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Reductive Alkylation Causes the Formation of a Molten Globule-Like Intermediate Structure in *Geobacillus zalihae* Strain T1 Thermostable Lipase

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Abstract A thermostable lipase from *Geobacillus zalihae* strain T1 was chemically modified using propionaldehyde via reductive alkylation. The targeted alkylation sites were lysines, in which T1 lipase possessed 11 residues. Far-UV circular dichroism (CD) spectra of both native and alkylated enzyme showed a similar broad minimum between 208 and 222 nm, thus suggesting a substantial amount of secondary structures in modified enzyme, as compared with the corresponding native enzyme. The hydrolytic activity of the modified enzymes dropped drastically by nearly 15-fold upon chemical modification, despite both the native and modified form showed distinctive α-helical bands at 208 and 222 nm in CD spectra, leading us to the hypothesis of formation of a molten globule (MG)-like structure. As cooperative unfolding transitions were observed, the modified lipase was distinguished from the native state, in which the former possessed a denaturation temperature (T_m) in lower temperature range at 61 °C while the latter at 68 °C. This was further supported by 8-anilino-1-naphthalenesulfonic acid (ANS) probed fluorescence which indicated higher exposure of hydrophobic residues, consequential of chemical modification. Based on matrix-

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assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis, a small number of lysine residues were confirmed to be alkylated.

Keywords Chemical modification · Reductive alkylation · Circular dichroism · Thermostable lipase · Molten globule

Introduction

Enzymes are natural catalysts made up of complex proteins, which have great impacts in our daily life. One of the most interesting and well-investigated class of enzymes are lipases (triacylglycerol hydrolases, E.C. 3.1.1.3). Lipases are unique molecules as they are activated at oil–water interfaces, termed "interfacial activation", and their activity is enhanced on insoluble lipid substrates [1]. Crystallographic studies revealed that lipases have a lid covering its active site [2]. At an oil–water interface, the lid was displaced to open by rotating around its hinge regions to expose the active site. The enzyme-substrate complex is then stabilized by a combination of electrostatic and hydrophobic interactions between the lid's hydrophobic region and the interface [3]. Nowadays, lipases stand amongst the most important biocatalysts carrying out reactions in both aqueous and nonaqueous media, offering potential applications that are literally boundless [4]. Lipases are frequently used because they catalyze various useful reactions for instance, hydrolysis, esterification, transesterification, and polyesterification reactions, and act as chiral catalysts in the production of various fine-chemicals and intermediates [5]. The diverse functions and the enzyme specificity, both stereospecificity and regiospecificity make lipase one of the most important biocatalyst in biotechnological applications [6, 7]. Due to the harsh environment in industrial processes, various efforts are being made to improve the enzymes efficiency to exploit their environmental friendly efficacy via alkylation, protein engineering, directed evolution, and immobilization [8–16]. Palomo et al. have reported a successful enantioselective alteration of porcine pancreas lipase using a combination of both immobilization and glutaraldehyde treatment [12].

As most biocatalysts are inherently labile, therefore, the stability in extreme operational conditions is of vital importance [17]. Enzymes derived from thermophilic bacteria are more resistant to denaturation than their mesophilic counterpart; thus thermostable lipases deriving from thermophilic bacteria have been receiving much more attention for their potential applications in the detergent, oil and fat, dairy, pharmaceuticals, and fine chemical industries [18]. In addition, chemical or bioprocess reactions at elevated temperatures will assist higher diffusion rate, increased solubility of substrates and other hydrophobic substrates, and reduced risk of contamination [19]. Besides that, enzymatic reactions in nonaqueous medium are also gaining interests as compared with aqueous solutions due to enhanced enzyme thermostability, efficient product recovery, and the possibility to perform reactions that are suppressed in aqueous solutions [5–7]. Despite all the advantages offered, enzymes are insoluble and often displayed low specific activities, requiring the use of large amounts of enzyme, and thus economically unfavorable [20]. A strategy to increase the solubility, activity, and stability of enzymes in organic solvents is by attaching hydrophobic molecules on the enzymes surface through chemical modification, i.e. reductive alkylation [8].

The lipase used in the present study was derived from *Geobacillus zalihae* strain T1 isolated from a palm oil mill effluent in Malaysia. T1 was selected for further studies as T1 has the highest lipase production rate, 0.15 U/ml^{-1}. The T1 lipase gene was then cloned and over-expressed in pGEX vector in a prokaryotic system [21]. Subsequent studies showed

that strain T1 was indeed a novel species and recognized as *G. zalihae* strain T1 [22]. The molecular mass of T1 lipase was determined to be approximately 43 kDa by gel-filtration chromatography. T1 lipase had an optimum temperature and pH of 70 °C and 9.0, respectively. It was also found to be stable up to 65 °C with a half-life of 5 h 15 min at pH 9.0. Similar to other lipases in the family, T1 lipase utilized serine and aspartate residues in catalysis, as its activity was strongly inhibited by 5 mM PMSF and 1 mM Pepstatin [23].

