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# MOLECULAR REASSESSMENT OF RELATIONSHIPS WITHIN *Vicieae* USING ELECTROPHORETIC AND IMMUNOCHEMICAL TECHNIQUES

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## ABSTRACT

An array of electrophoretic and immunochemical techniques was used to investigate legumins, vicilins, and albumins of seed storage proteins of pea *(Pisum sativum)*, bean *(Vicia faba)*, lentil *(Lens esculentum)* and chick pea *(Cicer arientinum)* to delimit the boundary of the tribe *Vicieae* and to clarify the systematic position of the genus *Cicer*. The band patterns of the legumins of these species were broadly similar in having bands at Mr 60,000 under non-reducing conditions and two sets of bands, at Mr approximately 40,000 and 20,000 under reducing conditions. And the band patterns of the vicilins were quit similar showing bands at Mr approximately 71,000 (convicilin) and 50,000 (vicilin). Serologically, the legumins of *V. faba* and *L. esculentum* gave a total identity with *P. sativum* legumin antiserum under non-reducing conditions, whereas the legumin of *C. arientinum* exhibited a partial identity, which was attributed to the failure of the low molecular sub-unit pair (Mr 33,000) to react with *Pisum* legumin antiserum. The vicilins of *V. faba, L. esculentum* and *C. arientinum* had a partial identity with vicilin of *Pisum sativum*. The electrophoretic patterns of *V. faba, L. esculentum* and *C. arientinum* albumins were markedly different. However, they gave a positive reaction with *Pisum* major albumin antiserum (Mr 25000). Immunochemically, the vicilin and legumin of *Cicer* were more related to those of *Vicieae* than to those of *Phaseoleae, Glycineae, Cajaneae, Diocleae*. Thus, the data presented in this work suggested the classification of *Cicer* under *Vicieae* rather than a separate tribe *Cicerideae*.

*Key Words*: Immunodiffusion, legumins, tribe *Vicieae*, vicilins, Western blotting

## RÉSUMÉ

Une sélection des techniques électrophorétique et immunochimiques était utilisée pour examiner les légumines, vicilines, et albumines des protéines des graines de petit pois *(Pisum sativum)*, haricot *(Vicia faba)*, lentille *(Lens esculentum)* et culin *(Cicer arientinum)* pour limiter les frontières de la tribu *Vicieae* et pour clarifier la position systématique de la variété *Cicer*. Les bandes prototypes des légumines de ces espèces étaient généralement similaires en ayant des bandes à Mr 60.000 sous les conditions de non atténuation et deux groupes des bandes, à Mr 40.000 et 20.000 approximativement sous les conditions d'atténuation. Et les bandes prototypes des vicilines étaient un peu similaires montrant des bandes à Mr 71.000 (conviciline) et 50.000 (viciline) approximativement. Sérologiquement, les légumines de *V. faba* et *L. esculentum* ont donné une identité totale avec la légumine antisérum de *P. sativum* sous les conditions de non atténuation, tandis que les légumines de *C. arientinum* ont exhibé une identité partielle, laquelle, était attribuée à l'échec de la sous unité paire moléculaire basse (Mr 33.000) de réagir avec le *Pisum sativum* antisérum. Les vicilines de *V. faba, L. esculentum* et *C. arientinum* avaient une identité partielle avec la viciline de *Pisum sativum*. Les prototypes électrophorétiques des albumines de *V. faba, L. esculentum* et *C. arientinum* étaient clairement différents. Cependant, ils avaient donné une réaction positive avec le *Pisum*, albumine antisérum majeure (Mr 25.000). Les immunochimiques, la viciline, et légumine de *Cicer* étaient plus relatées a celles de *Vicieae* que celles de *Phaseoleae, Glycineae, Cajaneae, et Diocleae*. Ainsi, les données présentées dans ce travail suggèrent la classification de Cicer sous Vicieae plutôt qu'une tribu *Cicadaire* séparée.

*Mots Clés*: Immunodiffusion, légumines, tribu *Vicieae*, vicilines, tache occidentale

## INTRODUCTION

Vicieae was first delineated as a "section", including *Aphaca*, *Cicer*, *Clymenum*, *Ervum*, *Lathyrus*, *Lens*, *Nissolia*, *Orobus*, *Pisum* and *Vicia*. Later on, it was given tribal rank (De Candolle, 1825), whose members include *Cicer* L., *Ervum* L., *Faba* L., *Lathyrus* L., *Orobus* L, *Pisum* L., and *Vicia* L. However, the number of genera into which the species of *Vicieae* are grouped has tended to decrease with time. In 1865, Bentham recognized six genera in the tribe *Vicieae*: *Cicer* L., *Vicia* L., *Lens* Miller, *Lathyrus* L., *Pisum* L. and *Abrus* L. The morphological, anatomical and karyological data advocated that *Abrus* should be excluded from the tribe *Vicieae* and placed in its own tribe *Abreae* (Streicher, 1902; Popov, 1928; Senn, 1938; Dormer, 1946; Hutchinson, 1964; Heywood 1971).

