ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 14 (2001) 85–94

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Stabilization mechanism of MPEG modified trypsin based on thermal inactivation kinetic analysis and molecular modeling computation

Ziding Zhang[1], Zhimin He*, Mingxia He

Laboratory of Enzyme Technology, Chemical Engineering Research Center, Tianjin University, Tianjin 300072, PR China

Received 24 July 2000; accepted 18 October 2000

Abstract

Thermal inactivation kinetic analysis and molecular modeling computation were jointly utilized to illuminate the detailed stabilization mechanism of trypsin caused by methoxypolyethylene glycol (MPEG) modification. First, trypsin was modified by MPEG (molecular mass 350 Da) to enhance its thermal stability. As expected, the modified trypsin was more stable against temperature than the native form. Second, a new kinetic model, which has the ability of taking the thermal denaturation and autolysis effects of proteases into account, was established and used to analyze the thermal inactivation process of the native and modified trypsin. The kinetic analysis showed that the increased thermal stability of MPEG modified trypsin is the joint result of a reduction in autolysis and a decrease in thermal denaturation. Finally, the molecular modeling technique was also employed to calculate some structural information change, i.e. solvent accessible surface, intramolecular hydrogen bond and root mean square fluctuation, between the native and modified trypsin. The results of molecular modeling computation demonstrated that (i) the steric hindrance caused by MPEG chain would result in the decreased rate of autolysis, (ii) the decreased rate of thermal denaturation should be ascribed to the increased number of hydrogen bond, not the result of the increased molecular rigidity.

Keywords: Trypsin; Chemical modification; Methoxypolyethylene glycol; Thermal stability; Thermal inactivation kinetics; Molecular modeling

Abbreviations: BAPNA: benzoyl D,L-arginine *p*-nitroanilide; CVFF: consistence valence force field; DMSO: dimethylsulfoxide; MD: molecular dynamics; MPEG: methoxypolyethylene glycol; NPC: *p*-nitrophenyl chloroformate; RMSD: root mean square distance; SASA: solvent accessible surface area; Trypsin-MPEG: trypsin modified by MPEG; TNBS: 2,4,6-trinitrobenzene sulfonic acid

*Corresponding author. Tel.: +86-22-2740-3154; fax: +86-22-2740-4757.

E-mail address: zhe@tju.edu.cn (Z. He).

[1]Present address: Department of Molecular Biophysics, Center for Chemistry and Chemical Engineering, Lund University, Box 124, SE-221 00 Lund, Sweden.

1381-1177/01/$ – see front matter
PII: S1381-1177(00)00241-1

1. Introduction

As an effective and simple approach to improve enzyme function, chemical modification has become an intensively studied medical and biotechnological application. Among the investigated modifiers, methoxypolyethylene glycol (MPEG) is outstanding as regards both the number of reports and the improvements in enzyme function achieved [1,2]. The basic idea of MPEG modification is to synthesize an "activated" MPEG containing a reactive terminal group that can be readily coupled with a functional group on the protein, such as the ε-amino group of a lysine residue [1–3]. In addition to increasing enzyme thermal stability, MPEG modification can also result in minimal loss of activity, reduced immunogenicity and antigenicity, facilitating the development of a number of MPEG-modified therapeutic enzymes [2].

Recently, the stabilization effects caused by MPEG modification have been widely reported. Several enzymes, such as trypsin [4,5], lipase [6,7], subtilisin [3], penicillin G acylase [8], horseradish peroxidase [9], and α-chymotrypsin [10] were involved. The reason of increased thermal stability of a modified enzyme was often explained by (i) the formation of a highly hydrogen-bonded structure around the enzyme caused by MPEG chain [4], (ii) a MPEG-induced increase in enzyme structural rigidity [3], (iii) a reduced autolysis rate for MPEG modified protease [3]. Additionally, the change of hydrophobic and electrostatic characteristics of enzyme surface caused by MPEG modification has also been applied to explain the stabilization effect [9,10]. It should be pointed out, however, the above explanations are still uncertain to some extent owing to lack of enough convincing experimental tests. Therefore, the detailed elucidation of molecular mechanism responsible for the stabilization effect of MPEG modification is of great importance from both a scientific and a commercial point of view [11].

