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Isolation, fractionation and characterisation of proteins from *Mucuna* bean

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Abstract

The chemical composition and fractional distribution of protein isolates prepared from species of *Mucuna* bean were studied. Using six different extraction media, the yield of protein based on the Kjeldahl procedure varied from 8% to 34%, and the protein content varied from 75% to 95%. When the yields were high, the colour of the isolates generally tended to be dark and unsatisfactory. Hence, the use of chemical treatments and high pressure processing were explored.

The solubility maxima for the protein isolates in water were found to occur at pH values of 2.0 and 11.0, while the pH corresponding to minimum solubility (i.e. isoelectric region) occurred at pH values of 4.0 and 5.0. The total essential amino acid in the isolates ranged from 495 to 557 mg g^{-1} protein, which compares favourably with the recommended level for pre-school and school children. Methionine and cysteine were the limiting amino acids. A key nutritional attribute of the protein isolates was its high lysine content. The isolate can therefore complement cereal-based foods which are deficient in lysine.

The proteins mainly consisted of albumins, glutelins and globulins. Prolamins were only present in trace concentration (<0.3%). Gel filtration chromatograms of the isolates indicated the presence of major protein fractions with molecular weights of 40 and 15 kDa, while gel electrophoresis (SDS-PAGE) indicated a major broad zone with molecular weights of 36 ± 7 and 17.3 ± 3 kDa.

Keywords: *Mucuna* species; Protein isolate; Isoelectric precipitation; Protein fractions

1. Introduction

The wide prevalence of protein-calorie malnutrition in developing countries is a global concern (Olsen, 1975). The continuous increase in population and inadequate supply of protein has increased the occurrence of malnutrition in developing countries (Siddhuraju, Vijayakumari, & Janardhanan, 1996). Recent studies have shown that malnutrition among children in developing countries is mainly due to the consumption of cereal-based porridge which is bulky, low in energy and density, and high in antinutrients (Michaelsen & Henrik, 1998). Plant protein products are gaining increased interest as ingredients in food systems throughout many parts of the world; the final success of utilizing plant proteins as additives depends greatly upon the favourable characteristics that they impart to foods. In the developed countries, plant proteins are now regarded as either versatile functional ingredients or as biologically active components, rather than as essential nutrients (Marcello & Gius, 1997). This evolution towards health and functionality, which is mainly driven by the partial replacement of animal foods with legumes, has been shown to improve nutritional status (Guillon & Champ, 1996) due to lower cholesterol

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level in plant foods, and increased level of fibre intake which reduces the risk of bowel diseases, including cancer, and also reduction in osteoporosis incidence (Sirtori & Lovati, 2001).

The world production of legume seeds, "the poor man's meat", as producers call them, was about 58 million tons in 1994 by FAO estimation. Of these, the major part, 40 million tons, was produced by developing countries, and only 8.5% of this amount was consumed outside its production area (Heiser, 1996). Renewed awareness, in developing and developed countries alike, of the potentials of the legumes seeds has called for new research approaches to the exploitation of the legume seeds.

Mucuna bean, an under-utilized legume, is not only rich in proteins but also in carbohydrates, fats, minerals and other nutrients. It is, however, limited by the presence of antimetabolic/antiphysiological substances, such as protease inhibitors, phenolic substances, non-protein amino acids, particularly 3,4-dihydroxy-L-phenylalanine (L-Dopa), lectins, saponins, flatulence and non-starch polysaccharides (Siddhuraju, Becker, & Makkar, 2000; Vidivel & Janardhanen, 2001). The antinutrients in this legume have recently been studied (Adebowale, Adeyemi, & Oshodi, 2005).

For plant proteins to be useful and successful in food application, they should ideally possess several desirable characteristics, referred to as functional properties, as well as providing essential amino acids (Kinsella, 1976). These properties are intrinsic physicochemical characteristics, which affect the behaviour of proteins in food systems during processing, manufacturing, storage and preparation (Kinsella, 1976). This search for plant protein sources has therefore stimulated research interest in characterising and isolating proteins from under-utilized known legumes, such as winged bean (Sathe & Salunkhe, 1981), adzuki bean (Tjahjadi, Lin, & Breene, 1988) and cowpea (Sefa Dedek & Stanley, 1979a), tree nuts like macadamia (Bora & Ribeiro, 2004), oilseeds, such as safflower (Betschart, 1975; Sauvaire, Baccou, & Kobrehel, 1984), peanut (Kim, Kim, & Young, 1992) and cereals, e.g. corn (Lin & Zayaf, 1987).

Adebowale et al. (2005) presented the variability in chemical composition, amino acids and antinutrients of six varieties of *Mucuna*. This paper considers methods which can be used to produce protein isolates from *Mucuna* bean, including the use of high pressures to improve their colour. It considers the effects of the extracting medium on the yield and integrity of the isolate. The proteins present have also been characterized. Gel filtration chromatography and polyacrylamide gel electrophoresis were carried out on the isolates, and amino acid content and *in vitro* multi-enzyme digestibility of the proteins were also evaluated. This work is part of our comprehensive research aimed at the eventual incorporation of protein isolates into food products to produce natural, cost-effective and adaptable functional foods.

2. Materials and methods

2.1. Materials

Six varieties of *Mucuna* bean seeds: *deeringeana*, *rajada*, *pruriens*, *veracruz mottle*, *veracruz white* and *cochinchinensis*, were obtained from the International Institute of Tropical Agriculture/International Livestock Research Institute IITA/ILRI, Ibadan-Nigeria.

2.2. Methods

2.2.1. Preparation of defatted flour

Mucuna bean seeds were dehulled manually, using a pestle and mortar. The seeds were ground in a Christy Laboratory Mill (Cheff Food Processor, Japan) and thereafter sieved through a screen of 20 mesh sizes before extraction for 9 h with hexane in a Soxhlet apparatus (10% w/v hexane). The defatted flour was air-dried at room temperature (approximately 28 °C) and subsequently kept in air-tight plastic containers at 4 °C prior to use.

2.2.2. Preparation of protein isolate

The basic steps are as follows: the slurry (1:20 w/v ratio of flour weight in grammes to water volume in millilitres) at pH 6.37 was stirred for 2 h using a Gallenhamp magnetic stirrer (model SS-615), and the pH was adjusted to the desired value using 0.1 M NaOH or 0.1 M HCl. The extractant was centrifuged in a Sorvall RC5C automatic super speed refrigerated centrifuge at 10,000*g* for 15 min. After centrifugation and recovery of supernatant, three additional extractions were carried out with half the initial volume of water taken. The supernatants were pooled and precipitated at pH 4.0 or 5.0, the isoelectric point (IEP); the precipitate formed was recovered by centrifugation at 10,000*g* for 15 min. The precipitate from the same batch of *Mucuna* bean was washed twice with distilled water, adjusted to pH 4.0 or 5.0 with HCl, dialyzed against distilled water for 24 h at 4 °C and then freeze-dried. Extraction was carried out using one of the following media separately: ascorbic acid (0.5% w/v); EDTA + 0.5% ascorbic acid; cysteine (0.5%); sodium sulphite (0.25%) and distilled water, following the above procedure. A schematic diagram of the isolation procedure is shown in Fig. 1.

2.2.3. Preparation of protein isolates using high pressure

Slurries were prepared (1:20 w/v ratio of flour in grammes to water in millilitres) at pH 6.37. The slurries were pressurized for 20 min at 200, 300, 500 and 600 MPa in a Stansted "Food lab" high pressure apparatus (Stansted fluid power, Essex UK). After high pressure treatment, the isolate was prepared as described above.

