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Rational approach to sequential optimization of antioxidative whey protein hydrolysate production

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ABSTRACT

A two-step method for sequential optimization was designed based on the role of hydrolytic factors in the preparation of antioxidative whey protein hydrolysates (WPH). In the first step, the rates of reaction-related variables such as pH, temperature and the enzyme/substrate (E/s) ratio (w/w) were optimized to 1.5, 37°C and 1:100 for pepsin, 8, 42°C and 1:100 for trypsin, and 7.8, 37°C and 1:50 for pancreatin, respectively, with maximum degrees of hydrolysis of 4.5%, 14% and 15.2%, respectively. In the second step, hydrolysis was performed at the optimum conditions determined in the first step. The optimum hydrolysis times were 1.5 h for pepsin and 2 h each for trypsin and pancreatin based on their maximum reducing powers of 0.31, 0.55 and 0.62, respectively. In sequential hydrolysis, the optimized pepsin hydrolysates with subsequent action of trypsin and pancreatin individually were optimized at their respective E/s ratios of 1:50 and 1:100 (w/w) based on their respective degrees of hydrolysis of 13.6% and 14.8%, while the optimum hydrolysis times were 4.5 h and 2.5 h for reducing powers of 0.66 and 0.75, respectively. This study indicates that sequential optimization for the production of antioxidative WPH could be better than the traditional one factor at a time (OFAT) method.

Keywords

Hydrolysis of protein,
Whey protein hydrolysate,
Sequential optimization,
Degree of hydrolysis,
Antioxidant activity,
Reducing power

Introduction

Research in the field of antioxidative nutraceuticals is focussing on antioxidative protein hydrolysates effective against free radical-induced harmful oxidation in both food and the human body.

Lipid peroxides affect the organoleptic properties and storage quality of foods as reported in meat and meat products [1]. Similarly, free radical-induced oxidation of biomolecules results in cardiovascular disease, diabetes mellitus, neurological disorders and Alzheimer's disease [2, 3]. Synthetic antioxidants are used against free radicals such as oxidants, but have side effects. Hence, antioxidative protein hydrolysates are being increasingly explored in the search for natural antioxidants.

Whey proteins from bovine milk have been analyzed for antioxidative hydrolysates. Whey is a co-product of the cheese industry and consists of β-lactoglobulin, α-lactalbumin, immunoglobulins, serum albumin and proteose peptone [4], which are collectively known as whey proteins. Whey proteins are used to produce whey protein isolate and whey protein concentrate, which are enzymatically hydrolyzed to produce antioxidative whey protein hydrolysates (WPH) [5–12]. The antioxidant activity of protein hydrolysate depends on its peptide composition as influenced by the specific activity of the protease enzyme, which is predominantly determined by the hydrolysis conditions employed in the hydrolysis process [13–17]. Hence, optimization of hydrolysis conditions can be an economically effective approach to obtain protein hydrolysates with maximum antioxidant activity.

Response surface methodology is widely used as an optimization approach; however, it requires preliminary determination of experimental parameters in order to achieve the optimum response. The one factor at a time (OFAT) method is commonly used, but is a tedious and laborious technique requiring energy, time and resources. Nonetheless, it is the only method available for sequential optimization, and takes into account hydrolytic conditions where

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pH, temperature and the enzyme/substrate (E/s) ratio determine enzyme activity, while hydrolysis time directly affects the size and composition of peptides in hydrolysate [18]. The present study seeks to modify the OFAT method. A sequential optimization strategy with two sequential steps was designed: (i) the optimization of pH, temperature and the E/s ratio based on the rate of reaction as monitored by degree of hydrolysis; and (ii) the optimization of hydrolysis time based on the maximum antioxidant activity at optimized conditions.

