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Full Length Research Paper

Purification and characterization of the chitosanase from *Aeromonas* sp. HG08

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In this work, the characterization of a chitosanase-producing bacterium isolated from soil was reported and this strain was grouped under the genus *Aeromonas* by virtue of its morphological, physiological properties and 16S rDNA gene sequences. It is the first report that the genus *Aeromonas* could produce chitosanase. *Aeromonas* sp. HG08 could secrete the chitosanase (named AsChi) with molecular weight of 70 kDa. The optimum pH and temperature of AsChi was 6.0 and 55°C, respectively. The activity of AsChi was markedly enhanced by Mn^{2+} and inhibited by Fe^{3+}, Cu^{2+}, Ag^{+} and Hg^{2+}; additionally, the activity of AsChi was increased with the degree of deacetylation (DDA) of chitosan. Through viscosimetric assay, AsChi probably hydrolyzed chitosan in an endo-type fashion.

Key words: *Aeromonas* sp., chitosanase, purification, characterization.

INTRODUCTION

Chitooligosaccharides, obtained from chitosan by hydrolysis with chitosanase, have various potential applications in biomedicines, pharmaceuticals, agriculture, and food (Chiang et al., 2003). Chitosanase (EC 3.2.1.132) occurs in a variety of microorganisms, including bacteria and fungi (Jung et al., 2006).

The degree of chitosanolytic activity varies among different species. In order to obtain a novel bacterial chitosanase system possessing high chitosanolytic activity and great potential for the large-scale production of chitosan oligomers, a large number of microorganisms have been screened in our laboratory. In this study, a bacterial strain (named *Aeromonas* sp. HG08) with high chitosanase activity was isolated and the chitosanase (named AsChi) was purified and characterized.

MATERIALS AND METHODS

Bacteria and their cultivation

400 strains were isolated from soil by a selection medium of chitosan agar (w/v) (1% colloidal chitosan, 0.5% $(NH_4)2SO_4$, 0.2% $K_2HPO_4.3H_2O$, 0.5% NaCl, 0.1% $MgSO_4.7H_2O$, 0.1% yeast extract, 2% agar, pH 6.5). The screened strains were inoculated in the medium (1% powder chitosan, 0.05% $MgSO_4.7H_2O$, 0.07% $K_2HPO_4.3H_2O$, 0.03% KH_2PO_4, 0.5% NaCl, 0.3% yeast extract, 1.2% $(NH_4)_2SO_4$, 0.1% glucose, pH 6.5) at 30ºC with shaking at 150 rpm.

Characterization and identification of the strain HG08

Physiological tests were carried out by using the Biochemical Card of the Vitek system and API 20 NE (BioMerieux, France). Alignment of the strain was based on the sequence of 16S rDNA gene, amplified by using PCR technique. The 16S rDNA sequences were amplified with the primers fD1 (5'-ACATGCAAGTCGAACGG-3') and rD1 (5'-TACGGTTACCTTGTTA CGAC-3'). GenBank and RDP databases were used to search for 16S rDNA sequence similarities. 16S rDNA sequence analysis was performed with DNAMAN 4.03 software package by using the neighbor-joining method and the Jukes-Cantor distance correction matrix method (Saitou and Nei, 1987).

Enzyme assay

Chitosan with 95% degree of deacetylation (DDA) was used as the substrate in the chitosanase assay. Chitosanase activity was deter mined by quantitative estimation of the reducing sugars produced from chitosan. The reaction mixture contained 0.9 mL of 1% soluble chitosan, 0.1 mL of diluted enzyme solution and 1 mL of 0.2 M sodium acetate buffer (pH 5.8). The reaction tubes were incubated at 50°C for 15 min. The reducing sugars produced in the supernatant were measured by modified dinitrosalicyclic acid method

