Food Production, Processing and Nutrition

RESEARCH Open Access

Production of whey protein hydrolyzates and its incorporation into milk

Jenny Ann John[1,2*] and Bikash C. Ghosh[1]

Abstract

Whey proteins provide an excellent source of low-molecular-weight bioactive peptides with important functional properties and bioactivities like antihypertensive, opioid, and antimicrobial effects. Presence of peptide molecules with lower molecular weight has a great role in food for health promotion. In this investigation, the release of low-molecular-weight peptides from whey protein concentrate was attempted by using enzymatic digestion. The hydrolyzate was then incorporated into milk to obtain enriched milk (EM) with low-molecular-weight peptides. Based on sensory analysis of EM, electrophoretic and RP-HPLC studies, hydrolyzates of 10% protein (degree of hydrolysis 5%; enzyme/ substrate E/S, 1:50) were finally incorporated into milk at 20% (v/v) to develop an acceptable product enriched with low-molecular-weight peptides. EM had higher protein content, viscosity and emulsifying properties than control milk with 3% fat. It is recommended that EM should not be sterilized as it results in coagulation, but can be safely pasteurized and spray dried without any undesirable effects. Maximum ACE-inhibition activity was obtained in hydrolyzate, followed by EM. This study is expected to boost the opportunity for the dairy industry to venture further into the nutraceutical dairy market.

Keywords: Whey protein hydrolyzate, Enriched milk, Electrophoresis, ACE inhibition activity

Introduction

Safe and nutritious foods are of paramount importance to human health and well-being. The scientific knowledge on the relation between food and health is progressing well and new functional foods are appearing in the market. Preventing diseases by food intervention has become the most challenging field of research. Several diseases such as diabetes, obesity, cardiovascular disorders and allergy are linked to food intake (Kopp 2019). The various risk factors identified for these diseases may be reduced by adaptation of the diet and/or consumption of functional foods or nutritional supplements.

Functional foods are regularly consumed foods but may have been supplemented or enriched with health promoting substances like vitamins, minerals, bioactive peptides and alike. Of the various substances that are used for supplementation, peptides are increasingly used due to their unique functional properties. It has been proven that peptides that are released in vitro or in vivo from animal or plant proteins are bioactive and have regulatory functions in humans beyond their normal nutrition role (Hartmann and Meisel 2007). These properties of peptides are endowed by the specific amino acid sequence; generally low-molecular-weight bioactive peptides that contain 2–20 amino acid residues. Their presence has a great role in food for health promotion. The functional properties of the peptides include enhanced foaming, gelling, emulsification, and solubility, as well as decreased viscosity, among others. They also possess multifunctional properties like antihypertensive, opioid, antimicrobial, antioxidant, immunomodulatory and mineral binding effects (Hernandez-Ledesma et al. 2005; Nongonierma and FitzGerald 2015).

The extent of functionality of peptides depends on the way they are produced and the source of protein they are derived from. Protein hydrolysis can be accomplished by different means – enzymes, acids, microbes,

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alkalis etc. The enzyme used and the method of hydrolysis of protein will also influence the functionality of the peptides released (O'Keeffe and FitzGerald 2014). Of the different means of hydrolysis, enzymatic hydrolysis is much preferred for its specificity and nutritional application. Hence, in order to get peptides with desirable properties, controlled hydrolysis of protein has to be accomplished. However, optimization of conditions for enzymatic hydrolysis is a tedious task because several factors need to be considered and their effect studied before arriving at optimum conditions (Zheng et al. 2008). There have been numerous studies related to the field of bioactive peptides during the past two decades and these have been extensively reviewed (Meisel et al. 2006; Madureira et al. 2010; Martinez-Maqueda et al. 2012; Nongonierma and FitzGerald 2015). Similarly, attempts have also been made to optimize the enzymatic hydrolysis of whey proteins (Xia and Xia 2012; Naik et al. 2013; Zhidong et al. 2013).

Among the several proteins, milk proteins are most preferred. Recent evidences have shown the role of milk apart from supplying dietary proteins and essential amino acids to the body, is more complex as it also exhibits additional physiological bioactivity that is essential for growth and blood pressure control, among others. The average protein content of milk is 3.2–3.4% of which casein constitutes 80% and whey proteins, 20%. Though whey proteins account for only about 20% of the total protein fraction of milk, they are the wonder proteins for nutritional, clinical, dietetic, sports, and infant foods. As such, whey proteins are expected to provide beneficial low-molecular-weight bioactive peptides upon enzymatic hydrolysis. Enriching milk with hydolyzates containing these low-molecular weight peptides may further increase its bioactive properties. However, the major problem associated with supplementing foods with peptides is bitterness of certain peptide preparations, which is a limiting factor in their addition to food. The present study was therefore undertaken to optimize the parameters for whey protein hydrolysis and further enrich milk with these hydrolyzates. This study aims to understand the hydrolysis pattern of whey proteins with the variation in enzyme addition and degree of hydrolysis achieved. Knowledge of peptides formation patterns can help in the incorporation of hydrolyzates in milk or to formulate a food or beverage. However, the analysis of the nutritional aspects of WP hydrolyzates was beyond the scope of this study. The present study is expected to offer the dairy industry entry to the nutraceutical dairy market, which was hitherto restricted largely to the non-dairy segment. Moreover, due to the large-scale economy of the dairy industry, the study may create new opportunities for the industry to consider commercialization of minor milk components with potential biological activity.

Materials and methods

Materials

Fresh whole milk was standardized to toned milk at the Experimental Dairy of National Dairy Research Institute, Bengaluru, India. Toned milk is the milk prepared from cow or buffalo milk or the mixture of both, by standardizing to 3% fat and 8.5% solids-not-fat percentage (SNF) using skim milk. Whey protein concentrate (WPC), 70% protein (PROCON 3700) was obtained from M/s Mahaan Proteins Ltd., New Delhi. Flavourzyme (1000 LAPU/g, Novozymes South Asia Pvt. Ltd.), the enzyme produced by *Aspergillus oryzae,* was used for hydrolysis. Angiotensin converting enzyme (ACE) from rabbit lung was procured from M/s. Sigma Fine Chemicals Ltd. ACE of 0.1 unit was dissolved in 1 mL of distilled water and stored at −20 °C.The protein molecular weight marker for the electrophoretic study was purchased from M/s. Bangalore Genei. Analytical grade chemicals obtained from various companies such as M/s. Sigma Fine Chemicals Ltd. and used for chemical analysis. HPLC grade chemicals were used for HPLC analysis.

A digital pH meter of Digisun Electronics, Hyderabad (Model D1 707) was used for measuring pH during the investigation. The cooling centrifuge type C-24 from Remi Sales and Engineering Ltd., Mumbai, was used for centrifugation of samples. The Crepaco Homogenizer with 500 L/h capacity was used to homogenize milk at 500 and 1500 psi. The Mini Dual Vertical Electrophoresis Unit which could hold two 10 × 8 cm slab gel sandwiches was purchased from Tarsons Products Pvt. Ltd., Kolkata. The Agilent 1100 HPLC unit was used to separate different peptides in the hydrolyzate and enriched milk samples.

Hydrolysis of whey protein concentrate (WPC)

WPC solutions (5, 10 and 15% protein, w/v) were prepared. The solutions were pre-incubated at 50 °C and the pH was adjusted to 7 using 0.5 M NaOH, followed by the addition of Flavourzyme at different enzyme to substrate (E:S) ratios of 1:25, 1:50 and 1:100 (v/w). The solutions were regularly stirred. Hydrolysis was carried out at pH 7 and 50 °C to attain 5, 7, 9 and 11% degree of hydrolysis (DH) and the time was noted. The DH which is defined as the percentage of peptide bonds cleaved by the enzyme was determined according to the method of base consumption using pH stat technique of Adler-Nissen (1986). The total volume of 0.5 M NaOH consumed during the entire hydrolysis process to maintain the pH 7.0 was recorded using the digital pH meter. After reaching the desired degree of hydrolysis, the hydrolyzates were heated at 85 °C for 8–10 min to

inactivate the enzyme and to arrest further hydrolysis. The DH was calculated as follows and expressed as percentage.

$$Degree\ of\ hydrolysis\ (DH) = B \times N_b \times 1/\alpha \times 1/MP \times 1/htot \times 100$$

where, B = Base consumption in mL (NaOH); N_b = Normality of the base (0.5 N); MP = mass of protein in g; htot = Total number of peptide bonds in the protein substrate (meq/g protein for whey protein; htot = 8.8); α = Average degree of dissociation of the α-NH_2 groups. 1/α factor was considered as 2.27 at pH 7.0 and 50 °C.

