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RESEARCH ARTICLE

Chitinase from *Enterobacter* sp. NRG4: Its purification, characterization and reaction pattern

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Enterobacter* sp. NRG4 was shown to excrete chitinase into the culture supernatant when cultivated in medium containing chitin. A 60 kDa extracellular chitinase was purified to homogeneity and characterized. The enzyme hydrolyzed swollen chitin, colloidal chitin, regenerated chitin and glycol chitin but did not hydrolyze chitosan. The chitinase exhibited K_m and V_{max} values of 1.43 mg ml^{-1} and 83.33 µM μg^{-1} h^{-1} for swollen chitin, 1.41 mg ml^{-1} and 74.07 µM μg^{-1} h^{-1} for colloidal chitin, 1.8 mg ml^{-1} and 40 µM μg^{-1} h^{-1} for regenerated chitin and 2.0 mg ml^{-1} and 33.33 µM μg^{-1} h^{-1} for glycol chitin, respectively. The optimal temperature and pH for activity were 45ºC and pH 5.5, respectively. Mg^{2+}, K^+ and Ca^{2+} stimulated chitinase activity by 13, 16 and 18%, respectively whereas Cu^{2+}, Co^{2+}, Ag^+ and Hg^{2+} inhibited chitinase activity by 9.7, 15, 22 and 72.2%, respectively at 1 mM concentration. N-bromosuccinamide (NBS) at 1 mM and iodoacetamide at 10 mM concentration completely inhibited the enzyme activity. Dithiobisnitrobenzoic acid (DTNB) at 10 mM concentration inhibited chitinase activity by 97.2%. Chitin was hydrolyzed to chitobiose and N-acetyl D-glucosamine when incubated with the purified enzyme. The hydrolysis pattern of the purified enzyme indicated that the chitinase was an endochitinase.

Chitin is composed of repeating N-acetyl D-glucosamine residues and is a component of crustacean exoskeleton, diatoms, fungal cell walls, and squid pens. Chitin is a versatile and promising biopolymer with numerous industrial, medical and commercial uses. However, it is difficult to purify and modify chemically. Hence identification of chitin modifying enzymes and elucidation of their activities could facilitate the efficient production of specific chitin products. The biodegradation of chitin requires the synergistic action of several hydrolytic enzymes

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Table 1. Purification of chitinase by ammonium sulphate, DEAE-Sephadex column chromatography and Sephadex G-200 gel filtration.

Purification Step	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Cell free supernatant	45100	255	176.4	-	100
Dialysed (ammonium sulphate precipitation 30-75%)	31950	57	560.5	3.18	71.0
DEAE Sephadex	22500	5.5	4090.9	23.2	47.1
Gel filtration (Sephadex G-200)	14010	1.8	7783.3	44.12	31.1

for efficient and complete breakdown. The combined action of endochitinases (EC 3.2.1.14) and exochitinases [(chitobiosidases and β-N-acetyl hexosaminidase (EC 3.2.1.82)] results in the degradation of chitin polymer into the soluble N-acetyl D-glucosamine (Gkargkas et al. 2004). Chitinases are produced by different micro-organisms which generally present a wide multiplicity of enzymes that are mainly extracellular. They have received increased attention due to their wide range of biotechnological applications, especially in the production of chito-oligosaccharides and N-acetyl D-glucosamine (Pichyangkura et al. 2002), biocontrol of pathogenic fungi (Chernin et al. 1997; Mathivanan et al. 1998), preparation of sphaeroplasts and protoplasts from yeast and fungal species (Mizuno et al. 1997; Balasubramanium et al. 2003) and bioconversion of chitin waste to single cell protein (Vyas and Deshpande, 1991).

In the present investigation we report an endochitinase that was purified and characterized from a newly isolated *Enterobacter* sp. NRG4.

MATERIALS AND METHODS

Chemicals and substrates

Flake chitin was obtained from Hi-Media, India. Swollen chitin was prepared by the method of Monreal and Reese (1969), colloidal chitin by the method of Jeuniaux (1966),

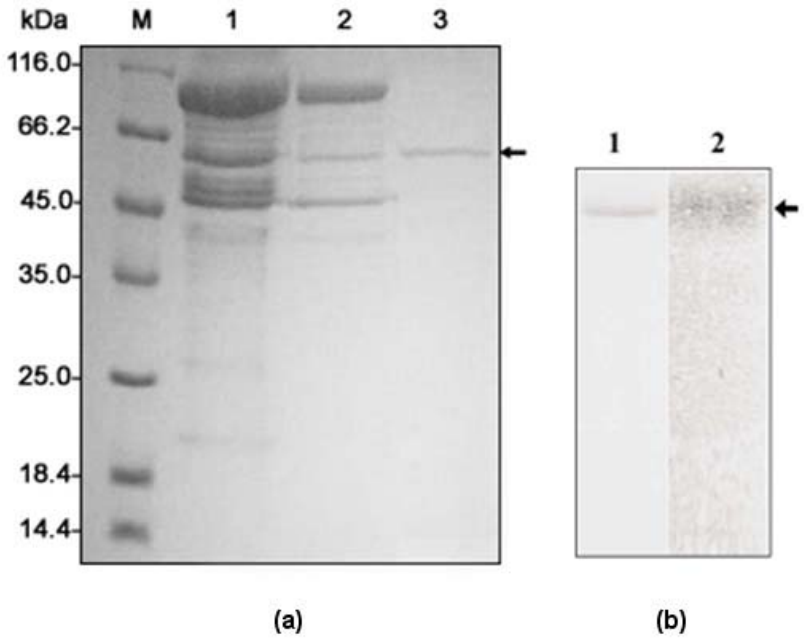

Figure 1. SDS-PAGE analysis of chitinase purified from *Enterobacter* sp. NRG4.
(a) Lane M, Molecular weight markers; Lane 1, Ammonium sulphate precipitated proteins (100 µg); Lane 2, Ion exchange purified proteins (30 µg); Lane 3, Sephadex G-200 purified chitinase (6 µg).
(b) Lane 1, Native-PAGE; Lane 2, Zymogram of purified chitinase of *Enterobacter* sp. NRG4.

