

A METHOD OF OBTAINING PROTEIN FRACTIONS

References to Related Application

- 5 This application claims priority to Singapore application number 10202251374A filed with the Intellectual Property Office of Singapore on 13 October 2022, the contents of which are hereby incorporated by reference.

Technical Field

- 10 The present invention generally relates to a method of obtaining protein fractions from a protein sample. The present invention further relates to a protein fraction obtained from the method.

Background Art

- 15 Plant proteins represent a promising solution to the escalating demand for proteins due to their long history of crop use and cultivation, lower cost of production, and better environmental sustainability. Beyond achieving high protein content, it has been recently observed that different plant protein fractions (e.g., albumin, vicilin and legumin) exhibit various techno-functionalities (i.e., solubility, emulsifying, foaming, and gelling), which could be captured for targeted food and nutrition applications.

- 20 Conventional methods of protein fractionation techniques use chromatography and buffer systems. However, these methods either require expensive instruments or involve multiple dialysis steps, which poses challenges for large-scale implementation due to cost and scalability issues.

Accordingly, there is a need for a method that efficiently extracts protein fractions from a protein sample that ameliorates one or more disadvantages mentioned above.

25 Summary

In one aspect, there is provided a method of obtaining at least one protein fraction from a protein sample comprising the steps of:

- (a) subjecting the protein sample to either an acidic condition or an alkaline condition to obtain a pH-treated sample;
- 30 (b) adjusting the pH of the pH-treated sample to another pH that is different from the pH used in the subjecting step (a) to obtain a pH-adjusted sample;
- (c) treating the pH-adjusted sample at an elevated pressure to obtain a plurality of protein extracts;
- 35 (d) isolating the plurality of protein extracts to form a solid component and a liquid component, wherein the solid component comprises a first protein fraction; and

(e) optionally precipitating the liquid component to obtain a second protein fraction.

Advantageously, the method may be conducted on a large-scale and therefore can be used to produce fraction-enriched protein ingredients on a large-scale and with high throughput due to the larger volumes of protein that can be processed at a time (for example, it is possible to process 3000 kg/h or 500L at a time). Advantageously, the method may be used to achieve large-scale separation of different protein fractions and in an efficient manner. Advantageously, the method may be used on plant proteins instead of conventional methods which are focused on dairy proteins. Advantageously, the treating step (c) in the method may be used to process samples in a short amount of time (such as from 5 to 15 minutes).

More advantageously, the method may result in protein fractions that may have at least one of (i) higher gastric digestibility, (ii) improved protein solubility, (iii) improved emulsifying properties, (iv) different isoelectric points, and (v) different protein composition as compared to conventional plant protein isolates that were obtained from conventional methods such as isoelectric precipitation only. In addition, conventional processes based on high-pressure processing only resulted in protein modification, where the functional properties of the protein were modified, but did not result in protein isolation or protein fractionation.

Where it is desired for the first protein fraction to have improved emulsifying stability (at acidic condition) and/or lower vicilin proportion and change in vicilin to legumin (7S/11S) ratio the subjecting step (a) may be carried out under acidic conditions. Where it is desired for the second protein fraction to have at least one of (i) higher gastric digestibility, (ii) improved protein solubility, (iii) improved emulsifying activity (at acidic pH), (iv) different isoelectric point and (v) higher albumin proportion and change in vicilin to legumin (7S/11S) ratio, the subjecting step (a) may be carried out under acidic conditions. Hence, advantageously, the first protein fraction may have improved emulsifying stability (at acidic condition) and/or lower vicilin proportion and change in vicilin to legumin (7S/11S) ratio when the subjecting step (a) is carried out under acidic conditions and the second protein fraction may have at least one of (i) higher gastric digestibility, (ii) improved protein solubility, (iii) improved emulsifying activity (at acidic pH), (iv) different isoelectric point and (v) higher albumin proportion and change in vicilin to legumin (7S/11S) ratio when the subjecting step (a) is carried out under acidic conditions.

Where it is desired for the first protein fraction to have improved emulsifying stability (at acidic condition) and/or higher albumin proportion and change in vicilin to legumin (7S/11S) ratio the subjecting step (a) may be carried out under alkaline conditions. Where it is desired for the second protein fraction to have at least one of (i) improved protein solubility, (ii) improved emulsifying activity (at acidic pH), (iii) different isoelectric point and (v) lower vicilin proportion and change in vicilin to legumin (7S/11S) ratio, the subjecting step (a) may be carried out under alkaline conditions. Hence, advantageously, the first protein fraction may have improved emulsifying stability (at acidic condition) and/or higher albumin proportion and change in vicilin to legumin (7S/11S) ratio when the subjecting step (a) is carried out under alkaline conditions and the second protein fraction may have at least one of (i) improved protein solubility, (ii) improved emulsifying activity (at acidic pH), (iii) different isoelectric point and (v) lower vicilin proportion and change in vicilin to legumin (7S/11S) ratio when the subjecting step (a) is carried out under alkaline conditions.

Further advantageously, since high-pressure processing units are used to generate the elevated pressure and such high-pressure processing units are present as stand-alone units, such high-

pressure processing units may be easily integrated into current plant protein manufacturing processes without requiring any complicated changes to the plant configuration.

In another aspect, there is provided a protein fraction obtained from the method as defined herein.

- 5 Advantageously, the protein fraction obtained from the method may have at least one of (i) higher gastric digestibility, (ii) improved protein solubility, (iii) improved emulsifying properties, (iv) different isoelectric points, and (v) different protein composition as compared to protein fractions obtained from conventional isolation methods.

Definitions

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

- 10 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.

- 15 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.

- 20 As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

- 25 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.

- 30 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.

35 Detailed Disclosure of Embodiments

Exemplary, non-limiting embodiments of a method of obtaining at least one protein fraction from a protein sample will now be disclosed.

