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Preparation of Yeast Hydrolysate Enriched in Cyclo-His-Pro (CHP) by Enzymatic Hydrolysis and Evaluation of Its Functionality

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ABSTRACT: In this study, we attempted to enrich cyclo-His-Pro (CHP) using enzymatic hydrolysis of yeast and to evaluate the functionality of yeast hydrolysate (YH)-enriched CHP. Flavourzyme offered a better performance in enhancing CHP content than other proteases. The CHP enrichment conditions were optimized as follows: addition of 1% Flavourzyme, 48-h incubation at 60°C, and pH 6.0. The CHP content significantly increased by 20-fold after ultra-filtration (UF). Maximal CHP translation was obtained after heating for 8 h at 50°C and pH 7.0. YH showed poor foaming capacity between pH 3.0 to 9.0. The emulsifying activities of YHs were slightly higher at near acidic pH. Increase in heating temperature and time resulted in decreased CHP content. The results indicate that YH is more heat stable after UF. Therefore, the CHP in YH after UF can be used as a food additive with physiological CHP activity and high heat stability.

Keywords: cyclo-His-Pro (CHP), flavourzyme, ultrafiltration, functionality, yeast hydrolysate

INTRODUCTION

In recent years, the role of dietary proteins as physiologically active components has been increasingly acknowledged. Such proteins or their precursors may occur naturally in raw food materials and exert their physiological action directly or upon enzymatic hydrolysis *in vitro* or *in vivo*.

Recently, several studies demonstrated that yeast hydrolysate (YH) displayed physiological effects such as anti-obesity, anti-stress, and immuno-potentiating activities (1). For these reasons, YH is receiving remarkable attention as a functional material in the diet food market. The continuing development of functional foods is likely to entail increased use of different protein sources known to contain bioactive components.

Cyclo-His-Pro (CHP) is a naturally occurring cyclic dipeptide consisting of histidyl and proline and is a metabolite of thyrotropin-releasing hormone (TRH). It is also synthesized through other biochemical processes and is found in many protein-rich, processed foods or peptide sources (2,3). Since plasma levels of CHP in humans are increased after glucose intake, CHP activity may be associated with glycemic control in diabetes (4-6). Furthermore, several studies have demonstrated that CHP decreases food intake, thus mimicking the action of leptin, which controls appetite (7,8). Furthermore, no evidence of toxicity or side effects associated with CHP oral administration has been reported (6,9).

Various products can be converted to value-added products by enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of proteins. Bioactive peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion. The possible regulatory effects of peptides are related to nutrient uptake, immune defense (10), opioid (11), antioxidant (12), and antihypertensive activities (13).

Furthermore, controlled enzymatic hydrolysis of proteins may modify and even improve the functional properties of proteins. Since enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as the polar and ionizable groups of protein hydrolysates, it directly affects the functional properties and their use as food ingredients (14). Thus, protein hydrolysates with bioactive properties and acceptable functional properties could be utilized in formulated food systems to provide high nutritional value, an attractive appearance, and a smooth mouthfeel.

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The objective of the present study was to investigate the optimum conditions to enrich CHP in YH and to evaluate its functional properties as a theoretical basis for the application of YH as functional food material.

MATERIALS AND METHODS

Materials

CHP purchased from Sigma Chemical Co. (St. Louis, MO, USA) was used as a reference material for analysis of CHP in the following commercial hydrolysate. Yeast was purchased from Jenico Co., Ltd. (Seoul, Korea), and YH was obtained from Neo Cremar Co., Ltd. (Sungnam, Korea). All hydrolysates were centrifuged at 3,000 *g*, and the supernatants were dried for further studies. For enzyme hydrolysis, Neutrase, Alcalase, Flavourzyme, and Protamax were obtained from Novo Korea (Seoul, Korea), and Ficin was obtained from Sigma Chemical Co. High-performance liquid chromatography (HPLC) grade acetonitrile was purchased from Burdick and Jackson (Muskegan, MI, USA). All other reagents were of analytical grade.

