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FRACTIONATION AND CHARACTERIZATION OF IPIL-IPIL (*Leucaena leucocephala* (Lam) de Wit.) SEED PROTEINS

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ABSTRACT

Defatted meal from ipil-ipil seeds containing 34.40% crude protein was fractionated using the Osborne method. Total soluble proteins amounted to 79.8% which was broken down as follows: 51.7% albumins, 14.0% globulins, 3.9% prolamins and 10.0% glutelins. The residue contained 11.9% protein.

All fractions except the prolamins were characterized for their physicochemical properties. Solubility tests showed that both albumins and globulins were least soluble at pH 7.0. When chromatographed through Sephadex G-150, albumins exhibited two peaks (MW = 243,700), globulins, two peaks (MW = 236,000) and glutelins three peaks (MW = 100,500). Electrophoretic properties revealed eleven bands for albumins, eight bands for globulins and two bands for glutelins. Polycarylamide gel electrophoresis in the presence of sodium dodecyl sulfate resolved eight bands for albumin (MW = 363,900), nine bands for globulins (MW = 368,000) and 3 bands for glutelins (MW = 114,500).

Amino acid profile indicated the presence of all essential amino acids. However, the amount of sulfur-containing amino acids and tryptophan were lower than those corresponding to the FAO protein pattern.

INTRODUCTION

The present global problem on protein-calorie malnutrition necessitates a need to look for non-conventional food protein sources.

Leguminous seeds, which are generally characterized by a relatively high protein content of 20-30%, are one of the alternative protein sources. Of the 13,000 species within the leguminosae order (Norton, 1976), only

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very few are widely cultivated as grain legumes.

Leucaena species, a leguminous tree, has been reported to contain 20-45% protein (Felker and Bandurstic, 1977; Espiritu, 1977; and Hibek, 1979). Despite the relatively high protein content of ipil-ipil seeds reported, there has been no studies on the physico-chemical properties of this protein. This paper reports the proximate analysis of ipil-ipil (Santa Elena) seeds, the fractionation of seed proteins by the Osborne method, and the physico-chemical properties of the fractions.

MATERIALS AND METHODS

Fresh, mature ipil-ipil *(Leucaena leucocephala* (Lam.) de Wit.) seeds were gathered around the campus of the University of the Philippines at Los Baños, College, Laguna. The seeds were air-dried at room temperature for six hours and sufficiently ground in a Wiley Mill to pass a 60-mesh sieve. The meal was then defatted with hexane using a Soxhlet extraction apparatus for 16 hours at 60°C.

Moisture, ash, and crude fiber contents were determined by the standard method of analysis (AOAC, 1970); crude protein by the semi-micro Kjeldahl procedure using selenium mixture as catalyst (Nx 6.25) and crude fat by extraction with petroleum ether in a Goldfish extractor for five hours.

Fractionation of Seed Proteins

Triplicate samples of the defatted ipil-ipil meal were subjected to successive extraction with each of the following solvents:deionized water (pH 7.0) for albumins; 0.5M sodium chloride solution for globulins; 80% ethanol for prolamins and 0.02M sodium hydroxide for glutelins. The residue containing non-solubilized proteins were retained for analysis. Figure 1 presents a schematic outline of the extraction procedure.

The nitrogen content of each fraction was determined using the method proposed by Lang (1958) for the microdetermination of Kjeldahl nitrogen in biological materials.

About ten milligrams of each lyophilized protein fraction was redissolved in a 10-ml sodium phosphate buffer (pH 7.0). The soluble protein was measured colorimetrically by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard protein. Absorbance was read at 650 nm using a Spectronic 20.

Solubility of Protein Fraction

Albumin and globulin (25-50 mg) fractions were each slurried in 20 ml distilled water, stirred for 15 minutes, using a magnetic stirrer and centrifuged at 15,000 rpm for 10 minutes. The solubilized proteins were determined

