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Limited proteolysis as a tool for the improvement of the functionality of sunflower (*Helianthus annus* L.) protein isolates produced by seeds or industrial by-products (solvent cake)

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Abstract

Protein isolates were extracted by different procedures, isoelectric precipitation and ultrafiltration, and also isolated by limited enzymatic treatment with trypsin. Their functional properties were studied, namely surface pressure, foam capacity and stability and emulsion mean oil droplet size and viscosity. The influence of pH value, the addition of NaCl and xanthan gum were also studied.

Results revealed that the limited proteolysis isolate was very effective in stabilizing emulsions and foams. Furthermore, the addition of NaCl enhanced foam ability while the addition of xanthan gum improved foam stability. The limited proteolysis isolate had better emulsifying and foaming properties than had the native isolates.

Keywords: Sunflower protein; Protein hydrolysis; Emulsifying properties; Foaming properties

1. Introduction

Sunflower seeds are mainly used for their oil content, which accounts 80% of the value of the sunflower crop. At the same time, there is an increasing interest in the use of sunflower protein (*Helianthous annus* L.) in human nutrition. Many investigators have examined its functional properties, nutritional value and possible food uses (Kabirullah & Wills, 1982; Rossi, Pagliarini, & Peri, 1985).

Sunflower oil extraction produces oil cake, which contains thermally denatured water-insoluble proteins with low functionality. The possibility of improving the functional properties of sunflower proteins contained in oil cake through their limited proteolysis is, therefore, economically justified and will be investigated in this research work.

Enzymatic protein hydrolysis is a common procedure for improving the solubility, as well as other functional properties, of sunflower proteins, because the proteins suffer denaturation during industrial oil extraction that reduces their solubility (Tsaliki, Pegiadou, & Doxastakis, 2004). High quality sunflower protein hydrolysates have been obtained in recent years, enabling use as food ingredients, in fortification of liquid foods and diets of surgical patients (Villanera et al., 1999a, 1999b).

Limited enzymatic treatment with trypsin can substantially influence the foaming properties of proteins. It has been reported that limited hydrolysis may improve foaming ability but may decrease foam stability (Bernardi, Pilosof, & Bartholomai, 1991; Bombara, Anon, & Pilosof, 1997; Chobert, Sitohy, & Whitaker, 1988; Vioque, Sanchez-Vioque, Clemente, Pedroche, & Millan, 2000). However, the decrease in molecular size resulting from

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hydrolysis can be expected to decrease the ability of the polypeptides at the interface to interact, to such extent that fewer viscoelastic films will be formed, which may cause a decrease in foam stability. On the other hand, protein extracted from thermally-treated cake has to be hydrolysed, to open the close-packed protein molecules and to allow them to exhibit their functionality. Because of the decreased foam stability of hydrolysed proteins, their use would require the addition of polysaccharides as stabilizers (Carp, Bartholomai, & Pilosof, 1994; Carp, Elizalde, Bartholoma, & Pilosof, 1997; Martinez, Baeza, Millan, & Pilosof, 2005). Previous studies on the effect of polysaccharides on foaming properties of intact food proteins have shown that foam stability is strongly increased (Cao, Dickinson, & Wedlock, 1990; Carp, Baeza, Bartholomai, & Pilosof, 2004).

Sunflower protein has been reported to exhibit emulsification ability. The potential use in food systems should be considered alongside other constituents that may intervene. The addition of salt may strongly affect protein solubility, even at the isoelectric point, due to changes in the molecular charges, which alter repulsion between protein molecules (Rossi et al., 1985).

The objective in this study was the prediction and evaluation of the functional properties of sunflower proteins, after limited enzymatic protein hydrolysis, through adsorption behaviour at the air–water interface, foaming characteristics and emulsion stability.

2. Materials and methods

2.1. Materials

Sunflower (*Helianthus annus* L.) protein isolates were prepared by isoelectric precipitation (A) and an ultrafiltration (B) extraction method from sunflower seeds. Sunflower protein isolate was prepared by acid extraction and isoelectric precipitation from sunflower solvent cake (C). Sunflower protein isolate was prepared by acid extraction and isoelectric precipitation from sunflower solvent cake with limited protein hydrolysis (D).

