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Purification and partial characterization of chitinase from a novel strain *Aeromonas* sp. PTCC 1691

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Abstract

A locally isolated and chitinase-producing *Aeromonas* bacteria strain PTCC 1691 was cultured and induced by powdered chitin for the production of chitinases**.** A chitinase enzyme was purified through ammonium sulfate precipitation, ion exchange chromatography and gel filtration (Sephadex G-50). Molecular weight of the purified chitinase (Chi-53) was estimated about 53 kDa through SDS-PAGE technique. The physicochemical properties of the chitinase was also determined and revealed that this enzyme was an acidic protein with a pI of 5.3. The optimal temperature for the chitinase activity was 55°C. Stability studies have indicated that this enzyme is stable over a pH range from 6 to 8, and the maximum activity was at pH 6.5. Among the metal ions, Cu^{2+} and Co^{2+} stimulated the enzymatic activity from 22 to 34%, whereas Hg^{2}+, Mg^{2+}, Br^{3+} and Ag^{+} inhibited the enzyme from 40 to 65%. The apparent Km and Vmax for colloidal chitin determined at 55°C and pH7 were 0.64 mg/mL and 2.3 µmol/µg/h, respectively.

1 1. Introduction

Chitin, a homopolymer of β, 1-4 linked N-acetyl D-glucosamine residues, is the most abundant natural polysaccharide after cellulose [1]. This polymer is widely distributed in nature in the exoskeletons of arthropods, the outer shell of crustaceans and also as a structural component of fungal cell walls [2]. Chitinases are glycosyl hydrolases, which catalyze the hydrolysis of chitin to low-molecular-weight products. These enzymes can be classified into two major categories: endochitinases and exochitinases. Endochitinases cleave the glycosidic linkages of chitin chain randomly at internal sites, generating low molecular mass oligomers of N-acetylglucosamine (GlcNAc). Exochitinases can be categorized as chitobiosidases and β-(1,4) N-acetyl glucosaminidases where chitobiosidases catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin, and β-(1,4) N-acetyl glucosaminidases cleave the chitin oligomeric products, generating monomers of GlcNAc [3]. Chitinases received increasing attention because of their applications in agriculture, medicine and biotechnology [4]. For example, they can be used for the biocontrol of plant-pathogenic fungi, preparation of single-cell proteins, production of pharmaceutically important chitooligosaccharides and N-acetyl D-glucosamine, isolation of protoplasts from fungi and yeast and bioconversion of chitin waste [2]. Chitinases are produced by a wide range of organisms such as fungi, plants, and insects, some vertebrates and bacteria [5-7]. Some chitinolytic bacteria, including *Aeromonas*, *Serratia*, *Pseudomonas*, *Streptomyces*, *Vibrio* and *Bacillus* have been isolated and their chitinase activity was also studied [8]. *Aeromonas* spp. is particularly efficient in the breakdown of chitin via chitinolytic enzymes and can be found in a variety of aquatic environments worldwide [9, 10]. In an earlier study, the authors have isolated *Aeromonas* sp. PTCC 1691 from the waste of shrimp shells that have the ability to produce high levels of extracellular chitinase in a simple medium containing chitin as the sole carbon source. They were able to use crude chitinolytic enzymes from this microorganism to prepare N-acetyl-D-glucosamine from α-chitin in good yield under simple and quite mild procedure [11, 12]. This paper describes the purification and partial characterization of a chitinase produced by *Aeromonas* sp. PTCC 1691.

2 Material and Methods

2.1 Preparation of colloidal chitin

Purified chitin was used for production of colloidal chitin according to the method of Roberts and Selitrennikoff (1988) [13] with minor modifications. Briefly, 2.5 g of commercial chitin from crab shells was added slowly to 45 mL of concentrated HCl under vigorous stirring for 2 h. Thereafter, the mixture was added to 250 mL of ice-cold ethanol (95 %) with rapid stirring and kept at room temperature for 16 h. The chitin pellets were collected by centrifugation and washed with 25 mM sodium phosphate buffer until the colloidal chitin became neutral (pH 7.0), then washed with ddH2O and finally kept at 4°C for further applications.

