Fractionation and Functional Characteristics of Proteins from Brebra (*Millettia ferruginea*) Defatted Seed Flour

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Abstract: Plant proteins play significant roles in human nutrition, particularly in developing countries where average protein intake is below standard. The aim was to analyze the protein yields, the solubility, fractionation and functional properties of the defatted brebra seed flour proteins. Protein analysis of defatted flour was determined by standard methods. The protein content was found to be 48.5± 0.20%. Its protein concentrate had 74.5 ± 0.01% protein. The minimum and maximum protein solubility of at pH 4 and pH 10 was 31.98 ±0.13% and 88.04 ±0.13%, respectively. SDS-PAGE analysis of this study represents the existence of 8 major proteins with molecular weight ranging from 66.2, to 14.4 kDa, of which 35.0 and 25.0 kDa of proteins were developed heavy bands. The major protein fraction was found to be albumin (58.4%). All tested samples have good foaming capacity and stability, emulsion capacity and stability, water and oil retention capacities and gelation properties. Defatted flour has high protein content with acceptable functional properties, in which makes it promising protein source in food applications. Therefore, it may be used as protein source for both humans and other animals.

Key words: Brebra · Fractionation · Functional characteristics · Protein content · Solubility

INTRODUCTION

Plant proteins play significant roles in human nutrition, particularly in developing countries where average protein intake is below the required amount. Because of inadequate supplies of food proteins, there has been a frequent search of protein from wild plants for use as both functional food ingredients and nutritional supplements [1]. The reason for searching such protein from nonconventional protein sources particularly from legumes is due to the high price of meat and fish almost in all the developing countries. Thus, the current major world concern in fulfilling the food need is to supply the protein required by the whole population now and in the future. Legumes such as peas, beans and lentils are high in protein content and have been used as an inexpensive protein source in the diets where animal proteins are either unaffordable or are considered detrimental to the health and nutrition conscience population [2].

In China, plant foods provide at least 50% of the dietary energy and nutrients [2]. Thus, plant protein products are gaining interest as ingredients in food systems throughout many parts of the world; the final success of utilizing plant protein additives depends greatly upon functional characteristics that they impart to foods. Since every storage protein of seeds has its unique characteristics, these functional properties can be optimized by knowing the chemical and physicochemical characteristics of these proteins. Characterization is the initial step for modification and manipulation according to the purpose and need. Therefore, the relationship of protein quality with processing parameters that affect the functional performance of protein products is worthy of extensive investigation.

Among protein functionalities, solubility of protein is a critical functional attributes required for its use as food ingredient, because solubility greatly influences other properties such as emulsification, gelation and foaming.
Solubility is affected by many factors, such as pH during extraction or solubilization [3]. Functional characterization of protein can be generalized as hydration, emulsification, textural and rheological [4]. Those characteristics can be measured through their nitrogen solubility, water absorption, swelling, gelation, fat adsorption, foaming, adhesion, fiber/texture, aggregation, dough formation and extrudability.

Investigation of proteins with new functional properties from various legumes and seeds in Africa has received some attention [5]. Increased interest in such plant proteins for feed and food led to the evaluation of wild especially legumes as high protein crop. The transformation of non-conventional sources of proteins is of great interest because it represents a potential source of inexpensive protein for feeding livestock and also for human consumption. The candidate plant in this study is *Millettia ferruginea* (Hochst) Baker (Abaca: Papilionoideae), which is endemic to Ethiopia and it is well known as brebra in Amharic. It is a multipurpose tree: provides shade for coffee in the coffee-growing regions; its pods are good sources of fuel and the wood is used to make tool handles and household utensils. The endogenous societies use this plant for mass fishing in rivers and as insecticide for pest control [6]. In spite of such investigations, the defatted flour seed content and other characteristics of protein in this legume plant is yet not studied. The objective of this study, therefore, was to analyze the protein yields, the solubility, fractionation and the selected functional properties of the defatted brebra seed flour proteins. The outcome of this investigation provides basic information to use in food formulation for both humans and other animals.