Materials and Methods

Whey protein isolate (WPI) processed using cross-flow microfiltration was purchased from Marin Agro Private Limited, New Delhi. Proteolytic enzymes including pepsin, pancreatin and trypsin, bovine serum albumin, methanol, ferric chloride, potassium ferricyanide and trichloroacetic acid were purchased from HiMedia, India. All other chemicals were of analytical grade.

Proximate composition of WPI

The moisture, protein, lipid and total ash contents were analyzed according to the methods of the Association of Official Agricultural Chemists [19]. The conversion factor of nitrogen to protein was 6.38 [20]. Total carbohydrates were determined by subtracting the sum of other estimates from 100.

Hydrolysis of WPI

WPI was suspended in distilled water to a substrate concentration of 8% (w/v) based on protein content and the suspension was adjusted to the hydrolytic temperature and pH with 2N HCl or 2N NaOH as appropriate for the enzymatic treatment. WPI was hydrolyzed by the individual addition of pepsin, pancreatin and trypsin, and by the sequential addition of pepsin with pancreatin and trypsin individually at their respective optimized hydrolysis conditions. Hydrolysis pH was maintained at the required value by continuous addition of 2N NaOH or 2N HCl. After the selected digestion time, hydrolysis was discontinued by heating the suspension at 80°C for 20 min to inactivate the enzyme. The suspension was then cooled to room temperature followed by centrifugation at 7000 rpm for 10 min and the supernatant was stored at –20°C. The degree of hydrolysis and antioxidant activity of hydrolysates were determined.

Sequential optimization design

The pH, temperature and E/s ratio were first optimized followed by optimization of hydrolysis time for the production of antioxidative WPH using pepsin, trypsin or pancreatin and sequential digestion of pepsin by trypsin and pancreatin individually.

Optimization of rate of enzymatic reaction based on degree of hydrolysis

In the first step, the optimum hydrolysis conditions (E/s ratio, pH and temperature) were determined following complete enzymatic hydrolysis for the required hydrolysis time at a fixed substrate concentration containing 8% (w/v) protein content. The E/s ratio was varied from 1:50 to 1:200 (w/w) depending on pH and temperature. Pepsin digestion was carried out at pH 1.5 and pH 2 at both 37°C and 40°C, pancreatin digestion was conducted at pH 7.8 and pH 9 at both 37°C and 40°C, and trypsin digestion was carried out at pH 8 and pH 9 at each of 37°C, 40°C and 42°C. The optimum E/s ratio for sequential hydrolysis was determined. Pepsin hydrolysates obtained by individual hydrolysis with the highest degree of hydrolysis were sequentially hydrolyzed by the addition of pancreatin and trypsin in E/s ratios varying from 1:50 to 1:200 (w/w) at the respective pH and temperatures optimized in individual hydrolysis. The degree of hydrolysis is defined as the percentage of cleaved peptide bonds in a protein hydrolysate [21]. It was measured using the pH stat method.

Optimization of extension of hydrolysis based on antioxidant activity

In the second step, hydrolysis time was optimized at the pH, temperature and E/s ratio already optimized in the first step. During hydrolysis, aliquots were taken every 30 min for analysis of reducing power in order to determine antioxidant activity. In this way, hydrolysis time producing the best hydrolysate reducing power was optimized for individual and sequential enzymatic treatment.

Reducing power

The method of Oyaizu [22] for determining reducing power was followed with some modifications. To 2.5 ml of the sample solution, 2.5 ml of sodium phosphate buffer (0.2 M, pH 6.6) was added, followed by the addition of 2.5 ml of 1% potassium ferricyanide. The reaction mixture was then incubated at 50°C in a water bath for 20 min. It was then cooled to 20°C and 2.5 ml of 10% trichloroacetic acid was added to terminate the reaction. The mixture was centrifuged at 3000 rpm for 10 min. Then 0.1 ml of the supernatant was made up to 2.5 ml with distilled water and 1 ml of 1% ferric chloride was added. The colour developed was read at 700 nm after 5 min reaction.

