Peptide Characteristics of Sunflower Protein Hydrolysates

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ABSTRACT: Protein isolate from sunflower seeds was used as the starting material for preparation of an extensively hydrolyzed peptide product. Protein was hydrolyzed using an endopeptidase (Alcalase), an exopeptidase (Flavourzyme), or both enzymes sequentially. Combined use of these proteases generated the highest degree of hydrolysis, 54.2%, and highest solubility, around 90%, between pH 2.5 and 7. Molecular weight profiles of the hydrolysates were characterized by gel filtration chromatography and denaturing electrophoresis. Amino acid compositions and solubilities of the different hydrolysates also were studied.

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KEY WORDS: Alcalase, enzymatic hydrolysis, Flavourzyme, sunflower proteins.

Enzymatic protein hydrolysis is a common procedure for improving functional properties of proteins. For example, some protein hydrolysates display high solubility across a wide range of pH and temperatures, in contrast to native proteins that have poor solubility at pH values around the isoelectric point and at high temperatures. The hydrolysis process generates smaller peptides with improved nutritional characteristics compared to the original protein. In fact, *in vitro* proteolysis is considered a predigestion of proteins that enhances absorption. It has been demonstrated that some short peptides are absorbed in the digestive tract at a higher rate than free amino acids (1). This suggests an advantage of protein hydrolysates with respect to equivalent amino acid mixtures in applications such as diets for surgical patients (2).

In recent years, the availability of industrial proteases, mainly from bacteria and fungi, has enabled production of protein hydrolysates in large scale. Most of these hydrolysates were obtained from milk and soybean proteins, although other sources such as meat, fish, eggs, cereals, and plant proteins have been used (3). Among plants, sunflower (*Helianthus annuus* L.) constitutes an interesting raw material for the preparation of protein hydrolysates. It is one of the more important oilseed crops cultivated in the world, representing the fourth-largest source of edible oil (4). Defatted sunflower meal contains about 30% protein, which could be used as a food ingredient (5). The accompanying high contents of fiber, soluble sugars, and polyphenols have limited the use of this protein source (6). Nevertheless, these undesirable components can be eliminated to a large extent if sunflower meal is processed into a protein concentrate and subsequently into a protein isolate (7).

Sunflower protein hydrolysates have been obtained in the past with proteases such as Kerase (5,8). In these cases the substrate for hydrolysis was a protein concentrate that still contained high amounts of undesirable components such as fiber. Recently, we have used a high-quality sunflower protein isolate as the starting material for the preparation of protein hydrolysates with the proteases Alcalase (endopeptidase), Flavourzyme (exopeptidase), and with Alcalase plus Flavourzyme sequentially (7). In the present work, protein compositions and solubilities of these hydrolysates are reported.

MATERIALS AND METHODS

Materials. Sunflower (*H. annuus* L.) meal, industrially produced by solvent extraction by Koipesol (Sevilla, Spain), was used as the protein source. A protein isolate, prepared as previously described, was used as substrate for the hydrolysis (7). Bromophenol blue, disodium EDTA, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), and D,L-α-aminobutyric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Acrylamide, *N,N′*-methylenebisacrylamide, and Coomassie Brilliant Blue G-250 were purchased from Serva (Heidelberg, Germany). Standards for electrophoresis and gel filtration were supplied by Amersham Pharmacia Biotechnology (Uppsala, Sweden). Diethyl ethoxymethylenemalonate was obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

Proteolytic enzymes.The enzyme preparations used were Alcalase 2.4 L and Flavourzyme 1000 MG (Novo Nordisk, Bagsvaerd, Denmark). Alcalase 2.4 L is a microbial protease from *Bacillus licheniformis* with endopeptidase activity. A main component of the commercial preparation is the serine

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protease subtilisin A. The activity of Alcalase 2.4 L is 2.4 Anson units (AU) per gram. One AU is the amount of enzyme that, under standard conditions, digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as one milliequivalent of tyrosine released per minute. Flavourzyme 1000 MG is a protease complex from *Aspergillus oryzae* that contains both endo- and exoprotease activities. Its activity is 1.0 Leucine aminopeptidase units (LAPU)/g. One LAPU is the amount of enzyme that hydrolyzes one micromole of leucine-*p*-nitroanilide per minute.

