Appl Biochem Biotechnol (2010) 160:1004–1016
DOI 10.1007/s12010-009-8530-2

Purification and Characterization of a Novel Heparin Degrading Enzyme from *Aspergillus flavus* (MTCC-8654)

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Received: 21 November 2008 / Accepted: 20 January 2009 /
Published online: 13 February 2009
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Abstract A heparinase-producing fungus was isolated, and the strain was taxonomically characterized as *Aspergillus flavus* by morphophysiological and 26S rRNA gene homology studies. The culture produced intracellular heparinase enzyme, which was purified 40.5-fold by DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-100 column chromatography. Specific activity of the purified enzyme was found to be 44.6 IU/μg protein and the molecular weight of native as well as reduced heparinase was 24 kDa, showing a monomeric unit structure. Peptide mass spectrum showed poor homogeneity with the database in the peptide bank. The enzyme activity was maximum at 30 °C in the presence of 300 mM NaCl at pH 7.0. In the presence of Co^{2+}, Mn^{2+} ions, and reducing agents (β-mercaptoethanol, dithiothreitol), enzyme activity was enhanced and inhibited by iodoacetic acid. These observations suggested that free sulfohydryl groups of cysteine residues were necessary for catalytic activity of the enzyme. The enzyme was also inhibited by histidine modifier, DEPC, which suggests that along with cysteine, histidine may be present at its active site. The enzyme showed a high affinity for heparin as a substrate with K_m and V_{max} as 2.2×10^{-5} M and 30.8 mM min^{-1}, respectively. The affinity of the enzyme for different glycosaminoglycans studied varied, with high substrate specificity toward heparin and heparin-derived polysaccharides. Depolymerization of heparin and fractionation of the oligosaccharides yielded heparin disaccharides as main product.

Keywords *Aspergillus flavus* · Glycosaminoglycans · Heparinase · Inducible · Purification · rRNA gene homology

Introduction

Heparin, a linear highly sulfated glycosaminoglycan produced by mast cells, is a widely used clinical anticoagulant and is one of the few biopolymeric carbohydrate drugs. Low

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molecular weight heparins (LMWHs) are derived from unfractionated heparin via controlled chemical or enzymatic breakdown [1]. LMWHs thus produced have enhanced efficacy (reduced rate of venous thromboembolism recurrence, thrombus extension, and mortality) and safety (lower rates of major bleeding) as compared to heparin [2]. Enzymatic depolymerization of heparin to generate LMWHs is done by commercially available heparinases from *Pedobacter heparinus*. However, other microorganisms like *Bacillus circulans*, *Sphingobacterium*, and *Bacteroides* are reported and patented for the production of heparinase [3–5]. Heparinases are useful for a variety of purposes, including sequencing of heparin and heparin-like glycosaminoglycans (HLGAGs), neutralization of heparin/heparan sulfate/LMWHs, inhibition of angiogenesis, and normalization of prothrombin and thromboplastin times of heparin containing plasma samples [6, 7]. The ability to inhibit neovascularization, tumor cell growth, and or metastasis is reported for both heparinases and the LMWHs [8, 9].

Heparinases employ two distinct mechanisms to break down HLGAGs: hydrolysis and β-elimination. The mammalian enzyme heparanase (of eukaryotic origin), an endo-β-glucuronidase, cleaves heparan sulfate utilizing a hydrolytic mechanism. It involves the addition of a water molecule to form saturated oligosaccharide products. Bacterial heparinases, in contrast, cleave their polysaccharide substrates through an elimination mechanism [10]. Fungi, especially Aspergilli, are known to produce heparinases but their purification, characterization, and mode of action have not been reported to date [11, 12]. In this paper, we are reporting the isolation and identification of a novel heparinase-producing fungal culture, *Aspergillus flavus* (MTCC-8654). Production and purification of a 24 kDa novel inducible heparinase enzyme is also being reported. Characteristics of this enzyme have been compared with the heparinases obtained from other microbial sources.

Materials and Methods

Microorganism, Culture Medium, and Heparinase Production

The heparinase-producing fungus, isolated from soil samples of basin and sub-basin of a large river of northern India, was characterized morpho-physiologically as well as by rRNA gene homology study at the Microbial Type Culture Collection, Chandigarh, India, and the culture has been deposited there with an accession number MTCC-8654. Universal primers, specific for 26S rRNA gene, were used for targeting conserved regions between internal transcribed spacers together with the divergent domains D1/D2 region in polymerase chain reaction (PCR) [13]. The amplicon was purified using a QIAquick PCR purification Kit (Qiagen, Crawley, UK) prior to sequencing to remove dNTPs, polymerases, salts, and primers. Purified amplicons were sequenced in an ABI PRISMTM dye terminator cycle sequencing reaction with AmpliTaq DNA polymerase and the forward 26S rRNA gene primer. Sequence data were compared with known rDNA sequences deposited in the NCBI and GenBank nucleotide databases using blastn alignment software. Phylogenetic tree was constructed to predict the species level characterization of the studied isolate. The 26 S rRNA gene sequence has been submitted with EMBL nucleotide database with accession number FM210756.

