Degradation of 2-phosphoglycerate by cytotoxin B of *Clostridium difficile*

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Cytotoxin B of *C. difficile* was highly purified by selective ammonium sulfate precipitation, Biogel A5m chromatography, phenyl boronate hydrophobic interaction chromatography and ultracentrifugation. The final cytotoxic product had a specific activity of 7.8×10^8 units/mg protein and showed a single protein band with an estimated molecular weight of 163000 when subjected to SDS-PAGE. Immunoelectrophoresis of the final product showed a single precipitin arc The addition of cytotoxin B to imidazole-HCl buffer (pH 7.4) containing $MgSO_4$, KCl and the substrate 2-phosphoglycerate resulted in the formation of phosphoenolpyruvate as demonstrated by spectrophotometric analysis. Phosphoglycerate conversion was absent when the cytotoxin was heat-inactivated of reacted with specific antitoxin prior to assay.

Cytotoxin; Enolase; 2-Phosphoglycerate

1. INTRODUCTION

Clostridium difficile, the etiologic agent of pseudomembranous colitis and antibiotic-associated diarrhea [1–4], produces two exotoxins (A and B). Toxin A, an enterotoxin, induces intestinal fluid accumulation in rabbit ligated ileal loops, an acute inflammatory response, and hemorrhagic edema [5–8]. In contrast, toxin B does not induce intestinal fluid accumulation but is a potent cytotoxin for numerous cell types [8–10]. Various molecular masses, ranging from 50 000 to 600 000, have been reported for cytotoxin B [5,11–13]. These differences in molecular mass could be due to complex associations of a single peptide, enzymatic cleavage of a protoxin(s), or the production of multiple forms.

Recently, Torres and Lonnroth [14] reported the presence of two forms of cytotoxin B produced by the same bacterial strain. In other studies Bisseret et al. [15] attempted the purification of cytotoxin B, reporting a single homogeneous protein with a molecular mass of 290 000. The protein had a molecular mass of 52 000 under denaturing conditions. Comparison of the amino acid sequence of tryptic peptides of toxin B with sequences of known proteins showed high similarities between the cytotoxin B peptides and enolases (EC 4.2.1.11). The current studies were intiated to isolate and highly purify cytotoxin B by separate methods and, more importantly, to determine whether cytotoxin B had the ability to act as an enolase.

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2. MATERIALS AND METHODS

2.1. *Culture conditions*

C. difficile MC201, obtained from S.J. Booth (University of Nebraska Medical Center, Omaha), was used for toxin production. The organism was grown (37°C, 48 h) in a one-liter round-bottomed flask containing 500 ml supplemented peptone, as described [11].

2.2. *Toxin purification*

A cell-free filtrate (0.45 μm, Millipore) was concentrated 3-fold by YM-100 ultrafiltration (Amicon). After 75% saturation with ammonium sulfate, the precipitate was collected by centrifugation and suspended in 8 ml of cold 67 mM K-phosphate buffer (KPB; pH 7.5). The sample was fractionated on a 2.5×90 cm column of Biogel A5m (BioRad) and 8 ml fractions automatically collected. Cytotoxic fractions were pooled and concentrated on a YM-5 membrane (Amicon).

The YM-5 retentate was applied to a phenyl boronate column (Amicon) and eluted with a 100 mM D-ribose gradient. Cytotoxic fractions were pooled, concentrated to 3.0 ml on a YM-5 ultrafiltration membrane and separated on an 11 ml sucrose density gradient (1.134–1.149 g/ml). The gradient was cushioned with 1 ml of 40% sucrose and completed at $205\,000 \times g$ for 48 h (4°C) in an IEC model B60 ultracentrifuge (SB282 rotor). The gradient was eluted and cytotoxic fractions dialyzed against 15 liters of 0.05 M KPB. Protein concentration was estimated by the method of Bohlen et al. [16].

2.3. *Toxin assays*

Mouse Y1 adrenal cells were used to determine the activity of cytotoxin B, as described [9]. Cytotoxic units were expressed as the reciprocal of the dilution which induced in excess of 90% cell rounding. The presence of enterotoxic activity was determined in the rabbit ligated ileal loop assay, as described [17].

