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Purification and characterization of a new chitinase from latex of *Ipomoea carnea*

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ABSTRACT

A novel enzyme with endochitinase/lysozyme activity was purified to homogeneity from latex of *Ipomoea carnea* subsp. *fistulosa* using latex collection, gum removal, ammonium sulphate precipitation, hydrophobic interaction, and anion exchange chromatography. The enzyme was glycosylated (5–6%) and homogeneous on SDS-PAGE; has a molecular mass of 30.06 kDa (MALDI-TOF) and an isoelectric point of pH 4.6. The enzyme exhibited chitinase activity for hydrolysis of glycol chitin and the chitinolytic activity was significantly inhibited by allosamidin and mercuric chloride. The enzyme is stable in the pH range of 4.0–9.5, 80 °C and the optimal activity was observed at pH 5.5 and 50 °C. The enzyme consists of 8 tryptophan, 14 tyrosine, 6 cysteine residues forming three disulfide bridges and the extinction coefficient was estimated as 21.35 M^{-1} cm^{-1}. The polyclonal antibody was raised in rabbit and immunodiffusion suggests that the antigenic determinants are unique. The first 15 N-terminal residues G-E-I-T-I-Y-W-G-Q-N-G-F-E-G-S exhibited high identity to other known plant chitinases. Owing to the economic purification, high yield, unique and extraordinary features, stability and behavior; the enzyme ICChII can be widely employed in agriculture, industry, environmental protection, and in recycling chitinous waste from arthropod shellfish and for chito-oligosaccharide production.

1. Introduction

Chitin, a linear homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues is the most abundant nitrogen containing organic compound found in fungi, animals, and plants [1]. Chitinases (EC 3.2.1.14) are classified as glycosyl hydrolases that catalyze the hydrolysis of β-1,4-glycosidic bonds of chitin. They are found in bacteria, fungi, higher plants, insects, and some vertebrates [2–4]. Chitinases have received increasing attention because of their broad applications in the fields of medicine, agriculture, biotechnology, waste management and industrial applications [5].

Plants defend themselves against pathogen invasion attack by activating synthesis of pathogenesis-related (PR) proteins. Chitinases are the most common among the PR proteins induced in response to fungal infection or treatment with fungal elicitors [6]. In addition their activity can be induced by bacterial, viral infections, wounding, hormones, heavy metal salts, and plant cell wall components [7]. Moreover chitinases also play a role in growth and development processes [8] including regulation of nodulation signal molecules [9], embryogenesis [10,11], and participate in programmed cell death [12]. In plants, chitinases have been widely used in the control of fungal and bacterial pathogens by means of genetic engineering. The over expression of chitinase in plant cell causes lysis of fungal hyphae and spore walls thereby reducing fungal growth [13]. Economically important plants like wheat, peanut, sugarcane, cotton and other ornamental plants engineered with chitinase genes showed a greater defense against the plant fungal pathogens such as *Rhizoctonia solani*, *Botrytis cinerea*, *Cercospora arachidicola*, *Alternaria solani*, etc. [14].

Plant chitinases are organized in five different classes numbered from I to V, based on amino acid sequence similarity of their catalytic domains and structure [15]. Classes I, II and IV share a homologous main catalytic domain. Classes I and IV possess a cysteine-rich domain involved in chitin binding in their N-terminal region which is lacking in class II [16]. Class III includes bifunctional lysozyme/chitinase enzymes [17] with no sequence similarity to plant chitinases from other classes. Class V chitinases show endochitinase activity but are unrelated to other plant

Abbreviations: BSA, bovine serum albumin; BLAST, basic local alignment search tool; DMSO, dimethyl sulfoxide; DTNB, 5,5μ-dithiobis (2-nitrobenzoic acid); ICChII, *Ipomoea carnea* chitinase I; ICChII, *Ipomoea carnea* chitinase II; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MES, 2-(*N*-morpholino) ethanesulfonic acid; MWCO, molecular weight cut off; NCBI, National Center for Biotechnology Information; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TEMED, tetramethylethylenediamine; TCA, trichloroacetic acid; TFMS, trifluromethane sulphonic acid.

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chitinases and share some similarity with bacterial exochitinases [18].

Chitinases have been identified from barley, bean, pea, rice, tobacco, and rubber tree [19]. Although, several plant chitinases has been purified and well characterized, but chitinase from the latex of a toxic and medicinal plant weed *Ipomoea carnea* has not been studied. In the present article, we described the isolation and characterization of a new chitinase from the plant weed *I. carnea* subsp. *fistulosa* (morning glory), a member of convolvulaceae family is a toxic plant (weed) found in abundance throughout Brazil and other tropical countries [20]. The toxic principles of the plant have been recently identified as two nortropane alkaloids, calystegines B2 and C1, and indolizidine alkaloid, swainsonine [21]. While screening different parts of *I. carnea* subsp. *fistulosa* (morning glory) plant for enzymatic activity, the latex exhibited a considerable amount of chitinase/lysozyme activity. Owing to its high purity, high yield, broad stability range, the morning glory chitinase may have immense industrial/biotechnological applications.