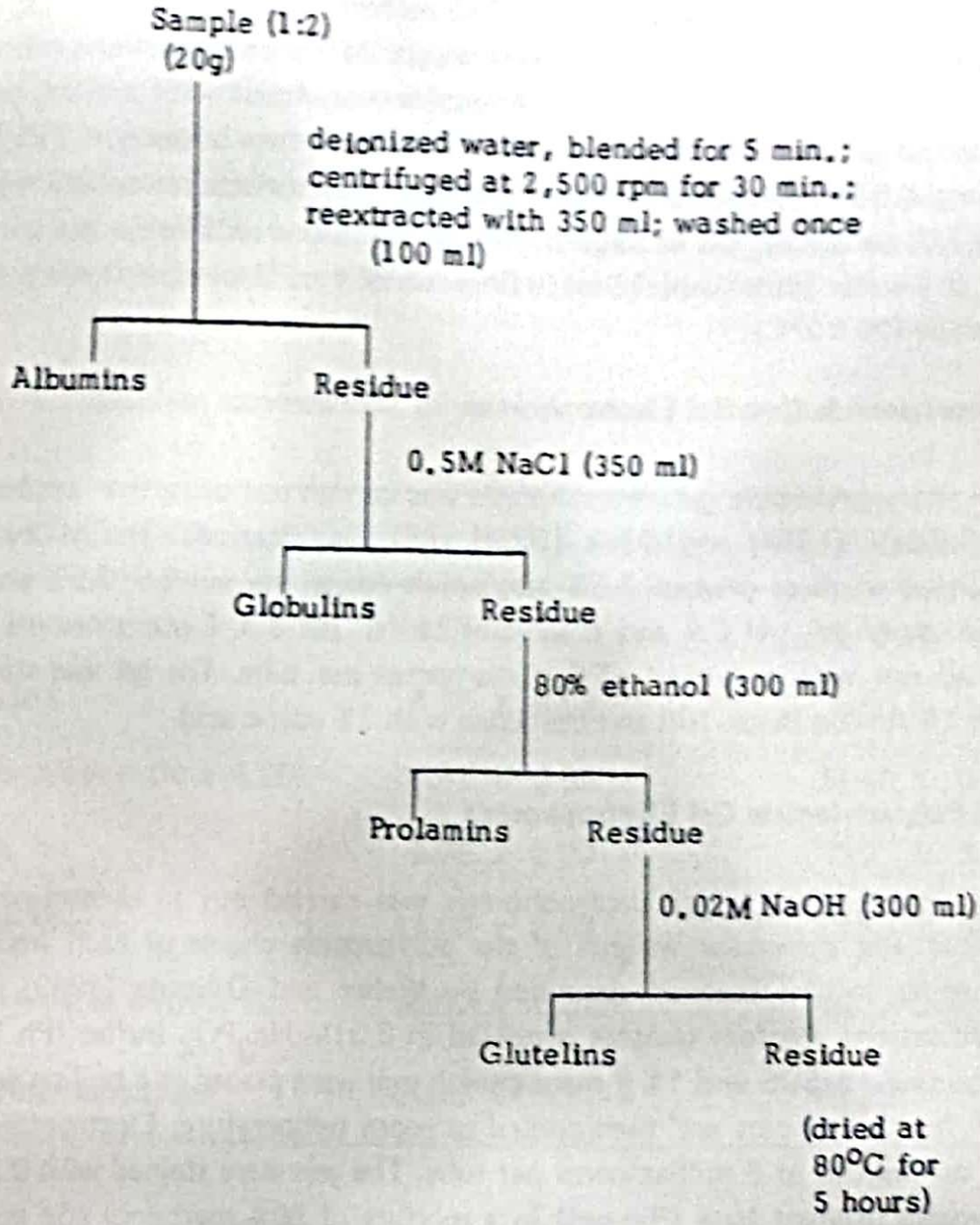

Figure 1. Fractionation procedure of ipil-ipil *(Leucaena leucocephala* (Lam.) de Wit.) seed proteins.

(Lowry et al., 1951). The pH of the solution was altered by the addition of 0.02 - 0.IN HCl or 0.02 - 0. IN NaOH.

Gel Filtration

Gel filtration on a Sephadex-G-150 column (1.5 x 90 cm (pH 7.0) was performed to determine the molecular weight of the three fractions, namely; albumins, globulins and glutelins. Albumins was eluted with sodium phosphate buffer (pH 7.0); globulins with sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride and glutelins with sodium phosphate buffer (pH 7.0) containing 0.5 M sodium chloride and 3 M urea. Elution was carried out at a water pressure of 16 cm, a flow rate of 1 ml/5 minutes at room temperature ($25 \pm 2^{o}C$).

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide gel eletrophoresis was carried out using the procedure of Ornstein (1964) and Davis (1964) with modifications. The gel system consisted of three phases: 7.5% acrylamide lower gel, pH 8.9; 2.5% acrylamide upper gel, pH 6.9 and trisglycine buffer, pH 8.3. Electrophoresis was carried out with a current of 6 milliamperes per tube. The gel was stained with 1% Amido Black 10B and destained with 7% acetic acid.

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out to determine the number and molecular weights of the polypeptide chains of each fraction according to the method described by Weber and Osborne (1969) with modifications. Protein samples dissolved in 0.01M Na_3PO_4 buffer (Ph 7.0) containing 1% SDS and 1% β-mercaptoethanol were placed in a boiling water bath for three hours and then cooled to room temperature. Electrophoresis was carried out at 8 milliamperes per tube. The gels were stained with 0.25% Coomasie brilliant blue (Bio-rad) in a mixture of 50% methanol and glacial acetic acid. After 6 hours, the gels were destained in an aqueous mixture of glacial acetic acid + methanol. Protein mobilities were measured and compared with standard proteins of known molecular weights.

Amino Acid Analysis

The protein samples were hydrolyzed with 6N HCl under vacuum for 24 hours at $110^{o}C$. The hydrolysate was analyzed in a Beckman Model 120-C amino acid analyzer using resin types of PA 35 and UR 30 and sodium acetate buffer. Tryptophan was analyzed according to the method of Knox et al. (1970). Cystine was determined as cysteic acid (Schramm et al., 1954).

RESULTS AND DISCUSSIONS

Proximate Composition of Ipil-ipil Seed Proteins

The proximate composition of ipil-ipil seeds presented in Table 1 showed carbohydrates (60.02% of undefatted meal) as the largest component. The crude protein of ipil-ipil seeds is fairly high and falls within the range (15-50%) of protein content of most legume seeds presently being exploited for both human consumption and as animal feeds. However, since the crude protein value includes both the protein nitrogen as well as the non-protein nitrogen the real protein content is expected to be lower.

Table 1. Proximate composition of ipil-ipil seeds (% moisture free basis).[a]

DETERMINATION	UNDEFATTED MEAL	DEFATTED MEAL
Ash	4.20 ± 0.0	4.15 ± 0.04
Nitrogen	4.86 ± 0.09	5.50 ± 0.02
Crude protein (N x 6.25)	30.42 ± 0.09	34.40 ± 0.13
Crude fat	5.36 ± 0.07	0.2 ± 0.08
Crude fiber	14.00 ± 0.08	13.59 ± 0.23
Nitrogen-free extract	46.02 ± 0.37	48.12 ± 0.48
Total carbohydrate	60.02 ± 0.10	61.20 ± 0.24

[a] *Mean of three replicates.*

Fractionation of Ipil-ipil Seed Proteins

Successive extraction with deinoized water (pH 7.0), 0.5M NaCl (pH 7.0), 80% ethyl alcohol and 0.2N NaOH solubilized 79.8% of the total seed protein. This amount consists of 51.7% albumins, 14.0% globulins, 10.0% glutelins and 3.9% prolamins. The residue was found to contain about 11.9% protein representing the amount not solubilized by the four solvents. About 8% protein remains unaccounted due to losses during the extraction process.

Figure 2 shows the solubility profile of albumins and globulins at pH 2.0 to 11.5. Both fractions have a minimum point of dispersion at pH 7.0.

