

Ebony (*Pithecellobium flexicaule* Benth) and proteins fractionation, solubilization, characterization and production of an isolate

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SUMMARY. Different combinations of pHs (2 to 12) and temperatures (25, 30 and 35°C) were tested to obtain a protein isolate from ebony (*Pithecellobium flexicaule*, Benth) seeds. Seed proteins contained 54.6% albumins, 32% globulins, 5.7% glutelins and 1.3% prolamins. The isoelectric points for albumins, globulins and glutelins were in the pH range of 2.3-2.7. The average molecular weight of albumins ranged from 92 to 100 kDa and for the four globulin subunits in the range of 28.4 to 57.3 kDa. For isolate production, proteins were sequentially extracted with distilled water and a 5% NaCl solution. The resulting supernatants were mixed. The best extraction was achieved at pH 11 and 25°C. 45.6% of the total seed protein was precipitated at pH 2.6 yielding an isolate with 90% protein (N x 6.25). The isolate contained high quantities of lysine, leucine, threonine and phenylalanine but were low in sulfur containing amino acids methionine and cysteine. The extraction process reduced tannins, phytates and trypsin inhibitor in 53, 70 and 70%, respectively. *In vivo* protein digestibility of the protein isolate was 85.4% and the corrected digestibility essential amino acid score was of 44% due to the lack of sulfur containing amino acids. In order to upgrade the protein quality of ebony isolate it is recommend to supplement with methionine or sulfur containing rich foods.

Key words: Ebony seed, protein extraction, protein isolate, amino acid composition, protein digestibility.

INTRODUCTION

The fast demographic growth plus the low economic resources in developing countries creates the necessity to look for new protein sources that can substitute animal proteins, complement the nutritional value of cereal-based foods and prevent malnutrition. The ebony (*Pithecellobium flexicaule* Benth) is an arboreous legume widely distributed in Texas and northeastern Mexico. Its cooked or toasted seeds are consumed by the population of rural and marginal areas of these Mexican regions. The seeds generally contain 35% protein and similar nutritional value and protein quality as

RESUMEN. Fraccionamiento, solubilización y caracterización de un aislado proteico obtenido de semillas de ébano (*Pithecellobium flexicaule*, B.). Diferentes combinaciones de pH's (2 to 12) y temperaturas (25, 30 y 35°C) se probaron para obtener un aislado proteico de semillas de ébano (*Pithecellobium flexicaule*, Benth). La composición de las semillas fue 54.6% albúminas, 32% globulinas, 5.7% glutelinas y 1.3% prolaminas, con los puntos isoeléctricos para las albúminas, globulinas y glutelinas en el rango de pH de 2.3-2.7. El peso molecular promedio de las albúminas fluctuó entre 92 y 100 kDa y para las cuatro subunidades de globulinas en el rango de 28.4 a 57.3 kDa. Para la producción del aislado, las proteínas se extrajeron con agua destilada y una solución al 5% de NaCl. Los sobrenadantes resultantes se mezclaron. La mejor extracción se obtuvo a pH 11 y 25°C. 45.6% del total de las proteínas precipitaron a pH 2.6 produciendo un aislado con 90% de proteína (N x 6.25). El aislado presentó alta cantidad de lisina, leucina, treonina y fenilalanina y baja cantidad de los amino ácidos azufrados metionina y cisteína. El proceso de extracción redujo el contenido de taninos, fitatos e inhibidor de tripsina en 53, 70 y 70%, respectivamente. La digestibilidad de la proteína del aislado *in vivo* fue de 85.4% y el valor corregido para la digestibilidad de los amino ácidos esenciales de 44% debido a la carencia de los amino ácidos azufrados. Se puede incrementar la calidad proteica del aislado suplementando con metionina o con alimentos ricos en aminoácidos azufrados.

Palabras clave: Semillas de ébano, extracción de proteínas, aislado proteico, composición de aminoácidos, digestibilidad proteica.

commercial legumes (1-2).

