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A combination of site-directed mutagenesis and chemical modification to improve diastereopreference of *Pseudomonas alcaligenes* lipase

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ABSTRACT

A combination of site-directed mutagenesis and chemical modification was employed to alter protein structure with the objective of improving diastereopreference over that achieved by simple site-directed mutagenesis. Conformational analysis using molecular dynamic (MD) simulation of *Pseudomonas alcaligenes* lipase (PAL) indicated that stronger steric exclusion and structural rigidity facilitated diastereopreference. A cysteine (Cys) residue was introduced using site-directed mutagenesis to construct variant A272C. The modifier 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was then reacted with the introduced Cys residue to provide stronger steric exclusion and structural rigidity. The modification was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Diastereopreference was improved significantly. The diastereomeric excess (de_p) of L-menthol increased from 35% with wild type PAL to 90% with A272C-DTNB modified PAL when the conversion ratio of L-menthyl propionate was nearly 100%. Conformation and kinetic parameter analysis showed that A272C-DTNB modified PAL exhibited stronger steric exclusion and increased structural rigidity around the modification site that inhibited the hydrolysis of non-targeted substrates. The combination of site-directed mutagenesis and chemical modification could be an effective method to alter protein properties and enhance diastereopreference through the combined effect of steric exclusion and structural rigidity.

1. Introduction

Lipases (triacylglycerol hydrolases, EC3.1.1.3) can be employed in chiral resolution to yield optically pure chemicals [1]. Optically pure compounds are important to chemical industries as they are needed in pharmaceuticals, agrochemicals, flavors and fragrances [2,3]. Lipase biocatalysis is a cost-effective and environmentally friendly method for chiral resolution of a racemic mixture [4]. When the target compound is not the natural substrate for the lipase, diastereopreference is usually not optimal [5]. To improve the optical purity of products, lipase requires structural modification.

Site-directed mutagenesis is widely used to modify the structure of lipases and other biocatalysts to improve their catalytic properties [6–10]. DNA-based techniques are limited to the exchange of one residue to the other from 19 natural ones. In the pre-genomic period, the chemical modification of proteins was studied as an approach to identify key residues [11–13], to alter substrate preference and specificity [12,14], and to improve stability and resistance to proteases [15–17]. Chemical modifications have more scope than site-directed amino acid changes. The principal disadvantage of chemical modification is lack of control. Uncertainty around the extent and exact location of the modifications makes it difficult to attribute changes in protein function [18].

Genomics and proteomics have allowed for novel methods that combine mutational and chemical modifications to alter protein structure and function [19]. The combination overcomes the disadvantages of both site-directed mutagenesis and chemical modification and can yield well-characterized homogeneous products [18,20]. As an effective method of structural modification, it sheds light on protein functions and provides insights into understanding the molecular mechanism of enzymes [19,21–25]. For previous studies, some of them fall short of full characterization of structural modifications and the mechanisms behind consequential alterations in protein function.

We previously proposed a diastereomeric recognition mechanism based on the combined effect of steric exclusion and structural rigidity in the resolution of racemic menthyl propionate (a molecule that has three chiral centers and eight isomers, Fig. 1) hydrolyzed by *Pseudomonas alcaligenes* lipase (PAL) to produce L-menthol (Scheme 1). Stronger steric exclusion and structural rigidity around the active site restricted the hydrolysis of a non-target substrate (L-neomenthyl propionate and D-isoneomenthyl propionate) and improved diastereopreference. Since Ala272 of wild type (WT) PAL was found in the most flexible region of complexes of PAL with non-target substrates, this site was identified as a hot-spot for the improvement of diastereopreference. Variant A272F was constructed and diastereopreference was improved significantly compared with WT PAL. To overcome the limitation of DNA-based technology and further improve diastereopreference, the strategy

Abbreviations: PAL, *Pseudomonas alcaligenes* lipase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); MALDI-TOF, matrix assisted laser desorption/ionization time of flight; WT, wide type; MD, molecular dynamic

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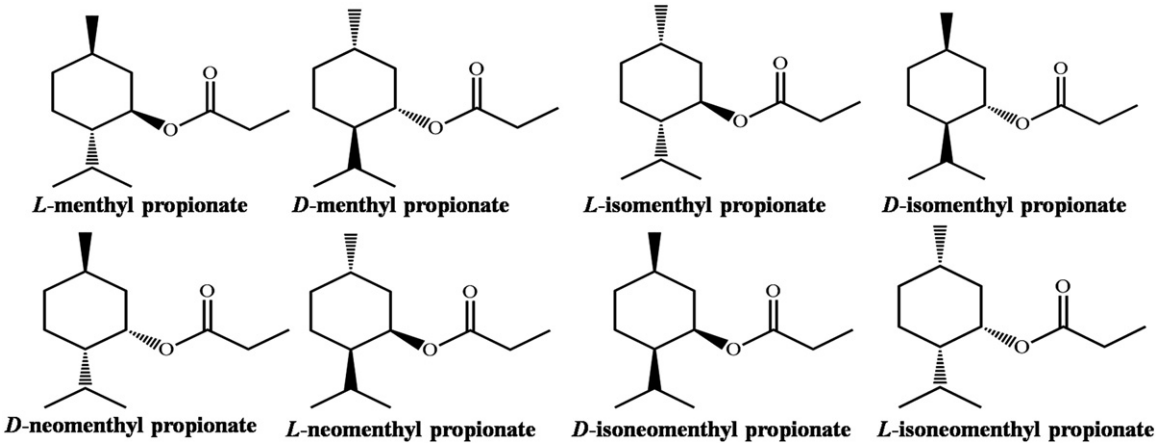


Fig. 1. The configuration of 8 isomers of menthyl propionate.

of combining site-directed mutagenesis and chemical modification was employed. Thiol groups provided by cysteines are particularly amenable to chemical modification [25]. The strategy involved the introduction of a single cysteine residue into the hot spot via site-directed mutagenesis. This was then covalently linked with DTNB to give chemically modified mutant lipase (Scheme 2). Through the combination method, the active site of modified PAL could exhibit stronger steric exclusion and structural rigidity than variant A272F, thus improving diastereopreference.