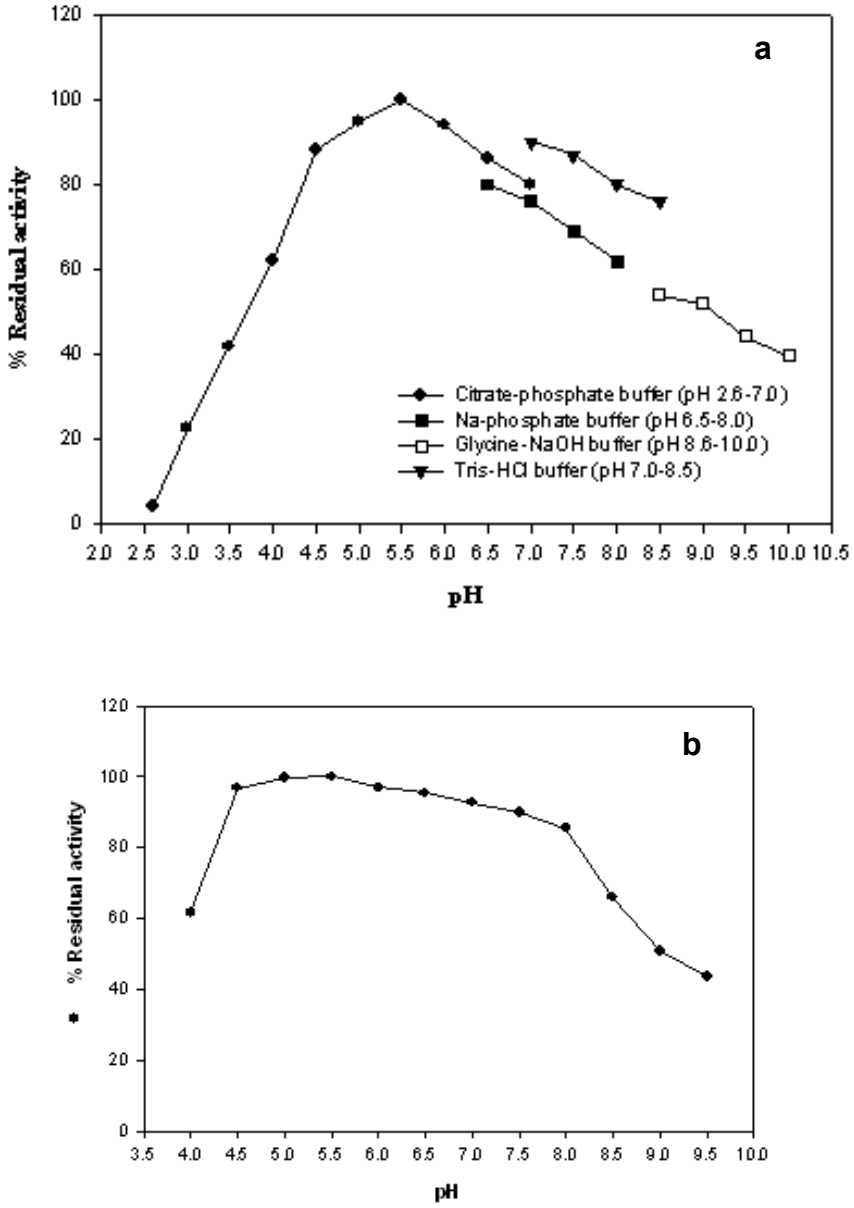

Figure 2.
(a) Effect of assay pH on purified chitinase activity.
(b) pH stability profile of purified chitinase from *Enterobacter* sp. NRG4.

regenerated chitin by the method of Molano et al. (1977) and glycol chitin by the method of Yamada and Imoto (1981). N-acetyl D-glucosamine was obtained from Fluka and chitobiose from Sigma, Co. All other reagents used were of analytical grade.

Micro-organism and culture conditions

Enterobacter sp. NRG4 isolated from degraded stalk of mushroom was selected as a potent chitinase producer (Dahiya et al. 2005). The culture medium was composed of 1.0% swollen chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 and 0.01% $MgSO_4.7H_2O$ (pH 8.0). The micro-organism was cultivated at 30ºC for 72 hrs with agitation at 150 rpm.

Enzyme and protein assay

The assay mixture contained 1 ml swollen chitin and 0.5 ml enzyme solution. After incubation at 45ºC for 15 min, it was centrifuged at 5000 x g for 10 min. The amount of N-acetyl D-glucosamine released in the supernatant was determined by the method of Reissig et al. (1955), using N-acetyl D-glucosamine as the standard. One enzyme unit

Table 2. Kinetic parameters for chitin hydrolysis by *Enterobacter* sp. NRG4 chitinase.

Substrate (1.0%)	K_m (mg ml^{-1})	V_{max} (µmole $µg^{-1}$ h^{-1})
Swollen chitin	1.43	83.33
Colloidal chitin	1.41	74.07
Regenerated chitin	1.8	40.00
Glycol chitin	2.0	33.33

was defined as the amount of enzyme that catalyzes the release of 1 µmol of N-acetyl D-glucosamine in 1 hr at 45ºC.

The protein concentration was measured using the method of Lowry et al. (1951) with bovine serum albumin as standard. For the purified enzyme, protein concentration was measured by determining the absorbance at 280 nm.

Purification of chitinase

The purification of chitinase was carried out in three steps. The cell free supernatant was precipitated with 30% ammonium sulphate. The resultant precipitate was centrifuged at 10,000 x g, 4ºC. Then ammonium sulphate concentration was increased to 75% saturation and it was left overnight at 4ºC. The precipitate was collected by centrifugation at 10,000 x g, 4ºC. It was dissolved in 50 ml of 25 mM Tris-HCl buffer pH 7.5 and dialysed against the same buffer.

