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CLOSTRIDIUM DIFFICILE TOXIN B: CHARACTERIZATION AND SEQUENCE OF THREE PEPTIDES

FRANÇOISE BISSERET

Institut de Bactériologie de la Faculté de Médecine, 3 Rue Koeberlé, 67000 Strasbourg (France)

GÉRARD KEITH

Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 Rue Descartes, 67084 Strasbourg Cedex (France)

BERTRAND RIHN*

Institut de Bactériologie de la Faculté de Médecine, 3 Rue Koeberlé, 67000 Strasbourg (France)

IRADJ AMIRI

Altergene Laboratoires, 4 Rue des Sarcelles, 67300 Strasbourg (France)

and

BRIGITTE WERNEBURG, RAYMONDE GIRARDOT, OLIVIER BALDACINI, GAYNOR GREEN, VU KHUE NGUYEN and HENRI MONTEIL

Institut de Bactériologie de la Faculté de Médecine, 3 Rue Koeberlé, 67000 Strasbourg (France)

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SUMMARY

The cytotoxin, also named toxin B, was isolated from a toxigenic strain of *Clostridium difficile*, purified to homogeneity and partially characterized. The purification procedure included ultrafiltration followed by anion-exchange chromatography. We noticed that a non-specific nucleic material eluted with the protein during the purification. The presence of these nucleic acids appeared to be important for the toxic activity of the protein. Some characteristics of the cytotoxin were examined, especially the amino acid composition and the sequence of three tryptic fragments.

INTRODUCTION

The anaerobic *Clostridium difficile* bacterium produces two toxins, an enterotoxin (toxin A) and a cytotoxin (toxin B), which have been implicated

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in the etiology of antibiotic-associated pseudomembranous colitis [1,2]. The present report deals exclusively with studies on toxin B, which is known to induce cellular lesions in many cell systems [3]. Different purification methods have been described for the cytotoxin [4–9]; however, there are discrepancies as far as the characterization of the toxin is concerned. The major difficulty in the purification of toxin B is the low recovery from chromatographic columns. The cytotoxin was previously purified in our laboratory and some of its physico-chemical properties have been determined [10].

We report here the purification of the cytotoxin using a synthetic culture medium to cultivate the bacteria. We noticed the presence of nucleic acids together with the toxin during the purification procedure. That observation has not previously been reported in the literature. These findings prompted us to investigate the nature and the role of this nucleic acid material. Also we report the amino acid composition of the protein and the sequence of three tryptic digestion fragments.

EXPERIMENTAL

Culture conditions and toxin purification

C. difficile (strain No. 68750) was isolated in our laboratory from a patient who had developed pseudomembranous colitis. It was grown under anaerobic conditions for 72 h at 37°C with stirring in a synthetic medium containing hemin (1 mg/l) (Haslam et al. [11]). The culture (20 l) was filtered and concentrated prior to fast protein liquid chromatography (FPLC, Pharmacia, Uppsala, Sweden) according to Rihn et al. [10]. After the anion-exchange FPLC step, the fractions containing the highest cytotoxic activity were pooled. The cytotoxic activity was detected using McCoy cells as described by Tytgat [12], the minimum toxic dose being defined as the lowest amount of protein able to round all 100 000 cells located in a culture well. The protein content was assayed by the method of Bradford [13], using bovine serum albumin as a standard.

Amino acid composition and tryptic digestion

The amino acid composition was determined in the fraction with the highest cytotoxicity. Amino acid analyses of the hydrolysate (6 *M* hydrochloric acid at 110°C for 20 h) were carried out on a high-performance liquid chromatographic (HPLC) apparatus (Waters Assoc., Milford, MA, U.S.A.; column: Picotag reversed-phase C_{18}, 300 mm×3.9 mm I.D., 4 μm, 80 Å) using a pre-column derivatization procedure with phenyl isothiocyanate. Results were expressed as residues per 100 amino acids.

