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Characterization of 31-kda, Tubulin α-1 chain like protein of *Euphorbia nivulia* Buch.-Ham. Latex

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ABSTRACT

A new latex protein named nivulian-I of plant origin has been isolated and purified from the latex of *Euphorbia nivulia* Buch.-Ham., a member of *Euphorbiaceae* family; by using centrifugation, acetone precipitation and ion exchange chromatography. This protein was characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis and mass spectroscopic analysis. Molecular mass of nivulian-I is 31486.985 Da. Peptide mass fingerprint revealed that, it fairly matches with Tubulin alpha-1 chain (TBA1_ELEIN) of *Eleusine indica* (L.) Gaertn, a member of *Poaceae* family. This investigation infers that nivulian-I may be a novel protein of plant origin.

KEYWORDS

Euphorbia nivulia
Buch.-Ham;
Euphorbiaceae;
Latex;
Nivulian-I;
Mass spectroscopy
(MALDI TOF);
SDS-PAGE.

INTRODUCTION

Plant latex is the milky juice, found in long branching tubes known as latex tubes, a characteristic tissue of laticiferous plants. This juice is white, yellow or pinkish in colour. It is a viscous fluid, colloidal in nature and a complex mixture of organic and inorganic compounds, waxy materials and hydrolytic enzymes. Among hydrolytic enzymes, protease is the major fraction and plays very important role in plant physiology[1]. Known ingredients of latex are proteins, alkaloids, tannins, terpenes, starch, sugars, oils, resins, gums and hydrolytic enzymes[2]. Plant latex is used in the Ayurvedic system of medicine for bronchitis, rheumatism and many other ailments. *Euphorbia nivulia* Buch.-Ham. a member of *Euphorbiaceae* family, is one of the most important latex bearing hedge plant found in North Maharashtra region of India, which is ethnomedicinally reported for skin disorders, wound healing, ear disorders, retention of urine, swelling, worm infection and asthma[3]. Wound healing activity of *E. nivulia* latex is experimentally investigated in mice and showed significant activity[4]. Several phytoconstituents have been previously reported from this latex bearing plant. Chemically latex contains macrocyclic diterpenes, tetracyclic triterpene and ingol diterpenes[5,6,7]. Phytoproteins have been extensively studied over the last few decades for their economic importance[8]. Recently, we reported the protein content of 21 latex bearing plants belonging to seven different laticiferous families. *E. nivulia* is rich in protein content as compared with other investigated members of *Euphorbiaceae* family[9]. In this investigation, we report on purification, identification and characterization of 31-kDa, Tubulin α-1 chain like protein i.e.

nivulian I of *E. nivulia* latex.

MATERIALS AND METHODS

Chemicals

Trypsin, Acetonitrile, Trifluoroacetic acid (TFA), Acetic acid, Sinapic acid, Bovine serum albumin, $(NH)_4HCO_3$ were purchased from Sigma Chemicals (St Louis MO, USA). DEAE Cellulose and Casein (Hammarsten type) were purchased from SRL Chemicals, Bangalore, India. Coomassie Brilliant Blue R-250, Protein molecular weight markers were purchased from Bangalore Genie, India. All other chemicals were obtained from commercial sources and were of the highest purity available.

Plant material, collection of latex and crude protein preparation

The comprehensive information about identification, collection, preservation and preparation of crude extract of protein sample of *Euphorbia nivulia* latex is described in our previous communication[9].

Proteolytic activity

Proteolytic activity was determined by the colorimetric assay using 1% casein (Hammarsten type) as a substratz[10]. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to μg of tyrosine / min / mg protein at 37^{0}C and protein content was measured using Bovine serum albumin as the standard protein[11].

Purification of Nivulian I

Preliminary purification step

For the initial isolation step, the crude latex was precipitated with ice chilled acetone (20-40 %) at 4^{0}C. The resulting precipitate was separated by centrifugation, washed with acetone and dissolved in 0.01 M ice chilled phosphate buffer (pH 6.0). This preparation, was named ''partially purified preparation'' of protein.

Chromatographic procedures

The partially purified preparation was applied (3ml) on to a column (1.8 x 30cm) of DEAE-Cellulose pre-equilibrated with 0.01 M phosphate buffer at pH 6.0. Elution of protein was carried out by batch wise addition of 40 ml portions of increasing molarities (0.0 –0.5 M) of NaCl in 0.01 M phosphate buffer (pH 6.0). Effluents were collected in 5.0 ml fractions at a flow rate of 20 ml/h. The absorbance at 280 nm as well as the proteolytic activity in all fractions was tested. The fractions showing a peak activity were pooled and dialyzed using dialysis tubing with a cutoff of 12000-14000 Da against three 2-L changes of 0.01M phosphate buffer (pH 6.0) over 16 h. The dialysate was centrifuged at 10000 rpm for 20 min at 4^{0}C to remove any insoluble material and was immediately subjected to rechromatography on DEAE cellulose column of same dimension and finally eluted with a 0.2 – 0.3 M NaCl linear gradient in the same buffer.

