Highly efficient renaturation of β-lactamase isolated from moderately halophilic bacteria

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Abstract Most, if not all, β-lactamases reported to date are irreversibly denatured at 60–70°C. Here, we found that a halophilic β-lactamase from the moderately halophilic bacterium *Chromohalobacter* sp. 560 was highly stable against heat inactivation: it retained ~75% of its activity after boiling for 5 min in the presence of 0.2 M NaCl, suggesting that the protein either incompletely denatures during the boiling process or readily renatures upon cooling to the assay temperature. Circular dichroism showed a complete unfolding at 60°C and a full reversibility, indicating that the observed activity after boiling is due to efficient refolding following heat denaturation. The enzyme showed optimal activity at 50–60°C, indicating that an increase in activity with temperature offsets the thermal denaturation. The gene *bla* was cloned, and the primary structure of the enzyme was deduced to be highly abundant in acidic amino acid residues, one of the characteristics of halophilic proteins. Despite its halophilic nature, the enzyme refolds in low salt media after heat denaturation.

Key words: Halophilic enzyme; Halophilic bacterium; β-Lactamase; Heat stability; Renaturation; Refolding

1. Introduction

Most halophilic microorganisms living in high salt environments can be classified as extremely halophilic archaea or moderately halophilic bacteria [1]. Extremely halophilic archaea, which are adapted to survive in extremely high salt environments such as the Great Salt Lake and the Dead Sea, accumulate concentrated salts in their cells. Proteins in these cells require the presence of a high concentration of salt for stability and activity [2,3]. In addition to being extremely halophilic, several enzymes from halophilic archaea were reported to show thermostable properties [4]. Moderately halophilic bacteria grow well over a wide range of salt concentrations, 3–15% [5], and sometimes even at close to saturated NaCl [6]. The cytoplasmic enzymes of their cells usually show less halophilicity than those from extremely halophilic archaea, since the salt concentration is lower in the cytoplasm of moderate halophiles than extreme halophiles. Extracellular proteins, such as those secreted into the medium and probably those in the periplasmic space, are exposed to external saline environments and must adapt to variable salinities. Although the halophilic nature of enzymes from both extremely and moderately halophilic microorganisms has been well described, the apparent thermostable properties of these enzymes, especially from moderately halophilic bacteria, have not.

β-Lactamases, localized to the periplasmic space in Gram-negative bacteria, are the major cause of bacterial resistance to β-lactam antibiotics. More than 200 species of β-lactamase have been obtained from natural and clinical isolates [7]. β-Lactamases can be classified into two groups. The first group corresponds to serine enzymes of three classes, A (penicillinase), C (cephalosporinase) and D (oxacillinase), while the second group comprises metalloenzymes (class B). Most, if not all, β-lactamases were reported to be denatured by heat treatment at 60–70°C [8–15].

We have been studying the mechanism by which moderately halophilic bacteria can survive upon exposure to xenobiotics in high salt environments. We reported that moderately halophilic bacteria are equipped with multi-drug efflux pump to excrete toxic compounds from cells (submitted elsewhere). Here, we found that *Chromohalobacter* sp. 560 produces a halophilic β-lactamase (HaBlap). The enzyme retains 75–82% of its activity after boiling for 5 min, making this the most thermostable β-lactamase reported to date. Nucleotide sequencing of the cloned HaBlap gene revealed that the enzyme is abundant in Asp and Glu residues, one of the characteristics of halophilic proteins. We report in this paper that the apparent thermostability of this enzyme arises from efficient renaturation after heat denaturation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The moderately halophilic bacterium used was strain 560 isolated from a salted squid gut specimen. The physiological properties and the nucleotide sequence of 16S ribosomal RNA genes (deposited in the DDBJ/EMBL/GenBank databases as AB105069) suggested that this bacterium belongs to the genus *Chromohalobacter* (to be described elsewhere). The bacterium was grown at 37°C in nutrient broth (1% beef extract and 1% polypeptone) supplemented with 2 M NaCl, pH 7.0.

