AFFINITY PURIFICATION AND CHARACTERIZATION OF CHELATING PEPTIDES FROM CHICKPEA PROTEIN HYDROLYSATES

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ABSTRACT

A chickpea protein hydrolysate produced with pepsin and pancreatin was used for the affinity purification of chickpea chelating peptides. Three chelating peptide fractions were obtained after affinity chromatography with immobilized copper. These peptide fractions showed a higher chelating activity and histidine contents than the original protein hydrolysate. Chelating activity was positively correlated with the histidine content of the purified fractions. Different subfractions were also obtained after gel filtration chromatography from the affinity purified peptide fractions. Some of these subfractions showed a higher chelating activity and histidine contents than the original fractions. These results suggest that a combination of high His contents, around 20–30%, and small peptide size provide the best chelating activities. Thus sequential purification with affinity and gel filtration chromatography is a useful procedure for the purification of chickpea peptides with high chelating activity. These results show that a range of chelating peptides are generated during digestion of the chickpea proteins that, after metal chelation, may prevent the generation of reactive oxigen species (ROI) and favour metal absorption.

KEYWORDS: Chelating peptides; Chickpea; Protein Hydrolysate; Pepsin; Pancreatin.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.), is an important source of proteins in human nutrition in developing countries. Its protein content range from 15 to 25% with a high nutritional quality. Chickpea is extensively grown in different parts of the world such as India, Mexico and the Mediterranean Region. About 20% of chickpea seeds are damaged during the harvest and processing, and considered as a by-product that is sold at low prices for livestock feeding (Ulloa, Valencia, & Garcia, 1988). However, these seeds may be an interesting source of proteins for the production of protein hydrolysates (Clemente, Vioque, Sánchez-Vioque, Pedroche, Bautista, & Millán, 1999) and bioactive peptides (Megías *et al*., 2004).

Bioactive peptides are small protein fragments, with beneficial biological activity, after they are released during gastrointestinal digestion or by previous *in vitro* protein hydrolysis (Gobbetti, Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002; Pihlanto & Korhonen, 2003; Vioque *et al.,* 2000). Bioactive peptides have been studied mainly in milk and derived products such as cheese or yogurt. In addition, their existence has been observed in other animal and also plant proteins such as soy, rice, chickpeas and even in fungi. Bioactive peptides with different functions such as antihypertensive, immunomodulatory, opioid, antioxidant, hypocholesterolemic, or metal chelating activity have been described (Vioque *et al.,* 2000; Gobbetti *et al.,* 2002).

Metals are capable of producing reactive oxygen species (ROS) causing damage to biomolecules, diseases such as cardiovascular disease, atherosis, cancer, neurological degenerative diseases, and others (Gaetke and Chow, 2003). In addition, ROS have a negative impact in flavor, texture, nutritive value,

and shelf life of food products and, under extreme conditions, produce toxins. Chelating peptides may prevent this pro-oxidant effect by metal ion chelation. Also, bioavailability of minerals represent a nutritional problem for different sectors of the population such as children, elderly, and women in the first world. Food fortification with the addition of minerals in the form of salts has been proposed to reduce these deficiencies (Zhu et al., 2006). However, this have some limitations such as ions low solubility, oxidative reactions and modification of the flavour and colour of the fortified food. Thus the addition of minerals in the form of chelates may improve minerals bioavailability. Chelating peptides may be an alternative to increase mineral bioavailability. Thus, the positive effect of chelating phosphopeptides, derived from milk proteins, on the absorption of minerals such as calcium (Cross, Huq, & Reynolds, 2007), zinc (Miquel & Farre, 2007) and iron (Bouhallab et al., 2002) have been reported. Hence, chelating peptides, purified from vegetables protein hydrolysates, may also facilitate the bioavailability of these minerals.

Bioactive peptides are usually purified from protein hydrolysates by several chromatographic steps including FPLC gel filtration and C_{18} RP-HPLC chromatography. Affinity chromatography is a powerful protein purification technique that relies on the formation of specific reversible complexes between the molecule to be purified and an immobilized ligand. After incubation of the affinity adsorbent with the mixture containing the molecule of interest and washing to remove unbound molecules, the molecules that are retained are recovered by using specific or nonspecific elution agents.

In previous works we have shown that sunflower and chickpea proteins hydroyzed with the microbial proteases alcalase and flavourzyme contained

peptides with chelating activity, indirectly measured as the ability to inhibit oxidation of β-carotene in the presence of copper (Megias et al., 2007a Megias et al., 2007b). In this work a chickpea protein hydrolysate produced with pepsin plus pancreatin has been used for the purification of chelating peptides by metal chelating affinity chromatography. Chickpea chelating peptides were further fractionated by size exclusion chromatography and characterized.

2. MATERIAL AND METHODS

2.1. Material

Chickpea seeds (*Cicer arietinum* L.) were purchased in a local market. Diethyl ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland). Amino acids standards, D,L-α-aminobutyric acid, trinitrobenzenesulphonic acid (TNBS), ethylendiamine tetra-acetic acid (EDTA), pepsin, pancreatin and pyrocatechol violet (PV) were provided by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Analytical methods

Nitrogen was analyzed in the defatted chickpea flour by Kjeldahl method in a Foss Kjeltec™ 2300 Analyzer equipped with a Digestion Tecator™ system (Höganäs, Sweden). Nitrogen was determined in protein isolates using a LECO CHNS-932 analyzer (Leco Corporation, St. Joseph, MI, USA). Protein content was calculated using 6.25 as conversion factor. Moisture and ash were determined using AOAC (1990) 942.05 and 945.39 approved methods,

