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Evaluation of Functional Properties of a Blood Protein

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Abstract: Aiming to evaluate the functional properties of a blood protein, the emulsifying ones were chosen to be studied in this work. Thus, the effect of the pH and of the tryptic hydrolysis on the emulsifying properties of bovine globin, extracted by the acidified acetone method, was studied. The emulsifying capacity (EC), the emulsifying activity index (EAI) and the emulsion stability (ES) were determined at pH varying from 3.0 to 8.0 and employing hydrolysis times from 5 to 60 min. The highest values for EC and ES were obtained at pH 5.0 and 6.0, respectively, corresponding to the range of high protein solubility. On the other hand, the EAI was higher at pH 3.0 and also at pH 7.0 and 8.0, where the protein is insoluble. The tryptic hydrolysis produced an increase in EC, in all pH range studied, while for the EAI the same effect was observed only in pH 4.0 and 5.0, and for ES at pH 7.0 after 60 min of hydrolysis.

Key words: Blood, bovine globin, pH, tryptic hydrolysis, emulsifying properties

Introduction

Bovine blood, originating from slaughter houses, is a by-produtct of the meat industry as well as a potential source of proteins, largely used in several countries in human diet (Autio *et al*., 1984). However, in Brazil, only a small part of the animal blood is used for this purpose (Penteado *et al*., 1979). Although the hemoglobin of bovine blood contributes to an increase in the nutritional value of food and has functional properties of great utility in industrialized products, its use in diets is restricted because of its strong color and flavor (Ockerman and Hansen, 1994). Nevertheless, this problem can be solved by removing the haem group, producing an isolated globin with great potential use in food; (Piske, 1982; Tybor *et al*., 1973).

The evaluation of the capacity of blood proteins in either forming or stabilizing emulsions is highly important from an industrial point of view since the manufacture of several foods, such as mayonnaise, patés and saussages involves an emulsification process. Moreover, the incorporation of proteins in food products may increase their nutritional value. On the other hand, considering that the action of proteins as emulsifiers is complex and depends on different factors (protein concentration, oil type, velocity and length of mixture, among others), it is important to study the behavior of proteins under different conditions (Crenwelge *et al*, 1974; Gauthier *et al*., 1993; Tybor *et al*., 1973).

The modification of protein structure by enzymatic hydrolysis has been used to improve functional properties. However, this result depends on the peptide size, and normally peptides containing more than 20 amino acid residues are needed in order to produce this advantageous effect (Brekke and Smith, 1985; Kinsella, 1984). The more common method involves a partial hydrolysis employing highly specific proteases in order to control the hydrolysis degree and, therefore, the size of produced peptides (Brekke and Smith, 1985; Wood, 1987). No report was found in the literature concerning the effect of the enzymatic treatment on the functional properties of bovine globin.

Our group studied the emulsifying properties of bovine casein aiming to use it as emulsifier in food industries (Duarte *et al*., 1988a). Considering the economical advantage of the replacement of this protein by blood ones, a factor that may be taken in consideration especially in developing countries, where milk proteins are imported and, consequently, are much more expensive than blood ones, we first decided to study the functional properties of plasma (Silva and Silvestre, 2003). Blood plasma is produced from simple centrifugation of whole blood and shows no problems concerning the development of undesirable flavour or color observed with red blood cell concentrate. (Autio *et al*., 1985; Ockerman and Hansen, 1994).

In this work, our interest was focused on the study of the emulsifying properties of another blood ingredient, bovine globin, extracted by the acidified acetone method, evaluating the effect of pH and tryptic hydrolysis.

Materials and Methods

Separation of red cells from bovine blood: The animals were killed in a slaughter house under federal inspection and the blood was collected directly from the carcass in vials containing the anticoagulant (2 mL of a 10% EDTA solution/100 mL of total blood) and the contact between the collecting recipient and the animal skin was avoided. The blood was immediately taken to the laboratory, where it was centrifuged (Jouan centrifuge, Br4i model) at 1,000 g for 15min, in order to separate the red cells (haemaceas). These cells were stored under refrigeration until the moment of the bovine

globin extraction (maximum of 24 h).

Extraction of bovine globin: The acidified acetone method (Tybor *et al.*, 1975) was used to extract globin. The haemaceas obtained as described in the item 2.1 were haemolysed by the addition of distilled water at the proportion of 1:1 and the pH adjusted to 4.0 with an ascorbic acid solution (2g/100 mL). Afterwards, the air was bubbled for an hour to oxidize the hemoglobin in colemetha-hemoglobin, the haem group being removed and the globin precipitated with the addition of an acidified acetone solution (in HCl, 99:1) at a proportion of 1:4. Next, it was filtered through a paper filter and the globin retained was washed in an ether-ethanol solution (3:1) and stored in portions of approximately 100 g in glass bottles covered with paper filters containing many small holes. After drying by forced ventilation at room temperature, the globin portions were sieved (24 mesh), weighed, transferred to glass bottles and frozen at -18°C until use.