For the preparation of the emulsion, corn oil was purchased from the local market and was used without further treatment. NaCl and xanthan gum (XG) of analytical grade (Merck, Germany) were also used. All the pH adjustments were done with 1 N HCl or 1 N NaOH (Merck, Germany); *n*-hexane was used for defatting. Sodium azide (Merck, Germany) was used as a preservative. Trypsin (Merck, Germany; activity 40 U mg^{-1}) was used for protein hydrolysis.

2.2. Isoelectric precipitation and ultrafiltration isolates

For the production of samples A and B, sunflower seeds were ground and the flour obtained was defatted. The defatted flour was diluted in distilled water (1:10, w/v) for 30 min and the pH was adjusted to 9.0. The solution was centrifuged at 5000*g* for 10 min, the supernatant was collected and the residue was re-dissolved in distilled water at a ratio of 1:5 (w/v). After a second centrifugation, both supernatant solutions were combined. The pH of the combined supernatants was adjusted to 4.5, centrifuged at 5000*g* for 10 min and the residue then lyophilised (A) (Alamanou & Doxastakis, 1995, 1997). Ultrafiltration isolates were prepared after the alkaline extraction of seeds, as described before, with the aid of an ultrafiltration apparatus, Pellicon XL filter, membrane PLCGC 10 K Regenerated cellulose 50 cm^2 MILLIPORE Corp. Bedford, UK; the residue was then lyophilised (B). Sample C was prepared by acid extraction and then isoelectric precipitation was applied, as described for the preparation of sample A.

2.3. Limited protein hydrolysis of sunflower sample C

Sample D was prepared by limited protein hydrolysis of sample C. In the optimal conditions, the yield of protein was about 90% (Ludescher, 1996). The samples were hydrolysed by about 10%. The dependence of solubility and yield of the isolate on its protein hydrolysis degree, at several very low concentrations of trypsin, was studied. The water solubility, at pH 4.5, of the modified protein was taken as the index for improving its functionality. The aim was maximal solubility and minimal loss of protein. The best results were obtained at 1/3400 enzyme/protein ratio; hydrolysis time 20 min at 30 °C and the solubility increased from 3% to 50% (Bulmaga, Shutov, & Vaintraub, 1989; Danilenko, Dianova, Braudo, Henning, & Schwenke, 1997; Danilenko et al., 1998).

2.4. Surface pressure properties

Surface pressure development with time at the air–water interface was derived from surface tension measurements conducted by applying the Du Nauy ring method with the aid of a tensiometer (Kruss K8, Hamburg, Germany), with a ring diameter of 2 cm and a glass vessel diameter of 4.8 cm. The tensiometer was operated manually. All the experiments were carried out at 25 °C. The surface pressure (π) was calculated by $\pi = \gamma_0 - \gamma_t$, were γ_0 and γ_t are the surface tension of the buffer (72.0 mN m^{-1}) and the protein solution at time t at the air–water interface, respectively (Yilmazer, Carrillo, & Kokin, 1991).

2.5. Emulsion preparation

Oil-in-water (50:50) emulsions were prepared by adding the oil to the protein solution (1%, w/v) while mixing with the aid of a mechanical stirrer. The pH of the aqueous phase was adjusted to 5.5 or 7.0. The resulting crude emulsion was then homogenized for 90 s using an Ultra Turrax T25 homogeniser (IKA Labortechnik, Germany) equipped with a S25KG-25F dispersing tool. A small amount of

sodium azide (0.1%, w/v) was added to the water phase as a preservative.

2.6. Average droplet diameter

The stability against coalescence of the oil-in-water emulsions stored at 4 °C was studied by particle size distribution measurements. Oil droplet size distribution was determined by integraded light scattering, using a Malvern Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). The refractive indices of the corn oil (1.473) and of the water (1.33) were used for the calculations. Measurements were performed at room temperature and the volume fraction of oil in the diluted emulsion was approximately 1:1000 in all cases. In the diluted emulsion, an amount of ~2% sodium dodecyl sulfate (SDS) solution was added in order to quench the interdroplet interactions and break up the flocks. The action of the SDS solution on the breakage of the flocks was confirmed by optical microscopy. Then, the droplet size distribution was determined using the index $d_{3,2}$, which is the volume-surface average diameter of the emulsion droplets

$$d_{3,2} = \sum_i n_i d_i^3 / i \sum_i n_i d_i^2 \quad (\mu m)$$

where, n_i is the number of droplets of diameter d_i (Walstra, 1983).

