 kDa, which were freeze-dried and kept in air-tight containers at −80 °C until further use.

### 2.3. Analysis of free amino acids, and anserine and carnosine in UF fractions

Analysis of free amino acids was performed using LC–MS, as described by Farvin, Baron, Nielsen, Otte, and Jacobsen (2010), with some modifications. The crude hydrolysate and the three fractions (>5 kDa, 3–5 kDa and <3 kDa) were analysed for the content of free amino acids and the dipeptides anserine and carnosine. Between 25 and 135 mg of sample material were dissolved in 1.0 mL water and the amino acids were derivatised using the EZ:Faast kit from Phenomenex A/S (Allerød, Denmark). Sample volumes of 2 μL were injected into the HPLC mounted with the reversed phase column EZ:Faast AAA-MS (250 × 3.0 mm; PhenomenexA/S Allerød, Denmark) and eluted at 35 °C with a flow rate of 0.5 mL/min. The mobile phase **A** was water containing 10 mM ammonium formate and mobile phase **B** was methanol containing 10 mM ammonium formate. The gradient was obtained by a linear increase from 60% to 83% **B** in 20 min. The column was re-equilibrated to 60% **B** until the end of the run (26 min). The eluate was transferred to the online mass spectrometer (Agilent 1100, Agilent Technology, Waldbronn, Germany) where amino acids were ionised using APCI (atmospheric pressure chemical ionisation) with a chamber temperature of 450 °C, and mass spectra were obtained by positive ion mode scanning from *m/z* 100 to 600. Amino acids were identified by comparison of masses of the obtained peaks with masses in the standard table from the EZ:Faast Kit, except for taurine. Detection of the taurine standard was not possible when using the Faast Kit, but could be detected by direct injection of the standard in the LC–MS. Hence, samples were analysed for taurine by direct injection. The levels of free amino acids, carnosine and anserine were quantified based on peak areas of known concentrations of the standards.

### 2.4. Amino acid composition of peptides in the crude hydrolysate and UF fractions

For determination of total amino acids, 50 mg of the hydrolysate and different fractions were hydrolysed overnight in 2 mL 6 M HCl in sealed ampoules. The samples were appropriately diluted and filtered through a 0.2-μm membrane filter before derivatisation of amino acids and analysis of amino acid content as described by Farvin et al. (2010).

### 2.5. Size exclusion fractionation of the low-molecular-weight UF fractions

The molecular weight distribution of the 3–5 kDa and <3 kDa fractions was analysed by applying 200 μL of the fractions (at 30 mg/mL protein) on a Sephadex G-25 Superfine column (2.6 × 64 cm) and eluting with a buffer containing 50 mM Na-phosphate, 50 mM NaCl, pH 7.2. For large-scale fractionation of these fractions, 5-mL samples containing 200 mg and 150 mg protein, respectively, were applied to the Sephadex G-25 Superfine column. Seven and ten sub-fractions were collected from the 3–5 kDa and <3 kDa fractions, respectively. Peptide content was measured with the Pierce BCA Protein Assay (Thermo Scientific; Rockford, IL) using BSA as standard. The 3–5 kDa sub-fractions were tested directly for antioxidant activity, whereas the <3 kDa sub-fractions, which had very low protein concentrations, were freeze-dried and re-dissolved in deionised water to obtain suitable protein concentrations.

### 2.6. Antioxidant activity of sub-fractions

The antioxidant activity of the sub-fractions was determined by three *in vitro* assays testing the DPPH radical-scavenging activity, reducing power, and $Fe^{2+}$ chelating activity, as described by Farvin et al. (2014). The sample concentration used for the assay was 0.5 mg/mL.

### 2.7. Characterisation and sequencing of peptides by LC–MS/MS

LC–MS/MS analyses were performed as described by Otte, Shalaby, Zakora, Pripp, and El-Shabrawy (2007) using an Agilent 1100 MSD Trap with Chemstation for LC 3D systems Rev. B.01.03 (Agilent Technologies 2001–2005) and Trap Control software version 5.3 (Bruker Daltonics GmbH 1998–2005). Aliquots (50 μL) of fractions were injected and eluted in 75 min using a gradient with 100% **A** (0.1% TFA in water) for 5 min followed by a linear increase to 60% **B** (0.1% TFA in 90% acetonitrile) over the next 70 min. Additional analyses of selected samples were performed with gradients of 0–40% **B** or 0–25% **B** in 75 min. Online MS/MS spectra were recorded using the range *m/z* 150–2000 and the target mass *m/z* 1521; in some cases another analysis was made with target mass *m/z* 322 or 622. AutoMS(2) spectra were recorded from two precursor ions with the Smart Parameter setting on.

Average mass spectra from entire chromatograms were made in order to detect dominating masses in interesting samples; these were then searched in the chromatogram in order to find their retention times. Mascot generic files generated from the whole chromatograms (for all AutoMS(n) compounds detected from 0 to 75 min) of interesting fractions were used to search Mascot for protein identification. The search was performed with the Swissprot or NCBInr database, no cleave, 1–3 positive charges, ESI-Trap and 1 Da tolerance. These spectra were also subjected to *de*

*novo* sequencing using PEAKS Online 2.0. Furthermore, individual MS/MS spectra obtained with the *m/z* 1521 target mass were analysed with BioTools software (Bruker Daltonics Bio Tools 3.0, Copyright® 1999–2004 Bruker Daltonik GmbH). Initially, to find sequences that were suggested to occur in the peptide, the RapiDe-Novo sequencing feature was used with hydroxyproline as an optional proline modification. Secondly, the Sequence Editor tool was used to match individual MS/MS spectra to theoretical MS/MS spectra of peptides with the same masses that occur in known cod protein sequences and collagen sequences from other fish species (since sequences from cod collagen were not present in UniProtKB). The sequences used were the following: beta-actin (B3GDZ9), beta-actin (G2PDJO), actin (Q91037), fast skeletal myosin heavy chain (Q8JIV5), myosin heavy chain fragment (Q98SS9), myosin heavy chain large fragment (Q8JIV4), myosin heavy chain (Q98954), fast skeletal muscle troponin I (Q8QG69), fast skeletal muscle troponin T (Q8JJ07), and Japanese ricefish α1a type II collagen (D6RUX4), Japanese ricefish α3 type I collagen (A8QX86), rainbow trout α1 type I collagen (O93485), rainbow trout α2 type I collagen (O93484), rainbow trout α3 type I collagen (O93486). Unfortunately, these sequences did not show the position of the hydroxyproline residues, so it was not possible to search for sequences containing hydroxyproline in these proteins.

### 2.8. Statistical analyses

The data obtained for *in vitro* assays were analysed by one-way analysis of variance (ANOVA). The statistical comparisons among the samples were performed with a Bonferroni multiple comparison test using the statistical package program Graphpad Prism 4 (Graphpad Software Inc., San Diego, CA). A $p$-value <0.05 was considered as being statistically significant.

