Zinc in lipase L1 from *Geobacillus stearothermophilus* L1 and structural implications on thermal stability

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Abstract Lipase L1 from *Geobacillus stearothermophilus* L1 contains an unusual extra domain, making a tight intramolecular interaction with the main catalytic domain through a Zn\textsuperscript{2+}-binding coordination. To elucidate the role of the Zn\textsuperscript{2+}, we disrupted the Zn\textsuperscript{2+}-binding site by mutating the zinc-ligand residues (H87A, D61A/H87A, and D61A/H81A/H87A/D238A). The activity vs. temperature profiles of the mutant enzymes showed that the disruption of the Zn\textsuperscript{2+}-binding site resulted in a notable decrease in the optimal temperature for maximal activity from 60 to 45–50 °C. The mutations also abolished the Zn\textsuperscript{2+}-induced thermal stabilization. The wild-type enzyme revealed a 34.6-fold increase in stabilization with the addition of Zn\textsuperscript{2+} at 60 °C, whereas the mutant enzymes exhibited no response to Zn\textsuperscript{2+}. Additional circular dichroism spectroscopy studies also confirmed the structural stabilizing role of Zn\textsuperscript{2+} on lipase L1 at elevated temperatures.

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1. Introduction

The lipases (EC 3.1.1.3) from thermophilic *Geobacillus* have already been classified as family I.5 among bacterial lipases based on their conserved sequence motif and biological properties [1–4]. These lipases have also attracted attention in relation to the industrial processing of lipids, as they possess a relatively high thermodynamic stability at elevated temperatures and in organic solvents [5–9].

Previously, we reported that the structure (PDB: 1KU0) of lipase L1 from *Geobacillus stearothermophilus* L1, the first reported lipase structure from a thermophilic microorganism, is composed of a main catalytic domain and an unusual extra domain [10]. The structure also shows a calcium-binding site in the main catalytic domain and a zinc-binding site in the unusual extra domain. Tyndall et al. also solved the crystal structure (PDB: 1JI3) of the *G. stearothermophilus* P1 lipase (95% identity) with very similar figures [11]. The superimposition of the main chains from these two lipases gives a root-mean-squared deviation of 0.69 Å. The two domains from both lipases tightly interact with each other through a Zn\textsuperscript{2+}-binding coordination mediated by D61 and D238 from the catalytic domain and H81 and H87 from the extra domain (Fig. 1A). Interestingly, the Zn\textsuperscript{2+}-coordinating amino acids are fully conserved in the primary structure of family I.5 lipases and family I.6 staphylococcal lipases (Fig. 1B) [10,11], indicating that the amino acids may play the same role in these lipases. In the 3D structures of lipase L1 and the *G. stearothermophilus* P1 lipase, the Zn\textsuperscript{2+} ion is about 19 Å away from the serine residue in the active site, ruling out any direct involvement of the Zn\textsuperscript{2+} ion in the enzyme catalysis. Taken together, these observations imply that the Zn\textsuperscript{2+} in lipase L1 may play a role in structural stabilization.

However, this notion of Zn\textsuperscript{2+} as a structural stabilizer has not yet been substantiated by any experimental data on lipase L1 or other lipases, even though Ca\textsuperscript{2+}-binding was shown to be important for the structural stabilization of several lipases, including lipase L1 [12,13].

Accordingly, this study investigated the role of Zn\textsuperscript{2+} in lipase L1 by mutating the zinc-ligand amino acids and demonstrated that the Zn\textsuperscript{2+} in lipase L1 conferred a conformational stabilization for enzymatic activity at elevated temperatures.

2. Materials and methods

2.1. Materials

The plasmid DNA was prepared using Qiagen miniprep kits (Qiagen, Inc.) and the PCR fragments were purified by agarose gel extraction using a QIAquick Gel Extraction Kit (Qiagen). The gel filtration PD-10 (Sephadex G-25) and GSTrap\textsuperscript{TM} HP columns were purchased from Amersham Biosciences Inc. All other chemicals used in this study were of analytical grade.

