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Iron and zinc availability and some physical characteristics from extruded products with added concentrate and hydrolysates from bovine hemoglobin

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Abstract

Four hydrolysates were obtained from bovine hemoglobin concentrate (BHC) and used to fortify extruded maize products. Extrusion was carried out with a Brabender single-screw extruder. Physicochemical properties from extruded products were measured. The iron availability was estimated by the dializability method, which measures the mineral dialyzed after a double digestion simulating physiological processes. The physicochemical properties of the extruded products were not affected by fortification, with the exception of total soluble solids. The enzymic hydrolysis increased the iron dializability with respect to the substrate. The highest value of iron dializability corresponded to the more hydrolysated sample. Extruded products fortified with BHC hydrolysates showed higher iron dializability than those fortified with BHC. However, iron dializability corresponding to BHC is lower than that expected from heme iron. Therefore, heme-iron availability is low when it is determined in the absence of meat proteins, and hydrolysis could increase potential iron availability.

Keywords: *Hydrolysis, availability, hemoglobin, fortification, extrusion*

Introduction

Iron deficiency is considered the commonest worldwide nutritional deficiency and affects approximately 20% of the world population (Martínez-Navarretea et al. 2002; Umeta et al. 2005). Iron is an important mineral in food because it has a role as a catalytic center for several metabolic functions. It is present as ferrous (Fe^{2+}) and ferric (Fe^{3+}) compounds and in organic forms as heme-iron (In et al. 2002). The availability of heme iron is superior to non-heme iron. The non-heme-iron absorption is usually poor and depends on intraluminal interactions with components of the diet (Vaghefi et al. 2004). Heme iron is provided by meat and blood-derived foods, and it is more efficiently absorbed than non-heme iron (King et al. 2000).

Most of diet iron is as the non-heme form and its low bioavailability from the predominantly cereal diets and blood loss due to parasitic infestations are causes of iron

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deficiencies and anemia in developing countries (Carpenter and Mahoney 1992). In the industrialized countries, iron anemia occurs among people having large iron requirements due to growth as infants, fetal demands as pregnant women, or iron loss as menstruating women and adolescent girls (Lebrun et al. 1998).

Zinc is one of the essential trace elements and, as such, a member of one of the major subgroups of the micronutrients that have attained such prominence in human nutrition and health (King 2000). Zinc plays a central role in cellular growth, differentiation, and metabolism. A low intake of zinc combined with poor availability and high demand during growth or reproduction results in zinc deficiency. That mineral deficiency has been associated with poor growth, depressed immune function, increased susceptibility to and severity of infection, adverse outcomes of pregnancy, and neurobehavioral abnormalities (Etcheverry et al. 2006). In many developing countries, zinc deficiency is due to the low consumption of animal-source foods, which are rich in zinc, and a high intake of cereals and legumes (López de Romaña et al. 2003).

Strategies to combat the deficiency of iron and zinc include control of parasitic infections, improving health, iron and zinc supplementation and fortification. Of these strategies, the fortification of staple foods is the most economical and convenient and has the advantage of not requiring changes in dietary habits (García-Casal et al. 2003). It consists of the addition of essential micronutrients to foods at a level higher than they usually contain. An important factor in the fortification is the selection of mineral source, which must be based on its bioavailability, its physical and chemical effects on the food and the cost (García-Casal et al. 2003).

In developing countries, cereal flours, especially wheat and corn, are often used as a vehicle for fortification because they are the staple food of the population. Cereal flours and grits are the main raw material for the production of extruded products (Fast 1991). Extrusion is a high-temperature, short-time process that can transform a variety of ingredients into intermediate or finished products such as precooked flours, expanded snacks, breakfast cereals, pastas and texturized protein. The effect of extrusion variables on the properties of extruded cereals has been studied extensively (González et al. 2002).

Although in principle the heme-iron bioavailability is high, it is necessary to study the availability of iron in non-meat products fortified with hemoglobin or its hydrolysates as the food matrix may interact with the peptide fractions and affect iron availability. Since the addition of iron could affect zinc bioavailability (Solomons and Jacob 1981), it is important to evaluate zinc availability in iron-fortified foods.