The method comprises the steps of:

(a) subjecting the protein sample to either an acidic condition or an alkaline condition to obtain a pH-treated sample;

5 (b) adjusting the pH of the pH-treated sample to another pH that is different from the pH used in the subjecting step (a) to obtain a pH-adjusted sample;

(c) treating the pH-adjusted sample at an elevated pressure to obtain a plurality of protein extracts;

(d) isolating the plurality of protein extracts to form a solid component and a liquid component, wherein the solid component comprises a first protein fraction; and

10 (e) optionally precipitating the liquid component to obtain a second protein fraction.

The protein sample may be protein ingredients such as protein flour, protein concentrates and so on.

15 The subjecting step (a) may comprise a step of forming a dispersion from the protein sample and then subjecting the dispersion to the acidic condition or the alkaline condition. The dispersion may be formed by dispersing the protein sample in an aqueous medium in the presence of water. Hence, the dispersion may be at acidic pH or alkaline pH.

20 The pH of the acidic condition in the subjecting step (a) may be selected from the range of about pH 1 to about pH 3, about pH 1 to about pH 2, or about pH 2 to about pH 3. The pH may be about 2. The acidic condition may be obtained by using an acid or an acidic solution. The acid is not particularly limited and may be hydrochloric acid, acetic acid, citric acid, lactic acid or a combination thereof.

25 The pH of the alkaline condition in the subjecting step (a) may be selected from the range of about pH 8 to about pH 10, about pH 9 to about pH 10, or about pH 8 to about pH 9. The pH may be about 9. The alkaline condition may be obtained by using an alkali or an alkaline solution. The alkali is not particularly limited and may be sodium hydroxide, sodium bicarbonate, potassium bicarbonate, or a combination thereof.

As the protein fractions may be used in food, the acid and the base are of food grade.

30 By subjecting the dispersion to the acidic condition or alkali condition, the protein may be extracted from the protein sample. Advantageously, extraction at different pH gives rise to proteins with different functional properties and different fraction-enriched protein ingredients.

35 The protein sample may contain protein(s) having a weight percentage in the range of about 5 to about 60 weight%, about 5 to about 10 weight%, about 5 to about 20 weight%, about 5 to about 30 weight%, about 5 to about 40 weight%, about 5 to about 50 weight%, about 10 to about 60 weight%, about 20 to about 60 weight%, about 30 to about 60 weight%, about 40 to about 60 weight%, about 50 to about 60 weight%, or about 10 to 55 weight%, based on the total weight of the protein sample.

The ratio of the protein sample to the aqueous medium in the dispersion may be in the range of about 1:5 to about 1:20, about 1:10 to about 1:20, about 1:15 to about 1:20, about 1:10 to about 1:20, or about 1:15 to about 1:20. The ratio may be about 1:5 or about 1:10.

5 In the step of forming the dispersion, the dispersion may be mixed to form a uniform dispersion. The dispersion may be mixed using magnetic stir bar stirring or shear mixing, but is not limited as such.

The mixing may be undertaken at a temperature in the range of about 25°C to about 50°C, about 25°C to about 30°C, about 25°C to about 35°C, about 30°C to about 40°C, about 25°C to about 45°C, about 30°C to about 50°C, about 35°C to about 50°C, about 40°C to about 50°C, or about 45°C to about 50°C. The temperature may be generated using a heat source
10 selected from a water bath or a heat exchanger.

The mixing may be undertaken at a duration in the range of about 30 minutes to about 24 hours, about 30 minutes to about 1 hour, about 30 minutes to about 5 hours, about 30 minutes to about 10 hours, about 30 minutes to about 15 hours, about 1 hour to about 24 hours, about
15 5 hours to about 24 hours, about 10 hours to about 24 hours, about 15 hours to about 24 hours, or about 20 hours to about 24 hours.

The method may further comprise a step of separating the pH-treated sample into a solid component and a liquid component. This may comprise centrifuging the pH-treated sample to form a solid component (being the pellet) and a liquid component (being the supernatant).
20 The method may further comprise a step of extracting the liquid component from the pH-treated sample (so as to collect the liquid component (which is rich in protein)), which is then subjected to the adjusting step (b).

In the adjusting step (b), where the pH of the pH-treated sample is adjusted to another pH that is different from the pH used in the subjecting step (a), the pH used in this step may be
25 adjusted to the range of about 3 to about 7, about 4 to about 7, about 5 to about 7, about 6 to about 7, about 3 to about 4, about 3 to about 5, or about 3 to about 7, provided that it is a pH that is different to the pH in the subjecting step (a). The pH may be about 7, or about 3. As an example, where the pH used in the subjecting step (a) is about 9, the another pH used in the adjusting step (b) may be about 7. In another example, where the pH used in the subjecting
30 step (a) is about 2, the another pH used in the adjusting step (b) may be about 2.

The pH-adjusted sample obtained from the adjusting step (b) may then be packaged into packaging that can withstand the elevated pressure in the treating step (c).

In the treating step (c), the elevated pressure may be a pressure in the range of about 300 MPa to about 800 MPa, about 300 MPa to about 400 MPa, about 300 MPa to about 500 MPa,
35 about 300 MPa to about 600 MPa, about 300 MPa to about 700 MPa, about 400 MPa to about 800 MPa, about 500 MPa to about 800 MPa, about 600 MPa to about 800 MPa, about 700 MPa to about 800 MPa, or about 450MPa to about 600MPa.

The elevated pressure may be generated using a high-pressure processing unit. The high pressure can be achieved by using a hydraulic fluid medium such as water that is transmitted
40 quasi-instantaneously and uniformly throughout the unit. The elevated pressure as described herein allows for aggregation of proteins in the protein sample. Uniquely, albumin protein is advantageously precipitated under the elevated pressure range. The treating step (c) may be

undertaken for a duration of about 30 seconds to about 15 minutes, about 30 seconds to about 1 minute, about 30 seconds to about 5 minutes, about 30 seconds to about 10 minutes, about 1 minute to about 15 minutes, about 5 minutes to about 15 minutes, about 10 minutes to about 15 minutes, or about 5 minutes.