Up to now, a series of approaches has been applied to illuminate the stabilization mechanism of an enzyme caused by chemical modification. Conventional approaches, including thermal inactivation kinetic analysis [9], thermodynamic analysis based on the Arrhenius equation [12] and calorimetric measurement by differential scanning calorimeter [3], have been used to assess the stabilization effect quantitatively. Owing to lacking of the molecular structural information, the stabilization mechanism deduced from these approaches is rather crude. Circular dichroism (CD) can be applied to monitor the conformational changes of the native and modified enzyme in response to elevated temperature [13,14], and hence to evaluate the thermal stabilization effects. However, the structural information obtained from this technique is still quite limited. As a powerful tool to study the relationship between structure and function of an enzyme, the molecular modeling technique has been applied in many biotechnological studies, such as the affinity ligand design for enzyme purification [15], investigation on enzymatic behavior in non-aqueous solvent [16] and molecular-modeling calculation of enzymatic enantioselectivity [17]. To further illustrate the stabilization mechanism of MPEG modification, molecular modeling technique may give considerable guidance and insight [18].

The highlight of the present work intends to illuminate the stabilization mechanism of MPEG-modified trypsin by combining the thermal inactivation kinetics analysis and molecular modeling computation. First, bovine pancreatic trypsin is modified with *p*-nitrophenyl chloroformate (NPC) activated MPEG (molecular mass 350 Da) and the thermal inactivation data for the native and modified trypsin are measured. Second, the thermal inactivation process of the native and modified trypsin is analyzed with a new kinetic model, which can consider both the thermal denaturation and the autolysis effects of trypsin. Finally, molecular modeling computation is employed to investigate the change of structural information related to enzyme stability, and hence to obtain the detailed molecular basis of enzyme stabilization caused by MPEG modification.

2. Experimental aspects

2.1. Materials

Bovine pancreatic trypsin (EC 3.4.21.4) with high purity was purchased from Gibco Co., MPEG (molecular mass 350 Da), NPC, benzoyl D,L-arginine *p*-nitroanilide (BAPNA) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were obtained from Sigma Corp. All other reagents and chemicals were of analytical grade.

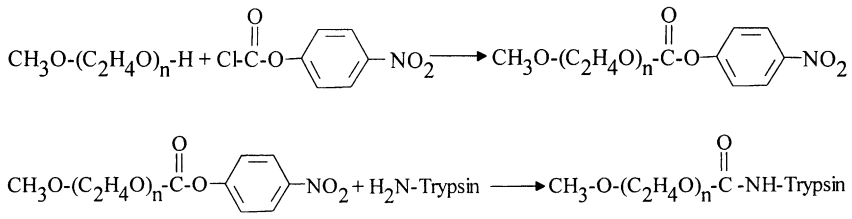

Fig. 1. Diagram of MPEG-modification reactions. For MPEG with the molecular mass of 350 Da, the value of n is ≈7.

2.2. Preparation of MPEG modified trypsin

MPEG (molecular mass 350 Da) was activated by NPC using the method of Veronese et al. [19] Typically, the activated MPEG was added in excess (10 molar excess over trypsin amino groups) to trypsin (2 mg/ml dissolved in 0.1 M sodium tetraborate buffer, pH 9.0). The resulting mixture was stirred at room temperature for 4 h, followed by extensive dialysis at 4°C for 24 h to remove excess activated MPEG. Finally, the aqueous solution of MPEG-modified trypsin (trypsin-MPEG) was obtained. The modification degree for trypsin-MPEG was determined by the TNBS method [20]. The reaction process of MPEG modification is also displayed in Fig. 1.

2.3. Enzyme activity assay

The amidase activity of trypsin was determined using BAPNA as the substrate as reported by Murphy and O'Fagain [21]. 2.6 ml BAPNA (1.66 mM; 18 mg dissolved in 1 ml of dimethylsulfoxide (DMSO) and added to 24 ml of 0.1 M Tris–HCl, pH 8.2, containing 20 mM $CaCl_2$) was equilibrated in a water bath at 30°C. Samples (0.2 ml) were incubated in a water bath at 30°C for 10 min. The reaction was then stopped with 0.4 ml 30% (v/v) acetic acid. The absorbance was read at 405 nm on a U-2001 spectrophotometer (Hitachi Company, Japan).

2.4. Thermal inactivation measurements

The data of thermal inactivation were measured by incubation of approximately 0.4 mg/ml native and modified trypsin in aqueous buffer (0.1 M Tris–HCl, pH 8.2) in 50 and 60°C water bath, respectively. Aliquots were removed at intervals and the residual activity determined using BAPNA as the substrate.

3. Theoretical aspects

3.1. Thermal inactivation kinetics analysis

Generally, trypsin is prone to thermal denaturation at high temperature. As a protease, trypsin can also hydrolyze itself (autolysis), leading to partial or complete inactivation [21]. Therefore, the effect of autolysis and thermal denaturation must be simultaneously considered when developing an understanding of the inactivation process. A two-step mechanism for trypsin inactivation was reported by Kawamura et al. [22]

1. Thermal denaturation: $E \leftrightarrow^{K_0} E_1 \xrightarrow{k_1} E_d$.
2. Autolysis: $E + E_1 \leftrightarrow^{K_p} EE_1 \xrightarrow{k_d} E + P$.