2.2.4. Fractionation of Mucuna protein

Fractionation of *Mucuna* protein was carried out as described by Sauvaire et al. (1984). The method was based on the classical Osborne protein fractionation procedure.

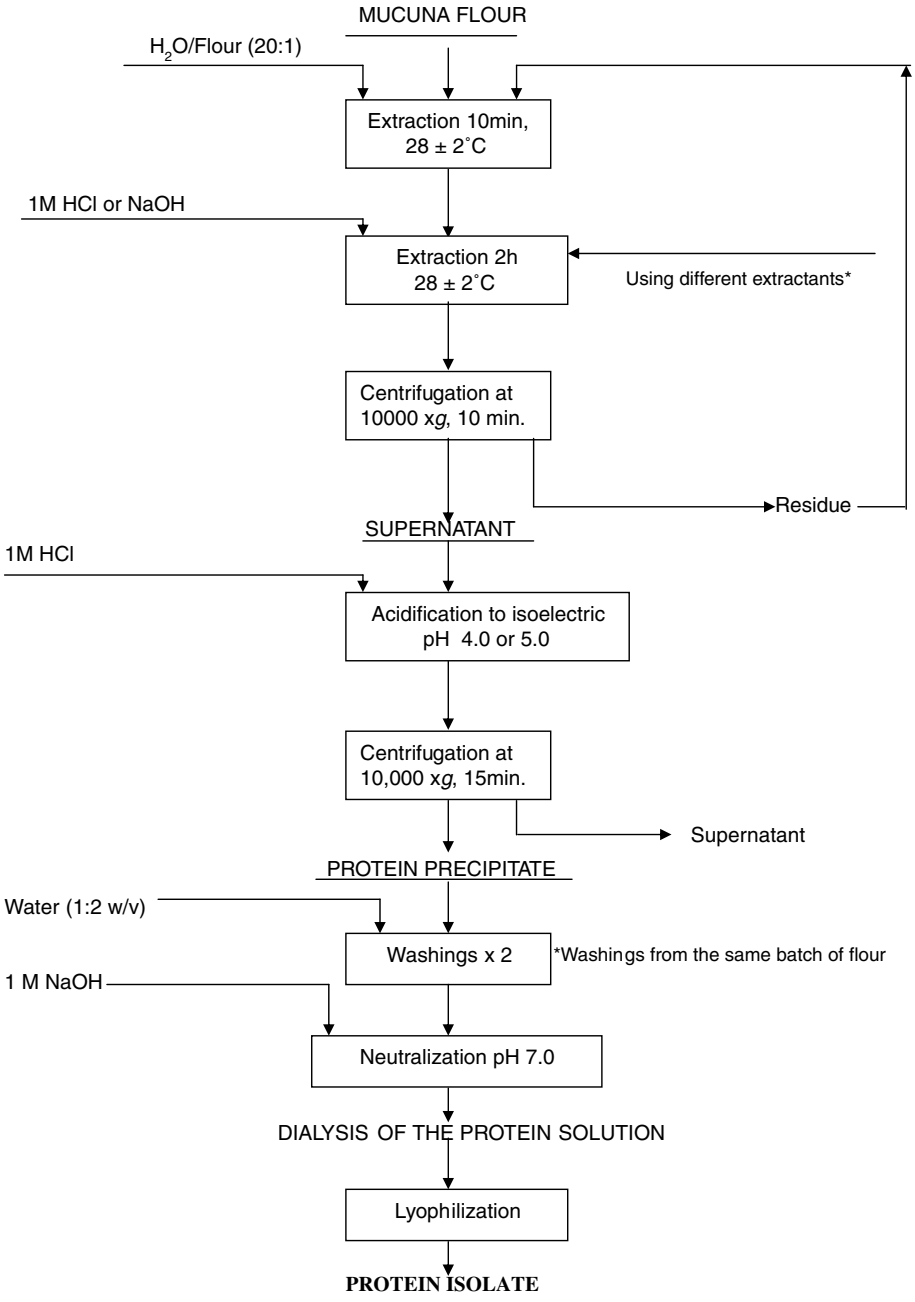

Fig. 1. Preparation of protein isolate. *Extractants: ascorbic acid (0.5% w/v); EDTA (0.25%); cysteine (0.5%); sodium sulphite (0.25%) and water.

Three solvents were used, consecutively, to extract the proteins of hexane-defatted *Mucuna* flour: 5% NaCl, 70% ethanol and 0.05 M NaOH. Defatted flour sample (20 g) was suspended in 400 ml of solvent (1:20 w/v) and the protein was extracted by stirring for 1 h, using a Gallenhamp magnetic stirrer (model SS-615). The extract was centrifuged at 5000*g* for 20 min and the supernatant filtered using Whatman filter paper. Two other extractions were carried out and the filtrates were pooled together and dialyzed against distilled water for 48 h at 4 °C and then freeze-dried. The NaCl combined extracts were fractionated into albumins and globulins by dialysis (MW cut-off = 12 kDa) for three days at 4 °C against distilled water. Recovered supernatants and precipitates, after centrifugation at 5000*g* for 20 min, were considered as the albumin and the globulin fractions, respectively.

2.2.5. Colour evaluation

Instrumental colour readings for the different isolates were obtained using a Hunter Lab Colour Quest instrument equipped with a D65 circumferential optical sensor and standard white tile using reflectance values of $X = 81.16$, $Y = 85.90$ and $Z = 92.41$ as references. The colour codes were: Lightness (L), redness ($+a$), greenness ($-a$), yellowness ($+b$) and blueness ($-b$). The L, a, b values of the standard white tile were, respectively, 94.25, −0.83

and 0.79, where $L = 100$ is associated with white and $L = 0$ is associated with black.

2.2.6. Protein solubility

Protein solubility was determined by the method of Sathe, Desphande, and Salunkhe (1982a) Sathe, Deshpande, and Salunkhe (1982b) with some modifications, as stated below. The suspensions (0.2%) of the flour in distilled water were adjusted to pH 2–11 using 1 M HCl and 1 M NaOH. Percent nitrogen in each supernatant was determined by the micro-Kjedahl method according to the method already described in AOAC (1990). The percentage of soluble protein was calculated as the percentage of nitrogen determined, multiplied by 6.25, and expressed on a wet weight basis according to the AOAC (1990).

2.2.7. Gel eletrophoretic studies

The molecular weight profile for the protein fractions was established using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970). One hundred milligrams of the sample were added to 2.5 ml of the buffer containing 0.5 M Tris–HCl (pH 6.8), 0.5% bromophenol blue, 10% glycerol and 2% SDS. Five percent (5%) 2-mercaptoethanol (2-ME) was added to another preparation to effect reduction of disulphide bonds. The preparation was centrifuged at 20,000*g* for 15 min at 4 °C and the supernatant was employed for electrophoresis. Protein samples (10 μl) were added onto the gel using a model 491 cell, Bio-rad Laboratories, California, USA. The electrode buffer contained a mixture of 0.025 M Tris buffer, 0.192 M glycine and 10% SDS to a final pH of 8.7. The separating gel had 12.5% final acrylamide concentration, while the stacking gel had 4.5%. Electrophoresis was carried out at a constant current of 60 mA at a maximum voltage of 600 V until the dye reached the bottom of the gel. The gel was stained with Coomassie brilliant blue G-250 and de-stained with 25% methanol solution. The gel was then transferred to 25% ammonium sulphate solution for storage. The molecular weights of protein subunits for each sample were determined using the broad spectrum molecular weight standard from SIGMA, USA containing the following: aprotinin, bovine lung, 6.5 kDa; α-Lactalbumin, bovine milk, 10.2 kDa; trypsin inhibitor, soybean, 20.0 kDa; trypsinogen, bovine pancreas, 24.0 kDa; carbonic anhydrase, bovine erythrocytes, 29.0 kDa; glycerol-3-phosphate dehydrogenase, rabbit muscle, 36.0 kDa; ovalbumin, chicken egg, 45.0 kDa; glutamic dehydrogenase, bovine liver, 55.0 kDa; albumin, bovine serum, 66.0 kDa; fructose-6-phosphate kinase, rabbit muscle, 84 kDa; phosphrylase b, rabbit muscle, 97 kDa; rabbit muscle phosphorylase b, 97.4 kDa; β-galactosidase, Escherichia coli, 116.0 kDa; myosin, rabbit muscle, 205.0 kDa.

