CHAPTER II

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Biological materials

Seeds of pigeon pea (Cajanus cajan) variety T-21 and TT-6 were supplied by Mr. S.E. Pawar (BARC). They were multiplied in the experimental field of this Research Centre. Mature seeds were used for the extraction of proteins. The different cultivars of pigeon pea were obtained from Dr. L.J. Reddy, International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad. The list of accessions used is given in Table 1.

2.1.2. Chemicals and reagents

The chemicals used were of the analytical grade. Fine chemicals were obtained from Sigma Chemical Company, Saint Louis, USA, with the exception of products listed below.


Protein molecular weight markers for gel filtration, thyroglobulin, ovalbumin and β-lactoglobulin (Schwarz/Mann, Orangeburg, SC, USA).
Table 1. List of pigeon pea accessions used

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Seeds of these were kindly provided by Dr. L.J. Reddy of ICRISAT.
TT-6 material was obtained from Mr. S.E. Pawar of BARC.
Cellulose acetate electrophoresis membranes lot No. C7M 20535 AG (Beckman Instruments Inc., Fullerton, CA 92634, USA).

Catalase (British Drug House, Poole, England).

N,N,N',N'-tetramethyl ethylene diamine (Eastman Organic Chemicals, Rochester, NY, USA). Riboflavin (Nutritional Biochemical Corporations, Cleveland, Ohio, USA).

Sulphosalicylic acid (GR) (Loba-Chemie Indoaustranal Co., P.O. Box 6136, Bombay, India).

POPOP and PPO, scintillation grade (Packard Instrument Company, Inc., Downers Grove, IL 60515, USA).

Membrane filters, pore size 0.45 μm (Sartorius membrane filter - GMBH, Gottingen, FRG).

Amido black 10B (E. Merck, Darmstadt, FRG).

Kjeltabs (Tecator, Box 70, S 263 01, Haganas, Sweden).

14C-uniformly labelled algal protein hydrolysate was obtained from the Isotope Division of this Research Centre.

2.2. METHODS

2.2.1. Developing seed and pod

The crop was grown at the experimental field during the normal growing season from June to December. No fertilizer was
applied to the plants. Roots were found to be well nodulated with native rhizobia. On the day of opening, the first two flowers in the inflorescence were tagged in a large number of plants. Only the first two flowers were allowed to develop in a tagged inflorescence. The untagged flowers elsewhere in the plant were allowed to develop normally. The tagged flowers and the pod bearing leaf were harvested at intervals of 7 days and each sample was designated for its maturity as the days after flowering (DAF).

Pod cover, seeds and pod bearing leaf were separated and their fresh weights were determined immediately. The samples were then dried in a circulating air oven at 90°C for 24 hours to determine the dry matter.

2.2.2. Determination of total nitrogen content

Seed meal or an aliquot of protein sample was digested with concentrated sulphuric acid and kjeltabs in Tecator digestion tubes at 350°C for one hour. The digest was diluted with distilled water and then taken in a PREGL (Parnas-Wagner) type of distillation flask. To this, alkali (5% Na₂S₂O₃ in 40% NaOH) was gently added. After steam distillation, ammonia was collected in 20 ml of 2% boric acid containing 2 drops of indicator (1:5 (v/v) mixture of 0.2% methyl red and 0.2% bromocresol green in 95% ethyl alcohol).
Distillation was continued till no more ammonia was liberated from the digest as indicated by a pH paper. The ammonia in the boric acid solution was titrated against 0.01 N HCl. Each ml of 0.01 N HCl is equivalent to 0.14009 mg of nitrogen.

Besides the Kjeldahl method the nitrogen was also estimated by a colorimetric method on a Technicon autoanalyser II following the procedure described in the manual (for reference see end of this chapter).

A conversion factor of 6.25 was used to convert % nitrogen values into % protein.

