CHARACTERIZATION OF PROTEINS IN *Costus pictus* D.Don

By

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THESIS

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2008

DECLARATION

I hereby declare that the thesis entitled **"Characterization of proteins in *Costus pictus* D. Don"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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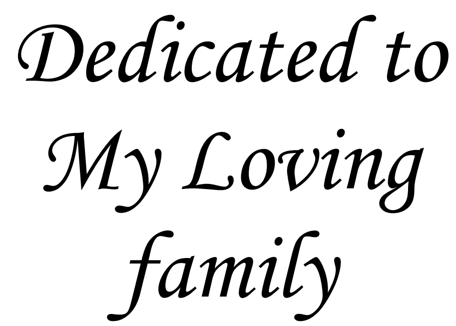
Dedicated to
My Loving
family

Costus pictus D.Don

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ABBREVIATIONS

APS	**Ammonium persulphate**
BSA	**Bovine Serum Albumin**
β	**Beta**
$CaCl_2$	**Calcium chloride**
cm	**Centimeter**
CPBMB	**Centre for Plant Biotechnology and Molecular Biology**
^{o}C	**Degree Celsius**
Da	**Dalton**
DAP	**Days After Pollination**
DEAE	**Diethyl amino ethyl**
DNA	**Deoxyribo Nucleic Acid**
EDTA	**Ethylene Diamine Tetra Acetic acid**
ELISA	**Enzyme Linked Immunosorbant Assay**
γ	**Gamma**
g	**Gram**
hr	**Hour (s)**
HPLC	**High Performance Liquid Chromatography**
K	**Kilo**
KAU	**Kerala Agricultural University**
Kg	**Kilogram**
m	**Meter**
M	**Molar**
Mg	**Milligram**
ml	**Millilitre**
mm	**Millimeter**
mM	**Millimolar**
μg	**Microgram**
μl	**Microlitre**
μM	**Micromole**
NaCl	**Sodium chloride**
$(NH_4)_2SO_4$	**Ammonium sulphate**
ng	**Nanogram**
nm	**Nanometer**
PAGE	**Poly Acrylamide Gel Electrophoresis**
PBS	**Phosphate Buffered Saline**
pH	**Hydrogen ion concentration**
ppm	**Parts per million**
%	**Percentage**
Rm	**Relative mobility**
RNA	**Ribo Nucleic Acid**
RNase	**Ribonuclease**
rpm	**Rotations per minute**
SDS	**Sodium Dodecyl Sulphate**

sec	**Second (s)**
TCA	**Trichloro acetic acid**
TEMED	**N, N, N, N- Tetramethylene ethylene diamine**
Tyr	**Tyrosine**
Trp	**Tryptophan**
SDS	**Sodium Dodecyl Sulphate**
U	**Unit**
UV	**Ultra Violet**
V	**Volts**
w/v	**Weight by volume**

Introduction

1. INTRODUCTION

Costus pictus D.Don is a Mexican plant brought to India during 2002-2003. It is also called as spiral ginger/ stepladder plant due to the spiral appearance of its stems. It is commonly known as insulin plant in the central part of Kerala and diabetic patients take raw leaves of the plant as a folk remedy for hyperglycaemia (Benny, 2006).

Diabetes mellitus has emerged as one of the challenging health problems of the 21st century. A survey conducted by the International Diabetes Federation Task Force on insulin has revealed that as of today more than 30 countries in the world are unable to ensure a continuous supply of insulin to diabetic patients. Under such a situation, there is an increasing need for natural alternatives for blood sugar control (Nair, 2005).

Before the introduction of insulin in 1922, the treatment of diabetes mellitus relied heavily on dietary measures, which included the use of traditional plant therapies. An antidiabetic agent could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and/or by mimicking insulin action (Gray and Flatt, 1999).

Khan and Anderson (2003) reported that flesh and seeds of jamun fruit, dried seeds of bitter gourd, water extract of fenugreek, black tea and green tea have good insulin potentiating function. Gray and Flatt (1999) reported that the traditional antidiabetic plant *Viscum album* (Mistletoe) has insulin secreting activity.

Balaji (2005) reported that active constituents in the leaf extract of *Costus pictus*, which are soluble both in water and alcohol medium could help in controlling hyperglycaemia in alloxan induced albino rats. The widely used practice for treating hyperglycaemia is taking insulin through injection. As insulin

is a protein, comparison of its characters to plant proteins of hypoglyceimic plants may open new doors to the field of antidiabetic treatments.

There are reports for the presence of insulin in plants like *Bauhinia variegata, Vigna unguiculata* and *Canavalia ensiformis* (Ellis and Eyster, 1923; Filho *et al.*, 2003; Vencio *et al.*, 2003; Azevedo *et al*., 2006).

With the completion of human genome project, now the attention is turning to the next step i.e. how to utilize this genetic data for the better understanding of diseases and develop targeted therapeutics more efficiently. As proteins, rather than genes convey most cellular functions, understanding protein expression and protein function is crucial to the identification of new targets for drug development (Goetz *et al.*, 2004).

Protein study is important in the biological function because of its involvement as

- catalysts that maintain metabolic processes in the cell,
- structural elements both within and outside the cell,
- signals secreted by one cell or deposited in the extracellular matrix that are recognized by other cells,
- receptors that convey information about the extracellular milieu to the cell,
- intracellular signaling components that mediate the effects of receptors,
- key components of the machinery that determines which genes are expressed and whether mRNAs are translated into proteins,
- manipulation of DNA and RNA through processes such as DNA replication, DNA recombination, RNA splicing or editing.

Protein characterization involves purification, quantification and identification tasks. The first and indispensable step in the series of studies, which is aimed at exploring protein function is the purification of the protein of interest. Proteins can be separated from one another on the basis of solubility, size, charge and binding ability. To understand the physiological context of a protein,

antibodies are choice probes for locating proteins *in vivo* and measuring their quantities.

Considering the above, the following aspects were targeted in the present study.

1. Isolation and purification of proteins and its quantification at different stages of purification.
2. Ammonium sulfate precipitation, molecular sieving, and ion exchange chromatography for the purification of proteins and evaluation of purity of proteins through SDS-PAGE analysis.
3. Comparison of the chromatographic behaviour and electrophoretic banding pattern of the isolated proteins with insulin.

Review of Literature

2. REVIEW OF LITERATURE

2.1 About the Plant

2.1.1 Botany of *Costus pictus*

Costus pictus D.Don commonly known as spiral ginger, step ladder or insulin plant native of Mexico. The plant belongs to the family Costaceae. The plant has spiral phyllotaxy and papery like flowers. It has red painted stem, glossy linear leaves. The flowers are borne in a terminal cone, yellow in colour with an orange-red tip, which lasts for three to four days. Flowers do not produce any aroma. Usually plant grows up to 2-3 m and spread to 1.5-2m. The plant is propagated through stem cuttings and rhizomes. In Kerala it is grown in gardens as an ornamental plant (Benny, 2004b).

2.1.2Morphology

Costus pictus is a perennial species with tall and red spotted stems, which can be used as cut foliage. It blooms basally in spring and terminally in summer. Height of the plant is generally one to four meters. Plant has thin stems and narrow leaves and it needs partial shade for growth. It requires moist and well-drained soil.

2.1.3 Distinguishing characteristics

Reddish yellow flowers and green inflorescence without callus and short ligules are the most important distinguishing characteristics. It has narrow leaves with undulating margins and produces inflorescence basally in the spring and terminally in summer and fall. It is placed between *Costus laevis* and *Costus malortienaus.* It differs from *Costus malortienaus* by leaf shape and indumenta. It seems to be one of the coldest hardy and vigorous plant of all *Costus* species.

2.1.4 Natural distribution

Costus pictus is commonly found from Mexico to Costa Rica in rain forests and clearings. It is also found in hill forests along water sources and roads down to sea level, mainly between 300m and 1800m.

2.1.5 Medicinal properties

Brown (1995) reported that *Costus pictus* root has anodyne, anti-bacterial, anti-spasmodic, aphrodisiac, carminative, skin stimulating, stomachic and anti-asthmatic properties.

The raw leaves of *Costus pictus* are commonly used as a folk remedy to reduce glucose level by diabetic patients. The hypoglycaemic properties of the plant have been reported both in streptozotocin and alloxan induced diabetic rats. Oral feeding of *Costus pictus* D. Don crude extract up to 1g/kg body weight did not produce any toxic effect (Benny, 2004 a; Benny *et al.,* 2004 a&b).

Balaji (2005) reported that the active constituents in the leaf extract of *Costus pictus*, which are soluble, both in water and alcohol medium could help in controlling hyperglycemia in alloxan induced albino rats. He noted that the plant extract not only effective in preventing the loss of body weight but also in increasing the body weight of diabetic rats. It increased the hemoglobin content (35%) but decreased the glycosylated haemoglobin (34%) after 28 days of treatment. Plasma glucose was found to decrease from the pre-treatment value in all the treatment groups (74%). He also reported that the plant extract also possess hypolipidaemic property. There was a substantial reduction in plasma total cholesterol level (42%). All the treatment groups showed an enhancement in plasma protein content over 60 days of treatment (34%).

Diuretic activity of the aqueous extract of the traditional medicine *Costus pictus* was demonstrated by measuring increases in the excretion of both K^+ and Na^+ in the urine of treated rats. The results revealed that *Costus pictus* induced a natriuretic effect similar to furosemide. But water excretion was not modified to the same degree as that of Na^+ and K^+ excretion. The aqueous extract induces an increment in sodium and potassium clearance similar to the one obtained with furosemide suggesting that it represents significant diuresis, where the extract does not affect potassium clearance to the same degree as measured here. The use of this herbal should be accompanied by potassium intake (Camargo *et al.*, 2006)

Jothivel *et al*. (2007) reported that the methanol extract of *Costus pictus* (MECP) leaf was investigated for its anti-diabetic effect in Wistar Albino rats. Diabetes was induced in Albino rats by administration of single doses of alloxan monohydrate (120 mg/kg).The methanol extract of *Costus pictus* at a dose of 120 mg/kg was administered as single dose per day to diabetes-induced rats for a period of 21days. The effect of MECP leaf on blood glucose, plasma insulin, serum lipid profile (cholesterol, triglycerides, phospholipids, Very Low Density Lipoprotein (VLDL), Low Density Lipoprotein (LDL), and High Density Lipoprotein (HDL), serum enzymes, total protein and liver glycogen were measured in the diabetic rats. Histopathological studies of liver, pancreas and kidney were also carried out. MECP elicited significant reductions of blood glucose, lipid parameters except HDL and serum enzymes and significantly increased HDL level. MECP also caused significant increases in plasma insulin levels in the diabetic rats. It significantly increased total protein and liver glycogen in diabetic rats. Histopathological observations revealed that leaf is nontoxic and regenerates the toxic effect of alloxan. From the above results, it is concluded that MECP possesses significant anti- diabetic effects in alloxan induced diabetic rats.

2.1.6 Biochemicals in *Costus pictus*

Benny (2006) studied chemical and physical properties of the extract. Proteins were estimated by Lowry's method and carbohydrates by Anthrone method. The fibre content was estimated by gravimetric method and total fat by extracting with hexane. Calcium content was estimated by permanganometric method. Further analyses on the chemical constituents were carried out by permanganometric titration, Thin Layer Chromatography and HPLC for organic acids. Physical and chemical properties of crude extract of *Costus pictus* is given in table 1. It was found that the sour taste of fresh leaves as well as the crude extract is due to the presence of oxalic acid.

Lincy (2007) also conducted biochemical characterization of *Costus pictus* and reported the presence of amylase and reducing sugars. It contains monosaccharides of ketohexose origin. More than 70 per cent of all proteins are glycoxylated in the plant. Qualitative tests revealed that *Costus* has low concentration of protein with aromatic amino acids and/or free amino acids including tryptophan. It also contains hydrolysable tannins.

2.1.7 Cytology

Vovides and Lascurain (1995) reported that a polyploidy series exists in *Costus*. Chromosome numbers are: *Costus pictus*, *Costus scaber*, *Costus pulverulentus* 2n=18.

Table 1. Physical and chemical properties of methanol extract of *Costus pictus*

Sl.No	Parameters	Description / Results
1	Colour and appearance	Pale brown to deep brown free flowing powder
2	Taste	Sour
3	Moisture content	4.8 %
4	pH (1 % Solution)	3.7
5	Acid value	304
6	Solubility in water (2% solution w/v)	62.5 %
7	Solubility in methanol (2 % solution w/v)	44.8 %
8	Proteins	49 %
9	Carbohydrates	33 %
10	Fiber	Negligible
11	Ash	19 %
12	Fat	2.5 %
13	Calcium	<1 %
	Heavy metal analysis	
14	Vanadium	10.12 ppm
15	Zinc	343.78 ppm
16	Cadmium	6.91 ppm

2.2 Immobilization technique

Leaf bits of *Costus pictus* were inoculated in the ½ MS + 1ppm 2, 4-D + 0.5 ppm BA, ½ MS+ 1 ppm BA+ 3 ppm NAA medium for callus induction. The Costus buds were kept first in *Costus speciosus* multiplication medium that is ½ MS+ 3 ppm BA to obtain *in vitro* leaves for callus induction. Suspension cultures were obtained by agitating the callus cells in ½ MS liquid medium+ 0.5 ppm 2, 4-D at 90 rpm on rotary shaker. The alginate beads formed were kept for 30 minutes in $CaCl_2$ for better complexation. Immobilized cells in ½ MS liquid media showed the presence of phenolics, terpenoids, plant acids and amino acids in qualitative tests (Lincy, 2007).

2.3 Protein characterization

With the completion of the human genome project, now the attention is turning to the next step i.e. how to utilize this genetic data for the better understanding of diseases and to develop targeted therapeutics more efficiently. As proteins rather than genes convey most cellular functions, understanding protein expression and protein function is crucial to the identification of new targets for drug development. As we now enter this new, post genomic era, proteins will move in to the focus of attention (Goetz *et al.*, 2004).

2.3.1 Purification of proteins

To become familiar with the nature of an enzyme, the enzyme must be purified to near homogeneity. To attain the goal of pure protein, the cardinal rule is that the ratio of enzyme activity to the total protein is increased to the limit. Generally a lot of trial and error is involved in identifying and maintaining the stability of desired protein (Santha *et al.*, 1998).

2.3.2 Quantification of protein

Kumar *et al*. (1995) reported that for the partial purification and characterization of UDP–Glucose pyrophosphorylase from immature grains of wheat, estimation of protein after each step of purification was carried out according to Lowry *et al*. (1951).

Quantification of protein is possible with a spectrophotometer. Absorption of radiation in the near UV range by protein depends on Tyr and Trp content (and to a small extent on the amount of phe and disulphide bonds). Therefore the A_{280} varies gently between different proteins. The advantage of this method is that it is simple and the sample is recoverable. The method has some disadvantages, including interference from other chromophores, and moreover, the specific absorption value for a given protein must be determined (Aitken and Learmonth , 1996).

Chatterjee and Sanwal (1999) reported that protein from *Lantana camara* was estimated by the method of Lowry *et al*. (1951) as modified by Khanna *et al.* (1969) on TCA precipitate with BSA as the standard.

Dried cowpea seed powder was digested and the nitrogen content was estimated by micro kjeldahl method. The total protein content was calculated by multiplying the nitrogen content by a factor of 6.25 per cent. It varied from 22 to 27.5 per cent on dry weight basis at different stages (Singh and Koundal, 2000).

Oliveira *et al*. (2002) reported that in the purification and physiochemical characterization of a cotyledonary lectin from *Luetzelburgia auriculata*, absorbance at 280 nm (Ultra spec III spectrophotometer Pharmacia LKB) was used to determine protein content of column elutes.

Insulin like protein extracted from empty pods and seed coats of Cowpea (*Vigna unguiculata*) was measured by an ELISA assay using an anti-human insulin antibody. The highest concentration (about 0.5 ng/mg) of this protein was found in seed coats 16 and 18 DAP (days after pollination) and total protein in the extracts was measured by the method of Bradford using ovalbumin as standard (Filho *et al*., 2003).

The proteins (bovine insulin and insulin) isolated from empty pods of *Vigna unguiculata*,10 days after pollination, were detected by absorbance at 280 nm on Reverse Phase HPLC separation (Vencio *et al.*, 2003).

Benny (2006) screened *Costus pictus* for its physical and chemical properties and estimated protein content as 49 per cent by Lowry's method.

2.3.3 Molecular weight determination of proteins using SDS- PAGE/ PAGE

Inouye (1971) reported that internal standard like 1-Dimethyl Amino Naphthalene 5-Sulphonyl (DANS) derivatives of proteins was applied for molecular weight determination of envelope proteins of *Escherichia coli* using Poly Acrylamide Gel Electrophoresis (PAGE).

The apparent molecular mass of acid soluble phosphoprotein in rat fat cells was determined by SDS-PAGE as 22 kDa by method of Laemmli (1970), adapted to a Bio-Rad Protean Mini Gel System using 15 per cent (w/v) poly acrylamide gel (Diggle *et al.,*1995).

An easy way to analyze the subunit composition of enzyme is to denature the protein by the presence of detergent Sodium Dodecyl Sulphate and run a poly acrylamide gel on denatured polypeptide (Campbell, 1995).

Das *et al*. (1996) reported that the subunit character of Arginine Decarboxylase from *Brassica campestris* was confirmed by running it in 10 per cent SDS-PAGE with marker proteins. A single band of molecular weight 60 kDa was obtained at pH 8.3 confirming the enzyme as a tetramer having identical subunits.

Electrophoresis is widely used to separate and characterize proteins by applying electric current. Electrophoretic procedures are rapid and relatively sensitive requiring only micro weight of proteins (Sadasivam and Manickam, 1996).

Sawhney *et al.* (1996) reported that lectin from *Lathyrus sativus* is a tetramer, consisting of two types of subunits of which the heavier subunit consisting of two polypeptides of molecular weight of about 21 kDa and 16 kDa while the smaller subunits consists of two polypeptides of about 5 kDa as revealed by SDS-PAGE on a 10 per cent gel.

Singh and Koundal (2000) reported that on analytical gel electrophoresis of globulin and legumin in 12.5 per cent SDS-PAGE, legumin resolved into three major protein bands of 40, 22 and 20 kDa and globin proteins resolved into a number of bands ranging from 14 to 18 kDa, which contains all the three fractions legumin, vicilin and convicilin.

