Biochimie 95 (2013) 2123–2131

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Purification and characterisation of recombinant *Bacteroides fragilis* toxin-2

D.D. Kharlampieva [a,*], V.A. Manuvera [a], O.V. Podgorny [a,b], S.I. Kovalchuk [a,c], O.V. Pobeguts [a], I.A. Altukhov [a], D.G. Alexeev [a,d], V.N. Lazarev [a,d], V.M. Govorun [a,c,d]

[a] *Research Institute for Physico-Chemical Medicine of the Federal Medical and Biological Agency of Russian Federation, Malaya Pirogovskaya Str. 1a, Moscow 119435, Russia*
[b] *Koltzov Institute of Developmental Biology of the Russian Academy of Sciences, Vavilov Str. 26, Moscow 119334, Russia*
[c] *Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Miklukho-Maklaya Str., 16/10, Moscow 117997, Russia*
[d] *Moscow Institute of Physics and Technology (State University), Institutskiy per. 9, g. Dolgoprudny, Moscovskaya obl 141700, Russia*

ARTICLE INFO

Article history:
Received 18 March 2013
Accepted 6 August 2013
Available online 15 August 2013

Keywords:
Bacteroides fragilis
BFT
Fragilysin

ABSTRACT

Fragilysin (BFT) is metalloprotease that is secreted by enterotoxigenic *Bacteroides fragilis*. Studying the mechanism of BFT interaction with intestinal epithelial cells requires a pure protein sample. In this study, we cloned DNA-fragments coding for the catalytic domain of fragilysin-2 and profragilysin-2 into an *E. coli* expression vector. Purification methods for the recombinant fragilysin-2 catalytic domain and profragilysin-2 were developed. In addition, we obtained mature active fragilysin-2 from recombinant proprotein by limited tryptic digestion. We tested the biological activity of the recombinant protein samples and revealed that E-cadherin was cleaved when HT-29 cells were treated with mature fragilysin-2 but not with profragilysin-2. Azocoll, azocasein and gelatin were not proteolytically cleaved by mature fragilysin-2. Proteins released in culture medium after HT-29 cells treatment with mature active BFT-2 were identified.

1. Introduction

Bacteroides fragilis is a gram-negative bacillus bacterium present in normal intestinal flora. *B. fragilis* participates in carbohydrate fermentation and biotransformation of bile acids [1]. However, there are toxigenic *B. fragilis* strains termed enterotoxigenic *B. fragilis* or ETBF. In 1984, Myers and colleagues presented evidence that certain strains of *B. fragilis* were associated with diarrhoeal disease in new-born lambs [2]. In 1987, enterotoxigenic strains of *B. fragilis* were isolated from stool specimens of humans with diarrhoea [3]. In further studies, the virulence factor of enterotoxigenic *B. fragilis* (named fragilysin or BFT – *B. fragilis* toxin) was isolated. It was demonstrated that BFT stimulates E-cadherin cleavage and destruction of tight junctions in intestinal epithelium [4]. Additionally, ETBF persistence is associated with inflammatory bowel disease and colorectal cancer. Other studies have shown that BFT induces activation signal pathways leading to proto-oncogene C-myc and cytokines synthesis [5]. Fragilysin treatment of intestinal epithelial cells stimulates shedding of the 80-kDa E-cadherin ectodomain, but other cell membrane proteins were not identified [6]. It was proposed that fragilysin binds to specific intestinal cell receptors [7].

BFT is synthesised as a preproprotein consisting of a signal peptide and two domains. During maturation, the signal peptide and N-terminal domain are cleaved, and the C-terminal domain is the mature form of the toxin [8]. Three isoforms of the toxin with several differences in their primary structures were identified. The catalytic C-terminal domain is more variable than other parts of the molecule. Differences in the sequences of BFT-1, BFT-2 and BFT-3 were observed in 2–5 positions of the prodomain and in up to 25 positions in the catalytic domain. Formation of an amphipathic structure was predicted for the C-terminal amino acid residues of BFT-2. This predicted structure is found only in BFT-2. Oligomerisation of BFT-2 may occur, allowing membrane insertion of the toxin and pore formation [9]. Nevertheless, there are no experimental data to support or refute this hypothesis.