Albumins were most soluble at pH 2.0 (94.2%). As the pH was increased the solubility of the fractions decreased until pH 7.0 when minimum solubility was observed. Precipitates were visibly observed in the solution. At pH 8.0, the fraction became more soluble and solubility remained the same through pH 9.0 and 10.0. Then as the pH was increased further, more proteins were solubilized.

Similarly, the globulins were most soluble at pH 2.0. At pH 7.0 only 26.3% of the proteins were soluble. As the pH was increased from 8.0 to 10.0, the solubility of the proteins increased from 28.3% to 41.6%. However, at pH 11.5 globulins were less soluble (62.5%) as compared to albumins which were 84.2% soluble.

Table 2. Result of the fractionation of ipil-ipil (Santa Elena) seed proteins.[a]

FRACTION	Amount of protein (g)	Percentage protein recovered
Initial protein of meal (d.b)	6.88	
Total soluble protein	5.49 ± 0.3	79.8
Albumin	3.56 ± 0.4	51.7
Globulin	0.96 ± 0.2	14.0
Prolamin	0.27 ± 0.0	3.9
Glutelin	0.69 ± 0.2	10.0
Nonsolubilized residue	0.82 ± 0.2	11.9
Total crude protein recovered	6.31 ± 0.1	91.7

[a] *Mean of three replicates.*

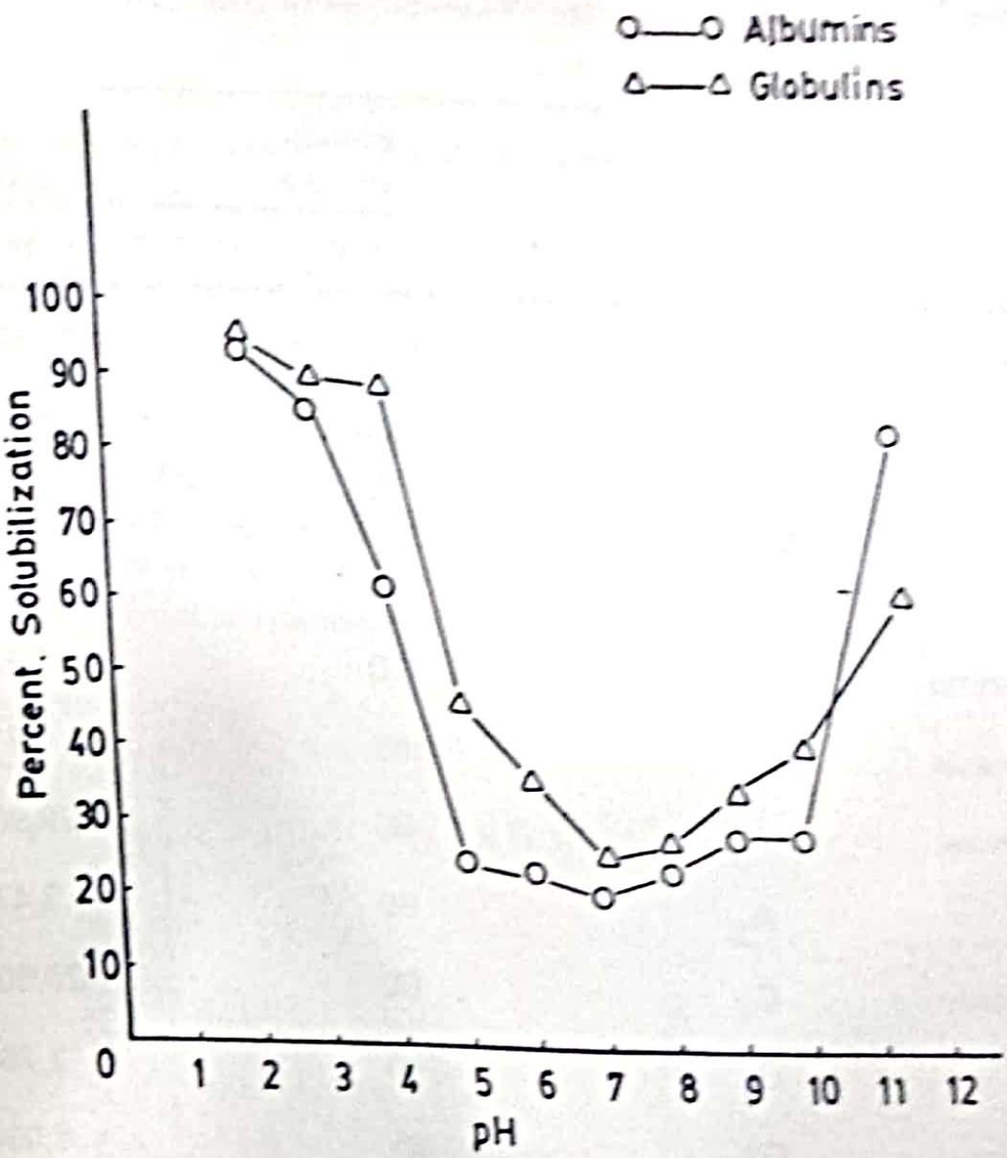

Figure 2. Solubility profiles of lyophilized ipil-ipil (Santa Elena) protein fractions.

Gel Filtration

Each of the 50 mg lyophilized fraction was dissolved in 0.5 ml 0.1 M sodium phosphate buffer (pH 7.0), stirred and applied to a Sephadex G-150 column. As shown in Figure 3, the albumins were characterized by two peaks. The first peak eluted at 60 ml had an estimated molecular weight of 209,000 daltons and the second eluted at 95 ml has a molecular weight of 34,700 daltons (Table 3). The estimated molecular weight of this fraction is 243,700 daltons.

The same number of peaks was observed with globulins (Fig. 4). One peak with an estimated molecular weight of 209,000 daltons eluted at 60 ml and the other eluted at 100 ml had an estimated molecular weight of 27,600 daltons. Thus, the globulins with a total estimated molecular weight of 236,600 daltons was smaller than the first fraction.

The glutelins had the lowest estimated molecular weight of 100,500

Table 3. Gel filtration of ipil-ipil seed proteins.