## 3. Results and discussions

### 3.1. Characterisation of UF-fractions

#### 3.1.1. Free amino acids, anserine and carnosine

The levels and composition of free amino acids and naturally-occurring dipeptides may give further detailed information regarding the antioxidant activities of protein hydrolysates. The contents of free amino acids in the crude cod hydrolysate and the different UF-fractions are shown in Table 1. In general, the relative content of the different amino acids (expressed as % of total free amino acids) was similar in all fractions. The predominant amino acids in all fractions were Ala, Gly, Glu and Ser, which constituted approximately 16–18%, 16–17%, 12–15% and 10–11% of the total free amino acids content, respectively. This is in line with the findings of Limin, Feng, and Jing (2006) showing that Glu was one of the most abundant free amino acids in muscles of marine fishes. As expected, the <3 kDa fraction had the highest total content of free amino acids among the UF-fractions, whereas the >5 kDa fraction had the lowest content. When comparing the absolute concentrations of amino acid in the different fractions, the <3 kDa fraction had a higher content of 13 out of the 19 amino acids than the two other fractions. This could be explained by its higher total content of free amino acids. Interestingly, the 3–5 kDa fraction had similar or higher concentrations of Ala, Arg, Ser, and Gly than the <3 kDa fraction despite its lower total content of free amino acids. The >5 kDa fraction did not contain a higher concentration of any amino acid than the two other fractions.

Neither the dipeptide carnosine (β-alanyl-histidine) nor taurine were detected in any of the samples, while anserine (N-methylcarnosine) was found in all samples. Taurine is present in fish muscle in relatively high concentrations (Shiau, Pong, Chiou, & Tin, 2001). Therefore, it was somewhat surprising that it was not detected. Perhaps taurine, which can bind to other biomolecules due to its zwitterionic properties (Wright, Tallan, & Lin, 1986), was bound to larger peptides and was not extracted together with free amino acids.

**Table 1**
Free amino acid content (% of total free amino acids) of the crude cod hydrolysate and the UF-fractions from this.

| Amino acid | Fractions | | | |
|---|---|---|---|---|
| | Crude | >5 kDa | 3–5 kDa | <3 kDa |
| Lys | 7.3 | 8.0 | 8.1 | 8.7 |
| Ala | 16.6 | 17.4 | 17.7 | 15.8 |
| Arg | 5.0 | 5.4 | 5.3 | 4.8 |
| Cys | 0.2 | 0.6 | 0.5 | 0.8 |
| Leu | 5.6 | 5.7 | 5.3 | 5.8 |
| Met | 2.3 | 2.0 | 2.1 | 2.7 |
| Phe | 2.3 | 2.6 | 2.3 | 2.7 |
| Pro | 3.9 | 3.5 | 3.7 | 4.1 |
| Thr | 4.0 | 3.5 | 4.2 | 3.8 |
| Tyr | 2.6 | 2.6 | 2.3 | 3.2 |
| Asp | 3.5 | 2.6 | 2.5 | 2.8 |
| Ser | 10.7 | 11.1 | 11.7 | 9.7 |
| Glu | 14.8 | 12.5 | 11.7 | 12.4 |
| Hyp | 0.7 | 0.7 | 0.7 | 0.6 |
| Val | 2.3 | 2.2 | 2.1 | 2.5 |
| His | 0.9 | 1.0 | 1.0 | 1.4 |
| Trp | 0.3 | 0.5 | 0.4 | 0.4 |
| Ile | 1.2 | 1.2 | 1.2 | 1.4 |
| Gly | 16.0 | 16.7 | 17.1 | 16.5 |
| Taurine | – | – | – | – |
| Total | 100.0 | 100.0 | 100.0 | 100.0 |
| Carnosine[1] | – | – | – | – |
| Anserine[2] | 49.4 | 20.7 | 31.5 | 31.6 |

[1] β-alanyl histidine as mg/100 g.
[2] β-alanyl-*N*-methylhistidine as mg/100 g.

Several amino acids have been reported to show antioxidant activity (Karel, Tannenbaum, Wallace, & Maloney, 1966; Marcuse, 1960, 1962). His, Thr, Lys, and Met were reported to have antioxidant activity in sunflower oil emulsions (Riison, Sims, & Fioriti, 1980). Proline has been reported to have antioxidative capacity equivalent to that of butylated hydroxyanisole (BHA) in sardine oil (Revankar, 1974). Good antioxidant activity has also been reported for His and Trp in both linoleic acid and methyl linoleate systems (Marcuse, 1962). His exhibits strong radical-scavenging activity, due to the presence of the imidazole ring (Yong & Karel, 1978). The higher radical-scavenging activity of the <3 kDa fraction compared to the other fractions may thus be due the presence of higher absolute amounts of His, Lys and Met than in the other fractions (Table 1). However, His also has a strong tendency to invert to a pro-oxidative compound at higher concentrations (Marcuse, 1962). Moreover, the antioxidant role of His in different lipid oxidation systems is not consistent. Erickson, Hultin, and Borhan (1990) found that His stimulated the oxidation of flounder sarcoplasmic reticulum, while Karel et al. (1966) reported that His inhibited lipid oxidation in a freeze-dried model system. The ability of His to accelerate Fe-dependent peroxidation has also been reported (Din, Schaur, & Schauenstein, 1988). This is in agreement with the poor performance of the <3 kDa fraction compared to the other fractions in 5% oil-in-water emulsions when oxidation is induced by iron (Farvin et al., 2014). Park, Nakamura, Sato, and Matsumura (2012) reported that Arg, Trp, Met, Met + Arg, and Met + Trp have pro-oxidative effects in emulsion systems, while Met + His has antioxidative effects. So the antioxidative or pro-oxidative effect of amino acids is not solely determined by the individual amino acids but also by the combination of these.