Das *et al.* (2002) reported that for the purification and characterization of urease from dehusked pigeon pea seeds, a seven per cent native PAGE of the purified sample showed a single band on staining with Coomassie Brilliant Blue corresponding to the jack bean protein band. A 10 per cent SDS-PAGE of the enzyme gave a single band on staining with Coomassie Brilliant Blue. The native molecular mass estimated by gel filtration was approximately 540 kDa. The subunit molecular mass of urease has been determined as 90 kDa. Presence of a single protein-staining band following SDS-PAGE suggests that the pigeon pea

urease is made up of subunits of identical molecular mass and is therefore a homo hexameric protein.

Oliveira *et al*. (2002) reported that SDS-PAGE of *Luetzelburgia auriculata* agglutinin both in the presence and absence of β-mercaptoethanol showed a major band of 29 kDa and two minor ones of around 15 kDa, which are not linked by a disulphide bridge.

Kapoor *et al.* (2003) reported that subunit molecular mass of β-1, 3-glucanase from *Brassica juncea* L. infected with *Albugo candida* was revealed 17.7 kDa by SDS-PAGE.

Vencio *et al. (*2003) reported that when the empty pods of cowpea (10 days after pollination) extracts containing the insulin antigen were submitted to SDS-PAGE (15% acrylamide gel) a protein band in the same position as a bovine insulin band with a molecular mass of approximately 6 kDa was detected.

To investigate the presence of insulin like molecules in the leaves of *Bauhinia variegata*, a simple extraction procedure was employed using the sample buffer utilized for SDS-PAGE. After electrophoresis, elution of the appropriate protein band (6 kDa) from the gel followed by amino acid sequencing showed the presence of a protein with partial sequence identity to bovine insulin (Filho *et al*., 2003).

Yamasaki (2003) reported that molecular weight of the purified β-amylase enzyme was estimated to be 58,000 Da based on its mobility on 7.5 per cent SDS-PAGE.

Although protein analysis technologies are developing fast, the current standard method for protein sizing is still SDS-PAGE (Goetz *et al.*, 2004)

De *et al.* (2004) reported that α-amylase from *Bacillus amyloliquefaciens* NCIM 2829 was homogenous with a molecular mass of 67.5 kDa as judged by SDS-PAGE. Protein bands were visualized by silver nitrate staining. The molecular mass of purified amylase was estimated from its position relative to the standard proteins.

Gupta *et al.* (2004) reported that PAGE as well as SDS-PAGE was run for determination of homogeneity and molecular weight of native lectin and their subunits respectively. Purified lectin is a hetero tetramer of 156 kDa in size, consisting of four polypeptides (molecular weights of 32, 36, 42 and 46 kDa) as detected on SDS-PAGE.

Purified β-galactosidase I from carambola fruit was analyzed using PAGE and SDS-PAGE. Poly Acrylamide Gel electrophoresis was done according to the method of Laemmli (1970) for SDS-PAGE and Reisfeld *et al.* (1962) for native PAGE using 12 per cent and 15 per cent acrylamide respectively. Results indicate that β- galactosidase I comprise two polypeptides with the size of 48kDa and 36kDa, suggesting that the native protein is an aggregate of two non-identical sub-units (Balasubramaniam *et al*., 2005).

To determine the molecular weight and subunit composition of purified adenosine nucleosidase from *Coffea arabica* young leaves, SDS-PAGE was carried out according to Laemmli (1970). Acrylamide gels (12% w/v) were silver stained and protein standards of low molecular weight were used for protein molecular weight determination. It revealed one major protein band of 34.6 kDa. Considering the native molecular weight estimated by gel filtration (72 kDa), it is suggested that the enzyme is composed of two identical subunits (Campos *et al*., 2005).

The molecular weight of peroxidase enzyme purified from *Brassica oleracea* var. Acephala was found to be 95 kDa by SDS-PAGE in which there

were three and eight per cent acrylamide for stacking and running gel respectively containing 0.1 per cent SDS (Gulcin and Yildirim, 2005).

The molecular weight of denatured nitroreductase I from *Klebsiella* sp. C1 was determined as 27 kDa by SDS-PAGE analysis with molecular weight standards (Kim and Song, 2005)

The subunit molecular mass of the recombinant Serine Hydroxy Methyl Transferase (SHMT) from *Streptococcus thermophilus* was determined by 12 per cent SDS-PAGE using Mini Protean II (Biorad) equipment according to the manufacturer's instruction. Gels were stained with Coomassie blue. Recombinant SHMT appeared as an intense protein band with an apparent molecular mass of about 45 kDa (Vidal *et al.*, 2005).

The apparent molecular weight of a novel fibrinolytic enzyme from *Rhizopus chinensis* 12 was found to be 18.0 kDa, which was determined by SDS-PAGE using 12 per cent poly acrylamide gel containing one per cent SDS and bromophenol blue as a marker and stained by Coomassie staining (Ian *et al.*, 2005).

Singh *et al.* (2006) reported that molecular weight of purified oxalate oxidase from NaCl stressed grain sorghum seedlings was 117 kDa which is almost similar to that from plants grown in normal condition, 120 kDa. The number of subunits as determined from SDS-PAGE was similar to that of normal.

2.3.4 Ammonium sulphate fractionation

High concentration of inorganic salts decreases the solubility of proteins, which may be due to dehydration of proteins. The phenomenon is known as salting out.

Mehta *et al.* (1995) reported that for the purification of Phosphoenolpyruvate (PEP) carboxylase, the crude extract was precipitated between 30 to 70 per cent $(NH_4)_2SO_4$ fraction, much higher enzyme activity was recovered than that obtained in the crude extract.

The crude extract of immature grains of wheat (*Triticum aestivum* L.) was fractionated using 50 to 80 per cent saturation with ammonium sulphate to precipitate UDP-glucose Pyrophosphorylase enzyme protein. The precipitates were separated by centrifugation at 10000 X g for 30 minutes. The pellet was suspended in minimum volume of extraction buffer and desalted by passing through a column of Sephadex G-25 and later used for DEAE cellulose anion exchange chromatography (Kumar *et a*l., 1995).

In the purification and characterization of lectin from *Lathyrus sativus*, lectin content in various ammonium sulphate fractions was found to be maximum in the 30 to 50 per cent fraction. Sixteen-fold purification was achieved with the ammonium sulphate fractionation of crude extract with 42 per cent recovery of the proteins from 4 ml of ammonium sulphate fraction (Sawhney *et al*., 1996)

Das *et al*. (1996) reported that for the purification and characterization of arginine decarboxylase from *Brassica campestris*, the homogenate of germinated seedlings after centrifugation was fractionated with solid $(NH_4)_2SO_4$ at 20 to 40 per cent saturation. The precipitate after dispensing in a homogenizing buffer was subjected to acetone fractionation.

Oliveira *et al*. (2002) reported that fractionation of the crude extract by precipitation with ammonium sulphate (40-60% saturation) increased specific activity of cotyledonary lectin from *Luetzelburgia auriculata*.

Andriotis and Ross (2003) reported that phytase from dormant *Corylus avellana* seeds was separated from the major soluble acid phosphatase by

successive $(NH_4)_2SO_4$ precipitation. For that 30 to 75 per cent $(NH_4)_2SO_4$ saturated fraction was analyzed by native PAGE.

Kapoor *et al*. (2003) reported that for the induction, purification and characterization of β-1, 3 Glucanase from *Brassica juncea* L. infected with *Albugo candida*, $(NH_4)_2SO_4$ fractionation with 30 to 60 per cent $(NH_4)_2SO_4$ was adopted as one of the methods for purification.

For the purification of β-amylase in germinating millet seeds, the peak that exhibited β-Amylase activity was collected and $(NH_4)_2SO_4$ was added to give 0.9 saturation. The precipitate was collected by centrifugation and dissolved in 20 mM NaOAc buffer, pH 4.5. After dialysis with 20 mM NaOAc buffer, the dialysate was applied to a CM cellulofine column (3.4 cm x 15 cm) equilibrated with the same buffer (Yamasaki, 2003).

Gupta *et al*. (2004) reported that lectin from the crude extract of seeds of *Delonix regia* has been purified by ammonium sulphate fractionation. The supernatant of the extract was fractionated for protein using 0 to 50, 50 to 65, 65 to 80 and 80 to 100 per cent $(NH_4)_2SO_4$ saturation. The 65 to 80 per cent precipitate that gave hem agglutination was collected and dissolved separately in the minimum quantity of PBS.

Ammonium sulphate precipitation of the crude extract followed by dialysis was attempted as an initial purification step for β-galactosidase from carambola fruit to remove other proteins as well as to reduce sample volume for subsequent chromatographic procedures (Balasubramaniam *et al*., 2005).

Campos *et al*. (2005) reported that for the purification and characterization of adenosine nucleosidase from young leaves of *Coffea arabica* the crude extract was successively fractionated with ammonium sulphate at 20, 40, 60 and 80 per

cent of saturation. Fractions with adenosine nucleosidase activity were loaded on to a Mono Q HR5/5 column equilibrated with buffer.

Gulcin and Yildirim (2005) reported that between 0 to 10, 10 to 20, 20 to 30, 30 to 40, 40 to 50, 50 to 60, 60 to 70, 70 to 80 per cent ammonium sulphate was used for precipitation carried out in a homogenate on an ice bath for the purification and characterization of peroxidase from *Brassica oleracea* var. *Acephala*. After precipitation, the mixture was centrifuged and the precipitate was dissolved in about three ml 0.3 M phosphate buffer. The concentrated sample was dialyzed and was used to load on CM Sephadex A-50 column.

In the purification and characterization of a novel fibrinolytic enzyme from *Rhizopus chinensis* 12, solid ammonium sulphate was added to the supernatant containing the fibrinolytic enzyme to make 40 per cent saturation. The mixture stood overnight at 4^0C and centrifuged at 7000 g for 30 minutes at 4^0C to remove particle material. The supernatant was adjusted to 70 per cent ammonium sulphate saturation by further addition of solid ammonium sulphate and kept overnight at 4^0C. The precipitate was collected by centrifugation at 10000 g for 20 minutes at 4^0C for further purification (Ian *et al*., 2005).

In the recombinant production of serine hydroxymethyl transferase from *Streptococcus thermophilus*, a fraction containing the recombinant protein was brought to 40 per cent saturation with ammonium sulphate at 4^0C for 24 hr. The suspension was centrifuged at 13,000 g for 30 minutes. The supernatant was discarded and the precipitated material was collected, resuspended in ice-cold ammonium sulphate (40%) and stored at 4^0C in a dark receptacle (Vidal *et al*., 2005)

De *et al*. (2004) reported that in the purification and characterization of α-amylase from *Bacillus amyloliquefaciens* NCIM 2829, 48 hr grown culture was centrifuged at 10,000 X g for 15 minutes at 4^oC and the supernatant was brought

to 30 to 60 per cent $(NH_4)_2SO_4$ saturation. The precipitate after dissolving in 5 mM phosphate buffer pH 7.2 and dialysis applied to DEAE sepharose column (2.5 cm X10 cm).

For the purification of peroxidase enzyme from *Solanum melongena*, filtered juice was saturated up to 60 per cent with ammonium sulphate and centrifuged at 4,000 g for 20 minutes at 4^0C. The precipitate was discarded and the supernatant was saturated up to 90 per cent by further addition of ammonium sulphate (Vernwal *et al*., 2006).

Demir *et al*. (2006) reported that for the purification of polyphenol oxidase from Van apple (Golden Delicious), the samples were fractionated with solid ammonium sulphate. The precipitate from 20 to 80 per cent saturation was dissolved in a minimal volume of 0.1 M sodium phosphate buffer (pH 6.8). The active fractions were applied to DEAE cellulose column.

Solid ammonium sulphate was added to the supernatant of leaf extract of NaCl stressed grain sorghum seedlings for the purification of oxalate oxidase to give saturation of 80 per cent. The resulting solution was kept overnight and centrifuged at 10000 x g for 30 minutes. The pellet was collected, washed carefully, re-dissolved in minimum volume of 0.1 M sodium phosphate buffer (pH 7.0) and tested for activity and protein (Singh *et al*., 2006).

2.3.5 Ion exchange chromatography

Thornber *et al*. (1965) reported that for the isolation and partial characterization of Fraction I protein from spinach – beet chloroplasts, the protein in the chloroplast washes were run into a DEAE cellulose column equilibrated in 0.025 M NaH_2PO_4-Na_2HPO_4 buffer, pH 7.0. The column was then washed with the same buffer (300 ml) and the proteins were eluted using a salt gradient.

Protein zones were examined by analytical ultra centrifugation and by PAGE in 0.03 M Tris-0.01 M cysteine HCl buffer, pH 8.0.

Crude enzyme extract containing β-xylosidase (50 ml) was applied to a DEAE Sephadex A 25 column (2.2 cm x 42 cm) pre-equilibrated with 50 mM phosphate buffer (pH 6.2). Elution was done initially with two bed volume of the same buffer and finally with a (0-500 mM) linear gradient of KCl (500 ml) in the same buffer at a flow rate of 25 ml/hr. All column fractions were monitored for β-xylosidase activity (Chakrabarti and Ranu, 1995).

Kumar *et al.* (1995) reported that for the purification and characterization of UDP–glucose pyrophosphorylase from immature grains of wheat, the pre-treated DEAE cellulose was packed in a glass column (40 cm x 2.6 cm). The ammonium sulphate fraction obtained after passing through Sephadex G-25 was loaded onto the DEAE cellulose column pre-equilibrated and concentrated by precipitation with ammonium sulphate (80% saturation). The precipitates were separated by centrifugation and suspended in 2ml of extraction buffer (50 mM Tris-HCl, pH 9.0)

For the purification and characterization of phosphoenol pyruvate carboxylase from developing seeds of Brassica, the enzyme preparation obtained after $(NH_4)_2SO_4$ fractionation was loaded onto the DEAE cellulose column (30 cm X 2.5cm) previously equilibrated with buffer. The enzyme was eluted with step-wise gradient of 0 to 0.2 M NaCl added buffer at a constant flow rate of 36 ml/hr. The fractions were collected and analyzed for protein and enzyme activity. The active fractions eluted as a single peak were pooled and concentrated by osmosis against solid sucrose (Mehta *et al*., 1995).

Chatterjee and Sanwal (1999) reported that DEAE-cellulose chromatography was attempted for the protein precipitated between 30 to 75 per cent of the sample extract of *Lantana camara* for the purification of *Cuscuta*

reflexa cellulase. The protein was eluted using a linear NaCl gradient (0-1 M). The active fractions eluted between 0.44 M to 0.84 M NaCl constituted a major peak with a minor peak eluted between 0.35 M to 0.43 M NaCl.

The urease enzyme from the gel filtration column was loaded on a DEAE-cellulose column of dimensions 20 cm X 1.6 cm equilibrated with four bed volumes of running buffer (0.1M Tris- acetate buffer, pH 6.8). The flow rate was 30 ml/hr. Urease was absorbed on the column material and unabsorbed proteins were washed out with several volumes of running buffer till the washings were free of protein. A discontinuous KCl gradient was applied for enzyme elution. Fractions with high A_{280} were assayed for urease activity and protein. Fractions with high specific activity were pooled (Das *et al*., 2002).

Crude extract of *Brassica juncea* infected with *Albugo candida* was used for purification of β-1, 3-glucanase using conventional techniques of $(NH_4)_2SO_4$ fractionation (30-60% saturation). These fractions were loaded for ion exchange chromatography on DEAE cellulose. Purity of enzyme preparation was judged on native PAGE performed on 10 per cent resolving gel and subunits molecular mass of enzyme was judged by SDS-PAGE on 7.5 per cent gel (Kapoor *et al*., 2003).

For the purification of β-Amylase in germinating millet seeds, the enzyme solution was dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0 and then applied to a DEAE –cellulofine column (15 cm x 3.4 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0. After the column was washed with the same buffer, a linear gradient of NaCl (0-1 M) in the same buffer was applied. The peak that exhibited β-amylase activity was collected and precipitated with $(NH_4)_2SO_4$ (Yamasaki, 2003).

Balasubramaniam *et al*. (2005) reported that two peaks of β-galactosidase activity (peak A and B) were obtained on passing crude extract from ripe fruits of carambola through CM-sepharose column. On passing through a DEAE sepharose

column (15 cm x 2.5 cm) at pH 7.2 peak A was further resolved into two activity peaks, the unbound β-galactosidase I and bound β-galactosidase II. The second peak was also resolved in to two activity peaks, comprising the unbound β-galactosidase IV on the same anion exchange

For the purification of nitroreductase I, pellets of *Klebsiella* sp. C1 were suspended in 20mM Tris buffer (pH 7.0). The suspension was transferred to a 327ml column of DEAE–Sepharose CL-6B exchange resin. The column was developed with a 0.25M NaCl gradient. The collected fractions were analyzed for nitroreductase activity and the active fractions were pooled (Kim and Song, 2005).

Demir *et al*. (2006) reported that for the purification of polyphenol oxidase from Van Apple (Golden Delicious), the filtrate of fruit juice after fractionation with solid ammonium sulphate, the precipitate from 20 to 80 per cent saturation was dissolved in a minimal volume of the sodium phosphate buffer (pH 6.8). The active fractions were applied to DEAE cellulose column (1 cm x 5 cm) previously equilibrated with sodium phosphate buffer (pH 6.3). The fractions containing PPO activity were appropriately combined, dialyzed against extraction buffer and distilled water. After overnight dialyzing, the solution was collected as PPO.

In the purification of oxalate oxidase from NaCl stressed grain sorghum seedlings, ammonium sulphate precipitated 80 per cent fraction, after dissolving in 0.1M sodium phosphate buffer pH 7.0 and loaded on to Sephadex G-200 column, three ml fractions were collected. Fractions with highest specific activities were pooled and loaded on to DEAE sephacel column (2.5 cm x 18 cm) previously equilibrated with 0.01 M potassium phosphate buffer (pH 6.7). The enzyme was eluted in a linear gradient of KCl (0-0.6 M) in the same buffer. Total volume of buffer was passed and 3 ml fractions were collected at the rate of 0.3 ml /minute. The active fractions were pooled and treated as a purified enzyme (Singh *et al*., 2006).

2.3.6 Gel filtration chromatography

In gel filtration chromatography, for desalting purposes, the sample size may be as large as 10 to 25 per cent of the bed volume, but for separation of macromolecules a sample size of 1 to 5 per cent is advisable (Santha *et al.*, 1998).

Mehta *et al.* (1995) reported that during the purification and characterization of phosphoenol pyruvate carboxylase from developing seeds of Brassica, the purified enzyme after passing through DEAE- cellulose was applied to sepharose CL-6B column (52 cm x 2.6 cm) previously equilibrated with buffer. The column was eluted with buffer at a flow rate of 18ml/hr and fractions of 3 ml each were collected. The active fractions eluted as a single peak were pooled and stored at 4°C. The molecular weight of purified PEP carboxylase as determined from gel filtration through Sepharose CL-6B was found to be about 400 kDa.