Protein Maker/ Protein Fraction		Elution Volume (ml)	Molecular Weight ($\times 10^4$)
Blue Dextan - 2000		55	200
Catalase		60	24,000
Bovine Serum Albumin		75	6,700
Egg Albumin		85	4,500
Lysozyme		110	1,430
Bacitracin		165	410
Albumins	A_1	60	20,900
	A_2	95	3,470
Globulins	G_1	60	20,900
	G_2	100	2,760
Glutelins	$G1_1$	85	6,030
	$G1_2$	100	2,760
	$G1_3$	115	1,260

daltons among the three fractions characterized. This protein showed three distinct peaks at elution volumes of 85 ml with MW 60, 300; 100 ml with MW 27,600 and 115 ml with MW 12,600 (Fig. 5).

All fractions were observed to be dependent on the buffer systems. Three buffer systems were employed as eluant during the chromatographic run: a) 0.1M sodium phosphate buffer (pH 7.0); b) 0.1M sodium phosphate butter (pH 7.0) containing 0.5M sodium chloride; and c) 0.1M sodium phosphate buffer (pH 7.0) containing both 0.5M sodium chloride and 3M urea. The albumins were observed to readily dissolve in three solvents. The second fraction was soluble in the second and third solvents but not in the first. The third fraction was not soluble in the first two solvents but readily dissolved in 0.1M sodium phosphate buffer (pH 7.0) containing 0.5M sodium chloride and 3M urea.

Polyacrylamide Disc Gel Electrophoresis

The electrophoretic patterns obtained from the various fractions are presented in Figure 7. Each electrophoretogram revealed a wide variation in the intensities of bands.

The albumins exhibited eleven bands with varying intensities having Rm values ranging from 0.11 to 0.84.

Eight bands have been observed in globulins; three major bands (Rm = 0.19, 0.28, and 0.38), one dark blue band (Rm =0.38), three blue bands (Rm 0.47, 0.53 and 0.56), the polypeptides that make up this fraction did not differ very much in their molecular sizes, shapes and charges.

The glutelins had one major band (Rm =0.27) and other very broad fast-moving diffused bands. This diffusion may be caused by the presence of several cross linked polypeptide chains of different charges (Alberty, 1953).

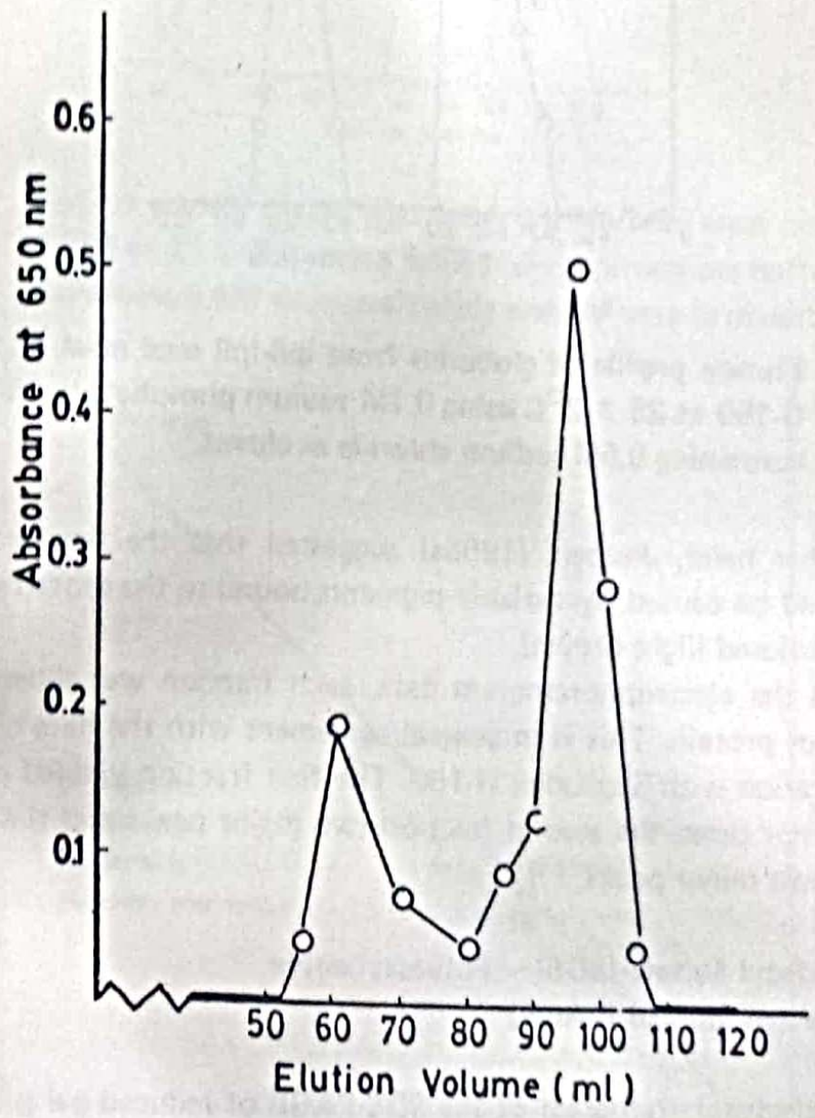

Figure 3. Elution profile of albumins from ipil seed meal on Sephadex G-150 at 25 ± 2°C using 0.1M sodium phosphate buffer (pH 7.0) as eluant.

