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RESEARCH ARTICLE

Physicochemical Characterization of Hydrolysates of Whey Protein Concentrates for Their Use in Nutritional Beverages

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Abstract Whey protein concentrates containing 50 and 60% protein were manufactured and were hydrolyzed for 0.5, 1, 2, 3, 4, and 5 h with 5 commercial enzymes (flavourzyme, protease A, protease M, protease S, and trypsin). Functional properties such as degree of hydrolysis (DH), non-protein-nitrogen (NPN), 5-hydroxymethyl-2-furfural (HMF), solubility, and free-sulfhydryl (FSH) levels were measured. In food applications functional efficiency of whey protein hydrolysates (WPHs) depended on hydrolysis time, protein composition and enzymatic specificity. WPHs treated with protease A were found to be suitable for applications that require extensively hydrolyzed (>2 h) WPHs, because they had high solubility, DH, HMF, and FSH contents. Proteases S and M hydrolysates delayed the Maillard reaction and had high DH in mild hydrolysates (≤2 h) of WPHs. Aggressive hydrolyzed WPHs of protease A, and mild hydrolysates of proteases S and M are preferred in beverage fortification for maximum functional efficiency.

Keywords: whey protein hydrolysates, nutritional beverage, HMF, protease A

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Introduction

Lately, with the growth of the food-processing industry, the demand and market for functional proteins has risen. The protein content of beverages and foods are adjusted according to the desired nutritional value, cost, physical and sensory properties, and shelf life of the product. Nutritional beverages are popular among consumers, who use them to fulfill their dietary requirements. Consumer acceptance of these products depends on their ability to maintain the appearance, texture, and flavor during storage and consumption (1). Whey protein concentrates (WPCs) are a major source of proteins and are of significant commercial value to beverage manufacturers owing to their easy availability, cheap manufacturing costs, favorable functionality, and high nutritional value.

Enzymatic hydrolysis of WPCs enhances their suitability for use in the food-processing industry. Hydrolysates also have numerous health benefits, including opioid, anti-hypertensive, antibacterial, mineral-binding, antithrombotic, and anti-gastric activities (2,3). Therefore, there is a higher interest to incorporate whey protein hydrolysates (WPHs) into nutritional beverages in the food industry (4). The functional properties of WPHs vary with processing conditions such as temperature, type of cheese coagulant, and the hydrolysis process. Enzyme selection plays a vital role in whey protein hydrolyzing process. Numerous enzymes have been used in the food-processing industry. Depending on the type of WPH required and its application, different enzymes can be used to achieve optimal results (5,6). Degree of hydrolysis (DH) of WPHs decides the unmasked bioactive peptides that are available in beverages and directly influenced to the improvement of nutritional value in a food or beverage. The higher DH improves the nutritional value of proteins and resulted small peptides and amino acids participate in many body functions before

being absorbed (7). Considering the solubility of WPHs is also an important factor to maintain the evenness of texture and flavor of the beverages and to avoid undesirable turbidity and sedimentation, especially during long time storage (8). The industry faces the problems in bitterness of WPHs applications which most responsible by levels of the hydrophobic groups in non-protein nitrogen (NPN). During the hydrolysis process, the exposed peptides and amino acids are reacting with sugar and this process called as Maillard reaction. This reaction produces number of by-products including 5-hydroxymethyl-2-furfural (HMF) that can be preferable or not. The Maillard reaction is responsible for aroma, color, flavor, and texture of a product and is useful in food designing (9,10). Levels of HMF as an indicator, decides the keeping quality of the beverage as well as a flavor and color enhancing compounds with caramel taste (11). The free-sulfhydryl/thiol groups (FSH) groups affect many of the characteristics of proteins found in beverages, including antioxidant action, browning inhibition, emulsifying activity, extrusion, flavor and cooked off-flavor, foaming and whipping, health promotion, microbiological safety, protein texturization, and solubility (12).