2.2.3. Determination of soluble amino nitrogen

The method described by Mitra et al. (1976) was used to estimate soluble amino nitrogen. Samples (0.5 - 1 g) were homogenised with ten volumes of 80% ethanol at room temperature and centrifuged at 12,000 x g for 15 minutes. The pellet was reextracted twice with 10 ml of 80% ethanol. The pooled supernatant solutions were evaporated to dryness in a boiling water bath and the residue was dissolved in 2-3 ml of 0.1 M citrate buffer, pH 2.2 and filtered. An aliquot of 0.2 ml was assayed for amino nitrogen after neutralisation by the ninhydrin method of Moore and Stein (1954). DL-Leucine was used as a standard.
2.2.4. Labelled amino acid incorporation into proteins

Incorporation of labelled amino acids into proteins during seed development was followed using the method of Chen and Osborne (1970) with some modifications as described by Mitra et al. (1976). Freshly harvested seeds were sliced into pieces and incubated in small petri plates at 25°C for 60 minutes in a reaction mixture consisting of 4.8 ml of 5 mM Tris-HCl buffer, pH 7.6 containing 20 mM KCl, 2 mM MgCl₂, 20 µg/ml sucrose, 20 units of penicillin and 0.2 ml of ¹⁴C-uniformly labelled amino acid mixture (algae protein hydrolysate 20 µCi; specific activity 42 µCi/m atom C). After incubation the slices were washed with 50 ml of 5 mM Tris-HCl buffer, pH 7.6 containing 20 mM KCl, 2 mM MgCl₂ and 0.5 mg/ml Cas-amino acids and then with 50 ml of distilled water over a Buchner funnel lined with Saran Screen (National Filter Media Corporation, Salt Lake City, Utah, USA). Washed slices were homogenised in 5 ml of chilled 5% trichloroacetic acid (TCA), heated for 15 minutes at 90°C in a water bath and then cooled to 4°C. An aliquot of 0.5 ml was filtered through Sartorius membrane filter (pore size 0.45 micron), washed with 30 ml of 5% TCA taking 10 ml each time. Dried membrane filters were counted in a Beckman liquid scintillation spectrometer LS-100 using 0.05% POPP and 0.4% PPO in toluene.
2.2.5. Estimation of protein in solution

Protein was estimated either by the method of Lowry et al. (1951) or by the biuret method (Layne, 1957). A standard graph was prepared using solutions of bovine serum albumin (BSA) (fraction V) as the standard protein. BSA concentrations were estimated from
\[
\frac{\text{Absorbance at } 280 \text{ nm}}{\text{cm}} = 6.6 \text{ (Casey, 1979).}
\]

2.2.6. Isolation of protein bodies

Protein bodies were isolated from 16 hour soaked seeds essentially according to the method described by Miflin et al. (1981) with some modifications. 5 g cotyledons were ground in 7 ml of buffer, pH 7.5 consisting of 0.05 M Tricine, 0.1 M potassium acetate, 0.01 M magnesium acetate and 20% (w/w) sucrose. The ground material was filtered through four layers of cheese cloth and 2 ml of the filtrate was layered onto a discontinuous gradient (25 ml). The discontinuous gradient consisted of 3 ml of 68%, 5 ml of 65%, 4 ml of 60%, 5 ml of 55%, 4 ml of 50% and 4 ml of 45% (w/w) sucrose made in extraction buffer. The gradients were centrifuged at 24,000 rpm in a SW 25.1 rotor in an L5 65B Beckman ultracentrifuge at 4°C for 4.5 hours. The gradients were then fractionated using a Pharmacia peristaltic pump P-3. About 0.9 ml fractions were collected. Sucrose concentration was estimated in alternate fractions using a Fisher refractometer.
I ml of Tricine buffer, pH 7.5 was added to each fraction and an aliquot of 0.5 ml was taken for protein analysis by biuret method. Appropriate fractions were pooled and protein bodies were pelleted by high speed centrifugation. Protein bodies were kept frozen till further use.

2.2.7. Isolation of protein from protein bodies

The protein body pellet was suspended in 2 ml of 0.1 M borate buffer, pH 8.2 and was stirred for about 60 minutes in cold. The suspension was centrifuged for 10 minutes at 12,000 x g and the supernatant was dialysed in cold against 0.15 M sodium phosphate buffer, pH 7.2. The contents of the dialysis bag after transferring into a tube were kept frozen till further use.