Moisture, fat, acidity and ash

The moisture (%), fat (%) and titratable acidity (% lactic acid) contents in the enriched milk were determined as mentioned in Indian Standards Institution IS: (SP: 18) (1981) and ash as per Indian Standards Institution IS: (SP: 18) (1980).

Protein

The total nitrogen content of the milk was determined by Micro Kjeldahl method (Menefee and Overman 1940) as elaborated by Sáez-Plaza et al. (2013) and ISO (2014).

Viscosity

The kinematic viscosity of milk was determined using simple Ostwald viscometer as described by Matz (1962).

Sensory analysis

The enriched milk was analyzed for sensory characteristics by a trained panel of 10 judges, from among the scientists and staff of National Dairy Research Institute, Bengaluru. The 9-point hedonic scale was used in order to assess the sensory acceptability of the product. The samples were coded for judging and the criticisms expressed by judges were recorded.

Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis

The Tris-Tricine gel electrophoresis of hydrolyzate, toned milk and enriched milk was carried out to detect low molecular weight peptides in the samples as described by Schagger and Jagow (1987), using acrylamide/bisacrylamide mix (49.5% T; 3% C) in a Mini Dual Vertical Electrophoresis Unit as mentioned in the previous section. Maximum voltage used was 500 V and maximum temperature 45 °C. Anode buffer (0.2 M Tris, pH 8.9) and Cathode Buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.2 ± 0.2) were used for the run.

Reverse phase high performance liquid chromatography (RP- HPLC)

Reverse phase high performance liquid chromatography (RP-HPLC) of hydrolyzate and enriched milk samples was carried out as explained by Bordin et al. (2001) with some modifications. The hydrolyzates and enriched milk samples were resolved on RP-HPLC at Hindustan Lever Research Centre (HLRC), Unilever, Bengaluru. The specifications of the instrument were: System, Agilent 1100 HPLC system; Pumps, Binary Pump G1312A; Column, C18, Bonda Pak, Waters, 10 μm, (3.9 X 390 mm); Injector, Autoinjector G1313A; Thermostat, Thermostat G1316A; Detector, Diode array detector G1315B; and Software, Agilent Chemstation Rev.A.08.03 (847). The mobile phase and other operating conditions used during the analysis were as follows: Mobile Phase, Water + Acetonitrile + Trifluoroacetic acid (90 + 10 + 0.1); Flow rate, 1 mL/min; Wavelength, 220, 280 nm; Temperature, 25 °C; and Injection volume, 20 μL.

The HPLC column was equilibrated with the mobile phase till baseline stabilized. Samples were filtered through a 0.45 μm filter before HPLC injection. The flow rate was adjusted to 1 ml/min and the peptides were eluted from the column at room temperature. Elution of peptides was monitored at 220 and 280 nm and the peptide profiles of the samples were observed on the monitor.

Angiotensin converting enzyme (ACE) inhibition assay

Angiotensin converting enzyme (ACE) inhibitory activities of WPC, protein hydrolyzate (5%DH) from 10% whey protein solution, toned milk and enriched milk were measured as described by Hernandez-Ledesma et al. (2003) with slight modifications in incubation time (37^0 C) and volume of substrate (110 μL of substrate). Hippuryl-Histidyl-Leucine; HLL) was used for the analysis.

The extent of inhibition was calculated as follows:

$$\text{Inhibition of ACE (\%IACE)} = \frac{[(A - B) - (C - D)]}{(A - B)} \times 100$$

where, A = absorbance in the presence of ACE (substrate + ACE)

B = absorbance of the reaction blank (substrate alone); C = absorbance in the presence of ACE and the hydrolyzate/inhibitor; (substrate + ACE + hydrolyzate); D = absorbance of the sample blank (substrate + hydrolyzate).

Statistical analysis

Data obtained from the various experiments during the process of standardization and storage studies were analyzed statistically using Microsoft Excel. Statistical

comparisons were made using analysis of variance (ANOVA) methods and Tukey's Multiple Comparison Test as described by Snedecor and Cochran (1994). Data were expressed as the predicted means of each assay ± standard deviation (SD). Results were considered to be statistically significant at $p < 0.01$.

Results and discussion

Experiments were conducted to assess the feasibility to manufacture milk enriched with bioactive peptides obtained from the hydrolyzates of WPC. Results obtained during process of standardization to attain a maximum amount of short-chain bioactive peptides (less than 10 kDa), its incorporation into milk, and evaluation of the quality characteristics of enriched milk are presented in the following subsections.

Standardization of procedure for enzymatic hydrolysis of WPC

The concentration of protein solution, the enzyme to substrate ratio and the optimum degree of hydrolysis for generation of a maximum yield of low-molecular-weight peptides were the major parameters that were standardized. Flavourzyme, with both exo- and endopeptidase activity, capable of hydrolyzing the protein into molecules with less than 3 kDa, was employed in this investigation.

Effect of concentration of protein solution and degree of hydrolysis on hydrolysis time

The duration of hydrolysis increased with increase in protein concentration and degree of hydrolysis as shown in Table 1. Both of these factors have a significant ($p < 0.01$) influence on the time of hydrolysis. Thus, it can be concluded that the time taken for hydrolysis of a lower protein concentration solution to higher DH can be equivalent to the time taken to hydrolyze higher protein concentration to a lower DH.

The 5% protein solution took almost the same time as 10% solution to reach 5% DH. The aim of the study was to enrich milk with protein hydrolyzate to the maximum possible level of bioactive peptides without much alteration of TS in milk. Therefore, 5% protein solution at all levels of hydrolysis was eliminated to avoid dilution of milk for the further studies.

The degree of protein hydrolysis is an extremely important factor. Extensive hydrolysis imparts bitterness by elaborating smaller peptides and amino acids in the hydrolyzate. Hence, the necessity to exercise a strict control on the extent of hydrolysis has been well recognized (Haque and Mozaffar 1992). Moreover, it also influences the functional properties of the hydolysates. In the study of protein hydrolyzates of yellow stripe trevally (*Selaroides leptolepis*), Klompong et al. (2007) observed that as DH increased, the interfacial activities (emulsion activity index, emulsion stability index, foaming capacity, foam stability) of hydrolyzates decreased ($p < 0.05$), possibly caused by the shorter chain length peptides. The method used most frequently to limit the DH is by stopping the reaction at a desired time by heat treatment at 85 °C for 15 min.

Table 1 Effect of concentration of protein solution and degree of hydrolysis on the hydrolysis times

Concentration of protein solution (%) (P)	Degree of hydrolysis DH (%) (D)	Hydrolysis time (min)
5	5	23.66 ± 1.15^{a}
	7	55.67 ± 3.06^{b}
	9	78.66 ± 3.51^{c}
	11	124.33 ± 6.03^{d}
10	5	29.66 ± 2.52^{e}
	7	105.67 ± 3.05^{f}
	9	297.00 ± 6.56^{g}
	11	427.00 ± 12.12^{h}
15	5	55.00 ± 3.00^{i}
	7	121.00 ± 3.61^{j}
	9	308.66 ± 7.02^{k}
	11	550.67 ± 19.01^{l}

Results expressed as Mean ± S. D, $n = 4$; Small letter superscripts (a, b...,) differ significantly ($p < 0.01$) across the % protein and degree of hydrolysis

It is also important to produce hydrolyzate with a maximum content of low-molecular-weight peptides in the shortest possible time period to avoid the possibility of any microbial spoilage. It was observed that there were a lot of variations in the time of hydrolysis to achieve DH of 11% in the three different protein solutions (Table 1). Protein solution of 10% with 11% hydrolysis was achieved after about 7 h whereas it took more than 9 h in 15% protein solution with 1:50 E/S ratio. Sometimes it may not be possible to achieve DH of > 11%. This is probably because all the specific bonds to be acted upon by Flavourzyme were already hydrolysed and more enzyme would be needed for their further breakdown. Since 11% DH in both 10 and 15% solution took more than 7 h, no further studies were carried out in these cases.

