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Physicochemical and Flavor Characteristics of Flavoring Agent from Mungbean Protein Hydrolyzed by Bromelain

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ABSTRACT: Enzymatic bromelain mungbean meal protein hydrolysate (eb-MPH) was produced from mungbean meal protein isolate (MPI). Enzymatic bromelain, with a known protease activity of 98,652 (unit/g), was used at concentrations of 0, 2, 6, 10, 14 and 18% (w/w) and with hydrolysis times of 0.5, 3, 6, 12, and 24 h. The pH and temperature were controlled at 6.0 and 50 °C, respectively. It was found that the best treatment combination for eb-MPH production by response surface methodology (RSM) was 18% bromelain and a hydrolysis time of 3 h, resulting in the greatest degree of hydrolysis (% DH) and percent yield, with values of 61.04 and 45.63%, respectively. Results also showed that the phenylalanine, tyrosine and leucine contents of the optimally produced eb-MPH were 20.88, 14.50 and 10.93%, respectively. Twelve volatile compounds were identified using gas chromatography mass spectrometry in eb-MPH; benzaldehyde, 2-pentylfuran and furfural were the predominant odorants.

KEYWORDS: physicochemical, bromelain, flavoring agents, protein hydrolysate, mungbean meal protein isolate, volatile compounds

INTRODUCTION

Flavoring agent was produced from hydrolyzed vegetable protein (HVP) that was prepared by hydrolysis of plant proteins, such as soybean, wheat and maize.[1] Hydrolysates are composed of free amino acids, smaller peptides, salt, and many savory compounds. Acid hydrolysis is a general process for the production of HVP, often using hydrochloric acid because it works quickly and produces a fully hydrolyzed and savory product. Commercially, acidic hydrolysate is obtained from treating the protein with 4−6 M hydrochloric acid (HCl) at 100−130 °C for 2−24 h followed by neutralization with sodium hydroxide (NaOH). During acid hydrolysis, high salt content (up to 40%) and some carcinogenic compounds, such as mono- and dichloropropanols and monochloropropanediols, can be produced. These carcinogenic compounds have been a serious concern in many countries. Weak acid and enzymatic hydrolysis, which is a mild hydrolysis reaction, could help avoid the formation of these carcinogens. The conditions of weak acid hydrolysis, low acid and low temperature lead to partial hydrolysis that generates a mixture of free amino acids and small and large peptides, which are necessary for flavor formation.[2] Weak acid hydrolysate is produced from hydrolyzing the protein with 0.1−1 M HCl at lower 95 °C for 12 or 36 h.[3] Enzymatic hydrolysis involves a much milder hydrolysis technique, a pH adjustment to pH 5−7, depending on the optimum of enzymes, and a hydrolysis time of 10−24 h at 50−55 °C.[4] Enzymatic hydrolysis also breaks peptide bonds of protein sources using commercial proteases, such as Flavourzyme, Alcalase, Novozymes, cucurbita, pomiferin, hieronymin, trypsin, papain and bromelain.[5] Enzymatic hydrolysis not only produces fewer carcinogens but also offers several advantages over chemical methods, such as providing reaction selectivity and mild deamidating conditions with a neutral pH at room temperature. Enzymatic hydrolysate gives meaty and savory flavor which it has to meet the consumer preference.[3]

The enzymatic reaction of protein hydrolysates generates either free amino acids or a large amount of short peptides. Various reports have indicated that amino acids and short chain peptides produce a unique taste profile. Indeed, glutamic acid and glutamic acid-rich oligo peptides from protein hydrolysis resulted in an umami flavor, which is very typical of traditionally produced HVP. However, amino acids and peptides from HVP production are also important in flavor formation because they are precursors in a variety of Maillard reactions that produce an extensive range of volatile flavors.[2]

Mungbean is an excellent source of protein, and its essential amino acid compositions compare favorably with that of soybean, kidney bean and FAO/WHO reference protein. In Thailand, mungbean meal is a byproduct obtained from the mungbean noodle industry. Because mungbean meal consists of a high protein content (approximately 72%), most meal is used as an ingredient for animal feeds (approximately 54,000 tons per year).[6] However, mungbean protein could be isolated for use in various food products: emulsifier and foaming and water absorption agents.[7] Mungbean protein isolate has been successful produced by alkaline extraction rather than neutral or acidic extraction. There is very little research reported on the production of mungbean protein hydrolysate. Previously reported was an acidic mungbean protein hydrolysate produced with 6 M HCl at 120 °C for 6 h. Under these conditions, the protein hydrolysate produced the highest amino nitrogen, amino nitrogen to total nitrogen ratio and odor score.[6] Therefore, the objectives of the present work were to produce hydrolysate vegetable protein from mungbean meal protein isolate (MPI) and to characterize

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the physicochemical properties and aroma of enzymatic bromelain mungbean protein hydrolysate (eb-MPH).

MATERIALS AND METHODS

Materials. Mungbean meal was obtained from Sittinan Co., Ltd. (Bangkok, Thailand). Bromelain (98,652 unit/g) was kindly provided by Hong Mao Biochemicals Co., Ltd. (Rayong, Thailand). All chemical reagents were of analytical grade (Mallinckrodt Chemicals, USA).

Preparation of Defatted Mungbean Meal (DMM). Mungbean meal was defatted twice with a mungbean meal:hexane ratio of 1:1 (w/v) under room temperature (approximately 25 °C) for 24 h. Defatted mungbean meal (DMM) was filtered with filter paper (Whatman No. 1) followed by air drying overnight in a fume hood. Next, DMM was sieved through a 40 mesh screen (U.S. Standard sieve), sterilized using an autoclave machine (Hirayama HVE-25/50, Japan) under $(1.0-1.3) \times 10^5$ Pa at 121 °C for 20 min and stored at 4 °C before use. The DMM obtained from mungbean meal contained approximately 11% moisture on a wet basis. For further studies, the weight of DMM was calculated on a dry basis.