The dialysed protein was subjected to ion exchanger, DEAE-Sephadex column (1.5 x 12 cm). The adsorbed chitinase was eluted by a linear gradient of NaCl from 0 to 0.25 M in the same buffer. Chitinase activity was assayed in each 5.0 ml fraction at a flow rate of 42 ml h^{-1}. In final step, the active fractions were pooled, concentrated by polyethylene glycol and dialysed against Tris-HCl, pH 7.5 and loaded onto a gel filtration column (2.2 cm x 90 cm), Sephadex G-200 and flow rate was maintained at 20 ml h-1. The molecular weight was estimated from a standard curve obtained from the proteins with their molecular weights known (68 kDa, bovine serum albumin, 45 kDa, ovalalbumin and 30 kDa, casein).

The purified protein was loaded onto SDS-PAGE (12%) as described by Laemmli (1970) to determine the protein profile. Native PAGE was carried out with the aim to study the zymography pattern of chitinase. The detailed procedure was exactly similar to the SDS-PAGE in which SDS, mercaptoethanol and the heating step during protein sample preparation were eliminated. The native PAGE gel was run with purified chitinase preparation. Half of the gel was cut and stained to locate the position of single band and the other half of the gel was placed over chitin agar plate (1.0% swollen chitin in citrate phosphate buffer + 1.5% agar) and incubated overnight at 45ºC to find the zone of clearance.

Characterization of purified chitinase

The purified chitinase was characterized with respect to its optimum pH, temperature, stability at different temperatures and pH values, effect of metal ions, surfactants, organic solvents on activity and stability.

Chitinase activity was assayed at different pH values (pH 2.6 to 10.0) using different buffers 50 mM such as citrate-phosphate buffer (pH, 2.6-7.0), sodium phosphate buffer (pH, 6.5-8.0), tris-HCl buffer (pH, 7.0-8.5) and glycine-NaOH buffer (pH, 8.6-10.0). To determine pH stability, chitinase preparations in buffer at different pH ranging from 4.0-9.0 were kept at room temperature for 2 hrs. Thereafter chitinase activity was assayed under standard conditions.

Chitinase activity was assayed at different temperatures ranging from 35-60ºC at pH 5.5 in citrate phosphate buffer (50 mM). To determine thermostability, chitinase preparation was incubated at temperature ranging from 40-55ºC for different time intervals up to 3 hrs. Chitinase activity was assayed at 45ºC and pH 5.5.

The effect of substrate concentration on chitinase activity was determined at different concentrations of chitin, varying between 0.25 mg ml^{-1} to 16 mg ml^{-1} (w/v). The K_m and V_{max} values were determined by Lineweaver-Burk's plot.

The effect of metal ions on enzyme activity was studied by incorporating these metal ions such as $MgSO_4.7H_2O$, KCl, $CaCl_2$, $2H_2O$, $CuCl_2$, $2H_2O$, $HgCl_2$, $AgNO_3$, $CoCl_2$, $2H_2O$, $ZnSO_4$, $FeCl_3$ and $FeSO_4$ in reaction mixture at 1 mM to100 mM concentration. Effects of these metal ions on enzyme stability were studied by incubating the purified enzyme in 25 mM Tris-HCl buffer, pH 7.5, with these metal ion salts at room temperature for 1 hr and subsequently determining the residual enzyme activity under standard assay conditions.

Allosamidin was added to the enzyme solution in the concentration range from 1 to 100 µg ml^{-1} and incubated at room temperature for 1 hr. Thereafter, residual enzyme activity was determined under standard assay conditions. The effect of sugars such as N-acetyl D-glucosamine, glucosamine HCl, galactosamine and glucose was studied by incorporating these sugars at 1 mM and 10 mM concentration in the reaction mixture and subsequently determining the enzyme activity.

Substrate binding was determined by incubating the enzyme with 10 mg substrate in citrate phosphate buffer (50 mM, pH 5.5) for 30 min at 0ºC with intermittent

shaking followed by centrifugation at 4ºC and residual activity was determined in the supernatant under standard assay conditions.

Chemical modification of chitinase was done using several reagents such as para chloromecuribenzoate (PCMB), N-bromosuccinimide (NBS), 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB), iodoacetamide and methylene blue. The effects of these modifiers were tested by incubating the enzyme with varying concentrations (0.1 mM to 10 mM) of the modifiers in the reaction mixture.

Hydrolysis pattern of purified chitinase

The mode of action of chitinase was determined by viscometric assay (Otakara, 1961). Purified chitinase (60 µg) was added to 60 ml substrate solution (5g L^{-1} glycol chitin in 50 mM citrate phosphate buffer, pH, 5.5) and the mixture was incubated at 45ºC for digestion. Aliquots (10 ml each) were removed at intervals and subjected immediately to viscosity measurement on an Ostwald viscometer. After incubation of the enzyme with 1.0% swollen chitin at 45ºC for 5 hrs, the hydrolytic products of chitin were resolved by high performance liquid chromatography (HPLC) (Shimadzu, USA). The HPLC system was fitted with LC1oAT HPLC pumps and a SPD-M10A detector. A reversed phase 100 NH_2 (4 x 250 mm) column from Shimadzu was used. The samples were eluted with 75% (v/v) aceton1itrile in water with a flow rate of 1.0 ml min^{-1}, and the injection volume was 20 µl. The aliquots

