Technical

Isolation, Amino Acid Composition and Molecular Weight Distribution of *Eucalyptus kirtoniana* Seed Proteins

S. Laskar*, S. Thakur and **B. Basak**
Natural Product Laboratory, Chemistry Department, The University of Burdwan, Burdwan 713104, West Bengal, India

Proteins were extracted from the deoiled seeds of *Eucalyptus kirtoniana* in aqueous solutions of various pHs or by different concentrations of NaCl, Na_2SO_3, $CaCl_2$ and $MgCl_2$ at pH 8.0. Amino acid analysis of the isolated protein identified 16 amino acids, nine of which were essential. Gel permeation chromatography on Sephadex G-200 revealed the presence of four components in the protein fraction, and their molecular weights were determined by two comparable standard methods. SDS-PAG electrophoresis demonstrated that each protein isolate from different salt solutions was composed of six fractions whose molecular weights were estimated to be 131,800; 108,300; 93,300; 51,300; 38,000 and 25,700 daltons.

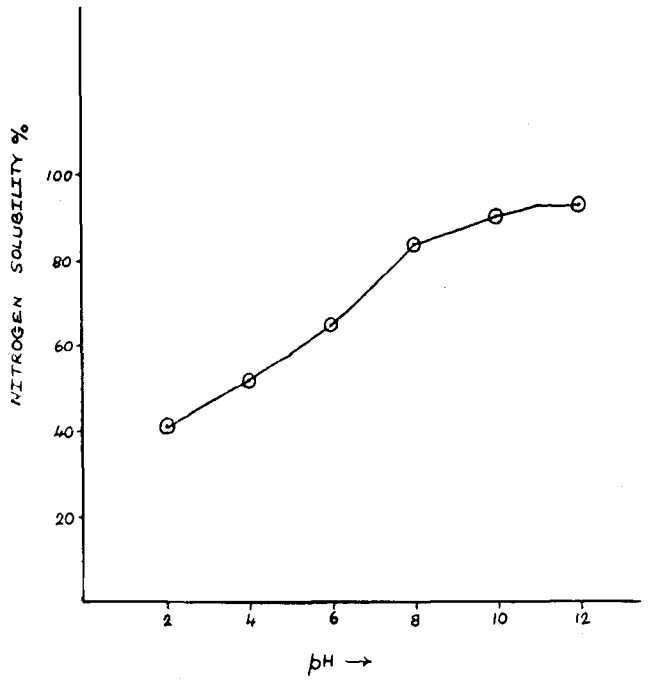

FIG. 1. Solubility of *Eucalyptus kirtoniana* seed protein at different pHs.

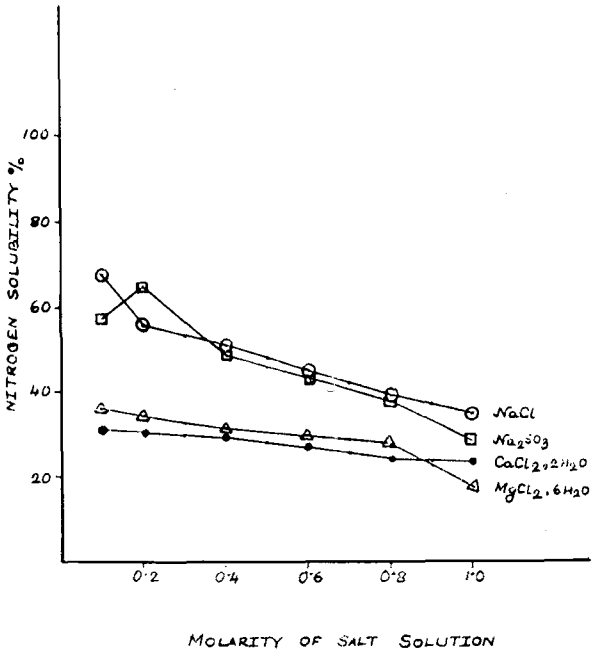

FIG. 2. Solubility study of *Eucalyptus kirtoniana* seed protein in different salt solutions of various concentrations.

