Nutritional and Antigenic Characterization of an Enzymatic Whey Protein Hydrolysate

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The present work was mainly undertaken to describe a method of characterizing protein hydrolysates suitable for use in enteral formulas. A commercial enzymatic whey protein hydrolysate was studied. Its chemical composition, molecular weight distribution, nutritional qualities (assessed by the measurement of the digestive and metabolic nitrogen utilization in growing rats), and potential antigenicity (by *in vitro* and *in vivo* tests) were determined. The large majority of this hydrolysate consisted of small peptides with only a small fraction (7.42%) higher than 10^3 Da but lower than 3×10^3 Da. The nutritional quality of the whey protein hydrolysate was good as it resulted in high nitrogen protein utilization and biological value as compared to the protein of reference (casein plus 5% DL-methionine). However, the protein efficiency ratio was slightly lower for the whey protein hydrolysate as compared to the control protein. The antigenicity of the whey protein hydrolysate was about 5 orders of magnitude lower than that of β-lactoglobulin, and it showed therapeutic and prophylactic properties in guinea pigs sensitized with either the native whey protein or the hydrolysate. The chemical, nutritional, and low antigenic properties of the whey protein hydrolysate make it appropriate for inclusion in enteral formulas, although clinical trials should first be carried out.

Keywords: *Whey protein hydrolysates; nutritional value; antigenicity*

INTRODUCTION

Peptide-based diets are widely used in the nutritional recovery of patients suffering from a variety of gastrointestinal malabsorption syndromes (Brinson *et al.*, 1989), patients with specific metabolic disorders (De Freitas *et al.*, 1993), and infants suffering from cow's milk allergy and other food protein intolerances (Walker-Smith *et al.*, 1989).

Peptides may be absorbed slightly better and more quickly than amino acids or whole protein according to several human and animal studies (Matthews, 1977; Grimble and Silk, 1989; Zaloga, 1990). This is due to the fact that di- and tripeptides can be directly absorbed from the gut by uptake systems that are independent of those used by amino acids (Matthews, 1977).

Moreover, recent studies have found that rats grow more rapidly when fed with isocaloric and isonitrogenous quantities of a peptide diet as compared to intact protein and amino acid based diets (Zaloga *et al.*, 1991). Poullain *et al.* (1989) measured nitrogen retention in rats that were refed after starvation with a diet containing the protein source as intact protein, protein hydrolysates, or free amino acids; the greatest nitrogen balance was shown when the hydrolyzed protein was used.

Apart from the nutritional qualities, it is critical to study the potential antigenicity of the protein hydrolysates. Thus, it is very important to check that the oral administration of the protein hydrolysate does not induce sensitization to major proteins present in the starting material or to residual peptides using appropriate animal models (Pahud *et al.*, 1988; American Academy of Pediatrics, 1989; Leary, 1992).

The present study was mainly carried out to describe the composition, amino acid profile, molecular weight distribution, nutritional quality, and antigenic properties (measured by *in vitro* and *in vivo* assays) of an enzymatic whey protein hydrolysate.

MATERIALS AND METHODS

An enzymatic whey protein hydrolysate (WPH) was provided by Puleva, Nutritional Division (Granada, Spain). This hydrolysate was produced by enzymatic hydrolysis of whey protein using fungal and bacterial proteases, followed by heat treatment and ultrafiltration. The protein content was analyzed according to the Kjeldahl procedure (AOAC, 1980) and calculated by subtracting the mineral nitrogen from the total nitrogen and multiplying the result by 6.38. Water content was determined by oven-drying at 105 ± 1 °C until a constant weight was obtained. Ash was analyzed according to AOAC (1980) methods. Lactose content was determined enzymatically according to the FIL-IDF (1991) method. The sample analyzed was a composite of several production batches.

Molecular Weight Distribution. The molecular weight of the WPH was investigated by HPLC, performed in a Waters (Milford, MA) liquid chromatograph on two Toyosoda TSK-2000 SW columns, connected in series. The injection volume was 5 μL, and the elution buffer was 6 M guanidinium hydrochloride at a flow of 1 mL/min. The column effluent was detected by absorbance at 280 nm. The columns were standardized with the following molecular weight standards: bovine serum albumin (67 000), ovalbumin (44 000), chymotrypsinogen (25 000), ribonuclease A (13 700), insulin (6000), insulin chain A (2530), sleep-inducing peptide (849), *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide (451), tryptophan (205), phenylalanine (165), and glycine (75) (Knights, 1985). Likewise, the free amino groups in the WPH were analyzed by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method using glycine as standard (Snyder and Sobocinski, 1975). The osmolarity of the WPH was measured as previously described by Parrado *et al.* (1993).

