Effect of heating temperature and time on the formation of 11S globulin nanofibril from Bogor nut (Vigna subterranean (L.) Verdc.) for food ingredients

D Sarastani1,2, D Fardiaz2, M T Suhartono2, H N Lioe2 and N Purwanti3

1 School of Vocational Studies, IPB University (Bogor Agricultural University), Bogor 16151, Indonesia
2 Department of Food Science and Technology, IPB University (Bogor Agricultural University), Bogor 16680, Indonesia
3 Department of Mechanical and Biosystem Engineering, IPB University (Bogor Agricultural University), Bogor 16680, Indonesia

E-mail: dewi_astani@yahoo.com

Abstract. Bogor beans (Bambara groundnuts) are thought to originate from Bambara, Timbuktu, Mali, West Africa. Introduced to Indonesia in the 20th century, and the name of the Bogor nut was given because it was widely sold in the city of Bogor. This research aims to study the formation of nanofibrils from 11S globulins to increase the benefits of Bogor nut as a food ingredient. Temperature treatment (65, 75, 85 °C) and heating time (6.12, 18, 24, 30 hours) at a concentration of 1% (w/w) protein solution, at pH 2, was applied to observe nanofibril formation. The formation of nanofibril was observed by measuring the viscosity of protein solutions before and after heating with a rheometer. A transmission electron microscope observed the morphology of nanofibrils formed at various heating times. The results showed that 11S globulin nanofibril formation was achieved at 85 °C heating temperatures. The morphology of 11S globulin nanofibrils from a 1% protein solution (w/w) with a heating temperature of 85 °C, is visible and significant in number after 24 hours of heating. The increase in the viscosity of the nanofibril solution from the protein solution indicates that the nanofibril from Bogor nut 11S globulin has potential as a food thickener.

1. Introduction

Bogor nut (Vigna subterranean (L.) Verdc.) is a Bambara groundnut originating from the Bogor region. In Indonesia, Bogor nut has a role in food diversification programs; even so, the availability of Bogor nut in the commercial market is very limited. While Bogor bean plants or Bambara groundnuts are plants that grow in hot climates, tolerate drought, and can produce yields even though they are planted in infertile soils where other legumes cannot grow well [1]. Furthermore, Bogor nut has a high production capacity of 4 tons of dry seeds/ha, if planted under optimal conditions [2]. Therefore, a study about diversification of the benefits of Bogor nut is needed to increase its availability in the commercial market.

Nuts are a source of protein that has the potential to be used as raw material for nanofibrils, a modified protein. This modification is due to changes in protein conformation from globular to fibrillar, and this modification of protein is intended to improve its functional properties as a food ingredient. The protein
content of Bogor nut, which is quite high (17.51 to 22.1% w/w) has the potential to be a source of protein to be isolated. The fat content is relatively low, which is around 5.0 to 11.16% [3], so this facilitates the process of isolation to obtain protein isolates.

Fibril proteins are globular proteins that are structurally composed of polypeptide piles in the form of β-sheets and form fibrils with the cross-β core structure. The formation of fibrils occurs through non-covalent interactions between beta-sheets, and these interactions cause the monomer pile to form very strong fibrils. A common characteristic of fibrils is straight, fibrous due to the intertwining of several beta-sheet filaments [4]. The aggregation of fibrillar proteins has unique characteristics that can modify the rheological properties and textures of food formulations with very efficient quantities so that fibrils are very potential as food ingredients. The structural properties of nanofibrils combined with the chemical properties at fibril surfaces make nanofibrils an interesting food ingredient. This fibril is considered as a potential new ingredient because of its resistance and rheological behavior in solution [5]. Fibrils can function as thickening agents, foam formers, foam stabilizers, or as highly effective gelling agents [6]. The objective of this research was to study the formation of nanofibrils from 11S globulins to increase the benefits of Bogor nut as a food ingredient.