Enzymes have been modified in many ways [8–16] in order to enhance their properties in all sorts of circumstances as well as for structural study purposes [24–29]. With advancements in enzymology, manipulation of enzyme structures was seen as a new way to probe or enhance their biochemical properties. Thus, modifying a protein could change its physical structure in ways that will unfold its properties and benefit structural studies. The chemical modification of protein has some distinctive applications, as has been widely used as a tool for differentiating essential side chains in the active center, determination of buried or exposed groups, modification of non-essential groups, mainly ε-amino groups of lysine for immobilization on insoluble matrices [30], localization of individual amino acids in protein, and their participation in maintaining the protein's native conformation, conversion to molten globule (MG) structures [24] and structure-function relationships [25]. Reductive alkylation has been the study of choice as it is a well-established method for the chemical modification of amino groups in proteins and is a convenient method to convert amino groups into their respective alkylamino derivatives. It involved two separate reactions, starting with condensation of an amino group of an enzyme and a carbonyl compound (modifier) to form an imine. Neither the initial adduct nor the Schiff base formed is very stable in dilute aqueous solution but extensive modification of protein amino groups can be obtained by reduction of the Schiff base to a stable secondary amines [31]. Many simple aldehydes and ketones react rapidly and reversibly with amino groups of protein.

Several effects of alklyation on protein activity, stability, and conformation have been reported, such as changes in enantioselectivity [14], solvent- and thermostability [8, 15]. However, adverse effects too have been reported, changes in the tertiary structure of protein [26] and formation of a MG [27] have lead to decreased in activity or stability. The term "molten globule" was first used by Ohgushi and Wada to represent a new structural state of the globular protein in acid-denatured cytochrome *c* [32]. Molten globule is basically an intermediate state resembling the compact state, with significant amount of secondary structures, but disordered tertiary structure and a significant exposure of hydrophobic surface. In general, MG can be found in a wide range of structures with different degrees of compactness and residual secondary structures. Some of these forms may contain as much secondary structures as the native state but lack a tertiary order [27, 33]. In 1990, evidence from Ptitsyn et al.'s analysis of protein molecules from diverse structural types supported the concept of the "MG" state as a major intermediate of the protein folding whereas Arai et al. suggested a close similarity between the MG states observed at equilibrium under mild denaturing conditions and those formed during the early stages of protein folding [34, 35]. Thus, characterization of this intermediate state is an important step towards understanding the complexity of the protein folding mechanism [36–38]. Stable MG can be obtained under equilibrium conditions which have similar structural and energetic properties as kinetic folding intermediates, hence making them an excellent model system for understanding the fundamental principles of protein structure and function [33].

In this work, we report the effect of chemical modification via reductive alkylation on thermostable lipase, which showed the properties of a MG. Previous reports on modified enzymes showed uncertain properties, with some being successfully enhanced, while a handful were diminished. By using T1 lipase as a model, this study seeks to investigate the

effect of reductive alkylation on a thermostable lipase. Thus far, studies had only been carried out on mesophilic counterparts [8, 13–16].

Materials and Methods

Materials

Source of Enzyme

The *G. zalihae* strain T1 lipase gene was cloned and expressed by Leow et al. [21]. The thermostable T1 lipase was purified to homogeneity before chemical modification was done. All the chemicals used in the study were of analytical reagent grade.

Methods

Purification of T1 Lipase

A total of 400 ml of recombinant culture was harvested by centrifugation (10,000×*g*) and resuspended with 40 ml of phosphate buffer saline (PBS) pH 7.4, containing 5 mM of dithiothreitol (DTT) and sonicated for 2 min using Branson 250 sonifier (output, 2; duty cycle, 30). The crude cell lysate was loaded on a Glutathione Sepharose HP (Amersham, Sweden) column containing 10-ml column volume equilibrated with PBS (pH 7.4) at a flow rate of 0.25 ml/min. The column was washed with the same buffer until no protein was detected. The bound lipase was eluted with thrombin cleavage buffer (50 mM Tris–HCl, 300 mM NaCl, and 1 mM $CaCl_2$), pH 8.0 supplemented with 10 mM reduced glutathione. The fusion protein was subjected to thrombin cleavage at 16 °C for 20 h and gel filtration (buffer exchange) with Sephadex G-25 (Amersham, Sweden) at a flow rate of 1 ml/min. The cleaved glutathione S-transferase tag and thrombin enzyme were further removed by using Glutathione Sepharose HP, Glutathione Sepharose 4FF (Amersham, Sweden) and Benzamidine FF (Amersham, Sweden) in series after dialysis with 2 L of PBS (pH 7.4) at 4 °C [23].