To date, the generic limits in the tribe *Vicieae* are still a matter of debate. This debate is essentially concentrated on the position of the *Cicer* within the tribe. Following anatomical, morphological, pollen grain morphology, karyological, isoflavonoid, and isoenzymatic data, it was suggested that *Cicer* must be assigned to a separate tribe, the *Ciceritleae* (Kupicha, 1977; Gapochka 1984). This was recently supported by further morphological, anatomical, karyological, and chemotaxonomical characteristics (Endo and Ohashi, 1997) and molecular phylogenies based on matK sequences (Wojciechowski *et al.*, 2000). Further, immunological and electrophoretic data of the total seed proteins of the members of the tribe *Vicieae* including *Cicer* displayed high similarity, supporting that *Cicer* should be included in *Vicieae* (kloz and Turkova, 1963; Simola, 1969; Tarlakovskaya, 1974; Gavriljuk, 1974; Tarlakovskaya, 1975; Cristofolini, 1981; Sammour, 1985). Furthermore, the data of DNA/DNA hybridization of 5 day-old seedlings of the members of the tribe *Vicieae* supported this conclusion (Sammour, 1991).

Therefore, this study aimed to use electrophoretic and immunological properties of the globulins (legumin, vicilin and convicilin) and albumins of seed storage proteins of members of the tribe Vicieae to further examine the relationships of the genus *Cicer* with the tribe *Vicieae*.

## MATERIALS AND METHODS

**Sources of samples.** All samples were obtained as dried seeds. *Vicia faba* L, Var. Giza 3, pea *(Pisum sativum)*, Var. Progress 9 and Victory, lentil *(Lens esculentum)*, Var. Giza 9, chick pea *(Cicer arientinum* L.), Var. Partly 2, were obtained from Agriculture Research Center, El Dokki, Egypt; Jack bean *(Canavalia ensiformis)* from Sigma Chemical Co., Poole, Dorest. U.K.; soybean *(Glycine max.)*, kidney bean *(Phaseolus vulgaris)*, scarlet runner bean *(Phaseolus coccineus)*, hyacinth bean *(Dolichos lablab)*, pigeon bean *(Cajanus cajan)* and sweet pea *(Lathyrus odoratus)* from the seed herbarium of Dr. P. Gates, Department of Biological Science, Durham University, DH1 3LE, UK; cowpea *(Vigna unguiculata* (L.) Walp) variety from the International Institute of Tropical Agriculture, c/o LW Lambourn & Company LTD, Carolyn House, 26 Dingwall Road, Croydon CR9 3EE, UK. All chemicals used in this experiment were purchased from Sigma Chemical Co., Poole, Dorest. U.K.

**Preparation of meals.** Seeds were freeze dried for 30 min to remove surface moisture, finely ground using a Janke and Kunkel water-cooled mill and the flour was sieved through a 365 µm mesh Endicott sieve. Defatting was carried out by two hexane extractions (10 mL hexane/g meal) for 30 min at 0°C. After filtering off the hexane, the meal was air dried under vacuum and kept at 4°C for further use.

**Preparation of extracts.** The air-dried meal was extracted with 50 mM sodium borate buffer pH 8.0 (10 mL buffer/g meal) at 4 °C for 1 h, then centrifuged at 10,000 g for 20 min. The resulting precipitate was discarded and the supernatant was used for ammonium sulfate fractionation, gel electrophoresis or to prepare globulin fraction.

**Ammonium sulfate preparation.** Total protein extract was fractionated by ammonium sulfate precipitation at 50, 70 and 90% relative saturation. The precipitates were collected by centrifugation at 10,000 g, then re-dissolved in 20 ml 0.05 M sodium borate buffer, pH 8.0. The supernatant at 90% relative saturation with ammonium sulfate

and the re-dissolved precipitates were extensively dialysed against 5 mM sodium borate buffer pH 8.0.

**Separation of the globulin fraction.** Total protein extract was extensively dialyzed against 33 mM sodium acetate buffer, pH 4.7 for 24 hours. The precipitated globulin proteins were separated from albumin proteins still in solution by centrifugation at 12,000 g, re-dissolved by suspending the pellet in water, and then dialyzed against 0.05 M sodium borate buffer, pH 8.0, prior to freeze drying.

**Separation of the albumin fraction.** Total extract was extensively dialyzed against 33 mM sodium acetate buffer, pH 4.7. The supernatant which contains albumin was separated from the globulin by centrifugation at 12,000 g, extensively dialysed against distilled water and then freeze-dried.

**Purification of legumins and vicilins.** Legumins and vicilins were purified following well established procedures (Croy *et al.*, 1980; Matta *et al.*, 1981). Total seed proteins were subjected to ammonium sulphate precipitation. The proteins precipitated in the range of 70 - 90% relative saturation were chromatographed on a column of hydroxylapatite. The proteins eluted in the second peak were shown to contain legumins. Legumins were further purified on a column of sephacryl S-300. On the otherhand, the proteins precipitated at 90% relative saturation were applied to a column of sephacryl S-300. The vicilins were recovered in the first peak.

**Preparation of antiserums.** The *Pisum* vicilln antibodies, *Pisum* legumin antibodies which used for immunodiffusion and Western blotting analysis of *Vicieae* legumins and *Vicieae* vicilins were prepared in rabbit according to the modified method of Hammer and Murphy (1993).

