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Characterization of a Recombinant Thermostable Dehalogenase Isolated from the Hot Spring Thermophile *Sulfolobus tokodaii*

Philip G Bachas-Daunert · Stacy A. Law · Yinan Wei

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Abstract A putative dehalogenase, L-HAD$_{ST}$, from the thermophile *Sulfolobus tokodaii*, was cloned and expressed in *Escherichia coli*. The recombinant enzyme catalyzes the stereospecific dehalogenation of L-2-haloacids with similar levels of activity as its homolog from mesophiles. L-HAD$_{ST}$ remains fully active after being incubated for 4 h at 70 °C and tolerates extreme pH conditions ranging from 4 to 10. Furthermore, it can be purified conveniently without the usage of any chromatography method. The high expression yield and easy purification procedure make the recombinant dehalogenase an excellent candidate for biotechnological applications.

Keywords Thermostable · Extremophile · L-2 Haloacid dehalogenase · Recombinant

Introduction

Life in extreme environments is manifested by the presence of microorganisms capable of surviving under harsh conditions. To maintain normal cell function, proteins from extremophiles usually have extraordinary stabilities. Enzymes extracted from extremophiles, as well as the extremophiles themselves, have found wide applications in the food, textile, paper, biotechnology, bioremediation, and chemical industries. For example, *Taq* polymerase derived from *Thermus aquaticus* is one of the best-known and first characterized thermostable DNA polymerase. Its ability to catalyze the polymerization of nucleotides into DNA strands at high temperatures has greatly improved and simplified the polymerase chain reaction (PCR), which is recognized as a major contribution to the fields of molecular biology and biotechnology [1, 2].

S. A. Law · Y. Wei (✉)
Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA
e-mail: yinan.wei@uky.edu

P. G. Bachas-Daunert
Paul Laurence Dunbar High School, 1600 Man O' War Blvd, Lexington, KY 40513, USA

Dehalogenases, enzymes that catalyze the cleavage of carbon–halogen bonds, are of particular practical interests because of their potential biotechnological applications in the bioremediation of halogenated environmental pollutants [3–14] and the production of enantiomerically pure precursor materials for compound synthesis in the pharmaceutical, fine chemical, and medical industries. In the current study, we cloned, over-expressed, and characterized a dehalogenase from the thermophile *Sulfolobus tokodaii*. The genome of *S. tokodaii* strain 7 has been sequenced [15], upon which two putative dehalogenases were identified (ST1839 and ST2570). Based on sequence homology analysis (Fig. 1a), these two putative dehalogenases belong to the L-2-haloacid dehalogenase (L-2-HAD) family. The L-2-HAD catalyzes the stereospecific conversion of L-2-haloalkanoates to the corresponding D-2-hydroxyalkanoates (Scheme 1) [4]. The L-2-HADs from different sources have high degrees of sequence similarity, with similar sizes ranging from 25 to 28 kDa and optimal activity at pH 9–11 [16].

In this study, we characterized ST2570, which we renamed as L-HAD$_{ST}$, because it had higher degree of sequence similarity with several extensively studied L-2-HAD examples (Fig. 1a). We cloned and expressed L-HAD$_{ST}$ in a tag-free form with high yield (60 mg purified protein per liter of culture). The recombinant protein had no vector-derived residues, as the sequence of L-HAD$_{ST}$ naturally started with a Met. A convenient two-step, heat-acid treatment purification protocol was established, which facilitated the production of large quantities of purified protein in a simple and timely manner. The protein can tolerate high heat and extreme pH conditions, which makes it an ideal candidate for various biotechnological applications.

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L-DEX   MDYIKGIAFDLYGTLFDVHSVVGRCDEAFPGRGREISALWRQKQLEYTWLRSLMNRYVNF
DhlB    M--IKAVVFDAYGTLFDVQSVADATERAYPGRGEYITQVWRQKQLEYSWLRALMGRYADF
ST2570  M--I-ILAFDIFGTVLDTSTVI---------------QEFRNKQLEYTWLLTIMGKYVEF
ST1836  ------------------------------------------------------------

L-DEX   QQATEDALRFTCRHLGLDLDARTRSTLCDAYLRLAPFSEVPDSLRELKRRGLKLAILSNG
DhlB    WSVTREALAYTLGTLGLEPDESFLADMAQAYNRLTPYPDAAQCLAELAP--LKRAILSNG
ST2570  EEITKITLRYILKVRGE---ESKFDEELNKWKNLKAYEDT-KYLKEISEIA-EVYALSNG
ST1836  ------------------------------------DS-LLLEEISYRA-RIFIITNL
                                              :    * *:     .   ::*