2. Materials and methods

2.1. Chemicals and reagents

Glycol chitosan, chitin, p-nitrophenyl N-acetyl-β-D-glucosaminide, calcofluor M2R fluorescent brightener 28, allosamidine, MES buffer, DTNB, N,N′-methylene-bis-acrylamide, TEMED, Coomassie brilliant blue R-250, Freund's complete, incomplete adjuvants, potassium sodium tartrate, 3,5-dinitrosalicylic acid, and agarose were obtained from Sigma Chemical Co. (USA); ampholine pH 4–6 and SDS-PAGE standard from BioRad (USA); orthophosphoric acid, periodic acid, Schiff's reagent was from Loba Chemie Pvt. Ltd. India, acrylamide, tris buffer, ammonium sulphate, TFMS, sodium metabisulphite, anisole, pyridine, and triton X, were purchased from Spectrochem Pvt. Ltd. (India).

2.2. Isolation and purification of chitinase

All steps for purification of the enzyme were carried out at 4 °C and the solutions were prepared using milli Q water (Millipore). Fresh latex secretions by superficial incisions made on young stems and apical buds of *I. carnea* plants were collected into 0.05 M MES buffer, pH 6.0 and stored at −20 °C for 24 h. The latex was thawed at room temperature and centrifuged at 12,000 × *g* (Sorvall RC-5C plus) for 20 min to remove all insoluble cell debris. Clear supernatant was used as the crude proteins for further investigations.

The crude protein solution was allowed for 85% ammonium sulphate saturation and resultant precipitate was collected by centrifugation at 12,000 × *g* for 30 min; redissolved in 0.05 M MES buffer pH 6.0 (buffer A) and followed by dialysis against the same buffer. The dialysate was adjusted with 4.0 M ammonium sulphate in 0.05 M MES buffer to achieve a final concentration of 0.05 M buffer containing 1.5 M ammonium sulphate and subjected to a hydrophobic interaction chromatography (HIC) on ether-Toyopearl 650S (ETP) (2.0 cm × 7.0 cm) column pre-equilibrated with buffer A containing 1.5 M ammonium sulphate. The column was washed with an excess of the same buffer containing in 1.5 M ammonium sulphate to remove loosely bound proteins if any. The bound proteins were eluted by a linear gradient of ammonium sulphate from 1.5 M to 0 M in the same buffer. The eluted active fractions possessing chitinase activity were pooled, desalted, dialyzed and subjected to anion exchange chromatography on Q-sepharose fast flow (Sigma) pre-equilibrated with tris buffer pH 8.0 and bound proteins was eluted with a linear gradient of buffer containing 0.0–0.5 M NaCl. The homogenous peak I fractions from SDS-PAGE analysis were pooled, concentrated, desalted and stored in 50 mM MES buffer pH 6.0 at 4 °C for all biochemical characterization and N-terminal amino acid sequencing. The enzyme was named as ICChII (*I. carnea* chitinase II).

2.3. Enzyme assay

The enzymatic activity was assayed with several substrates using methods of, fluorometric, spectrophotometric and in-gel diffusion assay.

2.3.1. Colloidal chitin chitinase assay

Chitinase activity was measured by reduction of 3,5-dinitrosalicylic acid, in presence of the amino-sugar N-acetyl-D-glucosamine (NAG) released by the enzymatic hydrolysis of chitin [22]. One unit of chitinase activity was defined as the amount of enzyme required to produce 1 μmol of NAG in 1 h [23].

2.3.2. In-gel diffusion chitinase assay

Chitinase was visualized by SDS-PAGE with 5% stacking gels and 12% resolving gels [24] containing 0.01% glycol chitin synthesized from glycol chitosan [25]. After SDS electrophoresis, gel was incubated for 24 h at 37 °C in 100 mM sodium acetate, 1% (v/v) Triton-X 100, pH 5.0 to remove SDS and promote chitinase activity against glycol chitin followed by staining with 0.01% calcofluor white M2R (Sigma) in 0.5 M tris–HCl pH 8.9. Protein bands exhibiting chitinase activity were visualized under UV transilluminator.

2.3.3. Exochitinase activity assay

β-N-acetylglucosaminidase activity of enzyme was assayed by using p-nitrophenyl-β-D-N-acetylglucosaminide as substrate [26].

2.3.4. Lysozyme activity

Lysozyme activity of the purified enzyme ICChII was measured by the rate of lysis of *Micrococcus lysodeikticus* cell walls as described by Shugar [27]. The reaction was monitored continuously as decrease in light scattering at 450 nm using a Beckman DU Spectrophotometer at 37 °C. The reaction contained 0.3 mg of *M. lysodeikticus* cell walls in a volume of 2.9 ml in 0.1 M phosphate buffer, pH 7.0 and 0.1 ml enzyme. One unit is defined as the amount of enzyme that produces a decrease in absorbance of 0.001 min^{-1} under the specified condition.

