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Chemical test and cellular bioactivities and flavor enhancer properties of mung bean protein via glycation reaction

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Abstract. Mung bean meal is by-product of the vermicelli industry and contains a high protein content of 70 %. Therefore, this research study and development of flavor-enhancers derived from the mung bean meal protein isolated (MPI) reacted with reducing sugars as glucose (Glu) for generating Maillard reaction products (MRPs) under thermal process. Sensory characteristic, antioxidant activities and cellular bioactivities invested under different ratios of MPI:Glu and different reaction times. The optimal production process of MRPs was determined by response surface methodology (RSM) under the desirability criteria. The optimal ratio of MPI:Glu and time for generating high teriyaki odor, savory, DPPH inhibition, ABTS inhibition with low nut odor and bitterness were identified. Results showed that the MPI:Glu ratio of 1.5:1 and reaction time for 150 min (MRP) were the optimum conditions that contributed to the highest teriyaki and savory scores of 2.54 and 2.25, respectively. MRP explored bioactivities, both chemical test and cellular bioactivity. MRP demonstrated both low cytotoxicity and significant anti-inflammatory effects. MRP exhibited non-toxic in Vero cells at 10 mg/mL and it significantly suppressed nitric oxide (NO) release to 18.62 µM with non-toxicity in the murine macrophage; RAW 264.7 at a concentration of 1 mg/mL.

1 Introduction

The glycation reaction or Maillard reaction is a non-enzymatic reaction that occurs between a carbonyl group of reducing sugar and the amino groups of protein generated a heterocyclic compound [1] which contributed to the browning color and enhances flavor in food products [2]. Glycation is the chemical process to improve not only flavoring properties but also function and bioactivities such as solubility, emulsifying property, foaming property, and bioactivity [3].

Mung bean meal is a by - product of vermicelli industry and had a protein content of 70% (w/w) with gluten-free. It was mainly used as animal feed with less value [4]. However, mung bean meal was also rich in important amino acids for flavor, such as glutamic and aspartic acid, precursors of glutamate, and aspartate, an umami flavoring agent [5]. Cysteine and lysine contained in mung bean meal protein were the primary precursor of glycation to generate savory flavor, were also found [6, 7]. Normally, mung bean protein isolate has various functional properties, both food functional and bioactivation, e.g., foaming, emulsifying, antioxidant and antihypertension etc. [12]. The above-state rationale of glycation between protein and reducing saccharide was to obtain better functions. Therefore, this work was to improve the food sensory characteristics and bioactivity of mung bean meal protein isolate by conjugating protein with glucose for its future healthy food application.

2 Materials and Methods

2.1 Materials

Mung bean meal obtained from Sittinan Co. Ltd., Lat Lum Kaeo District, Pathum Thani, Thailand. Mung bean meal defatted (DMM) was extracted twice using hexane. The proximate composition of DMM 76.34% protein, 1.05% fat, 3.16% ash, 0.96% fiber and 18.49% carbohydrate (dry weight). Chemical reagents were of analytical grade from Thermo Fisher Scientific (Waltham, MA, USA) and Sigma Aldrich (St. Louis, MO, USA).

2.2 Preparation of mung bean protein isolate

DMM was mixed with deionized water (ratio 1:15) adjusted pH to 9 by using 2M sodium hydroxide. Allowed to stand for 2 hours, then separated the supernatant with Whatman No.1 filter paper and kept the filtrates to adjusted pH to 4 with 2M hydrochloric acid [11]. Centrifuged at 8,000 rpm for 5 minutes.

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Separated and collected the MPI precipitated then freeze-dried and store at -20 ºC for further experiment.

2.3 Preparation of Maillard reaction products (MRPs)

MRPs were prepared by glycation between MPI (>76.34% of protein) and glucose (Glu). The experiment was performed in 3x4 Factorial. MPI was mixed with Glu and dissolved in deionized water 40 mL with ratio MPI:Glu of 1:1.5, 1:1, and 1.5:1. Each ratio was reacted at 99 ºC for 0, 90, 120, and 150 min, stopped the reaction in ice bath rapidly, then freeze-dried and stored at -20 ºC.

