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ARACHIN POLYMORPHISM IN GROUNDNUT (*ARACHIS HYPOGAEA* L.)

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Key Word Index—*Arachis hypogaea*; Leguminosae; groundnut; storage protein; arachin; variation.

Abstract—Arachin, the major seed storage protein of groundnut, showed polymorphism. The polymorphic forms were due to differences in molecular size, net charge and polypeptide composition of the native protein. Purified arachin at low ionic strength resolved into monomeric and dimeric forms both on sucrose density gradient centrifugation and cellulose acetate membrane (CAM) electrophoresis. The dimers had more net negative charge compared with the monomers. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of arachin from the cultivar Spanish improved (SP) under non-reducing conditions showed three major components of M_r 70.7, 63.8 and 60.9 k. Arachin from Trombay Groundnut 1 (TG-1) showed three components of M_r 70.7, 63.8 and 59.5 k while in TG-18, a derivative of a cross between SP and TG-1, there were only two components of M_r 70.7 and 63.8 k. Data from two dimensional gel electrophoresis in the presence and absence of 2-mercaptoethanol shows that each of the above components in turn consists of subunit pairs that are held together by disulphide linkages. The M_rs of the major polypeptides for the three arachins is as follows: SP, 47.5, 45.1, 42.6 and 21.4 k; TG-1, 47.5, 45.1, 41.2 and 21.4 k; TG-18, 47.5, 45.1 and 21.4 k. Two dimensional gel electrophoresis (IEF and SDS-PAGE) indicates that the arachin subunits consist of two major groups—the acidic polypeptides (three in SP, TG-1 and two in TG-18) and the basic polypeptides (three in SP, TG-1 and two in TG-18). The acidic polypeptides did not show charge variation while the basic polypeptides were charge heterogeneous. Absence of both an acidic polypeptide along with a basic polypeptide in TG-18 suggests that the acidic and basic polypeptides are probably products of the same gene and arise as a result of post-translational cleavage. Antibodies raised against purified arachin from SP reacted with arachin from TG-1 and TG-18 showing similar antigenic determinants. The acidic polypeptides show considerable homology in their structure as revealed by peptide mapping patterns.

INTRODUCTION

In groundnut about 87% of the seed protein is globulin [1, 2] consisting of two major fractions, arachin and conarachin [3], which correspond to legumin and vicilin, respectively, of other legume seeds. Arachin comprises up to 73% of the extractable proteins [4]. The structure, composition, sub-cellular location, association–dissociation and quaternary structure of arachin have been investigated over the last three decades [1, 5–7]. Two forms of arachin having sedimentation coefficients of 9S and 14S and M_rs of 180 and 350 k, respectively, have been demonstrated [4]. The monomeric form (9S) undergoes reversible interconversion to 14S by changes in the ionic strength or pH while the native dimeric form (14S) remains unchanged. The native monomer/dimer ratio is cultivar specific [4]. The monomeric and the dimeric forms of arachin have similar amino acid composition and identical subunit pattern consisting of six major subunits [8]. The six subunits (S_1–S_6) are classified into two groups of hydrophilic (S_1, S_2 and S_3) and hydrophobic (S_4, S_5 and S_6) subunits. Apparently there are no disulphide bonds between the members of the two groups [4, 8].

Three polymorphic forms A, B and A_1 were reported by examining the electrophoretic pattern of single seeds [6]. The polypeptide composition of the three forms was as follows: A, $\alpha_2\beta_2\gamma\delta$, B, $\beta_4\gamma\delta$ and A_1, $\alpha_4\gamma\delta$ [9]. The M_rs of α and β subunits were 35 k and of γ and δ 10 k each. All the three native forms of arachin had a M_r of 170 k. They had similar amino acid composition. The A_1 form was isolated from an African variety and had faster mobility than those of A or B forms. However, the inheritance of the polymorphic forms of arachin could not be investigated. The total protein extract of cv. Early Bunch showed three molecular variants of arachin and the variation was attributed to subunit exchange among arachin polypeptides during protein preparation [10]. Genotype-specific polypeptide patterns were also observed following two-dimensional electrophoresis of total groundnut protein [11]. Some groundnut varieties lack an arachin polypeptide of M_r 36 k and its presence or absence was correlated with the degree of blanchability [12].