2.4. Protein assay

Protein concentration was determined by the method described by Bradford [28].

2.5. Polyacrylamide gel electrophoresis

Protein samples were analyzed by SDS-PAGE [29] under reducing conditions to check the homogeneity of the enzyme preparations. After electrophoresis, gel was stained with 0.1% (w/v) Coomassie brilliant blue R-250.

2.6. Carbohydrate content and deglycosylation

The carbohydrate content of the enzyme was determined using phenol–sulfuric acid method [30]. Carbohydrate content of ICChII was extrapolated from the standard plot generated from galactose measurement. The deglycosylation of the enzyme was done by chemical method using TFMS [31]. The electrophoresis was carried with glycosylated and deglycosylated protein sample by the method as described above. The gel was stained with Schiff's reagent specific to glycoprotein [32].

2.7. Isoelectric focusing

The isoelectric point of the purified enzyme was determined by isoelectric focusing on polyacrylamide disc gels as described [33].

2.8. Antigenic properties of the chitinase

Antibodies against the purified enzyme ICChII were raised in a rabbit as described [33]. The presence of antibodies was confirmed by immunodiffusion studies by Ouchterlony's double immunodiffusion as described by Ouchterlony and Nilsson [34]. Enzyme (ICChII) and the other three proteins purified from the latex of *I. carnea* were loaded in the peripheral well and anti-ICChII serum was loaded in the central well to check the specificity of raised antibody.

2.9. Mass spectrometry

Molecular weight of the purified enzyme was determined by Micromass TofSpec MALDI/TOF. Samples were dissolved at a concentration of 10 pmol/μl in 1:1 (v/v) 1% aqueous formic acid and methanol. Positive ionization was used for the sample analyses with an electro spray voltage of 1.0 kV. Nitrogen was employed as the API gas and data were acquired over the appropriate *m*/*z* range at a scan speed of 3.0 s in continuum mode. An external calibration was made using horse heart myoglobin (MW 16,951.5 Da) and data were processed using the MassLynx suite of software programs supplied with the mass spectrometer.

2.10. pH and temperature optima

The effect of pH on the enzymatic activity of the purified enzyme was determined in the range of pH 0.5–12.0. Similarly, the effect of temperature on the enzymatic activity of the purified enzyme was determined in the temperature range of 20–95 °C. The colloidal chitin was used as substrate for the activity assay as described above. A buffer of 50 mM was used throughout the experiment.

2.11. Temperature and pH stability of the chitinase

The thermal stability of the purified enzyme was estimated by determining the residual activity of the enzyme solution after incubation for 60 min at various temperatures from 20 to 95 °C. Similarly, the stability of the enzyme was estimated by determining the residual activity of the enzyme solution after exposure to

different pH from 0.5 to 12.0 for 24 h at room temperature. The enzyme was assayed as described above with colloidal chitin as substrate.

2.12. Effect of compounds on the activity of chitinase

For the effect of allosamidin and mercuric chloride on the chitinase activity, 10 μg of ICChII enzyme was incubated in the presence of increasing concentration of the inhibitors for 60 min at 37 °C and assayed with colloidal chitin as substrate as described above. A control assay was done with enzyme solution without inhibitors, and the resulting activity was considered to be 100%. All experiments were performed in triplicate.

2.13. Specific amino acid residue

The tyrosine and tryptophan contents of the enzyme were measured using method of Goodwin and Morton [35]. The formula given by Goodwin and Morton was used to estimate the tryptophan and tyrosine contents. The exposed and total cysteine residues of chitinase (ICChII) were estimated by Ellman's [36] method using DTNB where the release of thionitrobenzoate (TNB) due to reduction of thiol with DTNB was determined by increase in the absorbance at 412 nm. The molar extinction coefficient of TNB anion at 412 nm is 14,150 M^{-1} cm^{-1} was used for the calculation [37]. The number of disulfide bonds, in the protein, was deduced by comparison of the number of free and total cysteine residues. To validate these measurements, tyrosine, tryptophan and cysteine content of papain, ribonuclease A, BSA and lysozyme were determined under similar conditions.

2.14. Extinction coefficient

The extinction coefficient of the enzyme (ICChII) was determined by spectrophotometric method using formula given by Aitken and Learmonth [38].

2.15. Amino-terminal sequence analysis of chitinase

The purified enzyme (ICChII) dialyzed against distilled water was concentrated by freeze drying and 10 pm sample was applied on a protein sequencer (Applied biosystem procise sequencer) and the N-terminal was sequenced automatically by Edman's degradation. The N-terminal sequence obtained was submitted to NCBI-BLAST for optimal alignment with other known plant chitinases.