Preparation of Mungbean Protein Isolate (MPI). MPI extraction was modified from Thompson et al.[8] DMM was suspended in water with a DMM:water ratio of 1:15 (w/v) and adjusted to pH 9.0 with 2 M NaOH at 25 °C. After 2 h of extraction, the sample was centrifuged at 5000*g* for 15 min, 25 °C in a centrifuge (Sorvall Dupont, RS-SC Plus, USA). The process of extraction and centrifugation was repeated twice. The pellet was discarded, and the supernatant was adjusted to pH 4.0 with 2 M HCl at 4 °C and stored overnight. The sample was centrifuged at 10000*g* for 5 min. The MPI precipitate was washed with distilled water using 1:2 (w/v) MPI:water.

Proximate Analysis of DMM and MPI. The proximate compositions (moisture, protein, ash, fat and fiber) of DMM and MPI were analyzed following the AOAC method.[9] Carbohydrate was determined based on the following formula: carbohydrate = 100 − (percentage of moisture, protein, ash, fat and fiber). All analyses were performed in three replicates.

Determination of Amino Acids of MPI. MPI was prepared using hydrolysis with 6 M HCl at 110 °C for 15 h. The samples were analyzed using high-performance liquid chromatography (HPLC) (Waters Alliance 2695, USA) with the mobile phase composed of sodium acetate buffer at pH 4.90 and 60% acetonitrile. All the analyses were performed on a reversed phase Hypersil Gold column (4.6 × 100 mm, 5 μm). A fluorescence detector (Jasco FP2020, Japan), with excitation and emission wavelengths of 250 and 395 nm, was used for the detection. Identification and quantification of amino acids were accomplished by comparison with a standard mixture of amino acids.

Preparation of Protein Hydrolysates. MPI was dispersed with sterile water (10 g/100 mL) in 500 mL flasks equipped with a condenser. The pH was adjusted to pH 6.0 with 2 M NaOH or 2 M HCl. Enzyme solution was varied at the following bromelain concentrations: 0, 2, 6, 10, 14 and 18% (w/w) in distilled water. Both MPI and bromelain solutions were incubated in a water bath at 50 °C for 5 min before mixing. After 5 min, bromelain solution was mixed with MPI and incubated at 50 °C for 0.5, 3, 6, 12, 18, and 24 h in a water bath. The reaction was stopped by heating to 90 °C for 15 min. Enzymatic bromelain mungbean meal protein hydrolysates (eb-MPH) were filtered through Whatman No. 1 filter paper. The filtrate of eb-MPH was collected and analyzed for physicochemical properties (color measurement, total salt percentage, yield percentage and degree of hydrolysis). All analyses were performed in three replicates. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were performed using the SAS Program Version 6.0 (SAS Institute, 1997, USA).

Color measurement of eb-MPHs (CIE L^*, a^* and b^* value) was determined by analyzing 2 mL of eb-MPH in a glass cell (10 mm in diameter) with a colorimeter (Minolta C-10, Japan). The L^* is 100, which represents a perfect reflecting diffuser, and the minimum for L^* is zero, which represents black. Positive a^* is red, and negative a^* is green. Positive b^* is yellow, and negative b^* is blue. L^*, a^* and b^* values are converted to hue angles (H° = arctan $[b^*/a^*]$) where 0° = red-purple, 90° = yellow, 180° = bluish-green and 270° = blue and chroma ($c^* = [(a^*)^2 + (b^*)^2]^{1/2}$), which indicates the color intensity or saturation. Total salt percentage (% total salt) was detected using a salinity refractometer (S-28E, Atago Co., Ltd., Japan) and reported as the percentage of total salt. Yield percentage (% yield) of eb-MPHs were concentrated using a rotary evaporator (Buchi R-200, Switzerland) until the eb-MPH became pastelike. The percentage of yields was calculated as follows:

$$yield(\%) = \frac{weightofhydrolysatepaste}{weightofMPI} \times 100$$

Degree of hydrolysis (% DH) was determined using trichloroacetic acid (TCA) as described by Flavia et al.[1] Total protein ($N = 6.25$) was measured by the Kjeldahl method.[9] The DH values were calculated by the following equation:

$$\%DH = \frac{20\%\ \text{TCA soluble N}}{solubleN} \times 100$$

where

$$\text{total N} = \text{percentage of soluble nitrogen in sample}$$

$$\text{20\% TCA soluble N} = \text{percentage of soluble nitrogen in TCA solution}$$

Experimental Design for Optimization Using Response Surface Methodology (RSM). Color, % total salt, % yield and % DH were used as criteria for the selection of the optimum hydrolysis conditions. A 6 × 6 factorial completely randomized design (CRD) was used to obtain the combination of values that optimized the reaction. All treatments were performed in three replicates. To identify the optimum levels of two variables, the response surface methodology was applied. The two independent variables were enzyme concentration (x_1 = 0, 2, 6, 10, 14 and 18% w/w) and hydrolysis time (x_2 = 0.5, 3, 6, 12, 18, and 24 h). It was assumed that the estimated response surface (y) could be described with the aid of a second-order model with the following equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}{x_1}^2 + b_{22}{x_2}^2 \quad (1)$$

Variable y was a predicted response (color, % total salt, % yield and % DH), x_1 and x_2 were independent variables, b_0 was an offset term, b_1 and b_2 were linear effects and b_{12} was interaction effects.[10] The model evaluated the effect of each independent variable. Analysis of the experimental design and calculation of predicted data were performed using SAS program to estimate the effects of the independent variables. Surface plots were produced by Statistica Program 5.0 (StatSoft Inc., 1995, USA). The eb-MPH from the optimized hydrolysis treatment combinations was concentrated using a rotary evaporator (Buchi R-200, Switzerland) at 7.2×10^6 Pa and 40 °C for 1 h. The concentrated eb-MPH was further used for amino acid analysis, sensory analysis and identification of volatile compounds.