Enzymatic hydrolysis

Enzymatic hydrolysis of yeast for enrichment of CHP was carried out as detailed below. Selection of enzyme type study; an 8% yeast suspension was hydrolyzed for 48 h using 5 enzymes: Neutrase (endo-protease from *Bacillus amyloliquefaciens*), Alcalase (endo-protease from *B. licheniformis*), Protamax (*Bacillus* protease complex), Flavourzyme (endo- and exo-protease from *Aspergillus oryzae*), and Ficin (endo-protease from fig tree latex). The main characteristics of each enzyme are summarized in Table 1. The hydrolysis temperature was maintained at 50～60°C for crude enzymes and the enzyme/yeast substrate ratio was 0.5～1.5/100. Hydrolysis was performed in 0.01 mol/L phosphate buffer and pH was adjusted to the specific optimal values for each of the proteases (pH 8.0 for Alcalase, pH 7.0 for Neutrase, Protamex, Flavourzyme, and Ficin) before hydrolysis was initiated. Enzyme inactivation to stop the hydrolysis was done by heating at 90°C for 5 min.

Optimization of processing parameters for production of yeast hydrolysate

An 8% yeast suspension was hydrolyzed with 0.5～1.5% Flavourzyme for 48 h. The reaction was stopped by incubating the mixtures in a boiling water bath for 10 min. The hydrolysate was then centrifuged at 2,800 *g* for 10 min and the supernatant was used for analysis of CHP.

Next, the effect of incubation time on the CHP content and yield was examined by hydrolyzing the yeast suspension with 1% Flavourzyme for 8 h. To obtain the optimum pH condition for CHP content and yield, protease solutions in a range of pH values from 5.0 to 7.0 were used, with the protease concentration at 1% and incubation time at 48 h.

Fractionation of YH by ultra-filtration

The YH obtained by enzymatic hydrolysis was centrifuged and the supernatants were passed through a 0.2 μm cutoff membrane filter (Satocon cassette, Sartorius, Germany). Next, a part of the solution was removed and the filtrate was pumped through 30 kDa and 10 kDa molecular weight cut off membranes (Satocon cassette).

Transformation of YH by heat treatment

The YH fraction with molecular weight below 10 kDa was transformed by heat treatment in 0.01 mol/L phosphate buffer for 2～12 h. The pH of the solution was adjusted to 5.0～8.0 values before hydrolysis was initiated. The mixture was incubated at 40～60°C in an incubator for 2～8 h.

Analysis of CHP contents

The hydrolysate was centrifuged at 10,000 *g* for 20 min at 5°C and the supernatant was filtered through glass-wool. The supernatant (300 μL) was then deproteinised by adding 3 volumes (900 μL) of methanol (HPLC grade) and allowing the mixture to stand at 4°C for 15 min. The sample was centrifuged (12,000 *g*) for 3 min, and the supernatants were filtered through a 0.22 μm membrane filter. The CHP content in each hydrolysate was analyzed by HPLC (Varian 230, Varian Inc., Palo Alto, CA, USA) using a Hamilton PRP-1 RP 10 μm column (250 mm×4.1 mm i.d., Hamilton Company, Reno, NV,

Table 1. Characteristics of enzymes used for the hydrolysis of yeast extract rich in cyclo (His-Pro)

Enzyme	Main activity	Source	Optimum conditions	
			Temperature (°C)	pH
Alcalase	Protease	*Bacillus* spp.	50	8.3
Ficin	Protease	Latex of *Ficus carica* or *Ficus glabrata*	45～55	6.0
Flavourzyme	Endo-protease, exo-protease	*Aspergillus oryzae*	50	5.0～7.0
Neutrase	Protease	*Bacillus amyloliquefaciens*	45～55	5.5～7.5
Protamax	Endo-protease	*Bacillus* spp.	35～60	5.5～7.5

Information supplied by product sheets from manufacturer. Selected optima for experimental runs are shown in parentheses.

USA) and a Hamilton PRP 10 μm pre-column (25 mm× 1.3 mm i.d., Hamilton Company). The mobile phase was a mixture of acetonitrile and 0.75 g/L 1-heptanesulfonic acid in 0.004 mol/L aqueous trifluoroacetic acid (10:90, v/v), adjusted to pH 2.4 with NaOH before use. The flow rate was 0.5 mL/min. The sample (20 μL) was injected and monitored spectrophotometrically at 220 nm (15).

