Protein isolation techniques of beans using different methods: A review

S H Kusumah1,2, R Andoyo1 and T Rialita1
1 Departement of Agroindustrial Technology, Faculty of Agricultural Industrial Technology, Universitas Padjadjaran, Jatinangor 45363, Indonesia
2 Departement of Food Technology, Faculty of Engineering, Universitas Islam Al-Ihya Kuningan, Indonesia

Email: r.andoyo@unpad.ac.id

Abstract. The trend that is developing now is that more people choose to reduce their consumption of animal protein due to health reasons, and replace it with vegetable protein to meet their protein intake. Proteins obtained from beans can be separated from other components called protein concentrates and isolates. Bean protein isolation techniques can use extraction methods with acid-base solvents, salting out methods with salt solvents, and modification by enzymatic hydrolysis. This discussion is being able to clearly see the differences or similarities characteristics of bean protein isolates/concentrates by different isolation methods. The isoelectric point and protein solubility are the main concerns for maximizing the protein of the bean to be isolated. The use of protease enzymes can be useful for breaking peptide bonds so that isolate/concentrate of bean protein becomes easily digested.

Keywords: Protein Isolation, Extraction, Enzymatic Hydrolysis, Beans

1. Introduction
The trend that is developing now is that more people choose to reduce their consumption of animal protein due to health reasons, and replace it with vegetable protein to meet their protein intake. In Indonesia, overall there has been an increase in protein consumption per Indonesian population since 2013–2017 [1]. The protein consumed is mostly derived from vegetable proteins such as cereals, tubers, vegetables, beans, and fruits. The use of soy as an alternative king of vegetable protein is likely to continue for some time to come. However, some researchers have succeeded in isolating proteins from Indonesian local plant protein sources such as green beans [2], red beans [3], winged beans [4], lamtoro gung seeds [5], and koro benguk [6].

Proteins obtained from beans can be separated from other components called protein concentrates and isolates. Concentrates have a protein content of at least 70%, whereas protein isolates must contain more than 90% of protein from dry ingredients. Beans protein concentrates or isolates are obtained by depositing proteins at their isoelectric point. Some researchers set their isoelectric point at pH 3.7–5.5 to produce different yields and characteristics [7–9]. In addition to regulating isoelectric point, the use of temperature and extraction time variations [9] and acid type [10] in protein isolation techniques also determine functional characteristics and properties protein isolates produced.
Bean protein isolation techniques can use extraction methods with acid-base solvents [11], salting out methods with salt solvents [12], and modification by enzymatic hydrolysis [13]. Variations in protein isolation techniques are carried out to produce the best characteristics of these protein isolates/concentrates, so they can be applied in a variety of food systems. Protease enzymes added during the process of protein isolation can break down the structure of proteins more simply (oligopeptides) by breaking peptide bonds.

A simple protein structure will increase the digestibility of proteins in the food system. Protease is used to improve the nutritional and sensory quality of proteins, as well as protein function [14]. Therefore, the authors are interested in discussing techniques for isolating bean protein by a variety of different methods. Another benefit of this discussion is being able to clearly see the differences or similarities characteristics of bean protein isolates/concentrates by different isolation methods.

2. Protein isolation techniques of beans using acid-base extraction methods

Protein isolation in principle is based on two main processes, namely extraction and precipitation of protein [4,6,9]. Protein isolate made from local legumes can be made by extraction/acid hydrolysis and centrifugation methods, to supplement proteins with other components. Protein separation uses solvent acids to deposition proteins at isoelectric pH. Several studies on the isolation of bean protein using the acid-base extraction method are presented in Table 1.

