ELSEVIER

FEMS Microbiology Letters 136 (1996) 39–44

FEMS MICROBIOLOGY LETTERS

Identification and characterization of an extracellular protease activity produced by the marine *Vibrio* sp. 60

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Received 31 August 1995; revised 22 November 1995; accepted 25 November 1995

Abstract

The marine fish pathogen *Vibrio* sp. 60 has been used as a host for heterologous expression of the *Escherichia coli* heat-labile enterotoxin B-subunit and derivatives carrying a C-terminal extension. In this study, a chimeric enterotoxin B-subunit with an extension corresponding to the carboxy-terminal nine amino acids -Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu-COOH from the small subunit of herpes simplex virus type 1-encoded ribonucleotide reductase, is shown to be proteolytically cleaved in the extracellular medium by a single protease that is secreted by the host strain. Such protease behaves as a typical metalloprotease, being inhibited by EDTA but not by a serine protease inhibitor. Purification and amino acid composition analysis of the two proteolysis products revealed a specific cleavage of the peptide bond between amino acids glycine and alanine of the nine amino acid extension with loss of activity. The above observation is relevant for the biotechnological exploitation of *Vibrio* sp. 60.

Keywords: *Escherichia coli* heat-labile enterotoxin; *Vibrio* sp. 60; Heterologous expression; Protease

1. Introduction

Among Gram-negative bacteria, the Vibrionaceae exhibit the remarkable capacity to secrete proteins through their two membranes into the growth medium [1,2]. Proteins like cholera toxin, hemagglutinin and neuraminidase are, for example, important virulence factors secreted by *Vibrio cholerae*. One of the most abundant extracellular products of vibrios are proteases; in *V. cholerae*, activation of cholera toxin by limited proteolysis of the A-subunit is carried out by a protease sensitive to serine protease inhibitors [3]. Other proteases have been identified in the culture supernatants of various *V. cholerae* strains that could be of significance as virulence factors. Degradation of immunoglobulins, for example, could be important for the evasion of the host immune response and mucinase activity could allow penetration of the mucous layer which is essential for successful colonization of the lower intestine. Other *Vibrio* strains, like *V. anguillarum* and *V. alginolyticus*, produce a variety of extracellular proteases, some of which have been characterized [4,5].

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SSDI 0378-1097(95)00482-3

Escherichia coli heat-labile enterotoxin (Etx) is an hexameric protein toxin, produced by certain enterotoxigenic strains of *E. coli*, that is functionally and structurally related to cholera toxin [1,6]. The three-dimensional structure of Etx shows a doughnut-shaped pentamer of B-subunits (EtxB) with the toxic A1-subunit (EtxA1) non-covalently connected by the short A2 (EtxA2) polypeptide to the EtxB subunits [7]. Unlike *E. coli*, which translocates Etx into the periplasmic space, *Vibrio* and *Aeromonas* secrete the heterologous enterotoxin into the surrounding medium [8,9,2]. This property is desirable for the biotechnological exploitation of *Vibrio* for the production of great amounts of Etx, EtxB and its derivatives [10,11]. The non-toxic B-subunit of Etx (EtxB), for example, has provoked considerable interest as a protein-based, non-living carrier system for the delivery of antigens to the gut mucosa [12]. More recently, in an effort to provide a protein carrier for the delivery of an antiviral peptide, an EtxB-based fusion protein containing a C-terminal extension, corresponding to the nine carboxy-terminal amino acids -Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu-COOH of herpes simplex virus type 1 (HSV-1) ribonucleotide reductase 38-kDa subunit (EtxB-R2), has been shown to be active in inhibiting HSV-1 replication by targeting the active nonapeptide into the infected cells [13,14].

Vibrio sp. 60, a fish pathogen closely related to *V. anguillarum*, is a marine vibrio that does not encode for cholera toxin but produces several extracellular proteins including protease, amylase, DNase and hemagglutinin [15]. This strain was chosen as a host for plasmids encoding EtxB, or derivatives like EtxB-R2, under the control of the inducible *tac* promoter and was shown to secrete these proteins into the extracellular medium upon induction with IPTG [16,17].