The native enzyme (E) is first reversibly denatured to E_1, and then irreversibly denatured to the completely inactive form (E_d). Meanwhile, E_1 is also digested under the catalysis of the native enzyme (E) to the decomposed product (P) following the second reaction. $K_0 = [E_1]/[E]$, $K_p = [E][E_1]/[EE_1]$; k_1 and k_d are the rate constants for the thermal denaturation and autolysis, respectively. The enzyme residual activity (Ra) is then given by [3,22]

$$\mathrm{Ra} = \frac{k_a}{k_a\, e^{k_a t} + k_b[E]_0 \mathrm{Ra}\, e^{k_a t} - k_b[E]_0 \mathrm{Ra}} \quad (1)$$

where $[E]_0$ is the initial enzyme concentration, k_a and k_b represent the apparent rate constant of thermal denaturation and autolysis, respectively. The formulae of k_a and k_b are listed as follows:

$$k_a = \frac{k_1 K_0}{1 + K_0} \quad (2)$$

$$k_b = \frac{k_d K_0}{K_p(1 + K_0)^2} \quad (3)$$

To perform the thermal inactivation kinetics analysis, the experimental inactivation data for the native and modified trypsin were fitted to Eq. (1) and the values of k_a and k_b were determined using a non-linear regression based on the Newton–Marquart method.

3.2. Molecular modeling computation

3.2.1. Strategy of molecular modeling computation

In this paper, the molecular modeling computation is performed on SGI O_2 workstation using Insight II software [23], to relate the structure change between the native and modified trypsin to stabilization effects. The following strategies of molecular modeling computation are employed. First, the molecular force field is applied to build the tertiary structures of the native and MPEG modified trypsin based on the crystal structure. Second, conformational searches for the native and modified enzyme's structures are carried out using the molecular dynamics (MD) modeling. Finally, some useful structural information for native and modified trypsin is calculated from the results of MD modeling.

3.2.2. Molecular structural building

The crystal structure of trypsin, from the same resource as used in the experiment, was found with the PDB entry 2PTN through the Brookhaven Protein Data Bank [24]. The single-chain enzyme molecule has 223 amino acid residues. A diagram of trypsin crystal structure is displayed in Fig. 2. Since the experimental result showed that the enzyme activity has only a partial loss after the MPEG modification (see Section 4), the main chain structure of trypsin could not have a substantial change. Hence, it is reasonable to build the structure of modified enzyme from the crystal structure of native enzyme.

The consistence valence force field (CVFF) [25] was used to optimize the trypsin crystal structure. The total charge was set to the default assignment of the CVFF force field and a cut-off distance of 20 Å was used for both the electrostatic and van der Waals terms to save computer time. First, hydrogen atoms were added to the crystal structure of trypsin from the PDB database. Second, the minimization was performed using a conjugate gradient algorithm. In order to keep the enzyme structure from an unreasonable change, the minimization is carried out by fixing the C^α atoms and then by setting all atoms free. Such minimization did not significantly modify the general shape of trypsin (the root mean square distance (RMSD) of C^α atoms between the initial crystal structure and minimized structure is 0.36 Å). The resulting energy-minimized conformation was regarded as the three-dimensional structure of the native trypsin, and was used for the following structure building of the modified trypsin.

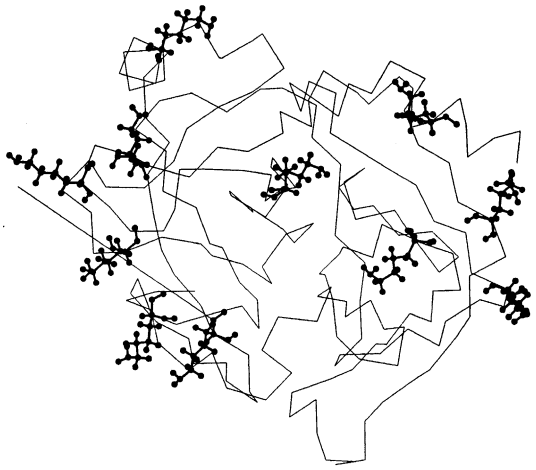

Fig. 2. A graph displaying the C^α trace of trypsin crystal structure, prepared using the Insight II molecular modeling software [23]. The residues displayed by the model of *Stick and Ball* are 11 Lys residues, which are more exposed to enzyme surface.

