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Affinity Purification of Copper Chelating Peptides from Chickpea Protein Hydrolysates

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Chickpea protein hydrolysates obtained with alcalase and flavourzyme were used for purification of copper chelating peptides by affinity chromatography using copper immobilized on solid supports. The chelating activity of purified peptides was indirectly measured by the inhibition of β-carotene oxidation in the presence of copper. Two protein hydrolysates, obtained after 10 and 100 min of hydrolysis, were the most inhibitory of β-carotene oxidation. Purified copper chelating peptides from these protein hydrolysates contained 19.7 and 35.1% histidine, respectively, in comparison to 2.7 and 2.6% in the protein hydrolysates. Chelating peptides from hydrolysate obtained after 10 min of hydrolysis were the most antioxidative being 8.3 times more antioxidative than the hydrolysate, while chelating peptides purified from protein hydrolysate obtained after 100 min were 3.1 times more antioxidative than its hydrolysate. However, the histidine content was higher in peptides derived from the 100 min hydrolysate (19.7 against 35.1% in 10 min hydrolysate), indicating that this amino acid is not the only factor involved in the antioxidative activity, and other factors such as peptide size or amino acid sequence are also determinant. This manuscript shows that affinity chromatography is a useful procedure for purification of copper chelating peptides. This method can be extended to other metals of interest in nutrition, such as calcium, iron, or zinc. Purified chelating peptides, in addition to their antioxidative properties, may also be useful in food mineral fortification for increasing the bioavailability of these metals.

KEYWORDS: Chelating peptides; chickpea; protein hydrolysate

INTRODUCTION

The search for functional components in foods has become a major area of research in recent years. These functional components include the bioactive peptides, which are small amino acid sequences in food proteins that have a beneficial biological activity after they are released during gastrointestinal digestion or by previous in vitro protein hydrolysis (*1*). For example, peptides with antihypertensive, immunomodulatory, opioid, antioxidant, hypocholesterolemic, or metal chelating activity have been described (*2*−*4*). Among metal chelating peptides, caseinophosphopetides have been widely studied. These peptides obtained after casein hydrolysis have been shown to bind and increase the absorption of metals such as calcium (*5*). Also, chelating properties of these peptides have been related to their antioxidative activity (*6*).

Copper is an essential trace element that plays a vital role as a cofactor for a variety of enzymes. Amino acids, such as histidine, methionine, and cysteine and small peptides, may bind to copper to allow absorption through an amino acid transport system, and mammals are in possession of specific copper chaperones that bind copper with high affinity (*7*).

However, similar to iron, copper is capable of producing reactive oxygen species inducing DNA strand breaks and oxidation of bases. Also, copper is a powerful catalyst of low-density lipoprotein (LDL) oxidation (*8*). This LDL oxidation may promote atherogenesis by enhancing the transformation of macrophages into foam cells and by developing vasoconstrictor and prothrombotic properties. Copper chelating peptides may prevent copper-induced LDL oxidation by metal ion chelation.

Chickpea protein isolates could be used for the production of protein hydrolysates with improved functional and nutritional properties (*9*, *10*). These protein hydrolysates could also be a source of bioactive peptides. Thus, we have reported the purification of angiotensin converting enzyme inhibitory peptides from chickpea protein hydrolysates (*11*, *12*).

Bioactive peptides are usually purified after several chromatographic steps. These may include enrichment in bioactive peptides by filtration followed by purification using gel filtration or ion exchange chromatography. Most often, high-performance liquid chromatography (HPLC) reverse-phase chromatography is used in order to further purify the bioactive peptides.

Affinity chromatography is a powerful protein purification method that relies on the specific reversible complexes that are formed between the molecule to be purified and a ligand bound to a suitable polymer support. After incubation of the affinity

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adsorbent with the mixture containing the molecule of interest and washing to remove unbound molecules, the molecules that are retained are eluted by using specific or nonspecific elution agents. Affinity chromatography may be useful in the purification of bioactive peptides. Thus, the immobilization of ACE on glyoxyl-agarose supports and the use of these derivatives for the purification of ACE inhibitory peptides were described in previous papers (*13*, *14*). Following a similar approach in this paper, we describe the affinity purification of copper chelating peptides from chickpea protein hydrolysates using copper-chelated solid supports.