Protein separation uses acid solvents to deposition proteins at isoelectric pH. The choice of an acidic atmosphere as pH during extraction is due to the fact that most amino acids will be positively or negatively charged. This kind of charge will repel each other which causes a minimum interaction between amino acid residues which means it will increase its solubility during extraction. The material is dissolved in dilute HCl solution to pH 4–5 and hydrolyzed, then the mixture is centrifuged so that deposits and liquid form, sediments mostly contain protein and non-protein components dissolved in liquid, the deposits are neutralized using dilute NaOH to pH 6–8 then filtered, dried and analyzed for protein isolates [15].

At its isoelectric point, a protein shows the smallest value of electrostatic repulsion, therefore the protein will have the lowest solubility and will eventually settle. These characteristics are very useful in the process of protein crystallization. When the pH of the solution reaches a certain isoelectric point, a protein will settle and separate from other proteins that have different isoelectric points. That principle is used in separating one protein from another protein. The difference in isoelectric points in proteins is based on differences in the constituent acids. Each amino acid has its own characteristics that distinguish between one another. There are amino acids that are positively charged, negative, or neutral when several amino acids combine to form proteins, then each amino acid charge will contribute to the total charge of the protein it composes [16]. At low pH, amino acids will be positively charged, while at high pH they will be negatively charged. At pH 4.0–5.0 (isoelectric pH), amino acids will be in a dipolar or in zwitter state. In this situation, the protein solubility is the smallest so that the protein will clot and settle [7,9].
Table 1. Several studies on the isolation of bean protein using acid-base extraction methods.

| Material                | Base Type | Extraction pH | Extraction Temperature | Extraction Time | Acid Type | Settlement pH | Result                                                                                     | Reference |
|-------------------------|-----------|---------------|------------------------|-----------------|-----------|---------------|--------------------------------------------------------------------------------------------|-----------|
| Winged Beans            | NaOH      | 10            | 50 ºC                  | 60 min          | HCl       | 3.45          | Levels of protein isolate 83.87% (dry weight), a kamb density value of 0.60 g/ml, the value of white degree 72.73%, water absorption and emulsion stability is quite good | [4]       |
| Koro Benguk             | NaOH and KOH | 10          | 55 ºC                  | 30 min          | HCl       | NaOH = 4.4; KOH = 4.6 | Base type/ characteristics yields | [6]       |
| Soybean Protein Isolates| NaOH      | 8.0           | Room temperature       | 90 min          | HCl       | 3.5           | There is a stable interaction between isoflavones and soy protein                           | [9]       |
| Green Bean              | NaOH      | 8.0           | 80 ºC                  | Acetic acid (CH₃COOH) | 4.5       |               | Yield 77.576 %                                                                 | [10]      |
| Mung Bean Flour         | NaOH      | 8.97          | 31.74 ºC               | 33.24 min       | HCl       | 4.4           | Yield 77.60%                                                                 | [11]      |
| Mung beans              | NaOH      | 9.1           | 40 ºC                  |                 | HCl       | 4.5           | The physicochemical properties (WHC, OAC, Solubility, Emulsification and emulsion stability) of MPI and albumin showed that MPI was more superior and could be considered as high quality of natural protein. Glu was the major component of amino acids in MPI and albumin, at approximately 16.21% and 20.78% respectively. | [17]      |
The value of the isoelectric point of a protein gives an important influence on the biochemical properties of the protein which can be used in the process of purification and electrophoresis. In electrophoresis, if the pH of the buffer solution is greater than the isoelectric point, the protein molecule will migrate towards the positive pole. While if the pH buffer is lower than the isoelectric point, then the protein molecule will migrate towards the negative pole. If the pH buffer is the same as the isoelectric point, then the protein will stay in place or not migrate at all [16].

One of the most important is protein solubility [18]. The isolate protein is made by depositing protein at its isoelectric point. Coagulation and precipitation are carried out by releasing, and releasing acid, to reach a certain pH (isoelectric pH), clumping occurs and deposits (proteins) are removed from the liquid (starch). Red beans and green beans have an isoelectric point at pH 4.4. The isoelectric point is a pH value where the protein has a negative amount of charge equal to the amount of positive charge, or in other words a protein that is neutral or not charged. At a pH value lower than the isoelectric point, the protein has a positive charge, and at a pH value greater than the isoelectric point, the protein will be negatively charged [19].