Protein Concentration

Determination of protein concentration was done according to the Bradford method with bovine serum albumin as standard. Absorbance was read at 595 nm [39].

Lipase Activity Assay

Determination of lipase activity was assayed colorimetrically [40]. The enzyme reaction mixture contained 2.5 ml olive oil emulsion, glycine buffer pH 9.0, 20 μl of 0.02 M $CaCl_2$ and 5 μl enzyme solution. The reaction was terminated after 30 min incubation (200 rpm, 70 °C) by adding 1 ml 6 N HCl and 5 ml of isooctane, followed by mixing the reaction mixture using a vortex mixer for 30 s. The upper isooctane layer (4 ml) containing the fatty acids were drawn off to a test tube for analysis. Copper reagent (1 ml) was added and again mixed using a vortex mixer for 30 s. The reagent was prepared by adjusting the solution of 5% (w/v) copper (II) acetate-1-hydrate to pH 6.1 with pyridine. Absorbance was read at 715 nm. Lipase activity was measured by measuring the amount of free fatty acid released

from the standard curves of free fatty acids. One unit of lipase activity was defined as 1 μmole of fatty acid released per min under standard assay condition.

Chemical Modification of T1 Lipase via Reductive Alkylation

Modification of T1 lipase with propionaldehyde was carried out in 50 mM borate buffer pH 9.0, at 4 °C. Unmodified lipase (10 mg) was dissolved in 20 ml borate buffer and stirred slowly for a minute. This was followed by addition of 20 μl of propionaldehyde to the enzyme solution and the mixture was stirred slowly for another minute. Reduction of the Schiff base formed to a stable secondary amine was achieved by adding 4–5 mg of sodium cyanoborohydride ($NaCNBH_3$). The second and third steps were repeated to obtain different degrees of modifications. The solution was then dialyzed against borate buffer for 48 h. The percentage of lysine modified was determined using the 2,4,6-trinitrobenzene-sulphonic acid (TNBS) method according to Habeeb [41].

Circular Dichroism

Circular dichroism (CD) spectra were recorded using a JASCO J-810 spectropolarimeter (Tokyo, Japan) at 20 °C. Far-UV CD spectra in the 190–250 nm regions were recorded with 1.0-mm path length cuvette containing 0.33 mg/ml of the native and modified T1 lipase. Final spectra were of average of 5 scans and corrected for buffer spectrum. The percentages of protein secondary structures were estimated from the far-UV CD spectra based on Yang et al. [42].

The thermal denaturation was monitored by following the ellipticity at 222 nm from 40 to 90 °C at a 1 °C/min heating rate. The concentration of both the native and modified lipase were 1 mg/ml with the top of the 10-mm cell completely closed using a cap. Data pitch, bandwidth, and response were set at 0.1°, 1 nm, and 8 s, respectively.

Fluorescence Spectrofluorometry

Intrinsic fluorescence of the enzyme solutions were measured on a Shimadzu RF-5301 (Shimadzu, Japan). The excitation wavelength was set at 295 nm and the emission was scanned between 310 and 500 nm.

As for the extrinsic fluorescence which utilized 8-anilino-1-naphthalenesulfonic acid (ANS) as fluorescence probe, the ANS emission was scanned between 400 and 650 nm with an excitation wavelength of 350 nm. All the experiments were carried out with a protein concentration of 25 μg/ml in 50-mM borate buffer, (pH 9.0). The final concentration of ANS in the enzyme solutions was 50 μM.

In-Gel Digestion and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry Analysis

The protein bands (from 1D SDS–PAGE) stained with Brilliant Blue R-250 (Sigma-Aldrich, USA) were excised and destained with 50% acetonitrile/100 mM NH_4HCO_3 (pH 8.0). The bands were dried briefly and 2 μl (1 μl at a time, twice) of *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Promega, USA) solution (0.2 μg/μl in 50 mM acetic acid) was applied to each gel piece, followed by incubation in 50 μl of 2.5 mM NH_4HCO_3 at 37 °C for 4 h. Prior to analysis, 5 μl of digested mixture were mixed with 5 μl of matrix solution (0.02–0.03 M of alpha-cyano-4-hydroxy-cinnamic acid in

0.1% TFA/50% acetonitrile) and then subjected to matrix-assisted laser desorption (MALDI) analysis. The MALDI spectra were obtained by Ettan matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) Pro (Amersham, UK) equipped with a nitrogen laser radiating at 337 nm to obtain the peptide mass fingerprint. The theoretical masses of the peptides were calculated using ExPASy PeptideMass [43, 44].