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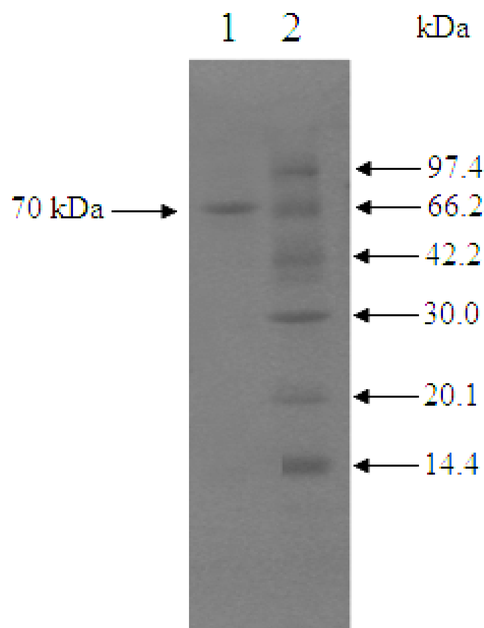

Figure 1. SDS-PAGE showing the purified chitosanase band from *Aeromonas* sp. HG-08. SDS-PAGE was performed with 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1, the purified chitosanase; Lane 2, standard molecular weight markers.

(Miller, 1959), with glucosamine-hydrochloride as the calibration standard. One unit of chitosanase was defined as the amount of enzyme that could liberate 1 μM of reducing sugar as GlcN per min under the conditions described above. Three replications were performed per analysis.

Protein assay and electrophoresis

Protein estimation was done by Lowry's method, using bovine serum albumin as the standard. The purity of enzyme was deduced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Viscosimetric assay

Viscosimetric assay was performed by the method of Muzzarelli and Peter (1997).

RESULTS

Characterization of *Aeromonas* sp. HG08

Chitosanase activity of 400 strains from soil was screened and the strain HG08 had the highest chitosanase activity (31 U/mL). HG08 was identified as the genus *Aeromonas* from its morphological, physiological properties and 16S rDNA sequences. Phylogenetic tree analysis by the neighbor-joining method demonstrated that strain GH08 was closely related to the genus *Aeromonas*. Its closest relative was *Aeromonas salmonicida* subsp. *salmonicida* which 16S rDNA accession number is AM296502 (level of similarity 99.1%). It is currently named *Aeromonas* sp. HG08.

Purification of chitosanases

The chitosanase (AsChi) was purified to homogeneity judged by SDS-PAGE (Figure 1). The molecular weight was estimated as 70 kDa. Estimation of the apparent molecular weight based on the elution volume on Sephacry S-100 columns yielded the same molecular weight as above, which indicated that it was monomeric (data not shown). The purification steps were summarized in Table 1.

Effects of pH and temperature on AsChi

The optimum activity of AsChi was at pH 6.2 (Figure 2). The maximum activity was observed at 55℃ (Figure 3). To determine the temperature stabilities of AsChi, the residual activity was measured after incubation of the enzyme at various temperatures for 2 h at pH 5.8 in the absence of substrate. 98, 92, and 85% of initial chitosanase activity (31 U/mL) retained after incubation at at 20, 30, and 40℃ for 2 h, respectively. The half-lives of AsChi at 50 and 60℃ were 30 and 13 min, respectively.

Effects of some compounds and metal ions on AsChi

The activity of AsChi with some compounds and metal was summarized in Table 2. The purified AsChi was slightly enhanced by β-mercaptoethanol. Treatment with EDTA and SDS was slightly inhibitory, and the PMSF had no effect. It was markedly enhanced by Mn^{2+} and inhibited by Fe^{3+}, Cu^{2+}, Ag^{+} and Hg^{2+} (Table 2), while it had no effect by other metals such as K^{+}, Na^{+}, Pb^{2+}, Mg^{2+}, Ba^{2+} and Zn^{2+} (date not shown).

Kinetic parameters

The double reciprocal plot of initial velocity versus the soluble chitosan concentration was deviated from linearity at a substrate concentration higher than 14 mg chitosan/mL. From the extrapolation of initial velocities at the concentration of chitosan from 1 to 14 mg/mL, K_m and V_{max} were estimated to be 7 mg chitosan/mL and 840 U/mg protein.