Legume seeds, mainly soybeans, are used as raw materials for production of protein concentrates and isolates. These products are playing an important role in human nutrition and as functional ingredients to improve processing properties of foods. Legume proteins are rich in globulins and albumins and generally have isoelectric points of 4.2 to 4.4 (3). These protein fractions are rich in lysine and other essential amino acids but generally low in sulfur containing amino acids; therefore, they complement protein quality of cereal-based foods. Several authors (4-,5) have researched the production of protein isolates with approximately 90% protein from wild

legumes such as lupins (*Lupinus albus* and *Lupinus mutabilis*) and Tepary beans (*Phaseolus acutifolius*). The protein isolation process eliminates polysaccharides, oligosaccharides and concentrates proteins based on solubility. These soluble proteins are precipitated by dropping the pH to their isoelectric point. The resulting isolates are utilized to upgrade protein concentration in foods and to impart different functionality to food systems such as water and oil absorption, foaming, emulsifying and gelation capacities (6).

The aim of this research was to characterize chemical composition, protein fractions and amino acid composition of ebony seeds and to determine the optimum pH and temperature conditions for the production of protein isolates.

MATERIALS AND METHODS

Ebony seeds

Mature and dried ebony seeds were collected from three different sites (General Escobedo, General Terán and Marin) located in the state of Nuevo León, México. The three sites have similar environmental and climatological conditions. A composite sample was utilized to perform extraction studies.

Protein fractionation.

Proteins from defatted flour were sequentially fractionated with distilled water (albumins), 5% NaCl solution (globulins), 70% ethanol (prolamins) and 0.05N NaOH (glutelins) according to the procedure of Sauvaire et al. (7).

Electrophoresis

Molecular weights of proteins present in each fraction were obtained after SDS-PAGE electrophoresis using the technique proposed by Laemmli (8). Before electrophoresis aliquots of protein fractions were diluted 1:1 in a buffer (pH 6.8, 3% tris, 20% B mercaptoethanol, 10% SDS, 0.02% bromophenol blue and 40% glycerol). 25-30 μ l were subjected to electrophoresis (Sigma Tech Ware) at 15°C. Protein bands were stained by overnight immersion in a 0.1% Coumassie blue R250 50% methanol and 10% acetic acid solution. Excess dye was removed by soaking for 8 hr in a 10% acetic acid solution. Molecular weight standards utilized were myosine (205 kDa), B galactosidase (116 kDa), phosphorilase b (97.4 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Isolate Production

The testa from raw ebony seeds was manually removed. The resulting cotyledons were ground in a Cofert mill (Model 518) into a flour that passed the 100 mesh sieve. The flour was defatted with n-hexane at 35-37°C for 6 h in a water

bath regulated to oscillate 80 cycles/min. The hexane was changed after 3 h. The resulting defatted flour was desolventized by drying at room temperature for 16 h and immediately stored in a refrigerator set at 4°C. Proteins were extracted at 25, 30 and 35°C at pH values between 2 and 12 according to the methodology of Bello and Okezie (9). Proteins were extracted sequentially first with distilled water and then 4 consecutive times with 5% NaCl solution. The supernatants of these extractions were composited. The pH was adjusted with 0.1N HCl or 0.1N NaOH. A mixture of 1:20 (w/v) flour:solvent was agitated at 150 rpm for 20 min at the preset temperature (Labline Incubator Shaker Orbit Model 3595) and then centrifuged (Beckman J2-21) at 25,000 x g for 20 min. The temperature of the centrifuge was regulated at the same predetermined extraction temperature. Supernatant protein concentration was determined in a UV spectrophotometer (Beckman DU 650) set at wavelengths of 280 and 260 nm. Protein isolates were obtained following the procedure described before. The optimum extraction was achieved at pH 11 and 25°C. Proteins were precipitated by adjusting the pH to 2.6 and centrifugation for 20 min at 25000 x g in a centrifuge set at 4°C. The supernatant was discarded and the resulting pellet lyophilized for 24 hr (Labconco Freezone 6). The dried isolate was stored in a desiccator under refrigeration.

