Protein Expression and Purification 62 (2008) 153–159

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Extracellular production of active vibriolysin engineered by random mutagenesis in *Escherichia coli*

Hiroyuki Sonoda *, Atsushi Sugimura

Medical Technology Research Center, Research Division, JCR Pharmaceuticals Co., Ltd., 2-2-10 Murotani, Nishi-ku, Kobe 651-2241, Japan

ARTICLE INFO

Article history:
Received 12 May 2008
and in revised form 4 August 2008
Available online 14 August 2008

Keywords:
Recombinant vibriolysin
Extracellular production
Escherichia coli
Random mutagenesis

ABSTRACT

Vibriolysin, an extracellular protease of *Vibrio proteolyticus*, is synthesized as a preproenzyme. The N-terminal propeptide functions as an intramolecular chaperone and an inhibitor of the mature enzyme. Extracellular production of recombinant vibriolysin has been achieved in *Bacillus subtilis*, but not in *Escherichia coli*, which is widely used as a host for the production of recombinant proteins. Vibriolysin is expressed as an inactive form in *E. coli* possibly due to the inhibitory effect of the N-terminal propeptide. In this study, we isolated the novel vibriolysin engineered by *in vivo* random mutagenesis, which is expressed as active mature vibriolysin in *E. coli*. The Western blot analysis showed that the N-terminal propeptide of the engineered enzyme was processed and degraded, confirming that the propeptide inhibits the mature enzyme. Two mutations located within the engineered vibriolysin resulted in the substitution of stop codon for Trp at position 11 in the signal peptide and of Val for Ala at position 183 in the N-terminal propeptide (where position 1 is defined as the first methionine). It was found that the individual mutations are related to the enzyme activity. The novel vibriolysin was extracellularly overproduced in BL21(DE3) and purified from the culture supernatant by ion-exchange chromatography followed by hydrophobic-interaction chromatography, resulting in an overall yield of 2.2 mg/L of purified protein. This suggests that the novel engineered vibriolysin is useful for overproduction in an *E. coli* expression system.

© 2008 Elsevier Inc. All rights reserved.

The marine microorganism *Vibrio proteolyticus* secretes the neutral protease, vibriolysin, which is a zinc-metalloenzyme with a molecular weight of 34.8 kDa [1,2]. It is synthesized as a preproprotein consisting of a signal peptide (24 amino acid residues), N-terminal propeptide (172 a.a. residues), 34.8 kDa mature protein possessing the proteolytic activity, and 10 kDa C-terminal propeptide [3]. Vibriolysin specifically hydrolyzes the peptide bond at the amino group side of the P1′ amino acid residue, which is usually a hydrophobic amino acid residue (e.g. X-Phe, X-Leu or X-Tyr). Vibriolysin is also active on various proteins such as casein, albumin, collagen, elastin, hemoglobin, fibrin, and fibrinogen.

It is well known that the N-terminal propeptide of many proteases functions both as an intramolecular chaperone in the folding process and as an inhibitor that prevents proteolytic activity of the mature enzyme [4–6]. The propeptide of *Pseudomonas aeruginosa* elastase, which is the one of the well-studied bacterial proteases, is also essential for the folding of the enzyme in the periplasm, and this folding allows for further processing of the proenzyme by autoproteolytic cleavage. After autoprocessing, the propeptide–enzyme complex is translocated across the outer membrane. The propeptide processed but associated noncovalently with the enzyme is degraded by its cognate mature enzyme after dissociation of the complex is induced by unclear mechanism [7–9]. Since vibriolysin is autoprocessed into an active form in an authentic host, *V. proteolyticus*, it is difficult to investigate whether the propeptide of vibriolysin inhibits the proteolytic activity of the mature enzyme or not. In the chimeric construct of the N-terminal propeptide of vibriolysin and the mature region of the PA protease from *Aeromonas caviae* T-64, the N-terminal propeptide of vibriolysin functioned as an intramolecular chaperone and inhibited the proteolytic activity of the PA protease [10].

