

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 April 2006 (06.04.2006)

PCT

(10) International Publication Number
WO 2006/034524 A1

- (51) International Patent Classification⁷: **A23J 1/14**
- (21) International Application Number:
PCT/AU2004/001583
- (22) International Filing Date:
16 November 2004 (16.11.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
2004905642 30 September 2004 (30.09.2004) AU
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- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF PRODUCING A LIQUID PHASE PROTEIN

(57) Abstract: A method of producing a liquid phase protein extract from a vegetable protein source, such as soy beans, by extracting soluble protein into an aqueous sodium bisulfite solution, filtering out the spent vegetable protein source to leave an aqueous protein extract, acidifying the aqueous protein to form a discontinuous liquid phase, and centrifuging to separate the phases thereby forming a lower layer of high concentration liquid protein.

WO 2006/034524 A1

TITLE

"METHOD OF PRODUCING A LIQUID PHASE PROTEIN"

THE FIELD OF THE INVENTION

THE INVENTION RELATES to a method of producing a liquid protein
5 phase from a vegetable protein source.

BACKGROUND

Interest has been growing in the use of soy protein in food products,
namely because a number of studies have shown that a soy-rich diet can
safely and effectively alleviate menopausal symptoms, while supporting bone
10 health and heart health, among other benefits. In 1999, the US Food and
Drug Administration (FDA) granted a health claim for the
hypocholesterolemic effects of soy protein. This interest has lead to an
increasing desire to incorporate soy proteins into foods and food
formulations.

15 Soy protein consists of glycinin (11S protein), β -conglycinin (7S
protein) and a mixture of minor proteins. Typically in manufacture, soy
protein is isolated by precipitating the whole soy protein from an aqueous
solution by acidification to the proteins minimum solubility, between pH 4 –
4.8, close to the isoelectric point (Virkar *et al.*, *Biotechnology and*
20 *Bioengineering*, 1982, 24, 871-887).

Glycinin and β -conglycinin have significantly different properties and
nutritional value and therefore it is often considered desirable to use one but
not the other protein in food preparation.

Soybean crops can be genetically modified to contain only glycinin or
25 only β -conglycinin. However worldwide, the application of genetically
modified ingredients in food products is limited by government regulations,
as well as consumer acceptance. An alternative to using genetically
modified crops is to separate proteins from natural soybeans by precipitation.

There have been a number of studies investigating the separation of
30 glycinin and β -conglycinin from soy flour, for example Thanh, V. H. and

Shibasaki K., *Journal of Agricultural and Food Chemistry*, 1976, **24**, 1117-1121 and Nagano, T., M., et al., *Journal of Agricultural and Food Chemistry*, 1992, **40**. Typically these methods involve an aqueous extract of soy protein from soy flour, which is acidified, typically to pH 5 — 6. At this pH, glycinin precipitates while β -conglycinin remains in solution, allowing for the glycinin to be removed prior to the β -conglycinin being obtained from the supernatant through further acidification of the extract and/or chromatographic methods.

U.S. Patent 4,368,151 in the name of Howard, P. A., et al. describes a method of preferentially precipitating the glycinin protein from an aqueous soy extract through the controlled addition of sodium bisulfite and sodium chloride in a pH range between 5.3-6.3. US 4,368,151 teaches that the controlled addition of both sodium bisulfite and sodium chloride are necessary to achieve precipitation of the glycinin protein in a sufficiently high yield.

U.S. Patent 4,370,267 in the name of Lehnhardt, W. F., et al. describes the selective extraction of glycinin protein from an isoelectrically precipitated mixture of glycinin and β -conglycinin. The glycinin and β -conglycinin protein mixture is dissolved in an aqueous environment, comprising sodium chloride and sodium bisulfite, at a pH between 5.0-5.6, adjusted using sodium hydroxide. The β -conglycinin is solubilised and removed in supernatant prior to drying to effect recovery of the β -conglycinin.

Whilst US 4,368,151 and 4,370,267 claim to provide processes which may be readily applied commercially, it has been found that the purity and yield of the protein extracts is low when the methods are scaled up to pilot-plant operations.

Bogracheva, T. Ya., et al., *Applied Biochemistry and Microbiology*, Vol 32, No 4, 1996, 429-433, describes a method of extracting glycinin and β -conglycinin from soy with a purity of greater than 97%. Defatted soy flour is extracted using water at pH 8.0, adjusted with sodium hydroxide, at a ratio of water to flour of 7:1. Mercaptoethanol is added to the extract prior to acidification using 0.2M HCl to pH 8-6. The acidified extract is then cooled to

8°C for 16 hours. It was found that at pH 6.6 two liquid phases formed in the protein extract, one phase having a relatively low protein concentration and the other being a concentrated protein extract. The concentrated protein extract was found to be 33% glycinin. The use of mercaptoethanol renders
5 this method unsuitable for manufacture of food grade protein extracts as mercaptoethanol is toxic to humans. Furthermore, the need to hold the extract at 8°C would render any scaling up of this process to commercial manufacture, cost prohibitive.

To date there is still no cost effective method of obtaining liquid phase
10 glycinin and/or β -conglycinin from soy protein that is suitable for commercial production.

OBJECT OF THE INVENTION

It is an object of the invention to overcome or alleviate one or more of the above disadvantages or to provide the consumer with a useful or
15 commercial choice.

SUMMARY OF THE INVENTION

In one form, although not necessarily the broadest or only form, the invention resides in a method of producing a liquid phase protein extract, including the steps of:
20 forming an aqueous sodium bisulfite solution at pH 8 – 9;
adding vegetable protein source to the aqueous sodium bisulfite solution at a ratio of approximately 1:10;
stirring at room temperature for approximately 35 – 60 minutes, to extract soluble proteins from the vegetable protein source into the aqueous sodium
25 bisulfite solution;
separating spent vegetable protein source from the aqueous sodium bisulfite solution to form an aqueous protein extract;
acidifying the aqueous protein extract to pH 5.7-6.8 to form a discontinuous liquid phase;
30 centrifuging the aqueous protein extract to coalesce the discontinuous phase and form an upper supernatant layer and a lower

layer of high concentration liquid protein phase; and

separating the lower layer of high concentration liquid protein phase.

Vegetable protein sources is herein taken to include soy beans, flax, barley, rye, wheat, cotton, corn, rape seed and the like, in the form of seeds, meal, flour or concentrates produced therefrom. Preferably the vegetable protein source is soybeans. Most preferably the vegetable protein source is selected from full-fat or defatted soy meal (also known as soy flakes), full-fat or defatted soy grits, full-fat or defatted soy flour.

The step of forming an aqueous sodium bisulfite solution preferably includes diluting or dissolving 30-40mM of sodium bisulfite in water and adjusting the pH through the addition of 1M sodium hydroxide (or other suitable base solution).

The aqueous sodium bisulfite solution preferably has an adjusted pH of 8.5.

The high concentration liquid protein phase preferably comprises approximately 75% to 100% glycinin. The high concentration liquid protein phase may further comprise approximately 0% to 13% β -conglycinin; and approximately 0% to 14.5% other proteins.

The supernatant layer or phase comprises between 1.7 to 2.4 wt% protein. More suitably the supernatant layer comprises between 0.3 to 0.9 wt% glycinin, 0.9 wt% β -conglycinin and 0.5wt% other soluble proteins.

The method may further include the step of recovering protein from the supernatant layer as solid precipitate by acidifying the supernatant layer to pH 4-4.5

In another aspect the invention provides the use of the high concentration liquid phase protein extract obtained from the above method in the preparation of food and food formulation, for consumption by both animals and humans.

Room temperature as used herein is used to describe the range of temperatures typically encountered within a laboratory or food processing

plant or assembly line. Room temperature is typically understood to mean a range of temperatures between 10-35°C. More preferably room temperature is considered to be the range of temperatures from 15-21°C.