2.2. DNA manipulation and cloning of the bla gene

DNA manipulation was carried out using standard procedures [16]. Chromosomal DNA was isolated by the method of Ausubel et al. [17]. Southern and colony hybridizations were conducted with the ECL Direct or AlkPhos Direct labeling and detection kit according to the manufacturer's instructions (Amersham Biosciences). Nucleotide sequences were determined by the dideoxy chain termination meth-

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od using a BigDye terminator cycle sequencing kit (Applied Biosystems).

A part of *bla* was amplified from strain 560 chromosomal DNA by polymerase chain reaction (PCR). The mixed primers used were designed based on two conserved sequences. One conserved region contains the active site Ser residue used to design the forward primer 5′-ACSCTGTTCGAGVTSGGCTC, while the other region contains the consensus motif including the KTG sequence used to design the reverse primer 5′-AARCCRCCSGTSSCRCCSGTCTTGT. The amplified 794 bp fragment was cloned into the *Eco*RV site of pBR322 (pBR-*bla*). A part of this fragment, 257 bp, was amplified by PCR using as forward primer 5′-ACCGACCGCGCATCGCTG and reverse primer 5′-CGGTCGGCTGGCCGTCCTT, for use in Southern and colony hybridizations. The 7 kb *Stu*I-digested fragment of strain 560 chromosomal DNA containing whole *bla* was finally cloned into the *Sma*I site of pRSGFP-C1 (Clontech) to obtain pRSGFP-*bla*. The determined nucleotide sequence of the *bla* region was deposited in the DDBJ/EMBL/GenBank databases with the accession number AB070219.

2.3. Construction of plasmids and expression of HaBlap and HaBlap fusion proteins

Plasmid pHS-*bla* (13.6 kb) was constructed as follows. The cloned *bla* gene was amplified by PCR using forward primer 5′-GGGAGGCCTAGCGCTAGG, containing a *Stu*I site (underlined), and reverse primer 5′-GGGACTAGTTGACGGTGCG, containing a *Spe*I site (underlined). The amplified fragment was digested with *Stu*I/*Spe*I and was inserted into blunt-ended *Bam*HI/*Spe*I sites of the halophile-*Escherichia coli* shuttle vector pHS15 [18]. The plasmid pHS-*bla* was transferred into a mobilizer strain *E. coli* S17-1 and the conjugation of S17-1 to *Chromohalobacter* sp. 560 was carried out by the filter mating method [19].

The cloned *bla* gene was amplified by PCR using forward primer 5′-GGGCATATGCAAGACGACGCGTCGGAC, which encodes an *Nde*I site (underlined), followed by the coding sequence starting at Gln22, and reverse primer 5′-GGGGATCCTTACGGCACGTCGATCGC, which contains the coding sequence up to the termination codon. The amplified fragment was ligated to *Nde*I/endfilled-*Bam*HI sites of pET15b (Novagen) to construct pET-*bla*, which encodes a fusion protein containing HaBlap preceded by a hexa-His tag. The plasmid pET-*bla* was introduced into *E. coli* BL21(DE3), and the synthesis of His-tag-HaBlap was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. The fusion protein was purified to homogeneity by Ni-NTA agarose (Qiagen) affinity chromatography according to the manufacturer's instructions.

The region encoding *bla* was amplified by PCR using forward primer 5′-GCAAGACGACGCGTCGGACAGGCAAACTCG, which contains the coding sequence starting at Gln22, and reverse primer 5′-CCCGCGGCCGCTTACGGCACGTCGATCGCC, which contains the coding sequence up to the termination codon of the *bla* gene followed by a *Not*I site (underlined). The amplified fragment was inserted into *Sma*I/*Not*I-digested pGEX-4T-1 (Amersham Biosciences) to construct pGEX-*bla*, which encodes a fusion protein containing HaBlap preceded by glutathione *S*-transferase (GST) and a thrombin cleavage site. The GST-HaBlap fusion protein was expressed and purified to homogeneity by Glutathione Sepharose (Amersham Biosciences) affinity chromatography.

