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COMPARATIVE EVALUATION OF PROTEIN EXTRACTION METHODS FROM FEW LEGUMINOUS SEEDS

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ABSTRACT:

SDS PAGE has been proved as one of the most important parameters for studying total protein as well as storage proteins in plants. However the consistency and accuracy of the protein based studies depend completely on the protein isolation. There have been a number of methods explained and used by different scientists in previous studies. Quality of a method however depends upon the amount of protein extracted as well as clarity and appearance of rare bands on SDS PAGE.

Seven different previously studied methods were compared for their protein extraction efficiency from the seeds of four different leguminous plants (*Pisum sativum*, *Vigna radiata*, *Cicer arientum* and *Vigna mungo)*. The efficiency was determined by the yield of protein as well as the clarity in the resolution of protein bands separated on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). In the study, the method thus used by Naushad et al., 2010 was found to be the best performing method as compared to other six methods under study.

Keywords: SDS PAGE, Protein isolation, Seed protein, Isolation method, Legumes, Plants.

[I] INTRODUCTION

In recent years, seeds of legumes have played a primary role in the search for vegetable sources of proteins owing to the high protein content of the seed, ranging from 20% in pea to 40% in lupin[1]. They can, therefore, be considered a good substitution to animal proteins in human diet, especially in the third world. However, the seed storage proteins of these legumes contain a low concentration of sulfur-containing aminoacids and plant breeders have to consider this problem in any improvement programs[2]. The legume crops can be improved after studying the genetic diversity in germplasm across different geographical area. Information about genetic diversity of germplasm is a useful tool in gene bank management and in planning experiments, as it facilitates efficient sampling and utilization of germplasm by identifying and/or eliminating duplicates in the gene stock and helps in the

establishment of core collection[3-5]. The protein profiling of germplasm and use of genetic markers have been widely and effectively used to determine the taxonomic and evolutionary aspects of several crops[3-8].

The technique of Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is commonly used for separation of seed storage proteins. Seed storage protein profiles have also been used to study evolutionary relation of several crop plants[9]. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is most economical simple and extensively used biochemical technique for analysis of genetic structure of germplasm. As seed storage proteins are largely independent of environmental fluctuations, their profiling using SDS-PAGE technology is particularly considered as a consistent tool for economic characterization of germplasm[10-11].

However there has been always confusion on isolation methods for protein as different scientists have used different methods for the same. The goal of this study is to compare and suggest the best performing protein isolation method from seeds belonging to fabaceae family (Legumes).

[II] MATERIALS & METHODS

2.1. Plant material

Four different types of leguminous seeds namely *Pisum sativum* (PlantA), *Vigna radiata* (PlantB), *Cicer arientum* (PlantC) and *Vigna mungo* (PlantD) were obtained from the local farmers near by Visakhapatnam, Andhra Pradesh. The seeds were collected dried, were rinsed with distill water to remove the dust particles and any other impurities and then dried again for the experiment.

2.2. Reagents

All the chemicals and reagents used were research grade.

2.3. Protein extraction

Seven different methods of protein extraction were carried out, using different buffer solutions.

2.3.1. Method1

Total proteins were extracted by adding 300mg of ground seeds in lml of 50mM tris-HCI (pH 7.5) and 0.5 M NaCI at 4^0C for 60 minutes. This was then frozen at -20^0C and thawed 3 times during 24h to disrupt the tissue and release the proteins (Miller et al., 1972) and centrifugations were at 10000 g for 15 min.

2.3.2. Method2

For extraction of proteins, seeds were grounded in 50 mM phosphate buffer (pH 7.8) and centrifuged in micro-centrifuge machine for 10 min at 14,000rpm (as used by Hameed et al., 2009). The supernatant was separated and used for protein profiling.

2.3.3. Method3

The method suggested previously (Damania et al., 1983) was used as method 3. The grains were ground to fine powder and 10 mg was weighed in 1.5 ml micro tube. 400 ml protein extraction buffer (Tris-HCl 0.05 M, pH 8, 0.02% SDS, 30.3% urea, 1% 2-mercaptoethanol) was added to each micro tube, kept overnight at 40°C and centrifuged at 13000 rpm for 10 min. The supernatant contain dissolved extracted protein ready for experiment purposes, which could be kept for longer time at 4°C.

2.3.4Method4

Seeds were grinded to fine powder with the help of mortar and pestle. We added sample buffer (used by Naushad et al., 2010) (400 µl) to a 0.02 g of fine seed flour as extraction liquid and bromophenol blue (BPB) follow the movement of protein in the gel. The active ingredients used for the extraction of protein buffer contained 0.5 M Tris-HCl (pH 8.0), 0.2% SDS, 5 M urea and 1% 2-mercaptoethanol. In order to know the movement of protein in the gel, a dye in the form of bromophenol blue was added to the extraction buffer. When all these chemicals are tightly put together than the solution needs to be purified and homogenate, we mixed the samples thoroughly by vortexing and centrifugation at 15,000 rpm for 5 min at room temperature. After

centrifuging samples, the crude proteins were recovered as clear supernatant on the top of the tube. Then the supernatant were transferred into new 1.5 ml eppendorf tubes and were stored at -20°C until gel electrophoresis.

