Sunflower Protein Hydrolysates for Dietary Treatment of Patients with Liver Failure

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ABSTRACT: A method is described to obtain hydrolysates with defined characteristics and a high Fischer ratio for patients with liver failure, using sunflower proteins (globulin fraction-II) as starting material. Protein with a branched chain amino acid (BCAA) concentration of 29.7 ± 1.7% is treated in a first step with immobilized chymotrypsin (raw hydrolysate-1). Subsequent ultrafiltration (cut-off 3 kDa) of the hydrolysate gives sunflower protein hydrolysate-I (SFPH-I). In a second step, SFPH-I is treated with immobilized carboxypeptidase-A at alkaline pH for quasi-selective removal of aromatic amino acids (AAA). This sequential two-step process, followed by size exclusion chromatography on a Sephadex G-15 column, yields a product (SFPH-II) with a BCAA concentration of 37.4 ± 2.2% and an AAA concentration of 0.5 ± 0.1%, which gives a very high Fischer ratio (≈75). The product, comprising mainly peptides with molecular weights in the range of 3500 to 750 Da and free amino acids, is hypoallergenic and shows no or only a trace of bitterness. Any bitterness can be completely removed by treatment with Flavozyme®, giving a hydrolysate that is composed mainly by tri- and dipeptides and free amino acids, and is termed highly hydrolyzed protein hydrolysate (HHPH). Both SFPH-II and HHPH can be used in enteral, parenteral, and oral nutrition for the treatment of patients with liver failure. This product presents all the conditions required for use in the treatment of patients with liver failure: high content in BCAA and low content in AAA, below 2%, and consequently, a very high Fischer ratio, ≅75.

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In developed countries, human nutritional deficiency diseases such as pellagra, goiter, rickets or scurvy are uncommon as a result of nutrition research programs and government interventions, leading to the creation of food products designed to supply essential nutrients. Current nutritional research and product development is focused on products helping to reduce or control diet-related chronic diseases such as atherosclerosis, cancer or liver failure, as well as special products for weight control and for hospitalized patients (1).

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Nutritional support in patients with liver disease is one of the most rewarding areas of treatment because of the clinically obvious improvement in well-being of the patient and the subsequent reduction of morbidity and mortality. Currently, treatments under investigation include the administration of insulin growth factors, branched-chain amino acids (BCAA: valine, leucine, isoleucine), structured lipids, and polyunsaturated fatty acids (2,3).

Protein hydrolysates enriched with up to 40–50% BCAA have been recommended for patients (both children and adults) with liver disease, because BCAA may reduce plasma concentration of aromatic amino acids (AAA), commesurated with improvement in encephalopathy (4). BCAA are preferentially taken up by muscle and are therefore theoretically available for peripheral metabolism even in advanced liver disease (5). Therefore, preparations of products with a high concentration of BCAA and a low content of AAA (high Fischer ratio) (6) are desirable in diet formulations for patients with liver disease. However, the preparation of proteic products with a high content in BCAA is not easy.

With regard to peptide size, short peptide hydrolysates with characteristic amino acid composition and defined molecular weight, and which are nonbitter and hypoallergenic, are highly desirable in both nutrition and food science for functional and nutritional purposes (7). Hydrolysates are better than free amino acids in two main features: (i) short peptides are absorbed in the digestive tract at higher rate (8), and (ii) the osmotic pressure of peptide is lower than that of the corresponding free amino acids (9). In this context, preparation of hydrolysates composed of short peptides with well-defined characteristics promises to be useful in producing physiologically functional foods for specific needs, such as in patients with malnutrition associated with cancer, burns, traumas, and liver failure, and for nutritional support of children with chronic or acute diarrhea or milk protein allergies (10).

Proteic products with a high BCAA concentration and low AAA content can be obtained in two ways. One is the enzymatic or chemical synthesis of peptides from their constituent amino acids (11). The other, which is the subject of our work, is the fractionation/separation and characterization of peptide groups from an enzymatic protein hydrolysate (7,12). The lat-

ter may be more economical than the former when the peptides are for larger-scale production as foodstuffs.