Based on the above and assessing the benefits of the fortification of cereals and the use of potential highly bioavailable heme iron, the aim of this study was to assess the availability of iron and zinc from bovine hemoglobin hydrolysates and expanded corn products fortified with these hydrolysates.

Materials and methods

o-Phthaldialdehyde, dithiotreitol, sodium dodecyl sulfate, bovine serum albumin, L-serine and Flavourzyme (F) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All reagents were analytical grade. The other enzymes—Protex 6L (P) and Fungal Protease Concentrate (FC)—were provided by Genencor S.A. (Arroyito, Córdoba, República Argentina).

Hydrolysates were prepared with commercial bovine hemoglobin concentrate (BHC) supplied by YERUVá SA (Esperanza, Argentina), for which the composition on a dry basis was: Protein: 96.62 g/100 g; fat: 0.052 g/100 g; ash: 2.77 g/100 g; iron: 1793.4 mg/kg and moisture: 3.36 g/100 g. The BHC composition was determined using AOAC (1995) procedures. Total iron and zinc were measured by atomic absorption spectroscopy after dry mineralization. Ash was removed with 20% HCl (v/v). An atomic absorption spectrometer (IL 551 device; Instrumentation Laboratory, Norwood, Massachusetts, USA) was used.

Hydrolysate preparation

Hydrolysates were obtained using an 800 ml batch thermostatized reactor. The reaction pH was continuously measured using an IQ Scientific Instruments pH-meter, and adjusted by adding base (NaOH) or acid (HCl) with a burette. The substrate concentration was 8% (w/w) in every case. Working conditions for the enzymes were: temperature 60ºC, pH 9.5, enzyme/substrate (E/S) ratio 0.1% for P; temperature 55ºC, pH 4.3, E/S ratio 0.1% for FC; and temperature 55ºC, pH 7.0, E/S ratio 1% for F.

Once the hydrolysis was finished, the enzyme was inactivated by thermal treatment following the manufacturer guidelines. BHC hydrolysates were prepared as follows:

- Simple hydrolysis systems:
  - Hydrolysis P, P enzyme (2 h).
  - Hydrolysis FC, FC enzyme (2 h).
- Sequential hydrolysis systems:
  - Hydrolysis P+F, P enzyme (2 h) + F enzyme (4 h); total reaction time, 6 h.
  - Hydrolysis FC+F, FC enzyme (2 h) + F enzyme (4 h); total reaction time, 6 h.

The hydrolysates were stored in different containers at –20°C, samples being thawed immediately before each test.

Free amino groups were measured using *o*-phthaldialdehyde, according to Nielsen et al. (2001), and the degree of hydrolysis (DH) was calculated as:

$$DH = \frac{(h - h_0)}{h_{tot}} \times 100\%$$

where h_{tot} is the total number of peptide bonds in the protein substrate (8.3 mEq/g protein), h is the number of peptide bonds cleaved during hydrolysis, and h_0 is the content of free amino groups of substrate.

Extruded products

Extruded products were obtained using maize grits, of which the composition on a dry basis was: Protein: 6.9 g/100 g; fat: 0.44 g/100 g; ash: 0.27 g/100 g; carbohydrates: 80.24 g/100 g and moisture: 12.1 g/100 g. The composition was determined using AOAC (1995) procedures. The maize grits were mixture with BHC and each hydrolysate in a proportion 0.5% (w/w). Extrusion was carried out with a 20 DN Brabender extruder in the following conditions: 4:1 compression

ratio screw, 160 rpm, 15% grit moisture, 175°C barrel temperature and 160°C die temperature.

The commercial maize grits were conditioned to the corresponding moisture 1 h before each run. The lyophilized hydrolysates were incorporated with the water at 0.5 g/100 g, on a dry basis. The feeding rate of the extruder was at full capacity.