Emulsifying properties

The emulsifying activity index (EAI) at various pH values was measured by the turbidometric method described previously (16). To form an emulsion, 1% (w/v) of the sample was dissolved in 50 mM citrate-NaOH buffer (pH 3.6), phosphate buffer (pH 7.6), and Tris-HCl buffer (pH 9.0). Twelve milliliters of the dissolved sample and 4 mL corn oil were homogenized in a blender (T25B, IKA® Werke GmbH & Co. KG, Staufen, Germany) at 12,000 rpm for 1 min. A 50-μL aliquot of the emulsion was taken from the bottom container at different time intervals and diluted in 5 mL of 0.1% sodium dodecyl sulfate. The absorbance of the diluted emulsion was measured at 500 nm. All experiments were conducted at room temperature. EAI was calculated according to the following equation:

$$\text{EAI } (m^2/g) = \frac{(2T \times D)}{(\varphi \times C \times 10^4)} = \frac{(4.606 \times A \times D)}{(\varphi \times C \times L \times 10^4)}$$

where T is turbidity, D is the dilution factor, φ is the volume fraction of the dispersed phase (oil), C is protein weight per volume of aqueous phase before the emulsion, A is the observed absorbance, and L is the path length of the cuvette.

Foaming properties

Foaming capacity and stability of the samples were measured according to a method described previously (17). A 1% (w/v) sample was dissolved in 50 mM citrate-NaOH buffer (pH 3.6), phosphate buffer (pH 7.6), and Tris-HCl buffer (pH 9.0) and then agitated for 2 min at 10,000 rpm in a blender (T25B, IKA® Werke GmbH & Co. KG). The whipped sample was immediately transferred into a graduated cylinder and the total volume was measured after 30 s. The foaming capacity was calculated according to the following equation:

$$\text{Foaming capacity } (\%) = \frac{\text{Vol. after whipping} - \text{Vol. before whipping (mL)}}{\text{Vol. before whipping (mL)}} \times 100$$

The whipped sample was then allowed to stand at 20°C for 3 min, and then the volume of whipped sample was recorded. Foam stability was calculated as follows:

$$\text{Foaming stability } (\%) = \frac{\text{Vol. after standing} - \text{Vol. before whipping (mL)}}{\text{Vol. before whipping (mL)}} \times 100$$

Heat stability of CHP in YH

For heat stability studies, 1% yeast hydrolysate solutions were prepared in 50 mM phosphate buffer (pH 7.6). YH (2 mL) were placed in glass tubes (10 mm i.d.×120 mm; AGB Scientific Ltd., Dublin, Ireland), sealed with silicone bungs, and immersed in a thermostatically controlled oil bath at 140°C and 160°C for 4 min. After heat treatment, thermal stability was determined by measuring the CHP content.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., Chicago, IL, USA). The statistical significance was assessed by a two-way analysis of variance (ANOVA). When significant effects were found, a one-way ANOVA was used to determine the differences between the groups involved. All data are presented as the mean± standard deviation (SD). Results were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Selection of enzyme

The enzymatic hydrolysis of proteins is a very efficient, specific reaction and can be carried out under mild conditions so that the nutritional quality of the amino acids is maintained (18). For example, alkaline hydrolysis of protein not only destroyed some essential amino acids (tryptophan, cysteine, or serine), but also resulted in change of amino acid structure from L-form into D-form which is not consumable by humans (19).