2. Materials and methods

2.1. Bacterial strains, plasmid, enzyme and reagents

Escherichia coli DH5α was used for gene cloning and sequencing. *E. coli* BL21 (DE3) and vector pET-30a(+) were used for gene expression. Pfu DNA polymerase and digestive enzyme Dpn1 were purchased from Takara Bio Inc.

DTNB was purchased from Sigma-Aldrich. Yeast extract and tryptone were purchased from Bio Basic Inc. Racemic menthyl propionate was synthesized in our own lab [26] (content proportions of isomenthyl propionate, neomenthyl propionate, menthyl propionate and isoneomenthyl propionate are 10%, 38%, 50%, and 2% respectively). Other chemical reagents were of analytical grade.

2.2. Site-directed mutagenesis

Substitution mutagenesis was performed using the QuikChange® site-directed mutagenesis method described by Fisher and Pei [27]. Two pairs of complementary primers were designed as follows: A272F primer F: 5′ CGCCACGGTGACCACTTCCTCAACGCCGCGCTC 3′, A272F primer R: 5′ GCGGTGCCACTGGTGAAGGCGTTGCTTCGCGAG 3′ and A272C primer F: 5′ CGCCACGGTGACCACTGTCTCAACGCCGCGCTC 3′, A272C primer R: 5′ GCGGTGCCACTGGTGACAGCGTTGCTTCGCGAG 3′.

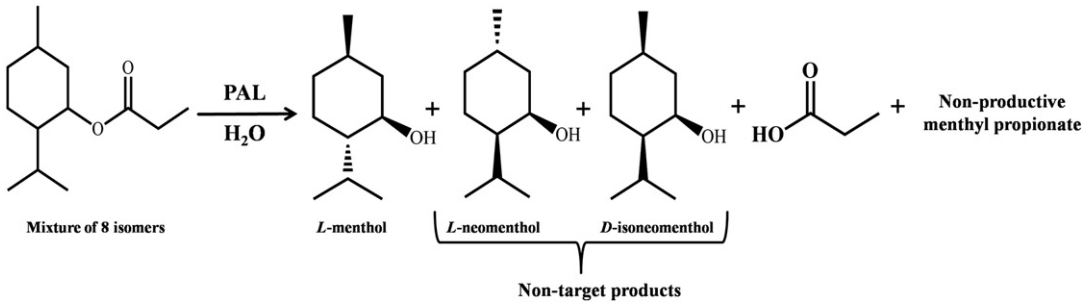
After amplification, the products were treated with Dpn1 in order to digest native parental plasmids.

2.3. Protein expression and purification

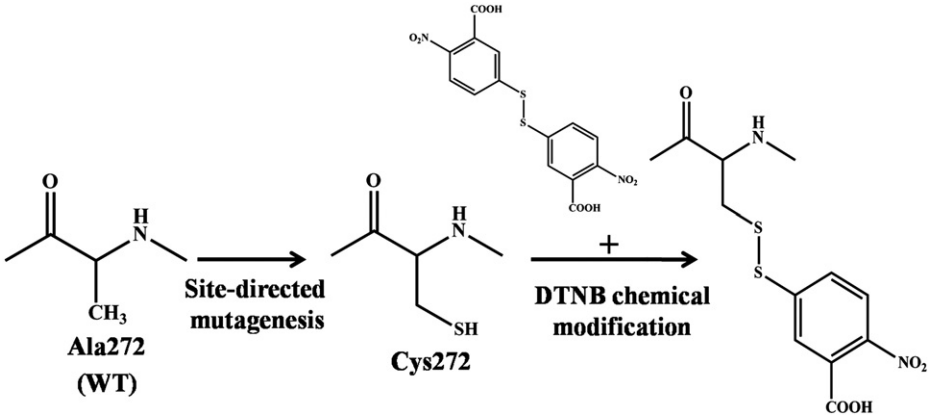
The digestion products were purified using a clean-up kit to remove the redundant primers, then, the products were transformed to *E. coli* BL21 (DE3) component cell by chemical method for each mutation at 42 °C for expression respectively. The *E. coli* BL21 (DE3) transformants grew overnight in Luria–Bertani (LB) medium containing 50 μg/mL Kanamycin at 37 °C. Cultured cells were induced by 0.5 mM IPTG at OD_{600} = 0.8 for 10 h at 18 °C. After induction, 100 mL volumes of cell culture were collected by centrifugation and resuspended in 20 mL Glycine (Gly)–NaOH buffer (50 mM, pH = 9.0), before undergoing ultrasonic cell fragmentation. As pET-30a(+) coding the 6×His tag, an affinity chromatograph of HisTrap™ FF column was employed to purify PAL. The concentrations of imidazole in the binding buffer and in the elution buffer were 50 mM and 250 mM respectively.

2.4. Chemical modification of variant A272C

The formation of disulfides on a protein can be accomplished by disulfide exchange, an effective protein modification method [28]. As the variant A272C has only one Cys residue, the modification reagent DTNB can be bonded to Cys residue specifically following the method described by Tsuneyuki [29].



Scheme 1. Resolution of racemic menthyl propionate to produce L-menthol catalyzed by PAL.



Scheme 2. The scheme of site-directed mutagenesis and chemical modification of PAL.

2.5. MALDI-TOF analysis of modified PAL

After purification, the variant A272C and A272C-DTNB modified PAL were dissolved in 75 μL of denaturing buffer (6 M guanidine hydrochloride, 0.5 M Tris hydrochloride, 2 mM EDTA, pH 8.0) and incubated at 37 °C for 30 min. The denatured protein solution was diluted 1:1 (v/v) with 100 mM ammonium hydrogen carbonate (pH = 7.8) and 20 ng of trypsin was added. Digestion was performed at 37 °C overnight. Finally, the sample was dried using a vacuum concentrator centrifuge and dissolved in 20 μL of acetonitrile with 0.1% TFA (1:9, v/v) for subsequent mass spectrometric analysis [30]. α-Cyano-4-hydroxycinnamic acid was used as the matrix and ions were extracted into the linear TOF spectrometer using an extraction potential of 20 kV in high-mass detection mode [31]. Peptide mass fingerprinting data was also employed to search in the NCBI non-redundant protein databases using Mascot software from Matrix Science®.

