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Immunolocalization and Immunochemical Cross reactivity of Chickpea Vicilin with other Globulins of Legumes

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Polyclonal antibodies were raised against purified whole Chickpea vicilin protein. The titre of anti-vicilin antibody was determined using ELISA. The anti vicilin antibody has shown significant reactivity with vicilin even at 10^{-4} dilution. The strong signals of anti- vicilin antibody were also confirmed by dot-bloating technique and western blot analysis. ELISA assessment of specificity of anti- vicilin antibodies has shown stronger cross reaction with pea and frenchbean than those of cowpea and Cluster bean. There was little homology with Brassica and BSA. The cross reactions with legumin and convicilin were also substantially high. Immunohistochemistry with cotyledonary section of chick pea using anti-vicilin antibodies indicated the sub-membranous fluorescence confirming the localization of protein bodies in membrane bound organelles.

Legumes /Immunology

Gram (*Cicer aretinum L.*) commonly known as chickpea or Bengal gram is the most important grain legume crop of India. Storage protein of Legumes seed are an important group of plant proteins that constitute a major source of dietary requirements for human livestock consumption. Seventy percent of the edible protein produce in the world comes from seeds (Spencer and Higgins, 1979; Singh et al.2007). Grain legume seeds, in general contains large amount of salt soluble proteins called globulin which is account for about eighty percent of the total seed protein (Danielson, 1949, Derbyshire et al.1976). Globulins are made up of two major components, Legumin (11S) and vicilin (7S). Grain legumes in general and chickpae in particular are deficient in sulphur containing amino acids, methionin and cystine (Koundal, 1983;Mandoakar, 1993). With a view to improving nutritional quality of chick pea by genetic engineering (Mandal and Mandal, 2000), efforts were made to characterize vicilin protein and also to raise antibodies against purified viclin. Immunological approaches based on specificity of interaction between antigens and antibody have been applied to characterize seed protein. Immunological studies using sensitive techniques like enzyme linked immunosorbent assay (ELISA), dot-blot and western blotting have been successfully used to indicate homology among the various protein classes

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Material and Methods

Plant materials

Seeds of chickpea (*Cicer arietenium* L.) Pusa 256 were treated with rhizobium culture and sown in the field according to standard agronomic practices (Sinha, 1977). The mature seeds were removed from the pod under cold conditions. Samples were freeze dried in liquid nitrogen and stored at -70 ˚C for further analysis. Mature seeds of Pea (*Psium satvium* L.), French bean (*Phaseolus vulgaris* L.) and cluster bean (*Cyamopsis psoralioides* DC) were obtained from local vegetable farm, Mehrauli, New Delhi.

Animals

New Zealand white rabbits were obtained from the small animal facility of the National Institute of Immunology, New Delhi.

Isolation of crude globulin

Globulins were extracted from mature seed of chickpea, cowpea, cluster bean French bean and pea as per the procedure described by Wright and Boulter, (1974).

Purification of Vicilin

Vicilin from chickpea mature seeds was isolated and purified as per procedure described by Scholz et al. (1983) and Jonnada et al. (2011). In this method, after removal of albumins, crude vicilin sample was subjected to zonal isoelectric precipitation using Sephadex-G-75 column. The first four fraction similar band pattern were pooled and dialysis against water and loaded on to con-A Sepharose affinity column. Lyophylized sample from affinity column was again purified using sephaeryl-2000 column as fine step.

Immunological characterization of vicilin from chickpea

In the present study, New Zealand white rabbits were immunized with whole vicilin protein to raise antibodies. The titers of anti-vicilin polyclonal antibody were determined using ELISA (fig.1). The pre-immune serum however, showed little but insignificant cross reactivity with vicilin. The anti-vicilin antibody has show sufficient signal at 10^{-4} dilution in two of the rabbits challenged with whole vicilin .The strong signals were also confirmed by dot blotting technique. In dot-blot technique antisera have recognised even as little as 30 ng of antigen whereas pre-immune sera have shown little or no signals (fig.2). Therefore, based on ELISA, dot blot and Western blot analyses, the sera recognised significantly with whole chickpea vicilin protein.

