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Whey protein hydrolysate: Functional properties, nutritional quality and utilization in beverage formulation

Rhicha Sinha [a], C. Radha [b], Jamuna Prakash [a], Purnima Kaul [b,*]

[a] Department of Studies in Food Science, and Nutrition, University of Mysore, Manasagangotri, Mysore 570006, India
[b] Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore 570020, India

Abstract

The objective of the study was to analyze the functional and nutritional properties of enzymatically hydrolyzed whey protein concentrate (WPC) and to formulate a beverage mix. WPC hydrolysates were produced using fungal protease and papain, at time intervals of 20, 40 and 60 min and were analyzed for proximate composition and functional properties. A beverage was formulated with hydrolyzed WPC, skim milk powder, cocoa, liquid glucose, sugar and vegetable fat and analyzed for physicochemical properties, sensory attributes and keeping quality. Results revealed that the protein content of WPC was 75.6% and decreased slightly on enzyme treatment (69.6%). The water absorption capacity of WPC was 10 ml/100 g and increased in enzyme treated samples from 16 to 34 ml/100 g with increase in the time of hydrolysis. Emulsion capacity (45 ml of oil/g of control WPC) showed a decreasing trend with increasing time of hydrolysis. Enzyme treatment slightly increased the foam capacity in three samples but lowered foam stability in all. The gel filtration pattern of enzyme treated samples showed an increase in low molecular weight fractions. The amino acid profile showed higher content of methionine in samples treated with enzymes, compared to the control. The *in vitro* protein digestibility of untreated WPC was 25% and increased in all treated samples to varying degrees (69–70%). Formulated beverage had 52% protein, 10% fat and 6.6% ash. There were no significant differences in the sensory attributes of formulated and commercial beverage. The formulated beverage could be stored well in a PET container for 30 days.

Keywords: Whey protein concentrate; Enzyme hydrolysis; Physico-chemical properties; Biologically active peptides

1. Introduction

Whey protein, a by-product recognized as a valuable food ingredient with important nutritional and functional properties is gaining acceptance as functional food ingredient. Commercial whey proteins are considered a GRAS substance for food product applications. It constitutes about 85–90% of the volume of the milk used for transformation into ripened cheese, and it retains about 55% of the milk nutrients. Much attention is focused on whey proteins. Not only is their biological value superior to most other proteins, but whey proteins also have a high content of sulfur-containing amino acids, which support antioxidant functions. Hydrolyzed whey protein-based formulas are beneficial for infants intolerant to cow's milk protein. During hydrolysis proteins are broken down into peptides of different sizes and free amino acids, as a result of cleavage of peptide bonds. Enzymes, acids or alkali can carry out this degradation. Acid and alkaline hydrolysis tend to be a difficult process to control and yield products with reduced nutritional qualities. Chemical hydrolysis can form toxic substances like lysino-alanine (Lahl & Windstaff, 1989). Enzymatic hydrolysis developed under mild conditions of pH (6–8) and temperature (40–60 °C) may also lead to the development of the biologically active nutritional components to promote health-giving opportunities for the use of dairy ingredients. Control of the molecular size of resultant peptides constitutes an essential step in the development of protein hydrolysates for dietary use. However, the degree of variability in composition, functionality and sensory properties of WPC has greatly limited

* Corresponding author. Tel.: +91 821 251 5331; fax: +91 821 251 6308.
E-mail address: purnimakaul@yahoo.com (P. Kaul).

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their use in food industry. The objectives of present study were to study the effect of enzymatic hydrolysis on the functional properties and amino acid composition of whey protein concentrate, to isolate biologically active peptide from hydrolyzed whey protein concentrate and to formulate a product incorporating whey protein concentrate and study the sensory and keeping quality of the product.