In this work, we described our experience in the preparation of protein hydrolysates for clinical nutrition, especially for patients with liver disease, which require protein nitrogen in the form of products rich in BCAA and poor in AAA, that is, products with a high Fischer ratio.

MATERIALS AND METHODS

Materials. Polyphenol-free sunflower proteins, in the form of sunflower protein isolates (SFPI) (80–90% protein), obtained from defatted sunflower meal as described previously (9), were fractionated into albumin and globulin by the method of Kortt and Caldwell (13). The globulin fraction was further fractionated in two main fractions, sunflower protein globular fraction-I (SFGF-I) and SFGF-II, by hydrophobic chromatography on a phenolic resin (PR) column (14). SFGF-II, because of its high contents in BCAA, was used as starting material for the preparation of hydrolysates with both a high content in BCAA and a high Fischer ratio.

Hydrophobic chromatography on PR. Hydrophobic chromatography was carried out on PR, prepared by packing prehydrated Duolite S-761 resin (Supelco, Bellefonte, PA) into a chromatography column (5.2 × 100 cm) and processed by standard procedure (14). The resin was washed with 2 vol of 2% (wt/vol) NaOH and rinsed with 2 vol of deionized water followed by 3 vol 1% (vol/vol) HCl and finally followed by deionized water until the effluent pH was 5.5. SFGF samples were applied to the top of the column and passed through the resin bed at 10 mL/min. Optical density of the effluent was monitored at 280 nm. The effluent before the appearance of the highest peak was collected as the SFGF-I and the effluent after the peak was collected as the SFGF-II. The PR column process was performed at room temperature, and the PR column was regenerated with 2 vol of 10% methanol followed by water until base line absorbance was obtained.

Enzymes. The enzymes chymotrypsin (Worthington Biochemical Co., Lakewood, NJ) and carboxypeptidase-A (Sigma Chemical Co., St Louis, MO) were immobilized by Dr. J.M. Guisan at the Instituto de Catálisis (Madrid, Spain). Agarose immobilized chymotrypsin (5000 U/mg), Sepharose immobilized carboxypeptidase-A (150 U/mg), and Flavozyme® (Novo Nordisk, Bagsvaerd, Denmark) (3000 U/mg) were used in this study for the hydrolytic processes.

Enzymatic hydrolysis. (i) First hydrolysis. SFGF-II was hydrolyzed batchwise in a fluidized bed reactor (15) with agarose-bead immobilized chymotrypsin at a constant pH of 8.0 (pH-stat) until a constant degree of hydrolysis (DH) of 12% is achieved.

(ii) Second hydrolysis. The hydrolysate originating in the first hydrolysis, sunflower protein hydrolysate-I (SFPH-I), was filtered through a 0.2-µm microfiltration membrane and a 10 kDa ultrafiltration membrane, and the filtrate was subjected to a second hydrolysis by recirculation through a column containing Sepharose immobilized carboxypeptidase-A at pH 9.0 until base consumption stopped. The product obtained was filtered through a 0.2-µm microfiltration membrane, and the filtrate chromatographed on a Sephadex G-15 column for the separation of peptides and some free amino acids [(tyrosine (Tyr) and phenylalanine (Phe)], which eluted at the end of the chromatogram (7). The peptide fraction constitutes SFPH-II.

(iii) Third hydrolysis. Highly hydrolyzed products were prepared by treatment of the appropriate substrate (i.e., SFPH-II) with Flavozyme®, a mixture of endo- and exoproteases, until a constant DH of 48–52% (15) was achieved.

Degree of hydrolysis. The DH, defined as the ratio (expressed as %) between the number of hydrolyzed peptide bonds and the total number of bonds available for hydrolysis, was measured by the pH-stat method (9).

Peptide fractionation. The hydrolysate obtained after the second hydrolysis was filtered by tangential flow microfiltration through an open channel cassette equipped with a 0.2-µm microfiltration membrane (Millipore, Bedford, MA), and subsequently fractionated by tangential flow ultrafiltration through a screen channel cassette equipped with a 3 kDa ultrafiltration membrane, installed in a Pellikon module (Millipore), into two fractions: ultraconcentrate-3 (UC3) and ultrafiltrate-3 (UF3).