MATERIALS AND METHODS

Material. Diethyl-ethoxymethyl-enemalonate was purchased from Fluka (Buchs, Switzerland). Amino acids standards, D,L-α-aminobutyric acid, trinitrobenzenesulphonic acid (TNBS), ethylendiamine tetra-acetic acid (EDTA), histidine, and β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). The enzymic complexes used were alcalase 2.4 L and flavourzyme 1000 mg provided by Novo Nordisk (Bagsvaerd, Denmark). Alcalase (2.4 L) is an endopeptidase from *Bacillus licheniformis*, with subtilisin carlsberg as the major enzymic component, having a specific activity of 2.4 Anson units (AU) per gram. Flavourzyme (1000 mg) is an exopeptidase and endoprotease complex with an activity of 1 LAPU (leucine amino-peptidase units) per gram. All other chemicals were of analytical grade.

Preparation of Chickpea Protein Isolates. Chickpea protein isolates were obtained as previously described with modifications (*15*). Chickpea flour (20 g) was extracted by stirring for 1 h in 200 mL of 0.2% NaOH, pH 12, at room temperature. After centrifugation at 8000*g* for 30 min, an additional extraction was carried out. The pH of the supernatant was adjusted to the isoelectric point (pH 4.3), and the precipitate formed was recovered by centrifugation as described above. The precipitate was washed with distilled water adjusted to the isoelectric point and freeze-dried until further use.

Preparation of Chickpea Protein Hydrolysates. Chickpea protein hydrolysates were obtained as previously described with modifications (*9*). Chickpea protein isolates were hydrolyzed with alcalase and flavourzyme using a hydrolysis reactor vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis parameters were as follows: protein isolate concentration, 5% (w/v); enzyme/substrate ratio, 0.3 AU g^{-1}; pH 8; temperature, 50 °C for alcalase; and enzyme/substrate ratio, 100 LAPU g^{-1}; pH 7; temperature, 50 °C for flavourzyme. Alcalase was added at time 0, and after 60 min, flavourzyme was added. Proteases in the aliquots and final hydrolysate were inactivated by heating at 80 °C for 20 min. Hydrolysates were clarified by filtration through 0.45 μm filters (Millipore, Bedford, MA) to remove insoluble substrate and lyophilized for storage at −20 °C.

Degree of Hydrolysis. The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS (*16*). 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.

Assay of β-Carotene Oxidation. The assay of β-carotene oxidation was carried out as previously described with modifications (*17*). A solution of β-carotene was prepared by dissolving 4 mg in 1 mL of chloroform. After the addition of 1 mL of Tween 20, chloroform was evaporated under nitrogen. Aliquots from this solution were dissolved in 100 mM, pH 7.4, phosphate buffer. A 10 μM solution of $CuSO_4$ was prepared, and 2 μL was added to each well. Final concentrations in the assay mixture were as follows: β-carotene, 119 μM; Cu^{++}, 0.1 μM; and different concentrations of peptides in a final volume of 200 μL. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm using a ThermoLab Systems Multiskan Spectrum microplate spectrophotometer (Vantaa, Finland).

Purification of Chelating Peptides. Chickpea protein hydrolysates with the highest antioxidative activity were selected for purification of chelating peptides. For this, the hydrolysates dissolved in 0.1 M sodium phosphate buffer, pH 7, were loaded on to Vivapure Metal Chelate Maxi spin columns (Vivascience, Sartorius, Madrid, Spain) that had been previously charged with copper following manufacturer instructions. After the column was washed with several volumes of phosphate buffer, chelating peptides were recovered from the column with 0.1 M sodium phosphate buffer, pH 3.5.