2. Materials and methods

Ultrafiltered whey protein concentrate, having a protein content of 75.6%, was obtained from Mahaan proteins, New Delhi. Other ingredients required for product formulation, namely cocoa powder, skim milk powder, sugar, vegetable fat and liquid glucose were purchased from a local market. All the chemicals used in this study were of analytical grade, from E. Merck, India, Ltd., Mumbai. Fungal protease, having activity of 550 TU/mg, was procured from Amano Pharmaceuticals, Japan, and papain having activity of 450 TU/mg was procured from Enzochem Company, Nasik, India. L-Tyrosine obtained from Sigma (St. Louis, MO, USA) was used as a standard for assay. Pepsin (from porcine stomach mucosa, activity 3000–4500 U per mg) and pancreatin (from bovine pancreas, activity equivalent to 8× USP specifications) enzymes, used to measure *in vitro* digestibility, were obtained from Sigma (St. Louis, MO, USA).

2.1. Enzymatic modification of WPC

Enzymatic hydrolysis was carried out using papain and fungal protease enzymes; both these enzymes work under mild conditions of pH (6–8) and temperature (40–60 °C). Papain preferentially cleaves at the hydrophobic amino acid residues, whereas fungal protease cleavage is non-specific. WPC was mixed with water in the ratio of 1:10 (w/v) to obtain a slurry. The pH of one batch of slurry was adjusted to 7.6 with 1 N NaOH for fungal protease (45 °C) and to pH 6.2 with 1 N HCl (55 °C) for papain. These were allowed to stand for 1 h and then enzymes were added to the slurries at 1:60 ratio of enzyme to substrate. Hydrolysis continued at time intervals of 20, 40 or 60 min at constant temperature. After the specified time the hydrolysates were placed in a boiling water bath for 10 min for enzyme inactivation. The hydrolysates were centrifuged at 6000 rpm for 20 min and the supernatant collected, lyophilized and used for determination of functional properties.

2.2. Functional properties

The functional properties of enzymatically hydrolyzed WPC were determined by standard methods as described briefly here. Water absorption capacity was determined according to the method described by Janicky and Walczak (1954). A 1.0 g of sample was placed in a centrifuge tube, 10 ml of distilled water was added, stirred with a glass rod and the weight of tube and the contents were recorded. After 30 min the suspension was centrifuged at 3000 rpm for 25 min. The supernatant was discarded and the tube inverted at an angle of 45° in an oven at 50 °C for 25 min, then transferred to a desiccator, cooled and weighed. The difference in the two weights gave the amount of water absorbed by the material. Emulsion capacity was determined by the methods described by Beuchat, Cherry, and Quinn (1975) and Beuchat (1977). To a known amount of sample (3 g), 50 ml of distilled water was added. The slurry was transferred to a blender and blended for 30 s at low speed. Refined groundnut oil was slowly added from a burette while the blending continued. The addition of oil was continued until there was a phase separation. Emulsion capacity was expressed as the amount of oil required to emulsify 1 g of protein.

Foam capacity and foam stability were measured according to the method described by Lawhon, Cater, and Mattil (1972). An equivalent of 3 g protein in the sample was accurately weighed out and mixed with 100 ml of distilled water. This was quantitatively transferred into a blender and whipped for 3 min at high speed. The slurry was poured immediately into a 250 ml measuring cylinder and the total volume of the liquid was measured immediately after 30 s. The difference in the volume was expressed as the volume of the foam. The foam stability was determined by measuring the fall in volume of the foam after 30 min. All the experiments were performed in triplicate and the results are the average of three values.

The molecular size of whey protein hydrolysate was determined by gel filtration using Sephadex G-75 column of dimensions 1.5×117 cm, pre-equilibrated with 0.05 M phosphate buffer (pH 7.4). Whey protein hydrolysate having a concentration of 20 mg protein was loaded on to the column and eluted at 20.0 ± 0.5-ml/h-flow rate. Fractions (3.0 ml) were collected and the absorbance measured at 280 nm. The elution profile of the whey protein concentrate, drawn by plotting absorbance against elution volume, was compared with enzyme treated samples.