L-DEX   SPQSIDAVVSHAGLRDGFDHLLSVDPVQVYKPDNRVYELAEQALGLDRSAILFVSSNAWD
DhlB    APDMLQALVANAGLTDSFDAVISVDAKRVFKPHPDSYALVEEVLGVTPAEVLFVSSNGFD
ST2570  SINEVKQHLERNGLLRYFKGIFSAESVKEYKPSPKVYKYFLDSIGAK--EAFLVSSNAFD
ST1836  EKKFVKEFLLRNNIDVFVQDVISAEEIKRYKPSPEFFDYLAKRANSVKGAITIISSNPQD
          . :.  : . .:   .. ::*.:  : :**    :    .  .       ::***  *

L-DEX   ATGARYFGFPTCWINRTGNVF----------------------EEMGQTPDWEVTSLRA
DhlB    VGGAKNFGFSVARVARLSQEALARELVSGTIAPLTMFKALRMREETYAEAPDFVVPALGD
ST2570  VIGAKNAGMRSIFVNRKNTIV----------------------DPIGGKPDVIVNDFKE
ST1836  IIVAKSMGFKAYWMNRSNLQY---P------------------FPANLQPDNILKSITS
           *:  *:    : * .                          **  :  :

L-DEX   VVEL-----FETAAG--KAEKG
DhlB    LPRL-----VRGMAGAHLAPAV
ST2570  LYEW-------------ILRYK
ST1836  LAESTAQHKVDSSMGDSALRCT
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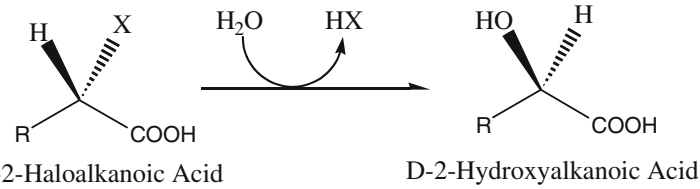
Fig. 1 Sequence alignment of L-2-HADs from *Pseudomonas* sp. YL (L-DEX), *X. autotrophicus* GJ10 (DhlB), and *S. tokodaii* (ST2570 and ST1836). *Asterisks* indicate identical residues, and *dots* indicate partially conserved residues. The critical residues for L-DEX activity are *highlighted*. ST2570 was renamed as L-HAD$_{ST}$ in the rest of the paper



L-2-Haloalkanoic Acid D-2-Hydroxyalkanoic Acid

Scheme 1 Reaction catalyzed by L-2-HAD

Materials and Methods

Construction of the Plasmid pHAD$_{ST}$

The gene of L-HAD$_{ST}$ was obtained by PCR using clone STLGR04873 as the template (National Institute of Technology and Evaluation, Japan) and primers: 5′-GAG *CAT ATG* ATC ATT CTA GCA TTT GAT ATC-3′ (forward) and 5′-GAG *GGA TCC* TCA CTT ATA TCG CAA AAT CCA TCC-3′ (reverse). Restriction enzyme digestion sites *Nde*I (forward primer) and *Bam*HI (reverse primer) are in italics. After amplification, the PCR product was subcloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) to generate plasmid pCR2.1-TOPO-HAD$_{ST}$. The plasmid pCR2.1-TOPO-HAD$_{ST}$ and the pET22b vector were both doubly digested with *Nde*I and *Bam*HI. Following digestion and agarose gel purification, the gene of L-HAD$_{ST}$ was ligated into the multiple cloning site of pET22b to generate plasmid pHAD$_{ST}$. The plasmid sequence encoding for the gene of L-HAD$_{ST}$ was verified by DNA sequencing (Davis Sequencing, Davis, CA, USA).

