Characterization of Proteins in Costus Pictus D. Don

Geena Paul and Augustin A
**Centre for Plant Biotechnology and Molecular Biology*
Kerala Agricultural University, Vellanikkara, Kerala, India

Abstract

Proteins in Costus pictus was purified and studied using PAGE, SDS- PAGE, Ammonium Sulphate fractionation, DEAE cellulose anion exchange chromatography and sephadex G-100 gel filtration chromatography. Tris buffer of 0.2M at pH 7.2 was found to be suitable buffer for extracting Costus pictus proteins. Ten percent acryl amide gel concentration for resolving and six percent for stacking gel could resolve proteins efficiently through SDS- PAGE and PAGE. Proteins of basic nature are precipitated with ammonium sulphate in the range of 30 to 50 per cent saturation and acidic proteins were precipitated at higher saturation of ammonium sulphate. Higher concentration of salt was required to elute proteins from DEAE column when protein fraction was salted out with 60 and 50-90 percent ammonium sulphate saturation. Molecular sieving was affected by both salt gradient as well as pH due to the presence of both acidic and basic proteins. 70 to 80 percent sequential precipitation gave a protein having same molecular weight as that of insulin. Purification, sequencing and further studies of this fraction have to be conducted for establishing the antidiabetic property and exploiting Costus protein by modern biotechniques.

Index Terms

Costus pictus, Ammonium sulphate precipitation, SDS-PAGE, PAGE, DEAE anion exchange chromatography, Molecular sieving.

I. INTRODUCTION

Costus pictus is commonly known as insulin plant in the central part of Kerala. Diabetic patients used to take raw leaves of the plant as a folk remedy for hyperglycemia. The studies of Benny et al (2004).[1], Balaji (2005)[2] and Jothivel et al.(2007)[3] are in agreement with its hypoglycaemic property. An anti diabetic plant protein could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and / or by mimicking insulin action. Characterization of a hypopglycaemic plant protein may open new dimensions to the fields of diabetic treatment. The project entitled 'Characterization of proteins in *Costus pictus* D.Don' was carried out at CPBMB, College of Horticulture, Kerala Agricultural University, Vellanikkara. The objective of the experiment was isolation, purification and characterization of proteins in *Costus pictus* and comparison with insulin.

II. MATERIALS

Sample plants of *Costus pictus* were maintained in the CPBMB field. Fresh and fully opened leaf samples were collected for analysis.

A) Chemicals, glass and plastic ware

Chemicals of good quality (AR/GR grade) procured from MERCK India Ltd., Sisco Research Laboratories and Himedia were used for the study. The Sephadex G-25 and dialysis tubes for desalting of protein sample and Sephadex G-100 for molecular sieving were obtained from Sigma-Aldrich, USA. Broad range protein molecular weight marker was procured from Bangalore Genei Ltd. The glassware required for biochemical studies were purchased from Vensil and Borosil India Ltd. The plastic ware was supplied by Tarson India Ltd. and Axygen, USA. The equipment items available at CPBMB were utilized for the present study.

III. METHODS

A) Estimation of protein content of Costus pictus

Protein content in the leaves of *Costus pictus* was estimated by Lowry's method (Lowry *et al*., 1951) [4] and also by spectrophotometry using Nanodrop ND 1000 spectrophotometer. Fourth and fifth physiologically mature leaves were collected fresh from the field on the day of analysis. The following five buffers at varying pH and concentrations were tried for selecting the most suitable buffer:

0.2 M Tris (Hydroxy methyl) Aminomethane buffer at pH 7.2 (Sadasivam and Manickam, 1996)[5].ii) 0.2 M Tris buffer containing citric acid (2.5 µM), ascorbic acid (6 µM), cysteine HCl (6 µM) and sucrose (0.5 M) at pH 7.2 (Harborne, 1984)[6].iii) Phosphate Buffered Saline solution (PBS) at pH 7.3.iv) 0.2 M phosphate buffer at pH 7.0 (Sadasivam and Manickam, 1996).v) Sodium citrate phosphate buffer at pH 2.8 (Parab, 2000).[7]

