A Novel Strategy for Thermostability Improvement of Trypsin Based on \( N \)-Glycosylation within the \( \Omega \)-Loop Region

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Introduction

The ability to engineer proteins for improved thermostability is an exciting and challenging field since it is critical for broadening the industrial applications of recombinant proteins. The approach of rational design has been proven to be a powerful tool to improve the thermostability of proteins [6, 7, 9]. Compared with directed evolution, the rational design methods are faster and universal, and have the potential to be developed into algorithms that can quantitatively predict the stabilities of the designed sequence [27]. Using the rational design method of biochemical dating, the thermostability of proteins has been thought to originate from the simultaneous effect of different interactions, such as hydrophobic interactions, disulfide bonds, salt bridges, and hydrogen bonds [36]. The critical point of designing thermostable proteins was to pinpoint the weak spots or the “unfolding nucleus.” Once the weak spots were identified, further thermostability could be achieved by optimizing the weak spot regions.

\( \Omega \)-Loops are found almost exclusively at the protein surface and they were shown to be involved in protein function and molecular recognition [10]. Moreover, these loops may also play significant roles in protein stability or folding. Single and multiple mutations in some \( \Omega \)-loops could have drastic effects on overall protein stability [15, 24]. Hydrogen-exchange experiments indicated that the \( \Omega \)-loop was the most susceptible element in cytochrome \( c \) denaturation [2]. By minimizing conformational flexibility through the hydrogen bonds and the existence of the Pro residue, the extra helix and adjacent loops in cytochrome \( c_{\text{SSS}} \) could help protect against protein denaturation [23].
Many methods have been applied to stabilize the thermostability of proteins. Among them, glycation is gaining increasing attention owing to the fact that it is a nontoxic method and can be easily performed. There is substantial evidence indicating that N-linked oligosaccharides are important for protein folding and stability [14, 22, 35]. With polysaccharides attached enzymatically to the asparagine, serine, and threonine side chains, naturally glycosylated proteins have increased structural stability. It would clearly be advantageous if improved structural stability could be achieved for nonglycosylated proteins by the covalent attachment of sugars or polysaccharides. As one of the members in the serine protease family, trypsin (E.C. 3.4.21.4) is commonly used as a tool for enzyme protein cleavage and digestion in the field of biotechnology. For instance, it is used for the activation of some zymogens and as a digester of proteins prior to the analysis of peptide fingerprints during mass spectrometry [11, 18]. When used for these purposes, the enzyme often suffers from autolysis and denaturation, which would be ideally solved by improved stability [11]. Moreover, the apparent increase in the thermostability of the trypsin could be used to digest native ribonuclease without the need for prior denaturation [25]. In this work, N-linked oligosaccharides have been introduced within the Ω-loop regions to enhance the thermostability of porcine trypsin. As a result, a more thermostable mutant protein was expressed in Pichia pastoris GS115, and its enzymatic properties, including its activity, secretion, thermostability, and temperature optima, were characterized and compared with the wild-type enzyme.

Materials and Methods

Microorganisms, Plasmids, and Reagents
Plasmid pPIC9K and the P. pastoris host strain GS115 were obtained from Invitrogen (Thermo-Fisher Scientific, USA). The Escherichia coli Top 10 host strain was from our laboratory collection. Phusion High-Fidelity DNA Polymerases were obtained from Thermo-Fisher Scientific. The restriction enzyme Sall was purchased from Takara (China). The BCA protein assay kit was purchased from Dingguo Changsheng Biotechnology Co., Ltd. (China). All other chemicals used in the experiments were of analytical grade.

Design and Selection of the Proper N-Glycosylation Sites
The crystal structure of porcine trypsin (PDB ID: 4AN7) was obtained from the RCSB Protein Data Bank (http://www.rcsb.org). The protein structure information, including its active sites, disulfide bond, β-sheet, and Ω-loop regions, were shown and analyzed using the Visual Molecular Dynamics (VMD) software. Molecular dynamics (MD) simulations were performed using GROMACS ver. 4.5.5 [33], implementing the Gromos96.1 (53A6) force field [28] to examine the flexible regions of the protein. The protein was solvated with the Simple Point Charge water molecule in a cubic box using the GROMACS software package. The minimum distance between any atom in the protein and the box’s walls was set to 1.1 nm. Sufficient Cl− ions were added to neutralize the positive charges in the system. The water box and the whole system were minimized using the steep descent method (1,000 steps) in addition to the conjugate gradient method (3,000 steps). Then, a 20 ps position-restrained MD simulation was performed at 300 K. Finally, an unrestrained MD simulation was performed on the entire system at 300 K for 10 ns. The Particle Mesh Ewald method was used to treat long-range electrostatic interactions and the cut-off value for van der Waals’ interactions was set at 1.0 nm. Trajectories were saved at every 1,000 steps (2 ps), and post-processing and analysis were performed using relevant tools in GROMACS. The root mean square fluctuation (RMSF) value of backbone atoms against each residue was calculated for the enzyme using g_rmsf. The proper N-glycosylation sites were chosen based on analysis of the crystal structure information and potential N-glycosylation sites (Asn-Xaa-Ser/Thr consensus sequences) within the Ω-loop region.