2.3.5. Method5

Protein extraction was carried out by homogenizing the cotyledons in 0.1 M Tris HCL buffer 7.5pH [16]. Samples of supernatant obtained after centrifugation at 17600g at 4^0C for 20 minutes were diluted with sample buffer and loaded in the gel.

2.3.6. Method6

Seed were routinely prepared by grinding seed sample in buffer used by R.H. Sammour, 1991. Total protein extracts were prepared by extracting appropriate protein of the seed with 0.2 M Tris /HCl, pH 6.8; distilled water and 0.125M Tris/borate, pH 8.9. All these extracts were carried for 24 h at 4°C and then centrifuged at 10.000 rpm for 20 min. The supernatant was used for electrophoresis.

2.3.7. Method7

The seeds were pounded in the mortar till they were powdered finely. 0.5 g seed flour and 1.5ml of buffer (suggested by Arulsekar and Parfitt, 1986), containing {0.05 M Tris base 6.5 g/L; 0.007 M citric acid (monohydrate) 1.5 g/L; 0.1% cysteine hydrochloride 1 g/L; 0.1% ascorbic acid (Na salt or free acid) 1 g/L; 1.0% polyethylene glycol (H 3500) l0.0 g/L; 1 mM 2-mercaptoethanol 0.08 mL/L, the final pH 8.0} were mixed and homogenized for 1 min. Homogenates in the tubes were transferred into eppendorf tubes and centrifuged at room temperature at 18000 rpm for 20 min. Supernatants were transferred into the new eppendorf tubes and pellets were discarded.

2.4. Protein estimation

The proteins were determined by Bradford's method (Bradford, 1976).

2.5. Electrophoresis (SDS PAGE)

One dimensional Sodium dodecyl sulfate polyacrylamide gel were prepared in a concentration of 8% resolving gel and 4.44% stacking gel as suggested by Sambrook et al. (1989). Electrophoresis was carried out according to Laemmli (1970) [20] after adding sample loading buffer. Protein bands were visualized by silver staining method as described by Blum et al.(1987) [20].

[III] RESULTS & DISCUSSION

Quantitative estimation by Bradford's protein estimation revealed that method 7 showed the best result with plants A, B and C with maximum amount of protein extracted. In plant D, however, method 4 was the best extraction method quantitatively (Table 1). When SDS PAGE was used to resolve the extracted proteins few unique revelations were observed.

While method 7 was found to maximum yielding protein extraction method in majority of the plants studied, method 4 was established as the best extraction method after electrophoretic study.

Also method 4 proved to be best yielding method in plants D. In addition it was the second best yielding method in all other plants studied. Moreover the SDS PAGE results made it quite clear that in method 4 maximum numbers of bands were observed with minimum background streaking effect. This proves the quality of the extracted protein by method 4.

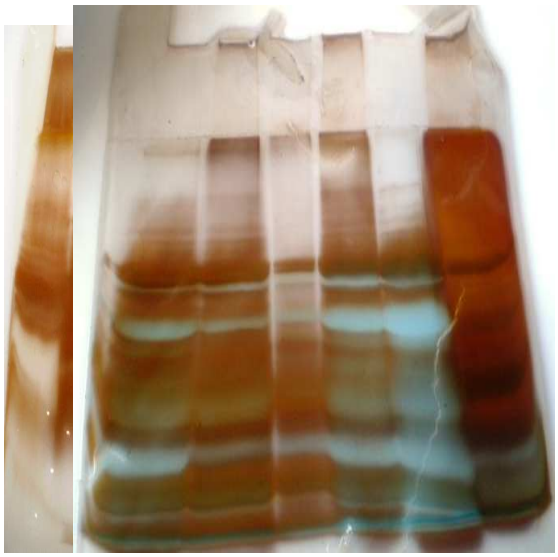
The seven different methods of protein extraction used in the present study comprised of different extraction buffer as well as different methods of cell disruption. It included method which depended upon freezing and thawing, method using grinding of seeds to rupture cells mechanically. Few methods also depended upon chemical lyses of cells using detergent. The best of the methods of protein extraction studied for plant seeds under study was determined as method 4. The method 4 actually was a combination of chemical as well as mechanical extraction procedure which may have resulted in the enhanced performance. Method 3 however used the same buffer as used in method 4 but it

did not used rough mechanical lyses and depended entirely on action of chemicals on powdered seeds. This explains the poor performance of method 3 and also proves that the extraction buffer alone is not responsible for the performance of a method but it also depends upon the cell rupture strategy.

