

A METHOD OF PREPARING A MEAT ANALOGUE

References to Related Application

- 5 This application claims priority to Singapore application number 10202251432E filed with the Intellectual Property Office of Singapore on 20 October 2022, the contents of which are hereby incorporated by reference.

Technical Field

- The present invention generally relates to a method of preparing a meat analogue.
10 The present invention further relates to a meat analogue prepared by the method.

Background Art

- There has been a growing interest in meat analogues due to greater awareness about healthy and sustainable foods. Meat production has been identified as a source of environmental change and natural resource depletion. Hence, consumers are looking
15 to reduce their meat consumption and have turned to options such as plant-based foods. Meat analogues are food products that resemble muscle meats, such as lean chicken breast, in terms of appearance, colour, texture, and structure. Meat analogues exhibit anisotropic (e.g., layered or fibrous) structures that give an appearance and taste-texture sensation similar to muscle meats. Meat analogues are predominantly
20 made from plant proteins, and common sources include wheat, soy, pea, peanut, lupin, and mung bean. Meat analogues can be produced by various methods involving rearranging protein fibres to produce textures and structures similar to meats. Conventional methods to prepare meat analogues include extrusion, freeze structuring, electrospinning, in-vitro animal cell culture, and shear cell technology.
25 In addition to these methods, traditional Asian meat analogues, known as seitan, are made using a relatively simple process of filtering gluten from wheat flour, kneading it into gluten masses, and cooking it in various sauces. Out of these conventional technologies, high-moisture extrusion processing has been the preferred and widely used technique of choice, notably by large-scale Asian manufacturers and especially
30 amongst Western manufacturers. This is because of its scalability and the consistent quality of the textured products produced.

- Another conventional and more affordable technique uses less sophisticated equipment known as protein elongation, which demonstrated potential in forming a product with textures having no significant difference from chicken. This technique
35 involves stretching and orientating the fine fibrils of a self-assembled porous zein network, resulting in anisotropic structures resembling meat fibres when a composite gel of soy protein is added. This method allowed greater control over the formation

of the fibrous structures, whereby the extent of stretching can further manipulate fibre width and the number of fibres.

Wheat gluten (WG) is a potential food material to form fibrous structures via protein elongation. WG is composed of a three-dimensional network of linearly cross-linked
5 glutenin subunits and gliadin protein. It forms a cohesive viscoelastic network connected by intramolecular and intermolecular disulphide bonds. The formations of interchain disulphide bond cross-linking are due to the aggregation of glutenins in WG. Networks of both WG and zein were observed to be able to self-assemble upon
10 hydration, where they exhibited three-dimensional networks with porous foam-like matrix microstructure at smaller length scales. There is a widespread use of WG in various Asian foods, but it is unclear whether WG is an appropriate substitute for zein in developing meat analogues (i.e., mixed protein composites) or not.

Accordingly, there is a need for a method of preparing a meat analogue that ameliorates one or more disadvantages mentioned above.

15 **Summary**

In one aspect, there is provided a method of preparing a meat analogue, comprising the steps of:

- (a) blending a mixture comprising wheat gluten and a protein source at a weight ratio in the range of about 50:50 to about 70:30;
- 20 (b) heating the mixture of step (a) in the presence of water at a temperature in the range of about 100 °C to about 140 °C to form a dough; and
- (c) cutting the dough of step (b) to form the meat analogue.

In another aspect, there is provided a meat analogue prepared by the method as described herein.

25 **Definitions**

The following words and terms used herein shall have the meaning indicated:

The word “substantially” does not exclude “completely” e.g. a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

- 30 Unless specified otherwise, the terms "comprising" and "comprise", and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, unrecited elements.

- 35 The term "about" as used herein typically means +/- 5 % of the stated value, more typically +/- 4 % of the stated value, more typically +/- 3 % of the stated value, more

typically, $\pm 2\%$ of the stated value, even more typically $\pm 1\%$ of the stated value, and even more typically $\pm 0.5\%$ of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Detailed Disclosure of Embodiments

Exemplary, non-limiting embodiments of a method of preparing a meat analogue will now be disclosed.

The method comprises the steps of:

(a) blending a mixture comprising wheat gluten and a protein source at a weight ratio in the range of about 50:50 to about 70:30;

(b) heating the mixture of step (a) in the presence of water at a temperature in the range of about 100 °C to about 140 °C to form a dough; and

(c) cutting the dough of step (b) to form the meat analogue.

The mixture is fully mixed or incorporated after the blending step (a). Therefore, the blending step (a) may comprise blending the mixture at a speed in the range of about 700 rpm to about 900 rpm, about 800 rpm to about 900 rpm or about 700 rpm to about 800 rpm. The blending step (a) may comprise blending the mixture at a speed of about 800 rpm.