- 5 The treating step (c) may further comprise a step of selecting an initial temperature used with the elevated pressure. The initial temperature may be in the range of about 2 °C to about 50 °C, about 4 °C to about 50 °C, about 6 °C to about 50 °C, about 10 °C to about 50 °C, about 20 °C to about 50 °C, about 30 °C to about 50 °C, about 40 °C to about 50 °C, about 2 °C to about 4 °C, about 2 °C to about 6 °C, about 2 °C to about 10 °C, about 2 °C to about 20 °C,
10 about 2 °C to about 30 °C, about 2 °C to about 40 °C, or about 4 °C to about 6 °C.

The treating step (c) may further comprise a step of depressurizing the dispersion from the elevated pressure.

- The depressurizing step may comprise depressurizing the dispersion from the elevated pressure instantly or at a rate in the range of about 0.5 MPa/s to about 60 MPa/s. During
15 depressurization, adiabatic heating of about 4°C/100MPa (when water is used as the hydraulic fluid medium) can occur, which is lost during depressurization.

- The isolating step (d) may form a solid component (being the pellet) and a liquid component (being the supernatant). The solid component may comprise the first protein fraction. The isolating step may be a centrifuging step. The centrifuging step may be undertaken at a rate
20 in the range of about 8,000 rpm to about 12,000 rpm, or about 10,000 rpm.

The centrifuging step may be undertaken for a duration in the range of about 8 minutes to about 12 minutes, or about 10 minutes.

- The solid component (being the protein extracts present in the pellets) comprising the first protein fraction may be re-dispersed in an aqueous medium and where required, the pH
25 adjusted to a neutral value (of about 7).

The first protein fraction with neutral pH may then be subjected to a drying step such as via freeze drying, spray drying, oven drying, supercritical drying or vacuum drying to obtain the plurality of protein extracts. The drying methods are not limited to the examples disclosed herein and any drying methods are part of this disclosure.

- 30 Advantageously, where the treating step (c) is followed by the isolating step (d), this allows for protein fractionation of the protein sample, which does not happen when only high-pressure processing is used (that is, without any subsequent centrifuging step) as using high-pressure processing only modifies the protein rather than fractionate the protein sample.

- The liquid component after the isolating step (d) may then be subjected to the precipitating step (e) to precipitate the second protein fraction. The precipitating step (e) may be isoelectric precipitation. Where it is desired to obtain two protein fractions from the protein sample, the precipitating step (e) is then not an optional step.
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- The method may further comprise a step of isolating the precipitated second protein fraction from the non-precipitated material. The isolating step may comprise centrifuging such that
40 the precipitated second protein fraction is obtained in the form of a solid component (being the pellet) and the non-precipitated material as a liquid component (being the supernatant).

The precipitated second protein fraction in the pellet may be re-dispersed in an aqueous medium and where required, washed with the aqueous medium and alternatively, if required, the pH adjusted to a neutral value (of about 7).

- 5 The method may further comprise a step of drying the precipitated second protein fraction. The drying step may be undertaken via freeze drying, spray drying, oven drying, vacuum drying or combinations thereof.

- 10 In the method, the protein sample may be selected from the group consisting of pea, lentil, faba bean, industrial hemp, chickpea, basil seed, pumpkin seed, almond, soy, quinoa, nuts, textured vegetable, tempeh, rice (such as white rice or brown rice), spirulina, peanut, legume, tofu, beans (such as faba beans or edamame), seitan, nutritional yeast and combinations thereof.

Exemplary, non-limiting embodiments of a first protein fraction or a second protein fraction will now be disclosed.

- 15 The first protein fraction or the second protein fraction may be obtained from the method as described herein.

The first protein fraction or the second protein fraction may comprise albumin.

The first protein fraction, or the second protein fraction may comprise, consist essentially of or consist of proteins or peptides selected from the group consisting of albumin, vicilin, legumin and combinations thereof.

- 20 The first protein fraction may comprise, consist essentially of or consist of albumin, vicilin and legumin. In the first protein fraction, the vicilin may have a weight percentage in the range of about 30 weight% to about 50 weight%, about 30 weight% to about 35 weight%, about 30 weight% to about 40 weight%, about 30 weight% to about 45 weight%, about 35 weight% to about 50 weight%, about 40 weight% to about 50 weight%, about 45 weight% to about 50 weight%, about 33 weight% to about 37 weight%, or about 45 weight% to about 49 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the first protein fraction. The legumin may have a weight percentage in the range of about 25 weight% to about 35 weight%, about 25 weight% to about 30 weight%, about 25 weight% to about 30 weight%, about 27 weight% to about 31 weight%, or about 28 weight% to about 32 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the first protein fraction. The albumin may have a weight percentage in the range of about 20 weight% to about 37 weight%, about 20 weight% to about 25 weight%, about 20 weight% to about 30 weight%, about 20 weight% to about 35 weight%, about 25 weight% to about 37 weight%, about 30 weight% to about 37 weight%, about 35 weight% to about 37 weight%, about 21 weight% to about 25 weight%, or about 31 weight% to about 35 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the first protein fraction.

- 40 The second protein fraction may comprise, consist essentially of or consist of albumin, vicilin and legumin. In the second protein fraction, the vicilin may have a weight percentage in the range of about 35 weight% to about 52 weight%, about 35 weight% to about 40 weight%, about 35 weight% to about 45 weight%, about 40 weight% to about 52 weight%, about 45 weight% to about 52 weight%, about 36 weight% to about 40 weight%, or about 47 weight%

- to about 51 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the second protein fraction. The legumin may have a weight percentage in the range of about 20 weight% to about 38 weight%, about 20 weight% to about 25 weight%, about 20 weight% to about 30 weight%, about 20 weight% to about 35 weight%, about 25 weight% to about 38 weight%, about 30 weight% to about 38 weight%, about 35 weight% to about 38 weight%, about 20 weight% to about 24 weight%, or about 33 weight% to about 37 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the second protein fraction. The albumin may have a weight percentage in the range of about 10 weight% to about 40 weight%, about 10 weight% to about 20 weight%, about 10 weight% to about 30 weight%, about 20 weight% to about 40 weight%, about 30 weight% to about 40 weight%, about 12 weight% to about 16 weight%, or about 36 weight% to about 40 weight%, based on the total weight of (where present) albumin, vicilin and legumin in the second protein fraction.
- Advantageously, the first and/or second protein fraction may have higher amount (or percentage) of albumin or lower amount (or percentage) of vicilin as compared to protein isolates obtained from a conventional method based on isoelectric precipitation only.