Tryptic hydrolysis of bovine globin: The method described by Chobert *et al.* (1988) was used. The globin was solubilized in a buffer solution (0.02 mol/L sodium phosphate and 0.01 mol/L citric acid), pH 8.0, to a protein concentration of 0.1 g/100 mL. Then, the trypsin (bovine pancreas, XIII type, TPCK treated, Sigma Chemical Co., St. Louis, Mo, USA), solubilized in the same buffer, was added to obtain a 0.1% enzyme:substrate ratio. The mixture was held in a water bath at 37°C, with stirring for 5, 10, 15, 30 and 60 min producing the T1 T2, T3, T4 and T5 hydrolysates, respectively. In all assays, the hydrolytic reaction was stopped by reducing the pH to 2.0, using hydrochloric acid (HCl). The hydrolysates were then freeze-dryed (Freezone® 4.5 model, Labconco, Kansas City, MI, EUA) and stored at – 18°C until the moment of use.

Sample preparation: The globin and its tryptic hydrolysates were solubilized in a buffer solution (0.02 mol/L sodium phosphate and 0.01 mol/L citric acid), at pH 7.0, to a concentration of 0.1 g of protein/100 mL of solution. After 30 min in a water bath at 35°C, the solutions were centifuged (Jouan, Br4i model, France) at 6,500 g for 10 min and then filtered (through paper filter Quanty, JP42 model, Curitiba, PR, Brazil). The filtrates were stored at -18°C until the moment of use.

Determination of optimum protein concentration: In order to determine the protein concentration to be used in all experiments, globin solutions were prepared in different concentrations varying from 0.025 to 3.0 g/100 mL in a buffer solution (0.02 mol/L sodium phosphate and 0.01 mol/L citric acid) at pH 7.0. In each case, the emulsifying capacity (EC) was determined according to the method described bellow. Then, a graph of the EC as a function of protein concentration was drawn.

Determination of emulsifying capacity (EC): For determining the emulsifying capacity, the method of Vuillemard *et al.* (1990), with modification by our group (Duarte *et al.*, 1998a,b) was used. Fifty mL of protein solution were homogenized using a mixer (Fisher, mod. 14057-5) at the highest speed. Corn oil (Mazzola) was added continuously during the emulsification process from a funnel into the mixture at a rate of 25 mL/min. During emulsification, the temperature was maintained at 25 ± 3°C by immersing the reaction vessel in an ice bath. The emulsifying capacity was determined by the interruption of the electric current detected by a 120 V lamp. The EC was calculated using equ. 1:

$$\text{1)} \qquad EC = \frac{EO\ (g) - BO\ (g)}{\text{Protein (mg)}}$$

where EO and BO are the amount of emulsified oil in the sample and in the blank, respectively. Blank is the buffer solution with no emulsifying agent.

Determination of emulsifying activity index (EAI): The method of Pearce and Kinsella (1978), with the modifications described by our group (Duarte *et al.*, 1998a,b), was used for determining the EAI. For preparing the emulsions, a volume of 30 mL of the protein solution and 10 mL of corn oil were shaken together in the same mixer cited above, at the highest speed for one minute. The temperature was maintained at 20°C. Aliquots (1 mL) of the emulsion were diluted (1/100) in a solution containing 0.1% SDS (sodium docecyl sulfate) and 0.1 M NaCl, homogeneized and the absorbance was read at 550 nm (spectrophotometer CECIL, CE 2041 model, UK). The EAI values were calculated using Eq. 2 proposed by Cameron *et al.* (1991)

$$\text{2)} \qquad EAI = \frac{2\,T}{(1-\theta).\,C}$$

where T is turbidity, θ is the volume fraction of the oil, and C is the initial protein concentration (0.1 g /100 mL). The turbidity was calculated by multiplying the absorbance by 2.203 and by the dilution factor (100) and then dividing this result by the optical path lenght of the cuvette (0.01 m).