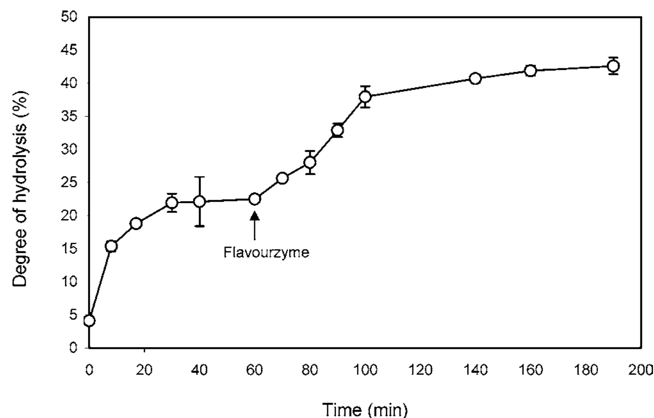

Figure 1. Time course of the hydrolysis of chickpea protein isolate by alcalase (added at time 0 min) and flavourzyme (added after 60 min). Data correspond to the average ± SD of three determinations.

Amino Acid Analysis. Samples (10 mg) were hydrolyzed with 4 mL of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110 °C for 24 h. Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by HPLC, according to the method of Alaiz et al. (*18*), using D,L-α-aminobutyric acid as an internal standard. The HPLC system consisted of a model 600E multisystem with a 484 UV−vis detector (Waters) equipped with a 300 mm × 3.9 mm i.d. reversed-phase column (Novapack C_{18}, 4 μm; 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.

HPLC C_{18} Chromatography. Purified chelating peptides were injected in a preparative HPLC reverse-phase column (C_{18} Hi-Pore RP-318, 250 mm × 10 mm i.d. Bio-Rad column). The injection volume was 100 μL, and the sample concentration was 20 mg/mL. Elution was achieved by a linear gradient of acetonitrile in water (0−30% in 50 min) containing 0.1% trifluoroacetic acid at a flow rate of 4 mL/min at 30 °C. Elution was monitored at 215 nm, and 11 fractions were collected for assay of antioxidative activity.

RESULTS

Obtaining and Antioxidative Activity of Chickpea Protein Hydrolysates. Chickpea protein hydrolysates were obtained by sequential action of the endoprotease alcalase and the endo/exoprotease flavourzyme. These are microbial proteases that are used in the food industry for the preparation of hydrolysates with improved functional or nutritional properties as compared to the original protein preparations. By starting the protein hydrolysis with the endoprotease alcalase, we achieve a predigestion so that the number of target sites for the action of the endo/exoprotease flavourzyme is increased. In this way, we obtained protein hydrolysates with a higher degree of hydrolysis than using alcalase or flavourzyme alone for the same time.

Figure 1 shows the kinetics of hydrolysis of chickpea protein isolate by these proteases. The rate of hydrolysis with alcalase is very high in the first 30 min, and after that, hydrolysis proceeds more slowly. On the other hand, flavourzyme activity is observed even after 2 h of incubation with the protease.

Aliquots of the protein hydrolysate were taken at intervals to determine their copper chelating capacity by inhibition of

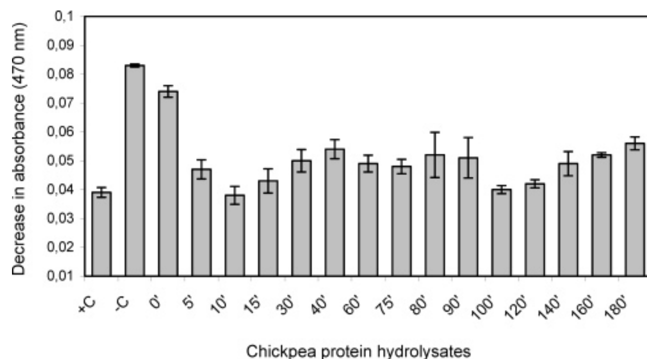

Figure 2. Antioxidative activity of chickpea protein hydrolysates obtained after hydrolysis with alcalase and flavourzyme as shown in **Figure 1** measured as inhibition of β-carotene oxidation (decrease in absorbance at 470 nm) in the presence of copper. Data correspond to the average ± SD of three independent experiments.