**Sample preparation.** Seed meals or purified proteins were dissolved in EDTA-Tris borate buffer, [60.5 g Tris, 6.0 g EDTA (disodium salt) and 4.6 g $H_3BO_3$, in each L].

**Gel preparation.** Agarose (1 g) was being boiled for five minutes in 50 ml distilled water. Meanwhile, 50 ml Tris-EDTA-borate buffer, pH 9.6 was kept at 65 °C. The boiled agarose was left at room temperature until its temperature reduced down to 65°C and then mixed with the warm buffer. The latter mixture was casting in plastic plates. The plates were stored at 4°C for 2-4 hours and then holes were made, using hexagonal array of outer wells around the central wells.

**Sample application.** Seed meals or purified samples were loaded in the outer wells and the antibody was loaded in the central well and left on leveled table at 4°C for 24 hours (unless otherwise mentioned). The arcs formed after the incubated time were pressed, washed, stained with staining solution (0.025% Commassic Blue R250, 7% acetic acid, 50% methanol in water), and destained with destaining solution (7% acetic acid and 50% methanol in water).

**Western blotting technique.** After SDS-PAGE separation, separated proteins were transferred to nitrocellulose paper by electro-blotting. The transferred proteins on nitrocellulose paper were immersed in specific antibodies, visualized by peroxidase-coupled antibodies and then stained with 4 chloro-1-napthol (Towbin *et al.*, 1979). Blotted proteins were separately detected by *Pisum* vicilin antibodies, *Pisum* legumin antibodies.

**SDS- Polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis for total protein extracts, globulins, albumins, vicilins, legumins were performed in 12% gels (Laemmli, 1970). Two dimensional SDS/PAGE were performed under non-reducing conditions in the first dimension and reducing conditions in the second dimension (Sammour, 1985).

## RESULTS

The seed globulins of *Vicia faba*, *Pisum sativum*, *Lens esculentum*, and *Cicer arientinum* were examined by using polyacrylamide gel electrophoresis, under reducing and non-reducing conditions (Fig. 1). The band patterns of all four species were broadly similar and showed bands at Mr approximately 71,000 and 50,000; these bands were not altered in the presence of 2-mercaptoethanol and had a typical size of convicilin and vicilin, respectively. Under non-

reducing conditions, bands at Mr 60,000 which disappeared in the presence of 2-mercaptoethanol, gave rise to two sets of new bands, at Mr approximately 40,000 and Mr 20,000, representing legumin: α and β subunits were observed. Other minor bands that showed much more marked species to species variation were also observed (Fig. 1).

The total protein extracts of *Pisum sativum*, *Lens esculentum* and *Cicer arientinum* were also analysed under non-reducing conditions in the first dimensions and under reducing conditions in the second dimensions (Fig. 2A-D). The second dimension patterns of different varieties of the same species were very similar in the number of the sub-units and the migration of the sub-units,

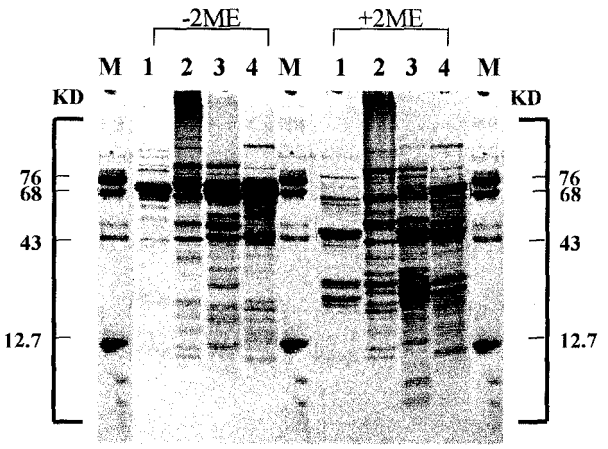


Figure 1. SDS-PAGE of globulin seed proteins analysed under non-reducing and reducing conditions. 1, *V. faba*; 2, *P. sativum*, 3, *C. arientinum*; 4, *L. esculentum*. M: molecular weight-standard proteins.

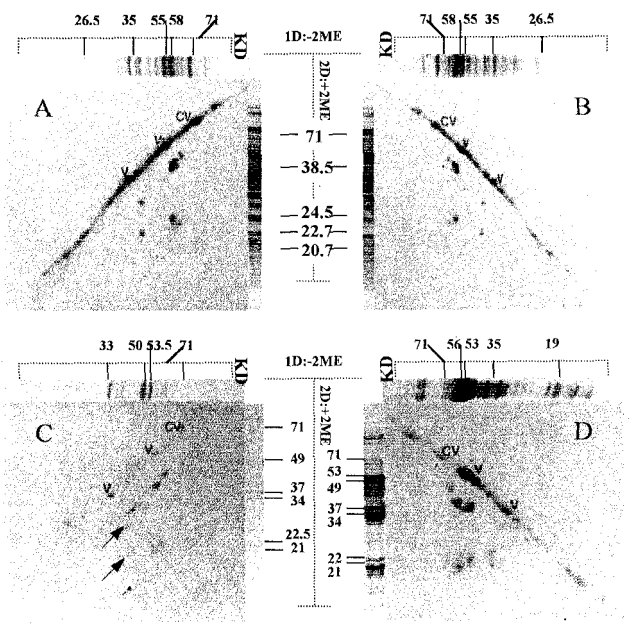


Figure 2. Two dimensional SDS-PAGE of total protein extracts. A, *P. Sativum* Var. Progress 9; B, *P. Sativum* Var. Victory; C, *C. arientinum;* D, *L. esculentum*. 1D: electrophoresis under non-reducing conditions in the first dimension. 2D: electrophoresis under reducing conditions in the second dimension.

