Characterization of Enzymatic Sunflower Protein Hydrolysates

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Sunflower meal (SFM) proteins are denatured during the process of oil extraction, which greatly limits their functional and nutritional properties. Protein hydrolysates were prepared from defatted undehulled SFM by enzymatic hydrolysis using Kerase—a microbial neutral protease—as hydrolytic agent. The resulting protein hydrolysates contained 78.9–84.8% protein and were highly soluble over a wide pH range (2–10). Their chemical composition, molecular weight distribution, and biological protein quality (protein efficiency ratio) were also studied. The properties of the resulting hydrolysates make them potentially useful in the food industry for dietetic purposes or as special food ingredients.

INTRODUCTION

Sunflower proteins have recently attracted considerable attention as potential food ingredients (Lusas, 1985; Gassman, 1983). However, the use of new protein sources has often been limited by the proteins' low biological value, undesirable organoleptic properties, and, in many cases, poor solubility, resulting in low functionality. The quality of sunflower meal (SFM) protein depends on the oil extraction process. The most usual process involves a mechanical pressing followed by a solvent extraction (prepress solvent extraction) (Fincher, 1958). This results in an SFM with a high content in insoluble proteins, originated during the extraction process (Cheftel, 1985). SFM protein is deficient in lysine and leucine and in several functional properties, including solubility near neutral pH and whipping and emulsifying properties (Sosulski, 1984). SFM protein contains undesirable or antinutritive components—mainly the hulls and polyphenols—which must be removed but does not contain toxic compounds (Gassman, 1983). However, these proteins could find applications in the food industry if appropriate modification processes were developed. One of the most efficient means of improving and expanding the functional properties of sunflower proteins is to subject them to enzymatic hydrolysis (Parrado et al., 1991; Palmieri et al., 1989; Kabirullah and Wills, 1981). In a previous work the authors described an enzymatic hydrolytic process for the production of soluble sunflower protein hydrolyzate (SFPH) (Parrado et al., 1991), using sunflower meal protein concentrate (SFPC) low in polyphenols and free in hulls as raw material. The aim of this work is to describe the composition, physicochemical, and nutritional characteristics of the SFPHs obtained by this process.

MATERIALS AND METHODS

Sunflower Protein Hydrolysates. Sunflower protein hydrolysates were prepared from defatted undehulled SFM provided by ARLESA (Sevilla, Spain). The SFM was fractionated as previously described (Bautista et al., 1990). The resulting protein concentrate, low in polyphenols, was then hydrolyzed batchwise with Kerase (CEPA, S.A., Madrid) in a pH-stat at pH 7.5 until a predetermined degree of hydrolysis was achieved, as described in a previous work (Parrado et al., 1991).

Chemical Analysis. The protein content was determined according to the Kjeldahl procedure. The crude protein content value was calculated by subtracting the mineral nitrogen from the total nitrogen and multiplying the result by 5.5 (Gassman, 1983). Mineral nitrogen was determined according to the method described by Bhatty et al. (1973).

Ash and moisture were analyzed according to AOAC (1980) methods. Chlorogenic acid was determined by measuring the absorbance at 328 nm at pH 5 and converting the value into millimolar units of chlorogenic acid by a standard curve (Dorrel et al., 1976).

Soluble carbohydrates were determined according to the method of Lane-Eynon recommended by AOAC (1980).

Analysis of Amino Acids. Peptides were hydrolyzed under vacuum at 110 °C in 6 M HCl containing 0.05% phenol for 24 h. Hydrolysates were derivatized with phenyl isothiocyanate (PITC), and the PTC amino acids were analyzed with a Waters HPLC system equipped with a C_{18} reversed-phase column (Waters, Millipore Corp.) as described by Bidlingmeyer (1984). Cysteine and methionine were determined by performic acid oxidation prior to their digestion in 6 N HCl and were measured as cysteic acid and methionine sulfone, respectively (Blackburn, 1968).

SFPH Molecular Weight Determination. SFPH samples were examined by SDS–PAGE according to the procedure of Laemmli (1970). The gel concentration was 15%, and the gel stain was Coomassie Brilliant Blue R-250. The electrophoresis was run at 25 mA for 0.75 mm thick gels and at 30 mA for 1.00 mm thick gels. The approximate molecular weight of the SFPH was determined using the Bio-Rad low molecular weight standards.