Earlier we reported four classes of arachin polypeptide patterns after screening about 90 genotypes by SDS-PAGE [13]. We have studied three representative polymorphic forms of arachin. The variations were due to differences in size and number of arachin polypetides. In this paper we report (i) charge and size variation of native arachin and the subunits, (ii) homology of the different arachin polypeptides and (iii) presence of disulphide linkages between the arachin polypeptides.

RESULTS

The electropherograms of the arachin fraction at various stages of purification are shown in Fig. 1. The identity of arachin and conarachin subunits was es-

tablished as in refs [13, 14]. Traces of conarachin were seen in calcium-precipitated arachin though it formed only a small fraction (Fig. 1, track b). This indicated that the precipitation of arachin by $CaCl_2$ was not complete (Fig. 1, track c). This small amount of conarachin contamination could be removed by ion exchange chromatography (Fig. 1, track d). Conarachin does not bind to DEAE-Sephacel at 0.15 M NaCl while arachin protein is eluted from the column around 0.22 M (monomer) and 0.26 M NaCl (dimer). Arachin protein after DEAE-Sephacel was passed through a Sephacryl S-300 column at high salt concentration to obtain a homogeneous preparation. A single protein peak having the same polypeptides as the protein from DEAE-Sephacel was obtained (Fig. 1, track e). The affinity purified arachin also showed a pattern similar to Fig. 1, track e.

After electrophoresis on CAM at low ionic strength (10 mM NaPi buffer) two well separated zones could be seen (Fig. 2, tracks a, b and c) but at high ionic strength (0.3 M NaPi buffer) there was only one zone (Fig. 2, tracks d, e and f). The faster migrating zone is the arachin dimer while the slower moving zone is the monomer. The dimer and monomer proteins were identified by electrophoresing purified monomer and dimer arachin proteins obtained after sucrose density gradient centrifugation following the methods described in ref. [4]. Thus in 10 mM NaPi buffer, pH 7.9, the arachin dimer had more net negative charge as compared to the monomer. The dissociation–association behaviour of arachin was also shown by sucrose density gradient centrifugation following the methods of ref. [4]. At low ionic strength the arachin resolved into two peaks corresponding to 9.6S and 14.1S while at high ionic strength only one peak with 14.1S was observed (data not shown). The arachin monomer and dimer from the three genotypes studied showed charge variations. Compared with SP and TG-18 dimers the TG-1 dimer had more net negative charge. Charge differences in the arachin monomer were also observed among the three genotypes. There were differences in the intensity of staining in the monomer and dimer of different genotypes.

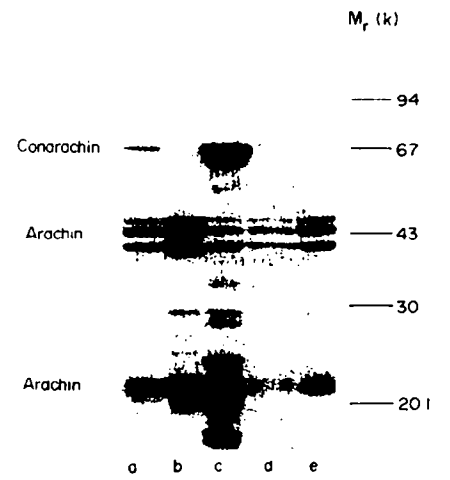

Fig. 1. SDS-PAGE (12%) of (a) 10% NaCl extract of seed meal, (b) crude arachin obtained by $CaCl_2$ precipitation, (c) crude conarachin obtained in the supernatant after $CaCl_2$ precipitation, (d) arachin after DEAE-Sephacel, (e) arachin after Sephacryl S-300. The numbers at the side indicate the position occupied by marker proteins of these M_rs.