for instance, *Pisum sativum,* Var Victory and Progress 9, (Fig. 2A and B). The second dimension patterns of *Pisum sativum, Lens esculentum* and *Cicer arientinum* showed the same number of the sub-unit pairs (five) (Fig. 2A-D). All the former species were broadly similar, though there are some minor differences with respect to the exact position on the gel and the number of components.

The legumins of all members of the *Vicieae* investigated in the present study have the same electrophoretic behavior on SDS-polyacrylamide gel electrophoresis, indicating that all of them are disulphide bonded sub-units, giving a high molecular weight, α subunit, and a low molecular weight, β sub-unit, on analysis by SDS-polyacrylamide gel electrophoresis, under reducing conditions (Fig. 3). All four species showed that almost all the sub-units had molecular weights between 60,000 and 35,000, except *Vicia faba* sub-unit pattern which had a number of subunits having molecular weights more than 60,000. Apparently, the sub-unit patterns of legumins of four species had two areas of considerable similarity, the first one was the region of sub-units which had a molecular weight around 55,000 and the other one had a molecular weight around 37,000. Under reducing conditions, the sub-unit pattern of the legumin of each species separated into high molecular weight sub-units (α or acidic sub-units) and low molecular weight sub-units (β or basic sub-units). The acidic sub-units of all four species dominated by a number of strongly stained sub-units, having Mr between 43,000 and 33,000. On the other hand, the basic sub-units were characterised by polypeptides had molecular weights between 24,500 and 20,000.

The sub-units of Mr 50,000, 33,000 and 14,500 were present in the sub-unit patterns of the vicilins of all four species (Fig. 4). In the Mr 50,000 region, the subunit patterns of all four species were similar except the vicilin of *Cicer arientinum* which had one more band with Mr 55,000. And in the Mr 33,000 region, the vicilins of *Lens esculentum* and *Cicer arientinum* showed different sub-unit patterns; the sub-unit pattern of *Lens esculentum* contained a large number of faint bands, whereas the sub-unit patterns of vicilin of *Cicer arientinum* had only one strong band that characterized this region. On the other hand, the vicilins of *Vicia faba* and *Pisum sativum* exhibited similar sub-unit patterns. In the third region which had molecular weights between 19,000 and 12,000, the sub-unit patterns showed clear resemblance amongst all four species. Generally, it can be concluded that vicilins of all four species share largely the same sub-unit composition.

The electrophoretic patterns of the seed albumins of *Pisum sativum, Vicia faba, Lens esculentum,* and *Cicer arientinum* differed markedly and only one putatively common protein species corresponding to the major albumin protein of *Pisum,* Mr approximately 25,000, would be identified in the electrophoretic profiles of the different species, with the exception of *Vicia faba*

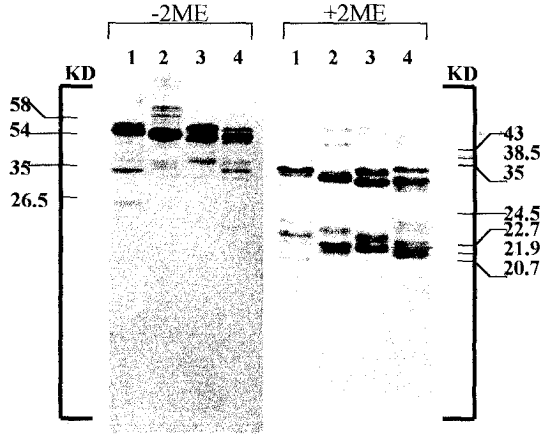


Figure 3. SDS-PAGE of legumins analysed under non-reducing and reducing conditions. 1, *P. sativum*; 2, *V. faba*; 3, *C. arientinum*; 4, *L. esculentum.*

(Fig. 5). However, when total protein extracts of all four species were examined by double antibody precipitation (Western blotting technique), the Mr 25,000 band of *Vicia faba* was shown on nitrocellulose paper (Fig. 5B), indicating that all four members of *Vicieae* contain Mr 25,000 band. Additionally, all four members of *Vicieae* showed electrophoretically and serologically a band that had a molecular weight which was slightly higher than the major albumin of *Pisum sativum*. However, this band is present in a very little amount (Fig. 5A).

When total protein extracts, purified legumins and purified vicilins of all four species were allowed to diffuse against *Pisum* anti-legumin antiserum and *Pisum* anti-vicilin antiserum separately in an Ouchterlony double immunodiffusion test, all gave a precipitin arc (data not shown). This indicated that there was a degree of similarity between the total protein extract, purified legumin and purified vicilin of *Pisum sativum* and those of the other members of *Vicieae*. Subsequently, comparative double immunodiffusion was used to study this degree of

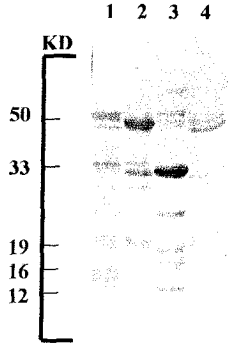


Figure 4. SDS-PAGE of vicilins analysed under non-reducing conditions. l, *P. sativum*; 2, *V. faba*; 3, *C. arientinum*; 4, *L. esculentum*.