The blending step (a) may comprise blending the mixture for a duration in the range of about 20 seconds to about 40 seconds, about 30 seconds to about 40 seconds or about 20 seconds to about 30 seconds. The blending step (a) may comprise blending the mixture for a duration of about 30 seconds.

The protein source may be any edible material (except wheat gluten) that comprises protein. The protein in the protein source may be concentrated and/or isolated before being used in the present method, thus the protein source may be in the form of a concentrate or an isolate. Non-limiting examples of the protein source include mung bean protein, soy protein, mycoprotein, pea protein, lentil protein, industrial hemp protein, chickpea protein, basil seed protein, pumpkin seed protein, almond protein, quinoa protein, nuts protein, textured vegetable protein, tempeh protein, rice (such as white rice or brown rice) protein, spirulina protein, peanut protein, legume protein, tofu protein, beans (such as faba beans or edamame) protein, seitan protein, nutritional yeast protein, and their concentrate, isolate and combinations thereof.

The mixture may comprise wheat gluten and the protein source at a weight ratio in the range of about 50:50 to about 70:30, about 60:40 to about 70:30 or about 50:50 to about 60:40. The mixture may comprise wheat gluten and the protein source at a weight ratio of about 60:40.

Advantageously, the meat analogue has an optimal fibre formation when the mixture comprises wheat gluten and the protein source at a weight ratio as described herein.

The blending step (a) may comprise blending a mixture comprising wheat gluten, the protein source and a mycoprotein.

The method may further comprise a step (a1) of treating the mycoprotein before the blending step (a).

The treating step (a1) may comprise adjusting a pH value of the mycoprotein. The treating step (a1) may comprise adjusting the pH value of the mycoprotein to about 12, followed by adjusting the pH value of the mycoprotein to about 7.

The treating step (a1) may alternatively or additionally comprise ultrasonication of the mycoprotein. The treating step (a1) may comprise ultrasonication of the mycoprotein for a duration in the range of about 20 minutes to about 40 minutes, about 30 minutes to about 40 minutes or about 20 minutes to about 30 minutes. The treating step (a1) may comprise ultrasonication of the mycoprotein for a duration of about 30 minutes.

Advantageously, incorporating treated mycoprotein in the meat analogue may improve macrostructure, microstructure and textures of the meat analogue prepared by the method and form better fibres within the meat analogue.

The mixture of the blending step (a) may comprise mycoprotein (where present) at a weight percentage in the range of about 10 weight% to about 20 weight%, about 15 weight% to about 20 weight% or about 10 weight% to about 15 weight%, based on the total weight of the mixture. The mixture of the blending step (a) may comprise mycoprotein (where present) at a weight percentage of about 15 weight% based on the total weight of the mixture. Advantageously, incorporating about 15 weight% of the mycoprotein in the mixture may improve macrostructure, microstructure and

textures of the meat analogue prepared by the method and form better fibres within the meat analogue.

The mixture may further comprise an additive. The additives may help to improve a taste or a texture (such as forming better fibres) of the meat analogue.

- 5 The additive may comprise flavouring, seasoning, sodium tri-polyphosphate or a combination thereof. The seasoning may be a vegetarian all-in-one seasoning.

The additive may comprise seasoning and sodium tri-polyphosphate at a weight ratio in the range of about 1:1 to 1:3, about 1:2 to about 1:3 or about 1:1 to about 1:2. The additive may comprise seasoning and sodium tri-polyphosphate at a weight ratio of about 1:2.

10 The mixture of the blending step (a) may comprise additive (where present at a weight percentage in the range of about 2 weight% to about 4 weight%, about 3 weight% to about 4 weight% or about 2 weight% to about 3 weight%, based on the total weight of the mixture. The mixture of the blending step (a) may comprise additive (where present) at a weight percentage of about 3 weight%.

In the heating step (b), proteins in the wheat gluten and the protein source are heated at a sufficiently high temperature to denature (or “melt”) the proteins. Accordingly, the temperature needs to be above a denaturation temperature of the proteins (which is typically above 70 °C for plant proteins).

- 20 Advantageously, when the proteins are denatured, they lose their quaternary, tertiary and secondary structures and the proteins become unfolded with covalent bonds broken and interactions between amino acid chains disrupted. Therefore, the heating step allows the meat analogue to have a better alignment of proteins during the cutting step and produce more fibrous structures. This is not possible when the heating step is undertaken at a temperature below 70 °C (e.g., at 60 °C).