**MATERIALS AND METHODS**

**Processing of Seeds and Defatting:** Matured brebra seeds were collected from matured dried pods and mechanically dehulled to remove the seed coats. The dehulled cotyledons were dried by oven to avoid excess moisture at 60°C for 24 h. The cotyledons were ground into fine flours and defatted by using hexane solvent. The extracted oil can serve as feedstock for the micro Kjeldhal method [7]. To determine non-protein N, 0.1 g of sample was added 1.5 ml of a 10% (w/v) aqueous trichloroacetic acid (TCA) solution, homogenized in a magnetic stirrer for 1 h at room temperature and centrifuged (15000 rpm for 40 min). The residue was re-extracted (2x), the supernatants were combined and the N was determined according to AOAC method [7]. Protein nitrogen plus non-protein nitrogen gives rise to total nitrogen.

**Determination of Protein Dispersibility Index:** Defatted brebra seed flour (3.5 g) was extracted twice with 50 ml distilled water for 30 min at room temperature in order to determine the protein dispersibility index of the sample. The extract was centrifuged at 3000 rpm for 30 min and the supernatant was used for the determination of a water-soluble protein. Protein dispersibility index (PDI) was calculated according to the method used by Anuonye et al. [8]. Protein dispersibility index (PDI) = (% protein solubility / % total protein content of the sample) x 100. For comparison purpose, total protein and protein dispersibility index of defatted soybean was determined by using the same process and procedure.

**Total Nitrogen and Protein Nitrogen:** The total nitrogen and protein nitrogen (TCA-insoluble) were estimated by the micro Kjeldhal method [7]. To determine non-protein N, 0.1 g of sample was added 1.5 ml of a 10% (w/v) aqueous trichloroacetic acid (TCA) solution, homogenized in a magnetic stirrer for 1 h at room temperature and centrifuged (15000 rpm for 40 min). The residue was re-extracted (2x), the supernatants were combined and the N was determined according to AOAC method [7]. Protein nitrogen plus non-protein nitrogen gives rise to total nitrogen.

\[
\text{Protein} \%(\%) = \frac{\text{(ml of} \ H_2\text{SO}_4 \ \text{for sample} - \text{ml of} \ H_2\text{SO}_4 \ \text{for blank)}}{\text{wt of sample}} \times \text{(N)} \times \text{appropriate conversional factor for sample (6.25).}
\]

In short, protein content of the sample was calculated by multiplying the nitrogen content by a factor of 6.25.
Protein Fractionation: Protein fractions were extracted according to their solubility in different solvents, as described by Landry and Moureaux [9]. Defatted brebra flour (3.5 g) was extracted twice with 50 mL distilled water for 30 min at room temperature. The extract was centrifuged at 3000 rpm for 30 min and the supernatant was used for the determination of a water-soluble protein (albumin). The residue was then extracted successively in a similar manner with 1.0 M NaCl, 70% ethanol or 0.2% NaOH. The supernatant of each extract was collected separately and used to estimate the salt (globulin), alcohol (prolamin) or alkali (glutelin) soluble fraction. The residue remaining after successive extractions represents the insoluble proteins.

Protein Solubility Profile at Different pH: The protein solubility at different pH was tested by taking 300 mL of sodium sulfate solution (0.25%) in flask and added 20 ± 0.2 g of sample flour in the entire solution [10]. Slurries were thoroughly mixed for 5 min and adjusted to pH 2.0, 2.5, 3, 3.5, 4.0, 4.5, 5, 5.5, 6.0, 8.0, 10.0 and 12 by adding appropriate amounts of 6N HCl or 6N NaOH to avoid substantial increases or differences in final volume. After 1 h of constant mixing at room temperature using a magnetic stirring bar, the slurry was centrifuged (Model J2-21M, Spinco Division of Beckman Instruments) at 3000 g for 20 min. Supernatant liquid was then filtered through Whatman No. 1 filter paper to obtain a clear extract. From the supernatant liquid, 15 mL was taken into a Kjeldahl nitrogen digestion tube in order to determine nitrogen content. Note: 15 mL of the supernatant is equivalent to 1.0 g of sample in the Kjeldahl method. Nitrogen content was measured by the micro-Kjeldahl method [7]. Protein solubility was expressed as percent of the protein content of the sample.

Soluble Carbohydrates: A soluble carbohydrate of the samples was determined according to the method described by Paredez-Lopez and Harry [11]. They were quantified using the phenol-sulfuric acid method of Dubois, et al. [12] with glucose as a standard.