2.6. Circular dichroism (CD) measurements

CD spectra were recorded on a JASCO® J-715 spectropolarimeter. The far-UV and near-UV CD spectra were taken at a protein concentration of 0.2 mg/mL and 0.4 mg/mL in Gly–NaOH buffer (50 mM, pH = 9.0) using a 1-mm path length cell. All spectra were collected from 190 nm to 240 nm for far-UV spectra and 250 nm to 330 nm for near-UV CD spectra. The background noise was corrected against Gly–NaOH buffer (50 mM, pH = 9.0) blank. The spectra of protein samples were subtracted by blanks. Molecular ellipticity was determined as $[\theta]_\lambda = [100 \times (\mathrm{MRW}) \times \theta_{obs} / (c \times l)]$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/mL and l is the length of the light path in cm. Noise in the data was smoothed using JASCO® J-715 software, including the fast Fourier transform noise reduction routine that allows enhancement of most noisy spectra without distorting their peak shapes [32].

2.7. Hydrolysis of racemic menthyl propionate

Hydrolysis was carried out in a 20-mL screw-capped tube containing 1880 μL enzyme (about 2 U enzyme activity) solution, 100 μL DMSO and 20 μL substrate (50 mM). The reaction was realized at 37 °C on a thermostatic rotator set at 200 rpm. The substrate and product were extracted from the reaction mixture with hexane, dried with Na_2SO_4 and then analyzed by gas chromatography (GC).

2.8. GC analysis

The samples extracted from the hydrolysis reaction were injected into an Agilent® 6890 GC instrument (Agilent Inc.) equipped with a CP-cyclodextrin-β-2,3,6-M-19, FS 50 m × 0.25 mm column of 0.25 μm film thickness (Varian Inc.) and a flame-ionization detector for analysis. N_2 was used as the carrier gas. The column, injection and detector temperatures were 130, 260 and 250 °C respectively.

Diastereomeric excess of L-menthol (de_p) was calculated as defined below, where [Lmenthol] and [Diastereomer] were the concentrations of L-menthol and its diastereomer (L-neomenthol and D-isoneomenthol) respectively. *n*-Dodecane was used as an internal standard to determine the concentration.

$$de_p = \frac{[\mathrm{Lmenthol}] - [\mathrm{Diastereomer}]}{[\mathrm{Lmenthol}] + [\mathrm{Diastereomer}]}$$

2.9. Determination of kinetic parameters of WT and A272C-DTNB modified PAL

The kinetic parameters K_m and K_{cat} were determined for PAL activity. Enzyme assay with 2 mL of free PAL was performed in Gly–NaOH buffer, pH 9.0 with racemic menthyl propionate concentrations from 5.0 mM to 75 mM. The data obtained were plotted according to the method of Lineweaver–Burk to determine K_m and K_{cat} graphically.

2.10. Model building

The starting three-dimensional model of PAL was constructed using the APM module of the program Sybyl-X 1.1 (Tripos®). This module employs the fold recognition method, which is based on the observation that the number of possible folds is limited. The X-ray crystallographic structure of cocaine esterase (PDB ID: 1JU3) was used as a template.

2.11. Covalent docking of substrate and molecular dynamic (MD) simulation

The tetrahedral intermediates of L-menthyl propionate, L-neomenthyl propionate and D-isoneomenthyl propionate were generated using Sybyl-X 1.1. Starting with the initial protein and substrate structure, the tetrahedral conformation of the substrate covalently bound to the catalytic serine (Ser133) was elucidated using the covalent docking program FlexX®. All parameters were set to the standard values.

All MD calculations were carried out using the Amber11® program and the ff99SB force field parameters. The simulation was carried out over 20 ns at constant temperature and pressure using the Berendsen algorithm with a coupling constant of 2 ps for both parameters. Electrostatic interaction was calculated using the Particle Mesh Ewald method with a non-bonded cutoff of 12 Å. The trajectories were studied over 20 ns and conformations of system were recorded every 2 ps for further analysis. Trajectory analysis was carried out using the PTRAJ module of

Amber11 package. A mass/weight average value was calculated for every residue. These parameters were related to the B-factors, which were calculated using the coordinates of 20 ns trajectories.

3. Results

3.1. Construction, expression, and purification of WT and mutant PAL

Substitution mutagenesis of A272F and A272C were performed by the QuikChange® site-directed mutagenesis method. After transformation, mutagenesis was confirmed by sequencing. To further purify and characterize native and mutant forms, PAL was overexpressed in the *E. coli* BL21 (DE3) host, and the purification of His_6-tagged fusion PAL was also performed by affinity (Ni-NTA Sepharose) chromatography.

The purified WT and mutant PAL had purities of >95% as per SDS-PAGE analysis in which PAL was present as a band between 40 and 60 kDa. MALDI-TOF mass spectroscopy determined the accurate molecular weight to be 58,094.3 Da.

3.2. The diastereopreference of WT, mutant and A272C-DTNB modified PAL

The de_p of L-menthol, hydrolyzed by WT, mutants and A272C-DTNB modified PAL was detected following different conversion ratios. As shown in Fig. 2, when the conversion ratio of L-menthyl propionate reached nearly 100%, the de_p of L-menthol hydrolyzed by WT PAL was 35%. For the variant A272F, the de_p of L-menthol increased to 54%. To confirm the effect of chemical modification, the de_p of L-menthol hydrolyzed by both A272C and A272C-DTNB modified PAL was determined. For the variant A272C, the de_p was 68%. After modification with DTNB, the diastereopreference improved significantly compared with WT and variant A272C. The de_p increased to 90% with A272C-DTNB modified PAL when the conversion ratio of L-menthyl propionate reached nearly 100%.

3.3. Kinetic properties

The kinetic parameters of WT and A272C-DTNB modified PAL are listed in Table 1. The K_m value of L-menthyl propionate for A272C-DTNB modified PAL (0.74 mM) was similar to that for the WT (0.63 mM). However, the K_m values of L-neomenthyl propionate and D-isoneomenthyl propionate for both WT and A272C-DTNB modified PAL were significantly different. The K_m value of L-neomenthol propionate for WT PAL was 1.57 mM. For the A272C-DTNB modified PAL, that increased to 15.29 mM. A significant difference in the K_m values of D-isoneomenthyl propionate was also observed. For WT PAL, it was 2.35 mM. That of A272C-DTNB modified PAL increased to 9.17 mM.