Statistical analysis

The means±SD for triplicate measurements were calculated. Differences between means were analyzed using Duncan's multiple range test at a 0.05% level of significance. Statistical analyses were conducted using SPSS version 12.

Results and Discussion

Nutrient analysis of whey protein isolate

Table 1 shows the nutritive value of whey protein isolate. Whey protein isolate contained 90 g of protein, 0.9 g of carbohydrate, 1.3 g of fat, 5.4% of moisture, 2.4 g of ash, 112 mg of calcium and 7 mg of iron per 100 g. The high protein content may be due to cross-flow microfiltration used in the isolation of whey proteins from whey, a co-product of the cheese industry. These results are supported by other similar findings on the chemical composition of WPI [23].

Sequential optimization strategy

Optimization of rate of reaction based on degree of hydrolysis

Enzymatic hydrolysis of proteins refers to the cleavage of specific peptide bonds using enzymes and the release of free amino acids, which are dissociated to release protons into the surrounding medium, altering the pH of the reaction mixture. The amount of alkali or acid added to the reaction mixture to maintain the required pH indicates the degree of hydrolysis and thus the rate of reaction. Hydrolysis conditions and values are given in Table 2.

Complete hydrolysis of WPI by pepsin, an aspartate protease, was carried out for up to 3 h with E/s ratios of 1:50, 1:100, 1:150 and 1:200 (w/w) at pH 1.5 and pH 2 and at 37°C and 40°C as depicted in Table 2. The degree of hydrolysis varied significantly from 2.6% to 4.5%, rising as the E/s ratio increased from 1:50 to 1:100 (w/w) with maximum values seen at pH 1.5 and 37°C. However, the decrease in the degree of hydrolysis at higher E/s ratios of 1:150 and 1:200 at other pH and temperature settings could be due to the enzyme becoming saturated with substrate. The optimum conditions for the hydrolysis of WPI by pepsin producing the highest degree of hydrolysis were an E/s ratio of 1:100, pH 1.5 and 37°C. Antioxidative whey fractions such as bovine serum albumin, β-lactoglobulin and small peptides have been isolated by a combination of gel filtration chromatography and pepsin treatment at optimum conditions similar to those found in the present study [11].

Complete hydrolysis of WPI by trypsin was carried out for up to 3 h with E/s ratios varying from 1:50 to 1:200 (w/w) at pH 8 and pH 9 and at 37°C, 40°C and 42°C, as shown in Table 2. The degree of hydrolysis varied from 10% to 14%, with an E/s ratio of 1:50, pH 9 and 40°C resulting in the lowest value and an E/s ratio of 1:100, pH 8 and 42°C ($p<0.05$) resulting in the highest value. The higher temperature of 42°C could increase the exposure of peptide bonds for cleavage, resulting in a high degree of hydrolysis [24].

As shown in Table 2, complete hydrolysis of WPI by pancreatin was carried out for 3 h with E/s ratios varying from 1:50 to 1:200 (w/w) at pH 7.8 and pH 8 and at 37°C and 40°C. The degree of hydrolysis varied accordingly ($p<0.05$). An increase in the E/s ratio decreased the degree of hydrolysis, with a higher degree of hydrolysis seen at pH 7.8 at both 37°C and 40°C. An E/s ratio of 1:50, pH 7.8 and 37°C were found to be optimum for the maximum degree of hydrolysis for obtaining pancreatin hydrolysates from WPI. The increase in degree of hydrolysis at pH 7.8 could be due to the maintenance of the ionic character of WPI for enhanced enzymatic activity [25].

Sequential hydrolysis focussed on optimization of the E/s ratio between 1:50 and 1:200 (w/w) for the sequential addition to the antioxidative pepsin hydrolysates of trypsin and pancreatin individually at their respective optimums of pH 8 and pH 7.8, and 42°C and 37°C. A hydrolysis time of 6 h was required to complete the subsequent action of trypsin and of 3 h for pancreatin; E/s ratios of 1:50 for trypsin and 1:100 for pancreatin were optimum based on the maximum degree of hydrolysis. The degree of hydrolysis depends on the individual and sequential actions of pepsin and trypsin for the preparation of peptides from whey proteins [26].