We also analysed three peptides resulting from the tryptic digestion of the protein. For this, 500 μg of toxin B were reduced, aminoethylated and citraconylated using the methods described by Raftery and Cole [14] and Dixon

and Perham [15], which were modified and adapted to microamounts. The modified protein was desalted by gel filtration on Sephadex G-25 (400 mm × 20 mm I.D., Pharmacia, Les Ulis, France), with 0.1 *M* ammonium bicarbonate as buffer, and recovered by lyophilization. The modified toxin B was then subjected to tryptic hydrolysis in 0.1 *M* ammonium bicarbonate at 37 °C for 4 h. The substrate/enzyme ratio was 50:1 (m/m). The action of protease was stopped by freeze-drying of the sample. The tryptic peptides were separated, after decitraconylation, on an HPLC apparatus (Beckman 421, Berkeley, CA, U.S.A.) using a reversed-phase column (Vydac C_4, 10 μm, 300 Å; Hesparia, CA, U.S.A.). Three fragments were sequenced on a fully automated protein sequencer Model 470 A (Applied Biosystems, Dr. Foster City, CA, U.S.A.).

Characterization of the co-eluted nucleic acids

Nucleic acids were isolated from purified cytotoxin after precipitation of the protein with ethanol at −20 °C. Proteins were removed by proteinase K digestion (200 μg/ml solution, 37 °C, 1 h.). Nucleic acids were then extracted in the aqueous phase using successively phenol, phenol–chloroform (1:1, v/v) and finally chloroform. A last extraction with diethyl ether was used to remove traces of phenol.

The 5′ ends of the extracted nucleic acids were labelled by T_4 polynucleotide kinase (10 U), using 0.1 mCi [^{32}P]ATP (3000 Ci/mmol, Amersham, Les Ulis, France) in the presence of 30 m*M* Tris–HCl (pH 8.0), 15 m*M* magnesium chloride, 15 m*M* 2-mercaptoethanol and 1 m*M* spermidine [16,17]. Labelled nucleic acids were then subjected to two-dimensional polyacrylamide gel electrophoresis (PAGE) [18,19]. The first-dimension gel was 25 m*M* citric acid (pH 3.5), 7 *M* urea, 10% acrylamide (N,N′-methylenebisacrylamide–acrylamide, 1:30). The analysis was performed at 300–500 V for 10–15 h at 4 °C.

The first-dimension band was cut out and included in the second gel slab. The second-dimension gel was 90 m*M* Tris–borate (pH 8.3), 7 *M* urea, 25 m*M* EDTA, 20% acrylamide (N,N′-methylenebisacrylamide–acrylamide, 1:25). The migration was started at a very low voltage (100 V), which was increased progressively to 500 V over 48 h. After migration, the gel was autoradiographed.

Effect of ribonucleases and deoxyribonucleases on the cytotoxic activity

Purification of toxin B was performed with and without ribonucleases and deoxyribonucleases. Each assay was realized using 20 l of culture, half for the blank and half with the following enzyme mixture: 5 U of ribonuclease A from bovine pancreas (111 Kunitz U/mg, Serva, Heidelberg, F.R.G.), 11 U of nuclease S_1 from *Aspergillus oryzae* (53 U/ml, Appligène, Strasbourg, France), 5 U of ribonuclease T_1 from *A. oryzae* (385 000 U/mg, Serva), 125 U of DNAse I from bovine pancreas (460 U/mg, Sigma, St. Louis, MO, U.S.A.) and 110 U of DNAse II from bovine spleen (400 U/mg, Sigma). These enzymes were

added twice in the synthetic medium, before inoculation and the day before purification.

RESULTS AND DISCUSSION

Purification and characterization of toxin B

Few authors have used a synthetic medium for the cultivation of *C. difficile*. Rolfe and Finegold [8] examined different media for their ability to support growth and toxin production, especially chopped meat glucose, peptone yeast extract, brain–heart infusion and also a synthetic medium. These authors prepared the synthetic medium by autoclaving all constituents except cysteine and sodium carbonate, which were filter-sterilized and added later. Under these conditions, they did not observe any toxin production. Our synthetic medium contained the same components as those of Rolfe and Finegold [8], but was prepared according to Haslam et al. [11] with a supplement of hemin. On FPLC, a gradient from 0 to 1 *M* sodium chloride allowed us to distinguish the enterotoxic (toxin A) from the cytotoxic (toxin B) activities. The latter eluted as a sharp peak at 0.54 *M* sodium chloride; 3 ng of this fraction were able to round 100 000 cells (Fig. 1). Purified cytotoxin, as assayed on PAGE (Fig. 2), was homogeneous in the presence or absence of sodium dodecylsulphate (SDS) in the gel buffer.