The culture was grown for 48 h on an orbital shaker at 180 rpm and at 28 °C in a 2-L flask containing 0.5 L optimized production medium [14] with the following composition (grams per liter): mannitol, 8.0; NH_4NO_3, 2; K_2HPO_4, 2.5; Na_2HPO_4, 2.5; $MgSO_4.7H_2O$, 0.5; chitin, 12.5; heparin, 0.2; trace salt solution ($NaMoO_4.2H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CaCl_2$), 10^{-4} M. All the medium components were procured

from HiMedia and other chemicals were of Sigma. Mycelial biomass was harvested by centrifugation at 4,500×*g* for 30 min at 4 °C. The biomass was washed twice with saline containing 10 mM Tris buffer pH 7.0 and then resuspended in the same buffer. The mycelial suspension was disrupted by a 10 min period of sonication for 30 s intervals on an ultrasonic processor at 80% output with cooling. Mycelial debris was removed by centrifugation at 6,000×*g* for 15 min at 4 °C and heparinase activity was assessed.

Heparinase Assay

Heparinase activity was determined by following the method of Linker and Hovingh [15], which is based on the estimation of the reaction product (uronic acid, ΔUA) absorption at 232 nm by using heparin (porcine intestinal mucosa, 12 kDa, Sigma) as a substrate. The assay solution contained 55 μL of heparin at a concentration of 20 mg/ml in 375 μL of 20 mM Tris buffer at pH 7.5 containing 50 mM NaCl and 4 mM $CaCl_2$. To the assay solution, enzyme was added at a protein concentration of ≤0.01 mg/ml. The samples were incubated at 30 °C for 5 min, and the reaction was stopped by addition of 2.5 ml of 0.05 M hydrochloric acid. The rate of formation of product (uronic acid, ΔUA) was monitored at 232 nm with respect to the control, and enzyme activity was calculated using a molar extinction coefficient of 3,800 M^{-1} cm^{-1} for unsaturated oligosaccharide products formed by heparinase (1 IU=1 μmol of ΔUA containing product formed/min) [16]. Specific activity was calculated by dividing the number of micromoles of product produced per minute by the quantity of protein in micrograms per milliliter. Protein concentrations were determined using the Folin Lowry assay and a bovine serum albumin standard curve.

Purification of Heparinase

Nucleic acid in the cell extract was precipitated with protamine sulfate at a concentration of 1 mg/mg protein. The supernatant was passed through a DEAE-Sephadex A-50 column (5×30 cm) that had been pre-equilibrated with 10 mM Tris buffer (pH 7.0). The column was then eluted with the same buffer until no heparinase activity could be detected. Active fractions were pooled, concentrated, and applied to a column packed with CM-Sephadex C-50 (2.6×45 cm) previously equilibrated with the 10 mM Tris buffer pH 7.0. Elution was performed using a step gradient of NaCl from 0 to 0.2 M in 10 mM Tris buffer (pH 7.0). Active fractions were pooled, desalted, concentrated, and applied onto a Sephadex G-100 column (1.8×30 cm). Tris buffer (10 mM) pH 7.0 was used to elute the enzyme.

Characterization of Purified Heparinase

Purity of the heparinase was confirmed by performing HPLC on μBondapak amino column (waters) of dimensions 0.46×25 cm using dual face programmable LC-10 AD pump from Schimadzu, SPD-10 A UV–Vis detector and a chromatopak C-R6 A integrating recorder. The HPLC was performed by using acetonitrile/water (60:40) as mobile phase with a flow rate of 0.5 ml and detection was done at 280 nm. Molecular weight of the purified enzyme was determined by ESI-MS.

Polyacrylamide Gel Electrophoresis and Peptide Mass Fingerprinting

Discontinuous SDS-PAGE (12.5%) was performed to determine apparent molecular mass of the purified heparinase, and the gels were stained with Coomassie brilliant blue R-250

(CBB R-250) or silver stain. The purified protein stained with CBB R-250 was excised for in situ gel digestion. The gel was washed with 50% acetonitrile in 0.1 M NH_4HCO_3 and vacuum-dried [17]. The gel fragments were reduced for 45 min at 55 °C in 10 mM dithiothreitol (DTT) in 0.1 M NH_4HCO_3. After cooling, the DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3. After 30-min incubation at room temperature in the dark, the gel fragments were washed with 50% acetonitrile in 0.1 M NH_4HCO_3 and dried in a SpeedVac evaporator. The dried gel pieces were swollen in a minimum volume of a digestion buffer that contained 25 mM NH_4HCO_3 and 12.5 ng/μL of trypsin (Sigma, sequencing grade) and incubated at 37 °C overnight. One volume of a solution composed of distilled water/acetonitrile/trifluoro-acetic acid (93:5:2) was mixed with the digestion mixture. The sample was then cleaned by zip tip clean up and matrix-assisted laser desorption/ionization-time of flight mass spectra was obtained. The obtained mass fingerprint data sets were analyzed using Mascot mass fingerprinting database.

