J. Biosci., Vol. 10, Number 1, March 1986, pp. 57–65. © Printed in India.

γ-Protein, a sulphur amino acid rich protein from pigeon pea (*Cajanus cajan* (L.) Millsp.)

T. G. KRISHNA and C. R. BHATIA
Biology and Agriculture Division, Bhabha Atomic Research Centre, Trombay, Bombay 400085, India.

MS received 9 May 1985; revised 14 November 1985

Abstract. A globulin protein comparatively rich in sulphur amino acids has been isolated from the seeds of pigeon pea. This protein termed *γ*-protein has a sedimentation coefficients of 7S and a molecular weight of about 90,000. Antibodies were raised against pure *γ*-protein. Using rocket immunoelectrophoresis it was observed that *γ*-protein was synthesised in the developing seeds, 21 days after flowering.

Keywords. Pigeon pea; seed globulin; *γ*-protein, sulphur amino acids.

Introduction

Grain legume seeds are next to cereals in importance as human food and animal feed. Though they contain a higher amount of protein than the cereals, sulphur amino acids are usually limiting in legumes. One of the approaches suggested for the improvement of seed protein quality in grain legumes is to identify proteins that are relatively rich in sulphur amino acids and to increase their amount by breeding. *γ*-Conglutin was reported to be one such protein in *Lupinus* (Blagrove and Gillespie, 1975). This suggested us to look for similar proteins in other legumes. In an earlier communication, we had reported the presence of a sulphur rich protein termed *γ*-protein from pigeon pea (Gopala Krishna *et al*., 1977). In this paper we report the isolation, characterisation and synthesis of *γ*-protein from pigeon pea seeds.

Materials and methods

Preparation of seed meal

Seed meal from mature dry seeds (cv. T-21) was prepared by grinding in a Udy cyclone mill fitted with a 40 mesh sieve. The finely milled meal was defatted with cold petroleum ether, air dried and stored at 4°C.

Protein extraction from seed meal

Defatted meal was extracted with 0·01 M sodium borate buffer, pH 8·2 in cold for 3 h with constant agitation. The ratio of seed meal to buffer used was 1:10 (w/v). The slurry

Abbreviations used: CAM, Cellulose acetate membrane; DAF, day after flowering; SDS, sodium dodecyl sulphate; M_r, molecular weight.

was passed through 4 layers of cheese cloth before centrifugation at 10,000 *g* for 30 min. The supernatant referred to as alkaline extract was used for globulin preparation.

Preparation of globulin

The alkaline extract was diluted with water to make the buffer concentration 0·05 M or lower. The pH of the solution was brought down to 4·8 with slow addition of 6 N HCl and the solution was continuously stirred. The precipitated globulin protein was collected by centrifugation at 12,000 *g* for 15 min. The protein pellet was dissolved in 0·05 M Tris-HCl buffer, pH 8 containing 0·5 M NaCl. The protein solution was loaded onto a Sepharose 6B column (2·8 × 92 cm) equilibrated with Tris buffer mentioned above. The column was eluted at a flow rate of 24 ml/h 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 rejected 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 protein pellet after centrifugation was dissolved in 0·15 M phosphate buffer, pH 7·2 and stored at 0°C.

γ-protein purification from globulin

The procedure followed for purification of γ-protein from globulin is shown in figure 1.

Estimation of protein

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 bovine serum albumin (fraction V) as the standard protein.

Cellulose acetate membrane electrophoresis

Electrophoresis on cellulose acetate membrane (CAM) was carried out in a Beckman microzone electrophoretic apparatus essentially according to the method of Blagrove and Gillespie (1975).

Immunological methods

Antibodies against *γ*-protein were raised in rabbits (Belgium strain; 4 month old) essentially according to the method described by Jurd (1982). The animals were bled according to the method of Gordon (1981). The serum was directly used without any purification.

Double immunodiffusion was carried out at 4°C for 16–24 h in 1 % (w/v) agarose gel following the method described in the LKB application note 249.