Free amino acids of the sample were identified with HPLC using the same conditions as MPI, but the sample was not hydrolyzed by HCl. The results were compared with the amino acids of MPI. Descriptive sensory analysis of eb-MPH aroma characteristics under optimum conditions was obtained by using twenty-five semipanelists and five trained panelists. All semipanelists had previously received training in descriptive sensory analysis with more than 20 h of experience in sensory analysis of various food samples. They were confirmed by the five panelists who were trained in savory application (Firmenich, Thailand). The eb-MPH

paste (5 mL) was prepared by dissolving in 10 mL of water at 60 °C. Scores of eb-MPH was qualitative descriptive, with a 9-point scoring test of bouillon odor, mungbean odor, soy sauce odor, bitter taste, sweet taste, sour taste, salty taste, bouillon taste, umami taste and overall acceptance. Sensory analysis was conducted based on a randomized complete block design (RCBD) with three replications. Means were compared with DMRT by SAS Program Version 6.0.

Determination of volatile compounds in eb-MPH produced by the optimized treatment combinations were detected using dynamic headspace gas chromatography mass spectrometry (DHS GC−MS). The eb-MPH sample was prepared from 4 mL of eb-MPH in a 20 mL vial for the headspace technique. Samples were heated at 70 °C for 10 min in a GC−MS heating block. Volatile compounds were separated using an Agilent Technologies GC-789DA (China) DB-WAX capillary column (30 m × 0.25 mm i.d.; coated film thickness: 0.25 μm). Five hundred microliters of headspace volume of samples was introduced with the splitless mode using an autoinjector (CTC CombiPal, Agilent Technologies, Switzerland). Temperature of the injection port was 240 °C. The oven temperature was maintained at 55 °C for 5 min, then increased to 180 at 10 °C/min, from 180 to 200 at 20 °C/min, and finally held at 200 °C for 10 min. A mass spectrometer (5975C inert XL EI/CI MSD with a triple-axis detector, Agilent Technology, USA) was interfaced with the chromatographic system at an interface temperature of 250 °C using a mass scan range of 35−450 amu. Helium was used as the carrier gas at a flow rate of 2 mL/min, and the pressure was 3.32×10^4 psi. The compounds were identified by first comparing their mass spectrum with those in Wiley 275 and the NIST library at percentage of quality match over 85%, and compared with previously published literature, followed by retention index (RI) values of eb-MPH, which were obtained from the comparison of eb-MPH with alkane standards (C_8−C_{20}).

RESULTS AND DISCUSSION

Proximate Composition of DMM and MPI. The proximate composition of DMM (from mungbean meal byproduct of a mungbean noodle factory) was 76.34% protein, 1.05% fat, 3.16% ash, 0.96% fiber and 18.49% carbohydrate (dry weight). The DMM was excellent for use as a protein source for protein hydrolysate production because it contained protein content greater than 30% (dry weight).[11] The MPI was derived from DMM alkaline extraction with 2 M NaOH at pH 9 for 2 h and precipitated with 2 M HCl at pH 4.0. Results showed that MPI had greater protein content (91.73%) compared with DMM. Fat, ash, fiber and carbohydrate contents (dry weight) in MPI were 1.64%, 1.51%, 0% and 5.12%, respectively. Results also showed that protein and fat contents in MPI were increased from the initial values; however, ash, fiber and carbohydrate contents were decreased. Fat content of MPI was slightly increased and was likely caused by the change of the proximate composition; the remaining fat was in the form of a residual protein−lipid complex. The little amount of fat in MPI does not impact the flavor of the protein hydrolysate.[12]

Enzyme Hydrolysis Optimization. The optimum hydrolysis treatment combination was determined using RSM. Statistical analysis indicated that two hydrolysis factors (enzyme content and hydrolysis time) had no significant influence on color value (L^*, $h°$ and chroma) or % total salt, whereas these factors had significant effects on % yield and % DH.

The L^*, $h°$ and chroma of the eb-MPHs are shown in Table 1. Results led us to conclude that the enzyme content and hydrolysis time had no significant effect on L^*, $h°$ or chroma of individual eb-MPHs ($p > 0.05$). All eb-MPHs had similar color, with an $h°$ value indicating brown-yellow-green. L^*, $h°$ and chroma values ranged from 36.0 to 36.83, 67.56 to 76.55 and 16.47 to 20.99, respectively, observed as a light color. As reported by Weir,[4] the enzymatic hydrolysate was usually lighter in color, whereas the acidic hydrolysate was dark brown in color.