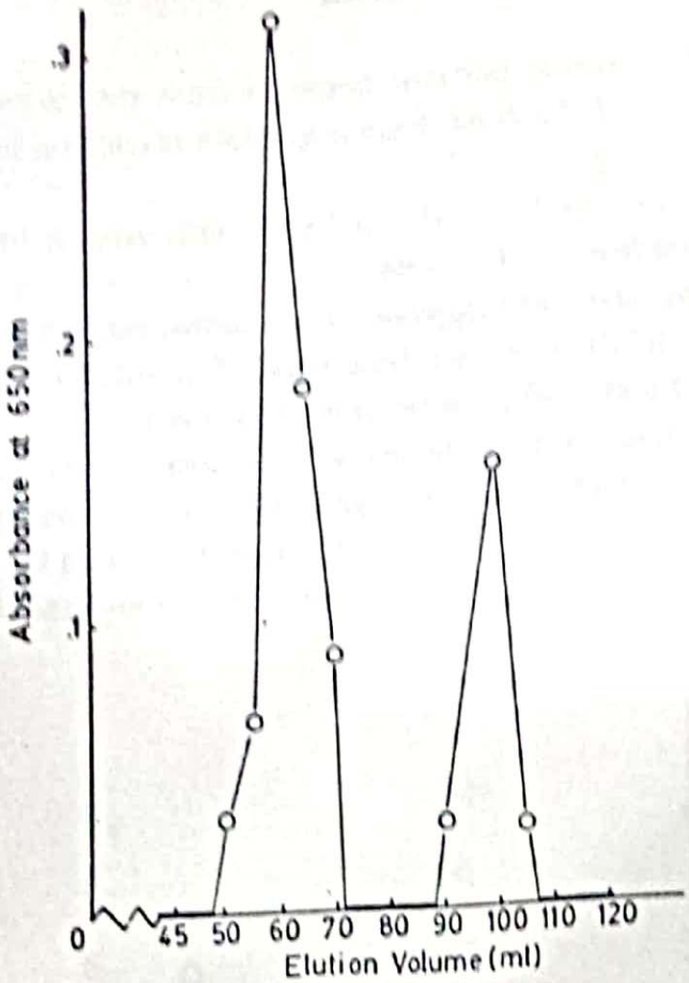

Figure 4. Elution profile of globulins from ipil-ipil seed meal on Sephadex G-150 at 25 ± 2°C using 0.1M sodium phosphate buffer (pH 7.0) containing 0.5M sodium chloride as eluant.

On the other hand, Joubert (1955a) suggested that the heterogeneity of charges could be caused by variable pigments bound to the proteins, and this fraction is colored (light brown).

From the electrophoretogram data, each fraction was shown to be a heterogenous protein. This is in general agreement with the data observed in the gel filtration with Sephadex G-150. The first fraction yielded one major and one minor peak; the second fraction two major peaks and the third one major and two minor peaks.

Sodium Dodecyl Sulfate (SDS) – Polyacrylamide Disc Gel Electrophoresis (PAGE)

The electrophoretogram of the SDS-PAGE of reduced gel proteins are presented in Figures 8, 9 and 10 which show the subunit constitution of the protein fractions and their molecular weights. Also, the subunit constitution of the protein fractions is summarized in Table 4.

Contrary to the result obtained from the polyacrylamide disc gel

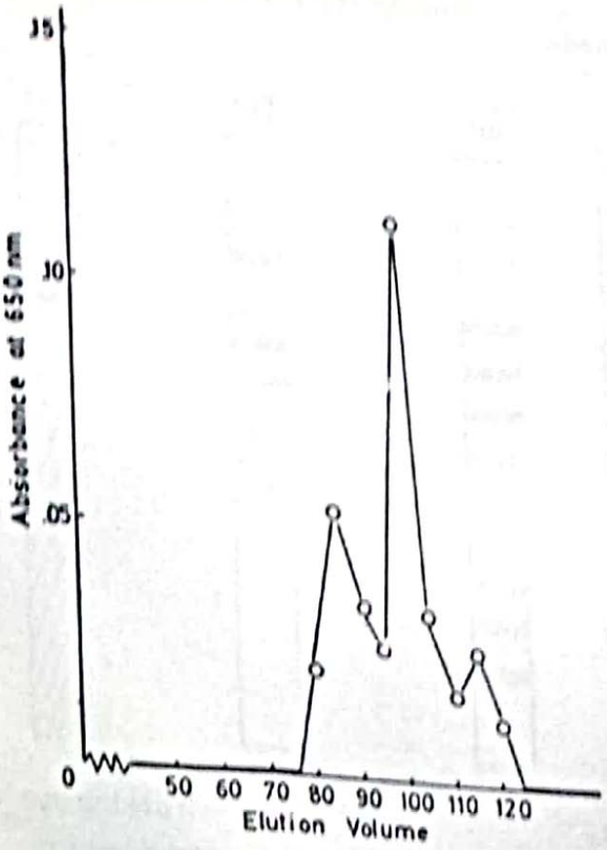

Figure 5. Elution profile of glutelins from ipil-ipil seed meal on Sephadex G-150 at 25 ± 2°C using 0.1M sodium phosphate buffer (pH 7.0) containing 0.5M sodium chloride and 3M urea as eluant.