Rocket immunoelectrophoresis was performed in a LKB-Multiphor apparatus according to the method described in the LKB application note number 249. The agarose gel contained 0·2 % (v/v) antiserum. 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 *g* for

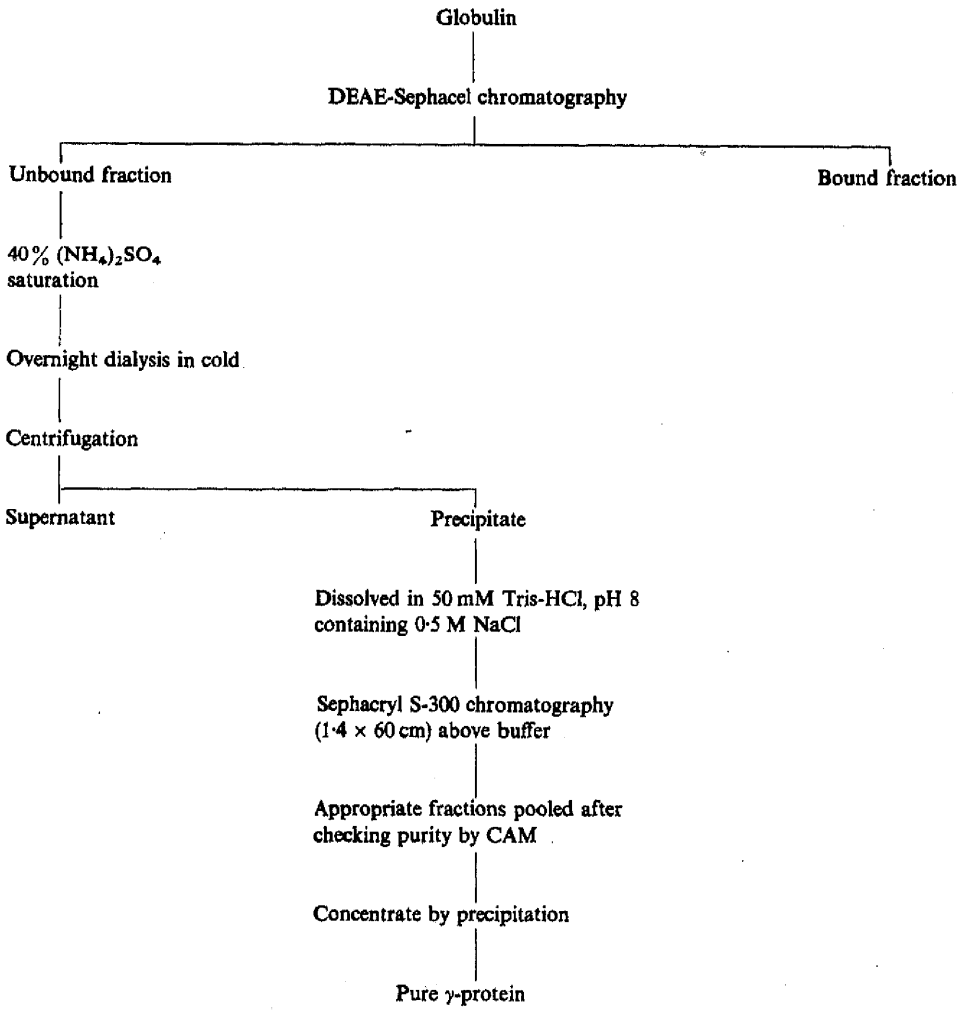

Figure 1. Outline of procedures used for preparation of γ-protein from globulin of pigeon pea seeds.

10 min. The supernatant was used for electrophoresis. For 7 and 14 DAF samples 10 μl of the supernatant and for all later samples 5 μl of the supernatant was used.

Sucrose density gradient centrifugation

Linear sucrose density gradients (10–30% (w/v) in 4·8 ml in volume) were prepared according to the method of Stone (1974). 2 mg protein sample (0·2 ml) in 0·05 M phosphate buffer, pH 7·2 containing 0·4 M NaCl was layered at the top. Centrifugation was for 16 h at 114,000 *g* and 4°C in a Beckman L5 65B ultracentrifuge. Sedimentation coefficients were determined as described by Martin and Ames (1961).

