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Production of Porcine Hemoglobin Peptides at Moderate Temperature and Medium Pressure under a Nitrogen Stream. Functional and Antioxidant Properties

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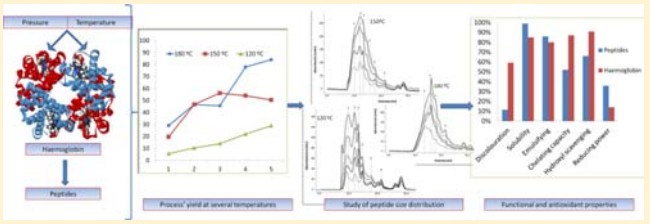
ABSTRACT: A new hydrolysis method for producing peptides from porcine hemoglobin has been developed. Current processes are based on the use of expensive enzymes or high hydrostatic pressures. In the present study, a cheap and effective process has been assayed to produce peptides from purified porcine hemoglobin. A solution of purified hemoglobin is heated at different temperatures and pressurized at 4 MPa while a stream of nitrogen is injected into the reactor. A total of 82% of initial hemoglobin was transformed into peptides presenting an average size of 3.2 kDa. Some preferential hydrolyzed bonds have been detected. The peptide size distribution was evaluated at different times and temperatures. It has been demonstrated that this technique produces large amounts of peptides possessing good antioxidant properties. Furthermore, functional properties are conserved, and a desirable decrease in color (80%) is achieved.



KEYWORDS: *porcine hemoglobin, nonenzymatic hydrolysis, antioxidant properties, functional properties*

1. INTRODUCTION

Slaughterhouses currently waste large volumes of blood. Rendueles et al.[1] have reported that this represents the most problematic byproduct of the meat industry due to the high volumes generated and the major polluting power of this waste blood. As regulations impede the discharge of blood to wastewater treatment plants for subsequent treatment, different removal techniques (drying, incineration, etc.) are usually employed to treat this byproduct. Moreover, seeing as proteins constitute one of the main components of blood and given that these molecules have an important economic value, it would seem logical to consider the feasibility of recovering the protein contained in slaughterhouse blood.[2] The most abundant protein in blood is hemoglobin, which represents 12−18% of the total composition of blood.

Hemoglobin can be easily purified and processed to produce several products, heme iron supplements,[3,4] peptides with different applications such as antimicrobial effects[5] and antioxidant effects,[6] iron-binding peptides,[7] and inhibitors of hypertension or regulators of glucose absorption,[8] or simply used as an ingredient to enhance the protein and amino acid content of food products. In the majority of these cases, the hydrolysis of porcine hemoglobin is carried out using different enzymes such as alcalase, trypsin, or pepsine. These methods require expensive chemical reagents and highly controlled conditions, pH, temperature, and enzyme/protein ratio, thereby notably increasing the cost of the process. The use of subcritical conditions to obtain amino acids has been reported in refs 9−11. The protein is hydrolyzed under moderately high pressures (15−27 MPa) and elevated temperatures (250−300 °C), but these methods are employed to produce large amounts of free amino acids. Recently, the combined use of enzymes and high hydrostatic pressures (HHPs) has been employed to hydrolyze hemoglobin, resulting in a decrease in incubation time[12] or more hydrolysis cleavage sites[13] due to the opening up of the protein structure.

It is known that HHPs at room temperature produce denaturation in proteins while keeping their molecular size constant.[14] If temperature is increased, pressure can be decreased to moderate values, and the proteins still remain unfolded, as has been shown by Smeller[15] in phase diagrams of biomolecules. The first step of thermal denaturation is loss of the quaternary structure (should it exist, as in hemoglobin), which is the most sensitive reaction to pressure.[16] Subsequently, the tertiary structure is unfolded in a direct relation with pressure and temperature. The completely denatured protein presents more bonds susceptible to hydrolysis.

Research into the production of peptides is related to the enhancement of functional properties or antioxidant properties when they are used as an ingredient in foodstuffs. It has been reported[17,18] that low molecular weight peptides (especially di- and tripeptides) with free amino acids have high nutritional and therapeutic values.[19,20] On the other hand, large molecular weight peptides (more than 20 amino acid residues) are presumed to be associated with an improvement in the functional properties of hydrolysates. The functional properties of native proteins from porcine blood have been previously

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studied,[21] so a comparison may be made. The peptides produced with this technique can broaden the range of applications of blood proteins.

The interest in peptides has increased in recent years, focusing mainly on peptides that present bioactivity. These have drawn attention due to their low molecular weight, which allows their easy absorption and high antioxidant activity, thus making low molecular size peptides desirable products. When the generation and scavenging of oxidative compounds is not balanced, antioxidative molecules are able to reduce the damage caused.[22] Peptides from several sources, always produced by enzymatic hydrolysis, have been isolated and subsequently tested as antioxidant compounds.

In this study, a new hydrolysis method has been used to produce peptides of low molecular weight: Three temperatures (120, 150, and 180 °C) were assayed at medium pressure (4 MPa) with nitrogen injection. The influence of temperature on the final average peptide size, the process yield, and the production of amino acids and ammonium compounds was evaluated, subsequently determining the functional properties and antioxidant activity of the peptides thus produced.