Determination of specificity of anti-vicilin antibody

The specificity of anti-chickpea vicilin antibodies using ELISA was determined by measuring the ability at different dilutions to cross react with globulin of chickpea, pea, frenchbean, cowpea, cluster bean and rubisco. The results are given in fig.3. The binding of anti-vicilin antibodies to protein fractions, Vicilin, legumin and convicilin of chickpea immobilized on microtitration plates at a concentration of 10µg ml^{-1} at different antibody levels was investigated by ELISA. Fig.4 illustrates the differential homologies among these three fractions i.e, vicilin, convicilin and legumin.

Immunofluorescence

The seeds of different developmental stages were preserved in liquid and cotyledon were separated with knife crystal and mounted in OCT embedding mature (Cellpath Plk, Hemel Hempesteat, VK) cotyledons were sectioned at 8 -16micron intervals in a crystal (Reichutjung, Germany) maintained at 180^0C. Sections were collected on glass slide and air dried. Section was fixed in chilled acetone for 20 min prior to staining cotyledon section were blocked with 1 percent BSA for 1hr at 37^0C. After washing with PBST twice, the section were treated with antibodies (1:5000) for 3hrs at 37^0C. After washing with PBST thrice, the section were incubated with FITC for 1hr at 37^0C. The slides were washed thoroughly with PBST and mounted in a mounting medium containing of glycerol and formal saline with added paraphenylene diamine as antifadent. The slides were examined under UV in a Nikon Microphot - FX, Japan.

Result and Discussion

SDS-PAGE analysis

The purified vicilin resolved on SDS-PAGE shown in the Fig.5. There are five major distinct bands corresponding to relative molecular weight (Mr) of 67 kDa and 27 kDa. There are low molecular weight bands. The similar result in vicilin storage protein of *Pisum sativum* were reported by Gatehouse et al.

(1981) .In agreement with our result, vicilin fraction of pea resolved by SDS - PAGE in 10 major sub unit with approximate Mr values of 12, 13 , 14, 18, 25, 30, 34, 50, 70 and 75 kDa (Bedenoch-Zones *et al*., 1981). The group of Mr 50 kDa is made up of a number of polypeptides which can be resolved into at least five components by insoluble focusing (Gatehouse et al., 1981 and Mandoakar, 1990).

Immuno-cross-reactivity

In recent years, immunological approach based on specificity of interaction between antigen and antibodies have been applied to characterize seed proteins. Antibody against cereal proteins (Singh et al.1983; Duranti et al. 1991; Donowan et al.1989); legume protein (Scholz et al.1983; Helbing and Manteuffel, 1987; Carter et al. 1992 and Leal and Mishra, 1993) have been successfully raised to characterization specific protein. Legume seed storage proteins as better immunogens which is a well established fact as many workers used immunological approaches for their characterization (Spencer et al. 1983; Carter at el. 1992; Helbing and Manteuffel, 1987).

Table-1: The percentages cross reaction of anti-vicilin antibody with globulin extract from different species (antibody dilution 10^{-3})

Globulin	Percentage of Cross-reaction
Chickpea	100
Pea	30
Frenchbean	26
Cowpea	15
Cluster bean	11
Rubisco	8
BSA	6

Table-2: The percentage cross-reaction of anti-vicilin antibody with other fraction of chickpea (antibody dilution 10^{-3})

Protein	Percentage of Cross-reaction
Vicilin	100
Legumin	42
Convicilin	23
BSA	06

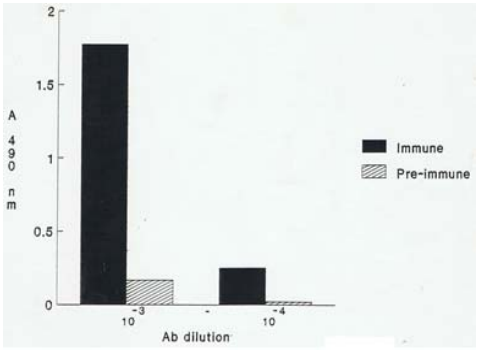


Figure 1: The titres of antivicilin antibody is determined using ELISA. The reactivity of antivicilin sera was significant even at 10^{-4} dillution when compare to pre-immune serum.