Construction of the Expression Vector and Site-Directed Mutagenesis
The wild-type porcine trypsinogen gene (GenBank Accession No. CS583166.1) was synthesized by GENEWIZ Inc. (China) and inserted into the pPIC9K vector. The recombinant plasmid pPIC9K-Try was linearized by Sall and integrated at the His6 locus on the competent Pichia genome through electroporation using a Bio-Rad Micropulser Electroporator (Bio-Rad, USA). The trypsinogen variants used in this study were constructed using the modified Quik Change method [38]. The mutagenic primers (synthesized by GENEWIZ Inc.) are listed in Table 1. The mutant plasmids were verified by DNA sequencing (GENEWIZ Inc.); they were then transformed into the P. pastoris host strain, GS115.

Expression and Purification of Enzymes in P. pastoris Recombinants
The selected clones were inoculated into 25 ml of buffered glycerol-complex medium and they were subsequently incubated overnight at 30°C with shaking at 250 rpm. When the OD600 reached 2-6, the cells were harvested via centrifugation at 5,000 × g for 5 min. To induce protein expression, the cell pellets were resuspended in 25 ml of buffered methanol-complex medium and transferred into a 100 ml culture flask. The culture was shaken for 96 h at 28°C and supplemented with 100% methanol to a final concentration of 1% (v/v) every 24 h. The fermentation supernatant was centrifuged and collected after being induced with methanol for 96 h. The supernatant was filtered through a 0.22 µm filter, and then was dialyzed through a Millipore 10 kDa cut-off membrane to exclude ions and salts, resuspended in buffer A (20 mM citric acid, pH 3.0), and loaded on a SP Sepharose HP (GE Healthcare, UK), which was pre-equilibrated with at least 5 column volumes
buffer A until the absorbance reached a steady baseline. The protein was eluted using a 0–500 mM sodium chloride gradient, and the active fraction of peak was pooled, dialyzed against the 50 mM Tris–HCl buffer (pH 7.8). Then, 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was applied to test the result of chromatography.

Table 1. Primers for the site mutation.

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Mutagenic primers</th>
<th>( T_m/°C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A73S</td>
<td>5'-AACAAATTTAATGCTTTAATTTATATTACATCC-3' (forward)</td>
<td>51.7</td>
</tr>
<tr>
<td></td>
<td>5'-GACATTAATATGTTTACACTTCCCAAAAC-3' (reverse)</td>
<td>51.3</td>
</tr>
<tr>
<td>N84S</td>
<td>5'-TCCAAATTTAATGCTTTTACCTTGGATAATGATATTATC-3' (forward)</td>
<td>55.5</td>
</tr>
<tr>
<td></td>
<td>5'-GACCATTAAAAATTGAGTGAATAATTTTACAG-3' (reverse)</td>
<td>54.4</td>
</tr>
<tr>
<td>R104S</td>
<td>5'-GCTACATTAAATTCATCTGTTGTACCTACATTTGTTG-3' (forward)</td>
<td>55.7</td>
</tr>
<tr>
<td></td>
<td>5'-AGATGAATTTAAGTGCAGAGACAAATTTAAC-3' (reverse)</td>
<td>56.0</td>
</tr>
</tbody>
</table>

*Nucleotides that were changed to encode Ser are underlined.

Deglycosylation of Mutant Trypsinogens

The N-glycosylation levels of the trypsinogen mutants (A73S, N84S, and R104S) were confirmed by deglycosylation using Endo H (New England Biolabs, USA) according to the manufacturer’s protocol. The enzyme samples (containing 50 \( \mu \)g protein of trypsinogen) were boiled for 10 min in denaturing buffer containing 0.4 M DTT and 0.5% SDS to fully expose the glycosylation sites, and deglycosylation was then performed by treatment with 1,000 NEB units of Endo H in 50 mM sodium citrate (pH 5.5) for 2h at 37°C. After the reaction, SDS-PAGE was performed to detect the separation of reaction products.