The dipeptides, anserine and carnosine, play a number of physiological roles, such as control of enzyme activities, neurotransmitter function and inhibition of oxidative reactions (Quinn, Boldyrevt, & Formazuyk, 1992). Wu, Shiau, Chen, and Chiou (2003) also

demonstrated that carnosine and anserine were antioxidants preventing lipid peroxidation in a linoleic acid system and also possessing DPPH radical-scavenging activity, reducing power, and ability to chelate copper and iron. Since carnosine was not detected in the present study, anserine, which was detected in all fractions, most likely contributed to the antioxidant activity of all fractions, including the crude protein hydrolysates, in the 5% oil-in-water emulsion (Farvin et al., 2014)

### 3.1.2. Amino acid composition

The amino acid compositions of the peptides in the different UF-fractions are given in Table 2. All the samples had high proportions of Gly, Glu, Lys, and. Ala. This is in line with the observations of Jensen, Larsen, Rustard, and Eilertsen (2013), who reported that the most abundant amino acid in cod muscle is Glu and that other abundant amino acids include Asp, Ala, Leu and Lys. The crude hydrolysate and the >5 kDa, and 3–5 kDa fractions had higher proportions of Gly, Pro (non-polar amino acids) and Asp (polar amino acid) than the <3 kDa fraction. The <3 kDa fractions in turn had higher proportions of the polar amino acids His, Ala and Met, and the non-polar amino acids Leu and Ile than the other fractions. Some amino acids reported to exert antioxidant effects, such as Lys, and Tyr (Marcuse, 1962), were abundant in all the peptide fractions. Since the levels of His, Ala, Leu, and Lys were higher in the <3 kDa fraction (Table 2), this fraction contained peptides including these amino acids, which probably contributed to its antioxidant activity. The antioxidant activity of peptides not only depends on the amino acid composition but also on the sequence and configuration of the peptides (Chen et al., 1998). Additional compositional and structural information of the peptides in these UF-fractions was obtained, after size-exclusion fractionation, from determination of their peptide profiles and amino acid sequences.

## 3.2. Characterisation of sub-fractions obtained by size-exclusion chromatography

In order to find out what causes the unique *in vitro* antioxidant activities of the 3–5 kDa and <3 kDa fractions, we further fractionated these fractions by size exclusion chromatography and collected 7 sub-fractions (F) from the 3–5 kDa fraction and 10 sub-fractions (F') from the <3 kDa fraction, as shown in Figs. 1a and 2a, respectively.

### 3.2.1. Antioxidant activity of sub-fractions

The antioxidant activity of the sub-fractions, i.e. the DPPH radical scavenging capacity, $Fe^{2+}$-chelating activity and reducing power are shown in Fig. 1(b–d) and Fig. 2(b–d). The $Fe^{2+}$-chelating activity and reducing power were not significantly different ($p > 0.05$) between the sub-fractions obtained from the 3–5 kDa fraction (Fig. 1b, c) indicating that peptides of varying sizes have the ability to bind iron. However, the DPPH radical-scavenging activity was significantly ($p < 0.05$) higher for sub-fraction F8 and significantly ($p < 0.05$) lower for sub-fraction F2, when compared to the other sub-fractions (Fig. 1d). In the case of the <3 kDa fraction, more than 95% chelating activity was observed in all sub-fractions, and there was no significant difference ($p > 0.05$) between sub-fractions (Fig. 2b). The DPPH radical-scavenging capacity and the reducing power showed some differences among the sub-fractions (Fig. 2c and d) with sub-fractions F6′ to F11′ showing higher DPPH radical-scavenging activity than F2′ to F5′ (Fig. 2d). Furthermore, sub-fraction F9′ showed significantly ($p < 0.05$) higher reducing power than the other sub-fractions (Fig. 2c).

**Table 2**
Amino acid composition (expressed as % of total amino acids) of crude cod hydrolysate and different UF-fractions from this.

| Amino acid | Fractions | | | |
|---|---|---|---|---|
| | Crude | >5 kDa | 3–5 kDa | <3 kDa |
| Lys | 8.4 | 8.5 | 8.7 | 8.8 |
| Ala | 7.7 | 7.8 | 8.2 | 9.2 |
| Arg | 4.9 | 6.9 | 6.8 | 4.7 |
| Cys | 0.3 | 0.2 | 0.2 | 0.1 |
| Leu | 6.9. | 5.8 | 6.0 | 8.1 |
| Met | 1.8 | 1.1 | 2.5 | 3.5 |
| Phe | 3.5 | 2.7 | 2.6 | 3.3 |
| Pro | 6.6 | 7.9 | 6.26 | 3. 7 |
| Thr | 2.2 | 2.1 | 2.2 | 2.0 |
| Tyr | 3.2 | 2.2 | 2.5 | 2.7 |
| Asp | 6.3 | 7.5 | 7.9 | 3.8 |
| Ser | 2.7 | 2.5 | 2.6 | 2.4 |
| Glu | 14.5 | 15.1 | 16.9 | 11.3 |
| Hyp | 1.1 | 1.8 | 1.1 | 0.4 |
| Val | 4.4 | 4.1 | 4.4 | 4.8 |
| His | 5.1 | 3.2 | 3.5 | 15.2 |
| Trp | – | – | – | – |
| Ile | 6.9 | 5.8 | 6.0 | 8.1 |
| Gly | 13.4 | 14.7 | 11.8 | 8.0 |
| Total | 100 | 100 | 100 | 100 |

Total amino acids includes free amino acids.

### 3.2.2. LC–MS Characterisation of sub-fractions

All gel filtration sub-fractions from the 3–5 kDa fraction contained a multitude of closely eluting peptides (with varying intensity according to the gel filtration profile shown in Fig. 1a), as can be seen from the peptide profiles shown in Fig 1e. The most interesting sub-fraction from the 3–5 kDa fraction was F8, which had a high DPPH radical-scavenging activity and the highest reducing power (Fig. 1c and d). We examined in more detail the profile of this sub-fraction together with the profile of F4 (Fig. 1f), which contained the highest concentration of peptides but had a lower antioxidant activity (Fig 1). The reason for the higher DPPH radical-scavenging activity of F8 in comparison to F4 could either be due to its higher content of hydrophilic amino acids and dipeptides (high peak with elution at ~2 min) or to the presence, among the multitude of peptides with low abundance (eluting between 10 and 70 min), of one or more very potent radical-scavenging peptides. An initial attempt to identify peptides using Mascot search for all detected AutoMS(n) compounds in both fractions did not result in any hits from fish or from major proteins expected in the fish samples (actin, myosin, collagen troponin etc.), so this did not lead to reliable identification of any of the peptides present in F4 and F8. In previous studies, low-molecular-weight compounds with DPPH radical-scavenging activity extracted from meat products, such as free amino acids and dipeptides, were found to elute near the void volume in RP-HPLC (Broncano, Otte, Peron, Parra, & Timon, 2012). Accordingly, the high peak at around 1.5 min (Fig. 1f) was higher in the profile of F8 (1.65 *versus* 1.35 AU for F4) and the shape was different, indicating a different composition of hydrophilic compounds in these samples. Comparison of the average mass spectra of material eluting from 1 to 5 min for F4 and F8 (data not shown) showed that some new compounds were present in F8, i.e. masses 175.1, 209.0, 246.0, 260.1, 274.1, 345.3, 361.1, 375.2, and 402.2. These masses might represent di- and oligopeptides and fragments thereof created during ionisation. The mass 175 could stem from free Arg or *y1* ions from peptides with C-terminal Arg. Neither masses for anserine nor other free amino acids present in the 3–5 kDa fraction (Table 1) were found in the average mass spectrum of the early eluting compounds in F8. Some di-, tri- and tetra-peptides that could fit with the other masses detected and their MS-MS spectra are given in Table 3.