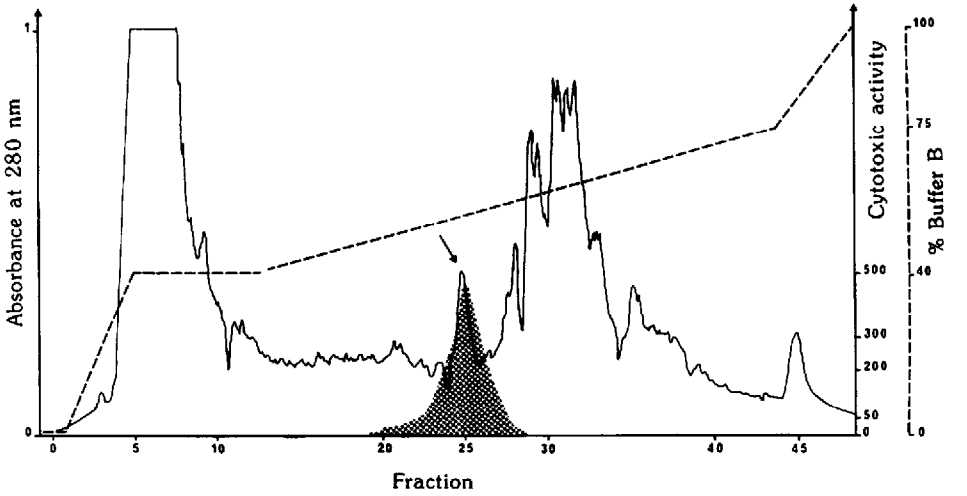

Fig. 1. Anion-exchange chromatography. Column, Mono Q, HR 10/10, Pharmacia, Les Ulis, France; buffer A, 10 m*M* Tris-HCl (pH 7.4); buffer B, buffer A containing 1 *M* sodium chloride; flow-rate, 4.0 ml/min; 100 mg of protein were loaded on the column. Enterotoxic activity was found in fractions 5, 6, 7 and 8; the shaded area indicates cytotoxic activity. For each fraction we used the inverse of the minimum toxic dose (μg) as a scale of cytotoxic activity. The arrow indicates the most toxic fraction (this fraction represents ca. 700 μg). Fraction 45 contains nucleic acids only.