In this study, we have identified a specific protease activity in the culture medium of *Vibrio* sp. 60 expressing EtxB-R2, following the observation that the hybrid protein, once concentrated from the culture supernatants, loses reactivity to a monoclonal antibody directed towards the carboxy-terminal nonapeptide. Using EtxB-R2 as the protease substrate, we were able to further characterize this proteolytic activity and to determine the peptide bond that was cleaved.

2. Materials and methods

2.1. Bacteria strains, plasmids and growth conditions

A rifampicin-resistant derivative of *Vibrio* sp. 60 (strain MVT606) was originally obtained from Dr. A. Ichige, University of Tokyo, Japan [15]. *Vibrio* sp. 60 was shown to express great amounts of EtxB in the culture supernatants when plasmids containing the *etxB* gene under the control of the inducible *tac* promoter were mobilized into the strain [16,17]. pMMB68 and pAM320 are broad-host range plasmids [18] containing the RSF1010 replicon that were engineered to express the B-subunit of heat-labile and its derivative EtxB-R2 containing a carboxy-terminal extension, respectively [17]. *Vibrio* sp. 60 with or without plasmids was grown in Luria broth (LB) supplemented with NaCl to a final concentration of 2% (w/v) and appropriate antibiotics, rifampicin (50 μg ml^{-1}) and carbenicillin (2 mg ml^{-1}), where necessary.

2.2. Expression and purification of EtxB and EtxB-R2

All *Vibrio* strains were inoculated from rifampicin/carbenicillin Bacto-agar plates into LB supplemented with NaCl but without antibiotics. Cells were grown up to an optical density of 0.05 at 600 nm in a rotary shaking incubator. Expression of EtxB and its derivatives was induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM final concentration) when the OD of the culture had reached approx. 0.2. After 5 h post-induction, cell-free supernatants were concentrated on a 10-kDa nominal weight exclusion polyethersulfone membrane with a cross-flow ultrafiltration apparatus (Filtron, Flowgen) and purified by hydrophobic interaction chromatography (Superose HR5/5, Pharmacia) and anion exchange chromatography (Neobar AQ 15/4, Flowgen) as previously described [19,20]. The same method was used for the purification of EtxB-R2 with the exception that 1 mM EDTA was added in the buffers and manipulations were performed at 4°C to minimize proteolysis.

2.3. Proteolytic activity assay

Total protease activity of *Vibrio* sp. 60 was detected on skim milk agar plates and in slab polyacrylamide gels containing gelatine as a copolymerized substrate. Specific peptidase activities on the oligopeptide extension were measured using peptide specific monoclonal antibody MAb8746 in a GM1 ELISA system. The supernatant was concentrated with a 35–65% $(NH_4)_2SO_4$ and the pellet was resuspended in 1/10th the original culture volume in ice-cold phosphate-buffered saline (PBS). Cleavage of the -R2 extension with time was monitored at various temperatures as well as in the presence of the protease inhibitor, PMSF (1 mM final concentration) or EDTA (1 mM final concentration). The extent of cleavage was calculated from the percentage of undegraded protein.

The assay was performed on 96-well ELISA plates coated overnight with soluble GM1 (Sigma) at a concentration of 1.5 $\mu g\ ml^{-1}$ in PBS. Samples at various time-points were diluted on the plates, probed with the anti-EtxB monoclonal antibody MAb118-8 or the monoclonal antibody MAb8746 directed towards the peptide extension and goat anti-mouse IgG peroxidase conjugate. Absorbance at 450 nm, reference filter 620 nm, was measured in an automated plate reader and Biolise software (Techgen, UK) was used to calculate the concentration of the sample as percentage of undegraded protein.

