

A METHOD OF SEPARATING PROTEIN FRACTIONS

References to Related Application

- 5 This application claims priority to Singapore application number 10202251369N 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 separating protein fractions from a protein sample. The present invention further relates to protein fractions 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 One conventional method of fractionating proteins uses chromatographic techniques to separate protein fractions. However, this method requires expensive instruments which are generally difficult to operate. In addition, chromatographic instruments are generally not able to produce protein fractions on an industrial scale.

- 25 Another conventional method uses buffer systems to separate protein fractions. However, this method involves multiple dialysis steps, which is highly time- and labour-consuming. In addition, it is difficult to obtain protein fractions on an industrial scale based on multiple dialysis steps.

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

30 Summary

In one aspect, there is provided a method of separating protein fractions from a protein sample comprising the steps of:

- 35 (a) dispersing the protein sample in a deep eutectic solvent and optionally in the presence of an aqueous medium to form a dispersion, wherein the deep eutectic solvent comprises a hydrogen bond acceptor and a hydrogen bond donor; and

(b) treating the dispersion of the dispersing step (a) to form a treated dispersion comprising a first protein fraction and a second protein fraction, wherein:

5 (i) the first protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated temperature to form the first protein fraction and a supernatant, and the second protein fraction is formed when the treating step (b) further comprises treating the supernatant at an elevated pressure or precipitating via pH adjustment; or

10 (ii) the second protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated pressure to form the second protein fraction and a supernatant, and the first protein fraction is formed when the treating step (b) further comprises treating the supernatant at an elevated temperature or precipitating via pH adjustment.

Advantageously, the deep eutectic solvent may separate a denaturation temperature of the first protein fraction, and a denaturation temperature of the second protein fraction. This allows the first protein fraction and the second protein fraction to be selectively denatured and aggregated by thermal or pressure treatments on a large scale. If a third protein fraction or a fourth protein fraction is to be obtained, the temperature selected for the thermal treatment will then be a value between the denaturation temperature of the first protein and that of the third protein fraction or the fourth protein fraction but one that is below the denaturation temperature of the second protein fraction.

In another aspect, there is provided a first protein fraction, a second protein fraction, a third protein fraction or a fourth protein fraction obtained from the method as described herein.

25 Advantageously, the first protein fraction, the second protein fraction, the third protein fraction and the fourth protein fraction may have an improved solubility when obtained from the present method as they have different compositions and structures compared with protein fractions obtained by conventional methods. The improved solubility allows the first protein fraction, the second protein fraction, the third protein fraction and the fourth protein fraction to be formulated into food products for different pH requirements.

Definitions

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

35 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.

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.

5 The term "about" as used herein typically means $\pm 5\%$ of the stated value, more typically $\pm 4\%$ of the stated value, more typically $\pm 3\%$ 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.

10 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.

20 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

25 Exemplary, non-limiting embodiments of a method of separating protein fractions from a protein sample will now be disclosed.

The method comprises the steps of:

- 30 (a) dispersing the protein sample in a deep eutectic solvent and optionally in the presence of an aqueous medium to form a dispersion, wherein the deep eutectic solvent comprises a hydrogen bond acceptor and a hydrogen bond donor; and
- (b) treating the dispersion of the dispersing step (a) to form a treated dispersion comprising a first protein fraction and a second protein fraction, wherein:
- 35 (i) the first protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated temperature to form the first protein fraction and a supernatant, and the second protein fraction is formed when the treating step (b) further comprises treating the supernatant at an elevated pressure or precipitating via pH adjustment; or
- (ii) the second protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated pressure to form the second protein fraction and a supernatant, and the first protein fraction

is formed when the treating step (b) further comprises treating the supernatant at an elevated temperature or precipitating via pH adjustment.

Where the treated dispersion further comprises a third protein fraction, the third protein fraction is formed by (c) adding an aqueous medium to the supernatant of the treating step (b), wherein the treating step (b) is treating the dispersion of dispersing step (a) at the elevated temperature only.

Where the treated dispersion further comprises a fourth protein fraction, the fourth protein fraction is formed by (c) adding an aqueous medium to the supernatant of the treating step (b), wherein the treating step (b) is treating the dispersion of dispersing step (a) at the elevated pressure only.

In the dispersing step (a), the deep eutectic solvent is a eutectic mixture comprising the hydrogen bond acceptor and the hydrogen bond donor. Therefore, the deep eutectic solvent has a melting point that is lower than a melting point of the hydrogen bond acceptor and a melting point of the hydrogen bond donor. As the deep eutectic solvent has a low melting point, it is in a liquid form at room temperature, such as 20 °C or 25 °C (i.e., the melting point of the deep eutectic solvent is lower than room temperature).

The hydrogen bond acceptor and the hydrogen bond donor are not particularly limited as long as they are able to form the deep eutectic solvent that allows for the separating of protein fractions.

The hydrogen bond acceptor may be a quaternary ammonium salt, an imidazolium salt or a combination thereof. Non-limiting examples of the hydrogen bond acceptor include choline chloride, *N*-ethyl-2-hydroxy-*N,N*-dimethylethaninium chloride, 2-(chlorocarbonyloxy)-*N,N,N*-trimethylethanaminium chloride, *N*-benzyl-2-hydroxy-*N,N*-dimethylethanaminium chloride and combinations thereof.

The hydrogen bond donor may be a compound that comprises a primary amino group, a secondary amino group, a primary amide group, a second amide group, a hydroxy group, a carboxylic acid group or a combination thereof. Non-limiting examples of the hydrogen bond donor include glycerol, ethylene glycol, 1,3-butanediol, 1,4-butanediol, urea, acetamide, 1-methyl urea, 1,3-dimethyl urea, 1,1-dimethyl urea, thiourea, benzamide, malonic acid, benzoic acid, adipic acid, oxalic acid, succinic acid, citric acid and combinations thereof.

The hydrogen bond acceptor and the hydrogen bond donor may have a molar ratio in the range of about 1:1 to about 1:5, about 1:2 to about 1:5, about 1:3 to about 1:5, about 1:1 to about 1:3, about 1:1 to about 1:2 or about 1:2 to about 1:3.

The deep eutectic solvent may be selected from:

(i) choline chloride and glycerol at a molar ratio of about 1:2;

(ii) choline chloride and ethylene glycol at a molar ratio of about 1:2; or

(iii) choline chloride and 1,4-butanediol at a molar ratio of about 1:3.

The aqueous medium may be water.

5 The dispersion may comprise water at a weight percentage in the range of about 0 weight% to about 95 weight%, about 50 weight% to about 95 weight%, about 0 weight% to about 90 weight% or about 50 weight% to about 90 weight%, based on the total weight of the deep eutectic solvent and water. The dispersion may comprise water at a weight percentage of about 50 weight%, about 60 weight%, about 70 weight%, about 80 weight% or about 90 weight%, based on the total weight of the
10 deep eutectic solvent and the aqueous medium. The weight percentage of water may be suitably selected to allow for an easy dispersion of the protein sample. The weight percentage of water may be alternatively or additionally selected to provide different thermal properties to the protein fractions such that they are readily separated by the present method.

15 As the protein fractions may be used in food, the deep eutectic solvent and the aqueous medium are of food grade. Additionally, the deep eutectic solvent and the aqueous medium may be derived from cost-effective materials (such as those that are commonly used and commercially available).

20 As the protein sample is dispersed (instead of being dissolved) in the dispersion, the dispersion may comprise undissolved solids. The solids may be present at a weight percentage in the range of about 10 weight% to about 30 weight%, about 20 weight% to about 30 weight% or about 10 weight% to about 20 weight%, based on the total weight of the dispersion. The protein sample may comprise the solids at a weight percentage of about 17 weight% based on the total weight of the dispersion.

25 Therefore, the protein sample may have a concentration in the range of about 0.1 g/mL to about 0.3 g/mL, about 0.2 g/mL to about 0.3 g/mL or about 0.1 g/mL to about 0.2 g/mL based on the total volume of the dispersion. The protein sample may have a concentration of about 0.2 g/mL based on the total volume of the dispersion.

30 The method may further comprise a step of heating the hydrogen bond acceptor and the hydrogen bond donor to form the deep eutectic solvent before the dispersing step (a).

The heating step may be undertaken at a temperature in the range of about 80 °C to about 100 °C, about 90 °C to about 100 °C or about 80 °C to about 90 °C.