Effect of enzyme to substrate (E/S) ratio and protein concentration on hydrolysis time

After the elimination of 5% protein solution and 11% DH, attempts were made to explore a suitable E/S ratio in 10 and 15% protein solutions for a DH of 5%. With the increase in enzyme concentration, hydrolysis time decreased in both protein solutions (Table 2). Enzyme concentration (E/S ratio) has a significant ($p < 0.01$) effect on the rate of hydrolysis, irrespective of protein

Table 2 Effect of E/S ratio and protein concentrations on the time to obtain DH of 5% and the effect of E/S ratio and DH on hydrolysis time in 15% protein solution

E: S	Hydrolysis time (min) to obtain 5%DH		Degree of hydrolysis (DH%)	Hydrolysis time (min) in 15% protein solution
	10% protein solution	15% protein solution		
1:25	13.66 ± 1.15^{a}	16.33 ± 1.53^{a}	5	15.33 ± 1.15^{A}
			7	30.66 ± 2.52^{B}
			9	145.67 ± 5.13^{C}
1:50	30.33 ± 2.52^{b}	55.00 ± 3.00^{d}	5	56.00 ± 3.61^{D}
			7	120.67 ± 4.04^{E}
			9	301.66 ± 7.64^{F}
1:100	93.00 ± 3.61^{c}	153.67 ± 3.51^{e}	–	–

Results expressed as Mean ± S. D, $n = 4$; Small letter superscripts (a, b..,) differ significantly ($p < 0.01$) across the % protein; capital letter superscripts (A, B..,) differ significantly ($p < 0.01$) between % degree of hydrolysis

concentration. Between the two protein solutions, the 15% solution took more time than 10% solution for achieving the same degree of hydrolysis using the same E/ S ratio. It was found that protein concentration also had a significant ($p < 0.01$) effect on the time of hydrolysis irrespective of the E/S ratio. Thus, the hydrolysis time increased with increased protein concentration, but decreased with increased E/S ratio. Among all, an optimum time of 30 min was found to be the most suitable hydrolysis period, considering its industrial application for time saving, analytical work and prevention of microbial spoilage during hydrolysis.

Table 2 indicates that protein solutions (10 and 15%) with three E/S ratio of 1:25, 1:50 and 1:100 took significantly ($p < 0.01$) different hydrolysis time for the same DH. However, there was no significant ($p > 0.01$) difference in hydrolysis time for 10 and 15% protein solutions with E/S of 1:25. This was probably because of the high concentration of enzyme used to achieve a lesser DH, which would result in all susceptible bonds being hydrolysed immediately, irrespective of the concentration of the protein solution. Therefore, the E/S ratio 1:50, which took 30 min to achieve the desired DH of 5% in 10% protein solution, was selected for further studies as already mentioned. The E/S ratio of 1:100 took more than 1 h to achieve a DH of 5% in both 10 and 15% solutions. This was not desirable and therefore, the E/S ratio of 1:100 was not considered in further studies.

Furthermore, E/S ratio of 1:50 for 10% protein solution was finally selected to achieve DH of 5% in 30 min. Since none of these combinations in 15% solution was found suitable for 30 min hydrolysis time, further studies were carried out to determine the suitable combination of DH and E/S ratio in 15% solution.

Effect of enzyme to substrate (E: S) ratio and degree of hydrolysis (DH) in 15% protein solution on hydrolysis time

In order to determine the suitable combination of E/S ratio and % DH, effect of two E/S ratios (1:25, 1:50) and three DH of 5, 7 and 9% in the 15% protein solution on the hydrolysis time was studied. Table 2 shows the difference in hydrolysis time in a 15% protein solution at different DH and E/S ratio. It is evident that DH had a significant ($p < 0.01$) effect on time for hydrolysis irrespective of E/S ratio. Although all the combinations were significantly different from each other, the combination that achieved the hydrolysis in 30 min was finally selected as it was the most suitable hydrolysis time, considering its advantages for industrial application as mentioned in the previous section. The choice of enzyme is determined by a combination of efficacy and economics. Within the limits of temperature and pH, a higher concentration of enzyme can be used to speed up the hydrolysis process. Here, economics is a factor, as the increased materials cost vs. speed of reaction must be considered. Therefore, 15% protein solution with a DH of 7%, which was achieved in 30 min using E/S ratio of 1:25 was selected in further studies. This is supported by the work done by Asselin et al. (1989) as they adopted the same E/S ratio for protein hydrolysis.

Identification of peptides present in hydrolyzates of 10 and 15% whey protein solution by Tricine SDS-PAGE

To study changes in the individual whey protein (WP) fractions during hydrolysis and to monitor the low-molecular-weight peptides formation, Tricine SDS-PAGE of 10 and 15% WP hydrolyzates were carried out.

Figure 1a, b, shows the peptide profiles obtained by hydrolyzing 10 and 15% protein solutions. The presence of low-molecular-weight peptides of < 10 kDa is evident

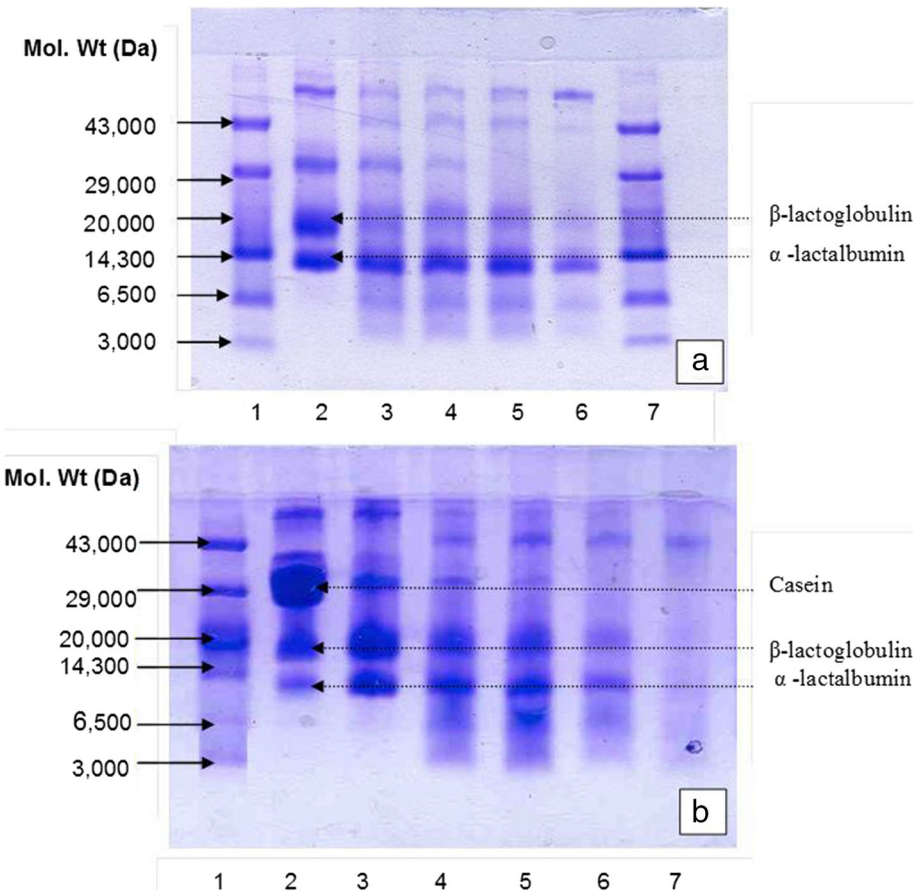

Fig. 1 **a** Tricine SDS-PAGE of hydrolyzates from 10% whey protein solution. Lane 1 - molecular weight standards; lane 2 - whole WPC; lane 3, 4, 5, 6 - hydrolyzates of DH 5, 7, 9, 11%, respectively; lane 7 - molecular weight standard **b** From 15% whey protein solution. Lane 1 - molecular weight standards; lane 2 - toned milk; lane 3 - whole WPC; lane 4, 5, 6, 7 - hydrolyzates of DH 5, 7, 9, 11%, respectively