2.2.8. Gel filtration chromatography

Gel filtration chromatography of the samples were carried out according to the modified method of Lqari, Vioque, Pedroche, and Millan (2001). Lyophilized samples (0.15 g) were dissolved in 10 ml of 0.05 M K_2HPO_4 and 0.15 M Na_2SO_4 buffer pH 7.0. Gel filtration was then carried out in the HPLC system equipped with Macrophere GPC 300A 7 μm (ALLTECH Associate Ltd.). Volume injection and concentration of samples were 200 μl and 1.6 mg protein per ml, respectively. The eluent used was the earlier-mentioned buffer at a flow rate of 0.3 ml min^{-1}. Protein elution was monitored at 280 nm and the approximate molecular masses were determined using blue dextran 2000E (2000 kDa), bovine serum albumin (67 kDa), ribonuclease (13.7 kDa), ovalbumin (43 kDa) and chymotrypsinugen A (25 kDa) as molecular weight standards.

2.2.9. Determination of amino acids in Mucuna protein isolates

Hydrolysis of the *Mucuna* isolate was effected as follows: lyophilized sample(20 mg) was weighed into a 16 × 125 mm screw-cap pyrex tube containing 2 ml of 6 M HCl (containing 1% phenol). The tube was flushed with nitrogen, capped and placed in the oven at 110 °C for 24 h. After hydrolysis, the residual HCl was removed under vacuum, using a gene vacuum EZ-2 personal evaporator at 55 °C. The dried sample was derivatized with diethyl ethoxymethylenemalonate. Amino acids were determined by reverse phase high performance liquid chromatography, RP-HPLC (DIONEX), consisting of a pump type P680, ASI-100T automated sample injector and PDA-100 photo diode array detector. L-α-Aminobutyric acid was used as internal standard. Data acquisition and processing were effected by a Chromeleon data system. Separations were attained with a 300 × 3.9 mm i.d reverse phase column (Nova pack C18, Waters) using a binary gradient system with 25 mM sodium acetate, pH 6.0 (A), and acetonitrile (B). The solvent was delivered to the column at a flow rate of 0.9 ml/min as follows: (i) time 0.0–3.0 min, linear gradient from (A:B) = (91:9) to (A:B) = (86:14); (ii) 3.013.0 min, elution with (A:B) = (86:14); (iii) 13.0–30.0 min, linear gradient from (A:B) = (86:14) to (A:B) = (69:31); (iv) 30.0–35.0 min, elution with (A:B) = (69:31). The column was maintained at 18 °C by a temperature controller. Amino acid composition was expressed as g amino acid per 100 g protein. Tryptophan was determined after hydrolysis of proteins according to the method of Aitken and Learmonth (1996).

2.2.10. In vitro protein enzyme digestibility

In vitro multi-enzyme digestibility was determined by the method of Hsu, Vavak, Satterlee, and Miller (1977). Fifty millilitres of aqueous suspension of the sample (6.25 mg sample per ml) in distilled water were adjusted to pH 8.0 with 0.1 M HCl and/or 0.1 M NaOH, while stirring on a water bath maintained at 37 °C. The multi-enzyme solution, containing 1.6 mg trypsin, 3.1 mg chymotrypsin and 1.3 mg peptidase, was maintained in an ice bath and adjusted to pH 8.0 with 0.1 M HCl and/or 0.1 M NaOH. The enzymes were purchased from SIGMA Chem.

Company (St. Louis, MO, USA). Five millilitres of multi-enzyme solution were added to the sample suspension with constant stirring at 37 ± 2 °C. The pH of the suspension was recorded 15 min after the addition of the multi-enzyme solution. *In vitro* digestibility was calculated using a regression equation of Hsu et al. (1977): $Y = 210.46 - 18.10X$, where Y = *in vitro* digestibility (%) and X = pH of the sample after a 15 min digestion with multi-enzyme solution. The enzyme activity was determined using casein of known *in vivo* apparent digestibility. Apart from this, a commercial protein isolate with known digestibility was also used as standard.

2.2.11. Extraction and determination of L-DOPA in the isolates

3,4-Dihydroxy-L-phenylalanine (L-DOPA) was determined using the method described by Myhrrman (2002). Five millilitres of distilled-deionised water was added to 0.5 g of the flour in a 16 mm (O.D.) × 100 mm glass culture tube (screw-cap style), and the tube was vortexed briefly to mix the contents and then placed in a boiling water bath for 6 min. The tube was centrifuged for 2 min at 3000*g* and the extraction repeated three times. The combined supernatant was diluted to 100 ml with dionised water, filtered through a 0.45 mm nylon membrane and cooled to approx 15 °C. The concentration of L-Dopa in the extract of the samples were determined by HPLC with UV detection at 279 nm on a SB-C18 column(4.6 × 150 mm, 3 μm particles). Two mobile phases were used at 1 ml min^{-1} and 30 °C. Mobile phase A consisted of buffer [0.1 M phosphoric acid, 1 mM 1-octanesulphonic acid, 2 mM EDTA–Na_2 adjusted to pH 3 with 20% (w/v) NaOH] and mobile phase B consisted of HPLC-grade methanol. Elution was carried out using nine parts of A and one part of B at 1.0 ml min^{-1}. Injection volume was 40 ± 1. Quantitation of L-DOPA was based on a standard curve developed using 1.00 mM standard L-DOPA dissolved in water.

2.3. Statistical analysis

All experiments in this study are reported as means of three replicate analyses. One-way analysis of variance (ANOVA) was carried out to compare the mean values of different *Mucuna* species. Differences in the mean values were determined using Duncan's multiple range test at $P < 0.05$ (SAS, 1990).