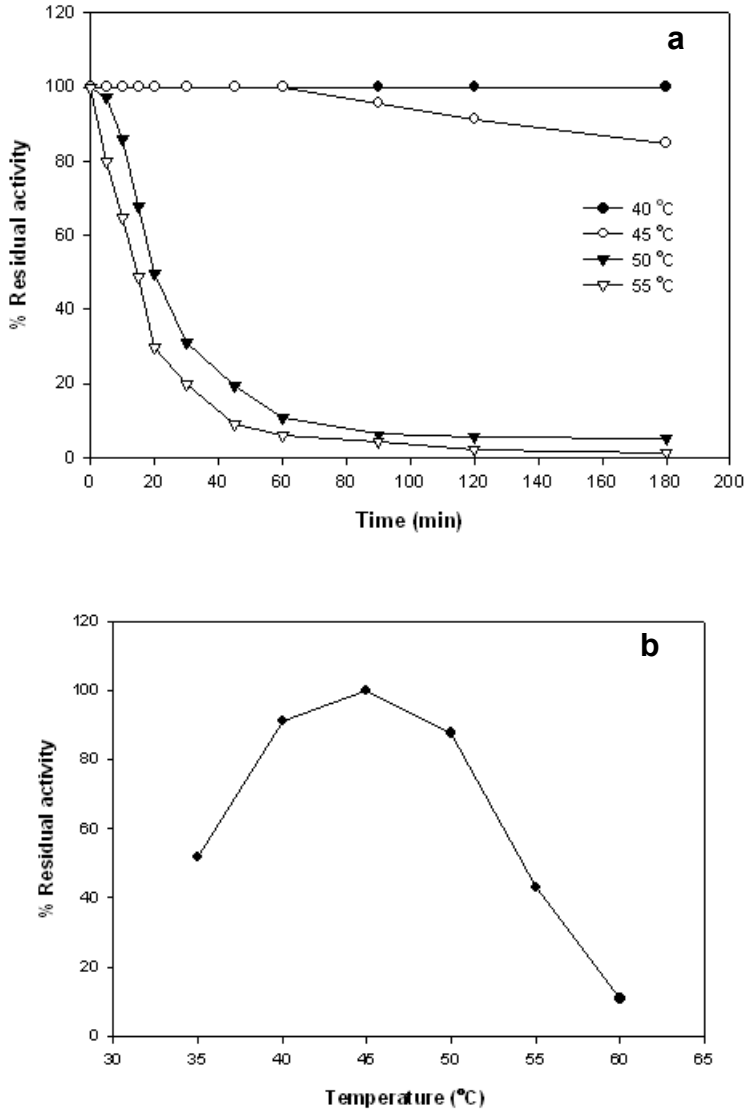

Figure 3.
(a) Effect of assay temperature on purified chitinase activity.
(b) Thermostability profile of purified chitinase from *Enterobacter* sp. NRG4.

were monitored for UV absorbance at 205 nm. N-acetyl D-glucosamine and chitobiose were used as standards. The peak areas of standard solutions and hydrolyzates were integrated by SPD-MXA real time software (Shimadzu, USA).

RESULTS

Purification of chitinase

With swollen chitin as the sole source of carbon, *Enterobacter* sp. NRG4 produced chitinase in the culture medium. The chitinase was purified using standard techniques *i.e.* ammonium sulphate precipitation (30-75%), DEAE-Sephadex ion exchange chromatography and Sephadex G-200 gel filtration chromatography. When cell free supernatant was subjected to fractional ammonium sulphate precipitation, chitinase activity was precipitated in 30-75% salt saturation. The yield of chitinase was 71% with a purification fold of 3.18 and specific activity of 560.5 U mg^{-1} protein. The dialyzed protein was loaded on DEAE ion exchanger. After elution with 0 to 250 mM NaCl gradient two major peaks of proteins were observed but chitinase activity was observed only in peak A. Here the yield of chitinase was 47.1% with a purification fold of 23.2 and specific activity 4090.9 U mg^{-1}. Using gel filtration, the chitinase was purified by 44.12 fold with specific activity of 7783.3 U mg^{-1} and the yield was 31.1%. The results of chitinase purification are summarized in Table 1. The molecular weight of the chitinase was estimated to be 60 kDa by SDS-PAGE (Figure 1a). It was consistent with the molecular mass determined by Sephadex G 200 gel filtration, suggesting that the purified chitinase is a monomer type. Native gel electrophoresis showed a single band which corresponded to chitinase activity as shown by the hydrolysis zone in the zymogram (Figure 1b).

Characterization of purified chitinase

The chitinase was maximally active at pH 4.5 to 8.0 thus exhibiting a broad pH optima (Figure 2a). Determination of pH stability of the chitinase indicated that the enzyme was stable between pH 4.5 to 8.0 and it retained 90% of its activity in this range (Figure 2b). The purified enzyme showed its maximum activity at 45ºC and was stable at 40ºC for 3 hrs (Figure 3).

With acid swollen chitin, colloidal chitin, regenerated chitin and glycol chitin the purified chitinase gave K_m of 1.43 mg ml^{-1}, 1.41 mg ml^{-1}, 1.8 mg ml^{-1} and 2.0 mg ml^{-1}, respectively and V_{max} were 83.33 µmole μg^{-1} h^{-1}, 74.07 µmole μg^{-1} h^{-1}, 40.00 µmole μg^{-1} h^{-1} and 33.33 µmole μg^{-1} h^{-1}, respectively (Table 2).

The enzyme showed activities towards swollen chitin, colloidal chitin, glycol chitin and regenerated chitin but exhibited no activity towards carboxymethyl cellulose, chitosan and *Micrococcus lysodeikticus* cell wall. When swollen chitin was used as substrate the activity was taken as 100. The activities with colloidal chitin, regenerated chitins, glycol chitin, flake chitin and crab shell chitin were 80.3, 44.7, 39.4, 5.9 and 2.3%, respectively. *Enterobacter* sp. NRG4 chitinase reduced the viscosity of glycol chitin significantly in 5 min due to cleavage of chitin long chains by the chitinase at 45ºC (Figure 4). Thus it was concluded