125 respectively. Soluble sugars were determined according to Dubois et al.,
126 (1956). Total fiber content was determined according to Lee et al. (1992).
127
128 **2.3. Preparation of chickpea defatted flour**
129 Chickpea seeds were ground and defatted with hexane in a soxhlet
130 extractor for 9 h. The resulting defatted chickpea flour was used as starting
131 material for production of protein isolates. For this, chickpea flour (20 g) was
132 suspended in 200 ml sodium sulphite (0.25%) pH 10, and extracted by stirring
133 for 1 h. After centrifugation two additional extractions were carried out.
134 Afterwards, the pH of the pooled supernatant was adjusted to the isoelectric
135 point (pH 4.3) and the precipitate formed was recovered by centrifugation. This
136 precipitate was washed with distilled water adjusted to pH 4.3 and freeze-dried
137 until further use.
138
139 **2.4. Preparation of chickpea protein hydrolysates**
140 Chickpea protein isolates produced as previously described (Sánchez-
141 Vioque et al., 1999) were used as substrate for the production of chickpea
142 protein hydrolysate. Chickpea protein isolates were hydrolyzed sequentially with
143 pepsin and pancreatin using a hydrolysis reactor vessel (B. Braun Biotech. Int.,
144 GMBH, Melsungen, Germany) equipped with astirrer, thermometer and pH
145 electrode. Hydrolysis parameters were as follows: protein isolates
146 concentration, 5% (w/v); enzyme/substrate ratio 1/20 (w/w); pH 2.5 (pepsin
147 hydrolysis) or 7.5 (pancreatin hydrolysis); temperature, 37 °C. Pepsin was
148 added at time 0, and after 180 min, pancreatin was added for another 180 min.
149 The hydrolysis was inactivated by heating at 80 °C for 20 min. Hydrolysates

were clarified by centrifugation, lyophilized and storage at -20 °C until further use.

2.5. Degree of hydrolysis

The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (Adler-Nissen, 1979). The total number of amino groups was determined in a sample of protein isolate hydrolyzed by treatment with 6 N HCl at 120 °C for 24 h.

2.6. Affinity chromatography purification of chelating peptides

100 mg of chickpea protein hydrolysate were dissolved in 10 mL loading buffer (50 mM pH 7.4 sodium acetate buffer, 0.5 M sodium chloride) and filtered through 0.22 µm. 10 mL of this hydrolysate solution were loaded on a HiPrep IMAC FF 16/10 (20 mL) column (GE) that was previously charged with copper according to manufacturer instructions and coupled to a FPLC AKTA-purifier system (GE). After loading the sample the column was washed with loading buffer until absorbance at 280 nm was stabilized. Then, chelating peptides retained to the column were eluted with a pH gradient from 7.4 to 4.0 in 50 mM sodium acetate, 0.5 M sodium chloride buffer at 1 mL/min flow. Eluted chelating peptides were collected and concentrated using a nanofiltration system (Amicon®, Millipore Corporation, Bedford, EEUU) with a nanofiltration membrane TFC-SR model 3 (Koch membranes).

2.7. Gel filtration chromatography of chelating peptides

Purified chelating peptide fractions were further fractioned by gel filtration chromatography using a Superdex-peptide column coupled to a FPLC AKTA-purifier system (GE). The eluent used was 0.75 M ammonium bicarbonate buffer at a flow rate of 0.5 ml min^{-1}. Elution was monitored at 215 nm and the proximate molecular masses of eluted peptides were determined using the following molecular weights standards from Pharmacia: blue dextran (2000 kDa), cytochrome C (12.5 kDa), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da).

2.8. Amino acid analysis of chickpea peptides

The amino acids composition of peptide fractions were deter- mined after amino acids derivatisation with diethyl ethoxymethyl- enemalonate by HPLC using D,L-a-aminobutyric acid as an internal standard (Alaiz, Navarro, Girón, & Vioque, 1992). The HPLC system consisted of a model 600E multisystem with a 484 UV–Vis detector (Waters Corporation, Milford, MA, EEUU) equipped with a 300 mm × 3.9 mm i.d. reversed-phase column (Novapack C18, 4 lm; Waters). A binary gradient was used for elution with a flow of 0.9 ml/min. The solvents used were (A) sodium acetate (25 mM) containing sodium azide (0.02% w/v), pH 6.0, and (B) acetonitrile. Elution was as follows: time, 0.0–3.0 min; linear gradient from A/ B (91/9) to A/B (86/14); 3.0–13.0 min, elution with A/B (86/14); 13.0–30.0 min, linear gradient from A/B (86:14) to A/B (69/31); and 30.0–35.0 min, elution with A/B (69/31). The column was maintained at 18 °C.

Tryptophan was determined after basic hydrolysis according to Yust et al. (2004). For this, samples were hydrolysed in 3 ml 4 M sodium hydroxide at 100

°C for 4 h. Hydrolysates were neutralized to pH 7 and diluted to 25 ml with 1 M sodium borate buffer (pH 9). Tryptophan was determined by HPLC-RP at 280 nm.

2.9 Copper chelating activity of chickpea peptides

Copper chelating activity was determined by copper chelate titration using pyrocatechol violet (PV) as the metal chelating indicator (Saiga et al. 2003). 100 µL of 0.1 µg/µL $CuSO_4$ in 50 mM sodium acetate buffer pH 6.0 was mixed with 25 µL of PV 4 mM in 50 mM sodium acetate buffer pH 6.0. The complex of PV and Cu^{2+} absorbs blue light at 632 nm, while PV dissociated with a metal ion in the presence of chelating agents does not show this absorption. Thus, copper chelating activity is proportional to the decrease in absorbance at 632 nm.

Chelating activity was calculated according to the following formula:

$$\% \text{ Chel.Act.} = (1 - Abs_{632nm}(PV + Cu^{++} + \text{sample})/ Abs_{632nm}(PV + Cu^{++})) \times 100$$

where Abs_{632nm} (PV + Cu^{++} + sample) is the absorbance at 632 nm of the PV + Cu++ complex in the presence of peptides samples, and Abs_{632nm} (PV + Cu^{++}) is the absorbance at 632 nm of the PV + Cu^{++} complex in the absence of peptides samples.