2.2.8. Preparation of seed meal

Seed meals were prepared from mature dry seeds by grinding them in Udy cyclone mill fitted with a 40 mesh sieve. The finely milled meal was defatted with cold petroleum ether. Defatted meal was then air dried and stored at 4°C. For some experiments protein was extracted from seeds soaked overnight. Seed coat was removed manually and the cotyledons were homogenised in buffer in a Braun mixer.
2.2.9. Fractionation of seed protein

The flour sample was successively extracted with 0.1 M borate buffer, pH 8.2, 0.1 N NaOH and 70% ethanol to separate the total seed proteins into salt soluble, glutelin and prolamin fractions respectively. The salt soluble fraction was separated into albumin and globulin fractions as described later (section 2.2.12.1).

The meal (2 g) was first extracted with 0.1 M borate buffer (20 ml) for 3 hours with continuous agitation and centrifuged at 12,000 x g for 15 minutes. The residue was reextracted with buffer and the supernatant of both extractions were pooled and designated as salt soluble protein. The residue after buffer extraction was homogenised in 0.1 N NaOH and 70% ethanol in a similar manner. After making up the protein extracts to a known volume, nitrogen was determined in an aliquot on a Technicon autoanalyzer II (section 2.2.2).

2.2.10. Nitrogen solubility of the seed meal at different pH

Defatted seed meal (250 mg) was weighed into Sorvall centrifuge tubes. 10 ml of distilled water was added to each tube and the pH was adjusted using HCl or NaOH from pH 3 to pH 11 with 1 pH unit increment, except in the range of pH 4 to pH 5 where the increment was 0.2 pH units. The tubes were agitated in a
wrist action shaker. Every hour the pH was checked and adjusted to the original pH. Variation to the maximum of about 0.2 pH unit was observed.

At the end of 5 hours the tubes were centrifuged at 12,000 x g for 30 minutes at 4°C. The supernatant solution was filtered using Whatman number 21 filter paper. In another set of experiment NaCl at a final concentration of 10% was used. Rest of the procedure was as described earlier. The filtrates were concentrated by evaporation and an aliquot was taken for nitrogen estimation using Technicon autoanalyser II (section 2.2.2).

2.2.11. Protein extraction from seed meal

2.2.11.1. Alkaline extraction

Defatted meal was blended with 0.1 M borate buffer, pH 9.2 in cold for 2.5 minutes in a Braun mixer at top speed. The mixer was operated each time for 30 seconds followed by a gap of 5 seconds. The ratio of seed meal to buffer used was 1 : 10 (w/v). The slurry was kept on a stirrer for about 3 hours then passed through four layers of cheese cloth and centrifuged at 10,000 x g for 30 minutes. The supernatant was passed through glass wool to remove traces of low density material that floated during centrifugation. The filtrate is referred to as alkaline extract.
2.2.11.2. Acidic extraction

Seed meal was suspended in 0.5 M NaCl (meal to solvent ratio 1:16 w/v) and the pH was adjusted to 3.5 with 6 N HCl. The extraction was continued for 4 hours in cold with stirring. The slurry was centrifuged at 10,000 x g for 30 minutes. The supernatant is referred to as acidic extract.

2.2.12. Preparation of globulin

2.2.12.1. Alkaline extract

The alkaline extract was appropriately diluted with distilled water so that the buffer concentration was equal to or less than 0.05 M. The pH of the solution was brought down to pH 4.8 with slow addition of 6 N HCl and the solution was continuously stirred. At this pH and buffer concentration, the globulin protein precipitated while the albumin remained in the solution. The precipitated globulin protein was collected by centrifugation at 12,000 x g for 15 minutes in cold. The protein pellet was dissolved in 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The protein solution was loaded on to a Sepharose 6B column (2.8 cm x 92 cm), equilibrated with Tris buffer mentioned above. The column was eluted at a flow rate of 24 ml/hr and 12 ml fractions were collected using an ISCO fraction collector. The column eluant was continuously monitored at 280 nm using a
LKB Uvicord II. The void volume fraction was collected and rest of the 280 nm absorbing fractions were pooled. Globulin protein was precipitated from the pooled fraction by overnight dialysis against 25 mM citrate buffer, pH 4.7. The precipitated globulin was collected by centrifugation at 12,000 x g for 15 minutes. The protein pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2 and was kept at 0°C.