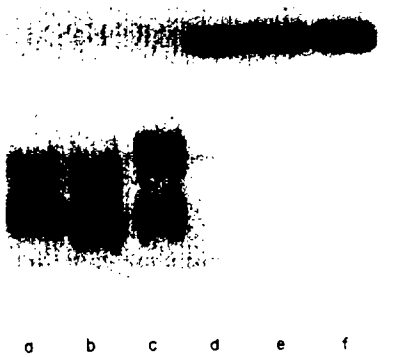

Fig. 2. Electrophoresis on CAM of arachin from SP, TG-1 and TG-18 at 10 mM NaPi buffer, pH 7.9 (a, b and c, respectively) and at 0.3 M NaPi buffer, pH 7.9 (d, e and f, respectively). Arrow indicates the origin. Migration was from cathode towards the anode.

The purified arachin protein was subjected to native PAGE to check the purity and to estimate the M_r of the native protein. The electrophoretic profile of purified arachin from the three genotypes on a native, continuous, gradient-PAGE is shown in Fig. 3. In all the three genotypes two main zones of protein could be seen. With reference to standard proteins the respective M_rs of the monomer and dimer were calculated as follows: TG-18, 220 k and 413 k; SP, 185 k and 378 k; TG-1, 170 k and 350 k.

When the arachin protein was subjected to SDS-PAGE under non-reducing conditions three major components in SP and TG-1 and two major components in TG-18 were observed (data not shown). The components of SP were estimated to have M_rs of *ca* 70.7, 63.8 and 60.9 k, while in TG-1 the M_rs were 70.7, 63.8 and 59.5 k. In TG-18 the two components had M_rs of 70.7 and 63.8 k. Thus compared to SP the third subunit in TG-1 is altered from 60.9 to 59.5 k and is absent in TG-18. The electrophoretic pattern obtained under reduced conditions is shown in Fig. 4. There were four major polypeptides in SP with M_rs of 47.5, 45.1, 42.6 and 21.4 k (Fig. 4, track a). In TG-1 the pattern was similar to SP except that the 42.6 k polypeptide was absent and there was a polypeptide of M_r 41.2 k (Fig. 4, track b). In TG-18 there were only three major polypeptides of M_r 47.5, 45.1 and 21.4 k (Fig. 4, track c).

Separation of SP arachin on two-dimensional electrophoresis is shown in Fig. 5. Each of the three subunits of M_r 60–70 k in the first dimension (unreduced condition) was resolved into two groups of polypeptides of M_r of *ca* 45 k and 21 k in the second dimension (reduced condition). This suggests that the polypeptides at 45 k were linked to the polypeptides at 21 k by disulphide linkages.

When urea was included in SDS-PAGE the polypeptide pattern in the regions of 45 and 21 k were altered (Fig. 6). The three polypeptides around 45 k region in SP and TG-1 resolved into five discrete bands while the 21 k polypeptide showed two distinct bands. In TG-18 the 45 k

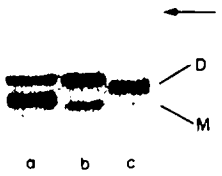

Fig. 3. Electrophoretic separation of arachin from (a) TG-18, (b) SP and (c) TG-1 on a 4–30% nondenaturing polyacrylamide gel in 10 mM NaPi buffer, pH 7.9. The arrow indicates the origin. D denotes the arachin dimer and M the monomer.

region showed three polypeptides corresponding to the slow mobility bands of SP and TG-1 and only one band in the 21 k region. Urea incorporation into SDS-PAGE also revealed some minor polypeptides which were not observed in SDS-PAGE.