2.16. Tryptic digestion for MS analysis and de novo sequencing by MALDI-TOF/TOF

Tryptic digestion of ICChII was done using the protocols [39,40] with minor modifications. After SDS-PAGE gel pieces were excised, washed, destained, dehydrated in acetonitrile, and further dried in a vacuum centrifuge. The gel pieces were cooled on ice and soaked with trypsin solution (20 μg/ml porcine trypsin in 40 mM NH_4HCO_3, 10% CH_3CN and 0.5% beta-octyl-D-glucoside) for overnight at 37 °C. Digested peptides were recovered from the gel by consecutive extraction for 10 min each in an ultrasonic bath. Extracts were pooled, dried, and peptides were dissolved in 5 μl matrix solution (alpha-cyano-4-hydroxy cinnamic acid, saturated solution in 40% CH_3CN, 0.1% TFA). Tryptic digested sample of ICChII was mixed with matrix and spotted on the polished stainless steel MALDI target plate for MALDI analysis on a MALDI-TOF mass spectrometer (Voyager DE-STR, Applied Biosystems, Foster City, CA) operating in reflector mode following parameters (20,000 V acceleration potential, 200 ns delay time, 67% grid voltage) optimized for peptide masses between 1000 and 2000 Da. Spectra were calibrated internally with trypsin peaks (842.5100, 2211.1046). 40 strongest monoisotopic peaks were subjected to profound using the database NCBI and *de novo* sequences of peptides were generated.

3. Results

3.1. Purification and characterization of the chitinase

A novel enzyme (ICChII) has been purified and characterized from the latex of *I. carnea*. After ammonium sulphate precipitation and dialysis, the crude protein extract was applied on an ether-Toyopearl column and the bound protein was eluted by a linear gradient of ammonium sulphate from 1.5 M to 0 M (Fig. 1a). Chitinase activity and some homogeneity on SDS-PAGE were observed in peak II fractions (65–95). Peak II fractions were pooled, desalted, and dialyzed against 50 mM tris buffer pH 8.0 and applied to Q-sepharose fast flow column. The bound protein was eluted with NaCl gradient 0.0–0.6 M (Fig. 1b). Chitinase activity and a homogenous protein with an apparent molecular

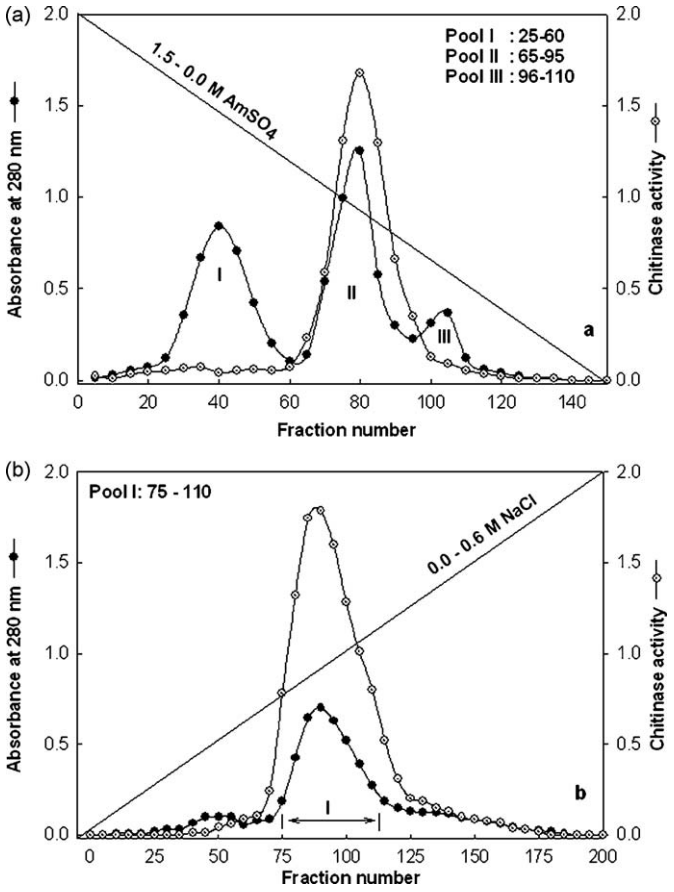

Fig. 1. Chromatography of chitinase (ICChII); (a) elution profile of ICChII by hydrophobic interaction chromatography on ether-Toypearl column. The bound protein was eluted with linear gradient of ammonium sulphate 1.5–0.0 M in 0.05 M MES buffer (pH 6.0). (b) The protein peak II fractions (65–95) of ether-Toypearl chromatography was pooled, concentrated and applied to anion exchange chromatography on Q-sepharose. Protein elution was carried out in 0.05 M tris buffer (pH 8.0) with NaCl gradient 0.0–0.5 M NaCl.

weight of 30–32 kDa on SDS-PAGE were observed in peak I fractions (Fig. 2a). Exact molecular weight estimated by MALDI-TOF was 30.06 kDa (Fig. 3). Peak I fractions (75–100) were pooled, concentrated, desalted, stored at 4 °C and used for experimental procedures. The purification profile is shown in Table 1 and the purified protein was named as ICChII.

3.2. Carbohydrate content and glycostaining

The carbohydrate content of enzyme using phenol–sulfuric acid method was estimated to be 5–6%. The deglycosylation of enzyme was performed by a chemical method using TFMS and a reduction of molecular weight around 2 kDa was observed on SDS-PAGE clearly visible by Coomassie and silver staining (Fig. 4a and c). In electrophoresis glycosylated enzyme produces pink stained proteins on gel (Fig. 4b) with Schiff's reagent whereas deglycosylated enzyme produces no color.