For industrial applications, it has been demonstrated that vibriolysin can be used as a bio-catalyst for the synthesis of aspartame precursors [11,12]. For this application, however, vibriolysin should be purified into a homogeneous form that excludes other enzymes, especially aminopeptidase, which is another major enzyme secreted from *V. proteolyticus*. Because of the similarity of aminopeptidase in charge and size properties, the effective removal of aminopeptidase from the culture medium containing vibriolysin is difficult. Griffin and Prescott reported that the production ratio of vibriolysin to aminopeptidase in the culture medium increased by using soybean protein as an organic component of the medium instead of casein [13]. Wilkes and Prescott achieved the effective separation of vibriolysin from aminopeptidase by performing chromatography on brushite ($CaHPO_4{\cdot}2H_2O$), although the

* Corresponding author. Fax: +81 78 991 4465.
E-mail address: sonoda-h@jcrpharm.co.jp (H. Sonoda).

1046-5928/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2008.08.001

vibriolysin preparations contained less than 0.5% of the original aminopeptidase activity [2,14]. Durham showed that alkaline treatment was effective for the irreversible inactivation of aminopeptidase without affecting the protease activity of vibriolysin. When the alkaline inactivation step was followed by ion-exchange chromatography, vibriolysin was purified homogeneously from the fermentation broth and the preparations contained only 0.1% of the original aminopeptidase activity [15]. In addition to vibriolysin's potential as a bio-catalyst, it has been reported that vibriolysin has medical applications. Durham et al. reported that vibriolysin was efficacious as a debriding agent against the burn wound eschar in a porcine model [16,17].

The expression system of recombinant proteins by *Escherichia coli* makes it easy to overexpress target proteins by using a strong promoter such as the T7 promoter. Furthermore, the use of *E. coli* as the production host eliminates the contaminant of aminopeptidases because it normally does not secrete a substantial amount of aminopeptidases in the culture medium. In therapeutic applications, *E. coli* has immense advantages over other microorganism expression systems, since the majority of therapeutic proteins have been produced in either *E. coli*, *Saccharomyces cerevisiae*, or mammalian cell culture systems [18,19]. However, it is reported that the expression of vibriolysin in *E. coli* exhibited less than 1/40th of the proteolytic activity observed in *V. proteolyticus* [3]. It is likely that *E. coli* is defective in at least one step of maturation observed in the authentic host. In this study, we isolated the novel vibriolysin, named NprV-R, which is expressed stably as an active form in *E. coli*. NprV-R has two point mutations, one in the signal peptide and the other in the N-terminal propeptide, which decrease the inhibitory effect. By using this novel enzyme, we succeeded in achieving the extracellular overproduction of active vibriolysin in *E. coli*.

Materials and methods

Materials

Azocasein and sea salts were purchased from Sigma (St. Louis, MO, USA). BugBuster Protein Extraction Reagent, Benzonase nuclease, and the plasmid pCDF-1b were purchased from Novagen (Madison, WI, USA). Oligonucleotides were prepared by Sigma Genosys (Tokyo, Japan). Restriction endonucleases, Blend Taq-plus DNA polymerase, and Ligation high were purchased from Toyobo (Shiga, Japan). A BigDye Terminator Cycle Sequencing Kit ver. 1.1 was obtained from Applied Biosystems (California, USA). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Wako (Kyoto, Japan). All the other chemicals used were of the highest quality commercially available.

Bacterial strains and growth conditions

For subcloning, the *E. coli* strain JM109 was used. Expression experiments were carried out in BL21(DE3). The transformants were grown in Luria–Bertani broth (LB[1] broth; 1% polypepton, 0.5% yeast extract, and 0.5% NaCl, pH 7.0) containing 30 μg/ml streptomycin at 37 °C until OD_{600} = 0.2–0.5. The target proteins were induced by the addition of 1 mM IPTG at final concentration, and the culture was continued at 37 °C for 20–24 h. *Vibrio proteolyticus* was cultured in a sea salt medium (2.6% sea salts, 2% tryptone) at 30 °C for 24 h.

