International Journal of Biological Macromolecules 54 (2013) 44–50

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

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

Channel forming outer membrane porin protein in halophile: Expressed as a soluble form in *Escherichia coli*

Hiroko Tokunaga [a], Masafumi Furukawa [a], Tsutomu Arakawa [b], Masao Tokunaga [a,*]

[a] *Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*
[b] *Alliance Protein Laboratory, 3957 Corte Cancion, Thousand Oaks, CA 91360, USA*

ARTICLE INFO

Article history:
Received 24 October 2012
Received in revised form
26 November 2012
Accepted 26 November 2012
Available online 3 December 2012

Keywords:
Halophilic
Acidic amino acid
Outer membrane
Porin

ABSTRACT

We have previously found that the N-terminal sequence of the outer membrane protein from moderate halophile is similar to the sequence of the well-known pore forming porin proteins from other Gram-negative bacteria. This highly expressed outer membrane protein was purified from *Halomonas* sp. 40 and reconstituted into liposome. It showed a permeability activity in the liposome swelling assay. Based on the N-terminal and internal amino acid sequences of this major outer membrane, we have cloned here the porin gene, *hopP* (*h*alophilic *o*uter membrane *p*rotein), from *Halomonas* sp. 40. The *hopP* gene encodes the porin precursor comprising 366 amino acid residues that include a 21 amino acid signal peptide. Mature porin (345 amino acids, 37,611 Da) is a highly acidic protein, just as is so for many halophilic proteins and was soluble when expressed in *Escherichia coli* with N-terminal His-tag. Purified recombinant His-porin was soluble even after heat-treatment at 95 °C for 5 min in the absence of salt. Circular dichroism analysis of His-porin showed conversion into a β-sheet rich structure by the addition of NaCl at 0.9–2.7 M.

1. Introduction

The moderately halophilic bacteria, one of the extremophiles, are defined as a group of microorganisms that are capable of growing over a wide range of salinities: optimally at 0.5–2.5 M, but sometimes even up to close to saturated NaCl [1,2]. Their intracellular ionic concentration was reported to vary from 0.04 to 3.52 M for Na^+ and from 0.002 to 0.89 M for K^+, depending on the strains and growth conditions [3]. These halophilic bacteria must adapt promptly to an abrupt change in environmental salinity. They precisely regulate their ion transport systems to adjust their intra-cellular ionic concentration following the changes in external salinity. One of such regulations is via control of permeability property and integrity of outer membranes in Gram-negative halophilic bacteria.

The outer membrane is a protective barrier for bacterial cells. One of the important roles of the outer membrane is to prevent the cells from harmful substances. Small hydrophilic nutrients and waste products are diffused through proteins present in the outer membranes. These proteins, called porin, are highly expressed in outer membranes. Since its identification by Nakae in 1976 [4], the properties and structures of general diffusion pores, porins, have been extensively studied [5–9].

We have previously shown highly abundant proteins in the outer membrane fraction of moderate halophiles, such as *Halomonas* and *Chromohalobacter* spp. [10]. These major proteins showed similarity in N-terminal amino acid sequence to porins from other Gram-negative bacteria. In order to characterize the properties of this major outer membrane protein from halophile, we have cloned the porin gene, *hopP* (*h*alophilic *o*uter membrane *p*rotein), from *Halomonas* sp. 40, expressed the gene in *Escherichia coli* and characterized the recombinant HopP porin protein. Although HopP is a channel-forming integral membrane protein, it was expressed as a soluble protein in *E. coli* with N-terminal His-tag (His-HopP), perhaps owing to its halophilic property, i.e., abundant acidic amino acids that have been shown to confer high aqueous solubility to halophilic proteins.

2. Materials and methods

2.1. Bacterial strains

Halomonas sp. 40 was grown in Nutrient broth containing 2 M NaCl as described previously [10] and used for purification of HopP protein and cloning of *hopP* gene. *E. coli* JM109 and BL21(DE3) were used for plasmid construction and expression of His-HopP protein. *E. coli* transformant cells were grown in LB-ampicillin (100 μg/ml).

* Corresponding author. Tel.: +81 99 285 8634; fax: +81 99 285 8634.
E-mail address: tokunaga@chem.agri.kagoshima-u.ac.jp (M. Tokunaga).

0141-8130/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijbiomac.2012.11.029

2.2. Purification of porin protein from Halomonas sp. 40

Porin (HopP protein) was purified from *Halomonas* sp. 40 cells according to the previous report [10] with a slight modification. The outer membrane (OM) fraction of *Halomonas* cells was obtained by differential detergent solubilization method described before. The OM fraction solubilized by 2.5% β-octylglucoside was applied to a HiTrap^{TM}QHP column (GE Healthcare) equilibrated with 0.25% β-octylglucoside in 20 mM Tris buffer (pH 8.0). The column was eluted with a 0–0.75 M linear gradient of NaCl in 20 mM Tris buffer (pH 8.0) containing 0.25% β-octylglucoside.

2.3. Determination of N-terminal and internal amino acid sequences

The N-terminal sequence of HopP protein was determined as follows. The purified HopP protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto Problot membranes. After staining with 0.2% Coomassie Brilliant Blue R250 in 40% methanol and destaining with 50% methanol, the protein band was cut out from the membranes and used for amino acid sequence analysis. The analysis of internal amino acid sequences was performed by in-gel digestion method [11] using endo-proteinase GluC (Wako Roche).