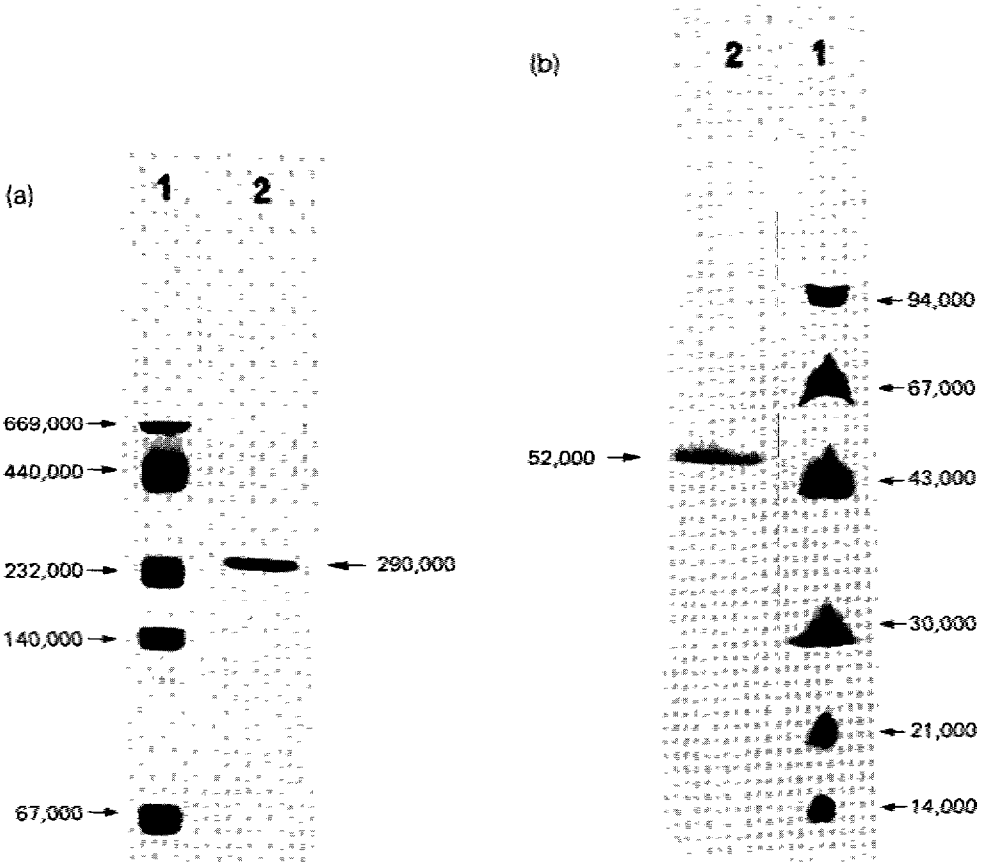

Fig. 2. Silver-stained PAGE of toxin B (8–25% acrylamide). Electrophoresis was performed according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden) (a) Without SDS: lane 1, high M_r calibration kit (Pharmacia): thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000), bovine serum albumin (67 000); lane 2, 1 μg of purified toxin. (b) With SDS: lane 1, low M_r calibration kit (Pharmacia): phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000), α-lactalbumin (14 400); lane 2, 1 μg of purified toxin.

The relative molecular mass (M_r) was estimated to be 52 000 under denaturing conditions and 290 000 under non-denaturing conditions, as was reported previously [7,10]. The dissociation of the cytotoxin into a single protein band at 52 000 supports its multimeric nature, as suggested by Rolfe and Finegold [8].

The amino acid composition of toxin B is presented in Table I. The content of aspartic and glutamic acids, glycine, alanine, leucine and valine is high, whereas that of methionine is low. Basic, acidic and non-polar hydrophobic amino acid residues accounted for 11.5, 47.7 and 40.8% of the total residues,

TABLE I

AMINO ACID COMPOSITION OF *C. DIFFICILE* TOXIN B

Amino acid		Residues per 100 amino acids
Aspartic acid[a]	(D+N)	11.62
Glutamic acid[b]	(E+Q)	12.24
Serine	(S)	4.30
Glycine	(G)	10.90
Histidine	(H)	1.05
Arginine	(R)	4.14
Threonine	(T)	4.71
Alanine	(A)	10.38
Proline	(P)	3.85
Tyrosine	(Y)	2.96
Valine	(V)	7.71
Methionine	(M)	1.54
Isoleucine	(I)	5.42
Leucine	(L)	9 18
Phenylalanine	(F)	2.74
Tryptophan	(W)	Not determined
Lysine	(K)	6.35
Cysteine[c]	(C)	0.95

[a]Includes aspartic acid and asparagine.
[b]Includes glutamic acid and glutamine.
[c]Determined as aminoethylcysteine.

respectively. Our results for the amino acid composition appear to be similar to those of Pothoulakis et al. [7].

Furthermore, no sequence data were known when this work was commenced, therefore we determined the primary structure of several tryptic peptides of toxin B. After chemical modification of the protein (by action of ethylene imine and citraconic anhydride), enzymic digestion and peptide separation, three informative fragments corresponding to peaks 4, 5 and 13 (Fig. 3) were selected for amino acid sequencing. The sequences are shown in Table II.

Surprisingly the comparison with a protein data bank showed high similarities between these sequenced toxin B peptides and the enolases (EC 4.2.1.11) from rat and *Saccharomyces cerivisiae* (Table III). Further work to check whether toxin B has enolase activity or not is in progress in our laboratory.