Protein Expression and Purification

Escherichia coli strain Rosetta 2(DE3)pLysS (EMD Biosciences, San Diego, CA, USA) was transformed with plasmid pHAD$_{ST}$ for protein production. The cells were grown in Luria–Bertani media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol to an OD$_{600}$ of 0.6, and then induced by adding 1 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG). Sixteen hours after the induction, the cells were harvested by centrifugation at 6,000×*g* for 10 min. The cell pellet was resuspended in buffer A (50 mM phosphate buffer, pH 7.0) and lysed by sonicating on ice for 5 min with 10 s on/off intervals. The cell debris was separated from the supernatant by centrifugation at 10,000×*g* for 15 min. The same centrifugation condition was used throughout the purification steps unless otherwise indicated. The supernatant was first incubated at 60 °C for 45 min, followed by centrifugation to remove the heat-denatured impurities. The pH of the supernatant was adjusted to 4.5 by the addition of concentrated sodium acetate buffer. The acidified solution was incubated at room temperature with gentle stirring for 40 min before being subjected to centrifugation to remove acid-denatured impurities. The final supernatant was dialyzed against buffer B (20 mM sodium phosphate buffer, pH 7.0, 0.02% *w*/*v* sodium azide) and stored at 10 °C. The purification scheme was illustrated in Fig. 2a. The purified dehalogenase was analyzed using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were separated on a 20% homogenous polyacrylamide Phast gel (Phast System, GE healthcare, Waukesha, WI, USA) and visualized through the Coomassie Blue stain. Molecular weight standards (Benchmark Protein Markers) were obtained from Invitrogen. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA).

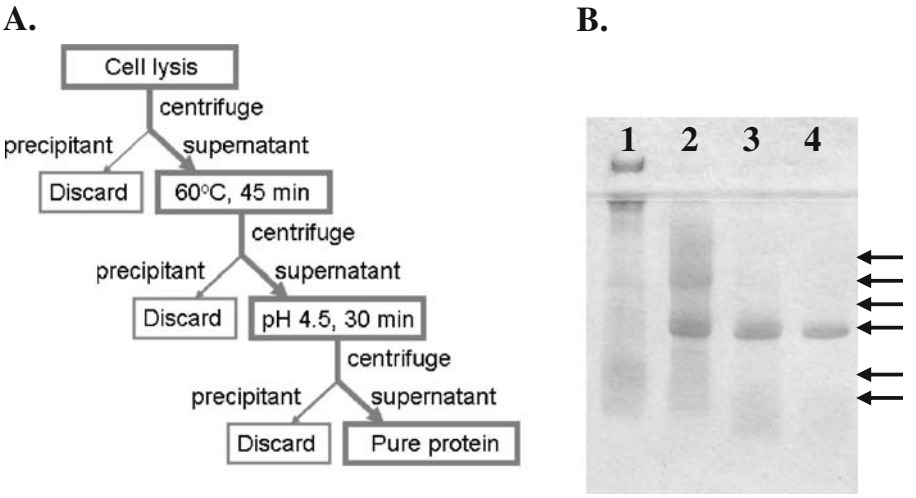


Fig. 2 L-HAD_{ST} expression and purification. **a** Schematic illustration of the simple protein purification procedure. **b** SDS-PAGE analysis of protein purification. *From top to bottom*, the *arrows* marked molecular weight of 10, 15, 20, 30, 40, and 50 kDa. *Lane 1* is the pre-induction cell lysate, showing no over-expression of L-HAD_{ST} before the addition of IPTG. *Lane 2* is the cell lysate after 16 h of induction. A prominent band at about 20 kDa can be observed, corresponding to the over-expressed L-HAD_{ST} (23 kDa). *Lanes 3* and *4* are the cleared cell lysate after heat treatment and acid treatment, respectively. After the purification, L-HAD_{ST} is highly pure as revealed by *lane 4*

Circular Dichroism Spectroscopy

Circular dichroism (CD) was performed on a JASCO J-810 spectrometer (JASCO, UK) with 1 nm bandwidth and a 0.1-cm pathlength cuvette. The L-HAD_{ST} was dialyzed overnight into a low salt buffer (5 mM phosphate buffer, pH 7.0) before the CD measurement. The wavelength scan was performed at 20 °C. The blank scan was performed with the exterior dialysis buffer and subtracted from the measured data. For the temperature denaturation experiment, the ellipticity was monitored at 222 nm from 0 to 99.5 °C using a 1 °C/min scan rate.

Enzyme Activity

The activity of the dehalogenase was determined by monitoring the rate of release of halide ions (chloride or bromide). The stock solutions of haloacid substrates were adjusted to neutral pH with concentrated sodium hydroxide. A 0.5-mL reaction mixture containing the indicated substrate and buffer (as described in the legends of the corresponding figures) was pre-equilibrated at the indicated temperature for 5 min before the addition of the protein. After the specified incubation time, the reaction was terminated by shifting the samples from the incubating water bath to ice plus the addition of 50 μL of 1 M nitric acid. The concentration of halide ions in the sample was determined using the Chloride Analysis Kit (Hanna Instruments, Woonsocket, RI, USA). To account for potential contributions from the background hydrolysis of the substrate, blank experiments in the absence of protein were performed in parallel. The quantity of halide in the blank experiments was determined and subtracted from the L-HAD_{ST} catalyzed reaction data.