2.7. Rheology of oil-in-water emulsions

A steady stress concentric cylinder rheometer (Brookfield DV-II, LV Viscometer, Brookfield Engineering Laboratories, USA), equipped with the SC4-25/13R small sample adapter, was used to determine the viscosity – rate of shear data of the emulsions after 1 or 30 days of storage. All the measurements were carried out at 25 °C.

2.8. Foam preparation and evaluation

The foams were prepared by air dispersion, with a mixer (Braun, Germany) for 5 min, in 100 ml of legume protein solution, which contained 1.0% (w/v) protein isolate, at pH value 5.5 or 7.0. The foams were then poured into a volumetric cylinder of 1000 ml and the initial foam volume, and the foam volume after 30 min, were measured. Foams were also prepared with the addition of 0.1% (w/v) XG or 0.25, 0.5 M NaCl solutions in order to study their effects on foam ability and stability, respectively.

For the evaluation of foam ability, foam volume at time 0 min is considered and, for the evaluation of foam stability, the aqueous phase volume at time 30 min is considered (Rahma & Rao, 1983).

2.9. Statistical analysis

All experiments were repeated at least three times and the data are the means of three values.

3. Results and discussion

Samples A, B, D at concentrations of 1% (w/v), were used for foam preparation at pH 5.5 and 7.0, and foaming ability and stability were studied (Fig. 1). Sample C, which consisted of completely denatured pure helianthinin, was practically insoluble and did not create any foam. Sample D at pH 7.0 exhibited very good foaming ability and relatively good stability, but not at pH 5.5. That could be due to the increased solubility of D isolate at pH 7.0 and the lack of solid particles, which lead to foam collapse (Dalgleish, 1999; German & Phillips, 1994). The hydrolyzed sample D seemed to produce higher foam volumes than did the non-hydrolyzed A and B. This could be due to the structural changes resulting from hydrolysis treatment, that is the cleavage of several positively charged short peptides from the C-terminus of the α-chain of the 11S protein (Henning, Mothes, Dudek, Krause, & Schwenke, 1997). Limited hydrolysis may improve foam capacity, because of the exposure of hydrophobic areas and increased molecular flexibility of polypeptides, that increases the affinity for the interface and the adsorption rate (Ispen et al., 2001). The observation that the hydrolyzed protein isolate had good foaming ability is in agreement with previous studies of Martinez et al. (2005) and Rahma and Rao (1983).

Foams with sunflower A, B, D, at pH 5.5 and 7.0, at concentrations of 0.25 M and 0.5 M NaCl were prepared (Fig. 2). Sample D at pH 7.0 in the presence of NaCl, exhibited very good foaming ability, with high foam volumes, higher even than those without NaCl. The studied samples showed better foam capacity in the presence of salt, which influences protein–protein interactions and their solubility by modifying their electrical charge and forcing them to orientate at the interface (Dalgleish, 1999; German & Phillips, 1994). On the other hand, at pH 5.5, D foams were very unstable, because they completely collapsed after 30 min. At this pH value, near the protein isoelectric point, solubility is limited, which retards partial denaturation of polypeptides at the interface, resulting in a thin and unstable film (Zayas, 1997).

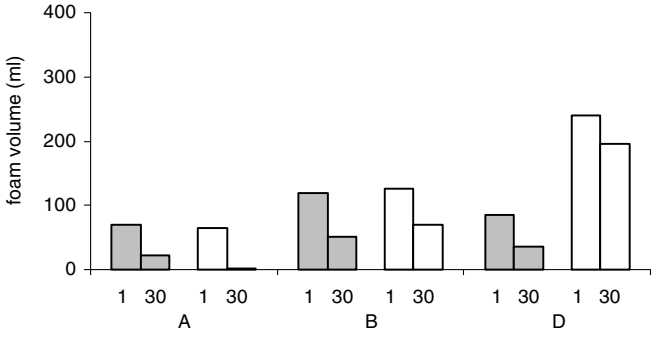

Fig. 1. Foaming ability (foam volume-ml-produced at time 1 min) and stability (volume-ml-remaining at time 30 min) of 1% (w/v) A, B and D sunflower isolates at pH 5.5 (gray) and 7.0 (white).