3. Results and discussion

3.1. Preparation of the isolates

The protein yield and protein content from the six extracting media are presented in Fig. 2. The results obtained for the six species of *Mucuna* were very similar. The protein yield varied from 8% to 34% and protein content varied from 77% to 95%. The protein yield was highest in the case of alkaline extraction using sodium hydroxide. A similar result was reported by Sefa Dedek and Stanley (1979a) during the extraction of Cowpea flour proteins with sodium hydroxide. Bello and Okezie (1989) noted that the use of sodium hydroxide, as base for pH adjustment, resulted in higher protein extractability from Winged bean flour. However, it has been established that higher pH, although more efficient in extracting proteins, should be avoided, due to the possibility of disruption of the protein structure and degradation of certain amino acids, thereby affecting the integrity of the protein isolate (Cerletti, Fumgall, & Ventunn, 1998; Lqari et al., 2001). Apart from these, *Mucuna* beans like some other legumes, are generally noted for the high solubility of their nitrogenous constituents in sodium hydroxide in view of their dominant albumin content (Smith, Earle, Wolff, & Jones, 1959). However, sodium hydroxide recorded the darkest colour of all the extracting media. Onigbinde and Onobun (1993) noted that the intensity of browning of cowpea was pH-dependent, with maximum browning occurring in the pH ranges 5 to 6, due to the release of acid-labile phenolic compounds, and 9–11, due to the release of alkali-labile phenolic compounds from the beans into the extracting media. This is consistent with the observed colour of isolates in the current study; the isolates darkened at pH 11 with sodium hydroxide. Extraction with distilled water gave the next highest yield to NaOH when compared with other salts. Although this may be seen to be encouraging in terms of economic significance, water could only extract albumins. The protein contents obtained using sulphite and ascorbic acid were slightly lower than those using sodium hydroxide extraction, but the former gave acceptable colour, as explained later.

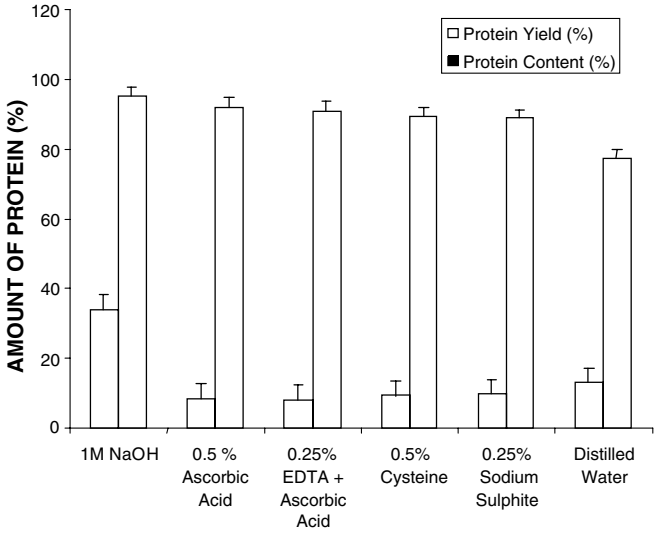

Fig. 2. Protein yield and content of *Mucuna* isolates.

3.2. Effects of high pressure processing and chemical treatments on the colour of protein isolates

3.2.1. General

Given that extraction media which produced higher protein yields also resulted in the formation of a dark colour

and that this might be due to the reactions involving polyphenol oxidase (PPO), the use of high pressures and chemical treatments were explored, in order to control the extent of such reactions.

3.2.2. Chemical treatments

The results of chemical treatments on the colour of the isolate are presented in Table 1. In this table, the L value represents the degree of lightness of the sample: a value of 100 represents the lightest colour while a zero value represents the darkest. In addition, the constant "a" represents green to red ($-a$ = green; $+a$ = red) and constant "b" represents blue to yellow ($-b$ = blue; $+b$ = yellow) of both the sample and standard. The value ΔE represents the total colour of the isolate and the standard which takes into account the values of the constants as given in Table 1. The result indicated that 0.25% sodium sulphite (L = 63.2) and 0.5% ascorbic acid (L = 61.86) produced the most acceptable colour of the isolates. Sulphites, reducing agents, such as ascorbic acid, quinine couplers and chelating agents are known to inhibit non-enzymatic browning by reacting with carbonyl intermediates, thereby preventing their further reaction to form brown pigments (Wedzicha, 1987). However, isolates extracted with NaOH at pH 11 recorded the darkest colour. These results show that treatment with sulphite appears to be one of the most effective ways of controlling browning. However, sulphites have been banned in certain categories of food products due to the health risk and their regulatory status for other categories is questionable (Sapers, 1993). The food Industry is looking for alternatives which have minimal health risk as a means for controlling browning (Sapers, 1993). The use of ascorbic acid in the extracting medium reduced the level of darkening. Ascorbic acid has long been used as an inhibitor of enzymatic browning in fresh-cut and frozen fruits such as peaches and apples and has been shown to be the best alternative to sulphite (Vamos-Vigyazo, 1981).

As indicated earlier, darkening of the protein isolates may be attributed to the reaction between protein and oxidized polyphenol (Gonclaves et al., 1997). Many phenolic compounds are oxidized by polyphenol oxidase (PPO). The most important substrates are catechins, cinnamic acid esters, 3,4 hydroxyphenylalanine (DOPA) and tyrosine (Sapers, 1993). *Mucuna* bean contains a large amount of L-DOPA that can react with PPO and reduce it to melanin, which produces a black colour (Balaban & Teixeira, 2002). The amount of L-DOPA detected in the isolates was between 0.12 and 0.17 g 100 g^{-1}. The formation of dark colour is objectionable in some foods as it adversely affects the marketability, sensory quality and even nutritional value of such products (Weemaes, Ludikhuhze, Vanderbroeek, & Hendricks, 1998).

3.2.3. High pressure processing of Mucuna bean

The result of the effect of high pressure processing on the colour of the protein isolates is presented in Table 2. The application of 200 MPa for 20 min did not improve the colour of the protein isolate. However, when the pressure was increased to 600 MPa, there was a slight improvement in the colour. The use of HPP during the isolation of the proteins from *Mucuna* flour helps to inactivate the PPO. In the same vein, sulphites and ascorbic acid are known to inhibit polyphenol oxidation, thereby limiting the reaction between proteins and oxidized polyphenols, which produces the dark colour (Gonclaves et al., 1997). Knorr (1994) reported that PPO is not always completely inactivated by high pressure processing. In the current study,

Table 2
Colour of protein isolates from high pressure processing performed at various pressures

Pressure (MPa)	L	A	B	ΔE
200	$36.02 \pm 0.10^{c*}$	0.65 ± 0.02	0.94 ± 0.01	58.25 ± 0.10
300	38.51 ± 0.01^{bc}	0.80 ± 0.01	1.70 ± 0.02	55.77 ± 0.01
500	43.78 ± 0.02^{b}	1.31 ± 0.01	3.55 ± 0.00	50.60 ± 0.01
600	47.07 ± 0.02^{a}	1.56 ± 0.00	3.94 ± 0.02	47.35 ± 0.10

Standard white tile with values: $L = 94.25$, $a = -0.83$, $b = 0.79$.
$\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b)^{1/2}$; where ΔL, Δa, and Δb are respective differences between standard and sample.
* Results are means ± SD of three replicate analyses. Means followed by different superscripts in each column are significantly different at $P < 0.05$.

Table 1
Colour of protein isolates from various chemical treatments

Sample	L	a	B	ΔE
White standard	94.25 ± 0.02	-0.83 ± 0.00	0.79 ± 0.01	na
Na_2SO_3 (0.25%)	63.24 ± 0.03	4.59 ± 0.10	14.87 ± 0.11	34.49 ± 0.02
Distilled water	62.85 ± 0.00	3.28 ± 0.10	15.09 ± 0.02	34.75 ± 0.00
Ascorbic acid (0.5%)	61.86 ± 0.01	4.76 ± 0.01	15.25 ± 0.03	35.91 ± 0.02
EDTA + ascorbic acid	55.54 ± 0.01	6.78 ± 0.03	12.25 ± 0.00	41.08 ± 0.13
Cysteine (0.5%)	55.01 ± 0.02	4.84 ± 0.02	10.81 ± 0.00	40.89 ± 0.03
NaOH (pH 11)	37.22 ± 0.02	1.22 ± 0.10	2.27 ± 0.01	57.09 ± 0.01
NaOH (pH 9)	41.13 ± 0.02	0.83 ± 0.01	1.05 ± 0.00	53.15 ± 0.00
NaOH (pH 11), followed by neutralisation	50.58 ± 0.11	2.63 ± 0.01	5.04 ± 0.02	44.01 ± 0.12
HCl (pH 2.0)	51.44 ± 0.10	1.18 ± 0.03	3.57 ± 0.03	42.95 ± 0.02

$\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ where ΔL, Δa and Δb are respective differences between standard and sample.
na: Data not available.

we found that the use of high pressure processing (HPP) considerably improved the colour of the isolates; however, the process did not completely eliminate the dark colour of the isolate. Apart from this, the high capital investment necessary is likely to discourage its adoption, particularly in developing countries.