2. Materials and methods

2.1. Materials
The materials used in this study were Bogor nut (Vigna subterranean L.) Jerde.) in the form of fresh pods, obtained from the local market (Bogor, Indonesia), and from the harvest of cultivated in Bogor, Indonesia. Chemicals for protein isolation, fractionation of protein, chemical analysis were purchased from Sigma (Sigma-Aldrich, Germany) and Merck (Merck, Darmstadt, Germany) with analytical grade.

2.2. Preparation of Bogor nut flour
Bogor nut flour was prepared from fresh pods through the following process stages: washing the pods, separating the seeds from the pods, stripping the epidermis, drying the seeds, grinding the beans, sifting the flour to obtain fine flour measuring 80 mesh. Drying the seeds using a cabinet dryer at a temperature of 60–65 °C for 6 hours. The was packed in plastic bags and stored in airtight containers at 4 °C. Bogor nut flour was defatted using a combination solvent (hexane: isopropanol = 2:1 v/v) with flour to solvent ratio 1:5, at 60 °C for 1 hour [7] with a few modifications.

2.3. Preparation of 11S globulin
Methods of protein isolation and fractionation of 11S globulin proteins from Bogor nuts were adopted from [8] with a few modifications. In this study, precipitation of samples was carried out by ultracentrifuge at a speed of 10000 g for 30 minutes. Protein fraction of 11S globulin was prepared by suspending defatted flour (145 g) in 30 mM Tris-HCl buffer pH 8 (1.5 L) that contained 10 mM 2-mercaptoethanol. This suspension was stirred for 1.5 h at room temperature. After centrifugation, the supernatant was adjusted to pH 6.4 with 6 M HCl to precipitate the fraction of 11S globulin. After stirring for 1 h, the suspension was centrifugated at 10000 g, 20 °C for 30 min. Furthermore, the precipitation of protein at pH 6.4 was resuspended and adjusted at pH 8. After overnight stirring, the protein resuspension was readjusted at pH 8 and followed by ultracentrifugation at 10000 g, 20 °C for 30 min. Then, the supernatant was dialyzed (MWCO 12000–14000, Spectrum Laboratories Inc., USA) vs.demineralized water for 24 h at 4 °C. Subsequently, the dialyzed protein solution was adjusted at pH 8 and directly was freeze-dried. The powder of 11S globulin was placed in an airtight plastic bottle and stored at 4 °C.

2.4. Formation of protein nanofibrils
The process conditions used for nanofibril formation in this study was protein solution concentrations of 1% w/w, protein solutions having a pH of 2, and shear flow from a protein solution of 150
strokes/minute or 2.5 shears per second. The heating temperature tested includes temperatures of 65, 75, and 85 °C, while the heating time is 6, 12, 18, 24, and 30 hours.

2.4.1. Preparation of protein solution. Powder of Bogor nut 11S globulin was suspended in demineralized water (the protein concentration of 1% w/w), the suspension was then set to pH 2 using 6 M HCl. After 1 h stirring at room temperature, the suspension was centrifugated by table top centrifuge at 3500 rpm, room temperature, for 15 min (Gemmy, model PLC-05, Gemmy Industrial Corp., Taiwan associated with Canning, Inc. USA) to remove undissolved protein. The stock of protein solution was ready to use.

2.4.2. Heating of protein solution. After putting protein solution, magnet stirrer in a bottle glass with hermetic lid, protein solution was heated at 85 °C for specific period of time (0, 6, 12, 18, 24, 30 h), in a shaking-water bath (Memmert, type WNB 14, Germany), 150 strokes per minute, with temperature controlled. After the heat treatment was achieved, the bottle was taken from the shaking device and immediately was cooled in an ice bath. The heated protein solution is ready to analyze.