Gel filtration was used for the estimation of molecular weight of β-xylosidase activity from *Aspergillus terreus*. A sephacryl–S -300 column (1cm x 50cm) was equilibrated with 20 mM phosphate buffer (pH 6.2) containing 50 mM KCl. Standard proteins were used as markers (Chakrabarti and Ranu, 1995).

For the partial purification and characterization of UDP-glucose pyrophosphorylase from immature grains of wheat, Sephadex G-100 gel was packed in an LKB column (50 cm x 2.6 cm). The column was equilibrated with the Tris-HCl buffer (50 mM, pH 8.5) in which the Sephadex was suspended. The concentrated enzyme from DEAE cellulose chromatography was loaded on to the Sephadex G-100 column and eluted with equilibration buffer at a flow rate of 15 ml/hr. The partially purified enzyme preparation was characterized for physical and kinetic properties of the enzyme (Kumar *et al.*, 1995).

Das *et al.* (1996) reported that for the purification and partial characterization of arginine decarboxylase from *Brasssica campestis*, the active

fractions from Sephadex G-25 were pooled, concentrated and applied to a Sephadex G-200 column (45 cm x 2 cm). Protein was eluted at a flow rate of 5 ml/hr. The active fractions were pooled and applied to DEAE Sephadex A 50 column.

Das *et al.* (2002) reported that during the purification of urease from dehusked pigeon pea seeds, the second acetone fractionated enzyme was loaded onto a gel filtration column (Sephadex G-200) of dimensions 1.6 cm x 20 cm. The column was equilibrated with four bed volumes of running buffer (0.1M Tris acetate buffer, pH 6.8) at flow rate of 18 ml/hr. The A_{280} was monitored of all fractions and activity and protein of the peak fractions assayed. High specific activity fractions were pooled.

The molecular mass of purified β-1,3-glucanase enzyme from Brassica juncea infected with *Albugo candida* was estimated by gel filtration through a Sephadex G-100 column which had previously been calibrated with BSA (66 kDa), trypsin (23 kDa) and lysozyme (14.3 kDa) (Kapoor *et al.,* 2003).

Size Exclusion Chromatography (SEC) is well suited for protein analysis applications like quantification, impurity testing, reaction monitoring, product purification, folding studies and the desalting and exchange of sample buffer. The protein's molecular mass may be simultaneously determined by calibrating SEC column retention times or elution volumes with an appropriate series of macromolecular standards or employing molecular mass sensitive detection method (Goetz *et al.,* 2004).

Balasubramaniam *et al.* (2005) reported that β-galactosidase I and β-galactosidase III that were obtained on fractionation with anion exchange chromatography on DEAE sepharose column were further fractionated by gel

filtration chromatography to remove contaminating proteins and both were individually eluted as a single peak through a Sephacryl S-200 HR column. The apparent native molecular sizes of β-galactosidase I, II, III, IV estimated by chromatography on a Sephadex G-100 column were 84, 77, 58 and 130 kDa respectively.

In the purification of NAD (P) H- dependent nitroreductase 1 from *Klebsiella* sp., the molecular mass of the native enzyme was measured by gel permeation chromatography (Sephadex G-100-120 resin) with molecular markers (Kim and Song, 2005).

Singh *et al*. (2006) reported that for the purification of oxalate oxidase from NaCl stressed grain sorghum seedlings ammonium sulphate precipitated pellet was redissolved in minimum volume of 0.1 M sodium phosphate buffer and loaded onto Sephadex G-200 column (20 cm x 52 cm) previously equilibrated with 0.01 M potassium phosphate buffer. The proteins were eluted in the same buffer at a flow rate of 0.3 ml/minute. Fractions were tested for activity and protein. Fractions with highest specific activities were pooled and loaded on to DEAE – sephacel column.

The dialyzed peroxidase enzyme from *Solanum melongena* fruit juice containing 6 mg/ml protein was loaded to a Sephadex G-100 column (2.6 cm x 60 cm) equilibrated with 100 mM phosphate buffer (pH 7.0). The enzyme activity was eluted as the first peak and impurities were eluted after that. The specific activity of enzyme, which was 0.28 IU/mg, before loading on the column, reached up to 3.13 IU/mg after elution (Vernwal *et al.*, 2006).

2.4 Hypoglycaemic property of plant proteins

Baldwa *et al.* (1977) reported that the insulin isolated from *Momordica charantia* i.e. V- insulin (vegetable insulin) consists of 17 amino acids from

qualitative amino acid analysis by paper chromatograph and quantitative analysis by an amino acid analyzer. Its three-dimensional structure was found to consist of two chains of amino acids bound together with sulfide bonds. Clinical trial in patients having diabetic mellitus with V-insulin revealed that it has hypoglycaemic effect when administered by the subcutaneous or the intramuscular route. The peak effect of vegetable insulin was seen after 4 to 12 hours as compared with insulin that is commonly used (2-3 hours). Though there were no anaphylactic reactions to vegetable insulin, further studies are required in order to evaluate its antigenic properties for long-term use.

Khanna *et al* (1981) reported that a hypoglycaemic peptide, polypeptide-p, has been isolated from fruit, seeds and tissue of *Momordica charantia* L. (Bitter gourd). Amino acid analysis indicates a minimum molecular weight of approximately 11,000 (166 residues). Polypeptide–p is a very effective hypoglycaemic agent when administered subcutaneously to gerbils, langurs and humans.

Azevedo *et al*. (2006) reported that they could isolate insulin like protein from the leaves of *Bauhinia variegata*. A chloroplast protein with a molecular mass similar to that of bovine insulin was extracted from 2 mm thick 15 per cent SDS- PAGE gels and fractionated with a 2 cm x 24 cm Sephadex G-50 column. The activity of insulin like protein on serum glucose levels of four-week-old Swiss albino diabetic mice was similar to that of commercial swine insulin used as control. Further characterization of this molecule by reverse–phase hydrophobic HPLC analysis as well as its anti-diabetic activity on alloxan-induced mice showed that it has insulin like properties.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled 'Characterization of proteins in *Costus pictus* D. Don' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2005-2007. Materials used and methodologies of this study are described below.

3.1 Materials

3.1.1 Plant materials

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

3.1.2 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 plasticware was supplied by Tarson India Ltd. and Axygen, USA.

3.1.3 Laboratory equipments

The equipment items available at CPBMB were utilized for the present study.

3.2 Methods

3.2.1 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) and also by spectrophotometry using Nanodrop ND 1000 spectrophotometer.

3.2.1.1 Lowry's method

A. Sample collection

Fourth and fifth physiologically mature leaves were collected fresh from the field on the day of analysis.

B. Selection of extraction buffer

The following five buffers at varying pH and concentrations were tried for selecting the most suitable buffer:

i) 0.2 M Tris (Hydroxy methyl) Aminomethane buffer at pH 7.2 (Sadasivam and Manickam, 1996).
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).
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).

Composition of buffers are given in Appendix I

C. Reagents

Reagent A – 2% Na_2CO_3 in 0.1 N NaOH
Reagent B – 0.5% $CuSO_4$ ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate
Reagent C - Mix 50 ml of A and 1 ml of B prior to use

Reagent D - Folin-Ciocalteau reagent (diluted in the ratio 1:2 with distilled water)
15% TCA – 15 g TCA in 100 ml water

D. Preparation of standard

Stock solution

Fifty milligrams of Bovine Serum Albumin (Fraction V) was weighed and dissolved in distilled water and made up to 50 ml in a standard flask.

Working standard

Ten ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of the solution contains 200 µg protein.

E. Procedure

i) Lowry's method (without TCA precipitation of protein)

One gram each of leaf sample was ground with 2 ml each of cold buffers in an ice-cold mortar. The extract was transferred to a centrifuge tube and 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. The volume was made up to 1 ml with the same buffers. Five test tubes containing 1 ml each of buffers were kept as blank. 5 ml reagent C was added to each tube including blank. Mixed well and allowed to stand for 10 minutes. Then 0.5 ml reagent D was added, mixed well and incubated at room temperature in dark for 20 minutes. Readings were taken at 660 nm.

ii) Lowry's method (with TCA precipitation of protein)

One gram each of leaf sample was ground with 2 ml of cold buffers separately in an ice-cold mortar. The extract was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 10 minutes. From the supernatant, 0.5 ml each

of extract was taken in five different test tubes. Equal quantity of 15 per cent TCA was added to each test tube. Samples were kept overnight at room temperature. On the next day, the sample was centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted and precipitates were dissolved in 1ml 0.1 N NaOH each. From the dissolved protein sample, took 0.5 ml of each sample in different test tubes. Volume was made up 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 that containing the blank. Mixed well and allowed to stand for 10 minutes. Then 0.5 ml of reagent D was added, mixed well and incubated at room temperature in dark for 20 minutes. Readings were taken at 660 nm (Augustin, 2005).

F. Preparation of standard curve

Standard curve was prepared from the working standards of protein with concentrations ranging from 40 to 200 µg.

Protein content in the sample was determined using the formula,

$$\text{Protein content (mg/g)} = \frac{\text{OD of the test solution x Conc.of std. x Total Volume}}{\text{OD of standard x Volume taken x Wt. of sample}}$$

3.2.1.2 Estimation of protein using Nanodrop ND 1000 spectrophotometer

The Nanodrop ND-1000 is a full spectrum (220-750nm) spectrophotometer that measures 1µl samples with high accuracy and reproducibility. It utilizes a patented sample retention technology that employs surface tension alone to hold the sample in place. This eliminates the need for cumbersome cuvettes and other sample containment devices and allows for clean up in seconds. ND-1000 has the capability to measure highly concentrated samples without dilution as compared to the samples measured by a standard cuvette spectrophotometer.

3.2.1.2.1 Procedure:

A 1 µl sample was pipetted on to the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought in to contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap was enclosed to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provided the light source and a spectrophotometer utilizing a linear CCD array was used to analyze the light after passing through the sample. The instrument was controlled by special software run from a PC, and the data was logged in an archive file on the PC. The following steps were followed to estimate the protein content.

1. The programme 'Protein A 280' was selected
2. With the sampling arm opened, the sample was pipetted on to the lower measurement pedestal.
3. The sampling arm was closed and initiated a spectral measurement using the operating software on the PC. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.
4. When the measurement was complete, the sampling arm was opened and wiped the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000 fold in concentration.
5. The report was saved.

3.2.2 Molecular weight determination by SDS-PAGE

Sodium Dodecyl Sulphate (SDS) is an anionic detergent with a net negative charge. Because the charge to mass ratio is nearly the same among SDS-denatured polypeptides, the final separation is dependent almost entirely on the differences in the molecular weight (MW) of polypeptides. The samples were

subjected to electrophoresis in a vertical electrophoretic unit (BIO-RAD) according to the procedure described by Laemmli (1970) with some modifications.

3.2.2.1 Preparation of reagents

A. Monomer solution
B. 4X Resolving gel buffer (1.5 M Tris-Cl, pH 8.8)
C. 4X stacking gel buffer (0.5 M Tris-Cl, pH 6.8)
D. Electrode buffer (0.025 M Tris, pH 8.3, 0.192 M Glycine)
E. 2X Treatment buffer (0.125 M Tris Cl)
F. Initiator (10% APS)
G. SDS (10%)

Composition of reagents are given in Appendix II

3.2.2.2 Casting of the gel units

The glass plates and spacers were thoroughly wiped with acetone and assembled properly as per manufacture's instruction. The assembly was held together and the clips closed simultaneously and clamped in an upright position.

3.2.2.3 Preparation of gel

1. **Resolving gel**

   Different combinations of resolving gel were tried out to select the optimum combination as mentioned by Parab (2000). Sufficient volume of resolving gel (separating gel mixture) was prepared by mixing the components given in Table 2.

Table 2. Different per cent of resolving gel tried with 6 per cent stacking gel

Components	7 %	7.5 %	10 %	15 %
Monomer	2.33 ml	2.49 ml	3.33 ml	4.99 ml
4X Resolving gel buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml
SDS 10%	0.28 ml	0.1 ml	0.1 ml	0.1 ml
Distilled water	5.06 ml	4.85 ml	4 ml	2.36 ml
APS 10%	100 µl	50 µl	50 µl	50 µl
TEMED	10 µl	5 µl	5 µl	5 µl

Mixed gently and carefully, poured the gel solution in the chamber between the glass plates with the help of micropipette up to desired height (leaving 2 cm at top). Distilled water was layered on top of the gel and left to set for 60 minutes.

2. Stacking gel (6%)

Stacking gel was prepared by mixing the following solutions.

Monomer : 0.67 ml

4x stacking gel buffer : 1.25 ml

SDS 10 per cent : 0.05 ml

Distilled water : 3 ml

APS10 per cent : 25 µl

TEMED : 30 µl

Water from the top of the gel was removed and the stacking gel mixture was poured, placed the comb in the stacking gel and allowed the gel to set for 30minutes. After the stacking gel has polymerized, the comb was removed without distorting the shapes of the well. The gel was carefully installed in the electrophoresis apparatus. Filled it with electrode buffer and removed any trapped air bubbles at the top of the gel.

3.2.2.4 Preparation of the sample

Different buffer systems 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 0.2 M Tris buffer (pH 7.2), 0.2 M Tris buffer containing ingredients (pH 7.2), sodium citrate phosphate buffer (pH 2.8), 0.2 M phosphate buffer (pH 7.0) and Phosphate buffered saline (pH 7.3). 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.

3.2.2.5 Running of the sample

The cathode and anode was connected to BIO-RAD power pack. The electrode buffer and plates were kept cool by using a cold cell so that heat generated during the run was dissipated and did not affect the gel and resolution. Current was turned on to 15 mA for initial 15 to 20 minutes until the samples ran through the stacking gel. Continued the run at 20 mA until the bromophenol blue reached the bottom of the gel. (The effect of current variation was tried for stacking gel and resolving gel to find the best one).

3.2.2.6 Staining and destaining

3.2.2.6.1 Staining with Coomassie brilliant blue

After the run, the gel was transferred to staining solution and kept overnight maintaining uniform shaking. The protein absorbs the Coomassie

brilliant blue. On the next day, the gel was transferred to a container with destaining solution and shook gently and continuously. The destainer was changed frequently until the background of the gel became colourless. The protein profile was viewed in Transilluminator and documented using the Alpha Imager TM1200 (Alpha Innotech Corporation).

Composition of protein staining solution and destainer are given in Appendix III

3.2.2.6.2. Silver staining

Silver staining was also carried out to study the nature of proteins contained in the leaf of *Costus pictus*.
Principle: The amino acids particularly aromatic amino acids in the protein reduce silver nitrate and form complexes with metallic silver of yellowish-brown to brown colour.
Procedure described by Sadasivam and Manickam (1996) was followed to stain the protein.

Reagents

1. Washing solution: Mixed 1 ml of formaldehyde (analytical grade, 37%), 40 ml of methanol and 60 ml of distilled water.
2. Sodium thiosulfate: Dissolved 200 mg in a litre of distilled water.
3. Silver nitrate solution (0.1%)
4. Developer: Dissolved sodium carbonate 3 g (w/v) in about 80 ml distilled water. Added 1 ml of the above sodium thiosulfate solution and 1 ml of formaldehyde and finally made up the volume to 100 ml with distilled water.
5. Stopper: 5 per cent acetic acid solution.

Procedure:

1. After electrophoresis, the gel was transferred to a clean plastic container and washed the gel in the washing solution with slow shaking for 10 minutes.

2. The wash solution was discarded and the gel rinsed with plenty of water for 2 minutes.
3. The gel was soaked in sodium thiosulfate solution for 1 to 2 minutes.
4. The gel was washed twice with water, each time 1 to 2 minutes and the wash water was drained
5. The gel was soaked in silver nitrate solution for 10 minutes in dark.
6. The gel was washed in water as in step 4.
7. Developer was poured to the plastic container and shook the gel slowly and gently. The proteins reduce silver nitrate to silver and yellow to dark brown colour bands appeared.
8. When sufficient intensity of band developed, the reaction was stopped by adding acetic acid solution.
9. The protein banding pattern was recorded by photography.

3.2.2.7 Molecular weight determination

Molecular weight of each band was determined by the comparison with the protein molecular weight marker (broad range protein molecular weight marker-PMWB GENEI of weight 3000 to 2,05,000 Da). Molecular weight determination was done using molecular weight determination tool in the Alpha imager. Position of wells and dye front were fixed. Molecular weights in Dalton were assigned to each band in the protein molecular weight marker as described in the catalogue of GENEI. The molecular weight and Rm value of bands in the sample were recorded.

3.2.3. PAGE (Poly Acrylamide Gel Electrophoresis)

PAGE separates native proteins. Separation of proteins is based not only on protein size but also on protein charge and shape. This system is recommended only if the biological activity of protein needs to be retained. By combining the information on the native molecular weight, the subunit molecular weight and the

number of different polypeptide chains from SDS-PAGE, it is possible to figure out what the sub-unit composition is.

3.2.3.1 Preparation of reagents

Reagents were prepared as mentioned in 3.2.2.1 without adding SDS to any of the stock solutions.

3.2.3.2. Preparation and casting of gel

The gel unit was assembled as described in section 3.2.2.2. Combinations of resolving gel and stacking gel were tried as mentioned in section 3.2.2.3, without adding SDS to any of the combinations.

3.2.3.3. Preparation of the sample

To select an appropriate buffer for PAGE, each gram of leaf sample was ground with 1 ml of 0.2 M Tris buffer (pH 7.2), 0.2 M Tris buffer containing ingredients (pH 7.2), Sodium citrate phosphate buffer (pH 2.8), 0.2 M phosphate buffer (pH 7.0), Phosphate buffered saline (pH 7.3). The extract was centrifuged at 12,000 rpm for 10 minutes.

Forty microlitre of the extract was mixed with 10 µl of 2X treatment buffer for PAGE. Five microlitre of insulin (human mixtard) and 10 µl marker (broad range protein molecular weight marker-PMWB GENEI of 3000 to 2,05,000 kDa) were also mixed with 10 µl of treatment buffer and 30 µl of distilled water and loaded in separate lanes for the comparison of molecular weight.