Determination of the emulsion stability (ES): The method of Chobert *et al.* (1988), as modified by our group (Duarte *et al.*, 1998a,b), was used for determining the emulsion stability. The stock emulsions prepared above were held at 20°C for 24 h. After stirring, aliquots were diluted in 0.1% SDS and turbidity was measured as described above (EAI, 20°C). The 24 h - old emulsions were then heated at 80°C for 30 min. After the aliquots were cooled to room temperature and stirred, the turbidity was again measured as described above

(EAI, 80°C). The ΔEAI% was calculated by the Eq. 3:

$$3) \qquad \Delta EAI\ \% = \frac{(EAI\ max - EAI\ min)\ 100}{EAI\ max}$$

where EAI_{max} is the maximum value obtained just after emulsion formation, and EAI_{min} is the lowest value obtained for the aliquots after 24 h-storage and 80ºC heating. ES values were calculated using Eq 4:

$$4) \qquad ES = \frac{1}{\Delta EAI\ \%}$$

Evaluation of pH effect: For studying the effect of pH on the solubility and on the emulsifying properties, the pH of globin solutions and of its typtic hydrolysates was adjusted to 3.0, 4.0, 5.0, 6.0 and 8.0, before the centrifugation and filtration steps.

Statistical analysis: All experiments were replicated three times. Analysis of variance was performed for the determination of optimal protein concentration, in order to investigate the presence of significant effects among treatments ($P < 0.05$). The Duncan test was applied to establish the differences among means (Pimentel-Gomes, 1990).

The effect of pH and hydrolysis time on EC, EAI and ES was analysed using split-plot design (in which the main plots were hydrolysis times and the values of pH the subplots). Analysis of variance for each property ($P < 0.05$) and then the Duncan test was applied to compare means (Pimentel- Gomes, 1990).

Results and Discussion

Optimal protein concentration: The results of optimum protein concentration are shown in Fig. 1. A maximum EC value was observed in 0.1g% and 0.2%g concentrations. After this point, the EC decreased until reach the minimum at 0.5g% concentration. Thus, 0.1g% was used in all subsequent analysis in this work. According to Pearce and Kinsella (1978), this value refers to the minimum concentration needed to obtain reproducible results for the emulsifying properties and must be established in each case because it depends on the type of protein involved in the emulsifying process.

The same value for the optimum concentration (0.1g%) was first found by our group for the commercial bovine casein (Duarte *et al.*, 1998a) and for the laboratory-prepared bovine plasma (Silva and Silvestre, 2003).

Other authors have been checking the effect of protein concentration on emulsifying capacity of bovine globin. After extracting globin using the same method described here, Caldironi and Ockerman (1982) found an EC maximum at a concentration of 0.2g%, in the presence of NaCl at pH 5.7. Crenwelge *et al.* (1974), working with the same type of globin, reported an optimun concentration of 0,404 g%, where the EC was measured by a sudden fall of the viscosity. For the globin extracted by a mixture of acetone and other solvents, Shahidi *et al.* (1984) found an EC maximum at 0,5 g%.

Effect of pH and tryptic hydrolysis on the emulsifying capacity: As shown in Fig. 2, the EC increased until reach a maximum at pH 5.0. After this point, the EC decreased and at pH 6.0 to 8.0, where globin shows the lowest solubility (Kuppevelt *et al.*, 1976), the EC was zero.

According to Mangino (1994), the EC measures the capacity of proteins to migrate to the water/oil interface and for doing that proteins must be sufficiently soluble. The pH affects protein charge and hence its solubility, which is minimum in the region of pI. Thus, in pH values close to pI, proteins show low emulsifying properties (Cheftel *et al.*, 1989; McClements, 1999).

Working with the same type of globin, but using a different method for measuring the EC (sudden fall of the viscosity), Crenwelge *et al.* (1974) reported a maximum and a minimum value for EC at pH 3.0 and 8.0, respectively.

In two previous studies of our group, working with other protein sources at the same pH and hydrolytic conditions used here, different results from those of globin were found. Thus, for the commercial bovine casein, we showed that even at the pH region of the lowest solubility (3.0 to 5.0), a moderate emulsifying capacity was observed (Duarte *et al.*, 1998a). In case of bovine plasma, pH had little effect on the EC which remained high and almost constant at all pH studied (Silva and Silvestre, 2003).

Regarding the effect of tryptic hydrolysis, one can see in Fig. 2 that it was advantageous for the EC mostly at pH bellow or above 5.0, for all times studied. Even at pH 5.0, the hydrolysis up to 15 min improved the EC of this protein.

Some authors have been considering the beneficial effect of moderate enzymatic hydrolysis on emulsifying properties of proteins and stated that besides improving protein solubility, it can also increase the number of contact points between proteins and the water/oil interface favoring the emulsion formation (Chobert *et al.*, 1988; Das and Kinsella, 1990; Gauthier *et al.*, 1993).