2.5. The viscosity of protein and nanofibril solution
Viscosity was measured by a controlled-stress rheometer (Anton Paar, Physica MCR 301, Austria). Approximately 5 ml of protein solution is placed in a concentric cylinder measuring system (CC 17) attached to the Peltier temperature device (C-PTD 200, Anton Par GmbH, Graz, Austria) for concentric cylinder system. Shear rate sweeps with the up ramp from 1 s⁻¹ to 500 s⁻¹, and down-ramp from 500 s⁻¹ to 1 s⁻¹ were performed at 30 °C. There are 38 data points for each ramp recorded, and each data point was measured for 15 seconds. The measurement of the sample was done twice, and the measurement results were displayed with a viscosity curve (viscosity vs. shear rate), and calibrated with the viscosity curve of the Ostwald model. Furthermore, the data were processed using Rheo-Plus Application to the Ostwald model as below.

\[ \tau = k \dot{\gamma}^n \quad \text{or} \quad \eta = k \dot{\gamma}^{n-1} \]

Where \( \eta \) is the viscosity (mPa.s), \( \tau \) is the shear stress (Pa), \( \dot{\gamma} \) is the shear rate (s⁻¹), \( k \) is the consistency coefficient (Pa.sⁿ), and \( n \) is the power-law index [9].

The flow behavior of the protein solution was indicated by \( n \) value. The power-law index, \( n = 1 \) shows that the flow behavior of protein solution is Newtonian, \( n < 1 \) shows that the protein solution behaves pseudoplastic (shear thinning), and \( n > 1 \) shows that the flow behavior of protein solution is dilatant (shear-thickening).

2.6. Morphology of nanofibril
TEM (Transmission Electron Microscope) was used to visualize the morphology of nanofibril. The fibril solution that will be observed was diluted (10 x) with HCl solution of pH 2. The aqueous solution of fibrils was dropped (3 drops) onto a carbon film (5 nm thickness) on a copper grid (porous 400 mesh) which was above filter paper to absorb excess liquid. A stain solution (a solution of 2% uranyl acetate in deionized water) was dropped on it and left to dry for 3 minutes. The excess of the stain solution was removed by dipping the grid several times (30 times) into deionized water. The grid was stored in an EM grid (electron microscope) before inspection, then imaged using an electron microscope (Hitachi, HT 7700 type, Japan) that operates at 80–100 kV. Scanning with a 5000 x magnification to get an overall of the fibril morphology, then a detailed of finer fibrils with higher magnification (10000 x–20000 x). Each sample is scanned in 2–3 areas.

2.7. Dissolved protein content from protein solution and nanofibril solution
Measurement of dissolved protein content was carried out by the Lawry method with absorbance readings at a wavelength of 600 nm. The difference of protein content in protein solutions before and after heating as a prediction of the protein amount that was converted to fibrils.
3. Results and discussion

3.1. Fresh bogor nut and bogor nut flour
Figure 1 shows that Bogor bean plants with pea pods (figure 1a). Fresh Bogor nuts had pods containing 1 to 2 seeds (figure 1b). After the pods were peeled, it will be obtained fresh seeds of Bogor nut that were round to oval. Fresh seeds had thick and smooth epidermis with white cream colour, light brown, reddish-brown, and purple-blackish. Although fresh seeds had epidermis with various colors (figure 1c), but after the epidermis had been peeled, the seeds were white colour (figure 1d). After drying and milling, it will result in the Bogor nut flour, which was white cream colour (figure 1e).

From previous work, it was obtained that the crude protein content of Bogor nut flour (21.65 g/100 g) was less high than that of soybean flour (44.7%)[10], and the fat content of Bogor nut flour (8.76 g/100 g) was also less high than that of soybean flour (23.9%) [10]. In this study, the protein and fat content of Bogor nut flour provide important information for the isolation process. The higher the protein content of Bogor nut flour, the greater the yield of the protein isolates obtained. While the smaller fat content of the flour, will provide an opportunity for easier protein isolation process, and lower costs for the isolation process.

3.2. Bogor nut 11S globulin
Based on the results of our previous study, among protein isolates, 11S globulin fraction and 7 S globulin from Bogor nuts, it was found that the nanofibril solution from 11S globulin gave the highest increase in viscosity compared to other nanofibril solutions. Therefore, the 11S globulin fraction is prioritized for further investigation.