The molecular weight of the samples was also investigated by gel filtration using an FPLC system (Pharmacia) equipped with a Superose 12 HR 10/30 column. The injection volume was 100 μL, and the elution buffer was 0.02 M sodium phosphate (pH 7) and 0.02% NaN_3. Elution was carried out at a flux of 0.75 mL/min, using a UV detector at 214 nm. The approximate molecular weight of the SFPH was determined using pig heart lactate dehydrogenase (145 900), henovotransferrin (78 000), bovine erythrocyte carbonic anhydrase (30 000), aprotinin (6500), and adrenocorticotropic hormone fragment 1–14 (1681 Da) as molecular weight standards.

Solubility. Solubilities of SFM, SFPC, and SFPH were determined by the protein dispersability index (PDI) method (Saeed and Cherian, 1988; Parrado, 1991). Briefly, a 1% aqueous solution was blended for 10 min, with either 2 N HCl or 2 N NaOH to adjust the pH. After a 15-min hold and readjustment, if necessary, of the pH, the sample was centrifuged at 1400g for 10 min. The nitrogen content of the supernatant was determined. Solubility is expressed as the percent of the total nitrogen of the original sample that was present in the supernatant.

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Table I. Composition of the Diets Used in Rat Feeding Studies

component	% dry matter[a] casein diet	sunflower diet (SFPH)[b]
protein source	12.6	12.6
cotton oil	7.6	8.0
vitamin mixture	1.0	1.0
salt mixture	4.2	3.4
cellulose	1.0	1.0
water	5.2	5.8
sucrose	68.4	69.0

[a] All percentages were calculated according to the AOAC (1975) method. [b] As protein source was used a SFPH of 18% degree of hydrolysis, free of insoluble residue.

Table II. Chemical Composition of Sunflower Meal (SFM), Protein Concentrate (PC), and Sunflower Enzymatic Protein Hydrolysate (SEPH)[a]

	protein, %	carbohydrates, %	polyphenols, %	ash, %
SFM	27.7 ± 2.3	19.70 ± 1.8	6.07 ± 0.8	5.75 ± 0.6
PC	58.3 ± 4.1	9.45 ± 0.8	1.60 ± 0.3	3.69 ± 0.4
SEPH[b]	78.9 ± 3.5	2.20 ± 0.2	1.10 ± 0.1	12.63 ± 1.0
SFPH[c]	84.8 ± 3.2	2.42 ± 0.2	1.05 ± 0.2	4.82 ± 0.5

[a] Each value represents the mean ± SD of three experiments. Results are expressed on dry matter basis. [b] 18.8% degree of hydrolysis; NaOH as base to keep pH constant. [c] 18.8% degree of hydrolysis; $(NH_4)OH$ as base to keep pH constant.

Osmolarity. The osmolarity of the hydrolysate was measured with an osmometer (OSMOMAT 030, Gonotec, Berlin) and is expressed in milliosmols per liter (mOsm/L).

Nutritional Evaluation of Sunflower Protein with Rats. Nutritional evaluation of sunflower protein was carried out using male Wistar rats as test animals. All animals were from the same colony and, after weaning, were allowed to adapt to the standard solid food and to environmental conditions providing normal development in all respects. Rats were ≥21 but ≤28 days old; the range of individual weights among animals used was ≤10 g. Groups were of 10 animals each. The reference group was fed with a casein diet and the test group with an SFPH (without insoluble residue) diet. The composition of the diets was according to AOAC (1975) (see Table I). Throughout the assay period each rat was kept in an individual cage and provided with appropriate assay diet and water *ad libitum*. During the assay period all of the cages were placed in an air-conditioned room at 20 °C with a 12-h light–dark cycle. The body weight of each rat was recorded on the first day of the assay period, and body weight and food intake of each rat were recorded every 4 days through the assay period (28 days). Protein efficiency ratio (PER) was calculated for each group according to the AOAC (1975) method as the ratio weight gain (g)/protein intake (g).

RESULTS AND DISCUSSION

To minimize the problems caused by the presence of a high concentration of polyphenols in raw SFM (see Table II), a sunflower protein concentrate (SFPC) low in polyphenols was used as starting material for the hydrolysis. The SFPC was hydrolyzed batchwise by treatment with Kerase in a pH-stat, using the following hydrolysis parameters: substrate concentration (S) = 10%; enzyme–substrate ratio ($E/S \times 100$) = 2%; pH 7.5; temperature (T) = 55 °C; and 0.15% $CaCl_2$ used as stabilizer for the enzyme. Hydrolysis was carried out until a degree of hydrolysis of 18.8 was achieved; working under these conditions, we are sure that maximum solubilization of insoluble sunflower proteins is reached (Parrado et al., 1991). The chemical composition of the resulting product (SFPH) is shown in Table II.