2.4. Assay of enzyme activity

Enzymatic activity was determined in a temperature-controlled cuvette containing 10 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl, 0.1 mM nitrocefin (Calbiochem) as a substrate, and ~40 ng of enzyme at 25°C, and the amount of hydrolyzed product was monitored at 486 nm. One unit was defined as the activity which forms 1 μmol product/min. We used a reaction temperature of 25°C for the standard assay conditions except in Fig. 3B. The temperature of the reaction mixture in the cuvette was measured directly by a digital thermometer (Sansho).

2.5. Purification of HaBlap from 560(pHS-bla) cells

Chromohalobacter sp. 560 harboring the plasmid pHS-*bla* was cultured in SW-10 medium [19] containing 200 μg/ml of streptomycin. Cells were suspended in 1/10 volume of 50 mM Tris–HCl buffer, pH 8.0, containing 0.5 M EDTA, 0.5 M sucrose, 1 M NaCl and 10 mM KCl, and incubated for 5 min on ice. Cells were collected by centrifugation at 6000×*g* for 10 min, and resuspended in the same volume of 0.3 M NaCl for osmotic shock treatment. After incubation for 45 s on ice, NaCl and $MgCl_2$ were added to final concentrations of 1.3 M and 1 mM, respectively, and a supernatant fraction was prepared by centrifugation at 6000×*g* for 10 min. This fraction was dialyzed against 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM $MgCl_2$, 25 mM KCl and 0.3 M NaCl, and applied to a column of ATP agarose equilibrated with the same buffer. The flow-through fraction was applied to a column of Resource Q (Amersham Biosystems) equilibrated with 50 mM Tris–HCl buffer, pH 8.0, containing 0.3 M NaCl, and the column was eluted with a linear gradient of 0.3–0.8 M NaCl. The β-lactamase activity eluted at ~0.47 M NaCl was collected, and further purified by preparative native gel electrophoresis with the Laemmli system [20] in the absence of sodium dodecyl sulfate (SDS). The gel was cut into pieces and proteins were extracted with 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl.

2.6. Heat and urea treatments of HaBlap

Native and recombinant HaBlaps in 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl were heat-treated at various temperatures from 25°C to boiling for 5 min and then promptly placed on ice prior to the assay of β-lactamase activity. His-tag-HaBlap was dialyzed against 6 M urea in 50 mM Tris–HCl buffer, pH 8.0, at 4°C for 20 h. Immediately after the dilution of urea to a final concentration of 0.1 M (60-fold dilution) with 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl, enzyme activity was monitored.

2.7. Circular dichroism (CD) spectroscopy

CD spectra were determined on a Jasco J-715 spectropolarimeter equipped with a PTC-348WI temperture 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 0.259 mg/ml. Five scans were accumulated. The mean residue ellipticity was calculated using a mean residue weight of 107.5. The solvent spectrum was subtracted from the sample spectrum to calculate the mean residue ellipticity. The helical content was estimated according to Greenfield and Fasman [21]. A thermal scan was carried out following a CD signal at 220 nm using a scan rate of 40°C/h. After the first scan, the sample was gently mixed with up-and-down turnover to avoid repetitive illumination of the same spot. The onset, mid and end temperatures of the transition were determined from the first derivative of the melting curve. Protein concentration was determined using an extinction coefficient of 1.06 at 280 nm for a 0.1% solution.

2.8. Preparation of anti-GST-HaBlap antiserum

To confirm the correct expression of GST-HaBlap, the purified fusion protein was digested with thrombin, and the amino-terminal amino acid sequence of the HaBlap portion was determined. It was confirmed that the amino-terminal sequence was identical to the predicted sequence encoded in pGEX-*bla*. Rabbits were immunized with the GST-HaBlap fusion protein and antiserum was obtained as described previously [22].