SDS-polyacrylamide Gel Electrophoresis: SDS polyacrylamide gel electrophoresis (SDS-PAGE) was done, using the method of Laemmli [13] with 12.5% (v/v) acrylamide resolving gel and 5% (v/v) acrylamide stacking gel. Half g sample was prepared from 1:16 (w/v) ratio of flour and distilled water, respectively and then sonicated by using sonicater (Soniprep 150, MSE). The portion of the sample was diluted into 1:1 ratio with distilled water in order to see clear bands in the electrophoresis gel. The whole sample was centrifuged at 10,000 rpm for 10 min. Sample was taken from the supernatant and mixed with Laemml sample buffer in 1:1 ratio. The prepared sample in this manner was denatured in boiling water for 10 min. Lastly, 25 µL of the denatured sample was loaded in the well. Electrophoresis was done at a voltage of 200 for 1 h. After electrophoresis, the gel sheets were stained for proteins with 0.2% Coomassie brilliant blue-R250 for an hour. Protein stain was destained with 7% acetic acid containing 30% methanol. Unstained protein molecular weight marker (Fermentas, SM0431) was used to determine the molecular weight of the samples.

Functional Properties of Brebra Seed Protein

Water and Oil Holding Capacity: Brebra flours (5.0 g) were thoroughly mixed, without pH adjustment, with 25 mL of deionized water or oil in 50 mL centrifuge tubes. Suspensions were stirred intermittently over a 30 min period at 25°C and then centrifuged at 12,000 rpm for 30 min at 25°C. The volume of decanted supernate was measured and the water and oil retention capacities were calculated as the number of mL of water and oil held by 1 g of protein sample. Triplicate samples were analyzed for each samples of flour.

Foaming Capacity: The foaming capacity was determined by the method of Lawhon et al. [14]. About 100 mL of distilled water were added to 3 g proteins. The mixture was homogenized for 5 min in a blender set at high speed at room temperature (25°C) and then transferred to a 250 mL-measuring cylinder. The volume of foam at 30 second was calculated and the increase in volume is expressed as a percent foam capacity (FC).

\[ FC (%) = \left(\frac{\text{volume after whipping} - \text{volume before whipping}}{\text{volume before whipping}}\right) \times 100 \]

Foam Stability: Foam stability (FS) was determined by measuring the decrease in volume of foam as a function of time up to a period of 120 min. The stable foam volumes were recorded at time intervals of 10, 30, 60 and 120 min. FS (%) = (Foam volume after time (t) / Initial foam volume) \times 100

Emulsifying Activity and Emulsion Stability: The procedure described by Volkert and Kelin [15] was used for both emulsification activity and emulsion stability. Emulsions were prepared with 1 gm of protein, 50 mL-distilled water at room temperature (25°C) and 50 mL of soybean oil. The mixture was emulsified for 30 min by magnetic stirrer at maximum speed. Each emulsified sample
was divided equally into 50 ml centrifuge tubes. Content of one tube was directly centrifuged at 3000 rpm for 30 min while the other centrifuged under the same conditions after heating in a water bath at 80°C for 30 min and cooling to 15°C. The height of the emulsified layer, as a percentage of the total height of material in the unheated tubes was used to calculate the emulsifying activity and stability using the following formulas:

\[ \text{EA} (\%) = \left( \frac{\text{height of emulsion}}{\text{height of whole layer}} \right) \times 100 \]

\[ \text{ES} (\%) = \left( \frac{\text{height of emulsion layer after heating}}{\text{height of whole layer}} \right) \times 100 \]

**Bulk Density:** Ten grams of the sample were placed in 25 ml-graduated cylinder and packed by gently, the volume of the sample was recorded and the bulk density is expressed as g protein/ml.

**Gelation Capacity:** Triplicate suspensions of 2-20% defatted brebra flour (dry w/v, at 1% increments) were prepared in 5 ml of deionized water and mixed thoroughly without pH adjustment. The slurries were heated in 125-20 mm screw-capped test tubes in a water bath at 95°C with intermittent stirring. After 1 h of heating, tubes were immediately cooled in tap water for 30s and then in ice water for 5 min to accelerate gel formation. All tubes were then held at 4°C for 3 h. Least gelation capacity (%) was determined as the concentration above which the sample remained in the bottom of the inverted tube.

**Statistical Analysis:** All data were expressed as mean ± standard deviation. Data were analyzed using one-way ANOVA using SPSS15.0. Duncan’s new multiple-range test was used to assess differences between means. A significant difference was considered at the level of p < 0.05.