3.3. Protein fractionation

The classification of seed proteins according to their solubility was developed by Osborne (1924), who distinguished four different fractions: albumin (water-soluble), globulin (salt-soluble), prolamins (alcohol-soluble) and glutelins (soluble in dilute NaOH). The results of protein fractionation are presented in Fig. 3. Albumin was found to be the major protein fraction in the *Mucuna* bean, regardless of the species. The ranges of the values obtained for albumins, globulins, prolamins and glutelins were 63.5–69.4%, 18.7–24.3%, 6.2–15.4% and 0.12–0.25% of the total extractable protein, respectively. This is similar to the composition of proteins in other legumes, such as *Vigna sesquipedalis* (Rajaran & Janadharanki, 1990), *Phaseolus lunatus* (Siddhuraju et al., 1996) and adzuki bean (Tjahjadi et al., 1988).

The stored proteins in legumes are of the globulin type. Due to the high levels of albumin in *Mucuna* beans, these proteins could play a prominent role in the legume. Low globulin content in the *Mucuna* bean might be due to its solubility in distilled water during fractionation. In *Mucuna*, the major protein fractions are water-soluble rather salt-soluble. Sefa Dedek and Stanley (1979a) reported that the water-soluble fraction accounted for over 67.5% of the total extracted protein. The authors further suggested that the high water-soluble protein fraction reported in cowpea might be due to the presence of salts in the seeds. However, albumins play an essential role in seeds because they are responsible for both enzymatic and metabolic activities in the seeds (Casey, Domoney, & Smith, 1993; Gueguen, 1999). In addition, albumins are of nutritional importance because of their high content of essential amino acids, lysine and sulphur amino acids (Bhatty, 1992; Murray & Roxburgh, 1984). This unique high solubility of *Mucuna* protein might be advantageous from the point of view of their use in food products.

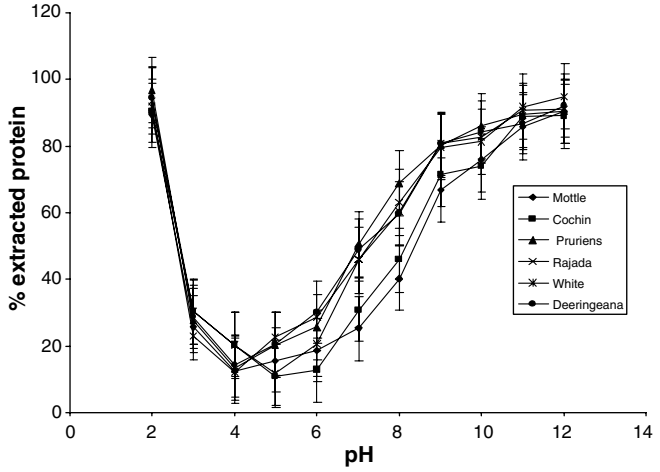

Fig. 3. Solubility curve of *Mucuna* protein isolates.

Our results showed that *Mucuna* had a low content of prolamins (0.12%). In fact prolamins were not detected in *M. rajada* and *M. cochinchinensis*. In general, legume seeds contain a very low percentage of prolamins (Boulter, 1977; Sosulski, Humbert, Bui, & Jones, 1976).

The amount of NaOH-soluble proteins (Glutelins) in *Mucuna* was between 18% and 25%. This was higher than similar legumes, such as soybean (Hu & Essen, 1981), peanut (Reichert & Mac-kenzie, 1982) and oil seeds, such as Fenugreek (Sauvaire et al., 1984). It was, however, lower than that recorded in *Myracrodron urundeuva* (Abdala et al., 2002) and safflower (Betschart, 1975).

3.4. Protein solubility

The solubility profile of a protein provides some insight into the extent of denaturation or irreversible aggregation and precipitation which might have occurred. It also gives an indication of the types of foods or beverages into which the protein could be incorporated. Factors such as concentration, pH, ionic strength and the presence of other substances influence the solubility of protein. The pH-dependent protein solubility profile of the isolates is presented in Fig. 4. Isoelectric point of the proteins was between 4.0 and 5.0. Generally, solubility was reduced as the pH increased until it reached the isoelectric point, followed by progressive increase in solubility with further increase in pH. Similar observations were reported by Sathe et al. (1982a, 1982b) for winged bean and Chickpea (Sanchez-Vioque, Clemente, Vioque, Bautista, & Millan, 1999).

The high solubility of these isolates in the acidic pH range indicates that these isolates may be useful in the formulation of acidic foods, e.g. protein-rich carbonated beverages (Kinsella, 1979), since protein solubility largely affects other functionalities, such as emulsification, foaming and gelation (Kinsella, 1976). The high solubility of the proteins indicates that they could have promising food applications.

3.5. Amino acid composition and in vitro multi-enzyme digestibility

The amino acid composition of the protein isolates is shown in Table 3. *M. cochinchinensis* recorded the highest total essential amino acids "TEAA" (557 mg g^{-1} protein) while *M. rajada* recorded the lowest TEAA (495 mg g^{-1} protein) at $P < 0.05$. In comparison with the results of amino acid content of soybean, displayed alongside, all the *Mucuna* protein isolates possess a higher TEAA. The nutritive value of proteins depends primarily on the capacity to satisfy the needs of nitrogen and essential amino