Column chromatography

A column of Sepharose 6B (1·6 × 56 cm) in 0·05 M Tris-HCl buffer, pH 8 containing 0·4 M NaCl was used for molecular weight determination by gel filtration. The column was eluted at a flow rate of 12ml/h and 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 volume (V_e) of standard proteins and a calibration graph was obtained by plotting V_e against the log molecular weight of the protein.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0·1 % sodium dodecyl sulphate (SDS) was carried out in 10% separating gel slabs (16 × 12 × 0·2 cm) following the method of Weber and Osborn (1969).

Amino acid analysis

The method of protein hydrolysis and analysis was as described earlier (Gopala Krishna *et al*., 1977).

Results

When globulin protein fraction in 15 mM phosphate buffer, pH 8 was loaded on a DEAE-Sephacel column equilibrated with the same buffer, the γ-protein did not bind to the ion-exchange resin. On adding ammonium sulphate to 40 % saturation the γ-protein precipitated. At this stage the γ-protein still had some contamination which were seen as fast moving zones on CAM. The contaminating protein could be removed

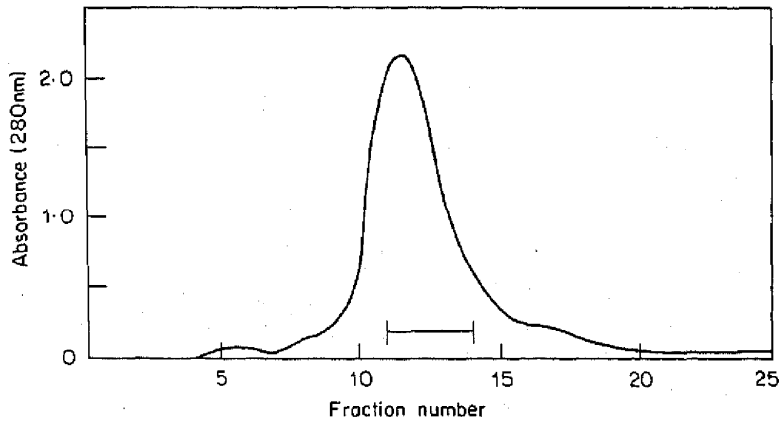

Figure 2. Elution profile of γ-protein from a Sephacryl S-300 column (1·4 × 60 cm). Column chromatography was performed in 0·05 M Tris buffer, pH 8 containing 0·5 M NaCl. The column was eluted at a flow rate of 11 ml/h and 4·4 ml fractions were collected. Absorbance at 280 nm was monitored in the different fractions. The horizontal bar indicates the fractions pooled for further analysis.

by passing through a Sephacryl S-300 column. The elution pattern of the protein is shown in figure 2. There was a small protein peak in the void volume and a major peak corresponding to the *γ*-protein fraction. The purity of each fraction in the peak region was checked by CAM electrophoresis.

The elctropherogram of globulin and the purified *γ*-protein after CAM electrophoresis is shown in figure 3. The purified *γ*-protein showed a single band and was free of the fast moving protein contaminants. The protein purity was also checked by gel electrophoresis in a continuous system. The *γ*-protein although gives one zone, does not travel very much at pH 7·2 because of the low charge to mass ratio (data not shown). Further evidence for the purity comes from the immunodiffusion studies where antibodies raised against purified *γ*-protein failed to crossreact with either purified legumin or vicilin (figure 4).

Precipitin patterns obtained after rocket immunoelectrophoresis of the protein extracts from seeds harvested at different periods after flowering are shown (figure 5).