3. RESULTS AND DISCUSSION

3.1. Production of chickpea protein hydrolysates

A chickpea protein isolate was used as a substrate for the production of chickpea protein hydrolysates. Table 1 shows the chemical composition of this protein isolate compared with the composition of the original defatted chickpea

225 flour. The protein isolate, with a protein content above 95% and minor amounts
226 of other compounds, represent an optimal substrate for the production of
227 chickpea protein hydrolysates and further purification of bioactive peptides. The
228 protein hydrolysate was produced by sequential action of the digestive enzymes
229 pepsin and pancreatin. Fig. 1 shows the kinetic of chickpea protein isolate
230 hydrolysis. The rate of hydrolysis with pepsin was very high during the first 10
231 min, and after that hydrolysis proceeded more slowly. After 180 min of
232 incubation, pepsin produced a protein hydrolysate with 16.3% degree of
233 hydrolysis, before pancreatin was added. A final protein hydrolysate, obtained
234 after sequential digestion of chickpea proteins with pepsin and pancreatin, had
235 a 27.03% degree of hydrolysis. At the end of the hydrolysis, the degree of
236 hydrolysis decreased from 31.5% to 27.0%, probably due to the plastein
237 reaction by which proteases catalyses a peptides condensation process,
238 resulting in protein materials of higher molecular weights (Wil-lians, Brownsell,
239 & Andrews, 2001).

240 **3.2. Purification of chickpea chelating peptides**

241 The chickpea protein hydrolysate produced after hydrolysis with pepsin
242 and pancreatin was used as substrate for the purification of chelating peptides
243 by affinity chromatography with immobilized copper. According to the
244 manufacturer's instructions (GE Healthcare) proteins or peptides bounded to
245 the metal charged Hi- Prep IMAC FF 16/10 column can be eluted with a linear
246 gradient from pH 7 to 4. Therefore, bounded chickpea chelating peptides were
247 eluted from the copper charged column with a pH gradient from 7.4 to 4 (Fig. 2).
248 Three chelating peptides fractions (F1, F2 and F3), that eluted with different
249 acidic pH values, were collected. The use of a cromatography column and a pH

gradient in the eluting buffer allowed the purification of different peptide fractions with increasing affinity for the solid support. This is in contrast to our previous results using metal chelate spin columns that pro- vided only a single fraction of chelating peptides after centrifugation of the columns for elution of the bounded peptides (Megías et al., 2007a, 2007b).

Table 2 shows the amino acid composition of the chickpea protein hydrolysate and the three purified chelating peptide fractions (F1, F2 and F3) from the copper affinity column. Fractions F1 to F3 showed different amino acid compositions that explain their different degrees of binding to the affinity col- umn. For example, F1 was especially rich in Arg and Lys and F3 in Ser. Also a positive correlation between His contents and elution volume from the affinity column was observed in these fractions (Fig. 3). Thus fraction F1, with His contents similar to the observed in the protein hydrolysate, eluted first at the beginning of the pH decreasing gradient. The high amounts of Arg and Ser may be the responsible of the interaction of this fraction with the solid support. After fraction F1, eluted fractions F2 and F3 with lower pH eluting values and increasing His contents. His, due to its imidazole ring, may be directly implicated in the peptide binding to the cop- per containing affinity column. Swain, Tabatabai, and Reddy (2001) also observed a positive correlation between the elution volume and His contents of chelating peptide fractions purified from a beef protein hydrolysate produced with pepsin plus pancreatin.

Chelating peptide fractions F1, F2 and F3 were further fractionated, by gel filtration chromatography, into subfractions of different molecular weights (Fig. 4). Table 3 shows the proximate molecular weight of these subfractions. Main subfraction of F1 fraction, F1B, has an average of 40 amino acids. On the

275 contrary in F2 main subfraction, F2B, was 11 amino acid long, and in F3 was
276 5 aminoacids long (F3C).
277 Amino acids composition (g/100 g proteins) of these subfractions was also
278 analysed (Table 2). Some subfractions were especially rich in certain amino
279 acids. This is the case of the high Asp values in F1A, Glu % in F2A and F3B,
280 high Ser values in F3C and F3D, Arg contents in F1F, Tyr contents in F1F,
281 Cys contents in F1F, Phe % in F1F and high Lys % in F1C and F1D. With
282 respect to His contents, highest amounts were observed in subfractions
283 belonging to fractions F2 and F3. Thus, F2D and F3E with 33.4% and 28.2%
284 His contents were the subfractions richest in this amino acid. Some of these
285 subfractions were poor in His, but their binding to the affinity column may be
286 explained by the presence in high amounts of other amino acids, such as Asp
287 or Glu, that have also been implicated in the chelating activity of peptides (Lu
288 et al., 2009)
289
290 **3.3. Copper chelating activity**
291 We have studied the copper chelating activity of chickpea puri- fied
292 peptide fractions. If the degree of binding to the affinity col- umn by chelating
293 peptides is proportional to their affinity for copper, then it is expected that
294 fractions more strongly bounded to the column would possess a higher
295 copper chelating activity. To test this hypotesis, the copper chelating activity of
296 fractions F1, F2 and F3 was assayed. F3 showed 45.3% chelating activity (Fig.
297 5A) followed by F2 and F1 with 36.7 % and 28.4 %, respectively. However, F1
298 had a similar chelating activity than the hydrolysate, that is in accordance with
299 their similar His contents (Table 2). The chelating activity of these peptide
300 fractions correlated positively with their elution volume from the affinity column
301 and with their histidine content (Fig. 5B).

Chelating activity of subfractions (F1A-F3D) purified by gel-filtration chromatography was also analyzed (Fig. 6). In general, sub- fractions of fractions F2 and F3 showed higher chelating activities. A range of different chelating activities were observed that are explained not only by the influence of the His contents, but also by the different peptide sizes. Thus in these subfraactivity, although this correlation was not as high as in fractions F1, F2 and F3 (Fig. 7A). On the other hand, no correlation was observed between the chelating activity and peptide size of these subfractions (Fig. 7B). Fractions with the highest chelating activity were those that were smaller and with high His contents. Hence, highest chelating subfractions were F2B (15.3% His and 1205 Da), F3E (28.3% His and 162 Da), F3D (17.6% His and 308 Da) and F2D (33.4% His and 105 Da). These results suggest that a combination of high His contents, around 20–30%, and small peptide size provide the best chelating activities.