2.2 Microorganism and culture conditions

Aeromonas sp. PTCC 1691 was previously isolated in our laboratory from the waste of shrimp shells sample collected from the north part of Iran using the chitin/agar plates as selective medium (Jami Al Ahmadi et al. 2008a) [12]. Cultivation of the isolate for growth and chitinase production was carried out in a liquid colloidal chitin (0.75 %) minimum medium containing (g/L) NaCl (0.25), Na_2HPO_4 (0.65), $(NH_4)_2SO_4$ (1.5), KH_2PO_4 (1.5), $CaCl_2$ (0.005), $MgCl_2$ (1.52), $MgSO_4$ (0.12), Triton X-100, 0.2% (v/v). The microorganism was incubated in shaken flasks at 30 °C, 150 rpm for 48 h. The culture was centrifuged at 12,000×g for 20 min (4 °C) to obtain culture supernatant which was used as source of chitinase.

2.3 Purification of chitinase enzyme

All purification processes were performed at 4°C. After cultivation, undegraded chitin and bacterial cells were removed by centrifugation at 12,000×g for 20 min (4 °C). The culture supernatant was precipitated with ammonium sulfate at 70 % saturation. The precipitate was dissolved in 50 mM sodium phosphate buffer, pH 7.0 and dialyzed overnight against 100 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was applied to a DEAE-cellulose column (2.0 × 25 cm) which equilibrated with Tris-HCl buffer (pH 8.0). A linear gradient of NaCl (0–1 M in 100 mM Tris-HCl buffer) was used for enzyme elution with a flow rate of 25 mL/h. The eluted fractions were assayed for enzyme activity. The prepared enzyme solution was loaded onto a Sephadex G-50 gel filtration column (2.5×120 cm) which was pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 mL/min and then eluted with the same buffer. Fractions collected were assayed for chitinase activity and protein content was determined by measuring absorbance at 280 nm.

2.4 Enzyme assays and protein estimation

Chitinase activity was measured with colloidal chitin as a substrate. The reaction mixture consisting of 1 mL of colloidal chitin (0.5 %) in 50 mM sodium phosphate buffer, pH 8.0 and 0.2 mL of enzyme solution. After incubation at 37 °C for 1 hour, the reaction was stopped by boiling, centrifuged and the reducing sugar released was determined by the modified Schales method with N–acetyl-D-glucosamine (GlcNAc) as a reference compound [14]. One unit of chitinase activity was defined as the amount of enzyme producing 1 µmol of N-acetyl-D-glucosamine per hour under the specified assay conditions. Protein concentrations were measured according to Bradford's method [15] using bovine serum albumin as standard.

2.5 Characterization of purified chitinase

2.5.1 Molecular weight estimation

The molecular mass of the purified chitinase was determined by SDS-PAGE according to the method of Laemmli (1970). The relative molecular mass of the enzyme was estimated using the standard molecular weight marker (29–205 kDa).

2.5.2 pI Estimation

Isoelectric focusing (IEF) was performed on purified chitinase samples according to Robertson et al. [16] with ampholine carrier ampholytes pH 3.5-10.0 at final concentration of 1%. Following electrofocusing, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the protein.

2.5.3 Effect of temperature and pH on chitinase activity and stability

Thermal stability was investigated by preincubation of enzyme samples at temperatures between 30 and 95°C for 30 and 60 min. To determine pH stability, the enzyme was incubated in different buffers with pH values of (4–10) at 4°C for 24 h. The remaining activity was measured by the standard assay. In order to determine the optimum temperature and pH for chitinolytic activity, the enzyme was assayed at various temperatures (10 to 80°C) and pH range (pH 4–10), respectively, under the assay conditions. The pH was varied using the following buffers with overlap pH: 0.1 M acetate buffer (pH 4–6), 0.1 M phosphate buffer (pH 5–7), and 0.1 M Tris buffer (pH 6.5-10).