35 The heating step may be undertaken using a heat source selected from water bath or heat exchanger.

The heating step may further comprise homogenising the hydrogen bond acceptor and the hydrogen bond donor via agitating, shaking or stirring.

The heating step may be undertaken for a duration in the range of about 0.1 hour to about 1 hour, about 0.5 hour to about 1 hour or about 0.1 hour to about 0.5 hour. The heating step may be undertaken until a transparent and homogenous liquid is formed.

- 5 The method may further comprise a step of pre-treating the protein sample before the dispersing step (a).

The pre-treating step may comprise the steps of:

(a1) mixing the protein sample with an aqueous medium in the presence of a base or mixing the protein sample with a basic solution; and

(a2) adding an acid to the protein sample.

- 10 In the mixing step (a1), the base may be sodium hydroxide, sodium bicarbonate, potassium hydroxide, calcium hydroxide, or a combination or a solution thereof. The basic solution may be a solution of sodium hydroxide.

The aqueous medium may be water.

- 15 The protein sample may have a pH value in the range of about 8 to about 11, about 9 to about 11, about 10 to about 11, about 8 to about 10 or about 8 to about 9, after the mixing step (a1). The protein sample may have a pH value of about 9 after the mixing step (a1). Advantageously, the protein fractions may have an improved solubility where the pH value is at least about 8. Further, the protein sample may comprise starch that starts swelling at a pH value above 11, which may result in contamination.
- 20 Therefore, the contamination may be advantageously avoided where the pH value is at most about 11.

The mixing step (a1) may be undertaken for a duration in the range of about 0.5 hour to about 2 hours, about 1 hour to about 2 hours or about 0.5 hour to about 1 hour. The mixing step (a1) may be undertaken for a duration of about 1 hour.

- 25 In the adding step (a2), the acid may be hydrochloric acid, citric acid, acetic acid, or a combination or a solution thereof.

The protein sample may have a pH value in the range of about 6.5 to about 7.5, about 7 to about 7.5 or about 6.5 to about 7 after the adding step (a2). The protein sample may have a pH value of about 7 after the adding step (a2).

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

The method may further comprise a step of characterising the dispersion of the dispersing step (a).

The characterising step may be undertaken via differential scanning calorimetry.

Thermal properties (such as one or more denaturation temperatures) of the dispersion may be determined during the characterising step. Thereafter, the elevated temperature in the treating step (b) may be adjusted according to the thermal properties. Where the protein sample contains at least two protein fractions, each protein fraction has a unique onset denaturation temperature and an end denaturation temperature. Therefore, each protein fraction does not have any substantial overlap in its range of denaturation temperatures as compared to the next protein fraction. This thus allows for the selection of the elevated temperature where the elevated temperature is a temperature that is above the end denaturation temperature of a previous protein fraction (such as the first protein fraction) but below the onset denaturation temperature of the next protein fraction (such as the second protein fraction). Where the protein sample contains more than two protein fractions, the elevated temperature is selected in a similar manner, for example, the elevated temperature is one that is above the end denaturation temperature of the second protein fraction but below the onset denaturation temperature of the third protein fraction, the fourth protein fraction and so on for the next pairs of protein fractions. Alternatively, where the protein sample contains more than two protein fractions, the elevated temperature can be one that is above the end denaturation temperature of the first protein fraction but below the lower of the onset denaturation temperature of the second, third and fourth protein fractions. Accordingly, the characterising step may comprise the steps of determining the end denaturation temperature of a previous protein fraction and the onset denaturation temperature of a next protein fraction; and selecting the elevated temperature based on a temperature between the end denaturation temperature of the previous protein fraction and the onset denaturation temperature of the next protein fraction.

Where elevated temperature is used in the treating step (b) after the characterising step, the elevated temperature may be a temperature that is generally in the range of about 80 °C to about 100 °C, about 90 °C to about 100 °C or about 80 °C to about 90 °C. Where the end denaturation temperature of the previous protein fraction and the onset denaturation temperature of the next protein fraction fall outside this range, the elevated temperature is then selected based on the temperatures determined from the characterising step and thus, the above range of the elevated temperature is only intended as a guide.

Where elevated temperature is used in the treating step (b), the treating step (b) may comprise treating the dispersion of the dispersing step (a) at the elevated temperature for a duration in the range of about 10 minutes to about 20 minutes, about 15 minutes to about 20 minutes or about 10 minutes to about 15 minutes. The treating step (b) may comprise treating the dispersion of the dispersing step (a) at the elevated temperature for a duration of about 15 minutes.

Where elevated pressure is used in the treating step (b), the elevated pressure may be a pressure in the range of about 400 MPa to about 800 MPa, about 450 MPa to about 800 MPa, about 600 MPa to about 800 MPa, about 400 MPa to about 600 MPa, about

400 MPa to about 450 MPa or about 450 MPa to about 600 MPa. The elevated pressure as described herein allows for aggregation of proteins in the protein sample. Uniquely, legumin protein is advantageously precipitated under the elevated pressure in the presence of the deep eutectic solvent.

- 5 Where elevated pressure is used in the treating step (b), the treating step (b) may comprise treating the dispersion of the dispersing step (a) at the elevated pressure for a duration in the range of about 0.5 minute to about 15 minutes, about 3 minutes to about 15 minutes, about 5 minutes to about 15 minutes, about 10 minutes to about 15 minutes, about 0.5 minute to about 10 minutes, about 0.5 minute to about 5 minutes, 10 about 0.5 minute to about 3 minutes or about 3 minutes to about 5 minutes. The treating step (b) may comprise treating the dispersion of the dispersing step (a) at the elevated pressure for a duration of about 5 minutes.

- Where elevated pressure is used in the treating step (b), the treating step (b) may comprise treating the dispersion of the dispersing step (a) at the elevated pressure at 15 a temperature 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 2 °C to about 6 °C, about 2 °C to about 4 °C or about 4 °C to about 6 °C.

- Where the treating step (b) comprises treating the dispersion of the dispersing step (a) at the elevated pressure, the treating step (b) may further comprise a step of 20 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, about 20 MPa/s to about 60 MPa/s, about 40 MPa/s to about 60 MPa/s, about 0.5 MPa/s to about 40 MPa/s or about 0.5 MPa/s to about 20 MPa/s.

- 25 The method may further comprise a step of isolating the first protein fraction and/or the second protein fraction from the treated dispersion. Where the treated dispersion further comprises a third protein fraction or a fourth protein fraction, the isolating step is also applicable to the third protein fraction or the fourth protein fraction and is undertaken after the adding step (c).

- 30 The isolating step may comprise centrifuging the respective treated dispersions obtained in the treating step (b) or after the adding step (c) to pellet the first protein fraction, the second protein fraction, the third protein fraction and/or the fourth protein fraction (as applicable).

- The centrifuging step may be undertaken at a rate in the range of about 8,000 rpm to about 12,000 rpm, about 10,000 rpm to about 12,000 rpm or about 8,000 rpm to about 10,000 rpm. The centrifuging step may be undertaken at a rate of about 10,000 rpm. 35

The centrifuging step may be undertaken 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 centrifuging step may be undertaken for a duration of about 10 minutes.

Where the treating step (b) further comprises precipitating via pH adjustment, the pH value may be adjusted to a value in the range of about 4 and about 5, about 4.5 to about 5 or about 4 to about 4.5. The pH value may be adjusted to about 4.5. As proteins (such as legume proteins) have a low solubility at a pH value in the range of about 4 and about 5, the adjusting of the pH value may precipitate proteins from the supernatant.

To adjust the pH value of the supernatant after being subjected to the elevated temperature or the elevated pressure, an acid may be added to the supernatant. The acid may be hydrochloric acid or a solution thereof.

In the adding step (c), the aqueous medium is added to the supernatant of the treating step (b). The aqueous medium is not particularly limited as long as it is able to break hydrogen bonds between the deep eutectic solvent and proteins in the protein sample. The aqueous medium may be water. The water may be deionised water.

The aqueous medium added in the adding step (c) may have a weight ratio to the supernatant from treating step (b) in the range of about 2:1 to about 6:1, about 4:1 to about 6:1 or about 2:1 to about 4:1. The weight ratio may be about 4:1.

After the adding step (c), the dispersion may be kept at a cold temperature for a duration in the range of about 1 week to about 3 weeks, about 2 weeks to about 3 weeks or about 1 week to about 2 weeks. The dispersion may be kept at the cold temperature for a duration of at least about 2 weeks.