2.2.12.2. Acidic extract

The acidic extract was initially dialysed against running tap water for 2-3 hours, to decrease the salt concentration. Later it was dialysed overnight against 25 mM sodium citrate buffer, pH 4.7. The precipitated globulin protein was collected by centrifugation at 12,000 x g for 15 minutes and the protein pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2.

2.2.13. Test for RNA and DNA

The procedure of Fleck and Munro (1962) was followed for checking the presence of ribonucleic acid. DNA was checked by the Indole method (Ceriotti, 1955).
2.2.14. Globulin fractionation

2.2.14.1. Isoelectric precipitation

The method of Danielsson (1949) and Bailey and Boulter (1970) was used with some modifications for the separation of globulin into different fractions. The protein solution was dialysed overnight against 33 mM sodium acetate buffer, pH 4.7 containing 0.2 M NaCl or against 25 mM sodium citrate buffer, pH 4.7 containing 0.2 M NaCl. Under these conditions the legumin fraction precipitated leaving the vicilin fraction in the solution. The precipitated legumin was collected by centrifugation at 12,000 x g for 15 minutes. The legumin protein pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2. The supernatant was dialysed against 0.15 M sodium phosphate buffer, pH 7.2, before further analysis.

2.2.14.2. Ammonium sulphate fractionation

The protein concentration was usually maintained around 2-3 mg/ml for ammonium sulphate fractionation. Separation of globulin was attempted by precipitations at definite concentrations of ammonium sulphate between 0-40%, 40-65% and 65-85% saturation. Required amount of finely ground ammonium sulphate was added slowly to protein solution with constant stirring at room temperature. After dissolution of ammonium sulphate the solution was left standing for 1-2 hours in cold. The precipitated
protein was collected by centrifugation at 12,000 x g for 15 minutes. The protein pellet was dissolved in minimum quantity of 0.15 M sodium phosphate buffer, pH 7.2 and dialysed against the same buffer for at least 2 days in cold. After dialysis the contents of the dialysis bag were kept at 0°C till further analysis.

2.2.14.3. Zonal isoelectric precipitation

The method as described by Scholz et al. (1974) was used with minor modifications.

Buffers

824 ml of 0.2 M disodium hydrogen phosphate and 176 ml of 0.1 M citric acid, pH 7.0 (phosphate buffer).

493 ml of 0.2 M disodium hydrogen phosphate and 507 ml of 0.1 M citric acid, pH 4.8 (citrate buffer).

Both buffers contained 0.02% sodium azide.

Sephadex G-50

Sephadex G-50 was allowed to swell in several volumes of citrate buffer at room temperature and washed several times with the same buffer to remove the fine particles. The column (2.4 cm x 75 cm) was packed to a height of 67 cm at room temperature using a peristaltic pump.
Protein loading and elution

The protein sample in phosphate buffer was placed at the top of the column. The sample volume was usually kept under 10 ml. The column was eluted with phosphate buffer and 5.6 ml fractions were collected using an ISCO fraction collector. The column eluant was either monitored continuously at 280 nm with a LKB Uvicord II or was checked manually at 280 nm in a Beckman DB-G spectrophotometer. pH of the fractions were measured in a Toshniwal digital pH meter.

Column washing

After each run the column was washed with 20 ml of 0.2 M NaOH and then with 20 ml of 0.2% sodium dodecyl sulphate (SDS). The column was equilibrated with citrate buffer before reusing.

2.2.15. Gel filtration

2.2.15.1. Globulin purification

A column of Sepharose 6B (2.8 cm x 92 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl was used for purifying globulin. The column was packed at a flow rate of 30 ml/hour using a peristaltic pump. About 30-40 ml of the alkaline extract was loaded on the column. The column was
eluted with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl at a flow rate of 24 ml/hour and 12 ml fractions were collected. The column eluant was monitored continuously at 280 nm as described in section 2.2.12.1.