in hydrolyzates with different % DH (Fig. 1a, Lanes 3, 4, 5, 6), whereas the control whole WPC (Fig. 1a Lane 2) did not show any of these low-molecular-weight peptides. However, in case of 15% protein hydrolyzates, the lower bands were lighter in the case of 9% DH (Fig. 1b, lane 6) and almost disappeared in the hydrolyzate with 11% DH (Fig. 1b, lane 7), which may be due to further breakdown of peptides into amino acids. This is important for reducing allergenicity of the products but was not intended as the main objective was to produce low-molecular-weight peptides of around 6 kDa or lower. Therefore, 15% protein solution with 9 and 11% DH was not considered as it further hydrolysed the peptides in the hydrolyzates. Thus, electrophoretic studies confirmed that in both 10 and 15% protein solutions, the desirable low- molecular-weight peptides (< 3 kDa) were present when hydrolyzed to DH of 5 and 7% and hydrolysis of β-lactoglobulin was faster than α-lactalbumin. Ghosh et al. (2017) have reported that higher degree of hydrolysis of whey proteins using Flavourzyme resulted in lighter bands for the two main protein fractions (α-lactalbumin and β- lactoglobulin) than control. Moreover, the number of peptide bands below α-lactalbumin fractions was higher, indicating formation of lower molecular-weight-peptides.

The results obtained here were in accordance with the findings of Schmidt and Poll (1991). They reported that peptides with estimated molecular weights between 2000 and 5000 Da were formed in appreciable amounts during the enzymatic hydrolysis of whey proteins. Between β-lactoglobulin and α-lactalbumin, the former seemed to be more prone to enzymatic hydrolysis than the latter, which is again in accordance with Schmidt and Poll (1991), who hydrolyzed different fractions of whey protein using trypsin. They found that α-lactalbumin was insensitive to the action of trypsin, while at the same time, β-lactoglobulin was rapidly hydrolyzed. Britten and Giroux (1994) hydrolyzed WPC solution (6% protein) using Rhozyme, a broad specificity protease from *Aspergillus oryzae* and observed that half of the β-lactoglobulin content was hydrolyzed when the degree of hydrolysis reached 5.1%. It is clear from our study

that α-lactalbumin is more resistant to attack by Flavourzyme than β-lactoglobulin. The enzyme did not attack α-lactalbumin and BSA.

Development of milk enriched with functional peptides

The hydrolyzates from 10 and 15% whey protein solutions were incorporated into toned milk (3% fat; 8.55% solids-not-fat) to enrich it with the functional peptides formed during enzymatic hydrolysis of the whey proteins. Milk incorporated with the WPC hydrolyzate is hereafter referred as 'Enriched Milk' (EM). The maximum level of hydrolyzate that could be incorporated into milk was assessed by sensory evaluation for its acceptability. Tricine SDS-PAGE were conducted to evaluate the presence of low-molecular-weight peptides and further confirmed by RP-HPLC study. The EM thus optimized was analyzed for physicochemical quality for a period of 72 h.

Optimization of whey protein hydrolyzate incorporation in toned milk (3% fat)

The hydrolyzates with DH of 5, 7, 9% prepared from WP solutions of 10 and 15%, were added into the milk at 5, 10 and 20% (v/v), pasteurized and evaluated separately by the sensory panel of judges. Milk enriched with hydrolyzates of 9% DH scored least for all sensory parameters as compared to those enriched with hydrolyzates of DH 5 and 7%, which showed that the sensory quality of the product decreased with the increase in % DH irrespective of the level of incorporation of the hydrolyzate. It was found that 10% protein solution with 5% DH was acceptable up to 20% (v/v), while the hydrolyzate with DH of 7% was acceptable only up to 10% (v/v) enrichment in milk. This showed that the sensory quality of the product decreased as the DH increased, irrespective of the level of incorporation of the hydrolyzate. Further, it was clear that milk enriched with hydrolyzate of 15% protein solution was acceptable only up to 10% (v/v) incorporation with DH of 7% and up to 5% (v/v) incorporation with DH of 9%.

Identification of peptides present in milk enriched at different levels with 10 and 15% protein hydrolyzates by Tricine SDS-PAGE

The electrophoretic pattern of proteins in toned milk and milk enriched with hydrolyzates from 10% protein solution, having DH of 5, 7, 9% and incorporated into milk at 5, 10 and 20% (v/v) is shown in Fig. 2c. The electropherogram (Fig. 2c) of the enriched milk samples (Lane 3–10) individually showed a total of seven bands. Prominent presence of bands in the middle part of each lane, whose migration rates were similar to the 3 prominent bands in toned milk (lane 2), thus confirming it as casein, ß-lactoglobulin and α-lactalbumin. However, these 3 bands, which were already present in the control, were found to be thicker in the enriched milk samples. The thickness and intensity of the bands increased as the level of incorporation of hydrolyzate into milk increased. Thus, the bands in lane 4, where hydrolyzate (DH 5%) was incorporated into milk at 20% (v/v) level was more prominent than those in lane 3, where hydrolyzate (DH 5%) was incorporated into milk at 10% (v/v) level. Similarly, the hydrolyzate (DH 7%) was incorporated into milk at 5, 10 and 20% (v/v) level and run through lanes 5, 6 and 7, respectively. It was found that band thickness and intensities were more in samples with 20% (v/v) incorporation of hydrolyzate (lane 7), followed by those with 10% (lane 6) and 5% (lane 5) incorporation. The same trend was observed in enriched milk samples incorporated with hydrolyzates of 9% DH. However, this sample had bitter taste and hence only 5% (v/v) incorporation of this hydrolyzate was found possible without affecting the flavour of the enriched milk. The casein band (29,000 Da) in all the samples was found to be uniform. It is evident that the intensity of band is inversely proportional to the degree of hydrolysis.

Two prominent bands corresponding to 6500 and 3000 Da of the marker proteins could be observed in the enriched milk (Fig. 2c, lane 4 to 7) which were absent in the control toned milk (lane 2). The new low-molecular-weight peptides are formed as a result of cleavage of higher molecular proteins by the enzyme.

Figure 2d depicts a similar trend for milk enriched with hydrolyzates from 15% protein solution. All enriched milk samples showed the presence of low-molecular-weight peptides (Fig. 2d, lane 3 to 10), while these were not present in the control milk. Hydrolyzates of 15% protein solution with DH of 9% (lane 8 to 10) were not desirable due to breakage of peptide bonds. Only 5% (v/v) of this hydrolyzate could be incorporated into milk for an acceptable product. Milk enriched with 10% (v/v) hydrolyzate of 15% protein solution, with DH of 7% conferred the highest amount of low-molecular-weight peptides incorporated, which was also organoleptically acceptable.

Protein hydrolyzates possess a physiological property of special importance. The gastrointestinal absorption of hydrolyzates, especially di- and tripeptides, seems to be more effective compared to both intact protein and free amino acids (Grimble and Silk 1989; Minami et al. 1992) at least in stressed situations with a reduced intestinal absorption area, an impaired digestive function and/or a high overload of protein intake. Enzymatic hydrolysis of proteins is a way to improve protein functionality. Although the degree of hydrolysis is an important factor for characterizing any protein hydrolyzate, it doesn't provide the complete information on peptide profile