BRIEF DESCRIPTION OF DRAWINGS

- 5 Embodiments of the invention will be described, by way of example only, with reference to the accompanying drawings in which:
- Fig 1: a graph showing the protein extraction kinetics of soluble soy protein;
- Fig 2: a graph showing total protein solubility, with no sodium chloride added;
- 10 Fig 3: a graph showing total protein solubility, with 0.2M sodium chloride added;
- Fig 4: a graph showing total protein solubility, with 0.5M sodium chloride added;
- 15 Fig 5: a graph showing glycinin solubility, with no sodium chloride added;
- Fig 6: a graph showing glycinin solubility, with 0.2M sodium chloride added;
- Fig 7: a graph showing glycinin solubility, with 0.5M sodium chloride added;
- 20 Fig 8: a graph showing β -conglycinin solubility, with no sodium chloride added;
- Fig 9: a graph showing β -conglycinin solubility, with 0.2M sodium chloride added;
- 25 Fig 10: a graph showing β -conglycinin solubility, with 0.5M sodium chloride added;
- Fig 11: a graph showing the shear behaviour of the high concentration soy protein extract;
- Fig 12: a graph of the calibration of UV Spectrometer for whole soy proteins;
- 30 Fig 13: SDS page results for supernatant at various pHs.
- Fig 14: a graph of the protein concentration profile during phase

separation.

- Fig 15: a micrograph of the liquid – liquid suspension, showing glycinin droplets;
- 5 Fig 16: a graph of discontinuous phase droplet formation in the aqueous protein extract, at pH 6.0, overhead impeller speed of 205rpm;
- Fig 17: a graph of discontinuous phase droplet formation in the aqueous protein extract, at pH 6.0, overhead impeller speed of 355rpm;
- 10 Fig 18: a graph of discontinuous phase droplet formation in the aqueous protein extract, at pH 6.0, magnetic stirrer speed of 215rpm; and
- Fig 19: a graph of discontinuous phase droplet formation in the aqueous protein extract, at pH 6.0, magnetic stirrer speed of 450rpm.
- 15

DETAILED DESCRIPTION

Whilst the below examples describe the use of defatted soy flour as the vegetable protein source for the method of the invention, it will be appreciated by the person skilled in the art that other vegetable protein

20 sources may be readily used in the method of the invention. However, the inventor notes that the invention has particular application as an economic process for producing liquid phase protein extract from full-fat and defatted soy flour, full-fat and defatted soy grits, defatted and full-fatted soy flour.

Example 1

25 An aqueous sodium bisulfite solution is formed by dissolving 40mM sodium bisulfite ($\text{Na}_2\text{S}_2\text{O}_5$, sometimes also referred to as sodium metabisulfite) in water and adjusting the pH to 8.5, through the addition of 1M sodium hydroxide (NaOH). The sodium bisulfite is added as an anti-bacterial agent, as well as providing a buffering effect to the soy protein

30 during extraction.

Defatted soy flour, 100g, is added to the aqueous sodium bisulfite

solution at flour to water ratio of 1:10. The addition of the flour to the aqueous sodium bisulfite solution forms a suspension, in which the aqueous sodium bisulfite solution extracts soluble soy proteins into the aqueous sodium bisulfite solution. The suspension is stirred for 1 hour at room temperature prior to being centrifuged to remove any spent soy flour and obtain an aqueous protein extract. The aqueous soy protein extract, comprises approximately 3.2 wt% protein.

The aqueous soy protein extract is acidified with 1N HCl (or other suitable acid solution) to pH 6.8 – 5.7, while stirring at room temperature until a discontinuous liquid phase is formed. This solution is centrifuged, resulting in the discontinuous liquid phase coalescing to form a lower layer of high-concentration liquid soy protein phase.

It will be appreciated that the amount of soy flour added to the aqueous sodium bisulfite solution may be greater than used in this example, so long as the ratio of flour to water is maintain at approximately 1:10.

The soy flour may be extracted with the aqueous sodium bisulfite solution for a period between 35 minutes and 1 hour, to achieve full extraction of the proteins from the flour.

The high concentration liquid soy protein phase may be separated from the upper supernatant layer, and used either directly in food stuffs or further processed prior to addition to food. Additional processing of the high concentration soy protein extract may include additional liquid-liquid extractions, isoelectric precipitations, enzymatic modification, filtration, drying and the like.

The supernatant layer may be further acidified to pH 4-4.5 to recover protein in solid precipitate form.

Example 2

The effect of various extraction parameters was investigated to determine the ideal extraction process. The process of Example 1 was

repeated altering the time allowed for the aqueous extraction of the soy flour, and observing the effect of salt (namely, sodium chloride, NaCl) on the process of obtaining a high-concentration liquid soy protein phase. The results are summarised below.

5 Protein Extraction Kinetics

The time required for extracting soy protein from the soy flour has been suggested in the literature to be 1 hour. However, it was found that by altering the time for aqueous extraction of the soy protein that complete protein extraction from the soy flour is achieved in approximately 35 minutes.

10 The results are summarised and represented graphically in Fig 1.

Protein Solubility - Effects of Salt and Temperature

The solubility of whole soy protein (glycinin + β -conglycinin + other minor proteins) is shown in Fig 2 to Fig 4, at range of variables, including pH 2-8; temperature 4 – 35°C and sodium chloride concentrations between 0-
15 0.5M. The legend for Figs 2-10 are such that 4C refers to 4°C; 21C to 21°C and 35C to 35°C. The concentrations of whole soy protein, glycinin and β -conglycinin were determined using the SDS-PAGE technique described in more detail in example 3 below.

Between 4 – 35°C, temperature has no significant effect on the
20 solubility profile. The addition of salt increases protein solubility and therefore leads to a decrease in yield of the concentrated soy protein extract. Furthermore, the discontinuous liquid phase does not occur in the presence of added NaCl. Whilst the role of NaCl is not fully understood it is believed that the salt alters the solubility and/or ionic attraction of glycinin present to
25 prevent the droplets of the discontinuous phase from forming.

The corresponding solubility profiles for glycinin and β -conglycinin are shown in Fig 5 to Fig 7 and Fig 8 to Fig 10, respectively. The results show that temperature has no significant effect on the solubility profile of glycinin, whilst it has a statistically significant but small effect on the solubility profile of
30 β -conglycinin. With added NaCl, the differential solubility between glycinin

and β -conglycinin at pH 5.7 – 6.8 is decreased thus separation decreased. Furthermore, at pH < 5.7 liquid-liquid separation does not occur, instead a solid precipitate is formed.

These results indicate that the process of the invention is not sensitive to temperature. The acidification of the aqueous protein extract to between pH 6.8 and 5.7 results in a dramatic decrease in glycinin solubility from 15mg/ml or 1.5wt% to between 3 and 9 mg/ml or 0.3 and 0.9 wt%, whilst having little effect on the β -conglycinin solubility, remaining constant at 9 mg/ml or 0.9 wt%. The addition of salt (NaCl) was found to increase protein solubility, more notably glycinin, and therefore decreases the formation of the discontinuous liquid phase and inturn the yield of high-concentration liquid protein phase. Furthermore the differential solubility of glycinin and β -conglycinin decreases with the addition of salt therefore diminishing the protein separation.

These results in respect to the adverse role of salt are contrary to suggestions made in previously published methods, such as US 4,368,151 and US 4,370,267.

The High-Concentration Soy Protein Liquid Phase

The coalesced discontinuous liquid phase, comprising high-concentration of liquid phase soy protein extract has a density at pH 6.2 of $1.125 \pm 0.001\text{g/ml}$. The high concentration soy protein extract is a non-newtonian fluid, displaying slightly shear-thinning behaviour, with a viscosity of 5 – 10 Pa.s under strain rates of 100 – 0.1 s^{-1} (see Fig 11).