25 The heating step (b) may comprise heating the mixture of the blending step (a) in the presence of water at the temperature in the range of about 100 °C to about 140 °C, or at a temperature in the range of about 120 °C to about 140 °C or about 100 °C to about 120 °C. The heating step (b) may comprise heating the mixture of the blending step (a) in the presence of water at a temperature of about 120 °C.

30 The heating step (b) may comprise heating the mixture of the blending step (a) in the presence of water for a duration in the range of about 8 minutes to about 12 minutes, about 10 minutes to about 12 minutes or about 8 minutes to about 10 minutes. The heating step (b) may comprise heating the mixture of the blending step (a) in the presence of water for a duration of about 10 minutes.

The heating step (b) may further comprise blending the mixture of the blending step (a) in the presence of water at a speed in the range of about 300 rpm to about 400 rpm, about 350 rpm to about 400 rpm or about 300 rpm to about 350 rpm. The heating

step (b) may further comprise blending the mixture of the blending step (a) in the presence of water at a speed of about 350 rpm.

- 5 In the heating step (b), the mixture of the blending step (a) and water may have a weight ratio in the range of about 8:11 to about 9:11, about 8.5:11 to about 9:11 or about 8:11 to about 8.5:11. The mixture of the blending step (a) and water may have a weight ratio of about 42:55.

The method may further comprise a step (b1) of cooling the dough of the heating step (b) after the heating step (b) but before the cutting step (c).

- 10 The cooling step (b1) may comprise cooling the dough of the heating step (b) for a duration in the range of about 3 minutes to about 7 minutes, about 5 minutes to about 7 minutes or about 3 minutes to about 5 minutes. The cooling step (b1) may comprise cooling the dough of the heating step (b) for a duration of about 5 minutes.

- 15 The cooling step (b1) may further comprise blending the dough of the heating step (b) at a speed in the range of about 150 rpm to about 250 rpm, about 200 rpm to about 250 rpm or about 150 rpm to about 200 rpm. The cooling step (b1) may further comprise blending the dough of the heating step (b) at a speed of about 200 rpm.

- 20 The cutting step (c) may comprise blending the dough of the heating step (b) at a speed in the range of about 2500 rpm to about 3500 rpm, about 3000 rpm to about 3500 rpm or about 2500 rpm to about 3000 rpm. The cutting step (c) may comprise blending the dough of the heating step (b) at a speed of about 3100 rpm.

The cutting step (c) may comprise blending the dough of the heating step (b) for a duration in the range of 1 minute to about 3 minutes, about 2 minutes to about 3 minutes or about 1 minute to about 2 minutes. The cutting step (c) may comprise blending the dough of the heating step (b) for a duration of about 2 minutes.

- 25 As the dough of the heating step (b) comprises protein fragments, the protein fragments may be stretched and pulled during the cutting step (c) so as to be aligned and agglomerated. Therefore, the meat analogue may comprise a strong anisotropic network of elongated protein fragments.

- 30 The method may further comprise a step (c1) of chilling the meat analogue after the cutting step (c).

The chilling step (c1) may comprise chilling the meat analogue at a temperature in the range of about 5 °C to about 10 °C, about 7 °C to about 10 °C or about 5 °C to about 7 °C. The chilling step (c1) may comprise chilling the meat analogue at a temperature of about 7 °C.

- 35 The chilling step (c1) may comprise chilling the meat analogue for a duration in the range of about 30 minutes to about 1 hour, about 45 minutes to about 1 hour or about 30 minutes to about 45 minutes.

In the chilling step (c1), the meat analogue may be wrapped in a layer of aluminium foil.

Alternatively or additionally, the method may further comprise a step (c2) of freezing the meat analogue after the cutting step (c) or the chilling step (c1) (where present).

- 5 The freezing step (c2) may comprise freezing the meat analogue at a temperature in the range of about -30 °C to about -10 °C, about -20 °C to about -10 °C or about -30 °C to about -20 °C.

- The freezing step (c2) may comprise freezing the meat analogue for a duration in the range of about 6 hours to about 10 hours, about 8 hours to about 10 hours or about 6
10 hours to about 8 hours. The freezing step (c2) may comprise freezing the meat analogue overnight.

The method may be undertaken in a multi-cooker.

Exemplary, non-limiting embodiments of a meat analogue will now be disclosed.

- The meat analogue may be prepared by the method as described herein. As mentioned
15 above, the meat analogue may comprise a strong anisotropic network of elongated protein fragments.

Brief Description of Drawings

- The accompanying drawings illustrate a disclosed embodiment and serves to explain the principles of the disclosed embodiment. It is to be understood, however, that the
20 drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

FIG. 1

[FIG. 1] is a schematic illustration of the protein elongation method to produce meat analogues.