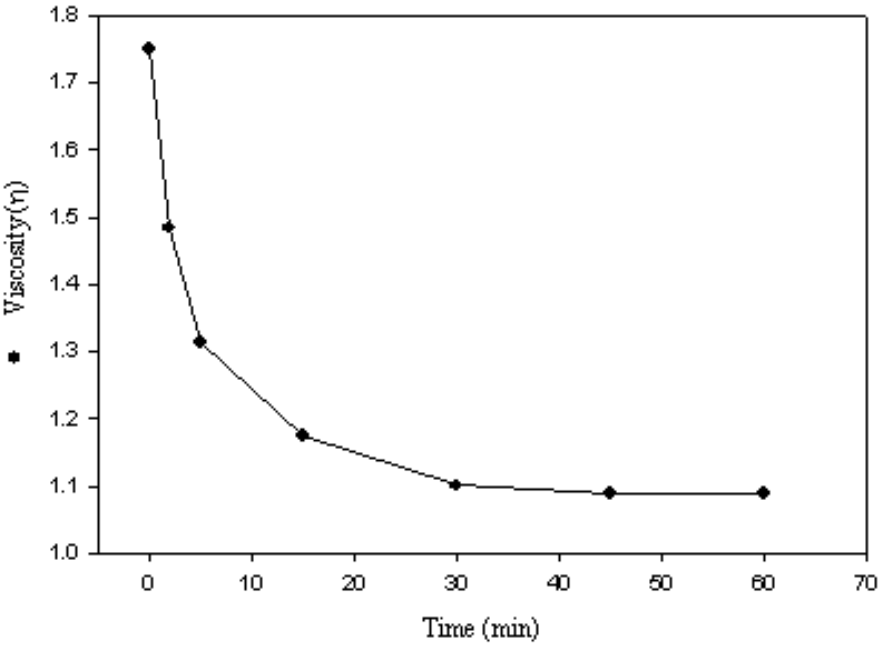

Figure 4. Hydrolysis of glycol chitin by purified chitinase.

Table 3. Comparison of the characteristics of purified chitinase from other reported *Enterobacter* sp.

Species	Chitinase	Mol. wt. (kDa)	Optimum pH	Optimum temp. (°C)	Substrate	Hydrolysis product/s	Inhibitors	Reference
Enterobacter sp. NRG4	Endochitinase	60	5.5	45	Swollen chitin	(GlcNAc) $(GlcNAc)_2$	Cu^{2+}, Co^{2+}, Ag^{+}, Hg^{2+}, NBS, DTNB, iodoacetamide	Present study
Enterobacte sp. G-1	Endochitinase	60	7.0	40	Colloidal chitin	$(GlcNAc)_2$ $(GlcNAc)_3$ $(GlcNAc)_4$	EDTA, PCMB	Park et al. 1992
Enterobacter aerogenes	N.D.	42.5	6.0	55	-	-	Hg^{2+}, Co^{2+} Mg^{2+}	Tang et al. 2001
Enterobacter agglomerans	Endochitinase	61	6.5	40	pNP-$(GlcNAc)_3$	pNP	-	Chernin et al. 1997

that the purified chitinase has endo-splitting activity. As shown in Figure 5 hydrolyzed products of enzymatic reaction of purified enzyme were $(GlcNAc)_2$ and N-acetyl D-glucosamine.

Chitinase exhibited a substrate binding capacity of 89.5, 26.2 and 15.2% for swollen chitin, flake chitin and carboxymethyl cellulose, respectively whereas no significant substrate binding was observed for pectin, starch, xylan, wheat bran and chitosan (Figure 6).

Mg^{2+}, K^{+} and Ca^{2+} stimulated chitinase activity by 13, 16 and 18%, respectively whereas Cu^{2+}, Co^{2+}, Ag^{+} and Hg^{2+} inhibited chitinase activity by 9.7, 15, 22 and 72.2%, respectively at 1mM concentration. At 100 mM concentration Cu^{2+}, Ag^{+} and Hg^{2+} completely inhibited chitinase activity when incubated at room temperature for 1 hr whereas Zn^{2+}, Fe^{3+}, Co^{2+} and Fe^{2+} inhibited chitinase activity by 98.3, 90.0, 89.5 and 83.7%, respectively.

Allosamidin, a known specific inhibitor of chitinase inhibited *Enterobacter* sp. NRG4 chitinase by 57.1 and 65.7% at a concentration of 50 and 100 µg ml^{-1}, respectively, with an IC_{50} value of 40 µg ml^{-1} (64 µM) (Figure 7). Study of end-products and sugars on chitinase activity showed that N-acetyl D-glucosamine, glucosamine HCl, galactosamine and glucose inhibited enzyme activity by 10, 8, 4 and 9.1% at 1 mM concentration and by 81.3, 19.0, 26.0 and 19.0%, respectively at 10 mM concentration of these sugars.

Iodoacetamide inhibited chitinase activity by 17.6, 66.2 and 84.5%, respectively at 0.1 mM, 1 mM and 5 mM concentration. DTNB inhibited chitinase activity by 1.5, 30.6, 77.5 and 97.2%, respectively at 0.1 mM, 1 mM, 5 mM and 10 mM concentration, respectively. NBS at 1 mM and iodoacetamide at 10 mM concentration completely inhibited the enzyme activity. PCMB did not affect the enzyme activity significantly. EDTA at 1 mM concentration inhibited chitinase activity by 11%.

DISCUSSION

An extracellular chitinase secreted by *Enterobacter* sp. NRG4 was purified to homogeneity by combination of ammonium sulphate precipitation, DEAE Sephadex ion exchange chromatography and Sephadex G-200 gel flitration chromatography. The chitinase showed a single band on 12% SDS-PAGE and Native PAGE indicating the complete purification of the enzyme. The molecular weight of the protein was found to be about 60 kDa by SDS-PAGE as well as by gel filtration chromatography. The chitinase from *Enterobacter* sp. NRG4 was active over broad pH range *i.e.* from pH 4.5-8.0, optimum being 5.5. Several workers have reported broad pH optima like pH 4.5-7.5 of chitinase from *Bacillus cereus* (Pleban et al. 1997), pH 5.0-8.0 for *Aeromonas hydrophila* H-2330 (Hiraga et al. 1997), pH 7.5-9.0 for *Bacillus* sp. BG-11 (Bhushan and Hoondal, 1998). The pH optima for other chitinases reported were pH 4.0 for *Aeromonas* sp. No. 10S-24 (Ueda et al. 1995), pH 5.0 for *Alcaligenes xylosoxydans* (Vaidya et al. 2001) and *Arthrobacter* sp. NHB-10 (Okazaki et al. 1999), pH 5.5 for *Bacillus* sp. WY22 (Woo and Park, 2003), pH 6.0 for *Enterobacter* sp. G-1 (Park et al. 1997), pH 5.4 and 6.6 for CHIT60 and CHIT100, respectively from *Serratia plymuthica* HRO-C48 (Frankowski et al. 2001), pH 6.3 for *Bacillus* sp. NCTU2 (Wen et al. 2002), pH 6.5 for *Vibrio alginolyticus* H-8 (Ohishi et al. 1996) and *Vibrio* sp. (Zhou et al. 1999), pH 7.0 for *Monascus purpureus* (Wang et al. 2002), pH 7.0-8.0 for *Bacillus* 13.26 (Yuli et al. 2004) and pH 10.0 for *Cellulomonas flavigena* NTOU1 (Chen et al. 1997).