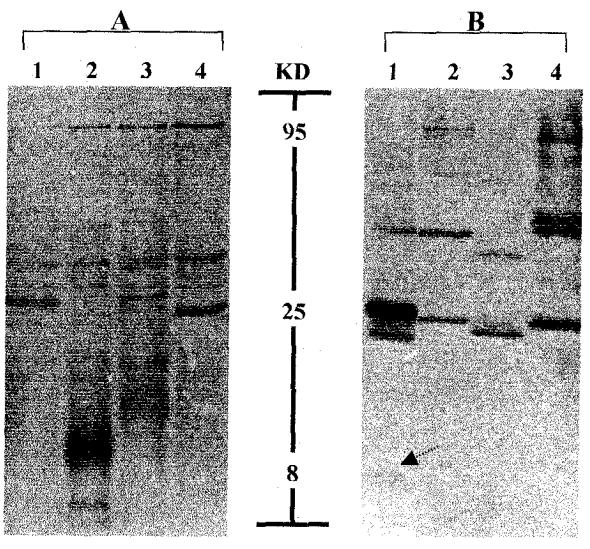


Figure 5A. SDS-PAGE of albumin seed proteins analysed under non-reducing conditions. 1, *P. sativum; 2, V. faba; 3, C. arientinum; 4, L. esculentum.*

Figure 5B. Reaction of albumin seed proteins of *P. sativum, V. faba, L. esculentum, C arientinum* (lanes 1-4) with anti (pea major albumin) IgG after transfer to nitrocellulose and staining with second antibody ("Western" blot).

similarity. The results showed that the legumins of *Vicia faba* and *Lens esculentum* have a total identity (Fig. 6). In contrast, legumin of *Cicer arientinum* shows a partial identity. Moreover, this work revealed that the vicilins of *Vicia faba, Lens esculentum* and *Cicer arientinum* had a partial identity with vicilin of *Pisum sativum.* However, the degree of this partial identity was lesser in case of *Cicer arientinum* (large spure produced) than the other two species (*Lens esculentum and Vicia faba*).

Total protein extracts of *Pisum sativum, Vicia faba, Lens esculentum* and *Cicer arientinum* were first subjected to electrophoresis in the presence of SDS-PAGE under reducing and non-reducing conditions, then transferred to nitrocellulose sheet by electroblotting. The immobilised protein on nitrocellulose sheet was detected with *Pisum* legumin anti-serum (Fig. 7) and *Pisum* vicilin anti-serum (Fig. 8). All members of *Vicieae* were found to contain convicilin-like protein. These convicilins gave cross reaction with *Pisun* vicilin antiserum on nitrocellulose sheets, having almost the same molecular weight of the convicillin of *Pisum sativum.* All sub-units of legumins of *Vicia faba* and *Lens esculentum* gave a very strong

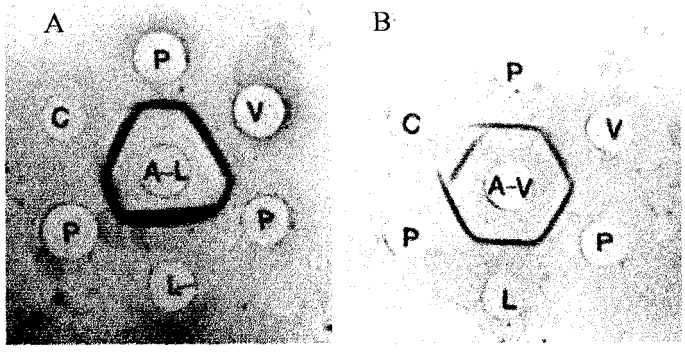


Figure 6A. Immunological reactions of anti (*Pisum*-legumin) antiserum (P) against *Pisum* legumin and other proteins; V, *Vicia faba* legumin; L, *Lens esculentum* legumin; C, *Cicer arientinum* legumin.

Figure 6B. Immunological reactions of anti (*Pisum*-vicilin) antiserum (P) against *Pisum* vicilin and other proteins; V. *Vicia faba* vicilin; L, *Lens esculentum* vicilin; C, *Cicer arientinum* vicilin.

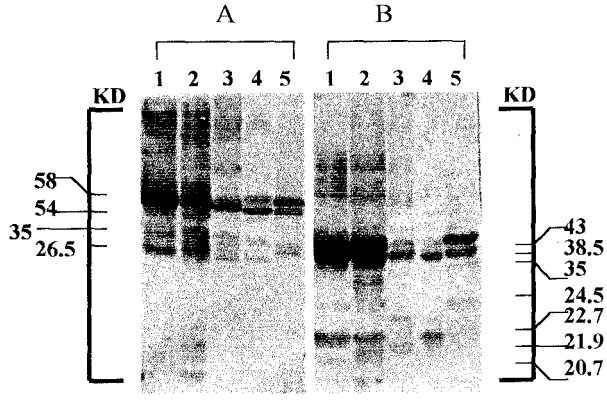


Figure 7A. Reaction of proteins with anti (*Pisum* legumin) IgG after transfer to nitrocellulose and staining with second antibody ("Western" blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin.

