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Characterization of Storage Proteins in Sunflower (*Helianthus annuus* L.)

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ABSTRACT: The seed protein extracts of seventeen sunflower lines (*Helianthus annuus* L.) analysed by SDS-Polyacrylamide gel electrophoresis revealed a large number of polypeptides of molecular weight ranging from 63,000-14,000. The bands in regions of MW 63-60,000, 58- 57,000, 55,000, 53-52,000, 46,000, 20,000 contained disulphide linkages. Under non reducing conditions these disulphide linkages were found to be inter as well as intra polypeptide in nature. Two-dimensional gel electrophoresis showed that bands of MW 63,000 – 60,000, 58-57,000, 55,000, 53,000, 51,000 and 46,000 consisted of two subunits – one large and one small – and thus represented polypeptide pairs. These by comparison with legumes could be considered as legume- like polypeptide pairs. Gel electrophoresis of the four protein fractions showed that globulins and albumins contained polypeptides with disulphide linkages. The glutelin and prolamin fraction had polypeptides in low mol. wt. region i.e., between MW 18,000 to 25,000. Globulins contained the major darkly staining bands. The albumin fraction had polypeptide pairs of MW 51,000 and between MW 41,000 to 30,000.

KEYWORDS: Sunflower, storage proteins, SDS PAGE, Protein fractions

I. INTRODUCTION

Sunflower finds an important place among the major edible oil yielding crops. Botanically known as *Helianthus annuus* L. and belonging to the family Asteraceae, the sunflower is one of the four most important oil crops globally and is grown on over 21 million hectares worldwide (Škorić et al., 2007). Oil content of the seed is known to vary from 30-46%. With 60% yield coming from Europe, the major sunflower producing countries are Russia, Argentina, China and U.S.A. Sunflower meets about 4% of edible oils of our country.

So far, its cultivation has been mainly limited to four southern states of Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu. Though, it has been gaining popularity in Punjab, Haryana and Uttar Pradesh also (Seetharam and Virupakshappa, 1989). Most of the Sunflower oil is represented by unsaturated fatty acids with linolenic acid component being almost absent. This composition makes the sunflower oil as medically accepted oil because it helps to reduce the cholesterol level in blood. Various breeding programs have generally aimed at developing hybrid varieties with a view to increasing seed yields, percentage of oil and resistance against certain important diseases. However, improvement of seed and oil quality has not been much emphasized as an important breeding objective.

Most commonly used as vegetable oil, sunflower oil also finds uses in industries like paints varnishes and plastics and to some extent in soaps and detergents. Sunflower proteins are also of great use in various ways. Sunflower meal left after extraction of seed oil has provided a very good source of proteins in animal foods. The protein content of dehulled seeds after oil extraction has been reported to be as high as 50% (Sosulski, 1979). The amino acids analysis has shown that sunflower proteins are rich in methionine but quite low in lysine. The rat feeding experiments with sunflower meal or blend with cereals also showed low protein efficiency ratio (Sosulski, 1979). Upcycled defatted sunflower seed flour (SUN), a by-product obtained from sunflower oil extraction, was used as an animal fat replacer to develop healthier frankfurters. (Grasso et al. 2021)

In addition to being known for value in bird feed, sunflower proteins have been found valuable in human foods as well. These have been used to supplement wheat flour in bakery for bread and cookies. Also the protein ingredients find uses in infant formulae and as meat and milk extenders. Zilic et al., 2010 observed the significant differences among the seed and kernel protein contents.



With increasing recognition of sunflower as an important protein source, a good number of reports/studies are available on sunflower seed proteins. However, this work has mainly involved use of only one line in various studies with sunflower cultivation finding base in Indian Agriculture, it was decided to analyse certain hybrid lines and other lines being used in Indian Programs. Here, we have shown the results of various studies on seed protein of different sunflower lines.