The composition of the high-concentration protein phase is shown in Table 1 below.

pH	Protein Content (wt%)	Composition (%)		
		β -Conglycinin	Glycinin	Other minor proteins
6.8	15.72	5.63	82.97	11.40
6.6	18.58	4.27	84.65	11.08
6.45	23.80	10.12	75.37	14.51
6.39	28.00	0.00	99.02	0.98
6.3	24.42	12.66	85.19	2.15
6.21	21.99	4.55	94.59	0.86
6.1	22.31	4.95	92.35	2.70
6	24.00	7.76	89.72	2.52
5.9	23.72	13.03	85.35	1.62
5.8	24.84	11.95	88.08	0.00
5.7	23.90	11.56	86.03	2.41

Table 1: Composition of the high-concentration protein phase

EXAMPLE 3

- 5 Additional tests were conducted on the solubility of whole soy protein and the compositions of an aqueous soy protein extract as well as the composition of the high concentrate soy protein extract.

Preparation of Protein Extract

- 10 In order to determine the protein content of soy flour, 100g of ground defatted soy flour flakes (courtesy of Solae, Inc.) is stirred into 1L of water at 20°C, prior to conducting a Kjeldahl analysis. The Kjeldahl nitrogen analysis reveals that the defatted soy flour contains approximately 53% protein.

- 15 A soy protein concentrated extract is prepared in a similar manner to example 1, with an aqueous 30mM sodium bisulfite solution, with a pH adjusted through the addition of 1N NaOH, to approximately pH 8.5.

- 20 The suspension is stirred with an overhead impeller at 270 rpm for 1 hr in a constant temperature water bath. The suspension is centrifuged for 25 mins at 10 000 rpm in a Beckman centrifuge to allow ready removal of the supernatant. The final pH of the aqueous protein extract is approximately 7.3.

Sodium bisulfite has a two-fold purpose; firstly, as a preservative to

inhibit bacterial growth and secondly, to function as a buffer to maintain a higher pH level during the extraction, which in turn will increase protein solubility and therefore increasing yield. The final pH of the extract without the addition of sodium bisulfite is approximately 6.3, compared to 7.3 when sodium bisulfite is added. The protein content of the extract has been found to be 32 – 37 mg/ml with sodium bisulfite present and 20 – 25 mg/ml without sodium bisulfite.

Determination of Protein Solubility and Composition

Protein solubility is determined by adding 1M HCl to the aqueous soy protein extract to the desired pH level. The temperature is kept constant at 4°C, 21°C and 35°C in a constant temperature water bath. To determine the effects of salt concentration, 0.2M and 0.5M NaCl is added to the protein extract. The solution is stirred with a magnetic stirrer and allowed to equilibrate overnight. After centrifugation, the supernatant is analysed for protein content using UV spectroscopy and for protein constituents using SDS-PAGE (Bio-Rad) coupled with image analysis (Bio-Rad).

UV Spectrophotometry

The total protein concentration of the supernatant is measured directly by measuring the absorbance of the solution at 280 nm. A calibration curve of absorbance (A_{280}) versus protein concentration, the slope of which is the extinction coefficient, is determined by a series of dilutions whose concentrations are determined by a Total Kjeldahl Nitrogen analysis, where protein content is taken as 6.25 times Kjeldahl nitrogen.

The calibration for the UV absorbance of soy protein at 280 nm is shown in Fig 12. The slope of this line, or the extinction coefficient is $1.913 \pm 3\%$. Surprisingly, a similar calibration curve cannot be found in literature for whole soy proteins but extinction coefficients are available for 7S and 11S proteins separately. Those curves are also plotted in Fig 12 for comparison.

SDS-PAGE and Image Analysis

The protein composition of the aliquot samples taken from the

supernatant at various pHs are determined using reduced sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini- Protean III cell, Bio-Rad 4 – 15% Tris-HCl ReadyGels and Bio-Safe Coomassie Stain. The intensity of the Coomassie stain is linearly proportional to protein content in the range of 8 – 29 μg . Samples or aliquots of the protein extract are therefore diluted to achieve protein content within this range. The image of the gel gives a qualitative measure of the protein composition of the sample. The protein composition is quantified by measuring the intensity density of the bands using Quantity One (Bio-Rad). This program measures the intensity density of each band on the gel and after subtracting the background intensity, measured intensity is linearly proportional to protein content in the range of 8 – 29 μg , the intensity percentage of each band equates to the protein composition of the sample by weight percentage.

Results and Discussion

Fig 13 shows an example of SDS-PAGE images, illustrating the equilibrium supernatant at several pH levels. A reduced SDS-PAGE means that the disulphide bonds are cleaved and the proteins are broken down to their subunits (polypeptide chains). Therefore, β -conglycinin is represented by its three subunits, α' , α and β and glycinin by A (acidic polypeptide) and B (basic polypeptide). The intensities of these bands are directly proportional to the protein content. Qualitatively, it can be seen that as pH is lowered, glycinin (A, B) diminishes from solution before β -conglycinin (α' , α , β) (see Fig 13 (Lanes 4 – 7). In lane 8 at pH 4.01, where the solubility profile (Fig 2) is at a minimum, the bulk of the glycinin and β -conglycinin has precipitated and the materials left are the other proteins (of lower MW).

Image analysis of the gels gives a quantitative measure for the composition of β -conglycinin, glycinin and other proteins in each supernatant sample (Table 2). These are multiplied by the total protein concentration in solution at the given pH, to give the solubility of β -conglycinin and glycinin as a function of pH. The results are shown in Figs 5 and 8.

Table 2. Protein compositions in supernatants of Fig 13

Lane	pH	β -Conglycinin (%)	Glycinin (%)	Other Proteins (%)
2	7.3	29.89	49.13	20.98
3	6.38	20.8	49.09	30.11
4	5.97	35.83	35.99	28.18
5	5.64	46.09	25.69	28.22
6	5.32	50.52	22.1	27.38
7	4.85	52.51	22.16	25.33
8	4.01	0	47.09	52.91
9	3.04	35.39	49.26	15.35

SDS-PAGE was performed on the redissolved droplet and showed that both β -conglycinin and glycinin are always present in the high concentration liquid phase ensuring that the solution is saturated with respect to both these protein species.

Between pH 5 and 6, the solubility of glycinin drops rapidly compared to that of β -conglycinin. At pH 5, the glycinin solubility is at a minimum whilst β -conglycinin solubility begins to drop dramatically until a minimum at pH 4. From these results, it can be concluded that the formation of glycinin droplets at pH 5 – 6 as a first step in soy protein separation is indeed appropriate. Furthermore, Figs 5 and 8 allow for careful selection of the initial protein concentration to improve the purity of coalesced glycinin extract. Since the typical ratio of glycinin to β -conglycinin in a whole soy protein extract feed is approximately 1:1, a simple dilution can result in a solution that is undersaturated with respect to β -conglycinin but supersaturated with respect to glycinin. For example, at 6 mg/ml of β -conglycinin and glycinin, β -conglycinin will remain completely soluble in the range of pH 5.8 – 5, whilst glycinin will form droplets. Thus the resultant glycinin fraction should be relatively free of β -conglycinin.

PHASE SEPARATION KINETICS

1M HCl is added to the aqueous soy protein extract to the desired pH level. Samples of the supernatant are taken at given time intervals and analysed for protein concentration using UV spectrophotometry to determine

the concentration profile during the phase separation process. Time 0 is the time at which the set pH is reached and the first sample is taken approximately 6 seconds after that. The results are shown in Fig 14.

The data in Fig 14 indicate that the phase separation kinetics is very rapid regardless of the pH level at which phase separation is taking place (liquid-liquid separation only occurs at pH > 5.7). The protein concentration of the supernatant at 6 seconds and 24 hours remains largely unchanged and any variations for the samples in-between are within the error of the experiment. This indicates that the solution has reached its equilibrium concentration almost instantaneously. It is thus confirmed that the solubility data in Figures 2 – 10 are equilibrium values as the experiments have been allowed to equilibrate overnight.