Protein isolate	**Amount of protein per gram of seed (in mg)**			
	Pisum sativum	*Vigna radiate*	*Cicer arientum*	*Vigna mungo*
Method1	0.146	0.173	0.148	0.170
Method2	0.200	0.210	0.190	0.230
Method3	0.080	0.092	0.060	0.010
Method4	0.280	0.360	0.300	0.480*
Method5	0.150	0.190	0.270	0.140
Method6	0.170	0.140	0.120	0.180
Method7	0.470*	0.590*	0.510*	0.440

Table 1: Amount of protein yield from plant seeds under study using different methods (* denotes the maximum protein yield)

PlantA:

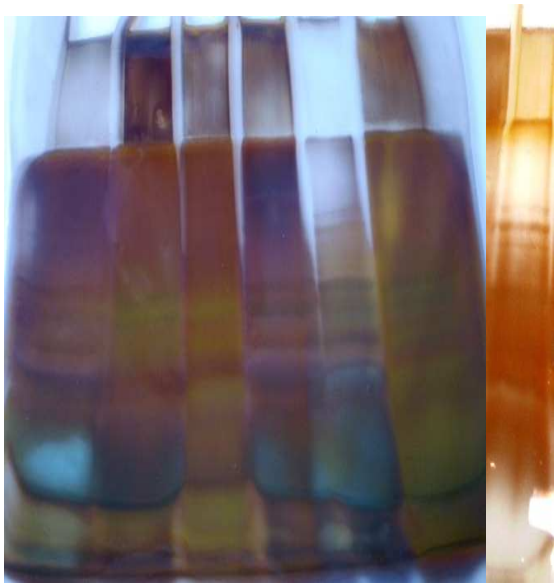


7 1 2 3 4 5 6

Fig: Protein bands of Plant A on SDS PAGE (Protein extracted by 1-7 different methods)

Another method (method 7) was found to be extracting maximum amount of protein in most of the plants studied was not found to be holding that good when studied on SDS PAGE. This can be explained as the extraction of proteins with same molecular weight and therefore not producing rare protein bands. This can be supported by presence of many very thick bands in method 7. Another negative side of method 7 is high streaking effect hindering the resolution.

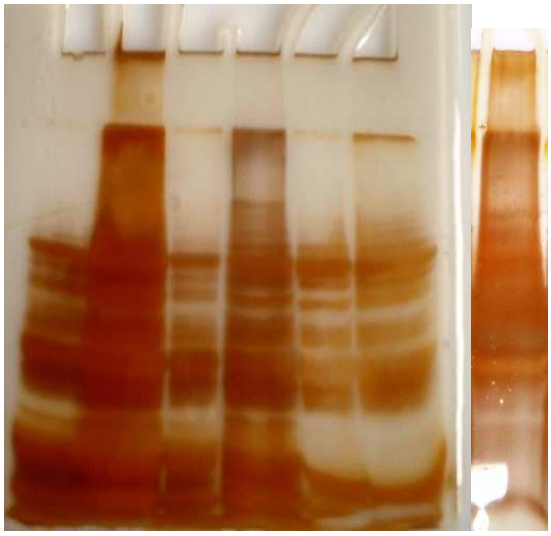
PlantB:



1 2 3 4 5 6 7

Fig: Protein bands of Plant B on SDS PAGE (Protein extracted by 1-7 different methods)

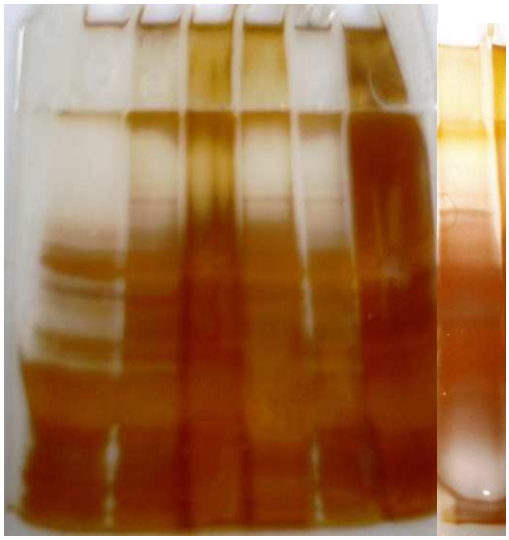
PlantC:



1 2 3 4 5 6 7

Fig: Protein bands of Plant C on SDS PAGE (Protein extracted by 1-7 different methods)

PlantD:



1 2 3 4 5 6 7

Fig: Protein bands of Plant D on SDS PAGE (Protein extracted by 1-7 different methods)

[IV] CONCLUSION

In conclusion, the method 7 (Naushad et.al, 2010) [15] was determined as the best method for protein extraction taking into consideration, the protein yield and the SDS-PAGE resolution. Although the study was conducted upon a set of leguminous plants, a similar result can be expected for protein isolation from seeds of other plants.

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