In this study, we evaluated the hydrolysates of WPCs with protein concentrates 50% (WPC-50) and 60% (WPC-60) for their DH, solubility levels, levels of NPN, HMF content, and concentration of FSH as parameters that enhance their applications in the beverage industry. In addition, we investigated the changes that occur in the functional properties of WPHs with different protein concentrates (50 and 60% protein) with different enzymes, towards the selection of most suitable WPH type for use in beverages such as nutritional drinks.

Materials and Methods

Preparation of WPC-50 and WPC-60 Fresh mozzarella cheese whey made by bovine milk was pasteurized at 70°C for 5 min and processed using a spiral-wound-type ultrafiltration (UF) unit (Danish Separation Systems [DSS] LabUnit M20; Alfa Laval Nakskov A/S, Nakskov, Denmark) using flat-sheet GR-60-PP UF membranes (DSS AS, Nakskov, Denmark) with an effective surface area of 0.036 m^2. The UF was carried out with inlet and outlet pressures of 6 and 2 bar, respectively. The molecular weight cut-off range of the membrane was 20 kDa. UF was continued until the retentate attained protein concentrations of 50 and 60% on a dry matter basis followed by the reduction of volume in to 95-97.5% (13). Volume reduction was determined by using the following formula.

$$VR = \frac{VP}{VO} \times 100$$

VR=the percentage volume reduction, *VP*=the volume of permeate removed (mL), and *VO*=original volume of the whey.

The retentate was spray-dried using a B-191 Mini spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) with inlet and outlet temperatures of 175 and 75°C, respectively. Finally, proteins levels were adjusted at 50 and 60% by mixing the resulted WPCs powder in several trials. The protein contents of each trial were analyzed using *Kjeldahl* method (14).

Enzymatic hydrolysis of WPC-50 and WPC-60 Sixty types of hydrolysates were prepared by varying the enzyme type, protein composition, and hydrolysis time using the enzymatic hydrolysis method (5). Both fungal and bacterial proteases were used for hydrolysis which use in food industry. Fungal proteases, protease A '2G' (EC 3.4.24.39; Amano Enzyme Inc., Nagoya, Japan), protease M 'G' (Amano Enzyme Inc.), and flavourzyme (EC 3.4.11.1; Novozymes, Copenhagen, Denmark) were derived from *Aspergillus oryzae*. *Bacillus stearothermophilus* protease S '2G' (E.C. 3.4.24.28; Amano Enzyme Inc.) and porcine stomach mucosa trypsin (EC 3.4.21.4; Wako Pure Chemical Industries Ltd., Osaka, Japan) were also used. The enzyme to substrate used as 1:25 (wt/wt). The WPCs were hydrolyzed with each of the enzymes for 0.5, 1, 2, 3, 4, and 5 h.

Analysis of the physicochemical properties of WPHs Functional properties of WPHs that affect the quality of beverages were studied. pH values were measured using a pH meter (Seveneasy; Mettler-Toledo International, Inc., Columbus, OH, USA). DH was estimated using 2,4,6-trinitrobenzene sulfonic acid (Sigma-Aldrich, St. Louis, MO, USA), as described previously by Adler-Nissen (15). Solubility was determined using a spectrophotometer (OPTIZEN 2120UV; Mecasys, Daejeon, Korea) by measuring the absorbance at 280 nm (5). pH values were used 2, 4, 6, 8, and 10 to evaluate the solubility at each level of 10% solution of WPH-50 and WPH-60. NPN and HMF contents were measured according the methods described by Lowry *et al.* (16) and Keeney and Bassette (17), respectively. Samples were dissolved in distilled water to 10% and 10 mL were digested with 5 mL of Oxalic acid for 1 h at 100°C. After rapid cooling in ice, added 40% Trichloacetic acid 5 mL and filtered through Whatman filter paper No. 42. Thiobarbituric acid 1 mL mixed with the 4 mL of filtrate and incubated at 40°C for 30 min. Absorbance measured at 443 nm using the spectrophotometer. HMF contents were calculated according to the following equation.

$$\text{HMF}=(\text{Absorbance}-0.055)\times 87.5$$

FSH values were determined using the Ellman's assay (Sigma-Aldrich), as described by Riener *et al.* (18). FSH concentration was calculated using the following formula. The c value obtained was used in all further calculations of FSH concentration in 10% WPH solution.

$$c = \frac{A}{bE}$$

c=concentration in moles/L (M), A=absorbance, b=path length in centimeters (a 1 cm spectrometric cuvette was used in this study), E=14,150 M/cm (absorbance of 2-nitro-5-thiobenzoic acid at 412 nm in the buffer system).