Physical properties

All extruded samples were air-dried in an oven at 50°C until a moisture content of 6% was reached, this moisture level being considered adequate for texture evaluation. Each dried sample was divided in several portions and kept in plastic bags hermetically sealed until their evaluation. Diameters were measured with a Vernier caliper on 10 pieces of sample, and radial expansion (E) was determined as the ratio $E = d.D^{-1}$, where d is the extruded diameter in millimeters (average of 10 determinations) and D is the die diameter (3 mm). Extruded specific volume (SV) was obtained by calculating the volume/weight ratio (m^3/kg), corresponding to an extruded piece of about 15 cm long. This procedure was applied to 10 pieces and the average is reported. The compressive strength was measured on extruded pieces of 6 cm in length, using a Universal testing machine mark (model 4411, Instron, Norwood, Massachusetts, USA), with a load cell of 4,905 N and a compression speed of 10 mm/min according to Park et al. (1993). Each determination was performed in quintuplicate. Total soluble solids from extruded were determined by the method of Anderson et al. (1969) modified by Gonzalez et al. (2002).

Determination of mineral dializability

The method developed by Miller et al. (1981), later modified by Wolfgor et al. (2002), was used. This method measures mineral dializability under controlled pH conditions after a digestion-simulating physiological process. In order to adjust the pH during the digestion and dialysis stage, and to obtain a uniform final pH in digest/dialysate systems, a PIPES buffer with molarity varying according to the matrix was used.

Dializability of mineral was calculated as the amount of dialysate mineral expressed as a percentage of total mineral content in the sample:

$$MD(\%) = \left(mgM_D / mgM_S\right) \times 100$$

where $MD(\%)$ is the percentage of mineral dialysate, mgM_D is milligrams of mineral dialysate, and mgM_S is milligrams of mineral of the sample. Dializability was used as an indicator of potential availability of iron and zinc and was named as follows: FeD% and ZnD%, respectively.

Statistical analysis

All analyses were performed in triplicate. The data were analyzed by one-way analysis of variance using the software Statgraphics Plus 3.0 (Warrenton, Virginia, USA). The LSD test was used to determine statistical differences among samples ($P < 0.05$), in all cases.

Results and discussion

Hydrolysis reactions

The degree of hydrolysis (DH) obtained with the different enzymatic systems was 8.33 ± 1.02, 8.43 ± 0.69, 19.84 ± 0.21 and 16.67 ± 0.11 for P, FC, P+F and FC+F, respectively. It is observed that the DH reached using sequential hydrolysis was significantly higher than those obtained using a simple hydrolysis. This increase in the DH efficiency is attributed to the actions of F enzyme; which is a mixture of endopeptidase and exopeptidase. This kind of mixture is able to hydrolyze proteins in a more efficient way than an endopeptidase (In et al. 2002).

Iron and zinc dializability from BHC and its hydrolysates

Iron dializability (FeD%). As shown in Figure 1, FeD% values corresponding to hydrolysates are higher than those from BHC, except for P. Besides this, sequential hydrolysis systems allow one to obtain higher FeD% values than those from simple hydrolysis systems and the highest value corresponded to the P+F hydrolysate. This can be attributed to the fact that the peptides produced by hydrolysis form iron-soluble complexes, which promote dialysis and that it is more efficient when hydrolysates are obtain by sequential hydrolysis. Similar results were reported by Vaghefi et al. (2002), for bovine hemoglobin hydrolysates obtained using Pepsin and Subtilisin proteases. For both enzymes, the DH reached was 15% (measured by Trinitrobenzene Sulfonic Acid (TNBS)) and the potential iron absorption was determined *in vitro* using duodenal and lumen tissues from rats.

The significant difference observed between FeD% values corresponding to P and FC hydrolysates could be explained taking into account the hydrolysis time used in our experiments (2 h), and that the nature of peptides obtained by the P and FC enzyme systems is different with respect to the molecular size distribution even