The MPEG modification is completed through the acylation of amino groups of lysine (Lys) located in the enzyme surface. The number of Lys residues is up to 14 in the native enzyme molecule. However, the surface area of each residue exposed to enzyme molecule surface is very different. Since our experimental study showed that about 11 Lys residues were modified for per MPEG modified trypsin molecule (see Section 4), for convenience, we suppose that the modification is performed on 11 Lys residues whose surface area are more exposed to the enzyme molecule surface. These 11 Lysines' positions in the primary sequence (also see Fig. 2) are Lys_222, Lys_109, Lys_145, Lys_239, Lys_159, Lys_188, Lys_60, Lys_169, Lys_87, Lys_224 and Lys_204, respectively. The initial structure of the modified enzyme is built by jointing the reaction group ($CH_3O(C_2H_4O)_7CO$–), whose initial conformation is regarded as a random chain, covalently with the amine

of these 11 Lys residues of trypsin. The optimization method is the same as the above minimization process in building the tertiary structure of the native trypsin.

As we know, the property of an enzyme is certainly affected by the surrounding solvent. To obtain more accurate results of molecular modeling computation, the water molecules should be considered for the following MD modeling [16]. In this study, two layers of water molecules are employed to solvate the native and modified trypsin. The thickness of inner and outer layer is set to 5 and 8 Å, respectively. The solvation is accomplished by placing the trypsin molecule in an equilibrated three-dimensional grid of water molecules [26] and removing those water molecules which overlap with atoms in the trypsin molecule being solvated [23]. Thus, two solvated systems for the native and modified trypsin are built. For the system of native trypsin, the number of water molecule is 1087 in inner layer and 995 in outer layer, respectively. For the system of modified trypsin, the number of water molecule in inner and outer layer is 1227 and 951, respectively. Before performing MD modeling, these two systems are also minimized by the CVFF force field.

3.2.3. Molecular dynamics modeling

The MD modeling is performed on the above two optimized aqueous systems of the native and MPEG modified trypsin by fixing the outer layer water molecules. The MD modeling is carried out at 300 K with a time step of 1 fs. Other modeling parameters are same as the above minimization process. The modeled enzyme molecules were first equilibrated for 30 ps followed by a production run of another 120 ps during which conformations were collected in preparing for the following conformation analysis. In the 30th-ps of equilibration process, the potential energy fluctuation (defined as σ/H, where σ and H indicate the standard deviation and average value of potential energy for 100 conformations sampled from the 30th-ps of equilibration procedure, respectively) for two systems are both $<1\%$. Therefore, the two systems are considered to be equilibrated, and the data collection from the next 120 ps MD modeling should be reliable.

3.2.4. Calculation of structural information

The structural information statistical analysis for the native and modified trypsin was performed on the conformations from the above MD modeling

1. The values of solvent accessible surface area (SASA) are calculated on 40 conformations (one average conformation for 3 ps MD modeling results). The SASA is defined as the area traced by the center of a solvent molecule rolling over the van der Waals envelope of an enzyme. In this paper, the SASA is computed by the method of Shrake and Rupley [27], and three different probe solvent sizes are selected with the radii of 1.4, 5.0 and 8.0 Å, respectively.
2. The number of hydrogen bond for the native and MPEG modified trypsin is also calculated on 40 conformations (one average conformation for 3 ps MD modeling results). A hydrogen bond is considered to be formed when the distance between the proton on the donor atom and the heavy atom acceptor is <2.5 Å, and the angle between the proton acceptor, the proton, and the proton donor is $>120^\circ$ [23].
3. The values of RMSD between the conformations from MD modeling (one average conformation for 3 ps MD modeling) and its reference conformation is calculated. It should be pointed out that the RMSD calculation is performed on all heavy atoms. And the reference conformation is defined as the average conformation from 120 ps MD modeling.

4. Results and discussion

4.1. Characterization of MPEG modified trypsin

Compared with the native trypsin, trypsin-MPEG showed slightly lower amidase activity (80%). TNBS determination of modification degree indicated that about 20% of the lysine residues remained, implying that 11 of 14 lysine residues per trypsin molecule had been modified.