2.9. Other methods

SDS–polyacrylamide gel electrophoresis [20] and Western blotting for proteins were performed using methods described elsewhere [23].

3. Results

3.1. Cloning of bla from Chromohalobacter sp. 560

We found β-lactamase (cephalosporinase) activity in a crude homogenate of *Chromohalobacter* sp. 560 cells. Remarkably, this enzyme in crude cell homogenates retained 75% of its activity after boiling for 5 min. To characterize the protein, we attempted to clone *bla* from strain 560 by PCR amplifying a part of the gene using information on conserved amino acid sequences of several β-lactamases and then Southern hybridization of chromosomal DNA using this fragment as a probe (see Section 2). We thus succeeded in cloning a *Chromohalobacter bla* gene (AB070219) encoding the precursor form of HaBlap (41 516 Da).

Fig. 1 shows the deduced amino acid sequence of the *Chro-*

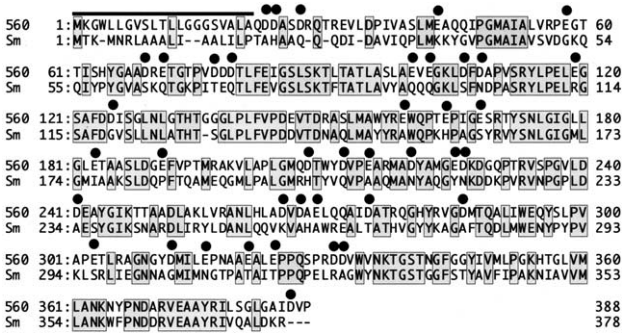

Fig. 1. Alignment of β-lactamases from *Chromohalobacter* sp. 560 and *Serratia marcescens*. The accession numbers for HaBlap and SmBlap are AB070219 and AB008455, respectively. Identical amino acid residues are boxed. The putative signal sequence of HaBlap, predicted with SignalP software, is shown by a thick line. Acidic amino acid residues of HaBlap found in non-identical regions between the two sequences are shown by closed circles.

mohalobacter HaBlap and its alignment with the *Serratia marcescens* β-lactamase (SmBlap, AB008455), which showed the highest similarity (49.5% identity, shown by the box in Fig. 1) to HaBlap in a database search. An abundance of acidic amino acids and relatively few basic amino acids are the general characteristics of halophilic proteins [24]. HaBlap is extremely abundant in acidic amino acids compared with SmBlap: ~22% of non-identical residues between the two sequences were found to be acidic amino acids (shown by closed circles in Fig. 1) in HaBlap. The ratio of basic to acidic amino acid residues in the overall sequence was 0.58 in HaBlap compared with 1.36 in SmBlap.

3.2. *Expression and purification of HaBlap in* Chromohalobacter *as a native protein and in* E. coli *as fusion proteins*

We constructed a HaBlap expression vector, pHS15-*bla*, by inserting the *bla* gene into the halophile-*E. coli* shuttle vector pHS15, and introduced it into strain 560 via conjugation with an *E. coli* mobilizer strain carrying pHS15-*bla*. The transformant 560(pHS15-*bla*) cells were found to have a ca. five-fold higher level of β-lactamase activity than 560(pHS15) cells. Western blotting of HaBlap using anti-GST-HaBlap antiserum, prepared in this study, confirmed this finding (not shown). We purified HaBlap 146-fold from crude homogenate of 560(pHS15-*bla*) by osmotic shock fractionation, followed by passage through an ATP column to remove major chaperones, Q Sepharose column chromatography, and preparative native gel electrophoresis. As shown in Fig. 2A,B, lane 1, the final preparation still contained a major contaminant (shown by the asterisk in Fig. 2A): the content of HaBlap in the final preparation was about 25% as estimated from densitometric measurements. The specific activity of the partially purified HaBlap (protein amount normalized for its purity as shown in Fig. 2A, lane 1) was calculated to be 650 ± 26 U/mg protein at 25°C. HaBlap showed high activity in the presence of 0–3.0 M NaCl: relative activity levels were 92, 100, 97, 94, 93, and 74 at 0, 0.5, 1.0, 1.5, 2.0 and 3.0 M NaCl, respectively. The enzyme was stable during storage at 4°C without NaCl.