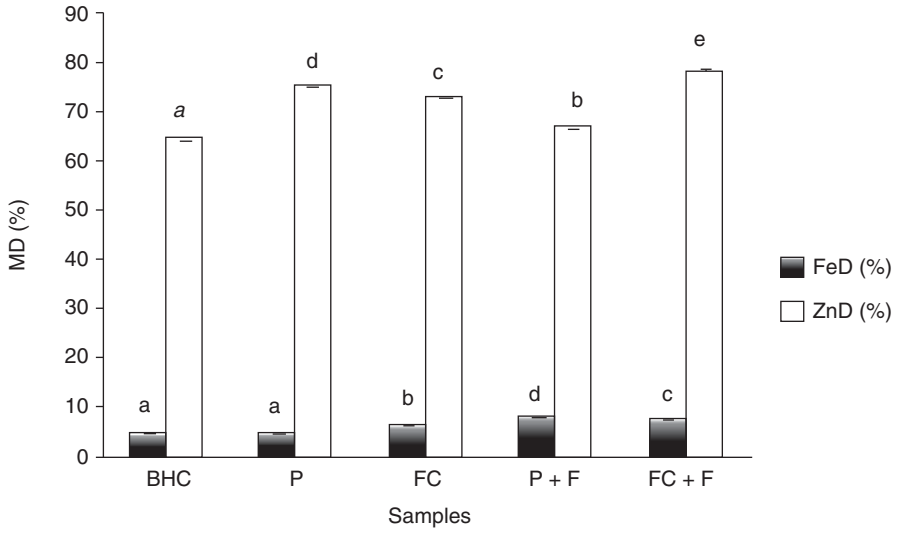

Figure 1. Iron and zinc dializability (FeD% and ZnD%, respectively) from BHC and its hydrolysates (simple hydrolysis systems: P and FC; and sequential hydrolysis systems: P + F and FC + F). Different letters indicate significant differences between samples ($P < 0.05$).

though they have similar DH. Drago et al. (2008), working with gluten protein, have shown that FC produces a mixture of low and high molecular weight peptides due to the combined activity of exopeptidase and endopeptidase—in comparison with P enzyme, which has only endopeptidase activity. As a result, the size and the amounts of peptides produced by P enzyme are not enough to give a noticeable effect on FeD %, while FC enzyme produced peptides that are able to form a soluble iron complex, increasing FeD%.

The value of the FeD% corresponding to BHC is lower than expected from heme iron. It has been established that the addition of meat to a meal increases iron availability. However, the heme-iron availability from a meal made with blood in the absence of meat is less than one-half of the corresponding value obtained when meat is added (Hallberg 1981). As an example, the availability of heme iron from a meat-based meal is approximately 25% while in the absence of meat the maximum value obtain was 10%, decreasing as the doses in the experiment increased. It was explained that peptides formed during meat protein digestion (mainly those contained cystein) could prevent heme-group polymerization and increase heme-iron absorption (Bouglé and Bouhallab 2005).

Therefore, heme-iron availability is low when it is determined in the absence of meat proteins, and in general, an increase in the DH of substrate could increase potential iron availability.

Zinc dializability (ZnD%). Figure 1 also shows the ZnD%. The values corresponding to hydrolysates are higher than those for the BHC, indicating the beneficial effect of substrate hydrolysis. However, no relationship was found between ZnD% and the hydrolysis system used, since the highest value was obtained for FC+F and the lowest one for P+F. This would indicate that the peptides promoting iron dialysis are not the same that those promoting zinc dialysis. It is important to point out that iron and zinc are in different chemical forms in the substrate, since most of the iron is part of the heme group and zinc is associated with other components like plasma proteins and different anions such as citrate (Arver 2009). It was observed in *in vivo* experiments that certain amino acids present in peptides favor the formation of soluble complexes with zinc, which promote absorption at an intestinal level. Among these amino acids, Met, Cys and histidine are mentioned. It is known that hemoglobin contains high amounts of histidine, three times higher than hydrolyzed casein (Aubes-Dufau et al. 1995). Thus, histidine could play an important role in zinc dializability and hydrolysis would

Table I. Physical properties of extruded products.