2.2. Site-specific mutagenesis

The lipase L1 gene was subcloned into the BamHI and EcoRI sites of the pGEX-4T1 expression vector (Amersham Biosciences), yielding plasmid pGSTBSL1. Oligonucleotide 5’-GCAGGATCCGCTCATCCTC-CACGGCGCAATGATGC-3’ was the forward primer, where

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*Abbreviations:* CD, circular dichroism; GST, glutathione S-transferase; HEPES, N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; TBH, tributyrin; TPEN, N,N,N’,N’-tetraakis (2-pyridyl-methyl) ethylenediamine; TX100, Triton X-100

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sequence GGATCC created a BamHI site, while oligonucleotide 5'-GCAGAATTCTTAAGGCCGCAAACTCGCCAG-3' was the reverse primer, where sequence GAATTC created an EcoRI site downstream of the gene’s transcription termination signal.

The site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis procedure (Stratagene) and the GeneAmp PE9700 thermal cycler (Perkin-Elmer) with mutagenesis primers synthesized by GenoTech Corp., Korea. The primers were used individually or in combination to introduce single, double, or quadruple mutations, respectively. Plasmid pGSTBSL1 was used as the template for the mutagenesis. The following oligonucleotides were used as the mutagenic primers: D61A, 5'-GCACGATTCGGCCGCAACTCGCCAG-3'; H81A, 5'-GCACGATTCGGCCGCAACTCGCCAG-3'; H87A, syntheses.
5’-GGGCGGCAACCGGCGGCGTGTTGCGGCGC-3’; D238A, 5’-ATCGTACGCGCGTGGATGGTCACGAG-3’. The mutations were confirmed by complete sequencing of the gene.

2.3. Protein expression and purification

Escherichia coli BL21 (DE3) (Stratagene) was transformed with the expression plasmids. The proteins were expressed in E. coli in an LB medium supplemented with 100 µg/ml ampicillin by induction with 1 mM IPTG at 30 °C for four hours. After centrifugation at 4000 × g for 20 min, the cell pellets were resuspended in a PBS buffer (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, and 2.7 mM KCl, pH 7.3). The cell homogenate was prepared by passing the cell suspension twice through a French pressure cell at 15 000 lbf/in2. After two consecutive centrifugations at 4500 × g and 10 000 × g for 10 min at 4 °C, the soluble fraction was loaded onto a GSTrap™ HP column (5 ml) pre-equilibrated with the PBS buffer at a flow rate of 1 ml/min using an AKTA™ FPLC (Amersham Biosciences). The column was washed with the PBS buffer to remove impurity proteins. And then thrombin (1 unit/100 µg of substrate protein) was injected into the column to release the lipase from the GST-tag. After incubating the column at 22 °C for 16 h, the glutathione S-transferase (GST)-free proteins were eluted with the PBS buffer, pooled and dialyzed against a N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid (HEPES) buffer (20 mM HEPES–KOH, pH 7.5) at 4 °C. The wild-type and its mutant enzymes were purified to homogeneity, as determined by an SDS-PAGE analysis, and the protein concentrations measured using a Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

2.4. Preparation of Zn2+-replenished and metal-free enzymes

The purified enzymes (100 µM, in 20 mM HEPES–KOH, pH 7.5) were incubated in 20–200 µM ZnCl2 for 30 min at 37 °C. To prepare metal-free enzymes, the purified enzyme was incubated in 2 mM N,N,N’,N’-tetras (2-pyridylmethyl) ethylenediamine (TPEN) for 4 h at 37 °C. The Zn2+-replenished and metal-free enzymes were then concentrated and extensively dialyzed against the 20 mM HEPES–KOH buffer, pH 7.5, to remove the unbound Zn2+ or excess TPEN and TPEN-Zn2+ complex. Thereafter, the enzymes were applied to a PD-10 column (Amersham Biosciences) equilibrated with a metal-free HEPES buffer (5 g of Chelex and 100 ml of 20 mM HEPES–KOH, pH 7.5, incubated for 1 h at 37 °C) before Zn2+ content and enzyme activity measurements. The metal contents in lipase L1 and its mutants were confirmed using inductively coupled plasma-atomic emission spectrometry (ICP-AES), where 1-ml samples were used for the ICP-AES analysis. The metal-free HEPES buffer was used as a control to determine the metal content.