The present study shows the importance of optimum hydrolysis conditions for facilitating enzymatic activity for the maximum degree of hydrolysis. However, the decrease in the degree of hydrolysis for some hydrolytic conditions may be due to restricted enzyme activity due to the formation

Nutrients	Values
Protein (g)	90.0±1.750
Carbohydrates (g)#	0.9±0.001
Fat (g)	1.3±0.200
Moisture (%)	5.4±0.400
Ash (g)	2.4±0.020
Calcium (mg)	112.0±3.000
Iron (mg)	7.0±0.500

*Values are the mean±SD of three determinations on a dry weight basis
#Calculated as follows: 100–(protein+total fat+ash+water)

Table 1 - Proximate composition of whey protein isolate (per 100 g)*

Enzymatic treatment	pH	Temperature (°C)	E/s ratio (w/w)*			
			1:50	1:100	1:150	1:200
Pepsin	1.5	37	3.0±0.2^{bc}	4.5±0.3^{a}	3.5±0.4^{b}	3.2±0.3^{b}
		40	2.8±0.4^{c}	3.4±0.5^{a}	3.0±0.3^{b}	2.6±0.5^{c}
	2.0	37	3.5±0.5^{c}	4.2±0.5^{a}	4.4±0.4a	3.9±0.3^{b}
		40	3.0±0.4^{bc}	3.1±0.5^{ab}	3.3±0.3a	2.9±0.2^{c}
Trypsin	8.0	37	10.5±0.5^{ab}	10.8±0.2^{a}	10.4±0.5^{bc}	10.2±0.4^{c}
		40	11.6±0.6^{c}	12.8±0.4^{a}	12.6±0.5^{ab}	12.4±0.3^{b}
		42	14.0±0.2^{b}	14.5±0.4^{a}	13.8±0.6^{c}	12.6±0.2^{d}
	9.0	37	10.2±0.4^{c}	10.6±0.5^{ab}	10.8±0.3^{a}	10.4±0.5^{bc}
		40	11.8±0.6^{b}	12.4±0.3^{a}	11.6±0.3^{b}	11.0±0.4^{c}
		42	12.4±0.5^{c}	13.5±0.2^{a}	13.0±0.5^{b}	12.6±0.5^{c}
Pancreatin	7.8	37	15.0±0.5^{a}	14.0±0.3^{b}	13.5±0.4^{c}	12.0±0.5^{d}
		40	14.4±0.3^{a}	13.8±0.4^{b}	12.6±0.7^{c}	12.4±0.5^{d}
	8.0	37	13.5±0.5^{a}	12.0±0.4^{b}	11.5±0.4^{c}	11.0±0.3^{cd}
		40	12.5±0.6^{a}	11.8±0.7^{b}	10.8±0.5^{c}	10.6±0.4^{c}
Sequential hydrolysis#						
Pepsin	1.5	37	–	4.5±0.3	–	–
Subsequent trypsin	8.0	42	13.6±1.2^{a}	12.7±1.0^{b}	12.4±0.8^{bc}	12.0±1.1^{d}
Subsequent pancreatin	7.8	37	13.8±1.3^{b}	14.8±0.9^{a}	14.3±0.7^{ab}	13.7±0.5^{b}

*Hydrolysis values (%) are the mean±SD of three determinations. The superscript letters indicate a significant difference within the same row (p<0.05)
#In sequential hydrolysis, the E/s ratio was optimized at the pH and temperature optimized for individual enzymes
Hydrolysis time was 3 h for pepsin, trypsin and pancreatin respectively. Pepsin hydrolysate was subsequently hydrolyzed with trypsin for 6 h and pancreatin for 3 h individually
E/s enzyme/substrate ratio

Table 2 - Effect of hydrolysis conditions on degree of hydrolysis of whey protein isolate*

of reaction products, the low availability of specific peptide bonds, and inhibition and deactivation of the enzyme during hydrolysis [27, 28].