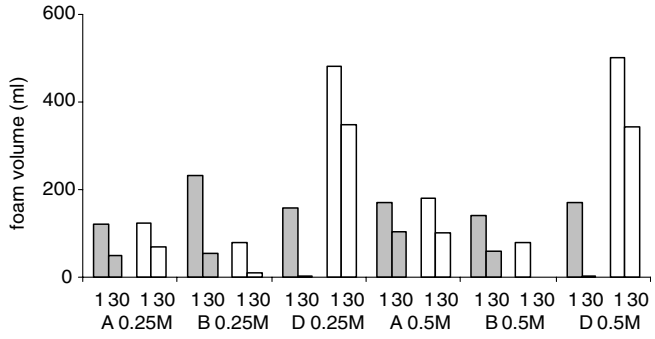

Fig. 2. Foaming ability (foam volume-ml-produced at time 0 min) and stability (volume-ml-remaining at time 30 min) of 1% (w/v) A, B and D sunflower isolates at pH 5.5 (gray) and 7.0 (white), with the addition of 0.25 M and 0.5 M NaCl.

Foams of samples A, B, D at pH 5.5 and 7.0, with 0.1% (w/v) XG were studied (Fig. 3). Sample D, especially at pH 7.0, showed high foam capacity and stability, even better than those of the plain foam without the polysaccharide. XG, being highly hydrophilic and without any significant amount of hydrophobic bonds, is not adsorbed at the interface (Yilmazer et al., 1991), but it can enhance foam stability, by increasing protein adsorption and serum viscosity (Tolstoguzov, 1993, 1994a, 1994b), thus thickening the aqueous phase and creating a network structure. At pH 5.5, a slight reduction of the foaming ability and stability was observed. XG, as an anionic polysaccharide, is negatively charged at pH 7.0 and protein is negatively charged as well at this pH above the isoelectric point. Electric repulsion, alongside thermodynamic incompatibility of the two macromolecules, forces the protein toward the interface, formulating thicker interfacial films. Sample B, even in the presence of 0.1% xanthan did not exhibit satisfying foaming properties. Especially at pH 7.0 it deteriorated in 30 min, maybe because this ultrafiltrated sample also included albumins, dissoluble at pH 7.0.

Since foams exhibit better results at pH 7, surface pressure measurements were made at this pH. In a protein solution, as the first protein molecules arrive at the surface, they tend to unfold, projecting their hydrophobic residues, apolar amino acid side chains, into the air phase and spreading their charged residues, polar with disulphide bridges, into the aqueous phase with the neutral residues in "trains" along the surface (Dickinson, 2003; Tolstoguzov, 1997). This process increases the surface pressure. Sample D (Fig. 4), having lower molecular weight, more flexibility and altered molecular structure, exhibited higher surface pressure than did C. This is a possible reason for sample C not being capable of producing foams.

Since D showed the better foaming properties, it was also studied at lower protein concentration (10^{-3}%) (Fig. 5). The addition of salt increased surface pressure as well as its foaming ability, as confirmed by the foaming stability data (Fig. 2), maybe because ions eliminate protein charge. When molecules are charged, penetration in the interface of the newly arriving molecules is more difficult, due to the electrical potential established at the interface by already adsorbed molecules (Tolstoguzov, 1997).

The addition of XG also increased the surface pressure to a less extent than did addition of NaCl and so improved the foaming ability (Fig. 3). This increase was not immediate, but it had an initial retardation until it reached the steady state, which gave stability to the foam. As more molecules arrive at the surface, they become increasingly difficult to unfold because the high surface coverage creates an energy barrier to their adsorption (Tolstoguzov, 1997).

