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Fractionation of *Leucaena* seed-kernel proteins based on their solubility characteristics

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Leucaena leucocephala seeds were found to be rich in proteins, but they contained a toxic amino acid, mimosine. Protein-isolation processes rendered the seed proteins relatively free of anti-nutritional factors. Proteins of *L. leucocephala* (K8) seed kernels were separated into different fractions on the basis of their solubility, by extracting successively and directly, with various solvents, i.e. water, sodium chloride (5%), and sodium hydroxide (0·05 M). Globulins were found to be the major proteins, followed by albumins, glutelins, and prolamins. The proteins in these fractions were further precipitated, after determining their isoelectric points (pH 2–2·5). The protein and mimosine contents of the soluble extracts (before protein precipitation) and their isoelectric protein precipitates, supernatants, and insoluble residues, were determined. Sodium chloride (5%) was found to be the solvent of choice, since it resulted in maximum protein yield. Furthermore, most of the extracted mimosine was left in the supernatant after the protein had been precipitated out.

INTRODUCTION

Leucaena leucocephala, one of the fastest-growing leguminous plants (Jagan Mohan Rao & Azeemoddin, 1988), has a high concentration of protein in its leaves and seeds (C.S.I.R., 1962; Jagan Mohan Rao & Azeemoddin, 1988; Yadav & Yadav, 1988). However, this plant as a food source is not yet fully utilized, owing to the presence of a toxic, non-protein amino acid, mimosine, which causes several adverse reactions in ruminants and non-ruminants (Ter Meulen *et al.*, 1979).

A number of reports (Kadade & Evans, 1964; Wolf, 1975) claim that the preparation of seed-protein concentrates/isolates results in rendering the seed proteins (navy beans and soybeans) fairly free of certain inherent toxic constituents, such as trypsin inhibitors and hemagglutinins. The bulk of the trypsin-inhibitor and hemagglutinating activity was found in the whey-protein fraction, which gave poor net-protein-utilization values in rats (Grant *et al.*, 1986). No similar attempts have been made in the case of *Leucaena* seed proteins.

Seed proteins, such as oat, coconut, chickpea, pigeon pea, soybean, fenugreek seed, and low-tannin sorghum, have been fractionated on the basis of their solubility (Peterson & Brinegar, 1986; Samson *et al.*, 1971; Singh & Jambunathan, 1982; Singh *et al.*, 1988; Derbyshire *et al.*, 1976; Sanvaire *et al.*, 1984; Taylor *et al.*, 1984). The distributions of different protein fractions, based on solubility, in the various anatomical parts of cereal and legume seeds have also been studied (Kapoor & Gupta, 1977; Schofield & Booth, 1983; Singh & Jambunathan, 1982). The isoelectric points of plant proteins show wide variations, with values ranging from pH 0·5 to 8·22 (Free & Satterlee, 1975; Samsom *et al.*, 1971; Satterlee *et al.*, 1975; Sumner *et al.*, 1981; Thanh & Shibasaki, 1976; Thomas, 1934; Wang & Kinsella, 1976), depending on the protein source, as well as the solvent in which the protein has been extracted.

In the present work, *L. leucocephala* seed-kernel proteins were fractionated, on the basis of their solubility in different solvents, and isolated by an isoelectric-precipitation technique. The distributions of protein and mimosine in the isoelectric protein precipitate, supernatant, and insoluble residue were measured, so as to arrive at an optimum method for preparing *Leucaena* protein isolate, with minimal residual mimosine content.

MATERIALS AND METHODS

Seeds of *Leucaena leucocephala* K8 were obtained from Bharatiya Agro Industries Foundation (BAIF), Urulikanchan, District Pune, India.

The seeds were soaked in hot water (initial temperature, 80°C) for approximately 24 hours and then manually dehulled. The moist seed kernels were sun-dried for approximately 48 h and finely ground to pass through a 40-mesh sieve.

Table 1. Distribution of total proteins, total soluble proteins and mimosine in various protein fractions of *L. leucocephala* K8 seed kernels*

	Protein fraction†	Protein (N × 6·25)			Mimosine	
		g/100 g seed kernel	Percentage total protein (%)	Percentage total soluble protein (%)	g/100 g seed kernel	Percentage total mimosine (%)
(*a*)	Water-soluble	14·3 ± 0·01	28·4	29·3	4·57 ± 0·02	91·5
(*b*)	NaCl-soluble	21·9 ± 0·09	43·5	44·9	0·25 ± 0·01	5·01
(*c*)	NaOH-soluble	12·6 ± 0·14	25·0	25·8	0·15 ± 0·03	3·00
	Insoluble residue	1·57 ± 0·07	3·11	—	0·02 ± 0·01	0·48

* Results expressed on dry-weight basis as mean ± S.D. of five determinations.
† 1 g kernels were extracted three times with 20 ml portions of each of the solvents *a*, *b*, and *c*, sequentially.