Statistical analysis All experiments were repeated at least three times and the mean values were estimated by the one-way analysis of variance method. The experimental results were analyzed using the Statistical Analysis System software. Duncan's multiple range test was used to determine the differences between the mean values obtained for individual experiments.

Results and Discussion

Degree of hydrolysis of WPHs DH can be used as an indicator for the comparison of different proteolytic processes (19,20). WPCs hydrolyzed with proteases S and M showed significantly higher DH levels than those treated with other enzymes (Fig. 1). However, hydrolysis with protease M did not result in an increase in the DH level with time. The DH values of WPC-60 treated with protease S (2-5 h, 26.4-28.77%), and trypsin (2-5 h, 24.4-25.24%) did not show any obvious increments after 2 h of hydrolysis. Except for protease A-treated WPC-50, all the other hydrolysates showed only a slight increase in their DH values. However, in both concentrates, protease A hydrolysates showed a sharp increase in DH value as the hydrolysis time increased. However, because the DH values continued to increase with hydrolysis time within the experimental range tested, the maximum achievable DH value for protease A hydrolysates could not be predicted. These results suggested that protease A may be used to achieve high DH values. On the other hand, protease S and protease M are more suitable for the hydrolysis of WPCs that hydrolyzed in 2 h.

Non-protein nitrogen contents of WPHs NPN is responsible for the bitter taste of WPHs, which is one of the major reasons that protein hydrolysates are used sparingly in the food-processing industry. A graph depicting the effect of enzymatic hydrolysis on the NPN content of WPHs is shown in Fig. 2. Protease A-treated WPH-50 had the highest NPN level (1.31-1.674). Treatment with protease S also resulted in a comparatively high NPN level (1.38-1.56). In spite of their high NPN levels, these protein hydrolysates are reported to be less bitter to taste, attributable to their treatment with a neutral protease of microbial origin (21). Protease S-treated WPH-60 had a higher NPN content (1.42-1.81) than that treated with protease A (1.19-1.79). NPN content of hydrolysates not only depends on the hydrolyzing enzyme, but also on the protein concentration in the WPCs. For both WPC-50 and WPC-60, treatment with protease M resulted in the lowest NPN levels (WPH-50: 0.5-5 h, 1.11-1.25; WPH-60: 0.5-5 h, 1.24-1.48). This trend was also observed for protease M-treated WPH-35 in our earlier studies (5). Irrespective of the enzyme, hydrolysis of WPCs for more than 4 h led to accelerated rates of NPN production.

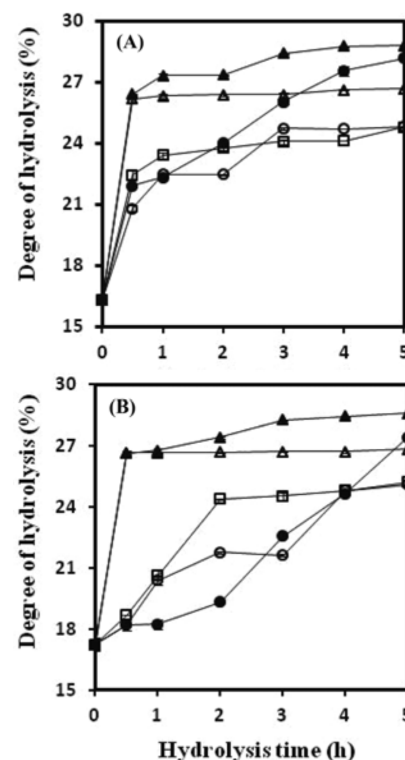

Fig. 1. The effect of enzymatic hydrolysis on the degree of hydrolysis of WPHs. (a) WPHs with 50% protein and (b) WPHs with 60% protein. ○, flavourzyme; ●, protease A; △, protease M; ▲, protease S; □, trypsin.