2.5. Lipase activity assays

The lipase activity was evaluated using the pH-stat method [16]. An olive oil emulsion was prepared by emulsifying 5 ml of olive oil in 495 ml of a 20 mM NaCl, 1 mM CaCl2, and 0.5% (w/v) gum arabic solution at maximum speed for 2 min in a Waring blender. Subsequently, the pH of the substrate solution was adjusted to 8.0 using a NaOH solution. The reaction was started by adding an appropriate amount of the enzyme solution (10–50 µg of protein/mg) in the presence of either 0.1 mM ZnCl2 or 0.1 mM TPEN for 5 min at 37 °C. The reaction was monitored using a TBN-TX100 micellar

2.6. Circular dichroism spectroscopy

The circular dichroism (CD) spectra were recorded using a J-720 spectropolarimeter (JASCO, Tokyo, Japan) equipped with a thermostatically controlled cuvette based on a 0.1-cm path length at a scan rate of 100 nm/min. The thermal denaturation was monitored by following the ellipticity at 222 nm at a 1 °C/min heating rate from 30 to 80 °C. Before taking the measurements, the proteins (0.45 mg/ml) were pre-equilibrated in 20 mM HEPES (pH 7.5) at 25 °C for 20 min. The thermal denaturation of the lipase L1 was monitored by following the ellipticity at 222 nm at a 1 °C/min heating rate from 30 to 80 °C.

3. Results and discussion

3.1. Comparison of temperature profile for wild-type and mutant enzymes

A previous X-ray crystallographic study by the current authors indicated that the Zn2+ in lipase L1 is tightly bound to the interface between the catalytic and the extra domains through coordination with D61 and D238 from the catalytic domain and H81 and H87 from the extra domain, suggesting that Zn2+ may play a structural role in the enzyme conformation. Thus, to investigate the role of Zn2+ in this protein, single (H87A), double (D61A/H87A), and quadruple (D61A/H81A/H87A/D238A) mutants of lipase L1 were constructed and purified by GST-affinity chromatography, as described in Section 2. The activities of the purified proteins were assayed at temperatures ranging from 10 to 80 °C using olive oil as the substrate (Fig. 2). The wild-type enzyme showed an optimum catalytic activity at 60 °C, while the mutant enzymes were optimally active between 45 and 50 °C. As shown in Fig. 2, none of the mutations significantly affected the enzyme activity at ambient conditions, excluding the participation of Zn2+ in the enzyme catalysis. To evaluate the effect of Zn2+ on the three mutant enzymes, H87A, D61A/H87A and D61A/H81A/H87A/D238A, their activities were determined in the presence or absence of Zn2+ using a TBN-TX100 micellar
substrate. The wild-type enzyme exhibited a notable 14-fold increase in enzyme activity in the presence of Zn$^{2+}$ at 60°C, indicating a zinc-induced structural stabilization for catalytic activity at a high temperature (Table 1). In contrast, the mutant enzymes did not exhibit any significant increase in their activities in the presence of Zn$^{2+}$.

### 3.2. Role of Zn$^{2+}$ in enzyme stability

The thermostabilities of the wild-type and mutant enzymes were evaluated by incubating the enzymes at 60°C for different periods of time in the presence and absence of Zn$^{2+}$. The residual activities of the incubated enzyme solutions were measured at 37°C. The thermal inactivation kinetics for the wild-type and mutant enzymes are shown in Fig. 3, where the linearity of the data for the wild-type and mutant enzymes indicated that the thermal inactivation process for the enzymes followed first-order kinetics. The wild-type and mutant enzymes had half-lives of about 1 min in the absence of Zn$^{2+}$. The addition of Zn$^{2+}$ dramatically enhanced the half-life of the wild-type enzyme by 34.6-fold. However, the half-lives of the mutants were not significantly changed in the presence of Zn$^{2+}$. Therefore, these results strongly support the notion that the Zn$^{2+}$ in lipase L1 participates in structural stabilization for an active conformation.