Advantageously, a duration of at least about 2 weeks allows proteins present in the dispersion to aggregate and settle out of the dispersion substantially completely. This makes subsequent collection steps (e.g., via centrifugation) of the proteins easier.

The dispersion may be kept at the cold temperature by being refrigerated at a temperature of about 2 °C to about 6 °C, about 4 °C to about 6 °C or about 2 °C to about 4 °C. The dispersion may be refrigerated at a temperature of about 4 °C.

The method may further comprise a step of purifying the first protein fraction and/or the second protein fraction after the treating step (b). The method may further comprise a step of purifying the third protein fraction or the fourth protein fraction after the adding step (c).

The purifying step may comprise washing the first protein fraction, the second protein fraction, the third protein fraction or the fourth protein fraction with water.

The method may further comprise a step of drying the first protein fraction and/or the second protein fraction after the treating step (b). The method may further comprise

a step of drying the third protein fraction or the fourth protein fraction after the adding step (c).

The drying step may be undertaken via freeze drying, spray drying, oven drying, vacuum drying or combinations thereof.

- 5 The method may further comprise a step of recycling the deep eutectic solvent from the treated dispersion of the treating step (b) or from the dispersion after the adding step (c).

Advantageously, the deep eutectic solvent obtained from the recycling step may be reused in the method.

- 10 In the method, the protein sample may be selected from the group consisting of pea, lentil, 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.

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

The first protein fraction, the second protein fraction, the third protein fraction or the fourth protein fraction may be obtained from the method as described herein.

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

- 25 The first protein fraction may comprise, consist essentially of or consist of albumin, vicilin subunit, convicilin subunit, legumin α and β subunits. In the first protein fraction, total legumin (including all subunits) may have a weight percentage in the range of about 45 weight% to about 55 weight%, about 50 weight% to about 55 weight% or about 45 weight% to about 50 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the first protein fraction. Total vicilin (including all vicilin and convicilin subunits) may have a weight
30 percentage in the range of about 45 weight% to about 55 weight%, about 50 weight% to about 55 weight% or about 45 weight% to about 50 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the first protein fraction. Albumin may have a weight percentage in the range of about 3 weight% to about 5 weight%, about 4 weight% to about 5 weight% or about 3 weight% to about
35 4 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the first protein fraction.

The second protein fraction may comprise, consist essentially of or consist of albumin, vicilin subunit, convicilin subunit, legumin α and β subunits. In the second

protein fraction, total legumin (including all subunits) may have a weight percentage in the range of about 35 weight% to about 45 weight%, about 40 weight% to about 45 weight% or about 35 weight% to about 40 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the second protein fraction. Total vicilin (including all vicilin and convicilin subunits) may have a weight percentage in the range of about 45 weight% to about 55 weight%, about 50 weight% to about 55 weight% or about 45 weight% to about 50 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the second protein fraction. Albumin may have a weight percentage in the range of about 6 weight% to about 10 weight%, about 8 weight% to about 10 weight% or about 6 weight% to about 8 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the second protein fraction.

The third protein fraction may comprise, consist essentially of or consist of legumin α and β subunits. In the third protein fraction, total legumin (including all subunits) may have a weight percentage in the range of about 85 weight% to about 95 weight%, about 90 weight% to about 95 weight% or about 85 weight% to about 90 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the third protein fraction. Total vicilin (including all vicilin and convicilin subunits) may have a weight percentage in the range of about 6 weight% to about 10 weight%, about 8 weight% to about 10 weight% or about 6 weight% to about 8 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the third protein fraction.

The fourth protein fraction may comprise, consist essentially of or consist of legumin α and β subunits and vicilin subunit. In the fourth protein fraction, total legumin (including all subunits) may have a weight percentage in the range of about 70 weight% to about 80 weight%, about 75 weight% to about 80 weight% or about 70 weight% to about 75 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the fourth protein fraction. Total vicilin (including all vicilin and convicilin subunits) may have a weight percentage in the range of about 20 weight% to about 30 weight%, about 25 weight% to about 30 weight% or about 20 weight% to about 25 weight%, based on the total weight of (where present) legumin, albumin, vicilin and convicilin in the fourth protein fraction.

As protein fractions obtained via conventional methods may consist essentially of convicilin and vicilin subunit, the first protein fraction, the second protein fraction, the third protein fraction and the fourth protein fraction obtained from the present method differ in composition and structure compared with protein fractions obtained via conventional methods. Therefore, the first protein fraction, the second protein fraction, the third protein fraction and the fourth protein fraction may advantageously have an improved solubility.

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 flow chart on a general extraction process of protein samples according to the present disclosure.

FIG. 1A

[FIG. 1A] is a flow chart on the extraction process of pea protein from pea protein concentrate using ChCl/glycerol (molar ratio = 1:2).

FIG. 2

[FIG. 2] is a flow chart on the extraction process of lentil protein from lentil protein concentrate using ChCl/1,4-butanediol (molar ratio = 1:3).

FIG. 3

[FIG. 3] is a flow chart on the extraction process of faba bean protein from faba bean protein concentrate using ChCl/ethylene glycol (molar ratio = 1:2).

FIG. 4A

[FIG. 4A] shows thermal characteristics of pea protein concentrate (PPC) dispersed in betaine/glycerol (1:2) of 50% hydration level.

FIG. 4B

[FIG. 4B] shows thermal characteristics of PPC dispersed in ChCl/glycerol (1:2) of 50% hydration level.

FIG. 4C

[FIG. 4C] shows thermal characteristics of PPC dispersed in ChCl/ethylene glycol (1:2) of 50% hydration level.

FIG. 4D

[FIG. 4D] shows thermal characteristics of PPC dispersed in ChCl/1,4-butanediol (1:3) of 50% hydration level.

FIG. 5A

[FIG. 5A] shows thermal characteristics of PPC dispersed in neat betaine/glycerol (1:2) of 0% hydration level.

FIG. 5B

[FIG. 5B] shows thermal characteristics of PPC dispersed in neat ChCl/glycerol (1:2) of 0% hydration level.

FIG. 5C

- 5 [FIG. 5C] shows thermal characteristics of PPC dispersed in neat ChCl/ethylene glycol (1:2) of 0% hydration level.

FIG. 5D

[FIG. 5D] shows thermal characteristics of PPC dispersed in neat ChCl/1,4-butanediol (1:3) of 0% hydration level.

- 10 **FIG. 6A**

[FIG. 6A] shows thermal characteristics of lentil protein concentrate (LPC) dispersed in water.

FIG. 6B

- 15 [FIG. 6B] shows thermal characteristics of LPC dispersed in betaine/glycerol (1:2) of 50% hydration level.

FIG. 6C

[FIG. 6C] shows thermal characteristics of LPC dispersed in ChCl/glycerol (1:2) of 50% hydration level.

FIG. 6D

- 20 [FIG. 6D] shows thermal characteristics of LPC dispersed in ChCl/ethylene glycol (1:2) of 50% hydration level.

FIG. 6E

[FIG. 6E] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 50% hydration level.

- 25 **FIG. 7A**

[FIG. 7A] shows thermal characteristics of LPC dispersed in neat betaine/glycerol (1:2) of 0% hydration level.

FIG. 7B

- 30 [FIG. 7B] shows thermal characteristics of LPC dispersed in neat ChCl/glycerol (1:2) of 0% hydration level.

FIG. 7C

[FIG. 7C] shows thermal characteristics of LPC dispersed in neat ChCl/ethylene glycol (1:2) of 0% hydration level.

FIG. 7D

[FIG. 7D] shows thermal characteristics of LPC dispersed in neat ChCl/1,4-butanediol (1:3) of 0% hydration level.

FIG. 8A

- 5 [FIG. 8A] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 90% hydration level.

FIG. 8B

[FIG. 8B] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 80% hydration level.

- 10 **FIG. 8C**

[FIG. 8C] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 70% hydration level.

FIG. 8D

- 15 [FIG. 8D] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 60% hydration level.

FIG. 8E

[FIG. 8E] shows thermal characteristics of LPC dispersed in ChCl/1,4-butanediol (1:3) of 50% hydration level.

FIG. 9A

- 20 [FIG. 9A] shows thermal characteristics of faba bean protein concentrate (FBPC) dispersed in water.

FIG. 9B

[FIG. 9B] shows thermal characteristics of FBPC dispersed in betaine/glycerol (1:2) of 50% hydration level.