3.2.3.4 Running of the sample

Running of the sample was carried as described in section 3.2.2.5

3.2.3.5 Staining and destaining

Done as described in section 3.2.2.6.1

3.2.4. Partial purification of protein through ammonium sulphate fractionation

3.2.4.1 Preparation of saturated ammonium sulphate solution

Crystal ammonium sulphate was added slowly to about 1000 ml of distilled water with stirring to maximum saturation. It was kept hot and added more ammonium sulphate till the crystal became insoluble. Cooled the solution to room temperature, removed the excess crystals and neutralized with ammonia solution.

3.2.4.2 Preparation of protein sample

Nine lots of 5 g leaf sample was collected (fourth or fifth) and ground in 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. The supernatant was collected.

3.2.4.3 Precipitation of protein using saturated ammonium sulphate solution

The procedure suggested by Chaykin (1966) was followed with some modifications for protein precipitation.

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.

3.2.5. Ion exchange chromatography

In ion exchange chromatography, molecules are separated by differences in their molecular charge. An ion exchanger saturated with one kind of ion is used as the stationary phase. The mobile phase is a solution containing the same ions with which the ion exchanger is saturated. When the ions of the sample are transferred to the column, they displace part of the original ions and remain on the ion exchanger. Later they are displaced by successive ions and in this way move through the column. The traveling speed of the substance is determined by the substance's degree of ionization.

There are two types of ion exchangers. Cation exchangers posses negatively charged groups and therefore, attract positively charged molecules. The anion exchangers have positively charged groups, which attract negatively charged molecules.

Anion exchange chromatography with DEAE cellulose

3.2.5.1. Sample preparation

Samples (20 g) were ground in 25 ml 0.2 M Tris buffer, centrifuged the extract at 12,000 rpm for 15 minutes and supernatant was collected. Saturated

ammonium sulphate solution was added to the supernatant to make it 10 per cent saturation and kept overnight at $0^{o}C$. On the next day, centrifuged the mixture at 12,000 rpm for 20 minutes and removed the precipitate. To the remaining supernatant, saturated ammonium sulphate was added to make it 60 per cent saturation. Precipitate was dissolved in 2 ml 0.2 M Tris buffer and dialyzed against the same buffer for 24 hours with two buffer changes. In another set 100 g leaf sample was ground in 80 ml 0.2 M Tris buffer and 50 to 90 per cent fraction was made as described above. The precipitate was dissolved in 3 ml buffer and dialyzed against the same buffer.

3.2.5.2. Packing of the column

Pre-swollen DEAE cellulose from GENEI was previously saturated with 0.2 M Tris buffer and used for the packing of column of 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.

3.2.5.3.Procedure

When the 0.2 M Tris buffer has just sunk in the column, 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. As soon as the sample sunk in the column, 0.2 M Tris buffer (pH 7.2) was poured into the column without disturbing the surface layer and collected 2 ml fractions for 60 per cent and 1 ml fraction for 50 to 90 per cent ammonium sulphate precipitated protein fraction separately. Protein in the column was 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 fractions of high peak values were used for 10 per cent SDS-PAGE. NaCl gradient at pH 8.0 and 6.5 were also tried for their efficiency in eluting protein in the column containing 50 to 90 per cent ammonium sulphate precipitated fraction.

3.2.6 Gel filtration chromatography (size exclusion chromatography)

In gel filtration chromatography, molecules are separated on the basis of molecular size in solution. A porous gel in the form of small insoluble beads is commonly used to separate proteins by size. Small molecules that can enter the pores of beads are retarded as they pass down a column containing the gel, but large molecules that are unable to enter the pores pass through the column unimpeded. Therefore the largest molecules will elute first and smallest molecules will elute last.

3.2.6.1. Sample preparation

1. Samples for gel filtration chromatography were prepared as described in 3.2.5.1 in the case of 60 per cent ammonium sulphate saturated fraction.
2. Protein fraction of 50-90 per cent was dialyzed and applied to DEAE column.

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.

3.2.6.2 Packing of the column

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.

3.2.6.3 Procedure

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. Fractions of high peak values were separately used for electrophoresis.

Results

4. Results

The results of the study conducted on Characterization of proteins in *Costus pictus* D.Don at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter under different subheads.

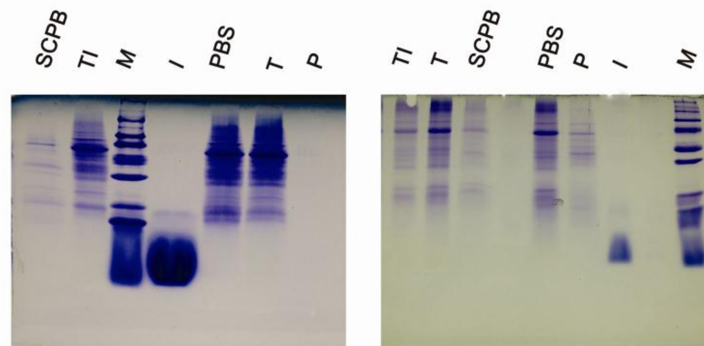
4.1. Estimation of protein content of *Costus pictus*

Protein content in the leaves of *Costus pictus* was estimated by Lowry's method and Nanodrop ND 1000 spectrophotometer. Five different buffers were tried for selecting an efficient one for protein extraction. Protein content recorded by each method is given in Table 3.

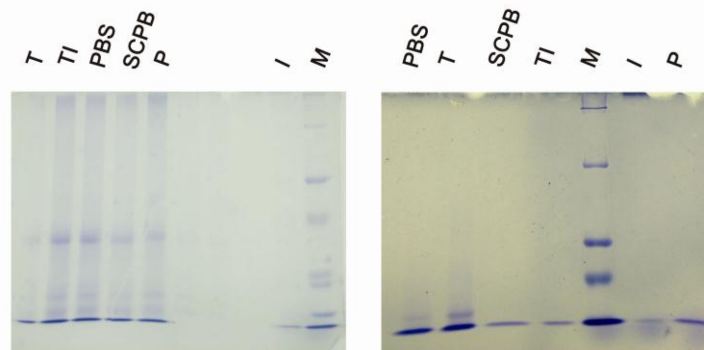
Nanodrop method gives direct measurement of protein in mg/ml which was converted to mg/g for presenting the results. It is evident from the observations that the extraction of protein with PBS and sodium citrate phosphate buffer gave maximum quantity of protein at 280 nm in Nanodrop and at 660 nm by Lowry's method (before precipitation). But on precipitating proteins with TCA, a considerable reduction in protein/protein analogues was recorded and maximum protein content was observed in the extraction with Tris buffer.

4.2. Effect of extraction buffer and per cent acrylamide in SDS-PAGE PAGE on *Costus pictus* sample

Each one gram leaf sample of *Costus pictus* was ground separately with 1 ml each of 0.2 M Tris buffer (pH 7.2), 0.2 M Tris buffer containing ingredients (pH 7.2), sodium citrate phosphate buffer (pH 2.8), 0.2 M phosphate buffer (pH 7.0) and phosphate buffered saline (pH 7.3).



a. 15 % [15 mA (S) & 30 mA(R)] b. 15 % [20 mA (S) & 20 mA (R)]



c. 7.5 % [20 mA (S) & 20 mA (R)] d. 7.0 % [20 mA (S) & 20 mA (R)]

Plate - 1

Standardization of acrylamide percentage for SDS PAGE

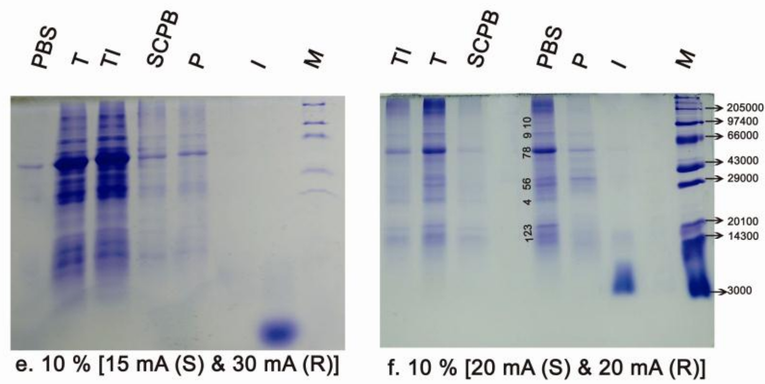
S - Stacking gel, R - Resolving gel

T - Tris buffer (pH 7.2), TI - Tris + ingredients buffer (pH 7.2),

SCPB - Sodium Citrate Phosphate Buffer (pH 2.8),

PBS - Phosphate Buffered Saline (pH 7.3),

P - Phosphate Buffer (pH 7), I - Insulin, M - Marker



e. 10 % [15 mA (S) & 30 mA (R)]

f. 10 % [20 mA (S) & 20 mA (R)]

Plate - 1

Standardization of acrylamide percentage for SDS PAGE

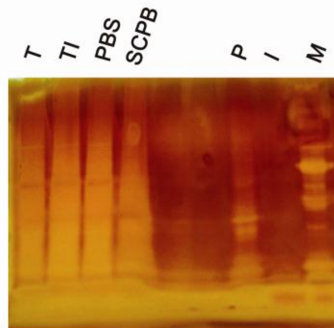


Plate - 2

Costus pictus protein on SDS PAGE with silver staining

S - Stacking gel, R - Resolving gel

T - Tris buffer (pH 7.2), TI - Tris + ingredients buffer (pH 7.2),

SCPB - Sodium Citrate Phosphate Buffer (pH 2.8),

PBS - Phosphate Buffered Saline (pH 7.3),

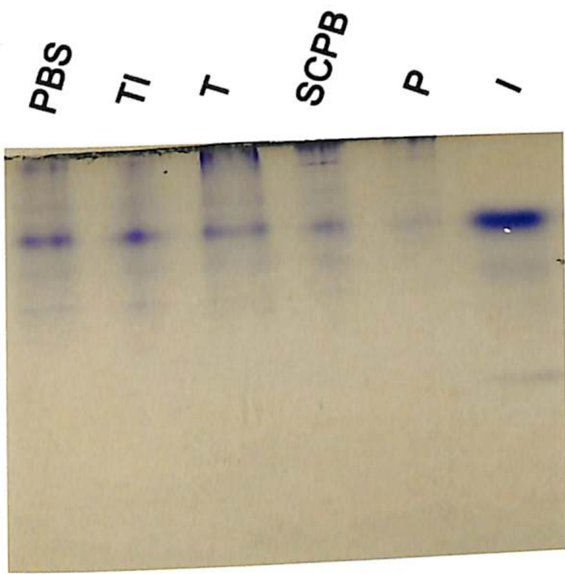
P - Phosphate Buffer (pH 7), I - Insulin, M - Marker

Table 3. Protein content in the leaf sample of *Costus pictus*

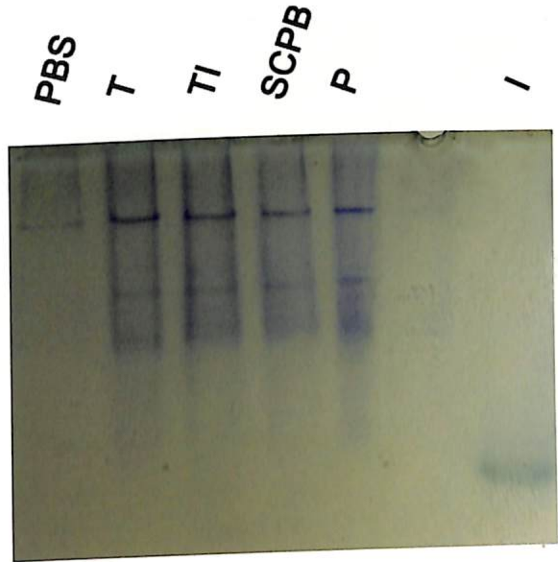
Buffer	Protein content (mg/g)			
	Nanodrop	Lowry's method		
		Before TCA precipitation	After TCA precipitation	Impurity factor
Tris buffer	2.79	2.47	0.30	8.23
Tris+ ingredients	1.73	1.78	0.13	13.69
PBS	3.11	2.50	0.24	10.42
Phosphate buffer	2.08	1.71	0.07	24.43
Sodium citrate phosphate buffer	3.10	2.73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

Table 4. Effect of buffers and concentration of acrylamide in the banding pattern of protein in SDS PAGE

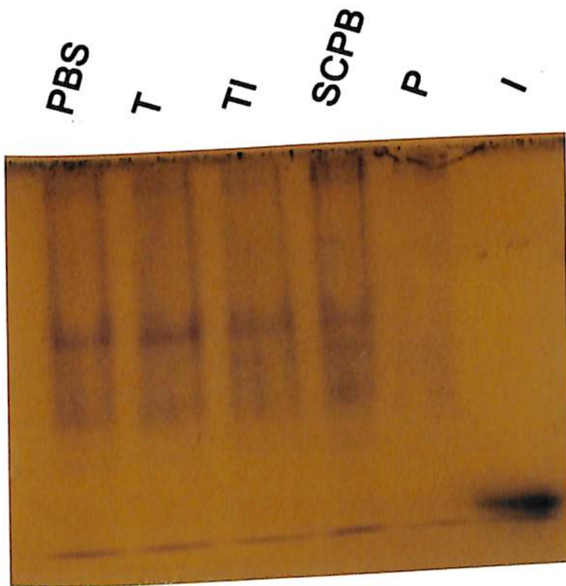
Buffer	No. of bands present in varying acryl amide concentration (SDS-PAGE)			
	15%	10%	7.5%	7%
Tris buffer	10	10	4	2
Tris+ ingredients buffer	8	10	4	1
PBS	10	10	4	2
Sodium citrate phosphate buffer	5	7	4	1
Phosphate buffer	5	7	1	1



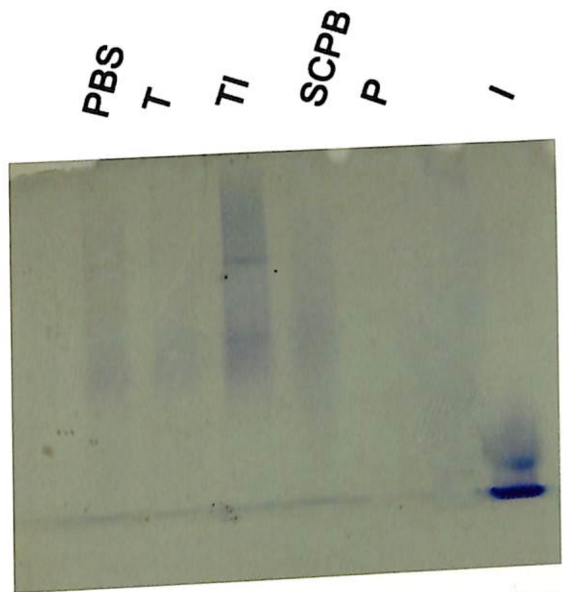
a. 15 % [20 mA (S) & 20 mA (R)]



b. 10 % [20 mA (S) & 20 mA (R)]



c. 7.5 % [20 mA (S) & 20 mA (R)]



d. 7.0 % [20 mA (S) & 20 mA (R)]

Plate - 3

Standardization of acrylamide percentage for PAGE

S - Stacking gel, R - Resolving gel

T - Tris buffer (pH 7.2), TI - Tris + ingredients buffer (pH 7.2),

SCPB - Sodium Citrate Phosphate Buffer (pH 2.8),

PBS - Phosphate Buffered Saline (pH 7.3),

P - Phosphate Buffer (pH 7), I - Insulin, M - Marker

The extraction with 0.2 M Tris buffer and PBS was found on par in giving maximum number of clear and distinct bands in all gel combinations tried (Table 4 & 5). Protein concentration was also maximum in the above extraction buffer (Table 3).

Concentration of stacking gel used was six per cent and varying resolving gel concentration of 15 per cent (Plate1 a & b), 10 per cent (Plate 1e & f), 7.5 per cent (Plate1 c) and seven per cent (Plate 1 d) were tried. Constant current of 20 mA throughout the run i.e. both for stacking and resolving gel showed more resolution of bands than 15 mA for stacking gel and 30 mA for resolving gel.

Ten per cent resolving gel of SDS-PAGE with 20 mA throughout the run was found best in producing clear and distinct bands with Coomassie Brilliant Blue R-250 (Plate 1 f). Silver staining of 10 per cent SDS-PAGE gel gave less number of bands in protein sample (Plate 2 and Table 8).

Ten per cent resolving gel with 20 mA throughout the run was found to give clear and distinct bands in PAGE (Plate 3 b) than other combinations tried (Plate 3 a, c & d).

4.3. Molecular weight determination.

Band number, molecular weight and Rm value of protein marker used are presented in Table 6 and that of proteins isolated in 10 per cent SDS-PAGE (Plates 1f & 2) is presented in Table 7 and Table 8. The approximate molecular weight and Rm value of proteins separated in 10 per cent PAGE are given in Table 9.

The protein having molecular weight of 58,131 Dalton and Rm value of 0.392 was found prominent on staining with Coomassie Brilliant Blue R-250.

Table 5. 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 6. Molecular weight and Rm value of marker proteins in SDS-PAGE

Band No.	Molecular weight (Da)	Rm value
1	3000	0.968
2	6500	0.921
3	14,300	0.886
4	20,100	0.814
5	29,000	0.607
6	43,000	0.496
7	66,000	0.339
8	97,400	0.250
9	2,05,000	0.146

Table 7. Molecular weight and Rm value of proteins of crude extract from *Costus pictus* in SDS-PAGE on staining with Coomassie Brilliant Blue R-250

Band No.	Molecular weight (Dalton)	Rm value
1	15,905	0.866
2	18,460	0.834
3	20,870	0.796
4	26,398	0.667
5	28,460	0.619
6	35,313	0.557
7	51,200	0.439
8	58,131	0.392
9	80,177	0.298
10	1,20,476	0.222

Table 8. 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 9. 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 10. Effect of varying concentration of ammonium sulphate (direct precipitation) in protein content and banding pattern of leaf sample from *Costus pictus*

Fraction	Protein content (mg/g)	No. of bands present	Band Nos.
10%	1.54	1	1
20%	1.46	4	1,4,6,9
30%	1.4	4	1,4,6,9
40%	1.56	4	1,4,6,9
50%	1.1	3	1,4,9
60%	1.82	5	1,4,5,6,9
70%	2.17	3	1,4,9
80%	1.86	4	2,3,7,10
90%	5.66	6	2,3,7,8,10,11

4.4. Ammomium sulphate fractionation and partial purification of proteins

Protein was salted out with saturated ammonium sulphate solution for each lot of sample extract. 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent ammonium sulphate saturation were applied separately. Salted out protein in each sample was estimated (Table 10).