Chemical Composition. As can be observed, the main feature of SFPH with a 18.8% degree of hydrolysis is its high content of protein (78.9–84.8%). Protein concentrations of this order are also found in other SFPHs with a degree of hydrolysis ≥12.5% (results not shown). The second component is the ash, composed mainly of Na^+ and Cl^- ions that come from pH adjustment during hydrolysis. Although these ions are acceptable in human nutrition, too high an osmolarity can cause diarrhea (Hegarty et al., 1982). The SFPHs with different degrees of hydrolysis at a concentration of 20 g/L give an osmolarity of 83–131 mOsm/L. Products with this osmolarity can be used for parenteral diets, in which the osmolarity must not exceed the limits of 600 mOsm/L (Koretz and Meyer, 1980). As shown in Figure 1, the osmolarity of the SFPHs increased during hydrolysis. If an SFPH of lower ash content is desirable, hyperfiltration could be used—operating by diafiltration (Olsen and Adler-Nissen, 1981)—or ammonium hydroxide could be used as base to keep the pH constant during the hydrolysis, removing it as gaseous ammonia during subsequent processing of the product (Archer et al., 1973) (see Table II).

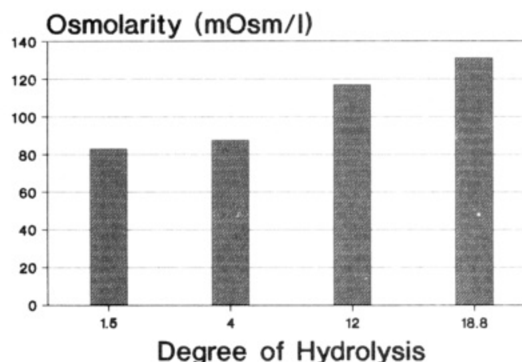

Figure 1. Osmolarity of the different hydrolysates at various degrees of hydrolysis.

Solubility. Solubilities of SFPHs and the raw materials (SFM and SFPC) at various pH values are shown in Figure 2. As expected, protease action increases the solubility of sunflower proteins. Compared with SFM and SFPC, which are very insoluble due to denaturation of their proteins during the industrial process of oil extraction (Cheftel et al., 1985), SFPHs are totally soluble over a wide pH range (2–12) (Parrado et al., 1991). This is an important feature, which could increase the use of these hydrolysates in many food and nonfood applications.

Molecular Weight Distribution. Molecular weight patterns of different SFPHs determined by electrophoretic and molecular filtration methods are shown in Figures 3 and 4. These results show that SFPHs with a degree of hydrolysis ≥12.0% are composed mainly of low molecular weight proteins and peptides; similar results are also reported by other grups (Palmieri et al., 1989; Kabirullah and Wills, 1981). No differences are seen between SFPHs with different degrees of hydrolysis. The electrophoretic pattern (Figure 3) shows that the hydrolysates at 1.50% and 4% degrees of hydrolysis comprise one large protein band of molecular weight higher than 90 000, three bands of molecular weight 22 400, 19 000, and 17 700, and several low molecular weight peptides that comigrated with the front dye. At higher degrees (12% and 18.8%) only the low molecular weight proteins and peptide bands are present, and the >90 000 protein band is not detected.

Similar results were obtained by gel filtration as shown in Figure 4. In these experiments we observed that the hydrolysates at 4% and 10% degrees of hydrolysis are composed of large proteins of approximately 300 000 (probably helianthin) that eluted with the void volume, and several low molecular proteins of 25 500–13 200 and