Substrate specificity

The activity of AsChi with various substrates was sum-

Table 1. Purification process of the chitosanase from *Aeromonas* sp. HG-08.

Steps[a]	Total activity ($\times 10^2$ U)	Total Protein (mg)	Specific activity ($\times 10^2$ U/mg)	Yield (%)	Purification folds
Crude extract	18	45	0.4	100	1
0–70%$(NH_4)_2SO_4$	15	20	0.75	83.3	1.88
Q-Sepharose Fast Flow	10	2.0	5	55.6	12.5
Sephadex G-75	8.5	1.0	8.5	47.2	21.25

[a]The chitosanase was purified by the following chromatographic procedure. All purification steps were performed at 4℃. Step 1: The bacterial cells were pelleted out from the culture medium by centrifugation (10 000×g, 30 min). Anhydrous ammonium sulfate was added to the supernatant to 70% saturation. Then the precipitate was dissolved in 50 mL of 50 mM Tris-HCl buffer, pH 7.8 (buffer A) and dialyzed against buffer A. Step 2: The dialyzed solution was loaded onto a Q-Sepharose Fast Flow column (2.0 × 20 cm) previously equilibrated with buffer A. The column was washed with 500 mL buffer A, and then eluted with a 400 mL of a 0-1.0 M NaCl linear gradient in buffer A at a flow rate of 0.40 mL/min. Step 3: The active fractions from step 2 were resuspended in buffer A containing 0.1 M NaCl (buffer B) and layered onto Sephadex G-75 column (1.6 × 80 cm) previously equilibrated with buffer B. The column was eluted with buffer B at a flow rate of 9 mL/h.

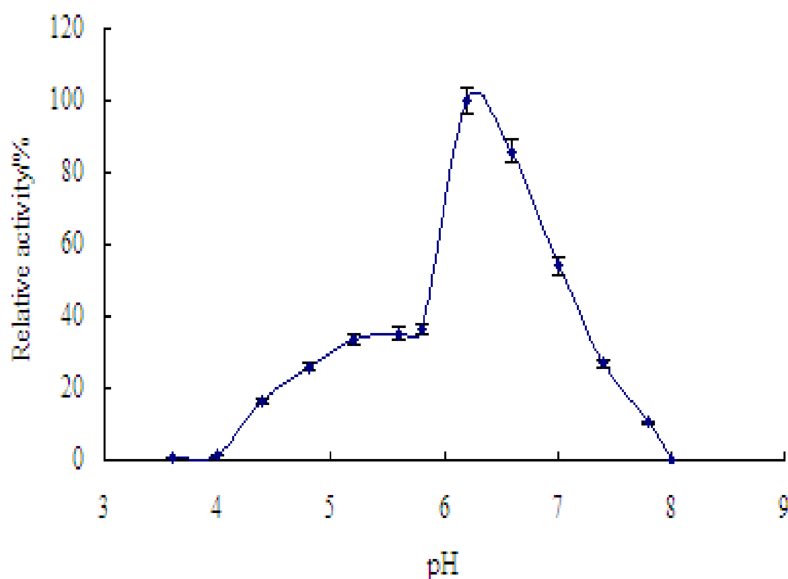

Figure 2. Effect of pH on the activity of the chitosanase. Different buffers (200 mM each) including sodium acetate (pH 3.6-5.8), sodium phosphate (pH 5.8-8.0) or Tris-HCl (pH 8.0-10.0) were added into the reaction mixture to create a range of pH values. The relative activity was expressed as percentage ratio of the specific activity (U/mL) of the purified chitosanase at pH 3.6 - 8.0 to the optimum pH (31 U/mL for the chitosanase). Values were means of three replications ± standard deviation.

marized in Table 3. It degraded chitosan with the DDA of 78 -100% effectively. The activity of AsChi was increased with the DDA of chitosan, which indicates that AsChi could probably split only the β-l, 4-glycosidic linkage of GlcN-GlcN. AsChi could not hydrolyze chitosan with the DDA of 50-70%, colloidal chitin, carboxymethyl cellulose sodium, or carboxymethyl chitosan.