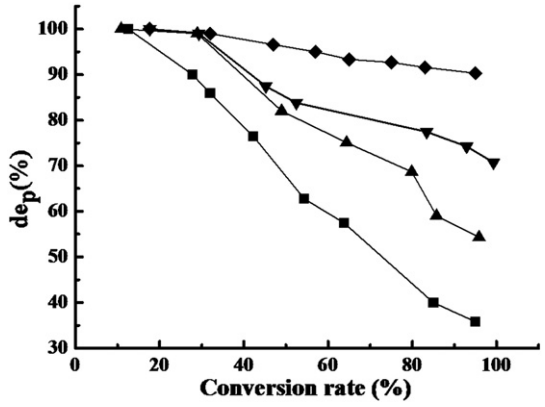


Fig. 2. The relationship between de_p and conversion ratio of L-menthyl propionate obtained from WT PAL (■), variant A272F (▲), variant A272C (▼), and A272C-DTNB modified PAL (♦).

Table 1
The Michaelis–Menten parameters for the PAL-catalyzed hydrolysis of L-menthyl propionate, L-neomenthyl propionate and D-isoneomenthyl propionate.

Substrate	WT PAL			A272C-DTNB modified PAL		
	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m ($mM^{-1}S^{-1}$)	K_m (mM)	K_{cat} (S^{-1})	K_{cat}/K_m ($mM^{-1}S^{-1}$)
L-menthyl propionate	0.63	7.63	12.11	0.74	0.26	0.35
L-neomenthyl propionate	1.57	0.26	1.66	15.29	1.13×10^{-2}	0.74×10^{-3}
D-isoneomenthyl propionate	2.53	0.83	0.36	9.17	0.19×10^{-1}	2.07×10^{-3}

The catalytic efficiency parameter of K_{cat}/K_m values of L-menthyl propionate, L-neomenthyl propionate and D-isoneomenthyl propionate exhibited a downward trend after DTNB-modification. For the A272C-DTNB modified PAL, the K_{cat}/K_m value of L-menthyl propionate was 35-folds lower than that of WT PAL. The K_{cat}/K_m values of L-neomenthyl propionate and D-isoneomenthyl propionate for A272C-DTNB modified PAL were 2243-folds and 174-folds lower compared to that of WT PAL respectively.

3.4. Characterization of variant A272C and A272C-DTNB modified PAL by MALDI-TOF mass spectroscopy

To verify that the Cys residue was reactive towards DTNB, variant A272C and A272C-DTNB modified PAL samples were treated with trypsin before undergoing peptide mass fingerprinting using MALDI-TOF mass spectrometry. As expected, the peptide fragment of residues 268–299, which contains the mutation site Cys272 (HGDHCLNEALGALGIPNEVIDQVGDWFDHYLK) was detected as a fragment of 3624.9 Da following the tryptic digestion of variant A272C. Additionally, a mass shift of 234 Da (corresponding to the weight of the fragment of DTNB covalently bonded to Cys and K^+) for this peptide fragment of A272C-DTNB modified PAL was detected at 3859.1 Da (Fig. 3). These data confirmed that DTNB was attached to the Cys residue. In addition, the peptide fragment at 3624.9 Da was almost insignificant, indicating that the degree of modification was sufficiently high.

3.5. Circular dichroism (CD) measurements

The far-UV CD spectra of WT, variant A272C and A272C-DNTB modified PAL are presented in Fig. 4A. The spectra were typical for α/β fold hydrolases, which comprise mainly α-helix and β-sheet protein. The CD spectra in the far-UV region obtained for WT, A272C and A272C-DTNB modified PAL did not exhibit obvious differences. The results indicated that the secondary structure did not change significantly among the three PALs. The near-UV CD spectra of WT, variant A272C and A272C-DNTB modified PAL are presented in Fig. 4B. To be different to some former researches [33,34], the mutation and introduction of disulfide bond did not change the CD spectra in near-UV region obviously, which indicated that the tertiary structure also did not change significantly among the three PALs.

3.6. MD study

MD simulation provided structural information relating to the interaction between A272C-DTNB modified PAL and the three substrates, L-menthyl propionate, L-neomenthyl propionate and D-isoneomenthyl propionate. A272C-DTNB modified PAL, in which the modified Cys residue faces the C4 stereocenter (Fig. 5D–F), displayed much stronger steric exclusion than the WT enzyme.

B-factor can provide information about local flexibility. Higher B-factor indicates higher structural flexibility [35]. To investigate changes