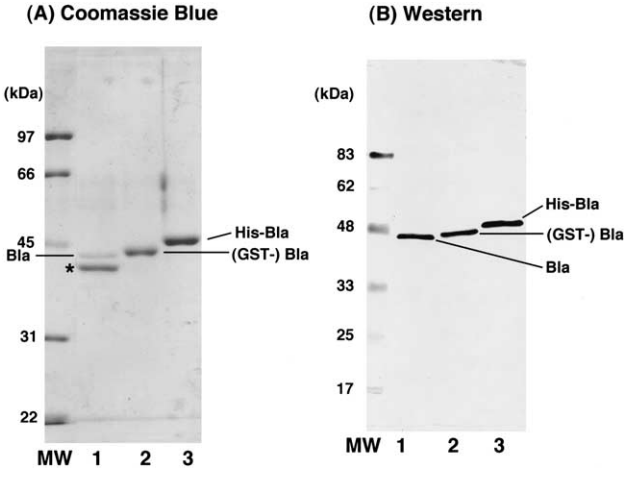

Fig. 2. SDS gel electrophoretogram and Western blot of native HaBlap and HaBlap fusion proteins. Gel was stained with Coomassie blue (A) and immunostained with anti-GST-HaBlap antiserum (B). Bla, native HaBlap; (GST-)Bla, the purified HaBlap portion of the GST-HaBlap fusion protein; His-Bla, His-tag-HaBlap. The asterisk in A, lane 1, shows a contaminant in the native HaBlap fraction.

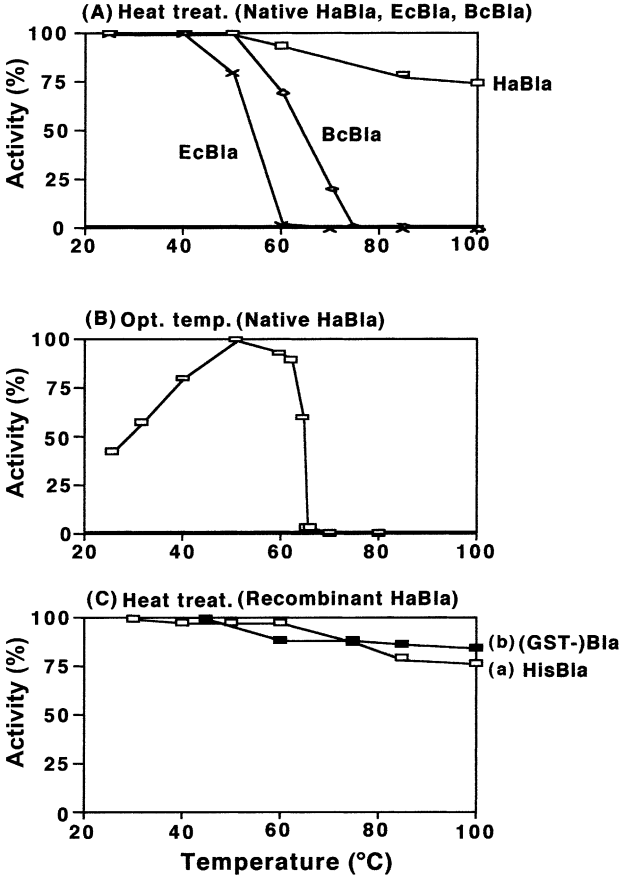

Fig. 3. Thermal stability and optimum reaction temperature of HaBlap activity. A: Native HaBlap (HaBla, 0.33 mg/ml), cephalosporinase (EcBla, 1.7 mg/ml) from *Enterobacter cloacae* (Sigma P4399), and penicillinase (BcBla, 1.98 mg/ml) from *Bacillus cereus* (Sigma P0389) were heat-treated at the temperature shown for 5 min, and residual activity was measured as described in Section 2. Both commercial enzymes were chromatographically purified and of the highest grade commercially available. B: Optimum reaction temperature for native HaBlap. C: His-tag-HaBlap (HisBla, 1.8 mg/ml) and the HaBlap portion of the GST-HaBlap fusion protein ((GST-)Bla, 0.84 mg/ml) were treated and assayed as described in A. Substrate and product were stable under the conditions we used.

