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Affinity Purification of Copper-Chelating Peptides from Sunflower Protein Hydrolysates

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Copper-chelating peptides were purified from sunflower protein hydrolysates by affinity chromatography using immobilized copper. A variety of protein hydrolysates were obtained by incubation with the proteases Alcalase and Flavourzyme for different periods of time. Chelating activity was indirectly determined by measuring the inhibitory effect of hydrolysates on the oxidation of β-carotene by copper. Copper-binding peptides purified from the two hydrolysates that inhibited oxidation by copper the most contained 25.4 and 42.0% histidine and inhibited β-carotene oxidation 8 and 3 times more than the original hydrolysates, which had 2.4 and 2.6% histidine, respectively. Thus, histidine content is not the only factor involved in antioxidant activity, and probably other factors such as peptide size and amino acid sequence are also important. This work shows that affinity chromatography can be used for the purification of copper-chelating peptides and probably other metals of nutritional interest such as calcium, iron, and zinc. In addition to their antioxidant potential, chelating peptides are of nutritional interest because they increase bioavailability of minerals.

KEYWORDS: Chelating peptides; sunflower; protein hydrolysate

INTRODUCTION

Bioactive peptides 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. Many bioactive peptides with different activities have been purified from plant and animal proteins, including peptides with antihypertensive, immunomodulatory, opioid, antioxidant, hypocholesterolemic, and metal-chelating activity (*1*−*3*). Metal-chelating peptides include the caseinophosphopetides, which are peptides produced by hydrolysis of casein that bind to metals such as calcium and increase their absorption (*4*). The antioxidative activity of these peptides has been related to their chelating properties (*5*).

Copper is an essential trace element that plays a vital role as a cofactor for a variety of enzymes. Binding to the amino acids histidine, methionine, and cysteine and to certain small peptides mediates absorption of copper through an amino acid transporter, and specific chaperones that bind copper with high affinity have been found in mammals (*6*). In addition to its role as an essential trace mineral, copper can also have a pro-oxidant activity similar to that of iron. Thus, copper can induce oxidative damage to DNA and low-density lipoprotein (LDL) (*7*). Copper-chelating peptides in the diet might prevent copper-induced in vivo oxidative damage, including LDL oxidation, which has been involved in atherogenesis because it facilitates the transformation of macrophages into foam cells in the arterial wall.

The production of sunflower protein hydrolysates with better functional and nutritional properties than the original native proteins has been described before (*8*, *9*). These protein hydrolysates can also be a source of bioactive peptides as reported for the purification of angiotensin-converting enzyme (ACE) inhibitory peptides (*10*).

Bioactive peptides are usually purified by using a combination of chromatographic techniques. These may include enrichment in bioactive peptides by filtration followed by purification using gel filtration or ion exchange chromatography. Most often, HPLC reverse-phase chromatography is used to further purify the bioactive peptides. Affinity chromatography is a powerful protein purification method 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. Affinity chromatography may be useful in the purification of bioactive peptides as described for the purification of ACE inhibitory peptides using ACE immobilized on a glyoxyl-agarose support (*11*, *12*). Following a similar approach, the affinity purification of copper-chelating peptides from sunflower protein hydrolysates using immobilized copper is described in this paper. These copper-chelating

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peptides were indirectly determined by measuring their inhibitory effect on the copper-induced oxidative degradation of β-carotene.

MATERIALS AND METHODS

Materials. Diethyl ethoxymethylenemalonate was purchased from Fluka (Buchs, Switzerland). Amino acids standards, D,L-α-aminobutyric acid, trinitrobenzenesulfonic acid (TNBS), ethylendiaminetetraacetic acid (EDTA), histidine, and β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Alcalase 2.4 L and Flavourzyme 1000 MG were provided by Novo Nordisk (Bagsvaerd, Denmark). Alcalase 2.4 L is an endopeptidase from *Bacillus licheniformis* containing subtilisin carlsberg as the major component and has 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 leucine amino-peptidase unit (LAPU) per gram. Defatted sunflower meal was supplied by MIGASA (Sevilla, Spain). All other chemicals were of analytical grade.