[1] *Abbreviations used:* LB broth, Luria–Bertani broth; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

DNA manipulation

Recombinant DNA techniques and methods were used, as described by Sambrook et al. [20]. Genomic DNA and plasmid DNA were prepared using the GenElute Bacterial Genomic DNA Kit (Sigma) and GenElute Plasmid Miniprep Kit (Sigma), respectively. The QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) was used for the extraction and purification of DNA from agarose gels. The nucleotide sequence was determined with a DNA sequencer model, the ABI 3100-Avant Genetic Analyzer (Applied Biosystems), using the dideoxy chain-termination procedure [21] with the BigDye Terminator Cycle Sequencing Kit (ver. 1.1). The nucleotide sequence data were analyzed using the DNASIS software (Hitachi, Tokyo, Japan).

Plasmid construction and preparation of the His-tagged mature peptide and N-terminal propeptide

The 1833 bp fragment containing the complete nprV gene and additional 3 bp (GCA; Ala) immediately after the first Met to generate the NcoI site was amplified using *V. proteolyticus* genomic DNA as the template and the oligonucleotides 5′-GCATAATCCATGGCAAATAAAACACAACGTCACATCAACTGGC-3′ (the underlined bases encode an NcoI site) and 5′-GCATAATGCGGCCGCGGATCCATTAGTCAGCACGCAAAGTTACACC-3′ (the underlined bases encode an NotI site). The PCR product was digested by NcoI and NotI, and inserted in pCDF-1b, to give the plasmid pCDF-nprV. The PstI–ClaI fragments of pCDF-nprV and pCDF-nprV-R, which was constructed by random mutagenesis as described below, were replaced each other, to give the plasmids pCDF-nprV-R2 and pCDF-nprV-R3 (see Fig. 3). The point-mutated fragments were generated by PCR using the following primers 5′-GCATAATCCATGGCAAATAAAACACAACGTCACATCAACTAACTGCTGGCTG-3′ (the underlined bases encode a NcoI site) and 5′-TTCACTACACCACAACCG-3′ for the nprV-R4, and 5′-GCATAATCCATGGCAAATAAAACACAACGTCACATCAACTAGCTGCTGGCTG-3′ (the underlined bases encode a NcoI site) and 5′-TTCACTACACCACAACCG-3′ for the nprV-R5. The respective amplified fragments were digested by NcoI and ClaI, and then substituted the corresponding region of pCDF-nprV, resulting in pCDF-nprV-R4 and pCDF-nprV-R5 (see Fig. 3). All of the sequences amplified by PCR and inserted into vectors were confirmed by nucleotide sequencing.

Two recombinant plasmids were constructed for the expression of the His-tagged mature peptide and N-terminal propeptide for the antiserum preparation. Based on the nucleotide sequence of the nprV gene [3], target regions were amplified by PCR using the oligonucleotides 5′-CGGGATCCGCACAAGCTGACGGTACTG-3′ (the underlined bases encode a BamHI site) and 5′-AAAACTGCAGTTATACATCGCCAGACGGAGG-3′ (the underlined bases encode a PstI site) for the mature region, and 5′-CGGGATCCGCAGAAATGATCAACGTAAATG-3′ (the underlined bases encode a BamHI site) and 5′-AAAACTGCAGTTAATGGTTCAGACCATCCCAAG-3′ (the underlined bases encode a PstI site) for the N-terminal propeptide. The respective PCR products were subsequently cloned into the BamHI–PstI restriction enzyme sites of pQE80L plasmid (Qiagen) and transformed into JM109 separately. The resulting transformants were grown in the LB broth containing 100 μg/mL ampicillin at 37 °C until OD_{600} = 0.5. After the addition of 1 mM IPTG at final concentration, incubation was continued at 37 °C for another 4 h. The inclusion body was isolated and purified using the BugBuster reagent and Benzonase according to the manufacturer's instructions. After solubilization overnight at 4 °C in PBS containing 6 M guanidine–HCl, the solutions were centrifuged at 15,000 rpm for 15 min. The clear supernatants were loaded onto a column packed with Ni–Sepharose FF (GE healthcare). After unbound proteins were washed off, the His-tagged mature peptide or N-terminal propep-

Download English Version:

https://daneshyari.com/en/article/2021523

Download Persian Version:

https://daneshyari.com/article/2021523

Daneshyari.com