4. CONCLUSION

We have shown that chickpea proteins can be hydrolyzed with pepsin plus pancreatin generating an extensive protein hydrolysate resembling that produced in humans during food digestion. We have also shown that this hydrolysate is rich in chelating peptides that can be fast and efficiently purified by affinity chromatography. Purified chelating peptides are a mixture of peptides with different amino acid compositions and sizes. We also observed that their His contents defined more strongly their chelating activity than their size. The most effective fractions were peptide fractions with His contents above 15%. The fraction size is not the determinant in the activity although, in general, smaller fractions showed higher chelating activity. Thus, chelating peptides, purified from food protein hydrolysates, may be useful in fortifying foods for

preventing oxidative activity of prooxidant metals and increasing their bioavailability while maintaining food quality and appearance.

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FIGURE LEGENDS

Figure 1. Time course of the hydrolysis of chickpea protein isolate with pepsin (added at time 0 min) and pancreatin (added after 180 min). Data correspond to the average ± SD of three determinations.

Figure 2. Affinity chromatography purification of chickpea chelating peptides using a copper charged agarose column.

Figure 3. Linear correlation between the His content (%) and volume elution of purified chickpea chelating peptide fractions F1, F2 and F3, from the copper affinity column.

Figure 4. Gel filtration chromatography fractionation of chickpea chelating peptide fractions F1, F2 and F3 purified from the copper affinity column.

Figure 5. **(A)** Copper chelating activity of chickpea protein hydrolysate and peptide chelating fractions F1, F2 and F3. Data correspond to the average ± SD of two independent experiments. **(B)** Linear correlation between copper chelating activity and His content of protein hydrolysate and chelating peptide fractions.

Figure 6. Copper chelating activity of chelating peptide fractions purified by gel filtration chromatography. Data correspond to the average ± SD of two independent experiments.

Figure 7. Correlation of copper chelating activity of chelating peptide subfractions purified by gel filtration chromatography with their His content **(A)** and their peptide size **(B)**.

Table 1. Chemical composition of defatted chickpea flour and protein isolate. Data expressed as g 100 g^{-1} of dry matter are the mean ± SD of two analyses.

Components	Defatted flour	Protein isolate
Moisture	5.0 ± 1.1	tr[a]
Ash	3.5 ± 0.1	1.3 ± 0.1
Fibre	25.4 ± 2.2	5.0 ± 0.0
Protein content[b]	20.0 ± 0.3	93.8 ± 3.3
Lipids	5.8 ± 0.1	tr
Soluble sugars	1.2 ± 0.0	tr
Carbohydrates by difference	39.1± 0.0	tr

[a] Trazes
[b] Total nitrogen x 6.25

Table 2. Percent amino acid composition of chickpea protein hydrolysate (PH) and peptide chelating fractions purified by affinity (F1-F3) and gel filtration chromatography (F1A-F3E).