II. METHODOLOGY

Biological Material

Various lines of *Helianthus annuus* used are as follows:

SH-8, MSFH-1, MSFH-30, APSH-11,7-1A, 6460, P271 R, CMS- 234A, KBSH-1, LDMRSH-3, KSP-15, TNAU-SUF-9, 300B, 341 B, 6D-1, MRHA-1, CMS-207A.

Chemicals and Reagents

Most of the chemicals used during the present investigations were those manufactured by Qualigens Fine Chemicals, Bombay, E. Merck (India) Limited (Bombay), Loba Chemieindo Australan Co. (Bombay), Glaxo Fine Chemicals (Bombay) and Sisco Research Laboratories Pvt. Ltd. Bombay. Acrylamide, Trizma base, 2- mercaptoethanol, coomassie brilliant blue, ammonium per sulphate (APS), sodium dodecyl sulphate (SDS), N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) and molecular marker proteins were all from Sigma Chemical Company, U.S.A. Visking tubing was from Medicell- International, London.

Methods

For various analyses involving use of seed meal, the seed meal was first defatted. One gram of defatted seed meal was then extracted with 10 ml of 50 mM borate buffer (pH 8.0) by continuous stirring for 2h at 4⁰C on a magnetic stirrer. This was followed by centrifugation at 23,000g in a Remi R-24 high speed refrigerated centrifuge at 4⁰C for 40 min. The supernatant 'A' contained albumins and globulins. The residue was then extracted with 10ml of 0.1 N NaOH and centrifuged at 14,000g for 20 min at 4⁰C. The supernatant 'B' represented glutelins. Further, extraction of the residue was carried out using 70% ethanol. Centrifugation at 9,000 g for 10 min at 4⁰C yielded prolamins in the supernatant 'C'. Supernatant 'A' was dialysed against 33mM acetate buffer (pH 4.8) and the proteins so precipitated were separated by centrifugation at 23,000 g for 40 min. This pellet representing the globulin fraction was dissolved in minimum volume of borate buffer. The supernatant 'D' obtained on dialysis of supernatant 'A' contained the albumins.

SDS- Polyacrylamide Gel Electrophoresis

The total protein extracts of all the lines were analysed in the discontinuous system of gel electrophoresis of Davis (1964) and under dissociating conditions following the formulations of Laemmli (1970). 14% polyacrylamide slab gels of 1mM thickness were formed by using 24x16 cm glass plates.

Reagents:

A. Separation gel

i)	Acrylamide stock (30 g acrylamide And 135 mg N, N1-methylene-bisacrylamide made to 100 ml with D.W.)	28.0 ml
ii)	1M Tris HCL (pH 8.8)	22.5 ml
iii)	Distilled water	7.0 ml
iv)	1% ammonium persulphate ----degassed----	1.5 ml
v)	10% SDS	0.5 ml
vi)	TEMED (-NNN'N'-Tetramethylethylene Diamine)	15 μ

B. Stacking gel

1.	Acrylamide stock (30 g acrylamide and 435 mg N, N1-methylene-bisacrylamide in 100 ml DW)	3.0 ml
2.	1M Tris HCL (pH 6.8)	2.5 ml
3.	Distilled water	13.8 ml
4.	1% ammonium persulphate	0.5 ml



---degassed---

- 5. 10% SDS 0.2 ml
- 6. TEMED (-NNN'N'-Tetramethylethylene Diamine) 15µ

Ammonium persulphate and SDS were prepared fresh every time.

To run a gel, Tris-glycine buffer was prepared having the following constituents:

Glycine (14.1 g); Trizma base (3.0 g); SDS (1.0g) were dissolved in water and volume made to one litre. In the upper reservoir 2-3 drops of 0.1% Bromophenol blue were added to make the marker front.

Proteins of known molecular weights supplied as 'Dalton mark VII-L' by Sigma were run and their relative mobilities on gels calculated. The mixture contained following standard proteins.