Total nitrogen determination. Samples (0.1 g) were used for the determination of total nitrogen by the micro-Kjeldahl method (9). Crude protein content was calculated with a conversion factor of 6.25.

Hydrolysis. The protein isolate (50 g resuspended in 1000 mL of water) was hydrolyzed batchwise by treatment with Alcalase and/or Flavourzyme in a pH-stat. Treatment with the proteases were for 3 h. When both proteases were used sequentially, Alcalase was added first and after 1 h of hydrolysis, Flavourzyme was added for another 2 h of hydrolysis. The hydrolysis curve was obtained by the pH-stat technique with the following hydrolysis parameters: substrate concentration, 5%; enzyme/substrate ratio, 0.3 AU/g for Alcalase and 50 LAPU/g for Flavourzyme; pH 8 for Alcalase and 7 for Flavourzyme; temperature, 50°C. The hydrolysis was conducted in a reaction vessel equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was stopped by dropping the pH to 5. Hydrolysates were clarified by centrifugation at $4{,}000 \times g$ for 30 min to remove insoluble substrate fragments, and the supernatants were lyophylized and freeze-dried for later uses.

Degree of hydrolysis. The degree of hydrolysis, defined as the percentage of peptide bonds cleaved, was calculated by the determination of free amino groups by reaction with TNBS according to Adler-Nissen (10). The total number of amino groups was determined in a sample that had been 100% hydrolyzed at 110°C for 24 h in 6 N HCl (10 mg sample in 4 mL HCl).

Gel filtration chromatography. Samples were passed through a PD-10 column (Amersham Pharmacia) to remove nonprotein components. Gel filtration was carried out in a fast protein liquid chromatography system equipped with a Superose 12 HR 10/30 column (Amersham Pharmacia). Injection volume was 200 μL. The eluent was 20 mM phosphate buffer, 0.5 M sodium chloride buffer, pH 8.3 at a flow rate of 0.4 mL/min. Elution was monitored at 214 nm to detect small peptides lacking aromatic residues. The molecular masses were determined with a calibration curve made with blue dextran 2000 (2000 kDa), catalase (240 kDa), α-amylase (200 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) as molecular weight standards.

Solubility curve determination. Protein isolate and hydrolysates (10 g) were extracted twice with 200 mL of 1N NaOH stirring for 2 hours at room temperature. Aliquots were taken for precipitation of the proteins at different pH values adjusted with HCl. The samples were centrifuged at $4{,}000 \times g$ for 30 min and the nitrogen content determined in the supernatant. With these values the percent solubility was calculated as the percent distribution of protein in the soluble and insoluble fractions.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (11). The gel system, containing 0.2% (wt:vol) SDS consisted of a 15% polyacrylamide-resolving gel (pH 8.8) and a 3% stacking gel (pH 6.8). The lengths of the resolving and stacking gels were 10 and 2 cm, respectively, with a gel thickness of 0.75 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gels in a 0.05% (wt/vol) Coomassie brilliant blue G-250 solution, in 45% methanol and 9% acetic acid solution.

Amino acid analysis. Samples (10 mg) were hydrolyzed with 4 mL of 6 N HCl. The solutions were sealed in tubes under nitrogen and incubated in an oven at 110°C for 24 h. Amino acids were determined in the acid hydrolysis, after derivatization with diethyl ethoxymethylenemalonate, by high-performance liquid cromatography (HPLC), according to the method of Alaiz *et al.* (12), with D,L-α-aminobutyric acid as internal standard. The HPLC system consisted of a model 600E multisystem with a 484 UV-Vis detector (Waters, Milford, MA). Separations were attained with a 300×3.9 mm i.d. reversed-phase column (Novapack C18, 4 m, Waters) with a binary gradient system. The solvents used were (i) 25 mM sodium acetate containing 0.02% sodium azide (pH 6.0) and (ii) acetonitrile. The solvent was delivered to the column at a flow rate of 0.9 mL/min as follows: time 0.0–3.0 min, linear gradient from A/B (91:9) to A/B (86:14); 3.0–13.0 min, elution with A/B (86:14); 13.0–30.0 min, linear gradient from A/B (86:14) to A/B (69:31); 30.0–35.0 min, elution with A/B (69:31). The column was maintained at 18°C with a temperature controller.