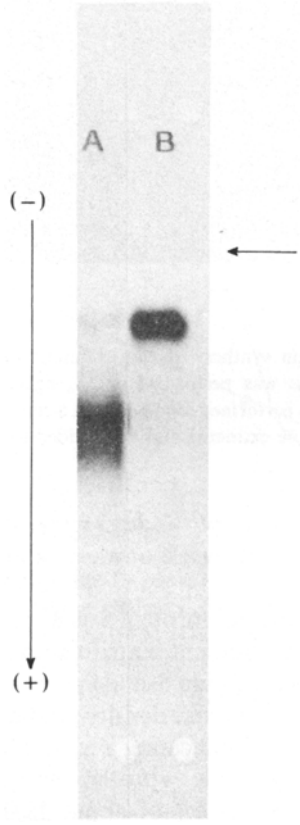

Figure 3. Electrophoresis on CAM of globulin and *γ*-protein from pigeon pea seed meal. Arrow indicates the origin. Migration was from cathode towards anode. The lanes are (A) globulin (B) *γ*-protein.

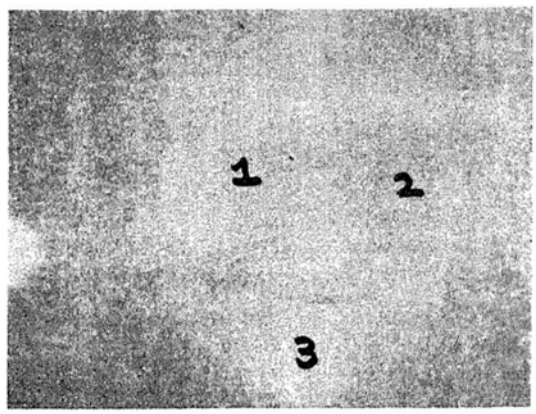

Figure 4. Specificity of antibodies raised against purified γ-protein. Double diffusion was performed in 1% agarose gel for 24 h. The central well contained the antibodies. The peripheral wells contained legumin (1), vicilin (2) and γ-protein (3).

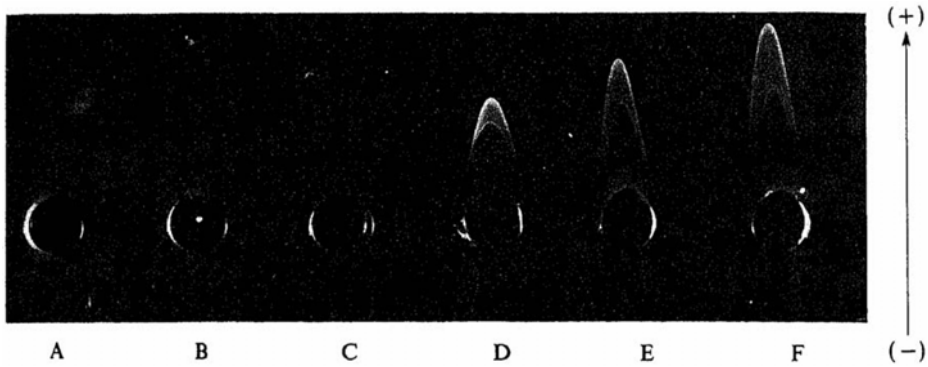

Figure 5. γ-Protein synthesis during different stages of seed development. Rocket immunoelectrophoresis was performed in 1% agarose gel containing 0·2% antisera (v/v). Electrophoresis was performed for 16–18 h at a constant voltage of 200 V. 7, 14, 21, 28, 35 and 42 day old seeds were extracted and were added to the wells (*a–f* respectively).

No precipitin band was observed upto about 3 weeks after flowering. The γ-protein was laid down in the seeds from 4th week onwards and the amount increased till seed maturity.

The molecular weight of the holoprotein was estimated by gel filtration chromatography and by sucrose density gradient centrifugation. On a Sepharose 6B column the γ-protein eluted as a single peak (figure 6) and the molecular weight was estimated to be about 90,000. The γ-protein on sucrose density gradient showed a peak (figure 7) with a sedimentation coefficient of 7S indicating a molecular weight (M_r) of about 130,000.