For commercial bovine casein our group found the same results as those of globin (Duarte *et al.*, 1998a). However, for plasma we showed that the tryptic hydrolysis reduced the EC at all pH values and reaction times, except for pH 4.0 after 5, 15 and 30 min of reaction (Silva and Silvestre, 2003). At that time, we considered that this result could be explained, at least in part, to the fact that plasma is not an isolated protein but a mixture of proteins and other constituents.

Effect of pH and tryptic hydrolysis on the emulsifying activity index: The maximum value for the EAI of globin

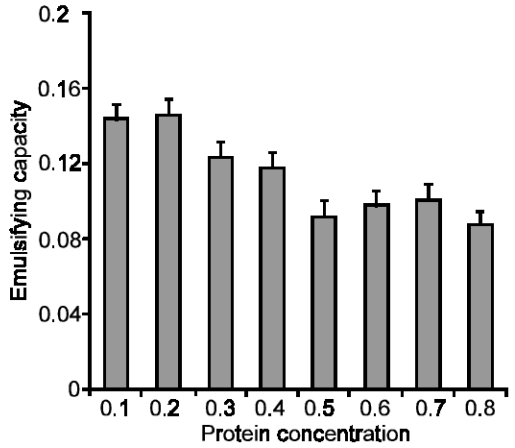

Fig. 1: Emulsifying capacity (g of oil/mg of protein) in function of the protein concentration (g%). Each value represents the mean of triple determination

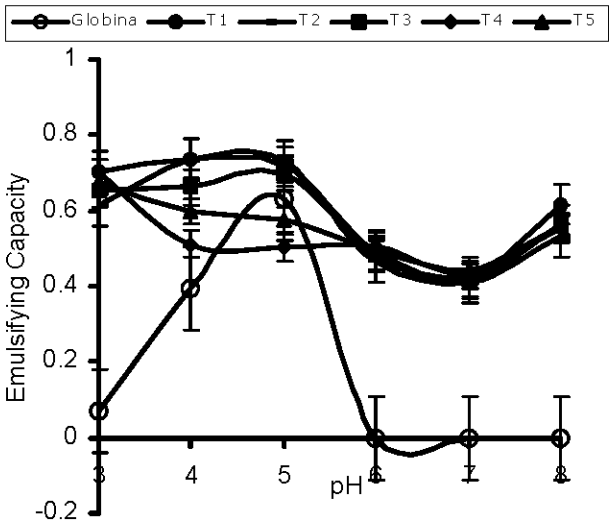

Fig. 2: Effect of tryptic hydrolysis and pH on the emulsifying capacity (g of oil/mg of protein) of bovine globin. T1, T2, T3, T4, T5: globin hydrolysates with hydrolysis time of 5, 10, 15, 30 and 60 min, respectively. Each value represents the mean of triple determinations

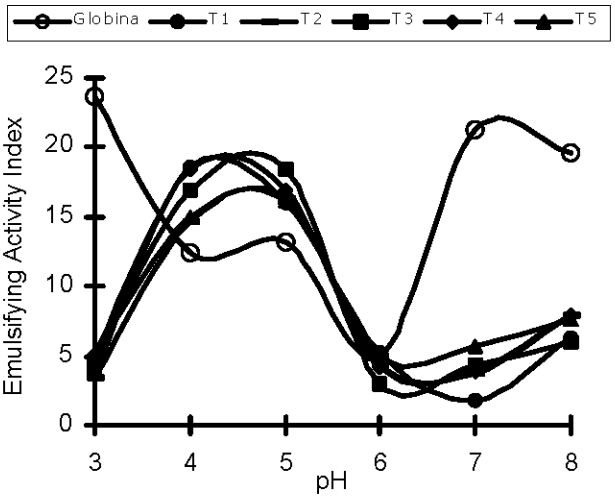

Fig. 3: Effect of tryptic hydrolysis and pH on the emulsifying activity index (m^2/g) of bovine globin. T1, T2, T3, T4, T5: globin hydrolysates with hydrolysis time of 5, 10, 15, 30 and 60 min, respectively. Each value represents the mean of triple determinations