aa	Asp[a]	Glu[b]	Ser	His	Gly	Thr	Arg	Ala	Pro	Tyr	Val	Met	Cys	Ile	Leu	Phe	Lys
PH	14.1±0.2	18.7±0.0	6.0±0.0	3.0±0.0	4.4±0.0	3.4±0.0	0.5±0.1	4.2±0.1	2.2±0.1	1.5±0.1	4.5±0.1	0.4±0.1	0.5±0.0	4.1±0.1	7.9±0.1	6.65±0.1	6.4±0.1
F1	12.8±0.1	17.3±0.1	3.4±0.0	3.2± 0.0	3.0±0.0	2.4±0.0	24.9±0.3	2.0±0.0	1.3±0.1	1.2± 0.0	1.6± 0.0	0.5±0.1	2.4±0.1	1.8±0.1	3.4±0.0	6.9±0.0	11.7±0.1
F2	14.1±0.6	19.1±0.2	6.2±0.1	17.4±0.1	4.4±0.0	3.1±0.1	5.8±0.2	4.0±0.0	1.3±0.0	0.9±0.0	3.7±0.1	0.2±0.0	1.2±0.0	2.0±0.0	6.9±0.0	4.7±0.1	5.0±0.1
F3	15.6±0.0	16.9±0.1	11.5±0.0	22.9±0.0	3.2±0.0	1.8±0.0	5.9±0.1	2.7±0.0	2.0±0.0	0.3±0.0	1.7±0.0	0.8±0.1	1.1±0.0	4.1±0.2	3.6±0.0	2.3±0.0	3.6±0.0
F1A	24.6±0.8	19.7±4.4	9.1±3.0	3.0±0.3	8.3±2.9	5.9±1.1	4.7±0.9	4.4±1.2	n.d	0.9±0.4	1.4±0.6	0.2±0.3	1.8±0.6	2.9±0.3	6.3±0.1	1.5±0.4	5.3±1.1
F1B	15.9±5.9	31.8±1.4	5.3±0.3	6.2±1.0	4.8±0.2	4.0±0.6	7.1±1.1	2.9±0.5	n.d	0.5±0.0	2.8±0.6	0.5±0.5	1.3±0.1	3.0±0.5	5.7±0.7	2.2± 0.6	6.1± 0.7
F1C	15.9±0.8	16.8±0.2	6.2±0.7	8.1± 0.1	6.3±1.3	3.1±0.4	10.5±1.5	3.9±0.4	n.d	1.2±0.7	2.5± 0.8	0.2±0.1	0.6±0.1	3.2±0.5	5.2±0.3	2.7± 0.5	13.7±0.3
F1D	10.8±1.7	11.8±0.9	5.1±0.7	8.1±0.2	4.8±0.5	2.8±0.8	18.2±3.4	4.1±2.1	n.d	1.9±1.2	1.6±0.2	1.3±1.6	0.8±0.0	2.0±0.7	4.5±0.4	3.5±1.3	18.4±8.7
F1E	9.3± 0.2	11.9±2.3	4.5±1.2	6.3±2.2	4.7±1.4	2.1±0.2	30.9±0.2	3.2±0.8	n.d	1.7±1.7	0.6±0.8	1.2±1.3	2.4±2.5	1.3±0.0	6.2±2.9	5.9±4.3	7.7± 1.1
F1F	6.0± 4.7	5.2±4.6	2.8±3.8	3.6±3.5	4.0±2.9	1.1±1.2	27.5±8.1	1.5±1.3	n.d	12.9±1.3	1.2±0.5	0.6±0.0	6.0±4.8	0.2±0.0	2.4±2.8	21.8±1.9	3.0±2.7
F2A	19.6±0.2	31.3±0.5	8.0± 0.8	8.8± 0.1	4.5±0.3	2.7±0.1	6.4±0.8	2.8±0.2	n.d	0.3± 0.0	0.9±0.9	0.1±0.0	0.2±0.1	2.4±0.3	5.7±0.0	2.2±0.1	3.8± 0.1
F2B	17.1±1.6	23.0±2.0	7.0±1.1	15.3±0.4	4.9±0.2	3.7±0.1	3.9±0.1	4.4±0.1	n.d	1.3± 0.1	1.7±1.5	0.9±0.1	0.5±0.2	2.9±0.1	7.4±0.0	2.7±0.1	3.8±0.5
F2C	14.9±0.8	15.0±2.6	6.4±0.2	23.7±0.7	5.2±0.2	2.0±0.3	5.2±0.9	3.9±0.2	n.d	2.0 ±0.4	2.5±1.7	0.3±0.0	0.5±0.2	1.8±0.2	6.2±0.3	4.9±0.7	5.5±0.4
F2D	10.5±1.8	12.1±0.9	6.6±0.3	33.4±2.3	3.9±0.3	0.9±0.3	7.1±0.9	2.8±0.1	n.d	1.5±0.0	3.4± 2.1	0.2±0.1	0.9±0.5	0.9±0.1	4.7±0.7	8.1±0.9	2.8±0.4
F3A	17.5±0.1	24.4±0.1	7.6±0.3	2.8±0.0	120.±0.1	2.0±0.1	6.3±0.1	3.7±0.0	n.d	0.0±0.0	1.4±0.3	1.0±0.1	0.0±0.0	2.4±0.3	9.9±0.2	2.8±0.0	6.0±0.1
F3B	21.3±0.4	29.6±0.5	9.3±0.3	6.3±0.2	6.8±0.1	2.0±0.0	5.7±0.1	1.1±1.5	n.d	0.0±0.0	0.5±0.0	0.8±0.0	1.6±0.0	1.8±0.0	6.8±0.0	1.1±0.0	5.1±0.1
F3C	20.4±0.7	23.7±0.5	15.0±0.1	8.5±0.1	4.4±0.1	3.0±0.1	5.1±0.1	1.4±2.0	n.d	1.2±0.1	1.7±0.1	0.1±0.1	0.1±0.2	6.3±0.2	4.1±0.1	1.8±0.0	3.0±0.1
F3D	15.3±0.0	15.0±0.1	11.4±0.2	17.6±0.1	5.6±0.0	2.5±0.1	5.2±0.0	4.7±0.1	n.d	0.1±0.0	2.3±0.1	0.0±0.0	0.0±0.0	3.9±0.0	5.7±0.1	4.1±0.0	5.4±0.0
F3E	8.3±0.0	11.0±0.3	8.5±0.1	28.3±0.4	4.6±0.1	2.4±0.0	9.7±0.1	4.0±0.0	n.d	1.4±0.0	2.3±0.1	0.1±0.0	0.1±0.1	1.5±0.0	7.4±0.1	5.2±0.0	4.8±0.2

Data correspond to the average ± SD of two independent experiments.

[a] Aspartic acid + asparagine.
[b] Glutamic acid + glutamine.
n.d. Not determined.

Table 3. Peptide size of gel filtration chromatography chelating peptide fractions. Number of amino acids residues has been calculated on an average molecular weight of 110 Da per amino acid.

Gel filtration peptide fractions	Molecular weight (Da)	Number of amino acid residues
F1A	12071	110
F1B	4436	40
F1C	478	4
F1D	296	3
F1E	102	1
F1F	62	1
F2A	2932	27
F2B	1205	11
F2C	281	2-3
F2D	105	1
F3A	5481	50
F3B	768	7
F3C	494	5
F3D	308	3
F3E	162	1-2

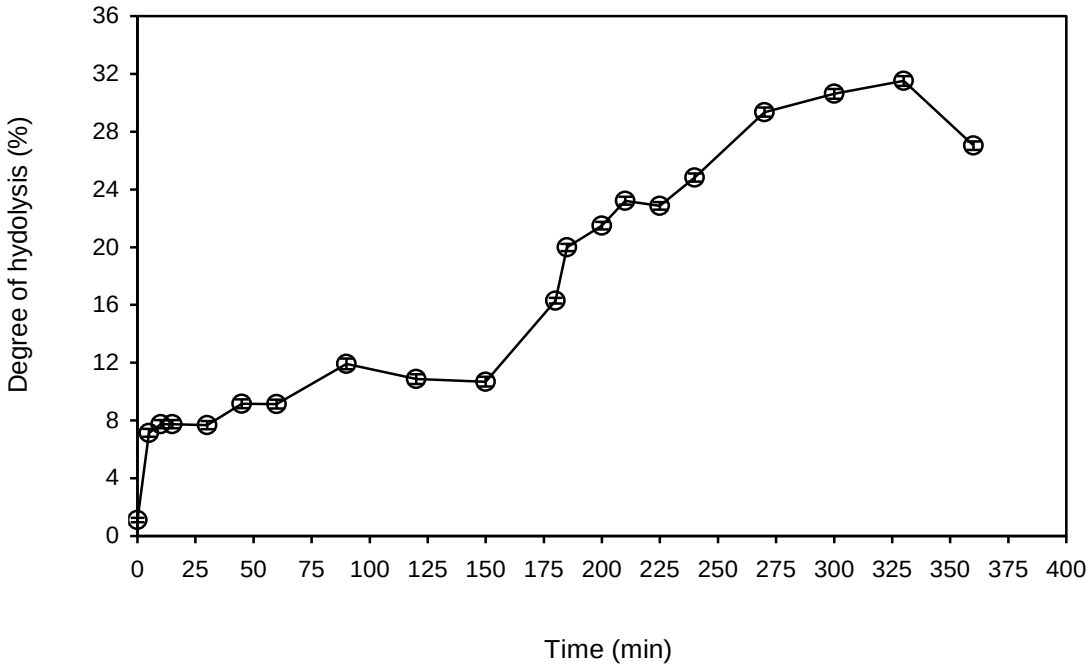

Degree of hydolysis (%)
0
4
8
12
16
20
24
28
32
36
0
25
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400
Time (min)