Figure 7B. Reaction of proteins with anti (*Pisum* legumin) IgG after transfer to nitrocellulose and staining with second antibody ("Western" blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin. All tracks were run under reducing conditions.

reaction under non-reducing conditions with *Pisum* legumin antiserum. However, sub-units of legumin of *Cicer arientinum* showed a slight difference, in that the lowest molecular weight sub-unit pair (Mr 33,000) failed to react with *Pisum* legumin antiserum under non-reducing conditions. Furthermore, under reducing conditions, three low molecular weight sub-units did not react: Mr 22,700, 21,900, 21,000. When *Pisum* vicilin antiserum was allowed to react with immobilized total protein of *Pisum sativum. Vicia faba, Lens esculentum* and *Cicer arientinum* on nitrocellulose sheet, some sub-units of vicilins of *Vicia faba, Lens esculentum* and *Cicer arientinum* showed no homology with vicilin of *Pisum sativun.* In addition, all low molecular weight sub-units of vicilin of *Cicer arientinum* which had molecular weights of 24,500, 23,000, 22,700, 21,500 and 21,000 did not react with *Pisum* vicilin antiserum, whereas some of the sub-units of vicilins of *Vicia faba* and *Lens esculentum* in the same range of molecular weight reacted. These data explain why vicilins of *Vicia faba, Lens esculentum* and *Cicer arientinum* featured different reaction of identity, when investigated by Ouchterlony double immunodiffusion technique.

Western blotting analysis of the total protein extracts of *Phaseolus coccineus, Phaseolus vulgaris, Vignia unguiculata, Dolichos lablab, Glycine max, Cajanus cajan* and *Canavalia ensiformis* showed that *Canavalia ensiformis* had a positive reaction with both *Pisum* vicilin and legumin antisera; *Vignia unguiculata, Dolichos lablab, Cajanus cajan* with *Pisum* vicilin antiserum; and *Glycine max* with *Pisum* legumin antiserum (Table 1).

## DISCUSSION

The position of *Cicer arientinum* is still a matter of debate. Whereas workers support the opinion that *Cicer arientinum* is a member of the tribe *Vicieae* ((Kupicha, 1977; Gapochka, 1984; Endo and Ohashi, 1997; Wojciechowski *et al.*, 2000); other showed that *Cicer arientinum* should be excluded from *Vicieae* and have its own tribe, the *Cicerideae* (kloz and Turkova, 1963; Simola, 1969; Tarlakovskaya, 1974; Gavriljuk, 1974; Tarlakovskaya, 1975; Cristofolini, 1981; Sammour, 1985; Sammour, 1991).

Many workers made comparative study amongst the members of the tribe *Vicieae*, on the basis of the electrophoreteic patterns of their storage proteins. In these studies, proteins were analysed by non-dissociating gel electrophoresis, in which the position on the gel to which a protein moves is dependent partly on its size and molecular weight and partly on its charge (Boulter *et al.*, 1967). In the light of this fact, the taxonomical comparison by non-dissociating gel electrophoresis may lead to misleading conclusions because there is a great possibility that proteins with different molecular weights may have the same electrophoretic mobilities on the gel (Derbyshire *et al.*, 1976). In this study dissociating gel electrophoresis, under non-reducing and reducing conditions, was used to compare the storage proteins of members of the tribe *Vicieae*. In 1970, Reynolds and Tanford

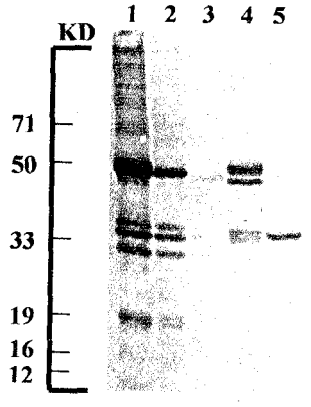


Figure 8. Reaction of proteins with anti (*Pisum* vicilin) IgG after transfer to nitrocellulose and staining with second antibody ("Western" blot). 1, *Pisum* total protein extract; 2, *Pisum* legumin; 3, *Vicia* legumin; 4, *Lens* legumin; 5, *Cicer* legumin.

demonstrated that under dissociating conditions all protein specificity was lost and mobility in the gel was a measure of molecular size alone. Therefore, comparison of seed proteins by using dissociating gel electrophoresis is to a large extent more convincing than that of the non-dissociating gel electrophoresis. However, this does not allow us to rely on only one technique to judge whether the taxa under study is homogeneous or not. By using dissociating gel electrophoresis, it was found that the band patterns of the globulin fractions of the total protein extracts of the seed meals of *Pisum sativum. Vicia faba, Lens esculentum* and *Cicer arientinum* were broadly similar. This result is conformed with those of Boulter *et al.* (1967), Jackson *et al.* (1967), Hennig and Schlesier (1994) who indicated that the electrophoretic patterns of globulin major storage proteins of all members of the tribe *Vicieae* including *Cicer arientinum* were similar.