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Table 1. Composition of the Diets Based on Casein + 5% DL-Methionine (Control) and on the Whey Protein Hydrolysate (WPH)

ingredient	control	WPH
casein (g/kg)	129.8	
whey protein hydrolysate (g/kg)		145.0
DL-methionine (g/kg)	6.3	
soy oil (g/kg)	50.0	50.0
corn starch (g/kg)	397.6	388.7
sucrose (g/kg)	300.0	300.0
cellulose (g/kg)	80.0	80.0
choline chloride (g/kg)	1.0	1.0
mineral mix[a] (g/kg)	35.0	35.0
vitamin mix[b] (g/kg)	0.3	0.3
chemical composition		
protein (%)	13.3	12.8
fat (%)	4.1	4.0
carbohydrates (%)	73.1	73.5
ash (%)	3.3	3.7
moisture (%)	6.2	6.0

[a] Contained the following (g/kg mix): $CaHPO_4$, 500.0; NaCl, 74.0; K citrate·H_2O, 220.0; K_2SO_4, 52.0; MgO, 24.0; $MgCO_3$, 3.5; Fe citrate, 6.0; $ZnCO_3$, 1.0; $CuCO_3$, 0.3; KIO_3, 0.01; $Na_2SeO_3 \cdot 5H_2O$, 0.01; $KCr(SO_4)_2 \cdot 12H_2O$, 0.56; sucrose, 118.03. [b] Contained the following (g/kg mix): thiamin hydrochloride, 6; riboflavin, 6; pyridoxine hydrochloride, 7; nicotinic acid, 30; calcium D-pantothenate, 16; folic acid, 2; D-biotin, 0.2; cyanocobalamin, 0.01; retinol acetate, 0.014; cholecalciferol, 0.025; DL-α-tocopherol, 5; menadione, 0.025; cornstarch, 927.726.

Amino Acid Composition. The WPH was further hydrolyzed to amino acids under vacuum at 110 °C for 24 h in 6 M HCl. Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, after performic acid oxidation. For the analysis of tryptophan, an alkaline hydrolysis was performed by adding barium hydroxide (Osborne and Voogt, 1978).

The released amino acids were analyzed with a Waters HPLC system equipped with a Pico-Tag C_{18} reversed-phase column (Scholze, 1985). Amino acids were derivatized with phenyl isothiocyanate prior to chromatographic separation.

Protein Quality Evaluation. Casein plus 5% DL-methionine (reference protein) and the WPH were assayed according to the Thomas-Mitchell method (Mitchell, 1923). Sixteen Wistar male rats at weaning (21 days old) from Interfauna Ibérica (Barcelona, Spain) were used as test animals. Rats were housed in metabolic cages in a room at 22 °C with a 12 h light–dark cycle. Two diets (12% protein) were prepared following Institute of Laboratory Animal Resources (1979) recommendations and differing only in the protein sources: casein plus 5% DL-methionine and the WPH. The composition of the diets is shown in Table 1.

The experimental design was as follows: diet and water were provided *ad libitum*, and the experiments were performed in two phases. The first period (6 days) was designed to calculate the nitrogen endogenous losses (feces and urine), and all animals were fed the casein plus 5% DL-methionine diet (4% protein) during this period. Only the data from the last 3 days of this period were recorded. After the initial period, rats were randomly assigned one of the two experimental diets (12% protein) and fed for 10 days, but only the last 7 days were used to record data. The indices used to estimate the quality of the protein sources were as follows: intake and body weight gain per day during the experimental period, true digestibility (TD) (absorbed nitrogen/absorbed nitrogen), net protein utilization (NPU) (retained nitrogen/ingested nitrogen), biological value (BV) (retained nitrogen/absorbed nitrogen), and protein efficiency ratio (PER) (body weight in grams/intake of protein in grams for the same period).