pH and Temperature Optima of the Dehalogenase Activity

For the pH study, 50 mM of sodium phosphate, Tris–SO_4, sodium glycine, and sodium-3-(cyclohexylamino)-1-propanesulfonate buffers were used for the pH ranges of 7.0, 7.5 to 8.5, 9.0 to 10.0, and 10.5 to 11.5, respectively. The reaction was allowed to proceed at 60 °C for 5 min. Then, the concentration of chloride in the reaction mixture was determined as described above. To determine the activity of the enzyme at different temperatures, the reaction mixture (with 50 mM sodium glycine buffer, pH 9.5) was incubated at the indicated temperature for 5 min and then followed by the chloride analysis. For the thermostability experiments, the protein was first incubated at the indicated temperature for 0.5, 1, 2, 3, or 4 h. Next, the activity of the protein was determined at 60 °C. L-2-Chloropropionic acid (L-CPA, 2 mM) was used as the substrate for these studies.

Results

Expression and Purification of L-HAD_{ST}

We have constructed a plasmid capable of producing L-HAD_{ST} in high yields in *E. coli*. Because of the extraordinary stability of L-HAD_{ST}, we were able to develop a facile method for its isolation and purification. The enzyme could be separated from the majority of the undesirable proteins in the cell lysate using two steps. The method involved a heat treatment step that removes 90% of the impurities, followed by an acidification step that eliminates the remaining impurities. This two-step method, which only took 2 h, yielded highly pure enzyme as demonstrated by the SDS-PAGE analysis (Fig. 2b). Small aliquots of the protein solution were sampled for the dehalogenase activity throughout the purification process. No significant decrease of the overall dehalogenase activity was observed, indicating that neither the heat nor the acid treatment affected the activity of the L-HAD_{ST}. This easy purification procedure made the production of this protein fast, cost effective, and suitable for large-scale production.

Catalytic Specificity of L-HAD_{ST}

As a putative L-2-HAD, L-HAD_{ST} should not catalyze the dehalogenation of D-2-haloalkanoic acid. We compared the release of chloride from L-CPA and D-2-chloropropionic acid (D-CPA) in the presence of L-HAD_{ST} (Fig. 3a). As expected from the sequence homology analysis, L-HAD_{ST} catalyzed the stereospecific removal of chloride from the L-conformer but not the D-conformer. To determine the K_M and V_{max} values, the catalytic reaction was performed at various L-CPA concentrations ranging from 0.125 to 6 mM (Fig. 3b). The kinetic parameters were derived from the linear fitting of the double reciprocal Lineweaver–Burk plot (Table 1). The K_M was found to be 1.7 mM. This is consistent with K_M values reported for other L-HAD enzymes, which are also in the mM range. For example, L-DEX YL has a K_M of 0.37 mM for L-CPA [17]. These K_M values indicate that the L-2-HADs have modest binding affinities, correlating well with their tolerance toward a broad range of substrates of different sizes [17]. The binding site of L-2-HAD is formed at the interface of the core domain and the cap domain. The flexibility between the two domains may be the fundamental reason for the relatively high K_M and wide substrate specificity.

The dehalogenase activity of L-HAD_{ST} toward 2-bromopropionic acid, 2-bromobutyric acid, and 2-bromohexanoic acid were examined to reveal the effect of substrate size on the