RESULTS AND DISCUSSION

Three different protein hydrolysates have been obtained, with the endopeptidase Alcalase, the exopeptidase Flavourzyme, and sequentially with Alcalase plus Flavourzyme (Fig. 1). With Alcalase, a hydrolysate with a 34.7% degree of hydrolysis was generated after 180 min. With Flavourzyme, a 42.2% degree of hydrolysis is achieved. As compared with Alcalase, Flavourzyme hydrolyzed the proteins more slowly, because the exopeptidase activity, and thereby the hydrolytic process, continued even after 120 min. When both proteases were used sequentially, adding Alcalase first and after 1 h adding Flavourzyme, the highest degree of hydrolysis is achieved, with 54.2%, after 3 h (7). In this case, the earlier addition of Alcalase generated a predigestion of the protein, producing more N-terminal sites for the action of Flavourzyme.

Amino acid composition of protein hydrolysates. The amino acid compositions of hydrolysates obtained with Al-

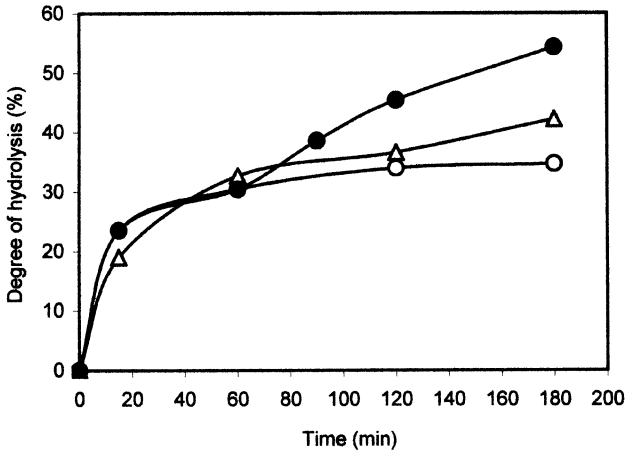

FIG. 1. Enzymatic hydrolysis of sunflower protein isolate with Alcalase (○), Flavourzyme (△), and Alcalase plus Flavourzyme (●).

calase and Alcalase plus Flavourzyme were very similar to the protein isolate, indicating that there were no losses of amino acids as a result of the hydrolytic process (Table I). Nevertheless, the hydrolysate obtained with Flavourzyme alone showed an amino acid pattern with important differences compared to the protein isolate. This is probably due to proteins from the protein isolate that are not solubilized by hydrolysis with Flavourzyme and eliminated by centrifugation after the hydrolytic process. The three types of protein hydrolysates prepared fulfilled the FAO/WHO requirements (13) for 2–5-yr-old children (1991) except for lysine which is limiting in sunflower proteins. The hydrolysate obtained with Flavourzyme was especially poor in this amino acid as well as in aspartic and glutamic acids, although its contents of histidine, tyrosine, and phenylalanine, 6.3, 6.3, and 10.4 g per 100 g of protein, respectively, were very high compared with the FAO pattern.

*Molecular weight characterization of hydrolysates***.** The most evident change observed in hydrolysates with respect to the protein isolates is the reduction in the molecular weight of proteins. The gel filtration profile of the protein isolate shows a main peak around 150 kDa corresponding to helianthin, the 11S globulin of sunflower storage proteins. The peptide size distribution in protein hydrolysates depended on the protease used. When sunflower protein isolate was hydrolyzed with the endopeptidase Alcalase, the 11S globulin disappeared after 60 min, and the profile of the resulting hydrolysate was characterized by peptides that eluted in the range of 20 kDa (Fig. 2). This protein profile was maintained until the end of the hydrolysis with Alcalase, after 180 min, suggesting that no more peptidic bonds were hydrolyzed. This agrees with the curves of hydrolysis which showed that after 60 min, the degree of hydrolysis remained practically unchanged (Fig. 1).

With the exopeptidase Flavourzyme, protein size also decreased to peptides in the range of 20 kDa (Fig. 3) after 60 min. But, because of the lower rate of hydrolysis with Flavourzyme, the reduction in protein size continued even at 180 min. Also, the exopeptidase activity of this protease generated a pool of free amino acids that eluted as a single peak (Fig. 3).