Characterization of Nivulian I

Electrophoresis (SDS-PAGE)

Chromatographically peak fraction of protein was analyzed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) with Tris-glycine buffer (pH 8.2) in 12% polyacrylamide gel according to Laemmli (1970)[12]. Detection was done by the Coomassie Brilliant Blue R-250 staining method and molecular weight was estimated by comparing relative mobility of marker proteins, namely Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa) and Lysozyme (14.3 kDa) (GeNei, Bangalore Genei, India).

Mass Spectrometry

Matrix-assisted laser desorption ionization / time of flight mass spectrometry (MALDI / TOF MS) was used for the determination of the molecular mass, as well as the degree of purity of active enzyme. MALDI / TOF mass spectra were acquired on a Bruker Daltoncs® model Ultraflex II Spectrometer, Germany equipped with a pulsed solid state nitrogen laser (337 nm), in linear positive-ion mode, using a 19-kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix i.e. sinapinic acid [prepared in 0.1% TFA (aq.) and 50% acetonitrile (2:1)]. 2 μl matrix solutions and 2 μl protein fraction were spotted on the sample plate, mixed them and allowed to evaporate to dryness. Proteins of known molecular masses were used as standards for calibration.

Peptide analysis

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For peptide mass fingerprinting, the target protein band was excised from the gel, destained and subjected to in-gel trypsin digestion[13]. Sample of trypsin digested peptides with α-cyano-4-hydroxycinnamic acid (matrix) were spotted on the MALDI plate and analyzed on a MALDI / TOF-TOF mass spectrometer. The peak list obtained was submitted to the Matrix Sciences program for protein identification (http://www.matrixscience.com). An additional match of the mascot peptide mass fingerprint of the protease with other existing protein sequences (http://www.ncbi.nlm.nih.gov/) was also performed.

RESULTS AND DISCUSSION

Euphorbia nivulia Buch.-Ham. belongs to the Euphorbiaceae family. Its members are characterized by secretory tissues (laticifers) which frequently include proteolytic and milk clotting enzymes. The young stem latex of *E. nivulia* possesses proteolytic and milk clotting enzyme in larger quantity as compared with other investigated laticiferous plants of Northern region of Maharashtra, India[9]. The crude enzyme of *E. nivulia* had highest proteolytic activity (12.64 U/min) against casein whereas the partially purified preparation retained 60.64% of its initial proteolytic activity and 46.8% of the protein content.

The protein extracted with 20-40% acetone precipitation showed maximum activity and hence this fraction of protein was subjected for chromatographic separation by DEAE cellulose column chromatography. The unbound materials to column as well as buffer wash of the column were devoid of any enzyme activity. The bound proteins were eluted from the column with salt gradient while the column elution profile resolved into three protein peaks as shown in Figure 1A. Fractions of all the peaks were assayed for proteolytic activity. The magnitude of activity of the fractions (25-29) of peak II was higher relative to the pooled fractions of peak I and III (data not shown). The dialysate of pooled fractions of peak II was used for further purification through rechromatography on DEAE-cellulose column.

The elution profile of rechromatographed column (Figure 1B) constitutes a large peak followed by small peak (fraction 31-36). The fractions of small peak were pooled. SDS-PAGE electrophoresis of this pooled fraction showed single protein band and its molecular mass is 31.27 kDa (Figure 2) and eventually match with mass spectral analysis, i.e. 31486.985 Da, (Figure 3). These results, clearly demonstrate that pooled fractions revealed single protein homogeneity to electrophoresis. The purified electrophorogram protein is named as nivulian I (31.27 kDa) according to nomenclature of plant latex origin protein and previous suggestions[14, 15]. Similar results have been reported in characterization of cereal protein, 32 kDa of Aleurain of *Hordeum vulgare* Linn[16] and latex protein, 30 kDa of Eumiliin of *Euphorbia milii* var. *hislopii*[17]. This suggests that, the biochemical characteristics of nivulian-I are very close to these proteins.