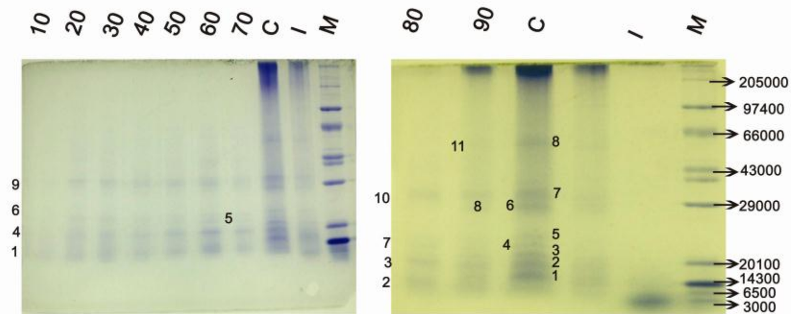
Salting out of protein was high at 70 per cent and 90 per cent ammonium sulphate saturation. Electrophoresis was carried out for each fraction in 10 per cent SDS-PAGE (Plate 4 a & b). The molecular weight and Rm value was tabulated in Table 12 and that of crude sample loaded is given in Table 11.

In another set, the supernatant obtained after protein precipitated in 10 per cent ammonium sulphate was progressively concentrated by increasing the concentration of ammonium sulphate to 20, 30 up to 90 per cent and the protein salted out at each stage was estimated (Table 13). Electrophoresis was carried out in 10 per cent SDS-PAGE (Plate 4 c & d) and molecular weight of each protein band was estimated (Table 14).

A band having molecular weight of 6500 Da and Rm value of 0.921 was present in 70 to 80 per cent saturation. No additional bands appeared in 60 to 70 per cent and 80 to 90 per cent fractions with Coomassie Brilliant Blue R-250. Silver staining of the gel (Plates 4 e & f) revealed a single band having molecular weight of 11,377 Da and Rm value of 0.899 uniformly in all the fractions up to 70 to 80 per cent saturation which did not appear in Coomassie Brilliant Blue R-250 staining.

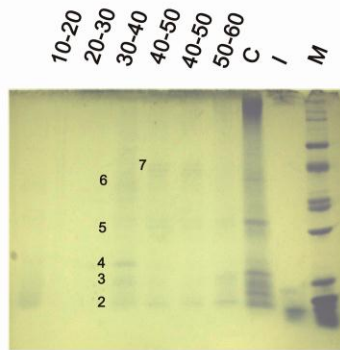
4.5. Anion exchange chromatography with DEAE cellulose

Ion exchange chromatography is based on the charge of the protein that needs to be purified. In anion exchange chromatography, the column is positively

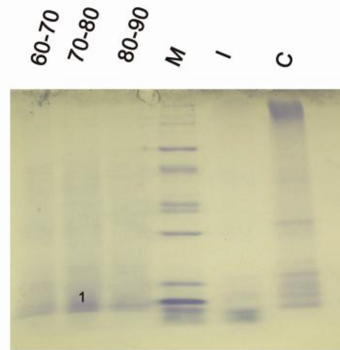


a. Direct precipitation

b. Direct precipitation



c. Sequential precipitation



d. Sequential precipitation

Plate - 4

Effect of $(NH_4)_2SO_4$ Saturation (Direct / Sequential) in salting out of proteins

I - Insulin, M - Marker, C - Crude sample

Table 11. Molecular weight and Rm value of crude sample used for ammonium sulphate precipitation

Band No.	Molecular weight (Da)	Rm value
1	7610	0.915
2	15,570	0.870
3	19,761	0.818
4	21,142	0.789
5	29,371	0.603
6	33,350	0.572
7	60,462	0.377
8	83,359	0.289

Table 12. Molecular weight and Rm values of proteins precipitated at varying concentration of ammonium sulphate (direct precipitation)

Band No.	Molecular weight (Da)	Rm value
1	9,367	0.908
2	10,995	0.901
3	17,196	0.850
4	18,275	0.836
5	21,222	0.787
6	21,954	0.770
7	22,884	0.748
8	29,000	0.607
9	31,297	0.589
10	34,107	0.566
11	59,948	0.380

Table 13. Effect of varying concentration of ammonium sulphate (sequential precipitation) in protein content and banding pattern of leaf sample from *Costus pictus*

Fraction	Protein content (mg/g)	No. of bands present in Coomassie staining	Band Nos.
10-20%	0.08	Zero	-
20-30%	0.06	Zero	-
30-40%	0.46	5	2,3,4,5,6
40-50%	1.34	4	2,3,5,7
50-60%	0.30	3	2,3,5
60-70%	0.48	Zero	-
70-80%	0.24	1	1
80-90%	0.96	Zero	-

Table 14. 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

charged and negatively charged proteins will bind to it. Salting out will release or elute the protein from the column. The technique uses a solution with high salt concentration. Column has a higher attraction for the charge of salts than for the charged protein and it will release the protein in favour of salts. Protein with weaker ionic interactions will elute at a lower salt concentration and that with stronger ionic interaction at higher ionic strength.

To study the nature of protein on DEAE cellulose, 60 and 50 to 90 per cent Ammonium sulphate saturated fractions were used.

1. **Sixty per cent ammonium sulphate fraction**

Column of size 30 cm x 1.2 cm was prepared for the anion exchange chromatography of 2 ml protein sample from 60 per cent ammonium sulphate saturated fraction. 2 ml fractions were collected and protein content of each fraction was estimated using the Nanodrop at 280 nm (Table 15). Fractions of high protein content on gradient elution were used for electrophoresis on 10 per cent SDS-PAGE (Plate 5) and molecular weights and Rm values are presented in Table 16.

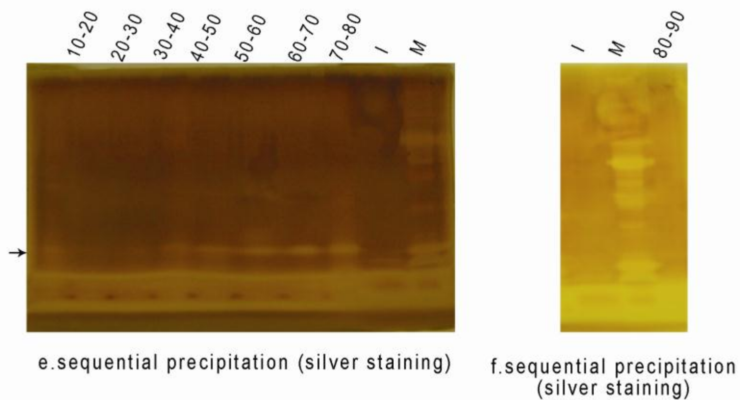
Protein content of fractions collected after washing the column with Tris buffer was negligibly small. For the elution of protein from the column higher salt concentration (>0.5 M NaCl) became needed and it recorded maximum elution at 0.9 M NaCl concentration and decreased there after (Table 15). Electrophoretic banding pattern was visible with Coomassie staining (Plate 5)

2. **50 to 90 per cent ammonium sulphate fraction**

15 cm x 1.2 cm column was used for the anion exchange chromatography of 1ml protein sample of 50 to 90 per cent ammonium sulphate saturation. Protein.

Table 15. Concentration of protein from the fractions of 60 per cent ammonium sulphate saturation in DEAE cellulose chromatography at pH 7.2

Fraction collected after washing the column with 0.2M Tris buffer (pH 7.2)	Protein content (mg/g)
Fraction 1	0.038
Fraction 2	0.025
Fraction 3	0.013
Fraction 4	0.013
Fraction 5	0.025
Fraction 6	0.000
Elution with 0.2 M Tris buffer containing NaCl (pH 7.2)	**Protein content (mg/g)**
0.1 M (Ist) NaCl	0.025
0.1 M (2nd)NaCl	0.050
0.2 M (Ist) NaCl	0.025
0.2 M (2nd)NaCl	0.025
0.3 M (Ist) NaCl	0.025
0.3 M (2nd)NaCl	0.013
0.4 M (Ist) NaCl	0.075
0.4 M (2nd)NaCl	0.088
0.5 M (Ist) NaCl	0.000
0.5 M (2nd)NaCl	0.000
0.6 M (Ist) NaCl	0.075
0.6 M (2nd)NaCl	0.150
0.7 M (Ist) NaCl	0.188
0.7 M (2nd)NaCl	0.175
0.8 M (Ist) NaCl	0.438
0.8 M (2nd)NaCl	0.638
0.9 M (Ist) NaCl	1.000
0.9 M (2nd)NaCl	0.988
1 M (Ist) NaCl	0.638
1 M (2nd) NaCl	0.413



e.sequential precipitation (silver staining)

f.sequential precipitation (silver staining)

Plate 4

Effect of $(NH_4)_2SO_4$ saturation (Direct/Sequential) in salting out of proteins

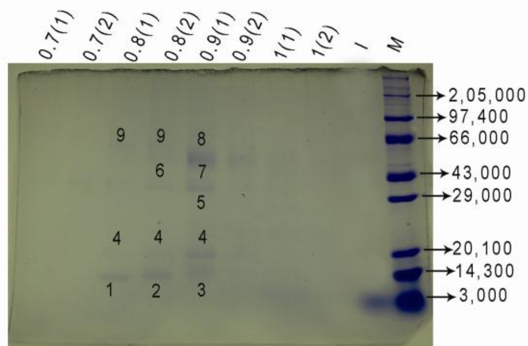


Plate 5

Electrophoretic pattern of protein fractions from 60 percent Ammonium Sulphate saturation eluted through DEAE cellulose colum at pH 7.2

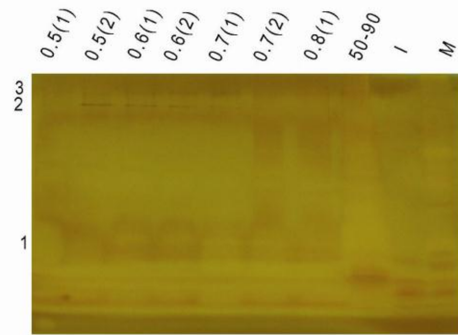
I - Insulin, M - Marker

Table 16. Molecular weight and Rm value of fractions from 60 per cent ammonium sulphate saturation on SDS-PAGE (10%) after DEAE cellulose column chromatography

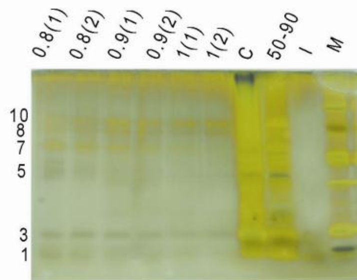
Fraction	No. of bands	Band No.	Molecular weight (Da)	Rm value
0.8 (1)	4	1	12,022	0.896
		4	19,560	0.821
		6	36,321	0.549
		9	62,081	0.366
0.8 (2)	4	2	13,693	0.889
		4	19,560	0.821
		6	36,321	0.549
		9	62,081	0.366
0.9 (1)	5	3	14,496	0.883
		4	19,560	0.821
		5	35,313	0.557
		7	40,079	0.462
		8	52,121	0.434
0.9 (2)	1	8	52,121	0.434

Table 17.Concentration of protein from the fractions of 50-90 per cent ammonium sulphate saturation in DEAE cellulose chromatography at pH 7.2

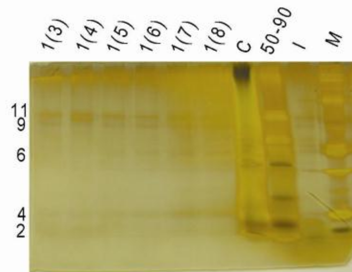
Fraction collected after washing the column with 0.2 M Tris buffer (pH 7.2)	Protein content (mg/g)
Fraction 1	0.037
Fraction 2	0.030
Fraction 3	0.023
Fraction 4	0.023
Fraction 5	0.045
Fraction 6	0.015
Elution with 0.2 M Tris containing NaCl (pH 7.2)	**Protein content (mg/g)**
0.1M (Ist) NaCl	0.000
0.1M (2nd)NaCl	0.000
0.2M (Ist) NaCl	0.023
0.2M (2nd)NaCl	0.015
0.3M (Ist) NaCl	0.000
0.3M (2nd)NaCl	0.007
0.4M (Ist) NaCl	0.053
0.4M (2nd)NaCl	0.075
0.5M (Ist) NaCl	0.195
0.5M (2nd)NaCl	0.241
0.6M (Ist) NaCl	0.211
0.6M (2nd)NaCl	0.173
0.7M (Ist) NaCl	0.158
0.7M (2nd)NaCl	0.098
0.8M (Ist) NaCl	0.135
0.8M (2nd)NaCl	0.083
0.9M (Ist) NaCl	0.098
0.9M (2nd)NaCl	0.090
1M (Ist) NaCl	0.083
1M (2nd) NaCl	0.060



a. Elution at pH 7.2



b. Elution at pH 6.5



c. Elution at pH 6.5

Plate 6
Electrophoretic pattern of protein fractions from 50-90 percent Ammonium Sulphate saturation eluted through DEAE cellulose colum at varying pH

I - Insulin, M - Marker, C- Crude sample

content of each fraction was estimated using Nanodrop at 280 nm. Fractions of high protein concentration were used for 10 per cent SDS-PAGE.

i) 2 ml fractions were collected on elution with 0.2 M Tris buffer containing NaCl (pH 7.2). The protein content estimated is given in Table 17. Fractions containing high protein content were used for SDS-PAGE analysis (Plate 6 a). The molecular weight and Rm value of bands observed in the electrophoresis is presented in Table18.

Table 18. Molecular weight and Rm value of fractions from 50-90 per cent ammonium sulphate saturation on SDS-PAGE (10%) after DEAE cellulose column chromatography at pH 7.2

Fraction	No. of bands	Band No.	Molecular weight(Da)	Rm value
0.5(2),0.6(1),0.6(2) 0.7(1),0.7(2) 0.8(1)	3	1	22,712	0.753
		2	1,10,262	0.231
		3	1,59,964	0.187

Protein content of fractions collected after washing the column with Tris buffer was negligibly small. The elution of protein from the column recorded maximum protein content at 0.5 M NaCl salt concentration and showed a gradual reduction in protein content for increase in salt concentration (Table 17).

ii) 1ml fractions were collected on elution with 0.2M Tris buffer containing NaCl (pH 8.0). The protein content is given in Table 19.

The protein content of fractions after washing the column with Tris buffer was negligibly zero. But none of the concentrations of salt up to 1 M NaCl could elute protein satisfactorily (Table 19).

Table 19. Concentration of protein from the fractions of 50-90 per cent ammonium sulphate saturation in DEAE cellulose column chromatography at pH 8.0

Fraction collected after washing the column with 0.2 M Tris buffer (pH 8.0)	**Protein content (mg/g)**
Fraction 1	0.008
Fraction 2	0.000
Fraction 3	0.016
Fraction 4	0.024
Fraction 5	0.016
Fraction 6	0.000
Fraction 7	0.024
Elution with 0.2 M Tris containing NaCl (pH8.0)	**Protein content (mg/g)**
0.1M (Ist) NaCl	0.000
0.1M (2nd)NaCl	0.000
0.2M (Ist) NaCl	0.000
0.2M (2nd)NaCl	0.000
0.3M (Ist) NaCl	0.000
0.3M (2nd)NaCl	0.024
0.4M (Ist) NaCl	0.032
0.4M (2nd)NaCl	0.008
0.5M (Ist) NaCl	0.000
0.5M (2nd)NaCl	0.000
0.6M (Ist) NaCl	0.032
0.6M (2nd)NaCl	0.024
0.7M (Ist) NaCl	0.032
0.7M (2nd)NaCl	0.072
0.8M (Ist) NaCl	0.024
0.8M (2nd)NaCl	0.048
0.9M (Ist) NaCl	0.072
0.9M (2nd)NaCl	0.144
1M (Ist) NaCl	0.088
1M (2nd) NaCl	0.032
1M (3rd) NaCl	0.104
1M (4th) NaCl	0.032
1M (5th) NaCl	0.040
1M (6th) NaCl	0.024
1M (7th) NaCl	0.024
1M (8th) NaCl	0.032

Table 20. Concentration of protein from the fractions of 50-90 per cent ammonium sulphate saturation in DEAE cellulose column chromatography at pH 6.5

Fraction collected after washing the column with 0.2 M Tris buffer (pH 6.5)	Protein content (mg/g)
Fraction 1	0.016
Fraction 2	0.000
Fraction 3	0.008
Fraction 4	0.000
Fraction 5	0.016
Fraction 6	0.008
Elution with 0.2 M Tris containing NaCl (pH6.5)	
0.1M (I^{st}) NaCl	0.000
0.1M (2nd) NaCl	0.000
0.2M (I^{st}) NaCl	0.008
0.2M (2nd) NaCl	0.024
0.3M (I^{st}) NaCl	0.000
0.3M (2nd) NaCl	0.040
0.4M (I^{st}) NaCl	0.040
0.4M (2nd) NaCl	0.072
0.5M (I^{st}) NaCl	0.088
0.5M (2nd) NaCl	0.072
0.6M (I^{st}) NaCl	0.032
0.6M (2nd) NaCl	0.056
0.7M (I^{st}) NaCl	0.104
0.7M (2nd) NaCl	0.160
0.8M (I^{st}) NaCl	0.080
0.8M (2nd) NaCl	0.272
0.9M (I^{st}) NaCl	0.488
0.9M (2nd) NaCl	0.464
1M (I^{st}) NaCl	0.464
1M (2nd) NaCl	0.416
1M (3rd) NaCl	0.320
1M (4th) NaCl	0.264
1M (5th) NaCl	0.136
1M (6th) NaCl	0.208
1M (7th) NaCl	0.248
1M (8th) NaCl	0.280

Table 21. Molecular weight and Rm value of fractions from 50-90 per cent ammonium sulphate saturation on SDS-PAGE (10%) after DEAE cellulose chromatography at pH 6.5

Fraction	No. of bands	Band No.	Molecular weight (Da)	Rm value
0.8(1), 0.8(2), 0.9(1), 0.9(2), 1(1), 1(2)	6	1	3,159	0.966
		3	13,216	0.891
		5	35,475	0.556
		7	51,840	0.436
		8	68,917	0.329
		10	71,962	0.321
1(3),1(4), 1(5),1(6), 1(7),1(8)	5	2	5,151	0.939
		4	14,690	0.881
		6	44,036	0.489
		9	70,996	0.324
		11	79,530	0.300

iii) 1ml fractions were collected on elution with 0.2 M Tris buffer containing NaCl (pH 6.5). The protein content is given in Table 20. SDS-PAGE analysis is shown in Plate 6 b & c and molecular weight and Rm value of each band is presented in Table 21.