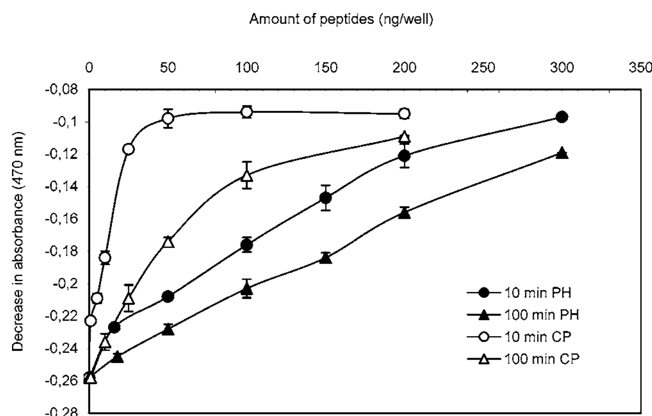

Figure 3. Antioxidative activity of protein hydrolysates obtained after 10 and 100 min of hydrolysis and of the corresponding chelating peptides purified by affinity chromatography, measured as inhibition of β-carotene oxidation (decrease in absorbance at 470 nm) in the presence of copper. Data correspond to the average ± SD of three independent experiments. Key: 10 min PH, protein hydrolysate obtained after 10 min hydrolysis with alcalase; 100 min PH, protein hydrolysate obtained after 100 min hydrolysis with alcalase and 40 min hydrolysis with flavourzyme; 10 min CP, chelating peptides purified from 10 min PH; and 100 min CP, chelating peptides purified from 100 min PH.

β-carotene oxidation in the presence of the metal. **Figure 2** shows the inhibition by the hydrolysates assayed of β-carotene oxidation in the presence of copper. As observed, chickpea protein hydrolysates obtained after 10 min of hydrolysis with alcalase and after 100 min of hydrolysis with alcalase plus flavourzyme (added at 60 min) showed the highest inhibition of β-carotene oxidation in the presence of copper. These hydrolysates possessed a 15.4 and 37.9% degree of hydrolysis, respectively. These chickpea protein hydrolysates with the highest inhibition of β-carotene oxidation were selected for further purification of their copper chelating peptides.

Purification and Antioxidative Activity of Copper Chelating Peptides Purified from Chickpea Protein Hydrolysates. Copper chelating peptides were purified by affinity chromatography from the selected hydrolysates using the Vivapure Metal Chelate Maxi spin columns as described in the Material and Methods. **Figure 3** shows the antioxidative activity of chelating peptides purified from the selected protein hydrolysates as compared with the antioxidative activity of the hydrolysates. As expected, purified chelating peptides protect β-carotene from oxidation by copper better than the original protein hydrolysates.

Table 1. Percent Amino Acid Composition of Chickpea Protein Hydrolysates Obtained after 10 Min of Hydrolysis (10 Min PH) and after 100 Min of Hydrolysis (100 Min PH) and the Corresponding Chelating Peptides Purified from Them (10 Min CP and 100 Min CP, Respectively)[a]

