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Purification and properties of an *N*-acetylglucosaminidase from *Streptomyces cerradoensis*

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Abstract

An *N*-acetylglucosaminidase produced by *Streptomyces cerradoensis* was partially purified giving, by SDS-PAGE analysis, two main protein bands with Mr of 58.9 and 56.4 kDa. The K_m and V_{max} values for the enzyme using *p*-nitrophenyl-*β*-*N*-acetylglucosaminide as substrate were of 0.13 mM and 1.95 U mg^{-1} protein, respectively. The enzyme was optimally activity at pH 5.5 and at 50 °C when assayed over 10 min. Enzyme activity was strongly inhibited by Cu^{2+} and Hg^{2+} at 10 mM, and was specific to substrates containing acetamide groups such as *p*-nitrophenyl-*β*-*N*-acetylglucosaminide and *p*-nitrophenyl-*β*-D-*N,N′*-diacetylchitobiose.

Introduction

Chitin, a *β*−1, 4-linked polymer of *N*-acetylglucosamine, is a structural component of the arthropod exoskeleton and is a common constituent of fungal cell walls. The complete degradation of chitin is performed by the chitinolytic system composed of chitinases (EC 3.2.1.14) and *N*-acetylglucosaminidases (E.C. 3.2.1.30) (Sahai & Manocha 1993). Chitinases can hydrolase the substrate by two possible mechanisms, identified by the products of hydrolysis: (a) endochitinases cleave internal bonds within chitin releasing chitotetraose, chitotriose and chitobiose; (b) exochitinases catalyses the release of chitobiose without the formation of oligo or monosaccharides. The *N*-acetylglucosaminidases cleave chitobiose, chitotriose and chitotetraose releasing *N*-acetylglucosamine.

Among bacteria, the actinomycetes are an important chitinase-producing group, especially those belonging to the genus *Streptomyces* (Broadway *et al.* 1995, El Sayed *et al.* 2000, Gomes *et al.* 2000, Ueno & Miyashita 2000). There is less work about *N*-acetylglucosaminidase from other organisms and few researches have been done to characterize this type of enzyme from *Streptomyces*. In this article we describe the partial purification and characterization of a *N*-acetylglucosaminidase produced by *Streptomyces cerradoensis*, isolated from Brazilian cerrado soils.

Materials and methods

Microorganism and enzyme production

Streptomyces cerradoensis obtained from solid culture media (ISP-2) were inoculated in Erlenmeyer flasks containing 50 ml YEME liquid media (Hopwood *et al.* 1985).

Samples of 50 μl of cells suspension from YEME media were added to Erlenmeyer flasks (250 ml) containing 50 ml media: 0.2% (w/v) K_2HPO_4, 0.3% (w/v) NaCl, 0.3% (w/v) KNO_3, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.04% (w/v) $CaCl_2 \cdot 2H_2O$, 0.002% (w/v) $FeSO_4$, 0.001% (w/v) $MnSO_4$, 1% (w/v) chitin, pH 7.0. The flasks were incubated at 30 °C with shaking (150 rpm) for 9 days.

N-*Acetylglucosaminidase assay (NAGase)*

Enzyme activity was assayed using *p*-nitrophenyl-*β*-*N*-acetylglucosaminide (*p*NP-GlcNAc) as substrate. The reaction mixture consisted of 50 μl enzyme solution, 350 μl 50 mM sodium acetate buffer (pH 5.5) and 100 μl 5 mM *p*NP-GlcNAc as described by Ulhoa and Peberdy (1993). One unit (U) of enzyme was defined as the amount of enzyme that released 1 μM *p*-nitrophenol per min.

Partial enzyme purification

Samples of the supernatant (150 ml) were applied to a SP-Sepharose chromatography column (2.2×15 cm), equilibrated with 50 mM sodium acetate buffer (pH 5.5), at 1 ml min^{-1}. The column was washed with the same buffer and the proteins eluted with a linear gradient of 0–1 M NaCl. Fractions containing NAGase activity were pooled, dialyzed against 50 mM sodium acetate buffer (pH 5.0), and applied to methyl-Sepharose column (1×5 cm). The adsorbed proteins were eluted at 4 °C with a decreasing linear gradient of $(NH_4)_2SO_4$ (1.2 M to 0). Fractions containing NAGase activity were pooled and stored at −10 °C. SDS-PAGE with 10 % (w/v) polyacrylamide was by the standard method of by Laemmli. The proteins were silver stained by the method of Blum *et al.* (1987).

Thin-layer chromatography

The end products of the enzymatic hydrolysis of *N,N′*-diacetylchitobiose (5 mM), *N,N′,N″*-triacetylchitotriose (5 mM) and colloidal chitin (0.2%, w/v) were analyzed by TLC according to Chung *et al.* (1995).

Results and discussion

Production of N-*acetylglucosaminidase*

Recently, we isolated a chitin-degrading actinomycete from soil sample, which was identified as belonging to *Streptomyces* genus. Sequence analysis of the gene encoding 16S rDNA (GeneBank Accession No. AY627277) showed that this actinomycete is a new species, named by us as *Streptomyces cerradoensis*. This strain showed in preliminary tests, high chitinase and *N*-acetylglucosaminidase (NAGase) activity when grown in chitin-containing medium. A main peaks of NAGase activity (0.061 U) was found after 120 h growth at 30 °C.