**RESULTS AND DISCUSSION**

**Protein Content, Fractionation, Solubility and Structure of Defatted Brebra Flour**

**Protein Content:** The crude protein content of brebra defatted flour was found to be 48.5±0.2 %, of which 98.3±0% and 1.7±0% were protein nitrogen and non-protein nitrogen, respectively. The amount of protein found in this study is almost comparable with that of defatted soybean [16] and 50% protein in the defatted peanut flour [17]. Moreover, percentage of protein in this sample (48.5±0.2%) (Table 1) is greater than amount of defatted sunflower meal reported by Parrado et al. [18].

<table>
<thead>
<tr>
<th>Functional property</th>
<th>Sample</th>
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<tbody>
<tr>
<td></td>
<td>Defatted brebra flour</td>
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<tr>
<td>Protein content</td>
<td>48.5 ± 0.20</td>
</tr>
<tr>
<td>Dispensibility index</td>
<td>58.4 ± 0.10</td>
</tr>
<tr>
<td>Protein concentrate</td>
<td>74.5 ± 0.01</td>
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<tr>
<td>Protein nitrogen</td>
<td>98.3 ± 0.00</td>
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<tr>
<td>Non-protein nitrogen</td>
<td>1.7 ± 0.00</td>
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<tr>
<td>Soluble carbohydrate</td>
<td>95.8 ± 0.02</td>
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ND = Not determined

The protein content of defatted brebra seed flour is comparable with different legume defatted flours and thus can be used as good source of protein for human consumption (Table 1).

Protein dispersibility index and amount of protein concentrate were 58.4±0.1% and 74.5±0.1%, respectively. For the sac of comparison, defatted soybean protein (44.3±0.04%), protein dispersibility index (58.2±0.3%) and protein concentrate (70±0.02%) were determined in this study. The protein concentrates of pea (*Pisum sativum* L.), faba bean (*Vicia faba* L. spp. minor) and lentil (*Lens culinaris* Medik.) were ranged from 59.2 to 70.6% [19], which is less than the protein concentrate of brebra seed flour (74.5±0.01%) in this study (Table 1).

The large quantity of (95.7±0.02%) carbohydrate in this study was soluble and thus can be easily metabolized by both humans and other organisms (including microorganisms, if it is used as medium). In our previous study, from non-defatted brebra seed flour 11.91 ± 0.2% was found to be carbohydrate.

Fig. 1 shows defatted brebra seed protein fractions on the basis of solubility. Defatted brebra seed flour protein was fractionated, on the basis of solubility, into albumin (58.4±0.2%), globulin (7.6±0.1%), prolamin (0.5±0.4%) and glutelin (8.5±0.03%). The results obtained indicated that about 75±0% of the total protein could be extracted by solvents and the remaining percentage (25±0.6%) accounted for the non-protein nitrogen and insoluble proteins. The albumin and glutelin content in this study is in close agreement with that of uncooked flour of lentil ranged from 56.26 to 64.00% and 2.10 to 3.5%, respectively [20] and as well as 54.62 ± 0.67% albumin and 5.77 ± 0.59% glutelin reported by Gonzalez et al. [21]. However, the content of globulin and prolamin in defatted flour of this finding were lower than lentil globulin (26.28 -29.5%) and prolamin (1.43 -1.96%).
albumins that comparable with egg white, blood, milk, leucosin in cereals and legumilin in legumes. On the other hand, all protein fractions of defatted brebra seed flour (except albumins) were less than that of bambara groundnut seed [22].

**Nitrogen Solubility Profile at Different pH:** Solubility is one of the most basic physical properties and prime requirements in functional food systems. All the results that are presented on Fig. 2 correspond to protein extractions that were carried out at room temperature and in medium containing sodium sulphite at different pH. As presented on Fig. 2, from 2 to 4.5 pH there is no statically significant (p < 0.05) difference in protein solubility, while at pH 10 protein solubility is significantly (p < 0.05) increased. The minimum nitrogen solubility was found to be 31.98±0.13% and 32.37±0% at pH 4 and 4.5, respectively. In this study, the isoelectric point of brebra seed protein was at pH 4. Brebra seed protein showed good solubility in both acid and alkaline pH regions, which is an important characteristic for food formulation. Generally, increasing pH from 4 to 10 considerably increased protein solubility (Fig. 2). The higher extraction of proteins at pH near their isoelectric point may be due to their composition. Aggregation of brebra proteins is not necessarily accompanied by complete insolubilization, as some albumins and globulins may not precipitate at their isoelectric point [23].