3.3. Enzyme activity assay

The chitinase activity with colloidal chitin as substrate and DNS for detection measures the amount of liberated reducing sugar. The chitinase activity was confirmed by the in-gel activity assay with glycol chitin as substrate and calcofluor as fluorescence brightener. Chitinolytic activity was detected as distinct single band on gels incorporated with glycol chitin stained with calcofluor white M2R

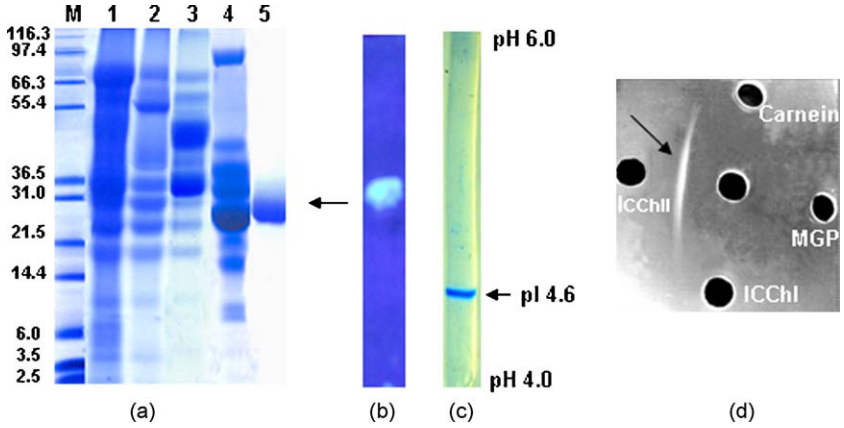

Fig. 2. Gel electrophoresis; (a) SDS–PAGE. Lane M, invitrogen mark 12 unstained molecular weight markers; Lane 1, crude protein bands of *Ipomoea carnea*; Lane 2, after 85% ammonium sulphate; Lane 3, unbound fractions from ETP; Lane 4, peak II fractions of ETP; Lane 5, purified chitinase ICChII from peak II fractions of Q-sepharose. (b) Detection of chitinolytic activity of ICChII when stained with calcofluor M2R. (c) Isoelectric focusing of chitinase ICChII against pH ampholytes 4–6. (d) Ouchterlony's double immunodiffusion in 1% agarose.

(Fig. 2b). Further, the exochitinase (β-N-acetylglucosaminidase) activity was assayed using p-nitrophenyl-β-D-N-acetylglucosaminide but no activity was observed suggesting that ICChII is an endochitinase.

For the estimation of randomly hydrolyzing and exochitinase (N-acetylglucosaminidase) activities, assay has been performed with several substrates. The fluorogenic substrate was more sensitive but assay using glycol chitin and colloidal chitin was used for most of the regular activity. In addition to endochitinase, this 30 kDa chitinase possess the lysozyme activity also Table 1.

3.4. Isoelectric focussing

Isoelectric focusing was performed in pH gradient 4–6; the enzyme (ICChII) migrated as a single sharp band focused at apparent isoelectric point (pI) 4.6 (Fig. 2c).

3.5. Polyclonal antibodies and immunoassay

The presence of polyclonal antibodies raised in rabbit was checked by immunodiffusion. The precipitin line is formed only against ICChII as a result of precipitation of antigen–antibody complexes near the equivalence zone. Antisera to ICChII did not cross-react with other proteins carnein [33], MGP [41] and ICChI [42] purified from the same plant suggesting that the antigenic determinants of ICChII are unique (Fig. 2d).

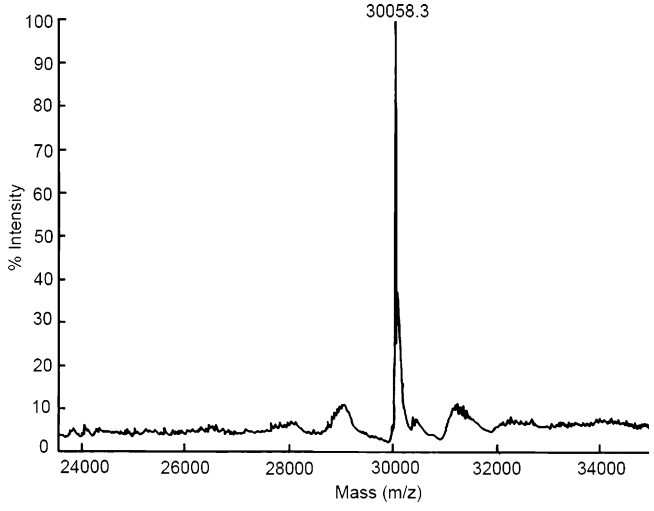

Fig. 3. Mass spectrometry of chitinase ICChII by MALDI-TOF.