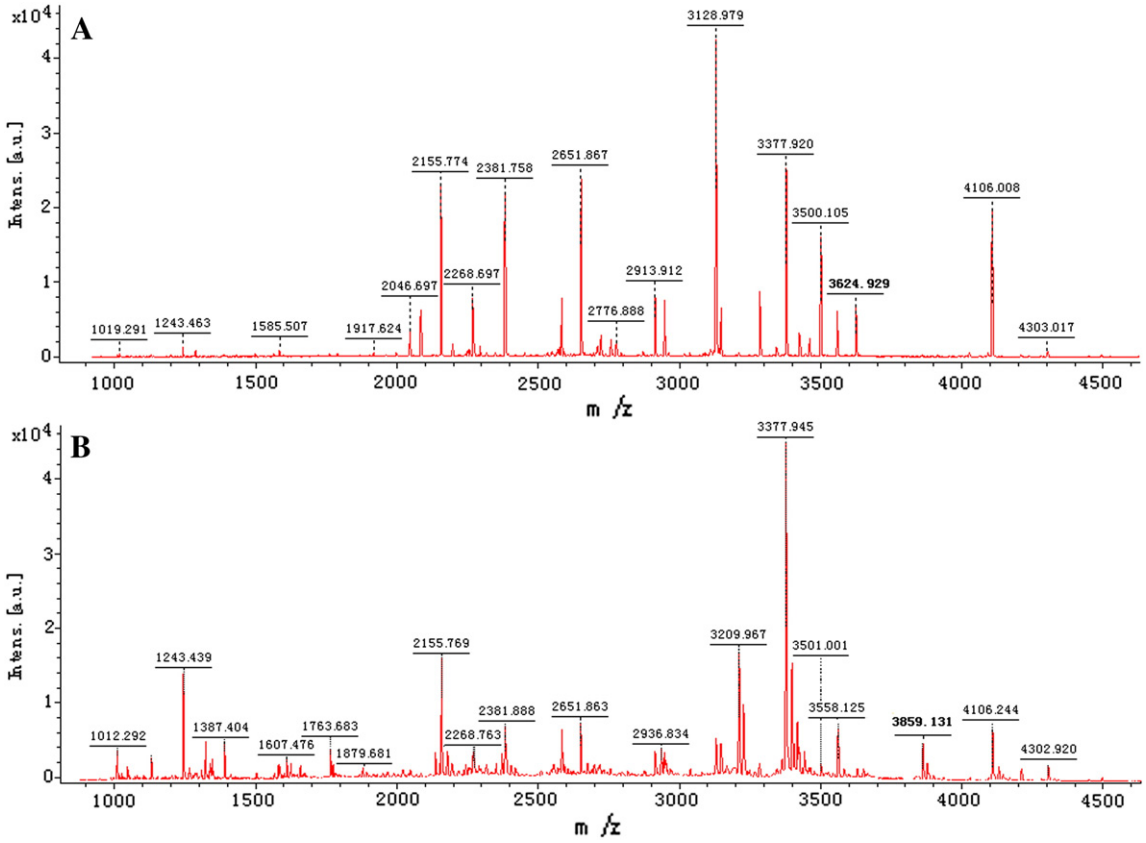


Fig. 3. MALDI-TOF mass fingerprint obtained by tryptic digestion of (A) variant A272C and (B) A272C-DTNB modified PAL.

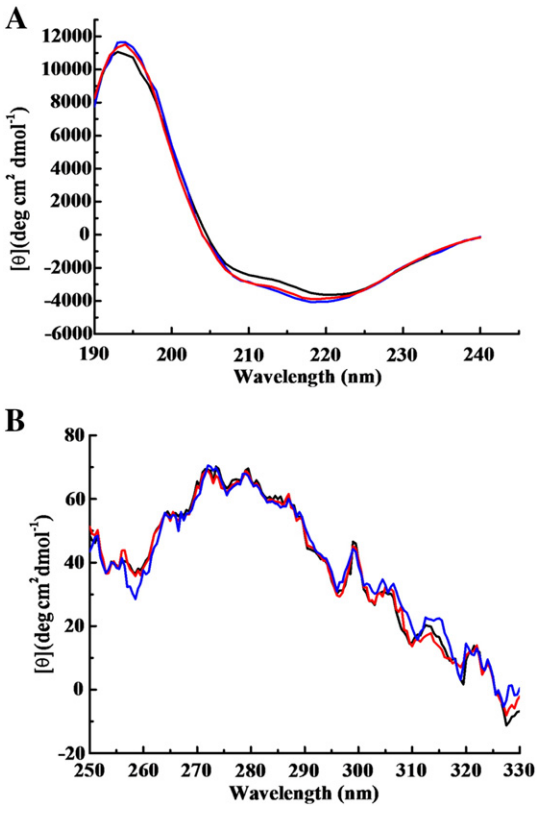


Fig. 4. The CD spectra for the WT PAL (black), variant A272C (red) and A272C-DTNB modified (blue) PAL. (A) The far-UV CD spectra. (B) The near-UV CD spectra.

in backbone flexibility, especially around the site of mutation and modification, MD simulation was used to calculate the B-factors of WT, A272F, A272C and A272C-DTNB modified PAL bound with each of L-menthyl propionate, L-neomenthyl propionate and D-isoneomenthyl propionate. As shown in Fig. 6A, when complexed with L-menthyl propionate, there were no obvious changes in B-factor among WT, A272F, A272C and A272C-DTNB modified PAL. However, the binding of L-neomenthyl propionate or D-isoneomenthyl propionate (Fig. 6B and C) leads to obvious variation of flexibility around the site of mutation or modification (residues 265–280). B-factor simulation revealed particularly high flexibility in WT PAL at this site. When residue Ala272 was replaced by Phe and Cys, the B-factor decreased significantly. More significantly, this region of A272C-DTNB modified PAL exhibited the lowest B-factor. This result indicates that the covalent linkage of modifier DNTB reduces the flexibility of the region round the active site of PAL.

4. Discussion

Residue Ala272 was identified as a hot spot for diastereopreference. MD simulation revealed that the region around Ala272 of WT PAL had a high density of residues with a higher B-factor when complexed with L-neomenthyl propionate and D-isoneomenthyl propionate. Docking analysis in previous research showed that this region faces and interacts with the isopropyl group of menthyl propionate (Fig. 5A–C). Stronger steric exclusion in this region helps to restrict the *S*-configuration of the stereocenter connected with isopropyl and a higher flexibility in this region is required to overcome the steric hindrance of isopropyl groups for non-target substrates to fit into the binding pocket of PAL [36]. Based on this analysis, we proposed that if the rigidity of this region was increased to such a degree that the