Composition of HMF (5-hydroxymethyl-2-furfural) of WPHs HMF is an intermediate product of caramelization in the Maillard reaction and the degradation of hexoses (22,23). Enzyme-modified spray-dried WPC samples are reported to have high HMF contents (19). Our studies suggested that WPCs with high protein concentrations had reduced levels of HMF (WPH-50: 51.47 μmol/L; WPH-60: 39.72 μmol/L). This is expected because the lactose content reduces as the protein concentration increases. For all samples tested in this study, HMF content increased

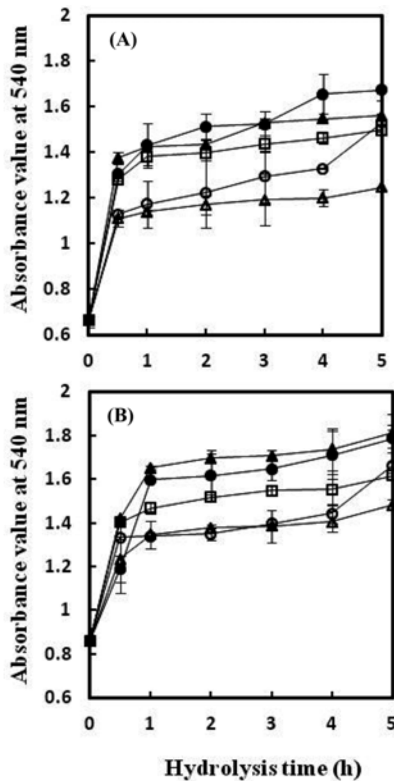

Fig. 2. The effect of enzymatic hydrolysis on the non-protein nitrogen content of WPHs. (a) WPHs with 50% protein and (b) WPHs with 60% protein. ○, flavourzyme; ●, protease A; △, protease M; ▲, protease S; □, trypsin.

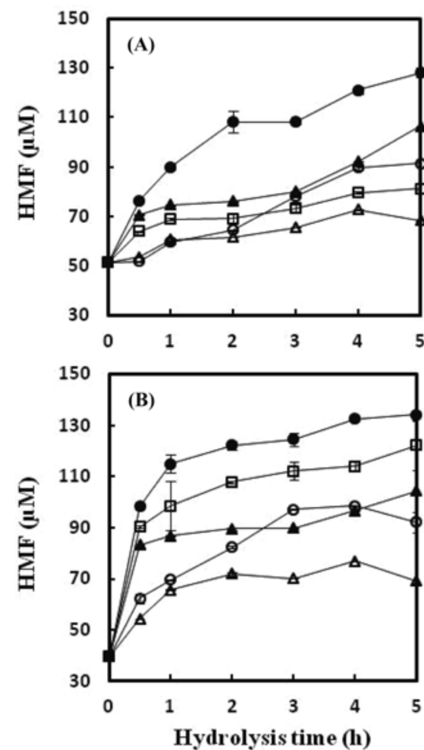

Fig. 3. The effect of enzymatic hydrolysis on the HMF content of WPHs. (a) WPHs with 50% protein and (b) WPHs with 60% protein. ○, flavourzyme; ●, protease A; △, protease M; ▲, protease S; □, trypsin.

with hydrolysis time (Fig. 3), this may be attributed to an increase in the amino acid concentration with hydrolysis time. Moreover, samples treated with protease A had the highest HMF content while those treated with protease M had the lowest HMF content. Treatment of both WPCs with flavourzyme and protease M for 2 h resulted in lower HMF levels. This suggested that hydrolysates of flavourzyme or protease M are more suitable for use as additives in fruit juices or nutritional beverages, which require weakly hydrolyzed WPHs.