3.6. Effect of pH and temperature

The optimum pH and temperature for the chitinase activity was 5.5 and 50 °C, respectively. The enzyme was remarkably stable in pH 4.0–9.5 and up to 80 °C (data not shown). The enzyme is fairly stable against temperature and pH and thus may find application in several industrial and biotechnological fields.

3.7. Effect of compounds on the activity of chitinase

The activity of chitinase enzyme was completely inhibited after incubation with allosamidin and mercuric chloride for 60 min, and it retained only 0.8% activity with 50 μM allosamidin, while retained 4.0% with 100 μM mercuric chloride (Fig. 5).

3.8. Specific amino acid residues

From biochemical methods, the tryptophan and tyrosine content of the enzyme ICChII was 8 (measured values 8.13) and 14 (measured values 14.27), respectively. The total sulfhydryl content of the protein was found to be 6 (measured values 6.16) forming three disulfides bridges. Under the similar experimental conditions ribonuclease, papain, proteinase K, and lysozyme gave the reported values.

3.9. Amino-terminal sequence

The N-terminal sequence of the ICChII was generated by Edmann automated degradation method. The first 15 amino residues, G-E-I-T-I-Y-W-G-Q-N-G-F-E-G-S showed high % identity with other known chitinases (Table 2). The first 15 N-terminal sequences showed an 80% similarity with *Dioscorea polystachya, Nicotiana tobacum and Vitis vinifera*. Moreover, ICChII has 73% N-terminal sequence similarity to many plant chitinases viz. *Hevea brasiliensis*, *Cucumis melo, Zea mays, Chamaecyparis glauca, Parkia platycephala, Gossypium hirsutum*, *Citrullus lanatus*, *Lupinus albus, Panax ginseng*, *Medicago truncatula*, *Rehmannia glutinosa* and *Vigna angularis*.

3.10. Tryptic digestion for MS analysis and de novo sequencing by MALDI-TOF/TOF

Attempts were made to identify the protein by peptide mass finger printing. For this, the protein was enzymatically digested

Table 1
Purification of chitinase (ICChII) from latex of *Ipomoea carnea*.

Procedure	Total protein (mg)	Chitinase activity				Lysozyme activity		
		Total activity[a] (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)	Total activity[b] (units)	Specific activity (units/mg)	Yield (%)
Crude extract	125	562.5	4.5	1.0	100.0	1475.0	11.8	100.0
Ammonium sulphate precipitation (85% saturation)	68	421.6	6.2	1.4	74.9	1219.0	17.9	82.6
HIC on ether toyperl (peak II fractions)	30	312.0	10.4	2.3	55.5	726.0	24.2	49.2
AEC on Q-Sepharose (peak I fractions)	8	135.2	16.9	3.8	24.0	552.0	69.0	37.4

[a] Chitinase activity was measured under standard assay conditions with glycol chitin as substrate as described in Section 2.
[b] One unit of lysozyme activity is defined as the amount of enzyme that produces a decrease in absorbance of 0.001 min^{-1} under the specified condition.

Table 2
Optimal alignment of N-terminal residues of *I. carnea* chitinase (ICChII) with amino acid sequences of other chitinases.

Accession	Source	Amino acid residues															Identity (%)
	I. carnea	**G**	**E**	**I**	**T**	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	**F**	**E**	**G**	**S**	100
BAC77768	*D. polystachya*	**G**	S	**I**	V	V	**Y**	**W**	**G**	**Q**	**N**	**G**	**F**	**E**	**G**	**S**	80
P29060	*N. tabacum*	**G**	D	**I**	V	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	80
CAO39704	*V. vinifera*	**G**	T	**I**	**T**	V	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	80
CAA09110	*H. brasiliensis*	**G**	G	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	T	73
ABL74451	*C. glauca*	**G**	G	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	T	73
2GSJ_A	*P. platycephala*	**G**	G	**I**	V	V	**Y**	**W**	**G**	**Q**	**N**	**G**	G	**E**	**G**	**S**	73
ABN03967	*G. hirsutum*	**G**	D	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	T	73
ABA26457	*C. lanatus*	A	G	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73
AAF64474	*C. melo*	A	G	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73
AAD53006	*C. moschata*	A	G	**I**	A	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73
ACF88326	*Z. mays*	**G**	N	**I**	A	V	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73
CAA76203	*L. albus*	A	G	**I**	V	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73
ABF82271	*P. ginseng*	**G**	G	**I**	S	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	G	**E**	**G**	T	73
AAQ21404	*M. truncatula*	**G**	K	**I**	S	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	T	73
AAO47731	*R. glutinosa*	**G**	K	**I**	S	**I**	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	T	73
BAA01948	*V. angularis*	**G**	G	**I**	S	V	**Y**	**W**	**G**	**Q**	**N**	**G**	N	**E**	**G**	**S**	73

Bold letters and shaded region indicates identical and conserved amino acid residues.

and resolved into a number of peptides. The masses of the peptides were determined and searched against relevant databases in NCBI. No significant hits were obtained based on peptide masses. Subsequently, the different peptide fragments of ICChII generated above were subjected to *de novo* sequencing by MALDI-TOF/TOF. The TOF/TOF spectra of representative peptides and their *de novo* sequence of match peptides are shown in Table 3. With MS/MS spectra we could get the *de novo* sequence of only 9 peptides and 10 remains unmatched. These sequences when submitted to BLAST (NCBI) showed that sequence of the protein is unique. No putative conserved domains have been detected using these sequences as search query. However, good score of 43 was obtained with sequence similarities to hevamive a 29 kDa endochitinase/lysozyme from *H. brasiliensis*.