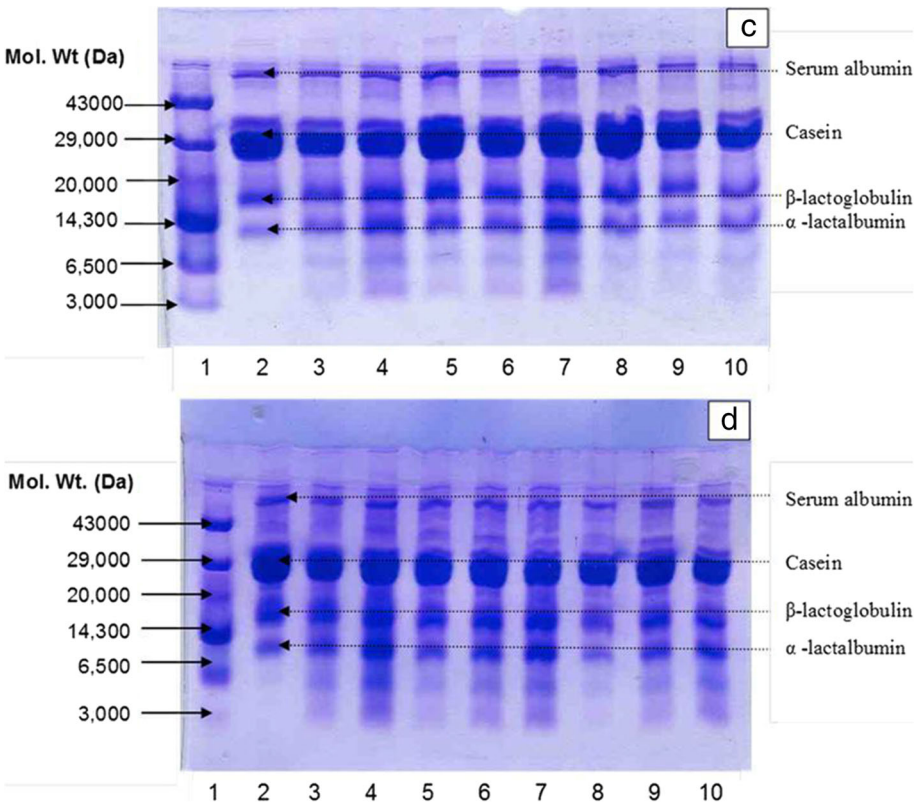

Fig. 2 c Tricine SDS-PAGE of milk enriched with hydrolyzates from 10% protein solution. Lane1 - Standard; lane 2 - toned milk; lane 3, 4 - milk enriched with 10 and 20% (v/v) hydrolyzate of DH 5%, respectively; lane 5, 6, 7 - milk enriched with 5, 10, 20% (v/v) hydrolyzates of DH 7%, respectively; lane 8, 9, 10 - milk enriched with 5, 10, 20% (v/v) hydrolyzate of DH 9%, respectively. **d** 15% protein solution. Lane1 - Standard; lane 2 - toned milk; lane 3, 4 - milk enriched with 10 and 20% (v/v) hydrolyzate of DH 5%, respectively; lane 5, 6, 7 - milk enriched with 5, 10, 20% (v/v) hydrolyzate of DH 7%, respectively; lane 8, 9, 10 - milk enriched with 5, 10 and 20% (v/v) hydrolyzate of DH 9%, respectively

(molecular weight distribution). Peptide profile is critical for clinical and food applications (Clemente 2000). Peptides are less hypertonic than free amino acid mixtures enabling good absorption of other dietary components and eliminating osmotic problems. It is generally accepted that mild hydrolysis is beneficial and extensive hydrolysis is detrimental (Haque and Mozaffar 1992). Thus, elimination of milk enriched with 9% hydrolyzate in our study has been supported. Lower DH of 5 and 7% were selected as these had higher amount of low-molecular-weight peptides that were not further broken down into amino acids.

Chorbert et al. (1988) found that the WP hydrolyzates in size exclusion chromatography showed major peaks near 18, 13, 9, 5 kDa after 2.5% hydrolysis, near 9 and 5 kDa after 3.9% hydrolysis and near 6 and 2 kDa after 5.3% hydrolysis. Larger peptides (2–5 kDa) are mainly used as functional ingredients or in personal care products. Medium size peptides (1–2 kDa) are used in clinical nutrition and sports nutrition. Electrophoretic analysis carried out in the present study showed that hydrolysis of 10 and 15% protein solution to DH of 5 and 7%, also resulted in the formation of peptides < 6.5 kDa.

Selection of samples having the highest amount of hydrolyzed proteins incorporated in milk

The acceptable level of hydrolyzate from 10 and 15% protein solution that could be incorporated into milk was standardized by sensory evaluation. The presence of low-molecular-weight peptides in these accepted samples was also confirmed by electrophoretic studies. Ideally, optimized enriched milk should have the maximum amount of low-molecular- weight functional peptides incorporated, with minimum bitterness. Among the accepted combinations, it was found that milk when incorporated with hydrolyzate from 10% protein solution, having DH of 5%, at 20% (v/v) level, it would contain 0.099% hydrolyzed protein and attain a total protein enrichment of 1.99%. Similarly, milk incorporated with hydrolyzate having DH of 7% from 15% protein solution at 10% (v/v) level; would contain 0.1049% hydrolyzed protein and attain a total protein enrichment of 1.495%.

Thus, it was found that samples of 10% protein solution, with DH of 5 and 15% solution with DH 7% are suitable for milk fortification with peptides,

Analysis of enriched milk

With the optimized conditions of hydrolysis for maximum incorporation of low-molecular- weight peptides with least bitterness, the hydrolyzate of 10% WP with DH of 5% was incorporated at 20% (v/v) level into milk and the fat was adjusted to 3%. Enriched milk (EM) was heated at 72 °C for 2–3 min, cooled, packed in polyethylene pouches and stored in refrigerator. The control and enriched milk were analyzed for physicochemical and sensory properties.

Physicochemical characteristics of enriched and toned milks

The physicochemical characteristic of toned and enriched milks are shown in Table 3. Higher protein content in enriched milk was due to the added proteins from the hydrolyzate. The hydrolyzate was prepared from a 10% protein solution and added at 20% (v/v) level. This accounted for the increase in protein content of enriched milk which also increases total solids contents. The pH of toned milk was around 6.4, while it was 6.6 for enriched milk. The enriched milk had higher pH value because of addition of 20% (v/v) neutralized hydrolyzate into the milk. pH of milk at 25 °C normally varies within a relatively narrow range of 6.5 to 6.7. There are many components in milk, which provide a buffering action as reported by Webb et al. (1974). The major buffering groups of milk are caseins and phosphate. Therefore, both the acidity and pH in enriched milk falls within the normal range of milk. Higher viscosity (2.146 cP) in enriched milk was probably due to the added hydrolyzate, as whey proteins were found to have good solubility and water biding capacity (Foegeding et al. 2002). Enriched milk showed lower rate creaming than toned milk indicating that added hydrolyzates showed emulsifying properties which might have prevented the rate of creaming in the final enriched milk. Some peptides had better emulsifying properties than milk proteins. Experiments showed that controlled enzymatic hydrolysis, particularly with trypsin; improve oil holding capacity by more than 600% (Haque 1993).

Table 3 Physico-chemical characteristics of enriched and toned milks

Attributes (%)	Samples	
	Toned milk	Enriched milk
Moisture	87.96 ± 1.05	87.32 ± 1.20
Protein	3.46 ± 0.11	4.70 ± 0.14
Ash	0.770 ± 0.014	0.775 ± 0.007
Fat	3.1 ± 0.07	3.0 ± 0.07
Lactose (by difference)	4.71 ± 0.06	4.20 ± 0.05
Acidity	0.15 ± 0.01	0.16 ± 0.01
pH	6.45 ± 0.05	6.64 ± 0.04
Viscosity (centipoises)	1.787 ± 0.003	2.146 ± 0.006
Rate of creaming (%)	90.9 ± 2.50	60.6 ± 1.80

Results expressed as Mean ± S.D; $n = 3$

Effect of heat treatments on the peptides profile in hydrolyzates and enriched milk

It was assumed that there may be changes taking place in the peptides present in hydrolyzate and enriched milks during condensing, spray drying and sterilization due to heat treatments. Therefore, an attempt was made to assess the changes in the peptides after different heat treatments by electrophoresis. Hydrolyzates of 10% WP solution with DH of 5% was subjected to different treatments like freeze drying, boiling for 30 min and spray drying. Similarly, toned milk enriched with 40% (v/v) of this hydrolyzate was also freeze dried, vacuum condensed up to 35–40% TS and spray dried. Toned milk enriched with 20 and 40% (v/v) hydrolyzate was homogenized at 1500 psi after preheating to around 65 °C. Thereafter, in-bottle sterilization of the enriched milk with and without the addition of 0.01% sodium citrate was carried out in an autoclave at 15 psi for 15 min (121 °C). Control toned milk did not coagulate, whereas enriched milk coagulated during sterilization. It is assumed that the added WP hydrolyzate in enriched milk may be the reason for the lower heat stability of the enriched milk. Early (1998) reported that casein tends to precipitate if milk is subjected to temperatures > 100 °C for a long time period. Even addition of sodium citrate did not improve the heat stability. Therefore, it is recommended that enriched milk samples should not be sterilized, but must be pasteurized to use as fluid enriched milk. However, enriched milk may be spray dried for longer shelf life. Subtil et al. (2014) successfully spray dried hydrolysed casein using gum Arabic as the carrier agent, in order to decrease the bitter taste.