The 11S globulin of Bogor nuts is a protein fraction obtained from the process of protein isolation through precipitation at pH 6.4. Its form was white powder. From the results of previous work found
that the yield of 11S globulin was 7.26 g/100 g, while the crude protein content was 80.72 g/100 g. In this study, both information is needed to estimate the amount of nanofibril yield obtained.

3.3. **Effect of temperature and heating time on 11S globulin nanofibril formation**

Presented in figure 2 is a steady shear viscosity. Each sample viscosity curve was calibrated with the Ostwald model's viscosity curve, and the standard deviation of each point is displayed with an error bar (not visible because of the y-axis in the log scale).

Figure 2 shows a graph of the viscosity of a protein solution before heating and a protein solution after heating, at various temperature levels (65, 75, 85 °C) and heating time (0, 6, 12, 18, 24, 30 minutes). Nanofibril growth from Bogor nut 11S globulin with heat treatment (temperature 65, 75, 85 °C) and treatment of heating time (0, 6, 12, 18, 24, 30 minutes) on 1% w/w protein solution, at pH 2, with shear flow 150 strokes/minute, can be monitored by changes in the viscosity of the protein solution [11].

![Figure 2](image)

**Figure 2.** Viscosity profile of unheated and heated protein solution (1% w/w) from Bogor Nut 11S globulin at various temperatures [65 (a); 75 (b); 85 °C (c)] and heating times [0, 6, 12, 18, 24, 30 hours], at pH 2. Viscosity was measured at 30 °C.

(■) Up-ramp of unheated protein solution; (→) Ostwald model, Up, of unheated protein solution
(◆) Up-ramp of heated protein solution (6h); (→) Ostwald, Up, of heated protein solution (6h)
(○) Up-ramp of heated protein solution (12h); (→) Ostwald, Up, of heated protein solution (12h)
(▲) Up-ramp of heated protein solution (18h); (→) Ostwald, Up, of heated protein solution (18h)
(●) Up-ramp of heated protein solution (24h); (→) Ostwald, Up, of heated protein solution (24h)
(◇) Up-ramp of heated protein solution (30h); (→) Ostwald, Up, of heated protein solution (30h)

Protein solutions that were heated at 65 °C (figure 2 a) and temperatures of 75 °C (figure 3 b) had a lower viscosity than the viscosity of protein solutions without heating. The heating time for 6 to 30 hours was not able to increase the viscosity of the protein solution above the viscosity of the protein solution without heating. Meanwhile, the viscosity of a protein solution heated at 85 °C for 18, 24, and 30 hours,
could increase the viscosity of protein solutions over protein solutions without heating. This is because the heating at 85 °C was heating that was far above the temperature of denaturation of Bogor nut (Bambara groundnut) protein. The denaturation temperature of the Bambara bean protein (*Vigna subterranean*) is 71.67 °C for white seeds and 73.56 °C for black seeds [12]. Heating above the temperature of denaturation is needed to ensure the opening of conformation of the globular structure of proteins (secondary, tertiary, quaternary).

Conformation changes in the structure of proteins play a very important role, because it provides an initial opportunity for the exposure of hydrophobic groups, thus triggering the formation of initial aggregates or called protofibrils [13], [14]. This aggregation process is influenced by interactions between proteins (denatured aggregates) due to the balance of hydrophobic interactions and electrostatic repulsion [14]. The opening of protein structures and being supported by conditions of pH 2, provide an earlier opportunity for the polypeptide hydrolysis process [15]. Furthermore, the formed peptide fragments will be arranged to form beta-sheets which are the core structures of the fibrils [16].

From the results of this study, information was obtained that from the three tested heating temperatures (65, 75, 85 °C), the heating temperature of 85 °C provided the best indication for nanofibril formation from Bogor nut 11S globulin. The indication is a significant increase in the viscosity of the heated protein solution than the ones of the unheated protein solution. Therefore, the temperature 85 °C furthermore used to the formation of nanofibrils from the Bogor nut 11S globulin.