- 25 **FIG. 9C**

[FIG. 9C] shows thermal characteristics of FBPC dispersed in ChCl/glycerol (1:2) of 50% hydration level.

FIG. 9D

- 30 [FIG. 9D] shows thermal characteristics of FBPC dispersed in ChCl/ethylene glycol (1:2) of 50% hydration level.

FIG. 9E

[FIG. 9E] shows thermal characteristics of FBPC dispersed in ChCl/1,4-butanediol (1:3) of 50% hydration level.

FIG. 10A

[FIG. 10A] shows thermal characteristics of FBPC dispersed in neat betaine/glycerol (1:2) of 0% hydration level.

FIG. 10B

- 5 [FIG. 10B] shows thermal characteristics of FBPC dispersed in neat ChCl/glycerol (1:2) of 0% hydration level.

FIG. 10C

[FIG. 10C] shows thermal characteristics of FBPC dispersed in neat ChCl/ethylene glycol (1:2) of 0% hydration level.

- 10 **FIG. 10D**

[FIG. 10D] shows thermal characteristics of FBPC dispersed in neat ChCl/1,4-butanediol (1:3) of 0% hydration level.

FIG. 11A

- 15 [FIG. 11A] shows thermal characteristics of FBPC dispersed in ChCl/ethylene glycol (1:2) of 90% hydration level.

FIG. 11B

[FIG. 11B] shows thermal characteristics of FBPC dispersed in ChCl/ethylene glycol (1:2) of 80% hydration level.

FIG. 11C

- 20 [FIG. 11C] shows thermal characteristics of FBPC dispersed in ChCl/ethylene glycol (1:2) of 70% hydration level.

FIG. 11D

[FIG. 11D] shows thermal characteristics of FBPC dispersed in ChCl/ethylene glycol (1:2) of 50% hydration level.

- 25 **FIG. 12A**

[FIG. 12A] shows thermal characteristics of pea protein in dispersed in ChCl/glycerol (1:2) at 60% hydration level.

FIG. 12B

- 30 [FIG. 12B] shows thermal characteristics of pea protein dispersed in ChCl/glycerol (1:2) at 60% hydration level with heat treatment.

FIG. 12C

[FIG. 12C] shows thermal characteristics of pea protein dispersed in ChCl/glycerol (1:2) at 60% hydration level with HPP treatment.

FIG. 13

[FIG. 13] shows SEC-HPLC chromatograms of pea proteins extracted by conventional alkaline-extraction/isoelectric precipitation (PPC-pH9-IEP); or extracted from the supernatants of heat-treated (PPC Heat ASP) or high-pressure treated (PPC HPP IEP) samples containing DES.

FIG. 14

[FIG. 14] shows the effect of pH (2.0 to 8.0) on protein solubility (%) of pea protein fractions obtained via different extraction methods.

FIG. 15

[FIG. 15] shows the effect of pH (2.0 to 8.0) on protein solubility (%) of faba bean protein fractions obtained via different extraction methods.

FIG. 16

[FIG. 16] shows SDS-PAGE (under reducing conditions) of pea protein obtained via different extraction methods.

FIG. 17

[FIG. 17] shows the iBright analysis of the SDS-PAGE (under reducing conditions) of the different sub-fractions of pea protein obtained via different extraction methods.

FIG. 18

[FIG. 18] shows SEC-HPLC chromatograms of the different pea protein fractions extracted using DES without any additional treatment.

FIG. 19

[FIG. 19] shows SEC-HPLC chromatograms of the different pea protein fractions extracted using DES alongside heat treatment.

FIG. 20

[FIG. 20] shows SEC-HPLC chromatograms of the different pea protein fractions extracted using DES alongside HPP treatment.

FIG. 21

[FIG. 21] shows SEC-HPLC chromatograms of lentil proteins extracted by conventional alkaline-extraction/isoelectric precipitation (LPC pH9-IEP); or using DES alongside heat treatment.

FIG. 22

[FIG. 22] shows SEC-HPLC chromatograms of faba bean proteins extracted by conventional alkaline-extraction/isoelectric precipitation (FBPC pH9-IEP); or using DES alongside heat treatment.

Detailed Description of Drawings

FIG. 1

[FIG. 1] is a flow chart on a general extraction process of protein samples according to the present disclosure. As shown in FIG. 1, a protein sample (100) may be pre-treated (102) with a base solution to form a mixture. The mixture may be centrifuged subsequently with the supernatant (104) being a solution of the desired proteins and insoluble solids (106) may be discarded.

The supernatant (104) may then be dispersed in a deep eutectic solvent (108) to form a dispersion (110). The dispersion (110) may be subject to a treatment at an elevated temperature (112) to produce a first protein fraction (116) or an elevated pressure (114) to produce a second protein fraction (118). The first protein fraction (116) may alternatively be obtained by treating the dispersion (110) at an elevated pressure (114) to form a supernatant (119) and the second protein fraction (118), followed by treating the supernatant (119) at an elevated temperature (112) or followed by adjusting a pH value of the supernatant to about 4.5 (120). The second protein fraction (118) may alternatively be obtained by treating the dispersion (110) at an elevated temperature (112) to form a supernatant (117) and the first protein fraction (116), followed by treating the supernatant (117) at an elevated pressure (114) or followed by adjusting a pH value of the supernatant to about 4.5 (120). A third protein fraction (124) may be obtained by treating the dispersion at an elevated temperature (112), followed by adding an aqueous medium to the supernatant (122). A fourth protein fraction (126) may be obtained by treating the dispersion at an elevated pressure (114), followed by adding an aqueous medium to the supernatant (122).

FIG. 1A

[FIG. 1A] is a flow chart on the extraction process of pea protein from pea protein concentrate using ChCl/glycerol (molar ratio = 1:2). Briefly, pea protein concentrate (127) may be mixed with distilled water at a 1:5 solid/liquid ratio and adjusted to a pH value of about 9 using NaOH (128). The mixture may then be stirred magnetically for 1 hour (130) and centrifuged at 10,000 rpm for 10 minutes (132). Insoluble solids (136) obtained after centrifugation may then be discarded and the supernatant (134) may be adjusted to a pH value of about 7 using HCl solution (138) and dispersed in a mixture of ChCl/glycerol (molar ratio = 1:2) of 40% hydration level (140).

The dispersion obtained may then be treated via HPP at 600 MPa for 5 minutes (142), heating at 100 °C for 15 minutes (144) or left untreated at this stage (146). The different samples obtained may then be centrifuged at 10,000 rpm for 10 minutes (148) and separated into a supernatant (150) and insoluble solids (152). The insoluble solids (152) may be washed with deionized water thrice (154) and freeze-dried (156) to form pellets labeled as PPC untreated/HPP/heat pellets (158).

The supernatant may be precipitated via addition of an anti-solvent (160). Where the anti-solvent is deionized water, the anti-solvent and the supernatant may have a weight ratio of about 4:1. After the addition of the anti-solvent, the supernatant may

be centrifuged at 10,000 rpm for 10 minutes (162) and separated into insoluble solids (164) and a supernatant (166). The insoluble solids may be further washed with deionized water thrice (168) and freeze-dried (170) to form solid products labeled as PPC untreated/HPP/Heat ASP (172).

- 5 The supernatant may be alternatively precipitated via adjusting its pH value to about 4.5 (174). After adjusting the pH value, the supernatant may be centrifuged again at 10,000 rpm for 10 minutes (176) and separated into insoluble solids (178) and a supernatant (180). The insoluble solids may be further washed with deionized water thrice (182) and freeze-dried (184) to form solid products labeled as PPC
10 untreated/HPP/Heat IEP (186).

FIG. 2

- [FIG. 2] is a flow chart on the extraction process of lentil protein from lentil protein concentrate using ChCl/1,4-butanediol (molar ratio = 1:3). Briefly, lentil protein concentrate (200) may be mixed with distilled water at a 1:5 solid/liquid ratio and
15 adjusted to a pH value of about 9 using NaOH (202). The mixture may then be stirred magnetically for 1 hour (204) and centrifuged at 10,000 rpm for 10 minutes (206). Insoluble solids (210) obtained after centrifugation may then be discarded and the supernatant (208) may be adjusted to a pH value of about 7 using HCl solution (212) and dispersed in a mixture of ChCl/1,4-butanediol (molar ratio = 1:3) of 40%
20 hydration level (214).