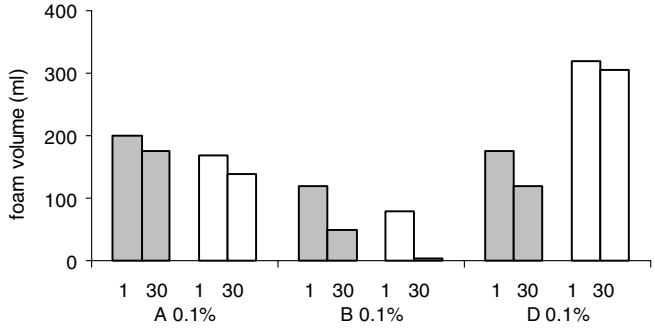

Fig. 3. Foaming ability (foam volume-ml-produced at time 0 min) and stability (volume-ml-remaining at time 30 min) of 1% (w/v) A, B and D sunflower isolates at pH 5.5 (gray) and 7.0 (white), with the addition of 0.1% (w/v) XG.

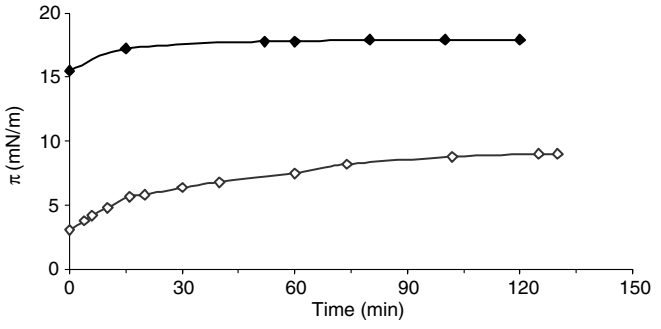

Fig. 4. Surface pressure increase with time of 10^{-2} (w/v) solutions of sunflower isolate C (–◇–) and D (–♦–) at pH 7.0.

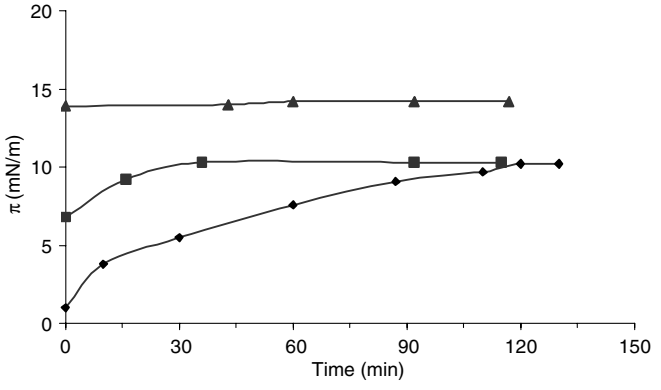

Fig. 5. Surface pressure increase with time of 10^{-3}% (w/v) solutions of sunflower isolate D (–♦–) or with the addition of NaCl 0.25 M (–▲–) or XG 0.25% (w/v) (–■–) at pH 7.0.

Xanthan gum also creates a network in the bulk, making the protein mobility difficult.

Samples A, B and D at concentrations of 1% (w/v), were used for emulsion preparation at pH 5.5 and 7.0, and emulsion stability, with respect to oil droplet diameter $d_{3,2}$, was studied for a period of 30 days (Fig. 6). Sample C could not create emulsions, not even when decreasing pH (8.5–8.0–7.0–5.5–3.0), and not even with the use of urea or salt. Samples A and D had smaller droplets at both pHs, in contrast to B.

Emulsions with samples A and D, at pH 5.5 and 7.0, with 0.5 M or 0.25 M NaCl were prepared (Fig. 7). Sample D showed smaller initial and final $d_{3,2}$ values than did sample A, at both pH values and salt concentrations studied. Sample B created unstable emulsions with large initial oil droplet size, at both salt concentrations (data not shown). Between the two concentrations of NaCl studied, at 0.5 M the system seemed to deteriorate more than at 0.25 M NaCl only for the sample A. In comparison to the plain emulsions, the presence of salt, led to the creation of less stable systems, for sample A. The presence of salt has been reported to enhance emulsion instability (Dickinson, Semenova, & Antipova, 1998; Makri & Doxastakis, 2006b; Mitidieri & Wagner, 2002). The screening of the electrostatic interactions, due to the presence of salt, reduces the height of the energy barrier and the oil droplets tend to aggregate. For