Activity Assay

The trypsin activity of the sample was estimated using Na-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as a substrate. Since there was an oligopeptide (DDDDK) in the N-terminus of the shortened porcine trypsinogen, enterokinase was required for its activation. The culture was processed by ultrafiltration using a Millipore filter (MW: 10,000 cut-off) and rebuffered by an equivalent volume of 67 mmol/l of sodium phosphate buffer (pH 7.6). Then, 100 \( \mu \)l of the processed sample was mixed with 2 \( \mu \)l of enterokinase and incubated at 25°C for 4 h. At continuous time points, sample mixtures (100 \( \mu \)l) were immediately mixed with 2 \( \mu \)l of assay buffer (67 mmol/l of sodium phosphate buffer at a pH of 7.6 at 25°C, containing 0.25 mmol/l of BAEE) and 100 \( \mu \)l of 1.0 mmol/l HCl. The change in absorbance at 253 nm was then monitored in a Shanghai Youke UV-765 PC spectrophotometer. One BAEE unit (U/ml) will produce a 1.0 mmol/l of sodium phosphate buffer at a pH of 7.6 at 25°C in a reaction volume of 3.2 ml (1 cm light path). The trypsin activity was calculated according to the following formula:

\[
\text{BAEE(U/ml)} = \frac{\text{AOD}_{253} \text{min} \times \text{df}}{0.001 \times 0.1}
\]

Far UV Circular Dichroism (CD) Spectroscopic Analysis

CD spectra were taken on a Jasco J-810 spectropolarimeter (Jasco, Japan), which was continuously purged with nitrogen. Measurement was performed at 25°C for a protein concentration of 0.5 mg/ml in 50 mM Tris–HCl buffer (pH 7.8) using the cell with 1.0 mm pathlength for far-ultraviolet CD spectra (190–260 nm). The values of the scan rate, response, and band width were 200 nm/min, 1.0 sec, and 1.0 nm, respectively.

Measurement of Thermostability

To study the half-lives of the thermal inactivation of the trypsin variants, the purified trypsins (200 \( \mu \)g/ml) in 67 mM of NaHPO\(_4\) (pH 7.6) were incubated at 50°C for different time intervals, ranging from 0 to 180 min; the trypsins were then cooled on ice for 30 sec. The enzyme activities were assayed under room temperature (25°C) and any remaining activity was recorded as a percentage of the original activity. The data were fitted to first-order plots and analyzed, with the first-order rate constant (\( k_d \)) measured by the linear regression of ln (remaining activity) versus the incubation time (t). The time required for the residual activity to be reduced by half (\( t_{1/2} \)) of the trypsin variants at 50°C was calculated by the equation

\[
t_{1/2} = \frac{\ln 2}{k_d}
\]

where \( df \) represents the dilution factor.
and $\theta_0$ are the corresponding values for the native and denatured states.

Effects of Temperature on Mutant Trypsins

The optimal temperature ($T_{opt}$) was determined by measuring the activity at a series of temperatures ranging 25°C–95°C at 5°C intervals. The percent age of the remaining activity was determined by considering the activity of the wild-type enzyme incubated at an optimal temperature as a control (100%).

Digestion of Native Ribonuclease A

Native ribonuclease A (Shanghai Sangon Biotech Co., Ltd, China) and wild-type porcine trypsin or glycated porcine trypsin were combined and incubated at 37°C for 3 h. The ratio of the protein to enzyme was 500:1. The digestions were analyzed by SDS-PAGE for each trypsin mutant and wild-type enzyme.

Results

A Three-Dimensional Model of Porcine Trypsin and the Selection of Proper N-Glycosylation Sites

The three-dimensional model of porcine trypsin was visualized and analyzed using the VMD software (Fig. 1). The soluble porcine trypsin is mainly composed of two $\beta$-barrels (fragments 1–96 and 122–221). The former (fragment 1–96) consists of only one disulfide bond, and fewer than five disulfide bonds are found in the latter (fragment 122–221). As disulfide bonds are covalent linkages, they play important roles in stabilizing the protein structure [1, 31]; hence, we may infer that the fragment 1–96 region may be more flexible.