TABLE 1

Nitrogen Solubility of *E. kirtoniana* Seed (deoiled) in Aqueous Solution at Various pHs and in Varied Concentrations of Different Salt Solutions at pH 8.0

pH of solution	Nitrogen solubility (%)	Molar concn of NaCl (pH 8)	Nitrogen solubility (%)	Molar concn of Na_2SO_3 (pH 8)	Nitrogen solubility (%)	Molar concn of $CaCl_2$ (pH 8)	Nitrogen solubility (%)	Molar concn of $MgCl_2$ (pH 8)	Nitrogen solubility (%)
2	41.27	0.1	67.62	0.1	57.53	0.1	30.72	0.1	36.01
3	46.50	0.2	55.76	0.2	64.99	0.2	30.29	0.2	33.38
4	51.82	0.3	53.50	0.3	56.50	0.3	29.50	0.3	32.25
5	58.35	0.4	50.93	0.4	48.72	0.4	28.98	0.4	30.72
6	64.99	0.5	48.10	0.5	46.00	0.5	27.65	0.5	30.10
7	74.00	0.6	45.00	0.6	43.20	0.6	26.50	0.6	29.95
8	83.85	0.7	41.99	0.7	40.00	0.7	25.20	0.7	29.00
9	87.00	0.8	38.64	0.8	38.64	0.8	24.15	0.8	27.67
10	90.03	0.9	36.50	0.9	32.75	0.9	24.00	0.9	22.15
11	93.00	1.0	34.23	1.0	26.78	1.0	23.72	1.0	17.12
12	93.00	—	—	—	—	—	—	—	—

*To whom correspondence should be addressed.

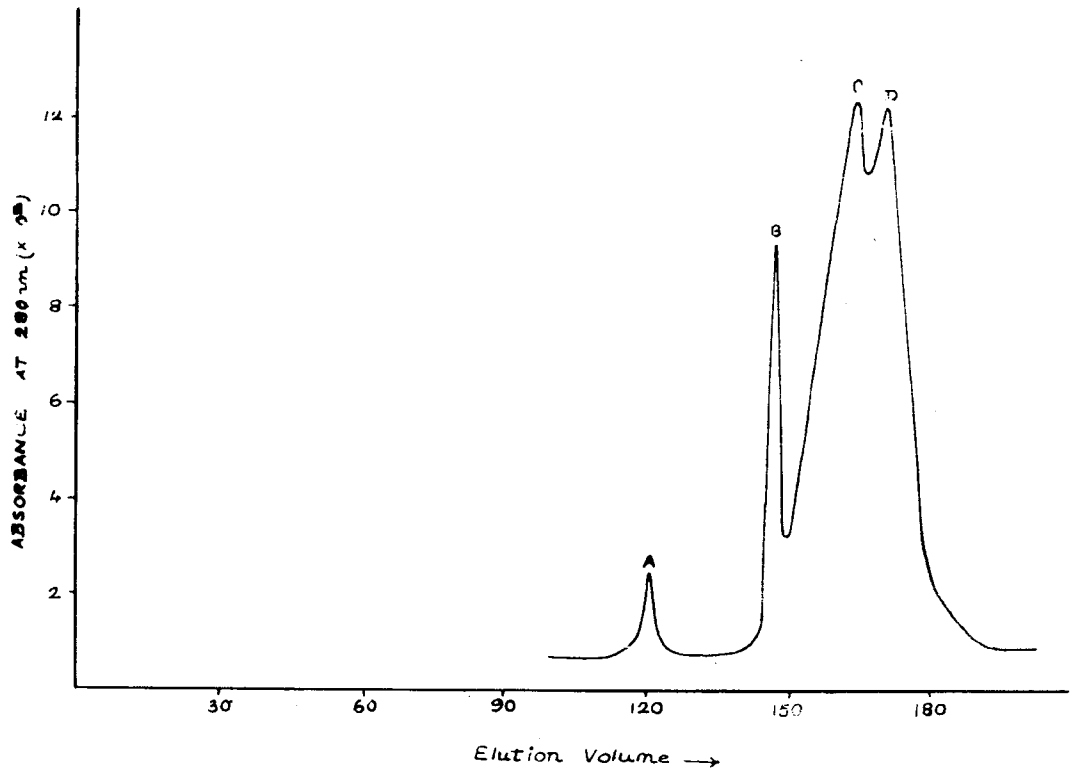

FIG. 3. Gel filtration of *Eucalyptus kirtoniana* seed proteins in Sephadex G-200 column (2.5 cm i.d. × 40 cm long).