The column was washed with Tris buffer after loading the sample and protein content was estimated. It showed values close to zero. The elution of bound protein could be possible by high salt concentration. The maximum protein was eluted at NaCl concentration of 0.9 M (Table 20). After electrophoresis, bands were visible only by silver staining. The proteins having molecular weight up to 79,530 Da was found.

4.6. Gel filtration chromatography (Size exclusion chromatography)

Sephadex G-100 was used for gel filtration chromatography. It can be used for the fractionation of molecules having molecular weight of 4000 to 1, 50,000 Da (Scopes, 1982). Andrews (1965) reported that Sephadex G-100 is probably the better gel for molecular weights up to ~ 1, 30,000.

6 cm x 1.2 cm column was used for the fractionation of 0.2 ml protein sample. Linear gradient of 0.1 to 3 M NaCl and 0.1 to 1 M NaCl in Tris buffer was used for eluting protein for 60 per cent fractions and 50 to 90 per cent fractions respectively and 1 ml fractions were collected. Protein content of each fraction was estimated using Nanodrop at 280 nm (Tables 22 & 24).

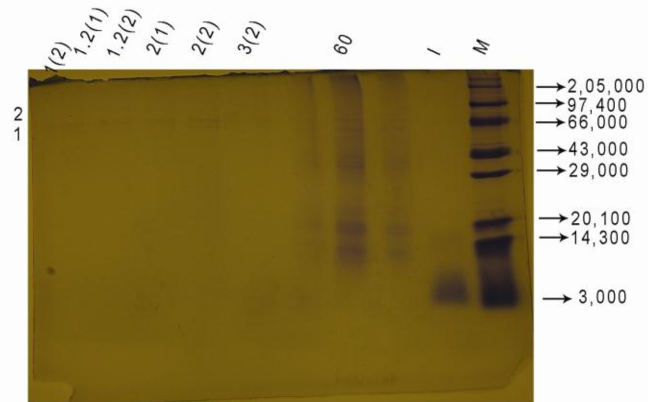
For 60 and 50 to 90 per cent ammonium sulphate saturated fractions, the fractions of high protein content were used for 10 per cent SDS-PAGE (Plate 7 a & b) and molecular weight of each band was recorded (Tables 23 & 25). Two light bands having molecular weight of 68,760 and 79,360 were found (Plate 7 a) for 60 per cent saturated fraction and of 23,098 Da and 21,142 Da for 50 to 90 per cent fraction. But it needed >1 M NaCl concentration to elute these proteins.

Table 22. Concentration of protein from the fractions of 60 per cent ammonium sulphate saturation in Gel filtration chromatography at pH 7.2

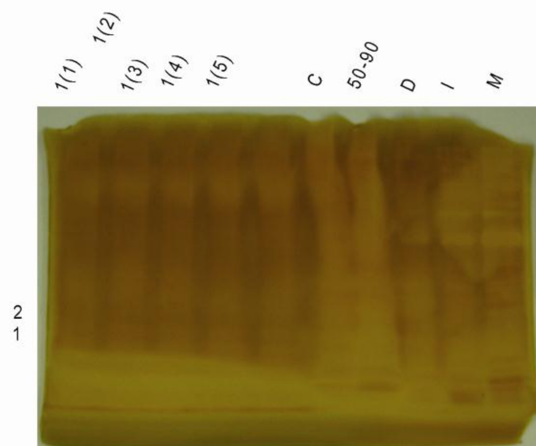
Fraction collected after washing the column with 0.2 M Tris buffer	Protein content (mg/g)
Fraction 1	0.038
Fraction 2	0.050
Fraction 3	0.075
Fraction 4	0.063
Fraction 5	0.050
Elution with 0.2 M Tris buffer containing NaCl (pH 7.2)	**Protein content (mg/g)**
0.1M (Ist) NaCl	0.025
0.1M (2nd) NaCl	0.050
0.2M (Ist) NaCl	0.050
0.2M (2nd) NaCl	0.038
0.3M (Ist) NaCl	0.013
0.3M (2nd) NaCl	0.025
0.4M (Ist) NaCl	0.000
0.4M (2nd) NaCl	0.000
0.5M (Ist) NaCl	0.000
0.5M (2nd)NaCl	0.000
0.6M (Ist) NaCl	0.000
0.6M (2nd)NaCl	0.000
0.7M (Ist) NaCl	0.025
0.7M (2nd) NaCl	0.025
0.8M (Ist) NaCl	0.000
0.8M (2nd) NaCl	0.000
0.9M (Ist) NaCl	0.013
0.9M (2nd) NaCl	0.000
1M (Ist) NaCl	0.013
1M (2nd) NaCl	0.038
1.2M (Ist) NaCl	0.038
1.2M (2nd)NaCl	0.050
1.5M (Ist) NaCl	0.000
1.5M (2nd)NaCl	0.000
2M (Ist) NaCl	0.025
2M (2nd) NaCl	0.025
3M (Ist) NaCl	0.013
3M (2nd) NaCl	0.025

Table 24. Concentration of protein from the fractions of 50-90 per cent ammonium sulphate saturation in Gel filtration chromatography at pH 6.5

Fraction collected after washing the column with 0.2 M Tris buffer (pH 6.5)	Protein content (mg/g)
Fraction 1	0.024
Fraction 2	0.000
Fraction 3	0.000
Fraction 4	0.000
Fraction 5	0.000
Fraction 6	0.008
Elution with 0.2 M Tris buffer containing NaCl (pH 6.5)	**Protein content (mg/g)**
0.1M (I^{st}) NaCl	0.032
0.1M (2nd) NaCl	0.008
0.2M (I^{st}) NaCl	0.016
0.2M (2nd) NaCl	0.024
0.3M (I^{st}) NaCl	0.000
0.3M (2nd) NaCl	0.000
0.4M (I^{st}) NaCl	0.008
0.4M (2nd) NaCl	0.016
0.5M (I^{st}) NaCl	0.000
0.5M (2nd) NaCl	0.016
0.6M (I^{st}) NaCl	0.000
0.6M (2nd) NaCl	0.000
0.7M (I^{st}) NaCl	0.000
0.7M (2nd) NaCl	0.000
0.8M (I^{st}) NaCl	0.000
0.8M (2nd) NaCl	0.008
0.9M (I^{st}) NaCl	0.008
0.9M (2nd) NaCl	0.000
1M (I^{st}) NaCl	0.032
1M (2nd) NaCl	0.024
1M (3rd) NaCl	0.024
1M (4th) NaCl	0.024
1M (5th) NaCl	0.024
1M (6th) NaCl	0.032
1M (7th) NaCl	0.032
1M (8th) NaCl	0.024



a. 60 percent Ammonium sulphate saturated fraction



b. 50-90 percent Ammonium sulphate saturated fraction

Plate 7

Electrophoretic pattern of protein fractions from 60 & 50-90 percent Ammonium Sulphate saturation eluted through Gel filtration colum

I - Insulin, M - Marker, C- Crude sample, D-Pooled fraction from DEAE

Table 23. 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 25. 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

Discussion

5. DISCUSSION

Costus pictus D.Don is a Mexican plant which is called spiral ginger/step ladder plant. It is commonly known as insulin plant in the central part of Kerala. There are reports which points out the hypoglycaemic action of *Costus pictus* (Balaji, 2005; Benny, 2006). An antidiabetic agent present in this plant could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and / or by mimicking insulin action. The widely used practice for treating hyperglycemia is taking insulin through injection. As insulin is a protein, comparison of its characters to plant proteins of a hypoglycaemic plant may open new doors to the field of antidiabetic treatments. In this context, 'Characterization of proteins in *Costus pictus* D.Don' was conducted at CPBMB and the results of the study are discussed below.

5.1. Effect of buffers and TCA in the estimation of protein from *Costus pictus*

Five different buffers were tried for selecting an efficient buffer system for protein extraction. The extraction of protein with PBS (pH 7.3), Tris buffer (pH 7.2) and sodium citrate phosphate buffer (pH 2.8) gave maximum quantity of protein at 280 nm in Nanodrop and at 660 nm by Lowry's method (before TCA precipitation). On precipitating proteins with TCA, a considerable reduction in protein /protein analogues was recorded. Maximum protein content was observed in the extraction with Tris buffer after TCA precipitation.

An alkaline pH (7.0 – 8.0) was used for extraction as reported by Sanwal (1979) since the isoelectric points of most of the proteins are acidic in nature. Higher protein content was recorded in the extraction with Tris buffer and PBS. 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 3). It may be due to the presence of various salts and their ionic concentration.

Lowry's method depends on the presence of aromatic amino acids and/ lysine in the proteins. In Lowry's method, Folin-Ciocalteau reagent reacts with tyrosine in proteins to give a strong dark blue colour (Folin and Ciocalteau, 1927). High readings in the protein estimation through Lowry's method (before TCA precipitation) and Nanodrop may be due to the interference of phenol in the crude extract and formation of complexes by the phenol with its protein by hydrogen bonding.

Warburg and Christian (1941) reported that as protein purification progresses and interfering compounds are removed, a simple 280 nm reading is sufficient. Proteins absorb at 280 nm solely because of tyrosine and tryptophan residues. Reading at 280 nm provides an accurate measure of the protein content for a pure protein, since no manipulation other than appropriate dilution is required.

Hence the direct reading of protein sample requires high purity. Leaf extract of *Costus* contains a lot of primary and secondary metabolite products. In addition to that phosphate impurities contributed much in the high value in Nanodrop method. It was evident in the analytical data of *Costus* (Table 3). TCA precipitation eliminated the impurities and precipitated protein alone. So precipitation of proteins using TCA can be adopted as a method before estimating actual protein content in the crude extract of *Costus pictus*.

5.2. Effect of extraction buffer and per cent acrylamide of SDS PAGE / PAGE in protein profile

For the resolution of separate components of a protein mixture and for analyzing the purity of a protein preparation electrophoretic method was used. Seven to fifteen per cent acrylamide was tried for the separation of protein from *Costus* sample

Out of the five buffers tried at varying pH for extracting proteins, the extraction with 0.2 M Tris buffer and PBS yielded maximum number of clear and distinct bands in the gel combinations tried for both PAGE and SDS-PAGE (Table 4 & 5). 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.

It was observed that PAGE at 10 per cent acrylamide was sufficient for separating complex protein mixtures of *Costus* sample. This method of gel electrophoresis allowed separation of native proteins according to difference in their charge density. 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 9, Plate 3 a & b). The possibility of protein aggregate formation at this stage due to high molecular weight proteins cannot be ruled out.

Proteins having molecular weight of 3,159 to 1, 59,964 Dalton were observed at different stages of purification and maximum number of bands in 10 per cent acrylamide concentration. As the concentration of acrylamide decreases, separation of components was found poor. It is an indication of the presence of closely related proteins of different molecular weight in *Costus* sample.

Singh and Koundal (2000) reported that qualitative analysis of globulin at different stages of seed development could analyze through SDS-PAGE and found that proteins resolved in to number of bands ranging from 14 to 80 kDa. Sawhney *et al.* (1996) also reported that the lectin from *Lathyrus sativus* is a tetramer with a heavier subunit having two poly peptides of molecular weight of

about 21 kDa and 16 kDa and smaller sub units of two polypeptides of about 5 kDa as revealed by SDS-PAGE on 10 per cent gel.

5.3. Staining behaviour of proteins in the *Costus pictus*

Proteins in *Costus pictus* responded differently to Coomassie and Silver stain. It was also evident from the result that (Tables 7 and 8) *Costus* contains proteins, which responded to both Silver and Coomassie staining. The response to silver staining was only to the protein salted out at 50 to 90 per cent ammonium sulphate fractions after DEAE anion exchange chromatography and gel filtration chromatography.

Switzer *et al*. (1979) introduced the silver staining technique for protein visualization with detection level down to the 0.3 to 10 ng levels. Protein detection in silver staining method depends on the binding of silver ions to the amino acid side chains, primarily the sulfhydril and carboxyl group of proteins, followed by reduction to free metallic silver (Switzer *et al.*, 1979; Oakly *et al.*, 1980; Merril *et al.*, 1981; Merril and Pratt, 1986). But several classes of highly negative charged proteins, including proteoglucans and mucins, which contain high levels of sulfated sugar residues and some of very acidic proteins were detected poorly by silver staining (Goldberg and Warner, 1997).

Tal *et al.* (1985) reported that the number of Coomassie R ligands bound to each protein molecule was approximately proportional to the number of positive charges on the protein, about 1.5 to 3 dye molecules / charge. There was a striking correlation between intensity of response to Coomassie dye and the basicity of a protein, which depends on the number of lysine, histidine and arginine residues and the NH_2 terminal amino group. The electrostatic association between Coomassie R and a protein must be involved in the sulfonic groups and basic amino acids rather than the positively charged nitrogen on the ligands with the carboxylates of the protein. Hydrophobic interaction of the dye molecule with

the polypeptide backbone adjoining the positively charged amino acid in the protein enhanced the binding effect.

The leaf protein in *Costus pictus* very well responded to Coomassie Brilliant Blue R-250 staining in the early stages of purification. Thus it may have proteins predominating with amino acids like lysine, histidine and arginine residues and the amino terminal amino group. Staining pattern also implied that proteins having amino acid side chains like sulfhydril and carboxyl groups are less in *Costus pictus* leaf sample. A protein rich in basic amino acid was found prominent at 58,131 Da having Rm value of 0.392.

5.4. Ammonium sulphate fractionation and partial purification of proteins

Differences in structure and amino acid sequence makes proteins differ in their salting in and salting out behaviour. This forms the basis for the fractional precipitation of proteins by means of salt (Sadasivam and Manickam, 1996). This basic principle was initially tried for the isolation of proteins from *Costus* sample.

Salting out of proteins with ammonium sulphate precipitation was used for further analysis. The proteins from *Costus* sample in a buffer solution was highly hydrated, in other words, the ionic groups on the surface of the protein attracted and bound many water molecules very tightly. When percentage of ammonium sulphate saturation was increased in the protein solution, they would have attracted the water molecules. Hence as the salt was added and the small ions bound water molecules, the *Costus* protein molecules were forced to interact with themselves and began to aggregate. At high salt concentration, the salt ions become solvated; the aggregation of hydrophobic patches (side chains of Phe, Tyr, Trp, Leu, Ile, Met, Val) on the Costus protein surface has taken place. The hydrophobic patches interacted intensively with each other getting aloofed from the highly polar water molecules. *Costus* proteins with greater number or bigger clusters of hydrophobic amino acid residues on their surfaces tend to aggregate

fast subsequently got precipitated before those smaller and fewer patches. Ammonium sulphate precipitation of the protein solution from *Costus* salted out different molecular weight proteins with hydrophobic patches.

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. Thus exposing these hydrophobic patches of amino acids, which bang against each other in the hydrophobic interaction and ultimately the protein precipitated since water became unavailable. The increase in number of protein during the course of adding ammonium sulphate was a clear evidence of unavailability of water. Some of the proteins were precipitated from the solution over a sufficiently narrow range of salt concentration (Table 13). Substantial quantity of a single protein (0.24 mg /g) was present in the protein solution of 70 to 80 per cent. It is evident in the electrophoresis of the same protein (Plate 4 d), which had an Rm value of 0.921 and molecular weight 6500 Da. It is having a similarity in molecular weight of insulin from other source.

So when enough salt was added, the protein of specific molecular weight began to precipitate. Effect of different per cent of ammonium sulphate in the protein solution is recorded in the Table 12 and Plate 4 a and b. Proteins were collected, centrifuged and redissolved in 0.2 M Tris buffer (pH 7.2) with low salt content. Large proteins were precipitated first and smaller ones were precipitated later except at 10 per cent saturation (Table 13 and Plate 4 c & d) indicating the presence of high hydrophobic patches.

5.5. Comparison of direct and sequential precipitation of proteins with ammonium sulphate.

Direct precipitation of protein at 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent ammonium sulphate saturation showed that salting out of protein was high at

70 per cent and 90 per cent ammonium sulphate saturation. Even though the number of bands in crude plant extract and 90 per cent ammonium sulphate saturation (direct precipitation) were same, the banding pattern and Rm values were different because of the presence of varying amino acid and their hydrophobic nature. But the protein salted out progressively to higher saturation of ammonium sulphate had no banding at 60 to 70 per cent and 80 to 90 per cent fractions on staining with Coomassie Brilliant Blue R-250. Protein bands appeared at 20 and 30 per cent direct ammonium sulphate saturation were not appeared in 10 to 20 and 20 to 30 per cent saturations as evident from the electrophoretic analysis. Scopes (1982) reported that in impure samples, co-precipitation occurs to an amount depending on the properties of the other protein present. The analytical data of ammonium sulphate precipitation was in agreement with this.

All the above four observations established the influence of ammonium sulphate and its concentration in the number and nature of hydrophobic patches, effect of ammonium sulphate salt in the water of solvation of protein and co-precipitation. It was more evident from the banding pattern of 90 per cent ammonium sulphate saturation. Because it contains lower to higher molecular weight of varying nature especially the response to different staining techniques.

Observations in this study revealed that effect of direct precipitation and varying concentration of ammonium sulphate were directly related to the protein expression in electrophoresis. Direct precipitation (10%) showed a band of small molecular weight 9,367 Da and Rm value of 0.908 with a content of 1.54 mg /g. While precipitating the protein directly at 20 and 30 per cent ammonium sulphate saturation, four bands with increasing molecular weight was recorded (Table 10). At the same time, there was no electrophoretic banding in serial increase of ammonium sulphate saturation from 10 to 30 per cent (Table 13). The observation showed the influence of ammonium sulphate in the aggregation of small molecule or the ability to regulate the breakage of long protein segment of *Costus* sample.

Further increase of ammonium sulphate in percentage of both direct and sequential precipitation revealed the presence of different higher molecular weight protein bands. Both studies of ammonium sulphate precipitation showed 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 resulted as an aggregation of proteins. So lower and higher percentage ammonium sulphate are not suitable to isolate a specific form of active protein from *Costus*. At the same time, it was observed that banding pattern was varied with 40 to 80 per cent ammonium sulphate or sequential increase of ammonium sulphate because of the variation in nature and properties of isolated proteins from *Costus* sample. Sensitivity and selectivity of silver staining was also evident in *Costus* sample. A very low molecular weight band was present in all fractions up to 80 per cent, indicating the slow release of the same or different proteins from the protein aggregate in a sequential manner from the *Costus* sample.

The difference between direct precipitation and sequential precipitation of proteins with ammonium sulphate may be due to the effect of salt and protein - protein interaction. In general, it can very well presume that the removal of SDS and selective precipitation by ammonium sulphate can be exploited for precipitating the desired protein for detailed studies and further use of selected proteins from *Costus* plant.