Characterization of the Catalytic Activity of Heparinase

The optimum pH for heparinase activity was determined using 20 mM Tris buffer at different pH (6.0–8.5). Heparinase was incubated with 1 mg of heparin at 30 °C for 5 min, and enzyme activity was determined by measuring the increase in absorbance at 232 nm. Enzyme activity was also measured at various ionic strengths (0–500 mM) of NaCl.

To investigate the effect of temperature (15–60 °C), heparinase was pre-incubated with 20 mM Tris buffer pH 7.5 for 5 min at each temperature without substrate. The substrate was added to the mixture, and activity was determined by measuring the increase in absorbance at 232 nm. Stability of the heparinase at −20, 4, and 28 °C was assessed for 72 h under standard enzyme assay conditions.

Effect of chloride salt of divalent metals and amino-acid modifiers on heparinase activity was assessed at 100 mM concentration with 20 mM Tris buffer, pH 7.5.

Kinetic Studies of Heparinase

The kinetic constant of heparinase was determined at 30 °C by measuring the initial rates of heparin degradation as a function of heparin concentration in the range 20 to 500 μM under standard reaction conditions. Reaction rates were measured by monitoring the increase of absorbance at 232 nm.

Substrate Specificity Studies

Complex glycosaminoglycans, heparin (porcine intestinal mucosa, 12 kDa), heparin (bovine, ≥140 USP units/mg), heparin sodium (low molecular weight, 6,000), sodium heparan sulfate (fast-moving fraction, porcine intestinal mucosa ≥90% in electrophoresis), hyaluronic acid, and chondroitin sulfate A, each at the concentration of 1 mg/ml, were incubated with heparinase under the standard assay conditions and the reactions were monitored at 232 nm. The final level of glycosaminoglycans depolymerization was expressed as percent activity, relative to heparin as a substrate.

Depolymerization of Heparin and Gel Permeation of Oligosaccharides

Heparin (2.5 mg in 20 ml of 0.25 M sodium acetate and 0.0025 M calcium acetate at pH 7.0) was digested by purified heparinase (44.6 IU/μg protein) for 48 h at 28 °C. The

reaction was monitored by increase in absorbance at 232 nm and stopped by heating at 100 °C, and the enzyme was removed by binding to CM-Sephadex C-50 at pH 3.5. Heparin fragments were separated on a Sephadex G-25 column (2.5×60 cm) equilibrated and eluted with 0.2 M NaCl, by taking heparin disaccharide (Sigma) as external standard.

Results

The fungal culture was able to grow on various media including czapekdox agar, *Aspergillus* differential agar (AFDA), Czapek yeast agar (CYA media), yeast extract sucrose (YES media), where it gave an orange-yellow-colored pigmentation on the reverse side with a blue-green fluorescent area surrounding colonies, as observed under far UV light. The mycelial color in the preliminary stages of growth was white, followed by appearance of yellowish sporulation after 36–48 h of growth, which on further incubation, turned to dark-green-colored asexual conidiospores and dark sclerotia, which were typical characters of *Aspergillus* section flavi [13]. Large sclerotia were formed near the center of the colony. The conidiophore were with globose vesicles and uniseriate. The strain was classified as L strain of *Aspergillus flavus* since it produced small numbers of large sclerotia [18].