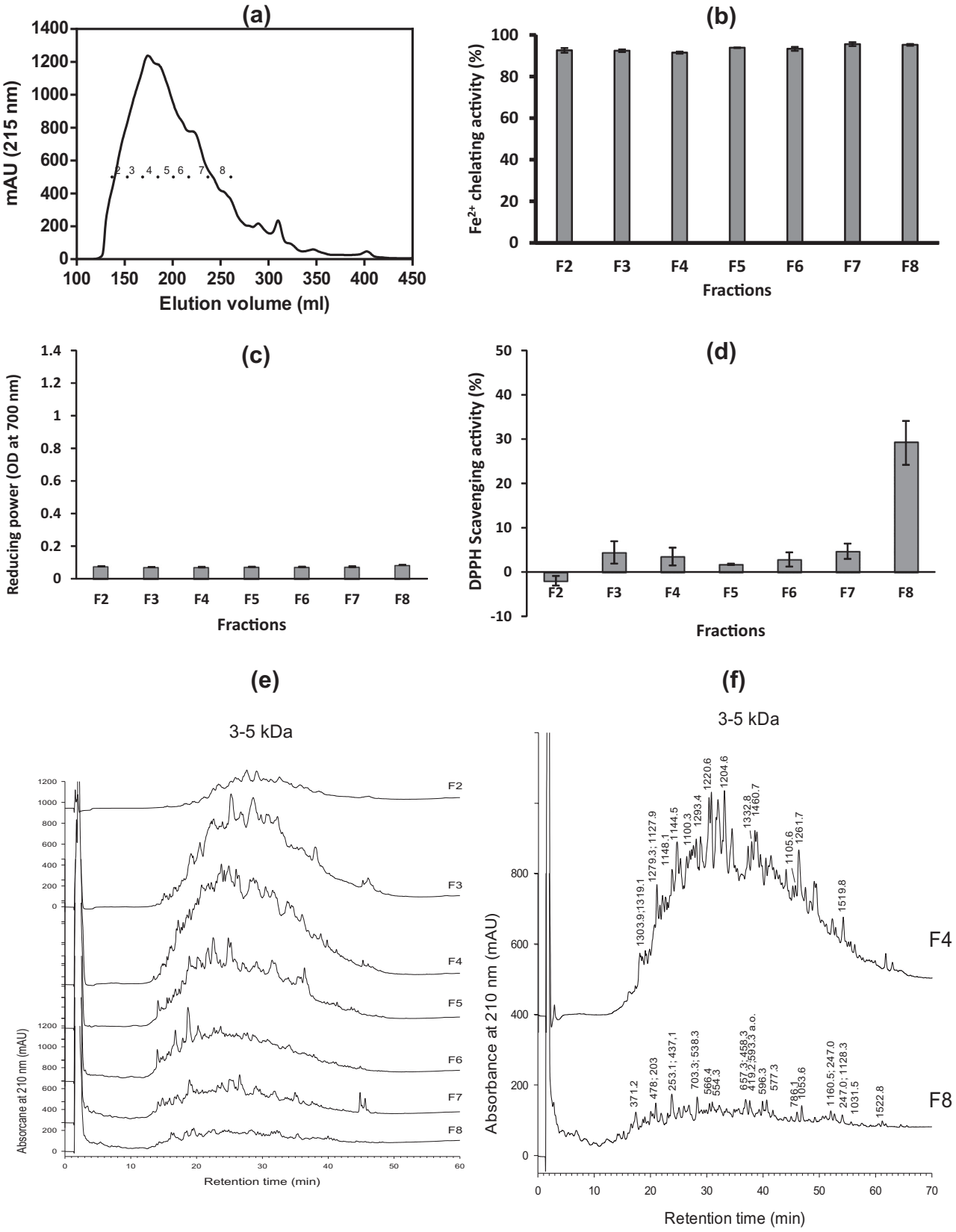


**Fig. 1.** Characteristics of the 3–5 kDa sub-fractions obtained from Sephadex G-25 chromatography with 50 mM Na-phosphate, 50 mM NaCl, pH 7.2 as eluent. (a) size-distribution profile with indication of the seven fractions collected. *In vitro* antioxidant activity measured as (b) Iron chelating activity (c) DPPH radical scavenging activity (d) Reducing power. Results are the mean values of triplicate determinations on the sample ± standard deviations. Peptide profiles using reversed-phase HPLC (e), and details of these for most active fractions (f).