The chitinase from the present strain was stable over wide pH range *i.e.* from pH 4.5 to 8.0. Other bacterial chitinase stable over broad pH range were pH 4.0 to 9.0 of *Aeromonas* sp. No. 10S-24 chitinase (Ueda et al. 1995), pH 6.0 to 9.0 of *Pseudomonas aeruginosa* K-187 (Wang and Chang, 1997), pH 5.0 to 8.0 of *Aeromonas hydrophila* H2330 chitinase (Hiraga et al. 1997), pH 4.0 to 9.0 for *Vibrio* sp. (Zhou et al. 1999), pH 6.8 to 8.0 of *Bacillus* sp. NCTU2 (Wen et al. 2002) chitinase and pH 4.0 to 8.5 of *Bacillus cereus* strain 65 (Pleban et al. 1997).

The temperature activity and stability profile of *Enterobacter* sp. NRG4 chitinase revealed that the enzyme was optimally active at 45ºC. It was stable at 40ºC for more than 3 hrs and for 1 hr at 45ºC. It retained 84.4% activity after 3 hrs at 45ºC. The temperature optima of *Enterobacter* sp. NRG4 was in accordance with other reports in literature such as *Arthrobacter* sp. NHBN-10 (Okazaki et al. 1999), *Vibrio alginolyticus* TK-22 (Ohishi et al. 1996). Chitinase from *Vibrio alginolyticus* TK-22 was stable at 40ºC for 30 min (Ohishi et al. 1996) and purified chitinase of *Vibrio* sp. P-6-1 was stable at 40ºC but completely inactivated at 55ºC in 30 min (Takahashi et al. 1993).

The K_m values of the *Enterobacter* sp. NRG4 chitinase against different substrates were 1.43 mg ml^{-1}, 1.41 mg ml^{-1}, 1.8 mg ml^{-1} and 2.0 mg ml^{-1}, respectively with swollen chitin, colloidal chitin, regenerated chitin and glycol chitin respectively, which are comparatively lower than the other reports in literature. The K_m values of chitinase from different organisms were, 2.88 mg ml^{-1} for *Enterobacter aerogenes* (Tang et al. 2001), 1.4 mg ml^{-1} and 0.8 mg ml^{-1} for chitinase C1 and C3 from *Vibrio alginolyticus* H-8 against squid chitin (Ohishi et al. 1996), 3.0 mg ml^{-1} for *Alcaligenes xylosoxydans* chitinase (Vaidya et al. 2003) and *Bacillus* sp. WY22 chitinase (Woo and Park, 2003), 12 mg ml^{-1} for *Bacillus* sp. BG-11 chitinase (Bhushan and Hoondal, 1998).

Ethylene glycol chitin, glycol chitin and colloidal chitin are useful substrate for enzyme assays of endo-type chitinase