2.3. Nutritional quality

WPC and formulated beverage mix were analyzed for moisture, protein, fat, total ash, acid insoluble ash by standard techniques (Ranganna, 1986). Amino acid analysis of WPC and samples hydrolyzed for 40 min was carried out using the method of Bidling Meyer, Cohen, and Tarwin (1984). Available lysine was determined by the FDNB reactive lysine method of Carpenter (1960), modified by Booth (1971). *In vitro* digestibility of all the samples was determined by the method of Akeson and Stahman (1964). A known amount of sample was incubated with 1.5 mg pepsin in 15 ml of 0.1 N HCl at 37 °C for 3 h. After neutralization with 2 M NaOH and addition of 4 mg pancreatin in 7.5 ml of phosphate buffer (pH 8.0), 1 ml toluene was added to prevent microbial growth, and the solution was incubated for an additional 24 h at 37 °C. After 24 h the

enzyme was inactivated by addition of 10 ml of trichloroacetic acid, to precipitate the undigested protein. The volume was made upto 100 ml and centrifuged at 5000 rpm for 20 min. The protein content of the clear supernatant was determined by Kjeldahl's method. *In vitro* digestibility was expressed as the percentage of the total protein solubilized after enzyme hydrolysis. The data were used for calculating certain nutritional indices like chemical score (Block & Mitchell, 1949), essential amino acid index (Oser, 1951), predicted biological value (Oser, 1959), and nutritional index (Crisan & Sands, 1978).

2.4. Presence of bioactive peptides

To identify the biologically active peptides, the anti-bacterial activity and anti-hypertensive activity of enzymatically hydrolyzed WPC were determined. The inhibitory activities of the proteolytic hydrolysates against the indicator bacteria were determined by agar well infusion assay (Varadaraj, Nirmala Devi, Keshava, & Manjrekar (1993)). Agar infusion assay has been widely used as a qualitative test for anti-bacterial activity (Davidson & Parish, 1989; Nostro, Germano, Angelo, Marino, & Canntelli, 2000). The plating medium used was brain heart infusion agar. The indicator bacteria used were strains of *Staphyloccus aureus* FRI 722, *Bacillus aureus* F4810, *Yersinia enterocolitica* MTCC 859, *E. coli* MTCC 1610 and *Bacillus laterosporus*. Aliqots of 75 ml of hydrolysate were introduced into the wells. Plates were examined for zone of inhibition after incubation for 24 h at 37 °C, including an initial incubation for 3 h at 60 °C. The zone diameter was measured in mm and recorded. Antibacterial activity in the culture filtrate was defined as the reciprocal of the highest dilution showing definite inhibition of indicator bacteria and was expressed as activity units per ml (AU/ml). For determining the anti-hypertensive activity, the action of angiotensin-converting enzyme (ACE) was studied. ACE-inhibitory peptides are haemodynamic regulators and exert an anti-hypertensive effect (Meisel & Bockelmann, 1999). *In vitro* ACE-inhibitory activity is quantified by means of hippuric acid formation (Blanca, Martin, & Pueyo, 2003; Ryan, 1988). Hippuryl-His-Leu was used as the substrate to yield hippuric acid and His-Leu. One millilitre of 1×10^{-3} M substrate was incubated with ACE having