	10 min PH	10 min CP	100 min PH	100 min CP
Asp[b]	15.1 ± 0.15	11.0 ± 0.5	15.2 ± 0.05	12.8 ± 0.55
Glu[c]	21.0 ± 0.0	7.0 ± 0.05	20.2 ± 0.05	5.8 ± 0.5
Ser	6.7 ± 0.0	7.3 ± 0.95	6.7 ± 0.0	6.9 ± 1.8
Hys	2.7 ± 0.05	19.7 ± 0.1	2.6 ± 0.0	35.1 ± 0.35
Gly	4.5 ± 0.0	4.4 ± 0.05	4.5 ± 0.0	4.0 ± 0.2
Thr	3.8 ± 0.0	2.0 ± 0.05	3.9 ± 0.05	2.2 ± 0.05
Arg	9.3 ± 0.05	18.2 ± 0.05	9.3 ± 0.0	15.6 ± 0.9
Ala	4.5 ± 0.0	3.1 ± 0.0	4.5 ± 0.0	2.0 ± 0.1
Tyr	1.9 ± 0.0	1.8 ± 0.05	2.0 ± 0.05	0.8 ± 0.0
Val	4.2 ± 0.0	3.7 ± 0.35	4.5 ± 0.0	3.7 ± 0.45
Met	0.0 ± 0.0	1.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.05
Cys	0.6 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
Ile	3.9 ± 0.0	1.5 ± 0.05	4.2 ± 0.0	1.2 ± 0.0
Leu	8.6 ± 0.0	6.9 ± 0.05	8.6 ± 0.0	2.9 ± 0.05
Phe	6.5 ± 0.05	4.4 ± 0.05	6.5 ± 0.0	2.1 ± 0.05
Lys	6.7 ± 0.0	7.6 ± 0.05	6.8 ± 0.0	4.2 ± 0.03

[a] Data correspond to the average ± SD of three independent experiments. [b] Aspartic acid + asparagine. [c] Glutamic acid + glutamine.

From this graphic was calculated the slope of the straight line fragments of the curves. These slopes show that protein hydrolysate obtained after 10 min of hydrolysis is 1.2 times more antioxidant than that obtained after 100 min of hydrolysis. Chelating peptides purified from protein hydrolysate obtained after 10 min of hydrolysis were 3.1 times more antioxidant than chelating peptides purified from protein hydrolysate obtained after 100 min of hydrolysis. Also, chelating peptides purified from protein hydrolysate obtained after 10 min of hydrolysis were 8.3 times more antioxidant than its parent protein hydrolysate. Thus, these chelating peptides were the most antioxidative in the presence of copper.

Table 1 shows amino acid composition of protein hydrolysates obtained after 10 and 100 min of hydrolysis and of the corresponding purified chelating peptides. Chelating peptides increase the contents of two amino acids, histidine and arginine, with respect to the original protein hydrolysates. Chelating peptides purified from protein hydrolysate obtained after 100 min of hydrolysis show the highest content in histidine, representing 35.1% of total amino acids. Apart from histidine, the other amino acid that increases its content in the purified chelating peptides is arginine.

Further Characterization of Chelating Peptides Obtained after 10 min Hydrolysis with Alcalase. The antioxidative activity of these chelating peptides was compared with that of a chelating agent such as EDTA and with free histidine. **Figure 4** shows that these chelating peptides are more antioxidant than free histidine although less than the same amounts of EDTA. From this graphic was calculated the slope of the straight line fragment of the curves. Comparing the slopes shows the result that EDTA was 1.3 times more antioxidative than the chelating peptides and that these peptides were 4.1 times more antioxidative than free histidine.

The antioxidative activity in the absence of copper of these chelating peptides and of the protein hydrolysates was compared. This antioxidative activity will result from inhibition of β-carotene oxidation by other factors such as light or oxygen. **Figure 5** shows that in the absence of copper, the original protein hydrolysate was more effective than their purified chelating peptides in the inhibition of β-carotene oxidation. From this graphic was calculated the slope of the straight line fragment

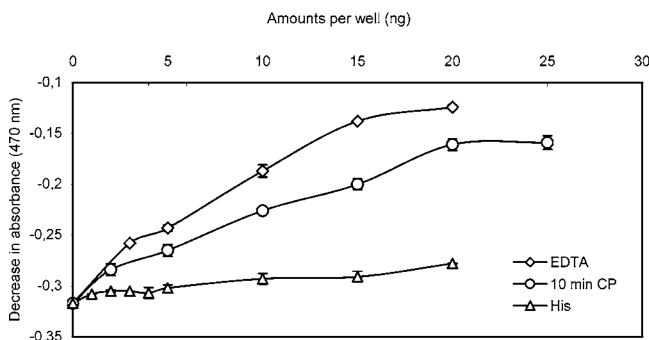

Figure 4. Antioxidative activity of chelating peptides (10 min CP) purified from the protein hydrolysate obtained after 10 min of hydrolysis with alcalase as compared with the antioxidative activity of EDTA and free histidine in the presence of copper. Data correspond to the average ± SD of three independent experiments.