Solubility of WPHs at different pH levels The solubility of WPHs was found to be higher than that of untreated WPCs, which may be attributed to an increase in the number of ionizing groups (NH_4^+, COO^-) and hydrophilicity with enzymatic hydrolysis. We were analyzed the solubility levels of WPCs and their hydrolysates in different pH as 2, 4, 6, 8, and 10 pH levels. Table 1 presents a comparison of the solubility levels of WPC-50 and its hydrolysates, WPH-50, obtained after hydrolysis with flavourzyme, protease A, protease M, protease S, and trypsin. Untreated WPC-50 had solubility between 33.65% (at pH 4) and 66.61% (at pH 10) while untreated WPC-60 had solubility between 22.85% (at pH 4) and 62.45% (at pH 10). Our analysis showed that WPCs, and their hydrolysates were least soluble at pH 4. Solubility in the pI range decreased as the protein concentration of the hydrolysates increased. Hydrolysates of WPC-60 showed a higher solubility than those of WPC-50. WPHs from both concentrates showed highest solubility in slightly acidic and alkaline media (≥pH 6), since microbial proteases are reported to be optimally active in neutral and alkaline conditions (24).

Of all the enzyme hydrolysates, those obtained upon treatment with proteases A and S (both active at neutral pH) had the highest solubilities. Although fungal proteases are active in a wide range of pH (14), flavourzyme hydrolysates did not have high solubility. Protease M is an acidic enzyme with optimum activity at pH 4.5-5. WPHs treated with protease M also had a high solubility at pH 4 (65.34-100%), albeit slightly lower than that of WPHs treated with protease A. Therefore, protease A may be most suited for use in fruit beverages such as orange juice and yoghurt, which have a pH range of 3.5-4 (25). Protease A is a metalloendopeptidase that uses a metal ion (often Zn^{2+}) in its catalytic mechanism (21). Therefore, protease A may be most suitable for the hydrolysis of WPHs due to high mineral content in their media. WPH-60 treated with protease A, and protease M for ≥2 h became completely soluble in media with pH between 6 and 10.

Table 1. Solubility levels (%) of whey protein hydrolysates with 50% protein content in different pH values