2. MATERIALS AND METHODS

All reagents (chloroform, sodium citrate, leucine, Nessler's reagent, sodium hydroxide, hydrochloric acid, sodium chloride, cotton oil, potassium ferrocyanide, trichloroacetic acid, ferric chloride, EDTA–Fe^{2+}, safranin O, H_2O_2, and ferrozine) were used in analytical reagent grade and were supplied by Sigma-Aldrich.

2.1. Hemoglobin Purification. Fresh blood was collected from the Junquera-Bobes slaughterhouse (Noreña, Asturias, Spain) in plastic bottles with the addition of pure sodium citrate at 2% (w/v) of total sampled blood. The whole blood was centrifuged at 10000*g* for 5 min at 5–10 °C (Kubota 6700 centrifuge), and the plasma thus obtained (around 60% of total blood volume) was separated by decantation. The hemoglobin was extracted by osmotic shock by adding distilled water to the red cells (1:1, v/v). Finally, chloroform was added (1:4, v/v, chloroform/aqueous red cell solution), and the solution was gently stirred and centrifuged (10000*g*, 10 min) to remove plasma membranes from the media. The supernatant is composed of an aqueous phase containing the hemoglobin, which was recovered, freeze-dried (Telstar Cryodos), and stored at −20 °C until use.

2.2. Hydrolysis Setup. Lyophilized hemoglobin was diluted in distilled water to obtain a final concentration of 50 mg/mL; 400 mL of this solution was then placed in a jacketed reactor. The hydrolysis parameters were established at a 120, 150, or 180 °C temperature and a 250 rpm stirring rate. A pressure of 4 MPa was achieved using a nitrogen stream of 1000 mL/min, controlled by a precision electrovalve (Brooks mass flow controller 5850). The injected gas was previously saturated with water to avoid sample evaporation and heated to keep the reactor temperature constant. A back-pressure valve allowed pressure control in the system; the excess of gas expelled via this valve was bubbled through 500 mL of acid solution to retain ammonium compounds. A scheme of the reactor is shown in Figure 1.

Samples were taken at different times of hydrolysis over a period of 6 h. The samples were cooled using a water-cooled glass spiral.

2.3. Peptide Size. An AKTA FPLC system (GE Healthcare, Uppsala, Sweden) was employed to determine the peptide size distribution using a size exclusion column (Superdex Peptide 10/300 GL). The buffer was composed of 50 mL of Trizma (pH 7.6, 1 M) and 950 mL of distilled water. Samples were filtered through 0.45 μM poly(vinylidene fluoride) (PVDF) membranes (Millipore) before injection. The elution flow was adjusted to 1 mL/min, and the results were recorded at a wavelength of 280 nm. The column was previously calibrated with standard mass markers supplied by Sigma: blue dextran (2 000 000 Da), lysozime (14 400 Da), cytochrome *c* (12 400 Da), aprotinin (6600 Da), and cianocobalamin (1355 Da) (R^2 = 0.99). The obtained peptides presented a molar extinction coefficient different from that of hemoglobin, so calibrants for peptide concentration were prepared. Freeze-dried peptides solved in water were employed as calibrants (0–40 mg/mL; R^2 = 0.99). The obtained chromatograms were processed using Unicorn 5.1 software.

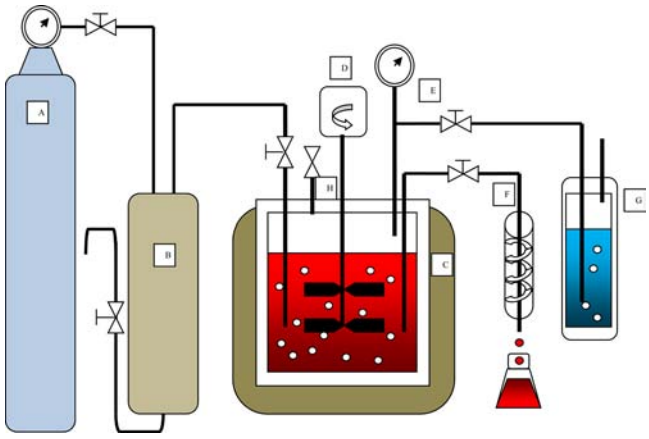

Figure 1. Scheme of the experimental setup: (A) gas tank, (B) humidifier, (C) jacketed reactor, (D) agitation, (E) back-pressure valve, (F) sample cooler, (G) bubbler, (H) rupture disk.

2.4. Amino Acid Quantification. The amino acids produced in the hydrolysis process were quantified by the method developed by Rosen,[23] which is a spectroscopy method based on the appearance of colored compounds when amino acids and ninhydrin react. Leucine (0.04–0.4 μM) was used to generate the calibration curve.

2.5. Ammonia Quantification. Small amounts of ammonium compound were formed during the process, the method developed by Nessler being employed to determine the exact concentration. A calibration curve created from ammonium chloride solution in the range of 1–14 ppm was used. A 10 mL volume of sample and 1 mL of Nessler's reagent (supplied by Sigma) were mixed together, and the absorbance was measured at 410 nm (Thermo Scientific, Helios γ UV–vis) after 10 min of reaction at room temperature.