***In Vitro* Antigenicity**. The antigenicity of whey proteins and the WPH was measured by an immunosorbent inhibition assay described by Knights (1985) and modified by us as follows. The assay was performed in 96 well flat-bottom microtiter plates (Costar, Cambridge, MA). A 200 μL aliquot of a solution of bicarbonate/carbonate buffer (pH 9.6), containing β-lactoglobulin (50 mg/L), was added to the inner 60 wells of the plate. After incubation overnight at 4 °C, the plate was washed with phosphate buffer solution (pH 7.4), containing 0.1% gelatin and 0.05% Tween 20 (PBSGT). Serial 10-fold dilutions of the samples (whey protein or WPH) were mixed with 200 μg/L of rabbit IgG anti-β-lactoglobulin, raised and purified from serum by affinity chromatography by the authors (Boza *et al.*, 1994), and the mixture was added to the wells (200 μL/well). Antibodies to β-lactoglobulin were used because β-lactoglobulin is considered to be the most potent allergen in the whey (Gjesing and Lowenstein, 1984). The plate was then incubated for 2 h and then washed as above with PBSGT, before 200 μL of diluted peroxidase-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO) was added to each well. The plate was incubated for a further 2 h at 37 °C and again washed as above with PBSGT. Finally, 150 μL of the substrate solution consisting of 1 mg/mL of tetramethylenebenzidine in citrate buffer (pH 4.0), containing hydrogen peroxide (33% w/v, 0.25 μL/mL buffer), was added to the wells. After 30 min of incubation, the reaction was stopped by addition to each well of 50 μL of 2.5 M H_2SO_4. The yellow color that developed was measured at 450 nm in an Easy Reader EAR 400 spectrophotometer (SLT Labinstruments, Salzsburg, Austria).

***In Vivo* Antigenicity**. Weaned Dunkin-Hartey guinea pigs (Biocentre, Barcelona, Spain) were housed in a room at 20 °C with a 12 h light–dark cycle and fed *ad libitum* a cow's milk protein free solid diet (Sanders, Madrid, Spain). Test diets consisting of solutions of whey protein or WPH (both at concentrations of 35 g/L) were given instead of water over 2 weeks. Two control animals only drank during all of the study. Then, animals were kept on the chow diet and received water for 6 days. On day 21, the occurrence of hypersensitivity was then tested by intravenous challenge for systemic anaphylaxis of a 35 g/L whey protein (native or hydrolyzed) solution in a 9 g/L NaCl solution. A small incision was made in the foreleg under Nembutal anesthesia and 0.5 mL of the test solution was injected into the accessory cephalic vein (Pahud *et al.*, 1988). Animals were then observed for signs of anaphylactic sensitivity. Fatal anaphylaxis was considered as positive. All solutions used in the study were sterilized by UHT treatment (140 °C, 4 s) and stored at −20 °C before use. The protocol of the study was approved by the Animal Ethics Committee of the University of Granada.

Statistical Analysis. Statistical differences for food intake, body weight gain, and digestive and metabolic nitrogen utilization between the protein and the protein hydrolysate dietary groups were evaluated using unpaired *t*-tests ($p < 0.05$ was considered statistically significant) (Dixon *et al.*, 1990).

RESULTS AND DISCUSSION

Analytical Composition. WPH provides a high content of protein equivalent (79.32%). The ash content was 6.14%, which is slightly higher compared to that observed in whey protein concentrates. It consisted mainly of K^+ and Na^+, which probably came from pH adjustment during hydrolysis. Despite this fact, the content of these ions is acceptable for inclusion of this hydrolysate as nitrogen source in milk formulas, and they do not represent any osmolarity problem.

Molecular Weight Distribution. Figure 1 shows the chromatographic separation by gel filtration of the WPH. This WPH is a very extensive hydrolysate indeed according to its molecular mass distribution, in which there is only a small fraction (7.42%) with molecular mass higher than 1000 Da but lower than 3000 Da. The large majority of this hydrolysate consists of small peptides and free amino acids (92.55%). The content of the latter was not high if we take into account the TNBS method results which showed a free NH_2 group concentration of 2.86 μmol/L, expressed as micromoles per liter of glycine in a 1 mg/L WPH solution. Thus, the WPH would appear to have a high percentage of small peptides (two to five residues) and a low content