Enzyme	pH	Hydrolysis time (h)						
		0	0.5	1	2	3	4	5
Flavourzme	2	60.73 (0.91)$^{a1)}$	80.01 (0.47)^{a}	83.11 (1)^{a}	84.6 (1.02)^{a}	87.88 (2.55)^{a}	88.09 (1.13)^{a}	89.58 (1.06)^{a}
	4	33.65 (0.98)^{a}	46.94 (0.37)^{ab}	48.05 (1.61)^{a}	49.64 (0.86)^{ab}	50.99 (0.47)^{ab}	56.77 (1.46)^{a}	62.47 (0.48)^{b}
	6	61.36 (0.66)^{a}	96.87 (0.51)^{ab}	97.44 (1.5)^{ab}	97.43 (7.45)^{b}	98.96 (1.2)^{ab}	100 (0.55)^{a}	100 (0.35)^{b}
	8	63.54 (0.81)^{a}	89.68 (1.56)^{ab}	90.66 (2.02)^{b}	91.45 (2.86)^{ab}	93.44 (0.87)^{ab}	95.78 (0.55)^{a}	96.88 (0.85)^{bc}
	10	66.61 (0.35)^{a}	78.99 (1.64)^{b}	82.71 (1.74)^{b}	86.94 (2.83)^{ab}	88.65 (2.56)^{b}	91.95 (0.58)^{a}	94.77 (0.68)^{c}
Trypsin	2	60.73 (2.84)^{a}	82.03 (1.1)^{a}	83.25 (0.92)^{a}	83.37 (1.56)^{a}	86.1 (2.6)^{a}	90.03 (1.09)^{a}	91.03 (1.3)^{a}
	4	33.65 (2.81)^{a}	49.57 (0.48)^{a}	50.61 (0.45)^{a}	51.68 (1.29)^{a}	51.83 (2.58)^{a}	61 (0.21)^{a}	61.62 (1.53)^{a}
	6	61.36 (1.99)^{a}	94.45 (0.7)^{a}	95.57 (1.6)^{a}	95.63 (0.27)^{ab}	99.31 (1.17)^{a}	100 (0.63)^{a}	100 (1.26)^{a}
	8	63.54 (1.48)^{a}	90.16 (0.63)^{a}	91.48 (1.88)^{a}	92.11 (1.92)^{ab}	92.28 (1.16)^{a}	96.19 (0.76)^{a}	97.09 (1.02)^{a}
	10	66.61 (2.22)^{a}	93.39 (1.85)^{a}	98.77 (1.64)^{a}	98.82 (0.62)^{b}	99.71 (1.08)^{a}	99.92 (0.68)^{a}	100 (1.48)^{a}
Protease A	2	60.73 (1.69)^{a}	73.38 (0.91)^{a}	73.57 (0.31)^{a}	77.58 (0.06)^{a}	99.64 (0.28)^{a}	100 (0.03)^{a}	100 (0.1)^{a}
	4	33.65 (0.99)^{ab}	53.83 (1.7)^{a}	65.72 (1.74)^{a}	68.13 (0.03)^{a}	99.52 (0.13)^{a}	100 (0.04)^{a}	100 (0.21)^{a}
	6	61.36 (0.51)^{ab}	98.98 (2.67)^{a}	98.49 (2.11)^{a}	100 (0.76)^{ab}	100 (0.04)^{a}	100 (0.3)^{a}	100 (0.06)^{a}
	8	63.54 (0.51)^{b}	89.38 (3.12)^{a}	90.88 (1.54)^{a}	97.3 (0.45)^{ab}	100 (0.01)^{a}	100 (0.27)^{a}	100 (0.14)^{a}
	10	66.61 (0.04)^{b}	91.44 (2.92)^{a}	93.59 (1.97)^{a}	99.53 (1.37)^{b}	100 (0.49)^{a}	100 (0.02)^{a}	100 (0.16)^{a}
Protease M	2	60.73 (0.07)^{a}	64.28 (0.03)^{a}	65.82 (0.06)^{a}	68.29 (0.08)^{a}	73.21 (0.1)^{a}	73.48 (0.14)^{a}	77.63 (0.11)^{a}
	4	33.65 (0.03)^{a}	65.34 (0.01)^{a}	79.77 (0.06)^{a}	96.02 (0.01)^{a}	98.87 (0.18)^{ab}	98.87 (0.08)^{ab}	100 (0.2)^{a}
	6	61.36 (0.04)^{a}	86.04 (0.08)^{a}	77.23 (0.05)^{a}	79.01 (0.12)^{b}	82 (0.16)^{ab}	87.62 (0.03)^{ab}	92.63 (0.2)^{a}
	8	63.54 (0.14)^{b}	75.97 (0.08)^{b}	76.24 (0.08)^{b}	80.02 (0.06)^{b}	84.88 (0.07)^{b}	84.98 (0.17)^{ab}	89.53 (0.04)^{a}
	10	66.61 (0.08)^{b}	63.71 (0.18)^{b}	71.59 (0.13)^{b}	76.09 (0.08)^{b}	76.8 (0.04)^{b}	82.02 (0.49)^{b}	85.45 (0.03)^{a}
Protease S	2	60.73 (0.02)^{a}	68.94 (0.01)^{a}	71.1 (0.01)^{a}	74.64 (0.03)^{a}	88.64 (0.03)^{a}	91.33 (0.08)^{a}	95.51 (0.07)^{a}
	4	33.65 (0.03)^{a}	43.47 (0.01)^{a}	51.33 (0.01)^{ab}	55.41 (0.01)^{a}	67.72 (0.03)^{a}	75.69 (0.07)^{ab}	86.44 (0.06)^{a}
	6	61.36 (0.04)^{b}	89.42 (0.01)^{a}	89.84 (0.02)^{bc}	92.15 (0.02)^{ab}	94.55 (0.02)^{a}	99.41 (0.01)^{abc}	100 (0.02)^{a}
	8	63.54 (0.02)^{bc}	78.99 (0.07)^{ba}	82.71 (0.01)^{bc}	86.94 (0.06)^{ab}	88.65 (0.02)^{a}	91.95 (0.06)^{cb}	94.77 (0.05)^{a}
	10	66.61 (0.03)^{c}	89.61 (0.14)^{b}	92.42 (0.01)^{c}	93.78 (0.07)^{b}	98.84 (0.02)^{a}	98.97 (0.06)^{c}	99.01 (0.02)^{a}