Figure 2: Dot-blot analysis of antivicilin polyclonal sera with different concentration of vicilin developed with diamino benzedene ((DAB). a: 10 mg/ml, b: 5mg/ml, c: 2.5mg/ml, d: 1.25 mg/ml, e: 0.66 mg/ml, f: 0.33 mg/ml.

Determination of anti-vicilin antibodies

The specificity of antibody was determined through ELISA by measuring the ability of the serum at a dilution of $1x10^{-3}$ to cross react with various globulins and given in table 1. The anti-vicilin antibody showed differential crossreaction with different protein studied here. The anti-cicer vicilin antibody recognised whole chickpea globulin and vicilin protein equally well as it was expected. The anti-vicilin antibody showed strong cross reaction with pea (*Pisum sativum*) and French bean (*Phaseolus vulgaris),* and cluster bean (*Cymopsis psoralioides*) gave only weak cross reaction.There was very little binding with crude rubisco of leaves of Indian mustered (*Brassica joncea*) which is non leguminous plant. A negligible cross reaction was observed with BSA. The titers of anti-cicer vicilin antibodies were comparable to that of glycinin antibodies (Carter et al. 1992) and gliadin antibodies (Skerritt et al.1984). The cross-reaction with pea and french bean were high at 26 percent and above. The cross reaction of other globulin tested (Cow pea and cluster

bean) were less significant giving values of 15 percent and below. Virtually a little binding with crude rubisco and BSA.

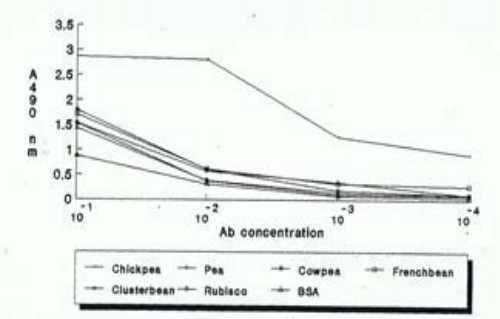


Figure 3: ELISA binding of antivicilin antibody to different plant proteins immbolized on to a microtitration plate at 10 µg/ml.

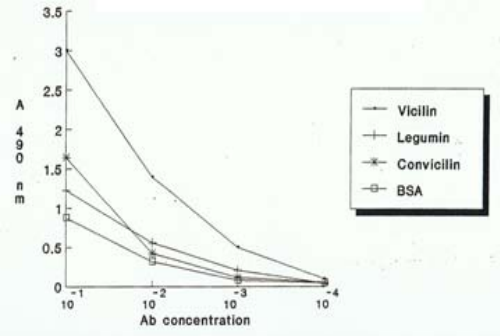
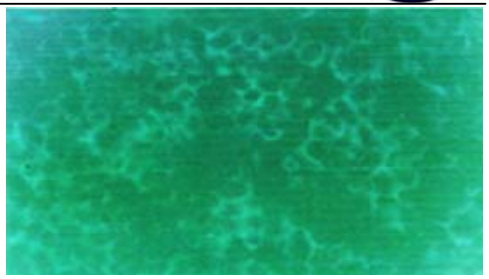
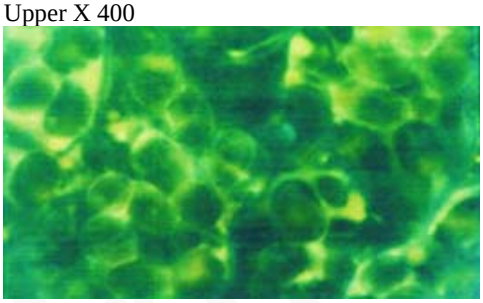


Figure 4: ELISA binding of antivicilin antibody to other storage protein fractions of chickpea immbolized on to a microtitration plate at 10µg/ml.