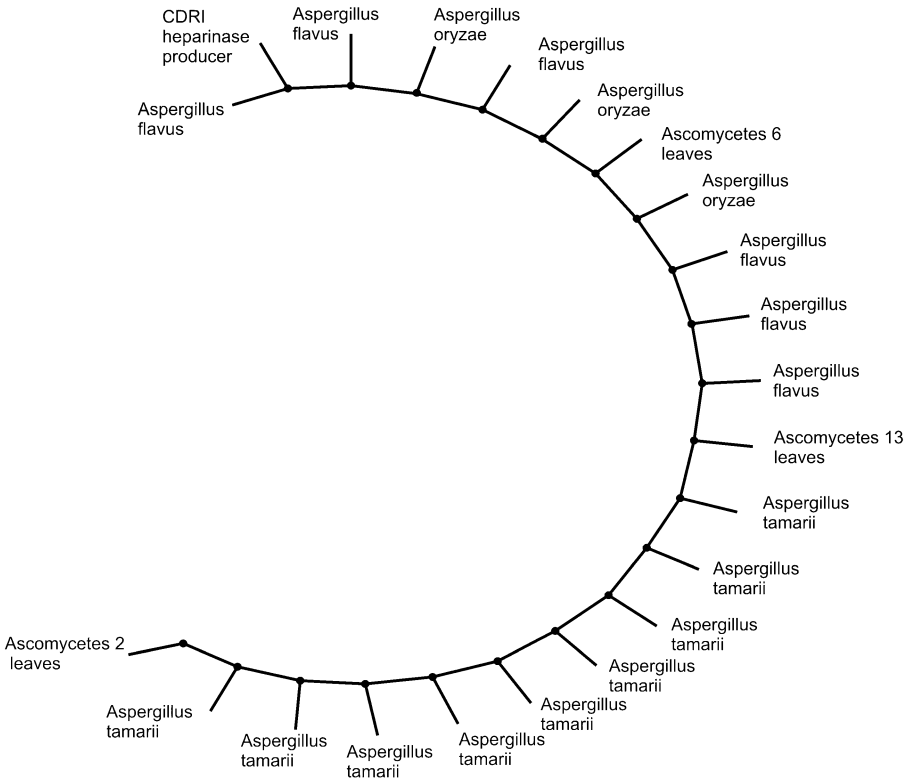
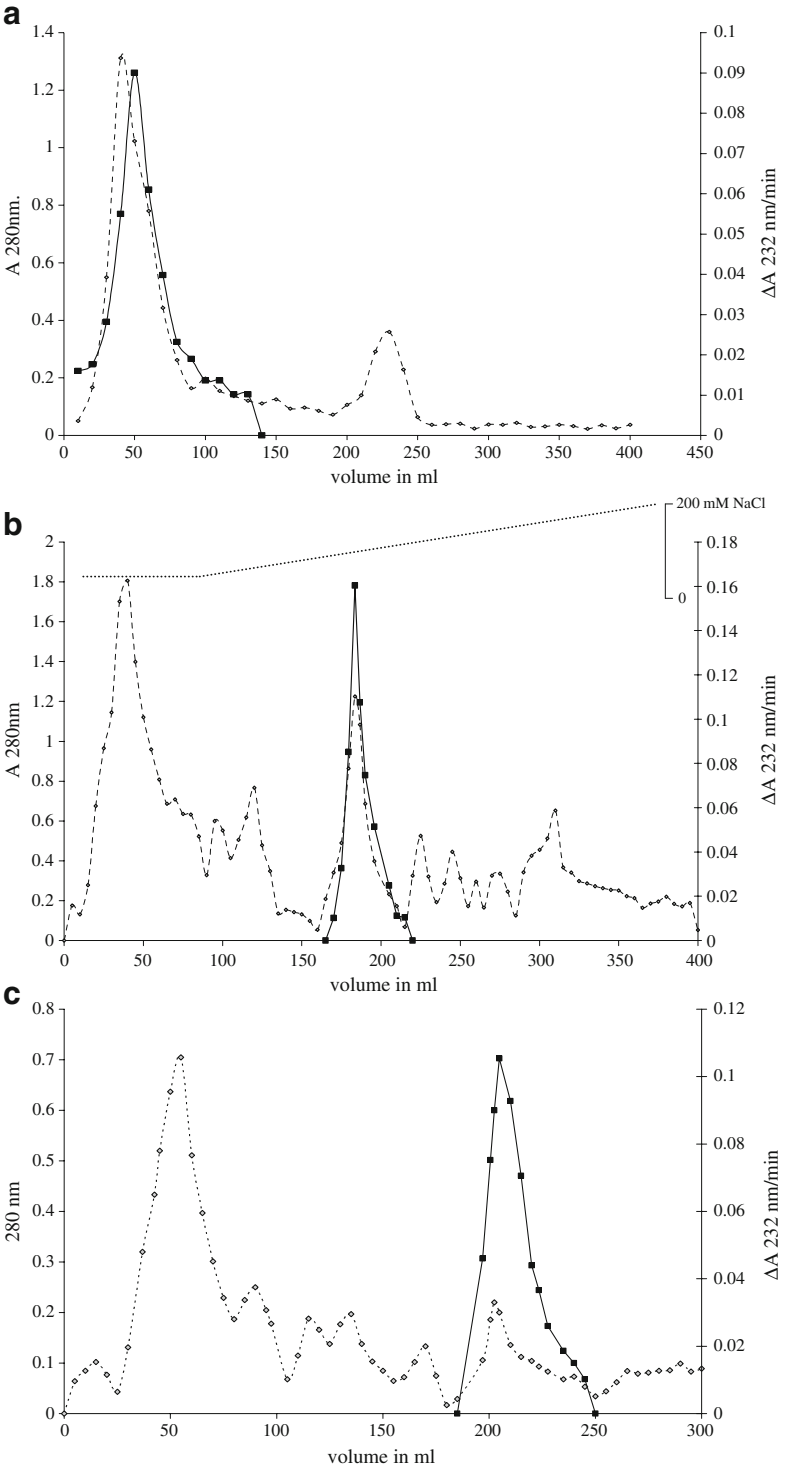