2.6. Functional Properties. *2.6.1. Solubility.* The solubility of samples was studied in an aqueous medium. Samples were prepared by dissolving 0.5 g of the sample in 10 mL of distilled water. The solution was centrifuged at 2400*g* for 30 min in a Kubota model 6700 centrifuge. The pH was varied to test its effect on solubility. Different pH solutions were prepared by adding HCl or NaOH (1 N) to the solution to obtain the desired pH, the tested pH ranging between 3 and 8. The amount of soluble protein was determined by evaluating the decrease in absorbance at 280 nm (Thermo Scientific, Helios γ UV–vis). All experiments were carried out in triplicate. Solubility was calculated according to the following equation:

$$S = \frac{\mathrm{Pd}}{\mathrm{Pt}} \times 100 \quad (1)$$

where *S* is the solubility (%), Pd the absorbance of soluble peptide, and Pt the absorbance of the total peptide used in the assay.

2.6.2. Gelling Properties. The protein samples were frozen at −80 °C for 24 h and dried in a lyophilizer (Telstar Cryodos) setup to prepare protein gels for the analysis of their gelling properties.

The gelling properties of the samples were measured in two ways: analyzing the lowest gelation concentration (LGC)[24] and the temperature of gelification (Tgel). A range of concentrations must be tested to determine the LGC of each sample. In this study, the tested range was 1–20% (w/v) aqueous solutions of freeze-dried peptides. The pH was adjusted to 6 using NaOH or HCl, and the solutions were heated in a thermostatic bath at 85 °C for 30 min. After heat-induced gelation, samples were cooled and stored at 4 °C for 24 h. The LGC is the lowest concentration of protein in the tested range in which the test tube is inverted and the gel does not slide down the tube; i.e., it is consistent.

Table 1. Mass Percentage of Soluble Peptide Recovered with Respect to Initial Hemoglobin Employed[a]

	120 °C					150 °C					180 °C				
	40 min	100 min	220 min	300 min	400 min	75 min	105 min	165 min	280 min	345 min	90 min	180 min	270 min	360 min	420 min
>10 kDa	2.8	5.0	7.4	12.2	17.6	10.4	17.0	23.8	25.8	21.4	6.4	6.8	4.2	6.2	5.6
10−6 kDa	0.4	1.4	1.6	2.4	3.0	2.2	3.6	5.8	7.8	7.0	3	3.8	3.6	7.4	7.8
6−1 kDa	2.0	1.8	2.0	3.2	4.0	3.2	5.2	9.0	9.0	13.6	9.2	17.2	16	26.2	29.8
<1 kDa	0.2	2.4	2.8	4.0	4.4	3.8	5.0	7.8	11.4	8.4	10.6	18.4	21.8	38.2	40.6

[a]Each molecular mass is represented at a different time and temperature.

The Tgel measurements were carried out on a rheometer (Haake MARS) fitted with a plate−plate system and a 1 mm gap between plates. Freeze-dried peptides were dissolved in water to obtain a final protein concentration of 10% (w/w), and the pH was adjusted to 6, as in the LGC tests.[25]

Temperature sweeps at 2 °C/min were recorded from 20 to 90 °C, the samples being covered with mineral oil to prevent evaporation. The deformation (γ) was adjusted to 1%, and the oscillation frequency was 1 rad/s. The storage modulus (G') and elastic modulus (G'') were recorded. Tgel is determined when a crossover between the plots of G' and G'' takes place.[26]

2.6.3. Emulsifying Properties. The emulsifying capacity was determined by the Inklar and Fortuin method.[27] Freeze-dried peptides were used in concentrations of 4 and 10 mg/mL in a final volume of 10 mL. The solvent used to prepare the peptide samples was water with NaCl (0.075%, w/v); this salt increases the protein's emulsifying capacity because protein folding is induced. Subsequently, 13 mL of cotton oil was added to each sample. After the sample was stirred for 15 min, it was centrifuged at 1200*g* for 10 min (Kubota 6700 centrifuge). Triplicates were obtained of all experimental results. Emulsification was calculated as

$$E = \frac{\text{Ve}}{\text{Va}} \times 100 \quad (2)$$

where E is the emulsification (%), Ve the volume of the emulsified oil (mL) and Va the volume of the added oil (mL).

The formation of an emulsion was confirmed by particle size determination using a Zetasizer Nano-ZS (Malvern Instruments) system. Emulsion stability was determined by means of a Turbiscan Lab Expert (Formulaction Co., L'Union, France). Emulsion samples were placed without dilution in a cylindrical glass of 40 mm length, and the transmitted light (180° from the incident light) and backscattered light (45° from the incident light) were monitored as a function of time and cell height for 24 h at 30 °C. The migration velocity was determined from the generated transmission and backscattering profiles.[28]

2.6.4. Sample Discoloration. Discoloration was evaluated by measuring the absorbance (Thermo Scientific, Helios γ UV−vis) of solutions of native hemoglobin and hydrolyzed peptides at the same concentration. Samples of 5 mg/mL were diluted to 1:50 in distilled water. Absorbance measures were carried out in duplicate at 407 nm[12] on a spectrophotometer.

2.7. Antioxidant Properties. *2.7.1. Reducing Power.* The reducing power of the peptides was determined following the method of Yen and Chen,[29] with slight variations. Different concentrations of freeze-dried peptides were dissolved in a phosphate buffer (0.2 M and pH 6.6) and then mixed with 2.5 mL of potassium ferrocyanide (1%, w/v). The mixture was incubated for 20 min at 50 °C in a temperature-controlled water bath. Once the mixuture had been incubated, 2.5 mL of trichloroacetic acid (TCA) (10%, w/v) was added to precipitate peptides, followed by centrifugation (5000*g* for 10 min) to separate the precipitated peptides. Subsequently, 2.5 mL of the supernatant was collected and mixed with 0.5 mL of ferric chloride (0.1%, w/v). Finally, the absorbance was measured at a wavelength of 700 nm (Thermo Scientific, Helios γ UV−vis). Increases in absorbance show the reducing power of the peptides.