Table 1. Physicochemical Properties of eb-MPHs: Lightness, Hue Angle, Chroma and % Total Salt of eb-MPH Prepared from MPI Hydrolyzed by Bromelain

enzyme concn (%)	time (h)	lightness	hue angle	chroma	% total salt
0	0.5	36.2	71.57	19.12	3.40
	3	36.8	69.19	20.99	3.37
	6	37.1	70.87	19.9	3.30
	12	36.2	70.02	16.81	3.33
	18	36.2	68.88	17.02	3.33
	24	35.8	72.65	18.93	3.37
2	0.5	36.0	73.74	18.75	3.37
	3	36.1	69.78	18.15	3.27
	6	36.4	70.12	18.74	3.27
	12	36.4	69.72	18.16	3.43
	18	36.0	70.11	18.74	3.30
	24	36.3	68.43	16.47	3.27
6	0.5	36.4	71.57	19.12	3.37
	3	36.4	68.20	17.80	3.23
	6	36.2	67.56	18.61	3.33
	12	36.3	69.04	18.96	3.27
	18	36.0	72.58	20.97	3.40
	24	36.8	69.30	17.58	3.27
10	0.5	36.2	70.87	19.93	3.30
	3	36.1	71.94	17.75	3.37
	6	36.1	71.93	17.75	3.27
	12	36.3	69.72	18.16	3.33
	18	36.1	70.82	17.95	3.43
	24	36.9	70.42	17.38	3.40
14	0.5	36.2	70.87	19.93	3.33
	3	36.2	70.82	17.95	3.27
	6	36.3	70.82	17.95	3.23
	12	36.3	69.72	18.16	3.27
	18	36.0	70.82	17.95	3.37
	24	36.3	67.56	18.61	3.37
18	0.5	36.1	72.98	19.54	3.27
	3	36.1	72.97	19.54	3.37
	6	36.3	70.82	17.95	3.37
	12	36.3	70.11	18.74	3.33
	18	36.0	71.94	17.75	3.27
	24	36.3	76.55	17.10	3.33
F-test		ns	ns	ns	ns
CV (%)		0.50	9.22	3.88	3.73
LSD		0.29	0.24	0.29	0.2

[a] ns means ($n = 3$) in the same column with different letters are not significantly different ($p > 0.05$) according to DMRT.

The % total salt of eb-MPHs was 3.27−3.43% (Table 1). These results showed that the enzyme content and hydrolysis time did not affect the % total salt of eb-MPHs ($p > 0.05$). However, the % total salt of eb-MPHs was not significantly different from the % total salt of MPI. The total salt produced from enzymatic hydrolysis was lower than that from acid hydrolysis. Although acid hydrolysis is a commonly used process for

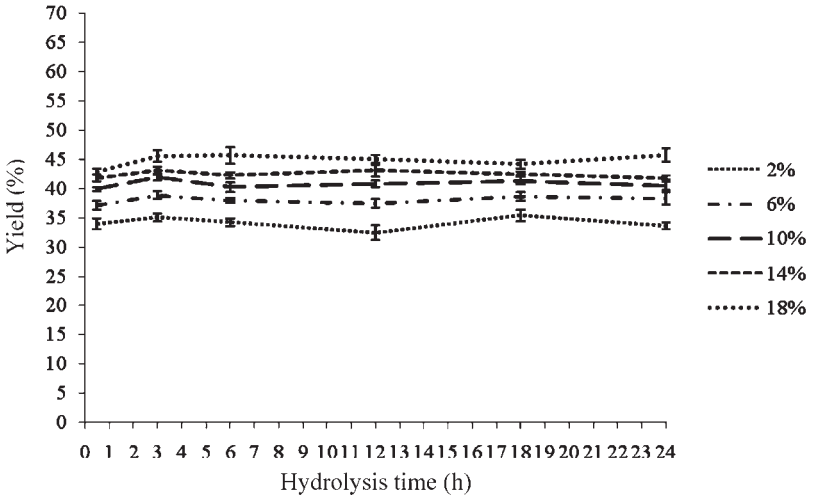

Figure 1. Yield (%) prepared by 2, 6, 10, 14 and 18% enzyme and hydrolysis time 0.5, 3, 6, 12, 18, and 24 h.

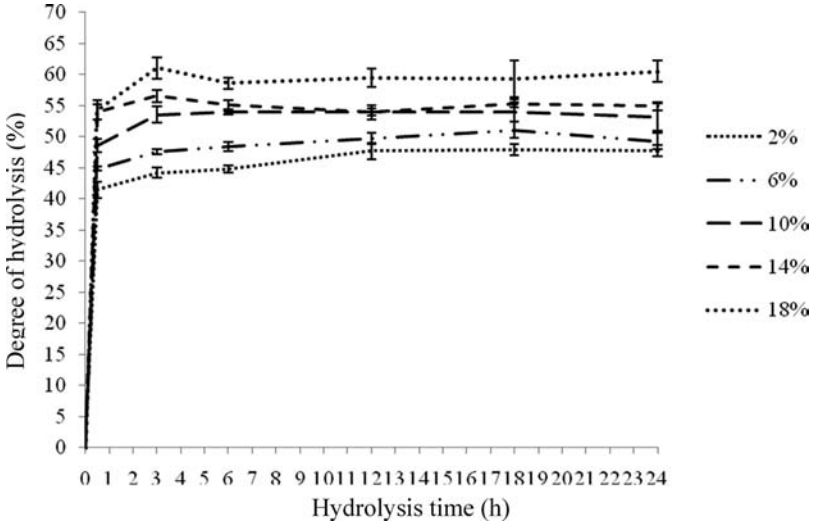

Figure 2. Degree of hydrolysis (%) prepared by 2, 6, 10, 14, and 18 enzyme and hydrolysis time 0.5, 3, 6, 12, 18, and 24 h.

the production of protein hydrolysates, it results in a very high salt content (about 40%).[13] High dietary salt (consumed as common salt and sodium chloride) is associated with increased blood pressure and adverse cardiovascular health.[14] Thus, eb-MPH is an alternative choice to replace high salt products.