2.4. Analytical methods

Partially degraded EtxB-R2 from anion exchange chromatography was further purified by high performance liquid chromatography (HPLC) using a reverse-phase C4 column (Vydac, 4.6 × 150 mm) and a 25–50% acetonitrile gradient in water–TFA 0.05%. The peaks corresponding to intact and cleaved EtxB-R2 were identified by 15% SDS-PAGE. Amino acid analysis was performed using the Millipore Waters workstation (Milford, MA) and the Pico-Tag C_{18} column (4.6 × 150 mm). Lyophilized samples of protein fragments (about 100 pmol), contained in heat-treated borosilicate tubes (4 × 50 mm), were acid hydrolysed for 1 h at 150°C using 200 ml of 6 N HCl, containing 0.1% (w/v) phenol, derivatized with phenylisothiocyanate and the resulting phenylthiocarbamoyl(PTC)-derivatives of amino acids were separated and quantitated by HPLC.

N-terminal sequence analysis was performed with an Applied Biosystems peptide/protein sequencer model 477A equipped with on-line PTH-analyzer model 120A. Standard manufacturer's procedures and programs were used with minor modifications.

3. Results and discussion

3.1. Production and purification of EtxB and EtxB-R2 in Vibrio sp. 60

Vibrio sp. 60 harbouring plasmids pMMB68 and pAM320, with the *etxB* and *etxB-R2* genes under the control of the inducible *Tac* promoter, secreted EtxB and the fusion protein EtxB-R2 in the culture medium upon induction with 1 mM IPTG yielding up to 30 and 15.5 mg of undegraded products from 1 l of culture, respectively. EtxB could be easily purified to homogeneity from the culture cell-free supernatants by concentration on a 10-kDa nominal weight exclusion polyethersulfone membrane followed by hydrophobic interaction and anion exchange column chromatography as previously described.

The high yield of EtxB production in the culture supernatants of *Vibrio* sp. 60 harbouring plasmid pMMB68 matched the massive production of extracellular proteases in comparison with other *Vibrio* strains as detected on skim milk agar plates. At least three major proteases were visible in slab polyacrylamide gels of the concentrated culture medium containing gelatine as a co-polymerized substrate.

3.2. Proteolytic degradation of EtxB-R2

Concentration of *Vibrio* sp. 60 culture supernatants containing EtxB-R2 at room temperature on the 10-kDa membrane resulted in the recovery of a protein that was reactive in GM1 ELISA and Western-immunoblot with the anti-EtxB antibody MAb118-8 but not with the anti-peptide antibody MAb8746. A time-course of proteolysis was developed to investigate the nature of this degradation: culture supernatants containing undegraded EtxB-R2 were concentrated in ammonium sulfate and incubated at 30°C in the presence of 1 mM EDTA, an

inhibitor of metalloproteases, or 1 mM PMSF, an inhibitor of serine proteases. The persistence of the carboxy-terminal peptide was monitored on a GM1 ELISA with the monoclonal antibody MAb8746 that reacts specifically with the peptide NH_2-Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu-COOH. As shown in Fig. 1, rapid proteolysis of the carboxy-terminal extension was obtained in this assay with a $t_{1/2}$ of approx. 15–30 min. This effect was efficiently prevented by incubation at 4°C or by addition of 1 mM EDTA, whereas PMSF was completely ineffective. Immunoreactivity of EtxB and EtxB-R2 with the anti-EtxB monoclonal antibody MAb118-8 under the same experimental conditions was not affected (data not shown). Proteolysis of the attached peptide probably occurred because the carboxy-terminus of EtxB-R2 is accessible to proteolytic degradation being exposed on the surface of the protein, whereas the properly folded EtxB oligomer is extremely stable and resistant to proteolysis. EDTA did not inhibit total protease activity secreted by *Vibrio* sp. 60 growing on skim milk agar plates, indicating that at least two different types of proteases are present in the culture medium.