Sephadex G-15 chromatography. Lyophilized raw hydrolysate-2 dissolved in 10% ethanol (vol/vol) at a concentration of 50 mg/mL, was poured onto a Sephadex G-15 column (4.0 × 25 cm), with a V_0 of 31 mL and a V_t and 1,275 mL. Elution was performed with the same solvent (10% ethanol, vol/vol) at 120 mL/h flux. Absorbance was measured at 220 nm. The main peak at the beginning of the chromatogram was collected as SFPH-II.

Molecular weight distribution. Molecular weight distribution of the hydrolysates was determined by gel permeation chromatography on a Superose 12-HR 10/30 column (Pharmacia/LKB) according to the procedure described previously (7). Molecular weight markers used were tryptophan (M_r 204), insulin chain B fragment 22-30 (M_r 1086) and insulin chain A (M_r 2533).

Amino acid composition. Amino acid composition and free amino acids content were determined by high-performance liquid chromatography (HPLC) using the method of Bidlingmeyer *et al.* (16). Performic acid oxidation was used to determine cystine and methionine (17), and basic hydrolysis to determine tryptophan (18).

Antigenicity. Immunologically active proteins in the hydrolysates were defined by referring to the unhydrolyzed SFPI proteins, using an inhibition enzyme-linked immunosorbant assay (ELISA) (19). SFPI protein antigen solutions at 5, 10, 50, 100, 500, 1,000, 2,000, and 4,000 ng/mL were prepared by using whole SFPI protein, and these were used to establish a standard curve by plotting logarithm of % inhibition of the ELISA signal (A/A_o) vs. logarithm of SFPI protein concentration. Hydrolysate antigen concentration was determined by reference to the standard curve and expressed as micrograms of immunoabsorbed sunflower protein (IASFP) per gram of product.

TABLE 1
BCCA and AAA Content (%) and Fischer Ratio of SFPI and of Sunflower Globulin and Albumin Fractions[a]

	SFPI	Sunflower globulin fraction*	Sunflower albumin fraction
BCAA	19.2 ± 1.2	32.1 ± 1.7	16.3 ± 1.8
AAA	10.6 ± 0.8	5.3 ± 1.1	6.7 ± 0.9
Fischer ratio	1.8	6.1	2.4

[a]SFPI, sunflower protein isolate; BCAA, branched-chain amino acids [valine (Val) + leucine (Leu) + isoleucine (Ile)]; AAA, aromatic amino acids [phenylalanine (Phe) + tyrosine (Tyr)]; Fischer ratio, (Val + Leu + Ile)/(Phe + Tyr).

Sensory evaluation. The freeze-dried hydrolysate samples were reconstituted to 3% (by weight) in tap water. Five trained taste panelists were selected for their sensitivity to bitterness and their ability to detect bitterness differences in triangular tests with caffeine solution of varying concentrations. All taste panel evaluations took place in a uniformly lit room equipped with individual booths for the panelists. The strength of bitterness was indicated on a six-point scale. The caffeine solutions used as bitterness standards were as follows: 0, not bitter (no caffeine); 1, trace of bitterness (0.025%); 2, slightly bitter (0.05%); 3, bitter (0.1%); 4, very bitter (0,2%); 5, extremely bitter (0.3%). The data from the scoring test were treated by analysis of variance and Tukey's studentized range tests using the Systat program (v.5.1; SPSS Inc., Chicago, IL) with significance of difference defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

The analysis of amino acid composition of 36 different vegetable protein sources, such as soy, sunflower, lupines, brassica, pea, chick-pea, cow-pea, faba, wheat, barley, maize, rye, lucerne and the like, shows that the BCAA concentration varies from 11.2% in lucerne to 20.3% in maize (20). Such concentrations are too low to allow one to use these sources as starting material for the production of high-concentration BCAA products required for the treatment of patients with liver failures (4). However, analysis of the amino acid compositions of the albumin and globulin fractions of sunflower, soy, lupines, brassica, chick-pea, and pea obtained by the method of Korrt and Caldwell (13), shows that in the case of sunflower proteins, the globulin fraction (SFGF) has a significantly higher BCAA content (25.8 ± 1.5%) than the SFPI (15) and the albumin fractions (19.2 ± 1.2% and 16.3 ± 1.8%, respectively) (see Table 1). Fractionation of the SFGF by hydrophobic chromatography on a PR resin (Fig. 1) yielded two main fractions, SFGF-I and SFGF-II. Analysis of the amino acid composition of these fractions shows that SFGF-II had a content of 32.1 ± 1.7% in BCAA, and a AAA content of 5.3 ± 1.1% (Table-1). Because of its high BCAA content, we selected SFGF-II as the starting material for the production of BCAA-rich hydrolysates with a high Fischer ratio (BCAA/AAA).