Electrophoretic studies were carried out to evaluate changes in peptide profiles of these samples. Figure 3 illustrates the electropherograms of WPC, hydrolyzates, toned and enriched milk samples after different treatments. Control samples of whole WPC, hydrolyzate, toned milk enriched with 40% (v/v) of this hydrolyzate and toned milk were run through lanes 2, 3, 7 and 8, respectively; while treated samples were run in lanes 4,5 and 6. The electropherogram reflected minimal changes taking place in the samples due to heating and freeze drying of the hydrolyzates. Similarly, no differences were observed in the lower molecular weight peptides of freeze dried milk (lane 6) and enriched milk (lane 7),

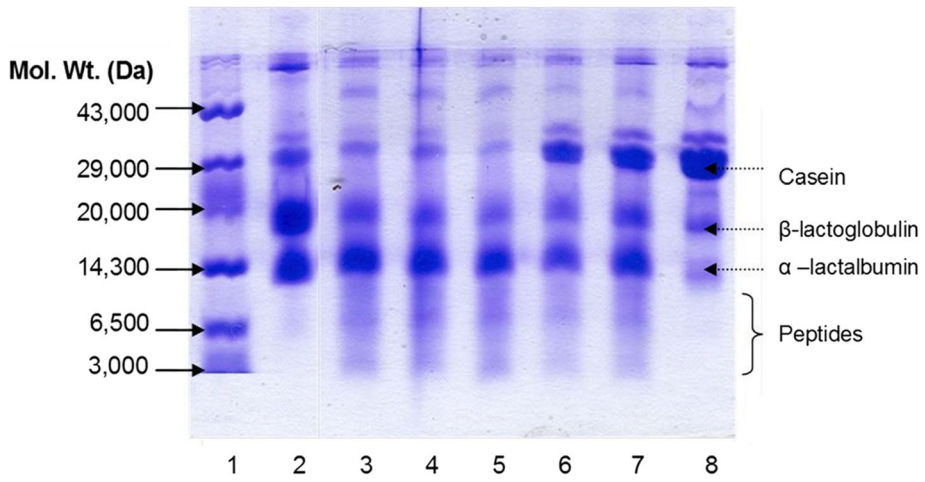

Fig. 3 Tricine SDS-PAGE of WPC, hydrolyzate, toned and enriched milk samples after different treatments. Lane 1 - Standard; lane 2 - whole WPC; lane 3, 4, 5 – hydrolyzate, boiled, freeze dried hydrolyzate, respectively; lane 6 -freeze dried enriched milk; lane 7 - enriched milk; lane 8 - toned milk

indicating once again no changes taking place in the lower molecular weight peptides of the enriched milk.

In our study, neither freeze drying nor heating showed any significant changes in peptide profile. This may be because the peptides are mostly in their primary structure and heat denaturation does not involve the breaking or changes in peptide bonds and thus could not be observed electrophoretically. The hydrolyzate and enriched milks were heated to more than 70 °C and denaturation of whey proteins commence at temperatures greater than 65 °C followed by aggregation and precipitation. In milk systems, these denatured whey proteins remain in suspension, becoming attached to the casein micelles. This may be the reason for the protein bands of α-lactalbumin, β-lactoglobulin to appear to be less prominent after the different treatments in the present electrophoretic studies. Thus, in the present electrophoretic study, no changes were observed in the peptide profiles of the hydrolyzates and enriched milks after subjecting to different treatments like freeze drying, boiling, concentration and spray drying, which showed that the enriched milk could be spray dried without any significant loss in the peptides in the dried product.

Reversed-phase high-performance liquid chromatographic (RP-HPLC) analysis

To confirm peptide profiles obtained by electrophoresis, five samples were analyzed by RP-HPLC technique. Figure 4e shows the superimposition of RP-HPLC Chromatograms of WPH, Toned Milk (TM) and Enriched milk with 20% hydrolyzates (EM_{20}). All the chromatograms showed some common peaks, which eluted between 3 and 35 min. However, in the WPH chromatogram, 3 major peaks were seen with retention time at 8.5, 10.36 and 20.2 min. These 3 peaks are absent in the chromatogram of TM, but can be clearly seen in the chromatogram of EM_{20}. Thus, it is confirmed that these 3 peaks corresponding to the new peptides formed have been contributed by the WPH in the enriched milk. The number of peaks in EM was more than in TM.

Similarly, Fig. 4f exhibits the superimposition of RP-HPLC chromatograms milk enriched with the 40% hydrolyzate (EM_{40}). The peak areas of the 3 peptides contributed by the WP hydrolyzate increased in EM_{40}. Thus, increasing the level of incorporation of hydrolyzate from 20 to 40% clearly shows the increase in the amount of peptides in enriched milk. This higher level (40%) of hydrolyzate incorporation was found to give an acceptable product of enriched milk without perceivable bitterness with 1% starch as a masking agent along with flavour and 7% sugar (results not shown).

Hernandez-Ledesma et al. (2005) separated and characterized whey proteins by RP-HPLC. Similarly, analytical identification and separation of peptides in various food products including protein hydrolyzates, dairy and meat products have been attempted by several investigators through RP-HPLC technique according to their hydrophobicity, molecular size and net charge (Fenelon et al. 2000). In the present study, the presence of peptides in the hydrolyzate and its higher presence in the enriched milk than milk were confirmed by RP-HPLC analysis.

ACE inhibitory activity

ACE inhibitory activity of the tested samples are shown in Fig. 5. Maximum ACE inhibition (% IACE) was observed in WP hydrolyzate, followed by enriched milk (EM_{20}), toned milk and WP solution. The difference in