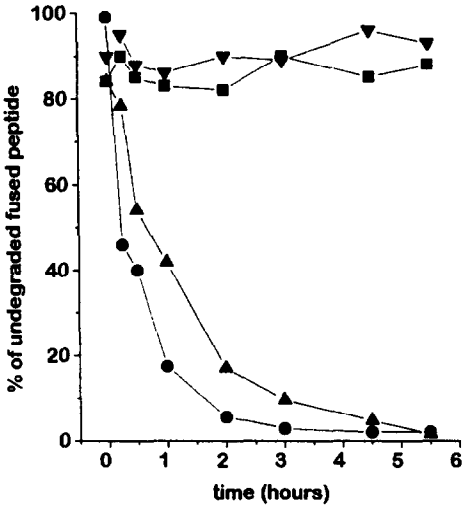

Fig. 1. Proteolytic degradation of EtxB-R2 produced in *Vibrio* sp. 60. Concentrated samples of extracellular medium from *Vibrio* sp. 60 cultures producing EtxB-R2 were monitored for the persistence of the fused peptide using a GM1 ELISA and the monoclonal antibody MAb8746 directed towards the -R2 extension. The effect of different temperatures (30°C, ●; 4°C, ▼) and protease inhibitors (EDTA 1 mM, ■; PMSF 1 mM, ▲) were evaluated.

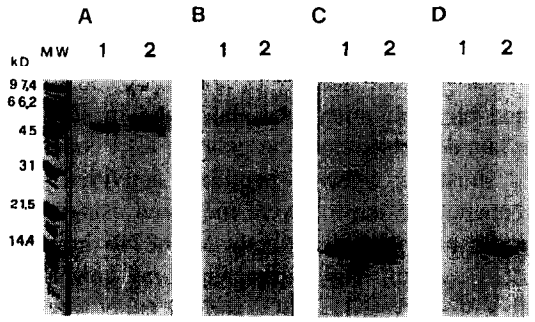

Fig. 2. Western-immunoblot analysis of EtxB (lane 1) and EtxB-R2 (lane 2) produced in *Vibrio* sp. 60 after concentration and purification of the samples. (**A**, **B**) Samples were not boiled prior to SDS-PAGE to show the pentamers and probed with the anti-EtxB monoclonal antibody MAb118-8 (**A**) and the anti-R2 monoclonal antibody MAb8746 (**B**). (**C**, **D**) Samples were boiled prior to SDS-PAGE to show the monomers and probed with a mouse anti-EtxB polyclonal antibody (**C**) and MAb8746 (**D**). The reactive polypeptide indicated by 1 in lane 2 of (**A–D**) corresponds to undegraded fusion protein EtxB-R2, whereas the polypeptide indicated by 2 which appears in lane 2 of (**A**) and (**C**) correspond to cleaved EtxB-R2. Biorad low molecular mass marker was used.

3.3. Purification of the proteolytic fragments and amino acid composition analysis

Purification of EtxB-R2 with the method previously described for EtxB, with the addition of 1 mM EDTA in all the buffers and working at 4°C, yielded 9 mg of protein per litre of culture. The purified material showed a duplets of bands on 13.5% SDS-PAGE gels that migrated at the position of the intact EtxB-R2 protein and at a position that is intermediate between EtxB-R2 and EtxB, consistent with the attached peptide being degraded. The Western-immunoblot analysis of the purified material shown in Fig. 2 confirmed that the upper band reacted with both anti-EtxB and anti-peptide antibodies, whereas the lower band reacted only with anti-EtxB antibodies. Only the two species were visible in SDS-PAGE, demonstrating that a single cleavage event was responsible for the peptide being lost.