The effect of adding Flavourzyme to proteins that had already been hydrolyzed by Alcalase for 60 min resulted in an aditional reduction in peptide size and a critical increase in

TABLE 1
Amino Acid Composition of SPI, APH, FPH, and AFPH[a]

	Amino acid composition (g/100 g of protein)				
Amino acids	SPI	APH	FPH	AFPH	FAO
Aspartic acid[b]	10.7 ± 0.1	10.9 ± 0.1	3.6 ± 0.1	10.1 ± 0.3	
Glutamic acid[c]	22.0 ± 0.5	21.4 ± 0.5	13.3 ± 0.3	21.8 ± 0.3	
Serine	5.1 ± 0.2	5.0 ± 0.1	6.2 ± 0.2	5.0 ± 0.1	
Histidine	3.0 ± 0.1	3.2 ± 0.1	6.3 ± 0.7	3.1 ± 0.1	1.9
Glycine	4.5 ± 0.1	4.6 ± 0.1	6.9 ± 0.1	4.3 ± 0.1	
Threonine	3.6 ± 0.2	3.7 ± 0.1	4.1 ± 0.2	3.6 ± 0.1	3.4
Arginine	11.6 ± 0.2	11.4 ± 0.1	17.7 ± 0.3	11.2 ± 0.0	
Alanine	3.7 ± 0.1	3.9 ± 0.1	2.2 ± 0.1	3.8 ± 0.1	
Proline	5.1 ± 0.4	2.7 ± 0.6	0.1 ± 0.1	5.0 ± 0.5	
Tyrosine	3.2 ± 0.2	3.4 ± 0.1	6.3 ± 0.4	3.3 ± 0.2	6.3[d]
Valine	4.1 ± 0.2	5.2 ± 0.1	5.1 ± 0.6	4.8 ± 0.2	3.5
Methionine	2.9 ± 0.2	2.7 ± 0.4	3.3 ± 0.2	2.8 ± 0.1	2.5[e]
Cysteine	2.0 ± 0.1	1.6 ± 0.0	2.3 ± 0.1	1.8 ± 0.1	
Isoleucine	3.9 ± 0.1	4.8 ± 0.1	4.7 ± 0.2	4.6 ± 0.2	2.8
Leucine	6.8 ± 0.1	7.1 ± 0.1	6.6 ± 0.1	6.9 ± 0.1	6.6
Phenylalanine	5.2 ± 0.2	5.5 ± 0.1	10.4 ± 0.7	5.2 ± 0.1	
Lysine	2.6 ± 0.1	2.9 ± 0.1	0.9 ± 0.1	2.7 ± 0.1	5.8

[a]Results are the average of fine independent determinations. SPI, sunflower protein hydrolysate; FPH, Flavourzyme protein hydrolysate; APH, Alcalase protein hydrolysate; AFPH, Alcalase plus Flavourzyme protein hydrolysate.
[b]Aspartic acid + asparagine.
[c]Glutamic acid + glutamine.
[d]Tyrosine + phenylalanine.
[e]Methionine + cysteine

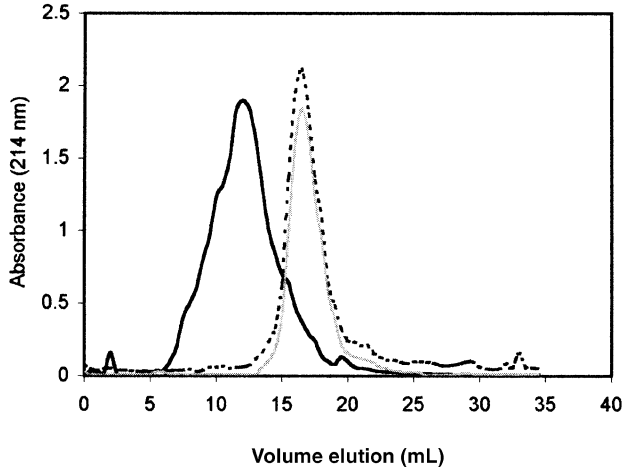

FIG. 2. Gel filtration chromatograph of sunflower protein isolate (0 min, —) and Alcalase protein hydrolysates after 60 min (-----) and 180 min (grey line).

