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Studies on Genetic Diversity in Chickpea (*Cicer arietinum* L.) using total Seed Protein Separation by SDS-PAGE

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ABSTRACT Thirty Indian cultivated chickpea genotypes were investigated to study the genetic diversity. All the genotypes were separated and compared by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Relative intraspecific similarities of protein patterns were estimated by Nei and Li equation and a cluster analysis was performed. A total of 39 bands were observed with Rm value ranging from 0.11 to 0.95. A minimum of 14 and a maximum of 34 bands were observed. The *kabuli* varieties have more high molecular weight proteins than the *desi* genotypes. Protein content was highest (0.2394 mg/g seed) in one *kabuli* genotype, HK-89-131 and one *desi* genotype, H-92-67 and minimum in GL-94022 (0.0194 mg/g seed). Two broad clusters of all chickpea genotypes were obtained in the dendrogram at a dissimilarity value of 25%.

Keywords: chickpea, SDS-PAGE, seed protein, genetic relationship

Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n=2x=16) annual grain legume and is traditionally grown in many parts of the world. India is the largest producer of chickpea, accounting for 75% of the world production. However, chickpea productivity is not high enough to fulfill the requirements of increasing population. One major reason for the low productivity of cultivated chickpea, (*Cicer arietinum*) is its narrow genetic base and its sexual incompatibility with other *Cicer* wild types in natural interspecific crosses.

A knowledge of genetic diversity and relatedness in the germplasm is a prerequisite for crop improvement programs. The basic criterion of phylogenetic relationships is gene homology, which in many cases cannot be measured directly because of reproductive barriers between species [1]. The fractionation of "non-essential" seed storage protein [2] by polyacrylamide gel electrophoresis (PAGE) is used as an additional tool for assessing species relationships in many major crop plants [3]. Such information supplements evidence from comparative morphology, breeding experiments and cytogenetic analysis of interspecific hybrids.

In the cultivated chickpea, water-soluble (albumin) and electrolyte-soluble (globulin) fraction constitute approximately one-third and two-third respectively, of the total seed storage protein [4]. Seed protein electrophoresis is increasingly being utilized as an additional approach for varietal identification and characterization. The uniformity of seed protein profile, its additive nature and negligible effect by environmental conditions or seasonal fluctuations makes it a unique and powerful tool [5]. Seed proteins are mainly storage proteins and are not likely to be changed in dry mature seed. In addition, intrinsic changes in the plant such as chromosomal rearrangements or even doubling of the chromosome numbers leave no or very small effect on the seed protein profile [6]. The successful exploitation of electrophoresis for plant variety identification relies on the fact that proteins are the products of structural genes. Proteins can thus be regarded as markers for the structural genes that encode them. Hence, a comparison of the protein composition of individuals or populations became a comparison of the underlying variation in the gene expression. By considering sufficient protein markers

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Table 1. List of chickpea lines studied for SDS-PAGE

	Genotype	Pedigree
1.	C-235	(H 91-40 X H 91-38) X H 91-38
2.	Gaurav	C-235 X E 100Ym
3.	Gora Hisari (K)	L 144 X P 6613
4.	CSG-8962	Selection from GPF 7035
5.	GL 94022 (K)	GL 769 X PBG 1
6.	H 86-143	H-208 X E100Ym
7.	HK 89-131(K)	(L 550 X E100YM) (ICC 32 X ICCL 82001)
8.	H 90-231	H 208 X E100YM
9.	H 91-36	H 82-2 X E100YM
10.	H 92-68	(BG 261 X DA 1)(BG 261 X P 6098) (L 144 X Plot No. 8230)
11.	H 92-67	(GG 588 X H 81-73) (BG 257 X H 81-73)
12.	H 96-99	(H 91- 40 X H 91-38) X H 91-38
13.	H 97-253	(H 91-35 X PDG 84-16) H 82-2
14.	H 97-254	GNG 146 X H 91-36
15.	H 97-255	GNG 146 X H 91-36
16.	H 98-57	H 82-2 X *C. reticulatum*
17.	H 98-58	-do-
18.	H 98-59	-do-
19.	H 98-78	H 9145 X ICCV 89445
20.	H 208	(S 26 X G24) F3 X C 235
21.	HC-1	(H 91-40 X H 91-38) H 91-38
22.	HC-3	L550 X E100 YM
23.	HK 94-134(K)	H82-5 X K100YM
24.	HK 98-155	Selection from different collections
25.	L 550(K)	PB 7 X Rabat
26.	ICCV 96030	NA
27.	Katila	NA
28.	NARC 9006	NA
29.	PDG 84-16	NA
30.	H 85-78	H 208 X P 2740 X H 82-5

a large portion of the genome can be covered. So analysis of proteins composition can be considered to be an analysis of gene expression and can be used as an ideal means of varietal discrimination [7].