Fig. 1 Phylogenetic tree illustrating 99.6% homology of the soil-isolated heparinase-producing fungus with *A. flavus* NRRL20521 (EF661547). This tree was produced using BLAST pairwise alignments. It is a forced, un-rooted tree, where nodes are pushed away from one another for better presentation


a
A 280nm.
ΔA 232 nm/min
volume in ml
b
200 mM NaCl
0
A 280nm
ΔA 232 nm/min
volume in ml
c
280 nm
ΔA 232 nm/min
volume in ml

◀ **Fig. 2** Elution profiles from **a** DEAE-Sephadex A-50, **b** CM-Sephadex C-50, and **c** Sephadex G 100 column chromatography (*diamonds* 280 nm; *squares* 232 nm/min)

The 26S rDNA amplicon corresponded to 636 bp, and the gene sequence homology analysis identified the organism having 99.7% homology with *A. flavus* strains, accession numbers EF661563 and EF661547, and 99.5% homology with *Aspergillus oryzae*, accession number EF634406 (Fig. 1).

Purification of Heparinase

The heparinase of *A. flavus* is an intracellularly produced enzyme released by sonication, and the nucleic acids were precipitated by protamine sulfate. The enzyme was purified by sequential chromatographic steps on DEAE-Sephadex A-50, CM-Sephadex C-50, and gel-filtration (Fig. 2). Heparinase passed through the DEAE-Sephadex A-50 column without binding to the matrix and was enriched 2.4-fold with respect to the cell extract (Table 1). The enzyme was further purified by using CM-Sephadex C-50 column chromatography at 40 mM NaCl in 10 mM Tris buffer, pH 7.0 and loaded onto Sephadex G-100, where it was enriched by 40.5 times with respect to crude extract. The purified enzyme gave a single band on SDS-PAGE (Fig. 3) and a single peak at 6.8 min retention time in HPLC column chromatography (Fig. 4). The molecular weight of fungal heparinase was ~24,000 Da for reduced enzyme and 23,380 Da for native enzyme as determined by SDS-PAGE and ESI-MS, respectively. Similarity search in the EBSCO database revealed that the peptide mass fingerprint of the enzyme exhibited no significant homology to any other reported microbial peptides.

Characterization of the Catalytic Activity of Heparinase

Heparinase activity increased with increasing NaCl concentration with maxima at 300 mM (Fig. 5). The optimal heparinase activity was found at pH 7.0, and the heparinase was highly active within the temperature range of 25 to 35 °C but its activity was lost rapidly above 35 °C (Fig. 5). The enzyme was stored for 72 h at −20 °C and at 4 °C and was found to be stable with <12% and <22% loss in activity, respectively. The catalytic activity of the enzyme was maintained for ~72 h, at 28 °C with 40.4% loss in activity. The heparinase activity was highest in the presence of Co^{2+} and Mn^{2+}, but inhibited by Pb^{2+}, Cd^{2+}, Ba^{2+}, and Zn^{2+} (Table 2). Among amino-acid modifiers, reducing agents (DTT, β-mercaptoethanol) and

Table 1 Purification summary of heparinase of *Aspergillus flavus*.

Purification step	Total units (IU)	Total protein (μg)	Specific activity (IU/μg protein)	Fold purification
Crude	504,631.6	467,408.7	1.1	1.0
Protamine sulfate	463,719.3	367,186.8	1.3	1.2
DEAE-Sephadex A-50	22,512.3	8,739.5	2.6	2.4
CM-Sephadex C-50	4,575.4	501.4	9.1	8.3
Sephadex G-100	4,084.2	91.5	44.6	40.5

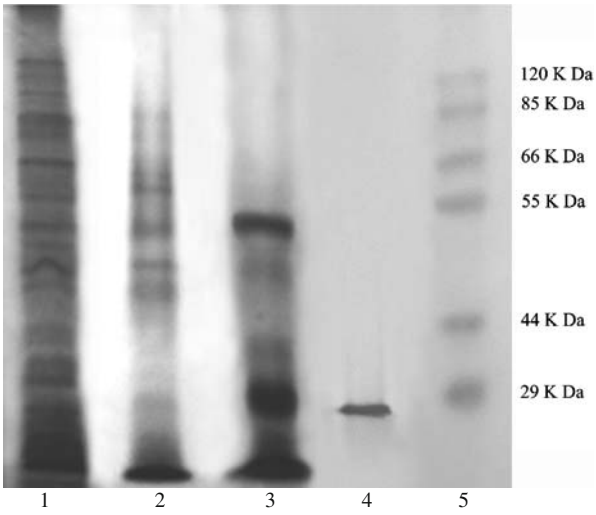


Fig. 3 SDS-PAGE of the purified heparinase at various steps of purification. *Lane 1* Preparation after crude extract, *lane 2* preparation after anion exchange (DEAE-Sephadex A-50), *lane 3* preparation after cation exchange (CM-Sephadex C-50), *lane 4* purified heparinase preparation after gel permeation (Sephadex G100) column chromatography, *lane 5* molecular weight markers

EDTA, a metal ion chelator, enhanced the enzyme activity (Table 2), whereas, cysteine modifier (IAA) and histidine modifier (DEPC) inhibited the enzyme activity.