2.5.4 Effect of Metal ions and EDTA

To determine the effect of different metal ions on chitinase activity, each metal ion (K^+, Na^+, Al^{3+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Ba^{2+}, Br^{2+}, Ag^+, Mn^{2+}, Hg^{2+} and Zn^{2+}) was added into reaction mixture with a final concentration at 5 mM and then the chitinolytic activity was determined under optimum assay conditions and compared to metal ion-free experiments (controls).

The inactivating effect of EDTA on the enzyme was determined by treatment of the enzyme with different concentrations of EDTA. The residual activity of the treated enzyme was determined by assaying the enzyme under standard assay conditions and compared to control (EDTA-free experiments).

2.5.5 Kinetic Parameters for Colloidal Chitin Hydrolysis by Chi-53

Different concentrations of substrate colloidal chitin (0.2, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.001% w/v) were incubated with partially purified enzyme. The enzymatic reaction was performed for 40 minute and with the final volume of 50 µL, finally the enzyme activity was measured and the Michaelis-Menten constant (*Km*) and maximal reaction velocity (*Vmax*) were calculated by Lineweaver- Burk curve by plotting 1/[substrate] (μM^{-1}) on the X-axis and 1/(Sp.activity) (ΔO.D/mg pr/h) on the Y-axis [17, 18].

2.5.6 Effect of substrate type on Chi-53 activity

The chitinase enzyme was active against chitin and chitosan. To analyze the effect of substrate on chitinase activity, chitin and chitosan (0.5% (w/v) as substrate) were prepared and then enzymatic assay was performed for 15 minutes.

2.5.7 Determination of Km and Vmax with chitosan as substrate

Km and Vmax for the purified chitinase were calculated using chitosan as different substrate. The substrate with various concentrations (0.2, 0.1, 0.05, 0.025, 0.01, 0.005 and 0.001% w/v) was prepared and the enzymatic assay was performed with final volume of 100 µL for 100 minute.

3. Results and discussion

3.1. Chitinase Purification

The chitinase (Chi-53) was purified partially using a two-step method that included ammonium sulphate precipitation followed by gel filtration (Sephadex G-50) to remove low molecular weight proteins and desalting. Using this scheme, one chitinase band which had a molecular size of 53 kDa in SDS-PAGE, and isoelectric point of 5.35 was purified (Figure 1). It was previously confirmed that microbial chitinases weigh from 20 to 120 kDa [19] and also show a broad range of isoelectric points (pI 4.5-8.5) (Hamid et al. 2013).The isoelectric point of Chi-53 was in the acidic range which was similar to that of the pI of chitinase from *Bacillus circulans* (5.1) and found in most bacterial chitinases [8].

