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Cloning, expression, purification and activation by Na ion of halophilic alkaline phosphatase from moderate halophile *Halomonas* sp. 593

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ABSTRACT

We have succeeded in the cloning of alkaline phosphatase gene, *haalp*, from moderate halophile *Halomonas* sp. 593. A deduced amino acid sequence showed a high ratio of acidic to basic amino acids, characteristic of halophilic proteins. The gene product was efficiently expressed in *Escherichia coli* BL21 Star (DE3) pLysS, but in an inactive form. The purified recombinant HaALP was separated into four fractions by gel filtration. When they were dialyzed against 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer containing 3 M NaCl, one of these four fractions was activated to almost full activity. This fraction contained a folding intermediate that was converted to the native structure by the salt. Among the additional salts tested, i.e., KCl, KBr, LiCl, $MgCl_2$, $(NH_4)_2SO_4$, and Na_2SO_4, only Na_2SO_4 was effective, suggesting the importance of Na ion.

Introduction

Halomonas sp. 593 [1] belongs to moderately halophilic bacteria, which can grow under a wide range of NaCl concentrations, even at the saturated level, but optimally at 0.5–2.5 M [2]. They accumulate compatible solutes in cytoplasm to adapt to the external high salt concentration of culture media. While the cytoplasmic enzymes of halophilic bacteria may not require high salt concentrations, the extra-cellular proteins must be active and functional in the high salt concentration, in which moderately halophilic bacteria grow [3].

Most halophilic enzymes are highly acidic, and hence charge repulsion is believed to be one of the major factors responsible for instability in low salt media. Salts exert their effects by shielding electrostatic charges on the protein surface [4]. In addition, in case of malate dehydrogenase from Haloarchaeal *Haloarcula marismortui*, it was reported that there are specific ion-binding sites, in particular four strong chloride-binding sites at the dimer–dimer interface of the tetrameric enzyme [5–8].

Alkaline phosphatase (ALP) is a well-studied periplasmic enzyme [9–11]. Compared to the extensive study on ALP from extremely halophilic archaea [12–15], reports on the ALP from moderately halophilic bacteria are almost limited to *Vibrio* species [16–18]. Recently, the structure of alkaline phosphatase has been characterized, revealing differences among various ALP species in the divalent metal ions that are involved in catalysis and structural details [9,14,17,19–22]. Moreover, in dimer interface of HSAP from *Halobacterium salinarum*, a network of interactions utilizing two Na ions and four Mg ions might be responsible for resistance of HSAP to high salt and elevated temperatures [14].

We have previously described the purification and characterization of ALP from *Halomonas* sp. 593 (HaALP) [23]. Gel filtration and cross-linking analysis showed that the functional structure of the HaALP is a dimer. The partially purified HaALP required at least 0.3 M NaCl for its stability and regained the activity, which was lost under low salt conditions, by the addition of NaCl at higher concentrations. Here we have cloned the corresponding gene, succeeded in the expression and refolding of recombinant protein in *Escherichia coli* and further clarified the effects of different ions on the folding of HaALP.

Materials and methods

Bacterial strains and culture

Halomonas sp. 593 was grown in Nutrient Broth containing 2 M NaCl at 37 °C for 3 days [23]. For cloning and expression of the *haalp* gene, *E. coli* JM109 and *E. coli* BL21 Star (DE3) pLysS was used in LB-ampicillin (amp) and LB-ampicillin-chloramphenicol (amp-cm), respectively.

Cloning of the haalp gene from Halomonas sp. 593 and structural modeling

Chromosome DNA was extracted from *Halomonas* sp. 593 according to Murray and Thompson [24]. Based on the N-terminal

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amino acid sequence (AEVKNVILMIGDGMGPQQVGLLETYANQAPDS IYD) of HaALP [23], APMF2 (ATGGGCCCSCARCARGTSGG) was designed as a forward primer. Reverse primer AMPR (ACCATSAGG AAGAAGCCRTC) was designed on the basis of conserved amino acids sequence derived from *Vibrio parahaemolyticus* (UniProt ID: Q87MR7) and *Vibrio vulnificus* YJ016 (UniProt ID: Q7MIW5). These primers were designed based on codon usage data of *Halomonas elongata*. These primers amplified about a 600 bp fragment from *Halomonas* sp. 593 chromosomal DNA and its sequence was determined. Since the sequence had a 66.83% identity to ALP from *V. vulnificus* YJ016, new primers (APFfra1: GTTTTTCCCTGACGCA TCCC and APRfra1: GCGGCCTGCGTCATCTCGTG) were designed to amplify the probe for Southern blotting. About 3000 bp *Nco*I-fragment of *Halomonas* sp. 593 chromosomal DNA was hybridized with this probe and used for inverse PCR to determine the *haalp* gene sequence. An enhanced chemiluminescence direct labeling kit (Amersham Biosciences) and ABI PRISM 3100 Genetic Analyser were used. The *haalp* gene sequence was deposited in the DDBJ databases with the accession no. AB271127. The structure of the HaALP was modeled based on the crystal structure of VAP (PDB ID: 3e2d) [17] using a computer program MOE (Chemical Computing Group Inc.).