- The dispersion obtained may then be heated at 81.5 °C for 15 minutes (216). After heating, the dispersion may then be centrifuged at 10,000 rpm for 10 minutes (218) and separated into a supernatant (220) and insoluble solids (222). The insoluble solids (222) may be washed with deionized water (224) and freeze-dried (226) to form
25 pellets labeled as LPC Heat Pellet (228).

- The supernatant may be precipitated via addition of an anti-solvent (230). Where the anti-solvent is deionized water, the anti-solvent and the supernatant may have a weight ratio of about 4:1. After the addition of the anti-solvent, the supernatant may be centrifuged at 10,000 rpm for 10 minutes (232) and separated into insoluble solids
30 (234) and a supernatant (236). The insoluble solids may be further washed with deionized water thrice (238) and freeze-dried (240) to form solid products labeled as LPC Heat ASP (242).

- The supernatant may be alternatively precipitated via adjusting its pH value to about 4.5 (244). After adjusting the pH value, the supernatant may be centrifuged again at
35 10,000 rpm for 10 minutes (246) and separated into insoluble solids (248) and a supernatant (250). The insoluble solids may be further washed with deionized water thrice (252) and freeze-dried (254) to form solid products labeled as LPC Heat IEP (256).

FIG. 3

- 40 [FIG. 3] is a flow chart on the extraction process of faba bean protein from faba bean protein concentrate using ChCl/ethylene glycol (molar ratio = 1:2). Briefly, faba bean protein concentrate (300) may be mixed with distilled water at a 1:5 solid/liquid ratio and adjusted to a pH value of about 9 using NaOH (302). The mixture may then be stirred magnetically for 1 hour (304) and centrifuged at 10,000 rpm for 10 minutes

(306). Insoluble solids (310) obtained after centrifugation may then be discarded and the supernatant (308) may be adjusted to a pH value of about 7 using HCl solution (312) and dispersed in a mixture of ChCl/ethylene glycol (molar ratio = 1:2) of 40% hydration level (314).

- 5 The dispersion obtained may then be heated at 97 °C for 15 minutes (316). After heating, the dispersion may then be centrifuged at 10,000 rpm for 10 minutes (318) and separated into a supernatant (320) and insoluble solids (322). The insoluble solids (322) may be washed with deionized water thrice (324) and freeze-dried (326) to form pellets labeled as FBPC Heat Pellet (328).
- 10 The supernatant may be precipitated via addition of an anti-solvent (330). Where the anti-solvent is deionized water, the anti-solvent and the supernatant may have a weight ratio of about 4:1. After the addition of the anti-solvent, the supernatant may be centrifuged at 10,000 rpm for 10 minutes (332) and separated into insoluble solids (336) and a supernatant (334). The insoluble solids may be further washed with
- 15 deionized water thrice (338) and freeze-dried (340) to form solid products labeled as FBPC Heat ASP (342).

- The supernatant may be alternatively precipitated via adjusting its pH value to about 4.5 (344). After adjusting the pH value, the supernatant may be centrifuged again at 10,000 rpm for 10 minutes (346) and separated into insoluble solids (348) and a
- 20 supernatant (350). The insoluble solids may be further washed with deionized water thrice (352) and freeze-dried (354) to form solid products labeled as FBPC Heat IEP (356).

Examples

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

Example 1 – Preparation of Deep Eutectic Solvents

- Food-grade deep eutectic solvents (DES) were prepared by mixing a hydrogen bond acceptor (e.g., choline chloride (ChCl), purchased from Sigma-Aldrich Pte Ltd, Singapore) and hydrogen bond donor (e.g., glycerol, purchased from Sigma-Aldrich
- 30 Pte Ltd; ethylene glycol, purchased from Sigma-Aldrich Pte Ltd, Singapore; or 1,4-butanediol, purchased from Sigma-Aldrich Pte Ltd, Singapore, etc.) in a specific molar ratio (e.g., choline chloride and glycerol in a molar ratio of 1:2, choline chloride and ethylene glycol in a molar ratio of 1:2, choline chloride and 1,4-butanediol in a
- 35 molar ratio of 1:3, etc.) and heating (at a temperature ranging from 80 to 100 °C) using a heat source (e.g., water bath, heat exchanger, etc.) with continuous agitation (e.g., shaking water bath) until a transparent, homogeneous liquid is formed. The DES remained in liquid form after cooling to room temperature.

Example 2 – Extraction Process of Pea Protein Using Choline Chloride / Glycerol at A Molar Ratio of 1:2 (60 % Hydration)

An overview of the protein extraction process from pea protein concentrate is shown in FIG. 1A.

- 5 Briefly, pea protein concentrates (VITESSENCE Pulse 1550, purchased from
Ingredion Singapore Pte Ltd, Singapore) was rehydrated with deionized water (at a
solid to liquid weight ratio of 1:5) and adjusted to an alkaline pH (between pH 8.0 -
11.0, preferably pH 9.0) using NaOH solution (1.0 M, prepared from food grade
10 NaOH pellets 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 the supernatant collected is
adjusted to pH 7.0 using HCl solution (1.0 M, prepared from food grade HCl solution
purchased from Thermo Fisher Scientific, Singapore). The total solid content of the
15 supernatant was measured (through weight difference via oven drying at 105 °C for
24 hours) and the amount of moisture present was used to determine the amount of
DES to be added to produce the DES system of the desired hydration level as
described below.

- Pea protein concentrate was dispersed in ChCl/glycerol at a molar ratio of 1:2 and
water content of 60 weight % to make up a protein dispersion of 20% solids by
20 weight. The protein dispersion was weighed (20 mg) into hermetically sealed
aluminium crucibles and the thermal characteristics of the pea protein in the hydrated
DES solution were analysed using DSC from 20 °C to 150 °C at a heating rate of 10
°C/minute.

- Pea protein concentrate was rehydrated with denionised water at a 1:5 solid to liquid
25 weight ratio and adjusted to pH 9.0 with NaOH solution before stirring for 1 hour
using a magnetic stirrer. The protein solution was then centrifuged at 10,000 rpm for
10 minutes and the supernatant collected was adjusted to pH 7.0 using HCl solution.
The total solid content of the supernatant was determined through weight difference
via oven drying at 105 °C for 24 hours. The supernatant had a moisture content of
30 88.25% and this amount of water was used to determine the amount of DES to be
added to produce a hydration level of 60%.

- The temperatures to be used for fractionating proteins dispersed in DESs were
determined via Differential Scanning Calorimetry (DSC). DESs of different
hydration levels (0 to 100%) were made by mixing deionized water with DES
35 uniformly (by hand, magnetic stirrer, shear mixing, etc.). The dispersion of pea
protein concentrate were weighed into hermetically sealed aluminium crucibles and
thermal characteristics of the proteins in different DES systems of different hydration
levels were determined using DSC.

- The thermal characteristics obtained from DSC showed that ≥ 2 different protein
40 fractions present can be well-separated when dispersed in specific DES systems with
specific hydration levels. Particularly, pea protein concentrate dispersed in a DES
system containing ChCl/glycerol at a molar ratio of 1:2 and deionized water of 60
weight % showed 2 well-separated protein fractions – the first protein fraction had
an onset denaturation temperature of 82.6 °C and end denaturation temperature of
45 99.8 °C while the second protein fraction had an onset denaturation temperature 101.6

°C and end denaturation temperature of 113.6 °C. The different denaturation temperatures could then be utilised for protein fractionation.

5 The pea protein-DES mixture was split into 2 equal volumes where one portion was heated at 100 °C in a water bath for 15 minutes for protein fractionation. As described above, this temperature was selected based on the end denaturation temperature of the first protein fraction and onset denaturation temperature of the second protein fraction. The heated mixture was subsequently centrifuged at 10,000 rpm for 10 minutes and the pellet (PPC Heat Pellet) was collected for further analysis. The remaining portion was poured into a flexible packaging and sealed before it was
10 processed at 600 MPa, 5 °C for 5 minutes. Adiabatic heating of about 4 °C/100 MPa for water occurred, which was lost upon depressurisation. The mixture was also centrifuged at 10,000 rpm for 10 minutes and the pellet (PPC HPP Pellet) was collected for further analysis.

15 The supernatant obtained from centrifugation was split into 2 equal volumes where the remaining proteins were precipitated using either isoelectric precipitation or anti-solvent precipitation. For isoelectric precipitation, the supernatant was adjusted to pH 4.5 using HCl solution and centrifuged at 10,000 rpm for 10 minutes with the pellet collected (PPC Heat/HPP IEP). For anti-solvent precipitation, deionised water was added to the supernatant in a weight ratio of 4:1 and the mixture was left to stand at
20 4°C for 2 weeks. The resulting mixture was then centrifuged at 10,000 rpm for 10 minutes with the pellet collected (PPC Heat/HPP ASP).