Preparation of Sunflower Protein Isolates. Sunflower protein isolates were obtained according to the method of Sánchez-Vioque et al. (*13*) with modifications. Defatted sunflower meal (20 g) was extracted by stirring for 1 h in 200 mL of 0.2% NaOH (pH 12) at room temperature. The pellet obtained by centrifugation at 8000*g* for 30 min was extracted once more under the same conditions. The pH of the combined supernatants was adjusted to an isoelectric point of 4.3, and the resulting precipitate was recovered by centrifugation as described above and freeze-dried after washing with water.

Preparation of Sunflower Protein Hydrolysates. Sunflower protein hydrolysates were obtained according to the method of Clemente et al. (*14*) with modifications. Sunflower 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); temperature, 50 °C; and enzyme/substrate ratios and pH were 0.3 AU g^{-1} and pH 8 for Alcalase and 100 LAPU g^{-1} and pH 7 for Flavourzyme. Alcalase was added at time 0 and Flavourzyme 60 min later. Proteases 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 (*15*). The total number of amino groups was determined in a sample of protein isolate hydrolyzed by treatment with 6 mol/L HCl at 120 °C for 24 h.

Assay of β-carotene oxidation was carried out in a 96-well plate as previously described with modifications (*16*). Tween 20 (1 mL) was added to a solution of β-carotene in chloroform (10 mg in 1 mL), and the chloroform in the resulting mixture was evaporated under nitrogen. Aliquots were dissolved in 100 mM (pH 7) phosphate buffer and added to wells containing 10 μL of 50 μM $CuSO_4$. Final concentrations were 298 μM β-carotene and 2.5 μM Cu^{2+} in a final volume of 200 μL containing different concentrations of peptides. The degradation of β-carotene was monitored by recording the decrease in absorbance at 470 nm using a microplate spectrophotometer.

Purification of Chelating Peptides. Selected sunflower protein hydrolysates were used for purification of chelating peptides. The hydrolysates, dissolved in 0.1 M sodium phosphate buffer (pH 7), were loaded on Vivapure Metal Chelate Maxi spin columns (Vivascience, Sartorius, Madrid, Spain) that had been previously charged with copper following the manufacturer's instructions. After the column had been washed with several volumes of phosphate buffer, chelating peptides were eluted from the column using 0.1 M sodium phosphate buffer (pH 3.5).

Amino Acid Analysis. Samples (10 mg) were treated with 4 mL of 6 mol/L HCl in tubes sealed under nitrogen for 24 h at 110 °C for hydrolysis. Amino acids were determined by high-performance liquid chromatography (HPLC) of the derivatives obtained by reaction with diethyl ethoxymethylenemalonate, according to the method of Alaiz et al. (*17*).

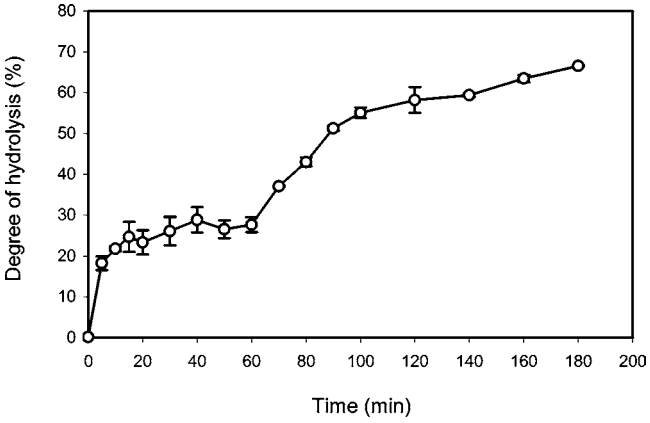

Figure 1. Time course of the hydrolysis of sunflower protein isolate by Alcalase (added at time 0 min) and Flavourzyme (added 60 min later). Data correspond to the average ± SD of three determinations.