These results also indicate that the phase separation kinetics is more rapid than previously suggested as previously published methods require the solution to be stored overnight after adjusting the pH of the aqueous protein extract.

GLYCININ DROPLET FORMATION

Droplet Formation Experiments

The droplet formation experiments are carried out in a 250ml glass beaker. The solution is stirred with either a magnetic stirrer or an overhead 4-blade 45° axial flow impeller at given speeds. 1M HCl is used to adjust the pH of 150ml of whole soy protein extract, or aqueous protein extract, to pH 6. Temperature is held constant at 21°C using a constant temperature water bath. Several samples are taken between 30 seconds and 15 minutes and droplet size distribution is measured by laser light scattering using the Malvern Mastersizer (Malvern, UK). The slurry sample is diluted and suspended in neutral RO water in the Malvern cell during the measurement. RO water is found not to affect the droplet size distribution in the Malvern cell for up to 12 minutes.

Particle/Droplet Size Analysis

The droplet/particle size distribution is measured by laser light scattering using the Malvern Mastersizer (Malvern UK). The slurry sample is diluted and suspended in neutral pH RO water during the measurement. RO
5 water is found not to affect particle size distribution during measurement times of up to 12 minutes.

Results and Discussion

Fig 15 is a typical example of glycinin droplets formed in the liquid-liquid suspension upon acidification of the supernatant. The droplets of
10 glycinin have a morphology quite distinct from the precipitations obtain from isoelectric precipitation of whole soy described in the literature to date.

The formation of glycinin droplets was originally thought to be a precipitate, in light of the teaching of the literature, however whilst surprising additional investigation confirmed the glycinin droplet formation.

15 The liquid phase glycinin in the discontinuous phase of the suspension is in the form of spherical droplets ranging from approximately 2 μm to 10 μm . These droplets coalesce upon centrifugation of the suspension to form the lower layer of the protein extract.

Figs 16 and 17 show the formation of the discontinuous phase, or
20 glycinin phase, at pH 6 with the solution being stirred with an overhead impeller at 205 and 450rpm. Figs 18 and 19 show the formation of the discontinuous phase, or glycinin phase, at pH 6 with the solution being stirred with a magnetic stirrer at 215 and 450rpm. As the phase separation kinetics is very rapid, any change in the size distribution would be due to
25 droplet breakage and/or coalescence only as growth is not expected over the time range observed. The lack of any significant changes in the size distribution indicates that droplet breakage and coalescence is unlikely. It can be readily seen from Figs 16 to 19 that the droplet size is not influenced
30 of shear conditions. It is believed that the reason that droplet break-up does

not occur is due to the viscosity ratio of the discontinuous or discrete phase to the supernatant or continuous phase is greater than 4. The droplets formed by the process of the invention are highly viscous and have a viscosity ratio is in the order of magnitude of 1000.

- 5 The method of the current invention provides the surprising effect of being able to readily obtain high concentration soy protein extracts with a high percentage of glycinin, which may be readily applied to the preparation of food and food formulations. Furthermore the current method avoids use of the more toxic sulphur containing compounds used in some of the prior art
- 10 methods, such as mercaptoethanol. In addition, the method of the current invention does not require the lengthy overnight storage of extract solutions and/or holding solutions at low temperatures that all add to the inconvenience and cost of the prior art processes.

- It should be appreciated that various other changes and modifications
- 15 may be made to the invention described without departing from the spirit or scope of the invention.

CLAIMS

1. A method of producing a liquid phase protein extract, including the steps of:
forming an aqueous sodium bisulfite solution at pH 8 – 9;
5 adding vegetable protein source to the aqueous sodium bisulfite solution at a ratio of approximately 1:10;
stirring at room temperature for approximately 35 – 60 minutes, to extract soluble proteins from the vegetable protein source into the aqueous sodium bisulfite solution;
10 separating spent vegetable protein source from the aqueous sodium bisulfite solution to form an aqueous protein extract;
acidifying the aqueous protein extract to pH 5.7-6.8 to form a discontinuous liquid phase;
centrifuging the aqueous protein extract to coalesce the discontinuous
15 phase and form an upper supernatant layer and a lower layer of high concentration liquid protein phase; and
separating the lower layer of high concentration liquid protein phase.
2. The method of claim 1 wherein the vegetable protein source is selected from one or more of soy beans, flax, barley, rye, wheat, cotton,
20 corn, rape seed and the like, in the form of seeds, meal, flour or concentrates produced therefrom.
3. The method of claim 1 wherein the vegetable protein source is soybeans.
4. The method of claim 1 wherein the vegetable protein source is
25 selected from full-fat or defatted soy meal, full-fat or defatted soy grits and full-fat or defatted soy flour.
5. The method of claim 1 wherein the step of forming an aqueous sodium bisulfite solution includes diluting or dissolving 30-40mM of sodium bisulfite in water and adjusting the pH through the addition of sodium
30 hydroxide.
6. The method of claim 1 wherein the aqueous sodium bisulfite solution

has an adjusted pH of 8.5.

7. The method of claim 1 wherein the high concentration liquid protein phase comprises approximately 75% to 100% glycinin.

8. The method of claim 1 wherein the high concentration liquid protein
5 phase further comprises approximately 0% to 13% β -conglycinin; and approximately 0% to 14.5% other proteins.

9. The method of claim 1 wherein the supernatant layer or phase comprises between 1.7 to 2.4 wt% protein.

10. The method of claim 1 wherein the supernatant layer comprises
10 between 0.3 to 0.9 wt% glycinin, 0.9 wt% β -conglycinin and 0.5wt% other soluble proteins.

11. The method of claim 1 wherein room temperature is in a range of temperatures between 10-35°C.

12. The method of claim 1 wherein room temperature is in the range of
15 temperatures from 15-21°C.

13. The method of claim 1 further including the step of recovering protein from the supernatant layer as solid precipitate by acidifying the supernatant layer to pH 4-4.5.

14. A liquid phase protein obtained by:
20 forming an aqueous sodium bisulfite solution at pH 8 – 9;
adding vegetable protein source to the aqueous sodium bisulfite solution at a ratio of approximately 1:10;
stirring at room temperature for approximately 35 – 60 minutes, to extract soluble proteins from the vegetable protein source into the aqueous
25 sodium bisulfite solution;
separating spent vegetable protein source from the aqueous sodium bisulfite solution to form an aqueous protein extract;
acidifying the aqueous protein extract to pH 5.7-6.8 to form a discontinuous liquid phase;
30 centrifuging the aqueous protein extract to coalesce the discontinuous

phase and form an upper supernatant layer and a lower layer of high concentration liquid protein phase; and separating the lower layer of high concentration liquid protein phase.

15. The liquid phase protein of claim 14 comprising spherical droplets
5 in said discontinuous liquid phase.
16. The liquid phase protein of claim 14 comprising spherical droplets ranging in size from 2 μ m to 10 μ m in said discontinuous liquid phase.
17. The liquid phase protein of claim 14 comprising greater than approximately 75% glycinin.
- 10 18. The liquid phase protein of claim 14 comprising approximately 100% glycinin.
19. A food formulation containing the liquid phase protein of claim 14.