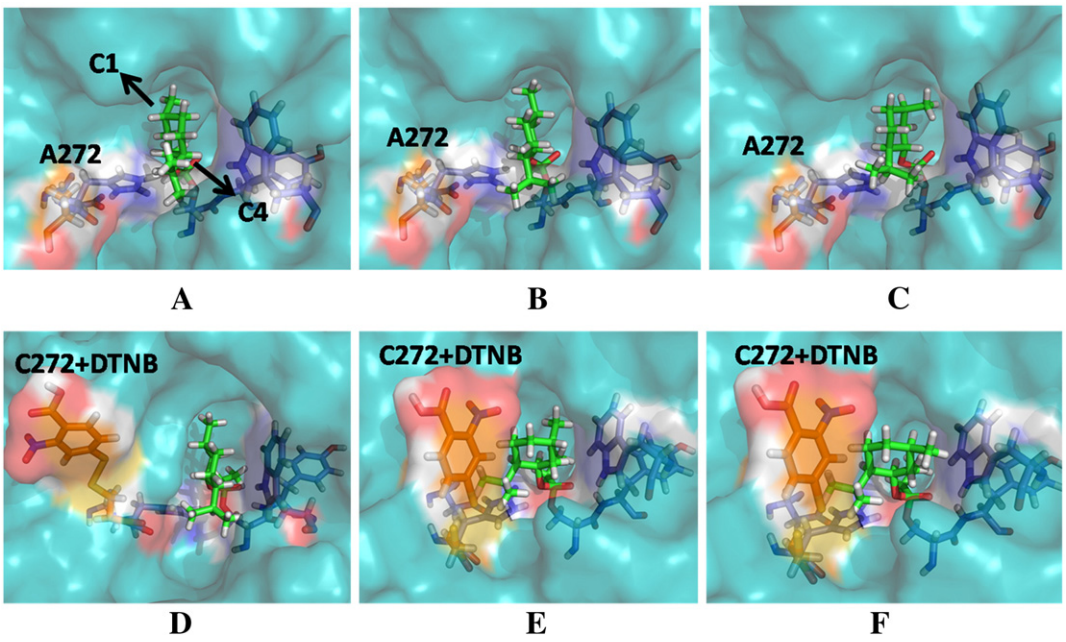


Fig. 5. Representations of models of menthyl propionate diastereomers covalently bound to catalytic Ser133. (A) WT PAL and L-menthyl propionate, (B) WT PAL and L-neomenthyl propionate, (C) WT PAL and D-isoneomenthyl propionate, (D) A272C-DTNB modified PAL and L-menthyl propionate, (E) A272C-DTNB modified PAL and L-neomenthyl propionate, (F) A272C-DTNB modified PAL and D-isoneomenthyl propionate.

steric exclusion effect could not be overcome, the hydrolysis of non-target substrates would be inhibited and diastereopreference further improved.

To increase the steric exclusion effect and decrease the flexibility of the region, an amino acid with a bulky side chain such as Phe could be substituted for Ala272. Instead, Cys was substituted for Ala272 and

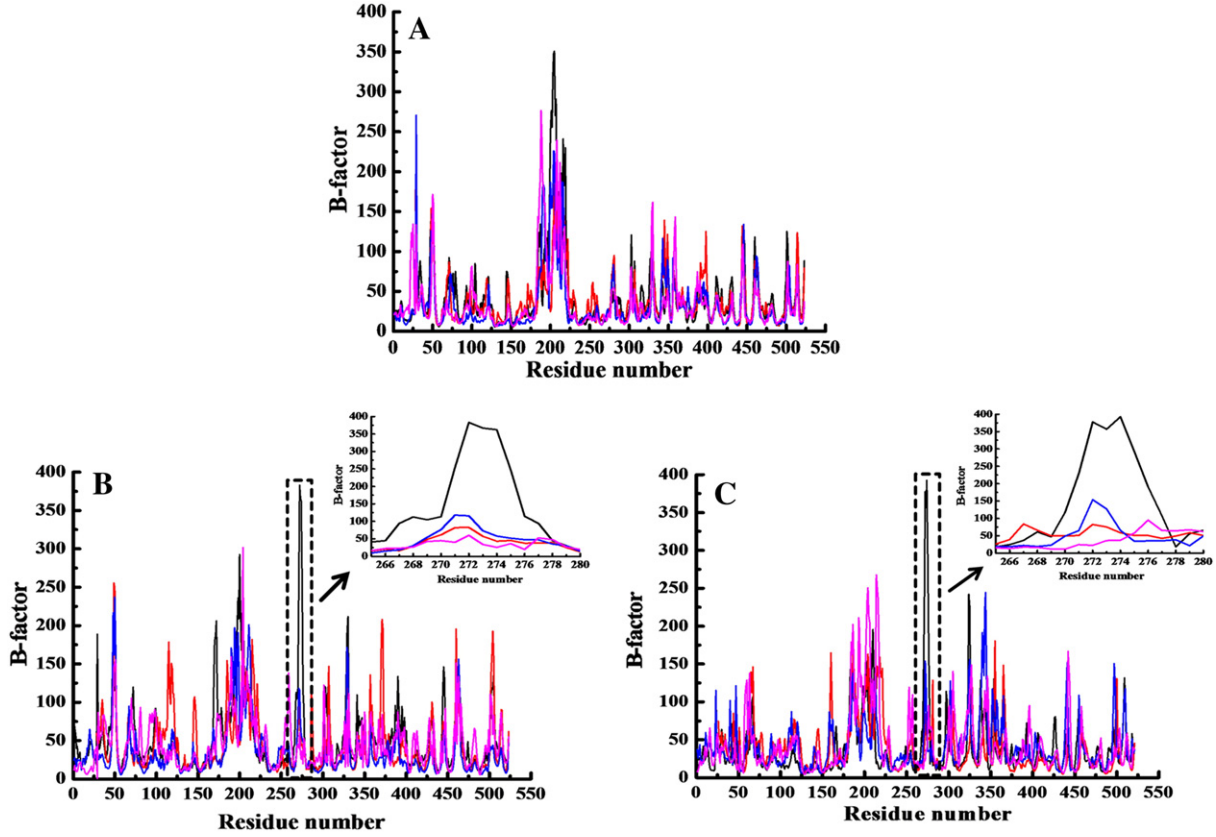


Fig. 6. Calculated B-factor of PAL residues from MD simulation. For WT PAL (black), for variant A272F (blue), for variant A272C (red), for A272C-DTNB modified PAL (pink). (A) WT, variant A272F, variant A272C and A272C-DTNB modified PAL complexed with L-menthyl propionate, (B) WT, variant A272F, variant A272C and A272C-DTNB modified PAL complexed with L-neomenthyl propionate, and (C) WT, variant A272F, variant A272C and A272C-DTNB modified PAL complexed with D-isoneomenthyl propionate.

subjected to DTNB-modification. MALDI-TOF mass spectroscopy evidenced the effective covalent attachment. The CD spectroscopy in far and near-UV regions was employed to investigate the secondary and tertiary structures of WT, variant A272C and A272C-DNTB modified PAL (Fig. 4A, B). The results indicated that the secondary and tertiary structures did not change significantly among the three PALs. The mutation and DTNB modification target (A272 or C272) were on the surface of PAL. The mutation and DTNB chemical modification were hardly to affect the secondary structure and the interactions of secondary structures. The differences of diastereopreference do not originate because of secondary and tertiary structure changes. As shown in Fig. 5D–F, the DTNB modification facilitated much stronger steric exclusion to the isopropyl groups of non-targeted substrates when compared with WT PAL. Diastereopreference analysis showed that A272C-DTNB modified PAL yielded the highest de_p, which was 2.57 times that yielded by WT PAL.