2.4 Sensory analysis

Quantitative descriptive analysis (QDA) of MRPs was obtained by using 20 from semi-trained panelists. All semi-trained panelists had previously received training in descriptive sensory analysis with more than 20 hours of experience in sensory analysis of various food samples. The quantitative descriptive, with a 5-point scoring test (1-5 point), where 1-point score not detectable, a 2-point score indicate slight, a 3-point score indicate moderate, 4-point score indicate strong and 5-point score indicate the strongest of nut odor, caramel odor, teriyaki odor, soy sauce odor, savory, bitterness, sweetness, saltiness, and umami. Sensory analysis was conducted based on a randomized complete block design (RCBD).

2.5 Antioxidant activities

The DPPH and ABTS radical scavenging activity of MRPs was analyzed according to the previous method [2,6]. DPPH and ABTS radical scavenging activity were calculated in the radical scavenging activity equation (Eq. 1)

$$Redical\ scavenging\ activity\ (\%) = ((A_{blank}–A_{sample})/A_{blank})\times 100 \qquad (1)$$

where A_b is the absorbance of blank.
A_s is the absorbance of sample.

The effective MRPs concentration which inhibited 50% of DPPH and ABTS (EC50) was performed the regression plot of concentration against percentage scavenging activity by GraphPad Prism Version 10.0 (GraphPad Software San Diego, Ca, USA).

2.6 Optimum condition of MRPs

The optimal condition for Maillard reaction products of MPI and glucose by Response surface methodology (RSM). The independent variables were the ratio of MPI:Glu (X_1) and reaction time (X_2), which affected sensory characteristics and antioxidant activity. The interaction was calculated by the quadratic equation (Eq. 2)

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_{12} + b_2^2x_2^2 \qquad (2)$$

where: b_0 was the constant regression coefficient of the model. b_1 and b_2 were the linear regression coefficients of the model. b_{11} and b_{22} were the quadratic regression coefficients of the model. b_{12} was the interaction regression coefficient.

The experiment plan was designed and statistically analysed for the effects of the independent variables using the SPSS statistic Version 20.0.0 (IBM, 2011, USA). A p-value less than 0.05 was considered statistically significant ($p<0.05$). STATITCA Version 10.0 (StatSoft, Inc., 2011, USA) was used to build the surface plots of each variable.

2.7 Fourier transform infrared (FTIR) analysis

MRPs were investigated in chemical composition by FTIR. Each sample was mixed with KBr pelleted. The FTIR spectra was investigated at IR ranges 400-4000 cm-1 by Fourier Transform Infrared Spectroscope (PerkinElmer, Spectrum 100, USA).

2.8 Cellular bioactivities

2.8.1 Determination of cytotoxicity

The Vero cell (1×10^3 cells) was cultured in a 96-well plate at 37°C for 16-18 hours with 5% CO_2. Subsequently, the cells were exposed to fresh medium containing 10 mg/mL sample to each well for an additional 72 hours. The negative and positive controls consisted of 10% DMSO and medium, respectively. At the indicated time, the medium was removed followed by adding of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) and the mixture was incubated for 3 hours. Afterwards, dimethyl sulfoxide (DMSO) was added to dissolve the insoluble formazan crystals for 15 min. Cytotoxicity was determined using excitation and emission wavelengths of 570 and 630 nm, respectively [9].

2.8.2 Determination of anti-inflammatory

The murine macrophage; RAW 264.7 (1×10^5 cells) was cultured into a 96 well plate at 37°C for 16-18h with 5% CO_2. The MRP was made by dilution with a culture medium until the concentration of 1 mg/mL was achieved and then added to each well for 1 h. A positive control was Diclofenac (75 µg/mL). After that, the cell was provoked by E. coli LPS (1 µg/ml) and continually incubated for another 24h. Finally, medium from each well was collected to measure NO production by Greiss reagent (Promega). The absorbance of the resultant solution was measured at 550 nm. Sodium nitrite ($NaNO_2$) was used as a standard [9].

2.9 Statistical analysis

The experimental data was analyzed by using IBM SPSS statistics 27.0 (SPSS Institute, USA). The statistical significance of differences between samples was accepted with confidence at 95% ($p \leq 0.05$).