Optimization of hydrolysis time based on reducing power

The hydrolysis time needed to produce a high reducing power was optimized by conducting complete hydrolysis of WPI for the required time at optimum pH, temperature and E/s ratio as in the first step described above.

Reducing power refers to the anti-oxidative mechanism of donating a hydrogen or electron to free radical scavengers or chelating metals like Fe^{3+} to counteract free radical-induced oxidation and hence is used to evaluate the potential antioxidant activity of antioxidants [29]. The ability of WPH to reduce the Fe^{3+} ferricyanide complex to the ferrous (Fe^{2+}) form was analyzed and the absorbance of the resulting Prussian blue colour formation was observed at 700 nm (the stronger the absorbance, the higher the reducing power).

Fig. 1a shows the reducing power of WPH at 30 min intervals during complete hydrolysis of WPI by pepsin carried out for 3 h at the optimum conditions of an E/s ratio of 1:100, pH 1.5 and 37°C. An increase in hydrolysis time from 0.5 h to 1.5 h resulted in an increase in reducing power from 0.18 to 0.31; however, a further increase in hydrolysis time up to 3 h decreased the reducing power to 0.21. Hence a hydrolysis time of 1.5 h was found to be optimum for obtaining WPH by pepsin at optimum conditions. The high reducing power was due to the specific cleavage of peptide bonds between aromatic amino acids such as phenylanalanine, tryptophan and tyrosine, which have been widely reported to have antioxidant activity. The aromatic ring of phenylalanine in peptides could help scavenge hydroxyl radicals [30]. The reducing power of tryptic hydrolysates sampled every 30 min during complete hydrolysis continued for 3 h with the optimum conditions of an E/s ratio of 1:100, pH 8 and

42°C as shown in Fig. 1b. Reducing power increased to 0.55 after 2 h of hydrolysis, after which it declined to 0.45 by the end of hydrolysis. This could be attributed to the release of antioxdiative peptides consisting of arginine and lysine from tryptic cleavage of WPI [31].

Fig. 1c shows the reducing power of pancreatin hydrolysates obtained every 30 min during 3 h hydrolysis at the optimum conditions of an E/s ratio of 1:50, pH 7.8 and 37°C. The maximum reducing power of 0.62 was observed after 2 h of hydrolysis, but this decreased to 0.52 by 3 h. This may be due to the release of oligopeptides with lysine and arginine through pancreatin cleavage of proteins. It has been suggested that alkaline amino acids such as histidine, arginine and lysine have strong antioxidant activity [32, 33].

In sequential hydrolysis, WPI already hydrolyzed by pepsin at optimum conditions was subsequently hydrolysed with trypsin and pancreatin for 6 h and 4 h, respectively, to achieve complete hydrolysis as shown in Fig. 1d,e. Hydrolysis times of 4.5 h for subsequent action of trypsin and 2.5 h for pancreatin were found to be optimum based on maximum reducing powers of 0.64 and 0.75, respectively. Loach hydrolysates were prepared by simulated gastrointestinal digestion using pepsin and pancreatin and their radical scavenging activity against DPPH, hydroxyl and superoxide radicals reported [34].

Hence, hydrolysis time can be optimized to control the extent of cleavage of peptide bonds in order to release peptides with specific sequences in WPH for enhanced antioxidant activity. The degree of hydrolysis varies with hydrolysis time and its influence on antioxidant activity has been reported in protein hydrolysates from peanut and loach [35–37].