To further characterize the proteolytic cleavage the two purified fragments were separated by reverse-phase HPLC, two peaks were eluted that corresponded on SDS-PAGE to fragment number 2 (which eluted first) and fragment number 1 (which eluted last) shown in Fig. 2. These two fragments were

Table 1
Amino acid analysis of EtxB, intact EtxB-R2 (fragment 1) and degraded EtxB-R2 (fragment 2)

Amino acid	Amino acid analysis		
	EtxB	EtxB-R2 (1)	EtxB-R2 (2)
Asx	**n. d. (10)**	**10 (11)**	**8.3**
Glx	n. d. (13)	13.2 (13)	13
Ser	8.1 (9)	7.7 (9)	7.9
Gly	3.8 (3)	4.1 (4)	4.1
His	2.2 (2)	1.97 (2)	2.1
Arg	3.6 (3)	3.3 (3)	3.35
Thr	7.9 (11)	8.45 (11)	8.3
Ala	**5.4 (6)**	**7.5 (8)**	**6.55**
Pro	2.6 (3)	2.95 (3)	2.9
Tyr	3.7 (4)	4.75 (5)	4.7
Val	**3.0 (4)**	**4.2 (6)**	**3.25**
Met	3.8 (4)	4.2 (4)	3.9
Cys	n. d. (2)	n. d. (2)	n. d.
Ile	7.9 (12)	8.1 (12)	8.0
Leu	**4.2 (5)**	**6.4 (7)**	**5.3**
Phe	2 (2)	2 (2)	2
Lys	9.2 (9)	9.5 (10)	9.6

Theoretical values are given in parenthesis and are calculated from the amino acid sequence of EtxB and EtxB-R2 deduced from the nucleotide sequence. The amino acids that have been lost after proteolysis are shown in bold.

Table 2
Carboxy-terminal sequences of EtxB, intact EtxB-R2 (fragment 1) and degraded EtxB-R2 (fragment 2)

EtxB	...Glu-Asn
EtxB-R2 (1)	...Glu-Lys-Leu-Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu
EtxB-R2 (2)	...Glu-Lys-Leu-Tyr-Ala-Gly

Glutamic acid in position +102 is the penultimate amino acid of mature EtxB.

processed to single amino acids and the composition of each fragment determined. In Tables 1 and 2 are shown the amino acid composition of the two polypeptides that differ only for one of each of the following amino acids: alanine, valine, asparagine, aspartic acid and leucine. This is consistent with the last six carboxy-terminal amino acids EtxB-R2 being cleaved off by a single proteolysis between amino acid Gly and Ala. The valine-valine bond is not efficiently hydrolysed by the method used and this might explain why only one more valine instead of two is detected for intact EtxB-R2 (Tables 1 and 2).

N-Terminal sequencing of the two fragments confirmed that proteolysis occurred only at the carboxy-terminus, showing the N-terminal sequence NH_2-Ala-Pro-Gln-Ser-Ile-Thr-Glu-Leu-Cys identical to mature EtxB.

4. Concluding remarks

In this work we took advantage of the observation that the fusion protein EtxB-R2, carrying a C-terminal extension of nine amino acids, was degraded to a smaller polypeptide that loosed cross-reactivity with a monoclonal antibody specific for the heterologous peptide. The protease responsible of this activity was shown to cleave between amino acids glycine and alanine of the carboxy-terminal extension and to be inhibited by EDTA. Since the cleaved polypeptide is inactive, production of EtxB-R2 in *Vibrio* sp. 60 is seriously affected by this protease activities and so might be for other fusion proteins that could be also susceptible to proteases not yet identified. Such an observation could have a profound effect on the biotechnological exploitation of *Vibrio* sp. 60 for the production of recombinant EtxB and derivatives. Further biochemical characterization of the protease will be necessary for a better evaluation of the substrate specificity in order to find suitable inhibitors.

Vibrios are the most common bacteria found in sea water and can cause gastroenteritis, sepsis and soft tissue infections after exposure to sea water or consumption of raw food. The role of the proteolytic activity we have identified in the pathogenicity of *Vibrio* sp. 60 for humans or marine animals remains to be established [21,22].

Acknowledgements

Financial support for this work was from the Istituto Superiore di Sanità of Italy (G.P.). We acknowledge also a travel grant to A.M. from the Consorzio Italiano Biotecnologie.

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