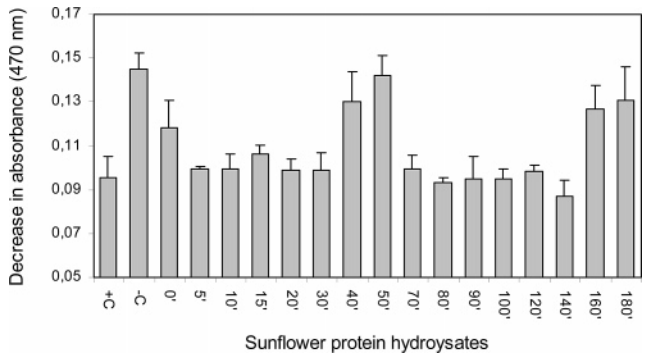

Figure 2. Antioxidative activity of sunflower protein hydrolysates obtained by treatment with Alcalase and Flavourzyme as shown in **Figure 1**. The copper-induced oxidation of β-carotene, which results in a decrease in absorbance at 470 nm, was determined in the presence of the hydrolysates. +C, β-carotene alone; –C, β-carotene plus copper. Data correspond to the average ± SD of three independent experiments.

HPLC C_{18} Chromatography. Chelating peptides previously purified by affinity chromatography were injected (100 μL, 20 mg/mL) in a preparative HPLC reverse-phase column (C_{18} Hi-Pore RP-318, 250 mm × 10 mm i.d. Bio-Rad column) kept at 30 °C. A linear gradient of acetonitrile in water (0−30% v/v in 50 min,) containing 0.1% trifluoroacetic acid at a flow rate of 4 mL/min, was used, and the eluent was monitored by measuring absorption at 215 nm.

RESULTS

Production of Copper-Chelating Sunflower Protein Hydrolysates. Sunflower protein hydrolysates were obtained by treatment with the endoprotease Alcalase followed by treatment with the endo/exoprotease Flavourzyme. As shown in **Figure 1**, hydrolysis by Alcalase was essentially over after 15 min, but hydrolysis by Flavourzyme took longer. This procedure yields degrees of hydrolysis higher than those obtained using either Alcalase or Flavourzyme alone (*18*). Alcalase and Flavourzyme are microbial protease preparations that are used by the food industry to improve the functional and nutritional properties of protein preparations.

Samples of the protein hydrolysates taken at different times were indirectly assayed for their copper-chelating capacity by determination of their inhibitory effect on the copper-induced oxidation of β-carotene (**Figure 2**). Although all of the samples inhibited β-carotene oxidation, those taken between 5 and 30 min and between 70 and 140 min had the highest inhibitory effects. Two of these hydrolysates, those obtained 30 (A30) and