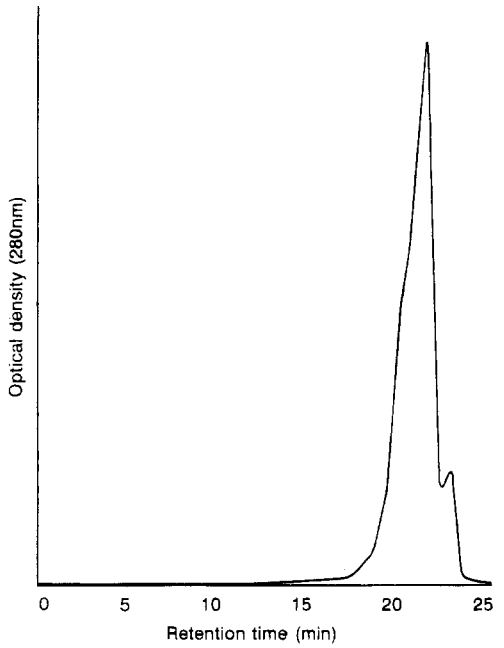

Figure 1. Chromatographic separation of the whey protein hydrolysate for the determination of its molecular weight distribution.

Table 2. Amino Acid (AA) Composition (Grams per 100 g of Protein) of the Whey Protein Hydrolysate

AA	g/100 g	AA	g/100 g	AA	g/100 g
Asp + Asn	8.0	Cys	2.6	His	1.4
Glu + Gln	17.1	Pro	7.5	Arg	1.8
Val	5.9	Lys	9.9	Ser	5.7
Ile	5.7	Phe	2.3	Gly	3.4
Leu	8.5	Tyr	2.1	Thr	7.1
Met	2.2	Trp	2.2	Ala	6.6

of free amino acids. The low content of free amino acids provides an osmolarity of 239 mOsm/L in a 10% (w/v) solution.

Amino Acid Composition. Table 2 gives the amino acid profile of the WPH. The essential amino acid content is nearly 50% (43.8%), indicating that it is a very high quality source of protein. The amino acid composition is very similar to the average amino acid composition of whey proteins (Boza *et al.*, 1994).

Controlled enzymatic hydrolysis by endo- and exoproteases is more gentle than acid hydrolysis; the former has the advantage of preserving the qualities of the native protein, without alterating the amino acid profile. Moreover, this kind of hydrolysis, by means of an adequate control of the factors involved in the process (type and amount of enzymes, temperature, pH) (Jost *et al.*, 1987), can render a protein hydrolysate rich in di- and tripeptides (directly absorbed in the gut) with a low content of free amino acids, thereby better controlling the osmolarity of the final product.

Protein Quality Evaluation. Table 3 shows the parameters studied to estimate the protein quality of the whey protein hydrolysate compared with casein plus 5% DL-methionine (reference protein). Daily food intake was very similar in both experimental and control groups, as well as the true digestibility, showing that the hydrolysate was well accepted by rats while no potential adverse effects of it were observed. Body weight gain was slightly lower in the experimental group as compared to the control one, but differences were not significant. There were no significant differences in NPU or in BV between the WPH and the protein of reference. However, the PER was significantly higher ($p < 0.05$) in the control group compared with that observed in rats fed the diet based on hydrolyzed whey proteins. The whey protein hydrolysate studied here has a very good quality as a source of nitrogen and has very similar nutritional value to that found in the whole whey protein (Boza *et al.*, 1994). The lower PER values observed in rats fed the experimental diet compared with those observed in the control animals could be due to the slightly lower protein content of the former diet (12.8–13.3%).

***In Vitro* and *in Vivo* Antigenicity.** As shown in Figure 2, the inhibition curve for the WPH was approximately 5 orders of magnitude higher than that for the whey protein and β-lactoglobulin.

Challenge with whey protein in guinea pigs sensitized to whey protein resulted in a 100% incidence of fatal shock (five of five animals). In contrast, animals of the same group challenged with WPH did not evidence any reaction (zero of five animals tested), showing the therapeutical characteristics of the hydrolysate.

Animals drinking WPH solution did not show any anaphylactic reaction when they were challenged with either native (zero of five) or hydrolyzed whey protein (zero of five animals), indicating the prophylactic properties of the hydrolysates. Challenge with WP or WPH in control guinea pigs did not result in any positive reaction.