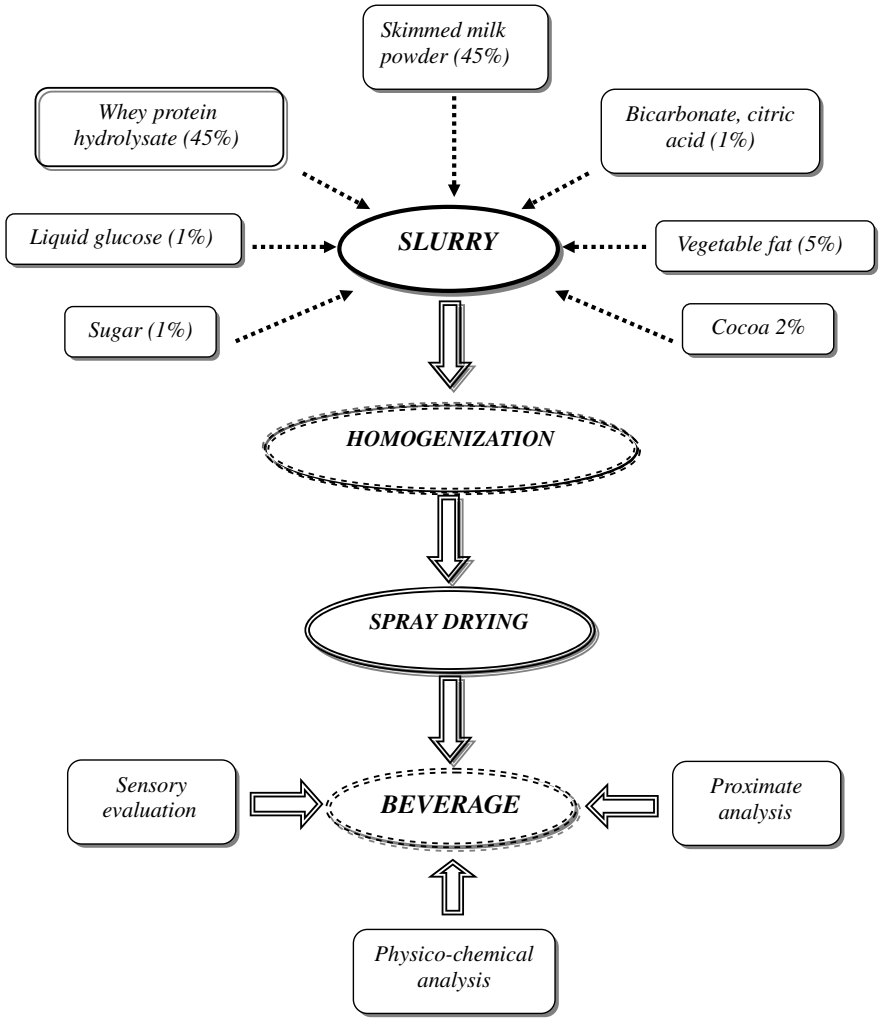

Fig. 1. Flow diagram for the preparation of a chocolate-flavored whey protein concentrate beverage.

different concentrations of hydrolysates, at 37 °C for 1 h. The reaction was stopped by addition of 1 ml ethyl acetate. This was shaken thoroughly to extract hippuric acid from the unhydrolyzed substrate into the ethyl acetate layer. The mixture was allowed to stand for 45 min till the extraction was complete. A sample of the ethyl acetate layer was collected and the solvent was removed by evaporation in a boiling water bath. The residue containing hippuric acid was dissolved in water and quantified in terms of its absorption at 228 nm. The experiments were performed in triplicate.

2.5. Beverage development

The basic ingredients used in the beverage product were whey protein hydrolysate, skim milk power (SMP), cocoa, liquid glucose, sugar and vegetable fat (Fig. 1). WPH and SMP were incorporated in the product in 1:1 ratio. Cocoa was added for desirable flavour and colour. Bicarbonate and citric acid were added at a combined level of 1%. The ingredients were mixed with water to give a slurry, which was homogenized. The homogenized slurry having 25–30% solid content was spray dried (Bowen, BLSA) at a feed rate of 70 ml/min. The inlet temp was 105 °C. The total drying time was 1 h. Temperature and vacuum were constantly maintained. The beverage thus obtained, having a solids content of 96–97%, was immediately packed in an airtight polythene bag and two PET containers. One of the PET containers was stored under refrigeration and the second one and polythene bag were stored at room temperature. Shelf life of the mix was studied for a month. Analysis of free fatty acids, peroxide value (Watson, 1994) and sensory attributes were carried out every 10 days. For sensory analysis 25 panel members were presented with coded samples of beverage along with a scorecard (ISI, 1972) and asked to evaluate the products on a scale of 1–5 (representing excellent – 5, very good – 4, good – 3, fair – 2 and poor – 1) for of appearance, taste, flavour, consistency and overall acceptability. A commercial beverage sample was used for comparison. All assessments were done in triplicate and means and standard deviations calculated. The sensory analysis data were subjected to statistical evaluation using computer software SPSS (version 10). The scores were subjected to students T test (initial evaluation of commercial and formulated beverage), analysis of variance (stored products) and a post test for ANOVA, Bonferroni P value' to find out if there were any significant differences between the samples.