2.7.2. Ability To Neutralize the Hydroxyl Radical. The method employed was that developed by Wang et al.[30] Several concentrations of peptides (from 200 to 5000 mg/L) were assayed in a total volume reaction of 4.5 mL. The sample was incubated in the presence of EDTA−Fe^{2+} (0.22 mM), safranin O (0.23 μM), and H_2O_2 (60 μM) in a potassium phosphate buffer (pH 7.4, 0.15 M) for 30 min at 37 °C. The absorbance was then measured at 520 nm (Thermo Scientific, Helios γ UV−vis). The hydroxyl radical decolors safranin O, so the higher the ability of the peptides to neutralize the radical, the lower the detected decrease in absorbance. The results were expressed as follows:

$$\text{scavenging effect (\%)} = [(A_1 - A_B)/(A_C - A_B)] \times 100 \quad (3)$$

where A_B is the absorbance of the blank without protein, A_c the absorbance of the control without H_2O_2, and A_1 the absorbance of the samples with protein.

2.7.3. Ferrous Ion Chelating Ability. The method reported by Dinis was followed,[31] mixing 5 mL of sample with 0.1 mL of 2 mM $FeCl_2$ solution and 0.2 mL of 5 nM ferrozine solution. After incubation at room temperature for 10 min, the absorbance was measured at 562 nm. The complex formed by Fe^{2+}/ferrozine has a strong absorbance at this wavelength. A high chelating ability is revealed at low absorbance. The results were calculated as follows:

$$\text{chelating ability (\%)} = \left[\frac{(A_0 - A_1)}{A_1}\right] \times 100 \quad (4)$$

where A_0 is the absorbance of the blank sample and A_1 the absorbance of the tested sample.

3. RESULTS AND DISCUSSION

3.1. Analysis of Peptide Size. To compare the influence of temperature on the peptide size, the molecular mass was monitored throughout the hydrolysis process. To systematize the study, four fractions of soluble peptides were established on the basis of molecular mass according to their volume elution: >10, 10−6, 6−1, and <1 kDa. The results of average peptide sizes obtained from two independent replicates of each temperature are shown in Table 1.

Temperature has an influence on two of the parameters measured in the obtained peptides: the process yield (given as the percentage of total peptides recovered with respect to the initial hemoglobin employed) and final average molecular size.

Temperature has a major influence on the size of the obtained peptides. In all the experiments, peptides were detected in all the molecular sizes studied. It should be noted that native hemoglobin was not detected at any time or temperature due to the fact that the first bonds broken in this protein are the hydrophobic bonds of the four main subunits of hemoglobin, which confer the quaternary structure on this protein.[15] Temperatures below 150 °C produce peptides of an average molecular size of around 10 kDa, the most abundant peptides being larger than 10 kDa. These results suggest that this fraction is formed by subunits of poorly hydrolyzed hemoglobin. Temperatures of 180 °C afforded an average

peptide size of 3.2 kDa, the majority of peptides (40.6% of the total) being smaller than 1 kDa in this case.

As regards the yield of the process (the evolution of which is shown in Figure 2), it can be seen that this decreases considerably at lower temperatures. In the hydrolysis carried out at 120 °C, the final recovery of soluble peptides was 30% of the initial hemoglobin employed, while a yield of 50% was obtained at 150 °C and 84% at the highest temperature assayed. The amount of soluble peptides detected increased with the process time. The process yield is higher compared to that obtained under subcritical water hydrolysis conditions,[9,10] in which recoveries of 60% of initial protein as free amino acid were reported. Moreover, the amount of substrate employed was 5 times lower: in the present study, 50 g/L protein solutions were employed, while the maximum concentration used in previous studies was 10 g/L. This means that this technique is able to process large amounts of proteins, a mandatory requirement in industrial processes.