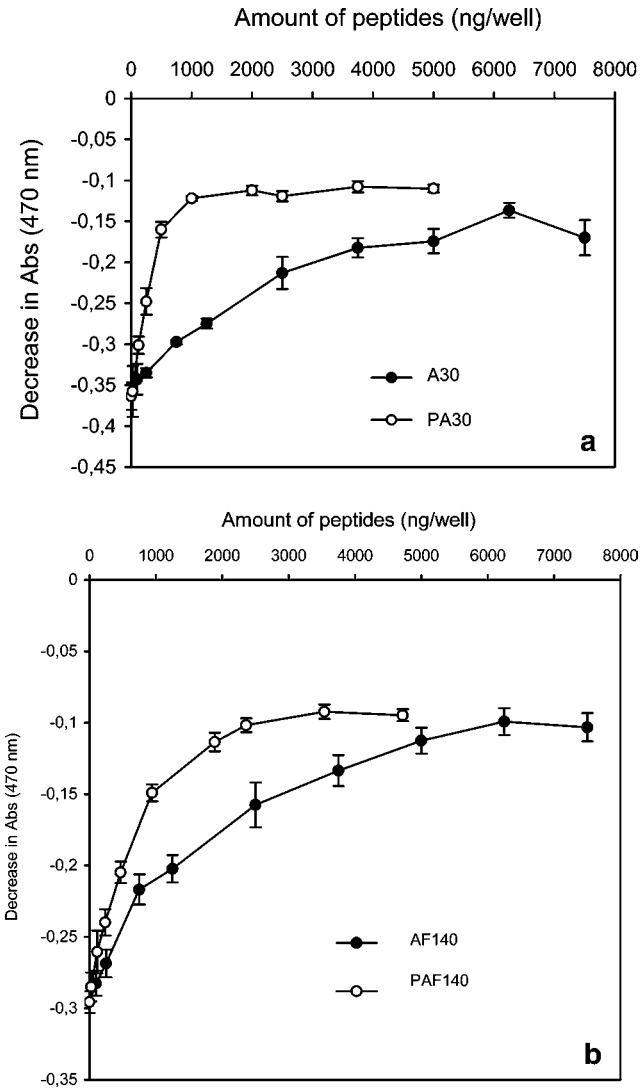

Figure 3. Antioxidative activity of hydrolysate A30 (**a**), hydrolysate AF140 (**b**), and the corresponding peptides (PA30 and PAF140) purified by affinity chromatography. Data correspond to the average ± SD of three independent experiments.

140 min (AF140) after the addition of Alcalase and Flavourzyme, respectively, were used for the purification of copper-chelating peptides.

Purification of Copper-Chelating Peptidic Fractions. Purification of copper-chelating peptides was carried out by affinity chromatography using copper immobilized in spin columns as described under Material and Methods. The antioxidative activity of the purified peptide fractions was much higher than that of the parent hydrolysates as shown in **Figure 3**. The slope of the straight portion of these graphs was used as a numeric estimate of the antioxidant effect. Thus, the antioxidant effect of hydrolysate AF140 was 2-fold higher than the effect of hydrolysate A30, although the peptides purified from A30 had twice the inhibitory effect as the peptides purified from AF140. These peptides had an inhibitory effect 7 times higher than that of the original A30 hydrolysate. The contents in histidine and arginine were higher in the purified peptides than in the corresponding hydrolysates (**Table 1**) and reached maxima of 42 and 22% total amino acids in PAF140, respectively.

Further Characterization of the Peptides Purified from Hydrolysate A30. The antioxidant activity of the peptides purified from hydrolysate A30 was compared with the activities of the chelating agent, EDTA, and the free amino acids histidine and arginine (**Figure 4**). The purified peptides and EDTA showed similar antioxidative activities, saturating at 500 ng per well, which was higher than the antioxidant activity of free histidine, saturating at 250 ng, and arginine, which did not show any protective effect against copper-induced β-carotene oxidation.

To find out whether factors other than the chelation of copper are involved in the antioxidant effect of the peptides, the antioxidative activity in the absence of copper was also studied (**Figure 5**). In the absence of copper, oxidative degradation of β-carotene is most likely the result of autoxidation initiated by light in the presence of oxygen or the result of oxidative degradation of pre-existing peroxides, although the presence of catalyzing trace metal ions cannot be ruled out. As shown in **Figure 5**, the peptides that were purified by affinity chromatography using immobilized copper are also more antioxidant than the original A30 hydrolysate in the absence of copper. Chelating peptides saturate at 100 ng per well, whereas the protein hydrolysate saturates at 500 ng per well.

Table 1. Percent Amino Acid Composition of Hydrolysates A30, AF140, and the Corresponding Chelating Peptides PA30 and PAF140[a]