4.2. Thermal inactivation kinetic analysis

The results of thermal inactivation kinetic analysis are displayed in Fig. 3 and the calculated kinetic parameters are listed in Table 1. As seen from Fig. 3, the MPEG modified trypsin is significantly more stable against temperature than the native form. In

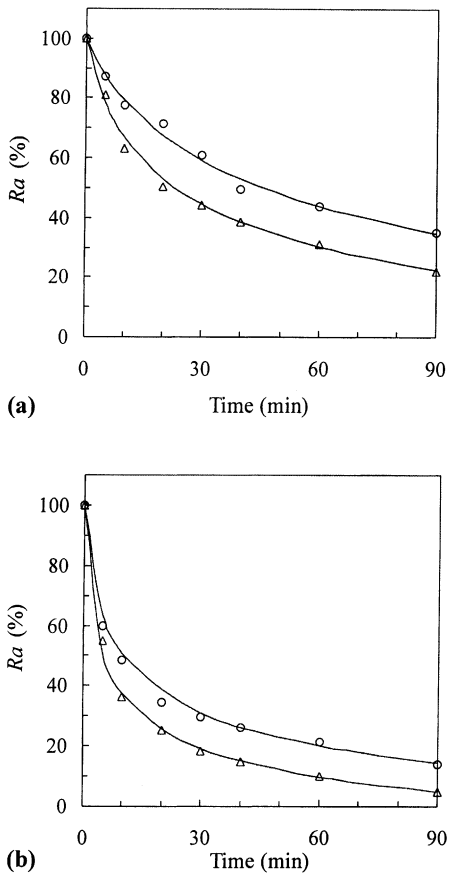

Fig. 3. Thermal inactivation of the native and modified trypsin (0.4 mg/ml) at 50°C (a) and 60°C (b), respectively. The activity is shown as percentage values relative to initial activity of samples. Symbols represent the experimental data, native trypsin (△), trypsin-MPEG (○). Solid curves are the results of fitting the data to the Eq. (1).

addition, the thermal inactivation profiles for the native and modified trypsin can be adequately simulated by Eq. (1). On the one hand, the value of k_a can reflect the rate of thermal denaturation. As given in Table 1, the kinetic value k_a significantly decreased after modification regardless of 50 or 60°C, which meant the thermal denaturation rate is decreased for MPEG modified trypsin. This result is consistent with our previous thermal inactivation kinetic analysis for the native and MPEG modified trypsin at low concentration based merely on the first-order model [5]. On the other hand, the value of k_b can reflect the rate of autolysis. As shown in Table 1, k_b value is also obviously decreased after modification, which meant the MPEG modified trypsin is less prone to autolysis. In our previous study [5], the Coomassie protein assay was used to measure autolysis characteristics of the native and MPEG modified trypsin. The autolysis measurement of the Coomassie protein assay is consistent with our present kinetic analysis result qualitatively. In a word, the present thermal inactivation analysis has clearly demonstrated that the increased thermal stability for the modified trypsin should be ascribed to a reduced autolysis rate and a lower thermal denaturation rate.

The thermal inactivation kinetic studies of an enzyme are widely reported in the literatures recently, with view to describe the inactivation process quantitatively and then provide further insight into the mechanism of enzyme inactivation [14,28,29]. To perform the thermal inactivation kinetic analysis, the key problem is how to select a suitable model. As reported in [30], the first-order model seems to be more popular for its simplicity. However, large deviation from the first-order kinetics is often observed [30]. Alternatively, the other models, i.e. the series-type model [29] and enzyme heterogeneity inactivation model [28], are also used to analyze the process of thermal inactivation.

For the proteases, the kinetics of thermal inactivation seems to be more complicated due to the autolysis effect. Generally, the thermal inactivation can be described by the first-order kinetic model at low concentration, since the autolysis under this condition can

Table 1
Kinetic parameters of thermal inactivation for native and MPEG-modified trypsin

Enzyme	50°C		60°C	
	k_a (min^{-1})	k_b ($ml\,mg^{-1}\,min^{-1}$)	k_a (min^{-1})	k_b ($ml\,mg^{-1}\,min^{-1}$)
Native trypsin	0.0109	0.082	0.0288	0.758
Trypsin-MPEG	0.0069	0.058	0.0129	0.391

be negligible [3,21]. This opinion can also be deduced from Eq. (1). As reported in literatures, the first-order model is often employed to describe the thermal inactivation data of proteases at low concentration [3,5,21]. However, at high concentration, the inactivation kinetics showed a polyphasic characteristic owing to the obvious autolysis effect, which meant that the inactivation could not be described by the first-order model. The kinetic model reported here, which has the ability of taking both the thermal denaturation and the autolysis effects into account, is very successful to describe the thermal inactivation process of the native and modified trypsin at high concentration. And the most important advantage of this model is that it can provide quantitatively information on thermal denaturation and autolysis, which would be helpful to our further analysis of the mechanism of thermal stabilization induced by MPEG modification.

4.3. Molecular modeling computation

4.3.1. Values of SASA

As shown in Table 2, the average value of SASA for trypsin is significantly decreased after modification regardless of the size of probe solvent. Compared with the native trypsin, the average value of the modified trypsin decreased 28, 43 and 49% for the probe radii of 1.4, 5.0 and 8.0 Å, respectively. This finding indicated that the steric hindrance between trypsin and other molecules is increased after MPEG modification. The steric hindrance is the result of the shielding effect caused by the MPEG chain around trypsin surface. So, the value of SASA can be applied to describe the effect of steric hindrance quantitatively.