Out of 89 trypsin digested peptide fragments of nivulian I (Figure 4), only five peptides i.e. 17.10 % sequence was hit in Swiss Port (protein sequencing database) by Mascot peptide fingerprint search engine with Tubulin alpha-1 chain (TBA1_ELEIN) of *Eleusine indica* with 70 score ($p<0.05$) (Figure 5). The mass spectrum showed several protonated ions $[M+H]^+$ of the peptide fragments. As listed in TABLE 1, the ions at 1132.631, 1396.829, 1473.901, 1872.078 and 2673.365 were the trypsin digested five peptides corresponding to residues 113-121, 85-86, 230-243, 353-370 and 281-304 and these are designated as T-1, T-2, T-3, T-4 and T-5 respectively. As depicted in TABLE 1, peptide mass profiles were obtained from the database search that identified Tubulin α-1 chain of *E. indica* from desired spot of protein of SDS-PAGE. The TABLE 2 summarizes the amino acid composition of obtained peptide fragments (T-1 to T-5) of Nivulian-I by trypsin digestion. OH-group containing amino acid i.e. threonine (T) was only found in T-3 peptide where as aromatic amino acid i.e. tryptophan (W) was absent in all sequenced peptides. This data deduced that the nivulian I may be a novel euphorbain protein as it is characterized is comparison with the protein of *Euphorbia lathyris* L. latex[18].

CONCLUSIONS

A new latex origin protein, named nivulian-I (MW: 31.27 kDa) is reported from the latex of *E. nivulia*, for the first time by simple purification procedures. Tryptic digestion profile of nivulian-I, concludes the exclusive

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TABLE 1 : Calculated and observed ions of trypsin digests of Nivulian-I

Peak Number	Amino acid sequence		Sequence	$[M+H]^+$	
	From	To		Calculated	Observed
				m/z	
25	113	121	EIVDLCLDR	1132.567	1132.631
36	85	96	QLFHPEQLISGK	1396.758	1396.829
46	230	243	LVSQVISSLTASLR	1473.864	1473.901
65	353	370	CGINYQPPSVVPGGDLAK	1871.932	1872.078
85	281	304	AYHEQLSVAEITNSAFEPSSMMAK	2673.217	2673.365

TABLE 2 : Amino acid composition of peptides obtained from Nivulian-I by trypsin digestion

Amino acid	T-1	T-2	T-3	T-4	T-5
Gly (G)	--	1	--	3	--
Ala (A)	--	--	1	1	4
Vsl (V)	1	--	2	--	1
Leu (L)	2	2	3	3	1
Ile (I)	1	1	1	1	1
Ser (S)	--	1	4	1	4
Thr (T)	--	--	1	--	1
Cys (C)	1	--	--	1	--
Met (M)	--	--	--	--	2
Asp (D)	2	--	--	1	--
Asn (N)	--	--	--	1	1
Glu (E)	1	1	--	--	3
Gln (Q)	--	2	1	1	1
Phe (F)	--	1	--	--	1
Tyr (Y)	--	--	--	1	1
Try (W)	--	--	--	--	--
Lys (K)	--	1	--	1	1
Arg (R)	1	--	1	--	1
His (H)	--	1	--	--	--
Pro (P)	--	1	--	3	1
Total	09	12	14	18	24
Position	113-121	85-86	230-243	353-370	281-304
Peak No	25	36	46	65	85

nature of protein and it may be a novel protein.

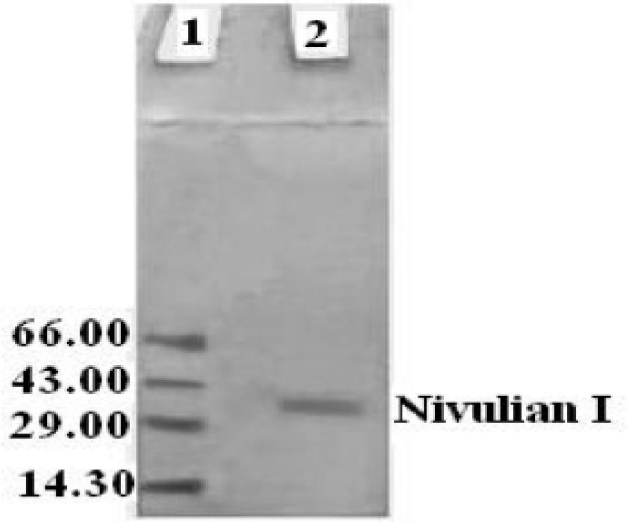

Figure 2 : Electrophoretic behaviour of Nivulian-I: Lane-1, molecular weight markers; Lane-2, Nivulian-I