3. Results and discussion

3.1. Functional properties

The functional properties of enzymatically hydrolyzed WPC are presented in Table 1. The water absorption capacity of WPC increased on enzymatic hydrolysis with fungal protease and papain and was highest for a hydrolysis time of 60 min. This can be attributed to dissociation of proteins into smaller subunits, which have more water binding sites (Castimopoolas, Funk, & Meyer, 1970). The emulsification capacity, in contrast, reduced on enzyme hydrolysis in both samples. The emulsifying capacity of proteins is related to their capacity to lower interfacial tension between the hydrophobic and hydrophilic components in foods. Similar results on emulsifying capacity were also obtained by other researchers and have been attributed to dry heat treatment (Rahma & Mostafa, 1998), partial hydrolysis (Sekul, Vinnett, & Ory, 1978), and enzyme modification (Bhagya & Srinivasan, 1989).

The foam volume of the control was found to be less than that of the treated samples. Enzymatic hydrolysis of whey proteins caused an increase in the foam volume initially and then a decrease with time. In the fungal protease treated sample the foam volume was found to be similar at both 20 and 40 min of hydrolysis, with a significant decrease after 60 min, while in the sample treated with papain there was a gradual decrease in foam volume with increase in hydrolysis time. The foam stability of the control was greater than that of the treated samples. Samples treated with both fungal protease and papain showed gradual decrease in stability with an increase in proteolysis. The foam stability at 60 min was almost negligible.

Table 1
Protein content and functional properties of whey protein concentrates

Whey protein concentrate	Protein (g/100 g)	Water absorption (ml/100 g)	Emulsion capacity (ml oil/g protein)	Foam volume (ml/g)	Foam stability (ml/g)
Control	75.6 ± 0.02	10 ± 0.1	45 ± 0.7	25 ± 2.0	20
Fungal protease treated					
20 min	69.0 ± 0.02	16.0 ± 0.1	40 ± 1.0	35 ± 1.8	10
40 min	70.2 ± 0.02	24.5 ± 0.1	36 ± 1.0	35 ± 2.0	5
60 min	71.5 ± 0.05	32.5 ± 0.1	35 ± 0.8	25 ± 1.0	2
Papain treated					
20 min	70.3 ± 0.05	18.0 ± 0.1	38 ± 1.2	30 ± 1.5	10
40 min	72.2 ± 0.02	26.2 ± 0.2	32 ± 1.0	25 ± 1.8	5
60 min	74.6 ± 0.05	34.0 ± 0.1	30 ± 0.8	20 ± 1.2	2

The above results show that a limited amount of hydrolysis is desirable to increase foaming but foam stability is greatly decreased as a result of such hydrolysis. This is probably due to an initial increase in the polypeptide content, which allows more air to be incorporated. However, the polypeptides do not have the strength required to give a stable foam. The decrease in foam stability manifests itself primarily in the initial 30 min of reaction (Kuehler & Stine, 1974). Further hydrolysis is likely to result in peptides, which lack the ability to stabilize the air cells of the foam.

The homogeneity of WPC and enzyme treated samples was determined using a Sephadex G-75 column which was pre-equilibrated with 0.05 M phosphate buffer at pH 7.4. The gel filtration profile of untreated WPC is shown in Fig. 2. In the case of samples treated with fungal protease (Fig. 3a–c) it can be seen that the unhydrolyzed portion of the sample is eluted in the void volume and with an increase in hydrolysis time the peaks get hydrolyzed. On hydrolysis for 60 min the main unhydrolyzed peak also begins to hydrolyze. The papain treated samples (Fig. 4a–c) also show an increase in hydrolysis. The number of peaks decreases progressively as the time of treatment of the sample with the enzyme increases.