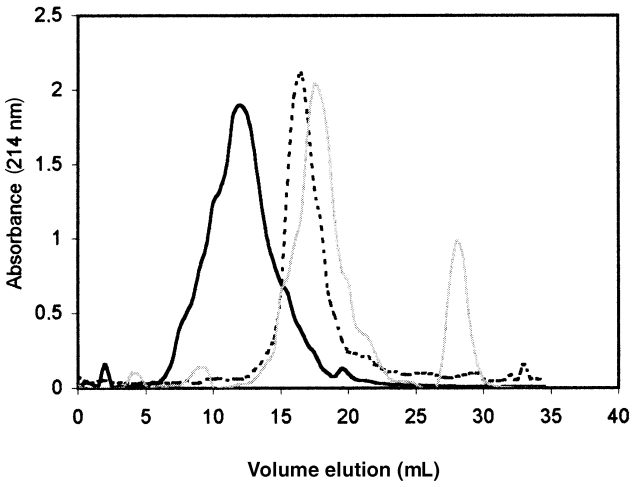

FIG. 4. Gel filtration chromatograph of sunflower protein isolate (0 min, —) and Alcalase plus Flavourzyme protein hydrolysates after 60 (-----) min and 180 min (grey line).

the content of free amino acids as compared with Alcalase or Flavourzyme digestion alone (Fig. 4).

The different hydrolysates obtained were also studied by SDS-PAGE. Hydrolysates generated with Flavourzyme were characterized by the conservation of protein bands in the range of 20–30 kDa that were resistant to the action of this protease (Fig. 5). These bands probably correspond to β subunits of the 11S storage protein of sunflower. These subunits are in the inner part of helianthin and are hardly cleaved by Flavourzyme because of their low accessibility (14,15). On the other hand, higher molecular weight proteins, between 30 and 67 kDa, gradually disappeared during the incubation with Flavourzyme.

Alcalase hydrolyzed quickly the peptidic bonds of the proteins, and protein bands practically disappeared after 15 min of reaction. Even upon loading a high amount of protein only a band with a molecular weight around 20 kDa that remained resistant to hydrolysis by Alcalase was visible (Fig. 6). SDS-PAGE profile of peptides hydrolyzed with Alcalase plus Flavourzyme was similar to the one obtained with Alcalase alone.

Solubility of protein hydrolysates. Good solubility of proteins is required in many functional applications, especially emulsions, foams and gels, because soluble proteins provide a homogeneous dispersability of the molecules in colloidal systems and enhance the interfacial properties (16). A direct consequence of enzymatic hydrolysis is the increase of solubility of the resulting peptides over a wide range of pH, temperature, nitrogen concentration, and ionic strength conditions (17). This increase is particulary high at the acidic isoelectric point of the original protein. Thus, the low solubility of sunflower protein isolate was greatly improved with Al-

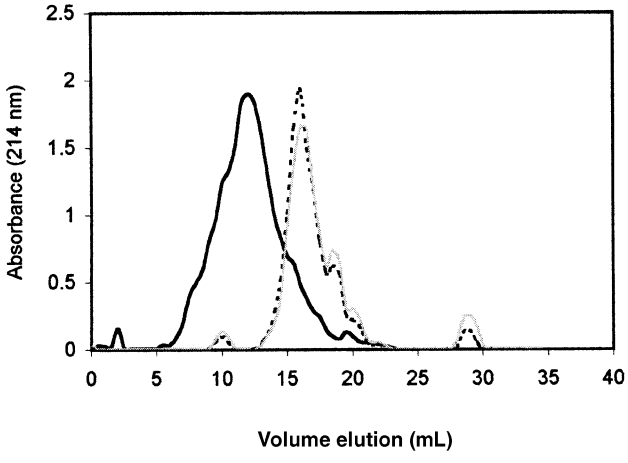

FIG. 3. Gel filtration chromatograph of sunflower protein isolate (0 min, —) and Flavourzyme protein hydrolysates after 60 (-----) min and 180 min (grey line).