Figure 1 shows that the enzyme content and hydrolysis time directly influenced the % yield of eb-MPHs ($p \leq 0.05$). Yield percentage at a hydrolysis time of 0 to 3 h in all enzyme concentrations increased rapidly and then increased slightly after 6 h. The eb-MPH that was produced from 18% enzyme in 3 h produced the highest % yield (45.63%), but it was not significantly different from hydrolysis times of 6, 12, 18, and 24 h. Results also showed that hydrolysis time influenced % yields in early periods, similar to % DH (Figure 2). The % DH of all enzyme concentrations was markedly increased during the first 3 h, thereafter reaching a plateau. Degree of eb-MPH hydrolysis was measured from soluble protein content in trichloroacetic acid solution. A rapid increase in % DH occurred during the initial time of hydrolysis because the bromelain was in excess and saturated the substrate and resulted in the cleavage of large numbers of peptide bonds in MPI. After 3 h, % DH was quite stable because of product inhibition.[15] The % DH of bromelain in this report was similar to soy protein isolates and defatted sesame flour hydrolysis by Flavourzyme, Protamex, Neutrase and Alcalase.[1,16] Table 2 summarizes the estimated regression coefficients of second-order polynomial models. The ANOVA of the regression coefficients of the response surface model in terms of % yield and % DH was evaluated.

For % yield, regression coefficients showed that enzyme concentration and hydrolysis time had linear and quadratic effects on the yield values. A statistically significant interaction ($p \leq 0.05$) on the yield values was observed between enzyme concentration (x_1) and hydrolysis time (x_2). The interaction term also remained in the model when the respective equation model was accurate. The large value for the estimated regression coefficient for enzyme concentration ($b_1 = 0.998$) indicated that it was the most important linear variable influencing the DH values. Positive value implied that the DH values increased with increasing enzyme concentration, which was similar to the results presented by other reports.[17] The best explanatory model

Table 2. Model Regression Coefficients Estimated by Multiple Linear Regressions for the % Yield and % DH of eb-MPHs[a]

	coefficient	
factor	% yield	% DH
constant	31.997*	40.251*
linear		
x_1	0.998*	0.958*
x_2	0.220*	0.598*
quadratic		
x_1^2	−0.017*	−0.002
x_2^2	−0.006*	−0.015*
interactions		
$x_1 \times x_2$	−0.005*	−0.009*
R^2	0.957	0.948
adjusted R^2	0.948	0.911

[a] A model in which x_1 = enzyme concentration, x_2 = hydrolysis time. *Significant at $p \leq 0.05$.

equation for the % yield obtained from enzymatic bromelain mungbean meal protein hydrolysate is shown in eq 2:

$$y = 31.997 + 0.998x_1 + 0.220x_2 - 0.05x_1x_2 - 0.017x_1^2\text{-}0.006x_2^2 \quad (2)$$

The % DH, enzyme concentration (x_1) and hydrolysis time (x_2) had linear effects on the DH values, while only hydrolysis time had quadratic effects (x_2^2). The interaction effect was significant ($p \leq 0.05$). Of the linear variables affecting the DH value, enzyme concentration had a higher regression coefficient ($b_1 = 0.958$) than hydrolysis time ($b_2 = 0.598$). The best explanatory model equation for the DH value for enzymatic bromelain mungbean meal protein hydrolysate is as follows:

$$y = 40.251 + 0.958x_1 + 0.598x_2 - 0.09x_1x_2 - 0.015x_2^2 \quad (3)$$

Results of the ANOVA for % yield and % DH in Table 2 demonstrate that both statistical models are significant at a 95% confidence level ($p \leq 0.05$). Equations 2 and 3 fit the experimental data with an acceptable determination coefficient (% yield, $R^2 = 0.957$ and % DH, $R^2 = 0.948$). A three-dimensional plot (Figure 3a,b) based on the proposed models showed that increasing hydrolysis time and enzyme content increased the % yield and % DH. Optimal treatment combinations for producing eb-MPH were determined to obtain maximum % DH and % yield (color and % total salt had no significant difference). Second-order polynomial models obtained in this research were utilized in order to determine the optimum treatment combinations. The zone of optimization as shown in the overlay plot depicts the optimal enzyme concentration to be 16−18% and hydrolysis time to be 3 h (Figure 3). Therefore, 18% enzyme and hydrolysis time of 3 h was the optimum condition for producing eb-MPH because this condition resulted in the highest % DH and highest % yield with the least hydrolysis time.

To confirm the validity of the experimental strategies and to gain a better understanding of % yield and % DH, two additional experiments (enzyme content and hydrolysis time) were conducted. The chosen treatment combinations for enzyme content and hydrolysis time, determined with the statistical model, and the experimental results are listed in Table 3. The measured