The strategy used in our laboratory for the preparation of hydrolysates with a high Fischer ratio is summarized in Scheme 1. SFGF-II was hydrolyzed batchwise in a fluidized bed reactor, by treatment with agarose-bead immobilized chymotrypsin in the pH-stat mode using the following hydrolysis parameter: substrate concentration = 8%; enzyme/substrate ratio =5000 U/g; pH = 8.0; temperature = 60°C. The reaction was continued until a constant DH was achieved (DH = 10–12%, depending of the lot of SFPI) (15). The obtained product constitutes the raw hydrolysate. Under these conditions, as chromatographic and electrophoretic studies show, these raw hydrolysates are composed of small proteins of molecular weight below 30 kDa and peptides of low molecular weight (15). In some cases (those of DH < 10), a large protein band with an approximate relative molecular weight of 300 kDa (probably helianthinin) was also observed. At DH = 12 only the low molecular weight proteins and peptides were observed (15). Size exclusion chromatograms of these hydrolysates on a Sepharose 300 column showed a broad peak at the beginning and two sequential peaks at the end of the chromatogram. Electrophoretic and chromatographic analy-

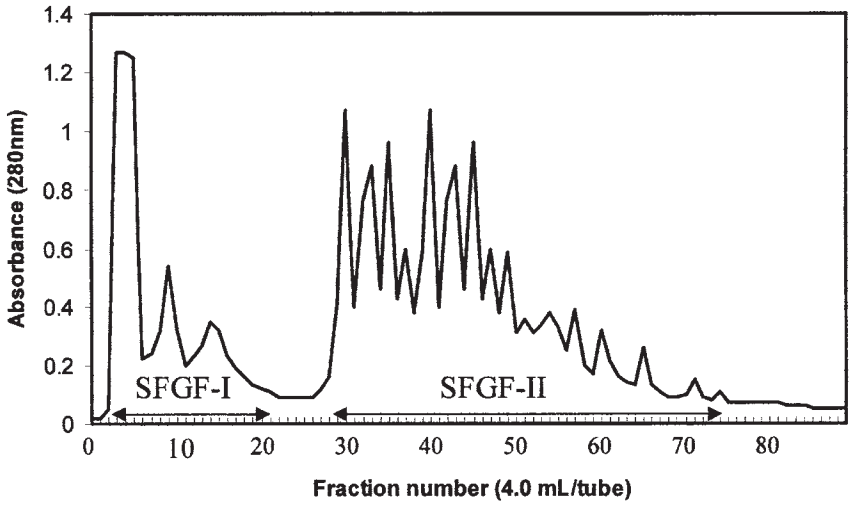
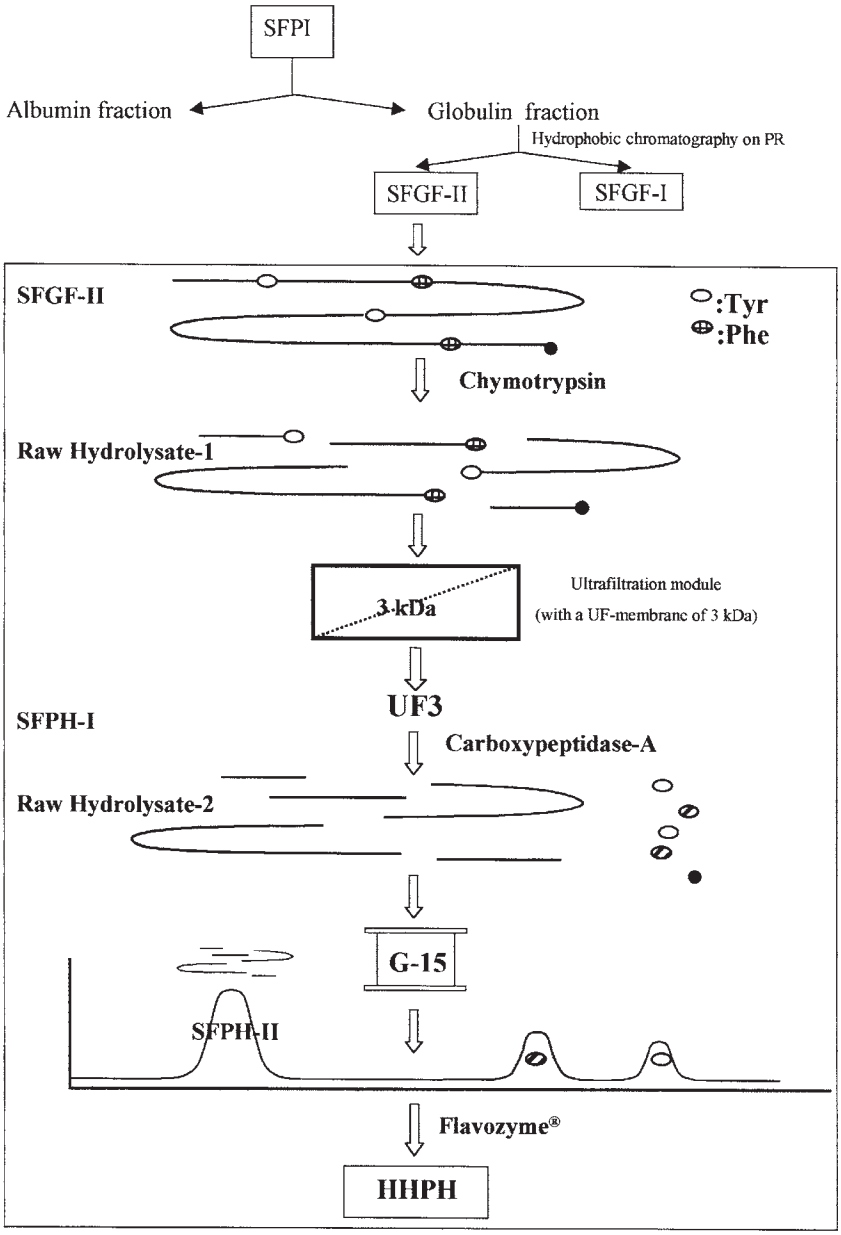