Acid type and The filtrate determines the ability to coagulate proteins, and the protein deposits formed. Citric acid is a weak acid, and has the power of coagulation, and produces low protein content, low, 54.73% - 61.94%. So it can only cause protein denaturation in smaller amounts with low electronegativity. The addition of acetic acid in proteins can cause protein denaturation. This happens because acetic acid cannot be fully ionized with its electronegativity properties which are smaller than hydrochloric acid. The addition of acetic acid with a pH of 4.5 gives optimal results on the protein content of mung bean isolates. The addition of hydrochloric acid (HCl) which is strong acid results in the presence of H + ions excess, which indicates the turbidity and the presence of more deposits in the heating process [10].

Separation of isolates/concentrates of beans protein through the extraction method begins by extracting the protein in an alkaline solution. A stronger alkaline solution will extract more protein. The results of the treatment in the form of extracts containing dissolved protein and residues which are mostly carbohydrates. The centrifugation stage is carried out to separate the two. Then the next step is to adjust the pH to an isoelectric pH with the addition of acids and will produce protein deposits that are separated from the liquid. The resulting precipitate is washed and dried to obtain protein isolates/concentrates. The best parameters in the isolation of mung bean protein are a liquid ratio of 10%, extraction temperature 31.74, extraction pH 8.97, settlement pH 4.4, extraction time 33.24 minutes [11].

The physicochemical properties (WHC, OAC, solubility, emulsification and emulsion stability) of MPI and albumin showed that MPI was more superior and could be considered as high quality of natural protein. Glu was the major component of amino acids in MPI and albumin, at approximately 16.21% and 20.78% respectively. A relatively greater quantity of Asp, Leu and Phe were demonstrated in the result, too. Conversely, the contents of val were lowest in MPI and albumin, at approximately 0.14%, 0.10% respectively, followed by sulfur-containing amino acids (i.e. methionine and cysteine) [17].

3. Protein isolation techniques with enzymatic hydrolysis methods

Hydrolysis is defined as breaking many bonds into smaller, simpler bonds. In hydrolysis, a bond between two atoms is broken. Peptide bonds that build polypeptide chains in proteins can be hydrolyzed using acids, bases, or enzymes. Breaking peptide bonds using enzymes is a biochemical hydrolysis process that will produce a reaction product in the form of one molecule with a carboxyl group and other molecules having an amine group [20]. The addition of protease enzymes in the process of protein isolation can break down proteins into oligopeptides thereby increasing the amount of oligopeptides in HPF products.

The protein isolation technique of beans carried out by enzymatic hydrolysis is slightly different from the process of isolation through deposition extraction. The beans are ground, then mix with distilled water according to a certain ratio of ingredients to the water. Then, transfer the mixture to the water bath with continuous stirring and adjust the pH of the mixture using NaOH. The protease enzyme is added
to the mixture and maintains the hydrolysis temperature during the enzymatic hydrolysis process. Then, deactivate the enzyme by increasing the temperature to 100 °C for 5 minutes. The mixture is centrifuged at 4,500 rpm for 30 minutes to produce supernatant and sludge. The precipitate was analyzed for protein content by the Kjeldahl method [21].

Hydrolysis is defined as solving many bonds into smaller and simpler bonds. In hydrolysis, a bond between two atoms is broken down. Peptide bonds that build polypeptide chains in proteins can be disconnected (hydrolyzed) using acid, base or enzyme breakdown of peptide bonds in strong acidic or basic conditions is a chemical hydrolysis process and the breakdown of peptide bonds using enzymes is a biochemical hydrolysis process peptide hydrolysis reaction will produce a reaction product in the form of one molecule with a carboxyl group and other molecules having an amine group. The addition of the protease enzyme in the process of isolating the peanut protein is expected to increase the number of oligopeptides so that HPF products become easily digested [20].