Lowry's method was tried by both with TCA precipitation and without TCA precipitation. One gram each of leaf sample was ground with 2 ml each of cold buffers in an ice-cold mortar. The extract was centrifuged at 12,000 rpm for 10 minutes. From the supernatants, 0.5 ml each of extract was transferred to five different test tubes separately. Equal quantity of 15 per cent TCA was added to each test tube. Samples were kept overnight at room temperature. On the next day, it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted and precipitates were dissolved in 1ml 0.1 N NaOH separately. From the dissolved protein samples, took 0.5 ml of each sample was made up the volume to 1 ml with the same buffer. One ml of 0.1N NaOH was kept as blank. Five ml of reagent C was added to each test tube including the blank. Mixed well and allowed to stand for 10 minutes, added 0.5 ml of reagent D and incubated at room temperature in dark for 20 minutes. Readings were taken at 660 nm (Augustin, 2005)[8] . A 1 µl sample was used for estimation by spectrophotometry using Nanodrop ND 1000 spectrophotometer.

B) Molecular weight determination by SDS-PAGE

Six buffer systems (Table 1) were tried to find the most suitable buffer for maximum extraction of protein. Each 1g leaf sample of *Costus pictus* was ground with 1ml each of different buffers. The extract was centrifuged at 12,000 rpm for 10 minutes. Forty microlitre of the extract was mixed with 10 µl 2X treatment buffer and heated at 100^0C for two minutes for denaturation of the protein and loaded in the wells. Five microlitre of insulin (Human mixtard) and 10 µl of marker (Broad range protein molecular weight marker-PMWB GENEI of 3000 to 2,05,000 kDa) were also mixed separately with 10 µl of treatment buffer and 30 µl of distilled water, heated at 100 ^{0}C for two minutes and loaded on other two separate lanes for the comparison of molecular weight.

The samples were subjected to electrophoresis in a vertical electrophoresis unit (BIO- RAD) according to the procedure described by Laemmli(1970)[9] with some modifications. Different combinations of resolving gel were tried out with stacking gel (6%) to select the optimum combination. The effect of current variation was tried for stacking gel and resolving gel to find the best one. Protein samples after electrophoresis were subjected to staining with both Coomassie brilliant blue and silver staining (sadasivam and Manickam, 1996) in different experiments. Molecular weight and Rm value were determined by molecular weight determination tool in the Alpha imager.

PAGE

Samples were prepared as that used for SDS- PAGE except SDS and denaturing agents from all steps.

C) Partial purification of protein through ammonium sulphate fractionation

The procedure suggested by Chaykin (1966)[10] was followed with some modifications for protein precipitation. Nine lots of 5 g leaf sample (fourth or fifth) was collected and ground with 10 ml 0.2 M Tris buffer. Samples were ground in ice-cold mortar and pestle. The extract was centrifuged at 12,000 rpm for 15 minutes and collected the supernatant. Saturated Ammonium sulphate solution was added drop by drop to the supernatant prepared separately at 4^0C so as to get 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent saturation separately. The mixture was kept at 4^0C overnight for precipitation of protein. On the next day, it was centrifuged at 12,000 rpm for 20 minutes. The precipitates were collected and dissolved in 1ml 0.2 M Tris buffer. In another set, supernatant of 10 per cent ammonium sulphate was further increased to 20 per cent saturation and salted out the protein, and the saturation was increased to 30, 40, 50, 60, 70, 80 and 90 per cent and collected the protein at different levels of ammonium sulphate saturation. Protein precipitates were dissolved in minimum quantity buffer and subjected to dialysis for 24 hours with two changes of buffer. Protein content of each fraction was recorded and electrophoresis was done with Ten per cent SDS-PAGE.