A pure protein sample is required to study the mechanism of BFT interaction with intestinal epithelial cells. A method for isolating fragilysin from cultural medium was previously described [10]. The method utilised a multistage procedure consisting of ammonium sulphate precipitation, dialysis, preparative ion-exchange chromatography on Q-Sepharose, hydrophobic interaction chromatography

* Corresponding author. Tel./fax: +7 499 255 28 46.
E-mail address: harlampieva_d@mail.ru (D.D. Kharlampieva).

0300-9084/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.biochi.2013.08.005

on phenyl-agarose and high-resolution ion-exchange chromatography on MonoQ to characterise the low yielding protein of interest (32 μg from 1 L of cultural medium). A modification to this method was developed [11]. This modified method decreased the number of purification steps; however, only 80 μg of proteinase was recovered from 1 L of cultural medium. Moreover, because the bacterium is anaerobic, its cultivation in a laboratory requires special equipment.

In the heterological system of *E. coli*, only isoform 3 of fragilysin was obtained and characterised [12]. Recombinant BFT-2 was obtained in a homological expression system [13], but this method cannot be used to determine the function of the prodomain because only the mature form of the protein was found in the cultural medium. Moreover, unlike BFT-1 and BFT-3, the proteolytic activity of BFT-2 *in vitro* has not been studied [10,12,14].

In this study, we cloned DNA-fragments coding for the catalytic domain of fragilysin-2 (CD-BFT) and profragilysin-2 without the signal peptide (proBFT) into an *E. coli* vector with regulated expression. After induction of heterological expression, the polypeptides of interest accumulated in the insoluble cell fraction. Furthermore, we purified the recombinant catalytic domain of fragilysin-2 and profragilysin-2 by metal-chelate chromatography. Methods to solubilise profragilysin-2 and process it *in vitro* by limited tryptic digestion were developed. As result of profragilysin-2 limited tryptic digestion mature fragilysin-2 was obtained (mBFT-2). E-cadherin cleavage in the HT-29 cell line after mBFT treatment was demonstrated. In addition, we studied the proteolytic activity of recombinant fragilysin-2 *in vitro* using azocoll, azocasein, gelatin and recombinant E-cadherin. Proteins released in culture medium after HT-29 treatment with mBFT-2 were identified.

2. Materials and methods

2.1. Materials

Inorganic salts (Merck, Germany; Sigma, USA); the molecular weight markers GeneRuler DNA Ladder Mix and Unstained Protein Molecular Weight Marker (MBI Fermentas, Lithuania); and the medium compounds tryptone, yeast extract, and agar (Helicon, Russia) were used.

2.2. Strains and plasmids

E. coli B834(DE3) (Novagen, USA), Top10 (Invitrogen, USA) and plasmid vectors pET15b, 22b (Novagen, USA), pBAD/GIII (Invitrogen, USA) and pGEM-T-easy (Promega, USA) were used.

2.3. Oligodeoxyribonucleotides

Oligodeoxyribonucleotides with the following structures (Litech, Russia) were used in this study:

bftNdeF 5′-CATATGGCAGTACCTTCTGAACCTAA.
bftBamR 5′-GGATCCCTAATCGCCATCTGCTATTT.
T7 5′-TAATACGACTCACTATAGGG.
T7t 5′-GCTAGTTATTGCTCAGCGG.
pBft-Bgl 5′-ATATAGATCTATGGCATGTTCTAATGAAGCTGAT.
C-Bft-Sal 5′-TTAAGTCGACATCGCCATCTGCTATTTCCCA.
cadPro F 5′-ATATAGATCTCAGGAGCCGGAGCCCTGCCA.
cadXho R 5′-ATATCTCGAGGTCGTCCTCGCCGCCTCCGT.

2.4. Polymerase chain reaction (PCR)

PCR was performed in an Eppendorf Mastercycler thermal cycler (Eppendorf, Germany) with a reaction volume of 20–25 μl with 2–3 mM $MgCl_2$, 0.125–0.2 mM of each dNTP, 67 mM Tris–HCl (pH 8.3), 16.7 mM $(NH_4)_2SO_4$, 0.5 unit Taq-polymerase (MBI Fermentas, Lithuania), 1–10 ng DNA and 5 pmol of each primer. The thermal cycling conditions (°C/sec) were 95/120 for 1 cycle and 95/10, 55–60/10, and 72/20–150 for 25 cycles.