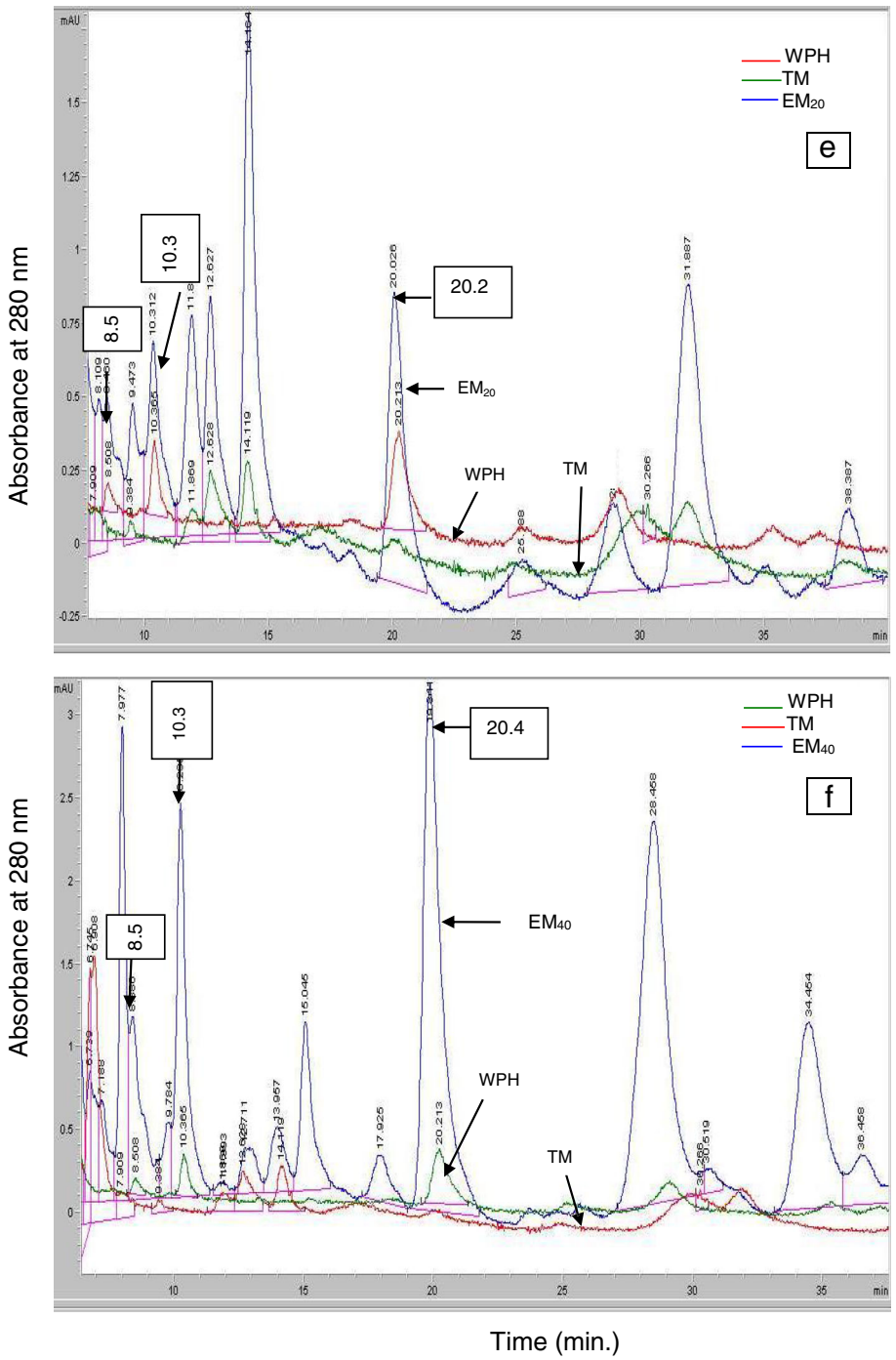

Fig. 4 e RP-HPLC profile of whey proteins and peptides in WPH, toned milk and enriched milk with 20% (v/v) hydrolyzate (EM_{20}) and **f** 40% (v/v) hydrolyzate (EM_{40})

the % IACE among the samples were significantly ($p < 0.01$) different. The high ACE-inhibitory activity of the hydrolyzate could be due to the presence of peptides released due to enzymatic hydrolysis of whey proteins. Goudarzi et al. (2012) reported the release of ACE inhibitory peptides from whey proteins due to both enzymatic hydrolysis and fermentation processes. The enriched milk had the next highest ACE inhibitory activity. This may be due to the addition of hydrolyzates into the milk indicating ACE inhibitory peptides were still intact and active, even after the pasteurization process. Slightly higher activity in toned milk than whole whey

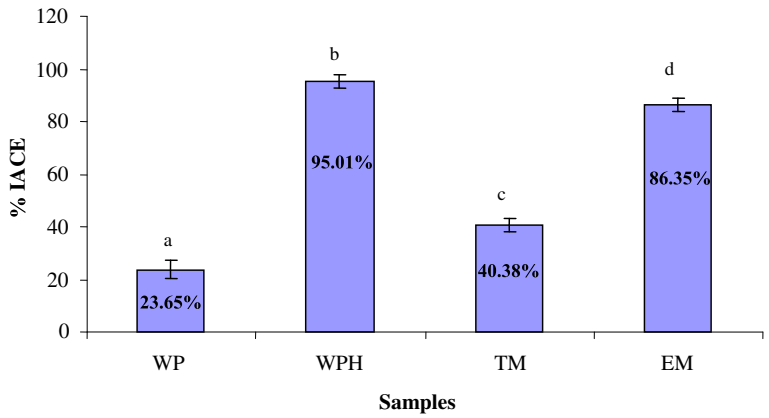

Fig. 5 ACE inhibitory activity in WPC, its hydrolyzate, toned and enriched milks. Different letters on top of the bars indicate significant differences ($p < 0.01$). Error bars indicate standard deviation from means ($n = 3$)

protein solution may be because of casein and other milk components.

Angiotensin-I-converting enzyme (ACE) is a key enzyme in the regulation of peripheral blood pressure (Tavares et al. 2011a, b). A strong ACE inhibitory activity of more than 70% (at 1 mg/ mL) was obtained after digestion of casein with trypsin, thermolysin (Koli 2004). Hernandez-Ledesma et al. (2003) reported the % IACE value of whey from full fat yogurt, skimmed yogurt and infant formula to be 43.4, 39.5 and 87.2%, respectively. Pihlanto-Leppälä et al. (2000) characterized the peptides with ACE inhibitory property from whey protein digests. They found that peptides in hydrolyzates have more ACE inhibition activity in comparison with the whole WPC. Furthermore, Foltz et al. (2009) showed that the tripeptide Ile-Pro-Pro, an ACE-inhibitory peptide, selectively escapes from intestinal degradation and reaches the circulation undegraded, which is a promising finding.

Thus, WP hydrolyzate contains peptides that are potential ACE-inhibiting agents. The present study also showed that 10% protein hydrolyzate with DH of 5% had very good ACE-inhibiting activity and when incorporated into milk at an acceptable level of 20% (v/v), can increase the ACE-inhibiting activity of the milk, thus improving its biological property of increased antihypertensive activity.

Conclusion

Generation of low-molecular-weight peptides using controlled enzymatic hydrolysis of whey protein concentrate is a good approach to obtain functional ingredients which can be incorporated in the food to enhance health benefits. This study elaborates the conditions to obtain maximum number of lower molecular weight peptides from whey proteins, which can be used in milk to increase its beneficial effect. Either hydrolyzates of 10% WP with DH of 5% could be incorporated up to 20% (v/v) or hydrolyzates of 15% whey protein with DH of 7% could be incorporated up to 10% (v/v) into toned milk without any bitter taste. This is our recommendation/suggestion to acquire maximum number of small peptides in the hydrolyzate for mixing in the 3% fat milk. Further research is required on debittering techniques that would further allow increasing the amount of peptides supplement. This study is expected to boost the opportunity for the dairy industry to venture further into the nutraceutical dairy market.

Abbreviations

ACE: Angiotensin converting enzyme; ANOVA: Analysis of variance; DH: Degree of hydrolysis; EM: Enriched milk; EM_{20}: Milk enriched with 20% hydrolyzates; EM_{40}: Milk enriched with 40% hydrolyzates; E:S: Enzyme to substrate ratio; IACE: Inhibition of ACE; LAPU/g: Leucine Amino Peptidase Units per gram; NaOH: Sodium hydroxide; RP-HPLC : Reversed- phase high performance liquid chromatography; SD: Standard deviation; SDS: PAGE-Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; SNF: Solids-not-fat; TM: Toned milk of 3% fat and 8.5% solids-not-fat; WP: Whey protein; WPC: Whey protein concentrate; WPH: Whey protein hydrolyzate

Acknowledgements

The first author thanks ICAR -National Dairy Research Institute, Bengaluru for providing financial assistance and all necessary facilities to carry out present research work.

Declarations

The manuscript in part or in full has not been submitted or published anywhere.

Authors' contributions

JAJ designed and carried out the experiments, collected and analyzed data, and prepared the draft of the manuscript. BCG conceptualized the study, designed experiments, supervised the study, read and edited the manuscript. All authors read and approved the manuscript.

Funding

This research work was supported by ICAR -National Dairy Research Institute, Bengaluru through an approved research programme to first author.

Availability of data and materials

All data supporting this study are included in this manuscript. Further details are available upon request from the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication
Not applicable.

Competing interests
Authors declare that they have no competing interests.