D) Anion exchange chromatography with DEAE cellulose

Pre-swollen DEAE cellulose from GENEI was previously saturated with 0.2 M Tris buffer and used for packing the column 30 cm x 1.2 cm for protein of 60 per cent fraction and 15 cm x 1.2 cm column for 50 to 90 per cent fraction of protein. 2 ml of dialyzed protein sample in the case of 60 per cent and 1 ml in the case of 50 to 90 per cent ammonium sulphate saturated fraction were applied into the separate columns and eluted with the same buffer containing a linear gradient of 0.1 to 1 M NaCl (pH 7.2). Protein content in each fraction was estimated using Nanodrop at 280 nm. The protein fractions of high peak values were used for 10 per cent SDS-PAGE.

E) Gel filtration chromatography (size exclusion chromatography)

Protein fractions (high peak values) of DEAE column were pooled and precipitated proteins with solid ammonium sulphate (90% saturation) and dissolved in 0.3 ml of 0.2 M Tris buffer (pH 7.2). Further dialysis was carried out to remove excess ammonium sulphate. Two gram Sephadex G-100 was swollen with 30 ml distilled water. Swollen Sephadex G-100 was saturated with 0.2 M Tris buffer of pH 7.2 for 60 per cent saturation and pH 6.5 for 50 to 90 per cent. The size of the column was 6 cm x 1.1 cm for both

samples. 0.2 ml of protein sample was applied to the column saturated with 0.2 M Tris buffer. Excess Tris buffer was applied to remove excess sample and other small particles. The gradient application was proceeded with 0.2 M Tris buffer containing a linear gradient of NaCl solution i.e. 0.1 to 3 M for 60 per cent and 0.1 to 1 M for 50 to 90 per cent ammonium sulphate saturated fractions. One millilitre fractions were collected and recorded the protein content of each fraction separately. Electrophoresis of protein fractions of high peak values were carried out separately.

IV. RESULTS AND DISCUSSION

A) Estimation of protein content of Costus pictus

The extraction of protein with PBS and sodium citrate phosphate buffer gave maximum quantity of protein at 280 nm in Nanodrop and at 660 nm by Lowry's method (before precipitation) but on precipitation of proteins with TCA, a considerable reduction in protein/protein analogues was recorded and maximum protein content was observed in the extraction with Tris buffer**.** Most of the proteins in *Costus* may have free acidic amino acid at surface level. But high protein content was also recorded in the extraction with sodium citrate phosphate buffer at pH 2.8 **(Table 1)**. It may be due to the presence of various salts and their ionic concentration. TCA precipitation eliminated the impurities and precipitated protein alone. High readings in the protein estimation by Lowry's method (before TCA precipitation) and Nanodrop may be due to the interference of phenolics in the crude extract and formation of complexes with phenolics by hydrogen bonding.

B) Effect of extraction buffer and per cent acrylamide in SDS-PAGE and PAGE on Costus pictus sample

The extraction with 0.2 M Tris buffer and PBS was found on par in giving maximum number of clear and distinct Protein bands in all gel combinations tried **(Table 2 & 3)** in SDS- PAGE and PAGE. Though the protein content extracted in the sodium citrate phosphate buffer (pH 2.8) was high, it gave less number of protein bands on 10 per cent SDS-PAGE analysis on staining with Coomassie Brilliant Blue R-250. It may be due to the clustering of protein at low pH and the action of SDS being not affected much in the *Costus* sample to break the disulphide bridges of high molecular weight proteins. Ten per cent resolving gel of SDS-PAGE and PAGE with 20 mA throughout the run was found best in producing clear and distinct bands with Coomassie Brilliant Blue R-250 **(Fig:1).** Silver staining of 10 per cent SDS-PAGE gel gave less number of bands in protein sample **(Fig: 2 and Table 4).**

PAGE at 10 per cent acrylamide was sufficient for separating complex protein mixtures of *Costus* sample. Tris buffer and PBS were found suitable for maintaining the protein in its native state also. It was evident from the result that the leaf protein contains only less number of native proteins with high molecular weight **(Table 5, Fig 3 a & b).** The possibility of protein aggregate formation at this stage may be due to high molecular weight proteins. Separation of proteins components was low in less % of acrylamide. It is an indication of the presence of closely related proteins of different molecular weight in *Costus* sample.