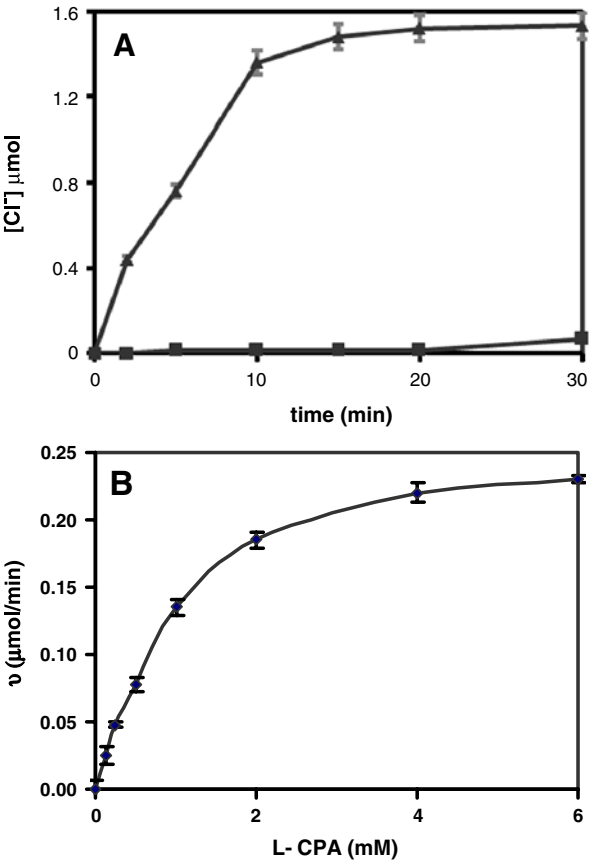


Fig. 3 Dehalogenation of L-CPA and D-CPA catalyzed by L-HAD$_{ST}$. **a** A 0.5-mL reaction mixture containing 2 mM of substrate (*triangles* L-CPA or *squares* D-CPA) in a 50 mM glycine buffer, pH 9.5, was pre-equilibrated at 60 °C before adding 0.005 mg of purified L-HAD$_{ST}$. After the indicated incubation time, the reaction was stopped, and the concentration of chloride was determined. Hydrolysis of the substrate in the absence of L-HAD$_{ST}$ was measured in parallel and subtracted. L-HAD$_{ST}$ stereospecifically catalyzed the dehalogenation of the L-conformer. **b** Kinetic parameters of L-HAD$_{ST}$ catalyzed L-CPA dehalogenation. The experiment was performed as described above except that the incubation temperature was 70 °C, and the incubation time was 5 min. Each data point represents the average of three measurements. *Error bars* indicate one standard deviation (n=3). For some data points, the standard deviations were so small that they were masked by the data marker

catalytic reaction (Table 1). A standard substrate for the characterization of haloalkane dehalogenases, 1,4-dibromobutane, was also included in this study. We found that when the substrate chain length increased from three to six carbons, the substrate-binding affinity also increased (K_M decreased from 2.1 mM for 2-bromopropionate to 0.41 mM for 2-bromohexanate), presumably due to improved binding of longer alkyl chains at the active site. In contrast, the catalytic activity decreased with the increase of substrate size (k_{cat} for 2-bromopropionate and 2-bromohexate were 53.2 and 25.4 μmol/min mg, respectively). The L-2-HAD family enzymes are quite tolerant to the size of their substrates. Liu et al. [17] examined the catalytic activity of L-DEX toward a series of 2-chloroalkanoic acids differing in the number of carbon atoms (three to six atoms) in their alkyl chain. Designating the

Table 1 Kinetic parameters of L-HAD$_{ST}$ toward different substrates.

Substrate	K_M (mM)	V_{max} (μmol/min)	k_{cat} (μmol/min·mg)
L-2-Chloropropionic acid	1.7	0.35	69.0
2-Bromopropionic acid	2.1	0.27	53.2
2-Bromobutyric acid	1.7	0.21	41.2
2-Bromohexanoic acid	0.41	0.13	25.4

activity toward L-CPA to be 100, the activity of L-DEX is about 25 toward 2-chlorobutyric acid (four carbons), and 3 for 2-chlorohexanoic acid (six carbons). The specific activity of L-HAD_{ST} also decreased for substrates of larger size. However, the decline was much slower. When the chain length increased from three carbons (2-bromopropinic acid) to six carbons (2-bromohexaoic acid), the relative activity decreased by about 50%. In addition, we also confirmed that L-HAD_{ST} is not active toward 1,4-dibromobutane.

Thermal Stability of L-HAD_{ST}

CD spectroscopy was employed to examine the thermal stability of the newly isolated L-HAD_{ST}. As expected for the L-2-HAD family proteins, L-HAD_{ST} had a CD spectrum typical of a protein with mixed α-helical and β-strand structures (Fig. 4a). Thermal denaturation of L-HAD_{ST} was monitored at 222 nm as the decrease of the ellipticity (Fig. 4b). The secondary structure of L-HAD_{ST} only began to unfold at about 95 °C, as indicated by the sharp decrease of the ellipticity, correlating with the loss of α-helical secondary structure. The protein did not fully denature at 99.5 °C, preventing the recording of its thermal denaturation profile to completion.