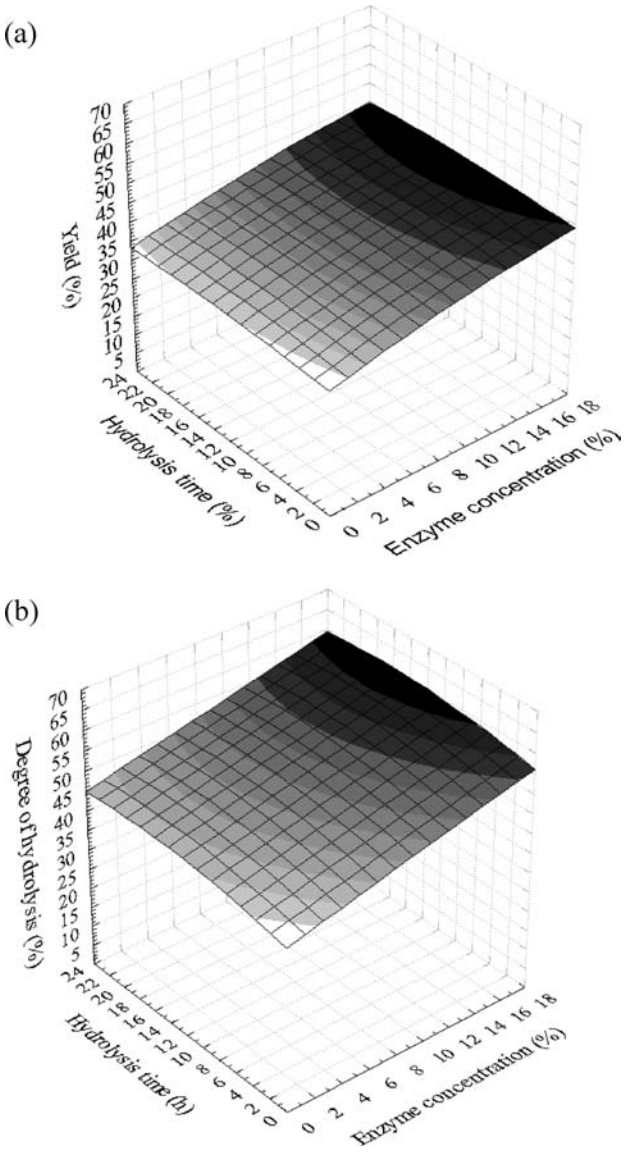

Figure 3. Response surfaces for the effects of enzyme concentration and hydrolysis time on yield and degree of hydrolysis (%) of eb-MPHs: (a) yield (%) and (b) degree of hydrolysis (%).

% yields and % DHs were close to the predicted values for % yield and % DH using RSM. These results confirm the predictability of the model for producing enzymatic bromelain mungbean meal protein hydrolysate with these experimental conditions.

Determination of Amino Acids of MPI and eb-MPH. The amino acids obtained by treating MPI with concentrated hydrochloric acid at 100−130 °C for 24 h until completely hydrolyzed are shown in Table 4. Seventeen amino acids were identified. These amino acids were free amino acids and amino acids in peptide chains. Amino acids present at the greatest concentrations were glutamic acid and aspartic acid. In 1908, Ikeda (a Japanese scientist) was the first who found the salt of glutamic acid, which had the unique taste of umami. Umami is described as savory, meat or broth-like taste, and the word means delicious in Japanese.[18] Cysteine was also found in MPI, and it contributed sulfur to the reaction as an important route to the creation of the many sulfur containing aromatic chemicals.

Table 3. Observed and Predicted Values of the Confirmation Experiments

		% yield[b]		% DH[b]	
trial	conditions[a]	obsd	predicted	obsd	predicted
1	$x_1 = 2$, $x_2 = 0.5$	33.88 ± 0.43	34.03	41.50 ± 1.30	42.45
2	$x_1 = 6$, $x_2 = 18$	38.59 ± 0.76	38.85	51.08 ± 1.29	50.93
3	$x_1 = 18$, $x_2 = 18$	45.17 ± 1.92	44.85	59.34 ± 2.9	60.48

[a] x_1 represents enzyme concentration. x_2 represents hydrolysis time. [b] Mean ± SD ($n = 3$).

Table 4. Amino Acid Composition of MPI and Free Amino Acid Content of eb-MPH Hydrolyzed by 18% Bromelain for 3 h

	MPI		eb-MPH	
amino acid	amino acid content[a] (mg/100 g of protein)	amino acid (%)	free amino acid content (mg/100 g of protein)	free amino acid (%)
aspartic acid	2413.66	13.83	55.68	1.45
glutamic acid	3967.50	22.73	161.64	4.22
serine	961.83	5.51	188.90	4.93
glycine	695.87	3.99	86.25	2.25
histidine	523.35	2.99	196.29	5.12
arginine	432.18	2.48	377.59	9.85
threonine	590.73	3.38	244.54	6.38
alanine	794.99	4.55	202.19	5.27
proline	914.29	5.24	25.45	0.66
cysteine	215.72	1.24	0	0
tyrosine	729.82	4.18	555.96	14.49
valine	726.09	4.16	113.60	2.96
methionine	135.21	0.77	94.13	2.45
lysine	1262.33	7.23	273.66	7.13
isoleucine	569.02	3.26	39.55	1.04
leucine	1337.15	7.66	418.92	10.92
phenylalanine	1187.99	6.80	800.97	20.88
total	17457.73	100	3835.32	100

[a] Amino acids in MPI were in free amino acid form and amino acids in peptide chains.