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 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 overview of a general method used to prepare protein fractions from a protein sample.

FIG. 2

[FIG. 2] is an image of SDS-PAGE profile under reducing conditions of pea proteins extracted using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 3

[FIG. 3] is a bar graph showing the protein composition of samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by SDS-PAGE under reducing conditions. Results are expressed in terms of the band intensity of each sub-fraction as a percentage of the total band intensity of the proteins of interest.

FIG. 4

[FIG. 4] is a bar graph showing the vicilin to legumin (7S/11S) ratio of samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by SDS-PAGE under reducing conditions.

FIG. 5A

[FIG. 5A] is a line chart showing the solubility of pea proteins extracted under alkaline conditions using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 5B

[FIG. 5B] is a line chart showing the solubility of pea proteins extracted under acidic conditions using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 6A

[FIG. 6A] is a line chart showing the surface charge (zeta potential) of pea proteins extracted under alkaline conditions using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 6B

[FIG. 6B] is a line chart showing the surface charge (zeta potential) of pea proteins extracted under acidic conditions using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 7A

[FIG. 7A] is a bar graph showing emulsifying activity (EAI) of pea proteins extracted using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 7B

[FIG. 7B] is a bar graph showing emulsifying stability (ESI) of pea proteins extracted using conventional IEP method (pH9-IEP and pH2-IEP), by HPP (pH9-pH7-600MPa and pH2-pH3-600MPa) or HPP-IEP methods (pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP).

FIG. 8A

[FIG. 8A] shows the average protein digestibility of the PPC samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by BCA assay after gastric digestion.

FIG. 8B

[FIG. 8B] shows the average protein digestibility of the PPC samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by BCA assay after intestinal digestion.

FIG. 8C

[FIG. 8C] shows the average protein digestibility of the PPC samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by OPA assay after gastric digestion.

FIG. 8D

- 5 [FIG. 8D] shows the average protein digestibility of the PPC samples extracted by different methods (IEP, HPP, HPP-IEP) as determined by OPA assay after intestinal digestion.

Detailed Description of Drawings**FIG. 1**

- 10 [FIG. 1] is a flow chart on the general extraction and precipitation process of protein samples to form at least one protein fraction according to the present disclosure. As shown in FIG. 1, a protein sample (100) may be dispersed in water and subsequently undergo protein extraction which can be undertaken at alkaline condition (102) or acidic condition (103). The pH-treated sample may be centrifuged and with the supernatant collected (104) subsequently to form a liquid component, which is then subjected to isoelectric precipitation (106) by
- 15 adjusting the pH to obtain comparative samples (122, 128). For actual samples (that is, following the method of the present disclosure), the pH-treated sample may be subjected to an adjusting pH step (116, 117) to form a pH-adjusted sample that is then treated at elevated pressure (108) and thereafter centrifuged once more (110) to collect its supernatant (112) and protein pellet (114), where the protein pellet (114) forms samples 126 and 132 (containing
- 20 respective first protein fractions). The supernatant collected (112) may then be subjected to isoelectric precipitation via pH adjustment (106) to form samples 124 and 130 (containing respective second protein fractions). The precipitated proteins (122, 124, 126, 128, 130, 132) may be centrifuged once more and the final pellets collected are neutralised to pH 7 (118) before drying and storage (120).

25 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**30 General Protein Extraction**

- The general process as shown in FIG. 1 is followed here. A protein sample (100) in the form of plant protein materials (such as protein flours, protein concentrates or protein isolates from plant sources) were rehydrated with deionized water (at a weight:volume ratio of the solid:liquid of 1:5 or 1:10) and subjected to protein extraction at an alkaline pH (102, where
- 35 the pH is between 8 to 10, preferably pH 9) using NaOH solution (1.0 M, prepared from food grade NaOH pellets purchased from Thermo Fisher Scientific, Singapore) or at an acidic pH (103, where the pH is between 1 to 3, preferably pH 2) using HCl solution (1.0 M, prepared from food grade HCl solution purchased from Thermo Fisher Scientific, Singapore) before mixing for 1 hour (using magnetic stirring, shear mixing, etc.). The protein solution was then

centrifuged/decanted at 10,000 rpm for 10 minutes and where required, the supernatant collected (104) is either subjected to an isoelectric precipitation (106) by adjusting the pH to obtain comparative samples (122, 128) or adjusted to a pH adjusting step using HCl to arrive at pH 7 (116) or NaOH solution to arrive at pH 3 (117). The pH-adjusted samples are then
5 contained in a flexible packaging and subjected to elevated pressure (108) in the form of high-pressure treatment at a range of 300 to 800 MPa, preferably between 450 to 600MPa, with a pressure hold time of 30 seconds to 15 minutes, preferably 3 to 5 minutes. After high-pressure treatment, the vessel was depressurised instantly or at a controlled rate between 0.5 and 60 MPa/s. The initial processing temperature was 2 to 50 °C, preferably 4 to 6 °C.
10 Adiabatic heating of about 4 °C/100 MPa for water occurred, which was lost upon depressurisation. The treated mixture was then centrifuged (110) at 10,000 rpm for 10 minutes and the protein pellet (114) was collected as respective first protein fractions (126 or 132).

The proteins remaining in the supernatant (112) after the centrifugation step as described
15 above was recovered using isoelectric precipitation (106). In isoelectric precipitation, the supernatant was adjusted to isoelectric point (between pH 4 to 5, preferably 4.5) using HCl solution (1.0 M, prepared from food grade HCl solution purchased from Thermo Fisher Scientific, Singapore). The subsequent proteins precipitated (124 or 130) containing
20 respective second protein fractions were obtained via centrifugation at 10,000 rpm for 10 minutes.

