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α-Glucosidase of *Capnocytophaga ochracea* ; Its Cellular Location, Dissolution, Purification, and Partial Characterization

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Summary

α-Glucosidase activity was detected in the crude extract of cell and envelope of *Capnocytophaga ochracea* ATCC 33596. The enzyme associated with the envelope was effectively dissolved by cetyltrimethylammonium bromide. The enzyme was purified from both fractions by combination of ammonium sulfate precipitation, ion-exchage chromatography, gel filtration, and isoelectric focusing. Properties of both enzymes were quite similar : they were basic proteins, metalloenzymes, and most active at pH7.0. They hydrolyzed only *p*-nitrophenyl-α-D-gluconopyranoside.

Introduction

Genus *Capnocytophaga* has been implicated as pathogenic agent of periodontal disease as well as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (2, 7). However, the biolological properties, especially production and characterization of substances which may be responsible for pathogenic action have not sufficiently been investigated. Laughon et al reported that *Capnocytophaga* elaborated phosphatase, enzyme hydrolyzed trypsin synthetic substrate, and glucosidase (4). Recently, isolation and characterization of bacteriocin of *C. ochracea,* which may play an ecological role in oral flora were reported (5). More information would be necessary for examination of behavior of this genus in the oral cavity. In this report, we describe about partial biological properties of α-glucosidase of *C. ochracea.*

Methods

Capnocytophage ochracea ATCC 33596 was cultured in General Anaerobic Medium (Nissui Seiyaku, Tokyo) in anaerobic glove box filled with gases ($N_2+H_2+CO_2$; 85 : 10 : 5) at 37°C for 4 days. α-glucosidase activity was assayed using *p*-nitrophenyl-α-D-gluconopyranoside (PNP-α-glucoside) as substrate. The reaction mixture contained 1.0 ml of 2.5 mM PNP-α-glucoside in 50 mM Tris-maleate buffer (pH7.0), 0.1ml of enzyme sample, and 1.9ml of 50mM Tris-maleate buffer (pH7.5). After incubation of the reaction mixture at 37°C for 30min, 0.5ml of 3.0M Na_2CO_3 to stop the reaction, and the released *p*-nitrophenol was determined by measurement of the absorbance at 420nm. One unit of activity was defined as the amount that increased 0.1 absorbance unit per 1 min.

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SDS-polyacrylamide gel electrophoresis for examination of purity and determination of molecular weights was carried out by the method of Laemmli (3). Gels were stained with Coomassie brilliant blue R250. Marker proteins for molecular weight estimation were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydlase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4kDa). Cells were harvested by centrifugation at 10,000g for 10 min, washed twice with saline, and suspended in 50 mM Tris-hydrochloride buffer (pH8.0). The cells were disrupted by sonication at 9 kHz for 20min. After sonication, the sonicated sample was centrifuged at 5,000g for 10min to remove the unbroken cells. The supernatant was centrifuged at 120,000g for 60min and the resultant precipitate (envelope) and supernatant (S120 fraction) were kept at −40°C until used.

Results and Discussion

α-Glucosidase activity was detected in S120 fraction and envelope but in the culture supernatant significant amounts of the activity could not be obtained. Attempts were made to dissolve the enzyme from the envelope. It could not be extracted by neutral saline (0.5 to 1.5M NaCl solutions). Of the detergents tested (Triton X-100, polyoxyethylene 10-tridecyl ether, cetyltrimethylammonium bromide), cetyltrimethylammonium bromide was most effective for extraction. Almost all activity was dissolved with this detergent. On the other hand, about 60 to 70% the activity could be extracted by the other two detergents. The envelope was suspended in 50mM Tris-maleate buffer (pH7.5) and cetyltrimethylammonium bromide was added to a concentration of 1% and gently stirred at 37°C for 3.5h. Then this mixture was centrifuged at 120,000g for 60min and the supernatant was dialyzed against 50mM Tris-hydrochloride buffer (pH8.0).

α-Glucosidase activity was found in S120 fraction and dissolved envelope fraction, but no significant activity was found in the culture supernatant. The ratio of activity in S120 fraction and in the dissolved envelope fraction was about 3 : 1. S120 fraction was applied to a column of QAE-Sephadex A25 equilibrated with 50 mM Tris-hydrochloride buffer (pH 8.0) and washed with this buffer. In this step α-glucosidase activity was recovered in the washings of the column. The active fractions were combined and dialyzed against 50 mM Tris-hydrochloride buffer (pH 7.5) and applied to a column of CM 32 equilibrated with the same buffer. Then the proteins in the column were eluted by linearly increasing concentration of NaCl (0 to 500 mM). The active fractions were combined, concentrated using rotary evaporater, and dialyzed against 50 mM Tris-hydrochloride buffer (pH 7.5) containing 200 mM NaCl. The dialyzed sample was applied to a column (2.6 by 95 cm) of Sephacryl S-300 equilibrated with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 200 mM NaCl and eluted with the same buffer saline. The active fractions obtained from gel filtration was dialyzed against 150 mM glycine solution and futher purified by electrophoresis on an isoelectric focusing column (110 ml) with pH range of 5 to 11 (Fig. la). The electrophoresis was carried out at 600 V for 48h. The peak of the activity found at pH 8.9 was dialyzed against 50 mM Tris-maleate buffer (pH7.5) and this was the purified enzyme from S120 fraction. Similarly, α-glucosidase in the dissolved envelope fraction was purified using the methods described above. It was obtained at pH of 9.0 in the electrophoresis on isoelectric focusing (Fig. 1b). α-glucosidase purified from S120 fraction and dissolved envelope fraction were referred to as S120 enzyme and envelope enzyme, respectively.