[1)]Mean values (SD). Averages that do not differ at 0.05 significance are indicated by the same letter within a raw. 0.05 of probability.

FSH Composition of WPHs β-Lactoglobulin, the major globular protein present in WP, has one free FSH group and two disulfide groups, located in the interior of the protein. Even low concentrations of FSH can significantly influence beverage properties. The FSH content of WPHs with different protein concentrations are shown in Fig 4. Although the FSH content is relatively low, its presence is considered highly functional. Irrespective of the hydrolytic enzyme and the protein concentration, just 30 min of hydrolysis resulted in a significant decrease in the FSH content. This can be cause by the sulfhydryl-disulfide interchange of proteins and increase S-S bonds by exposing the thiol groups after the enzymatic hydrolysis. Untreated WPC-50 and WPC-60 had FSH levels of 25.4 and 25.6 mM, respectively. Hydrolysis time did not appear to have any correlation with the FSH concentration of WPHs. Hydrolysis with flavourzyme resulted in lower concentration of FSH contents compared to the other enzymes. Hydrolysates treated with protease A for >3 h had increased FSH concentrations. This suggested that treatment of WPC with protease A reduces S-S bonds, leading to increased numbers of S-H bonds. Previous studies have reported that metalloendopeptidases, such as protease A, are not inhibited by -SH groups (26). Enzymatic hydrolysis can sometimes unmask certain concealed peptide sequences as the hydrolysis proceeds.

In conclusion, the characteristics of the hydrolysates obtained varied according to the specificity of the proteolytic enzyme, hydrolysis time, and protein composition. Therefore, these characteristics are important factors that need to be considered before the fortification of WPH into beverages. Maintain fresh taste and to improve the keeping quality, Maillard reaction should be delayed. It can be controlled by careful enzyme selections like protease S and M to hydrolyze WPCs. WPHs treated with protease M were less bitter, because the produced NPN was low compared to the other enzymes. WPHs treated with protease A were suitable for beverage applications that required extensively hydrolyzed (>2 h) WPHs which requires more nutritional benefits than the fresh taste.

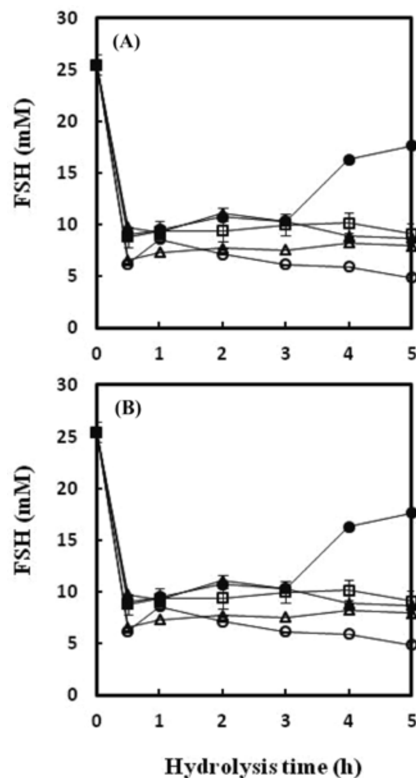

Fig. 4. The effect of enzymatic hydrolysis on the FSH levels of WPHs. (a) WPHs with 50% protein and (b) WPHs with 60% protein. ○, flavourzyme; ●, protease A; △, protease M; ▲, protease S; □, trypsin.

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