Soybeans Oligopeptides (SOP) showed strong stability for proteolytic digestion by pepsin and trypsin. The similarity of the SOP chromatogram before and after pepsin and trypsin digestion was 99.1% and 94.9% as analyzed by evaluation software, indicating that no clear SOP degradation occurred during in vitro digestion. These results indicate that SOPs may not be hydrolyzed into free amino acids in the digestive tract and hence stored in bioactive form after being given as food ingredients. SOP significantly inhibits lipid peroxidation in the linoleic acid oxidation system (IC50 0 1.2 ± 0.09 mg/mL). SOP has inhibition activity of angiotensin I conversion enzyme (IC50 0 1.1 ± 0.06 mg/mL), and antihypertensive effect in spontaneous hypertensive mice at a dose of 200 mg/kg [20]. There have been many studies on protein isolation using the enzymatic hydrolysis method, presented in table 2.

Deffated bean meal (DBM) contains a higher protein (± 40%) so that it can be used as a source of protein/isolate protein. Research related to the use of legume proteins focused on the study of physico-chemical characteristics, functionality, and allergies has been carried out. There are only a few studies that examine proteins in their use for the human diet. Bioactive peptides have been found from the results of enzymatic hydrolysis in several food proteins including mineral-bound peptides, immunomodulatory peptides, antibacterial peptides, antithrombotic peptides, and antihypertensive peptides [21].

Functional oligopeptides refer to the peptides produced with enzymolysis or microbial fermentation technique and with peanut meal or peanut protein as the main material, with a molecular mass of 5000 Da or less. Amino acid analysis results have shown that the free amino acid content of peanut functional oligopeptide is 66.4 mg/g. It can be seen that the product is mainly oligopeptide with a lower amino acid content, despite high hydrolysis of complex enzymolysis. By using the enzymatic method to hydrolyze protein in mild conditions, the polypeptides produced have a very high nutritional value. Oligopeptides with a molecular mass lower than 1000 Da can be easily absorbed by the human body and have strong functional activity. This indicates that the hydrolysis process selected for oligopeptide preparation should maximize the DH of protein while guaranteeing a high yield of oligopeptides [13].