FIG. 1. Hydrophobic chromatography on phenolic resin of sunflower globulin fraction (SFGF), yielding sunflower protein globular fraction-I (SFGF-I) and SFGF-II.



SCHEME 1

ses of these peaks showed that the broad peak comprised proteins and peptides, and that the two sequential peaks at the end of the chromatogram comprised low molecular weight peptides, tri- and dipeptides, and a small amount of free amino acids. A detailed analysis showed that proteins and peptides of molecular weight >5 kDa represented 10–15% of the product (15). These data suggest the possibility of fractionating hydrolysates by ultrafiltration (7). Fractionation with an ultrafiltration membrane of cut-off 3 kDa yielded two fractions—UC3 and UF3—constituting 14.6 ± 1.4% and 85.4 ± 1.4%, respectively (21), of the total protein nitrogen. From a practical and quantitative point of view, the more interesting fraction is UF3, characterized by having a low content of free amino acids and a very high percentage of peptides with molecular weight < 3 kDa. A detailed analysis of this fraction by fast protein liquid chromatography (FPLC) showed a main peak with relative molecular weights in the range 3000–750 Da, and two minor peaks corresponding to free amino acids (15). The chemical composition and the content in BCAA and AAA of the resulting product—sunflower protein hydrolysate-I (SFPH-I)—are shown in Table 2.