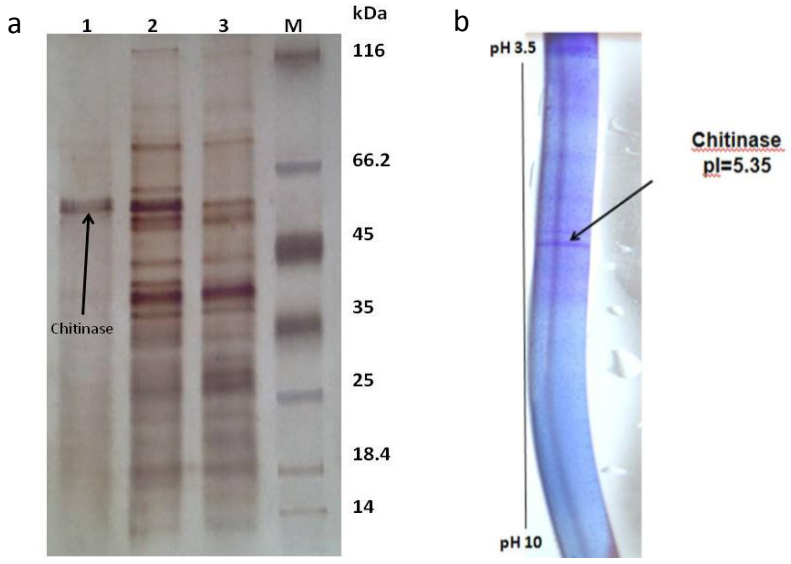


Figure 1: (a) Silver -stained SDS-PAGE analysis, Lane 1: purified Chitinase, Lane 2: ammonium sulfate fractionated, Lane3: culture supernatant, Lane M: protein ladder (b) Native IEF pattern of the purified chitinase from *Aeromonas* PTCC 1691

3.2. *Enzyme characterization*

The optimal temperature for the chitinase activity was 55 °C (Figure 2a). The purified enzyme retained more than 80% of its activity after 1 h at temperatures of up to 50°C (Figure 2b). However, at 80°C it was completely inactivated after 1 h. Previous reports have also shown that chitinases from *Bacillus subtilis* [20] and *Alcaligenes Xylosoxidans* [21] have a similar optimum temperature of 50-60°C. In contrast, a thermostable chitinase has been previously purified from *Humicola grisea* that exhibited optimal activity at 70°C [22].

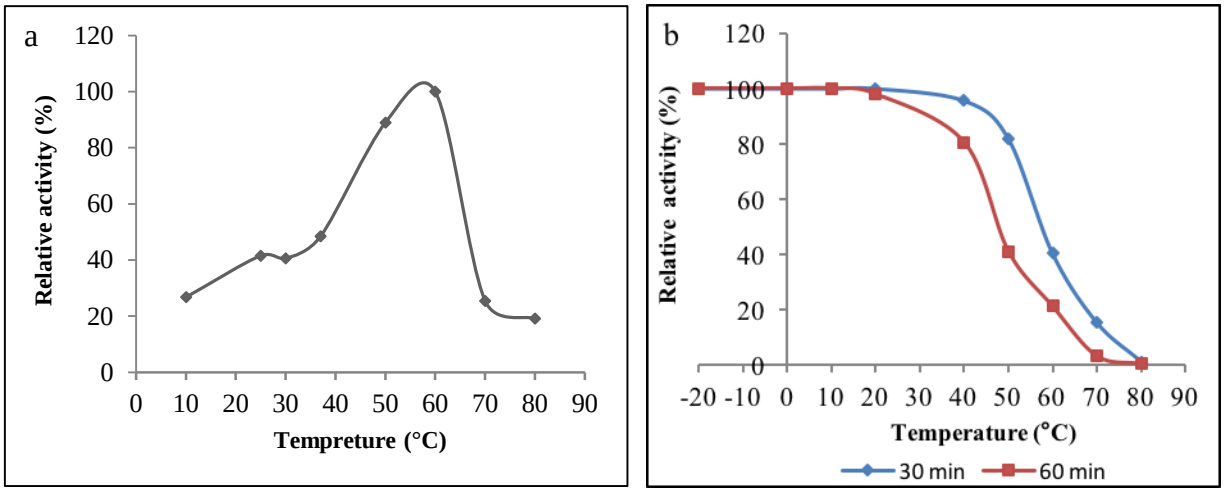
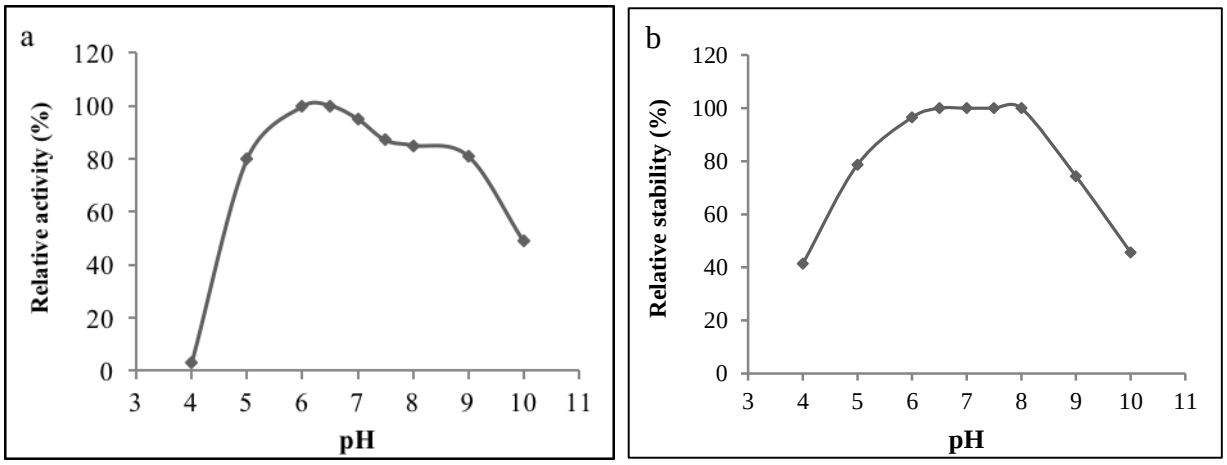