Figure 1

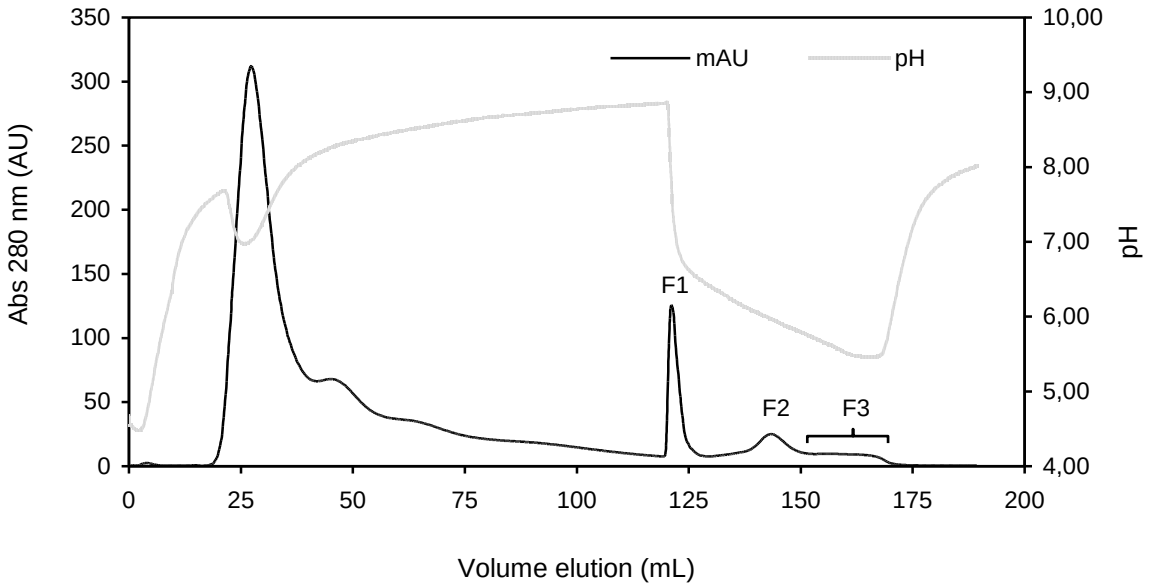

mAU
pH
Abs 280 nm (AU)
pH
Volume elution (mL)
F1
F2
F3
350
300
250
200
150
100
50
0
10,00
9,00
8,00
7,00
6,00
5,00
4,00
0
25
50
75
100
125
150
175
200

Figure 2

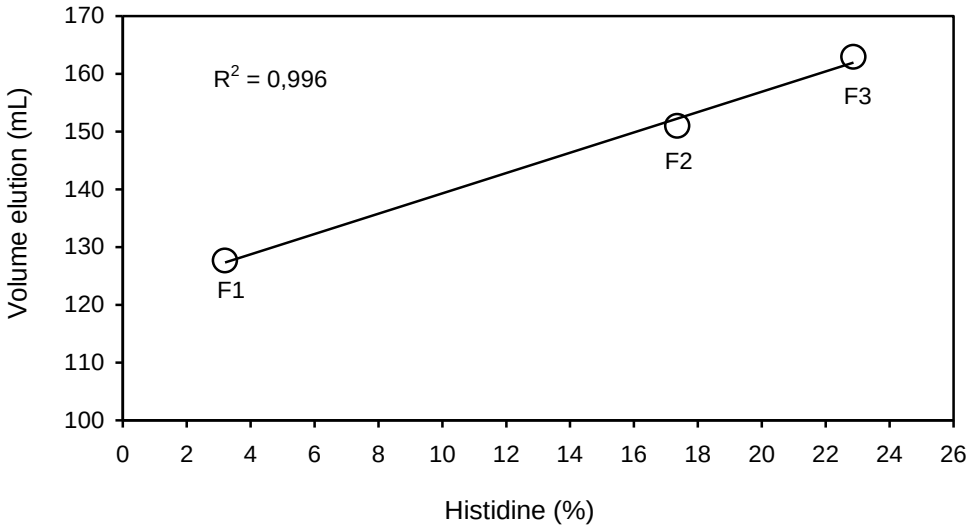
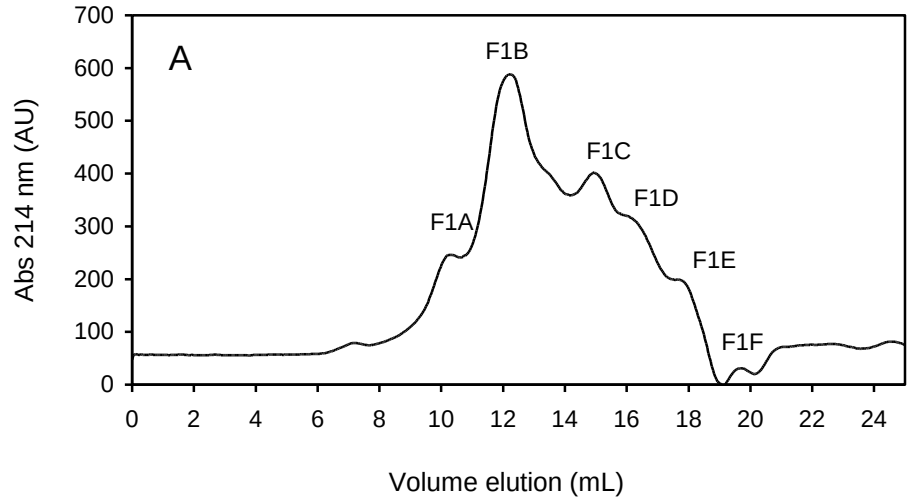
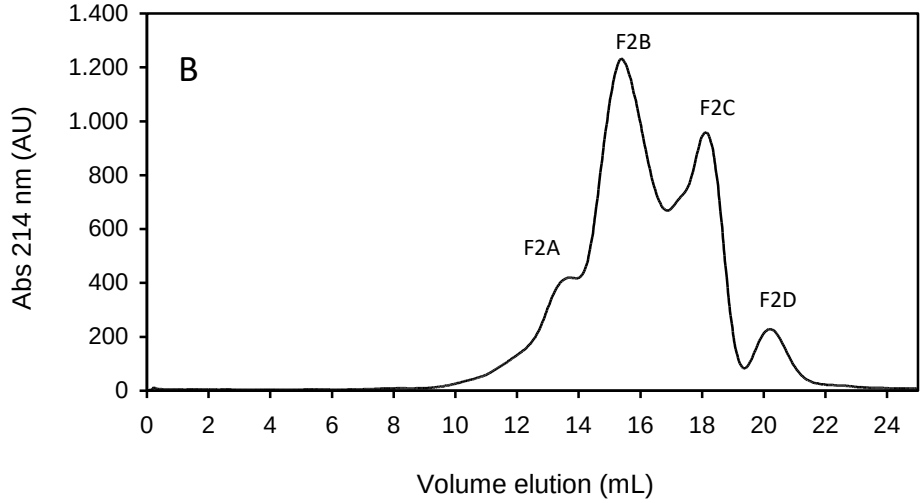