As known form Eq. (3), decreased value of k_d and increased value of K_p would result in reduced autolysis rate. The computational results of SASA for the native and MPEG modified trypsin indicated that the MPEG chains attached on the enzyme surface could produce remarkable steric hindrance to prevent trypsin from association each other by increasing the value of K_p, and hence the modified trypsin was more stable against autolysis. As reported by Murphy and O'Fagain [21], additionally, trypsin cleaves proteins and peptides at the carboxylic side of the basic amino acid arginine and lysine. Therefore, modification of lysine residues could also decrease the value of k_d and hence provides some protection against autolysis.

Table 2
The average value of SASA for native and MPEG modified trypsin[a]

Enzyme	Average value of SASA (Å^2)		
	1.4 Å	5.0 Å	8.0 Å
Native trypsin	10734	10855	13005
Trypsin-MPEG[b]	7748	6204	6661

[a] The average value of SASA for 40 different conformations from MD modeling.
[b] The MPEG chain is not included for the calculation of SASA.

Table 3
The average value of the number of hydrogen bond for native and modified trypsin

Enzyme	Average value of number of hydrogen bond	
	Average[a]	σ[b]
Native trypsin	59	7
Trypsin-MPEG	65	6

[a] The average value of the number of hydrogen bond for the 40 different conformations from MD modeling.
[b] The standard deviation of the 40 computational results for the number of hydrogen bond.

4.3.2. Number of hydrogen bond

Compared with the native trypsin, as shown in Table 3, the average number of hydrogen bond for modified trypsin is obviously increased (the value of added hydrogen bond is about 6). The added hydrogen bonds are mainly formed between MPEG chain and trypsin surface. As an important force to keep the structural stability of an enzyme molecule, the hydrogen bonds are correlated with the enthalpic effects of the enzyme unfolding process [31,32]. Generally, an increased number of hydrogen bond corresponds to the increased value of the unfolding enthalpy change, and hence the increased stability [31]. However, the different hydrogen bond's contribution to enzyme stability is different. Those hydrogen bonds, which forms in the enzyme folded state but destroyed in enzyme unfolded state, seem to have larger contribution to enzyme stability [32]. For the MPEG modified trypsin, the added hydrogen bonds seem to be more prone to be destroyed in its defolded state owing to the hydrophilization of MPEG chain. Therefore, the increased hydrogen bonds caused by MPEG modification would have substantial contribution to decrease the thermal denaturation rate of trypsin. As reported

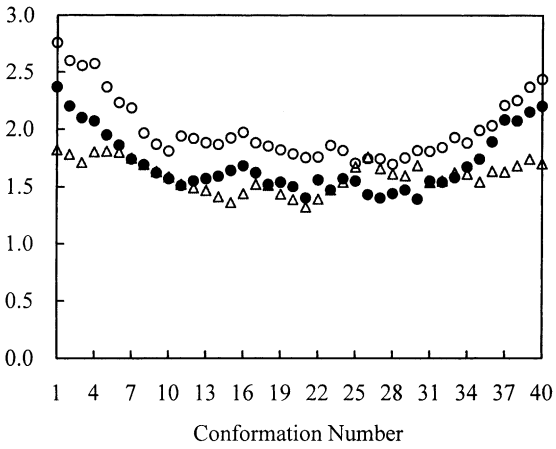

Fig. 4. The value of RMSD (Å) for native and MPEG modified trypsin. Native trypsin (△); trypsin-MPEG including MPEG chain (○); trypsin-MPEG not including MPEG chain (●).

by Gaertner and Puigserver [4], the decreased thermal denaturation could have been the result of a highly hydrogen-bonded structure around trypsin surface caused by MPEG modification. Our computational result for the number of hydrogen bond verified their opinion in further.

4.3.3. Values of RMSD

The computational results of RMSD for the native and modified trypsin are displayed in Fig. 4. Compared with the native trypsin, as seen from Fig. 4, the MPEG modified trypsin seems to have larger value of RMSD. When the MPEG chain is considered, the increased value of RMSD is more obvious. The average value of RMSD is also given in Table 4. The value of RMSD can reflect the structural fluctuation, and hence indicate the structural flexibility of an enzyme molecule [16,33]. As known from the computational results of RMSD, the modified enzyme seems to be more flexible than the native form. Therefore, the molecular rigidity is decreased after MPEG modification. The decreased molecular rigidity should be ascribed to the introduction of flexible MPEG chain.