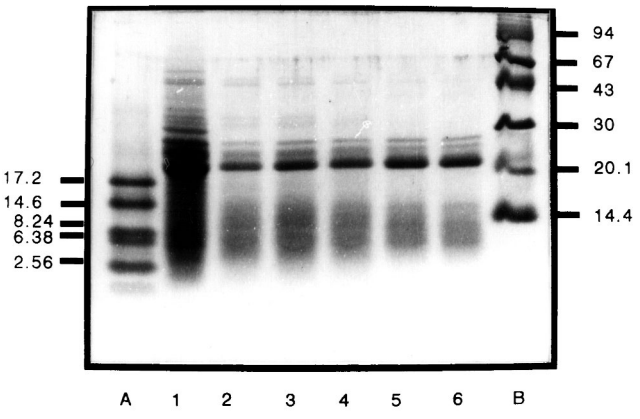

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile of sunflower protein isolate (lane 1) and Flavourzyme hydrolysates after 15 (2), 30 (3), 60 (4), 120 (5), and 180 min (6). Molecular weight markers (kDa) are indicated on the left (A) and right (B).

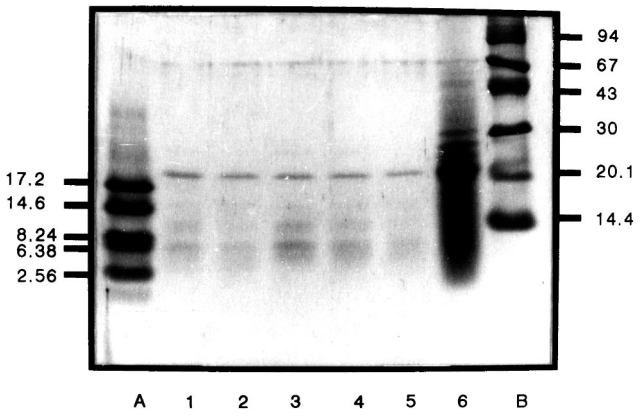

FIG. 6. SDS-PAGE protein profile of sunflower protein isolate (lane 6) and Alcalase hydrolysates after 15 (1), 30 (2), 60 (3), 120 (4), and 180 min (5). Molecular weight markers (kDa) are indicated on the left (A) and right (B). For abbreviation see Figure 5.

calase (Fig. 7). As expected, the greatest increase in solubility was observed around the isoelectric point of the original protein. Thus, the low solubility of the protein isolate, around 5–10%, between pH 3 and 5 increased to almost 70% after 15 min of hydrolysis with Alcalase. This high solubility as a result of protein hydrolysis has been observed in wheat protein hydrolysates even with a degree of hydrolysis of 2% (18,19). The nitrogen solubility of Alcalase hydrolysate increased with the degree of hydrolysis and, after 60 min, the percentage of soluble product was around 80%. This percentage was maintained until the end of hydrolysis with Alcalase.

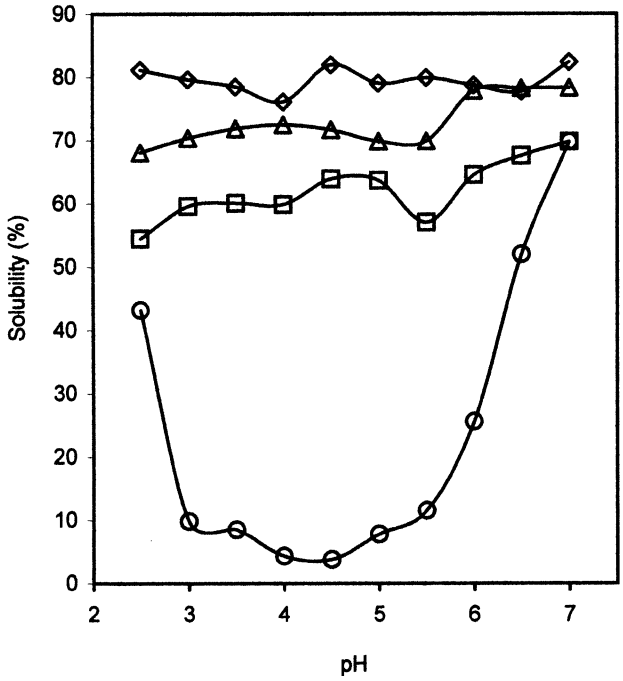

FIG. 8. Solubility at different pH values of sunflower protein isolate (0 min, ○) and Flavourzyme protein hydrolysates after 15 (□), 60 (△), and 180 min. (◇).