25 FIG. 2

[FIG. 2] shows visual observation of cross-sectional view of mixed plant protein composites at different wheat gluten to mung bean protein isolate (WG-MBPI) ratios of 80:20, 60:40, 40:60 and 20:80, and scanning electron micrograph of the cross-sectional view of mixed plant protein composite at WG-MBPI ratio of 60:40.

30 FIGS. 3A to 3G

- [FIG. 3A] and [FIG. 3B] are scanning electron micrographs of untreated and treated mycoprotein (MCP) powders at 500x and 250x magnification, respectively. [FIG. 3C] to [FIG. 3G] are scanning electron micrographs of the cross-sections of meat
35 analogues at 60:40 WG-MBPI ratio at 1000x magnification of control, 15-UT, 30-UT, 15-T and 30-T, respectively.

FIG. 4

[FIG. 4] shows the amount of protein solubilised from meat analogues at WG-MBPI ratio of 60:40 with the incorporation of untreated and treated MCP at 15 and 30% w/w induced by different extracting solutions P, PU, PD, and PS. Data represent the mean, and error bars represent the standard deviation. Overall one-way ANOVA was found to be significant ($p \leq 0.05$). Values bearing different lowercase alphabets within each extracting solution are significantly different from each other ($p \leq 0.05$) according to Tukey's posthoc test.

FIG. 5

[FIG. 5] shows visual observation of cross-sectional view of mixed protein composites at WG-MBPI ratio of 60:40 with the incorporation of untreated and treated MCP at 15% and 30% w/w.

Examples

Non-limiting examples of the invention will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

Example 1 – Preparation of Meat Analogues by Protein Elongation Method

Various combinations of wheat gluten (WG, Bob's Red Mill, 70% protein, 20% carbohydrate, 3.5% moisture, 3.5% ash and 3% fat, purchased from Phoon Huat Pte Ltd, Singapore) and Mung bean protein isolate (MBPI, Munptein™ Type B, 80% protein, 10% moisture and 8% ash, purchased from ETprotein, China) as the protein source were prepared together with vegetarian all-in-one seasoning (purchased from Unilever, Singapore), sodium tri-polyposphate (purchased from Kimic Chemtech (S) Pte Ltd, Singapore) and ultrapure water (purified using Milli-Q equipment from Millipore Corporation, Massachusetts, USA) as shown in Table 1.

Table 1. Formulation of mixed plant protein composites at different wheat gluten to mung bean protein isolate (WG-MBPI) ratios.

Sample	Formulation (% w/w)			
	Water	Wheat gluten	Mung bean protein	Other ingredients*
80%WG (20%MBPI)	55	33.6	8.4	3
60%WG (40%MBPI)	55	25.2	16.8	3

40%WG (60%MBPI)	55	16.8	25.2	3
20%WG (80%MBPI)	55	8.4	33.6	3

* include vegetarian all-in-one seasoning (1%) and sodium tri-polyphosphate (2%).

- The minimum amount of WG in the formulation was 60%WG, as a proper dough was not obtained at 20% or 40%WG. This was because MBPI had no/insufficient binding/texturizing agent (WG) to hold the dough together. 60%WG was better than 80%WG as it had better fibre formation visually. The WG-MBPI ratio of 60:40 was selected as the optimal ratio to form the meat analogues using a multi-cooker (TM6, Thermomix®, France) based on preliminary studies, as shown in Table 2. Meat analogues were prepared as shown in FIG. 1. The dry ingredients for each formulation were weighted (102) into the multi-cooker receptacle and were mixed at 800 rpm for 30 seconds (104). Water was then added to the receptacle and blended at 350 rpm for 1 minute, followed by heating to 120 °C for 10 minutes to form the dough (106). The dough was then left to cool while mixing at 200 rpm for 5 minutes (108), with the lid of the multi-cooker receptacle removed to facilitate the cooling process. The dough was cut at 3,100 rpm for 2 minutes (110) to stretch and pull small protein fragments to form a secondary dough via the high rotational speed of a knife blade (112). The stretching and pulling would align the protein networks, allowing the proteins to agglomerate and form stronger networks (114). The dough was then wrapped in a layer of aluminium foil and left to chill at 7 °C overnight before further analysis.
- Untreated or treated mycoprotein (MCP) was also included in some formulations. MCP powder was obtained by freeze-drying meat-free pieces (containing 95% of MCP, Quorn™, UK, purchased from NTUC FairPrice Co-operative Ltd, Singapore) in a laboratory-scaled freeze dryer (4KXL, VirTis, USA) for 72 hours at -80 °C and 1 Pa, followed by blending in a powder grinder (WSG60, Waring, USA) for 1 minute at 20,000 rpm. The MCP obtained was hydrated (2.5% w/w) with ultrapure water and magnetically stirred at 25 °C overnight before drying further to form untreated MCP.