Enzyme species are one of the most important influencing factors in enzymolysis. Five species of proteases are selected for peanut protein hydrolysis in an experiment. Under the recommended conditions for each protease, the TCA-NSI of neutral proteases 1398, FM, Protamex, and N120 p in peanut protein hydrolysis is 45–53% and DH is 9.4–12.1%; while TCA-NSI and DH of alcalase in peanut protein hydrolysis are respectively 63.80 ± 0.47% and 15.01 ± 0.88%, significantly higher than those of other proteases (p < 0.01). The effect of pH on enzymatic reactions is mainly reflected in its effect on enzyme activity. The experiment has analyzed the changes in TCA-NSI and DH when pH changes from 6 to 9. With the rise of pH, TCA-NSI and DH are rapidly increased. When the pH of the system reaches 8.0, TCA-NSI and DH reach 68.61 ± 1.23% and 16.36 ± 0.33%, respectively; when the pH of the system reaches 9.0, TCA-NSI and DH are lower than at pH 8.0, with a decrease of 1.59% and 0.84%, respectively [13].
| Material          | Enzyme Type & Enzyme Concentration | Result                                                                                                                                                                                                                                                                                                                                 | Reference |
|-------------------|-----------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Lamtoro Gung Seed | Sheep rumen fluid enzyme concentration (10 ml/100 gr) and 48 hour incubation time | Content of dry weight protein 56.30%, oil absorption 1.58 ml/gr, froth 11.56%, whereas for water absorption 3.42 ml/gr, kamba density 0.56 gr/ml, emulsion power 52.75%, moisture content 6.39% | [5]       |
| Peanut            | Enzyme Type: AS.1398, FM, Protamex, N120P, dan Alcalase; Dose 3642 U/g; Enzyme Concentration 1–10% | Alcalase has the best characteristics in the hydrolysis of peanut protein with a degree of hydrolysis (DH) and 15.01 ± 0.88%. The highest DH value was produced with an optimal enzyme concentration of 4%, an optimal pH of 8.0–8.5, an optimal temperature of 55 °C, and an optimal time of 120 minutes. | [13]      |
| Soybean Protein Isolates | 200,000 U/kg Protex 13 FL/protein enzyme-substrate | Soybean Oligopeptides (SOP) products consist of short-chain peptides and maintain high stability against digestion. SOP has a strong antihypertensive effect. | [20]      |
| Red Bean          | Enzyme protex 6L 1% - 5%          | The optimal results are pH 8.4, the amount of enzyme additives 3.5%, hydrolysis temperature 56 °C, hydrolysis time 3.5 hours, ratio of material & water 1:6.8, and yield 73.34%. | [21]      |
| Red kidney beans  | Acalase® (2.4 AU/g)               | Proteolysis of KBPI at 300 MPa for 15 min with an addition of 1% Alcalase produced the highest degree of hydrolysis (23.9%) and the antioxidant activities (30.1% DRSA). | [22]      |
| Mung beans        | Bacillus licheniformis alcalase (2.4 L), Aspergillus oryzae flavourzyme (500 U/g), porcine pancreas trypsin (93 U/mg), porcine gastric mucosa pepsin (250 U/mg solid) and pancreatin | The mixture of flavourzyme and pancreatin produced the highest DH but the hydrolysates had lower calcium and iron-binding capabilities when compared to hydrolysates from other enzymes. The calcium and iron-binding capacities of MBPHs were significantly increased after UF (MWCO 5 kDa) separation. The molecular weight of calcium and iron-binding peptides based on size exclusion results were smaller than 1 kDa. | [23]      |
| Soybean flour     | Pepsin (800–1000 U/mg protein), pancreatin (4xUSP), angiotensin-I converting enzyme (peptidyl-di- peptidase A, EC 3.4.15.1, 5.1 U/mg) | DSPI and DSPH exhibit similar antioxidant capacity and different ACE inhibitory activity with each other. DSPI presents ORAC values of 3.6 ± 0.2 µmol TE/mg protein and iACE (IC50) of 70 ± 4 µg protein/mL. DSPH presents ORAC values of 3.9 ± 0.1 µmol TE/mg protein and iACE (IC50) of 52 ± 4 µg protein/mL. | [24]      |
4. Conclusion

Isolation of bean protein can be carried out by an acid-base extraction method combined with enzymatic hydrolysis. The isoelectric point and protein solubility are the main concerns for maximizing the protein of the bean to be isolated. The use of protease enzymes can be useful for breaking peptide bonds so that isolate/concentrate of bean protein becomes easily digested.

References

[1] Badan Pusat Statistik 2017 Consumption of Calorie and Protein of Indonesia Population and Province

[2] Ekafitri R and R H F Faradilla 2011 Pemanfaatan Komoditas Lokal Sebagai Bahan Baku Pangan Darurat, PANGAN 20(2) 153–61 2011

[3] Ananditi R B K, Siswanti, Nurhartadi E and R Hapsari 2016 Formulaisi Pangan Darurat Berbentuk Food Bars Berbasis Tepung Millet Putih (Panicum millaceum L.) dan Tepung Kacang Merah (Phaseolus vulgaris L.) J. Agritech. 36(1) 23–9

[4] Budijanto S, Sitanggang A B and Murdiati W 2011 Karakterisasi Sifat Fisiko-Kimia dan Fungsional Isolat Protein Biji Kecipir (Psophocarpus tetragonolobus L.) [Characterization of Physicochemical and Functional Properties of Winged-Bean (Psophocarpus tetragonolobus L.) Protein Isolate Jurnal Teknologi dan Industri Pangan 22(2)