2.4. Preparation of proteoliposomes and permeability assay

Proteoliposomes were prepared according to the method described by Nakae et al. [12] with some modifications. Egg yolk phosphatidylcholine (7.9 μmol) was dried at the bottom of a conical centrifuge tube under N_2 gas stream and kept in an evacuated desiccator for 1.5 h. The dried lipid film was suspended in 0.5 ml distilled water and subjected to sonic oscillation with a microtip for 4 min (SMTcompany, dial setting 2). The porin from *E. coli* B strain or HopP protein, 18 μg each, was added to the prepared lipid suspension and subjected to sonic oscillation for 1 min. These mixtures were dried again under N_2 stream and kept for 2.5 h in the evacuated desiccator. The film was resuspended in 0.5 ml of 1 mM MOPS solution, pH 7.0, containing 18.5% dextran T4 (MW, 4000–6000). These liposomes were diluted into isotonic sugar solutions (arabinose, glucose, N-acetylglucosamine, sucrose and raffinose). The permeability of these saccharides on the proteoliposomes thus prepared was determined from the change in optical density at 450 nm. Diffusion rate was calculated as described by Nakae et al. [12]. The rates were normalized to the rate of arabinose (100%).

2.5. Structure modeling and electrostatic potential map of HopP and E. coli B porin proteins

Tertiary structures of HopP was modeled based on the crystal structure of *E. coli* porin (PDB:2zfg) using MOE. Surface electrostatic potential map was generated with MolFeat v.4.5.

2.6. DNA manipulations and cloning of the hopP gene

Chromosomal DNA of *Halomonas* sp. 40 was isolated by the procedure of Maniatis et al. [13]. To amplify the DNA fragment containing a part of the *hopP* gene, several mixed primers were designed based on the N-terminal and internal amino acid sequences. PCR was done using a forward primer, 5′-GCSACSGTSTAYAAYCARGA (encoding the N-terminus to 7th amino acid residues), and a reverse primer, 5′-ACRTASGTSAGYTGRTCSGT (encoding TDQLTYV in the internal sequence), resulting in amplification of a 437 bp fragment encoding a part of the *hopP* gene from *Halomonas* sp. 40 chromosomal DNA. Following the cloning of this fragment into the *Eco*RV site of pT7Blue vector, southern hybridization 386 bp probe was amplified using forward primer, 5′-ACGGCACCAAGCTCGACATCTA and reverse primer, 5′-ACCGACAGCAACGACAGAGGAA. A 2.9 kbp *Nco*I fragment and a 2.4 kbp *Sma*I fragment, hybridized with these probes, were cut out from the agarose gel, self-ligated, and amplified by inverse PCR. The resultant nucleotide was then sequenced. An *Nco*I fragment was found to contain *hop*P and its downstream region, and a *Sma*I fragment was found to contain *hop*P and its upstream region. The 2353 bp fragment containing *hop*P was directly amplified from *Halomonas* sp. 40 chromosomal DNA by PCR using forward primer 5′-AGAAACTCGATCGGCTGCAG-3′ and reverse primer 5′-TTCTCGCCGACGAACTGAAT-3′. The nucleotide sequence of the amplified fragment was confirmed by direct sequencing. The *hop*P sequence thus determined was deposited in the DDBJ/EMBL/GenBank databases with the accession number AB183012 (registration 2004.June, open 2010.January). Online database search was carried out using the 'GenomeNet Database Service' operated by the Institute for Chemical Research, Kyoto University (http://www.genome.ad.jp/).

2.7. Expression and purification of His-HopP protein in E. coli

The DNA fragment encoding mature HopP was amplified by PCR using a forward primer (5′-AGATATACATATGGCCACCGTCTATAAC) that encodes an *Nde*I site (underlined), followed by the coding sequence starting at Ala22, and a reverse primer (5′-CCCTCGAGTTAGAACATGTAGGTCAC) that contains the coding sequence up to the termination codon. The amplified fragment was ligated to *Nde*I/XhoI sites (underlined) of pET15b (Novagen) to construct pET-*hopP*, which encodes a HopP protein preceded by a hexa-His-tag. The plasmid pET-*hopP* was introduced into *E. coli* BL21(DE3), and the expression of His-HopP at 30 °C was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration of 0.2 mM). The recombinant HopP protein was purified to homogeneity by Ni-NTA agarose (Qiagen) affinity chromatography according to the manufacturer's instructions. The purified protein was dialyzed against 50 mM Tris buffer (pH 8.0) and used for the following experiments.

2.8. Size-exclusion chromatography of His-HopP

Gel filtration experiments were carried out using GE Healthcare Superdex 200 (10/300GL) column, equilibrated with 50 mM Tris buffer (pH 8.0) containing 0.1 M NaCl or 2 M NaCl at a flow rate of 0.7 ml/min. Elution was monitored by absorbance at 215 nm.

2.9. Circular dichroism (CD) measurement

CD spectra were determined on a Jasco J-715 spectropolarimeter equipped with a PTC-348WI temperature controller and a Peltier cell holder. Far UV CD spectra and thermal scans were obtained using a 0.1 cm cell. The protein concentration used was 0.2 mg/ml. The protein concentrations were spectrophotometrically determined using the absorbance value of 1.42 for 1 mg/ml solution. Five scans were accumulated. The mean residue ellipticity was calculated using a mean residue weight of 109. The extinction coefficient and mean residue weight were calculated from the amino acid composition of the deduced DNA sequence. The solvent spectrum was subtracted from the sample spectrum. The α-helical content was estimated according to Greenfield and Fasman [14].