- The treated MCP was obtained by adjusting the pH of the hydrated MCP to 12, stirring the dispersion for 30 minutes, followed by additional pH adjustments where required, as well as ultrasonication. The dispersion was then changed back to pH 7 and stirred for an additional 30 minutes, followed by any pH readjustments, where required. Both samples were frozen at -20 °C overnight before lyophilizing for 96 hours at -80 °C and 1 Pa. The freeze-dried samples were blended for 1 minute at 20,000 rpm and then placed into a desiccator until further use.

Table 2. Formulation of meat analogues at different wheat gluten-mung bean protein isolate (WG-MBPI) mixture to mycoprotein (MCP) ratios.

Sample#	Formulation (% w/w)				
	Water	WG-MBPI (ratio of 60:40)	Untreated MCP	Treated MCP	Other ingredients*
Control	55	42.0	0	0	3
15-UT	55	35.7	6.7	0	3
30-UT	55	29.4	12.6	0	3
15-T	55	35.7	0	6.3	3
30-T	55	29.4	0	12.6	3

* include vegetarian all-in-one seasoning (1%) and sodium tri-polyphosphate (2%).

- 5 # Control: meat analogues at WG-MBPI ratio of 60:40; 15-UT: 15% replacement of WG-MBPI with untreated MCP; 15-T: 15% replacement of WG-MBPI with treated MCP; 30-UT: 30% replacement of WG-MBPI with untreated MCP; 30-T: 30% replacement of WG-MBPI with treated MCP.

Example 2 – Physicochemical Properties of Meat Analogues

Protein content analysis

- 10 The protein content of meat analogues was determined by the Kjeldahl method using Tecator™ Digester 8, Tecator™ Scrubber, and Kjeltect™ 8200 Auto Distilling Unit (FOSS Analytical, Hoganas, Sweden). A nitrogen conversion factor of 6.25 for MCP and MBPI, and 5.7 for WG was used.

Moisture content analysis

- 15 The moisture content of meat analogues was determined using the air-oven method. Approximately five grams of samples were cut into small pieces and weighed into numbered pans. Drying was conducted in an oven (111 Eco Line, Venticell, Germany) at 105 °C for 24 hours, and the weight of the pans was recorded after cooling in a desiccator for 2 hours.

pH analysis

- 20 The pH of meat analogues was analysed using a benchtop pH meter (SevenCompact™ pH/ Ion S220, Mettler-Toledo, Switzerland) to measure the pH of the samples as described by Chiang, Loveday, Hardacre and Parker (2019). pH calibration was done using buffer solutions (pH 4, 7, and 10). The pH values were taken after blending the samples at 12,000 rpm, with ultrapure water at 20% w/w concentration, for 1 minute using a high-shear mixer (T25 digital Ultra Turrax®, IKA, Germany).
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Data Analysis

All experimental work was carried out in three replicates, where the results were reported as means $\pm$ standard deviations. Data were analysed using the one-way analysis of variance (ANOVA), followed by Tukey's pairwise comparison of means ($p \leq 0.05$) with Minitab[®] 21.1.1 statistical software (Minitab Inc., USA). Figures were plotted and exported using Origin 2020 software (OriginLab Corp, Massachusetts, USA).

Results of Measurement

The protein, moisture, and pH of the meat analogues at different MCP ratios are presented in Table 3. Untreated and treated MCP were incorporated into WG-MBPI meat analogues at ratios of 15:85 and 30:70 prepared using the protein elongation method. Meat analogues with 30% w/w untreated or treated MCP showed lower protein content, as MCP powder was found to have a lower protein content of $54.76 \pm 0.44\%$ than the WG-MBPI mixture (data not shown). No significant difference was observed between the moisture contents of all meat analogues prepared (see Table 3), which was due to the incorporation of the same amount of water in the analogue formulations. Individually, the water holding capacity (WHC) of WG was reported to be around 1.58 g water/g protein while the WHC of MBPI was around 3.33 g water/g protein. It is also possible that the blend of WG-MBPI at the ratio of 60:40 resulted in a similar WHC as MCP, resulting in no significant difference observed in the moisture contents in all meat analogue samples (Table 3). Among the samples, control had the highest pH while samples containing untreated MCP have the lowest pH. As the native pH of the untreated MCP solution was 5.60 ± 0.16 , when added into the meat analogue formulation, the pH of 15-UT and 30-UT samples decreased with increasing concentrations of untreated MCP added (Table 3). In the case of treated MCP, MCP was adjusted to pH 7 during the pH shift method. Hence, the pH of meat analogues made (i.e., 15-T and 30-T) remained near neutral (Table 3).