Proximate compositions of raw flour, defatted flour and resulting protein isolates were determined according to standard AOAC (10) procedures.

Amino acid composition

All amino acids, except tryptophan and sulfur containing, were determined after a 6N HCL hydrolysis as suggested by Moore and Stein (11) in a Pico Tag unit. Amino acids were separated in a Technicon (Model NC-2P) autoanalyzer equipped with ionic exchange columns. Sulfur containing amino acids were obtained after performic acid hydrolysis. For tryptophan analysis, samples were hydrolyzed with 5N NaOH and partially hydrolyzed potato starch that acted as a reducing agent. All amino acids were quantitated after ninhydrin reaction in a spectrophotometer set at 490 and 570 nm.

Antinutritional compounds

Tannins were quantitated as catequin equivalents by the HCl-vanillin method proposed by Preece et al. (12) and Desphande and Cheryan (13). Phytic acid content was determined by the modified extraction procedure of Wheeler and Ferrel (14) in which 3% TCA is used to precipitate proteins and 1% FeCl₃ in 3% TCA used to precipitate phytates. The quantification of phosphorus-phytate was determined colorimetrically in a spectrophotometer (Beckman DU 650). Trypsin inhibitors were determined

according to the methodology of Kakade et al. (15).

In vivo protein digestibility

In vivo protein digestibility of ebony protein isolate was determined according to the methodology proposed by the FAO/WHO (16). Sixteen Sprague Dawley male weanling rats were divided into two groups. Each group consisted of two blocks of 4 rats blocked by initial weight. One group was fed a protein isolate based diet and the other a protein free diet. The experimental diet was balanced to contain 10% protein, 10% ether extract, 5% crude fiber, 3.5% AIN mineral mixture 76 and 1% AIN 76 vitamin premix. The diet was adjusted to 100% with corn starch. Animals were housed individually in metabolic cages designed to minimize diet spillage. Feces were collected for 5 consecutive days and feed intake recorded. Fecal moisture and nitrogen were determined using AOAC (10) procedures.

Statistical Analysis

In order to study the effect of pH and temperature on protein extraction a bifactorial ANOVA procedure was utilized. Means were compared using Tukey's test ($\alpha = 0.05$). A non linear regression analysis that related pH in the range of 1 to 7 and precipitated protein of albumins, globulins and glutelins were performed to predict isoelectric points of each protein fraction.

RESULTS AND DISCUSSION

Ebony seeds contained 36 and 25% protein and ether extract respectively. The hexane treatment of the raw flour decreased the oil content to a level below 2% and therefore concentrated protein and nitrogen free extract (Table 1).

TABLE 1
Chemical composition of raw, hexane extracted
and a protein isolate obtained from ebony seeds
(g /100 g dry sample)^a

	Raw flour	Hexane extracted flour	Protein isolate
Protein (N x 6.25)	36.1 ± 0.17	52.45 ± 0.71	89.98 ± 0.41
Ether extract	25.43 ± 0.34	1.67 ± 0.18	0.35 ± 0.01
Crude fiber	3.64 ± 0.14	3.82 ± 0.17	1.69 ± 0.04
Ash	3.95 ± 0.06	5.20 ± 0.22	1.23 ± 0.03
NFE ^b	30.17 ± 0.36	36.86 ± 0.88	6.75 ± 0.37
Moisture	3.39 ± 0.09	4.59 ± 0.18	4.46 ± 0.07

^aEach value is the average of three observations ± standard deviation

^bNitrogen free extract, calculated by difference.