The use of chymotrypsin in the first hydrolysis leads to peptides with AAA and bulky hydrophobic amino acids at the C-terminus. Ultrafiltration through a 3 kDa UF membrane yields SFH-I. This hydrolysate has a protein concentration of 94.3 ± 1.8% and a BCAA and AAA composition and Fischer ratio similar to the starting material (Table 2). The use of immobilized carboxypeptidase A at alkaline pH permits the quasi-se-

TABLE 2
Protein, BCAA, and AAA Composition (%) and Fischer Ratio of SFGH-II, SFPH-I, SFPH-II, and HHPH

	SFGF-II	SFPH-I	SFPH-II	HHPH
Protein (%)[a]	93.1 ± 2.1	94.3 ± 1.8	94.0 ± 2.3	93.2 ± 2.9
BCAA	32.1 ± 1.7	33.0 ± 2.3	37.4 ± 2.2	37.6 ± 3.1
AAA	5.3 ± 1.1	5.1 ± 1.3	0.5 ± 0.1	0.5 ± 0.1
Fischer ratio	6.1	6.5	74.8	75.2

[a]Dry basis. SFGF-II, sunflower protein globulin fraction-II; SFPH-I and SFPH-II, sunflower protein hydrolysates-I and -II; HHPH, highly hydrolyzed protein hydrolysate.

lective elimination of AAA—mainly Phe and Tyr (21). The separation of peptides from free amino acids—mainly Phe and Tyr—would result in a marked increase of the Fischer ratio. This separation was carried out by size exclusion chromatography on Sephadex G15 according to a procedure described in previous works of our laboratory and others (7). The product obtained—SFPH-II, with a protein concentration of 94.0 ± 2.3% and a concentration in BCAA and AAA of 37.4 ± 2.2% and 0.5 ± 0.1%, respectively—has a very high Fischer ratio, 74.8. Characterization of this product by size exclusion HPLC showed that it was composed mainly of peptides (83.3%) of relative M_r range from 3500 to 750 Da and free amino acids (16.7%). This product presents all the conditions required for use in the treatment of patients with liver failure: high content in BCAA and low content in AAA, below 2% (22), and consequently, a very high Fischer ratio, ≅75.

The highly hydrolyzed products (DH > 50%) needed for parenteral nutrition require a third hydrolysis. We used for that purpose Flavozyme®, a commercial mixture of exo- and endoproteases. Hydrolysis was carried out batchwise using soluble Flavozyme®. The hydrolysis parameters were the following: substrate concentration = 5%; enzyme-substrate ratio = 3000 U/g; pH = 7.0; temperature = 55°C. The product obtained, highly hydrolyzed protein hydrolysate (HHPH), had a protein content of 93.2 ± 2.9% and was composed of tetra-, tri-, and dipeptides, and free amino acids. As was expected, it also had a very high Fischer ratio (Table 2).

All the products obtained were tested for it antigenicity and bitterness, following well-established procedures.

Antigenicity. All the products obtained were tested for allergenic response induction using antisera against sunflower proteins. Although the initial raw hydrolysate-1, obtained by the treatment with chymotrypsin, shows an allergenic concentration of 12,416 µg IASFP/g product, the SFPH-I, obtained by ultrafiltration through a membrane of 3 kA, only shows a 6.07% of the initial antigen concentration. However, in the raw hydrolysate-2 and SFPH-II, the allergenicity is practically undetectable, 0.07 and 0.001%, respectively. While in HHPH the allergenicity is not detected. The main allergens are retained by the UF membrane (3 kDa) in the UC-3, indicating that they are proteins or peptides of molecular weight >3 kDa. The treatment with carboxypeptidase also drastically reduced antigenicity, probably due to the appreciable reduction of bound Phe and Tyr present in raw hydrolysate-2 and its low content in SFPH-II. It seems that these amino acids are implicated in the formation of antigenic determinants (Blanco, J., personal communication). Highly hydrolyzed protein hydrolysate (HHPH), obtained by hydrolysis with Flavozyme®, was nonallergenic.

Bitterness. All the products obtained were tested for bitterness as described in Materials and Methods section. Although SFPI (3%) was not bitter, raw hydrolysate-1 (3%) and SFPH-I (3%) were slightly bitter, as was raw hydrolysate-2 (3%). SFPH-II (3%) showed only traces of bitterness, and HHPH (3%) was not bitter, although the content in BCAA, which has hydrophobic side chains, was very high. The loss of bitterness in both of these products could be attributed to the drastic reduction in AAA content and their relatively low content of arginine and proline (results not shown) (22).

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