All the pellets (122, 124, 126, 128, 130, 132) obtained from the protein fractionation process were centrifuged once more, re-dispersed in deionised water at 1:1 weight ratio and their pH adjusted to pH 7 (118) and subjected to drying such as, for example, freeze drying, spray drying, oven drying, vacuum drying etc, and storage (120).

25 **Example 2 – Extraction Process of Pea Protein**

Protein extraction

An overview of the protein extraction/fractionation process from pea protein concentrate (PPC) is shown in FIG. 1. Protein sample (100) in the form of commercial yellow PPC (from Vitessence Pulse 1550, Ingredion, Illinois, United States of America) was dispersed in water
30 at a 1:5 solid to liquid weight ratio. The pH of the solution was adjusted to 9 using 1M NaOH (102) or 2 using 1M HCl (103) and stirred using a magnetic stirrer for 1 hour. The solution was then centrifuged at 10,000 rpm for 10 minutes, the supernatant was collected (104) and subjected to either isoelectric precipitation (IEP) or High Pressure Processing (HPP).

Protein extraction followed by IEP (comparative example)

35 Following from centrifugation of the solution and collection of the supernatant (104), the supernatant was adjusted to pH 4.5 using 1M HCl or 1M NaOH for isoelectric precipitation (106), followed by centrifugation at 10,000 rpm for 10 minutes. The pellet was recovered and re-suspended in water in a weight ratio of a 1:1 and the pH was adjusted to 7 using 1M NaOH (118).

40 The extracted protein was freeze-dried (120) and stored in a desiccator until further analyses. The lyophilized protein powder samples extracted at pH 9 and pH 2 were labelled pH9-IEP

(122) and pH2-IEP (128) respectively. The samples obtained from this example are used as comparative samples.

Protein extraction followed by HPP

Following from centrifugation of the solution and collection of the supernatant (104), the pH was adjusted to 7 (alkaline) using 1M HCl (116) or 3 (acidic) using 1M NaOH (117). The pH-adjusted samples collected were processed at elevated pressure (108) of 600 MPa, 5°C for 5 minutes. Thereafter, the mixture was also centrifuged (110) at 10,000 rpm for 10 minutes and the precipitated protein pellets (114) comprising respective first protein fractions were collected. The pellet was recovered and re-suspended in water in a weight ratio of a 1:1 and the pH was adjusted to 7 using 1M NaOH (118).

The extracted protein was freeze-dried and stored (120) in a desiccator until further analyses. The lyophilized protein powder samples extracted at pH 9 and pH 2 were labelled pH9-pH7-600MPa (126) and pH2-pH3-600MPa (132) respectively.

Protein extraction followed by HPP and IEP

The pH of the supernatant collected after centrifugation (110) was adjusted to pH 4.5 using 1M HCl or 1M NaOH for isoelectric precipitation (112), where the subsequent protein precipitated comprise respective second protein fractions followed by another round of centrifugation at 10,000 rpm for 10 minutes. The pellet was then collected, recovered and re-suspended in water in a weight ratio of a 1:1. Following which, the pH was adjusted to 7 using 1M NaOH (118).

The extracted protein was freeze-dried and stored (120) in a desiccator until further analyses. The lyophilized protein powder samples extracted at pH 9 and pH 2 were labelled pH9-pH7-600MPa-IEP (124) and pH2-pH3-600MPa-IEP (130) respectively.

It should be noted that the same process can be used for a variety of plant protein sources such as faba bean and lentil protein concentrates.

Example 3 – Protein Content and Yield

Determination of protein content by Dumas

Protein content of pea proteins obtained via the different fractionation methods was determined via the Dumas method using a nitrogen analyser (Dumatherm DT N Pro, Gerhardt, Germany). Protein content of the samples were then derived from the nitrogen content using a protein conversion factor of 6.25. Protein yield was calculated using equation (1).

$$\text{Protein yield (\%)} = \frac{x_e m_e}{x_c m_c} \quad (1)$$

where x_e is the protein content of the protein extract, m_e the mass of the protein extract, x_c the protein content of PPC and m_c the mass of PPC used.

The protein content and yield of proteins of isolated pea proteins by HPP and HPP-IEP methods were obtained. The protein content ranged from 75 to 87 % as shown in Table 1, which is comparable to conventional plant protein isolates (pH9-IEP and pH2-IEP). While

the protein yield of HPP and HPP-IEP methods were found to be lower than conventional plant protein isolates, the method enables further separation of protein fractions with superior functional properties such as better solubility and higher gastric digestibility compared to conventional plant protein isolates.

5 **Table 1.** Protein content and yield of fractionated pea proteins

Sample	Protein Content (%)	Protein yield (%)
PPC-pH9-IEP	85.71 ± 0.29	69.32 ± 0.23
PPC-pH9-pH7-600MPa	75.04 ± 0.28	59.42 ± 0.22
PPC-pH9-pH7-600MPa-IEP	87.12 ± 0.42	5.15 ± 0.03
PPC-pH2-IEP	87.37 ± 0.36	56.05 ± 0.23
PPC-pH2-pH3-600MPa	76.36 ± 0.01	32.67 ± 0.00
PPC-pH2-pH3-600MPa-IEP	76.40 ± 0.21	1.52 ± 0.00

Example 4 – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10 Reducing SDS-PAGE was carried out using the method of Laemmli on a continuous buffer system. Protein dispersions containing 4 mg of soluble protein in 3 mL of deionised water were prepared and mixed with NuPAGE™ LDS Sample Buffer (Thermo Fisher Scientific, MA, USA, catalog #NP0007) in the presence of dithiothreitol (DTT) (NuPAGE™ Sample Reducing Agent (10X)) (Thermo Fisher Scientific, MA, USA, catalog #NP0009), followed by heating at 70 °C for 10 minutes. Subsequently, each sample was loaded on a 10 % Bis-
15 Tris NuPAGE™ midi protein gel (Thermo Fisher Scientific, MA, USA, catalog #WG1201BOX). A protein ladder (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa) (Thermo Fisher Scientific, MA, USA, catalog #26620) was also loaded to serve as a molecular weight marker. Electrophoresis was run at a constant voltage (120 V) for 70 minutes with MES/SDS as the running buffer (NuPAGE™ MES SDS Running Buffer (20X))
20 (Thermo Fisher Scientific, MA, USA, catalog #NP0002) until the dye front reached the reference line. The gel was stained with InstantBlue Coomassie protein stain (Abcam, Cambridge, UK) and scanned using iBright™ FL1500 Imaging System (Thermo Fisher Scientific, MA, USA). The intensity of bands was analysed using iBright Analysis Software and the results are expressed in terms of the band intensity of each sub-fraction as a
25 percentage of the total band intensity of the proteins of interest, enabling comparison across samples (Gao et al., 2020). The proteins of interest in this example refer to the major sub-fractions in pea protein, which include legumin (11S), vicilin (7S) and albumin.