Albumins were the most abundant protein fraction in defatted ebony seeds followed by globulins (Table 2). Sathe and Salunkhe (3) mentioned that these fractions are the most prevalent in other legume seeds. Globulins are the main protein fraction in common beans (3), soybeans (17), lupinus (5) and leucaena (18) whereas albumins in adzuki beans (19) and fenugreek seed proteins (7). A large variation in albumin content within a particular legume seed has been reported (20,21). According to Bhatta (22) these discrepancies are due to the albumin extraction procedure and pH used during extraction. Some salt soluble globulins might have solubilized in the albumin fraction due to the neutral salt content of the ebony seed flour. Ebony seeds contained low quantities of glutelins and alcohol soluble prolamins. Sauvaire et al. (7) also found that legume seeds are low in prolamins.

TABLE 2
Protein fractionation and isoelectric points of proteins
from ebony seeds

Protein fraction	Experimental isoelectric point	% Total protein isoelectric	Calculated point
Albumins	2.7	54.62± 0.67	2.64
Globulins	2.3	32.22± 0.64	2.43
Glutelins	2.5	5.77± 0.59	2.62
Prolamins	ND	1.31± 0.19	ND
Residual Protein	ND	6.07± 0.05	ND

ND= Not determined

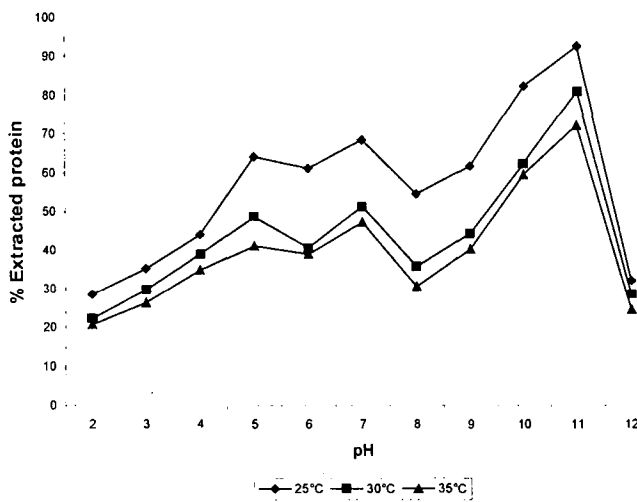
Temperature and pH significantly ($P < 0.01$) affected protein extraction. At all pHs tested the extracted protein progressively decreased when the temperature increased; therefore, the best extraction occurred at 25°C (Figura 1). The increased protein solubility at lower temperature is related to protein denaturation. For each temperature, the best extraction occurred at pH increasing gradually from pH 8 to 11. This is due to the electric charge and protein denaturation that increased at higher pHs. Bello and Okezie (9) working with winged beans (*Psophocarpus tetragonolobus* L) reported a maximum protein extraction at pH 12 and 30°C. The lowest protein extraction occurred in the pH range of 2 to 3 where the isoelectric points of these proteins generally lie.

Isoelectric points of albumins, globulins and glutelins were in the acidic pH (Figura 2) as previously detailed by Sethi and Kulkarni (18). Albumins, the most concentrated protein fraction in ebony seeds, had isoelectric points in the pH range of 2 to 4 where 76 and 94% of the proteins precipitated. The best precipitation occurred at pH 2.7. Likewise, from 80 to 98% of globulins precipitated in a pH range of 2.1 to 2.3. For glutelins, the pH value where proteins

precipitated the most was 2.5. In conclusion, the three protein fractions precipitated the most in the pH range of 2.3 to 2.7. Similar isoelectric point values are reported by Sethi and Kulkarni (18) for *Leucaena*. A non linear regression analysis that related protein precipitation with pH predicted very closely experimental isoelectric points of each protein fraction. The difference between experimental and predicted values for all fractions studied was less than 0.15 pH units (Table 2).