Table 4 shows the free amino acids in eb-MPH hydrolyzed by 18% bromelain for 3 h. Sixteen free amino acids derived by enzymatic hydrolysis were identified: one sulfurous (methionine), seven hydrophobic (alanine, valine, leucine, isoleucine, proline, methionine and phenylalanine), four hydrophilic (glycine, serine, threonine, and tyrosine), two acidic (aspartic and glutamic acid) and three basic (lysine, arginine and histidine). Phenylalanine was present at 800.97 mg/100 g, which was the highest content. Tyrosine, leucine, arginine and lysine were present at 555.96, 418.92, 377.59, and 273.66 mg/100 g of protein, respectively. Essential amino acids of phenylalanine and leucine resemble bitter taste, but tyrosine has no taste. Arginine has a sweet smell and taste, but lysine has fresh-green characteristics.[19] Cysteine was not found in eb-MPH, but it was found in MPI due to covalent linkage between two cysteines forming a disulfide bond of a cystine during hydrolysis. Glutamic acid (161.64 mg/100 g) was found in eb-MPH, but the contents were less than

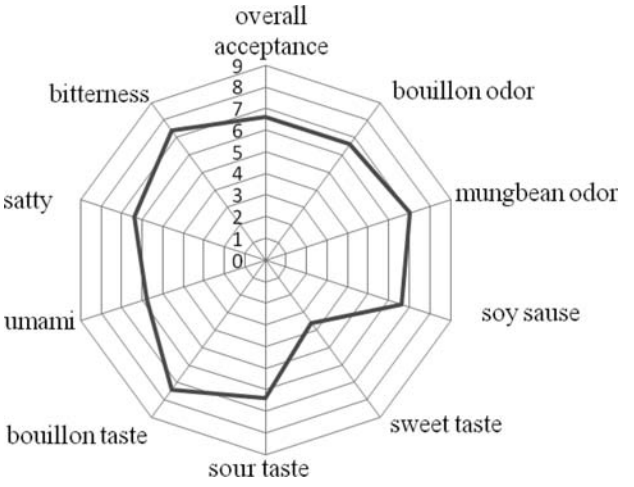

Figure 4. Sensory profile of eb-MPH from hydrolysis of MPI by 18% bromelain for 3 h.

MPI (Table 4) because MPI was hydrolyzed by concentrated HCl before analysis of the free amino acids, but eb-MPH was not. The most closely studied flavor enhancer is monosodium glutamate and sodium salt of glutamic acid. Glutamate is frequently used as a flavor enhancer in foods and enhances the savory flavors imparted by glutamic acid, which occurs naturally in protein foods (e.g., meats, seafood, stews, soups and sauces).[20] Recent studies have been reported that the amount of free glutamic acid in eb-MPH is in the range of general food enhancer (24−816 mg/100 g). Normally, the optimum amount of added glutamic acid to enhance the taste of food is at 100−800 mg/100 g by weight.[21] The flavor enhancing effect as umani has an important role in the overall development of a high quality savory flavor. However, glutamic acid alone does not contribute to making a meat flavor. One of the reasons that eb-MPH became very useful as meat flavor substitute flavorings is the fact that it has amino acid profiles that are similar to those of meat and it is reasonably inexpensive compared to meat extracts.[20] Therefore, the eb-MPH in this study could be used as condiment for direct food enhancer or precursor for thermal process flavoring.

The results of the analysis of eb-MPH showed a higher hydrophobic amino acid profile than hydrophilic. The reasons for these results could be that bromelain is an endoprotease, which is a sulfhydryl enzyme. The catalytic activity of stem bromelain has been shown to catalyze the hydrolysis of N^2-benzoyl-L-arginine ethyl ester and N^2-benzoyl-L-arginine amide via an acyl-enzyme mechanism. Bromelain has a similar interaction with papain, which is cleaved between a hydrophobic and suitable leaving group (alcohol, amine, amino acid or peptide), which are good substrates. In particular, substrates with phenylalanine at a hydrophobic position were found to be most effective.[22]

Sensory Analysis. Results from the sensory profiling showed that the characteristics of eb-MPH included salty, bitter, sweet, sour taste and odor. The assessors also described characteristics such as umami taste, mungbean odor, soy sauce, bouillon taste and odor and overall acceptance. The average score on a 9-point scale is shown in the profile wheel (Figure 4). The eb-MPH had bouillon odor, mungbean odor, soy sauce, bitter taste, sweet taste, sour taste, salty, bouillon taste, umami taste and overall acceptance with values of 6.6, 7.0, 6.6, 7.4, 3.6, 6.4, 6.4, 7.4,

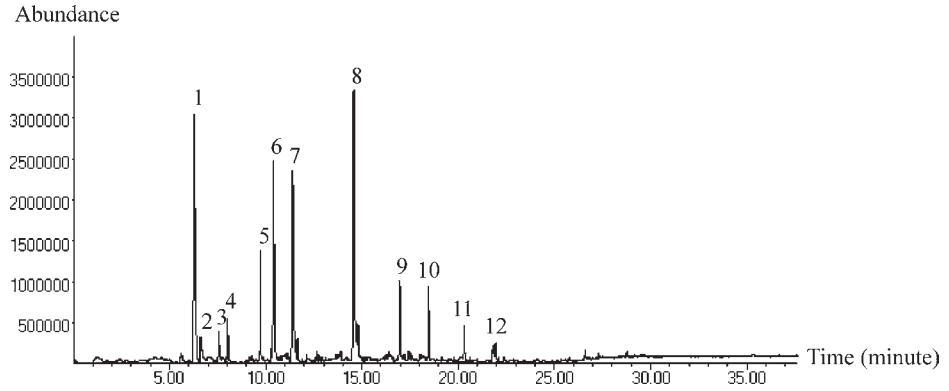

Figure 5. Flavor profile of eb-MPH from hydrolysis of MPI by 18% bromelain for 3 h. 1 = 2-pentylfuran, 2 = (*E*,*E*)-2,4-nonadienal, 3 = (*Z*)-2-(2-pentenyl)furan, 4 = (*E*)- 2-heptenal, 5 = 5-ethylcyclopent-1-enecarboxaldehyde, 6 = furfural, 7 = benzaldehyde, 8 = methoxy-phenyl-oxime, 9 = phenylethyl alcohol, 10 = isopropyl myristate, 11 = 2-phenoxyethanol, and 12 = *n*-hexadecanoic acid.