Michaelis–Menten constant was determined using a Lineweaver–Burk plot. The K_m and V_{max} of heparinase toward heparin were calculated as 2.2×10^{-5} M and 30.8 mM min^{-1}, respectively. This heparinase degraded heparin (porcine and bovine) and to a limited extent heparan sulfate. Heparinase activity for heparin was taken as 100%, and the relative

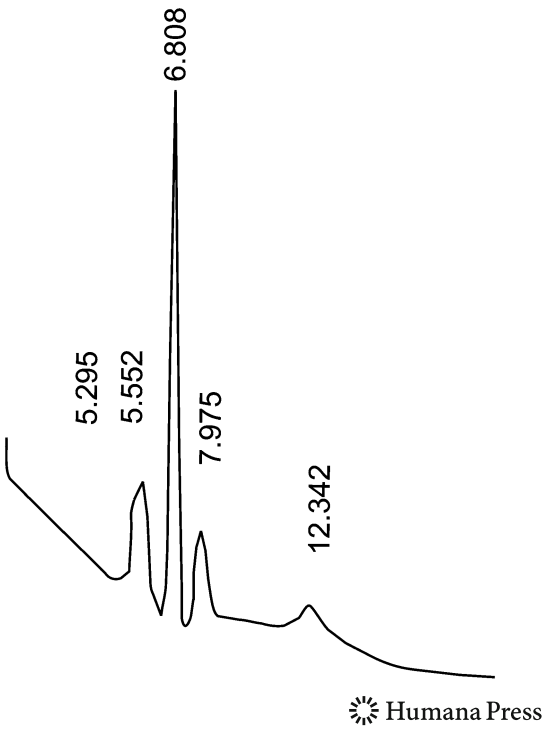


Fig. 4 HPLC profile of the purified heparinase from *A. flavus*

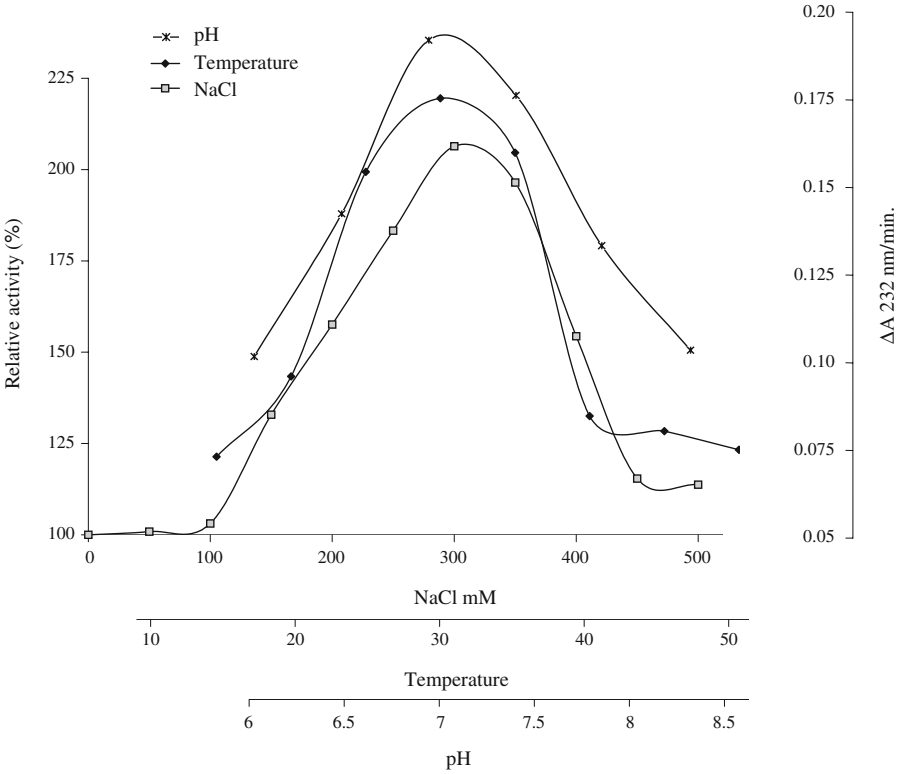


Fig. 5 Effect of pH and temperature on the catalytic activity of heparinase, represented as Δ *A* 232 nm/min. Effect of NaCl concentration on heparinase activity, represented as (percent) relative activity. Relative activity was calculated by taking the enzyme activity in the absence of NaCl as 100%

catalytic activity for heparan sulfate as substrate was 20.2%, but hyaluronic acid was not degraded (Table 3).