Protein Extraction Kinetics

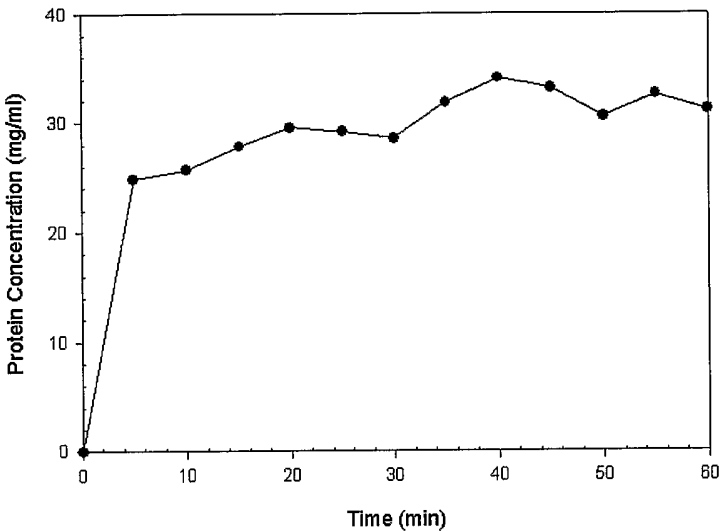


Fig 1: Protein Extraction Kinetics

Total Protein Solubility (0M NaCl)

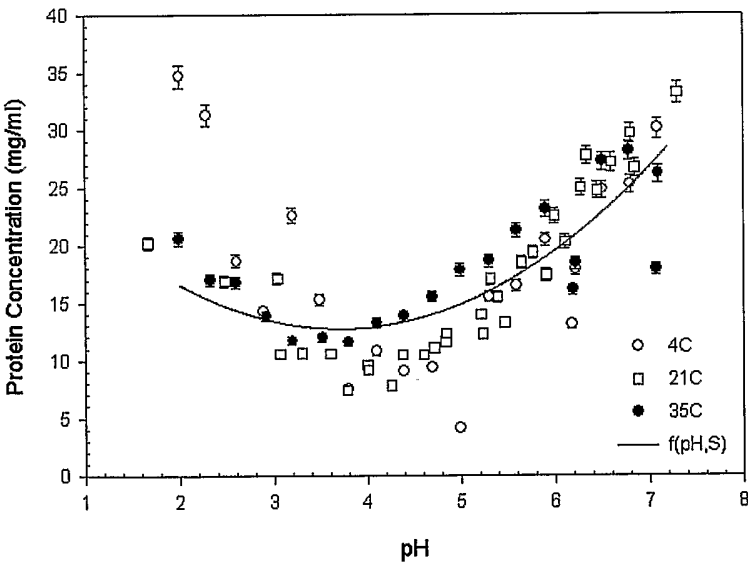


Fig 2: Total Protein Solubility, 0M NaCl added

2/10

Total Protein Solubility (0.2M NaCl)

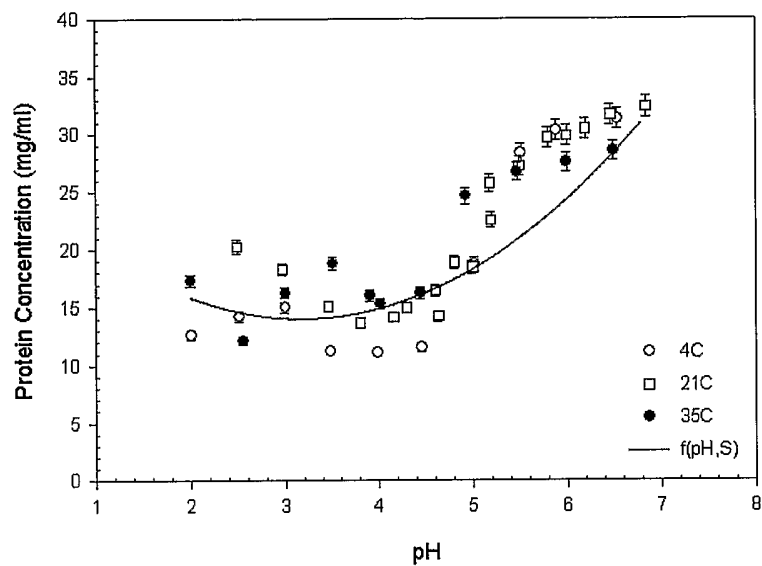


Fig 3: Total Protein Solubility, 0.2M NaCl added

Total Protein Solubility (0.5M NaCl)

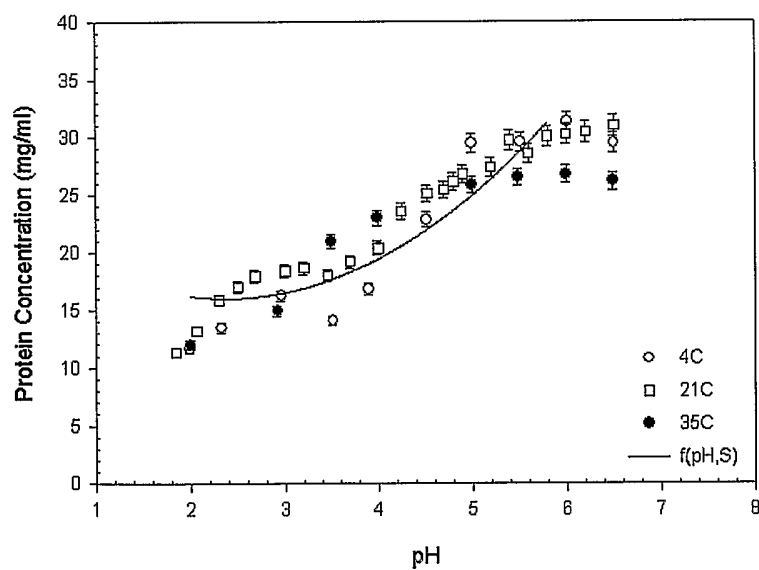


Fig 4: Total Protein Solubility, 0.5M NaCl added

3/10

Glycinin Solubility (0M NaCl)

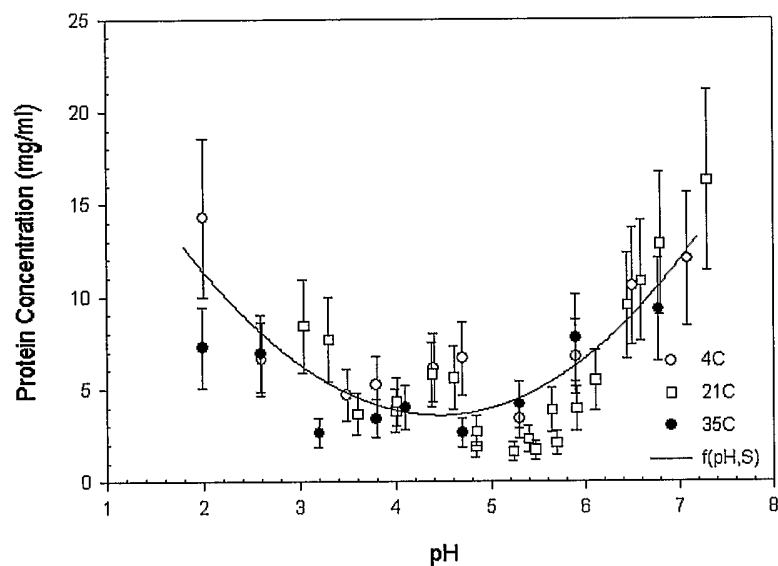


Fig 5: Glycinin Solubility, 0M NaCl added

Glycinin Solubility (0.2M NaCl)

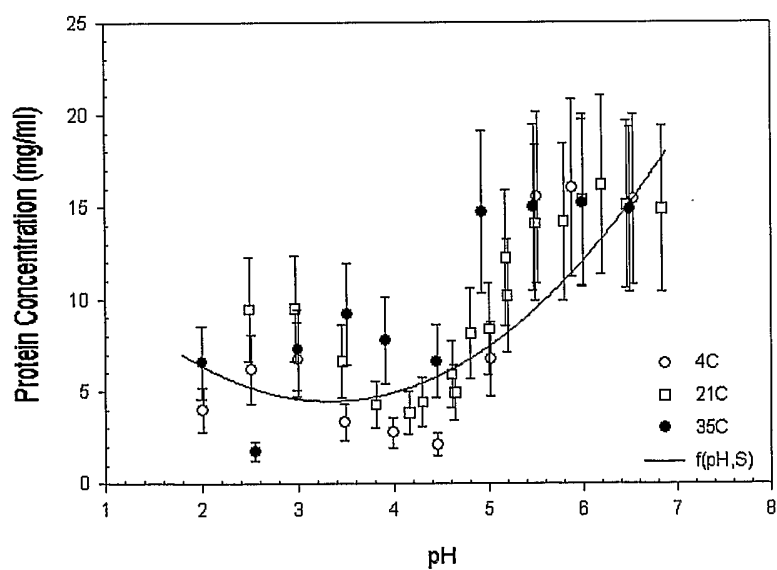


Fig 6: Glycinin Solubility, 0.2M NaCl added

Glycinin Solubility (0.5M NaCl)