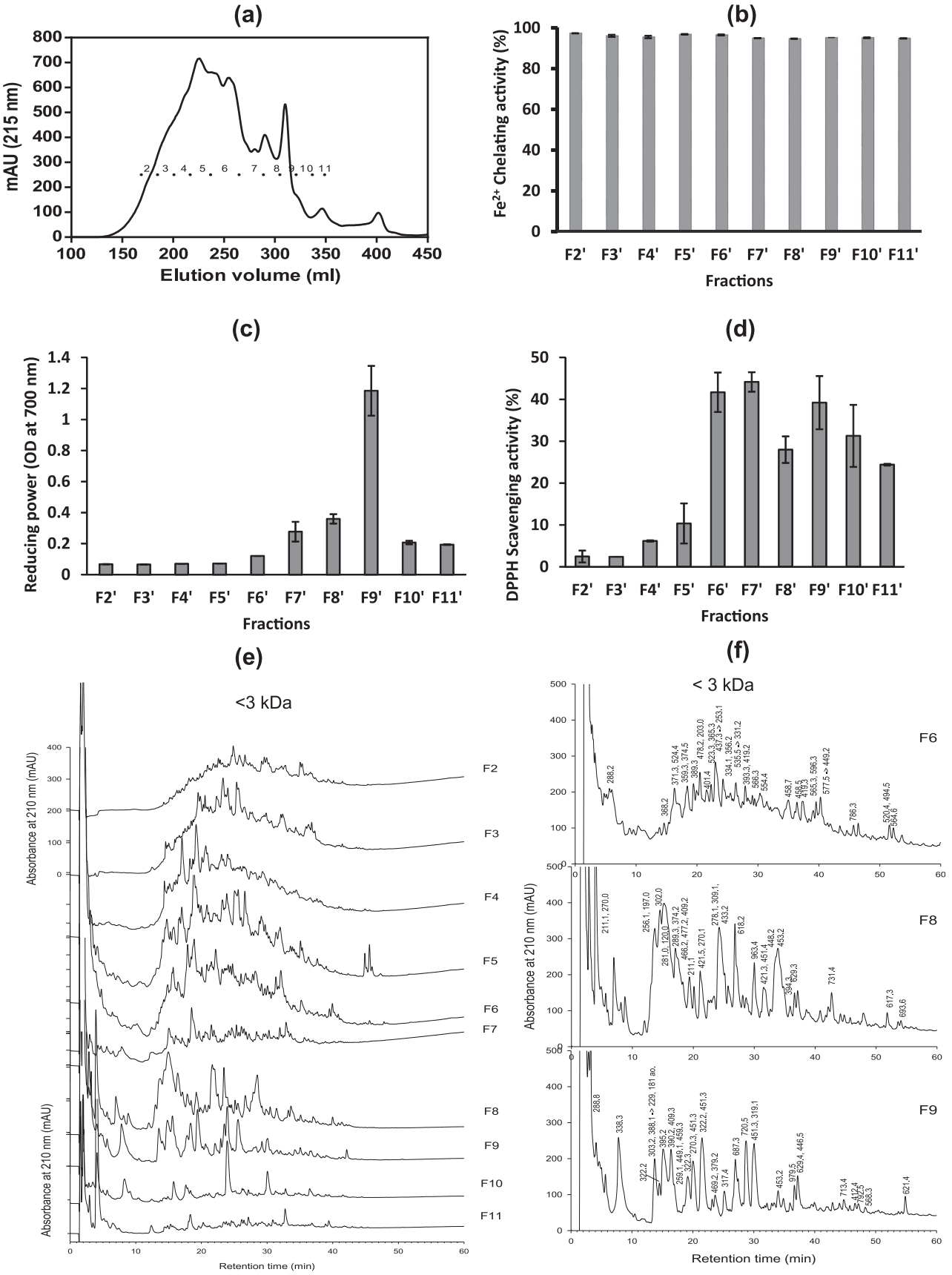


**Fig. 2.** Characteristics of the <3 kDa sub-fractions obtained from Sephadex G-25 chromatography with 50 mM Na-phosphate, 50 mM NaCl, pH 7.2 as eluent. (a) size-distribution profile with indication of the ten fractions collected. *In vitro* antioxidant activity measured as (b) Iron chelating activity (c) DPPH radical scavenging activity (d) Reducing power. Results are the mean values of triplicate determinations on the sample ± standard deviations. Peptide profiles using reversed-phase HPLC (e), and details of these for most active fractions (f).

**Table 3**
Major masses found in the sub fraction F8 from the 3–5 kDa UF-sample (Fig. 1a) and tentative peptide assignment. Masses that were dominating in both the average mass spectrum and in the highest peaks in the peptide profile (Fig. 1f) are highlighted.

| Mass obs. $[M+H]^+$ | Rt[1] (min) | MS(2) fragments[2] | Sequence present[3] and possible peptide with score[4] and source[5] | Mass theor. $[M+H]^+$ |
|---|---|---|---|---|
| **274.1** | 3.3 | 257.1, 175.0 a.o. | VGV (15) collagen, myosin, troponin | 274.2 |
| **260.1** | 3.4–3.9 | 242.1, 147.0, 129.1, 84.2 a.o. | LGA (13) or **P**GA[6] (13) collagen | 260.2<br>260.2 |
| **402.2** | 3.9 | 385.2, 256.1, 211.1 a.o. | -VAG; KVVG (25) or<br>AGVVG (23) collagen<br>or KVR (19) | 402.3<br>402.2<br>402.3 |
| **345.3** | 4.1–4.7 | 328.2, 310.2, 175.0, 158.1, a.o. | **P**GGV (24), I/LGGV[8] (24)<br>or IGR (16) | all<br>345.2 |
| **361.1** | 4.7 | 4.0: 344.1, 326.2, 175.0 | EGR (22) $\alpha_2$-collagen | 361.2 |
| **375.2** | 4.7 | 4.7: 358.3, 331.2, 296.1 256.1, 175.1 | -AGV; EAGV (41) or EAR (30) | both<br>375.2 |
| **524.3** | 17.5 | Many,e.g. 507.3 175.0 | -DGV;YADGV (54)<br>or VGPPGP (45)collagen | 524.2<br>523.3 |
| **371.2** | 17.5 | 353.2, 197.0, 147.0, 130.0 | -TGPP (10) collagen | 371.2 |
| **478.1** | 21.1 | 460.1, 276.1, 203.0 | -GPS;FKPS or KFPS (11) | 478.2 |
| **437.1** | 23.9 | 420.3, 253.1, 235.0, 156.0 | -APH; ALPH actin (33) | 437.2 |
| 253.1 | | (from 437)235, 156 | PH or HP | 253.1 |
| **538.3** | 28.2<br>29.0 | 520.2, 401.3 a.m.o. | -ATL/I, or –CCN; HPATL/I (36) | 538.3 |
| **703.3** | 28.4 | 685.3, 522.2, 504.1 a.o. | -DEY; GGYDEY (58) collagen | 703.3 |
| **566.4** | 30.2 | 548.3, 530.3, 447.3a.o. | -YG or -TTT; YGGGNV (93)<br>or YNGNV (51) | 566.3<br>566.3 |
| 554.3 | 30.9 | 536.5, 435.3, 322.1, 304.2, 209.1 | -AI/LT, -APT; HAI/LI/LT (43) | 554.3 |
| **668.3** | 36.7 | 651.2, 537.3, 520.3,a.o. | -HSHG; HSVRG**P** (103) or<br>PMGPPGL (56) collagen | 668.3<br>668.3 |
| 458.3 | 36.9 | 441.2, 345.3, 197.0 a.o. | -GGVN, -TI/LI/L; PTLL actin,<br>or I/LTI/LI/L (all 18) | 459.3 |
| **657.3** | 37.0 | 639.2, 253.0, 235.1 a.o. | -I/LSF and GYA-; GYALPH(66) β-actin | 657.3 |
| 593.3 | 37.8 | 576.2, 391.2, 244.1a.o. | -SDK or SDQ[7]; MGGSNK (43) n.i.[9] | 593.3 |
| **419.2** | 38.0 | 320.1, 263.1,a.o. | VGDE(19) actin | 419.2 |
| **263.1** | | (from 419) 215.2, 104.0, 87.1 | | |
| **596.3** | 40.0 | 578.3, 449.2, 336.1 a.o. | -GEM or -GME;FMGNAG (82) or MFG**P**E (66)or YDG**P**E (62)<br>or FIGME(58) actin, n.a.[10] | 596.3<br>596.2<br>596.3 |
| **565.3** | 40.1 | 547.2, 400.2, 382.1,a.o. | -GVF; WGGVF(90),<br>or GLGGNF(37)collagen | 565.3<br>564.3 |
| **577.3** | 40.7 | 559.3, 449.2, 318.2 | -GFP -GEM -GFI/L; MGEGAL/I(40) or O**P**GFI(19)<br>or EDKAD (20) troponin I n.a. | 577.3<br>577.3<br>577.2 |
| **786.1** | 46.1 | 769.3, 655.3 a.m.o. | TFYNEL (87) actin | 786.4 |
| 1053.6 | 47.0 | 1035.6, 922.4, 760.4 a.m.o | MYPGIADRM (160) actin | 1053.5 |
| 1160.5 | 51.9 | 1143.6, 900.9, 669.3a.m.o | -HVGF; MTKHDGMVAGN (477) n.a. | 1160.6 |
| **564.3** | 52.7 | No fragments | | |
| **1031.5** | 54.2 | 903.4, 845.4,826.7, 698.1, 680.0a.o. | -VEDA; GAGVQ/KEGVEDA (307) or similar | 1031.5 |
| **502.8** | 60.6 | 474.3, 389.1a.o. | -AK/QN; **P**GAK/QN[7] (11)<br>or **P**GAAGD (13) | 502.3<br>503.2 |
| 1522.8 | 61.3 | 1503.9, 1329.7, 921.5, a.m.o. | -NKE; **M**E**P**DMGVEPDNKE (1585) a.o., n.a. | 1522.6 |