Yamasaki (2003) brought the supernatant of millet seeds to 0.9 saturation with $(NH_4)_2SO_4$ for the purification of β-amylase. Andriotis and Ross (2003) also reported the purification of phytase enzyme from dormant *Corylus avellana* seeds using 30 to 75 per cent $(NH_4)_2SO_4$ saturated fraction. Singh *et al.* (2006) used the dissolved 0 to 80 per cent ammonium sulphate fraction for the purification of oxalate oxidase. Based on the above reports it is possible to go ahead for developing a technique to isolate a single protein from 70 to 80 per cent ammonium sulphate saturation of *Costus pictus* sample.

5.5. Anion exchange chromatography with DEAE cellulose

Ion exchange chromatography is based on the charge of the protein need to be purified. In anion exchange chromatography, column is positively charged and negatively charged proteins will bind to it. NaCl gradient was applied to elute Costus proteins from DEAE column. Column had a higher attraction for the charge of NaCl salts than for the charged *Costus* protein and released the protein in favour of binding the salts instead. Protein with weaker ionic interactions was eluted at a lower salt and with stronger ionic interaction at higher ionic strength. The gradient elution through DEAE cellulose has been widely used approach for enzyme purification (Boman, 1979). 60 and 50 to 90 per cent ammonium sulphate saturated fractions were used to study the nature of *Costus* protein on DEAE cellulose.

Proteins of *Costus* at 60 and 50 to 90 per cent ammonium sulphate saturation were applied to DEAE column after saturation with Tris buffer of 0.2 M at pH 7.2 and 6.5. The absence of protein in buffer elution indicated the presence of highly negatively charged proteins in the ammonium sulphate fractions of *Costus* sample.

For the elution of protein from 60 per cent ammonium sulphate fraction, high salt concentration (above 0.5 M NaCl) was needed for DEAE column and maximum protein was eluted at 0.9 M NaCl (Table 15 and Fig. 1a). It indicated that there was a strong ionic interaction between column and protein i.e. *Costus* protein is highly negatively charged. Staining behaviour also confirmed the same These bands were visible on Coomassie staining (Plate 5). Sodium chloride gradient at pH 7.2 gave a single peak of protein for 60 per cent ammonium sulphate at fraction No. 17 and 18. SDS-PAGE analysis of the fractions having high protein content revealed that it contained proteins having different molecular weight, which can be separated by adopting suitable techniques (Table 16).

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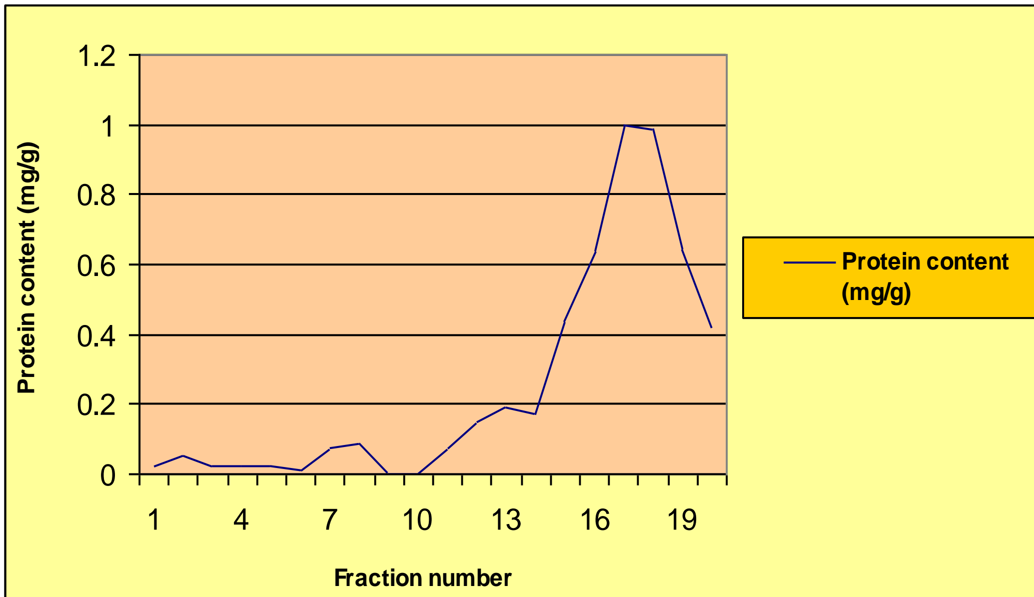
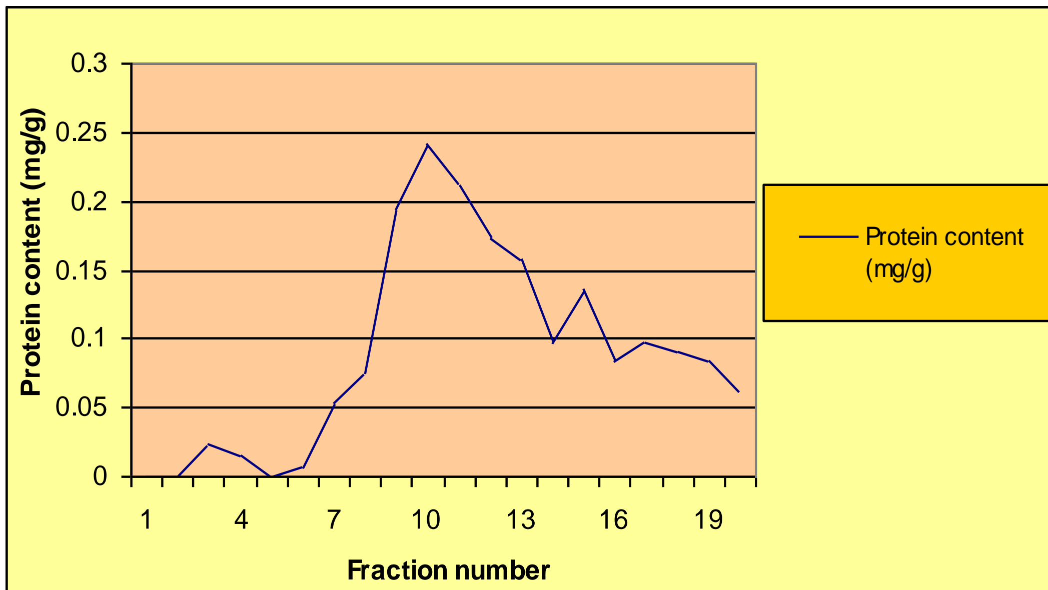


a. Protein fraction from 60 per cent ammonium sulphate saturation



b. Protein fraction from 50 to 90 per cent ammonium sulphate saturation

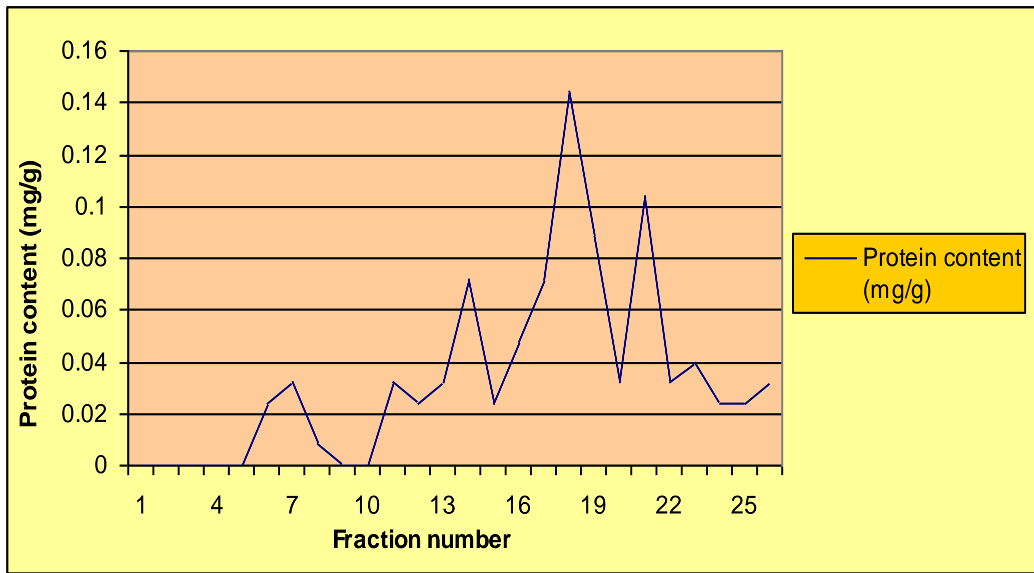
Fig.1 Protein content of different fractions from 60 /50 to 90 per cent ammonium sulphate saturation eluted through DEAE cellulose column at pH 7.2

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 8.0, none of the salt concentration (up to 1 M NaCl) could elute protein satisfactorily (Table 19). It appeared as a number of small peaks (Fig. 2a) and protein content was also negligibly small. It may be due to the degradation of protein at pH 8.0 and presence of other biomolecules might have contributed to it.

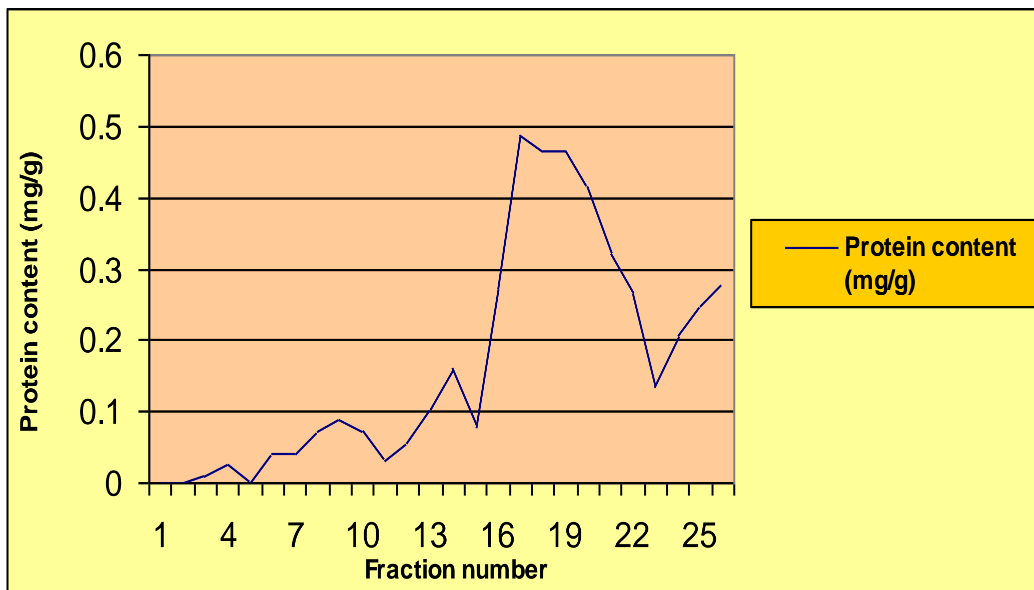
At pH 7.2, the elution of protein with 0.5 M NaCl salt concentration recorded maximum number of proteins of high molecular weight and showed a gradual reduction in protein content for higher concentration of salt (Table 17 and Fig. 1b). This indicated that a moderate ionic interaction between the column and *Costus* protein. Silver staining gave few bands of proteins with high molecular weight in the SDS-PAGE which was not expressed in Coomassie staining (Plate 6a and Table 18).

At pH 6.5, the elution of protein was possible only by increasing the salt concentration. Maximum protein was eluted at 0.9 M NaCl concentration (Table 20 and Fig. 2b). The protein was bound to column with strong ionic interaction. The fractions of high concentration were used for electrophoresis, which was visible only by silver staining. The proteins of molecular weight varied from 3,159 to 79,530 Da were found in the elution fraction of *Costus* protein (Table 21). Based on the observation it was found that the protein eluted at pH 7.2 was not separated at pH 6.5. Removal of ammonium sulphate and elution process gave way for the functioning of the free amino acids in the active form of *Costus*

The salt concentration needed to elute proteins will depend largely on isoelectric points. Some proteins require 1M salt to remove them, but rarely more. It is a common practice to apply high salt concentrations with DEAE cellulose, since iso electric points below the pKa value for aspartate / glutamate residues



a. Elution at pH 8.0



b. Elution at pH 6.5

Fig.2 Protein content of different fractions from 50 to 90 per cent ammonium sulphate saturation eluted through DEAE cellulose column at varying pH

proteins, which was evident from the difference of eluting proteins in the column by changing of pH .

(<pH 4.5-5) are much commoner than isoelectric points above pKa values for lysine (>pH 10), (Scopes, 1982). Yamasaki (2003) identified the peak that exhibited β-amylase activity, which was obtained on elution of DEAE cellulose column with a linear gradient of NaCl (0-1M).

For gradient work, slow pH changes are usually successful only if the buffering power is high. The observation of the effect of pH in *Costus* proteins also revealed that the net charge of a protein depended on pH. At its isoelectric point (pI), the net charge of a protein is zero and no binding to DEAE cellulose occurs. If pH > pI, the protein will have a negative charge and the protein will therefore bind to the anion exchange column. High pH values are not usually employed because of lack of protein stability above pH 9.0. DEAE adsorbents become uncharged and few proteins are basic enough to stick on to the ion exchanger at pH 8.

It was observed in the 50 to 90 per cent ammonium sulphate fraction that protein fractions of high molecular weight were obtained only after changing the pH of the eluting buffer from 8.0 to 6.5. At the same time 60 per cent fractions, proteins of molecular weight ranging from 12,000 to 63,000 Da were eluted at pH 7.2. So the weak anion exchanger – DEAE cellulose is suitable for isolating *Costus* proteins of interest.

DEAE ion exchange chromatography and electrophoresis are depending mainly on the electrical charge of the molecules. Boman and Bjork (1979) reported that if an electrophoresis gives an unsatisfactory separation in a buffer of a certain pH, one can expect chromatography on DEAE cellulose to give similar result, especially if the same pH and buffer are used. So the separation and fractionation of *Costus* proteins were carried out at different pH and it was so observed that pH 7.2 was effective for a clear separation of proteins in both SDS-PAGE and DEAE cellulose anion exchange chromatography. It was also observed that the protein fraction of 60 per cent ammonium sulphate in DEAE cellulose

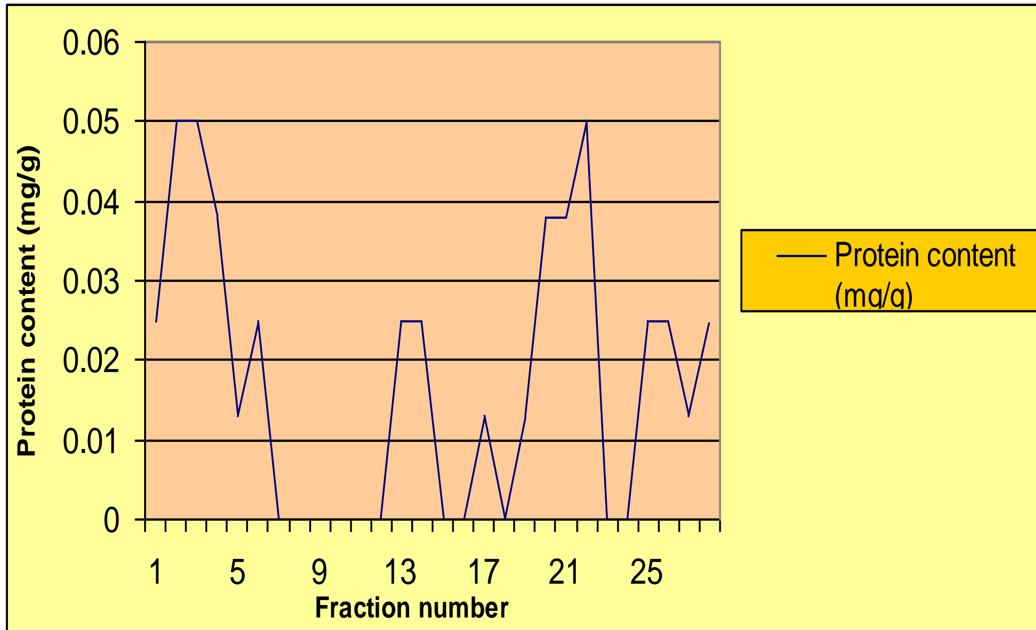
column at pH 7.2 and electrophoresis with 10 per cent SDS-PAGE gave clear banding pattern. At the same time protein fractions of higher ammonium sulphate saturation was not effective to get electrophoresis bands in the above conditions, especially at pH 7.2. Salt concentration and pH were affected in the elution pattern of *Costus* protein. So changing of pH at lower and higher levels was tried. Among the trials, it was found that the elution at pH 6.5 for higher concentration of ammonium sulphate was effective to fractionate protein aggregates through DEAE cellulose. So the report by Boman and Bjork (1979) is not in agreement with *Costus* protein, which may be due to the presence of varying number of amino acids of sulfhydil and carboxylic groups at surface level.

Sodium chloride gradient application in DEAE cellulose revealed that selected fractions contain more than one protein of different molecular weight. The hydrophobicity of the proteins in the sample as evident in ammonium sulphate precipitation was in agreement with the electrophoretic pattern of the fraction from DEAE cellulose. Selected fractions from DEAE cellulose column contained proteins of aromatic amino acids and lysine at higher rate or different proportion, which was suitable for clustering in the DEAE cellulose column, resulted in the presence of more than one protein in a single fraction. So it can very well presume that the nature of the true protein was maintained even at this stage of DEAE anion exchange chromatography.