FIGURE 1

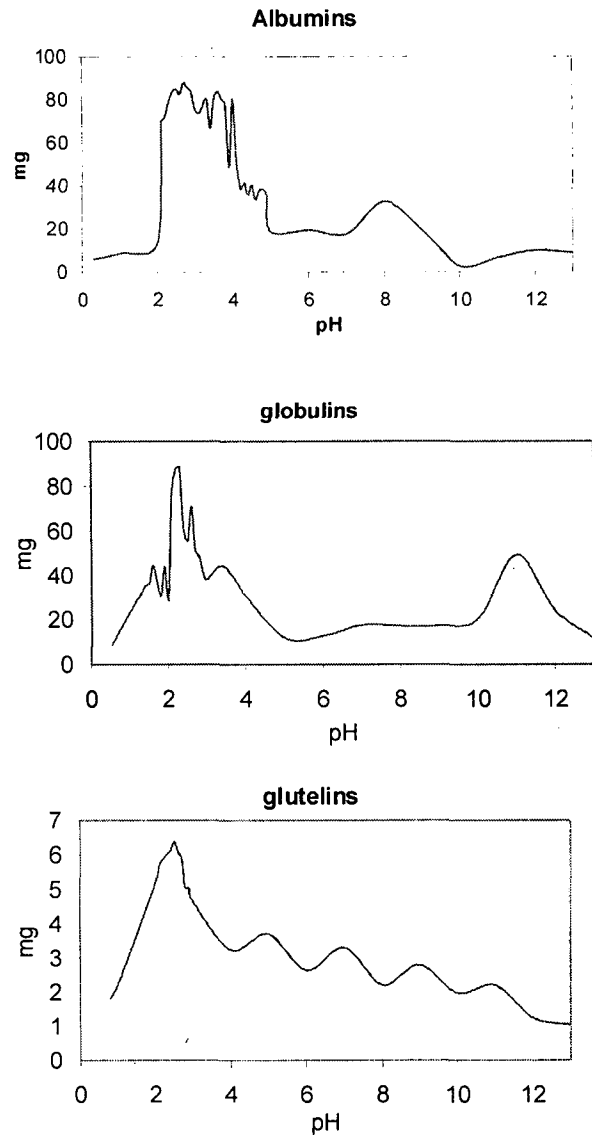
Effect of pH and temperature on the amount of extracted proteins from ebony seeds. Each value is the average of three replications.



SDS-PAGE electrophoresis of albumins indicated the presence of 5 subunits with molecular weight ranging from 51.4 to 100 kDa (Table 3). Four subunits with molecular weights ranging from 28.4 to 57.3 kDa were detected in globulins whereas only two subunits with molecular weights of 21.6 and 64.2 kDa in glutelins. In all fractions, lower molecular weight polypeptides with molecular weights ranging from 1.3 to 12.3 kDa were detected. These compounds may have resulted from the partial hydrolysis of larger subunits that occurred during lipid extraction. Chan and Phillips (23) working with cowpeas reported the existence of four albumin subunits, two with molecular weights of 99 and 91 kDa similar than the ones found in this study. Likewise, the same authors found similar globulins and glutelins bands as the ones found in this study.

FIGURE 2

Effect of pH on the amount of precipitated proteins from albumins, globulins and glutelins extracted from ebony seeds. Each value is the average of three replications



The protein content of the protein isolate reported in Table 1 was similar to counterparts obtained from soybeans (24), lupin seed (25), adzuki beans (19) and higher than isolates obtained from chickpeas (26), faba beans (27) and soybeans (28). The isolate yield (45.6%), calculated based on total protein content, was lower than the reported for soybeans (29), winged beans (30) and higher than adzuki beans (19). Protein precipitation was optimum at pH 2.6.