a.o = and others.
a.m.o = and many others.
[1] Retention time.
[2] Fragments obtained by collision induced dissociation, the major one is highlighted.
[3] Amino acid sequence suggested to be present in the peptide according to *de novo* analysis using BioTools software.
[4] Score is given in parentheses and is the value given by BioTools software for the match of fragments to this sequence.
[5] The protein in which the sequence occurs is given if a reasonable match is found.
[6] Modified residues are highlighted (**P** = hydroxylated Pro, **M** = oxidised Met).
[7] The mass for residues Lys and Gln are both 128 and cannot be distinguished.
[8] The mass for residues Ile and Leu are both 113 and cannot be distinguished.
[9] n.i. = not identified.
[10] n.a. = not all high mass peaks are assigned.

Due to the many possibilities, these small peptides could not be unambiguously identified. This would demand other methods, e.g., purification of each peptide and subsequent sequencing. The presence of Glu, Gly, Lys, Ala and Arg in the tentatively identified oligopeptides in F8 of the 3–5 kDa fraction (Table 3) is consistent with the amino acid composition of this fraction (Table 2). Of these amino acids, the charged residues Glu, Lys and Arg might confer antioxidant and metal-chelating activity to these early eluting oligopeptides (Saiga, Tanabe, & Nishimura, 2003). Acidic and basic amino-acid-containing peptides have been reported to possess very good antioxidant activity (Saiga et al., 2003).

Average mass spectra for all compounds eluting after 5 min in the two gel filtration samples F4 and F8 (data not shown) confirmed the separation according to size obtained by gel filtration (Fig. 1a), since F4 contained peptides with higher masses, mainly between 1000 and 1600 Da (Fig. 1f), whereas F8 contained mainly peptides with masses below 600 Da, and only a few with masses >1000 Da, all representing singly-charged peptides (Fig. 1f, Table 3).

**Table 4**
Major masses found in the sub-fraction F9′ from the <3 kDa UF-sample (**Fig. 2**a) and tentative peptide assignment. Masses that were dominating in both the average mass spectrum and in the highest of the peaks in the peptide profile (Fig. 2f) are highlighted. The major fragment resulting from MS (2) fragmentation of each peptide is underlined.

| Mass obs. $[M+H]^+$ | Rt[1] (min) | MS(2) fragments[2] | Sequence[3] and possible peptide with score[4] and origin | Mass theor. $[M+H]^+$ |
|---|---|---|---|---|
| 338.3 | 8.1 | 321.1, 278.1, 175.1 a.o. | RY(14) n.a.[5] | 338.2 |
| 288.1 | 13.7 | 271.1, 229.0, 180.0 | no match, n.i.[6] | |
| 395.2 | 15.1 | 378.2, 232.1, 157.1 a.o. | MTGS (38)collagen n.a.<br>or RGY (35)actin n.a. | both<br>395.2 |
| **390.2** | 16.4 | 372.2, 209.0 a.o. | HAY (23) | 390.2 |
| **409.3** | 16.4 | 392.2, 228.1, 183.0 a.o. | RAY(25) | 409.2 |
| 322.2 | 19.2 | 305.1,263.1,175.0 a.o. | RF (17) collagen, or FR(14) | 322.2 |
| **270.3** | 20.0 | 253.1, 211.1 a.o. | n.i. | |
| **451.3** | 21.2 | 416.3, 249.1, 175.1 a.o. | Y**P**R[7](23), YI/LR[8](23) or E**M**R[7](23) | All451.2 |
| 317.4 | 25.2 | 204.0 a.o. smallpeaks | n.i. | |
| **687.3** | 27.0 | 669.4, 559.3 a.o. | -PDK/Q;QSPPDK/Q[9](39) or AGSTHSQ/K (52) or K/QSTHSK/Q (47);n.i. | 687.3<br>687.4 |
| 720.5 | 28.7 | 702.4, 565.4 a.m.o. | PGPAGPAGP (186)collagen | 720.4 |
| 453.2 | 34.0 | 436.2, 288.2, 243.0, 157.0 a.o. | RMF(35), FMR (21)collagen or MRF (20)<br>or YVGD(18)actin | all<br>453.2 |
| 979.5 | 36.5 | 961.4, 850.4, 669.3, 454.3 a.m.o | -KYE; KWEPKYE (148) n.a. or<br>EPGAAPGVGPAG(27)collagenn.a.; n.i. | both<br>979.5 |
| 629.4 | 37.2 | 612.3, 482.3 a.o. | -YSF or YSM; FTK/QSF(61)<br>orEQGPAGA (67) collagen | both<br>629.3 |
| **446.3** | 37.2 | 429.3, 318.2, 242.1, 129.1 | -GGE, I/LGE or PGE; QLGE(29) collagen,GAIGE(28) collagen<br>or QIW(20) actin, all fit well | 446.2<br>446.2<br>446.3 |
| 792.4 | 47.3 | 775.9, 757.4, 645.4, 627.5, 599.5 a.o. | GDI/LF; RANGDI/LF (157) or RGSGRIF (134) or RGYHI/LF(123) or<br>VGGSGGVME (110) | all<br>792.4 |
| 621.4 | 55.0 | 605.4, 508.3, 462.2, 320.0 a.o. | -AVP or AVL/I; N**P**YVI/Lor **P**NYVI/L (16) or similar | 621.3 |

[1] Retention time.
[2] Fragments obtained by collision induced dissociation, the major one is highlighted.
[3] Amino acid sequence suggested to occur in the peptide according to *de novo* analysis using BioTools software.
[4] Score is given in () and is the value given by BioTools software for the match of the mass peaks to fragments from this sequence.
[5] n.a. = not all high mass peaks are assigned.
[6] n.i. = not identified.
[7] Modified residues are highlighted (**P** = hydroxylated Pro, **M** = oxidised Met).
[8] The mass for residues Ile and Leu are both 113 and cannot be distinguished.
[9] The mass for residues Lys and Gln are both 128 and cannot be distinguished.