As shown in Fig. 6, the backbone flexibility was ranked according to B-factor [36]. Enzymes are dynamic proteins with inherent flexibility that allows them to interact with substrates [35,37]. The binding of non-target substrates requires flexibility of the region around Ala272 of WT PAL, unlike the binding of L-menthyl propionate. The substitution of residues with larger side chains decreased the flexibility to some extent. DTNB-modification introduced the largest steric hindrance to PAL and decreased the flexibility to the lowest level. An analysis of kinetic properties showed a similar trend. When bound with L-menthyl propionate, WT and A272C-DTNB modified PAL exhibited similar K_m values. In contrast, the binding of non-target substrates produced increased K_m values due to decreased flexibility (Fig. 6B, C) and increased steric exclusion (Fig. 5D–F). The significant correlation of PAL flexibility with the K_m value of the non-target substrate can be explained by a need for induced fit of the active site to accommodate the substrate. One can speculate that the increased steric exclusion introduced by DTNB-modification of Cyc272 and encountered by non-target isomers prevents their fitting into the PAL's active site by restricting the orientation of the isopropyl group of the substrate.

What was interesting that the variant A272C exhibited better diastereopreference than that of A272F, although the side chain of cysteine is smaller than that of phenylalanine. The calculated B-factor (Fig. 6B and C) revealed that the structural flexibility of variant A272C was lower than that of variant A272F when bounded with non-target substrate. The increased structural rigidity may compensate the steric exclusion effect of A272C and also be a benefit for the variant A272C to achieve higher diastereoselectivity.

Differences in K_{cat}/K_m values evidence an improvement in the diastereopreference of A272C-DTNB modified PAL. For A272C-DTNB modified PAL, the K_{cat}/K_m values of L-neomenthyl propionate and D-isoneomenthyl propionate were further decreased than that of L-menthyl propionate relative to WT PAL. This result indicates that the hydrolysis of a non-target substrate was inhibited more than that of L-menthyl propionate. As evidenced by previous studies, an improvement in selectivity is often accompanied by a decrease in overall enzyme activity due to decreased affinity for the worst enantiomer or diastereomer.

In conclusion, this research demonstrates that the combination of site-directed mutagenesis and chemical modification in vitro is an effective method to modify a protein and further confirms the important roles of steric exclusion and structural flexibility.