The two dimensional (IEF-SDS-PAGE) separation of arachin from SP and TG-18 is shown in Fig. 7. In SP there were polypeptides around M_r 45 k with p*I*s of 4.7, 5 and 5.5 while in TG-18 there were only two polypeptides with p*I*s of 5 and 5.5. These polypeptides did not show any charge heterogeneity. There were three major polypeptides around M_r 21 k in SP (p*I* 6.3, 7 and 8.2) and only two major polypeptides in TG-18 (p*I* 7 and 8.2). These

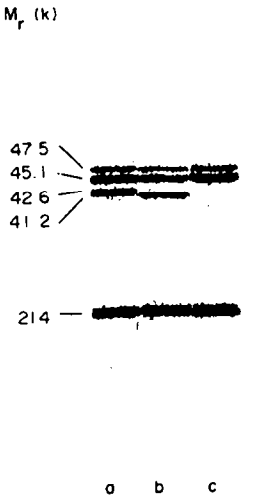

Fig. 4. SDS-PAGE (12%) of arachin from (a) SP, (b) TG-1 and (c) TG-18. The numbers at the margin indicate the M_r of the arachin polypeptides.

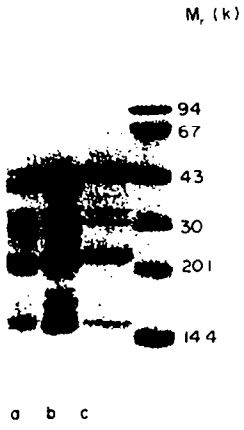

Fig. 6. Urea SDS-PAGE (12%) of arachin from (a) SP, (b) TG-1 and (c) TG-18. The numbers at the margin indicate the M_r of the standard proteins.

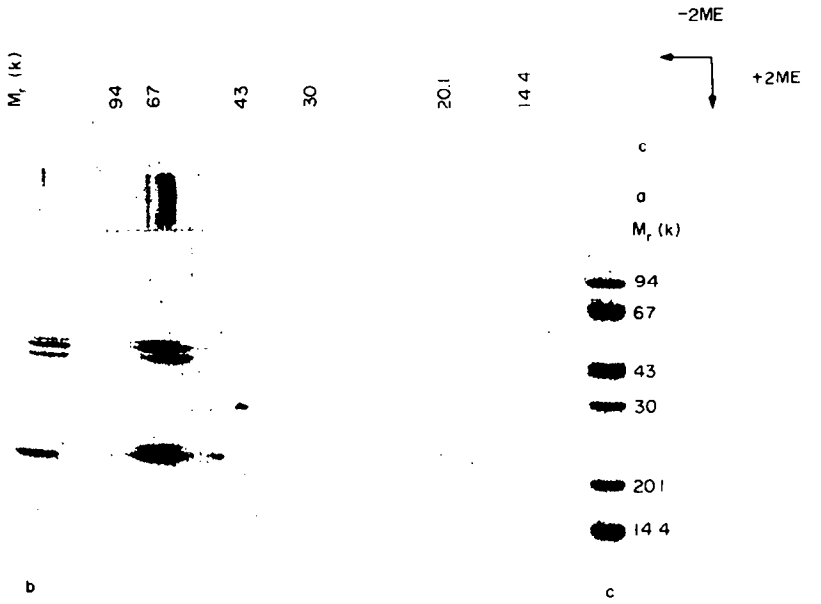

Fig. 5. Two-dimensional SDS-PAGE of SP arachin. First dimension (horizontal axis) unreduced (−2ME) and second dimension (vertical axis) reduced (+2ME) conditions. Lane (a) shows arachin after the first dimension. Lane (b) shows pattern of reduced arachin. Lane (c) indicates M_r markers.