C) Staining behaviour of proteins in the Costus pictus

The leaf protein in *Costus pictus* was very well responded to Coomassie Brilliant Blue R-250 staining in the early stages of purification. So it can very well presume that the amino acids like lysine, histidine and arginine residues are there in the amino terminal amino group of leaf protein in *Costus pictus*. Staining pattern also implied that proteins having amino acid side chains like sulfhydril and carboxyl groups are less in *Costus pictus* leaf sample.

D) Ammonium sulphate fractionation and partial purification of proteins

Salting out of protein was high at 70 per cent and 90 per cent ammonium sulphate saturation. Additional protein band of molecular weight of 6500 Da and Rm value of 0.921 was present in 70 to 80 per cent saturation which is having a similar molecular weight as that of the insulin from other source**. (table 6; Fig:5)**

No additional bands appeared in 60 to 70 per cent and 80 to 90 per cent fractions with Coomassie Brilliant Blue R-250. Even the smaller protein molecule of *Costus* sample was having sufficient quantity of hydrophobic patches which was evident from the precipitation of substantial quantity of protein in 10 per cent ammonium sulphate saturation. Both studies of ammonium sulphate fractional precipitation and direct salting out revealed that complete salting out of protein from *Costus* can be efficiently carried out with 80 per cent amonium sulphate even though the direct salting out of protein expressed some higher molecular weight protein banding pattern due to the aggregation of available proteins. So lower and higher percentage ammonium sulphate are not required to isolate a specific active protein from *Costus*.

E) Anion exchange chromatography with DEAE cellulose

Higher salt concentration (>0.5 M NaCl) was required for maximum elution of 60% ammonium sulphate fraction (0.9 M NaCl concentration) and

decreased the precipitation thereafter. It indicated the presence of a strong ionic interaction between column and protein i.e. *Costus* protein is highly negatively charged. For 50 to 90 per cent fraction, elution with linear gradient of sodium chloride containing Tris buffer at three different pH (8.0, 7.2 and 6.5) were tried. At pH 7.2, the elution of protein with 0.5 M NaCl salt concentration recorded maximum number of proteins of high molecular weight and showed a gradual reduction in protein content for higher concentration of salt. Aggregation of proteins in *Costus* can be considered as a characteristic of this plant because pH was the major factor that contributed to aggregation and elution of proteins in a different manner (protein aggregates are three in number at pH 7.2 where as eleven in number at pH 6.5, other factors being constant).

F) Gel filtration chromatography (Size exclusion chromatography)

For 60 and 50 to 90 per cent ammonium sulphate saturated fractions, the fractions of high protein content were used for 10 per cent SDS-PAGE **(Fig 4a & b)** and molecular weight of each band was recorded **(Tables7& 8).** Two light bands having molecular weight of 68,760 and 79,360 were observed (**Fig:4a**) for 60 per cent saturated fraction and of 23,098 Da and 21,142 Da for 50 to 90 per cent fraction but >1 M NaCl concentration was required to elute these proteins It was observed that protein subunits of basic protein having molecular weight of 68,760 and 79,360 Da were separated from 60 per cent ammonium sulphate saturation of *Costus* protein. It may be noted that 50 to 90 per cent sequence saturation of *Costus* sample had two acidic proteins of molecular weight 21,142 and 23,098 Da which were stained with silver stain **(Fig: 4b).** At the same time, there was no banding of basic protein in Coomassie staining. Based on the results, it can very well presume that basic proteins of high molecular weight proteins were salted out in the range of 30 to 50 per cent of ammonium sulphate saturation.