Characterization and role of the co-eluted nucleic acids

In the first experiments performed with a brain–heart culture broth, large amounts of nucleic acids were found to co-elute with the cytotoxic activity. To rule out the possibility of nucleic material coming from the culture medium we used a synthetic basal medium.

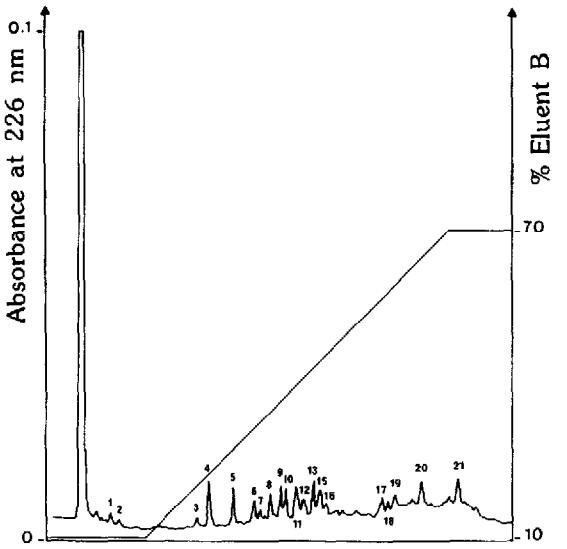

Fig. 3. Separation of tryptic peptides by HPLC. Column, Vydac C_4 (250 mm×4.6 mm I.D.). Eluent A, 0.1% trifluoroacetic acid (TFA); eluent B, 60% acetonitrile containing 0.1% TFA; flow-rate, 2.0 ml/min.

TABLE II

SEQUENCES OF THREE TRYPTIC FRAGMENTS OF TOXIN B

Fragment	Amino acid sequence
4	A-G-Y-T-A-V-I-S-H-R
5	V-A-K-Y-N-Q-L-L-R
13	M-G-A-E-V-F-H-S-L-K-K-V-L-G-E-K-G-L-A-S-G-V-G-D-E-G-G-F-A-P-N-L-G-S-N-I-R-(R)

TABLE III

COMPARISON BETWEEN THE FRAGMENT 13 OF TOXIN B AND α-ENOLASE OF RAT AND ENOLASE-2 OF *S CEREVISIAE*

Toxin B fragment 13	[1]	MGAEVFHSLKKVLGEK . . GLASGVGDEGGFAPNLGSN [35]
α-Enolase (rat)	[184]	IGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILEN [220]
Enolase-2 *S cerevisiae*	[185]	IGSEVYHNLKSLTKKRYGASAGNVGDEGGVAPNIQTA [221]

As determined by UV absorption, the highly toxic peak contains 2.5% nucleic acids (not shown). However, a second run of the latter toxic fraction on the same anion-exchange column gave a twenty-fold less active protein, con-

taining only 1% nucleic acids. In addition, this nucleic acid fraction could not be eliminated completely by further purification. Furthermore, additional enzymic removal of nucleic acids always significantly lowered the protein cytotoxicity, suggesting their importance for the cytotoxic properties.

For a better understanding of the role of these nucleic acids, we extracted them from the cytotoxin. The nucleic acid material associated with the toxin B migrates with a relative electrophoretic mobility similar to the xylene cyanol dye during 20% PAGE (Fig. 4a). Therefore an average length of twenty nucleotides could be estimated. This length was confirmed by a comparison of the amount of labelled end-nucleotide with the total nucleotide content according to Keith et al. [17]. Analysis of the nucleic acids after 20% PAGE, their enzymic digestion followed by thin-layer chromatography showed the presence of both ribo- and deoxyribonucleotides. Autoradiography after ^{32}P end-labelling of the nucleic acids and separation by two-dimensional PAGE revealed random, non-specific polydisperse nucleic acid material (Fig. 4b).