170
160
150
140
130
120
110
100
Volume elution (mL)
R2 = 0,996
F1
F2
F3
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Histidine (%)

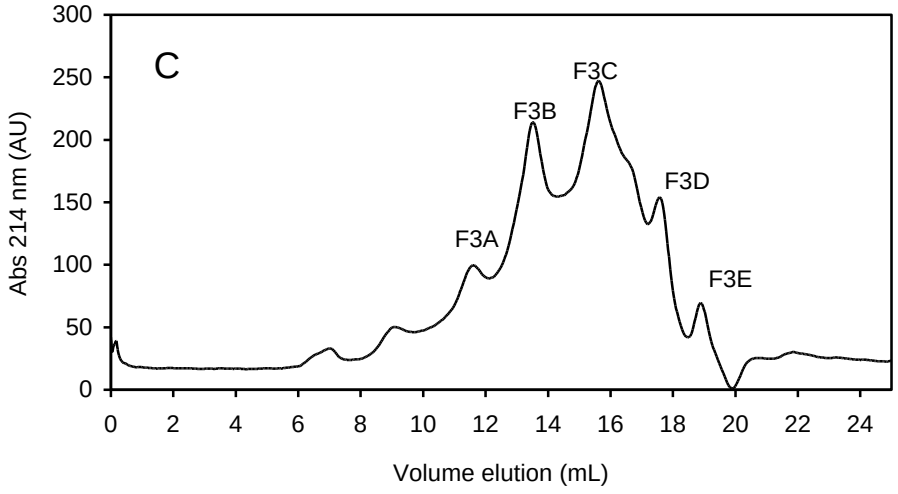
Figure 3



535

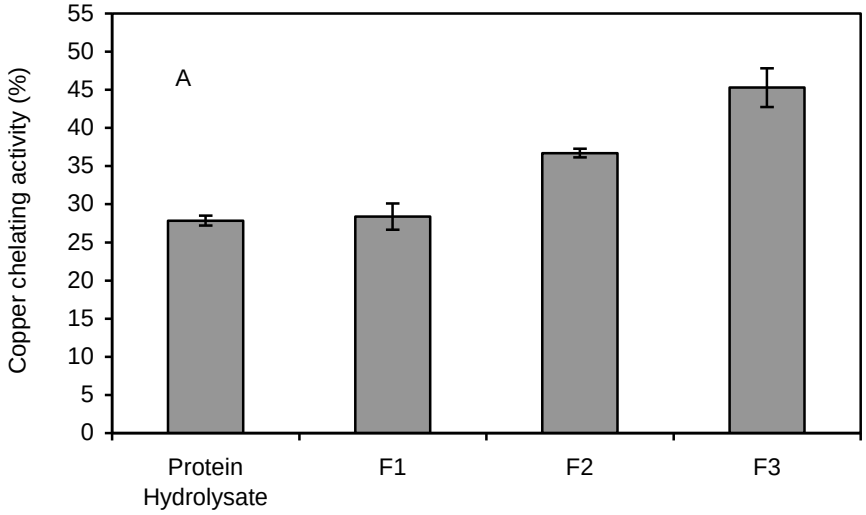


536



537

538 **Figure 4**


A
Copper chelating activity (%)
55
50
45
40
35
30
25
20
15
10
5
0
Protein Hydrolysate
F1
F2
F3

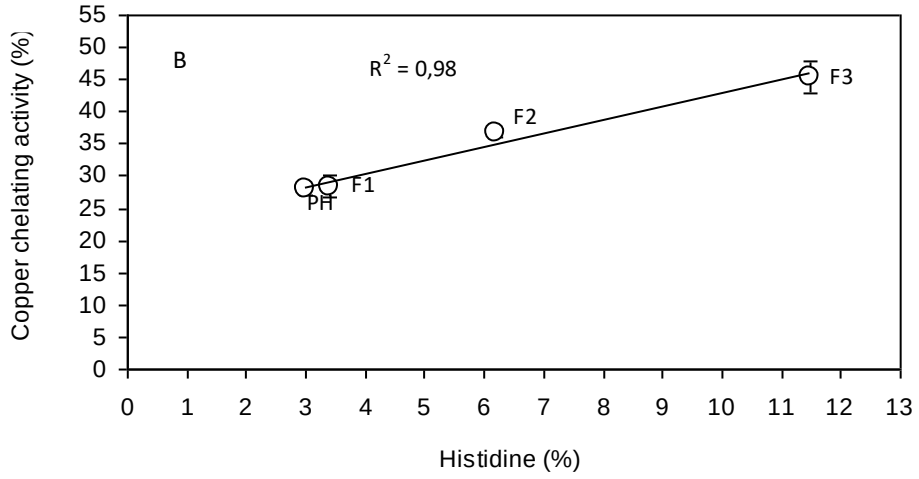

B
R^2 = 0,98
Copper chelating activity (%)
F3
F2
F1
PH
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Histidine (%)