V. CONCLUSION

The extraction with 0.2 M Tris buffer and PBS have been given maximum number of clear and distinct protein bands in all gel combinations tried **(Table 2 & 3)** in SDS- PAGE and PAGE. Though the protein content extracted in sodium citrate phosphate buffer (pH 2.8) was high, it gave less number of protein bands on 10 per cent SDS-PAGE analysis on staining with Coomassie Brilliant Blue R-250. It may be due to the clustering of protein at low pH and the action of SDS being not affected much in the *Costus* sample to break the disulphide bridges of high molecular weight proteins. It can infer that the possibility of protein aggregate formation may be due to high molecular weight proteins, Separation of proteins components was low in less percentage of acrylamide. It is also an indication of the presence of closely related proteins of different molecular weight in *Costus* leaf sample. Based on the above, it can very well presume that the amino acids like lysine, histidine and arginine residues are there in the amino terminal group of the leaf protein in *Costus pictus.* Staining pattern also implied that proteins having amino acid side chains like sulfhydril and carboxyl groups are less in *Costus pictus* leaf sample. Aggregation of proteins in *Costus* leaf can be considered as a characteristic of this plant because pH was the major factor that contributed to aggregation and elution of proteins in a different manner (protein aggregates are three in number at pH 7.2 where as eleven in number at pH 6.5, other factors being constant).

Most of the proteins in *Costus* leaf may have free acidic amino acid at surface level. At the same time high protein content was also recorded in the protein extract with sodium citrate phosphate buffer at pH 2.8 **(Table 1).** Higher salt concentration (>0.5 M NaCl) was required for maximum elution of 60% ammonium sulphate fraction (0.9 M NaCl concentration) and decreased the precipitation thereafter. It indicated the presence of a strong ionic interaction between column and protein i.e. *Costus* protein is highly negatively charged.

Additional protein band of molecular weight of 6500 Da and Rm value of 0.921 was present in 70 to 80 per cent saturation which is having a similar molecular weight as that of the insulin from other source**.** (table 6). Considering the above facts, there is possibility to exploit the antidiabetic property of *Costus* protein with modern biotechniques.

Table 1. Protein content in the leaf sample of Costus pictus

Buffer	Protein content (mg/g)			
	Nanodrop	Lowry's method		
		Before TCA precipitation	After TCA precipitation	Impurity factor
Tris buffer	**2.79**	**2.47**	**0.30**	**8.23**
Tris+ ingredients	**1.73**	**1.78**	**0.13**	**13.69**
PBS	**3.11**	**2.50**	**0.24**	**10.42**
Phosphate buffer	**2.08**	**1.71**	**0.07**	**24.43**
Sodium citrate phosphate buffer	**3.10**	**2.73**	**0.26**	**10.50**

Table 2. Effect of buffers and concentration of acrylamide in the banding pattern of protein in SDS PAGE

Buffer	No. of bands present in varying acryl amide concentration (SDS-PAGE)			
	15%	10%	7.5%	7%
Tris buffer	**10**	**10**	**4**	**2**
Tris+ ingredients buffer	**8**	**10**	**4**	**1**
PBS	**10**	**10**	**4**	**2**
Sodium citrate phosphate buffer	**5**	**7**	**4**	**1**
Phosphate buffer	**5**	**7**	**1**	**1**

Table 3. Effect of buffers and concentration of acrylamide in the banding pattern of protein in PAGE

Buffer	No. of bands present in varying acrylamide concentration (PAGE)			
	15%	10%	7.5%	7%
Tris buffer	**3**	**3**	**2**	**1**
Tris+ingredients	**3**	**3**	**2**	**3**
PBS	**3**	**3**	**2**	**1**
Sodium citrate phosphate buffer	**3**	**3**	**1**	**1**
Phosphate buffer	**1**	**3**	**1**	**1**

Table 4. Molecular weight and Rm value of proteins of crude extract from *Costus pictus* in SDS-PAGE on silver staining

Band No.	Molecular weight (Dalton)	Rm value
1	**15,540**	**0.870**
2	**38,423**	**0.532**
3	**60,006**	**0.379**
4	**1,19,149**	**0.223**

Table 5. Approximate molecular weight and Rm values of proteins of *Costus pictus* in PAGE on staining with Coomassie Brilliant Blue R-250

Band No.	Approximate molecular weight (Da)	Rm value
1	**41,108**	**0.511**
2	**58,675**	**0.389**
3	**1,48,096**	**0.201**

Table 6. Molecular weight and Rm value of proteins precipitated at varying concentration of ammonium sulphate (sequential precipitation)