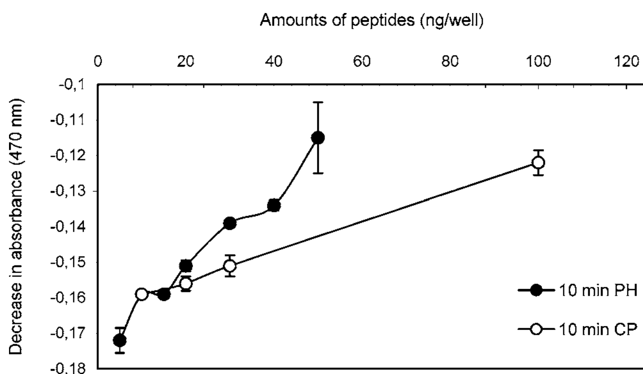

Figure 5. Antioxidative activity in the absence of copper of protein hydrolysate obtained after 10 min of hydrolysis with alcalase (10 min PH) and the corresponding purified chelating peptides (10 min CP). Data correspond to the average ± SD of three independent experiments.

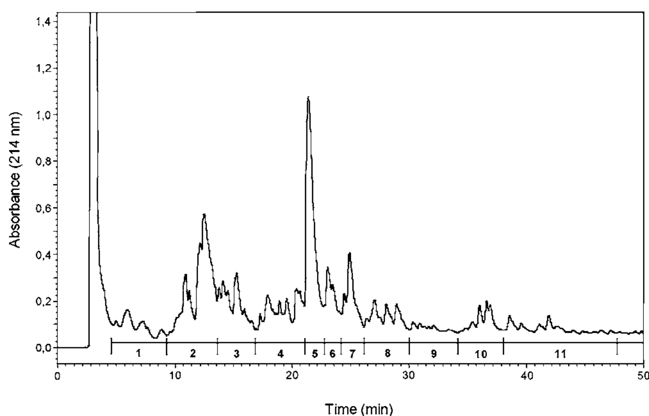

Figure 6. C_{18} reverse-phase HPLC profile of chelating peptides purified from protein hydrolysate obtained after 10 min of hydrolysis with alcalase. Eleven fractions were collected for further determination of antioxidative activity in these fractions.

of the curves. Comparing the slopes, it can be concluded that the protein hydrolysate obtained after 10 min of hydrolysis with alcalase is 2.8 times more protective against environmental oxidation of β-carotene than their purified chelating peptides.

For further characterization of these chelating peptides, they were analyzed by HPLC C_{18} reverse phase chromatography. **Figure 6** shows the peptide profile obtained. This chromatogram was divided into 11 fractions that were collected. **Figure 7** shows the protective activity of these fractions against β-carotene oxidation by copper. The third fraction showed the highest inhibition of β-carotene oxidation in the presence of copper. **Figure 8** shows the correlation between the protective effect against β-carotene oxidation by copper and the percent histidine content of fractions purified after HPLC C_{18} chromatography. As observed, there is a positive correlation between the histidine content and the antioxidative activity. Thus, fractions more antioxidative, such as 2 and 3, possess the highest histidine content, while less protective fractions such as 6, 7, or 11 are with the lowest histidine contents.