Two-dimensional Gel Electrophoresis

For two -dimensional gel electrophoresis of different protein samples, polyacrylamide gel electrophoresis was carried out under non-reducing conditions in the first dimension followed by the same under reducing conditions in the second. The gel strip with polypeptides separated under non reducing conditions was equilibrated for 2h with three changes of 0.25M Tris HCl (pH 6.8) buffer containing 2% SDS and 2% 2- mercaptoethanol. The gel strip so equilibrated was loaded on to a 2mm thick gel, the gel thicker than that used in the first dimension.

III. RESULTS AND DISCUSSION

Calibration curve for Molecular Weight Estimation

The 14% SDS- polyacrylamide gels were calibrated by running proteins of known molecular weight under reducing conditions. The relative mobilities of these standard proteins were found to be as follow

S. No.		Protein	Mol. Wt.	Relative Mobility
1.	G	Albumin, Bovine	66,000	0.29
2.	F	Albumin, Egg	45,000	0.51
3.	E	Glyceraldehyde-3-p-dehydrogenase	36,000	0.59
4.	D	Carbonic Anhydrase, Bovine	29,000	0.65
5.	C	Trypsinogen, Bovine Pancreas	24,000	0.75
6.	B	Trypsin Inhibitor, Soybean	20,000	0.84
7.	A	α- Lactalbumin, bovine milk	14,200	0.97

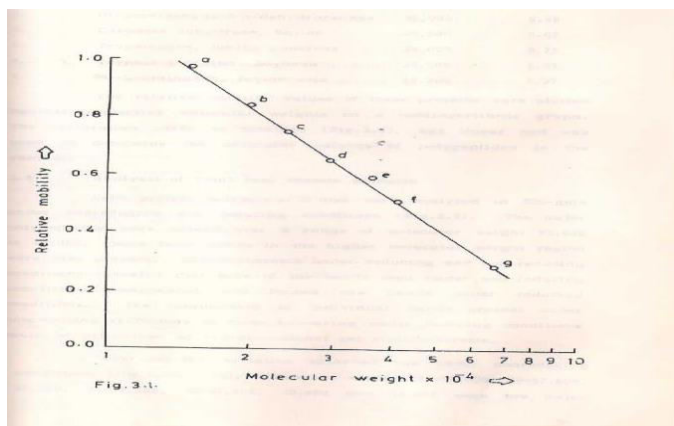


Fig 3.1: Calibration Curve for estimation of molecular weights on SDS-polyacrylamide gels.



The relative mobility values of these proteins were plotted against respective molecular weights on a semilogarithmic graph. The calibration curve so obtained (Fig.3.1) was linear and was used to determine the molecular weights of polypeptides in the samples.

Analysis of Total Protein Extracts

Seed protein extracts of 17 lines were analysed in SDS –gels under non-reducing and reducing conditions. The major polypeptides were spread over a range of molecular weight 63,000 to 14,000. Some faint bands in the higher molecular weight region were also present. Electrophoresis under reducing and non-reducing conditions revealed that some of the bands seen under non-reducing conditions disappeared and formed new bands under reducing conditions. The relationship of individual bands present under non-reducing conditions to those appearing under reducing condition could be determined by two-dimensional gel electrophoresis.

Most of the variation observed was under non-reducing condition. Polypeptides of mol. wt. 63-60,000; 58-57,000; 55,000; 53-52,000; 48-47,000; 20,500 and 19,500 were the major polypeptides. In presence of 2- mercaptoethanol, the bands of MW 63,000-51,000 disappeared and in their place new bands appeared at position between 29,000 to 46,000. Sharp bands of medium intensity could be seen as appearing at MW position 59,000; 56,000 and 51,000. Polypeptides of MW 39,000; 30,000 and 25,000 were represented by faint bands and remained uncharged in the presence of 2-mercaptoethanol.