Enzymatic hydrolysis for the enrichment of CHP was performed with various commercial proteases under optimal conditions. The CHP content in YH was 130.4± 8.7 μg/g. As shown in Table 2, the extraction yields of CHP increased notably upon treatment with various proteases. The proteases hydrolyzed the proteins and peptides in YH, released CHP from the proteins, and increased the extraction efficiency. Flavourzyme-treated hydrolysate contained the highest CHP content (674.8± 22.1 μg/g) of all the tested proteases. Since a high CHP content in the hydrolysate was necessary for its use as functional material, Flavourzyme was selected for optimizing the extraction process in the following tests. The possible reason for the high efficiency of Flavourzyme may be that it is a protease mixture containing both endo- and exo-protease activities. In food protein hydrolysis, endo-protease applications are dominant because

they can produce smaller peptides. Occasionally, the endo-proteases are combined with exo-proteases for complete degradation (18). In this study, the yeast cells treated with Flavourzyme, which has both activities (endo- and exo-protease), generated the highest level of CHP. The oral intake of protein hydrolysates is, however, limited by their often very bitter taste, so they can only be added to food products at relatively low concentrations without producing an unacceptable flavor. Flavourzyme was evaluated as an effective enzyme for the production of CHP with slightly bitterness from the present study (data not shown). Therefore, Flavourzyme was selected as a suitable enzyme for the production of CHP.

Table 2. Effect of different enzyme preparations on extraction yields of CHP

Hydrolysate	Yield (%)	CHP (μg/g)
Control	12.0±0.3^{e}	130.4±8.7^{e}
Alcalase	21.2±1.4^{c}	286.0±13.4^{d}
Ficin	33.9±1.0^{a}	468.1±15.4^{bc}
Flavourzyme	16.4±1.1^{d}	674.8±22.1^{a}
Neutrase	21.0±2.2^{c}	502.2±30.1^{b}
Protamax	28.4±1.2^{b}	430.2±28.3^{c}

Mean±SD.
Control: non-enzymatic treatment.
Values with different letters (a-e) within the same column are significantly different at $P<0.05$ by Tukey's multiple range test.

Optimization of YH enriched CHP

The effect of using different protease concentrations on CHP extraction at an incubation time of 48 h is shown in Fig. 1A. An increase in protease concentration up to 1% resulted in corresponding increase in the extraction contents of CHP and yields. Concentration of 1% produced almost the same extraction content of CHP and yield as a concentration of 1.5%, indicating that 1.0% protease concentration was sufficient for optimal hydrolysis of YH. Thus, 1% was selected as the suitable protease concentration for use.

Next, the effect of incubation time on the extraction content of CHP and yield was examined. An increase in the extraction contents of CHP and yields was observed with time (Fig. 1B). During the initial 48 h, the extraction of CHP was considerably enhanced. After further 18 h incubation, the extraction contents of CHP and yields were almost maintained at a steady level indicating that an incubation of 48 h is long enough for the protease to degrade the YH.