Electrophoretic bands (under reducing conditions) of pea protein extracted using conventional IEP method, by HPP or HPP-IEP methods are shown in FIG. 2. In general, the
30 electrophoretic profile of all samples exhibited a multitude of bands ranging from less than 10 kDa to 98 kDa. As shown in FIG. 3., all methods of protein extraction showed bands of the major sub-fractions present in pea protein, including that of the subunits of legumin, legumin α (~40 kDa), legumin β (~20-22 kDa), convicilin subunit (~70-75 kDa), vicilin subunits (~45-50 kDa, ~30-35 kDa and ~15-18 kDa) and a mixture of albumin and/or γ-
35 vicilin (≤ 10 kDa). With reference to the comparative samples extracted using conventional

- IEP method (pH9-IEP and pH2-IEP samples), there was a change in the protein composition in the samples extracted by HPP (i.e., pH9-pH7-600MPa and pH2-pH3-600MPa) and HPP-IEP (i.e., pH9-pH7-600MPa-IEP and pH2-pH7-600MPa-IEP) as shown by the decrease in the 7S/11S ratio in FIG. 4. This was explained by a decrease in the 7S fraction (i.e., vicilin+convicilin). There was an accompanying increase in the albumin fraction for the pH9-pH7-600MPa-IEP and pH2-pH3-600MPa samples. There was largely no change in the 11S fraction after extraction by HPP and HPP-IEP. Overall, this showed that protein extraction by HPP and HPP-IEP resulted in a modification of the protein composition of samples.

Example 5 – Protein solubility and ζ -potential

- Dispersions of extracted pea protein (1.0 wt. % protein) were prepared in water and adjusted to pH 2-8 using 0.1 M HCl, 1 M HCl, 0.1 M NaOH and/or 1 M NaOH. The solutions were stirred at room temperature (24°C) for 30 minutes and centrifuged at 10,000 rpm for 10 minutes. The protein content of the supernatant was determined using the dye-binding method of Bradford (1976) with BSA as the standard. Protein solubility was calculated using equation (2). The ζ -potential of the protein solutions at pH 2-8 was also measured using a Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) with disposable capillary cells (DTS1070, Malvern Instruments). Protein solubility and ζ -potential measurements were performed at least in duplicate.

$$\text{Solubility (\%)} = \frac{\text{Protein content of supernatant}}{\text{Total protein content}} \times 100\% \quad (2)$$

- The solubility of pea proteins extracted under alkaline condition and acidic condition was measured and shown in FIG. 5A and FIG. 5B respectively, while the surface charge of pea proteins extracted under alkaline condition and acidic condition was measured and shown in FIG. 6A and FIG. 6B respectively. In general, HPP-IEP method produced protein isolates (i.e., pH9-pH7-600MPa-IEP and pH2-pH7-600MPa-IEP) with improved solubility compared to conventional plant protein isolates (i.e., pH9-IEP and pH2-IEP), whereas pea proteins extracted by HPP (i.e., pH9-pH7-600MPa and pH2-pH3-600MPa) exhibited poorer solubility. Interestingly, pH9-pH7-600MPa-IEP extracts had high solubility (>70%) at pH 6, which is typically not observed in conventional plant protein isolates. These pea proteins could potentially be used in food applications which require high protein solubility at this pH. The isoelectric point of pea proteins extracted by HPP-IEP (i.e., pH9-pH7-600MPa-IEP and pH2-pH7-600MPa-IEP), defined at the pH where the zeta potential value is zero, was also found to be different from conventionally extracted pea protein isolates. For instance, the isoelectric point of pH9-pH7-600MPa-IEP was ~4.5, while conventional pH9-IEP was ~4.9. Also, the isoelectric point of pH2-pH3-600MPa-IEP was ~5.1, while conventional pH2-IEP was ~4.9.

Example 6 – Emulsifying property

- Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined using the turbidimetric method of Pearce and Kinsella (1978). Dispersions of extracted protein (1.0 wt. % protein) were prepared in water and adjusted to pH 3 and 7 using 0.1M HCl, 1 M HCl, 0.1M NaOH and/or 1M NaOH. Canola oil (5 mL) was added to 15 mL of protein dispersion and homogenized at 10,000 rpm for 1 minute with a high-speed disperser (T25 Ultra-Turrax, IKA, Germany). Each emulsion (50 μ L) was transferred from the bottom of the container

into 5mL 0.1% SDS solution at 0 and 10 minutes after emulsification. Absorbance was measured at 500 nm with a UV-Visible Spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). EAI and ESI were calculated using equations (3) and (4) respectively.

$$\text{EAI (m}^2\text{/g)} = \frac{2 \times 2.303 \times A_0 \times \text{DF}}{C \times l \times \phi \times 1000} \quad (3)$$

$$\text{ESI (min)} = \frac{A_0}{A_0 - A_{10}} \times t \quad (4)$$

- where A_0 = absorbance at 0 minute, A_{10} = absorbance at 10 minutes, DF = dilution factor (100), C = protein concentration (mg/mL), l = optical path (0.01 m), ϕ = oil volume fraction (0.25), t = 10 minutes.

The measurement of the emulsifying properties of all samples were carried out in triplicate except for pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP samples, which was performed once.