2.4. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*

Samples were analysed in a vertical slab unit (BioRad) using a gel thickness of 0.75 mm and an acrylamide concentration of 10%, as described [18]. The gels were silver stained [19] and molecular masses determined by comparison to standard reference proteins.

2.5. *Immunoelectrophoresis (IEP)*

Samples were subjected to IEP in 2% Noble Agar, as described

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Table I

Summary of procedures for the isolation of *Clostridium difficile* cytotoxin B[a]

Purification procedure	Biological activity[b]	Protein	Specific activity (units/mg protein)	Percent yield
Cell-free filtrate	5×10^8	5200 mg	9.6×10^4	100
XM-100 concentrate	4×10^8	2880 mg	2.4×10^5	80
$(NH_4)_2SO_4$ precipitate	4×10^8	190 mg	2.1×10^6	80
Biogel A5m	1×10^8	15 mg	6.7×10^6	20
Phenyl boronate	1×10^8	7 mg	1.4×10^7	20
Ultracentrifugation	7×10^7	90 μg	7.8×10^8	14

[a]The standard deviation was less than 5% for all values; data represents cytotoxic activity
[b]Data represent total recovery by each respective procedure

[20]. Goat antiserum against *C. difficile* culture filtrate was obtained from T.D. Wilkins (VPI, Blacksburg, VA).

2.6. *Assay of enolase activity*

Enolase activity was assayed by the spectrophotometric method of Warburg and Christian [21], as described [22]. The reaction mixture consisted of 50 mM imidazole-HCl buffer (pH 7.4) supplemented with 1 mM $MgSO_4$, 400 mM KCl and 1 mM 2-phosphoglycerate. After enzyme addition, the rate of phosphoenolpyruvate production was measured at 240 nm using a Beckman DB/GT spectrophotometer; maximum absorption (240 nm) for phosphoenolpyruvate was determined previously by scanning the product in a spectrophotometer. One unit of enzyme activity was defined as the amount required to convert 1 μmole of 2-phosphoglycerate to phosphoenolpyruvate per minute.

2.7. *Phosphoenolpyruvate formation*

The formation of phosphoenolpyruvate from 2-phosphoglycerate was determined by coupled enzymatic reactions, using lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) Briefly, the reaction system contained final concentrations of 0.05 M imidazole-HCl buffer (pH 7.6) supplemented with 0.12 M KCl and 0.062 M $MgSO_4$, 0.6 mM ADP, 0.18 mM NADH, 27 units lactate dehydrogenase, 3.9 units pyruvate kinase and 1×10^5 units cytotoxin B in a final volume of 3.0 ml. After 5 min for equilibration the reaction was initiated by the addition of 0.1 mM 2-phosphoglycerate. The decrease in optical density at 340 nm was observed as a measure of the formation of phosphoenolpyruvate as described (Boehringer Mannheim Biochemicals, Indianapolis, IN). Control reactions were completed in the absence of cytotoxin B or 2-phosphoglycerate.

2.8. *Statistical analysis*

All calculations were performed with an IBM/PC using a PHARM/PCS version 3.0 (Microcomputer Specialists, Elkins Park, PA) computer program.

3. RESULTS AND DISCUSSION

Cytotoxin B was highly purified by using ammonium sulfate precipitation, molecular sieve and hydrophobic interaction chromatography and sucrose density gradient ultracentrifugation (Table I). Culture cell-free filtrate was concentrated 3-fold by ultrafiltration and the proteins precipitated with ammonium sulfate. The resuspended precipitate was separated on a Biogel A5m column, resulting in a single protein peak at the column void volume and a bimodal peak during fractionation (Fig. 1). Toxic activity was located in the first portion of the bimodal peak.

Pooled and concentrated toxic fractions from the Biogel A5m column were separated by hydrophobic interaction chromatography using phenyl boronate (Fig. 2). Toxic activity was observed to elute in the apex of the absorption peak. All other fractions were void of toxic activity. Active fractions were then further separated by sucrose density gradient ultracentrifugation (Fig. 3). Elution of the gradient revealed the presence of a biphasic absorption peak with toxic activity associated with the latter phase. This method resulted in an 8125-fold increase in specific activity. Cytotoxin B (10^6 units) did not show enterotoxic activity in the rabbit ligated ileal loop model or suckling infant mouse assay.