To further probe the effect of prolonged incubation at high temperature on the function of L-HAD_{ST}, the protein was incubated for 0.5, 1, 2, 3, or 4 h at various temperatures ranging from 70 °C to 90 °C. Small aliquots of the protein were withdrawn and

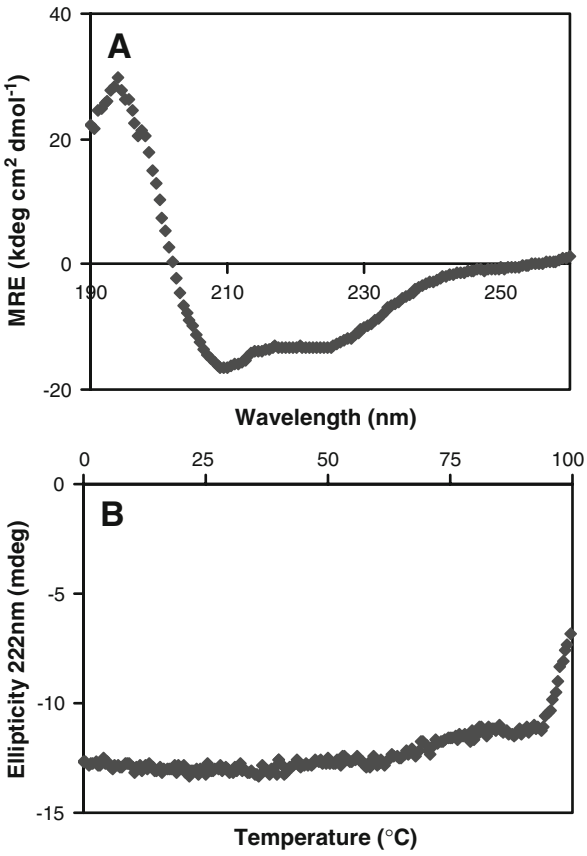


Fig. 4 CD measurements of L-HAD_{ST}. **a** Wavelength scan reveals a characteristic profile for proteins with a mixed α/β structure. The protein concentration was 0.125 mg/mL. **b** Change of ellipticity at 222 nm with increasing temperature. L-HAD_{ST} begins to unfold at 95 °C, as indicated by the sharp rise in the plot

immediately cooled to 60 °C. And then, the residual protein activity was determined through performing the dehalogenase activity assay at 60 °C. As shown in Fig. 5, the protein maintained 100% and 90% activity after a 4-h incubation at 70 °C and 80 °C, respectively. At 90 °C, the protein lost 70% of activity within the first 1 h and then gradually lost an additional 15% activity during the following 3 h.

Optimum pH and Temperature for the Activity of L-HAD$_{ST}$

Being an enzyme from an organism living at 80 °C, L-HAD$_{ST}$ is expected to have optimized activity at an elevated temperature. We found that the catalytic activity of L-HAD$_{ST}$ increased with temperature until it reached a maximum activity at approximately 70 °C and began to decrease at 85 °C. The protein retained 85% of its maximum activity at 90 °C (Fig. 6a).

The optimum pH for L-HAD$_{ST}$ is approximately 9.5 to 10 (Fig. 6b), which is consistent with pH optima of other L-HADs [16]. The *S. tokodaii* strain 7 thrives in very acidic conditions (pH 2–3), and yet L-HAD$_{ST}$ is totally inactive under acidic conditions. The identified high optimum pH is not unexpected, as L-HAD$_{ST}$ shares all the critical functional resides with L-DEX and DhlB (Fig. 1), indicating that they should have identical catalytic mechanisms. L-DEX and DhlB both work the best under alkaline conditions. Organisms thriving in extremely acidic or alkaline conditions have elaborated mechanisms to keep the pH homeostasis in their cytoplasm. As a consequence, cytoplasmic enzymes are not exposed to extreme pH [18]. The catalytic mechanism has been extensively studied for L-DEX, in which the carboxylate group of D10 (corresponding to D7 in L-HAD$_{ST}$) nucleophilically attacks the β-carbon of the substrate and causes the breakage of the carbon–halogen bond. And then, the hydrolysis of the ester intermediate yields the D-2-hydroxyalkanoic acid. Since all of the important catalytic residues are conserved in the sequence of L-HAD$_{ST}$, most likely it shares the same mechanism. L-DEX also has an optimal pH at around 9, despite the fact that an Asp was identified as the key catalytic residue. Two basic residues, R41 and K151 (corresponding to R23 and K128 in L-HAD$_{ST}$, respectively), present at the active site of L-DEX. The guanidino group of R41 is speculated to serve as an acceptor of the halide ion released from the substrate, which requires the guanidine side chain to be positively charged. In contrast, the side chain of K151 forms part of the hydrophobic pocket surrounding the alkyl group of the substrate. For this function, the K151 should remain preferably neutral [16]. The need to keep some of the residues protonated (such as R41) and others deprotonated (such D10 and K151) may be the reason