All the pellets obtained were washed with 4 parts of deionised water thrice and the final pellets were adjusted to pH 7.0 before undergoing freeze-drying at - 80°C for 7 days. The DES could be recycled via dehydration for further usage.

25 **Example 3 – Extraction Process of Lentil Protein Using Choline Chloride / 1,4-Butanediol at A Molar Ratio of 1:3 (60% Hydration)**

The protein extraction process from lentil protein concentrate is shown in FIG. 2.

Briefly, lentil protein concentrates (VITESSENCE Pulse 2550, purchased from
30 Ingredion Singapore Ptd Ltd, Singapore) was rehydrated with deionized water (at a solid to liquid weight ratio of 1:5) and adjusted to an alkaline pH (between pH 8.0 - 11.0, preferably pH 9.0) using NaOH solution 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 the supernatant collected is adjusted to pH 7.0 using HCl solution. The total solid content of the supernatant was
35 measured (through weight difference via oven drying at 105 °C for 24 hours) and the amount of moisture present was used to determine the amount of DES to be added to produce the DES system of the desired hydration level as described below.

Lentil protein concentrate was dispersed in ChCl/1,4-butanediol at a molar ratio of 1:3 and water content of 60 weight % to make up a protein dispersion of 20% solids
40 by weight. The protein dispersion was weighed (20 mg) into hermetically sealed aluminium crucibles and the thermal characteristics of the lentil protein in the hydrated DES solution was analysed using DSC from 20 °C to 150 °C at a heating rate of 10 °C/minute.

Lentil protein concentrate was rehydrated with denionised water at a 1:5 solid to liquid weight ratio and adjusted to pH 9.0 with NaOH solution before stirring for 1 hour using a magnetic stirrer. The protein solution was then centrifuged at 10,000 rpm for 10 minutes and the supernatant collected was adjusted to pH 7.0 using HCl solution. The total solid content of the supernatant was determined through weight difference via oven drying at 105 °C for 24 hours. The supernatant had a moisture content of 88.80% and this amount of water was used to determine the amount of DES to be added to produce a hydration level of 60%.

The temperatures to be used for fractionating proteins were determined via DSC as described in Example 2. Particularly, lentil protein concentrate dispersed in a DES system containing ChCl/1,4-butanediol at a molar ratio of 1:3 and deionized water of 60 weight % showed 2 well-separated protein fractions – the first protein fraction had an onset denaturation temperature of 70.9 °C and end denaturation temperature of 80.9 °C while the second protein fraction had an onset denaturation temperature 82.6 °C and end denaturation temperature of 93.0 °C. The different denaturation temperatures could then be utilised for protein fractionation.

The lentil protein-DES mixture was heated at 81.5 °C in a water bath for 15 minutes for protein fractionation. As described above, this temperature was selected based on the end denaturation temperature of the first protein fraction and onset denaturation temperature of the second protein fraction. The heated mixture was subsequently centrifuged at 10,000 rpm for 10 minutes and the pellet (LPC Heat Pellet) was collected for further analysis.

The supernatant obtained from centrifugation was split into 2 equal volumes where the remaining proteins were precipitated using either isoelectric precipitation or anti-solvent precipitation. For isoelectric precipitation, the supernatant was adjusted to pH 4.5 using HCl solution and centrifuged at 10,000 rpm for 10 minutes with the pellet (LPC Heat IEP) collected. For anti-solvent precipitation, deionised water was added to the supernatant in a weight ratio of 4:1 and the mixture was left to stand at 4°C for 2 weeks. The resulting mixture was then centrifuged at 10,000 rpm for 10 minutes with the pellet (LPC Heat ASP) collected.

All the pellets obtained were washed with 4 parts of deionised water thrice and the final pellets were adjusted to pH 7.0 before undergoing freeze-drying at - 80°C for 7 days. The DES could be recycled via dehydration for further usage.

Example 4 – Extraction Process of Faba Bean Protein Using Choline Chloride / Ethylene Glycol at A Molar Ratio of 1:2 (70% Hydration)

The protein extraction process from faba bean protein concentrate is shown in FIG. 3.

Briefly, faba bean protein concentrates (VITESSENCE Pulse 3600, purchased from Ingredion Singapore Pte Ltd, Singapore) was rehydrated with deionized water (at a solid to liquid weight ratio of 1:5) and adjusted to an alkaline pH (between pH 8.0 - 11.0, preferably pH 9.0) using NaOH solution 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 the supernatant collected is adjusted to pH 7.0 using HCl solution. The total solid content of the supernatant was

measured (through weight difference via oven drying at 105 °C for 24 hours) and the amount of moisture present was used to determine the amount of DES to be added to produce the DES system of the desired hydration level as described below.

- 5 Faba bean protein concentrate was dispersed in ChCl/Ethylene glycol at a molar ratio of 1:2 and water content of 70 weight % to make up a protein dispersion of 20% solids by weight. The protein dispersion was weighed (20 mg) into hermetically sealed aluminium crucibles and the thermal characteristics of the faba bean protein in the hydrated DES solution was analysed using DSC from 20 °C to 150 °C at a heating rate of 10 °C/minute.
- 10 Faba bean protein concentrate was rehydrated with deionised water at a 1:5 solid to liquid weight ratio and adjusted to pH 9.0 with NaOH solution before stirring for 1 hour using a magnetic stirrer. The protein solution was then centrifuged at 10,000 rpm for 10 minutes and the supernatant collected was adjusted to pH 7.0 using HCl solution. The total solid content of the supernatant was determined through weight difference via oven drying at 105 °C for 24 hours. The supernatant had a moisture content of 87.33% and this amount of water was used to determine the amount of DES to be added to produce a hydration level of 70%.
- 15

- The temperatures to be used for fractionating proteins were determined via DSC as described in Example 2. Particularly, faba bean protein concentrate dispersed in a DES system containing ChCl/ethylene glycol at a molar ratio of 1:2 and deionized water of 70 weight % showed 2 well-separated protein fractions – the first protein fraction had an onset denaturation temperature of 86.2 °C and end denaturation temperature of 94.8 °C while the second protein fraction had an onset denaturation temperature 99.3 °C and end denaturation temperature of 109.5 °C. The different denaturation temperatures could then be utilised for protein fractionation.
- 20
- 25

- The faba bean protein-DES mixture was heated at 97 °C in a water bath for 15 minutes for protein fractionation. As described above, this temperature was selected based on the end denaturation temperature of the first protein fraction and onset denaturation temperature of the second protein fraction. The heated mixture was subsequently centrifuged at 10,000 rpm for 10 minutes and the pellet (FBPC Heat Pellet) was collected for further analysis.
- 30

- The supernatant obtained from centrifugation was split into 2 equal volumes where the remaining proteins were precipitated using either isoelectric precipitation or anti-solvent precipitation. For isoelectric precipitation, the supernatant was adjusted to pH 4.5 using HCl solution and centrifuged at 10,000 rpm for 10 minutes with the pellet (FBPC Heat IEP) collected. For anti-solvent precipitation, deionised water was added to the supernatant in a weight ratio of 4:1 and the mixture was left to stand at 4°C for 2 weeks. The resulting mixture was then centrifuged at 10,000 rpm for 10 minutes with the pellet (FBPC Heat ASP) collected.
- 35

- 40 All the pellets obtained were washed with 4 parts of deionised water thrice and the final pellets were adjusted to pH 7.0 before undergoing freeze-drying at - 80°C for 7 days. The DES could be recycled via dehydration for further usage.

Example 5 – Characterisation of Thermal Properties

Thermal characteristics of proteins dispersed in different DES systems was analysed via Differential Scanning Calorimetry using DSC214 Nevio (NETZSCH, Germany). Protein dispersion of 20% solids by weight in DES was weighed (20 mg) into a hermetically sealed aluminium crucible and temperature was increased from 20 °C to 150 °C at a heating rate of 10 °C/minute. The protein dispersions were analysed against an empty aluminium crucible as reference. The onset and end denaturation temperatures of protein peaks were determined using Proteus Analysis software.