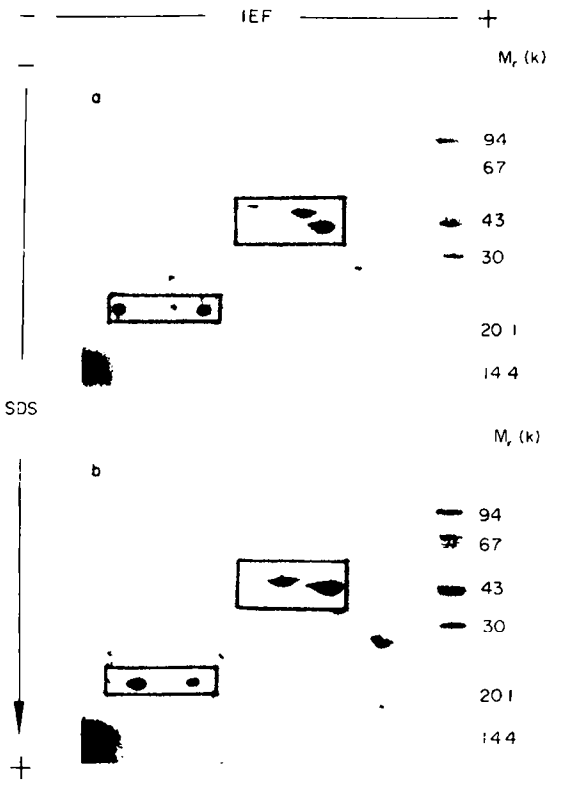

Fig. 7. Two-dimensional isoelectric focusing/SDS-PAGE of arachin from (a) SP, (b) TG-18. The major polypeptides are shown in the box. The track at the right hand side shows M_r markers.

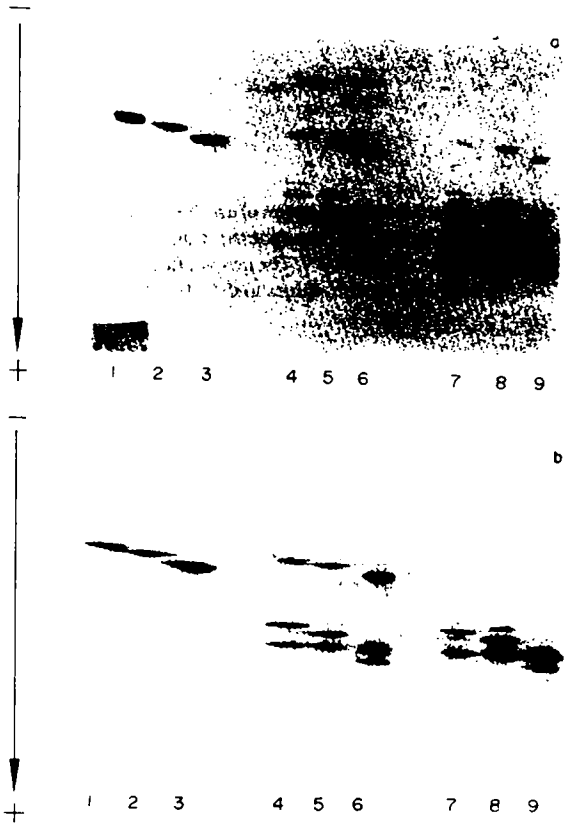

Fig. 8. Digestion of individual SP arachin polypeptides on 10–20% SDS-PAGE with (a) 1 μg (lanes 4–6) and 5 μg (lanes 7–9) of chymotrypsin (b) 1 μg (lanes 4–6) and 2.5 μg (lanes 7–9) of papain. Lanes 1, 2 and 3 indicate undigested individual polypeptides.

polypeptides, although they did not show size variation, did reveal charge heterogeneity.

Antibodies raised against purified arachin from SP showed precipitin formation against arachin from TG-1 and TG-18 indicating the presence of similar antigenic determinants (data not shown).

