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Zinc, a Thermostability Factor for Lipase L1 from *Geobacillus stearothermophilus* L1

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The lipases (EC 3.1.1.3) from thermophilic *Geobacillus* have been classified as family I.5 among bacterial lipases based on their conserved sequence motif and biological properties. 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. Tyndall *et al.* also solved the crystal structure (PDB: 1JI3) of *Geobacillus stearothermophilus* P1 lipase (95% identity) with very similar figures. The superimposition of the main chains from these two lipases gives a root-mean-squared deviation (RMSD) of 0.69 Å, and the two domains from both lipases tightly interact with each other through a Zn^{2+}-binding coordination mediated by D61 and D238 from the catalytic domain and H81 and H87 from the extra domain. Interestingly, the Zn^{2+}-coordinating ligand amino acids are fully conserved in the primary structure of family I.5 lipases and family I.6 staphylococcal, indicating that the amino acids may play the same role in these lipases. In the 3D structures of lipase L1 and the *Geobacillus stearothermophilus* P1 lipase, the Zn^{2+} ion is about 19 Å away from the catalytic serine residue in the active site, ruling out any direct involvement of the Zn^{2+} ion in the enzyme catalysis. Thus, from these observations, it is assumed that the Zn^{2+} in lipase L1 and P1 may play a role in structural stabilization. However, this notion of Zn^{2+} as a structural stabilizer has not yet been substantiated by any experimental data on lipase L1 or other lipases. Accordingly, to investigate the role of Zn^{2+} in lipase L1, the current study introduced mutations into the zinc-ligand amino acids, and demonstrated that the Zn^{2+} in lipase L1 conferred a conformational stabilization for enzymatic activity at elevated temperatures.

Temperature profiles for the lipolytic activity of the lipase L1 in presence and absence of metal ions were determined by the modified pH stat assay. In this case, instead of olive oil TBN-TX100 micelles was used as substrate solution.

The resulting temperature profiles are clear that the calcium dependence of lipase L1 is strongly influenced by the reaction temperature. The optimal temperature was 60 ℃ in the presence of calcium ions, whereas lipase L1 was optimally active at 50℃ in the absence of calcium ions. At 60 ℃, lipase L1 exhibited a notable increase in activity in the presence of calcium ion. Interestingly, lipase L1 exhibited similar profiles to the calcium ions in the presence of zinc ions. Zinc ions also affected enzyme activity.

Although none of the significantly altered enzyme activity by zinc and calcium at 40 ℃, the requirement for either zinc or calcium becomes virtually absolute at elevated temperatures. This observation suggests that zinc ions stabilize the active conformation of lipase L1, as well as calcium ions, in particular at higher temperatures where the enzyme is more susceptible to denaturation.

Thus, to investigate the role of Zn^{2+} in this protein, mutants of lipase L1 were constructed and purified. The activities of the purified proteins were assayed at temperatures ranging from 10 to 80 ℃ using olive oil as the substrate. While the optimal temperature for the wild-type enzyme was 60-65 ℃, most of mutant enzymes were optimally active at 45-50 ℃. The wild-type enzyme exhibited a notable 14-fold increase in enzyme activity in the presence of Zn^{2+} at 60℃, indicating a zinc-induced structural stabilization for catalytic activity at a high temperature. In contrast, the mutant enzymes did not exhibit any significant increase in their activities in the presence of Zn^{2+}.

The thermostability of the wild-type and mutant enzymes was evaluated by incubating the enzymes at 60 °C for different periods of time in the presence and absence of Zn^{2+}. 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.

CD spectroscopy was used to monitor the structural properties and thermostability of the wild-type and mutant proteins induced by 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℃, but showed a dramatic conformational change at 50℃. In contrast, the wild-type and mutant enzymes behaved differently in the presence of Zn^{2+}. The spectra of the wild-type did not reveal any significant conformational change even at 60℃ in the presence of the Zn^{2+}, implying that the Zn^{2+} stabilizes the enzyme conformation at high temperature. However, mutant H87A exhibited a remarkable instability at 60℃ in the presence of Zn^{2+}, although it showed a slight increase in stability at 50℃

The addition of Zn^{2+} to the wild-type enzyme elevated the T_m value from 51.1℃ to 69.3℃, 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 ℃, respectively, in the presence of Zn^{2+}, whereas in the absence of Zn^{2+} they were 49.3, 48.7 and 49.1℃, 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+}.

In conclusion, the above evidences strongly suggest that the Zn^{2+} 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.