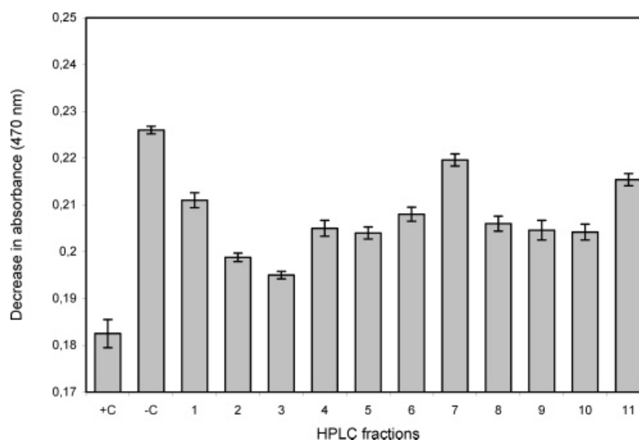

Figure 7. Antioxidative activity of fractions 1–11 collected from HPLC C_{18} chromatography of chelating peptides purified from protein hydrolysate obtained after 10 min of hydrolysis with alcalase. Key: +C, β-carotene alone; −C, β-carotene plus copper.

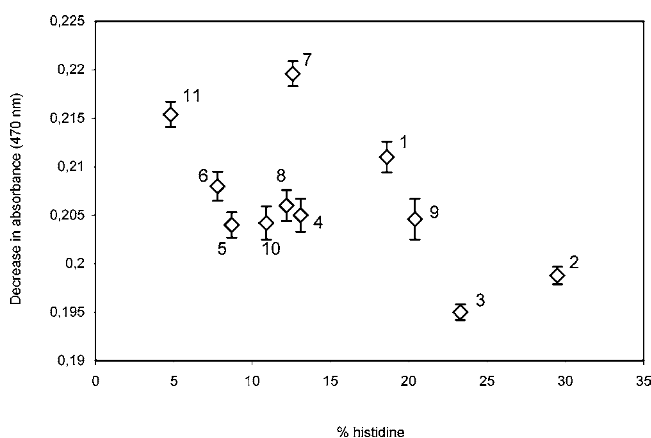

Figure 8. Correlation of the antioxidative activity and the histidine content of fractions (1–11) collected from HPLC C_{18} chromatography of chelating peptides purified from protein hydrolysate obtained after 10 min of hydrolysis with alcalase. Numbers in the graphic identified each fraction according to **Figure 6**.

DISCUSSION

As hydrolysis of chickpea proteins by alcalase and flavourzyme progresses, the degree of hydrolysis increases; that is, the peptide sizes decrease, conferring to protein hydrolysates with different functional, nutritional, and chemical properties with respect to the unhydrolyzed proteins. Thus, protein hydrolysates selected at several hydrolysis times had different activities in inhibiting β-carotene oxidation by copper. The protein hydrolysate obtained after 10 min of hydrolysis of chickpea proteins with alcalase was the most inhibitory of β-carotene oxidation. Further protein digestion with alcalase translated in hydrolysis of these peptides and decreases of their antioxidative activity. However, when flavourzyme, with a

different specificity, was added, a different pool of peptides was produced resulting in a new increase of the antioxidative activity up to a maximum at 100 min of hydrolysis. After this time, and similar to the hydrolysis with alcalase, the antioxidative activity decreases. This process of generation and degradation of bioactive peptides during the digestion of plant proteins with alcalase and flavourzyme has also been observed in the production of angiotensin converting enzyme inhibitory peptides during chickpea proteins hydrolysis with alcalase and flavourzyme (*11*).

Because the amino acid composition does not change as hydrolysis progresses (*9*), other characteristics such as peptide sequence and peptide size should be responsible for the differences in antioxidative properties. The copper chelating peptides that were purified from protein hydrolysates obtained after 10 min of hydrolysis were three times more antioxidative than the peptides obtained from protein hydrolysates generated after 100 min of hydrolysis. Although the two purified fractions were rich in histidine (**Table 1**), the former had a lower histidine content (19.7%) than the latter (35.1%), indicating that the protective effect against β-carotene oxidation is not only determined by the presence of histidine residues. Thus, the antioxidative potential of the peptides is also likely dependent on the peptide size. As an average, the peptides purified from the 10 min of hydrolysate are six or seven residues long, while the peptides purified from the 100 min protein hydrolysate are two or three residues long. Supporting this assumption is the observation that at equal histidine concentrations, free histidine is less effective in preventing β-carotene oxidation by copper than the peptides purified after 10 min of hydrolysis.