- 10 The EAI and ESI of the extracted pea proteins were measured at pH 3 and 7 and shown in FIG. 7A and FIG. 7B respectively. The emulsifying properties of pea proteins extracted by HPP and HPP-IEP (i.e., pH9-pH7-600MPa, pH2-pH3-600MPa, pH9-pH7-600MPa-IEP and pH2-pH3-600MPa-IEP) were generally comparable to that of conventionally extracted pea protein isolates (i.e., pH9-IEP and pH2-IEP). For example, HPP and HPP-IEP methods
15 produced pea protein extracts that had similar EAI values at pH 3 and pH 7 compared to conventional pea protein isolates. ESI values of HPP and HPP-IEP samples at pH 7 were also comparable to those of conventional pea protein isolates. Notably, pea proteins extracted by HPP exhibited improved ESI (50-100% increase) over conventional pea protein isolates at pH 3. This could be used for improved emulsion stability in acidic food applications.

20 Example 7 – INFOGEST static in vitro digestion

- A static in vitro digestion protocol according to the INFOGEST 2.0 was used to digest the PPC samples. This model consists of an oral, gastric and small intestinal phase with fixed digestion conditions for each phase. Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared 1.25x concentrated according to
25 Table 2 which allowed for subsequent addition of $\text{CaCl}_2(\text{H}_2\text{O})_2$, enzyme solution, bile solution, water, HCl and NaOH as required according to Brodkorb et al. (2019). Prior to the digestion experiments, the enzyme activities and bile concentration were determined following the assays as specified in the protocol (Brodkorb et al., 2019). The digestions were carried out in an incubator (HettCube 200, Hettich, Tuttlingen, Germany) maintained at 37°C
30 which housed a rotator (Rotator SB Stuart, Huberlab, Aesch, Switzerland) to ensure constant mixing (40 rpm) of the samples. Triplicate digestions were conducted.

Table 2. Preparation of electrolyte stock solutions of digestion fluids (1.25x concentrated)

Salt solution added	Stock conc. (M)	SSF (pH 7)			SGF (pH 3)			SIF (pH 7)		
		Vol. of stock added to prepare 0.4 L (1.25x) (mL)	of salt conc. in SSF (mM)	Final salt conc. in SSF (mM)	Vol. of stock added to prepare 0.4 L (1.25x) (mL)	of salt conc. in SGF (mM)	Final salt conc. in SGF (mM)	Vol. of stock added to prepare 0.4 L (1.25x) (mL)	of salt conc. in SIF (mM)	Final salt conc. in SIF (mM)

KCl	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH ₂ PO ₄	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO ₃	1	6.8	13.6	12.5	25	42.5	85
NaCl	2	-	-	11.8	47.2	9.6	38.4
MgCl ₂ (H ₂ O) ₆	0.15	0.5	0.15	0.4	0.12	1.1	0.33
(NH ₄) ₂ CO ₃	0.5	0.06	0.06	0.5	0.5	-	-
HCl	6	0.09	1.1	1.3	15.6	0.7	8.4
CaCl ₂ (H ₂ O) ₂	0.3	0.025	1.5	0.005	0.15	0.04	0.6

Protein isolates were dispersed in milli-Q water at 50 mg protein per mL and mixed overnight on a shaking plate. For the oral phase, the sample was diluted with SSF at a ratio of 1:1 (v:v) and was incubated for 2 minutes. As starch digestion was not studied, α -amylase was not added. For the gastric phase, the oral bolus was diluted with SGF at ratio of 1:1 (v:v), containing porcine pepsin (2000 U/mL of digesta, P7012, Sigma-Aldrich, Missouri, USA) and pH was adjusted to 3. After 120 minutes of incubation, gastric digestion was stopped by addition of Pefabloc®SC (2.5 mM final concentration, 76307, Sigma-Aldrich, Missouri, USA). For the intestinal phase, the digesta was diluted with SIF at ratio of 1:1 (v:v), containing porcine pancreatin (100 U trypsin activity/mL of digesta, P7545, Sigma-Aldrich, Missouri, USA), porcine bile (2.5 mM final concentration, B8631, Sigma-Aldrich, Missouri, USA) and pH adjusted to 7. After 120 minutes of incubation, intestinal digestion was stopped by addition of Pefabloc®SC (5 mM final concentration). The digesta samples were centrifuged at 13,000 x g (fixed angle 35° rotor) at 4°C for 15 minutes to separate the soluble and insoluble fractions before storage at -80°C for subsequent analysis.

Ultrafiltration of digesta

Prior to conducting the BCA and OPA assays, ultrafiltration of gastric and intestinal digesta were done to isolate digesta filtrate containing molecules smaller than 10 kDa. Ultrafiltration was done using Amicon® Ultra-4 centrifugal filters (Merck, Darmstadt, Germany) with molecular weight cut-off of 10 kDa, and a water rinse centrifugation step was carried out after centrifugation of digesta at 4000 x g (swinging bucket rotor) for 20 minutes.

Protein digestibility by bicinchoninic acid (BCA) assay

Quantification of peptides released after in vitro digestion was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instruction using the microplate protocol and are shown in FIG. 8A and FIG. 8B. Bovine serum albumin (BSA) at different concentrations was used as a protein standard and the concentration of the peptides in the digesta filtrates were determined from this standard curve. Duplicate absorbance measurements were read at 562 nm in a microplate reader (BioTek Cytation 5, Agilent Technologies, California, USA). Protein digestibility was calculated with respect to the initial protein input that make up these peptides.

Protein digestibility by o-phthaldialdehyde (OPA) assay

As shown in FIG. 8C and FIG. 8D, quantification of small peptides and free NH₂ groups released after in vitro digestion was determined using the o-phthaldialdehyde (OPA) assay according to Torcello-Gómez et al. (2020) with some modifications. Before the assay was conducted, 166 μ L of 5% w/v trichloroacetic acid was added to 100 μ L of digesta filtrate to

cause the precipitation of longer peptides that could interfere in the analysis. The supernatant collected from centrifugation at 10,000 g for 30 minutes was used for the assay. L-serine at different concentrations was used as a protein standard and the concentration of small peptides and free NH₂ groups in the digesta filtrates were determined from this standard curve. 200 µL of freshly prepared OPA working reagent was added to 10 µL of either L-serine standard, or supernatant or digestion blank using a 96-well UV transparent polystyrene plate. Duplicate absorbance measurements were read at 340 nm in a microplate reader (BioTek Cytation 5, Agilent Technologies, California, USA) after incubation for 15 minutes at room temperature in a dark environment. Protein digestibility was calculated with respect to the initial protein input that make up these peptides and free amino acids.