Figure 2: (a) Effect of temperature on activity, (b) Stability of purified chitinase purified from *Aeromonas* PTCC 1691

The activity–pH profile of Chi-53 was shown in Figure 3a. Enzymatic activity was optimal between pH 6 and 8, with a maximum at 6.5. Stability studies have indicated that the chitinase from the present strain was stable over a pH range from 6 to 8 (Figure 3b). Within this range, purified chitinase maintains more than 90% of its activity after 24 h at 4°C. Similarly, pH stability ranges of purified chitinases from *Bacillus* sp. [23] and *Enterobacter aerogenes* [24] were in the range 7 -8 and 5 -10, respectively.



Figur 3: (A) Effect of pH on the activity, (B) stability of purified chitinase from *Aeromonas* PTCC 1691

3.3. *Effect of different metal ions and reagents*

As shown in Table 1, Cu^{2+} and Co^{2+} stimulated the enzymatic activity of Chi-53 by 22 and 34%, respectively, whereas Hg^{2}+, Mg^{2+}, Br^{3+} and Ag^{+} inhibited the enzyme activity from 40 to 65%. The effect of other ions on Chi-53 activity was similar to that of the control. Chi-53 was also stimulated at the low concentration of EDTA (1mM) but inhibited by high concentrations of EDTA (15 and 20 mM). Our results were in line with those reported for other purified chitinases from different strains of bacteria, where Hg^{2+} [25-27] Mg^{2+} [28], Br^{3+} and Ag^{+} [29, 30] were found as inhibitors of chtinase activity, but contrary to our results enhancement of chitinase activity by Mg^{2+} was also observed in some reports [29, 31-33].

Table 1: Effect of 5 mM metal ions on the chitinolytic activity of Chi-53

Metal ions	Relative Activity (%)
Cu^{2+}	134
Co^{2+}	122
Al^{3+}	106
Na^{+}	99.5
Zn^{2+}–	97
Fe^{3+}	92.5
Mn^{2+}	90
K^{+}	90
Ca^{2+}	89
Ba^{2+}	80
Br^{+}	54
Ag^{+}	54
Hg^{2+}	49
Mg^{2+}	35
Blank(E+S)	100

3.4. Enzyme kinetic of Chi-53 with colloidal chitin as substrate

The Km and Vmax values were determined using a Lineweaver–Burk plot [34, 37] with (colloidal chitin) as substrate. The range of concentration was from 1 to 10 mg/L. The Chi-53 showed a Km of 0.64 mg/mL and Vmax 2.3 µmol/µg/h (Figure 4) which is comparatively lower than the other reports in literature. The Km values of chitinase from different organisms were, 1.41 mg/mL for *Enterobacter* sp. NPG4 [29], 8.3 mg/mL for *Serratia marcescens* B4A [35], 2.88 mg/mL for *Enterobacter aerogenes* [24] and 8.3 mg/mL for *Bacillus licheniformis* [36].