On SDS-polyacrylamide gels the γ-protein under dissociated but non reduced conditions gave a single band of M_r of about 52,000 while on reduction with 2-mercaptoethanol showed two subunits of molecular weight of about 32,000 and 20,000 (figured 8). Thus the interaction between the two subunits is through disulphide linkages.

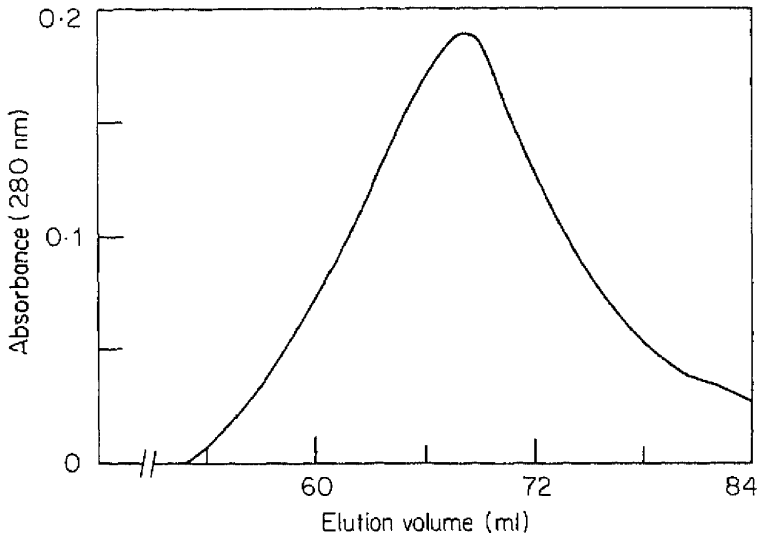

Figure 6. Elution profile of purified γ-protein from a Sepharose 6B column (1·6 × 56 cm). The column was eluted at a flow rate of 12 ml/h and 1·2 ml fractions were collected. Other details are as in figure 1.

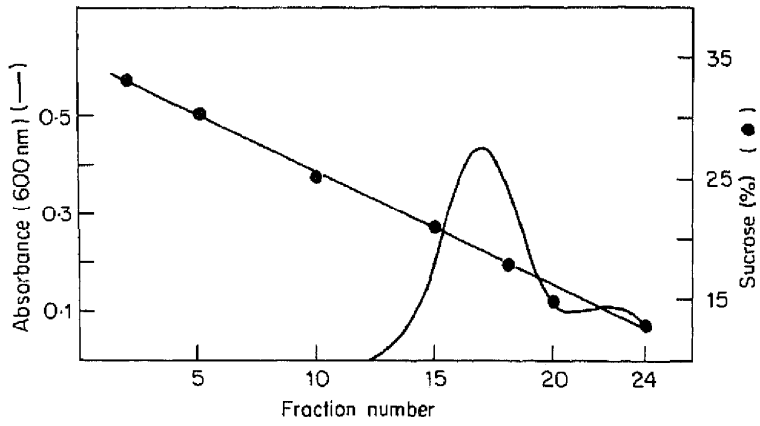

Figure 7. Separation of the purified γ-protein from pigeon pea seed meal according to its sedimentation velocity on sucrose gradients. Gradients were 10–30 % sucrose in 0·05 M phosphate buffer, pH 7·2 containing 0·4 M NaCl. Centrifugation was performed at 4°C for 16 h at 40,000 rpm in a Beckman ultracentrifuge. Protein in the fractions was estimated at 600 nm. (●), Indicates the sucrose gradient.

The sulphur containing amino acid composition of globulin and the purified fractions is shown in table 1. The γ-protein has about 4 and 3 times more total sulphur containing amino acids than legumin and vicilin respectively. Thus of the 3 globulin fractions, the γ-protein has the highest amount of total sulphur amino acids.