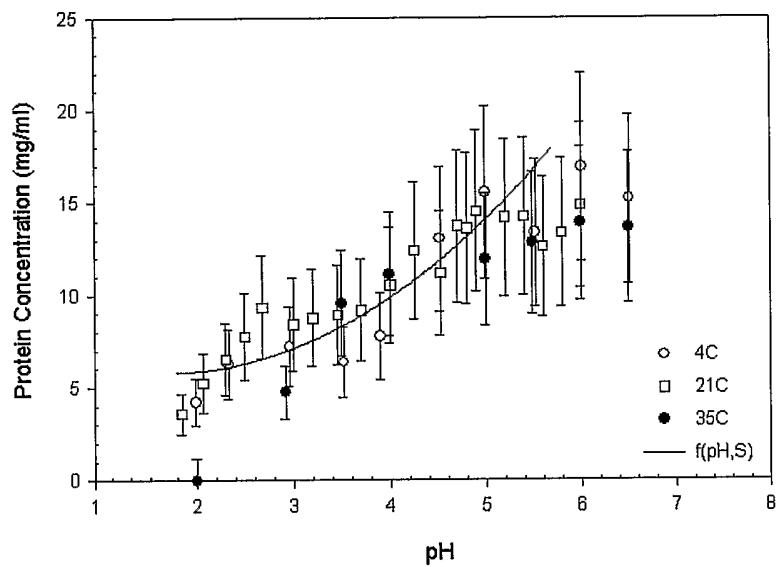


Fig 7: Glycinin Solubility, 0.5M NaCl added

β -Conglycinin Solubility (0M NaCl)

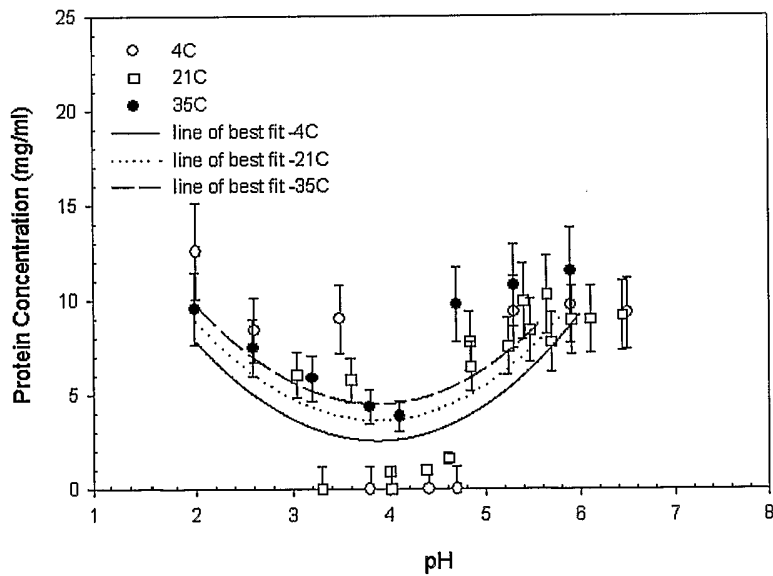
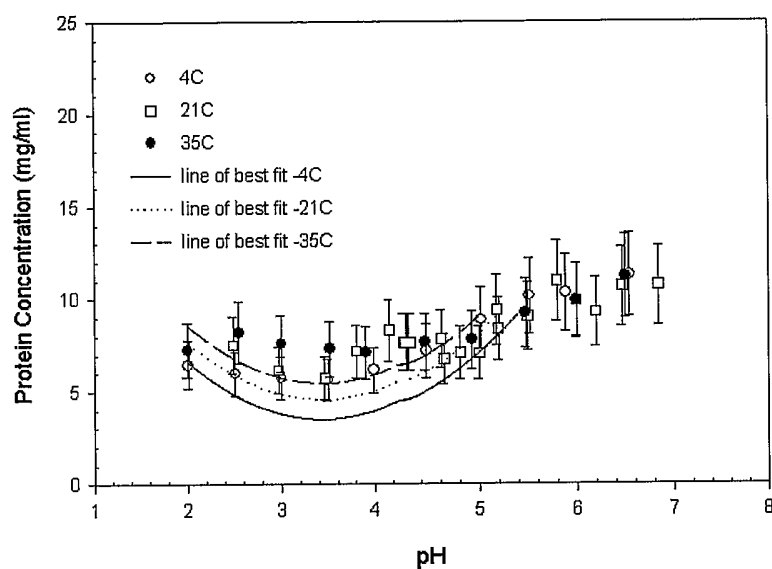
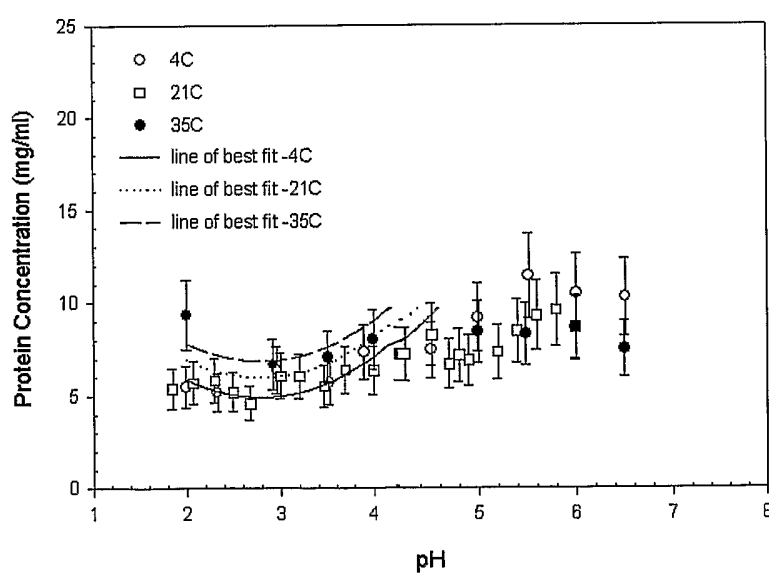


Fig 8: β -Conglycinin Solubility, 0M NaCl added

5/10

 β -Conglycinin Solubility (0.2M NaCl)Fig 9: β -Conglycinin Solubility, 0.2M NaCl added β -Conglycinin Solubility (0.5M NaCl)Fig 10: β -Conglycinin Solubility, 0.5M NaCl added