Conclusion

The antioxidant activity of WPH hydrolyzed by pepsin, trypsin and pancreatin was indicated by reducing powers of 0.31, 0.55 and 0.62, respectively, while the sequential action of pepsin with trypsin and pancreatin individually demonstrated reducing powers of 0.64 and 0.75, respectively. Optimization was shown to be necessary to produce antioxidative WPH as the antioxidant activity of WPH depends on the proteases, type of enzymatic treatment and hydrolysis conditions specific to enzymatic activity. Since the rate and duration of hydrolysis determine the ultimate peptide composition of hydrolysates, sequential optimization could be an appropriate strategy for obtaining WPH with a peptide composition with maximum antioxidant activity. Hence, the sequential optimization method described above may be

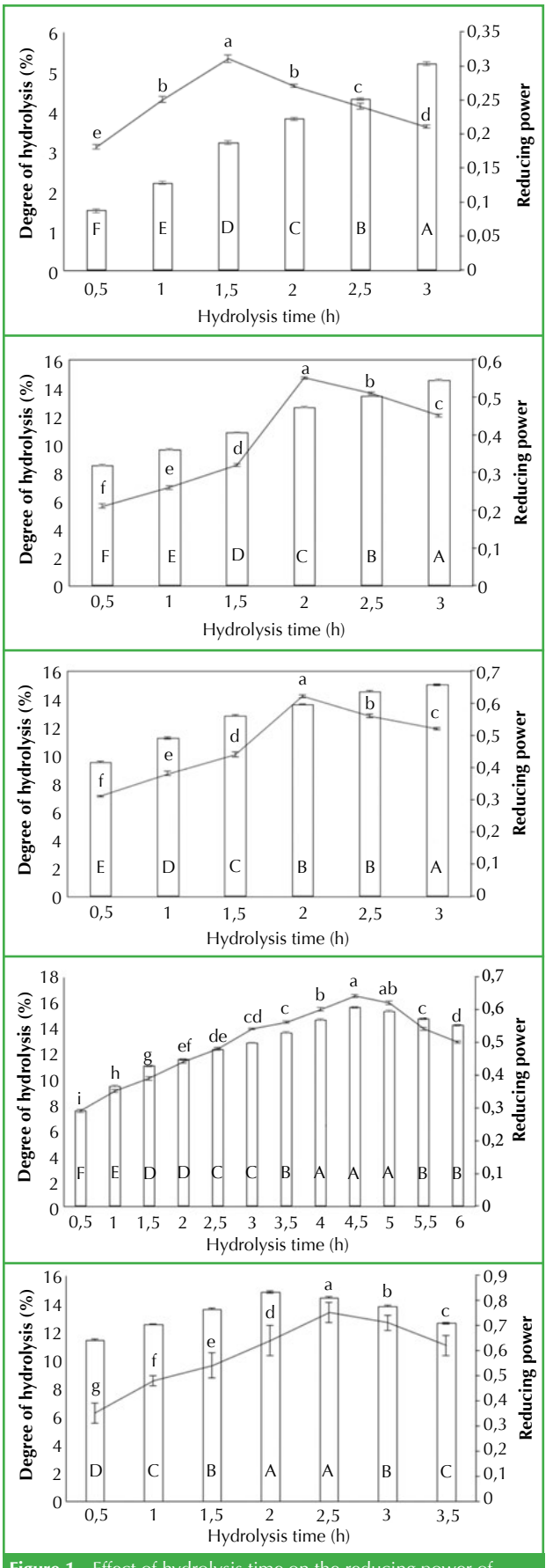

Figure 1 - Effect of hydrolysis time on the reducing power of whey protein hydrolysates following digestion by (a) pepsin, (b) trypsin, (c) pancreatin, (d) sequential digestion by pepsin with trypsin, and (e) sequential digestion by pepsin with pancreatin. Small letters indicate reducing power and capital letters degree of hydrolysis ($p<0.05$)

better than the traditional OFAT method. However, the molecular characteristics of WPHs such as molecular weight, amino acid composition and peptide sequences specific to enzymatic action, have yet to be explored in order to elucidate the mechanisms of antioxidant activity.

Human and Animal Rights

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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