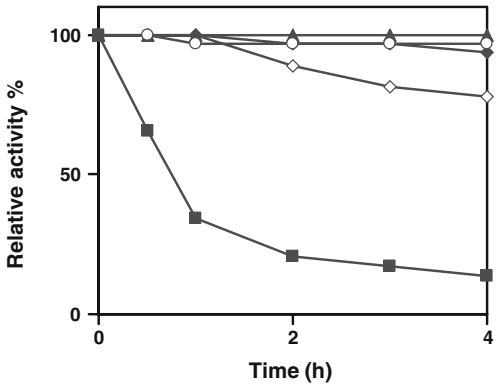


Fig. 5 Thermal stability of L-HAD$_{ST}$ as revealed by the decrease in activity after prolonged incubation at elevated temperatures. Protein samples were incubated at 70 °C (*filled triangle*), 75 °C (*open circle*), 80 °C (*filled diamond*), 85 °C (*open diamond*), or 90 °C (*filled square*). Each data point represents the average of three measurements. Standard deviation (not shown for the clarity of the plots) is usually within 5%

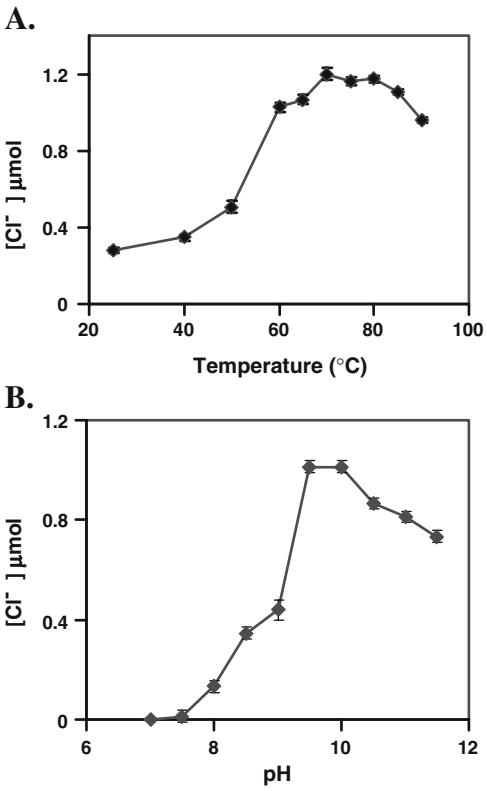


Fig. 6 Effect of temperature and pH on the activity of L-HAD$_{ST}$. A 0.5-mL reaction mixture containing 2 mM L-CPA in 50 mM indicated buffer was pre-equilibrated at the indicated temperature before adding 0.005 mg of purified L-HAD$_{ST}$. After 5 min, the reaction was stopped, and the concentration of chloride was determined as described in the "Materials and Methods" section. Hydrolysis of the substrate in the absence of L-HAD$_{ST}$ was measured in parallel and subtracted. Each data point represents the average of three measurements. *Error bars* indicate one standard deviation (n=3). **a** Effect of temperature. Glycine buffer, pH 9.5, was used throughout the experiment. **b** Effect of pH. Different pH buffer systems were used at various ranges as described in the "Materials and Methods" section. The incubation was performed at 60 °C. For some data points, the standard deviations were so small that they were masked by the data marker

behind the observed optimal pH. At lower pH, the protonation of K151 may disrupt the substrate binding and thus results in the diminished catalytic activity.

Discussion

We identified a putative dehalogenase from *S. tokodaii* strain 7 as a L-2-HAD. This was initially based on the high degree of sequence similarity with known L-2-HADs and then confirmed by the study of its substrate specificity. The most extensively studied members of the L-2-HAD family include the L-DEX from *Pseudomonas* sp. YL and the DhlB from *Xanthobacter autotrophicus* GJ10 [16]. High-resolution structures have been determined for these two enzymes in both substrate-bound and free states [19–21]. Several highly conserved residues that are critical for the L-DEX function have been pinpointed through structural and mutational studies [22, 23]. These catalytic amino acids are conserved in L-HAD$_{ST}$ as well, which has an overall sequence identity of 28% to L-DEX and 31% to DhlB (Fig. 1a). Both L-DEX and DhlB are dimers having a mixed α/β main domain and a four-helix bundle cap domain. The catalytic site is located in a cavity between the main and cap domains. While the main domain contributes the catalytic residues, the cap domain stabilizes the substrate and determines substrate specificity.