Partial purification of N-*acetylglucosaminidase*

The enzyme was partially purified from a culture supernatant by ion exchange chromatography and gel filtration. The fractions obtained after the procedure were free of chitinase activity. At the final steps, two main bands of protein were detected by SDS-PAGE with apparent molecular mass of 58.9 and 56.4 kDa (Figure 1). This

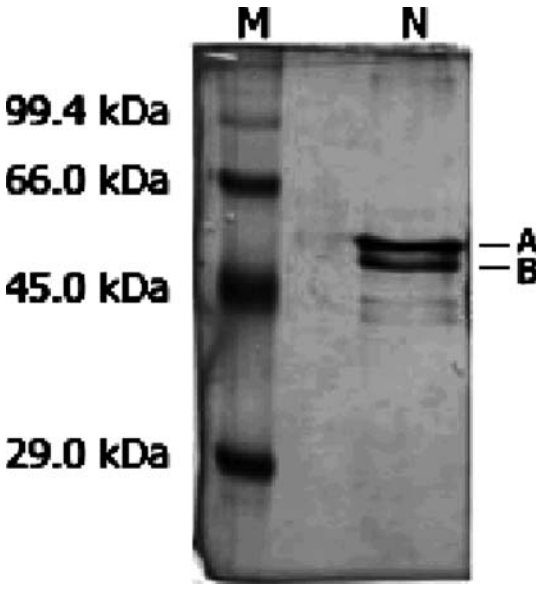

Fig. 1. Protein profile on SDS-PAGE at a concentration of 10% polyacrylamide. Lane M indicates the molecular mass markers and N shows the *N*-acetylglucosaminidase partially. Phosphorylase *b* (99.4 kDa), serum albumin (66.0 kDa), ovalbumin (45.0 kDa) and carbonic anhydrase (29.0 kDa) were used as molecular mass standards.

Table 1. Biochemical properties of the NAGase from *Streptomyces cerradoensis*.

pH Optimum	5.0
Temperature optimum (°C)	50
Temperature stability pH 5.5 over 30 min	
40 °C	30%
55 °C	12%
K_m (mM)	0.13
V_{max} (U mg^{-1} protein)	1.95
Inhibition by Hg^{2+} (10 mM)	100%
Inhibition by Cu^{2+} (10 mM)	78%

The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using sodium acetate (pH 3.5–5.5) and potassium phosphate buffer (pH 6.0–7.5). The effect of temperature on the enzymatic activity was determined at the pH optimum, in the range of 30–75 °C. The effect of temperature on the enzyme stability was analyzed by previously incubating the enzyme at 40 and 55 °C for 30 min. Michaelis–Menten constant (K_m) was determined by non-linear-regression analysis of data obtained by measuring the rate of ρNP-GlcNAc hydrolysis (from 0.02 to 0.6 mM). The inhibition of the NAGase activity by metal ions was determined through previous incubation of the enzyme samples with 10 mM $ZnSO_4$, KCl, $MgSO_4$, $CaCl_2$, $CuSO_4$ and $HgCl_2$ for 2 min. Results are means values of three replicates.

molecular weight was in a similar range to that produced by *S. thermoviolaceus* (Tsujibo *et al.* 1998) although, smaller NAGases (27 and 49.5 kDa) also have been isolated and characterized from *Streptomyces plicatus* (Tarentino *et al.* 1978, Trimble *et al.* 1982).

Enzyme characterization

The lower K_m (0.13 mM) indicates that the enzyme has high affinity for the substrate when it is compared with those reported for *S. thermoviolaceus* (0.43 mM) and for *S. hygroscopicus* (NA1: 0.12 mM and NA2: 0.76 mM) (Irhuma *et al.* 1991, Tsujibo *et al.* 1998). Most bacterial NAGases are optimally active in the range of pH 4–5 and between 50 and 60 °C (Trimble *et al.* 1982, Irhuma *et al.* 1991, Saito *et al.* 1998, Tsujibo *et al.* 1998). Concurring with this, the NAGase of *S. cerradoensis* displayed maximal activity at pH 5 and at 50 °C (Table 1). The enzyme, though, rapidly lost activity either at 40 or 55 °C (Table 1). Activity was not affected by Ca^{2+}, Mg^{2+} and Zn^{2+} but was inhibited by Cu^{2+} and Hg^{2+} at 10 mM. Almost all of the NAGases from *Streptomyces* are inhibited by Hg^{2+} indicating the importance of indole amino acid residues in the enzyme function (Irhuma *et al.* 1991, Trimble *et al.* 1982, Tsujibo *et al.* 1998).

Substrate specificity

The activity of the enzyme was observed only over substrates containing acetamide groups (Table 2). Similar results were observed for NAGase from other *Streptomyces* species, suggesting that acetamide groups are important to the recognition of chitin and its oligomers by these enzymes (Tsujibo *et al.* 1998). *N*-Acetylglucosaminide was released from *N,N′*-diacetylchitobiose and *N,N′,N″*-triacetylchitotriose after 24 h incubation, while this monomer was released from colloidal chitin only after 48 h incubation. Such a profile indicates that this enzyme acts at the extremity of the substrates releasing *N*-acetylglucosaminide and accords with the definition of NGAse as proposed by Sahai and Manocha (1993).

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Table 2. Specificity of *N*-acetylglucosaminidase to different substrates.

Substrate (5 mM)	Enzyme activity (U)	Relative activity (%)
p-Nitrophenyl-β-*N*-acetylglucosaminide	27 ± 4	100
p-Nitrophenyl-β-D-*N,N′*-diacetylchitobiose	6.5 ± 0.04	25
p-Nitrophenyl-β-D-glucopyranoside	0.07 ± 0.01	0.3
p-Nitrophenyl-β-D-galactopyranoside	0	0

The reaction mixture consisted of 50 μl of enzyme solution, 350 μl 50 mM sodium acetate buffer (pH 5.5) and 100 μl of specific *p*-nitrophenyl substrate (5 mM). One unit (U) of enzyme was defined as the amount of enzyme that released 1 μM *p*-nitrophenol per min.

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