Results and Discussion

Accessibility of Lysine Side Chain

Structural studies were initiated to gain insight into the effects of modifiers on the enzyme structure, in particular the modification sites. Based on the evaluation of the T1 crystal structure [PDB: 2DSN], T1 lipase possesses 11 lysine residues; Lys28, 84, 102, 138, 185, 207, 229, 251, 329, 344, and 345. From a total of 11 lysine residues, six residues (Lys28, 102, 207, 329, 344, and 345) were to be found at the random coil or turn, whereas, Lys84, 138, 185, 229, and 251 were positioned at the α-helix.

Reduced reactivity on the part of any group was often attributed to the positioning of the lysine residue in the structure of the protein and the exposure of active terminal amino group [28, 31, 45]. Random coil or turn structures have the most accessible residues as compared with other classes of secondary structures, α-helix, β-structures, parallel and antiparallel β-strands. However, there is segregation between the hydrophobic (I, L, V, M, W, F, and Y) and hydrophilic (K, R, N, D, Q, E, T, and S) residues. The former are 10–20% accessible and the latter are about 50%. For hydrophilic residues, there is an immense difference in solvent accessibility, i.e., lysine has 45% while serine has only 19% accessibility. Nevertheless, hydrophilic residues have an average of 35% accessibility [46]. As such, based on the analysis of the T1 lipase structure, highly exposed residues such as Lys84, 102, 138, and 251 were expected to react with modifiers with high reactivities and thus, would be successfully modified. However, some of the moderately exposed lysine residues such as Lys185, 344, and 345 may still stand a chance to be modified, depending on steric hindrance and dynamic factors [45].

Conformational Studies

Modification on lysine residues was carried out using small hydrophobic molecules, propionaldehyde via reductive alkylation method. Initial activity measurements showed the hydrolytic activity of T1 lipase has dropped drastically upon alkylation, a severe reduction of about 15-fold from its native activity of 440.57 U/ml (Table 1). Thus, the conformational features of both native and modified lipase were investigated using CD in the far-UV regions at 20 °C in 50 mM borate buffer, pH 9.0. However, both native and modified lipase showed similar distinctive α-helical bands at 208 and 222 nm (Fig. 1), thus leading to a MG-like structure hypothesis [47].

Further addition of propionaldehyde of up to 4.0% (data not shown) yielded the same results. Far-UV CD spectra revealed only slight changes in the secondary structures, yielded substantial α-helicity predictions of 22.8% and 19.4% for native and modified lipase, respectively. This was then followed by the T_m analysis of the modified lipase (Fig. 2), which showed a lower denaturation temperature, 61 °C when compared with the native lipase (68 °C). These simply imply a loosely disordered secondary structures of

Table 1 The secondary elements of native and alkylated T1 lipase with regards to their hydrolytic activity and T_m

Enzyme	Helix (%)	Beta (%)	Turn (%)	Random (%)	Lipase activity (U/ml)	T_m (°C)
Native lipase	22.8	45.0	1.7	30.5	440.57	68
Modified lipase	19.4	40.0	7.5	33.0	29.96	61

alkylation-induced state and the modified lipase was less stable than the native state. Since both states have comparable secondary structures, the lower T_m of modified lipase may be explained by loss of tertiary structure in the MG state. Tertiary structure is crucial for the stabilization of functional protein fold and a loss of them will accordingly destabilize the enzyme [48]. Similar phenomenon was also observed when chemical modification of lysine residues in glucose oxidase using citraconic anhydride were carried out [27, 33].

Intrinsic and Extrinsic Fluorescence Spectroscopy

Another approach was taken in the investigation of the reductive alkylation-induced MG structure, which involved intrinsic and extrinsic fluorescence. Figure 3 shows the intrinsic fluorescence of tryptophan residues of the modified lipases. The alteration of the microenvironment of the tryptophan residue(s) was supported by the enhanced fluorescence emission and accompanied by a red shift. These results showed that chemical modification had induced changes in the tertiary structure of the T1 lipase resulting in the exposure of tryptophan residues. X-ray small scattering studies have shown that MG acquired a range of structures, from relatively disordered structures to highly ordered structures [49]. This means reductive alkylation-induced MG is basically a semi-flexible structure and permits exposure of some hydrophobic groups in the protein structure similar to those reported by Hosseinkhani et al. [27] and Mossavarali et al. [33]. Thus, to test for a higher exposure of the hydrophobic sites after modification, ANS was used as a hydrophobic reporter group as it has a stronger affinity to the protein MG states when compared with its native state resulting in increased fluorescence intensity [50]. As expected, the alkylated lipase showed a clear enhancement of fluorescence intensity when compared with the native lipase (Fig. 4) due to the exposure of the hydrophobic interior of the enzyme.