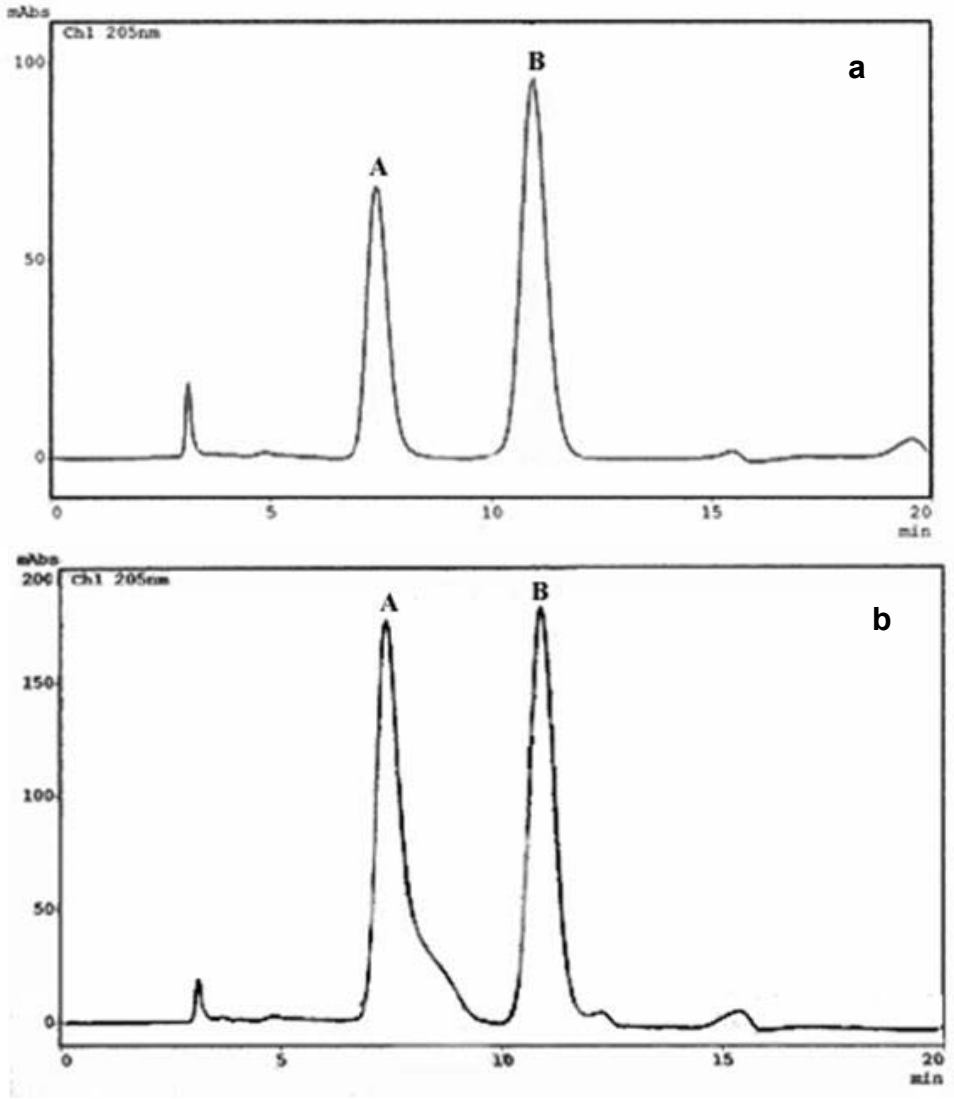

Figure 5. High Performance Liquid Chromatography of hydrolysis product/s of chitin by purified chitinase of *Enterobacter* sp. NRG4.
(a) Standards: A. N-acetyl D-glucosamine, B. chitobiose.
(b) Hydrolysed product/s of chitin after 5 hrs of enzyme-substrate reaction (1.0% swollen chitin + 10 U ml^{-1} chitinase).

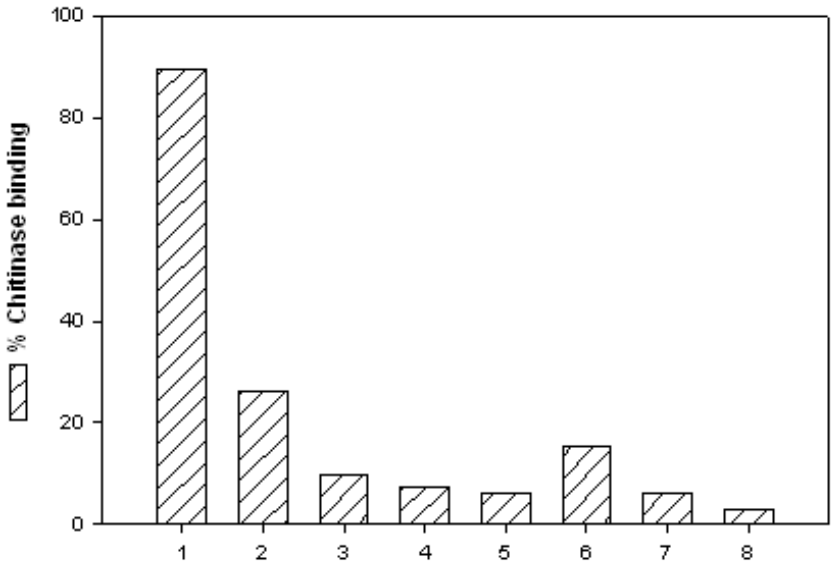

Figure 6. Substrate binding profile of chitinase at C for h. 1, Swollen chitin; 2, Flake chitin; 3, Wheat bran; 4, Chitosan; 5, Pectin; 6, Carboxymethyl cellulose; 7, Xylan; 8, Starch.

(Park et al. 1997). The hydrolysis pattern of purified enzyme indicated that chitinase from *Enterobacter* sp. NRG4 was an endochitinase. It exhibited high activity towards swollen chitin, colloidal chitin, regenerated chitin and glycol chitin as compared to flake chitin and crab shell chitin. It showed no activity towards carboxymethyl cellulose, chitosan and *Micrococcus lysodeikticus* cell wall. The hydrolysis products from swollen chitin were $(GlcNAc)_2$ and GlcNAc. *Enterobacter* sp. G-1 was also reported to secrete an endochitinase which showed high activity towards colloidal chitin and ethylene glycol chitin more than flake chitin or soluble CMC. It could not hydrolyze flake chitosan but showed 36 to 80% activity towards deacetylated chitosan compared with colloidal chitin. The products from colloidal chitin hydrolysis were mainly $(GlcNAc)_2$ with small amount of $(GlcNAc)_3$ and $(GlcNAc)_4$ (Park et al. 1997). Characteristics of purified chitinases from other reported *Enterobacter* spp. are summarized in Table 3. *Aeromonas* sp. chitinase I and II hydrolyzed colloidal chitin and ethylene glycol chitin effectively but the activity was significantly lower towards chitin and chitosan. No detectable activities towards *Micrococcus lysodeikticus* cell wall were observed (Ueda and Arai, 1992). Chitinase exhibited a substrate binding capacity of 89.5, 26.2 and 15.2% for swollen chitin, flake chitin and carboxymethyl cellulose, respectively. Lee et al. (2000) reported binding of *Pseudomonas* sp. YHS-A2 chitinase 78, 12, 0, 5 and 10% with colloidal chitin, chitin, carboxymethyl cellulose, crude chitosan and birch wood xylan, respectively.

Among metal ions, Mg^{2+}, K^{+} and Ca^{2+} stimulated chitinase activity by 13, 16 and 18%, respectively whereas Cu^{2+}, Co^{2+}, Ag^{+} and Hg^{2+} and inhibited chitinase activity by 9.7, 15, 22 and 72.2%, respectively at 1 mM concentration. Activation of chitinase by Ca^{2+} or Mg^{2+} is rare and reported in few cases only. At 100 mM concentration Cu^{2+} and Ag^{+} completely inhibited chitinase activity when incubated at room temperature for 1 hr. In *Pseudomonas aeruginosa*, Mg^{2+} and Na^{+} were inhibitory while Cu^{2+} activated the chitinase by 50% (Wang and Chang, 1997). Stimulatory effect of Ca^{2+} (30%) and Mn^{2+} (20%) at 1 mM concentration on *Pseudomonas* sp. YHS-A2 chitinase has been reported by Lee et al. (2000).