Figure 5

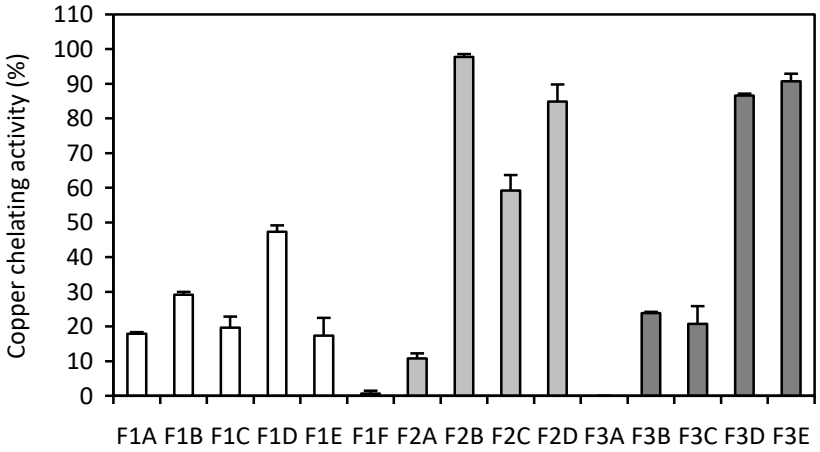
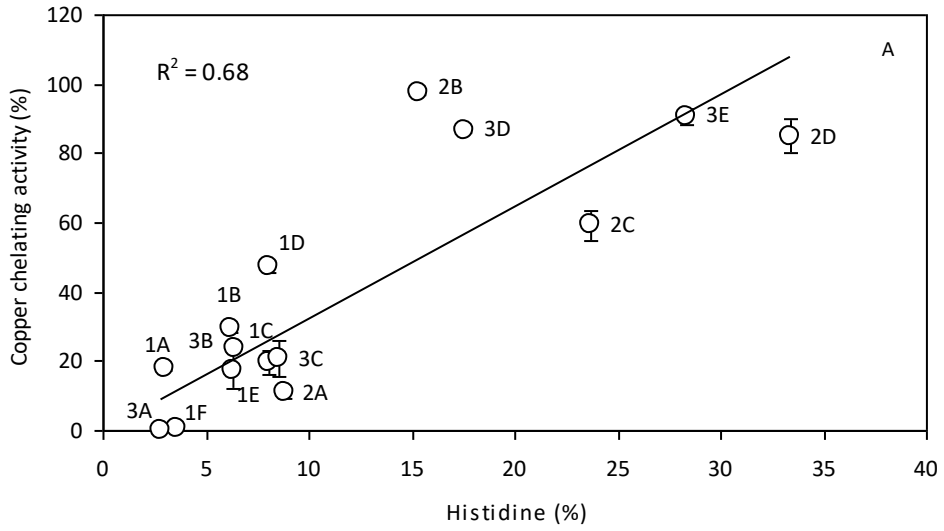

Copper chelating activity (%)
110
100
90
80
70
60
50
40
30
20
10
0
F1A F1B F1C F1D F1E F1F F2A F2B F2C F2D F3A F3B F3C F3D F3E

Figure 6


A
R^2 = 0.68
Copper chelating activity (%)
Histidine (%)
2B
3D
3E
2D
2C
1D
1B
1A
3B
1C
3C
1E
2A
3A
1F

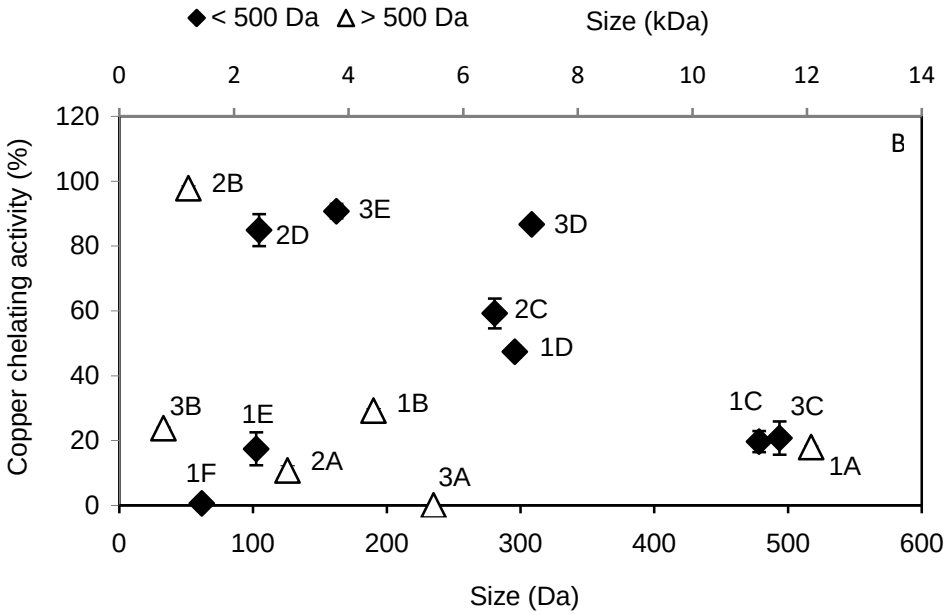

◆ < 500 Da △ > 500 Da
Size (kDa)
B
Copper chelating activity (%)
Size (Da)
2B
3E
2D
3D
2C
1D
3B
1E
1B
1C
3C
1A
1F
2A
3A

Figure 7

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