The relationship of bands present under non-reducing conditions with those observed under reducing conditions could be analysed by two-dimensional gel electrophoresis. Following subunit pairs were observed:

- 63,000= 51,000+ 20,500 and 20,000
- 60,000= 46,000+ 20,500 and 20,000
- 57,000= 41,000+ 20,000
- 55,000= 40,000+ 19,500
- 53,000= 34,000+ 20,500
- 51,000= 29,000+ (19,500 and 18,500)
- 46,000= 22,000+20,500

Polypeptides of MW 20,000; 50,000 and 53,000 moved as spots to position of higher MW 23,000; 59,000 and 56,000 respectively.

Variation in Polypeptide Patterns

Analysis of different lines showed a number of varying polypeptides patterns in different regions. The light band above the major dark polypeptide of MW 60,000 varied as band of MW 63,000 and 62,000. The major polypeptides of MW 60,000; 57-58,000 and 55,000 did not show any variation. Four different patterns occurred between MW 51,000-53,000.

These lines were as follows:

- MW 53,000= in lines MSFH-1, 6460 and KSP-15
- MW 53,000 and 51,000= in lines MSFH-30, 207B, TNAU-SUF-9, 300B, CMS-234A and CMS-207 A
- MW 52,000 and 51,000= in line MRHA-1 and 6D-1

In the region of MW 36,000, three patterns observed were as follows:

- MW 36,000= in line KBSH-1
- MW 37,500 and 36,000= SH-8, MSFH-30, 6460, TNAU-SUF-9, 207B, 300B, MRHA-1, CMS-207A and LDMRSH-3
- MW 37,500= MSFH-1, KSP-15, CMS-234A and 6D-1

In the lower mol. wt. region to dark bands of MW 19,500 and 20,500 were present in all lines and variation occurred with respect to polypeptide of mol. wt. above these. Whereas, some lines had polypeptides of MW 19,500 and 20,500 only, others had either polypeptide of MW 21,500 or MW 21,500 and 22,500 above these common bands. In the presence of 2- mercaptoethanol, there did not occur any variation in the polypeptides representing large and small subunits of subunit pairs.

SDS-PAGE of Seed Protein Fractions

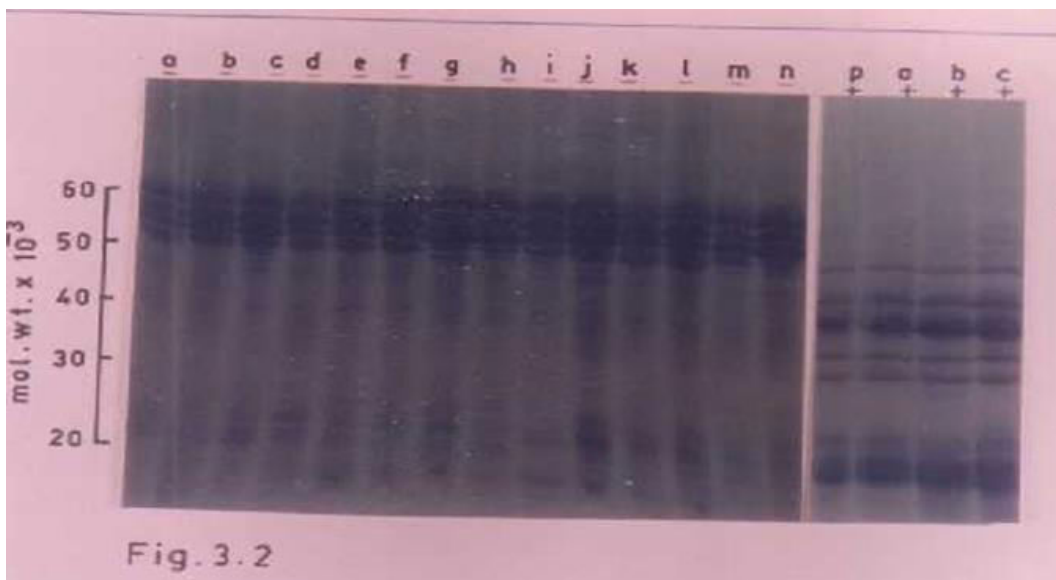
Polypeptides constitute the four protein fractions. Whereas, prominent polypeptides with maximum number were observed in globulin fraction followed by albumin fraction, those constituting the glutelins and prolamins were very faint and were present in the low mol. wt. region. These fractions were not fully dissolved, in the sample buffer and certain polypeptides with insoluble present in the background could not be resolved as sharp bands.