The peptide mapping pattern of the three SP acidic polypeptides is shown in Fig. 8. The patterns after chymotrypsin and papain treatment showed considerable similarity. The peptide mapping pattern of the arachin third polypeptide from SP (42.6 k) and TG-1 (41.2 k) is shown in Fig. 9. Each of the four enzymes used produced a specific cleavage pattern, as expected. However, a specific enzyme produced a nearly identical fragmentation pattern of the polypeptides. Of the four enzymes used, trypsin showed retarded enzymatic activity, probably because of the presence of 0.1% SDS. However, the same tryptic digestion pattern was observed in replicated runs. This caution was necessary in view of the recent finding of the distortion of the tryptic peptide pattern in the presence of varying amounts of SDS [15].

DISCUSSION

The amounts of monomer and dimer vary in the genotypes studied as judged by the band intensity (Fig. 2, tracks a, b and c). A similar observation was made earlier [4]. The biological significance of this variation in the relative amounts of monomer and dimer of arachin is not known. The dimers are resistant to proteolysis while the monomers are easily modified by autoproteolysis upon storage [16]. We have also observed that during storage of protein preparation arachin monomer can undergo limited proteolysis as seen on an SDS-PAGE while still retaining a 9.6S sedimentation coefficient on a sucrose density gradient. Therefore a fresh preparation of arachin is necessary to calculate the monomer and dimer ratios. In addition, proteolytic inhibitors like PMSF are necessary during protein extraction. A serine type proteinase activity has been reported in groundnut cotyledons [14].

The dissociation–association behaviour between dimer and monomer could be detected by CAM electrophoresis. The monomer and dimer could be resolved at low ionic strength while all the monomers could be associated into dimer form at high ionic strength. This observation is an extension of the earlier work where sucrose density gradient centrifugation was used at varying ionic strength and pH [4, 17].

Both the monomer and dimer show polymorphism at charge as well as size level. Earlier charge variation was detected in an African cultivar [9]. The charge variation could be due to altered amino acid composition. The M_r variation of the monomer is due to number, size and proportion of the constituent polypeptides of the arachin. Consequently, the differences are reflected in the respective dimers. It is apparent from the data that in TG-1 the

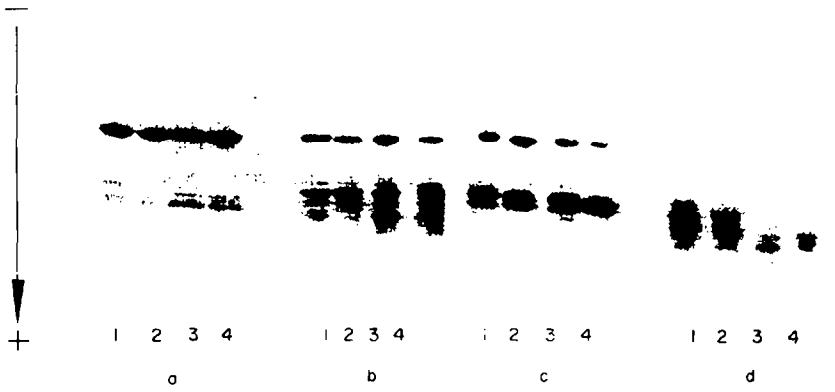

Fig. 9. Digestion of the third polypeptide of arachin from SP (42.6 k) and TG-1 (41.2 k) on 10–20% SDS-PAGE with (a) 2.5 μg trypsin (lane 1 SP and lane 2 TG-1) and 10 μg trypsin (lane 3 SP and lane 4 TG-1). (b) 2 μg of chymotrypsin (lane 1 SP and lane 2 TG-1) and 5 μg chymotrypsin (lane 3 SP and lane 4 TG-1). (c) 1 μg papain (lane 1 SP and lane 2 TG-1) and 2.5 μg papain (lane 3 SP and lane 4 TG-1). (d) 0.2 μg proteinase k (lane 1 SP and lane 2 TG-1) and 2 μg of proteinase k (lane 3 SP and lane 4 TG-1).

arachin polypeptide (41.2 k) is smaller in size compared with SP and is totally absent in TG-18. In addition to this size variation there is variability in the proportion of the different polypeptides of the arachin [13]. The arachin polymorphism observed has a genetic basis [13]. The TG-18 arachin polypeptide pattern is a result of a selection from a cross between SP and TG-1 [13].