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References

[1] L. Vincent, B. Sophie, P.G. Sophie, G. David, C. Juan, M. Pierre, S. Thierry, A. Isabelle, R.S. Magali, Control of lipase enantioselectivity by engineering the substrate binding site and access channel, Chembiochem 10 (2009) 2760–2771.
[2] D. Guieysse, J. Cort, S. Puech-Guenot, S. Barbe, A structure-controlled investigation of lipase enantioselectivity by a path-planning approach, Chembiochem 9 (2008) 1308–1317.
[3] K. Liebeton, A. Zonta, K. Schimossek, M. Nardini, D. Lang, K. Jaeger, Directed evolution of an enantioselective lipase, Chem. Biol. 7 (2000) 709–718.
[4] G.W. Zheng, J. Pan, H.L. Yu, M.T. Ngo-Thi, C.H. Li, J.H. Xu, An efficient bioprocess for enzymatic production of l-menthol with high ratio of substrate to catalyst using whole cells of recombinant *E. coli*, J. Biotechnol. 150 (2010) 108–114.
[5] M. Ivancic, G. Valinger, K. Gruber, H. Schwa, Inverting enantioselectivity of *Burkholderia gladioli* esterase EstB by directed and designed evolution, J. Biotechnol. 129 (2007) 109–122.
[6] R. Kobayashi, N. Hirano, S. Kanaya, I. Saito, M Haruki, Enhancement of the enzymatic activity of *Escherichia coli* acetyl esterase by random mutagenesis, J. Mol. Catal. B: Enzym. 67 (2010) 155–161.
[7] F. Bordes, E. Cambon, V. Dossat-Letisse, I. Andre, C. Croux, J.M. Nicaud, A. Marty, Improvement of *Yarrowia lipolytica* lipase enantioselectivity by using mutagenesis targeted to the substrate binding site, Chembiochem 10 (2009) 1705–1713.
[8] D. Zhu, Y. Yang, S. Majkowicz, T.H. Pan, K. Kantardjieff, L. Hua, Inverting the enantioselectivity of a carbonyl reductase via substrate-enzyme docking-guided point mutation, Org. Lett. 4 (2008) 525–528.
[9] Z. Amini-Bayat, S. Hosseinkhani, R. Jafari, K. Khajeh, Relationship between stability and flexibility in the most flexible region of *Photinus pyralis* luciferase, Biochim. Biophys. Acta 1824 (2012) 350–358.
[10] M. Chodorge, L. Fourage, C. Ullmann, V. Duvivier, J.M. Masson, F. Lefevre, Rational strategies for directed evolution of biocatalysts application to *Candida antarctica* lipase B (CALB), Adv. Synth. Catal. 347 (2005) 1022–1026.
[11] J. Olano, J. Soler, F. Busto, D. De Arriaga, Chemical modification of NADP-isocitrate dehydrogenase from *Cephalosporium acremonium* evidence of essential histidine and lysine groups at the active site, Eur. J. Biochem. 261 (1999) 640–649.
[12] P. Cuatrecasas, S. Fuchs, C.B. Anfinsen, The tyrosyl residues at the active site of staphylococcal nuclease. Modification by tetranitromethane, J. Biol. Chem. 243 (1968) 4787–4798.
[13] Z. Shriver, Y. Hu, K. Pojasek, R. Sasisekharan, Heparinase II from *Flavobacterium heparinum*. Role of cysteine in enzymatic activity as probed by chemical modification and site-directed mutagenesis, J. Biol. Chem. 273 (1998) 22904–22912.
[14] H. Yokosawa, S. Ojima, S. Ishii, Thioltrypsin. Chemical transformation of the active-site serine residue of *Streptomyces griseus* trypsin to a cysteine residue, J. Biochem. 82 (1977) 869–876.
[15] Y. Wang, R. Vazques-Duhalt, M.A. Pichard, Purification characterization, and chemical modification of manganese peroxidase from *Bjerkandera adusta* UANH8258, Curr. Microbiol. 45 (2002) 77–87.
[16] L. Biondi, F. Filira, V. Giormani, R. Rocchi, Modification of the properties of bovine pancreatic ribonuclease a by covalent attachment of D-gluconyl-glycine residues, Int. J. Pept. Protein Res. 15 (1980) 253–380.
[17] K. Khajeh, H. Naderi-Manesh, B. Ranjbar, M. Nemat-Gorgani, Chemical modification of lysine residues in *Bacillus* α-amylases: effect on activity and stability, Enzyme Microb. Technol. 28 (2001) 543–549.
[18] G. DeSantis, J.B. Jones, Chemical modification of enzymes for enhanced functionality, Curr. Opin. Biotechnol. 10 (1999) 324–330.
[19] E. Gaidamaviciute, D. Tauraite, J. Gagilas, A. Lagunavicius, Site-directed chemical modification of archaeal *Thermococcus litoralis* Sh1B DNA polymerase: acquired ability to read through template-strand uracils, Biochim. Biophys. Acta 1804 (2010) 1385–1393.
[20] G. DeSantis, P. Berglund, M.R. Stabile, M. Gold, J.B. Jones, Site-directed mutagenesis combined with chemical modification as a strategy for altering the specificity of the S1 and S1′ pockets of subtilisin *Bacillus lentus*, Biochemistry 37 (1998) 5968–5973.
[21] L.G. Martensson, B.H. Jonsson, Characterization of folding intermediates of human carbonic anhydrase II: probing substructure by chemical labeling of SH groups introduced by site-directed mutagenesis, Biochemistry 32 (1993) 224–231.
[22] P. Berglund, M.R. Stabile, M. Gold, J.B. Jones, Altering the specificity of subtilisin *B. lentus* by combining site-directed mutagenesis and chemical modification, Bioorg. Med. Chem. Lett. 21 (1996) 25025127.
[23] A. Tonazzi, N. Giangregorio, C. Indiveri, F. Palmieri, Identification by site-directed mutagenesis and chemical modification of three vicinal cysteine residues in rat mitochondrial carnitine/acylcarnitine transporter, J. Biol. Chem. 20 (2005) 19607–19612.
[24] P.S. Jois, N. Madhu, D.N. Rao, Role of histidine residues in EcoP15I DNA methyltransferase activity as probed by chemical modification and site-directed mutagenesis, Biochem. J. 410 (2008) 543–553.
[25] Y. Ivarsson, M.A. Norrgard, U. Hellman, B. Mannervik, Engineering the enantioselectivity of glutathione transferase by combined active-site mutations and chemical modifications, Biochim. Biophys. Acta 9 (2007) 1374–1381.
[26] R. Croteau, C.L. Hooper, Acetylation of (−)-menthol by a soluble enzyme preparation from peppermint (*Mentha piperita*) leaves, Plant Physiol. 61 (1978) 737–742.
[27] C.L. Fisher, G.K. Pei, Modification of a PCR-based site-directed mutagenesis method, Biotechniques 23 (1997) 570–574.
[28] J.M. Chalker, G.L. Bernardes, Y.A. Lin, B.G. Davis, Chemical modification of proteins at cysteine: opportunities in chemistry and biology, Chem. Asian J. 4 (2009) 630–640.
[29] N. Tsuneyuki, H. Akira, T. Atsushi, S. Tatsuya, DTNB modification of SH groups of isocitrate dehydrogenase from *Bacillus stearothermophilus* purified by affinity chromatography, J. Biochem. 81 (1977) 71–78.

[30] Z.M. Zehl, L. Ivana, A. Marija, R. Andreas, K.P. Biserka, Characterization of covalently inhibited extracellular lipase from *Streptomyces rimosus* by matrix-assisted laser desorption/ionization time-of-flight and matrix-assisted laser desorption/ionization quadrupole ion trap reflectron time-of-flight mass spectrometry: localization of the active site serine, J. Mass Spectrom. 39 (2004) 1474–1483.
[31] C. Chang, X. Liu, K. Chen, Molecular cloning, expression and characterization of a novel gene β-N-acetylglucosaminidase from *Bombyx mori*, Adv. Biosci. Biotechnol. 9 (2011) 123–127.
[32] K. Boren, P. Andersson, M. Larsson, U. Carlsson, Characterization of a molten globule state of bovine carbonic anhydrase III: loss of asymmetrial environment of the aromatic residues has a profound effect on both the near- and far-UV CD spectrum, Biochim. Biophys. Acta 1430 (1999) 111–118.
[33] N.P. Shroff, M. Cherian-Shaw, S. Bera, E.C. Abraham, Mutation of R116C results in highly oligomerized αA-crystallin with modification structure and defective chaperone-like function, Biochemistry 39 (2000) 1420–1426.
[34] P.R. Ramasarma, A.G. Rao, D.R. Rao, Role of disulfide linkages in structure and activity of proteinase inhibitor from horsegram (*Dolichos biflorus*), Biochim. Biophys. Acta 1248 (1995) 35–42.
[35] A. Schlessinger, B. Rost, Protein flexibility and rigidity predicted from sequence, Proteins 61 (2005) 115–126.
[36] K. Watanabe, T. Yoshida, S. Uejia, The role of conformational flexibility of enzymes in the discrimination between amino acid and ester substrates for the subtilisin-catalyzed reaction in organic solvents, Bioorg. Chem. 32 (2004) 504–515.
[37] M. Kokkinidis, N.M. Glykos, Protein flexibility and enzymatic catalysis, Adv. Protein Chem. 87 (2012) 182–212.