Table 3. Protein, moisture, and pH level of meat analogues at wheat gluten to mung bean protein isolate (WG-MBPI) ratio of 60:40 with the incorporation of untreated and treated mycoprotein (MCP) at 15 and 30% w/w.

Sample description	Physicochemical properties ¹		
	Protein content (%)	Moisture content (%) [#]	pH
Control	31.50 ± 1.02^a	55.98 ± 0.96	7.41 ± 0.02^a
15-UT	31.04 ± 1.03^a	54.75 ± 0.78	7.10 ± 0.03^c
30-UT	29.80 ± 1.56^{ab}	55.67 ± 0.64	6.87 ± 0.01^d

15-T	30.27 ± 1.08^{ab}	55.26 ± 0.76	7.29 ± 0.04^b
30-T	28.66 ± 0.90^b	55.75 ± 0.55	7.28 ± 0.03^b
p-values	0.002	0.065	0.000
F-values	5.65	2.53	175.37

¹ Data are presented as the mean and standard deviation of three replicates.

[#] Compound found to have no significant difference among the samples using ANOVA. Values bearing different lowercase letters under the same column were significantly different from each other ($p \leq 0.05$) according to Tukey's posthoc test.

- 5 Visual observation of cross-sectional view of meat analogues at WG-MBPI ratio of 60:40, with the incorporation of treated and untreated MCP at 15% and 30% are presented in FIG. 5. The samples were deformed manually to inspect for fibre formation. Among the meat analogues, the ones made with 15% treated and untreated MCP exhibited the most visible horizontally aligned macrostructures upon tearing,
- 10 with prominent fibrous structures observed. As the amount of MCP increased, its fibre formation was less defined. Meat analogues containing 30% treated and untreated MCP also displayed brittle and dense structures when torn apart. Hence, it can be concluded that the incorporation of 15% treated and untreated MCP is more suitable for producing meat analogues with better aligned macrostructures and
- 15 fibrous structures as compared to 30%.

Example 3 – Textural Properties of Meat Analogues

- A two-bite test was conducted to determine the textural properties of the meat analogues using a texture analyser (TA.XT Plus, Stable Micro Systems, UK). Here, samples were cut into a size of 15×15×10 mm, and then compressed using a P/75
- 20 probe to 50% of its original thickness at a speed of 1 mm/s for the first bite, returned to the original position over 5 seconds, followed by the second bite at 1 mm/s to 50% of the first compressed thickness. Hardness at 50% deformation, springiness and chewiness were obtained from the texture profile (force/ time) curves.

- The textural properties of meat analogues with different MCP ratios are presented in
- 25 Table 4 below. Textural property analysis was performed on the samples using a double compression test. With the addition of MCP, the hardness of the meat analogues increased, and its springiness decreased, indicating that the analogues became firmer. The springiness of the meat analogues was also significantly lowered with increasing amounts of MCP added, regardless of treatment. In addition, no
- 30 significant differences in hardness, springiness, and chewiness were observed between the meat analogues made with 30% treated or 30% untreated MCP, indicating that the functionalisation of the MCP did not have an impact on the texture of the meat analogues.

Table 4. Textural properties of meat analogues at wheat gluten to mung bean protein isolate (WG-MBPI) ratio of 60:40 with the incorporation of untreated and treated mycoprotein (MCP) at 15 and 30% w/w.

Sample description	Textural properties ¹		
	Hardness (N)	Springiness	Chewiness (N)
Control	22.16 ± 2.59 ^c	0.87 ± 0.03 ^a	14.63 ± 2.55 ^c
15-UT	31.65 ± 2.25 ^a	0.84 ± 0.02 ^{ab}	18.78 ± 1.22 ^a
30-UT	33.55 ± 0.98 ^a	0.81 ± 0.01 ^b	18.20 ± 0.87 ^{ab}
15-T	27.36 ± 0.87 ^b	0.84 ± 0.02 ^{ab}	16.10 ± 0.81 ^{bc}
30-T	33.51 ± 2.62 ^a	0.81 ± 0.04 ^b	18.72 ± 1.66 ^{ab}
<i>p</i> -values	0.000	0.002	0.000
F-values	34.52	5.65	8.40

¹ Data are presented as the mean and standard deviation of three replicates.