[5] D F Rosida and Y R A W 2014 Isolasi Protein Biji Lamtoro Gung (Leucaena leucocephala) Menggunakan Cairan Rumen Domba 8(1)

[6] A B N Sudrajat, N Diniyah and R Fauziah 2016 Fungsional Isolat Protein Koro Benguk (Mucuna pruriens), Pros. Semin. Nas. APTA 112–8

[7] X Kong, X Li, H Wang, Y Hua and Y Huang 2008 Food Chemistry Effect of lipoxygenase activity in defatted soybean flour on the gelling properties of soybean protein isolate 106 1093–1099

[8] V P Dia, W Wang, V L Oh, B O De Lumen and E G De Mejia 2009 Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity, Food Chem. 114(1) 108–15

[9] F Speroni, V Milesi and M Cristina 2010 Interactions between iso flavones and soybean proteins Applications in soybean-protein-isolate production LWT - Food Sci. Technol. 43 1265–70

[10] A Triyono 2010 Mempelajari Pengaruh Penambahan Beberapa Asam Pada Proses Isolasi Protein Terhadap Tepung Protein Isolat Kacang Hijau (Phaseolus radiatus L.) 4–5

[11] X Wang, M Jiang, L Li, Y Wang and S Sui 2011 Optimization of Extraction Process of Protein Isolate from Mung Bean Procedia Eng. 15 5250–8

[12] A Kumar, I Galaev and B Mattiasson 2011 Isolation and Purification of Proteins ed Hatti-Kaul R and Mattiasson B (Basel, NY Marcel Dekker)

[13] Q Wang et al 2016 Peanuts Processing Technology and Product Development ed Wang Q (Cambridge, Massachusetts Academic Press) 211–325

[14] N J D M Stonestreet 2011 Processing and Characterization of Sorghum Protein Concentrates

[15] N Hapsari 2000 Efektivitas Metode Pemisahan Dalam Produksi 6(1) 23–8

[16] T Lafarga, C Álvarez, G Bobo and I Aguiló-aguayo 2018 Characterization of functional properties of proteins from Ganxet beans (Phaseolus vulgaris L. var. Ganxet) isolated using an ultrasound-assisted methodology LWT - Food Sci. Technol. 98 106–12

[17] M Du et al 2017 Food Hydrocolloids Extraction, physicochemical characteristics and functional properties of Mung bean protein Food Hydrocoll. 1–10

[18] Arakawa T and S N Timasheff 1985 Theory of Protein Solubility Meth. Enzymol. 114 49–77

[19] H Lqari, J Vioque, J Pedroche and F Milla 2002 Lupinus angustifolius protein isolates chemical composition, functional properties and protein characterization Food Chem. 76 349–56

[20] M Cai, R Gu, C Li and Y Ma 2014 Pilot-scale production of soybean oligopeptides and antioxidant and antihypertensive effects in vitro and in vivo J. Food Sci. Technol. 51 1866–74

[21] Q Liu, L Jiang, Y Li, S Wang and M Wang 2011 Study on Aqueous Enzymatic Extraction of Red Bean Protein Procedia Eng. 15 5035–45
[22] N Al-ruwaih, J Ahmed, M F Mulla and Y A Arfat 2019 LWT - Food Sci. Technol. 100 231–6
[23] S Budseekoaid, C Takahashi, N Sirinupong and A M Alashi 2018 Structural and functional characterization of calcium and iron-binding peptides from mung bean protein hydrolysate J. Funct. Foods 49 333–41
[24] E R Coscuetra, D A Campos, H Osório and B B Nerli 2019 Enzymatic soy protein hydrolysis : A tool for biofunctional food ingredient production Food Chem. X 1 100006

Acknowledgment
The authors would like to thank the Ministry of Research, Technology and Higher Education, Universitas Padjadjaran.