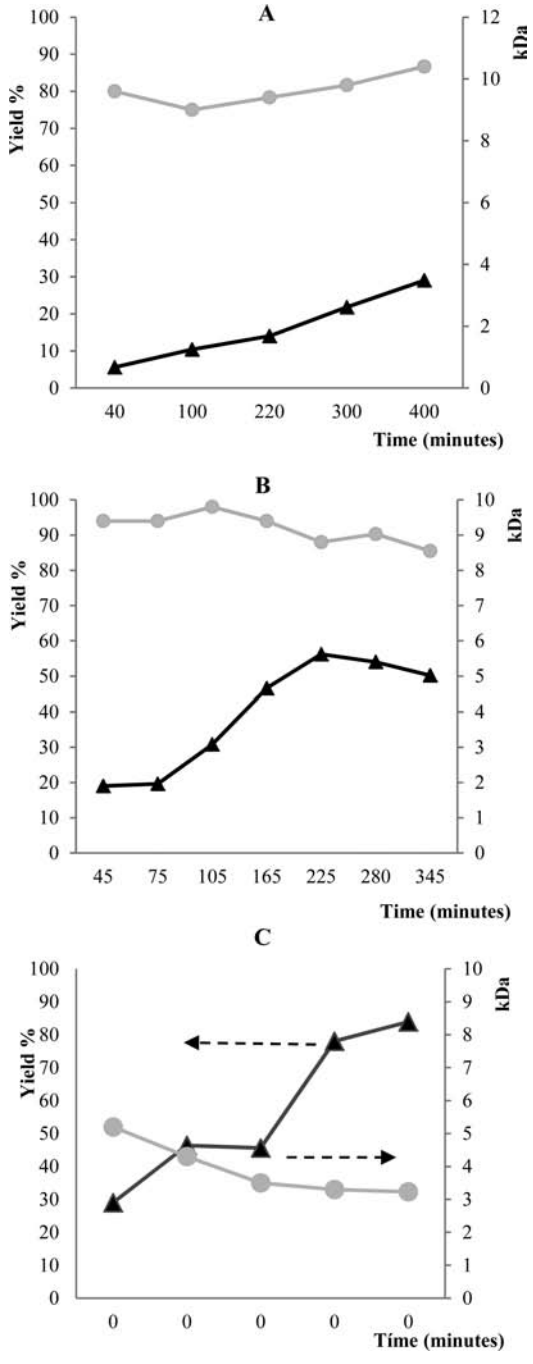

Figure 2. Process yield (▲) and average size (○) of peptides produced through the hydrolysis process at different temperatures: (A) 120 °C, (B) 150 °C, (C) 180 °C.

At a process temperature of 180 °C, at least 270 min is needed to obtain small molecular size peptides. At this time, the percentage of hydrolyzed hemoglobin is 45%. When the results are analyzed at 360 min, the average peptide size remains constant, though the yield increases to 80%. The average size of peptides remains constant at lower temperatures, though the yield increases with time.

The effect of temperature on yield and size can be explained by the fact that the degree of unfolding is higher at higher temperatures. The formation of aggregates was detected when the protein solution was heated at the beginning of each experiment. These aggregates are formed by protein−protein interactions and are nonsoluble. They remain at the lower temperatures assayed until the end of the experiment inside the reactor. These aggregates are barely hydrolyzed, especially at low temperatures; hence, the production of soluble peptides is lower at 120 and 150 °C than at 180 °C. At high temperatures (around 180 °C), these aggregates dissolve during hydrolysis, thus increasing the amount of soluble peptides.

The loss of spatial conformation of proteins is more intense at higher temperatures and leads to protein unfolding, thus enabling more peptide bonds to be exposed to hydrolysis. More aggressive conditions and more exposed peptidic bonds give rise to peptides of a smaller size.

3.2. Analysis of Chromatogram Profiles. The size exclusion chromatograms obtained at different temperatures are shown in Figure 3. Besides, the corresponding molecular size for each elution time is showed in Table 2. The higher peaks correspond to longer hydrolysis time. The chromatographic profiles change when the process temperature is modified. Independent experiments carried out at the same temperature indicate that each peak profile is characteristic of a specific temperature. The result is that higher yields and smaller peptides are produced at higher temperatures, while larger peptides and lower yields are obtained at low temperatures. The peptide size remains constant throughout the hydrolysis process in all cases, although the amount of peptides increases with time. This fact leads us to deduce that preferential targets of rupture may exist on the hemoglobin molecule for two reasons: some peptide bonds are more difficult to break down, and secondary structures are better preserved at lower temperatures. It has been reported[32] that peptide bonds formed by hydrophobic amino acids are more difficult to break down, especially those formed by Val, Ala, and Ile. Bonds of this kind often appear in hemoglobin (60% of its amino acids are hydrophobic), and higher temperatures could be needed to hydrolyze these hydrophobic bonds, which are more difficult to break down. Besides, some secondary structures remain intact at lower temperatures, and some peptide bonds cannot be hydrolyzed,[13] while unfolding by heating allows the exposure of more points of hydrolysis.

The retention times of the main peaks detected are very similar in the hydrolysis carried out at 120 and 150 °C. However, the peaks detected at 150 °C show a lower resolution, so the valleys between peaks are less defined. This is a result of the presence of intermediate-size peptides (less

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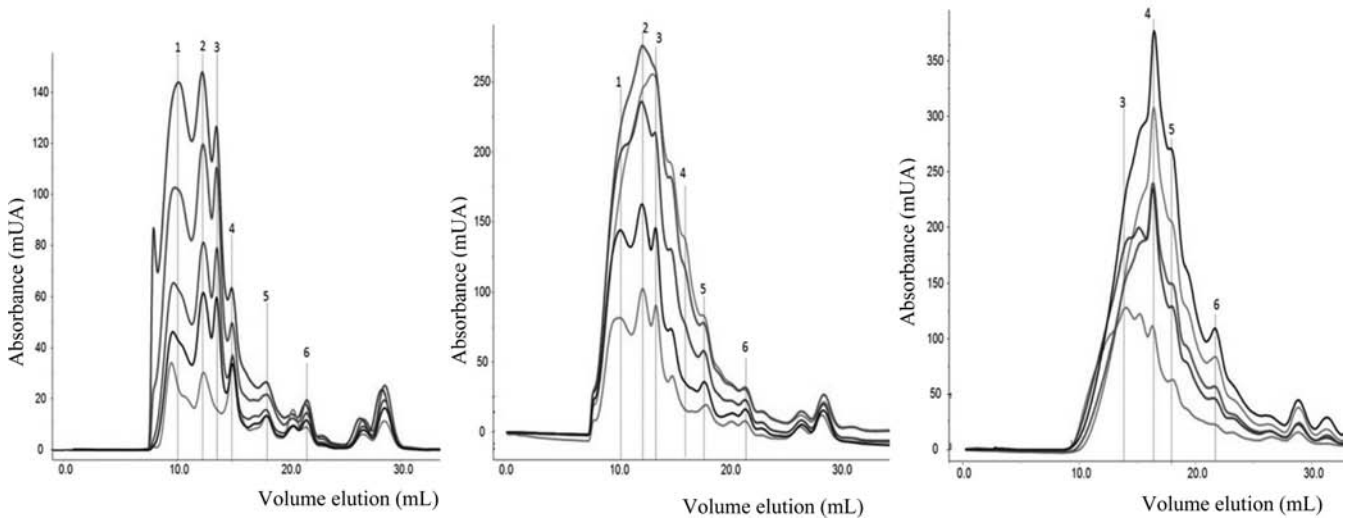