Solubilization and fractionation of *L. leucocephala* seed-kernel proteins

The proteins of Leucaena seed kernels were separated into different fractions on the basis of their solubility in various solvents (water, 5% sodium chloride, 70% ethanol, and 0·05 M sodium hydroxide), based on a modification of the procedure of Sauvaire *et al.* (1984). The various soluble-protein fractions were made up to a known volume, the insoluble residue was washed with distilled water, and the protein contents of the soluble-protein fractions and insoluble residue were determined, by using a micro-Kjeldahl nesslerization method (ISO, 1981).

Since the ethanol-soluble protein fraction was found to be very negligible in *L. leucocephala* cotyledons, only the protein and mimosine contents of water-, sodium-chloride- and sodium-hydroxide-soluble fractions were determined in the later studies.

Two sets of protein extraction were undertaken. In the first case, the samples were extracted three times with each solvent. In the second study, the samples were extracted with the same three solvents, but the number of extractions varied, i.e. five extractions with water, followed by two extractions with sodium chloride (5%) and by one extraction with sodium hydroxide (0·05 M). The mimosine contents of the protein fractions in both these studies were determined (Matsumoto & Sherman, 1951).

In a trial study, *Leucaena* seed kernels were extracted *directly* with 5% sodium chloride. Two such samples, after sodium chloride extraction, were separately extracted with distilled water and sodium hydroxide (0·05 M), and each of these fractions was tested for mimosine.

Isoelectric pH

The isoelectric pH was determined by measuring the pH of the sample at which maximum precipitation of protein took place on the addition of 0·1 N hydrochloric acid (concentrated HCl was used for the NaOH-soluble fraction).

Protein-isolation Studies

(*a*) A *Leucaena* K8 seed-kernel sample was extracted successively with distilled water (five extractions), 5% sodium chloride (two extractions) and 0·05 M sodium hydroxide (one extraction), and the three protein fractions were precipitated isoelectrically.
(*b*) This study formed the basis for the final selection of the method for the preparation of *Leucaena* protein isolate (LPI) from *Leucaena* seed kernels. The seed-kernel samples were directly extracted with the given solvents, separately. The protein contents of the isoelectric-protein precipitate, supernatant, and insoluble residue were determined for each of the protein extracts (ISO, 1981).

The mimosine contents of the isoelectric-protein precipitate, supernatants, and insoluble residues, in the above two studies, were determined (Matsumoto & Sherman, 1951).

RESULTS AND DISCUSSION

The seed-kernel proteins of *L. leucocephala* were fractionated, on the basis of their solubility, in various solvents (Sauvaire *et al.*, 1984). (A preliminary study showed that the ethanol-soluble protein fraction (prolamin) constituted a very small percentage (1·2%) of the total soluble proteins of *Leucaena* seed kernels and was thus not included in the further studies on protein fractionation and isolation.)

The initial fractionation study (Table 1) formed the basis for all the future work on *Leucaena* seed-kernel-protein fractionation and isolation. The major proteins of *Leucaena* seed kernel were of the sodium-chloride-soluble type (globulins, 43·5%), followed by the water-soluble (albumin, 28·4%), and sodium hydroxide-soluble (glutelins, 25·0%) proteins.

Further studies were conducted on the various protein fractions of *L. leucocephala* K8 kernel proteins, and the protein and mimosine contents of these various fractions and the insoluble residue were determined. These results are presented in Tables 1 and 2.

Table 2. Protein and mimosine in the various protein fractions of *L. leucocephala* K8 seed kernels*‡

Protein fraction§	Protein (N × 6·25)		Mimosine	
	g/100 g seed kernel	(%)[2]	g/100 g seed kernel	(%)[2]
Water-soluble	16·6 ± 0·36	31·0	3·10 ± 0·10	82·1
NaCl-soluble	20·9 ± 0·01	39·0	0·14 ± 0·01	3·7
NaOH-soluble	7·57 ± 0·10	14·1	0·48 ± 0·01	12·8
Insoluble residue	8·52 ± 0·09	15·9	0·05 ± 0·01	1·4

* Results expressed on dry weight basis.
‡ Results expressed as mean ± S.D. of 5 determinations.
§ The fraction with each of the solvents was as follows:
Water (20 × 5), NaCl (5%) (15 × 2), and NaOH (0·05 M) (20 × 1), where (20 × 5) means 5 extractions with 20 ml solvent (water) per gram of sample, each time.
(15 × 2), similar to above, i.e., 2 extractions with 15 ml NaCl per gram of sample, each time, and
(20 × 1) means one extraction with 20 ml NaOH.
(%)[2] percent of protein or mimosine.