Sample	Expansion	Specific volume $\times 10^{-3}$ (m^3/kg)	Maximum compression force (N)	Total soluble solids (%)
Maize (M)	1.038 ± 0.013^{A}	6.65 ± 0.13^{A}	57.78 ± 2.50^{A}	61.8 ± 0.8^{B}
M + CHB	1.04 ± 0.01^{A}	6.61 ± 0.19^{A}	58.51 ± 2.52^{A}	53.7 ± 0.8^{A}
M + P	1.038 ± 0.011^{A}	6.40 ± 0.36^{A}	59.61 ± 2.48^{A}	55.1 ± 0.8^{A}
M + FC	1.036 ± 0.01^{A}	6.35 ± 0.32^{A}	57.78 ± 2.49^{A}	53.7 ± 0.8^{A}
M + P + F	1.034 ± 0.011^{A}	6.44 ± 0.27^{A}	58.55 ± 2.53^{A}	53.4 ± 0.8^{A}
M + FC + F	1.036 ± 0.01^{A}	6.65 ± 0.20^{A}	59.35 ± 2.51^{A}	52.4 ± 0.8^{A}

Data presented as mean ± standard deviation. Values with different uppercase superscript letters indicate significant difference between samples ($P < 0.05$). BHC: bovine hemoglobin concentrate; P and FC: Simple hydrolysis systems; P+F and FC+F: sequential hydrolysis systems.

contribute to the formation of peptides containing amino acids that promote soluble zinc complexes.

Extruded samples

Physical properties Table I presents the results of the physicochemical properties obtained from extruded products. It is possible to observe that the expansion, the degree of cooking (assessed through the specific volume) and compressive strength were not affected by the addition of BHC or their hydrolysates ($P < 0.05$). These results are probably due to the fact that the addition of both BHC and its hydrolysates were in a low proportion (0.5% w/w), with respect to the maize. However, the total soluble solids from extruded products added with BHC and its hydrolysates were significantly lower than the control (M). The decrease in total soluble solids could be due to interactions between peptides and proteins from BHC with other components of the maize matrix.

Iron and zinc availability evaluation

Iron dializability (FeD%). Figure 2 shows the FeD% and ZnD% corresponding to the extruded samples. Regarding FeD%, iron was not detected in dialysates from extruded maize and maize fortified with BHC, P and FC. This could be explained considering protein aggregations that could take place between hydrophobic maize protein (zein) and the hydrophobic region present in BHC and peptides formed by P and FC. Liu et al. (1996) observed hydrophobic interactions in peptides from acid porcine hemoglobin globin hydrolysates. As a result, hemoglobin proteins and high molecular weight peptides could interact by hydrophobic forces that could decrease total soluble solids (as shown in Table I) and iron dializability. In the case of hydrolysates produced by P+F and FC+F enzymatic systems, the smaller peptides

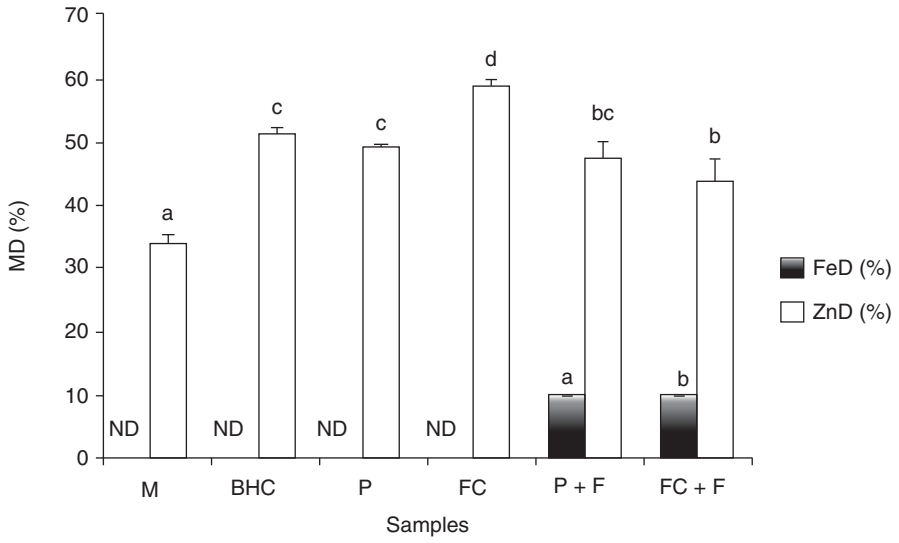

Figure 2. Iron and zinc dializability (FeD% and ZnD%, respectively) from extruded products with added BHC and its hydrolysates (simple hydrolysis systems: P and FC; and sequential hydrolysis systems: P+F and FC+F). Different letters indicate significant differences between samples ($P < 0.05$). ND, nondetected iron in dialysate.