In the course of afforestation, different species of the genus *Eucalyptus* (family Myrtaceae) are widely planted in different parts of India. It is used mainly as a valuable pulpwood, and its leaf oil is used as a medicine for diseases like bronchitis and asthma (1). Although their seeds contain an appreciable amount of oil and protein based on the survey conducted jointly by the Indian Oilseed Committee and Hindusthan Lever Limited (2), it has been observed that only 7% of the total seed is utilized, mainly for replantation.

One of the highly planted species (mainly in West Bengal, India) of this genus is *Eucalyptus kirtoniana*, seeds of which are available in plenty in local forests and practically unutilized at the present.

No proper attempts have been made to utilize the protein of this seed. To determine the extent to which the protein could be utilized, a brief chemical investigation of its solubility study, amino acid profile and molecular weight distribution has been undertaken. This is a part of our program to use nonconventional seed materials as an alternative source of protein to combat the increasing malnutrition problem prevailing in India.

TABLE 2

Amino Acid Analysis of *Eucalyptus kirtoniana* Seed Protein

Amino acids	g/16 g Nitrogen
Aspartic acid	11.93
Threonine[a]	4.34
Serine	4.52
Glutamic acid	12.90
Proline	6.77
Glycine	14.52
Alanine	8.71
Valine[a]	7.10
Methionine[a]	0.65
Isoleucine[a]	4.84
Leucine[a]	7.74
Tyrosine	1.93
Phenylalanine[a]	3.55
Histidine[a]	1.93
Lysine[a]	4.52
Arginine[a]	3.55

[a]Essential amino acid.

EXPERIMENTAL PROCEDURES

Materials. All reagents used in this investigation were of analytical grade. Reagents for SDS-Polyacrylamide gel electrophoresis, Sephadex G-200 (for gel filtration) and proteins used for standard calibration (BSA, Ovalbumin, Pepsin and Lysozyme) were purchased from Sigma Chemical Co., St. Louis, Missouri.

Extraction of proteins from E. kirtoniana *seeds and amino acid analysis.* Finely powdered *E. kirtoniana* seeds were extracted with petroleum ether (40–60 C) in a soxhlet extractor for 48 hr. Then the seeds were washed well with acetone and air dried. The nitrogen content of the seed was estimated by the micro-Kjeldahl method (3), and the protein content was determined (4).

Protein solubility profile of the deoiled seed was conducted under two different conditions. In case I, extraction was carried out at room temperature for 30 min using distilled water to deoiled seed (20:1, v/w) at pHs 2–12. The pH of extraction was adjusted to 2, 3,

TABLE 3

Determination of Molecular Weights of *Eucalyptus kirtoniana* Seed Proteins by Gel Filtration Procedure

Proteins	Elution vol/ void vol (V/V_o)	Mol wt determined from the curve (Fig. 4) (daltons)	Mol wt determined by equation [1] (daltons)	Literature mol wt[a] (daltons)
BSA[b]	1.880	—	—	66,000
Ovalbumin	2.071	—	—	45,000
Pepsin	2.195	—	—	34,700
Lysozyme	2.618	—	—	14,300
Eucalyptus kirtoniana seed proteins				
Component A	1.714	93,300	103,500	—
Component B	2.086	43,100	45,600	—
Component C	2.343	31,600	25,800	—
Component D	2.429	21,400	21,300	—

[a]Literature of mol wt of standard proteins, from Sigma Chemical Co., St. Louis, Missouri.

[b]Bovine serum albumin.

4, 5 and 6 by adding 0.5 M hydrochloric acid or adjusted to pH 7, 8, 9, 10, 11 and 12 by 0.5 M sodium hydroxide and the respective pHs of the extractants were maintained throughout the experiment (5, 6). In case II, the solubility profile of the seed protein was determined at a fixed pH (8.0) using varied concentrations (0.1-1.0 M) of NaCl, Na_2SO_3, $CaCl_2$ and $MgCl_2$ (5,6). The nitrogen content of each extract was monitored by the micro-Kjeldahl method (3).