In this study, the data showed that the electrophoretic patterns of albumin protein fractions of all four species differed markedly and only one possible common protein species corresponding to the major albumin protein of *Pisum* (Mr approximately 25,000) could be identified (Croy *et al.*, 1984; Gatehouse *et al.*, 1985; Rosa *et al.*, 2000). Though this band was not apparently present in the electrophoretic pattern of *Vicia faba*, it gave a faint reaction with anti-*Pisum* anti-major albumin when investigated by using "Western" blotting technique. Electrophoretically and immunochemically, all four members of *Vicieae* showed a band with molecular weight higher than that of *Pisum* major albumin. This band precipitated *in vitro* synthesis with antibodies raised against major albumin of *Pisum* (Gatehouse *et al.*, 1985). This data, in conjunction with the finding of Croy *et al.* (1984) showed that all members of the tribe *Vicieae* contained a major and high molecular weight albumins-like proteins. It also showed that *Lens esculentum* was the most closely related species to *Pisum sativum, Cicer arientinun,* and *Vicia faba* had a more distant relationship.

The second dimension patterns of *Pisum, Lens* and *Cicer* were broadly similar in terms of number of sub-unit pairs (six pairs), whereas the second dimension pattern of *Vicia faba* was dissimilar to other species (9 pairs) (Matta *et al.*, 1981). On the contrary the second dimension patterns of all four species seem to be greatly different, on the grounds of the number of the sub-units and their position on the gel. However, in light of the facts : 1) that legumin sub-unit pairs are divided into three types: the main type (Mr 53,000-54,000)

TABLE 1. Distribution of proteins imunochemically related to either legumin or vicilin of *Pisum sativum* in seeds of Leguminosae subfamily Papalionoideae

| Tribe | Species | Protein detected by anti-*P sativum* antiserum by "Western" blotting technique | |
|---|---|---|---|
| | | V (vicilin) | L (legumin) |
| Vicieae | *Pisum sativum* | + | + |
| | *Vicia faba* | + | + |
| | *Lens esculentum* | + | + |
| | *Cicer arientinum* | + | + |
| | *Lathyrus odoratus* | + | + |
| Phaseoleae | *Phaseolus coccineus* | - | - |
| | *Phaseolus vulgaris* | - | - |
| | *Vignia unguiculata* | ± | - |
| | *Dolichos lablab* | ± | - |
| Glycineae | *Glycine max* | - | ± |
| Cajaneae | *Cajanus cajan* | ± | - |
| Diocleae | *Canavalia ensiformis* | ± | ± |

+ = strong reaction
± = weak reaction
- = no reaction

corresponds to the conventional legumin sub-unit pairs of the Wright and Boulter (1974) model (the major legumin subunits of Casey, 1979), and the other two types (present in lesser amounts) designated "big" (Mr 55,000-58,000) and "small" (Mr 35,000) legumin sub-unit pairs; 2) that conventional legumin sub-unit is synthesised as precursor molecules with molecular weight of 60,000 daltons (Croy *et al* ., 1980; Casey *et al.*, 1984; Higgins, 1984; Hager *et al.*, 1995; Jung *et al.*, 1998); and 3) that the possibility that the other two types are synthesized as precursor molecules with Mr of 80,000 (Casey *et al.*, 1984 and Jung *et al.*, 1998), the second dimension patterns of the conventional legumin sub-units of all four species can be considered similar. Though the second dimension gel electrophoresis technique has the ability to analyze legumin fraction in total protein extracts, without the need for purification; thus saving a great deal of time and relatively high capital outlay, it has two disadvantages which, will probably limit its use on very large scale in molecular taxonomy. The first one is that its effectiveness range is associated with the range of distribution of sub-unit pairs. The second disadvantage is that it is not feasible to analyse multiple samples by this technique on the same gel.

Legumin has been defined as a disulphide bonded sub-unit pairs and seed storage protein of the globulin type, i.e., salt soluble but insoluble in water, Mr 300-400,000 (Wright and Boulter 1976). This operational definition of legumin was adopted by Matta *et al.* (1981) and generalized to include all legumin-like proteins by Pernollet and Mosse (1983) and Freitas *et al.* (2000) On these bases, the operational definition of legumin is stretched out to the legumins of *Cicer* and *Lens*, since they are seed storage protein of the globulin type, with a molecular weight of 300-400,000 daltons; disulphide bonded sub-unit pairs, separated into high molecular weight ( $\alpha$ as acidic) sub-units and low molecular weight ($\beta$ as basic) sub-units, under reducing conditions. The broad similarity amongst the legumin composition patterns of all four species supported the opinion that *Cicer* was a member of *Vicieae.*

The electrophoretic patterns of vicilins of *Vicia faba, Lens esculentum* and *Cicer arientinum* were found to consist of two groups of sub-units, one with molecular weight of 50,000 daltons and the other with molecular weight less than 50,000 daltons, like the vicilin sub-unit composition of *Pisum.* These different sub-units are encoded by different gene families, and often subject to post-translational modifications (Gatehouse *et al.*, 1983; Slightom *et al.*, 1983). By analogy, it was expected that vicilins of *Vicia faba, Lens esculentum* and *Cicer arientinum* had a similar quaternary structure of 7S family. This speculation was supported by the fact that the molecular weights of vicilins of all four species were almost the same. Though the lowest molecular weight sub-units of all four species were electrophoretically similar, immunochemically they did not exhibit a considerable homology with *Pisum* vicllin anti-serum by the Western blot. However, this did not undervalue or depreciate the suggestion that the vicilins of *Pisum, Vicia, Lens,* and *Cicer* are members of 7S family and are broadly similar. This suggestion was supported by comparison of nucleotide sequences, electrophoretic patterns and structural similarities of the vicillins of legume species , in particular to those of the tribe *Vicieae* (Sáenz de Miera and Pérez de la Vega 1998; Rosa *et al.*, 2000; Tiedemann *et al.*, 2000).