Distinguishing varieties is not always possible, though it is undoubtedly one of the most commonly used criterion. Thus, there is a need to develop alternative tests, which can distinguish varieties on the basis of stable biochemical properties of seed or seedlings. Of the techniques available, analysis of seed or seedling proteins and isozymes using electrophoresis are the most widely used, because of their reliability, rapidity and cost effectiveness [8]. Keeping these aspects in view, the present study was undertaken with the objective of using PAGE profiles of total seed storage proteins to further elucidate genetic relationships among chickpea lines, genotypes or different species.

MATERIALS AND METHODS

Seeds of thirty chickpea varieties of the genus *Cicer arietinum* L. (Table 1.) including both *desi* and *kabuli*, were obtained from the Pulses section, Dept. of Plant Breeding, CCS Haryana Agricultural University, Hisar.

The protein samples of dry mature seeds of the varieties were prepared by grinding five seeds per sample using pestle and mortar. 500 mg of dry seed meal was taken and dissolved in 500 µl protein extraction buffer (0.6 M Tris-HCl buffer, pH 6.6). Then dissolved in 1X sample buffer (Tris-HCl containing 2g SDS, 10 mg Pyronin-G, 0.75 ml of mercaptoethanol and 10 ml glycerol). The samples were kept at room temperature for 2 h and then in refrigerator overnight. The samples were then heated in boiling water bath at 40° C for 10 min., cooled and centrifuged at 15,000 rpm for 10 min. The clear supernatant was used as protein source for electrophoresis. The total protein content in the samples was estimated using Bradford's reagent.

Electrophoresis SDS-PAGE [Slab gel of 15% separating gel and 6% stacking gel] was performed following the procedure as described by Dadlani and Varier [9] using Tris-Glycine buffer (pH 8.3) containing 0.1% SDS at a constant current of 30 mA continuously till the tracking dye reached the bottom. 100 µg of protein was loaded from each sample. After electrophoresis, the gels were stained overnight in 1% Coomassie Brilliant Blue R-250 in 50% methanol and 15% acetic acid. The gels were destained in a solution of 25% methanol and 7% acetic acid.

RESULTS

The total soluble protein content in seeds of different chickpea varieties was estimated (Fig 1). The analysis of variance revealed significant differences among the varieties for protein content. Four genotypes had protein content below 10% i.e., the amount of total protein was minimum in the genotype, GL-94022 (0.0194 mg/g seed) followed by 0.0327 mg/g seed in the genotype CSG-8962, 0.058 mg in PDG-84-16 and then 0.0621 in H-97-253. The highest protein was present in two genotypes, HK-89-131(*kabuli*) and H-92-67 (0.2394 mg/g seed) each. Rest of the genotypes had protein content in the range of 10-20%.