The legumin type proteins are post-translationally cleaved to yield the disulphide-bonded acidic and basic polypeptides that are characteristic of the mature native protein [18–20]. However, in arachin, the presence of disulphide linkages has not been reported [6, 8]. From our data it is clear that the polypeptides of the arachin subunit are held by disulphide linkages. There is only one structural gene for a legumin subunit that codes for both the acidic and basic polypeptide [21]. A similar situation could exist in groundnut also. In the two dimensional pattern of TG-18 arachin, the absence of an acidic polypeptide is accompanied by the absence of a basic polypeptide. This is also observed in urea SDS-PAGE patterns where only one polypeptide is present in TG-18 (around M_r 21 k) compared with two polypeptides in SP and TG-1. The multiplicity of basic polypeptides of glycinin was also observed in urea SDS-PAGE [22]. Although the arachin protein from the three genotypes studied shows charge and size heterogeneity at the native protein level as well as at polypeptide level, they show immunological cross reactivity suggestive of similar antigenic determinants.

Among the pea and soyabean acidic polypeptides and the phaseolin polypeptides, a considerable degree of internal homology has been shown [21, 23]. This could be interpreted as a result of gene duplications from an ancestral gene [21]. It was shown earlier that there are at least four independent genes coding for the arachin subunits [13]. The present data show that the major differences are recorded in the acidic polypeptides while the basic polypeptides do not show any size differences. Since the 45.1 k polypeptide is present in all the 90 genotypes studied as well as in some wild *Arachis* species [13], it could be speculated that the gene coding for this polypeptide is the ancestral one from which the other genes could have been derived. The peptide fragmentation pattern indicates that not only there is similarity between the three arachin polypeptides of SP but also between the third polypeptide of arachin of SP and TG-1, although the genes coding for these polypeptides are non-allelic [13]. This is probably suggestive of a common origin for all the different arachin genes.

EXPERIMENTAL

The seeds of genotypes SP, TG-1 and TG-18 were obtained from Mr. S. H. Patil (BARC). Testa from mature seeds was removed manually and the seeds were defatted with *n*-hexane by grinding. The defatted meal was air-dried and passed through 40 mesh sieve before protein extraction.

Defatted meal was extracted with 10% NaCl (meal to solvent ratio 1:10) containing 2 mM PMSF. Extraction was done at room temp. (25°) for 3 hr with continuous agitation. The slurry was passed through 4 layers of cheesecloth and the filtrate was centrifuged at 20 800 *g* at 10° for 10 min. The supernatant was used for crude arachin preparation according to ref. [6] except that the protein in the final stage was loaded onto an ion exchange column instead of freeze drying.

Purification of crude arachin was performed on a DEAE-Sephacel column (2.6 × 13 cm). The column was equilibrated with 10 mM NaPi buffer, pH 7.9 containing 0.15 M NaCl. Crude arachin (300–450 mg) in the same buffer was loaded onto the column. The bound protein was eluted from the column with a linear NaCl gradient (0.15–0.4 M, total vol. 500 ml). The column was eluted at a flow rate of 26 ml/hr and 5 ml fractions were collected. The salt concn was determined by conductivity measurement in every 5th fraction after appropriate dilution. Protein in the fractions was determined as in ref. [24]. The appropriate fractions were pooled and arachin precipitated by dialysis in cold against 25 mM citrate Pi buffer, pH 4.7. Precipitated arachin was collected by centrifugation at 12 000 *g* for 15 min and the pellet dissolved in 10 mM NaPi buffer, pH 7.9.