	A30	PA30	AF140	PAF140
Asp[b]	11.0 ± 0.34	6.4 ± 0.42	11.4 ± 0.21	4.2 ± 0.14
Glu[c]	23.3 ± 0.33	9.7 ± 0.28	22.8 ± 0.21	10.4 ± 0.38
Ser	5.2 ± 0.5	6.7 ± 0.4	5.6 ± 0.35	2.1 ± 0.07
His	2.4 ± 0.18	25.4 ± 1.20	2.6 ± 0.21	42.0 ± 1.8
Gly	6.4 ± 0.25	5.7 ± 0.5	5.9 ± 0.28	2.8 ± 0.42
Thr	4.3 ± 0.4	3.1 ± 0.85	4.2 ± 0.07	0.8 ± 0.14
Arg	9.5 ± 0.68	15.9 ± 1.2	10.1 ± 0.14	22.5 ± 0.39
Ala	4.9 ± 0.23	4.5 ± 0.42	4.8 ± 0	2.1 ± 0.42
Pro	4.6 ± 0.86	0 ± 0	2.5 ± 0.14	0 ± 0
Tyr	2.2 ± 0.18	1.3 ± 0.28	2.4 ± 0	0.7 ± 0
Val	4.8 ± 0.13	2.7 ± 0.07	5.7 ± 0	3.8 ± 0.27
Met	0.8 ± 0.07	3.2 ± 0.7	1.3 ± 0.14	0 ± 0
Cys	0.9 ± 0.42	1.4 ± 0.5	0.8 ± 0.07	0 ± 0
Ile	3.9 ± 0.13	1.7 ± 0.14	4.4 ± 0	1.2 ± 0.28
Leu	7.5 ± 0.13	3.9 ± 0.35	7.1 ± 0.07	1.7 ± 0.5
Phe	6.5 ± 0.19	5.4 ± 0.21	5.6 ± 0	1.3 ± 0.14
Lys	3.1 ± 0.28	3.4 ± 0.21	3.2 ± 0	1.8 ± 0.07

[a] Data correspond to the average ×b1 SD of three independent experiments. [b] Aspartic acid + asparagine. [c] Glutamic acid + glutamine.

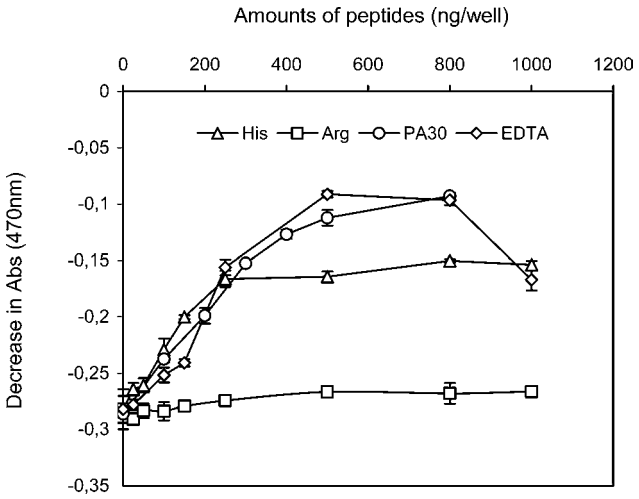

Figure 4. Antioxidative activity of purified peptides PA30, EDTA, and free histidine, as determined by their effect on the copper-induced oxidative degradation of β-carotene. Data correspond to the average ± SD of three independent experiments.

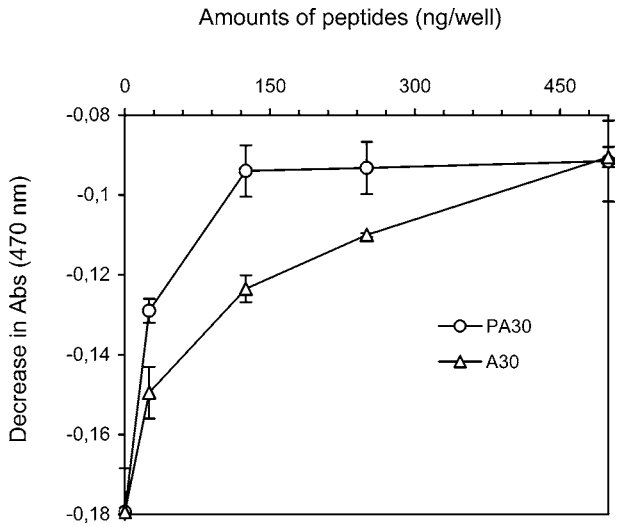

Figure 5. Antioxidative activity of hydrolysate A30 and the corresponding purified peptides, PA30, in the absence of copper. Data correspond to the average ± SD of three independent experiments.