The lower antigenicity of the WPH, as *in vitro* antigenicity measurement shows, was reflected in the guinea pig tests, where neither oral sensitization was induced nor an anaphylactic reaction provoked in animals previously sensitized with whey proteins. These results are in agreement with those reported by Pahud *et al.* (1985), who observed that even limited tryptic hydrolysates of whey proteins were unable to sensitize guinea pigs by the oral route. However, Knights (1985) indicates that even a small amount of peptides with molecular masses between 1300 and 5000 Da would prime the guinea pigs and provoke symptoms of anaphylaxis in animals sensitized by the U.S. Pharmacopeia test (intraperitoneal injection).

In this study it has been shown that the WPH used has (a) an excellent nutritive value according to its amino acid profile and NPU and BV values, (b) a good molecular weight distribution that allows its inclusion

Table 3. Food Intake, Body Weight Gain, and Digestive and Metabolic Utilization Obtained in Rats Fed the Casein (5% DL-Methionine (Control Diet) and the Whey Protein Hydrolysate Diet (WPH)[a]

diet	food intake (g/day)	body wt gain (g/day)	TD[b] (%)	NPU[c] (%)	BV[d] (%)	PER[e]
control	9.0 ± 0.0	4.1 ± 0.2	95.9 ± 0.8	82.0 ± 1.6	85.6 ± 1.5	3.4 ± 0.2
WPH	9.3 ± 0.0	3.6 ± 0.1	95.2 ± 0.5	74.8 ± 3.2	79.4 ± 2.3	2.9 ± 0.1[f]

[a] Results are expressed as mean ± SEM, $n = 8$. [b] TD, true digestibility, calculated by the following equation: $TD = [I - (F - EF)]100/I$. [c] NPU, net protein utilization, calculated as follows: $NPU = [I - (F - EF) - (U - EU)]100/I$. [d] BV, biological value, calculated by the following equation: $BV = [I - (F - EF) - (U - EU)]100/[I - (F - EF)]$. [e] PER, protein efficiency ratio, defined as body weight gain in grams divided by the intake of protein in grams for the same period (7 days). [f] Significant difference between control and WPH ($p < 0.05$). [g] Abbreviations: *I*, nitrogen intake; *F*, fecal nitrogen; EF, endogenous losses of nitrogen in feces; *U*, urinary nitrogen; EU, endogenous losses of nitrogen in urine.

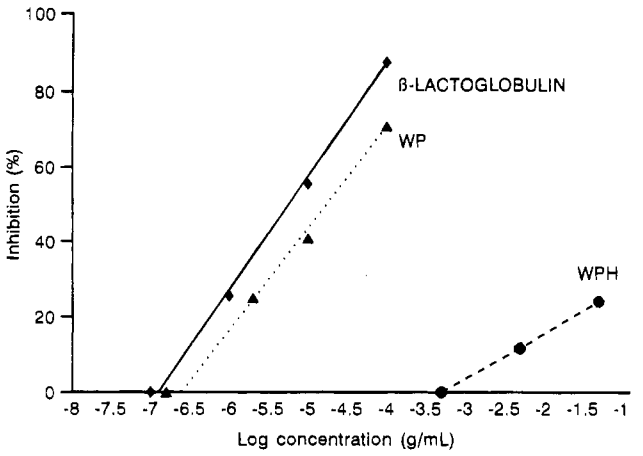

Figure 2. Inhibition of the binding of rabbit IgG anti β-lactoglobulin to β-lactoglobulin coated microtiter plates in the presence of β-lactoglobulin, whey protein (WP), and whey protein hydrolysate (WPH), assayed at different concentrations.

in formulas without increasing their osmolarity, (c) a very low residual antigenicity, as *in vitro* and *in vivo* methods used have shown.

In conclusion, a wide methodology for characterizing protein hydrolysates suitable for use in enteral formulas, including the study of the chemical composition, molecular weight distribution, amino acid profile, nutritional qualities, and antigenic properties has been described. However, before the inclusion of protein hydrolysates in enteral formulas, clinical trials have to be carried out to demonstrate their nutritional efficacy and their hypoantigenicity in the human being.

ABBREVIATIONS USED

BV, biological value; NPU, net protein utilization; PER, protein efficiency ratio; TD, true digestibility; WPH, whey protein hydrolysate.

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