In the first set of studies (Table 1), the sodium chloride fraction was seen to have the maximum protein (43·5%), followed by the water-soluble fraction, and the least was in the sodium-hydroxide-soluble fraction, with a small percentage of protein also present in the insoluble residue. These results are in close agreement with those reported by other workers (Derbyshire *et al.*, 1976; Peterson & Brinegar, 1986; Samson *et al.*, 1971; Satterlee *et al.*, 1975; Singh & Jambunathan, 1982; Singh *et al.*, 1988) for various seed proteins, though not comparable with the earlier reports (Kale, 1987; Tantung & Madamba, 1981) on *L. leucocephala* seed-protein fractions. Most of the mimosine was extracted with the first extraction solvent, water, i.e. in the water-soluble-protein fraction (91·5% of the total mimosine in K8 kernels), with only small amounts present in the later fractions, i.e. sodium-chloride-soluble and sodium-hydroxide-soluble fractions, and in the insoluble residue.

In the second set of studies (Table 2), the maximum extraction of mimosine in the water-soluble fraction was achieved by increasing the number of water extractions. This could decrease the concentration of mimosine in the sodium-chloride-soluble-protein fraction, in which the maximum soluble protein was found to be extractable (Table 1). It was observed that the percentage protein extracted in the water-soluble fraction increased on increasing the number of water extractions (Table 2), along with a corresponding decrease in the percentage proteins in the sodium-chloride-soluble fraction, 39·0%, as compared with 43·5% with three water extractions (Table 1). The maximum mimosine was extracted in the water-soluble-protein fraction (82·1%) (Table 2), with lower concentrations present in the other soluble-protein fractions and in the insoluble residue. Similarly, the mimosine content of the sodium-chloride-soluble fraction was reduced from 5·01% (Table 1) to 3·66% (Table 2) when the extraction procedure was changed.

Thus increasing the number of extractions with water from three to five was found to result in higher concentrations of protein and mimosine in the water-soluble fraction. This suggested that the method involving the use of three extractions with each solvent was a more favourable extraction method, particularly for *Leucaena* proteins, since the protein that was concentrated in the sodium chloride fraction by this extraction procedure (Table 1) had very low levels of mimosine, the latter, in turn, being concentrated in the water-soluble fraction.

The mimosine in the directly extracted sodium-chloride-soluble fraction was found to be comparable with and even slightly higher in concentration than the levels present in the directly extracted water-soluble-protein fraction, indicating that, when the sample was not first treated with water, but directly with 5% sodium chloride solution, the water-soluble fraction was also included in the sodium chloride solution, along with the sodium-chloride-soluble fraction. After the sodium chloride extraction, the residue, when extracted with water and then with sodium hydroxide solutions, and tested for mimosine qualitatively, gave near-negative results, indicating an almost quantitative extraction of mimosine in the sodium-chloride-soluble fraction.

This is of significance, since the major proteins in *Leucaena* seed kernel are of the water-soluble and sodium-chloride-soluble type. It was necessary to verify whether the mimosine was present along with the protein, once the latter was isolated and precipitated out, or whether the mimosine was retained in the non-precipitable whey fraction or supernatant.

The isoelectric pH values of the proteins from all the three soluble-protein fractions, i.e., water, sodium chloride, and sodium hydroxide, were found to be in the range 2–2·5.

The distributions of mimosine in the isoelectric-protein precipitate and supernatant of each fraction and the insoluble residue left over at the end of the stepwise reaction, are presented in Table 3. Most of the mimo-

Table 3. Mimosine content in the isoelectric-protein precipitate and supernatant of various protein fractions of *L. leucocephala* K8 seed-kernel flour*†

Soluble protein§	Fraction	Mimosine	
		g/100 g seed kernel	(%)[2]
Water soluble	Isoelectric ppt.	0·11 ± 0·01	2·63
	Supernatant	3·76 ± 0·11	89·74
NaCl-soluble	Isoelectric ppt.	0·10 ± 0·01	2·38
	Supernatant	0·02 ± 0·01	0·48
NaOH-soluble	Isoelectric ppt.	0·17 ± 0·01	4·06
	Supernatant	0·01 ± 0·00	0·24
Insoluble residue	------->	0·02 ± 0·00	0·47

* Results expressed on dry-weight basis.
† Results expressed as mean ± S.D. of five determinations.
§ This is a stepwise process, where the sample was first extracted with distilled water, followed by NaCl (5%), and NaOH (0·05 M) solutions.
[2] Percentage of total mimosine.

sine was retained in the non-precipitable supernatant fraction of the water-soluble extract; the supernatants of the other fractions and the protein precipitates and insoluble residue contained only a very small percentage of the total mimosine. A possible explanation for this phenomenon could be that mimosine, being a free amino acid, is not precipitated out along with the protein. Other workers (Grant *et al.*, 1986; Kakade & Evans, 1964) have also observed that some of the toxic constituents, such as trypsin inhibitors and hemagglutinins, in various seeds, such as soybeans and navy beans, seem to be concentrated in the supernatant (whey) fraction after the proteins have been precipitated out from the soluble seed-protein extracts.