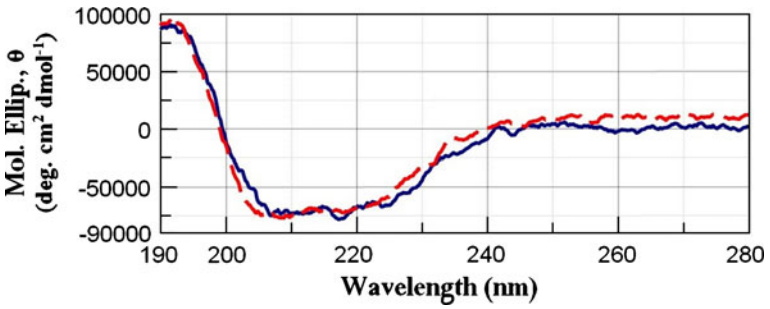

Fig. 1 Far-UV CD spectra of native and modified T1 lipase. Native (*solid line*) and modified (*dashed line*)

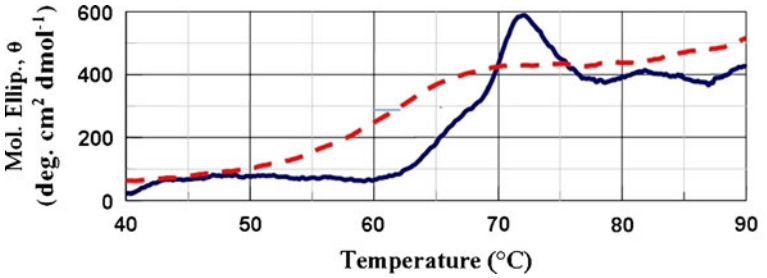

Fig. 2 Thermal transition curves obtained following the change in the CD signal at 222 nm for both native and alkylated lipase. Native (*solid line*) and modified (*dashed line*)

Detection and Localization of Modification Sites

MALDI-TOF MS was carried out to ascertain the modifications and the location of these modifications. Out of 11 lysine residues, only four lysines (Lys28, 84, 207, and 329) were clearly identified from the native spectrum (Fig. 5). There were found to originate from the 22–28, 63–84, 186–207, and 313–329 fragments of the T1 lipase, respectively. All these peptide fragments were identified based on the theoretical peptide masses and they are as good as the experimental masses. Lys84 which originated from the 63–84 fragment had a mass increase of 40.5 amu from its counterpart in the unmodified lipase due to the addition of a propionaldehyde to the lysine residue and the subsequent loss of a hydroxyl group (Fig. 6). Two other modified peptides found in the fragment of tryptic-digested alkylated lipase recorded a mass of 1,329.8 and 830.4 amu, respectively. These peptides were found to originate from the 93–102 and the 180–185 fragments of the T1 lipase and represented a modified site of Lys102 and Lys185, respectively. The same peptide fragments carrying Lys102 and Lys185 were also found in the unmodified state. These residues were partially modified due to the fact that the ε-amino group of Lys102 formed a hydrogen bond distance of 3.45 Å with a carboxyl group from the neighboring Val71, while the Lys185 formed two salt bridges with Asp182 (3.94 Å) and Asp222 (2.84 Å), which were all well within the

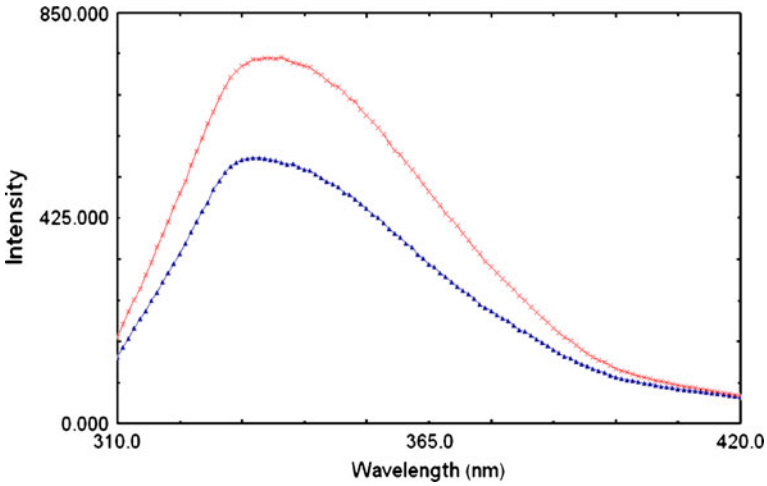

Fig. 3 Intrinsic fluorescence of native and alkylated T1 lipase. Native (*triangles*) and modified (*error marks*)