Amino acid analysis of the seed protein was performed on a Beckman Multichrome 4255 amino acid analyzer as described by Matsubara and Sasaki (7).

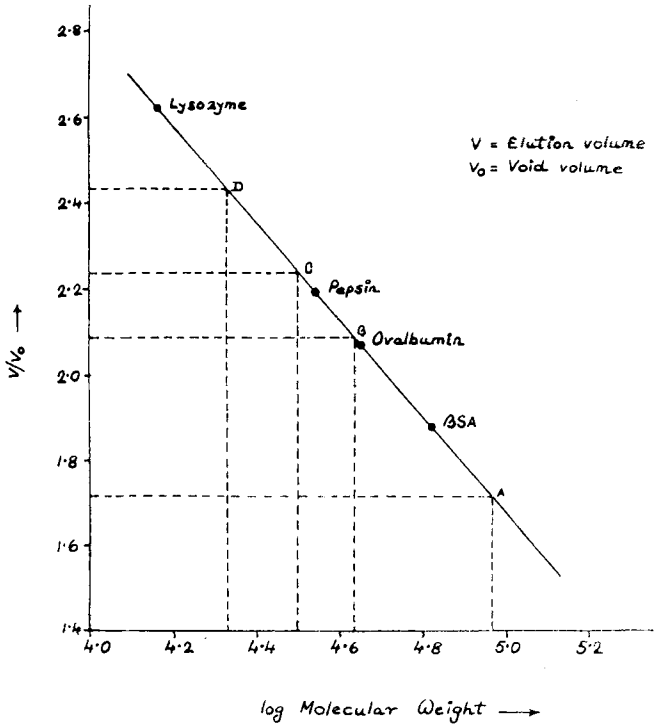

FIG. 4. Determination of molecular weight of *Eucalyptus kirtoniana* seed proteins by gel filtration.

Preparation of protein sample and gel filtration on Sephadex G-200. Seed protein extract in 0.1 M NaCl at pH 8.0 was dialyzed against 0.01 M phosphate buffer (pH 7.0) for 48 hr at 4 C. The protein was obtained by freeze drying the dialyzed solution. It was then dissolved in 0.01 M phosphate buffer (pH 7.0) containing 0.2 M NaCl to obtain a protein concentration of 4 mg/ml.

Gel filtration (8) was carried out on Sephadex G-200 gel in a 2.5 × 40 cm column and operated at 25 C. Two ml of the protein sample was applied and the eluting buffer was 0.01 M sodium phosphate (pH 7.0) containing 0.2 M NaCl. Two-ml fractions were collected at the rate of 0.4 ml/min and monitored at 280 nm.

SDS-PAG electrophoresis. Protein extracts in various salt solutions (0.1 M NaCl, 0.2 M Na_2SO_3, 0.1 M $CaCl_2,2H_2O$ and 0.1 M $MgCl_2,6H_2O$) at pH 8.0 were dialyzed against 0.01 M sodium phosphate buffer at pH 7.0. Samples containing 5 mg/ml protein were incubated with 1% SDS and 1% β-mercaptoethanol at 98 C for 5 min (9). The SDS-PAG electrophoresis was

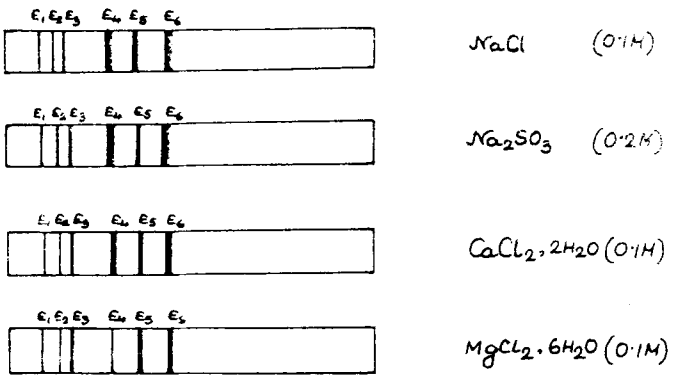

FIG. 5. Line diagram of SDS-PAG electrophoresis of *Eucalyptus kirtoniana* seed proteins using different salt solutions as extractant.