Table 4
The average value of RMSD for native and MPEG modified trypsin

Enzyme	Average value of RMSD (Å)
Native trypsin	1.60
Trypsin-MPEG (MPEG chain is included)	2.02
Trypsin-MPEG (MPEG chain is not included)	1.72

The structural rigidity can reflect its thermal stability of an enzyme molecule to a large extent [34]. Increased structural rigidity may make the enzyme molecule less flexible in aqueous solution, hence protecting it from unfolding and in turn decreasing the rate of thermal denaturation [34]. Generally, structural rigidity corresponds to the entropic effect of an enzyme unfolding process [31]. Increased rigidity would result in a decreased rate of enzyme thermal denaturation by decreasing the entropy change in the unfolding process. As reported in the literatures, the increased molecular rigidity is often used to explain the stabilization mechanism of immobilized and intramolecular cross-linked enzyme. Yang et al. [3] regarded the increased thermal stability of MPEG should be also partly ascribed to the increased structural rigidity caused by MPEG modification. Interestingly, our finding that the MPEG modified trypsin seems to be more flexible than the native trypsin is very different from their opinion.

Noticeably, the molecular rigidity is also an important notion related to enzyme activity. Generally, enzyme molecule is less structurally rigid (more flexible) than other proteins, since structural flexibility is very important to achieve its catalysis function. However, the structural flexibility of an enzyme molecule has an opposite effect on its thermal stability. Since the molecular rigidity of an enzyme is so strongly related to its function, how to describe the structural rigidity of an enzyme molecule quantitatively is of great importance. It seems to be very difficult to obtain the molecular rigidity information by experimental measurement, since the structural rigidity is related to the motion of an enzyme molecule [35]. It is also hard to accurately calculate the structural rigidity of an enzyme by the theoretical approaches [35]. Therefore, the RMSD value from MD modeling may become an effective parameter to describe the molecular rigidity of an enzyme, which has been confirmed by several research groups [16,33].

4.3.4. Possible results for larger molecular mass MPEG modified trypsin

Generally, the experimental study related to MPEG (molecular masses 2000 and 5000 Da) modification is more frequently reported in [6,7]. However, it

seems to be difficult to perform the molecular modeling computation for long chain MPEG modified trypsin at present. The main reasons should be due to that the conformation of long chain MPEG is very complicated and that the computer time will be drastically increased. Therefore, only the molecular modeling computation for MPEG (molecular mass 350 Da) modified trypsin is considered in this study. As pointed out in previous experimental work [4,5], the larger size MPEG modified trypsin seems to have lower thermal denaturation and autolysis rates, and hence have the more significant stabilization effect. This means that the stabilization strategies caused by different sizes of MPEG modification are basically identical, therefore, the present molecular modeling computation can also provide enough insight into the molecular basis of enzyme stabilization caused by larger molecular mass MPEG modification. As can be conceivable, the larger size MPEG modified trypsin would correspond to more obvious steric hindrance, larger number of intramolecular hydrogen bond and less structural rigidity.

5. Concluding remarks

In summary, the current study has elucidated the molecular basis of stabilization effect of trypsin induced by MPEG modification in further. The kinetic analysis of thermal inactivation showed that the increased thermal stability of MPEG-modified trypsin is the joint result of reduced autolysis and decreased thermal denaturation. The molecular modeling computation showed that (i) the steric hindrance caused by MPEG chain would result in the reduced autolysis, (ii) the decreased thermal denaturation for MPEG modified trypsin should be ascribed to the increased number of hydrogen bond, not the increased molecular rigidity. The above results should be also of general interest in understanding the molecular basis of chemically modification induced stabilization effect of an enzyme as well as the potential value for the development other enzyme stabilization strategy.

It should be pointed out that the present molecular modeling computation might be not perfect. For the convenience of structure building of the modified trypsin, for example, we assume that the modification is performed on 11 Lys residues which surface area are more exposed to the enzyme surface. Whether such a hypothesis is true or not needs to be proved by additional experimental studies. Furthermore, the current computation does not have the ability to assess if the stabilization effect caused by MPEG modification should be also contributed to the change of hydrophilic or electrostatic characteristics in enzyme surface. The ultimate goal of the present topic is to predict the stabilization effects caused by modification quantitatively by molecular modeling technique. Owing to theoretical prediction of stabilization energy of an enzyme molecule from its tertiary structure is still a hard problem of modern biochemistry [32], however, there is still a long way to reach this aim.

Acknowledgements

Research was sponsored by the grant no. 29776033 of National Natural Science Foundation of China. We would also like to thank the two anonymous referees whose constructive comments were very helpful for improving the paper.