Band No.	Molecular weight (Da)	Rm value
1	**6,500**	**0.921**
2	**13,311**	**0.890**
3	**21,181**	**0.788**
4	**23,519**	**0.734**
5	**34,722**	**0.562**
6	**63,586**	**0.345**
7	**66,000**	**0.339**

Table 7. Molecular weight and Rm value of fractions from 60 per cent ammonium sulphate saturation on SDS-PAGE (10%) after Gel filtration chromatography

Fraction	No.of bands	Band No.	Molecular weight (Da)	Rm value
1.2(1),1.2(2), 2(1)2(2),3(2)	**2**	**1**	**68,760**	**0.330**
		2	**79,360**	**0.301**

Table 8. Molecular weight and Rm value of fractions from 50-90 per cent ammonium sulphate saturation on SDS-PAGE (10%) after Gel filtration chromatography

Fraction	No.of bands	Band No.	Molecular weight	Rm value
1(1),1(2), 1(3)1(4),1(5)	**2**	**1**	**21,142**	**0.789**
		2	**23,098**	**0.744**

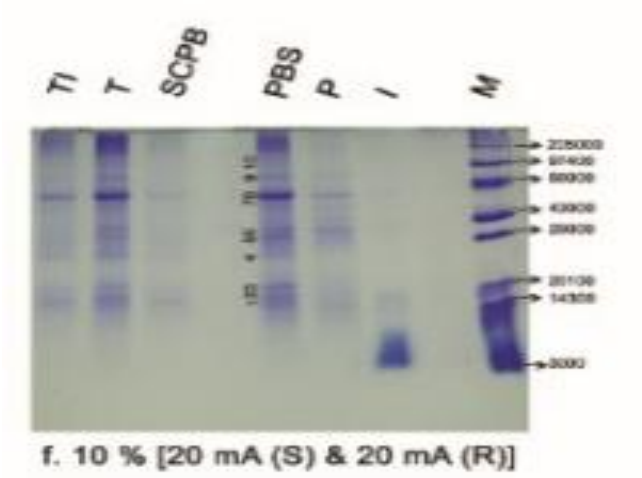


Fig. 1. Standardization of acryl amide percentage for SDS – PAGE

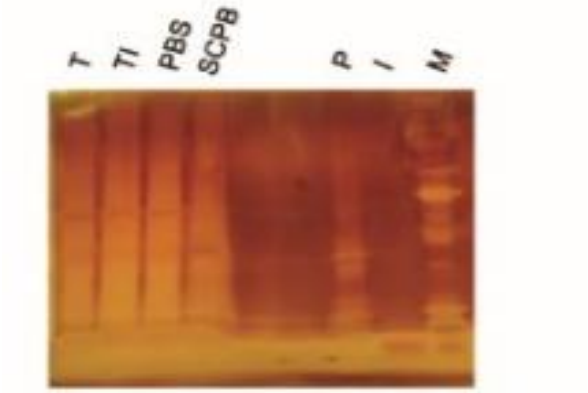


Fig. 2 . Costus pictus protein on SDS – PAGE with silver staining

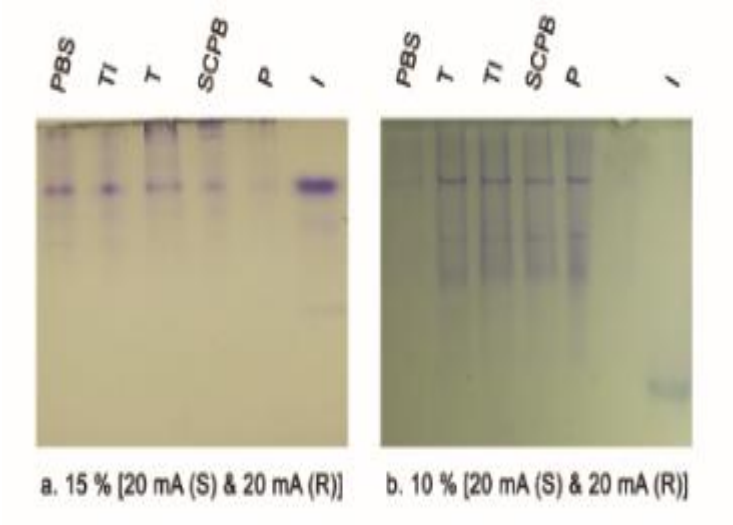
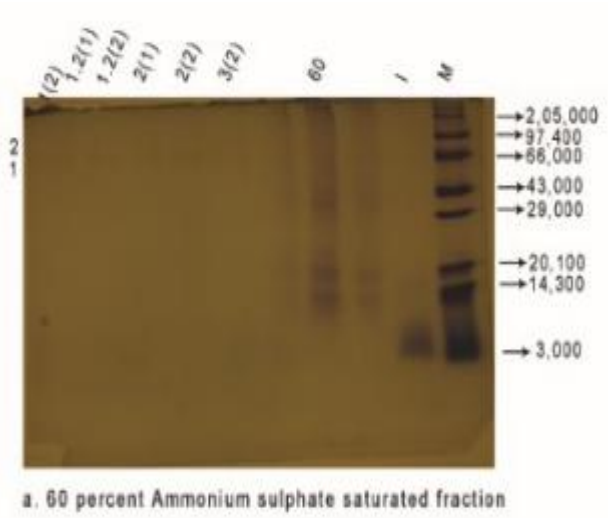


Fig. 3. Standardization of acryl amide percentage for PAGE



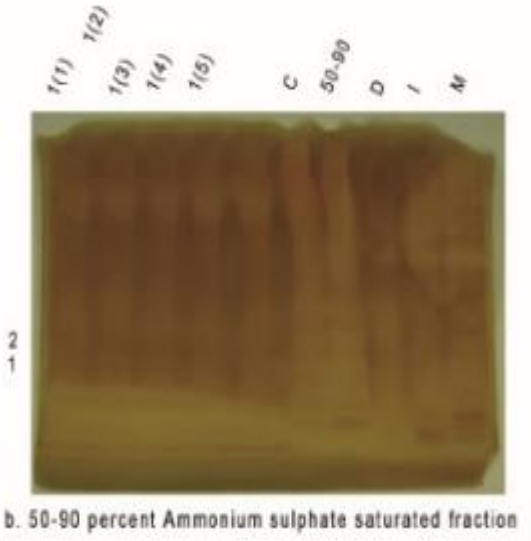


Fig. 4. Electrophoretic pattern of protein fractions from 60 &50-90% Ammonium sulphate Saturation eluted through Gel filtration column