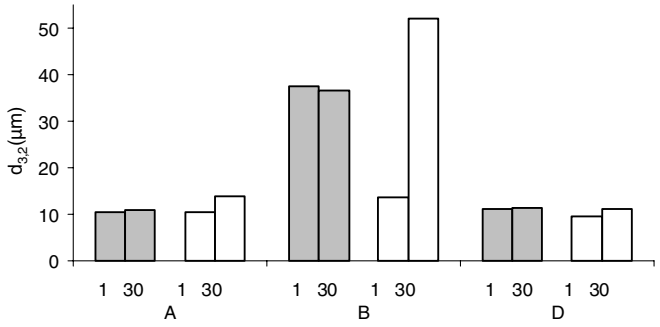

Fig. 6. Mean droplet diameters of emulsions prepared with 1% (w/v) A, B and D sunflower isolates at pH 5.5 (gray) and 7.0 (white), measured at 1st and 30th day of their preparation.

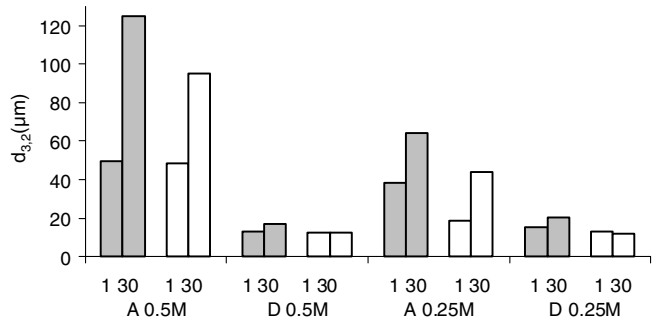

Fig. 7. Mean droplet diameter of emulsions prepared with 1% (w/v) A and D sunflower isolates at pH 5.5 (gray) and 7.0 (white), with the addition of NaCl 0.5 M or 0.25 M, measured at 1st and 30th day of their preparation.

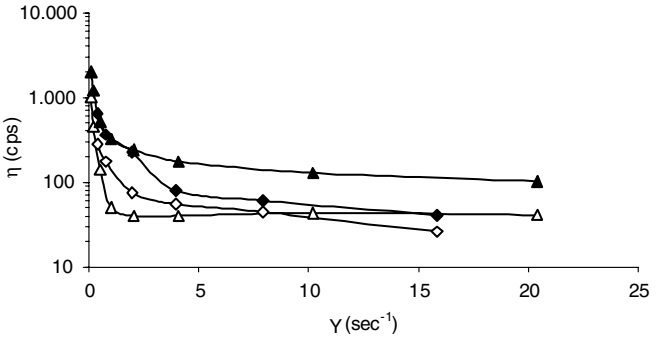

Fig. 8. Viscosity of emulsions prepared with 1% (w/v) A (–▲–) and D (–♦–) sunflower isolates at pH 5.5 (black symbols) and 7.0 (white symbols), measured at 1st day of their preparation.

emulsions made with D, the smaller influence of salt could be attributed to the suitable molecular configuration and structure, which is quite effective in enhancing steric repulsive forces, operating between the newly formed oil droplets, leading in return to their stabilization.

The viscosities of emulsions with sunflower A and D at pH 5.5 and 7.0, at concentrations of 1% were measured (Fig. 8). Viscosity of the emulsions at pH 5.5 was higher than at pH 7.0. At pH close to their isoelectric points the protein molecules are in a more compact form, and provide more space for the inter-droplet interactions to take place, resulting in an increase in the emulsions rheological properties (Makri & Doxastakis, 2006a). Moreover, the difference of the viscosity with respect to pH, was smaller for sample D in comparison to sample A. This could be an advantage in the exploitation of the protein in different pH environments.

4. Conclusions

Sample D was very effective in stabilizing emulsions and foams. Also, the addition of NaCl enhanced foam ability but not stability and the addition of XG enhanced its foam stability. The above conclusions were also confirmed by the surface pressure measurements. Sample D had better emulsifying and foaming properties than had A and B. This has to be attributed to the protein hydrolysis, which provides protein molecules with suitable extensibility and configuration and therefore improves functionality.

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