The globulin polypeptides, like those in the seed extract, were present in the range of MW 63,000 to 14,000. The major dark bands of MW 60,000- 51,000 and certain other bands seen under non-reducing condition disappeared when run in the presence of 2-mercaptoethanol. Under reducing conditions, the darkly staining bands were present in the region of MW 46,000-40,000; 34,000 and 20,500-19,500. The two-dimensional gel electrophoresis showed polypeptide- pairs as breaking into two spots and each moving off the diagonal. Thus, each subunit pair under non-reducing conditions consisted of larger and a smaller subunit. The molecular weights and polypeptide composition of subunit-pairs was similar to as described for those in the seed protein extracts. The only difference which could be observed was the lack of the pair of subunits 29,000+ 19,500.

The albumin fraction was also constituted by a large number of polypeptides in the range of MW 14,000 to 80,000. Under non-reducing condition the major polypeptides present had mol. wt. as 51,000; 47,000; 41,000-40,000 to 30,000 and 25,000. On analysis under reducing condition, new polypeptides of MW 29,000-19,500 could be seen. The two dimensional gel electrophoresis of albumin showed some of the darkly staining polypeptides moving along the diagonal. Whereas, others moved downwards as two spots off the diagonal. Following subunit pairs were observed:

- 51,000 = 29,000 + 19,500
- 41,000 = 25,000 + 20,500
- 35,000 = 22,500 + 20,000
- 30,000 = 20,500 + 19,500

Very faint bands could be analysed in the glutelin and prolamin fractions. Whereas, polypeptides of MW 25,500; 22,000; 21,000 and 18,000 constituted glutelins, prolamins had polypeptides of MW 25,000; 21,500; 20,000 and 19,000.



Track	Sunflower lines	Track	Sunflower lines
a	SH-8	H	300B
b	MSFH-1	I	CMS234 A
c	MSFH-30	J	KBSH-1
d	6460	K	6D-1
e	KSP-15	L	MRHA-1
f	TNAU-SUF-12	M	CMS 207A
g	207B	N	LDMRSH-3
		P	SH-2

‘-‘ stands for absence of 2- mercaptoethanol; ‘+’ stands for presence of 2- mercaptoethanol

Fig 3.2 SDS-polyacrylamide gel electrophoresis of Sunflower lines.