Enzyme activities are easily influenced by pH. Keeping

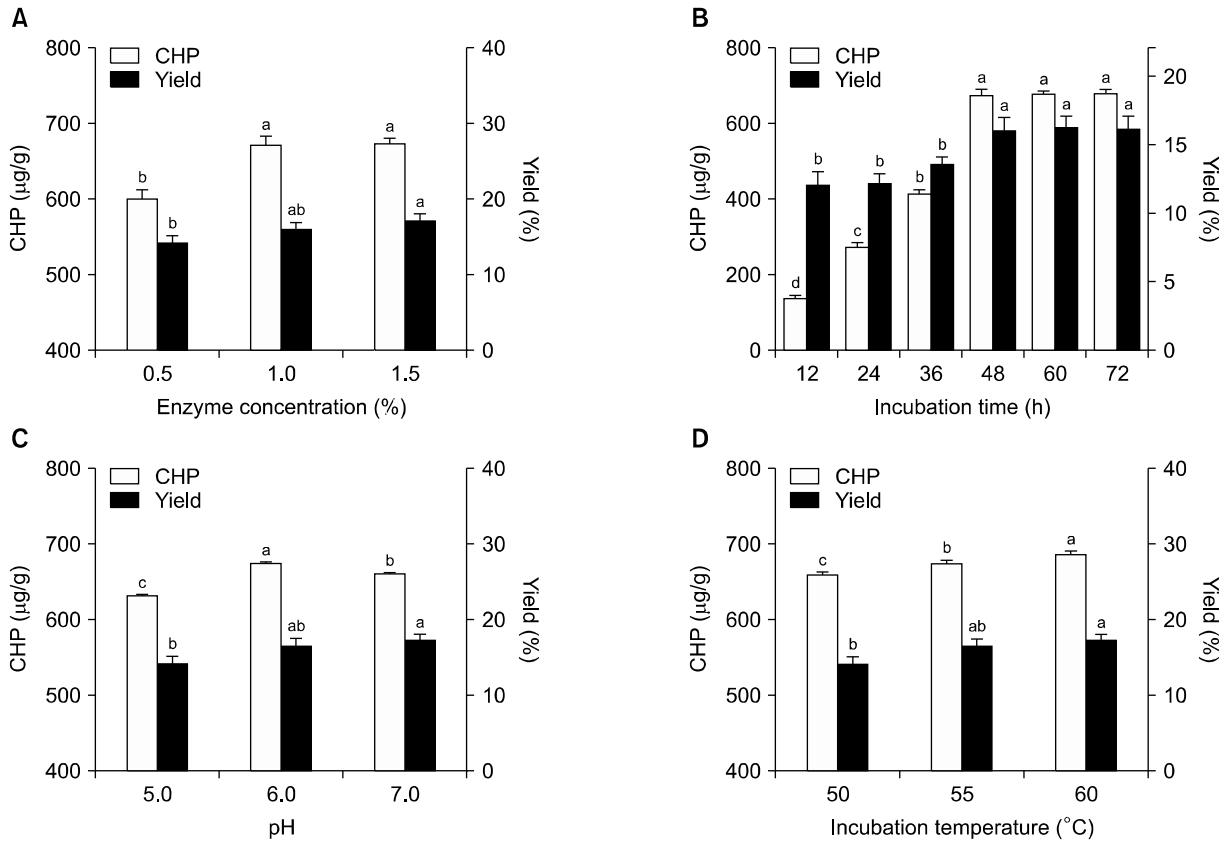

Fig. 1. Optimization of yeast hydrolysis by Flavourzyme for enrichment of CHP. (A) optimization of protease concentrations, (B) optimization of incubation time, (C) optimization of pH, (D) optimization of incubation temperature. For optimization of the hydrolysis conditions, CHP content and yield were measured in various conditions. Values with different letters (a-d) are significantly different at $P<0.05$ by one-way ANOVA with Tukey's multiple range test.

the protease concentration at 1% and incubation time at 48 h, we varied pH values of the protease solution in a range from 5.0 to 7.0 to investigate its effect on extraction contents of CHP and yields (Fig. 1C). The highest amount of CHP (673.4±2.1 μg/g) was obtained at pH 6.0. Thus, pH 6.0 was chosen as the optimum pH for obtaining optimal extraction contents of CHP and yields. CHP is as a metabolite of the hypothalamic TRH and is produced as a result of pyroglutamyl peptidase activity (20) and subsequent cyclization of the dipeptide His-Pro-NH_2 at 37°C via a non-enzymatic process, which shows maximal velocity at pH 6.0 to 7.0 (21). A variety of cyclic dipeptides have also been shown to exist in protein and polypeptide hydrolysates, as well as in cultures of yeast, lichens, and fungi. The high preponderance of cyclic dipeptides in fermentation broths and cultures appears to be consistent with many recent findings. These include very high levels of CHP in nutritional supplements derived from casein, a milk protein rich in His-Pro or Pro-His dipeptide sequences in its primary structure, and those undergoing enzymatic hydrolysis and thermal manipulations during the manufacturing process (2). CHP appears from a non-enzymatic process with a maximal velocity (tr2=140 min) at pH 6.0 to 7.0 and 37°C (22). Therefore, CHP could be derived via enzymatic hydrolysis of food protein from the metabolism of dietary proteins like yeast.

Likewise, temperature also affects enzyme activity. Reaction rates of enzymes are accelerated with increase in temperature up to an optimum, above which the enzymes are denatured. The effect of incubation temperature on the extraction contents of CHP and yields was studied with the other conditions set as mentioned above. As can be seen from Fig. 1D, the highest extraction contents of CHP and yields were obtained at 60°C. Therefore, 60°C was considered as the optimum incubation temperature.