Immunologically, total protein extracts of all members of *Vicieae* including *Cicer arientinun* gave a precipitin arc when they were allowed to diffuse against both *Pisum* legumin and vicilln antisera in an Ouchterlony double immunodiffusion test. Furthermore, purified legumins and vicilins of all four species showed the same results as well. These data were similar to the data obtained by earlier workers (Kloz and Turkova, 1963; Simola, 1969 and Dudman and Millerd, 1975). Though the antibodies used in each work were raised against antigens (legumin and vicilin) of different species; against legumin and vicilin of *Pisum sativum* (Kloz and Turkova, 1963), *Lathyrus* (Simola, 1969) and *Vicia* (Dudman and Millerd, 1975), all workers demonstrated that all members of *Vicieae* including *Cicer* contained legumin and vicilin-like protein. But, there was a discrepancy on the degree of relatedness amongst these proteins. Whereas Kloz and Turkova (1963) and Simola (1969) indicated that all members of *Vicieae* contained similar legumin and vicilin, Dudman and Millerd (1975) demonstrated that vicilin-like

protein of *Cicer* was absolutely dissimilar with other vicilin like protein of members of *Vicieae*, concluding that *Cicer* did not contain vicilin-like protein. In this study, comparative double diffusion technique was used to clarify this point.

The resulting data showed that all members of *Vicieae* had similar or identical legumins, except *Cicer* legumin which showed partial identity with Pisum legumin antiserum (small spure produced). On the other hand, the vicilins of *Vicia*, *Lens*, and *Cicer* showed partial identity with vicilin of *Pisum*, but the degree of this partial identity was lesser in the case of *Cicer* (large spure produced). It was clear that all members of *Vicieae* contained legumin and vicilin-like proteins, but both legumin and vicilin of *Cicer* were not in the same order of homology of other members with *Pisum* legumin and vicilin. These data does not support the earlier report of Dudman and Millerd (1975), indicating that *Cicer arientinum* has no vicilin-like protein.

The advent of "Western" blotting technique opens the door to conduct immunochemical studies of different species of seed proteins on the basis of their sub-unit composition. This technique, therefore, was used to study the degree of relatedness between different members of *Vicieae* in terms of the legumins and the vicilins of the globulin seed proteins of their members. Data showed that all sub-units of legumins of *Vicia faba* and *Lens esculentum* gave a very strong reaction, under non-reducing and reducing conditions, with *Pisum* legumin antiserum. However, legumin of *Cicer* exhibited a slight difference, that is the low molecular weight sub-unit pairs 33,000 failed to react with *Pisum* legumin antiserum, under non-reducing conditions and some bands of the basic sub-units did not react under reducing conditions. These data confirmed the results obtained using comparative double diffusion technique, putting a conceivable explanation for the reaction of partial identity with *Cicer* and reaction of identity with the rest of the members of the tribe *Vicieae*. On the contrary, some sub-units of vicilins of *Vicia*, *Lens* and *Cicer* did not react with *Pisum* vicilins antiserum when the total proteins of the members of the tribe *Vicieae* were immobilised on nitrocellulose sheet, for instance all the sub-units of the vicilin of *Cicer* which have a molecular weight less than 25,000. These data clarified the reason for which *Cicer* gave reaction of low partial identity (large spure produced) and both *Vicia* and *Cicer* gave reaction of high partial identity (small spure produced). However, this level of dissimilarity amongst both the 7S family and the 11S globulins of the members of the tribe *Vicieae* did not mean that structural similarity among each group was at low level, as reported by Freitas *et al.* (2000), since the low molecular weight sub-units represent a low percent of each protein species. Interestingly, all members of *Vicieae* which were investigated by Western blotting showed only one band, having nearly the same molecular weight of *Pisum* conviclin, 71,000 (Croy *et al.*, 1980).

Western blotting technique was also used to screen some representatives of the tribes *Phaseoleae*, *Glycineae* and *Cajaneae* for vicilin and legumin-like protein. The presented data revealed, for the first time, that there is immunological homology between *Pisum* globulin and those of some members of *Phaseoleae* and representatives of both *Glycineae* and *Cajaneae*, and this is not in agreement with all previous serological work (Kloz and Turkova, 1963 and Millerd, 1975). However, these weak reactions of homology would be predicted on the basis of known sequence homologies in the proteins (Ko *et al.*, 1993; Garcia *et al.*, 1997; Freitas *et al.*, 2000; Mendoza *et al.*, 2001).

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