The thermal characteristics of pea, lentil and faba bean protein in different DES systems of different hydration levels are shown in FIGS. 4 to 11. It was found that the addition of DES was able to cause a separation in the denaturation temperatures of different protein fractions. From FIGS. 4, 6 and 9, it was observed that the dispersion of proteins in the 4 different DES (Betaine/Glycerol at a molar ratio of 1:2, ChCl/glycerol at a molar ratio of 1:2, ChCl/1,4-butanediol at a molar ratio of 1:3 and ChCl/ethylene glycol at a molar ratio of 1:2) at 50% hydration level resulted in a separation of protein denaturation peaks when compared to the single peak observed when the proteins were dispersed in water.

The dispersion of lentil and faba bean proteins in DES systems of different hydration levels are shown in FIGS. 8 and 11 respectively. The usage of the same DES system at different hydration levels also resulted in different protein denaturation peak characteristics. Hence, both the type of DES used, and their hydration levels are key parameters that showed remarkable impact on the characteristics of the protein denaturation peaks.

The separation of protein denaturation peaks allowed for selective denaturation and aggregation of protein fractions as observed in FIG. 12. The dispersion of pea protein in ChCl/glycerol at a molar ratio of 1:2 at a 60% hydration level produced two separated protein denaturation peaks where the first protein denaturation peak had an end denaturation temperature of 99.8°C. With thermal treatment of the protein dispersion at 100°C for 15 min, the first protein fraction (7S) was selectively denatured and aggregated, leaving behind the other protein fraction that had a higher onset denaturation temperature. On the other hand, the second protein fraction (11S) was preferentially denatured and aggregated by high pressure processing. Such phenomena was first found by the inventors and reported herein.

While separation of protein denaturation peaks was not generally observed in neat DES systems of 0% hydration level as shown in FIGS. 5, 7 and 10, the usage of different DES caused shifts in the onset and end denaturation temperatures of the protein.

Example 6 – Protein Content and Yield

Protein content of 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.

The protein content of proteins extracted using the different DES fractionation processes ranged from 62.59 % to 94.67 % as shown in Table 1. Purity (measured as

“Protein Content” below) of proteins extracted from lentil and faba bean protein concentrates using DES were generally comparable or higher than that of proteins extracted using the conventional alkaline extraction-isoelectric precipitation method (pH 9-IEP).

- 5 **Table 1.** Protein content and yield of pea protein (PPC), lentil protein (LPC), and faba bean protein (FBPC) obtained from fractionation with DES.

Sample	Protein Content (%)	Yield (%)
PPC Heat Pellet	70.38 ± 0.06	32.09
PPC Heat IEP	88.70 ± 0.07	22.34
PPC Heat ASP	84.39 ± 0.06	22.93
PPC HPP Pellet	62.59 ± 0.33	1.50
PPC HPP IEP	83.45 ± 0.11	40.41
PPC HPP ASP	75.70 ± 0.67	43.00
PPC Untreated Pellet	65.57 ± 0.01	1.38
PPC Untreated IEP	77.69 ± 0.11	20.91
PPC Untreated ASP	79.45 ± 0.50	34.68
PPC pH 9-IEP	85.79 ± 0.29	69.32
LPC Heat Pellet	91.23 ± 0.38	27.84
LPC Heat IEP	90.55 ± 0.31	11.06
LPC Heat ASP	84.95 ± 0.06	5.60
LPC pH 9-IEP	86.35 ± 0.04	40.66
FBPC Heat Pellet	88.19 ± 0.18	20.56
FBPC Heat IEP	92.06 ± 0.15	14.80
FBPC Heat ASP	94.67 ± 0.14	13.85
FBPC pH 9-IEP	89.90 ± 0.54	50.33

Example 7 – Chromatographic Characterisations of Protein Extracted

- 10 Size Exclusion Chromatography (SEC) was used to determine the molecular weight distribution of proteins extracted. Protein dispersions of 1% w/v protein weight basis were prepared in potassium phosphate buffer (0.1 M, pH 6.6, prepared from potassium phosphate monobasic and potassium phosphate dibasic that were

- purchased from Sigma-Aldrich Pte Ltd, Singapore). The samples were shaken at 300 rpm at room temperature for 30 minutes using a PSU-10i orbital shaker (biosan, Latvia) before centrifugation at 10,000 rpm for 10 minutes. The supernatant collected was then filtered through a PVDF membrane of 0.22 µm pore size (Merck Millipore Ltd, Ireland). SEC- high performance liquid chromatography (HPLC) was performed using a Shimadzu Prominence LC-20AD system (Kyoto, Japan) equipped with a Yarra™ SEC-4000 column (Phenomenex, USA) and an SPD-M20A diode array detector. The liquid chromatography system also comprises of DGU-20A5R degassing unit, LC-30AD binary pump, SIL-30AC autosampler maintained at 4 °C and CTO-20A column oven at 35 °C. Elution was carried out at a flow rate of 0.4 mL minute⁻¹ with potassium phosphate buffer (0.1 M, pH 6.6) as the mobile phase and a protein standard mix of size 15 to 600 kDa was obtained from Sigma-Aldrich, Singapore for molecular weight determination. A fixed volume of 20 µL of protein sample was injected into the system and detected at 280 nm.
- SEC-HPLC was used to analyse the molecular weight distribution of the protein fractions obtained. As shown in FIG. 13, protein extracted from PPC Heat ASP produced a higher proportion of fractions that were either larger than 150 kDa (similar to 11S legumin fraction) or smaller than 13.7 kDa (albumins). On the other hand, protein extracted from PPC HPP IEP produced a large proportion of protein fraction that lied between 13.7 kDa and 150 kDa.

Example 8 – Solubility of Protein Extracted

- An aqueous mixture of 1% w/v, protein weight basis, was prepared and adjusted to different pH values from 2.0 to 8.0 using NaOH and HCl solutions (1.0 M, purchased from Thermo Fisher Scientific, Singapore). The samples were shaken at 300 rpm at room temperature for 30 minutes using a PSU-10i orbital shaker (biosan, Latvia). pH of the samples was monitored at the 15-minute mark and adjusted to their respective pH when required. The samples were subsequently centrifuged at 10,000 rpm for 10 minutes and the protein content in the supernatant was determined using the Bradford assay (Bradford, 1976). The Bradford assay involved adding Coomassie Brilliant Blue G-250 dye to a test sample comprising protein. When the Coomassie Brilliant Blue G-250 dye binds to proteins, absorbance maximum of the reagent shifts from 470 nm to 595 nm. Therefore, the higher the absorbance detected at 595 nm, the higher the protein concentration in solution. The solubility of the protein extracted was calculated using the formula below.

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

- The percent protein solubility of different pea and faba bean protein fractions across pH range of 2.0 to 8.0 are shown in FIGS. 14 and 15, respectively. As shown in FIG. 14, all pea protein fractions obtained had the lowest solubility at pH 5.0 and higher solubilities at the acidic and alkaline pH ranges. PPC Heat Pellet had the lowest solubility among all fractions across the entire pH range of 2.0 to 8.0. On the other hand, both PPC Heat IEP and PPC Heat ASP had higher solubility than PPC pH9-IEP which was extracted using the conventional alkaline extraction-isoelectric precipitation method.

- The percent protein solubility of different faba bean protein fractions across the pH range of 2.0 to 8.0 is shown in FIG. 15. All protein fractions generally displayed the

lowest solubility at pH 5.0 and higher solubilities at the extreme acidic and alkaline pH ranges. FBPC Heat Pellet had the lowest solubility among all the protein fractions across the entire pH range of 2.0 to 8.0. Proteins extracted from FBPC Heat ASP exhibited higher solubility than that extracted from conventional alkaline extraction-isoelectric precipitation in the acidic pH range of 2.0 to 5.0.

Example 9 – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Protein Extracted

Reducing SDS-PAGE was carried out using the method of Laemmli (Laemmli, 1970) on a continuous buffer system. Protein dispersions containing 4 mg of soluble protein in 3 mL of deionised water were prepared and shaken at 300 rpm at room temperature overnight using a PSU-10i orbital shaker (biosan, Latvia) before centrifugation at 10,000 rpm for 10 minutes. The supernatant collected was mixed with NuPAGE LDS Sample Buffer (4X, purchased from Thermo Fisher Scientific, Singapore) and NuPAGE Reducing Agent (10X, purchased from Thermo Fisher Scientific, Singapore) and made up to a total volume of 100 μ L using deionised water. The mixtures were then heated at 70 °C for 10 minutes. Subsequently, the samples were loaded on a NuPAGE 10% Bis-Tris Midi Gel. A protein ladder (PageRuler Plus Prestained (10 to 190 kDa)) was also loaded to serve as a molecular weight marker. Electrophoresis was run at a constant voltage (120 V) for 60 minutes with NuPAGE MES SDS running buffer (purchased from Thermo Fisher Scientific, Singapore). The gel was stained with InstantBlue Coomassie protein stain (Abcam, Cambridge, UK). The SDS-PAGE gel was then analysed using iBright™ FL1500 Imaging System (Thermo Fisher Scientific, MA, USA). iBright Analysis Software was used to analyse the intensity of bands and the band intensity of each sub-fraction was expressed as a percentage of the total band intensity of the proteins of interest to allow for comparison across samples (Gao et al., 2020). The proteins of interest include the major sub-fractions in pea protein, legumin (11S), vicilin (7S) and albumin.