Ultra-filtration and heat treatment for increased CHP content

Ultra-filtration has been used for the separation of a wide range of compounds (23,24). Ultra-filtration processes can also be fine-tuned to achieve high productivity and product purity at the same time (25). Yeast proteins removed from cell debris are cut irregularly into polymers of 100 kDa molecular weight or more, or low molecular weights of 1 kDa or less. CHP is a di-peptide, so we cut-off the unwanted proteins to increase the purity of the hydrolysate. CHP content were significantly increased, by 20.0-folds, in the Flavourzyme-treated sample (670.3±13.3 μg/g) when compared to the ultra-filtration-treated sample (12.1±0.2 mg/g) (data not shown).

Membrane technology is an efficient and ecological process for the extraction (concentration, purification, and fractionation) of valuable molecules from wastes or by-products in the agro-food industry. Membrane fractionation has allowed the enhancement of biological or functional properties of milk proteins, and so the technology has been progressively applied to other substrates such as soy proteins (26), hemoglobin (27), and wheat gliadin (28). Indeed, in this study, ultra-filtration was used as a convenient and simple fractionation tool to increase CHP content.

An additional, heat treatment was given to increase CHP content. Previous reports show that some peptides such as isoalliin can be chemically cyclized by heat treatment (29,30). The effect of heat treatment on translated CHP content and yields was studied with the other conditions set as mentioned above. As can be seen from Fig. 2, the highest CHP content was obtained at 50°C. Therefore, 50°C was considered as the optimum temperature

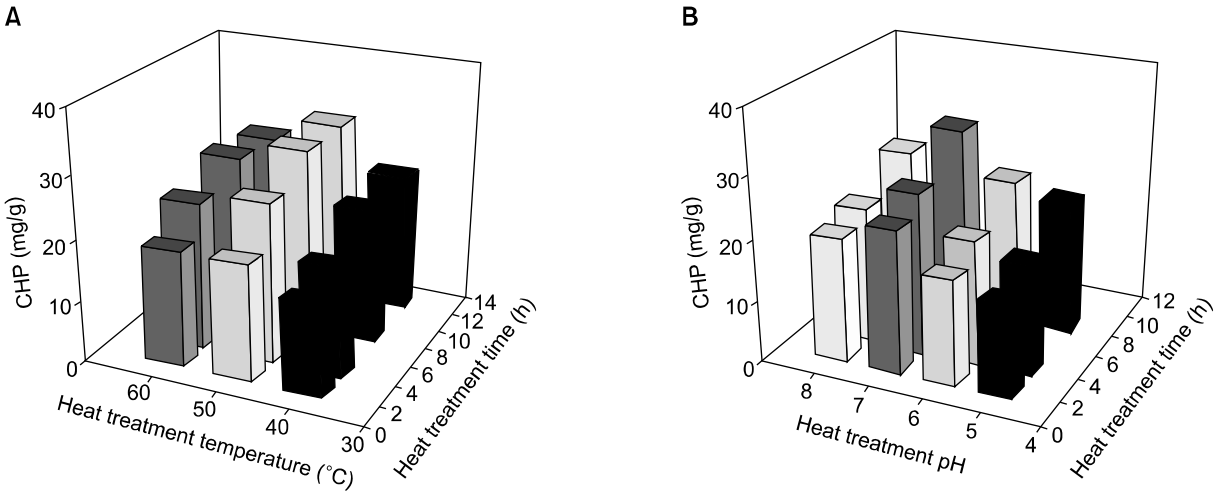

Fig. 2. Effect of heat treatment (A) temperature and (B) pH on the increase of CHP in Flavourzyme treated YH. Data were analyzed by two-way ANOVA; (A) temperature effect: $P<0.05$, time effect: $P<0.05$, There were no significant interaction (temperature and time). (B) pH effect: $P<0.05$, time effect: $P<0.05$, There were no significant interaction (pH and time).

for heat treatment. Keeping the heat treatment temperature constant at 50°C, we varied the pH values of the protease solution in a range from 5.0 to 8.0 to investigate its effect on translated contents of CHP and yields (Fig. 2). The highest amount of CHP was obtained at pH 7.0, which was chosen as the optimum pH for obtaining optimal CHP content. Next, the effect of heat treatment time on the translated CHP content was examined. An increase in the translated CHP content was observed with time. During the initial 8 h, the translation of CHP was considerably enhanced. After 8 h, the translated CHP content and yield were almost steady indicating that 8 h is sufficient for the protease to translate the YH into CHP.