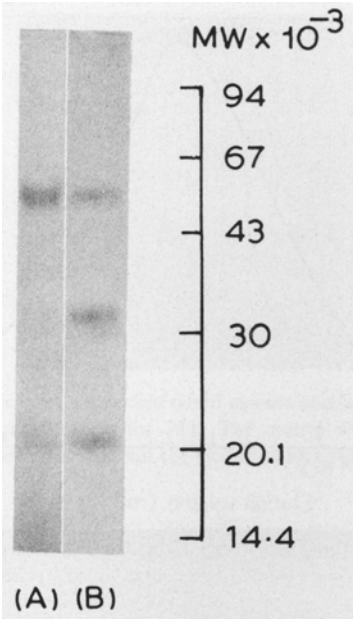

Figure 8. SDS-polyacrylamide gel (10%) electrophoresis of γ-protein in the absence of (A) and presence (B) of 2-mercaptoethanol. About 20 μg protein was loaded in each track after treatment with SDS. The numbers at the side indicate the position occupied by marker proteins.

Table 1. Amino acid composition of globulin and the γ-fraction (g/16 g N).

Amino acid	Globulin	γ-Protein
Lysine	4·40	3·17
Histidine	2·76	1·33
Ammonia	6·13	7·57
Arginine	4·40	2·59
Methionine sulfoxide	0·13	0·22
Aspartic acid	6·12	4·67
Threonine	2·67	2·26
Serine	3·43	2·85
Glutamic acid	11·15	5·03
Proline	3·32	2·70
Glycine	2·52	2·24
Alanine	2·99	2·28
Half Cystine	0·45	1·72
Valine	2·93	3·25
Methionine	0·81	0·85
Isoleucine	2·57	2·14
Leucine	5·27	3·65
Tyrosine	2·37	2·21
Phenylalanine	4·91	2·50
Methionine sulfone	0·18	0·33

Discussion

Pigeon pea is an important grain legume crop in India, parts of Africa and Latin America. We have purified and characterised a globulin protein in pigeon pea seeds which is termed *γ*-protein. This protein resembles the *γ*-congjutin from *Lupinus* (Blagrove and Gillespie, 1975). The molecular weight of *γ*-protein as estimated by gel filtration was 90,000 and this value is higher than the value obtained by adding the molecular weights obtained for the subunits by dodecyl sulphate gel electrophoresis. The *γ*-protein probably is present as a tetramer (M_r 100,000) consisting of two dimers (M_r 52,000) each which is made up of two subunits of molecular weights 31,900 and 20,000. Blagrove and Gillespie (1975) found that in aqueous solutions conglutin-*γ*, a protein resembling *γ*-protein, was made up of a number of polymeric species in contrast to other globulins. They could not find appropriate conditions of salt concentrations and pH to make conglutin-*γ* assume one polymeric size. A similar situation probably exists for the *γ*-protein of pigeon pea. However, under the experimental conditions employed for gel filtration, the *γ*-protein could be existing as a tetramer. This would account for the differences in the molecular weights observed in the different techniques.

Antibodies raised against the *γ*-protein has been used to develop a simple procedure to investigate the variation in the amount of *γ*-protein in the germplasm collection. So, now it is possible to use this technique in a breeding programme for selecting lines with increased amounts of *γ*-protein in pigeon pea.

References

Blagrove, R. J. and Gillespie, J. M. (1975) *Aust. J. Plant Physiol*., **2,** 13.
Gopala Krishna, T., Mitra, R. and Bhatia, C. R. (1977) *Qualitas plantarum*, **27,** 313.
Gordon, L. K. (1981) *J. Immunol. Meth*., **44,** 241.
Jurd, R. D. (1982) in *Gel Electrophoresis of Proteins: a practical approach* (eds B. D. Hames and D. Rickwood) (Oxford and Washington: IRL Press Ltd.) pp. 229.
Layne, E. (1957) in *Methods in Enzymology* (eds S. P. Colowick and N. O. Kaplan) (New York: Academic Press) Vol. 3, pp. 447.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem*., **193,** 265.
Martin, R. G. and Ames, B. N. (1961) *J. Biol. Chem*., **236,** 1372.
Stone, A. B. (1974) *Biochem. J*., **137,** 117.
Weber, K. and Osborn, M. (1969) *J. Biol. Chem*., **244,** 4406.