Table 5. Compounds of eb-MPH from Hydrolysis of MPI by 18% Bromelain for 3 h

compounds[a]	RI[a,b]	% rel peak area	odor descriptions[c]
2-pentylfuran	900	22.73	greenbean, butter
(*E*,*E*)-2,4-nonadienal	912	4.22	watermelon
(*Z*)-2-(2-pentenyl)furan	944	3.65	meat
(*E*)- 2-heptenal	962	1.26	intense green, sweet and fresh fruity apple skin
5-ethylcyclopent-1-enecarboxaldehyde	1031	6.88	cooked pork
furfural	1068	17.89	bouillon, sweet, caramel
benzaldehyde	1125	22.83	cooked meat, sweet, oily,
methoxy-phenyl-oxime	1344	5.84	unknown
phenylethyl alcohol	1513	8.64	honey, spice, rose, lilac
isopropyl myristate	1639	3.74	oily
2-phenoxyethanol	1824	0.53	rose
n-hexadecanoic acid	1959	1.79	creamy, lard

[a] All compounds were identified by comparison with mass spectra and retention index database. [b] RI (retention index) calculated with a DB-Wax stationary phase using a series of alkanes between C_8 and C_{20}. [c] Odor descriptions cited from www.flavornet.org and recent reports.

5.8 and 6.6, respectively. The characteristics of eb-MPH were bouillon, salty, umami and sweet taste; thus, it could be used as a precursor for processed flavor for savory food flavor production. Commercially, the hydrolyzed vegetable protein may be used directly or after further thermal processing. Further processing leading to different specific meat flavors was included. This depended on the chemical composition, which was very important.

The intensity of bitterness depends on % DH and protease specificity because hydrophobic amino acids responsible for bitterness can be liberated by endopeptidases. Therefore, with an increase in DH, more hydrophobic amino acids can be generated from interior peptide chains, resulting in increasing bitterness. A relatively high sensory bitterness score (Figure 4) found in eb-MPH was related to the concentration of hydrophobic free amino acids. This was clear from the bitter taste of eb-MPH. Not only free amino acids but amino acids in peptide form affect taste.[23] Many studies have attempted to decrease bitterness by hydrolyzing bitter peptides with efficient, cost-effective, and environmentally compatible exopeptidases.[24] These could be used by mixing with proteases to produce bitter protein hydrolysates in a single-step enzymatic reaction as well.

Determination of Volatile Compounds. Volatile compounds of concentrated eb-MPH were detected using the DHS-GC-MS technique. As reviewed in previous studies, there are no reports of volatile compounds in mungbean meal protein hydrolysate. Figure 5 shows the chromatogram of volatile compounds of eb-MPH. Results were compared with Wiley 275 and NIST library with a % quality match greater than 85%. The RI values were calculated with a DB-Wax stationary phase using a series of alkanes between C_8 and C_{20}. There are twelve volatile compounds (Table 5), and the flavor profile is consistent with the sensory evaluation in Figure 4. The predominant odorants were benzaldehyde, 2-pentylfuran and furfural with % relative peak areas of 22.83, 22.73 and 17.89, respectively. In this work, the volatile compounds occurred in small amount after inactivate enzyme reaction by heating (data not shown) and by concentration process. The findings on the effect of concentration on volatile compounds were in agreement with earlier reports.[25] The analysis also found (*E*,*E*)-2,4-nonadienal, (*Z*)-2-(2-pentenyl)furan, (*E*)-2-heptenal, 5-ethylcyclopent-1-enecarboxaldehyde, methoxy-phenyl-oxime, phenylethyl alcohol, isopropyl myristate, 2-phenoxyethanol and *n*-hexadecanoic acid.

Benzaldehyde is sweet and oily and is found in meat, bone meal and sukiyaki flavor.[26,27] The 2-pentylfuran, which is derived from cysteine, ribose, and polyunsaturated fatty acid reactions, provided green bean and butter flavors.[28] Furfural (bouillon, sweet

and caramel) is found in smoked dried meat.[29] Chevance et al.[30] reported that furfural was the major volatile odor compound in frankfurter. The odor characteristics of the volatile compounds in eb-MPH in this study were bouillon character, sweet smell, slightly green and fatty, as determined by sensory analysis.

In conclusion, the hydrolysis of mungbean meal protein isolate with bromelain under the treatment combination of this study (18% bromelain for 3 h) resulted in 61.04% DH and 45.63% yield. The enzyme content and hydrolysis time did not affect color or % total salt. After enzymatic hydrolysis, 16 free amino acids were found in the eb-MPH. The free amino acids present were aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, lysine, isoleucine, leucine and phenylalanine. The phenylalanine was present at the highest concentration in eb-MPH. The sensory characteristics of eb-MPH were bouillon, salty, sour taste and umami. The predominant odorants of eb-MPH were benzaldehyde, 2-pentylfuran and furfural, which all provided characteristics in the sensory analysis.

This study is the first to report producing enzymatic mungbean meal protein hydrolysate and the first report of amino acids, sensory characteristics and volatile compounds in protein hydrolysate from enzymatic mungbean meal. These findings suggest that eb-MPH could be used as a precursor in processed flavor for producing specific savory food flavors, such as beef, chicken and pork. Despite the fact that eb-MPH has a bitter taste, it could be masked by processed flavor or exoprotease enzymes could be used; further studies should be performed to evaluate these options. Furthermore, future work will investigate eb-MPH as a functional food because eb-MPH has essential amino acids and short chain peptides, which may lead to eb-MPH being a new selection in human nutrition for healthy consumers.

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