2.2.15.2. Molecular weight determination

Gel filtration for molecular weight determination was performed on a column of Sepharose 6B (1.6 cm x 56 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl. The column was packed in cold room under gravity flow and was eluted at a flow rate of 12 ml/hour; 1.2 ml fractions were collected. Effluent fractions were monitored at 280 nm in a Beckman DB-G spectrophotometer.

The column was calibrated by determining the elution volumes (Ve) of the following standard proteins: human thyroglobulin, lactate dehydrogenase, malate dehydrogenase, ovalbumin and \( \beta \)-lactoglobulin. The void volume (Vo) was determined using Blue dextran 2000. A calibration graph was obtained by plotting (Ve) against the log molecular weight of the protein.

5 mg of legumin, 4.5 mg vicilin and 5 mg of \( \gamma \)-protein were loaded separately on the column to determine the molecular weights of the holoproteins.
2.2.15.3. \( \gamma \)-protein purification

A column of Sephacryl S-300 (1.4 cm x 60 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl was used for purifying the \( \gamma \)-protein. The column was packed at room temperature at a flow rate of about 12 ml/hour. The same buffer at a flow rate of about 11 ml/hour was used for eluting the column; 4.4 ml fractions were collected. Protein was monitored at 280 nm in a spectrophotometer. The fractions around the peak were analysed by cellulose acetate membrane (CAM) electrophoresis before pooling.

2.2.16. Ion exchange chromatography

Fractionation of globulin protein and purification of individual fractions were attempted on a DEAE-Sephacel column. The column was prepared in a 50 ml plastic syringe (2.6 cm x 13 cm). DEAE-Sephacel was packed in the column using a peristaltic pump to a bed height of about 11 cm and at a flow rate of about 50 ml/hour. The resin in the column was regenerated as described in the Pharmacia booklet on ion exchange chromatography (for reference see end of this chapter). The resin was first washed with about 50 ml of 1 M NaCl and then with about 50 ml of 0.1 N NaOH. The column was washed thoroughly with water before equilibrating in 15 mM sodium phosphate buffer, pH 8.0.
Protein solution dialysed overnight against 15 mM sodium phosphate buffer, pH 8.0 was loaded on the column. After removing the unbound material, the column was eluted with a linear gradient of NaCl (0 to 0.4 M or 0 to 1 M) in 15 mM sodium phosphate buffer, pH 8.0. The column was eluted at a flow rate of 40 ml/hour and 5.2 ml fractions were collected. The concentration of NaCl was determined by conductivity measurement in every fifth fraction after appropriate dilution. Protein was monitored at 280 nm as mentioned earlier. Few fractions around the peak were electrophoresed on CAM and only after ascertaining the purity, the appropriate fractions were pooled and protein was precipitated by dialysis against 25 mM citrate buffer, pH 4.7. The protein was collected by centrifugation at 12,000 x g for 15 minutes and the pellet was dissolved in 0.15 M sodium phosphate buffer, pH 7.2 and kept frozen till further use.

2.2.17. Ultracentrifugation

2.2.17.1. Preparative ultracentrifugation

Linear sucrose gradients, 4.8 ml volume and 10 to 30% (w/v) concentration were prepared according to the method of Stone (1974) with some modifications. The stock solutions were 8% (w/v) and 34% (w/v) sucrose in 0.05 M sodium phosphate buffer, pH 7.2 containing 0.4 M NaCl. To a vertically held centrifuge tube 34% sucrose (2.4 ml) and 8% sucrose (2.4 ml) solution were
added carefully without disturbing the interphase. After covering the tube with parafilm (American Can Co., Greenwich, CT 06830, USA), the tube was tilted by 90° carefully to rest on its side. The tubes were left undisturbed for 4 hours and later were brought back to the vertical position.