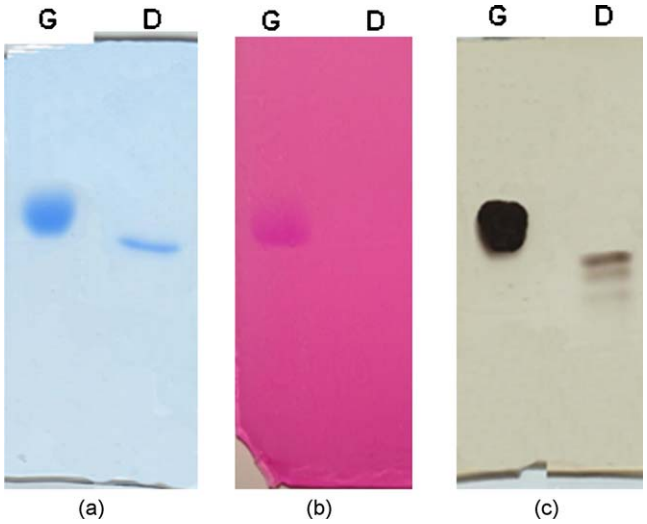

Fig. 4. Gel visualization of glycosylated and deglycosylated ICChII. (a) Coomassie stained gel; Lane G, glycosylated ICChII; Lane D, deglycosylated ICChII. (b) Schiff's reagent stained gel; Lane G, glycosylated ICChII; Lane D, deglycosylated ICChII. (c) Silver stained gel; Lane G, glycosylated ICChII; Lane D, deglycosylated ICChII.

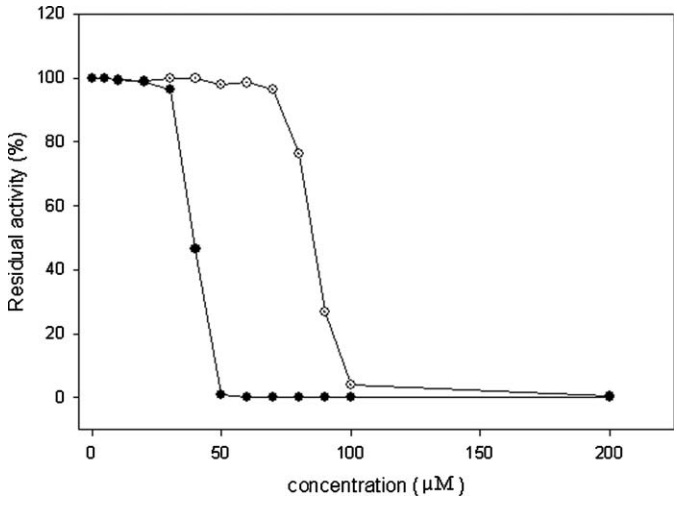

Fig. 5. Effects of compounds on the activity of enzyme ICChII. (●) With allosamidine and (○) with mercuric chloride. For determination of inhibition against these compounds, 10 μg of enzyme was incubated with increasing concentration of these compounds for 1 h and assayed for the residual activity. The maximum activity was considered as 100% activity.

Table 3
Sequences of some peptides obtained from peptide finger mass printing.

m/z PMF	Sequences	Amino acid position
2000.15	GEITIYWGQNGFEGSLTQ	1–18
1246.45	IVSNGIRSCQIG	59–70
804.02	KVMLSLGG	73–80
793.83	LDGIDFD	119–127
1535.68	YWDDLARYLSAY	133–144
688.78	LGTALNT	166–172
1348.61	PPDVLISRILPE	231–242
668.74	YSSSIL	264–270
1832.05	INNIINSWNRWTTSI	195–209