Depolymerization of Heparin and Gel Permeation of Oligosaccharides

Heparin was depolymerized till a constant absorbance at 232 nm was obtained. Since the enzyme retains 59.6% activity till 72 h at 28 °C, the depolymerization continued for 48 h without addition of fresh enzyme in the reaction. The mixture of oligosaccharide was fractionated by Sephadex G25 fine gel permeation chromatography using 0.2 M NaCl as eluent (Fig. 6). The chromatogram showed that heparinase of fungal origin cleaved heparin to generate lower molecular weight oligosaccharides with disaccharide as the major constituent, as shown by the heparin-disaccharide external standard.

Discussion

Aspergillus is an economically, ecologically, and medically important fungal genus. Although morpho-physiological methods are commonly employed in the systematics of *Aspergillus* sp., phylogenetic analysis of sequences of the ribosomal RNA gene cluster is useful for clarifying

Table 2 Effect of divalent metal ions and amino-acid-modifying agents (100 mM each) on heparinase activity.

Divalent cations/amino-acid modifiers	Residual activity (%)
Control	100.00
Co^{2+}	159.44
Cu^{2+}	126.85
Ni^{2+}	85.59
Ba^{2+}	63.78
Zn^{2+}	86.29
Pb^{2+}	77.17
Mg^{2+}	108.74
Ca^{2+}	89.29
Cd^{2+}	72.07
Mn^{2+}	140.94
Fe^{2+}	133.93
β-mercaptoethanol	153.06
DTT	137.12
DEPC*	34.44
Iodo acetic acid	40.82
EDTA	169.01

DTT dithiothreitol, *DEPC* diethylene pyrocarbonate, *EDTA* ethylene diamine tetra acetic acid

taxonomic relationships among Aspergilli [13]. However, rDNA homology is not able to distinguish between species belonging to the *Aspergillus* section *flavi*, mainly, *A. flavus*, *Aspergillus parasiticus*, and *A. oryzae* [19], so morpho-physiological characteristics and rDNA homology were employed to further consolidate the identity of the fungus as *A. flavus*.

Aspergillus nidulans is reported to produce heparinase but purification and characterization of the heparinase has not been reported [11, 12]. *A. flavus* produces heparinase inducibly at a volumetric yield of 2.2 IU/μg protein (Table 1), whereas *Pedobacter heparinus* produces 1.6 IU/μg protein of heparinase I for 1 L broth each [16].

The molecular weight of the purified heparinase was observed to be 23,380 Da, much lower than the 43,000 Da of heparinase I of *P. heparinus* and 46,000 Da of heparinase of *Bacteroides* [4, 16]. Similarity search in the EBSCO database revealed that the peptide

Table 3 Substrate specificity of the heparinase of *A. flavus* and heparinases of *Pedobacter heparinus*.

Substrates	Relative activity (%)			
	Heparinase of *A. flavus*	Heparinase of *Pedobacter heparinus*[a]		
		I	II	III
Heparin (porcine)	100	100	100	0
Heparin (bovine)	75.4	–	–	–
Heparan sulfate (porcine)	20.2	30	172	100
Heparin (LMWH, 6,000)	65.8	–	–	–
Hyaluronic acid	0	0	0	0
Chondroitin sulfate A	0	0	0	0

[a] Relative activity of heparinases of *Pedobacter heparinus* was obtained from Lohse and Linhardt [16]

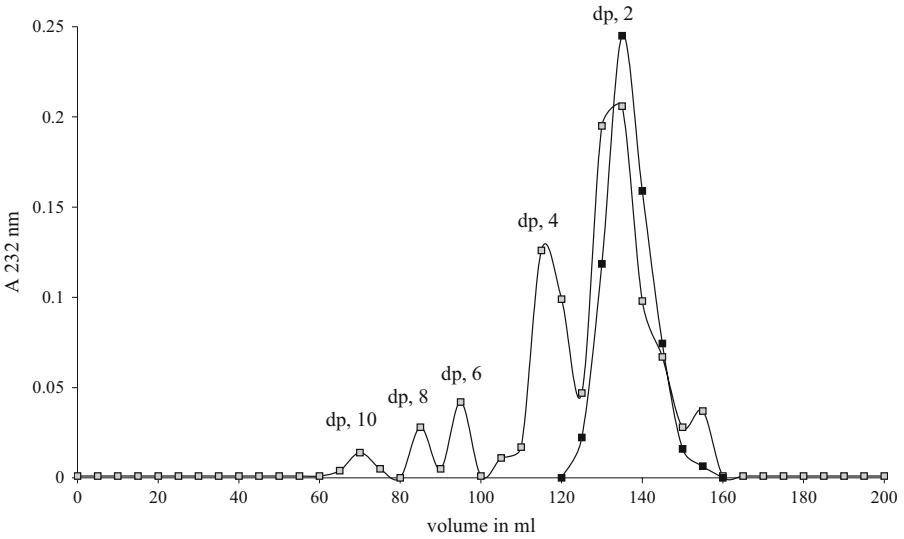


Fig. 6 Oligosaccharides formed separated by Sephadex G25 column chromatography as detected at 232 nm (*gray squares*) and standard heparin disaccharide (*black squares*)

mass fingerprint of the enzyme exhibited no significant homology to any other reported microbial peptides and is most likely a novel protein. All the three heparinases of *P. heparinus* as well as heparinase from *Sphingobacterium* differ in their peptide mass spectra, indicating that products of different genes are structurally dissimilar though all cleave heparin or HS by eliminase mechanism [5, 20].