### 3.3. Thermal denaturation of wild-type and mutant enzymes

CD spectroscopy was used to monitor the structural properties and thermostability of the wild-type and mutant proteins. Fig. 4 shows the CD spectra of the wild-type and mutant H87A lipases measured at various temperatures in the absence and presence of 0.1 mM ZnCl$_2$. The spectra obtained for the wild-type (Fig. 4A and C) and mutant enzyme H87A (Fig. 4B and D) were indistinguishable at 30°C in the absence and presence of zinc. The secondary structure did not appear to undergo any significant change, indicating that at an ambient temperature the structural conformation of the enzyme was not altered by the removal of Zn$^{2+}$. In the absence of Zn$^{2+}$, the wild-type and mutant enzymes behaved in similar fashion. They exhibited a fairly stable conformation up to 40°C, but showed a dramatic conformational change at 50°C (Fig. 4A and B). In contrast, the wild-type and mutant enzymes behaved differently in the presence of Zn$^{2+}$ (Fig. 4C and D). The spectra of the wild-type did not reveal any significant con-
formational change even at 60 °C in the presence of the Zn\(^{2+}\), implying that the Zn\(^{2+}\) stabilizes the enzyme conformation at high temperature (Fig. 4C). However, mutant H87A exhibited a remarkable instability at 60 °C in the presence of Zn\(^{2+}\), although it showed a slight increase in stability at 50 °C (Fig. 4D). As such, it was assumed that the mutant H87A enzyme was still able to retain a zinc-coordination facilitated by the remaining three ligands, D61, H81, and D238. The thermal transition curve obtained by plotting the unfolded fraction based on the ellipticity at 222 nm also supported the partial removal of the Zn\(^{2+}\)-binding site by the alanine substitution for H87. The addition of Zn\(^{2+}\) to the wild-type enzyme elevated the \(T_m\) value from 51.1 to 69.3 °C, indicating the importance of Zn\(^{2+}\) for maintaining structural stability at a high temperature. In contrast, the \(T_m\) values for the mutant enzymes H87A, D61A/H87A, and D61A/H81A/H87A/D238A were 54.4, 49.1, and 49.5 °C, respectively, in the presence of Zn\(^{2+}\), whereas in the absence of Zn\(^{2+}\) they were 49.3, 48.7 and 49.1 °C, respectively. These results suggest that the H87A mutation partially disrupted the Zn\(^{2+}\)-binding site, and led to reduced thermal stabilization by Zn\(^{2+}\), and that additional mutations in the Zn\(^{2+}\)-binding site completely abolished Zn\(^{2+}\)-binding and thermal stabilization by Zn\(^{2+}\) (Fig. 5).

Fig. 4. Effect of Zn\(^{2+}\) on structural conformation. The CD spectra of the (A) wild-type and (B) H87A proteins were measured at various temperatures in the absence of Zn\(^{2+}\) (0.1 mM TPEN). Panels (C) and (D) show the spectra of the wild-type and H87A proteins, respectively, in the presence of Zn\(^{2+}\) (0.1 mM ZnCl\(_2\)).

Fig. 5. Temperature-induced denaturation. The temperature-induced unfolding transitions of the wild-type (squares), H87A (circles), D61A/H87A (up triangles) and D61A/H81A/H87A/D238A (down triangles) mutant proteins were monitored by far-UV CD at 222 nm. The open and closed symbols indicate the presence of 0.1 mM TPEN and 0.1 mM ZnCl\(_2\), respectively. The data were fitted using an equation for the unfolded fraction \(f_u\), as described in Section 2.
Furthermore, the zinc content of the wild-type and H87A mutant enzymes was determined. The enzymes, to which either ZnCl₂ or TPEN was added, were extensively dialyzed against a 20 mM HEPES–KOH (pH 7.5) buffer and then applied to a PD-10 column equilibrated with a metal-free HEPES buffer. The protein solutions were analyzed by ICP-AES. The wild-type and H87A mutant enzymes had zinc contents of 1.07 ± 0.21 and 0.48 ± 0.19 mol of Zn²⁺/mol of protein, respectively, confirming that the H87A mutant possessed at least half of the zinc content. However, in the case of the zinc-free lipases, no metal ion was detectable except for a trace amount of Zn²⁺ (~0.05 mol per mol of protein).

In conclusion, the above evidences strongly suggest that the Zn²⁺ in lipase L1 plays a purely structural role to stabilize an active conformation at high temperature. Although further studies are still needed to analyze the distinct structural differences between lipase L1 and other lipase families that lack the extra domain, the results of this study provide a unique insight into the molecular mechanism of the metal-induced thermostability of lipase L1, thereby expanding current knowledge on structure–function relationships of protein families.

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References