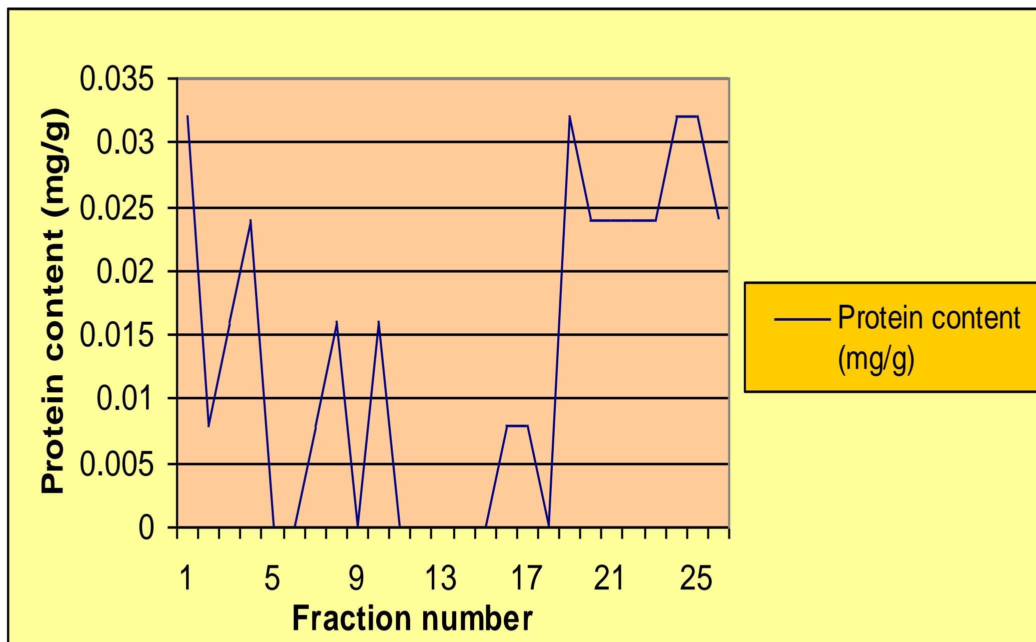
Aggregation of proteins in *Costus* can be considered as a characteristic of this plant because pH was the major factor that contributed to aggregation and elution of proteins in a different manner (protein aggregates are three in number at pH 7.2 where as 11 in number at pH 6.5, other factors being constant).

5.6. Gel filtration chromatography (Size exclusion chromatography)

Gel filtration chromatography separates proteins on the basis of their size. The column is packed with a matrix of fine porous beads. As the protein solution



a. 60 per cent ammonium sulphate saturated fraction



b. 50 to 90 per cent ammonium sulphate saturated fraction

Fig.3 Concentration of protein fractions from the sample of 60 and 50 to 90 per cent ammonium sulphate saturation in gel filtration chromatography

is poured on the column, small molecules enter the pores in the beads. When a mixture of molecules and ions dissolved in a solvent is applied to the top of the column, the smaller molecules and ions are distributed through a larger volume of solvent than is available to the larger molecules. Consequently, the large molecules move more rapidly through the column and in this way the mixture can be separated (fractionated) in to its components. These larger molecules are eluted first from the column. The smaller molecules have a longer path to travel as they get stuck over and over again in the maze of pores running from bead to bead. These smaller molecules therefore take longer to make their way through the column and are eluted last.

Sephadex G-100 is used for the fractionation of molecules having molecular weight of 4000 to 1, 50,000 Da (Scopes, 1982). Andrews (1965) reported that Sephadex G-100 is the better gel for molecular weights up to ~ 1, 30,000 Da.

60 and 50 to 90 per cent ammonium sulphate saturated fractions were used to study the gel filtration behaviour of protein in *Costus pictus*. Protein content of eluted fractions (Fig. 3) was estimated and higher protein content fractions were used for SDS-PAGE. De *et al.* (2004) reported that following $(NH_4)_2SO_4$ precipitation and chromatography on DEAE sepharose, the protein was subjected to gel filtration and the enzyme was eluted as a broad peak.

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. Coomassie staining of bands is shown in Plate 7a. 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 (Plate 7b). 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 were salted out in the range

of 30 to 50 per cent of ammonium sulphate saturation (Table 14). So in gel filtration, molecular sieving was affected by both salt gradient as well as pH, which in turn showed the presence of both the acidic and basic proteins (Table 26).

Table 26. Electrophoretic banding pattern of different protein fractions after gel filtration chromatography

Saturation of ammonium sulphate (%)	Electrophoretic bands		Reaction / Nature
	No. of bands	Molecular weight (Da)	
60% (Direct precipitation)	2	68,760	Basic
		79,360	
50-90% (sequential precipitation)	2	21,142	Acidic
		23,098	

5.7. Comparison with insulin

The hormone insulin is a protein consisting of two polypeptide chains, originally produced as a single molecule (preproinsulin) in the beta cells within islets of Langerhans of the pancreas. It is composed of 110 amino acids. This form is transformed in to proinsulin after the removal of 24 amino acids (the signal peptide). This folds and bonds to give the molecule similar to the final structure. From this molecule 33 amino acids (the C chain) is removed inside the golgibody to form a two-chain structure and again two amino acids are removed.

Insulin can form into granules consisting of hexamers due to the interaction between hydrophobic surfaces and is stored in this form inside the beta cells. Insulin may also form into dimers. But the active form is a single unit (monomer). These interactions have important clinical ramifications. Monomers

and dimers readily diffuse into blood, whereas hexamers diffuse poorly. Hence, absorption of insulin preparations containing a high proportion of hexamers is delayed.

Although the amino acid sequence of insulin varies among species, certain segments of the molecule are highly conserved, including the positions of the three disulfide bonds, both ends of the A chain and the C-terminal residues of the B chain. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among species.

Size and functional group of insulin from different sources are different. The structure and size of *Costus* proteins may also be differed but the functioning of such protein may be preproinsulin, proinsulin or signaling protein. In this context the antidiabetic property of *Costus* plant may be contributed by similar proteins of any other.

Recombinant DNA technology has contributed much in the large-scale production of insulin especially insulin analogues from *E.coli*. Recombinant insulin analogues are engineered such that lysine and proline residues on the C-terminal end of the B- chain are reversed. This modification does not alter receptor binding, but minimizes the tendency to form dimers and hexamers. Allergy to animal insulin is also avoided by the use of insulin analogues (http://www.vivo.colostate.edu/hbooks/pathways/endocrine/pancreas/insulin structure.html)

Observations from *Costus* proteins revealed that even the smaller protein molecules of *Costus* sample was having sufficient quantity of hydrophobic patches. Substantial quantity of a single protein was present in the protein solution of 70 to 80 per cent saturation. This molecule had an Rm value of 0.921 and molecular weight of 6500 Da which can be analyzed for studying the antidiabetic property. There are a good number of hydrophobic amino acids in the insulin

Table 27. Behaviour of proteins from *Costus pictus* at different stages of purification

Molecular weight (Da)	Native PAGE	Crude extract after SDS PAGE	Ammonium sulphate precipitation		After DEAE cellulose chromatography			After gel filtration chromatography
					60%	50-90%		
			Direct	Sequential	pH 7.2	pH 7.2	pH6.5	
<5,000							**3,159**	
5000-15,000		**7,610**	**9,367, 10,995**	**6,500, 13,311**	**12,022, 13,693, 14,496**		**5,151, 13,216, 14,690**	
15,001-25,000		**15,570, 19,761, 21,142**	**17,196, 18,275, 21,222, 21,954, 22,884**	**21,181, 23,519**	**19,560**	**22,712**		**21,142, 23,098,**
25,001-30,000		**29,371**	**29,000**					
30,001-50,000	**41,108**	**33,350**	**31,297, 34,107**	**34,722**	**36,321, 35,313, 40,079**		**35,475, 44,036**	
50,001-60,000	**58,675**		**59,948**		**52,121**		**51,840**	
>60,000	**1,48,096**	**60,462, 83,359**		**63,586, 66,000**	**62,081**	**1,10,262, 1,59,964**	**68,917, 71,962, 70,996, 79,530**	**68,760, 79,360**

(Valine, Leucine, Phenyl alanine, Proline, Isoleucine). All observations established the influence of ammonium sulphate and its concentration in the number and nature of hydrophobic patches in *Costus* protein. Ammonium sulphate was influencing in the aggregation of small molecule or the ability to regulate the breakage of long protein segment of *Costus* protein. Behaviour of *Costus* proteins in DEAE cellulose and gel filtration chromatography also revealed the tendency of the *Costus* proteins to aggregate each other. The above facts are illustrated in a tabular form (Table 27).

Future line of study

The ability to isolate and study a protein lies at the heart of much modern biotechnology. If a protein can be isolated and purified, it can be studied in isolation from other proteins and its enzymology or signaling capability can be studied in detail. It is this ability to independently isolate proteins and study them individually or in mixtures with complete control of their environment (salt, temperature, pH etc.) that lies at the heart of working *in vitro*. Although this *in vitro* approach can clearly be complemented by genetic approaches and *in vivo* approaches, there is really no substitute for it in the repertoire of experimental design available to the modern biologist.

In the present study, at 70 to 80 per cent sequential precipitation of protein, a protein having similar molecular weight of insulin (6500 Da) was observed. But its isolation and detailed study are to be conducted. After sequencing the protein, its action on cell cultures grown in glucose medium can be carried out. It can also be used for bioassay for its hypoglycaemic action, which can be applied subcutaneously or as nasal spray.

Based on the above facts, following observations can be established for the welfare of human being:

1. The biological activity (enzymatic activity, signaling capacity etc.) actually resides in this unique protein.
2. It is possible to study its enzymology, understand its affinity for particular substrates, or dissect its ability to catalyze enzymatic reaction.
3. It allows modifying specific residues to understand how these residues confer particular structures or allow the protein to operate as catalyst.
4. Nature and properties of this protein can be a powerful indication of how an organism regulates particular cellular tissue processes.
5. It is possible to produce reagents (antibodies) that are capable of determining the location of that protein *in vivo* which can give important support to interesting hypothesis and disprove incorrect speculations of diabetics.
6. By understanding the structure – function relationships of the protein, it is often possible to design specific reagents that can be used to test the function of this protein *in vivo.*
7. Understanding how particular amino acid residues are involved in the protein function, especially when it is combined with knowledge of the 3-D structure of the protein, helps to understand how particular sequences in this protein are involved in biological functions.

Summary

6. SUMMARY

The salient findings of the study "Characterization of proteins in *Costus pictus* D.Don" are summarized below.

1. Tris buffer of 0.2 M at pH 7.2 was found to be the suitable buffer for extracting proteins of *Costus pictus*.

2. Ten per cent acryl amide gel concentration for resolving and six per cent for stacking gel could resolve *Costus pictus* proteins efficiently through SDS-PAGE and PAGE.

3. Same protein fractions from *Costus pictus* responded to Coomassie staining and silver staining indicating the presence of acidic and basic proteins in the plant.

4. Salting out with ammonium sulphate was carried out directly and sequentially to precipitate protein from *Costus* plant.

5. Proteins of basic nature proteins in the *Costus pictus* are precipitated with ammonium sulphate in the range of 30 to 50 per cent saturation which was stained with Coomassie dye and acidic proteins were precipitated at high saturation of ammonium sulphate.

6. Aggregation of small proteins and the ability to regulate the breakage of long proteins were observed in the ammonium sulphate gradient application.

7. Salting out of *Costus* protein was efficiently carried out with 80 per cent ammonium sulphate saturation.

8. Lower and higher percent of ammonium sulphate were not feasible to isolate the desired protein

9. The presence of hydrophobic patches was observed in the fractions of direct precipitation with ammonium sulphate.

10. Sequential precipitation with ammonium sulphate was removing the proteins in a phased manner, which is almost same as affected by the presence of SDS in the crude sample.

11. Higher concentration of salt was required to elute proteins from DEAE column when protein fraction was salted out with 60 and 50 to 90 per cent ammonium sulphate saturation.

12. Salting out was substantially influenced by changing in pH and percentage of saturation. Proteins of 60 per cent ammonium sulphate saturation was eluted at pH 7.2 and 50 to 90 per cent saturation were eluted at pH 6.5.

13. Sodium chloride gradient application in DEAE cellulose revealed that selected fractions contain more than one protein of different molecular weight.

14. The hydrophobicity of the proteins in the sample was evident in ammonium sulphate precipitation which is in agreement with electrophoretic pattern of fraction from DEAE cellulose.

15. Aggregation of proteins in *Costus* can be considered as a characteristic of this plant because pH was one of the major factors contributed aggregation and elution of proteins.

16. On comparing with insulin, 70 to 80 per cent sequential precipitation gave a protein with similar range of molecular weight as that of insulin. Purification, sequencing and further studies of this fraction are to be conducted for establishing the antidiabetic property of the *Costus* plant.

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Appendices

Appendix I

Composition of buffers used for protein extraction

1) 0.2M Tris (Hydroxy methyl) Aminomethane buffer

A. 0.2M solution of Tris HCl / Tris buffer

Tris buffer-2.4228g/100ml distilled water or

3.152g Tris HCl /100ml water

B. 0.2N HCl

Concentrated HCl-0.23ml/100ml distilled water.

Mixed 50ml of ‘a’ and 44.2ml of ‘b’, diluted and corrected the pH to 7.2 and made up to 200ml.

2) Tris buffer containing ingredients

A. Citric acid (2.5mM)

Citric acid - 0.052g/100ml distilled water

B. L-ascorbic acid (6mM)

L-ascorbic acid- 0.105g/100ml distilled water

C. Cysteine HCl (6mM)

L-cysteine HCl -0.105g/100ml distilled water

Stock solutions of citric acid, L-ascorbic acid and L-cysteine HCl were prepared and pipetted out 0.1 ml of each to 0.2M Tris buffer; added 17.115g of sucrose adjusted pH to 7.2 and made up to 200ml with distilled water.

3) Phosphate buffered saline solution (PBS)

A.1.4mM KH_2PO_4 - 0.019g/100ml

B.8mM Na_2HPO_4 - 0.142g/100ml

C.140mM NaCl -0.818g/100ml

D. 2.7mM KCl -0.020g/100ml

Dissolved the ingredients in 80ml distilled water, adjusted the pH to 7.3 and made up the volume to 100ml.

4) Phosphate buffer

A. 0.2M solution of monobasic sodium phosphate - 2.78gNaH_2PO_4/100ml

B. 0.2M solution of dibasic sodium phosphate

$Na_2HPO_4.7H_2O$ -5.365g/100ml or $Na_2HPO_4.12H_2O$-7.17g/100ml

Mixed 39ml of 'a' and 61ml of 'b', adjusted pH to 7.0 and made up to a total volume of 200ml.

5) Sodium citrate phosphate buffer

A. Sodium citrate (85mM):

Sodium citrate tri basic dihydrate-1.2495g

B. $NaH_2PO_4.H_2O$ - 0.221g

Dissolved both in distilled water, adjusted pH to 2.8 and made up to 50ml.

Appendix II

Composition of reagents used for SDS PAGE

A. Monomer solution

Acrylamide : 30g

Bis acrylamide : 0.8g

Distilled water to: 100ml

Stored at 4°C in amber coloured bottle.

B. 4X Resolving gel buffer (1.5MTris-Cl, pH 8.8)

Tris base : 18.5g

Distilled water to: 100ml

Dissolved the Tris base in about 50 to 60 ml distilled water, adjusted the pH to 8.8 with concentrated HCl and stored at 4°C.

C. 4X stacking gel buffer (0.5M Tris-Cl, pH 6.8)

Tris base : 6g

Distilled water to: 100ml

Dissolved the Tris base in about 50 to 60ml distilled water, adjusted the pH to 6.8 with concentrated HCl and stored at 4°C.

D. Electrode buffer (0.025M Tris, pH 8.3, 0.192M Glycine)

Tris base : 1.525g

Glycine : 7.2g

SDS : 0.5g

Distilled water to : 500ml

E. 2X Treatment buffer (0.125M Tris Cl)

4X Tris Cl , pH 6.8 :2.5ml

Glycerol : 2ml

2-Mercaptoethanol : 0.2ml

Bromophenol blue : 0.2g

SDS (10per cent) :4ml

Distilled water : 10ml

F. Initiator (10% APS)

Ammonium persulphate: 0.1g

Distilled water : 1ml

This solution is prepared fresh, immediately before use.

G. SDS (10%)

Sodium dodecyl sulphate : 10g

Distilled water to : 100ml

Appendix III

Composition of protein staining solution and destainer

1. Protein staining solution

Coomassie brilliant blue R 250	:0.1g
Methanol	: 40ml
Acetic acid	: 10ml
Water	: 50ml

First dissolved the dye in methanol and then mixed acetic acid and water.

2. Destainer

Methanol	: 40ml
Acetic acid	: 10ml
Water	: 50ml

CHARACTERIZATION OF PROTEINS IN *Costus pictus* D.Don

By

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ABSTRACT OF THESIS

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ABSTRACT

Costus pictus D.Don is a Mexican plant brought to India during 2002-2003. Due to the spiral appearance of stems, it is called as spiral ginger/ stepladder plant. *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)., Balaji (2005) and Jothivel *et al.*(2007) are in agreement with its hypoglycaemic property.

Insulin is taking through injection for treating hyperglycemia. An antidiabetic plant protein could exert a beneficial effect in the diabetic situation by enhancing insulin secretion and / or by mimicking insulin action. As insulin is a protein, comparison of its characters to proteins of a hypoglycaemic plant may open new doors to the field of diabetic treatment.

The project entitled 'Characterization of proteins in *Costus pictus* D.Don' was taken up at CPBMB, College of Horticulture, Kerala Agricultural University, Vellanikkara. The objective of the experiment was the isolation, purification and characterization of proteins in *Costus pictus* and comparison with insulin.

Effect of extraction buffers, percentage acrylamide and quantification of protein were studied. Use of 0.2 M Tris buffer at pH 7.2 and 10% acrylamide in the electrophoresis were found to be the most suitable condition for studying the proteins in *Costus* plant.

The study revealed the presence of acidic and basic proteins in this plant. Proteins of basic nature in the *Costus pictus* are precipitated with ammonium sulphate in the range of 30-50 per cent saturation which were stained with Coomassie dye and acidic proteins were precipitated at high saturation of ammonium sulphate which were expressed in silver staining.

Salting out of *Costus* protein was efficiently carried out with 80 per cent ammonium sulphate saturation. Aggregation of small proteins and the ability to regulate the breakage of long proteins were observed in the sequential saturation of ammonium sulphate. The hydrophobicity of the proteins in the sample was evident in the ammonium sulphate precipitation. Same pattern was observed in the electrophoretic pattern of fractions from DEAE cellulose with NaCl gradient. Hydrophobic patches were obtained for small and large proteins of *Costus* plant. Aggregation of proteins in *Costus* can be considered as its characteristics. pH was one of the major factor contributed aggregation and elution of proteins.

On comparing with insulin, 70-80 per cent sequential precipitation gave a protein of similar range of molecular weight of insulin available in the market by Recombinant DNA Technology. The present study revealed that the gradient application of salt and pH can be efficiently utilized for isolating single protein from *Costus*. Purification, sequencing and further studies are to be conducted for establishing the antidiabetic property of *Costus* plant.

Costus protein may provide signals in the body fluid that may recognize by the cells in the pancreas for the production of insulin. It may also be possible to function as receptor that conveys information to produce insulin within the body. Plant protein may serve as intracellular signaling component that mediate the effect of insulin production and as a key component of the machinery that determines which genes are to be expressed and whether mRNA is translating in to insulin.