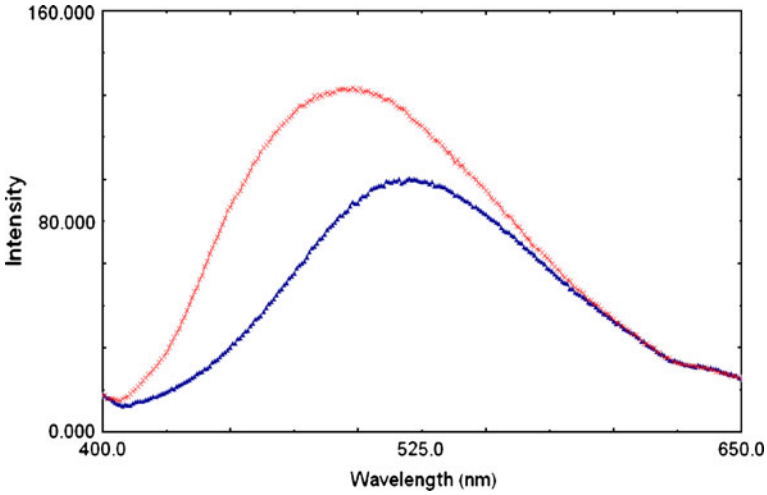

Fig. 4 Extrinsic fluorescence of native and alkylated T1 lipase in the presence of 50 μM of ANS probe. Native (*triangles*) and modified (*error marks*)

4.0 Å distance vicinity (Fig. 7) [51, 52]. Subsequently, it would be extremely resistant to modification due to the unavailability of the lone electron pair from ε-amino, which is essential in the reductive alkylation process and thus permitted only partial alkylation as encountered by Fujita et al. [16]. Scores of other non-modified lysines, i.e., Lys28, 207, and 329 were also detected in the spectra.

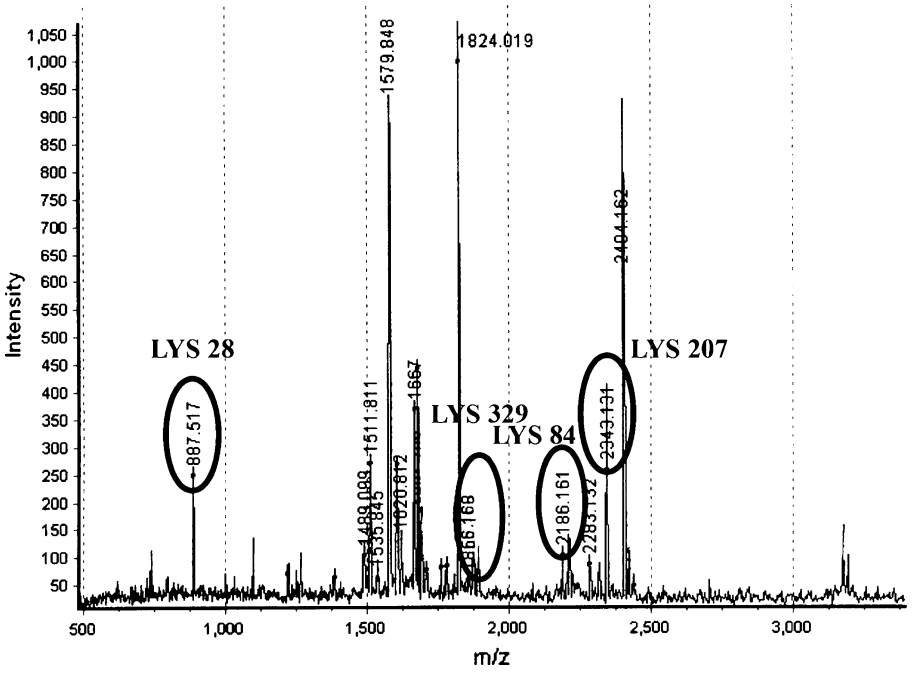

Fig. 5 MALDI-TOF mass spectrum for a tryptic digest of native T1 lipase. The identified unmodified peptide fragments (enclosed in *circles*)