Figure 3. Fast protein liquid chromatograms from the hydrolysis process at different temperatures: (A) 120 °C, (B) 150 °C, (C) 180 °C. The main peaks detected at each temperature are indicated.

Table 2. Elution Time of the Main Peaks Detected at Different Temperatures and Their Molecular Size

	elution time (min)					
	peak 1	peak 2	peak 3	peak 4	peak 5	peak 6
120 °C	10.4	12.4	13.6	16.0	17.9	21.5
150 °C	10.4	12.4	13.6	15.8	17.8	21.6
180 °C			13.5	15.9	17.9	21.6
size (Da)	35 800	11 700	6200	1500	740	110

Table 3. Evolution of Total Mass Amount of Free Amino Acids (inside the Reactor) and Ammonium Compounds (inside the Bubbler) during the Hydrolysis Process

120 °C			150 °C			180 °C		
time (min)	amt of Aa's (mg)	amt of ACs (mg)	time	amt of Aa's (mg)	amt of ACs (mg)	time	amt of Aa's (mg)	amt of ACs (mg)
0	0	0	0	0	0	0	0	0
40	135	0.50	45	136	0.58	60	741	0.93
100	188	0.87	105	189	1.02	90	959	1.19
220	272	1.17	165	226	1.37	180	1031	1.54
300	303	1.37	225	310	1.61	270	1548	3.49
400	319	2.27	285	300	2.64	360	1515	4.07
460	318	5.38	360	290	6.38	420	1508	7.85

abundant at 120 °C) which promote an overlapping effect. In the case of the peptides obtained at 180 °C, the hydrolysis is very aggressive, so fewer peaks are detected and correspond to low peptide sizes. All these data support the theory that the hydrolysis takes place in a specific number of preferential bonds inside the hemoglobin and that this number increases with temperature.

The chromatograms also show compounds of very low (less than 100 Da) and low (between 100 and 200 Da) molecular mass, which appear in all the experiments. These uncharacterized peaks may possibly represent free amino acids and degradation compounds.

3.3. Free Amino Acids and Ammonium. Free amino acids are produced from the ends of the peptides during the hydrolysis process. Other products are also formed, such as ammonium compounds and other volatile products[10] such as amides, aldehydes, hydrogen, methane, carbon dioxide, carbon monoxide, and alcohols. As amino acids and ammonium compounds are the main degradation compounds, their concentration was determined in the hydrolysis solution. Table 3 shows the total amounts of each compound during the hydrolysis process at different temperatures. This table presents the evolution of the amount of amino acids (Aa's) and ammonium compounds (ACs) in the reactor over time at each tested temperature. The total amount was calculated by multiplying the measured concentration by the total volume (400 mL inside the reactor for the amount of Aa's and 500 mL for the bubbler volume in the case of the amount of ACs).

The data show that the production of free amino acids and ammonium compounds increases at higher temperatures. However, the final amount of amino acids is not significant, being 0.6%, 0.56%, and 3% of the initial protein used or 2.13%, 1.15%, and 3.6% of the total protein nitrogen recovered (amino acids and peptides) at the different tested temperatures. Ammonium compounds, on the other hand, represent less than 0.01% of the initial protein used. From the fourth hour of hydrolysis on, the concentration of amino acids inside the reactor is constant or decreases due to the formation of other degradation compounds, while the amount of ammonium increases. After approximately 300 min, the amount of amino acids remains constant, indicating that the production of amino acids is less than or equal to their degradation into volatile compounds. Ammonium compounds, represented as ACs, accumulate considerably after 300 min of processing, as can be seen in the table. To avoid this degradation of produced amino acids, it is necessary to stop the hydrolysis when the amount of amino acids becomes constant.