Histidine, having a high chelating activity due to its imidazole ring, is directly implicated in peptide binding to the copper containing support. The higher histidine content in the shorter peptides is explained by the fact that their purification was based on their binding to immobilized copper. Thus, the same amount of histidine may be needed for binding to copper in shorter or longer peptides, making the shorter peptides richer in histidine in a weight basis as given in **Table 1**. The purified chelating peptides are also rich in arginine (**Table 1**), which is consistent with observations that arginine is more abundant in positions near histidine in the main pea seed storage protein (data not shown). Nevertheless, the possibility also exists that arginine residues favor binding to the metal as well.

The antioxidative activity of chelating peptides in the absence of copper and protein hydrolysate was compared. Results showed that protein hydrolysate was more antioxidant in the absence of copper, indicating that the protective effect of purified chelating peptides in the presence of copper is related to their chelating activity.

Peptides purified from protein hydrolysates obtained after 10 min of hydrolysis were further fractionated by HPLC chromatography in 11 fractions that were assayed for their antioxidant activity in the presence of copper. Results varied between fractions. In these fractions, belonging to the same protein hydrolysate, i.e., of similar peptide size, a positive correlation between the antioxidant activity and the histidine content was observed. Thus, with similar peptide size, the histidine content determines the antioxidant activity of peptides in the presence of copper. In conclusion, when chelating peptides of different sizes are compared, this characteristic is more important than the histidine content in determining the antioxidative activity in the presence of copper. However, when chelating peptides of similar size are compared, the effectiveness in preventing oxidation of β-carotene by copper is directly related to the histidine content of peptides.

Copper is a physiologically important metal ion that may play a significant role in the endogenous oxidative DNA and lipid damage involved in aging, neurodegenerative disorders, and cancer (*7*). Also, injury to tissues may release copper and copper ions have been detected in atherosclerotic lesions (*19*). Thus, copper chelating peptides purified in this work may be useful not only in preventing the oxidative activity of copper that may damage cells at the luminal space of the stomach but also if transported to the blood strain, which may prevent oxidation, for example, of LDL, that has been shown to be sensitive to oxidation by copper (*8*). Copper chelating peptides may also be useful in target organs, such as the brain, where oxidative process are involved in the development of diseases. For example, oxidative modification of LDL exists in the brain and has been implicated in the pathogenesis of neurodegenerative diseases (*20*).

The use of immobilized metals for the purification of recombinant proteins with histidine tails is extensively employed in molecular biology protocols. Immobilized metals may be useful also in the purification of other types of proteins or peptides (*21*), and different types of metal chelating peptides or proteins may be purified by changing the immobilized metal (Ca, Fe, Zn) to a solid support. Purified chelating peptides may be useful not only in preventing oxidative activity of prooxidant metals such as copper or iron but also in increasing bioavailability of these or other metals such as calcium or zinc. Food mineral fortification is employed to increase absorption of essential minerals such as iron or calcium (*22*). However, mineral supplementation frequently develops undesired secondary effects, such as food lipid oxidation or flavor and appearance deterioration. Thus, chelating peptides purified from food protein hydrolysates may be useful in increasing mineral bioavailability while maintaining food quality and appearance. This paper does not address the bioavailability of the chickpea chelating peptides. Thus, it is under investigation in our laboratory whether these peptides will be resistant to hydrolysis by proteases and peptidases in the digestive system and whether they can be absorbed from the digestive tract. Another important issue that will have to be addressed in the future is whether the action of physiological proteases and peptidases on chickpea protein leads to formation of chelating peptides as those resulting from hydrolysis by alcalase and flavourzyme.

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