Functional properties of YH before and after UF

Foaming properties of the YH before and after UF were measured in the pH range 3.0~9.0 (Table 3). YH showed poor foaming capacity from pH 3.0 to 9.0. In fact, YH after UF did not show much foaming capacity. Foaming capacity is strongly dependent on the ease with which a protein lowers the interfacial tension at the gas liquid interface. Proteins usually do this by unfolding and aligning themselves between the two phases. Coagulation of the unfolded proteins promotes foam stability by creating strong surface films at the solution-air interface (31). The decrease in foaming capacity and foaming stability by increasing the degree of hydrolysis to 42% is a consequence of structure loss (32). Therefore, YH showed poor foaming capacity because it contained smaller peptides after UF-treatment.

The emulsifying activities of YHs were slightly higher at near acidic pH, but decreased in neutral and alkaline conditions (Fig. 3). YH did not show any difference in emulsifying capacity before and after UF treatment.

Generally, the pH of protein solutions during emulsification affects their emulsifying properties by affecting charge (33). The mechanism of the emulsification process of hydrolysates is attributed to adsorption to the surface of freshly formed oil droplets during homogenization. This forms a protective membrane and prevents the droplets from coalescing. Hydrolysates are surface active materials and promote oil-in water emulsions because of their hydrophilic and hydrophobic groups (34). However, peptides with low molecular weights have low amphiphilicity, which is required for producing an emulsion. Although small peptides diffuse rapidly toward the interface, they are less efficient at stabilizing emulsions, because they cannot readily agglomerate to produce a fat-globule membrane due to charge repulsions (35).

Heat stability of CHP in YH before and after UF

To investigate the heat stability of CHP in YH, YH was treated at 130~150°C for 4 min. Fig. 4 shows the changes in CHP during heat treatment. Increase in heating temperature and time showed a corresponding decrease in CHP content. Especially, CHP contents were sharply decreased at 150°C in YH before UF treatment. Many other factors also influence the thermal behavior of a peptide including pH, ionic environment, protein concentration and hydrolysis (36). UF treatment might induce the charge distribution of YH and these differences in charge distribution might lead to differences in quaternary structure and protein stability.

In general, proteins are vulnerable to heat treatment, leading to aggregation and exposure of the hydrophobic domain (37). Nevertheless, peptides with smaller sizes are more stable to aggregation at high temperatures (38).

In the present study, Flavourzyme was found to be more efficient than the other enzymes for CHP enriched YH preparation. At optimized conditions, the extraction contents of CHP was 670.3±13.3 μg/g, which represents an increase of 515%, compared to untreated con-

Table 3. Foaming capacity and stability of Flavourzyme treated yeast hydrolysate-enriched CHP before and after ultra-filtration (UF)

pH	Foaming capacity (%)		Foaming stability (%)	
	Before	After	Before	After
3.6	12.5±2.5	0.0±0.0*	0.8±0.3	0.0±0.0*
7.6	10.8±1.4	0.0±0.0*	0.5±0.0	0.0±0.0*
9.0	12.5±2.5	0.0±0.0*	0.2±0.5	0.0±0.0

pH 3.6, citrate-NaOH buffer; pH 7.6, phosphate buffer; pH 9.0, Tris-HCl buffer.
Mean±SD.
Data were analyzed by two-way ANOVA; foaming capacity; pH effect: $P>0.05$, UF effect: $P<0.01$, pH and UF interaction: $P<0.05$. Foaming stability; pH effect: $P>0.05$, UF effect: $P<0.01$, pH and UF interaction: $P<0.05$.
*Indicates significant differences before vs. after UF by paired t-test, $P<0.05$.