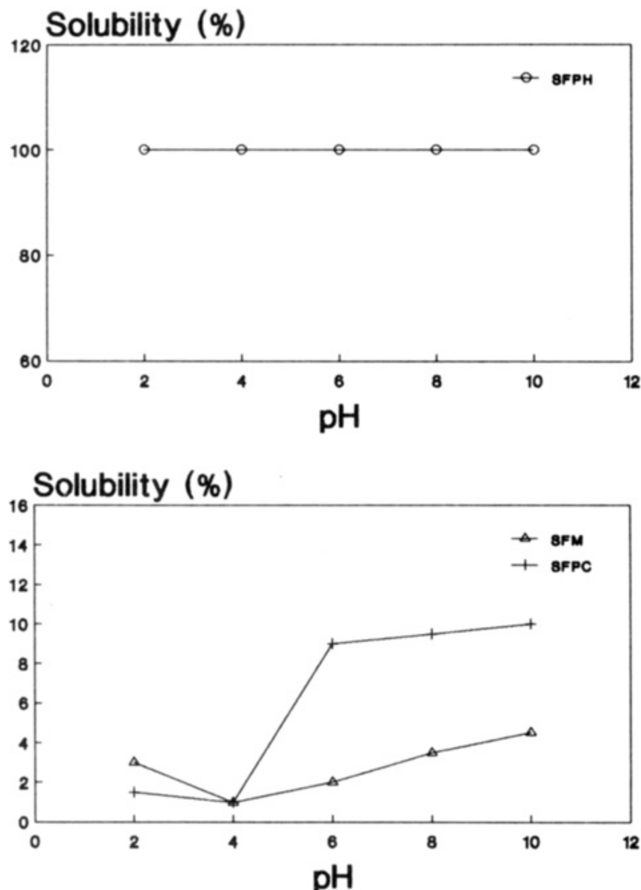

Figure 2. Solubility of SFPH, SFM, and SFPC at different pH values.

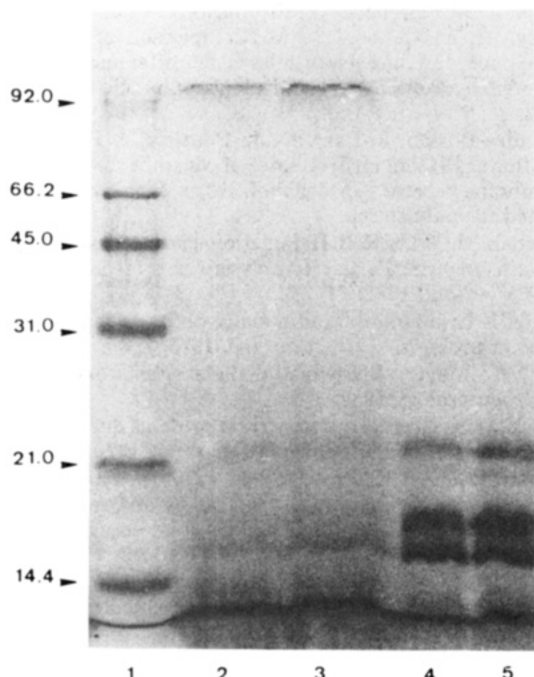

Figure 3. 10% SDS–PAGE of various hydrolysates at different degrees of hydrolysis. (Lane 1) Reference proteins (top to bottom): phosphorylase *b* (92 000/subunit), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 000), and lysozyme (14 400). (Lane 2) SFPH 1.5%. (Lane 3) SFPH 4%. (Lane 4) SFPH 12%. (Lane 5) SFPH 18.8%.

peptides of molecular weight <5000. At higher degrees of hydrolysis (18.8%), only the low molecular weight proteins

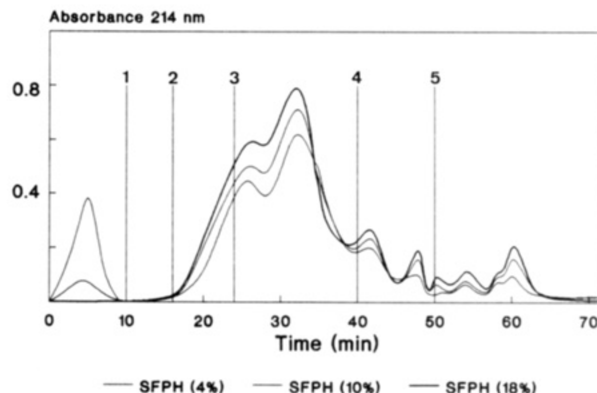

Figure 4. FPLC gel filtration patterns of hydrolysates at different degrees of hydrolysis (column, Superose 12HR 10/30; injection volume, 100 μL; buffer, 0.02 M sodium phosphate, pH 7, with 0.02% NaN_3; flux, 1.0 mL/min). Molecular weight standards: pig heart lactate dehydrogenase (145 900), henovotransferrin (78 000), bovine erythrocyte carbonic anhydrase (30 000), aprotinin (6 500), and adrenocorticotropic hormone fragment 1–4 (1681).

and peptides are present and the large proteins of 300 000 are not detected.

These results can be explained by the splitting of peptide bonds as a result of a series of simultaneous reactions. In the initial stage, the enzyme cleaves the soluble protein and is adsorbed onto the insoluble protein aggregates. The soluble proteins are hydrolyzed more quickly than the compact insoluble protein aggregates that are slowly solubilized by the adsorbed enzyme in the subsequent stages of the reaction. The limiting step of the proteolytic reaction is the adsorption of the protease onto the insoluble protein (results not shown), leading to a one-by-one type of mechanism (Linderstrøm-Lang, 1952). This indicates that no appreciable amounts of intermediary products will be present and the reaction mixture will consist of native protein and end products only. The same kind of reaction was described by Pace and Barret (1984).