- 5 # Compound found to have no significant difference among the samples using ANOVA. Values bearing different lowercase letters under the same column were significantly different from each other ($p \leq 0.05$) according to Tukey's posthoc test.

10 All meat analogues with MCP incorporated were also found to be softer and slightly chewier than the steamed chicken breast (hardness of 40.53 ± 6.14 , chewiness of 17.80 ± 5.75). This shows that all meat analogues made with WG, MBPI, and treated or untreated MCP using the protein elongation method could produce products that have similar mouthfeel and texture to chicken breast.

Example 4 – Microstructural Properties of Meat Analogues

15 Meat analogues (sectioned to 3×5 mm) were chemically fixated using 4% formaldehyde and 2% glutaraldehyde (EM grade, purchased from Electron Microscopy Sciences, USA) in 0.05M HEPES buffer (pH 7.4, purchased from ThermoFisher Scientific, Singapore) for 1.5 hours. The samples were then post-fixed with osmium tetroxide (1% in distilled water, purchased from ThermoFisher Scientific, Singapore) for 1.5 hours and dehydrated in ethanol (purchased from
20 ThermoFisher Scientific, Singapore) in a graded series of up to 100%. Samples were dried using critical point drying (Leica EM CPD030, Leica Microsystems, Germany) and mounted onto aluminium stubs with double-sided carbon tape. Platinum (Leica EM SCD050, Leica Microsystems, Germany) was sputter-coated onto the samples in a 4 nm layer, and SEM was performed using a field emission scanning electron
25 microscope (JSM-6701F, JEOL, Japan) operating at 10 kV. Images were collected

from three different regions of interest (ROI) for each sample and technical replicated at 1000× magnification.

- It was observed that the meat analogue without the addition of MCP (control) in FIG. 3C had filament-like fibre strands when viewed under SEM (depicted by arrows on the micrographs). This was likely due to the gluten network formation. Globules of proteins were also found in the control samples (depicted by circles). In mung bean, 85% of proteins present are storage proteins, namely globulin (60%) and albumin (25%). Hence, the globular proteins depicted in FIG. 3C was suspected to be globular proteins from MBPI.
- In the samples containing MCP, hollowed-out filament of fibre can be seen in FIGS. 3D – 3G, which are highlighted in circles. These hollowed-out fibres were concluded to be the MCP fibres, as they were not present in the control samples. In the 15-UT and 30-UT samples, the hollow filaments were present in a large number and were very distinct. In comparison, they were less pronounced in the 15-T and 30-T samples. There was also a larger amount of gluten fibres (arrows) formed in both the analogues made with the treated MCP, as compared to those with the untreated MCP. This could be due to the change in structural properties of the MCP after it had been treated with both the pH shifting and ultrasonication process. In addition, unlike in the 30-T samples whereby the gluten network fibre strands were still visible, the 30-UT samples also had less pronounced fibre-like structure. Although no difference was observed in terms of texture when the samples were torn apart manually (as shown in FIG. 5), the fibre formation when the meat analogues were viewed under SEM were better in the samples with treated MCP.

Example 5 – Chemical Bond Elucidation by Selective Solubilisation

- The protein-protein interaction of the meat analogues was analysed. Four extracting solutions (1–4) were used to dissolve specific chemical bonds within the samples to assess protein solubility: (1) P (PB; 0.1 M phosphate buffer consisting of KH_2PO_4 and K_2HPO_4 with a pH of 7.5, native state protein), (2) PU (PB+8M U; hydrogen bonds), (3) PD (PB+0.05 M DTT; disulphide bonds), and (4) PS (1.5 g/100 mL SDS, hydrophobic interactions) (all reagents were purchased from Sigma-Aldrich, Singapore).

- Meat analogues (0.5 g) were extracted with 10 mL of each extracting solution on a shaker (LSE™, Corning, Slovenia) at 400 rpm for 30 minutes. A high-shear mixer (T25 digital Ultra Turrax®, IKA, Germany) was used to blend the samples at 12,000 rpm for 30 seconds. The mixture was then shaken again for 30 minutes, followed by centrifugation at 2580×g for 10 minutes. The supernatant was transferred into an Eppendorf tube and centrifuged at 9300×g for 10 minutes. Bradford protein assay was carried out.

- Based on the intermolecular bond analysis (FIG. 4), the poor solubility in P for all samples indicates that proteins were denatured during heating. With other reagents

(e.g., urea, DTT and SDS) mixed with P, the amount of protein extracted from meat analogues increased, indicating that the aggregated protein in meat analogues had more than one type of chemical bonds. The amount of protein extracted from highest to lowest among the two-component solvents was PU > PS > PD, suggesting that the protein in meat analogues was mainly associated with hydrogen bonds, followed by hydrophobic interactions, then disulfide bonds. It was interesting to note that the amount of protein extracted by PD was lower as compared to other studies using WG:SPI composited meat analogues. This could be due to MBPI lacking sulphur-containing amino acids, which caused a lower amount of disulfide bonds formed during the development process.