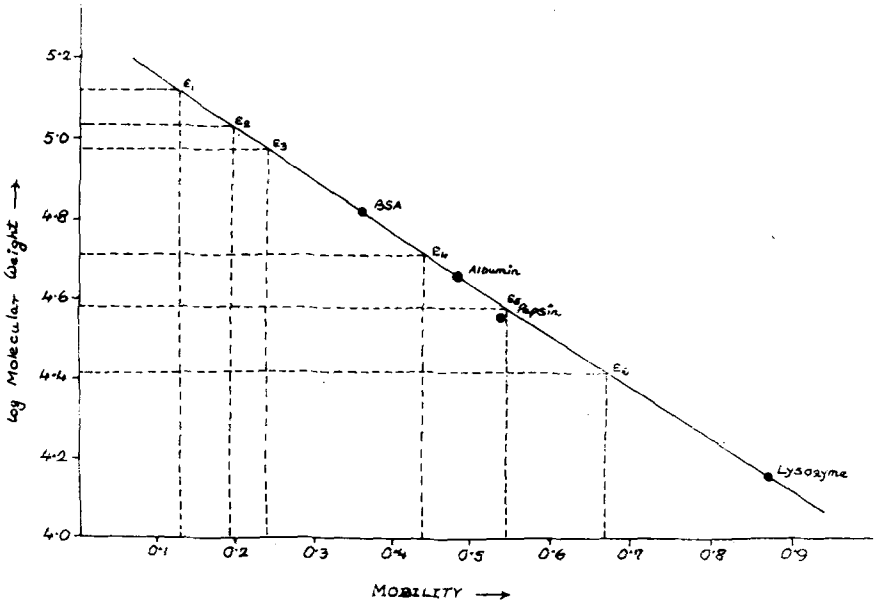

FIG. 6. Determination of molecular weight of *Eucalyptus kirtoniana* seed proteins by SDS-PAG electrophoresis using some standard proteins.

conducted by staining the gels in a solution of Coomasie brilliant blue (1.25 g Coomasie brilliant blue in 454 ml 50% MeOH and 46 ml HOAc) for 2 hr followed by destaining electrophoretically using HOAc-MeOH-H_2O (3:2:35) system (10). Then the molecular weights of protein bands so obtained were determined (10) from a linear curve obtained by plotting log mol wts against mobilities of standard proteins (BSA, Ovalbumin, Pepsin and Lysozyme).

RESULTS AND DISCUSSION

It was found that the nitrogen and protein contents in deoiled seeds were 2.07 and 11.80, respectively.

Figure 1 shows the nitrogen solubility profile of *E. kirtoniana* seed meal in aqueous solution of pHs 2-12.

TABLE 4

Molecular Weight Determination of *Eucalyptus kirtoniana* Seed Proteins by SDS-PAG Electrophoresis

Proteins	Mobility	Mol wt from the literature[a] (daltons)	Mol wt from Fig. 6 (daltons)
BSA[b]	0.36	66,000	—
Ovalbumin	0.48	45,000	—
Pepsin	0.54	34,700	—
Lysozyme	0.87	14,300	—
Eucalyptus kirtoniana seed proteins			
(E_1)	0.13	—	131,800
(E_2)	0.19	—	108,300
(E_3)	0.24	—	93,300
(E_4)	0.44	—	51,300
(E_5)	0.55	—	38,000
(E_6)	0.67	—	25,700

[a]Literature of mol wt of standard proteins, from Sigma Chemical Co., St. Louis, Missouri.

[b]Bovine serum albumin.

The seed protein solubility increased with an increase in pH (Table 1). Solubility study on the seed protein was also carried out in different concentrations (0.1-1.0 M) of NaCl, Na_2SO_3, $CaCl_2$ and $MgCl_2$ at pH 8.0, adjusted by the addition of 0.5 M NaOH solution.

The solubility profile of *E. kirtoniana* seed protein is different in various salt solutions (Table 1). NaCl and Na_2SO_3 are more efficient extractants than the other two ($CaCl_2$ and $MgCl_2$). In NaCl, $MgCl_2$ and $CaCl_2$, the solubility of protein increased with decreasing concentrations to 0.1 M, whereas for Na_2SO_3, solubility increased to 0.2 M (Fig. 2).