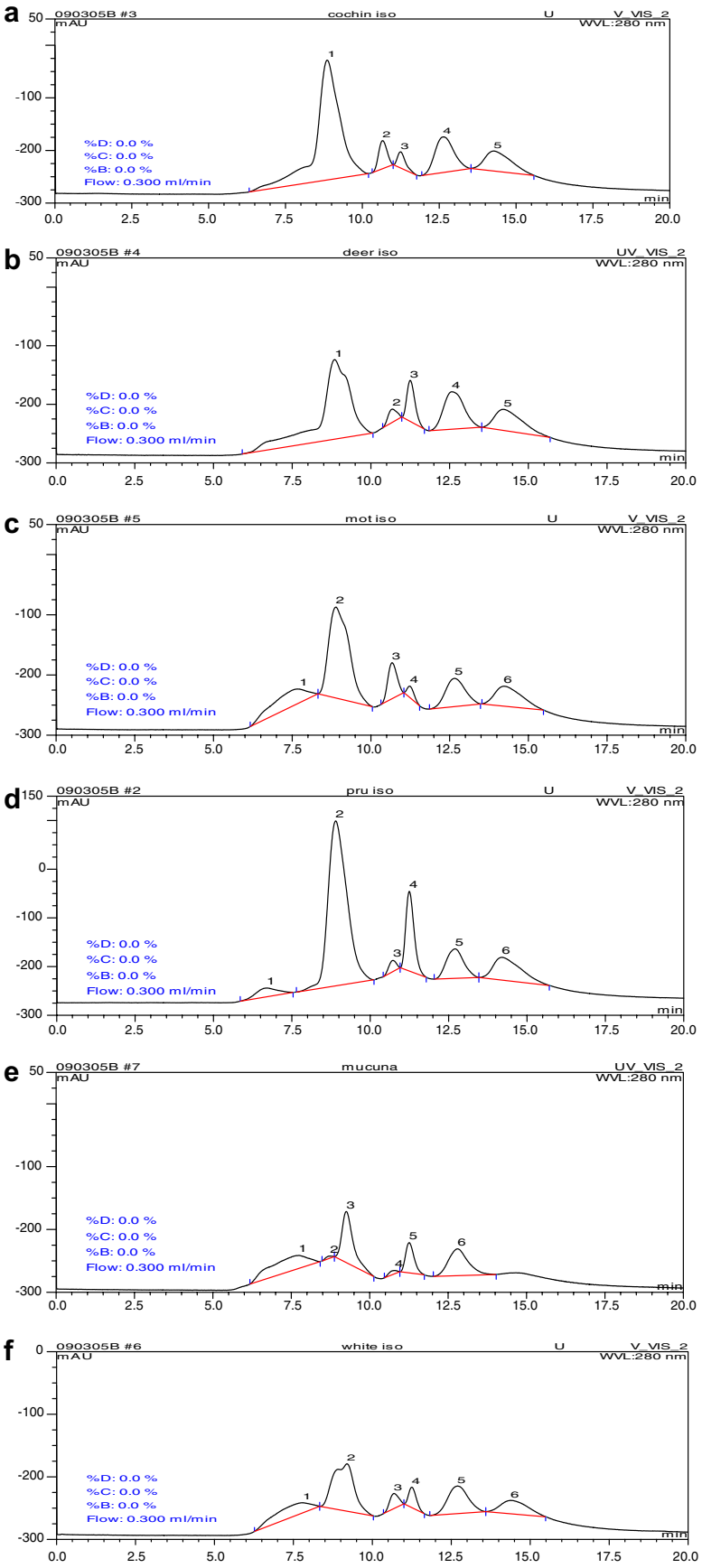

Fig. 4. Gel filtration chromatography of (a) *M. cochinchinensis* protein isolate, (b) *M. deerigeana* protein isolate, (c) *M. veracruz mottle* protein isolate, (d) *M. pruriens* protein isolate, (e) *M. rajada* protein isolate and (f) *M. varacruz white* protein isolate.

Table 3
Amino acid composition of *Mucuna* protein isolates (mg g^{-1} crude protein)[1]

Amino acid	*Mucuna* species						Soy beans[*]	FAO[**]
	M. pruriens	*M. cochinchinensis*	*M. rajada*	*M. vera cruz (M)*[‡]	*M. vera cruz (W)*[‡‡]	*M. deeringeana*		
ALA	63.9 ± 2.7^{d}	84.5 ± 3.6^{a}	67.8 ± 5.2^{c}	75.6 ± 4.2^{b}	66.9 ± 3.2^{c}	82.5 ± 4.6^{a}	42.3	
ARG[+]	90.6 ± 1.7^{a}	82.7 ± 1.8^{ab}	75.9 ± 1.0^{b}	61.6 ± 1.1^{c}	79.8 ± 1.2^{b}	90.5 ± 1.3^{a}	71.3	
ASP + ASN	171 ± 2.0^{a}	137.4 ± 2.2^{ab}	108 ± 2.5^{b}	126 ± 2.3^{ab}	87.5 ± 1.2^{c}	126 ± 2.0^{ab}	113	
CYS	Trace	8.4 ± 0.3^{a}	Trace	Trace	Trace	9.3 ± 0.5^{a}	17.0	
GLU + GLN	155.5 ± 12^{b}	165 ± 2.4^{ab}	151 ± 2.7^{b}	179.5 ± 3.6^{a}	125 ± 3.0^{c}	180 ± 2.4^{a}	169	19.0
GLY	50.5 ± 1.1^{a}	$52.2 \pm ^{a}$	33.8 ± 0.9^{c}	54.6 ± 1.0^{a}	41.2 ± 0.8^{b}	49.7 ± 0.7^{a}	40.1	
HIS	35.3 ± 0.8^{b}	27.8 ± 0.5^{d}	39.5 ± 0.6^{ab}	32.6 ± 0.9^{c}	47.7 ± 0.7^{a}	46.6 ± 0.4^{a}	25.0	34.0
ISOLEU[+]	92.3 ± 1.1^{b}	96.0 ± 1.2^{a}	87.3 ± 1.6^{c}	85.4 ± 2.0^{c}	85.3 ± 1.1^{c}	97.2 ± 1.3^{a}	46.2	40.0
LEU[+]	90.8 ± 1.0^{a}	91.4 ± 0.9^{a}	87.8 ± 1.5^{ab}	80.7 ± 1.6^{b}	85.7 ± 1.2^{ab}	90.5 ± 1.1^{a}	77.2	70.0
LYS[+]	46.4 ± 0.9^{c}	55.7 ± 0.7^{b}	34.6 ± 0.8^{d}	61.5 ± 0.9^{a}	62.6 ± 1.0^{a}	66.5 ± 0.9^{a}	60.8	58.0
MET[+]	Trace	9.2 ± 0.2^{b}	9.6 ± 0.3^{b}	Trace	Trace	12.7 ± 0.3^{a}	12.2	25.0
PHE[+]	$80.8 \pm .2^{ab}$	86.7 ± 1.1^{a}	79.4 ± 1.1^{ab}	77.6 ± 1.3^{ab}	67.4 ± 1.6^{b}	62.1 ± 1.5^{c}	48.4	60.0
PRO	151 ± 2.1^{bc}	145 ± 2.3^{c}	150 ± 2.1^{bc}	132 ± 2.5^{d}	129 ± 2.7^{d}	161 ± 2.8^{a}	48.6	
SER	41.5 ± 0.7^{c}	35.8 ± 0.9^{d}	22.6 ± 0.9^{e}	48.3 ± 0.6^{b}	52.6 ± 0.8^{b}	63.5 ± 0.9^{a}	56.7	
THR[+]	44.4 ± 0.9^{c}	50.8 ± 0.8^{b}	52.3 ± 0.9^{ab}	66.8 ± 1.0^{a}	65.3 ± 1.2^{a}	61.9 ± 0.9^{a}	37.6	40.0
TRY	22.3 ± 1.0^{b}	24.8 ± 0.8^{ab}	34.6 ± 0.8^{a}	22.6 ± 0.6^{b}	24.0 ± 0.5^{ab}	23.0 ± 0.5^{b}	33.9	60.0[§]
TYRO	$70.9 \pm .2.0^{b}$	65.5 ± 1.3^{c}	66.8 ± 1.1^{c}	70.8 ± 1.2^{b}	74.4 ± 1.4^{b}	85.5 ± 1.7^{a}	12.4	
VAL[+]	58.3 ± 1.0^{c}	71.0 ± 1.2^{a}	68.1 ± 1.3^{ab}	64.5 ± 1.2^{b}	60.7 ± 1.3^{b}	75.9 ± 1.4^{a}	45.9	50.0
TEAA	504	544	495	498.1	506.8	557	400	
TNEAA	761.2	745.5	674.2	742.6	649	827	558	
% TEAA	39.8	42.2	44.0	40.1	43.8	40.1	41.7	
% TNEAA	60.2	57.8	56.0	59.9	56.2	59.9	58.3	
In vitro PD[++]	92.6 ± 0.8^{a}	94.5 ± 0.7^{a}	90.6 ± 0.5^{a}	93.7 ± 0.3^{a}	91.1 ± 0.6^{a}	90.7 ± 0.8^{a}	NA[+++]	
L-DOPA (g 100 g^{-1})	0.48 ± 0.02^{d}	0.50 ± 0.01^{c}	0.54 ± 0.04^{c}	0.66 ± 0.01^{b}	0.86 ± 0.03^{a}	0.44 ± 0.02^{e}	–	

ALA – alanine; ARG – arginine; ASP – aspartic acid; ASN – asparigine; CYS – cysteine; GLU – glutamic acid; GLN – glutamine; GLY – glycine; HIS – histidine; ISOLEU – isoleucine; LEU – leucine; LYS – lysine; MET – methionine; PHE – phenylalanine; PRO – proline; SER – serine; THR – threonine; TRY – tryptophan; TYR – tyrosine; VAL – valine.