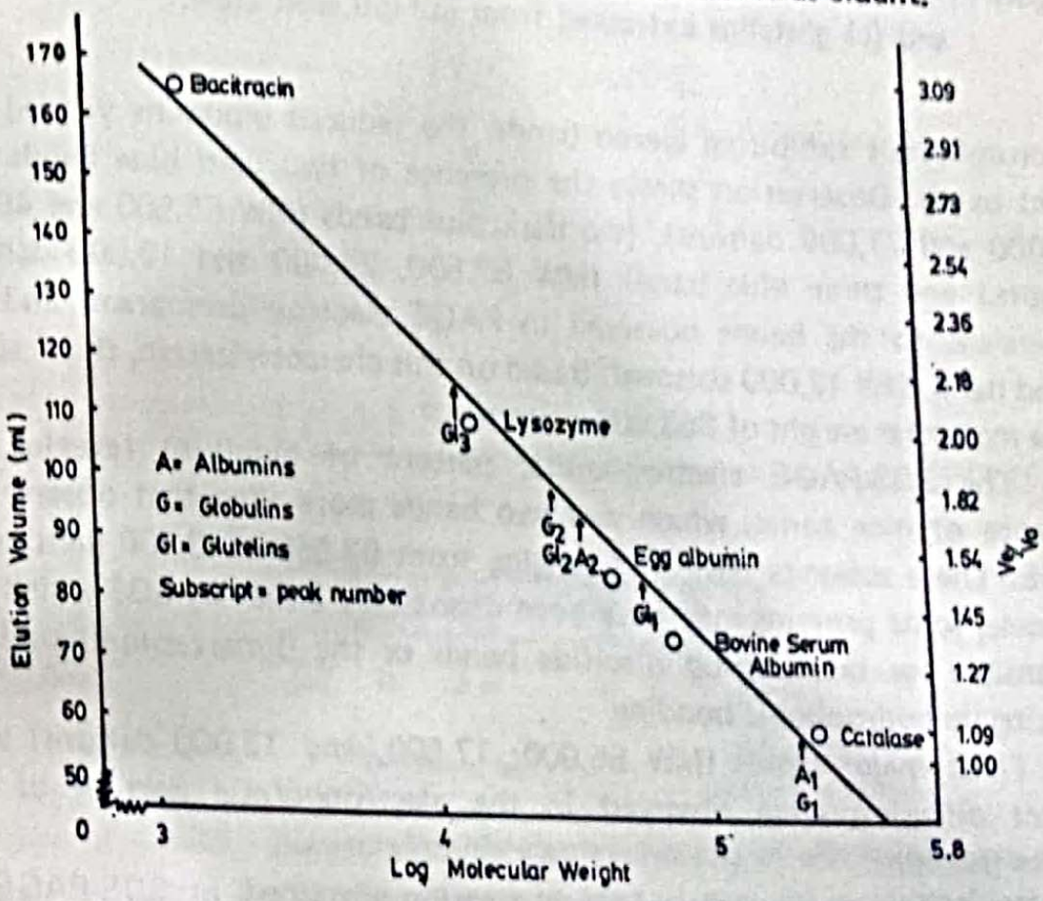

Figure 6. Plot of elution volume, Ve, against log molecular weight for standard proteins on Sephadex G-150 at 25 ± 2°C.

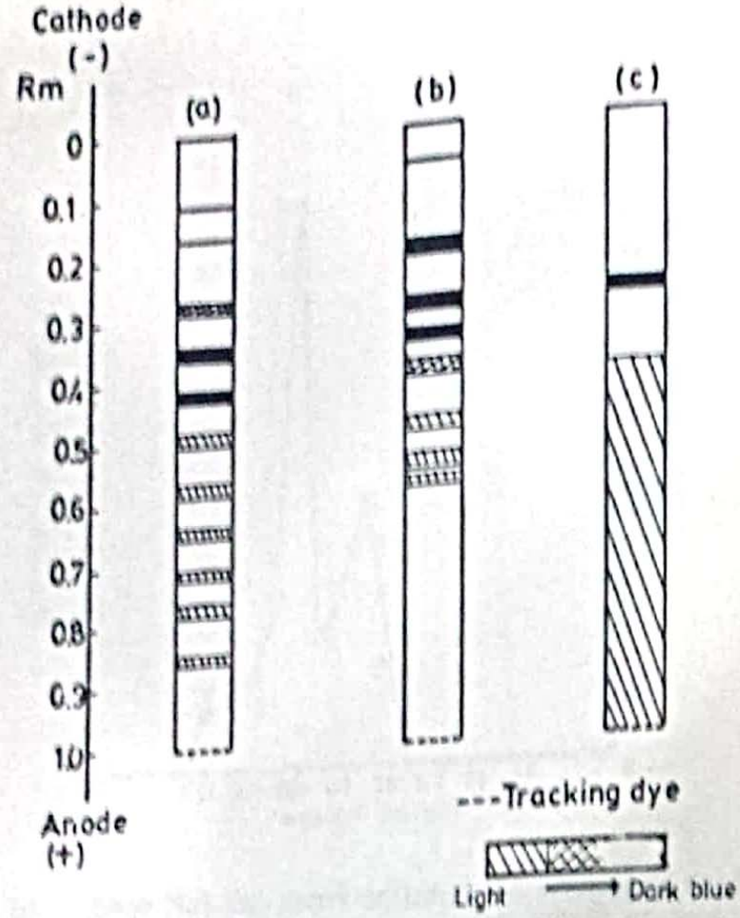

Figure 7. Analytical gel electrophoretogram of (a) albumins, (b) globulins and (c) glutelins extracted from ipil-ipil seed meal.

electrophoresis exhibiting eleven bands, the reduced albumins yielded only eight bands. Observation shows the presence of two light blue bands (MW 73,000 and 71,000 daltons), two dark blue bands (MW 55,500 and 46,000 daltons) and three blue bands (MW 62,500, 24,000 and 19,000 daltons) equivalent to the bands observed in PAGE electrophoretogram, and one broad band (MW 12,000 daltons). Based on this characterization, this fraction has a molecular weight of 363,900 daltons.

The SDS-PAGE electrophoretic pattern of globulins revealed the presence of nine zones, which was two bands more than that observed in PAGE. These subunits have MW ranging from 63,500 to 3,500 daltons. In this case, some proteins may have been dissociated either by SDS which was responsible for breaking up disulfide bands or the B-mercaptoethanol for breaking up hydrophobic bonding.

Three major bands (MW 85,000; 17,500, and 12,000 daltons) with distinct diffusion were observed in the electrophoretic pattern of the glutelin fraction.

The molecular weight of each fraction obtained in SDS-PAGE is generally higher than that obtained in gel filtrations. Albumins have MW 363,900 daltons; globulins, MW 313,500 and glutelins MW 114,500.

Table 4. Subunit constitution of the ipil-ipil protein fractions.