Amino acid analysis (7) of the seed protein revealed the presence of 16 amino acids of which nine were essential (Table 2). The seed protein contains a good amount of aspartic acid, glutamic acid and glycine. No tryptophan was found and, except for small amounts of methionine (0.65%) and histidine (1.93%), other essential amino acids were present in considerable amounts.

Gel filtration of *E. kirtoniana* seed protein was done only with the extract in 0.1 M NaCl at pH 8.0 which recovered maximum native proteins among all the salt solutions at that particular pH (pH 8.0). Gel filtration (8) of *E. kirtoniana* seed protein on Sephadex G-200 was conducted on a 2.5- × 40-cm column at 25 C. Four peaks were obtained (Fig. 3). The molecular weights of the components corresponding to the four peaks, i.e. A, B, C and D (Fig. 3), were determined (8) from a linear curve (V/V_o against log mol wt, Fig. 4), which is calibrated with reference protein standards (BSA, ovalbumin, pepsin and lysozyme). The molecular weight of each of the components (A-D) also was calculated by the equation (11) cited here:

$$\log \text{mol wt} = -0.959\,(V/V_o - 1) + 5.7 \qquad [1]$$

The mol wt of the components determined by two comparable methods (8,11) are not identical but found to be very close to each other (Table 3).

Extractable *E. kirtoniana* seed proteins in various salt solutions were determined by electrophoresis, and the MW of each fraction in each case was determined by SDS-PAG electrophoresis (10). Figure 5 demonstrates the SDS-PAG electrophoresis for the fractions of the seed proteins extracted in various salt solutions (0.1 M NaCl, 0.2 M Na_2SO_3, 0.1 M $CaCl_2$ and 0.1 M $MgCl_2$) at pH 8.0. The pH for extraction of proteins in salt solutions was adjusted to 8.0, although a high pH extracts more proteins (5). Each of the protein isolates consists of six fractions or bands (Fig. 5). Actually there is no differences in the number of protein fractions of various salt solutions. Relative mobilities for these fractions (components) were calculated and compared with a standard curve (Fig. 6). Molecular weights as determined on the basis of their mobilities (Table 4) were 131,800 (E_1), 108,300 (E_2), 93,300 (E_3), 51,300 (E_4), 38,300 (E_5) and 25,700 (E_6) daltons. The six fractions or bands thus obtained from SDS-PAG electrophoresis may be due to either six different proteins or different subunits of the same protein.

Because low mol wt proteins (may be useful for dietary purpose) are abundant (Fig. 3) in the investigated material and most of the essential amino acids (except tryptophan) are present therein, this material may be considered as a feed material after its toxicological status has been determined.

REFERENCES

1. Chopra, R.N., S.L. Nayar and I.C. Chopra, in *Glossary of Indian Medicinal Plants*, Council of Scientific and Industrial Research, New Delhi, 1956, p. 112.
2. Anonymous, in *Khadi and Village Industries Commission*, Bombay, India, 1973.
3. Vogel, A.I., in *Elementary Practical Organic Chemistry, Part III. Quantitative Organic Analysis*, 7th edn., Longman Group Ltd., London, 1971, pp. 652-657.
4. Holt, N.W., and F.W. Sosulski, Abstract 169, *Cereal Sci. Today 19*:409 (1974).
5. Guerra, M.J., and Y.K. Park, *J. Am. Oil Chem. Soc. 52*:73 (1975).
6. Laskar, S., S. Ghosh Majumdar and B. Basak, *Ibid. 62*:1266 (1985).
7. Matsubara, H., and R.M. Sasaki, *Biochem. Biophys. Res. Commun. 35*:175 (1969).
8. Whitaker, J.R., *Anal. Chem. 35*:1950 (1963).
9. Ganesh Kumar, K., and L.V. Venkatraman, *Phytochem. 17*:605 (1978).
10. Weber, K., and M. Osborn, *J. Biol. Chem. 244*:4406 (1969).
11. Leach, A.A., and P.C. O'Shea, *J. Chromatogr. 17*:245 (1965).

[Received October 27, 1986; accepted November 24, 1987]