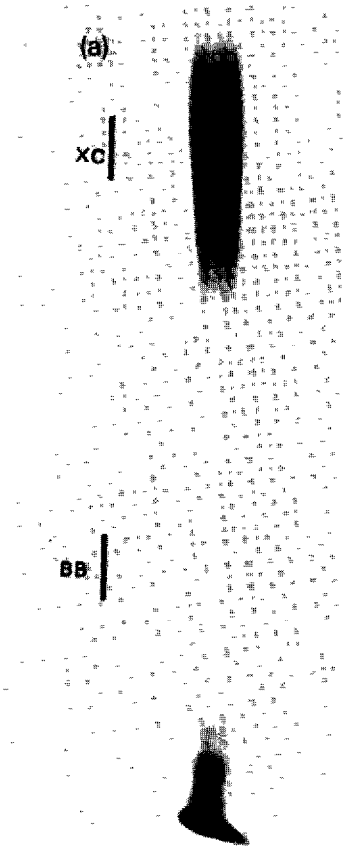

Fig. 4.

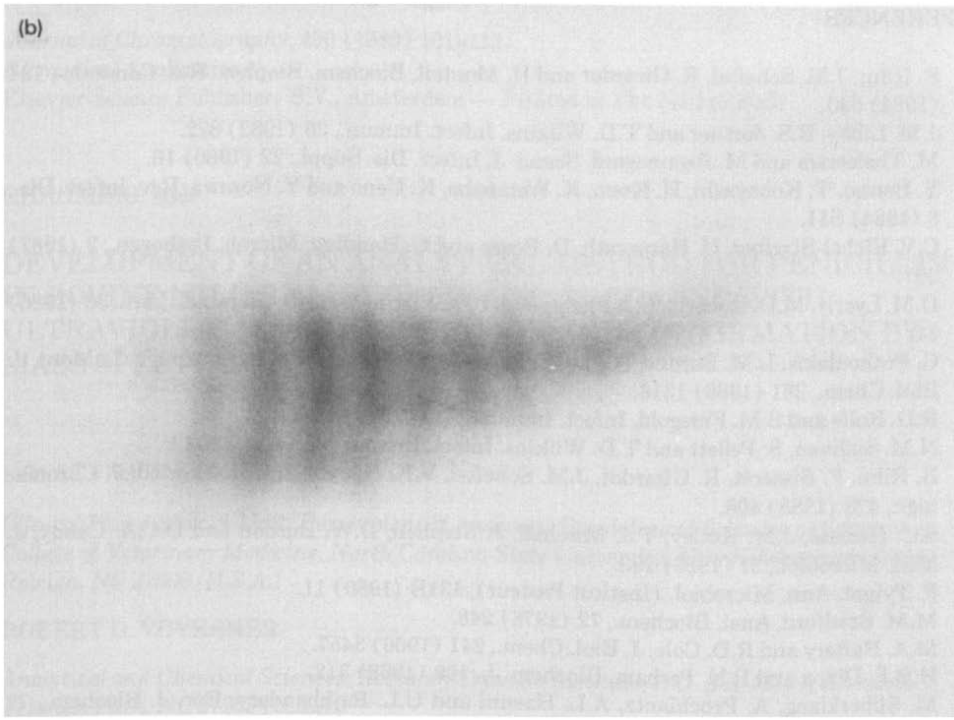

Fig. 4. (a) Autoradiography of nucleic acid material co-purified with toxin B. Conditions used for labelling and electrophoresis are described in Experimental. Sample, 1 μg of ^{32}P end-labelled nucleic acid (XC = xylene cyanol dye; BB = bromophenol blue dye). (b) Autoradiography of the two-dimensional gel. Sample, 2 μg of ^{32}P end-labelled nucleic acid.

The addition of nucleases to the culture medium suppressed the nucleic acid peak at 0.8 *M* sodium chloride in the chromatographic profile. Furthermore, the cytotoxic activity was three times lower in terms of specific activity, compared with non-treated medium: indeed, 10 ng of cytotoxin were required for rounding 100 000 cells instead of only 3 ng of toxin prepared in the absence of nucleases. This result was observed many times and again suggested a relationship between toxin B cytotoxicity and the presence of nucleic acid material.

To our knowledge this is the first report of co-elution of nucleic acids with *C. difficile* toxin B; investigations are in progress to elucidate the binding between the protein and the nucleic acids and to specify the role of these nucleic acids in the cytotoxic process.

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