All the major masses observed in both the average mass spectrum and the small distinguishable peaks in F8 (highlighted masses in Table 3) had a 10 times lower abundance in F4. These masses could thus represent peptides with antioxidant activity. Possible sequences of the major peptides in F8 are shown in Table 3. However, for most of the peptides many possible sequences could fit reasonably well to the fragment peaks, so an unequivocal identification was not attained. It is interesting to note, however, that many of the peptides tentatively identified in this sub-fraction contained His (H) and Tyr (Y), which are known to confer antioxidant properties to peptides (Chen et al., 1998). The higher DPPH scavenging activity of F8 fraction might stem from the peptides containing these amino acids in this sub-fraction. The radical-scavenging activity of protein hydrolysates from edible meat was attributed to the presence of His, Tyr and Met (Saiga et al., 2003). Since there were many peptides in each sub-fraction of the 3–5 kDa fraction, and the peptides could not be identified with certainty, the activity of the sub-fractions could not be related to particular peptides present in the fraction.

Most of the gel filtration sub-fractions from the sample with molecular weight <3 kDa also contained a multitude of closely-eluting peptides with varying intensity, as can be seen from their peptide profiles shown in Fig 2e. However, the later fractions (F8′–F11′) contained less and more separated peaks. The most interesting SEC sub-fraction from this UF fraction (<3 kDa) was F9′, which showed both a high DPPH radical-scavenging activity and a high reducing power. This is in contrast to sub-fractions F6′ and F8′, which showed high radical-scavenging activity but low reducing power. Sub-fractions F8′ and F9′ contained slightly more early-eluting compounds (~2 min) than F6′, in agreement with their content of lower molecular masses according to elution in gel filtration. The peptide profiles of F8′ and F9′ were clearly different but had some peptides in common (Fig. 2f), which might be the ones contributing to the radical-scavenging activity of these fractions. The dominating masses in F9′ found both in the average mass spectrum and in chromatographic peaks were 338, 288, 395, 322, 317, 720, 453, 979, 629 and 621 (Table 4). These were all more abundant in F9′ than in F8′ and were only present with low abundance in F6′. From the peptide profile (Fig. 2f, bottom panel) the peptides with retention times 22, 29–30, 37 and 55 min, with masses 322, 451, 720, 629 and 621, seem to be dominating in F9′ and might be among those with the high reducing power in F9′. According to the tentative identifications (Table 4), the peptides with masses 322 and 451 contain Arg and 322 supposedly also Phe, both of which can contribute to their antioxidant activity (Power, Jakeman, & FitzGerald, 2013). The peptides with masses 720 and 629 most probably contained fragments from collagen rich in Pro, Ala and Gly. This fits with the high concentration of Ala in the <3 kDa fraction (Table 2). Many collagen and gelatin-derived peptides have been reported to have antioxidant and anti-hypertensive or ACE inhibitory activity, partly due to their unique Gly–Pro–Hyp sequence in their structure (Kim & Mendis, 2006). The high His content of this UF fraction (Table 2) might be present in peptides eluting mainly in other gel filtration sub-fractions or being present as free histidine (Table 1). Because of different enzymes used, difference in species tested and lack of information about cod peptides in the literature, direct comparison of our results on cod peptide constituents to other studies is not feasible. The sequences suggested in Tables 3 and 4 do not seem to be identical to antioxidative peptides previously identified in hydrolysed pollack skin and meat or other fish hydrolysates (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011; Ryan et al., 2011) and thus represent novel antioxidative peptides from fish sources.

### 3.3. Relation between peptides in fractions and their antioxidative activities

In our previous study (Farvin et al., 2014), the antioxidant activity of the peptides derived from cod protein hydrolysate was evaluated by several assays evaluating different reaction mechanisms and in 5% oil-in-water emulsions. In these studies, the low-molecular-weight fraction <3 kDa was shown to have the highest radical-scavenging activity, reducing power and iron-chelating activity and the 3–5 kDa fraction showed higher inhibition of TBARS formation in liposome model systems and in 5% oil-in-water emulsions (Farvin et al., 2014). In the following section, we will try to relate the antioxidant activities of the fractions to their composition.

The antioxidant activity of proteins and peptides has been reported to be related to their amino acid composition, sequence/structure and hydrophobicity (Chen et al., 1998). A number of studies have observed a high correlation between certain amino acid residues and the antioxidant activity of peptides. The importance of these amino acid residues is believed to be related to their unique structural features. Aromatic amino acids, such as Tyr, His, Trp and Phe, and hydrophobic amino acids, including Val, Leu and Ala, as well as Met and Gly, have been reported to be critical for the antioxidant activities of peptides, although some of these amino acids, such as Gly, Met and Trp, have also been reported to show pro-oxidative effects under certain experimental conditions (Chen et al., 1998; Rajapakse, Mendis, Jung, Je, & Kim, 2005). Several studies have shown that a high content of hydrophobic amino acids is mainly responsible for the potent radical-scavenging and lipid-peroxidation inhibitory activities of peptide fractions, e.g., from jumbo squid-skin gelatin, hoki-skin gelatin and giant squid muscle (Mendis, Rajapakse, Byun, & Kim, 2005; Mendis, Rajapakse, & Kim, 2005; Rajapakse, Mendis, Byun, & Kim, 2005). Hydrophobic residues, such as Val, Leu and Tyr, can enhance the solubility of peptides in a lipid matrix, improving the accessibility to hydrophobic radical species or polyunsaturated fatty acids (Qian, Jung, & Kim, 2008) and potentially increase their concentration at water–lipid interfaces and allow close contact with lipid molecules, thus facilitating the scavenging of lipid-derived radicals through direct proton donation (Rajapakse, Mendis, Byun, et al., 2005; Saiga et al., 2003). Most of the peptides identified in the F8 sub-fraction of 3–5 kDa contained at least one of these amino acid residues (Table 3) which might be the reason for the better performance of the 3–5 kDa fraction in lipid containing system such as liposomes and in 5% oil-in-water emulsion (Farvin et al., 2014). Moreover, the F8 sub-fraction contained a number of peptides with His (peaks with retention time 17.5, 23.9, 28.2, 30.9, 36.7, and 37 min). The antioxidative activity of histidine-containing peptides can exceed that of histidine itself, a phenomenon that may partly result from increased hydrophobicity of the peptides, which increases the interaction between the peptides and fatty acids (Saiga et al., 2003). Phenylalanine, which is also an important constituent in the medium and late eluting peptides from the F8 sub-fraction of 3–5 kDa and also in the F9′ sub-fraction of <3 kDa (Tables 3 and 4), may also increase the hydrophobicity of peptides. A number of antioxidant peptides reported from different fish sources, such as grass carp muscle hydrolysate (Ren et al., 2008), horse mackerel visceral proteins (Kumar, Nazeer, & Jaiganesh, 2011), giant squid muscle (Rajapakse, Mendis, Byun, et al., 2005), sauce of fermented blue mussel (Rajapakse, Mendis, Jung, et al., 2005), also contained Phe. It is therefore presumed that the presence of hydrophobic amino acids in these fractions might have contributed to the inhibition of lipid peroxidation by increasing solubility of peptides in lipid, and thereby facilitating better interaction with radical species. Furthermore, Phe might also act as hydrogen donor, and also as a direct radical scavenger (Zhang et al., 2009). The higher radical-scavenging and reducing power of the F9′ sub-fraction might stem from such peptides (Table 4).