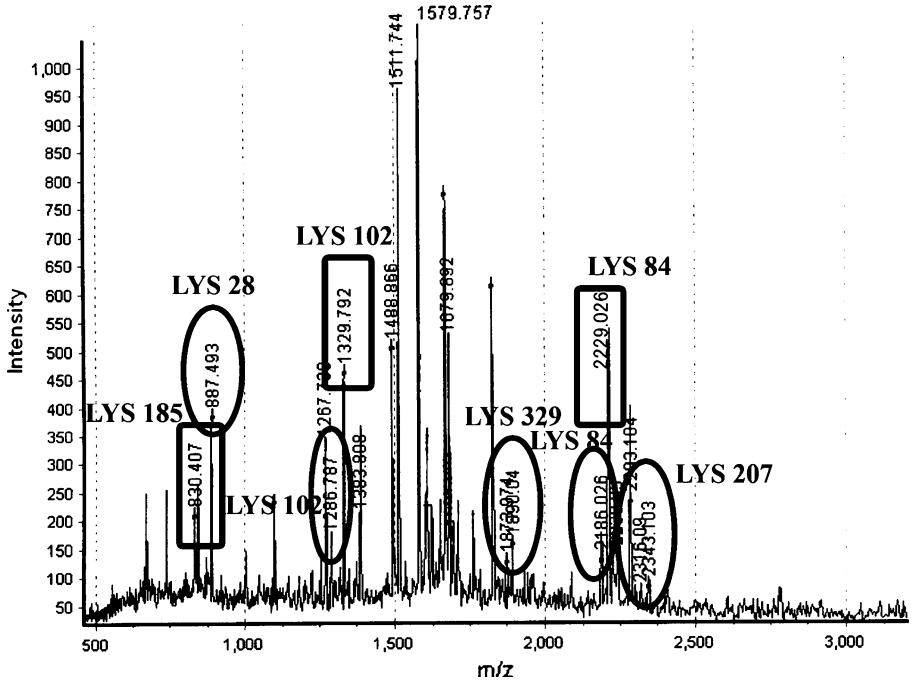

Fig. 6 MALDI-TOF mass spectrum for a tryptic digest of alkylated T1 lipase. The identified unmodified peptide fragments (enclosed in *circles*) and modified peptide fragments (enclosed in a *rectangle*)

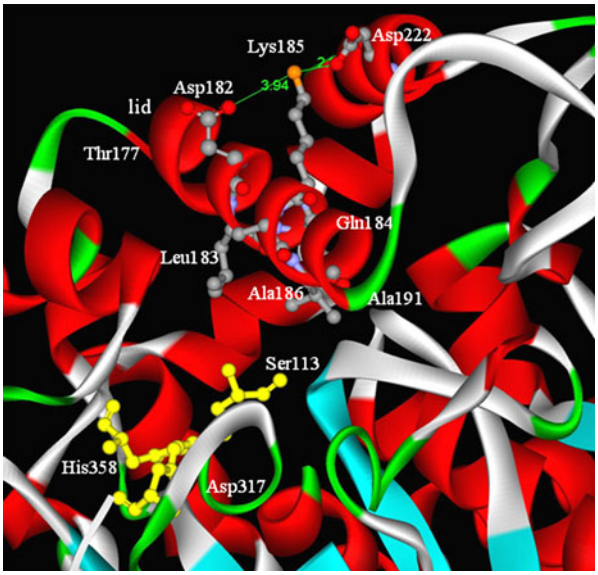

Fig. 7 Local milieu surrounding Lys185. The lid of T1 lipase stretching from Thr177 to Ala191 is located well above the catalytic triad (*yellow*). The ε-amino group lysine, amino group, and oxygen atom are shown in *orange*, *blue*, and *red*, respectively

Metal ions may be involved in enzyme catalysis or stabilization. High-temperature stability and structural stabilization of enzymes have always been associated with Zn^{2+} and Ca^{2+} metal ions, and they were well conserved among thermostable lipases [53, 54]. The Zn^{2+} metal ion in T1 lipase was coordinated in a tetrahedral environment through the Zn^{2+}-binding domain, which involved His81, His87, Asp61, and Asp238. The Zn^{2+} which was 19.27 Å away from the active center serine, suggested it did not contribute to enzyme catalysis but might probably be involved in maintaining structural stability at high temperature [55]. From the CD spectra results of both native and modified T1 lipase, it was clear that most of the secondary structures were maintained but there was a slight loss in the α-helix structure and reduction in thermostability. Given the close distance between Lys84 and the Zn^{2+}-binding domain, alkylation on the lysine could have disrupted local hydrogen bonds between the oxygen atom of His81 with the nitrogen atom and imidazole side chain of His85. As attachment of an alkyl chain on the lysine could also decreases the possibility of creating intramolecular interactions between lysine and its neighboring residues [56], alkylation at Lys84 could result in the destabilization of the secondary structure of α3 (Gly78 to His87), a short α-helix which included both His81 and His87 from the Zn^{2+}-binding domain. Through site-directed mutation, Choi et al. [55] have demonstrated that the disruption of the Zn^{2+}-binding site resulted in a notable decrease in the optimal temperature for maximal activity from 60 to 45–50 °C. Apart from thermostability, Zn^{2+} metal ion was also found to be an important factor in maintaining the stability of the secondary structures of protein, and thus helps maintain protein stability and functions [57].