3.4. Functional Properties. As stated previously, solubility, gelling properties, and emulsifying capacity were measured. Figure 4 shows a comparison of the functional properties of peptides obtained at 180 °C with the properties of native hemoglobin.[21]

It can be seen that the solubility of peptides is better than that observed in native hemoglobin over the entire pH range, except at very low pH. This parameter is one of the most

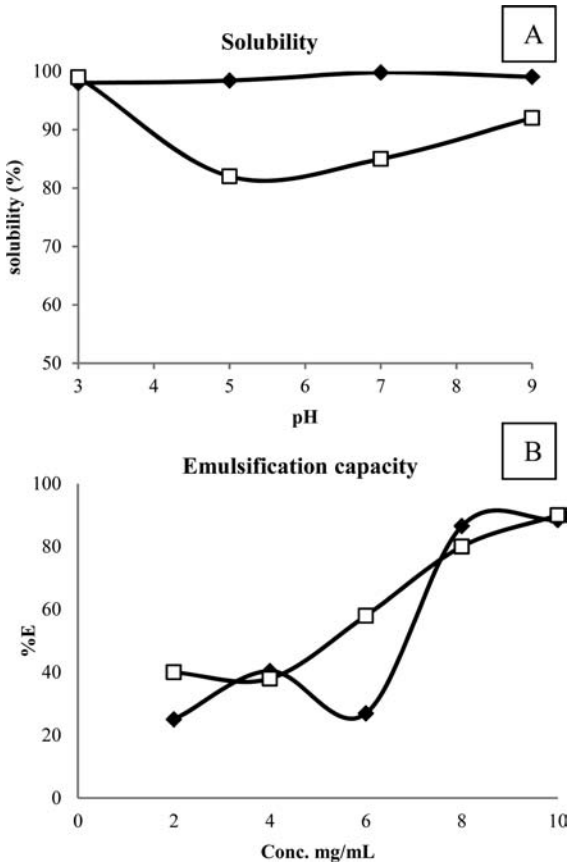

Figure 4. Solubility (A) and emulsifying capacity (B) of hemoglobin (□) and peptides (◆) depending on the medium pH.

important, seeing as the remaining functional properties are highly dependent on solubility.

Peptides and hemoglobin present a very similar emulsifying capacity. Some authors have reported that the emulsion capacity decreases when the degree of hydrolysis is increased.[33,34] However, this effect was not observed in the present study, probably due to the fact that the hemoglobin is composed of equal proportions of nonpolar and polar amino acids. Hence, the obtained peptides possess a major capacity to form amphipathic structures.

The emulsions obtained using peptides present a fluid texture, while the native hemoglobin forms solid emulsions. These results suggest that the obtained peptides are amphipathic in nature. Hemoglobin forms o/w (oil in water) type emulsions, while peptides seem to be able to form both o/w and w/o emulsions, although the discontinuous phase is present at very low amounts in both cases. Two different phases were thus detected, each one formed by a different type of emulsion. To verify that an emulsion was formed, the droplet size was measured, giving 3.2−5.9 and 0.6−3.7 μm in w/o and o/w emulsions, respectively, from each of the phases formed during the emulsifying process. These values are of the same magnitude as those obtained from hemoglobin emulsions (2.0−3.9 μm), thus indicating that the two phases obtained are effectively emulsions.

The stability of both emulsions was monitored every 3 h over a period of 3 days. In the case of o/w emulsions, destabilization took place in the first 18 mm of the bottom of the sample in the first 3 h, the backscattering in this zone increasing from 5% to 15% in 72 h. The rest of the o/w emulsion (18−40 mm height) did not show changes in backscattering, maintaining a constant value of 42%. The o/w emulsion thus separates into two different phases. When the w/o emulsion was analyzed, a gradual increase in backscattering was detected (5−15%) after 3 days of analysis, this effect being observed throughout the sample. These changes in backscattering suggest that the drops present in this emulsion increase in size, making the emulsions not completely stable after 3 days. Therefore, vigorous stirring would be needed to form the emulsion after several days.

The hydrophilic−lipophilic balance (HLB) is a parameter that shows which kind of emulsion a particular compound will form. A value from 7 to 11 indicates a w/o emulsion and a value from 12 to 16 indicates an o/w emulsion. It is likely that peptides with different HLBs are present in the sample, as hydrolysis produces peptides of various sizes and completely dissimilar sequences, leading to changes in their chemical properties and hence the formation of two emulsions.

As regards gelification properties, it was not possible to obtain gels within the range of concentrations studied (2−20%, w/w). The small sizes of the molecules prevent the precipitation and aggregation of peptides (the first step in gel formation), so gelification is not possible. This property could be applied in products which require a protein implement. Thermal treatment will thus be needed, and gel formation is not desirable in this case.

3.5. Discoloration Efficacy. The reduction in color was measured in the final product to evaluate the use of peptides as a food ingredient that does not color the final product. Peptides present a reduction of 80% with respect to native hemoglobin (hemoglobin absorbance 0.593, peptide absorbance 0.115). This process is found to be more effective than others used previously in which the absorbance of obtained peptides ranged between 0.800 and 0.220,[12] measured at the same concentration.

3.6. Antioxidant Properties. The peptides obtained at 180 °C were employed to evaluate their antioxidant properties due to the high process yield and the low molecular size obtained. Some authors[35,36] have reported that small-size peptides (<1 kDa) present the best antioxidant properties; in our study, these peptides represent more than 50% of all the peptides produced.

The ability to neutralize the hydroxyl radical and reducing power were evaluated. Figures 5 and 6 show the results for hydroxyl scavenging and reducing power, respectively.

The peptides present better hydroxyl scavenging than ascorbic acid, used as the control, in all the concentration

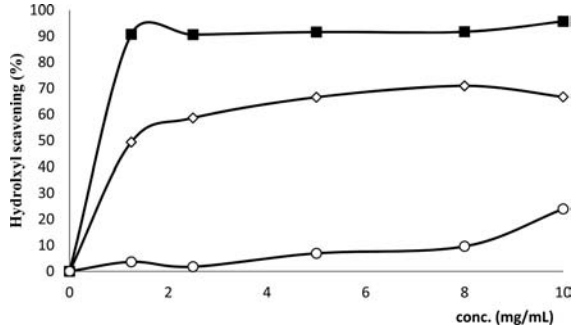

Figure 5. Hydroxyl scavenging produced by native hemoglobin (■), hydrolyzed peptides (◇), and ascorbic acid (○) depending on their concentration.