3.2. Chemical composition

The amino acid profiles of WPC and enzyme treated samples are given in Table 2. There was a significant increase in the methionine content of WPC, corresponding to 24.3% and 42.2%, after hydrolysis with fungal protease and papain, respectively. In addition higher values for glutamic acid, serine, glycine and threonine were found in the samples treated with fungal protease and papain, compared to the control.

All samples contained essential amino acids and the chemical score indicated tryptophan, leucine and lysine to be I, II and III limiting amino acids, respectively. The nutritional indices indicated that the control had a slightly higher value for available lysine (Table 3). The essential amino acid index and predicted biological values of all the samples were almost similar. The nutritional index of enzyme treated samples was slightly lower than the control.

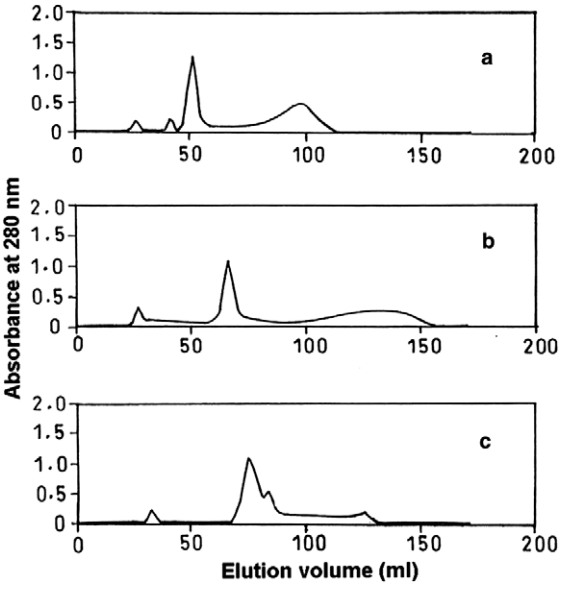

Fig. 3. Gel filtration profile of whey protein concentrate treated with fungal protease at (a) 20 min (b) 40 min and (c) 60 min.

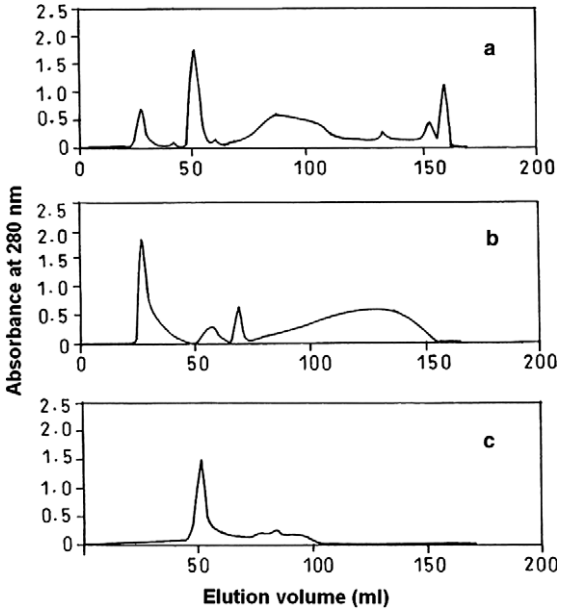

Fig. 4. Gel filtration profile of whey protein concentrate treated with papain at (a) 20 min (b) 40 min and (c) 60 min.

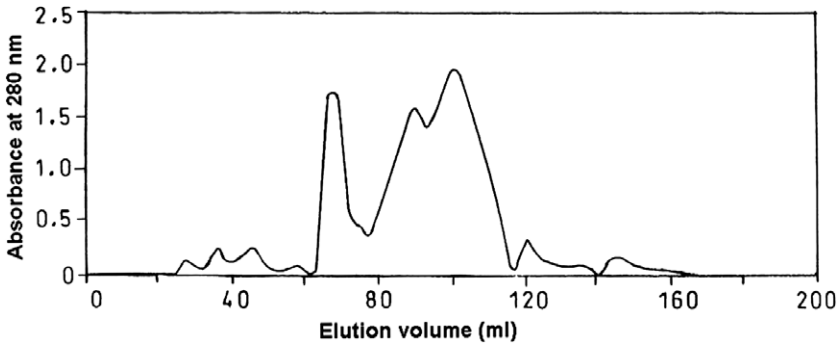

Fig. 2. Gel filtration profile of whey protein concentrate.