Protein samples (2 mg) in 0.2 ml of 0.05 M sodium phosphate buffer, pH 7.2 containing 0.4 M NaCl was layered on the gradient with a microsyringe. The gradients were centrifuged for 16 hours at 40,000 rpm in a SW 65 rotor at 4°C in a Beckman L5 65B preparative ultracentrifuge. The contents of the gradients were collected manually by puncturing the tubes and 3 drops were collected in each fraction. Sucrose concentration was estimated using 5 μl sample from every fifth fraction in a Fisher refractometer. Protein in the fractions was estimated by the method of Lowry et al. (1951). Bovine liver catalase and lysozyme were used as protein markers to determine the sedimentation coefficients as described by Martin and Ames (1961).

2.2.17.2. Analytical ultracentrifugation

Analytical ultracentrifugation of freeze dried globulin was carried out by Dr. M.W. Pandit, Centre for Cellular and Molecular Biology, Regional Research Laboratory, Hyderabad. Sedimentation velocity was determined at 60,000 rpm at 26°C. The S values were corrected to $S_{20,w}$.
2.2.18. Electrophoretic techniques

2.2.18.1. Cellulose acetate membrane electrophoresis

Electrophoresis on cellulose acetate membranes (CAM) was carried out in a Beckman micрозone electrophoretic apparatus essentially according to the method of Blagrove and Gillespie (1975). The electrophoretic buffers used were either 0.05 M sodium phosphate buffer, pH 7.2 or sodium barbital buffer, pH 8.6 (0.075 M), usually 2-4 μg protein in 0.15 M sodium phosphate buffer, pH 7.2 was applied on the membranes with the help of a sample applicator provided by Beckman Instruments Co. Electrophoresis was carried out at room temperature and at a constant voltage of 150 V for about 20 minutes. The current usually rose from about 4-5 mA to about 7-8 mA at the end of 20 minutes. The current was never allowed to exceed 9 mA. After electrophoresis the membranes were stained for about 5 minutes in 0.2% (w/v) Coomassie brilliant blue R-250 dissolved in methanol, acetic acid and water (30:10:60 v/v) and destained in the same solvent without the dye.

2.2.18.2. Agarose gel electrophoresis

LKB multiphor apparatus was used for agarose gel electrophoresis essentially following the procedure as described in the LKB manual (for reference see end of this chapter). 1% agarose gel was prepared in 0.05 M sodium phosphate buffer,
pH 7.2. Protein (10 - 20 µg) in 0.05 M sodium phosphate buffer, pH 7.2 was applied to the gel and electrophoresed at a constant voltage of 10 V/cm. The gel was cooled by circulating water at 5°C. Sodium phosphate buffer (0.05 M), pH 7.2 was used as the electrophoresis buffer. Electrophoresis was stopped when the bromophenol blue marker dye had migrated about 8 cm from the origin. The gels after electrophoresis were stained and destained as described for CAM (section 2.2.18.1).

2.2.18.3. Polyacrylamide gel electrophoresis

Acrylamide and bisacrylamide were used without any further purification. Throughout the present work the polyacrylamide gel concentrations (i.e., 10% or 5%) are referred to on the basis of both the monomer and cross linker concentrations (% T) and not on the acrylamide monomer concentration alone. % C wherever given represents the amount of crosslinker as a % of the sum of monomer and crosslinker.

Non-dissociating, discontinuous system, pH 8.3 (O'neil, 1964; Davis, 1964).

The stock solutions were the same as described by Davis (1964) except 'C solution' which contained 29.2 g acrylamide and 0.8 g bisacrylamide in a final volume of 100 ml. Polyacrylamide gels were prepared either in glass tubes (0.6 cm i.d. x 13 cm)
or in slabs (16 cm x 12 cm x 0.2 cm). A filter paper strip with a code number was inserted into the separating gel solution before polymerisation to enable identification of slabs.

Protein samples (20 - 40 μg/tube or track) were routinely loaded onto the stacking gel surface. A sample gel was never used. The samples were made dense by mixing the protein solutions with 20% (w/v) sucrose. Bromophenol blue marker dye was added into the top cathodic buffer compartment. Electrophoresis was performed at room temperature with a constant current of 4 mA/tube or 25 mA/slab till the marker dye had migrated to about 0.5 cm from the bottom of the gel surface.