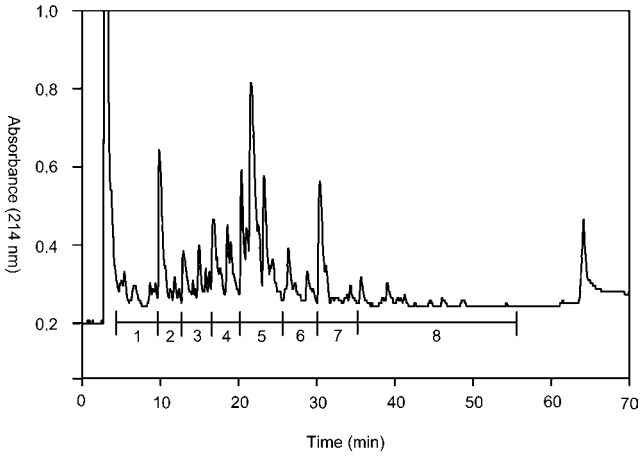

Figure 6. C_{18} reverse-phase HPLC profile of purified peptides PA30. Eight fractions were collected for determination of antioxidative activity.

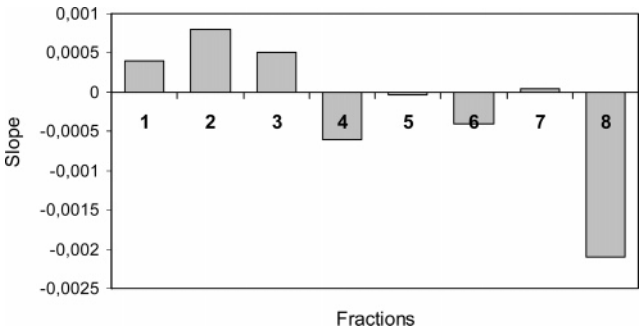

Figure 7. Antioxidative activity of fractions 1–8 collected as shown in **Figure 6** by HPLC reverse-phase chromatography of PA30.

Preparative HPLC C_{18} reverse-phase chromatography of the peptides purified by affinity chromatography was carried out to study the antioxidant effect of the resulting fractions. Thus, the affinity-purified preparation was further resolved and separated into eight fractions (**Figure 6**) that were assayed for antioxidant activity. As described above, the slopes of the straight portions of the graphs were used as an index of the antioxidant effect of each fraction. As shown in **Figure 7**, only fractions 1−3 had antioxidant activity. Fractions 5 and 7 had no effect on β-carotene oxidation, whereas fractions 4, 6, and 8 were prooxidant. **Figure 8** shows that there is a positive correlation ($R^2 = 0.36$) between histidine content and antioxidant activity in these fractions. Thus, fraction 2, having the highest antioxidant effect, also has the highest histidine content, whereas fraction 8, having the highest prooxidant effect, has the lowest histidine content.

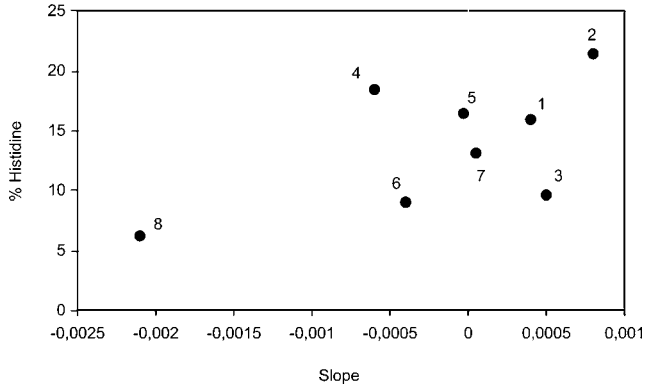

Figure 8. Correlation of antioxidative activity and histidine content in fractions 1–8 collected as described in **Figure 6** by HPLC reverse-phase chromatography of PA30. Numbers identify each fraction according to **Figure 6**.