Some of the peptides in our study match fully or partly with antioxidant peptides reported earlier. Bougatef et al. (2010) reported an antioxidant peptide with the sequence Gly-Gly-Glu. In our study also we could find this peptide as such or in part in the F9′ sub-fraction of <3 kDa (peaks with retention time 37.2 min in Table 4). Je, Park, and Kim (2005) have reported an antioxidant peptide with the sequence Leu-Pro-His-Ser-Gly-Tyr. In the present study the F8 sub-fraction of 3–5 kDa contains part of this sequence -Leu-Pro-His and -Pro-His (peaks with retention times 23.9 and 37 min). Hernandez-Ledesma, Miralles, Amigo, Ramos, and Recio (2005) identified eight antioxidant peptides from fermented milk, and seven of the eight peptides identified contained at least one proline residue, and six of them had more than two residues of proline. Similarly, hydrolysates of jumbo flying squid skin gelatin had a scavenging effect on radicals, probably because of the presence of Pro residues in the peptide sequence (Lin & Li, 2006). Accordingly, a number of the peptides mentioned in Table 3 and Table 4 contained Pro residues and which may potentiate their antioxidant activity.

The position of amino acids in the N- and C-terminus of the peptide is reported to be an important predictor of the antioxidant activity (Chen et al., 1998). The hydrophobic properties of the N-terminal amino acids are important and will increase the antioxidant activity of the peptides. It has been reported that many antioxidant peptides contain the hydrophobic amino acid residues Val or Leu at the N-terminus of the peptides and Pro, His or Tyr in the sequence (Chen et al., 1998; Uchida & Kawakishi, 1992). This is consistent with some of the peptides present in the sub-fraction F8 from the 3–5 kDa fractions containing Val at the N-terminal and Pro in their sequence (peak with retention time 17.5 Table 3). In addition, some peptides have histidine at the N-terminal (peaks with retention time 28.2, 30.2, 30.9, and 51.9 min in Table 3 and 164 min in Table 4), at which position it has been reported to be effective in metal ion chelation (Chen et al., 1998).

The electronic charge properties (i.e. net charge, molecular polarity) of the C-terminal amino acid are also an important predictor of antioxidant activity (Power et al., 2013). His and Pro have been described as the most important residues in the lipoprotein peroxidation-inhibitory activity of peptides isolated from soybean β-conglycinin hydrolysates (Chen et al., 1998). Chen, Muramoto, Yamauchi, and Nokihara (1996) designed 28 synthetic peptides following the structure of an antioxidative peptide (Leu-Leu-Pro–His-His) from digestion of soybean protein and compared their antioxidative activities against peroxidation of linoleic acid. It was revealed that the antioxidant activity of a peptide was dependent on the His residue at the C-terminus in the Leu-Leu-Pro-His-His domain, and that the activity was decreased by removing the His residue from the C-terminus. Similarly, in the present study also some of the peptides present in sub-fraction F8 from the 3–5 kDa fraction contain di-, tri- and tetra-peptides with C-terminal His residues with Leu or Pro in their sequence (peak with retention time of 23.9). The higher protection of 3–5 kDa against oxidation in liposomes and in 5% oil-in-water emulsion might be due to the presence of these kinds of peptides. Saito et al. (2003) also concluded that the amino acid sequence influenced the antioxidant activity, and showed that tripeptides containing Trp or Tyr at the C-terminus had strong radical-scavenging ability. The LC–MS/MS characterisation of sub-fractions from <3 kDa revealed that the sub-fraction F9′ contained tripeptides with Tyr (peaks with retention times 15.1 and 16.4 min) and Trp (peak with retention time 37.2) at the C-terminus. Similarly, the sub fraction F8 of 3–5 kDa also contained a tripeptide with Tyr at the C-terminal (peak with retention time 28.4 min). The higher radical-scavenging activity of <3 kDa and 3–5 kDa fractions might stem from these Tyr and

Trp containing tripeptides in these fractions. The antioxidative activities of Trp and Tyr may be explained by the special capability of phenolic and indolic groups to serve as hydrogen donors. The phenoxyl and indoyl radicals are much more stable and have longer lifetimes than simple peroxy radicals, so any reverse reaction or the propagation of the radical-mediated peroxidising chain reaction are inhibited (Saito et al., 2003). Similarly, the radical-scavenging activity has been related to the presence of specific amino acid residues, such as Met, Tyr, Arg and Pro in the peptides (Power et al., 2013). A number of peptide sequences identified in the F9′ sub-fraction of <3 kDa contained one or more of these amino acid residues (Table 3), which may be the reason for the higher radical scavenging activity of these fractions.

## 4. Conclusion

Overall, our findings suggest that the presence of both antioxidant peptides and free amino acids in the cod protein hydrolysates significantly contribute to the high oxidative stability of 5% fish oil-in-water emulsions containing such hydrolysates (Farvin et al., 2014). Free amino acids with antioxidative properties (e.g., Lys, Met and His) were present in all fractions, and the total content of free amino acids was higher in the <3 kDa fraction compared to other fractions. In addition, a large number of peptides were identified in the low-molecular-weight UF fractions (3–5 kDa and <3 kDa), among them some containing Lys, Arg and His. Further sub-fractionation according to size showed that the high DPPH radical-scavenging activity and reducing power in the 3–5 kDa and <3 kDa fractions may stem mainly from low-molecular-weight peptides with a high abundance of Glu, Gly, Lys, Ala, Arg, His, Tyr, Pro and Phe. All sub-fractions showed very good iron-chelating activity. These results point to the applicability of these naturally-occurring antioxidant peptides as an ingredient in other food products, in order to increase their oxidative stability. However, further studies are needed to evaluate their antioxidant activity in more complex food systems where the oxidation is induced by more than one mechanism.

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