As shown in Fig. 2, both preparations were found to be homogeneous on SDS-PAGE. The positions of bands were rightly the same and the molecular weight was calculated to be 70 kDa.

PNP-β-glucoside, PNP-α-frucoside, PNP-α-xyloside, and PNP-α-galactoside were not hydrolyzed by both purified enzyme preparation. Both enzymes were completely inhibited by 1 mM of EDTA. EGTA also inhibited by 96% and 94% the activity of S120 enzyme and envelope enzyme, respectively. However, *O*-phenanthrolin did not inhibited significantly both enzymes. No effect of Ca^{2+} or Mg^{2+} on the activity of both enzymes was observed. S120 enzyme and envelope enzyme exhibited maximum activity at pH 7.0. Both were inactivated by heating at 50℃ for 10 min.

Purification and characterization of α-glucosidase in crude extract and in envelope from *C. ochracea* have revealed that both enzymes show substantially identical properties. Different from glucosidase of *Bacillus* (1) and *Ruminococcus* (6), the pI of C. ochracea was very high, those values of the enzyme of the former and the latter were reported to be 4.6 and 4.4, respectively.

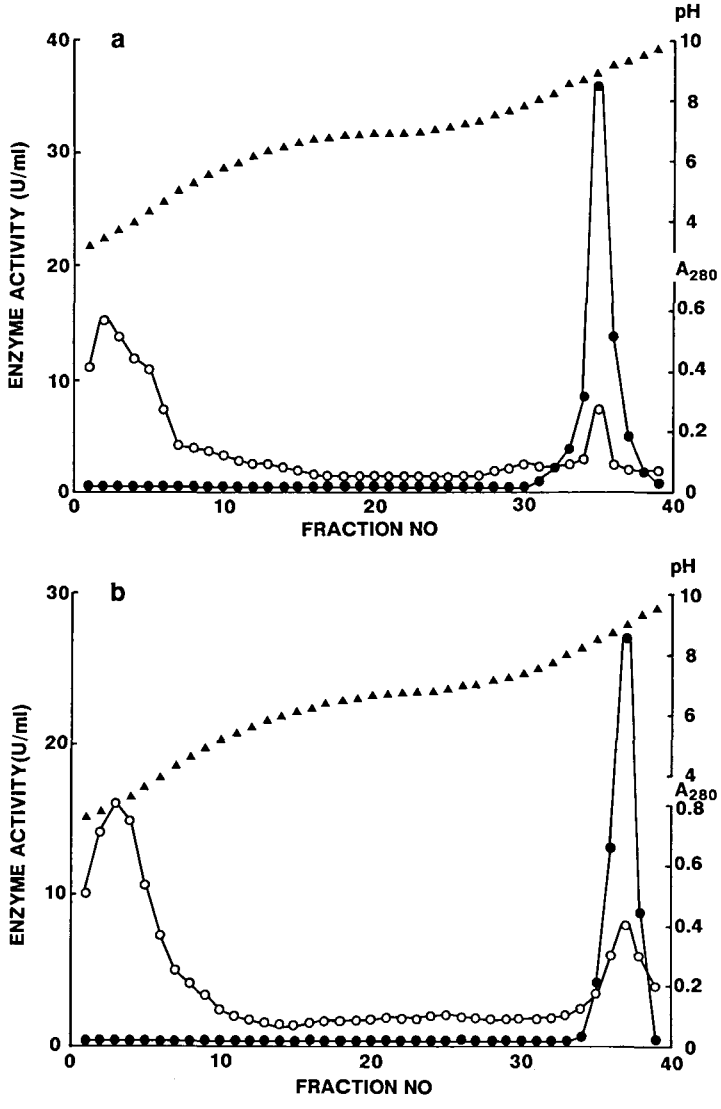

Fig. 1: Isoelectric focusing of α-glucosidase of S120 fraction (a) and envelope (b). Symbols; ●: α-glucosidase activity, ○: absorbance at 280 nm, ▲: pH

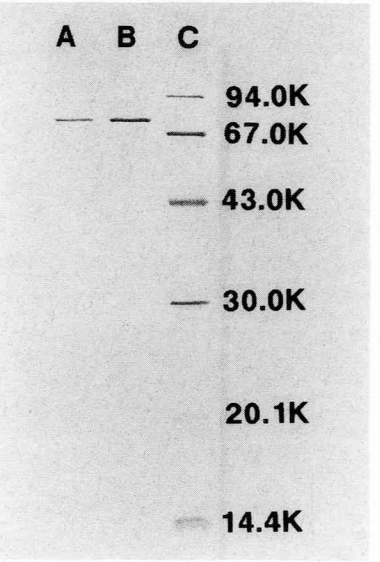

Fig. 2: SDS-PAGE of purified α-glucosidase of S120 fraction (Lane A), and envelope (Lane B). Standard proteins were run in Lane C.

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抄録：*Capnocytophaga ochracea* のα-グルコシダーゼ；その局在性，可溶化，精製および性状

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Capnocytophaga ochracea ATCC33596株の粗抽出液とエンベロープにα-グルコシダーゼ活性を検出した．エンベロープに結合したα-グルコシダーゼはセチトリメチルアンモニウムブロミドで効果的に可溶化された．両画分からのα-グルコシダーゼを硫安沈殿，イオン交換クロマトグラフィー，ゲル濾過，等電点電気泳動で精製した．精製された両酵素の性状は類似しており両者とも塩基性たんぱく質で，メタロ酵素であり，至適pHは7.0にあった．またパラニトロフェニル-α-D-グルコノピラノシドのみを加水分解した．