The presence of a constant product from an established degree of hydrolysis (12%) is a good feature for the continuous or periodic production of these hydrolysates in a membrane (ultrafiltration) reactor (Hernandez-Pinzon, unpublished results). The continuous or periodic elimination of product would also improve the process by reducing the inhibition by product of the protease (Parrado, 1991).

Amino Acid Composition. The main advantage of enzyme hydrolysis over acid and alkali hydrolysis is that the nutritive quality of the protein remains practically the same as that of the starting protein, with no destruction of amino acids. This is reflected in the great similarity of the amino acid profiles of both SFPC and SFPH.

The amino acid content of the different hydrolysates at different degrees of hydrolysis and that of the starting material are shown in Table III. The overall amino acid profile of the hydrolysates is not significantly different from that of the substrate, although SFPH is slightly richer in lysine than the starting material SFPC. Similar results have been described by Deeslie and Cheryan (1988) for soy isolates. This suggests that SFPC is a very unfolding substrate with many links susceptible to breakage by the protease—Kerase—and that they break randomly.

Nutritional Studies. Compared with most legumes and animal protein sources, sunflower protein hydrolysates are relatively low in lysine (4.05%). As this amino acid is generally considered an indication of the nutritional quality of a protein, its content is important. However,

Table III. Amino Acid Composition (Grams per 100 g of Protein) of Several Protein Hydrolysates and the Raw Starting Materials

amino acid	degree of hydrolysis 1.5%	4%	12%	18.8%	SFPC[a]
Asp + Asn	9.75	10.60	10.30	9.95	9.70
Glu + Gln	18.40	18.80	18.40	17.45	16.70
Ser	5.10	5.60	5.70	5.25	5.15
Thr	4.05	4.10	4.20	4.20	3.70
Ala	8.80	8.10	8.80	9.30	9.70
Pro	5.45	5.00	5.30	5.30	4.95
Cys	0.30	0.60	0.70	0.30	0.60
Leu	6.95	7.00	7.20	7.15	8.50
Ile	4.15	4.20	4.10	4.15	4.45
Gly	8.20	8.20	8.70	8.45	8.60
His	2.35	2.10	2.30	2.50	2.30
Arg	7.50	7.50	7.70	7.70	7.60
Tyr	2.30	2.20	2.30	2.30	2.05
Val	6.60	5.80	5.20	5.85	6.15
Met	2.35	2.70	1.60	2.50	3.15
Phe	3.55	3.60	3.50	3.60	3.75
Lys	4.25	4.00	4.00	4.05	3.65

[a] SFPC is the low-polyphenol protein concentrate.

Table IV. PER Results Obtained from Control Diet and SFPH[a] Diet

	uncorrected	corrected
casein	2.86 ± 0.15	2.5
SFPH	2.51 ± 0.29	2.2

[a] SFPH with a degree of hydrolysis of 18.8% (without insoluble residue).

the consideration of lysine alone to evaluate the nutritional quality of a protein can be insufficient (Kofrányi, 1973; Morup and Olsen, 1976). Therefore, we chose the protein efficiency ratio (PER) method to evaluate the nutritional quality of the SFPHs (see Table IV). The results show that the PER value of 2.2 obtained for the SFPH is acceptable, as plant protein must have a PER value higher than 1.8 to be permitted as a protein source for infants' diets and above 2.0 for use as a partial substitute in animal protein products (Vanderveen et al., 1977).

Conclusions. The use of Kerase as protease for the hydrolysis of SFPC at a degree of hydrolysis 18.8% leads to an SFPH that can be described as a brown-yellow powder with a density of 0.566 g/cm^3, highly hygroscopic, rich in protein (78.9–84.8%), and without antinutritional or undesirable components. SFPH amino acid composition is maintained similar to that of the starting material (SFPC) but with enhanced functional properties—mainly its solubility. In fact, the proteolytic treatment changed the pH solubility profile from the usual U-shape to a fairly flat plateau. Nutritional evaluation of SFPH with rats shows a corrected PER value of 2.2—very similar to that obtained for the standard diet formulated with casein (2.5). No particular adverse effects were observed with the SFPH diet, as seen from the measurement of various biochemical, hematological, and anatomical parameters (results not shown). All of these are strong arguments for the use of these hydrolysates in food applications. SFPHs increase the application of sunflower proteins, making it possible to incorporate them into many food systems, such as low-pH beverages and enteral or parental diets, and in nonfood applications, such as a nitrogen source for fermentation media formulation (Parrado et al., 1993).

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