DISCUSSION

Depending on how extensive hydrolysis is, treatment of sunflower proteins with Alcalase and Flavourzyme renders peptides of different size and sequence that have different functional, nutritional, and chemical properties than the original, unhydrolyzed proteins. Thus, the duration of the hydrolytic treatment determines the inhibitory effect of the resulting peptides on the oxidation of β-carotene catalyzed by copper. Hydrolysis with Alcalase initially translates into an increase in antioxidative activity, although at longer times of hydrolysis the antioxidant effect diminishes, probably due to hydrolysis of antioxidant peptides into smaller peptides and/or amino acids. Similarly, the addition of Flavourzyme led to an increase in the antioxidant effect of the hydrolysates that again goes down with longer incubation times with the protease. This process of generation and degradation of bioactive peptides during the digestion of plant proteins with Alcalase and Flavourzyme has also been observed during the production of angiotensin-converting enzyme inhibitory peptides (*19*) and copper-chelating peptides (*16*) by treatment of chickpea proteins with the same enzymatic preparations.

Because the amino acid composition of hydrolysates does not change as hydrolysis progresses (*14*), other factors such as peptide sequence and size should be responsible for the differences in antioxidative properties. The antioxidant effect of the copper-chelating peptides that were purified from hydrolysate A30 was 3 times higher than the antioxidant effect of the peptides purified from AF140. Although the two purified fractions were rich in histidine (**Table 1**), the former had a lower histidine content (25.4%) than the latter (42.0%), indicating that the protective effect against β-carotene oxidation is not only determined by the presence of histidine residues but also likely dependent on peptide size. As an average, the peptides purified from the hydrolysate A30 are three or four residues long, whereas the peptides purified from hydrolysate AF140 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 from A30.

Histidine, having a high chelating activity due to its imidazole ring, is directly implicated in peptide binding to copper. 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 on a weight basis as given in **Table 1**. Similar results were obtained during the purification of copper-chelating peptides from chickpea protein hydrolysates (*16*). The abundance of arginine in the purified chelating peptides is probably due to the abundance of this residue next to or near histidine residues, although it might also be due to a possible effect of arginine on binding to copper. It was interesting to observe that the purified peptides were also more effective than the original hydrolysates in the inhibition of the oxidative degradation of β-carotene in the absence of copper. A positive correlation between antioxidant activity and histidine content was observed in the peptides purified by reverse-phase chromatography from hydrolysate A30 Thus, it appears that histidine content determines the antioxidant activity in peptides of similar size, although this is not true when peptides of different sizes are compared.

The inhibition of the copper-catalyzed oxidative degradation of β-carotene has been used as an indirect method to measure the presence of copper-chelating peptides in the present work. In addition, this antioxidant property of the peptides is of importance by itself. Thus, copper is a physiologically important metal ion that may play a significant role in the endogenous oxidative degradation of DNA and lipid damage that have been involved in aging, neurodegenerative disorders and cancer (*6*). In addition, copper may be released in injured tissues and has been detected in atherosclerotic lesions (*20*). Thus, copper-chelating peptides such as those described in this paper may be useful not only in preventing oxidative activity of copper in the digestive tract but also in preventing oxidative damage to LDL in blood and tissues after copper is absorbed into the blood stream (*7*, *21*).

The use of immobilized metals for the purification of recombinant proteins carrying histidine tails is extensively employed in molecular biology protocols. Immobilized metals may be useful also in the purification of different types of metal-chelating peptides and proteins by immobilization of diverse metals such as calcium, iron, and zinc (*22*). Purified chelating peptides in this way may be useful in increasing bioavailability of metals such as calcium, iron, and zinc. Food mineral fortification is employed to increase absorption of essential minerals such as iron or calcium (*23*). However, mineral supplementation frequently leads to the development of undesired secondary effects such as lipid oxidation and deterioration of flavor and appearance in foods. Thus, chelating peptides purified from food protein hydrolysates may be useful in increasing mineral bioavailability while maintaining food quality and appearance.

It is under investigation in our laboratory whether the action of physiological proteases such as pepsin and pancreatin on sunflower proteins leads to the formation of chelating peptides such as those resulting from hydrolysis by Alcalase and Flavourzyme.

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