A total of 39 protein fragments were obtained after SDS-PAGE of total soluble seed protein ranging in molecular weight from 10 KDa to 110 KDa. The relative mobility of the fragments varied from 0.11 to 0.95. A standard protein marker of known molecular weight (14.4 KDa to 94 KDa) was also used along with the samples. All the protein fragments were grouped into four main groups A, B, C and D (Fig.2). The protein polypeptides in group A were of high molecular weight i.e., 66 -100 KDa. The proteins in group B are of the range 40-66 KDa, 26-40 KDa in group C and the lower molecular weight protein below 26 KDa are grouped under D. Band number 21 (~40 KDa) and band no. 28 (~27 KDa) are common to all the thirty genotypes. Band no. 7 (68 KDa) is also present in all except one genotype, H-97-253. Rest protein bands are polymorphic. Band number 1 and 2 are present only in one genotype, ICCV 96030 which are high molecular weight protein fragments (~110 & 100 KDa). Similarly, another fragment of 97 KDa at position 3 was present in ten genotypes only, out of thirty genotypes (Fig 3). The number of bands shows how much polypeptides are present in a protein. As equal amount of protein (100 µg) was loaded in all, thus the banding pattern shows that of which range are the proteins present in the respective varieties. One *kabuli* genotype L-550 has lowest number no. of protein fragments, 21 among the five *kabuli* ones. The other five chickpeas have more number of protein bands than the *desi* genotypes i.e., 34, 34, 29 & 30. Three genotypes, H-98-57, H-98-58 and H-98-59 having same parentage but they differ from each other with respect to the number of bands present. The maximum number of protein fragments were present in the genotype, HK-89-131 whereas the genotypes, GL-94022 and Katila had the lowest number of bands i.e., 14 and 16 respectively.

Cluster analysis:

Based on the dendrogram, thirty chickpea genotypes could be grouped into two main clusters a & b (Fig 4) while two genotypes, H-97-253 and H-97-255 are most diverse, thus are separated from the rest genotypes. Three genotypes, GL-94022, L-550 and Katila are grouped separately in cluster **b** as their pattern and number of bands is lowest from the others. Similarly, in cluster **a**, three genotypes, H-98-57, H-98-58 and CSG-8962 form a separate group. In the another broad group, three main subclusters; (i), (ii), (iii) are formed. Eight genotypes in one cluster, nine genotypes in 2nd cluster and 5 genotypes in the third cluster. In cluster (i), H-97-254 is most diverse from the rest in the same cluster, similarly ICCV-96030 in (ii) and H-96-99 in (iii) are diverse from the others. Out of five *kabuli* genotypes, Gora-Hisari and HK-89-131 are similar to each other, as their similarity correlation is 100% although their