Gel filtration was performed on a Sephacryl S-300 column (2 × 49 cm). The column was equilibrated with 0.3 M NaPi buffer, pH 7.9. The arachin protein obtained from the DEAE-Sephacel column was loaded onto this column. The column was eluted at a flow rate of 30 ml/hr and 6 ml fractions were collected. Protein in the fractions was estimated [24]. The appropriate

fractions were pooled and the protein was recovered by dialysis against 25 mM citrate Pi buffer, pH 4.7. The protein pellet after centrifugation was dissolved in 10 mM NaPi buffer, pH 7.9 and kept frozen in small aliquots till further use.

During the latter part of the work, arachin was routinely prepared from crude arachin by immunoaffinity chromatography. Anti-arachin antibodies raised against purified SP arachin was linked to CNBr-activated Sepharose 4B following the procedures recommended by Pharmacia. For bound arachin elution 0.15 M NaCl, pH 11 (adjusted with NH_3), was used instead of the usual 0.2 M glycine, pH 2.5. The arachin protein was precipitated by dialysis against 25 mM citrate Pi buffer, pH 4.7.

Electrophoresis on CAM was carried out in a Beckman microzone electrophoretic apparatus [25]. The electrophoretic buffers used were either 10 mM or 0.3 M NaPi buffer, pH 7.9. 2 μg protein was applied with the help of the sample applicator from a stock soln of 8 mg/ml. Electrophoresis was carried out at room temp. (25°) at a constant voltage of 200 V for 10 min (10 mM buffer) or 50 V for 40 min (0.3 M buffer). After electrophoresis the membranes were stained for about 5 min in 0.2% Coomassie brillant blue R-250 dissolved in MeOH–HOAc–H_2O (45:10:45) and destained in a soln consisting of MeOH–HOAc–H_2O (30:10:60).

Gradient gels, 4–30% polyacrylamide concn, were prepared using a gradient mixer (GM-1) and gel casting apparatus (GSC-2) from Pharmacia. The gel contained 10 mM NaPi buffer, pH 8. About 20 μg protein was loaded in each track. Electrophoresis was performed at a constant voltage for *ca* 2000 V-hr (Pharmacia GE 2/4 apparatus). Protein bands after electrophoresis was visualized by Coomassie brilliant blue R-250 staining. Reference proteins applied to the gradient gels were: thyroglobulin (669 k), ferritin (440 k), catalase (232 k), bovine serum albumin (67 k) and ovalbumin (45 k).

SDS-PAGE was performed in a 12% homogeneous, discontinuous polyacrylamide gel [26]. 20 μg protein pretreated (with or without 2-mercaptoethanol) was loaded in each track. Electrophoresis was performed at a constant voltage for 650–700 V-hr (Pharmacia GE 2/4 apparatus). Gels after electrophoresis were fixed, stained in 0.2% (w/v) Coomassie brilliant blue R-250 in MeOH–HOAc–H_2O (30:10:60) and destained in the same solvent without the dye. The apparent M_rs of the polypeptides were determined from a calibration graph prepared using the Pharmacia M_r calibration kit. SDS-PAGE in the presence of urea was performed according to ref. [22].

Two-dimensional gel electrophoresis was performed following the procedures given in the polyacrylamide gel electrophoresis manual by Pharmacia. For isoelectric focusing, protein samples were treated with 12 M urea and 10% 2-mercaptoethanol (1:1) for 2 hr at room temp. (25°). 30 μg protein was subjected to focusing in tubes (6 × 110 mm) containing 6% acrylamide (stock soln 29.2% acrylamide and 0.8% bis-acrylamide), 6 M urea and 5% pharmalyte (pH 3–10). The cathode contained 1 M NaOH and the anode was 0.1 M H_3PO_4. Prefocusing was for 2 hr at 250 V and focusing was performed for a total of 4500 V-hr. The gel in the second dimension was a gradient gel from 10 to 20%.

For peptide mapping of arachin acidic subunits, two cycles of electrophoresis was used. In the first cycle after brief staining gel pieces containing individual arachin polypeptides were obtained. In the second cycle the individual polypeptides were proteolytically digested on top of a second gel, essentially following the method described in ref. [27].

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