TEAA – total essential amino acids; TNEAA – total non-essential amino acids means followed by different superscripts in each row indicate significant differences at $P < 0.05$.

[*] Data from Chavan et al. (1999).

[**] Data from FAO/WHO/ONU. Energy and protein requirement (1985).

[‡] *M. vera cruz (M)* – Mottle.

[‡‡] *M. vera cruz (W)* – White.

[§] Tyrosine and phenylalanine.

[+] Essential amino acids.

[++] *In vitro* protein digestibility (digestibility of casien, standard protein is 94.9%: commercial soybean, 89.9%).

[+++] ND: Data not available.

[1] Results presented as means ± SD of triplicate analyses.

acids (Pellet & Young, 1980). Comparison of the amino acid composition in the *Mucuna* species with FAO/WHO/ONU reference values (FAO/WHO/UNU, 1985), indicates that the values reported here are higher than the values recommended for pre-school and school children. This implies that the isolates would be a good source of essential amino acids and could be used for the fortification of cereal-based foods which are particularly deficient in lysine.

The quality of dietary proteins can be measured in many ways. There is general acceptance that this value is a ratio of the available amino acids in the food or diet compared with the daily requirements. Comparing the provisional amino acid scoring pattern with the scores reported for *Mucuna* spp. in Table 4, it is evident that the amino acid scores are superior to the suggested reference standard. In fact, in most cases, the values obtained in the present study are almost double the suggested levels. However, methionine and cysteine were present at relatively low levels. This is an uncommon observation in legumes and has been reported by other investigators (Apata & Ologhobo, 1994). However the high lysine content of the *Mucuna* protein is a very important nutritional attribute, and probably more significant than the protein content, because it makes this legume a significant supplementary protein to cereal-based diets which are known to be deficient in lysine. The amino acid content of the isolates compares favourably with that of the flour (Adebowale et al., 2005). The differences observed were in the reductions of the lysine, cysteine and methionine contents of the isolates; this may be attributed to destruction of sulphur-containing amino acid through partial hydrolysis by alkali during isolate preparation. Similar observations have already been reported by earlier authors (Clemente et al., 1998; Lqari et al., 2001;

Table 4
Provisional amino acid scoring pattern and amino acid scores of *Mucuna* spp.

Amino acid	Suggested[A] level	Amino acid content (mg g^{-1}) Present work						Amino acid score[B] Present work					
		a	b	c	d	e	f	a	b	c	d	e	f
ILE	40	92.3	96.0	87.3	85.4	85.3	97.2	2.3	2.4	2.2	2.1	2.1	2.4
LEU	70	90.8	91.4	87.8	80.7	85.7	90.5	1.3	1.3	1.3	1.2	1.2	1.3
LYS	55	46.4	55.7	34.6	61.5	62.6	66.5	0.8	1.0	0.6	1.1	1.1	1.2
MET + CYS	35	–	17.6	9.6	–	–	22.0	–	0.5	0.3	–	–	0.6
PHE + TRY	60	151	152	146	148	142	148	2.5	2.5	2.4	2.5	2.4	2.5
THR	40	44.4	50.8	52.3	66.8	65.3	61.9	1.1	1.3	1.3	1.7	1.6	1.5
VAL	50	58.3	71.0	68.1	64.5	60.7	75.9	1.2	1.4	1.4	1.3	1.2	1.5

ILE – isoleucine; LEU – leucine; LYS – lysine; MET – methionine; CYS – cystine; PHE – phenylalanine; TRY – tryptophan; THR – threonine; VAL – valine.

a – *M. pruriens*; b – *M. cochichinensis*; c – *M. rajada*; d – *M. veravruz mottle*; e – *M. vera cruz white*; f – *M. deeringeana*.

[A] Suggested daily levels for adults (FAO/WHO, 1985).

[B] Amino acid score = mg of amino acid per g test protein/mg amino acid per g reference protein.

Sanchez-Vioque et al., 1999). Apart from this, there were increases in the levels of leucine, phenylalanine, proline and tryrosine in the isolates compared with the flours.

The digestibility of the protein was also examined and the results are presented along with the composition of the amino acids in Table 3. The digestibility of the protein isolates ranged between 90.6% and 94.5%. It is noteworthy that the digestibility of a protein and bio-availability of its constituent amino acids, are important factors characterising the protein quality (Hsu et al., 1977; Suman, Monterio, Ramanchandra, & Sudharshana, 1992). This is because not all proteins are digested, absorbed and utilized to the same extents. Differences in protein digestibility may arise from inherent differences in the nature of food protein constituents, which may be modified by the presence of antiphysiological factors. The removal of antinutritional factors in the isolates is expected to enhance digestibility. Besides, *Mucuna* proteins are partially denatured during the preparation of the isolates, making the proteins more accessible to digestive enzymes and improving the hydrolysis. Results obtained are similar to those observed in other seeds, for example 90% for rapeseed (Gonclaves et al., 1997) and 90–94 % for chickpea protein isolates (Sanchez-Vioque et al., 1999).

3.6. Evaluation of the sub-unit patterns in the isolates

In order to characterize the proteins in the isolates, the sub-unit patterns were studied by means of gel filtration chromatography and SDS-PAGE. The latter was performed in the presence and absence of a reducing agent, mercaptoethanol (ME). This allowed us to distinguish between free polypeptide chains and the chains linked by disulphide bridges.

The results of gel filtration chromatographic studies of the protein isolates are depicted in Fig. 4a–e. *M. pruriens* isolate consisted of two major fractions: peak 2, with a molecular mass of 40 kDa, and peak 4, with a molecular weight of 15.3 kDa. The molecular weights of other minor components were 913.5 (peak 1), 35.8 (peak 3), 3.9 (peak 5) and 1.2 kDa (peak 6). *M. rajada*, *M. veracruz white* and *M. veracruz mottle* had similar molecular weight patterns in the fractions. Only five peaks were identified in *M. cochnichinensis* and *M. deerigeana* isolates. Their molecular weights were roughly similar (Table 5).