3.4. Monitoring the formation of nanofibril from Bogor nut 11S globulin with a rheometer

The process conditions used for nanofibril formation from Bogor nut 11S globulin were 1% w/w protein concentration, protein solution had pH 2, the shear flow of protein solutions in bottles during heating was 150 strokes/minute, heating temperature 85 °C with heating period 6, 12, 18, 24, 30 hours. Figure 3 represents the apparent viscosity of the solution of 11S globulin during nanofibril growth from incubation of 0, 6, 12, 18, 24, to 30 hours, at shear rates of 5 s⁻¹ and 100 s⁻¹.

The pattern of developing apparent viscosity of a fibril solution at a particular shear rate (5 s⁻¹; 100 s⁻¹), as presented in figure 3, is a specific pattern in fibrillar protein formation as like the results of previous researchers [5], [10]. After the protein solution is heated above the temperature of denaturation for some time, then initially the viscosity of the fibril solution will be lower than the viscosity of the protein solution. This is due to the hydrolysis of globular structures from protein macromolecules, into peptide fragments as hydrolysis products. At the same time, some peptide fragment aggregated to form a beta-sheet arrangement as the fibril core structure. Continuous heating time (from 6 to 30 hours), will increase the aggregation of peptide fragments; as a result, the fibrils become longer, interactions between fibrils occur forming amyloid fibrils and increasing the viscosity of the fibril solution. This change in viscosity is caused by the content of beta-sheet structure in fibril solution. Supported by the results of [17] in the formation of fibrils from kidney beans, in the first 15 minutes of heating, the beta-sheets quantity in the fibril solution decreases from the protein solution. Increased heating time from 15 minutes to 60, 360, and 720 minutes, the beta-sheet quantity is increasing. The secondary structure of fibrillar proteins composed of beta-sheets, causes fibrils to have very large volumes [18]. Changes in the volume of fibrillar protein which is one contribution to the increase in viscosity of fibril solution.
Figure 3. The pattern of development viscosity of nanofibril solution from Bogor nut 11S globulin. Processed with 1% (w/w) protein solution, at pH 2, 85 °C for 0, 6, 12, 18, 24, 30 hours.

The results of this study show, with a protein concentration of 1% (w/w), in the first 6 hours heating (figure 3 red graph, apparent viscosity at a shear rate of 5 s⁻¹) the viscosity of the protein solution decreases, then the viscosity of the protein solution increases slightly at incubation 12 and 18 hours, but the increase in viscosity of the three heating periods has not been able to exceed the viscosity of protein solutions without heating. Significant increase in the viscosity of the fibril solution at heating for 24 and 30 hours (figure 3), can exceed the viscosity of the protein solution. Heating for 24 hours increased the viscosity of 11S nanofibril globulin solution by 2.5 times at a shear rate of 5 s⁻¹ and 2.9 times at a shear rate of 100 s⁻¹. By heating 30 hours, the viscosity of the nanofibril solution increases 5 times at a shear rate of 5 s⁻¹, and 4.5 times at a shear rate of 100 s⁻¹. This result is far less than the results of our previous work, with a concentration of 2% protein solution (w/w), obtained an increase in viscosity of the fibril solution dramatically reaching 71.4 times (at a shear rate of 100/s) by heating for 21 hours. The increase in viscosity in nanofibril solutions to the protein solution, caused by a change in the structure of proteins from globular to fibrillar.

3.5. Monitoring the growth of nanofibril from Bogor nut globulin with TEM

Figure 4 shows the results of monitoring the growth of nanofibrils during heating from 6, 12, 18 to 24 hours, with a protein solution of 1% (w/w), pH2, 85 °C heating temperature.
Figure 4. Morphology of 11S globulin nanofibril from Bogor Nut at various heating times (a. 6 h; b. 12 h; c. 18 h; d. 24 h). It is formed from 1% w/w protein solution at pH 2, 85 °C.