Received: 11 September 2020 Accepted: 11 February 2021
Published online: 07 May 2021

References

Adler-Nissen, J. (1986). A review of food protein hydrolysis – Specific areas. In *Enzymatic hydrolysis of food proteins*, (pp. 57–109). Barking: Elsevier Applied Science Publishers, Copenhagen.
Asselin, A., Herbert, J., & Amiot, H. (1989). Effects of *in vitro* proteolysis on the allergenicity of major whey proteins. *Journal of Food Science, 54*, 1037–1039.
Bordin, G., Raposo, F. C., Calle, B., & Rodriguez, A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. *Journal of Chromatography A*, *928*, 63–76.
Britten, M., & Giroux, H. J. (1994). Effect of pH during heat processing of partially hydrolysed whey proteins. *Journal of Dairy Science*, *77*, 676–684.
Chorbert, J. M., Habv, C. B., & Nicolas, M. G. (1988). Stability and emulsifying properties of casein and whey proteins modified enzymatically by trypsin. *Journal of Agricultural and Food Chemistry*, *36*, 883–892.
Clemente, A. (2000). Enzymatic protein hydrolyzates in human nutrition. *Trends in Food Science and Technology*, *11*, 254–262.
Early, R. (1998). Liquid milk and cream. In The Technology of Dairy Products, Ed., R. Early. Blackie Academic and Professional, London, U.K., 2nd edition, 38–45.
Fenelon, M. A., O'Connor, P., & Guinee, T. P. (2000). The effect of fat content on the microbiology and proteolysis in cheddar cheese during ripening. *Journal of Dairy Science*, *83*, 2173–2183.
Foltz, M., Van Buren, L., Klaffke, W., & Duchateau, G. S. M. J. E. (2009). Modeling of the relationship between dipeptide structure and dipeptide stability, permeability, and ACE inhibitory activity. *Journal of Food Science, 74*, H243–H251.
Foegeding, E.A., Davis, J.P., Doucet, D. & McGuffey, M.K. (2002). Advances in modifying and understanding whey protein functionality. *Trends in Food Science & Technology, 13*, 151–159.
Goudarzi, M., Madadlou, A., Mousavi, M., & Emam-Djomeh, Z. (2012). Optimized preparation of ACE-inhibitory and antioxidative whey protein hydrolyzate using response surface method. *Dairy Science and Technology*, *92*, 641–653.
Ghosh, B. C., Prasad, L. N., & Saha, N. P. (2017). Enzymatic hydrolysis of whey and its analysis. *Journal of Food Science and Technology*, *54*, 1476–1483.
Grimble, G. & Silk, D.B.A. (1989). Peptides in Human Nutrition. *Nutrition Research Reviews, 2*, 87–108.
Haque, Z. U. (1993). Influence of milk peptides in determining the functionality of milk proteins: A review. *Journal of Dairy Science*, *76*, 311–320.
Haque, Z. U., & Mozaffar, Z. (1992). Casein hydrolyzates II. Functional properties of peptides. *Food Hydrocolloids*, *5*, 559–571.
Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity: From research to food applications. *Current Opinion in Biotechnology, 18*, 163–169.
Hernandez-Ledesma, B., Alvarez, P. J. M., & Pueyo, E. (2003). Assessment of the spectrophotometric method for determination of angiotensin-converting enzyme activity: Influence of the inhibition type. *Journal of Agricultural and Food Chemistry*, *51*, 4175–4179.
Hernandez-Ledesma, B., Davalos, A., Bartolome, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolyzates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, *53*, 588–593.
Indian Standards Institution IS: (SP: 18) (1980). *ISI handbook of food analysis. Part I: General methods*. New Delhi: Indian Standards Institution.
Indian Standards Institution IS: (SP: 18) (1981). *ISI handbook of food analysis. Part XI: Dairy products*. New Delhi: Indian Standards Institution.
ISO 8968-1/2|IDF 020-1/2 (2014). Milk - Determination of nitrogen content - Part 1/2: Determination of nitrogen content using the Kjeldahl method.
Klompong, V., Benjakul, S., Kantachote, D., & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolyzate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry*, *102*, 1317–1327.
Koli, D. (2004). *Comparative studies on casein derived ACE-inhibitory and immunomodulatory peptides from cow and buffalo milk*. Karnal: Ph.D. Thesis submitted to National Dairy Research Institute.
Kopp, W. (2019). How western diet and lifestyle drive the pandemic of obesity and civilization diseases. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *12*, 2221–2236.
Madureira, A. R., Tavares, T., Gomes, A. M. P., Pintado, M. E., & Malcata, F. X. (2010). Invited review: Physiological properties of bioactive peptides obtained from whey proteins. *Journal of Dairy Science*, *93*, 437–455.
Martinez-Maqueda, D., Miralles, B., Recio, I., & Hernandez-Ledesma, B. (2012). Antihypertensive peptides from food proteins: A review. *Food and Function*, *3*, 350–361.
Matz, S. A. (1962). *Food texture*, (pp. 26–30). Connecticut: The AVI Publishing Company, Inc.
Meisel, H., Walsh, D. J., Murray, B., & FitzGerald, R. J. (2006). ACE inhibitory peptides. In Y. Mine, & F. Shahidi (Eds.), *Nutraceutical proteins and peptides in health and disease*, (pp. 269–315). Boca Raton: CRC.
Menefee, S.G. & Overman, O.R. (1940). A semi micro-kjeldahl method for estimation of total nitrogen in milk. Journal of Dairy Science, *23*, 1177–1185.
Minami, H., Morse, E.L. & Adibi, S.A. (1992). Characteristics and mechanism of glutamine-dipeptide absorption in human intestine. *Gastroenterology, 103*, 3–11.
Naik, L., Mann, B., Bajaj, R., Sharma, R. B., & Sharma, R. (2013). Process optimization for the production of bio-functional whey protein hydrolysates: Adopting response surface methodology. *International Journal of Peptide Research and Therapeutics*, *19*, 231–237.
Nongonierma, A. B., & FitzGerald, R. J. (2015). The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A review. *Journal of Functional Foods*, *17*, 640–656.
O'Keefe, M.B. & FitzGerald, R.J. (2014). Antioxidant effects of enzymatic hydrolysates of whey protein concentrate on cultured human endothelial cells. *International Dairy Journal, 36*, 128–135.
Pihlanto-Leppälä, A., Koskinen, P., Piilola, K., Tupasela, T., & Korhonen, H. (2000). Angiotensin I-converting enzyme inhibitory properties of whey protein digests: Concentration and characterization of active peptides. *Journal of Dairy Research*, *67*, 53–64.
Sáez-Plaza, P., Navas, M., Wybraniec, S., Michałowski, T., & Asuero, A. G. (2013). An overview of the Kjeldahl method of nitrogen determination. Part II. Sample preparation, working scale, instrumental finish, and quality control. *Critical Reviews in Analytical Chemistry*, *43*, 224–272.
Schagger, H., & Jagow, G. V. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, *166*, 368–379.
Schmidt, D. G., & Poll, J. K. (1991). Enzymatic hydrolysis of whey proteins. Hydrolysis of α-lactalbumin and β-lactoglobulin in buffer solutions by proteolytic enzymes. *Netherlands Milk and Dairy Journal*, *45*, 225–240.
Snedecor, G. W., & Cochran, W. G. (1994). *Statistical methods*, (8th ed.,). Calcutta: Oxford and IBH Publishing Co.
Subtil, S. F., Rocha-Selmi, G. A., Thomazini, M., Trindade, M. A., Netto, F. M., & Favaro-Trindade, C. S. (2014). Effect of spray drying on the sensory and physical properties of hydrolysed casein using gum arabic as the carrier. *Journal of Food Science and Technology*, *51*, 2014–2021.
Tavares, T., Contreras, M. D. M., Amorim, M., Pintado, M., Recio, I., & Malcata, F. X. (2011a). Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro effect and stability to gastrointestinal enzymes. *Peptides*, *32*, 1013–1019.
Tavares, T. G., Contreras, M. M., Amorim, M., Martín-Álvarez, P. J., Pintado, M. E., Recio, I., & Malcata, F. X. (2011b). Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolyzates obtained with cardoon extract. *International Dairy Journal*, *21*, 926–933.
Webb, B.H., Johnson, A.H. & Alford, J.A. (1974). Fundmental of Dairy Chemistry. 2nd Ed. Chapter I, AVI Publishing Co., Westport, CT.
Xia, Z., & Xia, M. (2012). Orthogonal optimization of enzymatic hydrolysis of whey protein. *Advanced Materials Research*, *550-553*, 1556–1560.
Zheng, H., Shen, X., Bu, G., & Luo, Y. (2008). Effects of pH, temperature and enzyme-to-substrate ratio on the antigenicity of whey protein hydrolyzates prepared by Alcalase. *International Dairy Journal*, *18*, 1028–1033.
Zhidong, L., Benheng, G., Xuezhong, C., Zhenmin, L., Yun, D., Hongliang, H., & Wen, R. (2013). Optimisation of hydrolysis conditions for antioxidant hydrolysate production from whey protein isolates using response surface methodology. *Irish Journal of Agricultural and Food Research*, *52*, 53–65.

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