Protein Fraction	Mobility	Molecular Weight (x 10)
Albumins	0.20	7.30
	0.22	7.10
	0.31	6.25
	0.38	5.55
	0.48	4.60
	0.70	2.40
	0.75	1.99
	0.83	1.20
Globulins	0.3	6.35
	0.37	5.65
	0.42	5.15
	0.46	4.80
	0.52	4.25
	0.71	2.35
	0.79	1.60
	0.86	0.85
	0.91	0.35
Glutelins	0.08	8.5
	0.77	1.75
	0.83	1.20

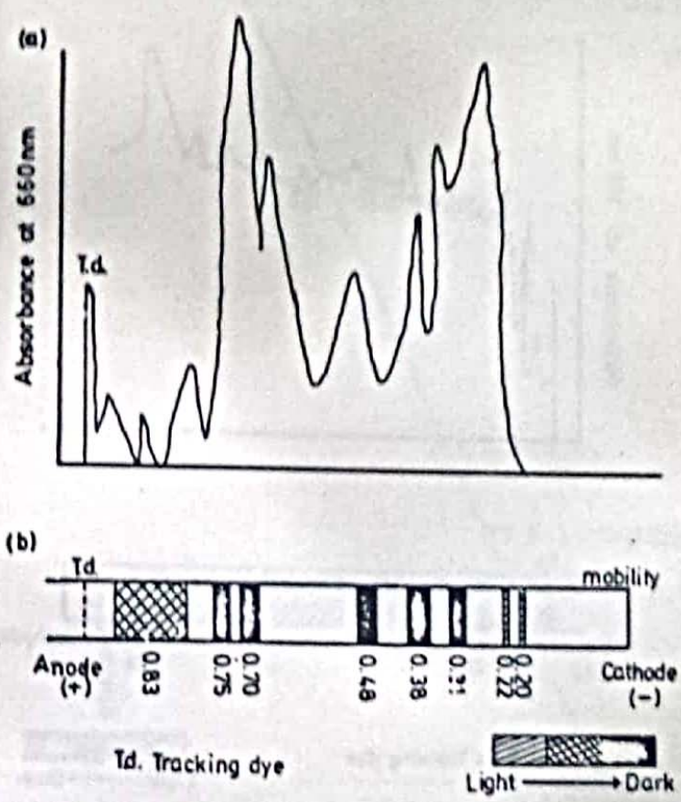

Figure 8. SDS - polyacrylamide electrophoresis of albumin fraction, showing its (a) densitometric tracing and (b) analytical gel electrophoretogram. Amount applied was 50 ug and electrophoresis was done at 8 ma/gel tube.

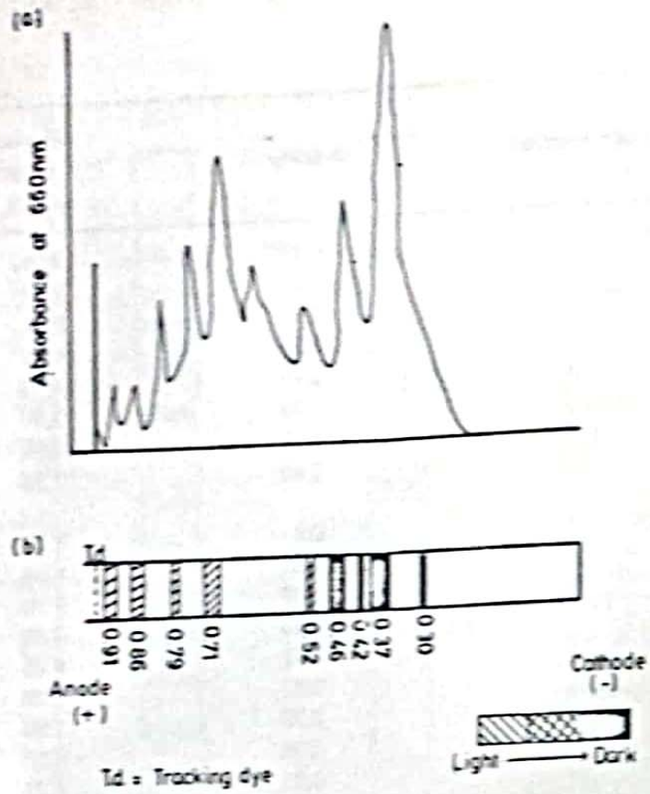

Figure 9. SDS - polyacrlamide electrophoresis of globulin fraction showing its (a) densitometric tracing and (b) analytical gel electrophoretogram. Amount applied was 40 ug and electrophoresis was at 8 ma/gel tube.

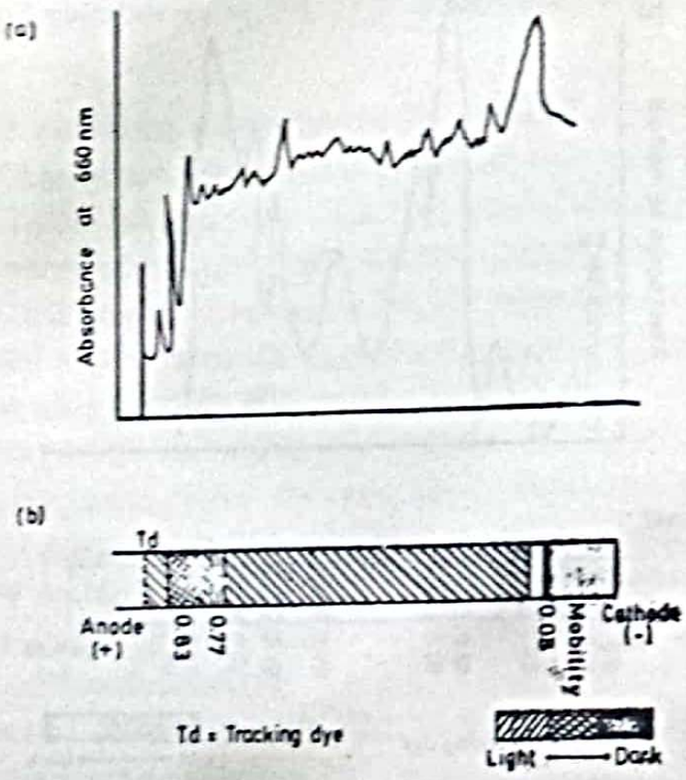

Figure 10. SDS - polyacrylamide electrophoresis glutelin fraction, showing its (a) densitometric tracing and (b) analytical gel electrophoretogram. Amount applied was 30 ug and electrophoresis was at 8 ma/gel tube.