In figure 4 a (heating the protein solution for 6 hours), there is a huge hydrolysis product, and there are not yet fibrils formed clearly. In 12 hours of heating (figure 4 b), there begin to appear to be quite a several hydrolysis products in little form. Figure 4 c (heating a protein solution for 18 hours) shows that it is seen the short fibrils are formed. On 24 hours of heating (figure 4 d), it is very clearly seen that there are many long fibrils. The visualization of the morphology of fibrils strengthens the explanation of changes in viscosity that occur during the formation and growth of 11S globulin fibrils. The results of figure 4 are consistent with those presented in figure 3 and figure 2 c.

From the results of this study, it was shown that changes in the globular structure of 11S globulin proteins to fibrillar structures caused an increase in the viscosity of protein solutions. In addition to fibril volume, other parameters that affect the viscosity of fibril solutions are fibril morphology, interactions between fibril [4], and fibril characteristics: length, flexibility, and fibril alignment [5].

3.6. Percentage of fibrils formed

Table 1 shows the percentage of fibrils formed in fibril formation at each heating time (6, 12, 18, 24 and 30 hours) at 85 °C. The percentage is calculated as the difference in the dissolved protein content in the protein solution to the fibril solution.

| Products      | Period of heating at 85 °C | Protein that might be converted to fibrils (%) |
|---------------|-----------------------------|----------------------------------------------|
| 11S Globulin  | 0 h                         | -                                            |
|               | 6 h                         | 8.04                                         |
|               | 12 h                        | 8.65                                         |
|               | 18 h                        | 16.99                                        |
|               | 24 h                        | 19.28                                        |
|               | 30 h                        | 27.83                                        |

The results showed the amount of dissolved protein that allows some or all to be converted to fibrils 11S globulin at heating 6, 12, 18, 24 and 30 hours as much as 8.04, 8.65, 16.99, 19.28, 27.83% respectively. This result is consistent with the visualization of fibrils seen in figure 4 (except for 30 hours heating, no image).
3.7. Flow behavior of protein solution and nanofibril solution

Seen in table 2, the flow behavior of protein solutions and fibril solution from Bogor nut 11S globulin are shear thinning. As the shear rate increases, the viscosity of the fibril solution decreases (shear thinning). Generally, polymeric materials are viscoelastic, non-Newtonian shear thinning [4].

Table 2. The parameters for Ostwald model from Bogor nut 11S globulin (formed at 1% w/w, pH 2, 85 °C).

| Products          | k (mPa.s) | n        | R²       | Flow behavior      |
|-------------------|-----------|----------|----------|--------------------|
| 11S Globulin 0 h  | 1.912     | 0.8566   | 0.984    | Shear thinning     |
| 11S Globulin 18 h | 1.957     | 0.9488   | 0.999    | Shear thinning     |
| 11S Globulin 24 h | 5.154     | 0.8557   | 0.999    | Shear thinning     |
| 11S Globulin 30 h | 12.698    | 0.7685   | 0.996    | Shear thinning     |

The k value of nanofibrils with 24 and 30 hours heating showed a significant increase compared to the protein solution (table 2). This increase is consistent with the graph of viscosity in figure 2 c. The value of k shows the consistency coefficient of the solution; the higher the value k indicates the solution is thicker [9].

3.8. The potential of nanofibril from Bogor nut 11S globulin as a food ingredient

With a protein concentration of 1% w/w, 11S globulin can form nanofibrils. Changes in globular structure into fibrillar can increase the viscosity of protein solutions significantly, so the potential of 11S globulin nanofibril as a food ingredient is very interesting to develop. Based on the results of our previous work, by increasing the initial concentration of protein (2% w/w), the viscosity of fibril solution increased 74.1 times from the viscosity of protein solution. Therefore, this can improve the functional properties of 11S globulin nanofibrils as a food thickener.

4. Conclusion

Important information obtained from this study is the 11S globulin protein from Bogor nut can form nanofibrils. The process of nanofibril formation can be achieved at 85 °C. With 1% w/w protein solution, 85 °C, pH 2, nanofibrils of 11S globulin begun to show significant growth at 24 hours heating. The formation of nanofibrils shows the globular structure of proteins can be modified into fibrillar structures. This conformational change can improve the functional properties of 11S globulin nanofibril proteins as food ingredients, so it is very interesting to develop.

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