Analysis of the final toxin sample by SDS-PAGE and immunoelectrophoresis revealed the presence of a

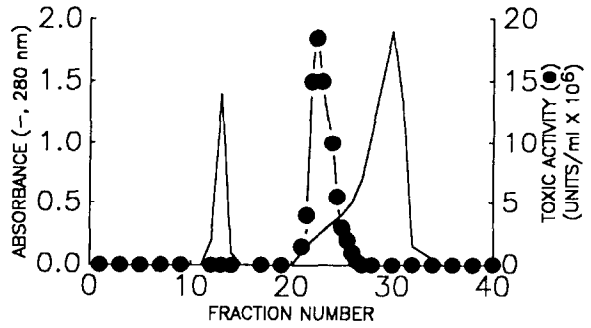

Fig. 1. Biogel A5m molecular sieve chromatogram.

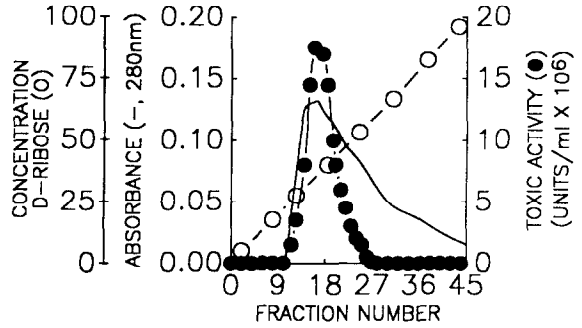

Fig. 2. Phenyl boronate hydrophobic interaction chromatogram. D-Ribose concentration is represented in mM.

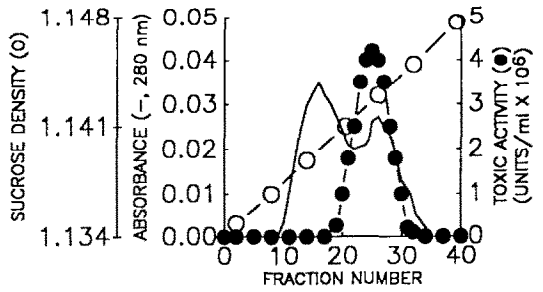

Fig. 3. Sucrose density gradient ultracentrifugation elution profile. Sucrose density is represented in g/ml.

single protein band with a molecular mass of 163 000 (not shown) and a single precipitin arc, respectively (Fig. 4). These results on molecular mass are similar to a single subunit complex of the 'form 2' cytotoxin recently described by Torres and Lonnroth [14]. Variable differences in the molecular mass of cytotoxin B reported in the literature [11–13] could be related to the association(s) of subunits. Additional studies are underway to determine the subunit composition of cytotoxin B.

In further studies the final toxin sample showed an enzymatic activity similar to enolase (EC 4.2.1.11) from *Saccharomyces cerivisiae*. Using 2-phosphoglycerate as substrate, the rate of toxin-mediated phosphoenolpyruvate formation was assessed spectrophotometrically at 240 nm (Fig. 5). As shown, cytotoxin B reacted similar to yeast enolase, resulting in an increase of phosphoenolpyruvate. The formation of phosphoenolpyruvate was confirmed by using the pyruvate kinase/lactate dehydrogenase enzyme system, which is coupled to the oxidation of NADH (Fig. 6). Under the stated conditions, cytotoxin B had an enolase activity of approximately 700 units per mg protein. The specific activities for chicken brain, chicken skeletal-muscle and human brain enolases are 85, 78 and 91 units per mg protein, respectively [23–25]. The catalytic effect of