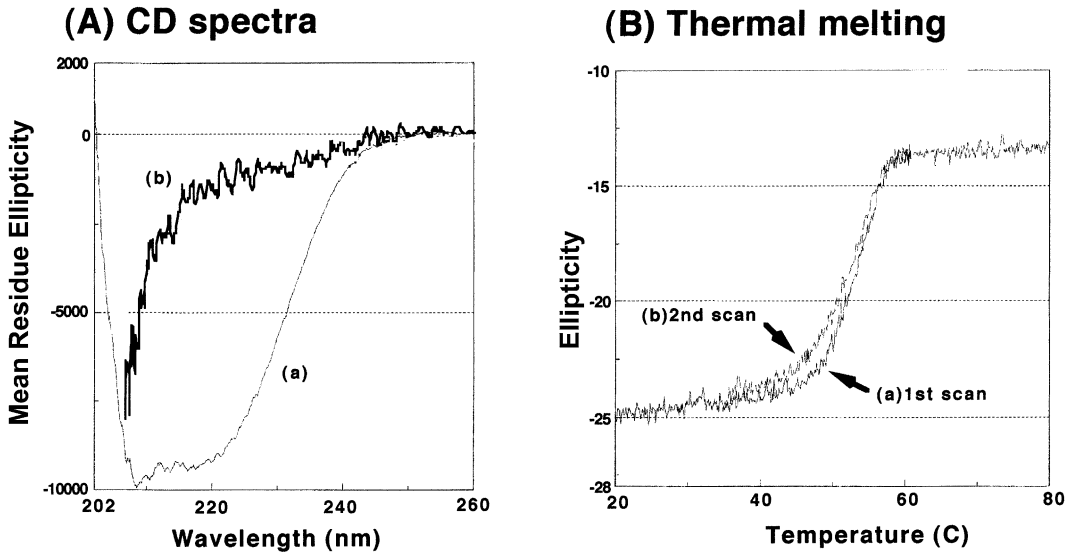

Fig. 4. CD spectra and thermal melting of His-tag-HaBlap. CD spectra were recorded as described in Section 2. A: Far UV CD spectra of His-tag-HaBlap in 50 mM Tris–HCl buffer, pH 8.0 (a, 0.199 mg/ml) and in 50 mM Tris–HCl buffer, pH 8.0, containing 6 M urea (b, 0.158 mg/ml) measured. B: First (a) and second (b) scanning profiles of His-tag-HaBlap (0.259 mg/ml) are shown.

We then tried to express HaBlap as either an amino-terminal GST or an amino-terminal His-tag fusion protein in *E. coli*. The *bla* gene was inserted into the GST fusion vector, pGEX-4T-1, and His-tag fusion vector, pET15b, to construct pGEX-*bla* and pET15b-*bla*, respectively. Both recombinant proteins were expressed in *E. coli*, and purified to homogeneity by affinity chromatography (Fig. 2A,B, lanes 2 and 3). The GST portion of GST-HaBlap was removed by thrombin digestion (Fig. 2A,B, lane 2), and the amino-terminus of HaBlap was confirmed to have the expected sequence. The GST-removed HaBlap (lane 2) and His-tag-HaBlap (lane 3) exhibited the same specific activity as native HaBlap. This result indicates that halophilic HaBlap was successfully expressed in non-halophilic *E. coli* cells.