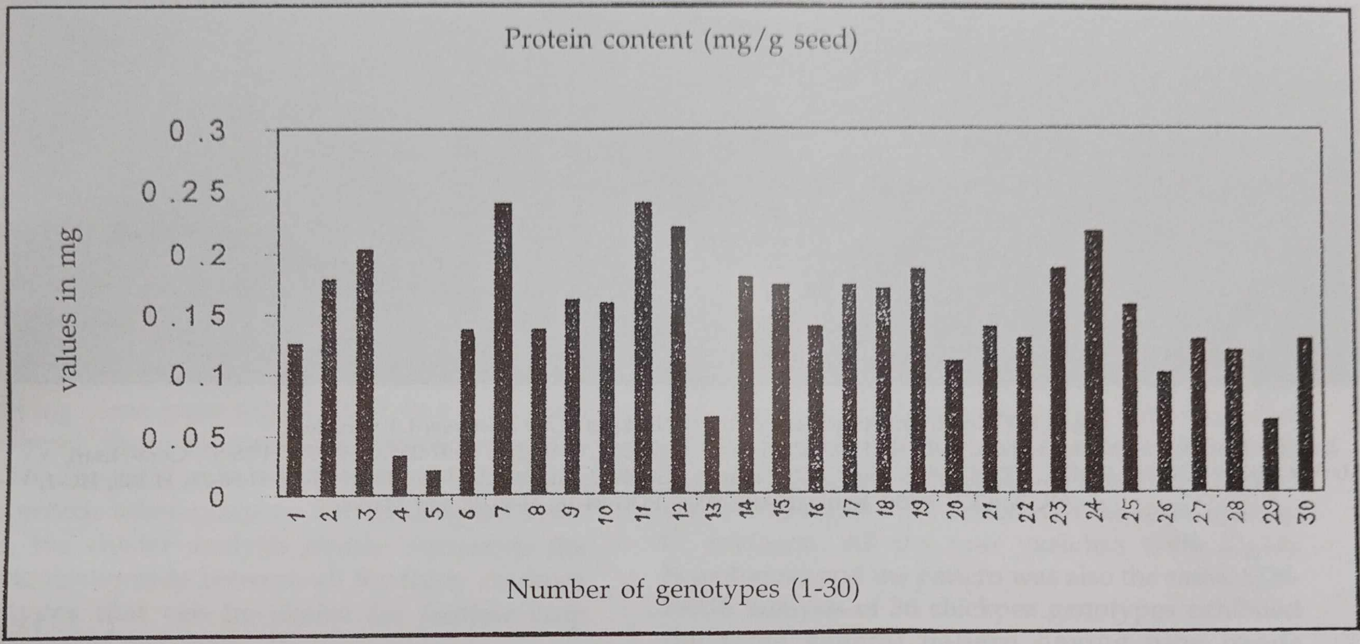

Fig 1. Total protein content (mg/g seed) profile of soluble seed proteins of different chickpea genotypes.
1 to 30: C-235, Gaurav, Gora Hisari (K), CSG-8962, GL-94022, H-86-143, HK-89-131 (K), H-90-231, H-91-36, H-92-68, H-92-67, H-96-99, H-97-253, H-97-254, H-97-255, H-98-57, H-98-58, H-58-59, H-98-78, H-208, HC-1, HC-3, HK-94-134 (K), HK-98-155 (K), L-550 (K), ICCV 96030, Katila, NARC 9006, PDG 84-16 and H-85-78

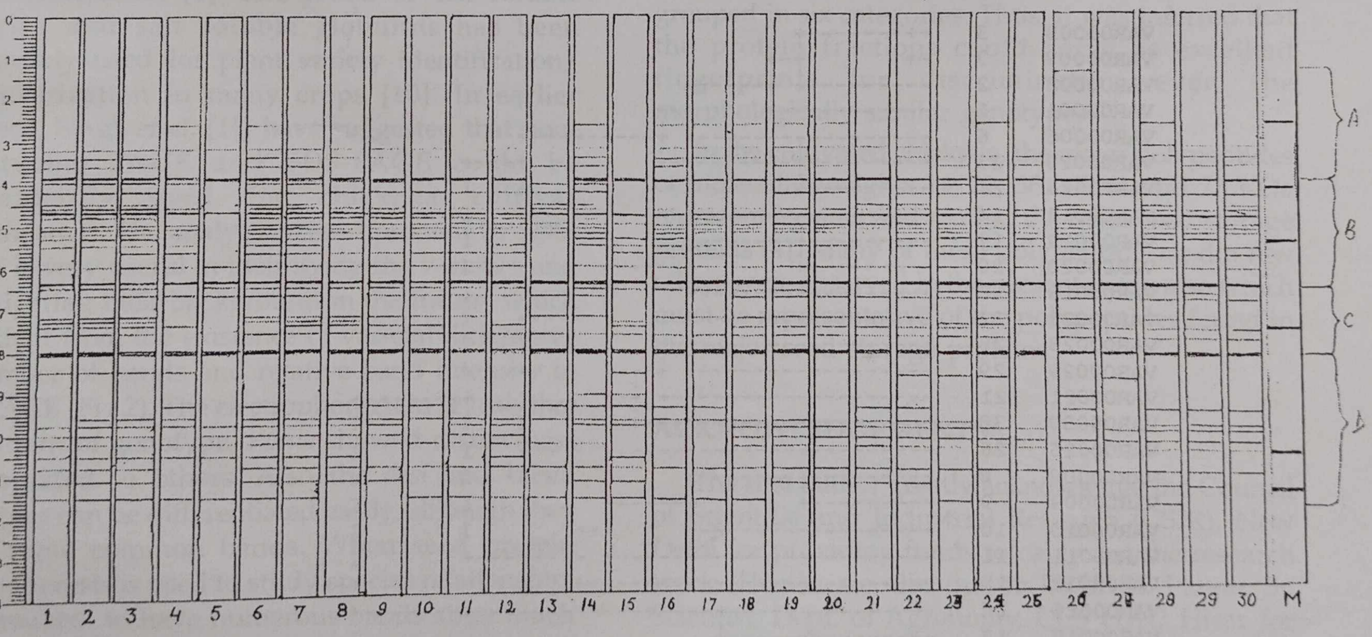

Fig 2. Electrophoregram of total soluble seed protein after SDS-PAGE among thirty chickpea genotypes