6/10

Shear Behaviour of the High-Concentration Phase

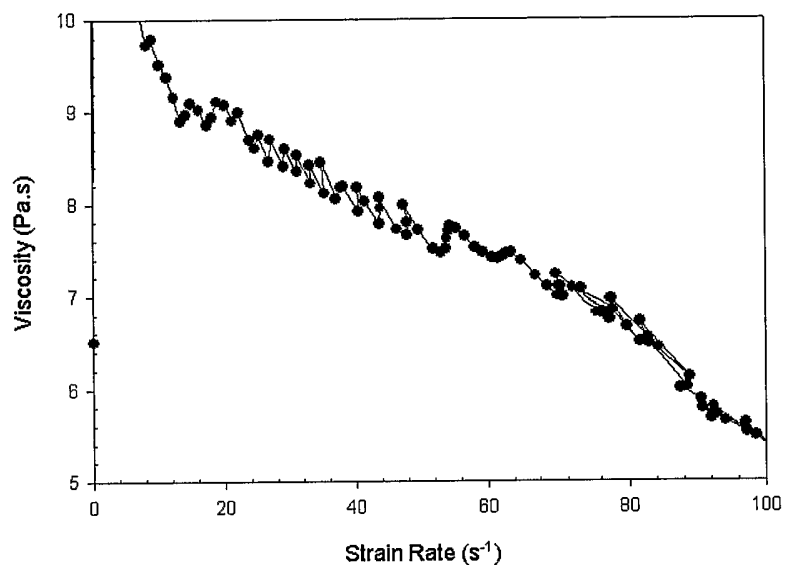


Fig 11: Shear Behaviour of the high-concentration protein phase

UV Absorbance of Soy Proteins at 280 nm

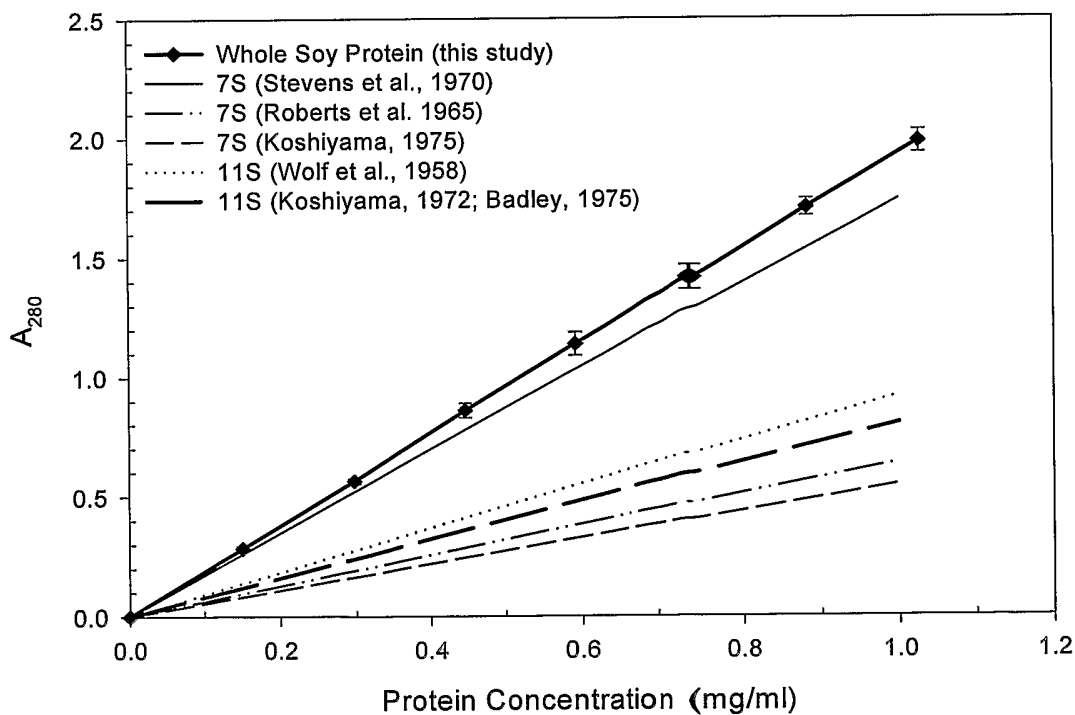


Fig 12: Calibration of UV spectrophotometer for soy proteins.

Estimated 95% confidence bars are shown for the data points.

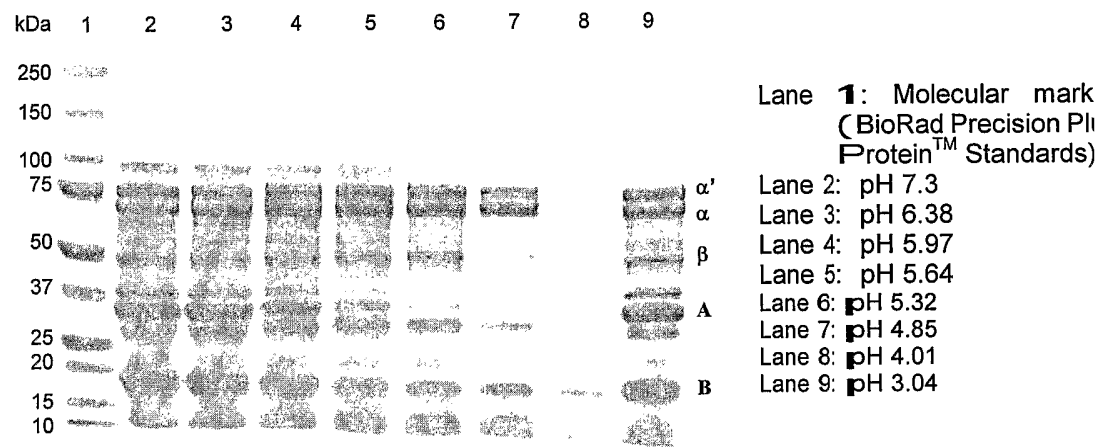


Fig 13: SDS-PAGE results for supernatants from solubility measurements.

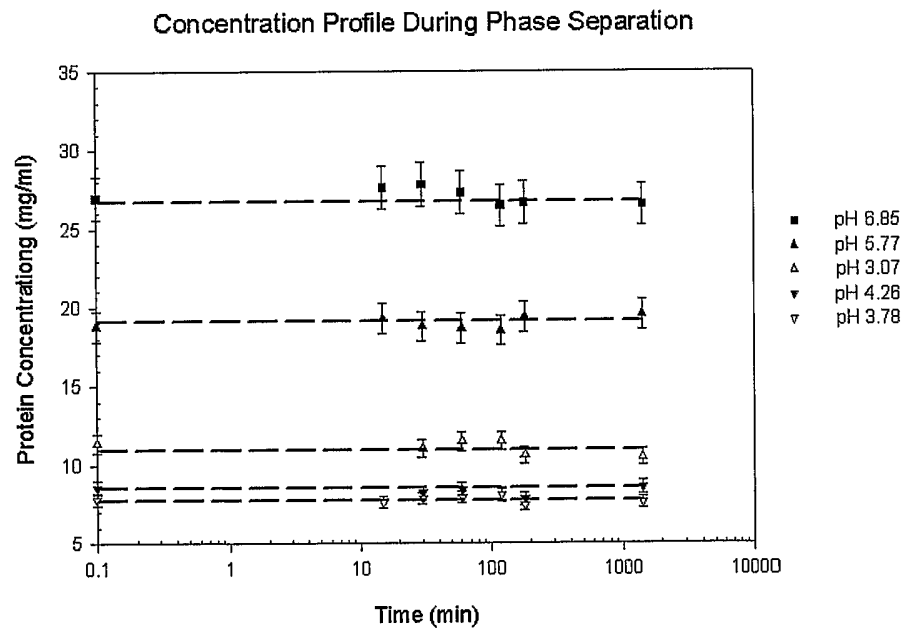


Fig 14: Concentration profile during the phase separation process

8/10

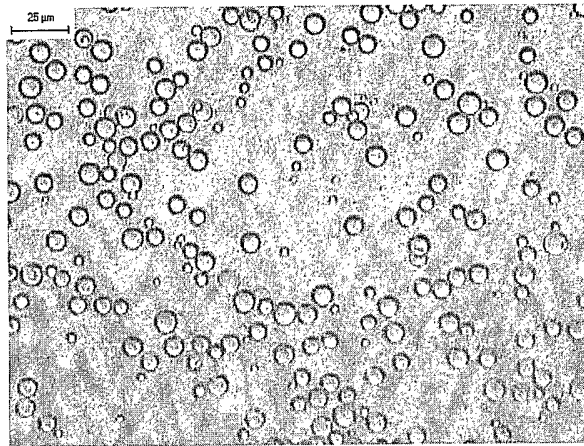


Fig 15: Micrograph of precipitated protein. Bar shown is 25μm .