3.3. Thermal stability and optimum reaction temperature of HaBlap activity

The partially purified HaBlap was heat-treated at various temperatures from 25°C to boiling for 5 min, and residual activity was measured at 25°C as shown in Fig. 3A. Surprisingly, the boiled HaBlap retained 75% of its activity, while *Enterobacter cloacae* β-lactamase (Sigma P4399, cephalosporinase) and *Bacillus cereus* β-lactamase (Sigma P0389, penicillinase) were denatured irreversibly at 60°C and 75°C, respectively (Fig. 3A). Since β-lactamase is generally reported to be irreversibly denatured at 60–70°C [8–15], HaBlap appears to be an exceptionally heat-stable cephalosporinase. As shown in Fig. 3C, HaBlap derived from GST-HaBlap and His-tag-HaBlap retained 82% and 75% of its activity, respectively, after boiling for 5 min. We then examined the optimum reaction temperature of HaBlap. As shown in Fig. 3B, HaBlap was most active at 51°C. It started to lose activity sharply above 60°C and was completely inactive at 70°C. During this experiment, we observed a very quick and fully reversible response of enzymatic activity to the respective reaction temperatures. For example, when the cuvette containing the reaction mixture at 65°C (showing 3.8% of full activity) was adjusted to 64°C by a temperature-controlled cuvette folder, HaBlap immediately exhibited the corresponding activity at 64°C (59.6% of full activity) shown in Fig. 3B. These results indicate that HaBlap lost its activity above 65°C, but renatured immediately upon a shift to lower temperatures.

We also observed a quick renaturation of urea-denatured His-tag-HaBlap. His-tag-HaBlap was denatured in 6 M urea/50 mM Tris–HCl buffer, pH 8.0 (CD spectrum shown in Fig. 4A-b), and renaturation was measured as the enzymatic activity recovered by dilution of urea to a final concentration of 0.1 M with buffer. A recovery of 57, 85 and 95% activity was attained 0.5, 2 and 4.5 min after the dilution of urea. His-tag-HaBlap showed no activity in the presence of 6 M urea.

3.4. CD measurement and thermal transition

To confirm the reversible renaturation of heat-denatured HaBlap, we measured CD spectra and thermal melting. Here, we used His-tag-HaBlap, since the amount of native HaBlap or HaBlap obtained from GST-HaBlap by proteolytic

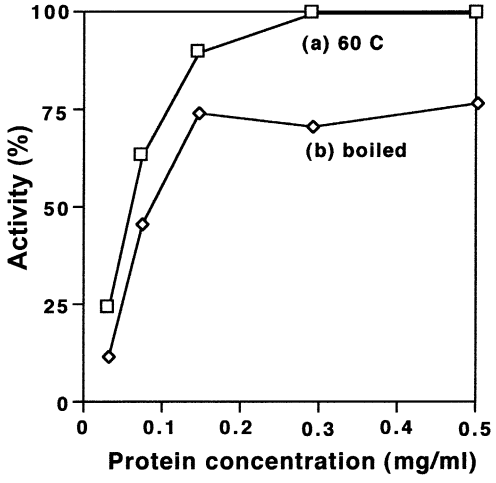

Fig. 5. Effects of protein concentration on thermal stability of His-tag-HaBlap. His-tag-HaBlap at several protein concentrations was heat-treated at 60°C (a) and boiling temperature (b), and its residual activity was measured.

cleavage was too small for CD measurements and following experiments. Fig. 4A-a shows the far UV CD spectrum. It is characterized by double minima at 208 and 220 nm, indicative of an α-helical structure. The α-helical content was calculated to be 20% based on Greenfield and Fasman [21]. If the His tag portion is random, then HaBlap has an α-helical content of ~21%. Fig. 4B shows the melting curve. In the first scan, the sample was heated from 20 to 60°C. It shows a highly cooperative transition, indicating that the protein is folded into a single globular structure. The onset, mid and end temperatures of melting are calculated to be 46, 54 and 60°C. The sample was cooled to 20°C and incubated for 10 min. The signal at 20°C was identical to that before heating, indicating that the melting of HaBlap was completely reversible when the enzyme was heated to 60°C. The second scan is shown in the same figure, again indicating a highly cooperative transition. The melting temperatures are 45-54-59°C, almost identical to those determined in the first scan. There appears to be a small difference in CD signal during thermal transition in the lower temperature range. From the identical signal at 220 nm after cooling and the observed recovery of activity, it is evident that the global structure and α-helix were regained after heating to 60°C and cooling. There may be a local change in helical packing, causing a segment of the α-helix to melt at lower temperature.