TABLE 3
Molecular weights (kDa) determined by SDS-PAGE
electrophoresis of albumins, globulins and glutelins
extracted from ebony seeds

Albumins	Globulins	Glutelins
100.0	57.3	64.2
92.1	46.2	21.6
62.4	33.5	5.7
55.8	28.4	4.7
51.4	12.3	3.4
12.3	5.7	
5.7	4.7	
4.7	3.4	
3.4	1.3	
1.3		

In vivo protein digestibility of the ebony isolate was 85.4%. Swamylingappa and Srinivas (29) and Sathe et al. (25) reported higher and lower in vitro protein digestibilities for a soybean isolate (88.4%) and a lupinus concentrate (75%). Torun (31) reported that soybean isolates have protein digestibilities ranging from 84 to 87%. It is recognized that protein isolates are highly digestible due to their low fiber content and trypsin inhibitors (32). Table 4 shows the essential amino acid pattern of the ebony protein isolate. The most limiting amino acids were the sulfur containing methionine and cysteine followed by isoleucine. The protein isolate provided approximately 50% of the sulfur containing amino acids required by a growing child. The rest of the essential amino acids exceeded the quantities required by growing infants. Interestingly, the ebony protein isolate contained higher amounts of lysine, leucine, phenylalanine, tyrosine, threonine and histidine than counterparts from soybeans (17), chickpeas (26), and a commercial soybean isolate (33). Due to the high quantities of lysine and threonine the experimental isolate can be used to supplement and upgrade protein quality of cereal-based foods. The corrected essential amino acid score of the ebony isolate, obtained by multiplying chemical score by the protein digestibility, was 44%. In order to upgrade the protein quality of ebony isolate it is recommend to supplement with methionine or sulfur containing rich foods. Processing raw ebony seeds into protein isolates reduced tannins, phytates and trypsin inhibitors in 53, 70 and 70%, respectively. The reduction in phytates and tannins can be due to solubility and pH changes used to extract and precipitate proteins. Saeed and Cheryan (34) determined the solubility of phytates in a pH range of 2 to 12. The least solubility (less than 20%) was observed at pH 11, value utilized to extract proteins in this study. Chen and Morr (28) reported similar phytate content in soybean isolate as the one found in this study. Desphande and Cheryan (35) found that

saline solutions such as the utilized in this study reduced condensed tannin solubility. The inhibitor is classified as globulin and is reported to possess a low molecular weight (8-20 kDa) and an isoelectric point of 4.2 (36). The hexane extraction treatment and the small size of the molecule explain the loss of this antinutritional compound. Swamlingappa and Srinivas (29) reported the reduction of 138 to 52 TIU/mg protein when full fat soybean flour was defatted with hexane at 28°C. According to Henn and Netto (33) the trypsin inhibitor content in soybean isolate is greatly reduced when proteins are extracted with salt solutions and precipitated at acidic pHs. These authors reported that soybean isolates contained from 5 to 95 TIU/mg protein.

TABLE 4
Comparison between the essential amino acid composition
of a protein isolate from ebony with the requirement
FAO/WHO for preschool children (mg/g protein)

Essential amino acid	Ebony protein isolate	FAO/WHO requirement ^a	% Requirement
Isoleucine	25.6	28	91
Leucine	81.6	66	>100
Lysine	75.2	58	>100
Methionine + cysteine	12.9	25	51
Phenylalanine + tyrosine	110.2	63	>100
Threonine	52.4	34	>100
Tryptophan	11.2	10	>100
Valine	35.2	35	100
Histidine	28.1	19	>100

^a FAO/WHO (19).

TABLE 5
Comparison of condensed tannins, phytates and trypsin
inhibitor contents between defatted flour and protein
isolate from ebony seeds

	Defatted flour	Protein isolate
Tannins (mg equiv. catechin/100g protein)	38.7 + 0.27	16.35 + 0.94
Phytates (mg phytic acid/g protein)	72.5 + 0.80	18.66 + 0.31
Trypsin inhibitor (TIU/mg protein)	616.7 + 0.60	139.44 + 0.51

^a Expressed on dry matter basis. Each value is the average of three replications + standard deviation.

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