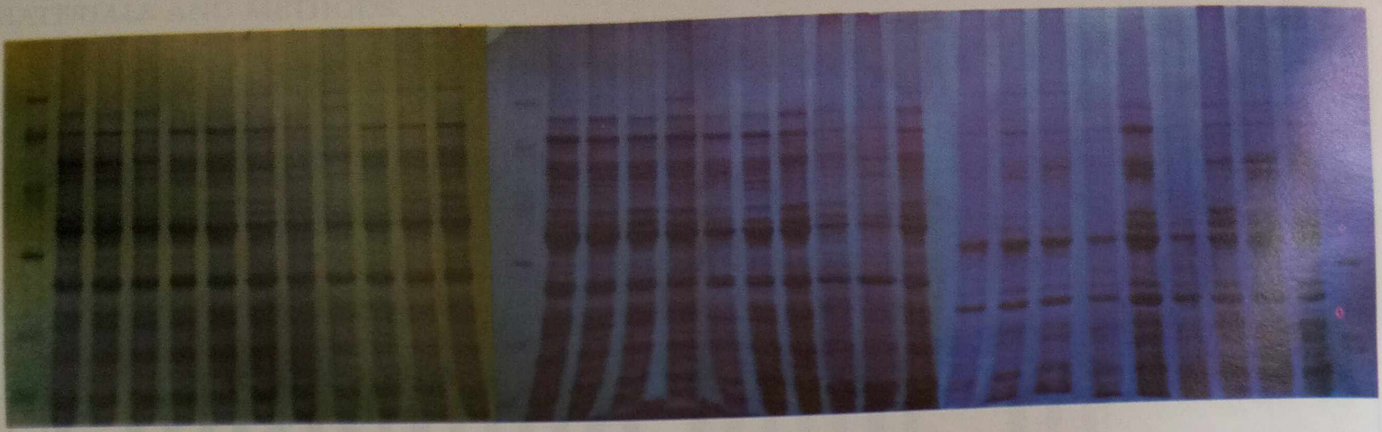

Fig 3. SDS-PAGE protein pattern of thirty chickpea (*Cicer arietinum* L.) varieties
L to R : Marker, H-90-231, H-91-36, H-92-68, H-92-67, H-96-99, H-97-253, H-97-254, H-97-255, H-98-57, H-98-57, Gora-Hisari, Marker, HK-89-131, HK-94-134, HK-98-155, L-550, C-235, Gaurav, CSG-8962, GL-94022, H-86-143, H-98-59, H-98-78, H-208, HC-1, HC-3, ICCV-96030, Katila, NARC-9006, PDG-84-16, H-85-78 and Marker

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* * * * * * H I E R A R C H I C A L   C L U S T E R   A N A L Y S I S *
* * * * *

 Dendrogram using Average Linkage (Between Groups)

                        Rescaled Distance Cluster Combine

    C A S E         0         5        10        15        20        25
  Label     Num     +---------+---------+---------+---------+---------+

  VAR00003    3    -+-------+
  VAR00007    7    -+       +-+
  VAR00002    2    ---------+ +-----+
  VAR00001    1    -----------+     +-+
  VAR00006    6    -----------------+ +---------+
  VAR00023   23    ---------+---------+         +---+
  VAR00024   24    ---------+                   I   I
  VAR00014   14    -----------------------------+   I
  VAR00019   19    ---------+-------+               I
  VAR00020   20    ---------+       +-----+         I
  VAR00018   18    -----------------+     I         I
  VAR00022   22    -----------+-----+     +-----+   I
  VAR00028   28    -----------+     +-+   I     I   I
  VAR00029   29    -----------------+ +-+ I     +---+
  VAR00021   21    -------------------+ +-+     I   +-------+
  VAR00030   30    ---------------------+       I   I       I
  VAR00026   26    -----------------------------+   I       I
  VAR00008    8    ---------+-------+               I       I
  VAR00009    9    ---------+       +-----+         I       I
  VAR00010   10    -----------------+     +-+       I       I
  VAR00011   11    -----------------------+ +-------+       I
  VAR00012   12    -------------------------+               +---+
  VAR00016   16    ---------------+-------------------------+   I
  VAR00017   17    ---------------+                         I   +---+
  VAR00004    4    -----------------------------------------+   I   I
  VAR00005    5    ---------------------------------+---------+ I   I
  VAR00025   25    ---------------------------------+         +-+   I
  VAR00027   27    -------------------------------------------+     I
  VAR00013   13    -------------------------------------------------+
  VAR00015   15    -------------------------------------------------+
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Fig 4. Association among thirty chickpea genotypes as revealed by SDS-PAGE of total soluble seed protein parentage is not common. On the other hand, three genotypes, H-98-57, H-98-58 and H-98-59 are having same parentage but these are grouped in two different clusters. H-98-57 and H-98-58 have 88% similarity thus their genetic distance is 12 whereas the genetic distance between H-98-57 & H-98-59 and H-98-58 & H-98-59 is 22 and 20 respectively. Similarly, HC-3 and HK-94-134 have one parent common but these two are grouped in different clusters with a genetic distance of 28. The genetic distance between H-97-253 & H-97-255 is 40% whereas between H-97-254 & H-97-255 is 36% inspite of having same parentage. Out of thirty chickpea genotypes there is maximum genetic distance between GL-94022 and H-97-253 (69%), which shows their genetic relationship on the dendrogram scale. Thus, the cluster analysis clearly represents the genetic relationship between all the thirty chickpea genotypes that can be useful for further crop improvement programs.