Gels after electrophoresis were stained either in 1% (w/v) amido black in 7% (v/v) acetic acid for 60 minutes or overnight in 0.2% (w/v) Coomassie brilliant blue R-250 in methanol, acetic acid and water (30:10:60 v/v). The gels stained with amido black were destained in 7% acetic acid and those stained with Coomassie brilliant blue in methanol, acetic acid and water (30:10:60 v/v). The gels were destained in a Hoefer diffusion destainer till the background became clear.
Non-dissociating, continuous system, pH 7.2.

Stock solutions

i) Monomer solution (30% T, 2.7% C)

This solution contained 29.2 g acrylamide and 0.8 g bisacrylamide in a final volume of 100 ml of distilled water. The solution after filtration was stored at 4°C in dark.

ii) 0.2 M sodium phosphate buffer, pH 7.2

31.2 g sodium dihydrogen phosphate (NaH₂PO₄) was dissolved in about 800 ml of distilled water and the pH was adjusted to 7.2 with dilute NaOH. The volume was made upto 1000 ml with distilled water.

iii) Catalyst

Ammonium persulphate at a concentration of 0.14 g/100 ml of distilled water was used. This solution was prepared fresh at the time of gel preparation.

The gels were prepared either in tubes or in slabs as described earlier. The gel solution was prepared by mixing the stock solutions in the following ratios:

- Monomer solution : 1.33
- Phosphate buffer : 2.00
- Catalyst : 4.00
- Distilled water : 0.67
After degassing the solution, TSMED (N,N,N',N'-tetramethylethylenediamine) was added at a concentration of 35 μl/100 ml of gel solution. The gel solution was quickly poured into the gel mould, overlaid with distilled water and allowed to polymerise. In this system the spacer gel was omitted. The electrophoretic buffer was 0.05 M sodium phosphate buffer, pH 7.2.

Protein loading, electrophoresis, staining and destaining were performed as described earlier.

**Dissociating, continuous system, pH 7.2.**

Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS was carried out in 10% separating gel slab (16 cm x 12 cm x 0.2 cm) following the method of Weber and Osborn (1969).

**Stock solutions**

1) 0.4 M sodium phosphate buffer, pH 7.2.

62.4 g of sodium dihydrogen phosphate (NaH₂PO₄) was dissolved in about 800 ml of distilled water and the pH was adjusted to 7.2 with dilute NaOH. The volume was made upto 1000 ml with distilled water.
ii) Monomer solution (30% T, 2.7% C)

29.2 g acrylamide and 0.8 g bisacrylamide were dissolved in a final volume of 100 ml of distilled water. The solution was filtered and stored at 4°C in a brown coloured bottle.

iii) Sodium dodecyl sulphate

10 g of SDS was dissolved in distilled water and the final volume was made up to 100 ml. This solution was stored at room temperature.

iv) Catalyst

Freshly prepared ammonium persulphate (15 mg/ml) solution was used as catalyst for polymerisation.

The gel solution was prepared in a side arm flask by mixing the stock solutions in the following proportions:

Stock solution i = 15 ml
Stock solution ii = 20 ml
Stock solution iv = 3 ml
Distilled water = 21.3 ml

After thorough degassing, 0.6 ml of SDS (stock solution iii) and 0.1 ml of TEMED were added. The solution after mixing was poured into the gel mould and a perspex comb was inserted to form the sample slots. The gel solution under these conditions polymerised within 30 minutes.
Protein samples (final concentration 0.5 mg/ml) were treated in a boiling water bath for 2-3 minutes in a sample treatment buffer (0.01 M sodium phosphate buffer, pH 7.2 containing 1% SDS and 5% 2-mercaptoethanol and 10% glycerol). In cases where disulphide bond reduction was not required 2-mercaptoethanol was omitted from the sample treatment buffer.