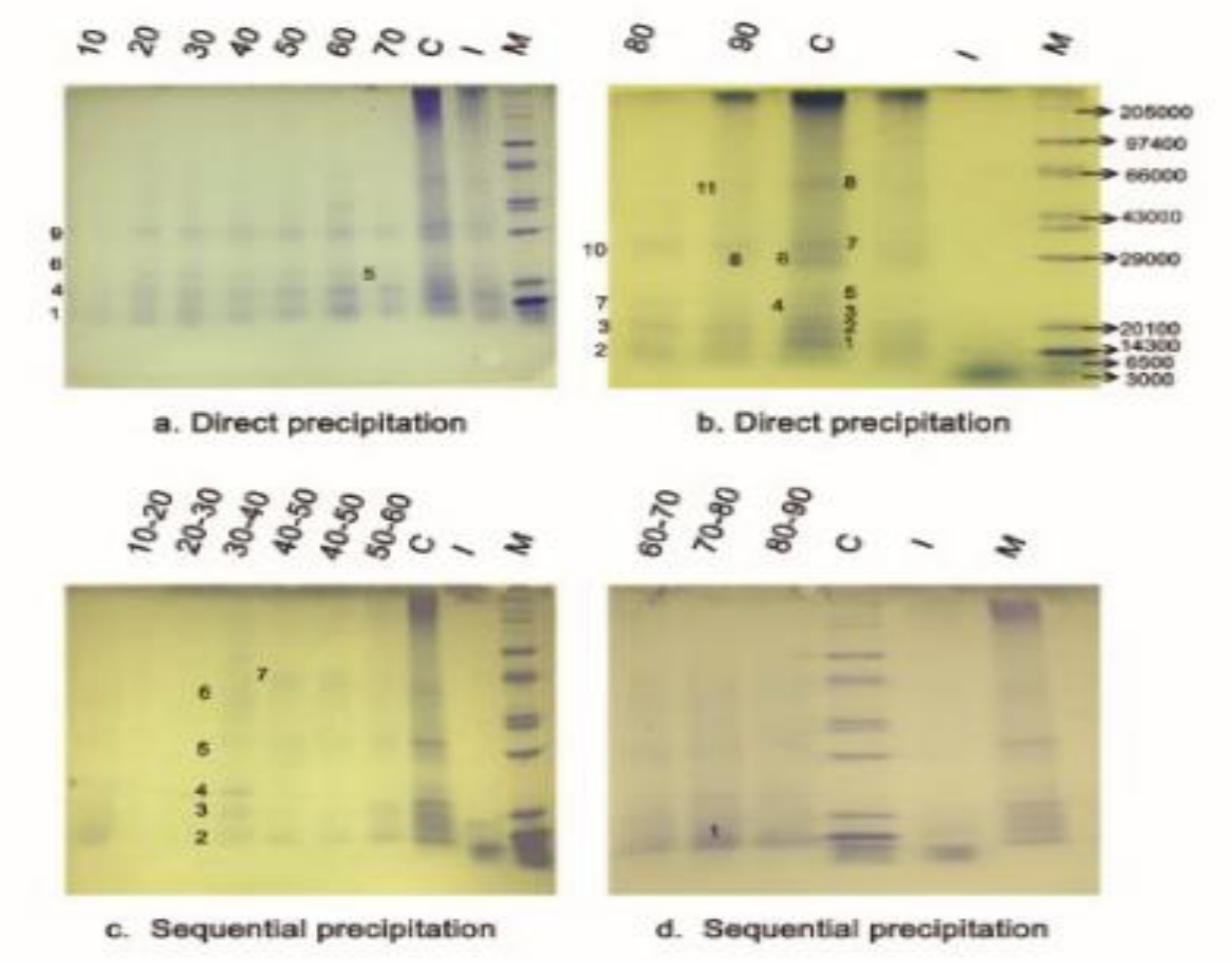


Fig. 5. Effect of Ammonium sulphate saturation in salting out of proteins

REFERENCES

[1] Benny, M. 2004a. Insulin plant in gardens. Natural Product Radiance 3(5): 349-350.

[2] Balaji,S. 2005.Hypoglycaemic efficacy of Scoparia dulcis and Costus species in Albino rats. MVSc.thesis, Kerala Agricultural University, Thrissur, 117p.

[3] Jothivel, N., Ponnusamy,S.P., Appachi,M., Singaravel,S., Rasilingam, D., Deivasigamani, K. and Thangavel, S. 2007.Anti-diabetic activity of methanol leaf extract of Costus pictus D.Don in alloxan–induced diabetic rats. J.Health.Sci. 53(6): 655-663.

[4] Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.1951. Protein measurement with the Folin phenol reagent. J.Biol.Chem.193: 265-275.

[5] Sadasivam, S and Manickam, A.1996.Biochemical Methods. New Age International Publishers, New Delhi, 255 p

[6] Harborne, J.B.1984. Phytochemical Methods. 2nd edition. Chapman and Hall Ltd, London. 288p.

[7] Parab, G.V.2000.Evaluation of pathogenesis related proteins in relation to Phytophthora foot rot in black pepper [Piper nigram L.]. MSc (Ag) thesis, Kerala Agricultural University, Thrissur, 100 p.

[8] Augustin, A.2005.Methods in Plant Biotechnology. Kerala Agricultural University, Thrissur. 334p.

[9] Laemmli, U.K.1970.Cleavage of structural proteinsT4. Nature.227: 680-685.

[10] Chakin, S.I.1966. In: Biochemistry Laboratory Techniques. Ist Ed. Roberte Kriegar Publishing Company Huntington, New york.22-34pp.