2.5. Cloning of DNA-fragments coding for BFT-2 catalytic domain and proBFT-2

DNA-fragments coding for the BFT-2 catalytic domain and proBFT-2 were amplified using pairs of oligonucleotides, bftNdeF – bftBamR and pBft-Bgl – C-Bft-Sal, as primers, respectively, and using enterotoxigenic *B. fragilis* DNA as a template. The resulting PCR product (for DNA-fragment coding for BFT-2 catalytic domain) was cloned into a T-vector, pGEM-T-easy, according to the manufacturer's instructions (Promega). The resulting vector was treated with endonucleases *Nde*I and *BamH*I (MBI Fermentas, Lithuania). The DNA fragment of interest was isolated by preparative electrophoresis and ligated into the pET15b expression vector previously hydrolysed by the same enzymes. PCR product for DNA-fragment coding for proBFT-2 and pBAD/GIII-B plasmid (Invitrogen, USA) were treated with endonucleases *Bgl*II and *Sal*I (MBI Fermentas, Lithuania) and ligated. The ligation mixtures were transformed into the *E. coli* Top 10 strain and plated on LB plates containing 100 μg/ml ampicillin. Clones containing the insert were selected using PCR with T7 – T7t and pBft-Bgl and C-Bft-Sal primers respectively. Then, the plasmid DNA was isolated from the selected clones. The resulting plasmids containing the insert and coding for the BFT-2 catalytic domain and proBFT-2, fused with the signal peptide of fd phage GIII protein, were named pET15b-bft and pBAD/GIII-prBft.

Nucleotide sequences of the cloned fragments were verified by sequencing using the BigDye Terminator Cycle Sequencing Kit (v. 3.1) and AbiPrism 3730xl (Applied Biosystems, CШA).

2.6. Isolation and purification of recombinant proteins

The *E. coli* B834(DE3) and Top10 strains were transformed with the plasmids pET15b-bft and pBAD/GIII-prBft, respectively, plated on an LB dish (150 μg/ml ampicillin) and incubated overnight at 37 °C. Then, 100 ml of LB medium containing 150 μg/ml ampicillin was inoculated with a single colony of B834(DE3)/pET15b-bft and grown at 37 °C to an OD_{600} of ~0.8. Expression was induced by adding IPTG to a final concentration of 0.5 mM. The cells were cultured for an additional 4–5 h at 30 °C, spun down (3000 g, 15 min) and washed with cold PBS.

300 ml of LB medium containing 150 μg/ml ampicillin was inoculated with a single colony of Top10/pBADGIII-prbft and grown at 37 °C in a shaker at 180 rpm for 16 h. The culture was then added to 2.7 L of TB medium (150 μg/ml ampicillin) in a BIOFLO 110 Fermentor/Bioreactor (New Brunswick Scientific, USA) and grown for 2 h at maximal aeration (OD_{600} ~ 2). Expression was induced by adding arabinose to a final concentration of 0.5 g/L. The cells were cultured for an additional 4–5 h at 37 °C, spun down (3000 g, 15 min) and then resuspended in 150 ml of TE buffer (10 mM TrisHCl and 1 mM EDTA at pH 8.0).

The cells were disrupted by sonication using a Branson Sonifier 250 (VWR Scientific, USA) sonicator according to the manufacturer's instructions. The lysate was purified by centrifugation (15,000× *g*, 25 min) and washed twice with 1% (v/v) Triton X-100. The pellet was stored at −20 °C.

To isolate proBFT-2, an aliquot of an inclusion body suspension from 1 L of the culture was centrifuged (50,000× *g*, 15 min) and dissolved in 20 ml of buffer A (20 mM Na^+-phosphate buffer, 8 M urea, 0.5 M NaCl and 10 mM imidazole at pH 7.4) with 0.01% (v/v) β-mercaptoethanol. The solution was purified by centrifugation (50,000× *g*, 15 min) and applied to a column filled with 5 ml of Ni Sepharose High Performance media (GE healthcare, USA). Then, the