3 Results and Discussion

3.1 Optimization of Maillard reaction products (MRPs) from mung bean protein isolate

The optimum conditions for producing MRPs from mung bean protein isolate and glucose were carried out by RSM. The influence of different ratios (X_1) and reaction times (X_2) on sensory characteristics, DPPH, and ABTS were statistically analyzed by SPSS. Then, nut odor, teriyaki odor, savory, bitterness, DPPH activity, and ABTS activity, which are significant interaction-dependent parameters ($p<0.05$), were selected to perform the quadratics equation as shown in Eq. 3, 4, 5, 6, 7 and 8, respectively. Determination coefficients (R^2) of nut odor, teriyaki odor, savory, bitterness, DPPH, and ABTS were 0.834, 0.838, 0.894, and 0.958, respectively and the R^2 value higher than 0.75 indicated a good fit of the model [2]. The regression coefficients of ratio (X_1) in dependent parameters were higher than time (X_2). This could be explained by reaction time's stronger influence on sensory characteristics and antioxidant activities than ratio. The equations were used to perform response surface plots to determine the critical points and effectiveness of nut odor (Figure. 1a), teriyaki odor (Figure. 1b), savory (Figure. 1c), bitterness (Figure. 1d), DPPH (Figure. 1e), and ABTS (Figure. 1f). The results showed that reaction time 0 min had the highest nut odor and bitterness because MPI and Glu was non-heated and the reaction wasn't reacted. After heated 90, 120, and 150 min the reaction was reacted teriyaki odor and savory were increased while nut odor and bitterness were decreased that indicated heterocyclic compounds had been formed. MPI:Glu ratio of 1.5:1 reaction time 150 min (G403) had the highest teriyaki odor, savory and the lowest nut score unless bitterness score but could be acceptable G403 also had the lowest EC50 of DPPH and ABTS value of 0.21 and 0.45 mg/mL, respectively compared to all conditions demonstrated that reaction time and ratio of MPI:Glu was contributed to both of single electron transfer (SET) and hydrogen atom transfer (HAT) that the main reaction of DPPH and ABTS [2].

All figures were overlayed and selected the range of ratio and reaction time, which obtained the low nut odor, high teriyaki odor, high savory, low bitterness, low EC_{50} value of DPPH, and low EC_{50} value of ABTS inhibition. Resulted showed that the optimal condition of glycation to produce MRPs was a ratio of MPI:Glu was 2.0-3.0 (1:1 to 1.5:1) and reaction time 120-150 min. Therefore, the optimal condition of MRPs by glycation was MPI:Glu 1.5:1, and reaction time 150 min. The models were validated by observed and predicted values (Table 1). The predicted values were calculated from the equation model close to the observed value derived from the laboratory experiment. The equation models obtained from this experiment fit and were acceptable for MRPs preparation under glycation.

$$Nut = 1.624 – 0.578\, X_1 + 0.002\, X_2 + 0.191 X_1^2 – 0.001\, X_1X_2 + 1.710\times10^{-5}\, X_2^2 \quad (3)$$

$$Teriyaki = 1.424 + 0.028X_1 + 0.002X_2 – 0.016X_1^2 + 0.001X_1X_2 + 4.113\times10^{-6}\, X_2^2 \quad (4)$$

$$Savory = 2.561\times0.319\, X_1 + 0.000X_2 + 0.083X_1^2 + 1.190\times10^{-5}\, X_1X_2 + 1.053E\text{-}5\, X_2^2 \quad (5)$$

$$Bitterness = 1.270 – 0.936\, X_1 – 0.001\, X_2 + 0.191\, X_1^2 – 0.001\, X_1X_2 + 1.710\times10^{-5}\, X_2^2 \quad (6)$$

$$DPPH = 0.876 – 0.366\, X_1 + 0.001\, X_2 + 0.085\, X_1^2 + 0.000\, X_1X_2 – 1.164\times10^{-5}\, X_2^2 \quad (7)$$

$$ABTS = 2.924 – 1.472\, X_1 - 0.010\, X_2 + 0.281\, X_1^2 + 0.002\, X_1X_2 + 1.067\times10^{-5}\, X_2^2 \quad (8)$$