formed could be also more hydrophilic and could permit the formation of iron-soluble complexes with higher dializability. It is important to point out that the FeD% values obtained in our study for maize with added P+F and FC+F samples (around 10%) are higher than those reported in the literature for maize base food. Regarding this, it is well known that the iron content in maize grits is low (Senser and Scherz 1999).

Several reports have shown that the iron dializability corresponding to maize base product is also low. Wolfgor et al. (1996) observed values of 4.8% for FeD% from breakfast cereal prepared with commercial corn flakes and milk. In another study, Wolfgor et al. (2002), measured a 2.9 FeD% for corn flakes without milk. In a human study, García-Casal et al. (2003), working with corn flakes, determined a rate 2.96% of absorbed iron. Also, Bovell-Benjamin et al. (2000) found a low iron bioavailability (1.4%), from maize-based porridge, in a human study.

The fortification of foods with bovine hemoglobin is currently under investigation. In Chile, cookies have been developed with added hemoglobin (Walter et al. 1993), and other researchers have suggested its use to increase the bioavailable iron from hamburger meat and other foods in which soy protein has been used to replace part of the meat protein. Different processes for producing heme iron from hydrolysis of bovine hemoglobin, and thereby for obtaining a peptide-enriched prosthetic group, have been developed. These peptides have higher iron content than hemoglobin (Liu et al. 1996), and contribute to maintain the heme group in a soluble form, facilitating its availability for absorption. Duarte et al. (1999) have indicated whole blood or red blood cells as supplemental sources of iron, mainly because of its high bioavailability. Other authors have proposed the use of hemoglobin or hydrolysates as a source of histidine for supplements for children, since it has a three-times higher histidine content than the casein hydrolysate (Aubes-Dufau et al. 1995). Also blood proteins are used as a food ingredient, both for their functional properties (Lee et al. 1993) and as a nutritive complement, mainly for cereal proteins (Wismer-Pedersen 1979).

However, hemoglobin addition to vegetable foods does not increase the FeD%. Weinborn et al. (2008) found that heme-iron bioavailability was negatively affected by vegetable protein like isolated soybean. Moreover, Hurrell et al. (1988) observed in a human study that hemoglobin addition to maize porridge did not increase iron absorption. These researchers explained their results on the basis of possible interactions between BHC and maize protein, which could avoid iron absorption.

Not only is heme-iron availability low when it is determined in the absence of meat proteins, but also hemoglobin interactions with other food components could decrease heme-iron availability.

Zinc dializability (ZnD%). Regarding ZnD%, it is observed in Figure 2 that the values are significantly higher than those of FeD% for all samples. This difference can be related, as it was mentioned before, to the fact that iron and zinc are associated with different chemical forms in the substrate. It is also observed that ZnD% corresponding to samples fortified with BHC and its hydrolysates are significantly higher than ZnD% from extruded maize grits, but no tendency was found among fortified extruded samples. Thus, heme-iron fortification did not affect zinc availability. We could suggest that some compounds naturally present in the BHC could be the cause of the higher ZnD% corresponding to the BHC and its hydrolysate-fortified extruded samples.

Conclusions

The potential availability of iron from BHC was low, and significantly lower than that from the BHC hydrolysates. For extruded products, it was not possible to detect iron in hydrolysates from maize fortified with BHC or hydrolysates obtained with simple systems (P and FC). Not only is heme-iron availability low when it is determined in the absence of meat proteins, but also hemoglobin interactions with other food components could decrease heme-iron availability.

Fortification with BHC hydrolysates with a DH of 16.67% or more allowed one to obtain an extruded product with a potential availability of 10%, which corresponds to an intermediate availability food.

Expanded maize product fortified with BHC or their hydrolysates improved the potential availability of zinc.

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