Expression of periplasmic enzyme HaALP in E. coli BL21 Star (DE3) pLysS

The DNA fragment encoding HaALP mature region was amplified by PCR using forward primer f (GCGGTGCCGGCCCATATGGCC GAGGTCAAG) containing *Nde*I site (underlined) and reverse primer r (CCGGATCCTCACTCGACCAGCGACTTGAGG) containing *Bam*HI site (underlined) and stop codon (box), and then ligated to *Nde*I/*Bam*-HI-digested pET3a to construct pHA. *E. coli* BL21 Star (DE3) pLysS harboring the pHA plasmid was grown in LB-amp-cm containing 0.4% glucose at 37 °C overnight and the 1% culture was added to 100 ml LB-amp. After OD_{600} reached 0.8 at a cell culture temperature of 18 °C, synthesis of HaALP was induced for 8 h by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were disrupted in 20 ml ice-cold 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer (without NaCl) by sonication (SMT UH-150 sonifier with a 5 mm tip) for 3 min with a 40% pulse, and soluble and pellet fractions were obtained by centrifugation at 12,000*g* for 15 min. When cells were disrupted in the presence of NaCl at high concentrations, most of the HaALP expressed was fractionated in the pellet fraction (see Fig. 1), probably due to the formation of non-specific protein aggregations by the enhanced salting-out effects. Therefore, protein purification was carried out in the absence of the salt or at low salt concentrations. After purification, an attempt was made to refold the HaALP in the presence of NaCl at high concentrations.

Purification of HaALP by ion exchange chromatography and separation of HaALP with different structures by gel chromatography

The soluble fraction of disrupted cells as above was applied to a HiTrap Q HP (1.6 × 2.5 cm, GE Healthcare) using an Akta prime chromatography system (Amersham, USA). The bound proteins were eluted with 100 ml of a linear gradient of NaCl from 0.3 to 0.9 M in 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer. The fractions containing HaALP were pooled and dialyzed against 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer. After dialysis, the pool was concentrated to 5 ml by Ficoll PM 400 (Sigma Aldrich, F4375) and then applied to a Hiload Superdex 200 pg (1.6 × 60 cm) gel filtration column equilibrated with 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer containing 0.15 M NaCl. The elution was pumped at 0.5 ml min^{-1} at 4 °C. The fractions eluted from the gel filtration column were dialyzed against 50 mM Tris–HCl (pH 8.0)/2 mM $MgCl_2$ buffer. To confirm the purity, 10% SDS–PAGE was carried out according to Laemmli [25]. The amount of protein was measured using BCA method as described by Smith et al. [26].

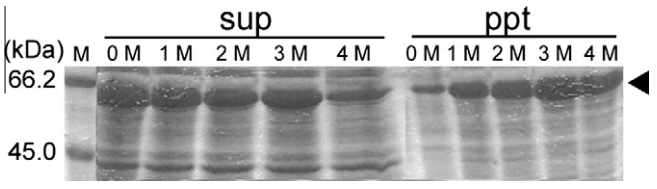

Fig. 1. SDS–PAGE of HaALP localization after sonication. NaCl at 0–4 M was added in sonication buffer. After centrifugation, soluble (sup) and pellet (ppt) fractions of crude homogenates were analyzed by SDS–PAGE.

HaALP assay

ALP activity was measured as described previously [23]. The increase in absorbance at 405 nm was monitored at 37 °C in a reaction mixture containing 0.97 M diethanolamine buffer, pH 10.25, 0.25 mM $MgCl_2$, and 10 mM *p*-nitrophenyl phosphate. One unit was defined as the rate of formation of 1 μmole product/min.

Refolding assay and stability in several salts

The purified samples (100 μg/ml) were dialyzed against 50 mM Tris–HCl (pH8.0)/2 mM $MgCl_2$ buffer containing different salts at 4 °C overnight. The activity was assayed after 1 day. For evaluating the stability, the activated samples (250 μg/ml) by 3 M NaCl were dialyzed against 50 mM Tris–HCl (pH8.0)/2 mM $MgCl_2$ buffer containing different NaCl concentrations at 4 °C overnight. The activity was measured daily.

Others

Native-PAGE was carried out using 8% or 10% polyacrylamide gel at 4 °C according to Laemmli [25] without SDS. The gel was stained with coomassie brilliant blue or incubated with 0.97 M diethanolamine (pH 10.25)/0.25 mM $MgCl_2$ buffer containing 2% 5-bromo-4-chloro-3-indolyl phosphate after washing the gel with 0.25% Triton X-100 at room temperature for 1 h to remove SDS [23].

Results and discussion

Cloning of the haalp gene from Halomonas sp. 593

Native (non-recombinant) HaALP has been partially purified from *Halomonas* sp. 593 and its N-terminal 35 amino acid was determined [23]. The N-terminal sequence showed a high degree of identity to ALP from *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Using primers designed from the sequence information of ALPs from these strains, the 2023 bp DNA fragment, including the *haalp* gene (DDBJ ID: AB271127), was obtained by inverse PCR. To confirm the sequence, this 2023 bp fragment was amplified again by PCR from *Halomonas* sp. 593 chromosomal DNA and the nucleotide sequence was determined. The deduced amino acid sequence from the *haalp* gene consisted of a pre-sequence of 29 amino acids (MTFCMKQKTAVGSLVGGMLLASVAVPASA) and 497 amino acid long mature sequence. The sequence showed a high similarity to the amino acid sequence of other ALPs. Recently, proteome analysis from six extreme halophilic and 24 non-halophilic bacteria showed a significant increase in Asp, Glu, Val and Thr, and a decrease in Lys, Met, Leu, Ile and Cys in the extreme halophiles [27]. Table 1 shows the amino acid composition of HaALP and other ALPs, i.e., *Vibrio* sp. G45–21 (VAP), Antarctic bacterium TAB5 (TAP),