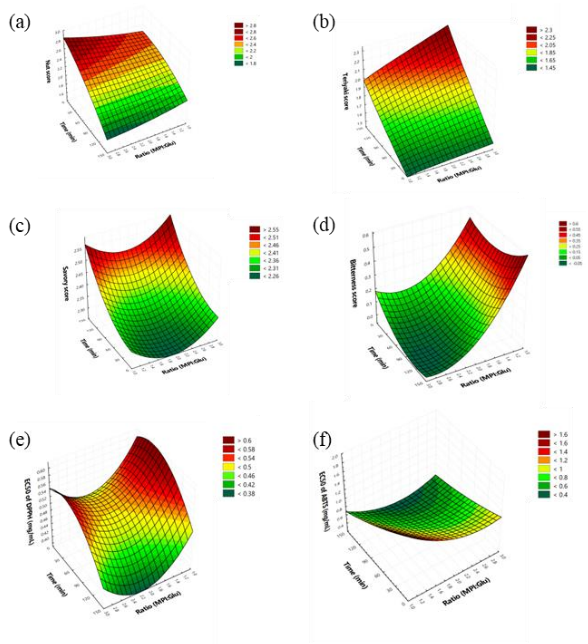

Fig. 1. The 3D surfaces plot of nut score (a), teriyaki score (b), savory score (c), bitterness (d), DPPH (e), and ABTS (f) of Maillard reaction products (MRPs) after production process by different ratio of MPI:Glu and reaction time (min).

Table 1. The predicted (Pre.) and experimental (Exp.) value of confirmation experiments

Response variables	Sample					
	G401		G402		G403	
	Pre.	Exp.	Pre.	Exp.	Pre.	Exp.
Nut score	2.11	1.98	2.07	1.96	2.00	1.60
Teriyaki score	1.98	1.88	2.03	1.92	2.10	2.25
Savory score	2.62	2.48	2.56	2.50	2.51	2.54
Bitterness	1.91	1.71	1.77	1.67	1.65	1.83
DPPH[a]	0.56	0.43	0.48	0.21	0.41	0.21
ABTS[b]	1.00	0.50	0.77	0.34	0.54	0.45

[a]DPPH and [b]ABTS radical scavenging activity was calculated as EC_{50} (mg/mL)

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3.2 FTIR spectrum of MRP

Mung bean meal and MPRs had protein as a main component, consist of peptides, carbonyl group, amino group, and carbohydrates. MPI used as a reference to compare with MRPs and glucose (Glu). MRP was chose for analysis of FTIR. The results showed that MRP had the strong peak O-H stretching of hydroxyl group at wavenumber 3500-3350 cm^{-1} a peak was decreased compared to MPI and Glu. The spectra exhibited bands in range of 2950-2850 cm^{-1} attributed C-H stretching of aliphatic and aromatic hydrogen another peak was C=O stretching at 1750-1650 cm^{-1} could be typical peak for protein. MRP spectrum had less intensity that could be an interaction between MPI and Glu, leading to Maillard reaction products. The absorptions in the 1400-1200 cm^{-1} associated with C-N stretching indicated MRP had C-N formation in the structure and C-O stretching at 1100-1000 cm^{-1} was evident the MRP had sugar derivatives compared to Glu. FTIR spectrum confirmed that MRP structure was changed compared to MPI and Glu after reaction [10].

3.3 Cellular bioactivities

3.3.1 Cytotoxicity of MRP

The *In Vitro* cellular bioactivity of mung bean protein isolate (MPI) and its Maillard reaction product derived from the optimal condition (MRP) were determined. The Vero cells were used to investigate cytotoxic activity in normal cells of MRP by MTT assay. The inhibitory effect was observed after 24h incubation. The survival rate of MRP were 82.10 %, this can define as MRP was low cytotoxicity in normal cells at a concentration of 10 mg/mL (Figure. 3a). Similarly, MPI was at the same concentration. The normal cell could survive 87.42%. Even though, cell viability in MRP was lower than MPI by approximately 5%, the number of healthy cells was over 80% [13]. Results indicated that MRP and MPI were not harmful for the normal cell at this concentration.