Table 2
Amino acid composition of whey protein concentrates

Amino acid (g/100 g protein)	Control	Fungal protease treated (40 min)	Papain treated (40 min)
Aspartic acid	10.42 ± 0.38	10.29 ± 0.3	10.77 ± 0.19
Glutamic acid	19.45 ± 0.89	20.53 ± 1.72	21.21 ± 0.06
Serine	4.68 ± 0.3	5.29 ± 0.38	5.05 ± 0.3
Glycine	1.74 ± 0.1	2.28 ± 0.07	2.16 ± 0.48
Histidine	2.20 ± 0.33	1.93 ± 0.27	2.18 ± 0.05
Arginine	2.32 ± 0.34	2.25 ± 0.22	2.44 ± 0.41
Threonine	4.80 ± 0.62	5.57 ± 0.16	5.36 ± 0.13
Alanine	4.50 ± 0.18	3.96 ± 0.75	4.09 ± 0.19
Proline	5.76 ± 1.31	6.02 ± 0.08	5.70 ± 0.04
Tyrosine	1.52 ± 0.42	2.41 ± 0.68	2.90 ± 0.11
Valine	6.54 ± 0.29	6.52 ± 0.25	5.09 ± 0.43
Methionine	3.41 ± 0.34	4.24 ± 0.91	4.85 ± 0.54
Cystine	0.6 ± 0.35	0.12 ± 0.06	0.20 ± 0.19
Isoleucine	5.65 ± 0.31	5.65 ± 0.1	5.44 ± 0.33
Leucine	13.29 ± 1.13	11.37 ± 0.97	11.28 ± 0.68
Phenylalanine	3.62 ± 0.22	3.57 ± 0.24	3.34 ± 0.13
Lysine	8.98 ± 0.86	7.88 ± 0.47	8.44 ± 0.22

Table 3
Nutritional quality of whey protein concentrates

Indices (%)	Control	Fungal protease treated (40 min)	Papain treated (40 min)
Available lysine	7.6 ± 0.1	6.8 ± 0.15	7.0 ± 0.12
Essential aminoacid index	97.4	97.5	98.3
Predicted biological value	94.5	94.6	95.4
Nutritional index	73.6	68.4	70.9
In vitro digestibility	25 ± 2.0	–	–
20 min	–	70.0 ± 3.0	68.9 ± 3.1
40 min	–	73.6 ± 2.5	70.2 ± 2.8
60 min	–	78.0 ± 2.0	75.1 ± 1.9
Essential amino acid (%)			
Leucine	62.7	71.8	71.7
Isoleucine	116.4	113.9	117.4
Valine	110.3	108.3	123.1
Tryptophan	52.1	51.0	50.5
Lysine	78.6	87.6	81.2
Threonine	106.9	90.2	92.9
Phenylalanine + tyrosine	190.4	160.2	152.2
Methionine + cystine	140.0	126.0	107.9
Protein score (%)	52.1	51.0	50.5
Sequence of limiting amino acid	I Tryptophan II Leucine III Lysine		

The *in vitro* protein digestibilities of the 60 min hydrolyzed samples increased threefold, compared to the control WPC (Table 3).

3.3. Anti-bacterial and anti-hypertensive activity of enzyme hydrolyzed WPC

No zone of inhibition was found, indicating that the proteolytic hydrolysates of WPC showed no antibacterial activity. The whey protein hydrolysate showed 59 ± 2.0% ACE inhibition at a concentration of 200 μg/ml. It is possible therefore that the hydrolysate/peptides reported herein could find application as neutraceuticals/functional food ingredients for the prevention/treatment of hypertension.