The data shown here reveal that all the protein fractions characterized electrophoretically are not homogenous. This observation has also been found with other legumes (Cerletti et al., 1978). Protein fractions that behave as a unique aggregate and contain a higher number of different peptides, several of which have quite similar molecular weights, are not unusual among legumes. For example, Mantenffel and Scholz (cited by Cerletti et al., 1978) found that *Vicia faba* is not a uniform protein, but a collection of different, although highly similar molecules.

Heterogeneity of the fractions may be observed in the presence of broad bands. Broadening of the bands may be caused by variable amounts of pigments bound to the protein or by the differences in the degree of amidation of aspartic acid and glutamic acid or by the differences in the amount of composition of bound carbohydrates (Glagrome and Gillespie, 1975).

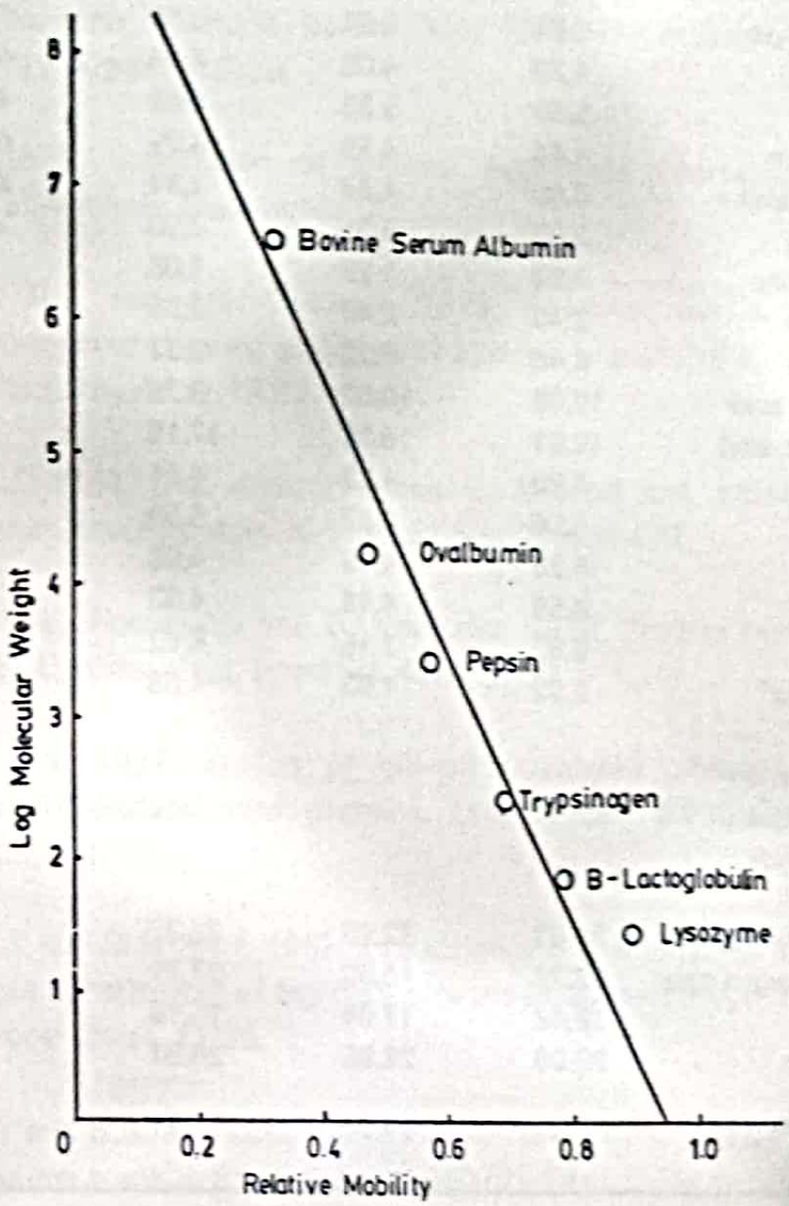

Figure 11. Standard curve for molecular weight determination by SDS gel electrophoresis.

Amino Acid Composition

The amino acid composition of the three protein fractions shown in Table 5 are compared with the essential amino acids of FAO reference

Table 5. Amino acid composition of the three fractions as compared to FAO reference protein.[a]

Amino Acid	Albumins (g/16 N)	Globulins (g/16 N)	Glutelins (g/16 N)	FAO Reference Protein (g/16 N)
Methionine	1.35	1.55	1.51	2.20
Threonine	4.02	3.49	4.14	2.80
Tryptophan	0.64	0.84	0.60	1.40
Valine	4.30	4.08	4.78	4.20
Lysine	5.59	5.33	4.62	4.20
Isoleucine	4.44	4.55	4.29	4.20
Phenylalanine	4.80	4.88	4.44	2.80
Tyrosine	2.96	3.31	3.00	2.80
1/2 Cystine	1.03	1.28	1.08	–
Histidine	2.43	2.49	2.35	–
Arginine	8.46	9.22	8.22	–
Aspartic acid	10.08	10.02	9.79	–
Glutamic acid	19.01	18.86	17.15	–
Glycine	4.50	4.33	4.51	–
Proline	4.09	4.52	5.50	–
Serine	5.20	4.49	4.98	–
Alanine	4.59	4.48	4.33	–
Leucine	6.82	7.15	6.62	–
Ammonia*	2.02	1.93	4.08	–
Classification Distribution of amino acids				
1. Hydrophobic	31.03	32.05	32.07	–
2. Uncharged polar	17.71	16.90	17.71	–
3. Basic	16.48	17.04	15.19	–
4. Acidic	29.09	28.88	26.94	–
TOTAL	94.31	94.87	91.91	–

[a] *Mean of two replicates*

**Not an amino acid*

(19.01, 18.86, and 17.15 for albumins, globulins and glutelins, respectively) followed by aspartic acid (16.08, 10.02 and 9.79). In terms of the classification distribution of amino acids, the hydrophobic group (Alanine, valine, leucine, isoleucine, proline, phenylalanine, trytophan and methionine) is predominant, followed by the acidic group (glutamic acid and aspartic acid). The basic amino acids consist of lysine, arginine and histidine; and the uncharged group is composed of glycine, serine, threonine, cysteine and tyrosine.

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