Viscosimetric assay

To investigate the cleavage pattern of chitosan by AsChi, a decrease in viscosity of 99% DDA of chitosan was investigated during enzymatic hydrolysis (Figure 4). AsChi decreased the viscosity extensively in the early phase of the reaction. It indicated that AsChi probably hydrolyzed chitosan in an endo-type fashion.

DISCUSSION

In this paper, the characterization of a novel chitosanase producing bacterium isolated from soil was reported for the first time. This strain was grouped under the genus

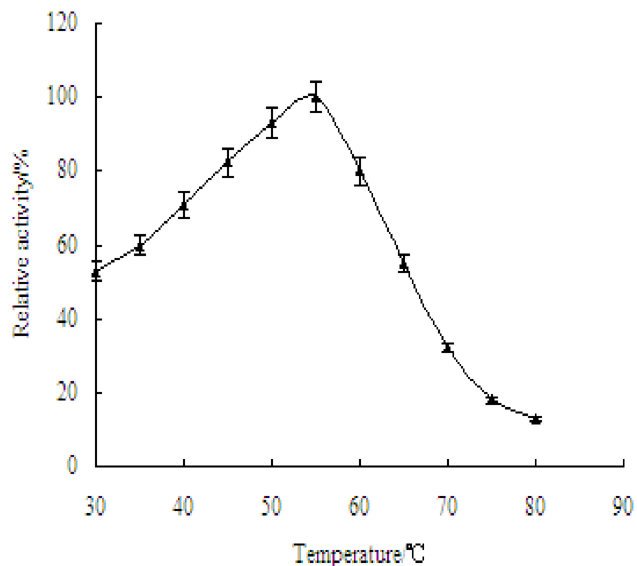

Figure 3. Effect of temperature on the activity of the chitosanase. The enzyme activities were measured under standard assay conditions (pH5.8, 15 min) at 30-80ºC. The relative activity was expressed as percentage ratio of the specific activity (U/mL) of the purified chitosanase at 30-80ºC to the optimum temperature (31 U/mL for the chitosanase). Values were means of three replications ± standard deviation.

Table 2. Effect of compounds and metal ions on the activity of the chitosanase.

compounds and metal ions	Relative activity/%	
	0.1 mM	**1 mM**
None	100±3.0	100±2.5
Mn^{2+}	104±3.5	146±1.3
Cu^{2+}	87±1.6	75±1.0
Hg^{2+}	67±2.2	28±1.3
Ag^{+}	97±1.6	63±2.5
Fe^{3+}	84±3.5	40±1.4
β-Mercaptoethanol	103±3.7	128±4.5
EDTA	90±2.9	82±2.8
SDS	93±3.0	79±3.1
PMSF	99±3.8	101±3.1

The chitosanase was assayed after adding metal ions and compounds, including Ag^{+}, Hg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, K^{+}, Na^{+}, Pb^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, β-Mercaptoethanol, EDTA, SDS and PMSF, to the reaction mixtures to a final concentration of 0.1 and 1 mM. The enzymatic activity was assayed under standard assay conditions (50℃, pH5.8 and 15 min). The relative activity was expressed as percentage ratio of the specific activity (U/mL) of the purified chitosanase with metals and compounds to those without metals and compounds (31 U/mL for chitosanase). Values were means of three replications ± standard deviation.

Aeromonas by virtue of its morphological, physiological properties and 16S rDNA gene sequences. There have

Table 3. Substrate specificity of the chitosanase.