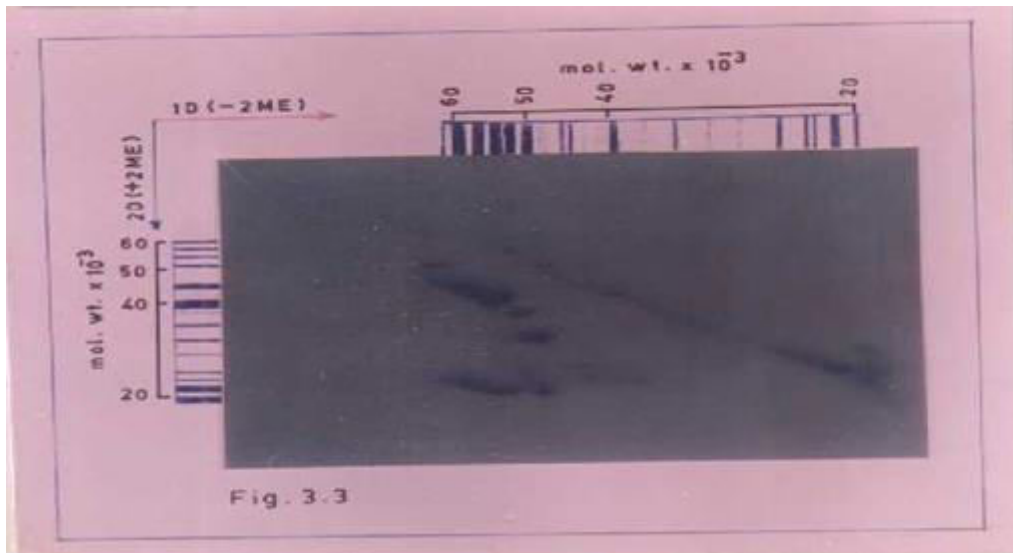


Fig. 3.3 Two dimensional gel electrophoresis of seed extract of sunflower line CMS-234A.

‘1D-2ME’ - SDS- polyacrylamide gel electrophoresis in the absence of 2- mercaptoethanol in the first dimension. ‘2D + 2ME’ - SDS- polyacrylamide gel electrophoresis in the presence of 2- mercaptoethanol in the second dimension.

Studies on proportion of four protein fractions showed that the salt soluble fraction is the dominant protein fraction with prolamins representing the fraction with lowest proportion. The other two fractions- albumins and glutelins had more or less the same proportion. This distribution of four protein fractions in sunflower proteins represents the same trend as found in legumes (Boulter and Derbyshire (1978), Dhankher et al. 1990; Chandna et al., 1991). This pattern of distribution (globulins: 63-68.6%, albumins and glutelins: approx. 15% each and prolamins: 4-6%) is quite similar to that observed for *Cicer arietinum*. Thakral and Matta, unpublished) *Lathyrus sativus* (Chandna et al. 1991) and *Vigna radiata* (Sushil Kumar, 1993).

The proportion of various seed protein fractions and their subunit composition is a very important factor in determining the grain quality and nutritional value of a given protein source. Maintenance of genetic uniformity of lines and hybrids is a prerequisite for successful production and placement of commercial hybrid seed on the market. Genetic purity of seed as specific seed trait is of great significance for seed science. The methods of electrophoretic separation of isozymes and seed storage proteins were in agreement, with differences ranging from 1% to 5% in 81% of the samples (Nikolić, Z. et al 2008). The reports of differences in ratio of protein fractions and their subfractions in cereals and legumes and findings of certain methionine rich/poor fractions in legumes and lysine poor fractions in cereals suggests



towards screening and breeding of lines with nutritionally richer protein fractions. Analysis of individual sunflower protein fractions for amino acids of importance thus, will make a significant study. Low lysine in cereals is suggested to be due to higher proportion of prolamin fraction which contributes 40-50% of total protein in many cereals. The sunflower globulins are represented mainly by 11S fraction and proportions of prolamins is very low. Also sunflower proteins are known to be poor in amino acids like lysine (as in cereals) but methionine level unlike legumes is high. It will be of interest to know the factors which lead to higher methionine and low lysine. As many as 17 sunflower lines were used for studying variation in polypeptide patterns on polyacrylamide gels. Variation could be observed only for a few polypeptides e.g. MW 51,000 and MW 36,000. This lack of variation in polypeptide patterns may be due to the fact that all these lines have been developed from the same genetic stock or these polypeptides have undergone very little variation after domestication of this crop. Most of the darkly staining bands were in the range of MW 51,000 to 60,000 and these disappeared in the presence of 2-mercaptoethanol. As many as 15 new bands could be seen under reducing conditions. This shows the occurrence of disulphide linkage in sunflower protein. Two dimensional gel electrophoresis under non-reducing conditions in the first dimension followed by that under reducing conditions in the second dimension was used to study the nature of these disulphide linkages and the relationship of bands seen under nonreducing conditions. whereas, all the bands of MW 51,000 to 60,000 resolved into two spots off the diagonal, some bands moved up as spots to a higher position, In this way the former represents the occurrence of intra polypeptide disulphide linkages. Reduction of intra polypeptide disulphide linkages results in complete unfolding of the polypeptide leading to its being resolved up at a higher mol. wt. position. Polypeptides lacking disulphide linkages move along the diagonal in the second dimension. Thus two -dimensional gel electrophoresis has proved an excellent tool for studying the polypeptides with and without disulphide linkages.