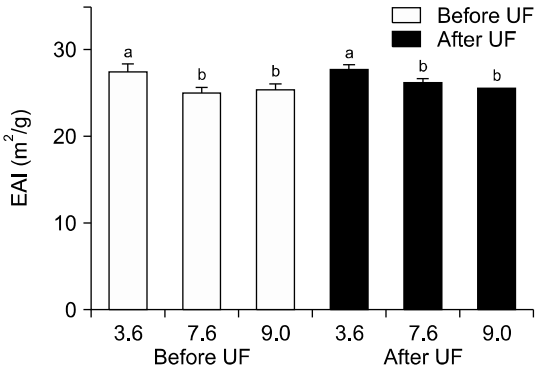

Fig. 3. Emulsifying capacity of Flavourzyme treated yeast hydrolysate before and after ultra-filtration (UF) treatment. Data were analyzed by two-way ANOVA; pH effect: $P<0.05$, UF treatment effect: $P>0.05$, There were no significant interaction (pH and UF treatment). Values with different letters (a,b) are significantly different at $P< 0.05$ by one-way ANOVA with Tukey's multiple range test.

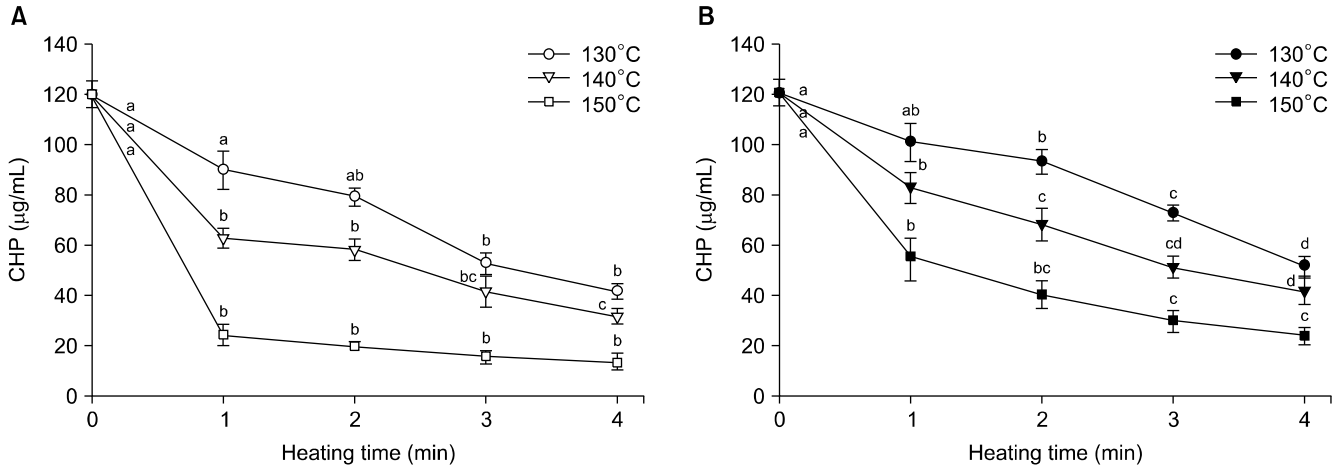

Fig. 4. Heat stability of Flavourzyme treated yeast hydrolysate before (A) and after (B) ultra-filtration treatment. Data were analyzed by two-way ANOVA; (A) temperature effect: $P<0.05$, time effect: $P<0.05$, temperature and time interaction: $P<0.05$. (B) temperature effect: $P<0.05$, time effect: $P<0.05$, temperature and time interaction: $P<0.05$. Values with different letters (a-d) are significantly different at $P<0.05$ by repeated measures ANOVA with Tukey's multiple range test.

trols. Subsequent UF treatment showed a 20-fold increase in CHP content. The emulsifying properties in YH after UF were superior to YH before UF, whereas the foaming properties were very poor in both YHs. YH with low MW after UF treatment showed superior heat stability to YH before UF. Therefore, the CHP in YH after UF can be incorporated in cooked food systems with heat stability and can be used as a food additive with physiological CHP activity.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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