Furthermore, MD simulations were performed using Gromacs ver. 4.5.5 to determine the most flexible region within the $\beta$-barrels. The RMSFs of the backbone atoms against each residue were calculated for the enzyme (Fig. 2). The higher the value of RMSF, the more flexible the protein structure. From the calculation results, the average RMSF value of residual number 57–67 and 78–91 was 0.128 and 0.096, respectively, larger than the rest of the protein. (The average RMSF value of the rest residual number within fragment 1–96 is 0.086.) Therefore, according to the result of the MD simulation, Fragments 57–67 and 78–91 revealed higher RMSF values and showed greater flexibility when compared with the rest of the protein within the fragment 1–96 region. Both of these two fragments contain an $\Omega$-loop, indicating that $\Omega$-loops are flexible regions in the protein.

In addition, we further analyzed the crystal structure information within two $\Omega$-loop regions and their potential N-glycosylation sites (Fig. 3). The $\Omega$-loop region (fragment 57–67) contains a Ca$^{2+}$-binding site, which might feature intrinsic stability and affect overall protein stability [10]. Therefore, we introduced the N-glycosylation site (N71 and N102) in its wing chain to avoid affecting the metal ion-binding domain. N80, N82, N84, and N88 are potential N-glycosylation sites within fragment 78–91. Considering that N84 and N88 are near the enzyme’s active sites, the
introduction of N-linked oligosaccharides could influence enzymatic activity owing to space steric hindrance. Thus, three potential N-glycosylation sites (N71, N82, and N102) were ultimately chosen to improve the protein’s thermostability.

**Identification of N-Glycosylation Levels of Trypsinogen Variants**

The mutant and wild-type trypsinogens expressed by *P. pastoris* were assessed by SDS-PAGE, as shown in Fig. 4. The N-glycosylation levels of the heterologous proteins were determined by the change in their apparent molecular mass following mutation. From the results, the proteins with molecular mass of ~26 kDa were considered non-glycosylated forms (Fig. 4, lane 1), and the proteins with higher molecular mass of ~28 kDa were considered glycosylated forms (Fig. 4, lanes 5 and 7). Furthermore, the N-glycosylation levels of the trypsin mutants were confirmed by deglycosylation using the Endo H (NEB), and the Endo H enzyme was also assessed by SDS-PAGE (approximately 28 kDa). From the results, the apparent molecular mass of the wild-type trypsin was not changed by deglycosylation (Fig. 4, lane 2), whereas those of N84S and R104S decreased to approximately 26 kDa after deglycosylation (Fig. 4, lanes 6 and 8). The SDS-PAGE of the A73S mutant did not show any bands (Fig. 4, lane 3), which indicated that the A73S variant was not expressed. After deglycosylation, the figure (Fig. 4, lane 4) just showed the Endo H enzyme. These results demonstrated that the introduction of an N-glycosylation site to N71 significantly inhibited trypsin expression.

**Thermal Stability of N-Glycosylation**

To determine the impact of N-glycosylation on trypsin stability, the kinetic stability of trypsin mutants was measured. Purified proteins were incubated at 50°C and the remaining activity was measured at various times. The results are shown in Figs. 5 and 6; the $k_d$ of the trypsin...
mutant, N84S, was less than that of the wild type at 50°C, whereas the $k_d$ of R104S was larger than that of the wild type, indicating that the wild-type trypsin had deactivated more rapidly than N84S and more slowly than R104S (Fig. 6). The mutant N84S retained 85% of its original activity after about 60 min at 50°C, whereas the wild-type and R104S retained residual activities of 77% and 53%, respectively (Fig. 5). The half-lives of the wild-type trypsin and the N84S mutant were 155.3 min and 333.2 min, respectively (Table 2). These results indicate that N-glycosylation at N82 contributes to the thermal stability of trypsin.

**CD Spectroscopy Analysis**

To determine the effect of N-glycosylation on recombinant porcine trypsin from *P. pastoris*, the secondary structures of recombinant trypsin mutants were analyzed by CD spectroscopy and compared with those of the wild type. As shown in Fig. 7, the spectrum of the wild-type trypsin showed a single minimum near 210 nm, indicating a β-sheet secondary structures, comparable to what has been reported for trypsin [12, 25]. Besides this, the average CD spectra of N84S and R104S were similar to that of the wild-type trypsin, suggesting that the mutations did not disrupt the secondary structure of trypsin and maintained a conformation close to the native protein.