A large number of darkly staining bands under non reducing conditions have been seen to have interpolypeptide disulphide linkages. Those staying along the diagonal were lightly staining. On the basis of studies in legumes, sunflower proteins, with disulphide linkages may represent the legumin like polypeptide pairs and the ones resolved as faint spots along the diagonal, the vicilin- like polypeptides.

Gel electrophoresis of four protein fractions showed the occurrence of a large number of polypeptides in the globulin and albumin fractions. The polypeptides in glutelins and prolamin fractions were restricted only to low mol. wt. region. Similar low mol. wt. polypeptides have been found in glutelins and prolamins of various legumes like *Vigna unguiculata* (Dhankher *et al.*, 1990), *Lathyrus sativus* (Chandna *et al.*, 1991), *Vigna radiata* (Sushil Kumar, 1993) and *Cicer* (Khalid *et al.*, 2023). The globulin fraction had polypeptide pairs in the range of MW 51,000-60,000 and MW 45,000. Polypeptide pairs (MW 51,000; 41,000-30,000) were also observed in albumin fraction. The polypeptide pair of MW 51,000 breaking into subunits of MW 29,000+ 19,500 and present in the seed extract thus represents the constituent of albumin fraction. This subunit pair and its constituent subunits lacked in the globulin fraction. Thus, the remaining bands (MW 63,000; 60,000, 57,000; 55,000; 53,000, 46,000) of seed extract under non reducing conditions may be representing the legumin like polypeptide pairs and those staying along the diagonal and representing vicilin-like(7S) polypeptides were very few and faint. These electrophoretic studies confirm the reports of occurrence of legumin-like 11S fraction only in the sunflower globulins (Joubert, 1955). In Hemp seeds edestin which is the 11S globulin fraction is most abundant and it has two subunits of MW approx 35,000 and 20,000 (Xin Sun *et al.* 2021). The 11S protein, as per model of legumin given by Wright and Boulter (1979), consist of six monomers of approx. MW 60,000. Each monomer further consists of two sub units of MW 40,000 and 20,000. Further work on legumin of *Vicia faba* and *Pisum sativum* showed much more heterogeneity and Matta (1981) classified legumin subunit pairs as belonging to three different classes. The earlier reported subunit pairs of MW 60,000 belonging to conventional and major legumin present in relatively high concentrations were designated as LegC. The subunit pairs of mol.wt. higher and lower than the conventional legumin and present in lower concentration were designated as LegB ('big' Legumin) and LegS ('small' legumin). Similar heterogeneity in molecular weights of subunit pairs has been observed in legumin of *Lathyrus sativus*, another member of the tribe viciae (Chandna *et al.*, 1991). The sunflower proteins seem to have all these three types of legumins. However, these are more similar to *Pisum* legumin in having 'big' legumin subunit pairs and subunits as present in *Vicia faba* (Matta *et al.*, 1981a) and *Lathyrus sativus* (Chandna *et al.*, 1991) were lacking.

The occurrence of disulphide bonded polypeptide pairs and sulphur rich polypeptides in albumins in legumes have been known earlier. It will be of great interest to study similar albumin polypeptides in sunflower proteins for their role and amino acid composition. Amino acid composition and other characterization studies of individual polypeptides belonging to major protein fraction may provide answer for the higher methionine and lower lysine in sunflower proteins.



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