Figure 5: Purified vicilin that was subjected to 12% SDS-PAGE. a: the five major bands corresponding to 67kDa, two co migrating 50kDa, 33kDa and 27kDa. M: Low range protein molecular markers.



Upper X 400



Lower X 800

Figure 6 Upper: Immunofluorescence studies of chickpea cotyledonary sections for vicilin protein. Submembranous localization of the protein is observed both in low (Upper X 400) and high (lower X 800) magnifications. Presence of vicilin could also be seen in the cytoplasm of many cells in the higher magnification.

The binding of anti-chickpea vicilin antibodies to the above tested globulin suggest that homologies between chickpea with pea, french bean, cow pea and cluster bean exist in their sequence relationship and also their structure. In conformity with our result a significant degree of homologies between 7S globulin of representative of different tribes of legume plants could be demonstrated by using antibodies raised against purified preparation of vicilin protein of *Vicia faba* and *Phaleous vulgaris* by western blot analysis (Helbing and Manteuffel,1987). Anti-phaseolin antibodies cross reacted unambiguously with crude globulin of *Pisum*, *Arachis*, *Beptisa*, *Canavalia*, *Lupinus*, *Vicia* and *Trifolium*. Anti-vicia vicilin antibodies showed additional cross reaction with Astralogous and Medicago. Robert et al. (1985) and Faijanski et al. (1985) were also made similar observation in assessing homologies between 7S globulin and more distantly related species. In the present study it was observed that anti-cicer vicilin antibodies showed little cross reactivity with Rubisco of Brassica. The similar observation was also

made by Helbing and Manteuffel, (1987) using anti-vicia vicilin and anti-phaseolin antibodies.

Specificity of anti-vicilin antibody to globulin fractions

The binding of anti-vicilin antibodies to globulin fractions of chickpea namely vicilin, Legumin and convicilin immobilized on microtitretion plates at concentration of 10µg /ml at different antibody levels was investigated by ELISA. At lower dilution (10^{-1}) convicilin showed higher binding than legumin. Whereas in subsequent higher dilution (10^{-2} and 10^{-3}) convicilin showed lower affinity then legumin. The percentage of cross-reaction was calculated at antibody dilutions of 1x 10^{-3} as given in table-2. Cross-reaction with chickpea legumin was high at 42 percent and 24 percent with convicilin. In a similar serological in *Pisum* by Gatehouse, (1984) demonstrated that convicilin has all the vicilin antigenic determinants and therefore must be closely related. It was confirmed subsequently through the sequence of gene encoding for convicilin. The gene sequence showed that convicilin was similar to vicilin but different by the insertion of a 121 amino acid sequence near the N-terminus of protein.

In agreement with the above observation, Jackson et al. (1969) indicated through tryptic peptide maps that there was a considerable overlapping in the peptide pattern of legumin a vicilin ranging from about 80 percent with *Pisum satium* and it is possible that the substantially similarity between finger print pattern can safely be interpreted as indicative of some degree of common structure. The amino acid homology of either oat globulin or rice glutalin with soyabean glycin or pea legumin is approximately 35 percent (Takaiwa et al. 1987 and Higuchi and Fukasawa,1987) this homology indicates that rice glutelin and soyabean glycinin may have evolved from a common ancestral gene (Higuchi and Fukasawa,1987). The binding of anti-chickpea vicilin to different legume globulins as tested in the present study, and its own legumin and convicilin proteins might suggest, homologies between these storage proteins in their sequence relationship and in their structure found to be existing. As suggested by Wright, (1988), there are similarities between 7S and 11S seed storage protein exists. However, Boroto and Dure, (1987) have proposed that all globulin fractions were derived from two ancestral genes: one for vicilin-like proteins and for legumin like proteins.