Therefore, the utilization of ultrasonication in combination with the pH shift method at pH 12>7 was successful in the functionalization of the MCP. The incorporation of the treated MCP at 15% was also deemed to be the most suitable in terms of the macro- and ultrastructural properties of the meat analogues obtained. This shows the potential application of MCP in meat analogues made with WG-MBPI composites.

Summary of Examples

By using the protein elongation method on composite mixtures of WG, MBPI, and MCP, meat analogues were successfully produced with good macrostructure and textural properties. The analogues had harder textures with the increase in both treated and untreated MCP concentrations. Results from intermolecular forces analysis suggested that the forces responsible for the formation, stabilisation and retention of the structures within the meat analogues were in the following order from strongest to weakest: hydrogen bonds > hydrophobic interactions > disulfide bonds. Meat analogues made with 15% treated MCP also showed the best fibre formation according to the SEM analysis conducted. This shows the potential of incorporating treated MCP in meat analogues at 15% to obtain good macro- and microstructure, as well as good textures.

Industrial Applicability

The method of the disclosure may be used in a variety of applications such as preparation of plant-based meat and other vegetarian dishes.

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

Claims

1. A method of preparing a meat analogue, comprising the steps of:
 - (a) blending a mixture comprising wheat gluten and a protein source at a weight ratio in the range of 50:50 to 70:30;
 - 5 (b) heating the mixture of the blending step (a) in the presence of water at a temperature in the range of 100 °C to 140 °C to form a dough; and
 - (c) cutting the dough of the heating step (b) to form the meat analogue.
- 10 2. The method of claim 1, wherein the blending step (a) comprises blending the mixture at a speed in the range of 700 rpm to 900 rpm.
3. The method of claim 1 or 2, wherein the blending step (a) comprises blending the mixture for a duration in the range of 20 seconds to 40 seconds.
- 15 4. The method of any one of claims 1 to 3, wherein in the blending step (a), the mixture comprises wheat gluten and the protein source at a weight ratio of 60:40.
5. The method of any one of claims 1 to 4, wherein the blending step (a) comprises
20 blending a mixture comprising wheat gluten, the protein source and a mycoprotein.
6. The method of claim 5, further comprising a step (a1) of treating the mycoprotein before the blending step (a).
- 25 7. The method of claim 5 or 6, wherein in the blending step (a), the mixture comprises mycoprotein at a weight percentage in the range of 10 weight% to 20 weight% based on the total weight of the mixture.
8. The method of any one of claims 1 to 7, wherein in the blending step (a), the
30 mixture further comprises an additive.
9. The method of any one of claims 1 to 8, wherein the heating step (b) comprises heating the mixture of the blending step (a) in the presence of water for a duration in the range of 8 minutes to 12 minutes.
- 35 10. The method of any one of claims 1 to 9, wherein the heating step (b) further comprises blending the mixture of the blending step (a) in the presence of water at a speed in the range of 300 rpm to 400 rpm.
- 40 11. The method of any one of claims 1 to 10, wherein in the heating step (b), the mixture of the blending step (a) and water have a weight ratio in the range of 8:11 to 9:11.
12. The method of any one of claims 1 to 11, further comprising a step (b1) of cooling the dough of the heating step (b) after the heating step (b) but before the cutting step (c).
- 45 13. The method of claim 12, wherein the cooling step (b1) comprises cooling the dough of the heating step (b) for a duration in the range of 3 minutes to 7 minutes.

14. The method of claim 12 or 13, wherein the cooling step (b1) further comprises blending the dough of the heating step (b) at a speed in the range of 150 rpm to 250 rpm.
- 5 15. The method of any one of claims 1 to 14, wherein the cutting step (c) comprises blending the dough of the heating step (b) at a speed in the range of 2500 rpm to 3500 rpm.
- 10 16. The method of any one of claims 1 to 15, wherein the cutting step (c) comprises blending the dough of the heating step (b) for a duration in the range of 1 minute to 3 minutes.
17. The method of any one of claims 1 to 16, further comprising a step (c1) of chilling the meat analogue after the cutting step (c).
- 15 18. The method of claim 17, wherein the chilling step (c1) comprises chilling the meat analogue at a temperature in the range of 5 °C to 10 °C.
19. A meat analogue prepared by the method of any one of claims 1 to 18.