3.4. Nutritional, sensory and keeping quality of formulated beverage mix

The proximate composition of WPC and that of the formulated beverage were similar for moisture, total ash and acid-insoluble ash. The protein content of the beverage was slightly lower and the fat content was higher than WPC, due to addition of vegetable fat (Table 4).

The formulated beverage was stored in different packaging materials in order to study the shelf life of the product. Fats undergo hydrolytic and oxidative deterioration during storage, which can be measured by free fatty acids and peroxide value. These were analyzed on the 0th, 10th, 20th and 30th day of storage in the formulated beverage.

Table 4
Chemical composition and keeping quality of beverage mix

Chemical composition (% dry basis)	Protein	Fat	Carbohydrate (by difference)	Total ash	Acid insoluble ash
Whey protein concentrate	78.2 ± 0.02	4.9 ± 0.05	9.9	6.5 ± 0.01	0.5 ± 0.01
Beverage mix	52.4 ± 0.02	10.4 ±0.05	30.0	6.6 ± 0.01	0.6 ± 0.01

Keeping quality	Days of storage			
	0 Day	10th Day	20th Day	30th Day
Free fatty acid (%)				
Beverage mix fresh	1.00	–	–	–
Beverage mix *b*	–	1.60	1.95	2.00
Beverage mix *c*	–	1.82	2.00	3.10
Beverage mix *d*	–	3.22	4.30	5.20
Peroxide value (mEq. O_2/kg fat)				
Beverage mix fresh	0.05	–	–	–
Beverage mix *b*	–	1.62	2.00	2.56
Beverage mix *c*	–	2.50	3.21	3.65
Beverage mix *d*	–	3.06	5.49	6.10

Beverage mix *b*: stored in PET container under refrigeration.
Beverage mix *c*: stored in PET container room temperature.
Beverage mix *d*: stored in polyethylene bags at room temperature.

FFA and peroxide values increased gradually with storage but a significant increase in both parameters was found in the product stored in polyethylene bags at room temperature. Higher values on the 30th day of analysis than on the initial day indicated that polyethylene permitted transmission of gases and thus was not a preferable packing material for the formulated product. Results show that the product was best stored in PET containers under refrigeration.

The freshly formulated beverage was compared with a commercial beverage on day 0. Subsequently formulated beverage mix stored in a PET container under refrigeration (beverage mix '*b*'), stored in a PET container at room temperature (beverage mix '*c*') and stored in polyethylene bags at room temperature (beverage mix '*d*') were compared for sensory attributes on the 10th, 20th and 30th day. There were no significant differences between the commercial and formulated beverages at day zero.

The sensory analysis data for stored samples was subjected to analysis of variance to find out the significant differences among the quality parameters. There were no significant differences in the appearance of the product. Significant differences were observed for taste, consistency and overall acceptability of the commercial and formulated products stored in polyethylene at room temperature. Significant differences were also observed in the flavour of products stored in PET containers under refrigeration and product stored in PET container at room temperature as well as those stored in polyethylene at room temperature. The analysis of products stored for 20 and 30 days showed no differences in the appearance and flavour of the product but differences were observed in the taste of products (b) and (d). Significant differences were observed in the consistency and overall acceptability of stored products compared with the commercial mix.

4. Conclusion

In future successful applications in food may take advantage of the biological activities of whey protein hydrolysates. *In vitro* digestibility tests offer a rapid and a simple way to mimic *in vivo* conditions. Here, *in vitro* digestibility results showed that the hydrolysates increased threefold in digestibility compared to the control. It has been reported that protein hydrolysates have better absorption than whole proteins (Clemente, 2000). *In vitro* digestibility can give us information about the stability of protein hydrolysates and how they could survive the digestion process. The bioactivities of peptides encrypted in major whey proteins are latent until activated by enzymatic proteolysis. However, evaluation of the efficacy of bioactive proteins/peptides in animal model and human clinical studies is needed to fully substantiate their role. In this paper, introducing hydrolyzed whey protein concentrate with anti-hypertensive peptides into a beverage appears to create an exciting link between food science and therapeutic nutrition.

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