The method of choice for comparing homologies between proteins is the amino acid sequence analysis (Boulter,1981). The antigenic homologies demonstrated by using anti-7S globulin antibodies confirmed the amino acid sequence data studied recently by several workers. They found considerable homologies between the amino acid sequence of 7S globulind of *Phaseolus* and Glycine (Schuler et al. 1983). *Canavalia* and *Pisum* (Lycett et al.1983). In agreement to recently published papers (Howes et al.1989; Skerritt et al. 1984; Carter et al. 1992, Juan De Dios et al. 2006; Nakamura, et al. 2004; Jonnada et al.,2011; Gangwar et al. 2006) these results confirm the suitability of ELISA in assessing homologies between 7S globulins of more distantly related species.

Immunofluorescence

Localization of vicilin was attempted in cotyledonary section in acryostat using anti-vicilin antibodies. Fig.6 shown the sub membranous fluorescence confirming the protein bodies embedded in membrane bound organelles. The similar type of submembranous fluorescence indicating the localization of protein bodies were reported from *Pisum. Vicia, Phaseolus, Glycin* and *Acacia* and Sesame (Juan De Dios, et al. 2006; Tai et al. 2001) Although, this was observed in majority of the section, clear globoid inclusion were also noticed in few section (Fig.7) as that of *Arachis* and *Cassia.* Intracellular antigens can be visualized by immunocytological techniques. At the light microscopic level intracellular antigen can be localized at the sub-cellular compartments.

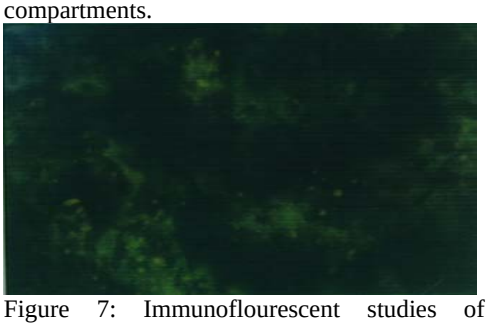


Figure 7: Immunoflourescent studies of cotyledonary sections using anti-vicilin antibodies. Vicilin is localized as globoid inclusions in the cytoplasm (Immunofluorescence x 400).

Conclusion

Polyclonal antibodies were raised against purified whole Chickpea vicilin protein. The titre of anti-vicilin antibody was determined using ELISA. The anti vicilin antibody has shown significant reactivity with vicilin even at 10^{-4} dilution. The strong signals of anti- vicilin antibody were also confirmed by dot-bloating technique and western blot analysis. ELISA assessment of specificity of anti-vicilin antibodies has shown differential cross reactivity with globulin of pea, cowpea, cluster bean, French bean and Rubisco of *Brassica*. Cross reaction with pea and French bean were stronger than those of cowpea and Cluster bean. There was little homology with Brassica and BSA. The cross reactions with legumin and convicilin were also substantially high. Immunohistochemistry with cotyledonary section of chick pea using anti - vicilin antibodies indicated the sub membranous fluorescence confirming the localization of protein bodies in membrane bound organelles. The result of present study indicate that vicilin of chickpea is similar to vicilin reported from *Pisum sativum* and *Vicia faba* in its protein composition. Strong cross reaction found among the vicilin, legumin and convicilin suggesting the possible existence of common sequence and structural features. It is further suggested that additional studies with the vicilin using 2- D gel electrophoresis further confirmation of its heterogeneity. Alternatively hetrologous cDNA probes from pea, French bean and *Vicia faba* may also be employed to isolate vicilin gene from DNA libraries of chickpea. Further bioinformatics tools can be used to ascertain the homologies confirming the common ancestral gene families.

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