4. Discussion

This paper described the purification and characterization of a hydrolase with chitinase and lysozyme activity from the secretions of a plant weed called morning glory. The molecular weight 30 kDa of the reported enzyme ICChII is comparable to other known plant chitinase (both intracellular and extracellular) with a molecular weight between 25 and 35 kDa [43]. The isoelectric point of the enzyme was 4.6, as determined by IEF electrophoresis. In addition to endochitinase activity, ICChII exhibits lysozyme activity, as observed in several other chitinases [17]. The enzyme contains carbohydrate moieties and stable in broad range of temperature and pH. Based on the N-terminal sequence alignment, ICChII chitinase shared identity and similarity to known class III chitinases. The important feathers and characteristics of the reported enzyme includes bifunctional nature viz. chitinase/lysozyme activity, acidic iso-electric point; extremely high stability against temperature and pH; monomeric; good yield; glycosylation; broad stability range; three disulfide bridges; hydrophobic amino acid residues 8 tryptophan, 14 tyrosine; and higher N-terminal identity with other known plant chitinases. The polyclonal antibodies raised for ICChII would be of immense importance in detecting and as a ligand for various future studies, and thus may demand in several industrial and biotechnological applications. The economic purification procedure combined with the easy availability of the plant weed may makes large-scale preparation of the enzyme allowing a broad study of its various aspects and hence probable applications in agriculture, industry, environmental protection fungal biotechnology and pharmaceutical fields. The biotechnological applications of chitinases are diverse and widely used in agriculture, industry, environmental protection, and employed in recycling chitinous waste from arthropod shellfish and for chitooligosaccharide production.

ICChII is fairly stable enzyme similar to hevamine, a glycosyl hydrolase family 18 chitinase isolated from the latex of *H. brasiliensis*. The structure of hevamine is well known and it consists of $(\beta\alpha)_8$ barrel fold [44]. Further unlike hevamine, ICChII is a glycosylated protein and upon deglycosylation it aggregates and loses activity indicating a possible role of glycans in structural integrity. Moreover, the glycoprotein nature of the protein could also be a contributing factor in high thermal stability [45]. In our experience, ICChII is stable for 6 months at 4 °C, under neutral conditions without any detectable loss in activity and thus it may find industrial and biotechnological application.

The majority of chitinases/lysozymes identified to date in plants have been basic proteins. A major difference between ICChII and hevamine chitinase is that the former is an acidic protein and the latter a basic one. However like ICChII, cucumber chitinase is an acidic protein but no lysozyme activity was found in highly purified preparations of cucumber chitinase, although lysozyme activity was present in crude extracts of cucumber leaves [17]. An extracellular acidic chitinase purified from yam tubers also lacked lysozyme activity [46]. An intact cell wall is of vital importance for the survival of bacteria. Compounds that interfere with the strictly regulated activity of the enzymes involved in the synthesis and degradation of the cell wall peptidoglycan have been found to be effective antibiotics. Whereas penicillin and related antibiotics inactivate the enzymes that make and break the peptide bonds in the peptidoglycan, only a few inhibitors are known for the enzymes that act on the glycan strands of peptidoglycan [47]. The reported chitinase is catalyzing the cleavage of the β-1,4-glycosidic bond between an N-acetylmuramic acid residue and N-acetylglucosamine residue in peptidoglycan which suggests other biological implications of ICChII enzyme.

The chitinase activity of ICChII was inhibited by allosamidin, a potent inhibitor that strongly inhibits glycosyl hydrolase family 18 chitinase, like the hevamine for which the 3D structure of the enzyme with a bound inhibitor is described [47]. However the allosamidin did not inhibit the yam chitinase. The similar inhibition profile suggests that ICChII may be a member of glycosyl hydrolase family 18 chitinase. Further, structure and sequence analysis is needed to classify ICChII. ICChII shows a high sequence similarity with hevamine as well as several other known plant chitinases. ICChII has 73% N-terminal identity with hevamine and other well known plant chitinases playing a role in plant defense. 97 amino acid residue of ICChII generated from tryptic fragments and *de novo* sequencing showed a good score with hevamine.

Many plant produce chitinases and other so-called pathogenesis proteins to deal with stressful conditions such as wounding and pathogen attack [48]. The presence of chitinases especially in the latex secretion is to retard attack by fungi and insects which contain chitin in their cell wall and exoskeletons. As chitin is not a component of plant, the presence of ICChII in high levels suggests a possible role of ICChII in protection of morning glory from the phytopathogens. The diversity and complexity of plant latex may have arisen from longstanding hostile relationships between plants and herbivores. In short, in a manner analogous to animal venoms, plant latexes are diverse mixtures and treasuries of defense proteins and secondary metabolites, yet unknown, playing important roles in inter-specific (plant-herbivore) interactions, and may prove applicable to agricultural and medical fields [49]. Therefore plant latex from *I. carnea* could be good target to discover defense proteins. The presence of large amount of chitinases in latex may also have role in the protection of embryo and the young seedlings and in addition may function as protectants of stored food reserves of the endosperm from fungi and insects. ICChII like other known chitinases may be considered as potential biocontrol agents and find application in isolation of fungal protoplast, preparation of bioactive chito-oligosaccharides, and reclamation of shellfish waste chitin and production of single cell protein [50].

Since, the chitinases/lysozyme appears to behave like a biological response modifier that elicits host responses by interacting with pathogens, therefore the elucidation of the catalytic mechanisms of ICChII as well as other biological functions reported for chitinase/lysozyme is now in progress with the aid of crystallization and high resolution 3D structure determination.

Clearly, much work remains to be done to determine the specific function of ICChII in *I. carnea* plant latex. Further gene sequence and structural determination may increase our understanding of the function of this chitinase in latex and result in the production of transgenic plants with increased resistance to fungal pathogens.

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