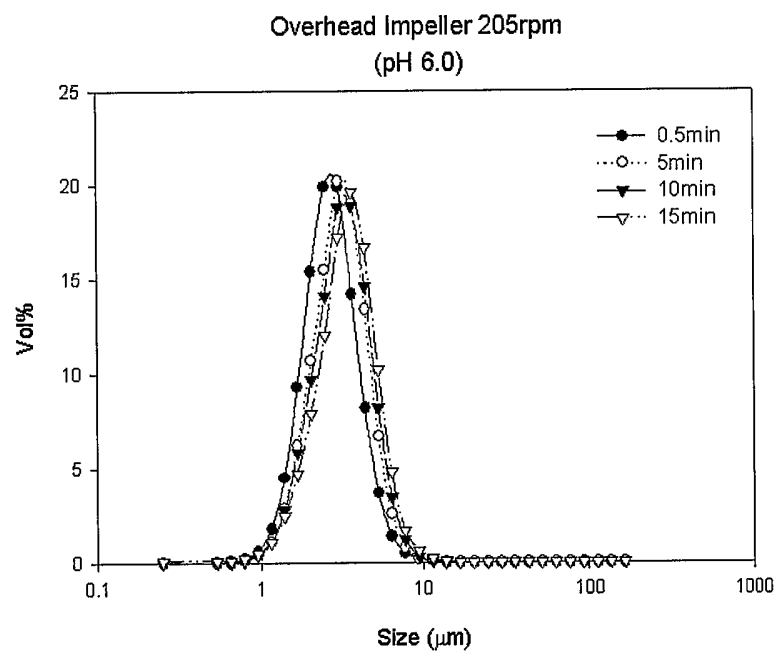


Fig 16: Droplet size distribution of suspension stirred with an overhead 4-blade 45° axial flow impeller at 205rpm.

9/10

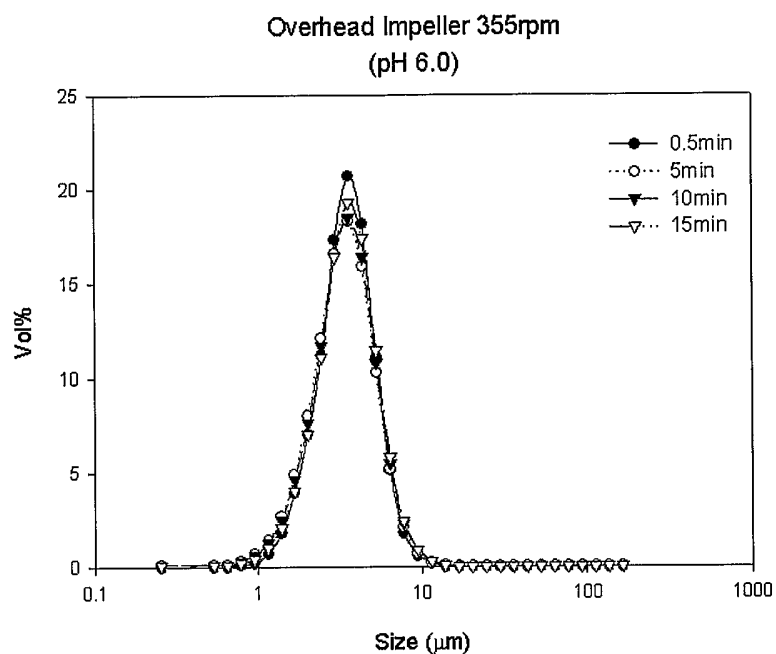


Fig 17: Droplet size distribution of suspension stirred with an overhead 4-blade 45° axial flow impeller at 355rpm.

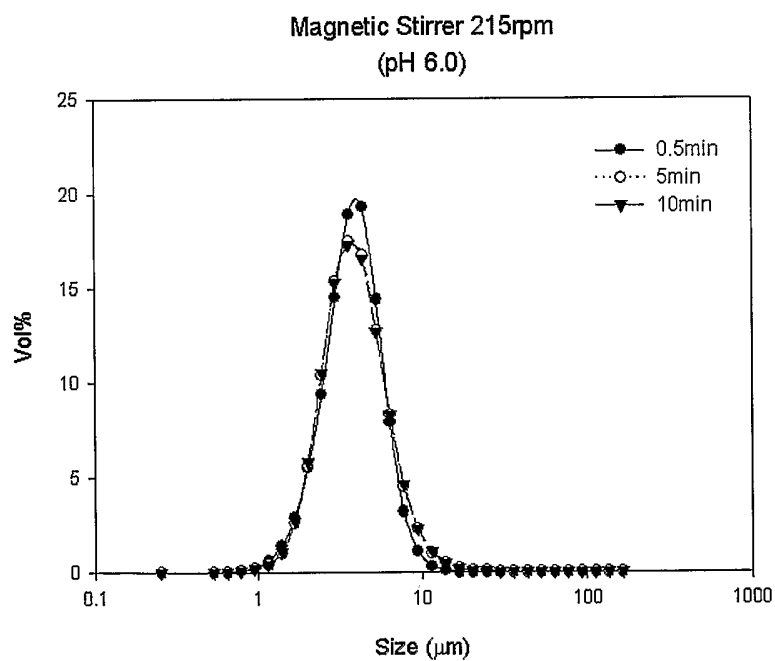


Fig 18: Droplet size distribution of suspension stirred with a magnetic stirrer at 215rpm.

10/10

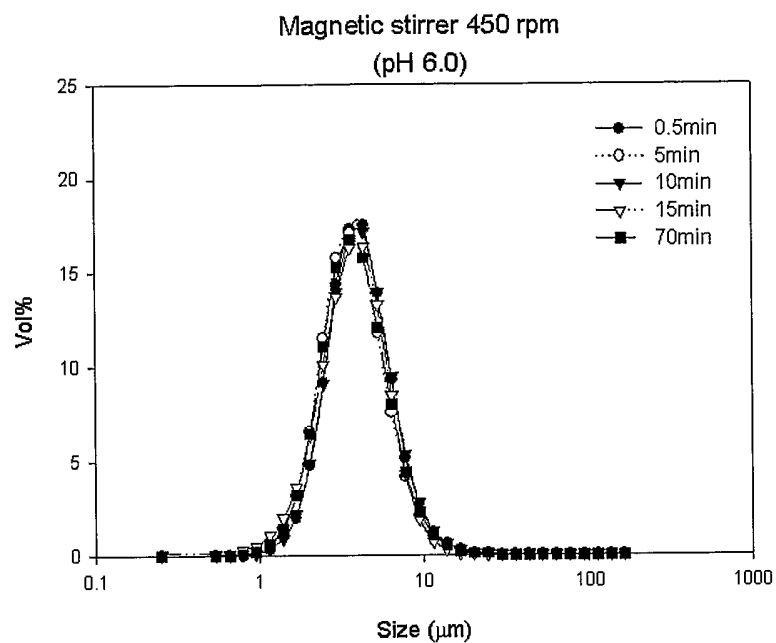


Fig 19: Droplet size distribution of suspension stirred with a magnetic stirrer at 450rpm.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2004/001583

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. ⁷: A23J 1/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC (7): A23J 1/12, 1/14, C07K 14/415

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DWPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4771126 A (Hirotsuka et al.), 13 September 1988 Whole document	1-19
X	US 4435438 A (Lehnhardt et al.), 6 March 1984 Whole document	1-19

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Further documents are listed in the continuation of Box C

☒

See patent family annex

* Special categories of cited documents:	
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
21 December 2004

Date of mailing of the international search report
11 JAN 2005

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2004/001583

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US	4771126	BE	904225	CN	86100959	JP	61187755
		JP	61236795				
US	4435438	CA	1182112	EP	0058276	JP	57132845
		US	4349576				
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							