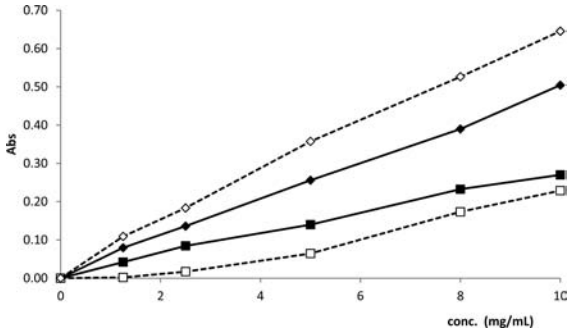

Figure 6. Reducing power at different reaction times of hemoglobin (15 min, ■; 75 min, □) and peptides (5 min, ◆; 70 min, ◇) depending on their concentration.

ranges used in the assay. The hydroxyl radical is one of the most reactive and can lead to severe damage to cells. Native hemoglobin is able to capture more hydroxyl radicals at all the tested concentrations. These data present the same magnitude of efficacy as those of other peptides obtained by enzymatic digestion.[22,37]

The reducing power (or ability to donate electrons) was evaluated as reported in the Materials and Methods. The increase in absorbance at 700 nm means higher reducing power. The evolution of this parameter was monitored over time. This revealed that peptides continued to donate electrons over time (increasing absorbance, probably because some peptides were not precipitated with the addition of TCA). However, native protein decreases over time (due to all the hemoglobin being precipitated with TCA). It can be seen that the produced peptides have a high reducing power compared to native hemoglobin. Other studies[6] reported that hemoglobin hydrolysates produced by enzymatic methods presented a low reducing power compared to native hemoglobin. Thus, this novel nonenzymatic hydrolysis technique is a promising way to obtain peptides with good reducing behavior.

3.7. Ferrous Chelating Ability. It has been proved that transition-metal ions are involved in many oxidation reactions in vivo. Ferrous ions can catalyze the Haber–Weiss reaction, generating hydroxyl radicals. These radicals react with adjacent biomolecules and cause severe damage. Compounds that interfere with the catalytic activity of metal ions thus produce a beneficial effect on antioxidant capacity.

Figure 7 shows the ferrous chelating ability of peptides, native hemoglobin, and EDTA as the control. In this case, the effect of the concentration is not lineal. As expected, the best

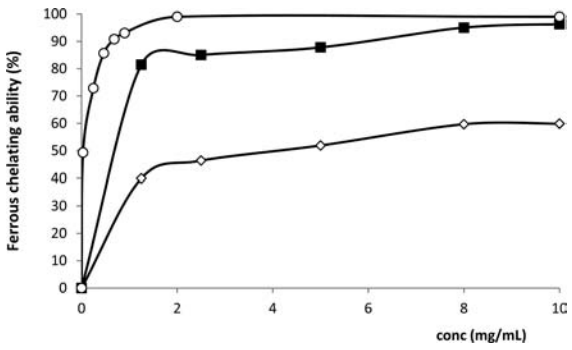

Figure 7. Ferrous chelating ability of EDTA (○), native hemoglobin (■), and peptides (◇) depending on their concentration.

results are produced by EDTA (close to 100%), followed by native protein (85–90%) and peptides (45–60%). The results obtained for peptides are equal to those of hemoglobin peptides obtained by two-step enzymatic hydrolysis and better than those of hemoglobin peptides obtained via the one-step process.[6]

3.8. Conclusions. A novel nonenzymatic technique has been developed to produce hemoglobin peptides using high temperatures (120–180 °C) at low pressures (4 MPa) under a nitrogen stream. Peptides presenting an average size of 3.2 kDa are obtained, a yield of 84% with respect to the initial hemoglobin employed. The main fraction of these peptides (40%) is composed of molecules smaller than 1 kDa, which afford good antioxidant properties.

It has been shown that the molecular size of the produced peptides depends on the temperature employed, with smaller peptides being produced at higher temperatures. Moreover, some specificity regarding hydrolysis targets is detected, resulting in different chromatographic profiles depending on the temperature of the hydrolysis process.

The solubility of peptides has been enhanced, obtaining values of 99% in a wide range of pH. On the other hand, the emulsifying capacity remains the same, although o/w and w/o emulsions are formed. Gelling properties are completely lost; solutions up to 20% (w/w) did not form gels. A marked decrease in coloration (80%) has also been achieved.

As regards antioxidant properties, hydroxyl scavenging is worse in peptides than native protein at high concentrations (more than 5 mg/mL), but is better at low concentrations. The reducing power increased in peptides compared with native hemoglobin, presenting a lasting effect over time which did not occur in the control samples. The iron chelating ability decreased in the peptides, but was still maintained at 50% activity.

Compared to hemoglobin peptides obtained via an enzymatic process, this method produces a higher yield of peptides from hemoglobin that present good antioxidant and functional properties and which are obtained in a cheaper and simpler way.

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Notes

The authors declare no competing financial interest.

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