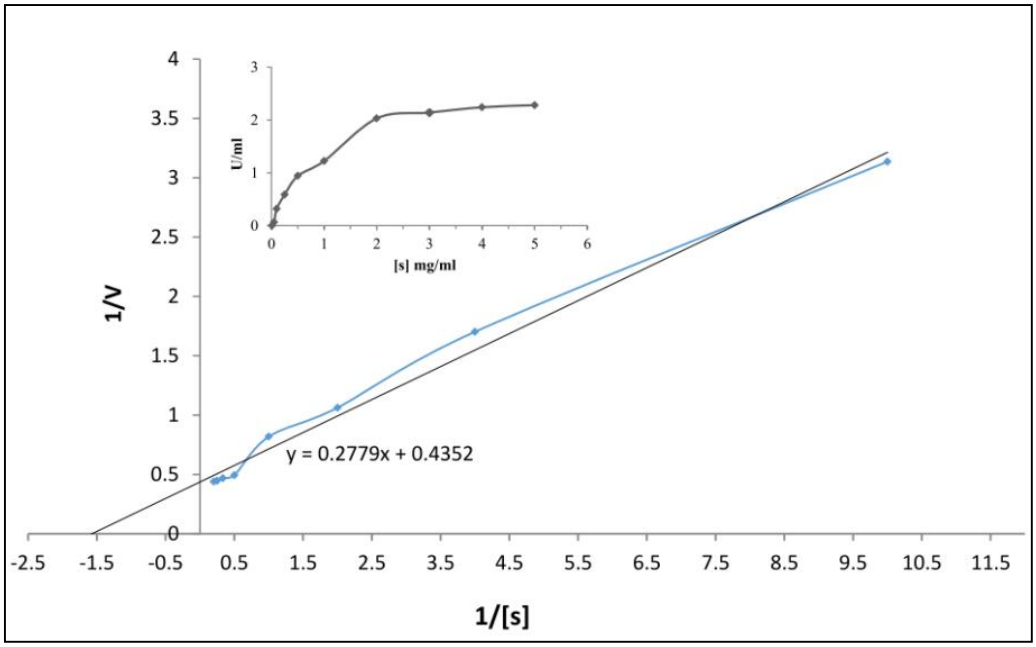


Figure 4: Lineweaver–Burk plots of the corresponding kinetic data of chitinase in the presence of colloidal chitin colloidal chitin as substrate (V—velocity and [S]—substrate concentration)

3.5. Effect of substrate type on chitinase activity

The Chi-53 was incubated with two different substrates (chitin and chitosan). As the results were shown in Table 2, the chitinase activity with chitosan was greater than chitin (about 3 times).

Table 2: Effect of substrate on the activity of purified chitinase

	Test	**Ctrl**	**C-T**	**µg/ml**	**µmol/ml**	**U/ml**
Chitosan (0.5%)	0.194	0.519	0.325	27.81818	0.125755	5.030185
Chitin (0.5%)	0.513	0.62	0.107	8	0.036165	1.446589

3.6. Kinetics of purified chitinase with chitosan as substrate

For chitosan hydrolysis, the measured affinity of binding between chitinase and chitosan (Km) and enzymatic reaction rate (Vmax) was measured with various concentration of chitosan and finally a Km value of 1.9006 mg/mL and Vmax value of 6.2112 µmol/µg/h were obtained (Data not shown).

Conclusion

The present study showed the purified chitinase from iranian-native bacteria, *Aeromonas* sp. PTCC 1691, could catalyze chitin degradation. Enzyme characterization indicated good stability in relatively wide range of pH and temperature of the bacterial chitinase. These characteristics indicated that this enzyme could be a suitable candidate as biocatalyst for use in biotechnological and pharmacological industries. This study could be extended to exhibit if Chi-53 has ability to suppress the growth of phytopathogenic fungi in vitro which could be an environmental friendly method and economically feasible.

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