Finally, *L. Leucocephala* K8 seed-kernel samples were directly extracted with each of the three solvents (water, 5% sodium chloride, and 0·05 M sodium hydroxide), and the protein and mimosine contents of the isoelectric-protein precipitate, supernatant, and insoluble residue in the case of each of the three soluble-protein fractions, were determined (Table 4A, 4B, and 4C).

In the case of all three fractions, the mimosine was concentrated in the supernatant (82·4%, 82·3% and 99·1% in water-soluble, sodium-chloride-soluble and sodium-hydroxide-soluble fractions, respectively), with the mimosine levels being very much lower in the isoelectric residue (0·52%, 9·20%, and 0·89% in water-soluble, sodium-chloride-soluble, and sodium-hydroxide-soluble fractions, respectively). Thus the very process of isolating the protein from the *Leucaena* K8 seed-kernel sample resulted in an appreciable decrease in the mimosine content of the sample. Moreover, the maximum protein, 34·2%, was isolated from the sodium-chloride-soluble fraction, with lower levels observed with sodium-hydroxide- and water-soluble fractions.

From the above results, on comparing the three solvents, sodium chloride seemed to be the best for extracting and isolating protein from *L. leucocephala* seed kernels, even though the protein isolate from this fraction contained higher concentrations of mimosine as compared with the protein isolates obtained from the other solvents, i.e. water and sodium hydroxide.

In conclusion, it could be said that *Leucaena* seed kernels contain extremely small amounts of prolamins, the major proteins being globulins, followed by albumins and glutelins. The isoelectric pH of the *Leucaena* seed-protein fractions studied, was found to be 2–2·5. Sodium chloride was found to be the solvent of choice,

Table 4. Protein and mimosine in the isoelectric-protein precipitates, supernatant, and insoluble residue of the various protein fractions of *L. leucocephala* K8 seed kernel flour*

Fraction	Protein (N × 6·25)		Mimosine	
	g/100 g seed kernel	(%)§	g/100 g seed kernel	(%)§
A. *Water-Soluble Fraction*‡				
Isoelectric ppt.	1·75 ± 0·62	2·44	0·02 ± 0	0·52
Supernatant	11·6 ± 0·75	16·1	3·19 ± 0·03	82·4
Insoluble residue	58·3 ± 1·02	81·4	0·66 ± 0·05	17·10
B. *NaCl (5%)-Soluble Fraction*‡				
Isoelectric ppt.	13·4 ± 0·11	34·2	0·25 ± 0·02	9·20
Supernatant	14·9 ± 0·30	38·0	2·24 ± 0·09	82·3
Insoluble residue	10·9 ± 0·09	27·8	0·23 ± 0·04	8·51
C. *NaOH (0·05M)-Soluble Fraction*‡				
[1]Isoelectric ppt.	[1]7·02 ± 0·08	32·7	[1]0·03 ± 0·01	0·89
Supernatant	14·5 ± 0·09	67·4	3·55 ± 0·06	99·1
[2]Insoluble residue	—	—	—	—

* Results expressed on dry-weight basis as mean ± S.D. of five determinations.
‡ Each sample was extracted only with one given solvent. It was not a stepwise extraction with various solvents for each sample. The number of extractions was (20 × 5) with each solvent, i.e. five extractions with 20 ml solvent per gram of sample, per extraction.
§ Percentage of protein or mimosine.
[1] Dialysed sample.
[2] Very insignificant amount of insoluble residue left over.

resulting in maximum extraction and isolation of proteins from *Leucaena* seed kernels, even though the mimosine in this protein precipitate was more than that with the other solvents. Furthermore, in all the soluble-protein fractions, most of the mimosine (82·4%, 82·3%, and 99·1% in water-soluble, sodium-chloride-soluble, and sodium-hydroxide-soluble fractions, respectively) was retained in the supernatant, i.e. the non-precipitable whey fraction, after the proteins had been precipitated out from the soluble-protein fractions. Thus protein isolation by isoelectric precipitation could itself be considered as one means of utilizing *Leucaena* seed proteins, with minimal interference from mimosine, owing to its toxicity complications.

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