More detailed information can be obtained with SDS-PAGE, as shown in Fig. 5a and b. A major band consisting of a broad zone, with molecular weights 36 ± 7 and 17 ± 3 kDa, appeared in all the samples under both reducing and non-reducing conditions. This indicated that these major polypeptides were free of interchain disulphide bonds and possibly represented typical subunits of vicilin-like storage proteins, as reported by earlier authors (Derbyshire, Wright, & Boutten, 1976; Rahman, Dubek, Mottes, Gornitz, & Schwenke, 2000). In addition, some minor polypeptide chains gave similar patterns under both reducing and non-reducing conditions. These included the polypeptide chain with 55; 84; 97 and 116 kDa. In contrast, two peaks, which occurred as minor fractions (molecular weights, 205 and 66 kDa), disappeared after reduction which resulted in the formation of a smaller polypeptide chain with 24 kDa. A similar observation was reported by Rahman et al. (2000), in their gel electrophoresis studies of mung bean. The authors reported similar disappearance of a polypeptide chain with molecular weight of 61.5 ± 1.3 kDa and the

Table 5
Fractionation of *Mucuna* bean protein

Isolates	Fractions (% extracted protein)			
	Albumin	Glutelin	Globulin	Prolamin
M. pruriens	67.0 ± 0.3[a]	22.1 ± 0.1	10.1 ± 0.4	0.12 ± 0.04
M. cochinchinensis	68.5 ± 0.2	20.1 ± 0.2	11.4 ± 0.2	ND[b]
M. rajada	63.2 ± 0.4	21.4 ± 0.3	15.4 ± 0.3	ND
M. veracruz white	69.4 ± 0.3	24.3 ± 0.1	6.2 ± 0.2	0.15 ± 0.01
M. veracruz mottle	65.2 ± 0.1	19.7 ± 0.2	14.9 ± 0.1	0.19 ± 0.02
M. deerigeeana	66.0 ± 0.2	18.7 ± 0.3	15.0 ± 0.2	0.25 ± 0.03

[a] Mean ± SD of three replicates expressed on dry weight basis.

[b] ND indicates not detectable (i.e. below detectable limits).

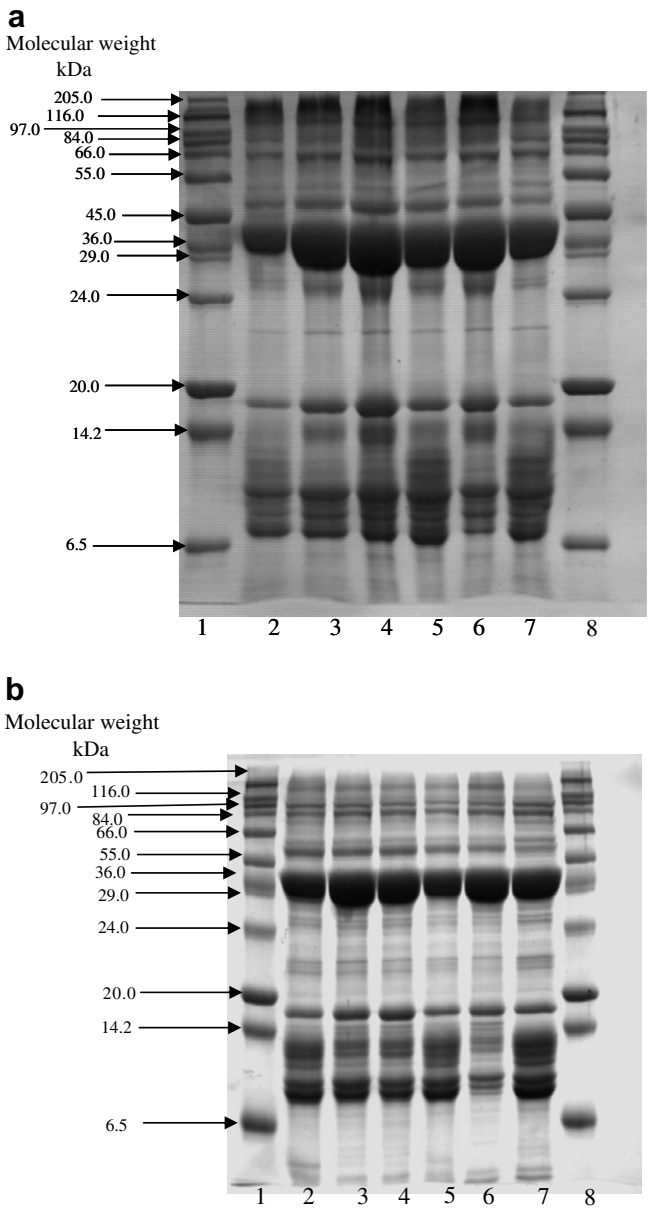

Fig. 5. SDS-PAGE (a) without and (b) with mercaptoethanol (ME) of six *Mucuna* protein isolates. Lanes 1 and 8 are standard markers; Lane 2: *M. veracruz mottle*; Lane 3: *M. vera cruz white*; Lane 4: *M. deerigeana*; Lane 5: *M. cochichinensis*; Lane 6: *M. rajada*; Lane 7: *M. pruriens*.

appearance of another polypeptide chain of molecular weight 23.3 ± 1.0 kDa. Therefore, in these studies, the parent subunit might belong to the family of legume-like 11 S-type storage proteins which are characterized by disulphide α–β subunits. A smaller polypeptide fraction also disappeared after reduction. Legume seeds have been shown to contain high molecular weight oligomeric storage proteins, which are the major components in protein isolates prepared from seeds. While some legumes, such as soy beans (Carsey & Domoney, 1984; Iwabuchi & Yamauchi, 1987; Thanh & Shibasaki, 1976) and faba beans (Muntz, Horstmann, & Schlesier, 1986), contain two major storage proteins, 11S legumin and 7S vicilin, there are a number of other legumes which contain a 7S fraction as the major protein component and other storage proteins in trace amounts. Among these are legumes such as *Phaseolus vulgaris* (Sakakibara, Aoki, & Noguchi, 1979), cowpea (Sefa Dedek & Stanley, 1979a, 1979b), pigeon pea (*Cajanus cajan*) (Krishna & Bhatia, 1985), jack bean (*Carnavalia gladiata*) (McPherson, 1980) and winged bean (*Psophacarpus angularis*) which contain 7S and 2.5S proteins (Yanagi, 1985).

The present results provide evidence that the *Mucuna* bean species belong to the latter group of legumes, having a 7S fraction as the predominant storage proteins. Other storage proteins, which might be assigned to the 11S legumins type, composed of larger and smaller units of a disulphide linked polypeptide chain, are present only in marginal amounts in the *Mucuna* bean isolates and extracts. This was supported by our preliminary studies of the isolation and characterisation of 7S and 11S proteins in the isolates.

4. Conclusions

The *Mucuna* protein isolates are good sources of proteins (92–96%). Besides, they register high levels of nutrient bioavailability, in addition to higher levels of amino acids, compared with recommended levels. The levels of antinutrients were either not detectable or reduced to tolerable limits.

Ascorbic acid was adopted as the extraction medium for this study, due to its positive effect on the isolate in terms of reduction of the dark colour, high protein yield, high protein content and relative safety in foods. Apart from this, it is cheaper, and relatively more acceptable and adaptable than are the other extraction media and processing techniques used in this study.

Although high pressure processing HPP considerably improved the colour of the isolates, the process did not completely eliminate the dark colour of the isolate. Apart from this, the high capital investment necessary is likely to discourage its adoption, particularly in developing countries.

The proteins might consists mainly of 7S-vicilins with a marginal amount of 11S-legumins, composed of larger and smaller units of disulphide-linked polypeptide chains.

Work is already in progress on two-dimensional gel electrophoresis, analysis of the ultracentrifuged fractions and N-terminal amino acid sequencing in order to elucidate the complete structure of the proteins. It is hoped that the studies will also throw some light on the nature of antinutrients in *Mucuna* spp.

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