As compared with Alcalase, Flavourzyme increased more slowly the solubility of sunflower proteins as a concequence of its different catalytic activity (Fig. 8). Thus, after 15 min of hydrolysis, the percentage of soluble protein was around

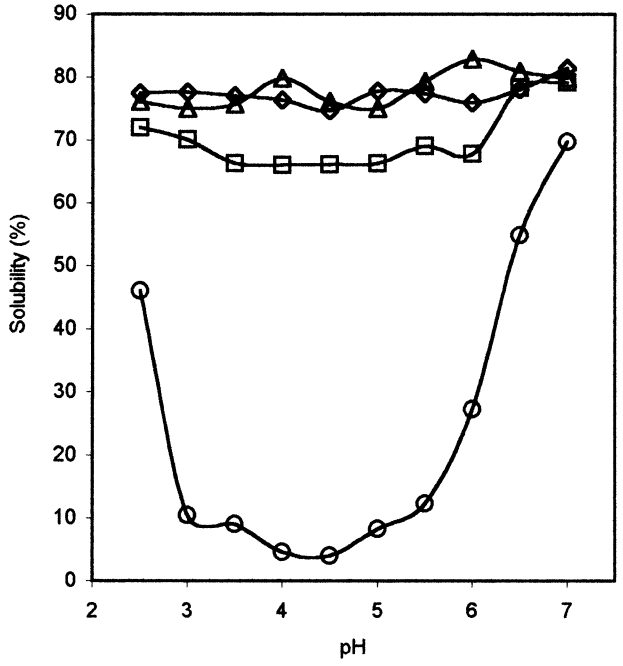

FIG. 7. Solubility at different pH values of sunflower protein isolate (0 min, ○) and Alcalase protein hydrolysates after 15 (□), 60 (△), and 180 min (◇).

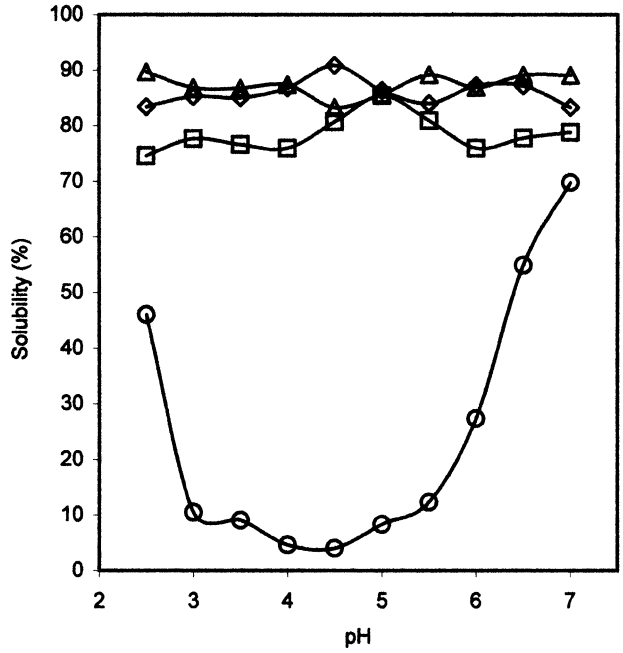

FIG. 9. Solubility at different pH values of sunflower protein isolate (0 min, ○) and Alcalase plus Flavourzyme protein hydrolysates after 30 (□), 90 (△), and 180 min (◇).

60%, but this value gradually increased, reaching 80% after 180 min of hydrolysis.

As expected, the highest solubility was obtained with the use of both proteases, reaching 90% solubility at the end of the hydrolytic process with Alcalase plus Flavourzyme (Fig. 9).

In conclusion, the use sequentially or in cascade of two proteases with different catalytic activities generated an extensive protein hydrolysate with the highest degree of hydrolysis and solubility. This hydrolysis maintained the amino acid composition of the original sunflower proteins and, owing to its high solubility, may increase the field of application of these proteins, increasing the value of defatted sunflower meal. For example, in the last years protein hydrolysates from plant sources have been used in the enhancement of savory flavors, supplementation of liquid foods or high energy beverages, production of hypoallergenic foods, or preparation of special diets for the treatment of specific illness (19).

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