Although a preliminary X-ray crystallography study had been reported for L-HAD$_{ST}$, no structure has yet been published [24]. The amino acid sequence of L-HAD$_{ST}$ was submitted to the online server of the structural modeling software LOOPP [25–27]. Based on

sequence similarity, LOOPP identified closely related homologous proteins and extracted their structures from the Protein Data Bank (PDB) protein database. Then, using each of the top five identified structures as templates, LOOPP generated five structural models for L-HAD$_{ST}$. The template structures were 1ZRN [28], 1QQ5 [29], 1ZRM [28], 1QQ7 [29], and 1JUD [19]. It is not surprising that the top five matches are all structures of L-2-HADs, including L-DEX, DhlB, and their complexes with substrates. The template structures are very similar, which instigates the high degree of similarity of the generated models. Figure 7a shows the stereo view of the superposition of the L-HAD$_{ST}$ model with the corresponding template structure of L-DEX. The core domain of the L-HAD$_{ST}$ model structure superimposes well onto the crystal structure of L-DEX, with a root-mean-square deviation of 0.72 Å for the backbone α carbon. There are two stretches of residues missing in the middle of L-HAD$_{ST}$ sequence when aligned with L-DEX (Fig. 1), between V18 and I19 and between G58 and E59. These are the locations where the model deviates the most from the template (Fig. 7a). Through the comparison of the template and model structures, it is apparent that the two missing pieces in the L-HAD$_{ST}$ sequence correspond to two exterior loops in L-DEX, which contact each other. The main force stabilizing these two loops is the hydrophobic interaction between the side chains of F29, I36, L77, and L79 (Fig. 7b). The lack of an extensive network of hydrogen bonding and charge–charge interaction indicates that the gain of enthalpy to fold these two loops is small compared to the large entropy loss to keep the loops in a fixed position. As a result, the overall effect of these loops on the protein stability is negative. Elimination of exterior loops without the loss of function is actually a commonly observed strategy employed by thermophiles to improve the structural stability of proteins [30].

In adaptation to the extreme habitat, L-HAD$_{ST}$ was evolved to drastically increase the structural stability and yet maintain the catalytic mechanism. Using L-CPA as the substrate, the specific activity of L-HAD$_{ST}$ is about half of what was reported for a mesophile counterpart [17]. However, the L-HAD$_{ST}$ may be more active than dehalogenases from mesophilic organisms for substrates of larger size, as its activity declined slower with the increasing substrate chain length. The dehalogenase does not depend on cofactors to perform catalytic activity. Furthermore, L-HAD$_{ST}$ has no intrinsic cysteine residues, which

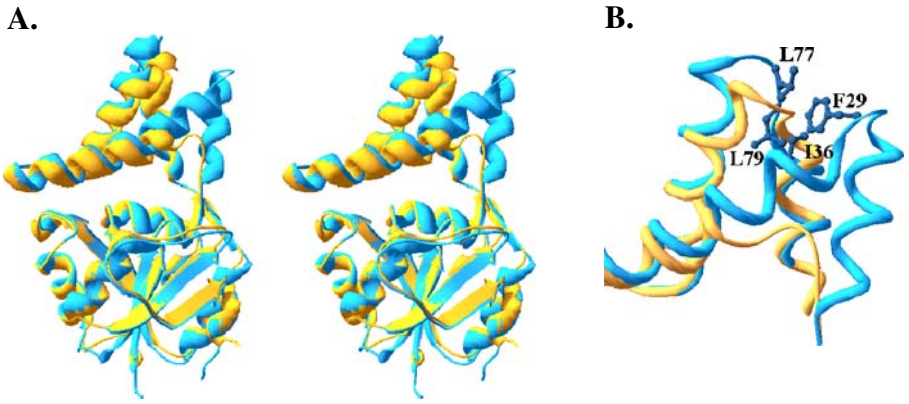


Fig. 7 Structural modeling of L-HAD$_{ST}$. **a** Stereo view of the superimposition of the template L-DEX structure (*blue* PDB ID: 1ZRN) and the calculated model structure of L-HAD$_{ST}$ (*yellow*). **b** Highlight of the two exterior loops. The colors of the ribbons are the same as in **a**. The residues involved in hydrophobic interactions are highlighted as *ball-and-stick models in dark blue*

is a convenient feature for immobilization studies. The extraordinary stability, high expression yield, and easy purification procedure make L-HAD_{ST} an excellent candidate as a model enzyme for biotechnological studies and applications. With the event of modern biotechnology and the continuous exploration of the oceans and extreme locations that were not accessible before, a myriad of new organisms and their respective protein enzymes that are capable of surviving and working in extreme conditions will be discovered. Our dehalogenase is one of such enzymes that could find broad applications in different areas of chemistry, biotechnology, and environmental engineering.

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