Results and Discussion

1. For Peptides < 10 kDa in the in vitro digesta

Based on the BCA analysis of digesta after the gastric phase of in vitro digestion, the protein digestibility of the pH2-pH3-600MPa-IEP extracted PPC samples was significantly greater than the pH9-IEP, pH9-pH7-600MPa and pH9-pH7-600MPa-IEP extracted PPC samples ($p>0.05$). This shows that the HPP-IEP proteins extracted under acidic conditions were faster digesting than conventional isolation methods.

Based on the BCA analysis of digesta after the intestinal phase of in vitro digestion, the protein digestibility of the PPC samples extracted by different methods were not significantly different.

2. For Peptides < 6 kDa and free amino acids in the in vitro digesta

Based on the OPA analysis of the digesta after the gastric phase of in vitro digestion, the amount of peptides smaller than 6 kDa and free amino acids released from the pH2-pH3-600MPa-IEP extracted PPC samples was significantly greater than the pH9-pH7-600MPa-IEP extracted PPC samples ($p>0.05$). Based on the OPA analysis of the digesta after the intestinal phase of in vitro digestion, there were no significant differences in the amount of peptides smaller than 6 kDa and free amino acids released from the PPC samples that were extracted by different methods. Thus, different extraction methods may not impact the overall protein digestibility (i.e. the peptide fragments that are likely available for uptake in the small intestine).

Although the protein digestibility of the extracted PPC samples were not significantly different at the end of intestinal digestion, the different amounts of released peptides after the gastric phase of in vitro digestion suggest that there are differences in the rate of digestion. The quicker appearance of certain bioactive peptides and amino acids have been reported to have metabolic effects such as influencing the post-prandial insulin secretion profiles.

Additionally, it is known that protein fractions <10 kDa possessed α -amylase inhibitory due to their narrower size range which allowed for easy binding to the catalytic sites of the α -amylase. The different digestibility of the extracted PPC samples have direct implications on the formation of enzyme-peptide complexes formed during digestion. Thus, there is potential application for PPC samples with faster protein digestibility to be used as ingredients in the diet to control the postprandial glucose response.

Industrial Applicability

The method and the protein fractions of the disclosure may be used in a variety of applications such as formulation of sports and medical nutrition, acidic beverages, and dairy analogs.

- 5 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.

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Claims

1. A method of obtaining at least one protein fraction from a protein sample comprising the steps of:
 - (a) subjecting the protein sample to either an acidic condition or an alkaline condition to obtain a pH-treated sample;
 - (b) adjusting the pH of the pH-treated sample to another pH that is different from the pH used in the subjecting step (a) to obtain a pH-adjusted sample;
 - (c) treating the pH-treated sample at an elevated pressure to obtain a plurality of protein extracts;
 - (d) isolating the plurality of protein extracts to form a solid component and a liquid component, wherein the solid component comprises a first protein fraction; and
 - (e) optionally precipitating the liquid component to obtain a second protein fraction.
2. The method of claim 1, wherein the subjecting step (a) comprises the step of dispersing the protein sample in an aqueous medium in the presence of water to form a dispersion.
3. The method of claim 2, further comprising the step of adjusting the pH of the dispersion to either acidic condition or alkaline condition, wherein the pH of the acidic condition is selected from the range of pH 1 to 3 and wherein the pH of the alkaline condition is selected from the range of pH 8 to 10.
4. The method of claim 2 or 3, wherein in the dispersing step, the protein sample comprises a protein at a weight percentage in the range of 5 to 60 weight%, based on the total weight of the protein sample.
5. The method of any one of claims 2 to 4, wherein in the dispersing step, the weight: volume ratio of the protein sample to the aqueous medium is in the range of 1:5 to 1:20.
6. The method of any one of claims 2 to 5, wherein the dispersing step further comprises the step of mixing the dispersion at a temperature in the range of 25°C to 50°C.
7. The method of any one of claims 2 to 6, wherein the dispersing step further comprises the step of mixing the dispersion for a duration in the range of 30 minutes to 24 hours.
8. The method of claim 7, further comprising the step of separating the dispersion into a solid component and a liquid component, the liquid component being subjected to the adjusting step (b).
9. The method of any one of claims 1 to 8, wherein in the adjusting step (b), the pH is selected from 3 to 7.
10. The method of any one of claims 1 to 9, wherein in the treating step (c), the elevated pressure is a pressure in the range of 300MPa and 800MPa.

11. The method of any one of claims 1 to 10, wherein the treating step (c) is undertaken for a duration of 30 seconds to 15 minutes.
12. The method of any one of claims 1 to 11, wherein the isolating step (d) comprises the step of centrifuging the plurality of protein extracts to form the solid component and the liquid component.
13. The method of claim 12, wherein the centrifuging step is undertaken at a rate in the range of 8,000 rpm to 12,000 rpm.
14. The method of claim 12 or 13, wherein the centrifuging step is undertaken for a duration in the range of 8 minutes to 12 minutes.
15. The method of any one of claims 1 to 14, wherein the precipitating step (e) comprises isoelectric precipitation.
16. The method of any one of claims 1 to 15, further comprising the step of isolating the precipitated protein fractions from non-precipitated material.
17. The method of any one of claims 1 to 16, further comprising a step of drying the precipitated protein fractions.
18. The method of any one of claims 1 to 17, wherein the protein sample is selected from the group consisting of pea, lentil, industrial hemp, chickpea, basil seed, pumpkin seed, almond, soy, quinoa, nuts, textured vegetable, tempeh, rice, spirulina, peanut, legume, tofu, beans, seitan, nutritional yeast and combinations thereof.
19. A protein fraction obtained from the method of any one of claims 1 to 18.