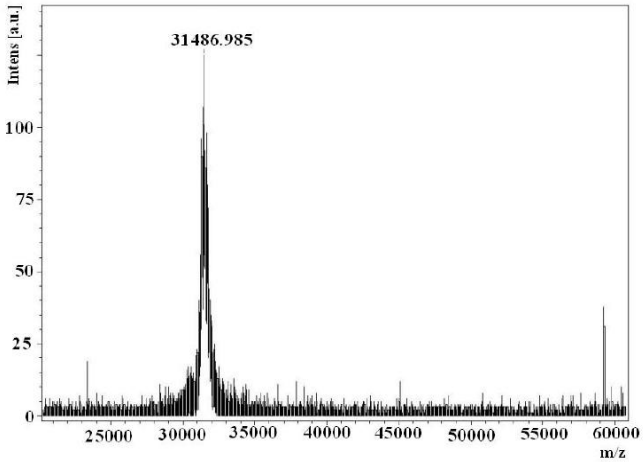

Figure 3 : Mass spectrometry of purified nivulian-I plant origin protein

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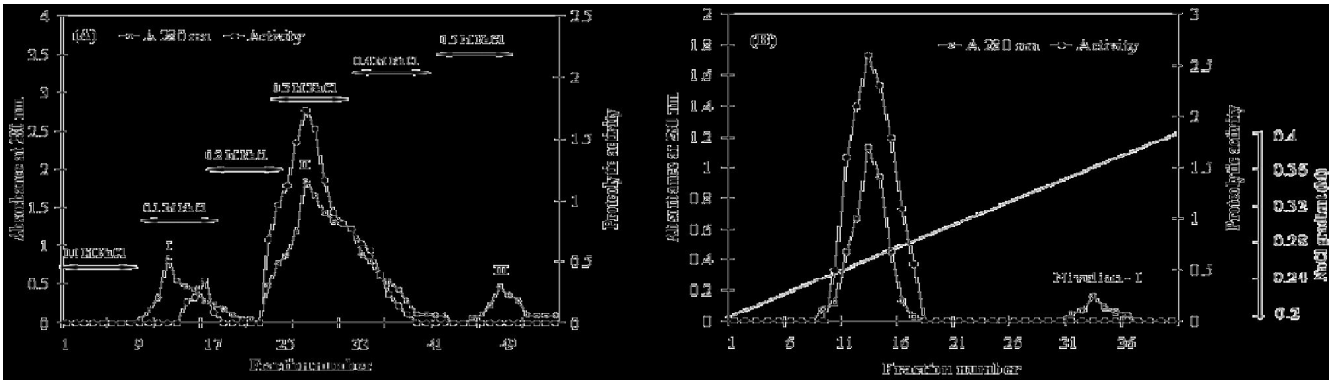

Figure 1 : Chromatographic purification of Nivulian-I protein on DEAE-cellulose column: (A) Chromatography of acetone precipitate fraction of protein on a DEAE-cellulose column. (B) Rechromatography of dialyzed fraction of protein on DEAE-cellulose column.

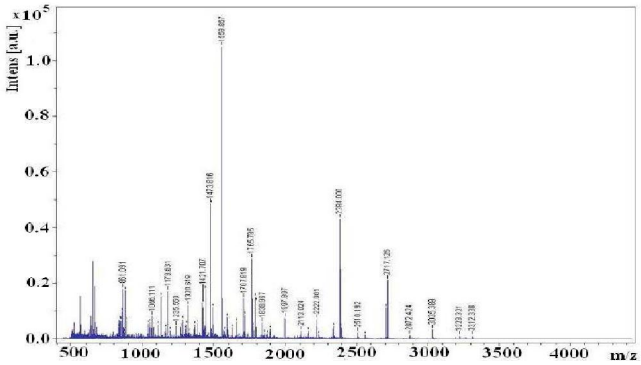

Figure 4 : MALDI-TOF mass spectrum of trypsin digested peptide map of nivulian-I

MRECISIHIG QAGIQVGNAC WELYCLEHGI QADGQMPGDK TIGGGDDAFN TFFSETGAGK 60
HVPRAVFVDL EPTVIDEVRT GTYRQLFHPE QLISGKEDAA NNFARGHYTI GKEIVDLCLD 120
RIRKLADNCT GLQGFLVFNA VGGGTGSGLG SLLLERLSVD YGKKSKLGFT VYPSPQVSTS 180
VVEPYNSVLS THSLLEHTDV AVLLDNEAIY DICRRSLDIE RPTYTNLNRL VSQVISSLTA 240
SLRFDGALNV DVNEFQTNLV PYPRIHFMLS SYAPVISAEK AYHEQLSVAE ITNSAFEPSS 300
MMAKCDPRHG KYMACCLMYR GDVVPKDVNA AVATIKTKRT IQFVDWCPTG FKCGINYQPP 360
SVVPGGDLAK VQRAVCMISN STSVVEVFSR IDHKFDLMYA KRAFVHWYVG EGMEEGEFSE 420
AREDLAALEK DYEEVGAEFD EGEEGDEGDE Y 451

Figure 5 : Peptides matched with Tubulin α-1 chain (TBA1_ELEIN) of *Eleusine indica*.

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