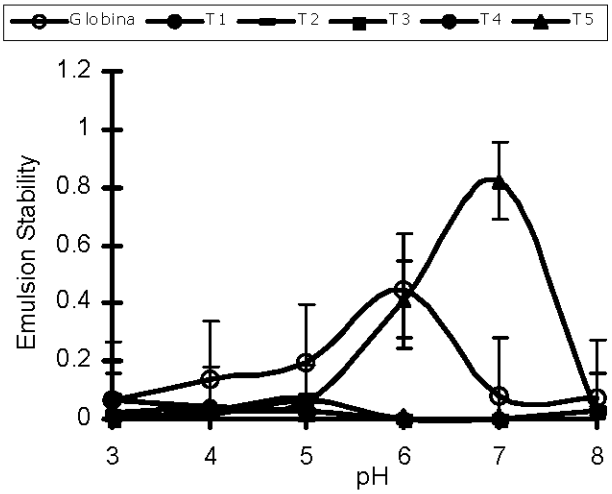

Fig. 4: Effect of tryptic hydrolysis and pH on the emulsion stability of bovine globin. T1, T2, T3, T4, T5: globin hydrolysates with hydrolysis time of 5, 10, 15, 30 and 60 min, respectively. Each value represents the mean of triple determinations

was reached at pH 3.0, and even at pH values situated at its pI region (7.0 and 8.0), contrarily to the EC (Fig. 3). Commercial bovine casein behaved similarly since it showed high EAI values at its pI region (pH 3.0 to 5.0), as shown before by our group (Duarte *et al.*, 1988a).

Considering that the EAI measures the protein capacity to stay at the water/oil interface after the emulsion formation, the low solubility could hinder the protein to pass to the aqueous phase and hence to improve its attachment to the interface water/oil, leading to an increase in the EAI.

This was confirmed by our results with bovine plasma, where the solubility remained high (between 70% and 80%) and almost unchangeable at all pH values (form 3.0 to 8.0) and the EAI was low at the same range of pH (Silva and Silvestre, 2003).

Contrarily to our results, a globin extracted by a different

method used here, employing carboxymethylcellulose, showed an EAI minimum at pH 7.0. According to AUTIO *et al.* (1984), the functional properties of proteins depend not only on their sources but also on the extraction conditions.

The tryptic hydrolysis was beneficial to the EAI of globin only at pH 4.0 and 5.0 (Fig. 3). This enzymatic treatment produced almost the same effect on the EAI of the bovine casein, as shown before by our group, since it increased its EAI at pH 3.0, 4.0 and 5.0 (Duarte *et al.*, 1998a). On the other hand, when we worked with plasma, it was shown that the tryptic hydrolysis did not improve the EAI at all pH and hydrolysis times (Silva and Silvestre, 2003).

Effect of pH and tryptic hydrolysis on the emulsion stability: No effect of pH on the emulsion stability (ES) was observed up to pH 5.0, since the ES remained unchanged (Fig. 4). Next, the ES increased sharply and reached the maximum at pH 6.0, close to its pI. Then, The ES decreased until the minimum value at pH 7.0 and 8.0, region where the solubility of this protein shows the lowest value. Also, we showed that casein had a high value for ES at pH 4.0 near to its pI, but the maximum value was reached at pH 7.0, where this protein is highly soluble (Duarte *et al.*, 1998a). Concerning plasma, the pH had no effect on ES all long the pH range studied (3.0 to 8.0) (Silva and Silvestre, 2003).

The effect of pH on the stability of emulsions shows a certain complexity. At pH values close to the pI, the proteins are able to form more firm and viscous interfacial films which are beneficial to the stability (Mangino, 1994; McClements, 1999).

Das and Kinsella (1990) reported that the results coming from different laboratories concerning the effect of pH on emulsion stability are contradictory. Some groups mention that the maximum value for ES was reached at the pI of the protein while others described the opposite. The absence of standardization of the methods used as well as the use of different protein concentrations which give rise to interfacial protein films with varied properties and forces, can partly explain this contradiction.

One can also observe in Fig. 4 that the tryptic hydrolysis influenced the ES of globin only at pH 7.0 (the pI region of this protein) and after 60 min of reaction. In case of both casein and plasma, our previous results showed that this enzymatic treatment had no effect on the ES, in all pH range studied (3.0 to 8.0) (Duarte *et al.*, 1998a; Silva and Silvestre, 2003).

The action of enzymatic hydrolysis in improving the stability of emulsions is associated to the increase of the solubility and of the hydrolysis degree (Das and Kinsella, 1990).

Finally, it is worth stating that no data concerning the effect of tryptic hydrolysis on the emulsifying properties of bovine globin was found in the literature.

Conclusions: The best results for the emulsifying properties of bovine globin, extracted by the acidified acetone method, was observed in the acid region (pH from 3.0 to 6.0), where this protein is highly soluble. In general, the tryptic hydrolysis was advantageous for these properties, especially for the emulsifying capacity which was improved in almost all pH region and reaction times.

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