In addition, to confirm the thermal effect on the conformational stability of mutant enzymes, their thermodynamic stability was measured. The proteins’ melting temperature ($T_m$) was assayed using CD spectroscopy (Fig. 8). The thermal denaturation of proteins was detected by monitoring ellipticity changes at 215 nm over the temperature range of 25°C–95°C. The $T_m$ value for the wild-type trypsin was 69.4°C, whereas the N84S and R104S mutants showed $T_m$ values of 78.5°C and 66.0°C, respectively.

### Table 2. Thermostability of the porcine trypsin mutants.

<table>
<thead>
<tr>
<th>Trypsin</th>
<th>$t_{1/2}$ (min)</th>
<th>$T_m$ (°C)</th>
<th>$T_{opt}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>153.35</td>
<td>69.4</td>
<td>75</td>
</tr>
<tr>
<td>N84S</td>
<td>333.24</td>
<td>78.5</td>
<td>80</td>
</tr>
<tr>
<td>R104S</td>
<td>98.32</td>
<td>66.0</td>
<td>65</td>
</tr>
</tbody>
</table>

**Fig. 6.** Investigation of the half-life of the wild-type and mutant trypsins.

**Fig. 7.** Circular dichroism spectra of the wild-type and mutant trypsins at 25°C and at a concentration of 0.5 mg/ml.

**Fig. 8.** Temperature-induced unfolding measured using CD spectroscopy for wild-type and mutant trypsins.
respectively (Table 2). The results demonstrated that the N84S mutant displayed superior thermostability when compared with that of the wild type, whereas R104S did not show improved thermostability.

**Digestion Specificity of N-Glycosylation**

The digestion specificities of the wild-type and N-glycosylation mutants were measured using RNase A as the enzyme substrate at 37°C. All enzymes, except for A73S, indeed digested native RNase A (Fig. 9), indicating that introducing N-glycosylation within the native trypsin had no effect on enzyme digestion specificity.

**Optimum Temperature for N-Glycosylation**

The optimum temperature for N-glycosylation for the wild-type trypsin, R104S, and N84S was 75°C, 65°C, and 80°C, respectively (Table 2). Furthermore, the wild type showed higher activity at low temperatures (50°C–65°C) than N84S, whereas the latter was more active at higher temperatures (70°C–85°C). The N84S mutant enzyme still retained 86% of its activity when compared with the wild-type enzyme (which retained 45% of its activity) after incubation at 90°C (Fig. 10), which could be extensively used to digest native proteins at high temperature without the need for prior denaturation during mass spectrometry.

**Discussion**

The selection of mutation sites in enzymes or proteins is the most critical step in the rational design method [36]. Inappropriate positions can affect folding and destroy the structure of an active protein [37]. Therefore, two main selection principles have been considered: one was that the chosen mutation sites should be situated away from the active center to guarantee that the overall structure and catalytic activity of the target enzyme were less affected. The other principle was that the N-glycosylation sites would be introduced in a flexible region in the protein. In the present work, MD simulation predicted that two Ω-loops represented the flexible region within the protein structure (Fig. 2). Several research studies have indicated that the Ω-loop was the most susceptible part in protein denaturation [2, 23]; thus, rigidifying the Ω-loop region within trypsin may help to enhance its thermostability.

Glycosylation is one of the most naturally occurring modifications of a protein’s covalent structure [34]. The effects of N-glycans on enzyme structure and activity have been well reported, due to their importance in biotechnological applications [4, 8, 26]. Several roles have been suggested for the carbohydrate moieties of proteins, one of which is the stabilization of protein conformation. Moreover, the great stabilization effect is achieved when the attached glycan is located at a more flexible region (e.g., a loop) of the protein [29]. Therefore, glycosylation site-directed mutations (A73S, N84S and R104S) were introduced within the Ω-loop region and its wing chain to improve the stability of trypsin. The SDS-PAGE analysis indicated that the N84S and R104S mutants showed obviously increased molecular masses when compared with the wild type (increasing from 26 to 28 kDa). It has been reported that the N-glycan chains of the glycoproteins secreted by *P. pastoris* are a high-mannose type (Man₉GlcNAc₂ or Man₉GlcNAc₃).
with a molecular mass close to 2 kDa [5, 14, 20], which may explain why the molecular masses of the wild-type and mutant proteins are displayed at intervals of ~2 kDa.