Discussion

There are a number of electrophoretic techniques and procedures to distinguish among closely related varieties, of the techniques available, analysis of seed or seedlings proteins using SDS-PAGE is most widely used because of their reliability, rapidity and cost effectiveness [8]. SDS-PAGE of Tris-soluble proteins and salt soluble globulins has been extensively used for plant variety identification/ characterization in many crops [10]. In earlier research, Singh *et al*, [11] have suggested that non-denaturing PAGE and SDS-PAGE could be conveniently used for chickpea cultivar identification. The analysis of seed proteins by SDS-PAGE is very useful in identifying the varieties and investigating their background in the future; which is evident from the existence of varietal differences in number of bands and relative band intensity in SDS-PAGE (Fig 2). The electrophoregram reveals that the pattern of genotype, *Katila* showed distinctness as compared to others. Also the *desi* and *kabuli* genotypes can be differentiated easily, although they share some common bands. When seed protein electrophoresis is used to study species relationship, it is desirable to have numerous bands since much more taxonomic information is encoded there than in a situation with fewer bands. In view of the fact that only limited information on species relationship, based on genome pairing, is available in the genus *Cicer*, the seed protein profile which reflects the genetic constitution of the species, should provide some clues. For example, *C. judacium* and *C. pinnatifidum* have been considered by various botanists to be variants of the same species. Of the total 74 bands observed in the profiles of these two species, only 26 could be matched. On the other hand, the close morphological resemblance (excluding seed coat texture) among *C. arietinum*, *C. reticulatum* and *C. echinospermum* are supported by their seed protein profiles.

The distribution of different class of proteins in seeds of 4 cultivars each of *kabuli* and *desi* chickpea using three different solvents and salt-soluble proteins have been characterized by electrophoresis [11]. There was very little difference between *kabuli* and *desi* types. Similarly, the results of Kharkwal [12] showed that *desi* (L-345, G-130 and H-214) and *kabuli* (C104) types are varieties of the same species, *C. arietinum*. All the four varieties were highly homologous and the pattern was also the same. SDS-PAGE analysis of 36 chickpea genotypes exhibited the same general pattern having nine bands corresponding to nine subunits [13]. The relative migration values of protein subunits ranged from 0.04 to 0.51 with molecular weight from 18,000 to 94,000 Dalton. The similarity in pattern indicated monophyletic origin. However, PAGE analysis of albumin fraction indicated that each protein subunit was under the control of separate genes and no two genotypes has a similar pattern. Globulin was also variable with 15 distinct patterns which could be grouped in six categories. Thus, it was inferred that the protein fractions could serve as excellent fingerprints for discriminating even the morphologically similar genotypes.

In the cultivated chickpea, the major polypeptides of molecular weights 14, 21, 36, 37, 41 and 73 KDa represent *legumin* and *vicillin*, the main seed storage proteins [4]. Many of these polypeptides and a few others were observed in the present study also which must be representative of the polypeptides found in the major seed storage proteins.

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