Modification at Lys185 proved to be damaging given the significance of the lid as structural basis of interfacial activation in lipases. Modification of Lys185 could have disrupted local hydrogen networks and reduced its ability to form intramolecular interactions, which are crucial during the interfacial activation [56, 58]. On top of that, modification at this point also disrupted the $i+3$ interactions, a crucial hydrogen bond between Arg182 and Lys185 in maintaining the helical structure. Although the enzyme's lid (Thr177 to Ala191), being the most dynamic part of the enzyme, could undergo a significant conformational change during the lid movement to facilitate the stabilization of enzyme-substrate intermediate, most of the helical structure was maintained for efficient substrate binding. Thus, an alkylated Lys185 would have prevented the lid to switch from the closed to the open form [28, 45]. Through molecular dynamics (MD), James et al. [58] noticed significant changes in the lid conformation of *Candida rugosa* lipase to cater different hydrophobic substrates during the catalytic process. Further analysis showed that Lys75 formed four hydrogen bonds to help maintain its lid from adopting an over-extended conformation [58]. Therefore, modification at the lid should be prevented to preserve its secondary helix structure.

Though Lys102 was located far away from the catalytic triad, it could have a rather global effect rather than a confined area. As demonstrated by Tejo [59], residues which were affected upon alkylation were mostly located far away from the alkylated lysine. Some of them were even located near the binding pocket which had no direct contact with the alkylated, surface exposed lysine. Through MD in carbon tetrachloride, Gly124 and Phe344 (residues in acyl-binding tunnel) were found to be more rigid upon the alkylation of lysines on the lid [59]. Oelschlaeger et al. [60] proposed a "domino-effect" mechanism in metallo-β-lactamases to explain the remote effect of a mutated residue on another distant residue(s). In metallo-β-lactamase IMP-6, Gly196 is not catalytically active but adjacent to His197, a zinc ligand critical for β-lactam hydrolysis. Gly196Ser mutation created an interaction between hydroxyl group of Ser196 with the side chain nitrogen of Lys33. The carbonyl

oxygen of Pro32, the direct neighbor of Lys33, tightly increased with Nδ of His197 at a distance of 2.8 Å. The flexibility of His197 was dependent on Lys33 and Pro32, thus, removal of the hydroxyl group of Ser196 would increase the flexibility of His197 through Lys33 and Pro32 [60].

Formation of a MG-Like Structure

The CD and hydrolytic activity analyses indicated that the chemically alkylated T1 had a modest change in its tertiary structure compared with the native state, which led to the theory of a MG-like structure formation as was shown by the diminishing activity while upholding a considerable amount of secondary structures (Table 1) and a noteworthy exposure of hydrophobic surface. The interior side chains may be mobile, more closely resembling a liquid than the solid-like interior of the native state. As α-helices and β-sheets are major structural element found in globular proteins, these core elements are held together when all the NH and C=O groups are joined with hydrogen bonds to provide a rigid and stable framework. Thus, decreasing of helical and β-sheet structures due to the diminishing of ionic bridges and hydrogen bonds contributed to the instability of the tertiary structure resulting in decreased catalytic activity [48].

It is clear that reductive alkylation is a simple and rapid method to manipulate an enzyme native structure. It has been widely employed to alter native properties, in order to fit into today's demanding needs. It has a high potential for stabilizing proteins [8, 12, 14, 15]. The higher exposure of hydrophobic residues due to chemical modification had been found to be useful for improving the interaction between the enzyme and the hydrophobic support [30]. Chemical modification on immobilized enzyme has also been found to be useful in promoting enzyme stability by multipoint cross-linking between the enzyme and the support [12]. As much as chemical modification could improve enzymes ability to work in non-native environment it also could diminish its catalytic activity, bringing unanticipated changes to the structure of protein which is a distinct phase on its own, a molten globule. In general, thermostable enzyme is believed to have a very compact and rigid structure to counter the harsh and extreme environments. However, the findings here pointed out that the rigid and compact structure of thermostable enzyme is sensitive to changes carried on its native structure via reductive alkylation. The effect of alkylation is not localized at the modified lysine but it has been passed on to other residues which are not modified or even far away from modified residues.

In moving forward with the current knowledge, this technique could bring us back to the very basics of protein structure and function to rediscover our crucial fundamental understanding of proteins. Many de novo designed proteins have turned out to be molten globules, which required substantial amount of time and efforts to convert into native-like protein molecules [61]. Many if not most of the enzymes could possibly have evolved from molten globules [62]. Thus, chemical modification could be used as a powerful tool for the understanding of protein structures and functions or foldings. Further studies of the molten globule-like state through X-ray crystallography method would be helpful to probe the stability of thermostable enzyme and its "vulnerable" points. As we already have the high resolution crystal structure of native T1 lipase [63], a direct comparison could be made to evaluate the effect of alkylation on the structure of T1 lipase. By applying random mutagenesis and powerful screening methods, it may be possible to substitute these residues, to produce enzymes with tailor-made properties, where molten globules could well be the starting point of tailored biocatalysts.

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