Serratia plymuthica activity was stimulated by 120, 150 and 240% in presence of 10 mM Ca^{2+}, Co^{2+} or Mn^{2+} and inhibited by 80% in presence of 10 mM Cu^{2+} (Frankowski et al. 2001). Chitinase from *Alcaligenes xylosoxydans* was inhibited by 25% by Cu^{2+} and Na^{+} at 5 mM but not by Ca^{2+}, Ba^{2+} or Mg^{2+} at the same concentration (Vaidya et al. 2003). *Enterobacter* sp. G-1 chitinase activity was not affected by addition of Ca^{2+} or NaCl to the enzyme solution (Park et al. 1997). Chitinase from *Enterobacter aerogenes* was stimulated by Zn^{2+}, Ba^{2+}, Ca^{2+} and Mn^{2+} and strongly inhibited by Hg^{2+}, Co^{2+} and Mg^{2+} (Tang et al. 2001). Hg^{+} and Hg^{2+} inhibited chitinase of *Streptomyces* sp. M-20 (Kim et al. 2003). Frankowski et al. (2001) reported stimulation of CHIT60 from *Serratia plymuthica* HRO-C48 by 10 mM Ca^{2+}, Co^{2+} or Mn^{2+} and inhibition in presence of Cu^{2+}. In contrast, Mn^{2+} and Ca^{2+} inhibited chitinase of *Bacillus* sp. 13.26 (Yuli et al. 2004). Ag^{+} and Hg^{2+} inhibited chitinase C1 and C3 from *Vibrio alginolyticus* H-8 (Ohishi et al. 1996). Hg^{2+} also inhibited chitinases from *Arthrobacter* sp. NHB-10 (Okazaki et al. 1999) and *Aeromonas hydrophila*

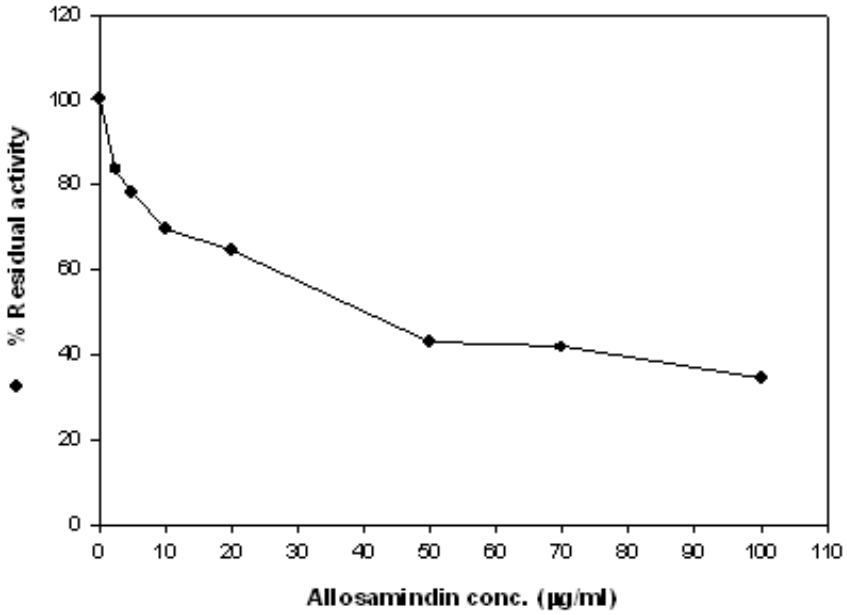

Figure 7. Effect of allosamidin on chitinase from *Enterobacter* sp. NRG4.

H 2330 (Hiraga et al. 1997). Chitinase from *Monascus purpureus* CCRC31499 was stimulated by Fe^{2+} and strongly inhibited by Hg^{2+} (Wang et al. 2002). Ag^{+} and Hg^{2+} inhibited chitinase from *Ralstonia* sp. A-471 (Sutrisno et al. 2004). *Enterobacter* sp. NRG4 chitinase was inhibited by 11% in presence of 10 mM EDTA. EDTA at 10 mM concentration inhibited chitinase of *Enterobacter* sp. G-1 by 42% (Park et al. 1997).

N-bromosuccinamide at 1mM and iodoacetamide at 10 mM concentration completely inhibited the enzyme activity. PCMB does not affect the enzyme activity much. DTNB and iodoacetamide inhibition suggested the role of cysteine residues in active site. NBS is protein oxidizing agent. The oxidizing reaction is specific for tryptophan and -SH groups and therefore suggested the role of tryptophan residues. There are few reports in the literature on effect of group specific reagents on chitinase activity. PCMB at 1 mM concentration inhibited chitinase of *Enterobacter* sp. G-1 by 24% (Park et al. 1997). PCMB was inhibitory for chitinase of *Aeromonas* sp. No. 10S-24 (Ueda et al. 1995). Chitinase from *Streptomyces* sp. M-20 was completely inhibited by PCMB (Kim et al. 2003). Chitinase of *Pseudomonas* sp. YHS-A2 was inhibited by 90% in presence of N-bromosuccinimide at 1mM concentration (Lee et al. 2000).

Allosamidin inhibited chitinase activity by 57.1 and 65.7% at 50 and 100 µg ml^{-1}, respectively. The IC_{50} value was 40µg ml^{-1} (64 µM). Other reported IC_{50} values were 48 µM for *Bacillus* sp. BG-11 chitinase (Bhushan and Hoondal, 1999) and 9.0 µM for chitinase from human serum and leucocytes (Escott and Adam, 1995).

Among various sugars and end products, chitinase was inhibited by 81.3% in presence of N-acetyl D-glucosamine at 10mM concentration whereas glucosamine HCl, galactosamine and glucose inhibited up to 19%. Chitinase of *Metarhizium anisopliae* was inhibited by 28, 21 and 79% in presence of glucose, N-acetyl D-glucosamine and D-glucosamine, respectively at 10 mM concentration (Pinto et al. 1997).

In conclusion, we have purified and characterized a chitinase from newly isolated *Enterobacter* sp. NRG4. The capability of this chitinase to hydrolyze chitin efficiently, lower end product inhibition, broad pH activity and stability makes the enzyme industrially significant for biotechnological applications, especially in production of chitobiose and N-acetyl D-glucosamine.

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