Electrophoretic bands (under reducing conditions) of pea protein fractions obtained via different extraction methods are shown in FIG. 16 and the analysis of the different sub-fractions of pea protein obtained via different extraction methods is shown in FIG. 17. The first protein fraction as shown by PPC HPP IEP, comprises of 51.08% total legumin, 48.41% total vicilin and 4.15% albumin. The second protein fraction as shown by PPC HPP Pellet and PPC Heat IEP, comprises an average of 51.02% total vicilin, 41.02% total legumin, and 7.96% albumin. The third protein fraction as shown by PPC Heat ASP, comprises of 92.24% total legumin and 7.76% total vicilin. The fourth protein fraction as shown by PPC HPP ASP, comprises of 73.69% total legumin and 26.31% total vicilin.

The different treatments allowed for sub-fraction enrichment that produced protein fractions that are distinct from that of the conventional extraction method of PPC pH 9 IEP. For the heat treated and HPP treated samples, protein recovered via isoelectric precipitation and anti-solvent precipitation yielded vastly different protein fractions. For example, the subunits of vicilin with low molecular weight (14 to 18 and 33 to 35 kDa) were observed in both PPC Heat IEP and PPC HPP IEP but not in PPC Heat ASP and PPC HPP ASP. On the other hand, similar electrophoretic patterns were produced in PPC Heat IEP and PPC HPP IEP, and likewise comparing PPC Heat ASP with PPC HPP ASP, suggesting that the precipitation method may have a greater effect on the protein fractions recovered when subjected to either heat or HPP

5 treatment. Generally, anti-solvent precipitation methods give legumin-rich fractions as observed in the third and fourth protein fractions (PPC Heat ASP and PPC HPP ASP), with more legumin present in the third protein fraction than the fourth protein fraction. However, protein recovered via isoelectric precipitation and anti-solvent precipitation in the untreated samples produced similar electrophoretic bands.

10 The effect of DES on protein fractions obtained can be observed by comparing PPC pH 9 IEP with PPC Untreated IEP. PPC Untreated IEP had less protein bands than PPC pH 9 IEP, especially in the low molecular weight range, suggesting that the interactions between DES and proteins prevented the proteins from being recovered via isoelectric precipitation. Both PPC Heat IEP and PPC HPP Pellet contained more 10 kDa molecular weight albumins than PPC pH 9 IEP as observed from the darker protein bands produced. This showed the advantage of the present process over conventional methods to extract albumins.

Industrial Applicability

15 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, high protein foods and dairy analogs.

20 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 separating protein fractions from a protein sample comprising the steps of:
 - (a) dispersing the protein sample in a deep eutectic solvent and optionally in the presence of an aqueous medium to form a dispersion, wherein the deep eutectic solvent comprises a hydrogen bond acceptor and a hydrogen bond donor; and
 - (b) treating the dispersion of the dispersing step (a) to form a treated dispersion comprising a first protein fraction and a second protein fraction, wherein:
 - (i) the first protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated temperature to form the first protein fraction and a supernatant, and the second protein fraction is formed when the treating step (b) further comprises treating the supernatant at an elevated pressure or precipitating via pH adjustment; or
 - (ii) the second protein fraction is formed when the treating step (b) comprises treating the dispersion of the dispersing step (a) at an elevated pressure to form the second protein fraction and a supernatant, and the first protein fraction is formed when the treating step (b) further comprises treating the supernatant at an elevated temperature or precipitating via pH adjustment.
2. The method of claim 1, wherein the treated dispersion further comprises a third protein fraction that is formed by (c) adding an aqueous medium to the supernatant of the treating step (b), wherein the treating step (b) is treating the dispersion of dispersing step (a) at the elevated temperature only.
3. The method of claim 1, wherein the treated dispersion further comprises a fourth protein fraction that is formed by (c) adding an aqueous medium to the supernatant of the treating step (b), wherein the treating step (b) is treating the dispersion of dispersing step (a) at the elevated pressure only.
4. The method of any one of claims 1 to 3, wherein in the dispersing step (a), the hydrogen bond acceptor is selected from the group consisting of choline chloride, betaine, *N*-ethyl-2-hydroxy-*N,N*-dimethylethaninium chloride, 2-(chlorocarbonyloxy)-*N,N,N*-trimethylethanaminium chloride, *N*-benzyl-2-hydroxy-*N,N*-dimethylethanaminium chloride and combinations thereof.
5. The method of any one of claims 1 to 4, wherein in the dispersing step (a), the hydrogen bond donor is selected from the group consisting of glycerol, ethylene glycol, 1,3-butanediol, 1,4-butanediol, urea, acetamide, 1-methyl urea, 1,3-dimethyl urea, 1,1-dimethyl urea, thiourea, benzamide, malonic acid, benzoic acid, adipic acid, oxalic acid, succinic acid, citric acid and combinations thereof.
6. The method of any one of claims 1 to 5, wherein in the dispersing step (a), the deep eutectic solvent is selected from:
 - (i) choline chloride and glycerol at a molar ratio of 1:2;
 - (ii) choline chloride and ethylene glycol at a molar ratio of 1:2; or
 - (iii) choline chloride and 1,4-butanediol at a molar ratio of 1:3.

7. The method of any one of claims 1 to 6, wherein in the dispersing step (a), the dispersion comprises water at a weight percentage in the range of 50 weight% to 90 weight% based on the total weight of the deep eutectic solvent and the aqueous medium.
- 5 8. The method of any one of claims 1 to 7, wherein in the dispersing step (a), the dispersion comprises solids at a weight percentage of about 17 weight% based on the total weight of the dispersion.
9. The method of any one of claims 1 to 8, further comprising a step of heating the
10 hydrogen bond acceptor and the hydrogen bond donor to form the deep eutectic solvent before the dispersing step (a).
10. The method of any one of claims 1 to 9, where elevated temperature is used in the
15 treating step (b), the elevated temperature is a temperature in the range of 80 °C and 100 °C.
11. The method of any one of claims 1 to 10, where elevated temperature is used in the treating step (b), the elevated temperature is conducted for a duration of about 15
20 minutes.
12. The method of any one of claims 1 to 11, where elevated pressure is used in the treating step (b), the elevated pressure is a pressure in the range of 400 MPa and 800 MPa.
13. The method of any one of claims 1 to 12, where elevated pressure is used in the
25 treating step (b), the elevated pressure is conducted for a duration of about 5 minutes.
14. The method of any one of claims 2 to 13, further comprising a step of isolating the first protein fraction, the second protein fraction, the third protein fraction and/or the fourth protein fraction.
- 30 15. The method of any one of claims 1 and 4 to 14, wherein the precipitating via pH adjustment comprises adjusting a pH value of the supernatant of the treating step (b) to a value in the range of 4 and 5.
- 35 16. The method of any one of claims 2 to 14, wherein the aqueous medium added in the adding step (c) has a weight ratio to the supernatant of the treating step (b) in the range of 2:1 to 6:1.
- 40 17. The method of any one of claims 2 to 16, further comprising a step of purifying the first protein fraction and/or the second protein fraction after the treating step (b) or the third protein fraction or the fourth protein fraction after the adding step (c).
18. The method of any one of claims 2 to 17, further comprising a step of drying the first protein fraction and/or the second protein fraction after the treating step (b) or the
45 third protein fraction or the fourth protein fraction after the adding step (c).
19. The method of any one of claims 1 to 18, 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.
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20. A first protein fraction or a second protein fraction obtained from the method of any one of claims 1 to 19 or a third protein fraction obtained from the method of any one of claims 2 and 4 to 19 or a fourth protein fraction obtained from the method of any one of claims 3 to 19.
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