**SDS-PAGE Analysis of Protein:** SDS-PAGE analysis showed the existence of 8 major protein bands within the molecular weight range of 14 to 66 kDa (Fig. 3) of which two bands with molecular weight of 25.0 and 35.0 kDa being the most dominant protein.

**Functional Properties of Brebra Defatted Seed Flour Protein:** This study presents the result of functional properties of defatted brebra seed flour in comparison with defatted soybean flour produced by the same procedure.

**Water and Oil Absorption Capacities:** Table 2 presents the results of water and oil absorption capacities of defatted brebra flour. There is significant difference in water holding capacity at (p < 0.05) between defatted brebra and soybean flour. The water retention of soybean (1.9±0.2 ml H₂O/gm protein) was further better than defatted brebra flour (1.6±0.00 ml H₂O/gm). However, the water absorption capacity of (1.6±0.00 ml H₂O/gm or
Emulsion Activity and Stability: Emulsion activity and stability are shown in Table 2. There is no significant difference in oil holding capacity at (p < 0.05) between defatted breba and soybean flour. The values for emulsifying activity of defatted breba flour were 42.6 ±0% and 46.9 ±0.3%, respectively. These values are comparable to 45.3 ± 0.7% noted by Adetuyi et al. [30] whose value of *Sorghum bicolor* L. stem flour was 36.1%. The emulsion activity and stability of defatted soybean in this study is within the range value of defatted breba flour. According to Elizalde et al. [31] report, emulsion stability is enhanced by high protein and oil concentrations and these factors are highly interrelated. They also reported that the emulsion stability depends primarily upon the water and oil absorption capacity. Moreover, stable emulsion may be formed by reduction of interfacial tension, formation of a rigid interfacial film and electrical charge.

Bulk Density: The bulk densities values of (0.63 ± 0.01 gm/ml) defatted breba flour as well as defatted soybean (0.6 ± 0 gm/ml) are depicted in Table 2. These values are higher than the values reported for rear cowpea (0.53 ± gm/ml) and bambara groundnut (0.59 ±0.5 gm/ml) [29]. Bulk density depends on combined effects of interrelated factors such as intensity of attractive inter-particle forces, particle size and number of contact points. Higher bulk density is desirable since it helps to reduce the pate thickness which is an important factor in convalescent and child feeding.

Foaming Capacity and Stability: In this study, there is no significant (P > 0.05) difference in the foam capacity between breba and soybean defatted flour that treated with same condition. The foaming capacity of defatted breba flour and soybean are presented as 7.7±0.5% and 8.4±0.4%, respectively, in Table 3. The values reported in this study are higher than the foaming capacity of *Sorghum bicolor* L. stem (6%) [30] but are almost comparable with quinoa (9%) [26] and red coat bambara groundnut (7.9±0.5%) [29]. However, values of the foam capacity of this study are lower than those of kersting’s groundnut flour (23.1%) and bambara groundnut (15.4%), scarlet runner beans flour (28.1± 0.5%) reported by [29].

The foam stability after 120 min of defatted breba flour and soybean in this investigation are presented in Table 3. All samples have been shown good foam stability including soybean.

Gelation Capacity: Gelation capacity of defatted breba seed flour is shown in Fig. 4. The least gelation concentration which is defined as the lowest protein concentration at which gel remained in the inverted tube was used as an index of gelation capacity. In the present study, the least gelation capacity for the sample is 16% w/v and is similar to that observed by Aremu et al. [29]
Fig. 4: Gelation capacity of defatted brebra seed flour at different sample concentration

in rear cowpea flour (16% w/v), quinoa flour (16%, w/v). The ability of protein to form gels and provide a structural matrix for holding water, flavors, sugars and food ingredients is useful in food applications and in new product development, thereby providing an added dimension to protein functionality. The lower the least gelation capacity is the better the gelating ability of the protein ingredient [32]. Thus, the low gelation concentration absorbed in this finding may be an asset in the use of this flour for the formulation of curd or as an additive to other gel-forming materials in food products.

CONCLUSION

Defatted brebra seed flour showed higher nutritional value and can serve as good source of plant protein compared to some legumes. The major protein in this study was found to be high amount of albumins like that of egg white, blood and milk. Defatted flour was found to be possessed good foaming capacity and stability, emulsion capacity and stability, water and oil absorption capacities and gelation properties. Therefore, it may be used as protein source for both humans and other animals, especially useful as supplements to low nitrogen foods such as cereals, tubers and maize gruel.

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