References

[1] Y. Inada, M. Furakawa, H. Sasaki, Y. Kodera, M. Hiroto, H. Nishimura, A. Matsushima, Trends Biotechnol. 13 (1995) 25.
[2] Y. Inada, A. Matsushima, M. Hiroto, H. Nishimura, Y. Kodera, Adv. Biochem. Eng. Biotechnol. 52 (1995) 129.
[3] Z. Yang, M. Domach, R. Auger, F. Yang, A.J. Russell, Enzyme Microb. Technol. 18 (1996) 82.
[4] H.F. Gaertner, A.J. Puigserver, Enzyme Microb. Technol. 14 (1992) 151.
[5] Z. Zhang, Z. He, G. Guan, Biotechnol. Tech. 13 (1999) 781.
[6] M. Basri, K. Ampon, Y. Wan, C.A.N. Razak, B. Salleh, J. Chem. Tech. Biotechnol. 64 (1995) 10.
[7] M.J. Hernaiz, J.M. Sanchez-Montero, J.V. Sinisterra, Enzyme Microb. Technol. 24 (1999) 181.
[8] D. Kazan, A. Erarslan, Appl. Biochem. Biotechnol. 62 (1997) 1.
[9] D. Garcia, F. Ortega, J.-F. Marty, Biotechnol. Appl. Biochem. 27 (1998) 49.
[10] M.A. Longo, D. Combes, J. Chem. Technol. Biotechnol. 74 (1999) 25.
[11] S. Janecek, Process Biochem. 28 (1993) 435.
[12] M.H. Rashid, K.S. Siddiqui, Process Biochem. 33 (1998) 109.
[13] J.A. Rupley, E. Gratton, G. Careri, Trends Biochem. Sci. 8 (1983) 18.
[14] T. Sasaki, M. Kobayashi, H. Kise, Biotechnol. Tech. 6 (1997) 387.
[15] N.E. Labrou, E. Eliopoulos, Y.D. Clonis, Biotechnol. Bioeng. 63 (1999) 322.

[16] M. Norin, F. Haeffner, K. Hult, O. Edholm, Biophy. J. 67 (1994) 548.
[17] T. Ke, B. Tidor, A.M. Klibanov, Biotechnol. Bioeng. 57 (1998) 741.
[18] Z. He, Z. Zhang, J. Protein. Chem. 18 (1999) 557.
[19] F.M. Veronese, R. Largajolli, E. Boccu, O. Schiavon, Appl. Biochem. Biotechnol. 11 (1985) 141.
[20] S.L. Snyder, P.Z. Sobocinski, Anal. Biochem. 64 (1975) 284.
[21] A. Murphy, C. O'Fagain, J. Biotechnol. 49 (1996) 163.
[22] Y. Kawamura, K. Nakanishi, R. Matsuno, T. Kamikubo, Biotechnol. Bioeng. 23 (1981) 1219.
[23] Insight II User Guide, Biosym/MSI, San Diego, 1995.
[24] F.C. Bernstein, T.F. Koetzle, G.J.B. Williams, E.F.J. Mayer, M.D. Brice, J.R. Rodgers, O. Kennard, T. Shimanouch, M. Tasumi, J. Mol. Biol. 112 (1977) 535.
[25] P. Dauber-Osguthorpe, V.A. Roberts, D.J. Osguthorpe, J. Wolff, M. Genest, A.T. Hagler, Proteins 4 (1988) 31.
[26] W.L. Jorgensen, J. Chandraseklar, J.D. Madura, R.W. Impey, M.L. Klein, J. Chem. Phys. 79 (1983) 926.
[27] A. Shrake, J.A. Ruple, J. Mol. Biol. 79 (1973) 351.
[28] G. Toscano, D. Pirozzi, M. Maremonti, L. Gianfreda, G. Greco, Catal. Today 22 (1994) 489.
[29] B. Tuccio, E. Ferre, L. Comeau, J. Chem. Technol. Biotechnol. 55 (1992) 17.
[30] S. Nath, Biotechnol. Bioeng. 49 (1996) 106.
[31] B. Robson, J. Garnier, Introduction to Protein and Protein Engineering, Elsevier, Amsterdam, 1986, p. 587.
[32] T. Albert, in: G.D. Fasman (Ed.), Prediction of Protein Structure and the Principles of Protein Conformation, Plenum Press, New York, 1987, p. 161.
[33] E.M. Pedone, S. Bortolucci, M. Rossi, M. Saviano, J. Biomol. Struct. Dyn. 16 (1998) 437.
[34] R.M. Daniel, Enzyme Microb. Technol. 19 (1996) 74.
[35] K.E.S. Tang, K.A. Dill, J. Biomol. Struct. Dyn. 16 (1998) 397.