Protein (20 - 30 μg) was loaded into each slot and electrophoresis was performed at room temperature at a constant current of 50 mA. The electrophoresis buffer was 0.1 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS. Electrophoresis was stopped when the bromophenol blue marker dye had reached the bottom of the gel after about 20 hours. The position of the marker dye in the gel was marked by introducing a piece of copper wire. The gels were fixed for about 60 minutes in a solution of 12.5% TCA containing 4% sulphosalicylic acid. They were later stained overnight in 0.2% (w/v) Coomassie brilliant blue R-250 in methanol, acetic acid and water (50:10:40 v/v). The gels were destained by diffusion in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid.

The following proteins were used to prepare the standard graph: α-lactalbumin (14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000) and phosphorylase a (94,000), their molecular
weights are given in parentheses. Mobilities were measured relative to the bromophenol blue marker dye and used to obtain approximate molecular weights of protein subunits as described by Weber and Osborn (1969).

2.2.19. Immunological methods

2.2.19.1. Raising of antibodies

Antibodies against γ-protein fraction were raised in rabbits (Belgium strain, 4 months old). Before injecting the antigen, the rabbits were bled to obtain preimmune serum. For obtaining antibodies 1 mg of γ-protein in 1 ml of 0.15 M sodium phosphate buffer, pH 7.2 was emulsified with 1 ml of complete Freund's adjuvant and was injected at multiple sites, subcutaneously. An identical booster injection was given 15 days later. The animals were bled from the central artery of the ear following the method of Gordon (1981) 15 days after the booster dose. After allowing the blood to clot at 4°C overnight, serum was collected by centrifugation at 12,000 x g for 10 minutes. The serum was transferred to small glass tubes and was kept frozen till further use. The serum was used directly without any further purification.
2.2.19.2. Double immunodiffusion

Double immunodiffusion was carried out at 4°C for 16 - 24 hours in 1% (w/v) agarose gel following the method as described in the LKB application note number 249 (for reference see end of this chapter).

2.2.19.3. Rocket immunoelectrophoresis

Rocket immunoelectrophoresis was performed in a LKB-multiphor apparatus according to the method described in the LKB application note number 249 (for reference see end of this chapter). The agarose gel contained 0.2% (v/v) antiserum. Electrophoresis was performed at a constant voltage of 200 V for 18 - 19 hours at 5°C.

Protein from 500 mg seed meal was extracted from each of the Cajanus accessions in 2 ml of 0.1 M borate buffer, pH 8.2. Extractions were made in centrifuge tubes in cold with constant stirring. Supernatant obtained after centrifugation at 12,000 x g for 10 minutes at 4°C was used for rocket immunoelectrophoresis. 15 μl of the supernatant was used.

2.2.19.4. Protein fraction in developing seeds

The seeds for this study were obtained from the crop grown during the year 1981. The seeds, 50 for 7 days after flowering (DAF), 20 for 14 DAF and 5 seeds each for 21, 28, 35
and 42 DAF were extracted in 2 ml of 0.1 M borate buffer, pH 8.2. The homogenate was centrifuged at 12,000 x g for 10 minutes in cold and the supernatant was used for rocket immunoelectrophoresis according to the method described earlier. For 7 and 14 DAF 10μl of the supernatant and for all later stages 5μl of the supernatant was used.

2.2.20. Amino acid analysis

Globulin protein and the purified fractions were hydrolysed in sealed pyrex glass tubes in 6 N HCl (3 mg protein/5 ml HCl) at 110°C for 24 hours. The amino acid analysis was carried out by Dr. Pershko on a Beckman amino acid analyser using the standard procedures at the Seibersdorf Laboratory of the International Atomic Energy Agency, Vienna.
List of manuals or application notes referred to under Materials and Methods sections:

1. Nitrogen estimation on Technicon autoanalyser II.
   Technicon Industrial Systems, Tarrytown, New York, 10591, USA.

2. Ion exchange chromatography - Principles and methods.
   (Printed March 1980-1). Pharmacia Fine Chemicals AB,
   Box 175, S-75104, Uppsala 1, Sweden.

3. Agarose gel electrophoresis with LKB-2117 multiphor.
   LKB-Produkter AB, S-16125, Bromma, Sweden.

4. Immunelectrophoretic techniques with the LKB-2117 multiphor.
   LKB-Produkter AB, S-16125, Bromma, Sweden.