The heparinase degraded heparin (porcine and bovine) and to a limited extent heparan sulfate, with a K_m for heparin (porcine) as substrate, similar to the K_m (1.8×10^{-5} M) of heparinase I [16]. This reveals that the enzyme has high substrate specificity toward heparin and heparin-derived polysaccharides, with limited or no specificity toward heparin-like polysaccharides.

The effect of temperature and pH on heparinase of *A. flavus* is similar to heparinase I with optima at 30 °C and pH 7.0, with 59.6% retention in activity under these conditions after 72 h of incubation. The pH optima of all the three heparinases from *P. heparinus* are reported to range between 30 and 35 °C, but heparinase I and III are very unstable at this temperature range, where heparinase I loses 80% of its activity in 5 h at 30 °C and heparinase III loses 80% of its activity in 3.5 h at 35 °C [20]. Heparinase II on the other hand is a stable enzyme and retains 70% of its activity toward both heparin and HS after 25 h at 35 °C [20]. Heparinase from *A. flavus* showed increase in enzyme activity with increasing NaCl molarity with maxima at 300 mM, whereas heparinases from *P. heparinus* and *B. circulans* are reported to be completely inhibited to as higher as 400 mM NaCl concentration [3, 20]. Heparinase I is reported to show an increase in activity in response to increased salt concentrations, with an optimum at 100 mM. But, heparinase II and III show a decrease in activity with increasing concentration of salt [20], although other heparinase from *Bacteroides* and gram-negative bacterium show high salt concentration optima of 500 mM and 223 mM, respectively [4, 21].

Reducing agents and EDTA are reported to enhance the enzymatic activity of heparinases from different sources [4, 20]. Different metals and heavy metals show varying

affect toward different heparinases, as heparinase I from *P. heparinus* and *Bacteroides heparinolyticus* is inhibited by Cu^{2+}, Hg^{2+} and heparinase from *Bacillus* is inhibited by Cu^{2+}, Cu^{+}, Zn^{2+}, Fe^{3+}, Hg^{2+}, Co^{2+}, and Ni^{2+}. Heparinase I from *P. heparinus* and *B. heparinolyticus* is activated by Ca^{2+} and Fe^{2+}, respectively, and heparinase from *Bacillus* is activated by Ca^{2+}, Mg^{2+}, and Ba^{2+} [22]. Cysteine modifier (IAA) and histidine modifier (DEPC) inhibited the heparinase from *A. flavus*, which suggested that cysteine and histidine, like that of heparinase I and II [10, 23, 24], may be present at the active site of this enzyme. In addition, both thiol reducing reagents (DTT, β-mercaptoethanol) enhanced enzyme activity, suggesting that disulfide linkage is not essential for the enzyme activity, which is also supported by the decrease in enzyme activity in the presence of IAA, a carboxy-methylating agent of the sulfohydryl group of cysteine.

The oligosaccharide generation from cleavage of heparin by heparinase of *A. flavus* resulted in increase in absorbance as measured at 232 nm. The complete depolymerization of heparin by heparinase from *A. flavus* yielded disaccharides as the main product along with the production of oligosaccharides ranging from dp 4 to dp 10. Studies of heparin depolymerization with heparinase I have reported similar substrate specificity and digestion profile [25, 26]. The products of heparin degradation with heparinases from *B. heparinolyticus*, *Bacillus* sp., and from an unclassified bacterium [21] are consistent with those found for degradation with heparinase I from *P. heparinus* [22]. This suggests that this 24 kDa enzyme may also be using a similar method as of heparinase I for cleaving hexosamine–glucuronic acid linkages in heparin.

Conclusion

The studies conducted at heparinase of *A. flavus* revealed that the substrate specificity, K_m and oligosaccharide generation profile resembled that of heparinase I of *P. heparinus*, but the two enzymes differed in molecular weight, peptide mass spectrum, and salt tolerance. Ability of mass production of a fungal culture and stability of the enzyme at room temperature make it useful for the generation of heparin oligosaccharides, active as antithrombotics. Further studies on the mode of action, structural details, and genomics of this heparinase shall be useful in elucidating the role of fungi in producing heparinases.

Acknowledgement This research was financially supported by grant from University Grants Commision, South Campus, Delhi University, Benito Juarez Marg, New Delhi-110021.

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