Furthermore, several research studies have indicated that N-glycosylation could significantly influence enzyme activity and secretion [13, 16, 30]. The results reported herein demonstrated that the addition of N-glycosylation at the N82 and N102 positions of the porcine trypsin improved the trypsin production level when compared with the wild-type enzyme, but attachment of an N-glycan at N71 site would drastically inhibit protein expression (Table 3). N-Glycosylation is a common form of post-translational modification in P. pastoris and it frequently affects proper protein folding and secretion [3]. Misfolded polypeptides are subject to endoplasmic reticulum-associated degradation, ubiquitinated, and degraded by the 26S proteasome in the cytoplasm [3, 19]. In this paper, the addition of N-glycosylation at the N82 and N102 positions of the porcine trypsin increased protein expression levels, implying that there is a positive role of N-glycosylation in the proper folding of the enzyme [13]. In contrast, introduction of an N-glycosylation site to N71 may influence the proper translation or correct folding of the enzyme, indicating that trypsin was not produced, or that the misfolded protein might have been rapidly degraded. These results are consistent with those of a few studies, which reported that the attachment of the N-glycans of glycoproteins exerted negative effects on their secretion [16, 30]. Furthermore, the enzymatic activity and digestion specificity of mutants have been studied. Our data indicated that there were no decreases in activity for the glycated trypsin (Table 3 and Fig. 9). Owing to the fact that the N-glycosylation sites added in trypsin are located far away from the substrate-binding pocket, the glycoproteins might have no significant influence on substrate specificity.

In addition, the role of the N-glycosylation sites at N82 and N102 on protein stability was investigated by measuring the half-lives of their thermal inactivation ($t_{1/2}$) and melting temperature ($T_m$). The recombinant glycated trypsin (N84S) exhibited better thermostability (after being incubated at 50°C for 60 min, it still retained 85% of its original activity) than the wild-type enzyme (Fig. 5). Moreover, the CD spectra of the native and glycated enzymes at ambient temperature (Fig. 7) confirmed that N-glycosylation site-directed mutations did not disrupt the secondary structure of trypsin, and there were no decreases in activity for the mutant enzymes (Table 3 and Fig. 9). Hence, we may infer that the addition of N-glycosylation at the N82 position increases the protein stability, based on the fact that the protein melting temperature ($T_m$) of glycated protein was significantly increased compared with that of the wide-type trypsin (Fig. 8). Glycosylated forms of a protein are often less susceptible to thermal inactivation than their unglycosylated counterparts, probably because glycosylation renders the protein more resistant to unfolding [21, 32]. Pham et al. [25] also claimed that glycation did increase the resistance to unfolding at higher temperatures (as reflected in the increased $T_m$ values), and this effect is magnified owing to the decreased rate of autolysis in the glycated enzymes. As for proteolytic enzymes such as trypsin and chymotrypsin, protein unfolding at elevated temperatures results in increased exposure of the susceptible sites to autolytic cleavage, which prevents refolding of the denatured protein molecules [25]. The slight decoration of N-linked glycosylation modification on the N82 positions of the porcine trypsin would increase the protein’s conformational stability, which in turn might result in a decreased rate of autolysis. Moreover, the N-linked glycan moiety is a negatively charged molecule with a large branched structure [17]; the thermostability of trypsin might be improved by hydrophilic sugar components providing repulsive interactions, which may prevent the formation of large aggregates during heating at high temperatures.

Glycoengineering is a new approach that is currently being applied to improve the thermostability and activity of a given enzyme. In this study, a new strategy to improve the thermostability of trypsin was proposed. MD simulation was used to investigate the molecular stability of trypsin, and oligosaccharides were added within the flexible region of the protein through native glycosylation modified by P. pastoris GS115. Based on this strategy, we successfully improved the thermostability of trypsin without altering its activity. This strategy provided a referential method through which to engineer the thermostability of other industrial enzymes; it also has the potential for therapeutic application.

<table>
<thead>
<tr>
<th>Protein concentration (µg/ml)</th>
<th>Protein concentration (µg/ml)</th>
<th>Protein concentration (µg/ml)</th>
<th>Protein concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative activity</td>
<td>Relative activity</td>
<td>Relative activity</td>
<td>Relative activity</td>
</tr>
<tr>
<td>Trypsin</td>
<td>WT</td>
<td>A73S</td>
<td>N84S</td>
</tr>
<tr>
<td></td>
<td>364.0</td>
<td>–</td>
<td>602.3</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>–</td>
<td>0.98 ± 0.02</td>
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</table>

Table 3. Protein concentration of trypsin and the activities of glycated trypsin derived from the hydrolysis of BAEE.
Acknowledgments

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