As shown in Fig. 4A-b, His-tag-HaBlap is completely denatured in 6 M urea, indicating that the observed activity after urea treatment is not due to the resistance of the protein to urea denaturation, but is due to efficient refolding as described above.

3.5. *Effects of protein concentration on thermal stability*

Fig. 5 shows the effects of protein concentration on thermal stability at 60°C and boiling. His-tag-HaBlap lost activity sharply when heat-treated at protein concentrations lower than 0.15 mg/ml. The stability was constant at a protein concentration of 0.3–2.9 mg/ml (Fig. 5 and not shown). By gel filtration, we found that this enzyme is monomeric. These findings clearly suggest that this protein is highly soluble with no tendency to aggregate even after denaturation.

4. Discussion

We have shown here that HaBlap is stable against heat treatment in that it regains enzyme activity after boiling. Since CD thermal melting revealed that the enzyme melts below 60°C, the observed recovery of activity after boiling is due not to its stability at high temperature, but to high reversibility of its thermal denaturation. We showed before that a halophilic NDK (HsNDK) from the extremely halophilic archaeon *Halobacterium salinarum* can refold after denaturation caused by heat treatment [25,26]. However, HaBlap refolds in low salt media, while HsNDK requires a high concentration of salt [25,26]. As shown in Fig. 1, HaBlap is highly acidic similar to HsNDK, yet the salt requirements of the two enzymes are very different. Although the reason for this is not clear, the observed refolding in low salt media is consistent with the natural environment of HaBlap, i.e. moderately halophilic. Since the amount of pure enzyme was limited in this study, we did not carry out systematic experiments to examine the effects of salt concentration on the properties of HaBlap in detail. While many enzymes isolated from moderately halophilic bacteria are reported to show maximum enzymatic activity in the presence of 0.1–0.6 M salt [6], HaBlap showed only slightly decreased activity at high salt concentrations, i.e. 93% and 74% in the presence of 2.0 M and 3.0 M NaCl, compared with the activity in 0.5 M NaCl. Thus, the effects of salt concentration on refolding efficiency of HaBlap should be interesting and to carry out such a study, we are now trying to improve the yield of pure HaBlap preparation.

Despite the difference in their requirement for salt, both HaBlap and HsNDK denature reversibly upon heat as well as urea treatment. This reversibility is due to an abundance of acidic amino acids, which prevent irreversible aggregation at high temperature. Although HaBlap may denature above 45°C, it is most active at 50–60°C, indicating that an increase in activity with temperature offsets the thermal denaturation. In other words, while the activity increases with temperature, the amount of folded, active protein decreases. Because of its stability at high temperature, as shown in Fig. 3, HaBlap is active over a wide range of temperatures. Such an activity–temperature profile may be of commercial value given that enzymes from thermophilic organisms, thermozymes, are only marginally active at low temperatures [27]. Structural rigidity is suggested to be responsible for their poor catalytic activity at low temperatures [28]. Under the extreme conditions of high temperature, it is possible that cofactors, substrates, or products become unstable, and that other side reactions occur [27]. In this sense, the thermostable enzymes from cells which grow at 'normal' temperatures may be more versatile, since they could function at low to high temperatures.

In conclusion, we described here that not only enzymes from extremely halophilic archaea but also those from a moderately halophilic bacterium exhibited apparent thermostability, and that the highly acidic molecular nature of halophilic proteins generates a high solubility without aggregation, and thus, ensures highly efficient renaturation after heat or urea treatment.

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