Substrates	Relative activity (%)
chitosan (100% DDA)	100±3.5
chitosan (99% DDA)	97±2.1
chitosan (95% DDA)	85±1.5
chitosan (86% DDA)	72±1.8
chitosan (78% DDA)	60±1.0
chitosan (70% DDA)	8±0.2
chitosan (50% DDA)	0
colloidal chitin (19% DDA)	0
carboxymethylcellulose sodium	0
carboxymethyl chitosan	0

The activities of the chitosanase with various substrates were determined. The relative activity was expressed as percentage ratio of the specific activity (U/mL) of the purified chitosanase with various substrates to the chitosan of 100% degree of deacetylation (DDA) (31 U/mL for chitosanase). Values were means of three replications ± standard deviation.

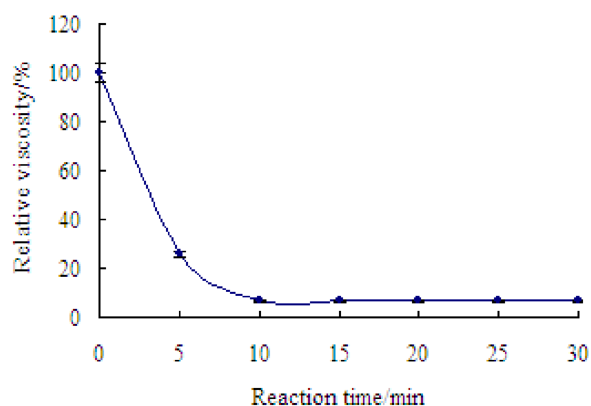

Figure 4. Viscosity variation during enzymatic hydrolyzation. Reduction in viscosity was determined with viscometer (Haake Rotovisco RV-20, M5). The reaction mixture (200 mL) contained 2% chitosan (degree of deacetylation of 99%) in 0.2 M sodium acetate buffer (pH 5.8), and 31 U of the chitosanase. Values were means of three replications ± standard deviation.

been some reports that the genus *Aeromonas* could produce chitinase, but it is the first report that the *Aeromonas* sp. HG08 could produce chitosanase, not chitinase. And the chitosanase of this strain could not hydrolyze chitin. So it is probably a novel strain that produces chitosanase.

It is noteworthy that the level of chitosanolytic activity of HG-08 (31 U/ml of culture fluid) was higher than those of the previously reported microorganisms. Hence, *Aeromonas* sp. HG-08 could be a potential selection to be used to produce chitosanolytic enzymes.

Most of the chitosanase from the previously reported microorganisms are characterized by their low apparent molecular mass, which is almost in the range of 10-50 kDa, except 100 kDa from *Rhodotorula gracilis*

(Somashekar and Joseph, 1992) and 76 kDa from *Mucor rouxii* ChA (Alfonso et al., 1992). The molecular mass from *Aeromonas* sp. HG-08 was estimated as 70 kDa, which is out of the above range. So it is a kind of novel chitosanase.

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REFERENCES

Alfonso C, Martinez MJ, Reyes F (1992). Purification and properties of two endo-chitosanases from *Mucor rouxii* implicated in its cell wall degradation. FEMS Microbiol. Lett. 95:187-194.

Chiang CL, Chang CT, Sung HY (2003). Purification and properties of chitosanase from a mutant of *Bacillus subtilis* IMR-NK1. Enzyme Microb. Tech. 32: 260-267.

Jung WJ, Kuk JH, Kim KY, Jung KC, Park RD (2006). Purification and characterization of exo-beta-d-glucosaminidase from *Aspergillus fumigatus* S-26. Protein Expr. Purif. 45: 125-131.

Miller GL (1959). The use of dinitrosalicylic acid reagent for the determination of reducing sugars. Anal. Chem. 31: 1321-1326.

Muzzarelli RAA, Peter MG (1997). Depolymerization of chitins and chitosans with hemicellulase, lysozyme, papain and lipases. In: Muzzarelli RAA, eds. *Chitin Handbook,* Institute of Biochemistry, Faculty of medicine, University, Italy, pp.160-161.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Somashekar D, Joseph R (1992). Partial purification and properties of a novel chitosanase secreted by *Rhodotorula gracilis.* Lett. Appl. Microbiol. 14: 1-4.