3.3.2 Anti-inflammatory of MRP

To investigate the suppression effect of NO release in RAW264.7 cells after pretreatment by MRP, MPI and control samples for 1 h and provoked by LPS for 24 h. The cell viability of all samples survived upper 80% at concentration of 1 mg/mL. MRP treatment showed a significant reduction of NO production 18.44 µM above 1 mg/mL and quite similar to NO production of MPI treatment (Figure. 3b). The NO release was 16.05 µM. Although NO production was not decreased in MRP treatment compared to MPI, Its NO value was significantly compared to LPS treatment with a NO value of 24.14 µM. The NO release of MRP treatment was higher than Diclofenac (12.76 µM), a commercial anti-inflammatory drug. Now, previous research lacks information related to the kinetics or metabolite profile, which explains anti-inflammatory in cells by Maillard reaction product. MRP may acted as inhibitor to block inflammatory cytokines and mRNA level of nuclear factor-kB in cell, resulted in low NO production [14]. NO equivalent treated by MRP was consistent with previous researches. Quinoa protein which was processed under glycation, significantly inhibited NO release. NO release of glycosylated quinoa protein (1 mg/mL) with xylose and mannose was 38.12 and 64.69 µM, respectively, whereas LPS treated was 88.24 µM [15]. Probably, MRP had the potential to be consumed as a functional food, which reduced inflammatory reactions. It could be developed and applied as an interesting source of potent anti-inflammatory food.

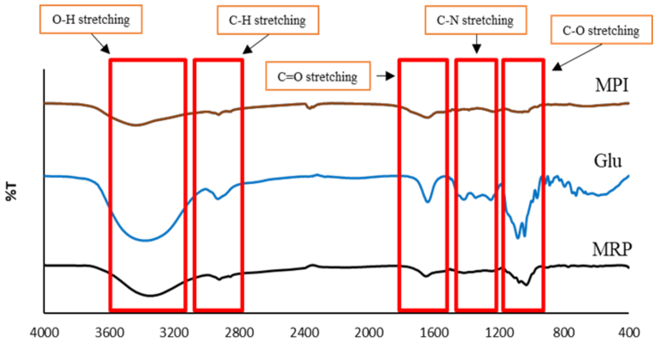

Fig. 2. FTIR spectrum of mung bean meal protein isolate (MPI), glucose (Glu), and Maillard reaction product (MRP).

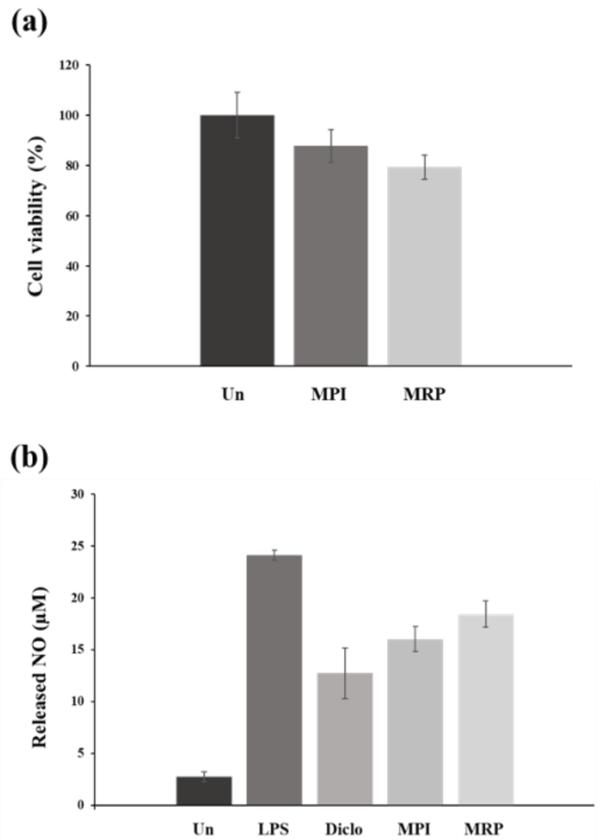

Fig. 3. Cytotoxicity of MRP compared to untreated (a). Anti-inflammatory activity of MRP compared to untreated, LPS and Diclofenac (b).

4 Conclusion

In this study, Maillard reaction product derived from mung bean meal isolated and reacted with glucose contributed to a potential functional agent that served teriyaki and savory characteristics, strong antioxidant activity in both DPPH radical and ABTS radical, and suppressed anti-inflammatory in cells. The MRP can be potentially used as a food flavoring with bioactivity includes antioxidant activity and anti-inflammatory activity.

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