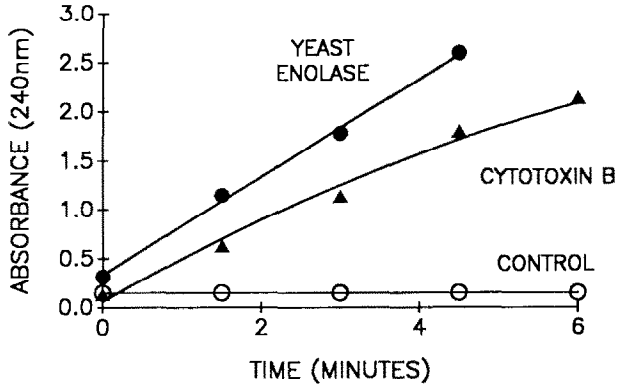

Fig. 5. Time course of enolase activity identified with isolated cytotoxin B. No difference was observed between the control (buffer) and enzyme reacted with heat or antitoxin.

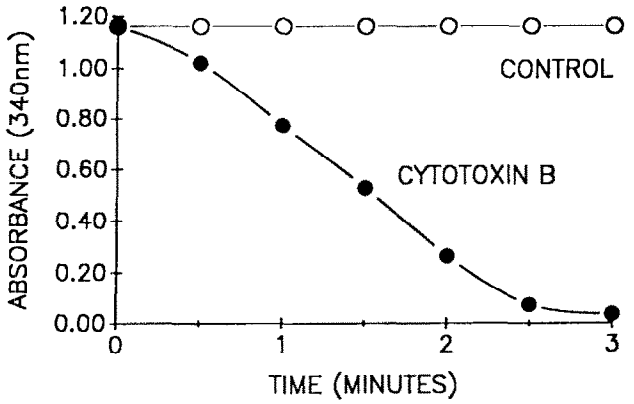

Fig. 6. Formation of phosphoenolpyruvate by cytotoxin B. The reaction was coupled to pyruvate kinase/lactate dehydrogenase enzymes, resulting in the oxidation of NADH.

cytotoxin B/enolase was inhibited by antitoxin and heat inactivation (Fig. 5). These results support the studies of Bisseret et al. [15] which indicate that the amino acid sequence of cytotoxin B is similar to enolases from rat and *S. cerivisiae*.

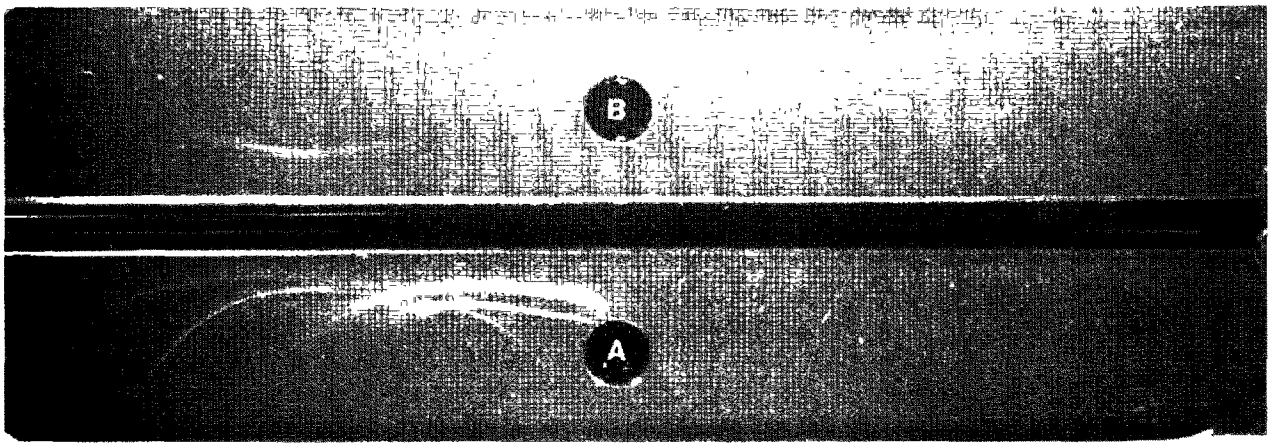

Fig. 4. Immunoelectrophoresis of *C. difficile* cytotoxin B fractionated by phenyl boronate hydrophobic interaction chromatography (A) and sucrose density gradient ultracentrifugation (B).

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