Improvement of Physicochemical and Enzymatic Properties of Bovine Trypsin by Non-enzymatic Glycation

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Bovine trypsin was modified with glucose through the Maillard reaction at 50°C and 65% RH for various periods (1 to 8 days). Tryptic activity against both benzoyl-L-arginine-p-nitroanilide and two protein substrates was enhanced with increases in the reaction period, and reached the maximum after a 4-day reaction. Although there were no big differences in pH dependency of trypsin activity between native and modified trypsins, the $K_m$ of the modified trypsin decreased to about half the native one. The modified trypsin retained its original activity almost completely after incubation in a buffer solution of pH 8.0 at 37°C for 72 h, while native trypsin was greatly inactivated. Furthermore, trypsin activity at high temperature, residual activity after heating, and differential scanning calorimetric analysis showed that the modified trypsin was more heat-stable than native trypsin.

A number of attempts have been made to improve physicochemical and functional properties of proteins and enzymes by various chemical modifications.1,2) Chemical glycation methods and reagents are also available to couple carbohydrates to proteins, resulting in the production of "neoglycoproteins".3) Chemical glycation to proteins have been done to immobilize enzymes,4) to change protein immunological properties,5) to increase the heat stability of enzymes,6) and to improve protein functional properties.7,8)

Protein amino groups react with reducing sugars under mild conditions through the Maillard reaction, resulting in protein glycation. The Maillard reaction in the early stage was reported to increase solubility and heat stability of proteins.9,10) We have also reported that ovalbumin gained high solubility and heat stability in the early stage of the Maillard reaction with glucose,11) and that immunological properties of ovalbumin and $\beta$-lactoglobulin were altered by the glycation through the Maillard reaction with glucose and lactose, respectively.12,13)

In this study, the Maillard reaction with glucose was used for a chemical glycation of bovine trypsin to improve physicochemical and enzymatic properties of the trypsin. The glycation was found to improve not only the protein heat stability but also catalytic properties of the trypsin.

Materials and Methods

Materials. Trypsin from bovine pancreas (Type II), lysozyme, and $\alpha$-lactalbumin were obtained from Sigma Chemical Co. Glucose and $\alpha$-N-benzoyl-L-arginine $p$-nitroanilide (BAPA) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka) and the Peptide Institute Inc. (Osaka), respectively.

Modification of trypsin. Trypsin (20 mg) and glucose (10 mg) (1:0.5, w/w) were dissolved in 10 ml of distilled water and the pH of the solution was adjusted to 7.7 with diluted NaOH solution. The trypsin concentration was measured by the value of $A_{280} \times 1.54$. Samples of the mixed solution were pipetted into each tube (1 mg protein/tube). The separated solutions were freeze-dried and kept at 50°C and 65% relative humidity (RH) for various periods to accelerate the Maillard reaction.14) The powdered samples were stored at -80°C before use.

A sample of the trypsin and glucose mixture was kept at -80°C immediately after the lyophilization, and used as a control. These was no distinction between the control and native trypsins for all physicochemical and enzymatic properties examined in this study.

Measurement of trypsin activity. BAPA solution (100 $\mu$g/ml) was prepared with the Tris-HCl buffer (pH 8.0) containing 50 mM CaCl2. The BAPA substrate solution (700 $\mu$l) warmed at 37°C was added to a spectrophotometer cuvette kept at 37°C. The native or glycerated trypsin was dissolved in the same buffer (1 mg/ml) and the enzyme solution (20 $\mu$l) was added to the cuvette. The change in the absorption at 420 nm was monitored by a spectrophotometer with an autorecorder. The trypsin activity was represented as the initial reaction rate ($dA_{420}/dt$). The concentration of the catalytic product by trypsin was calculated using the absorption coefficient of p-nitroaniline at 420 nm (ε 38,000).

Both lysozyme and $\alpha$-lactalbumin solution (1 mg/ml) were prepared with the same buffer solution as in the BAPA solution. The native or glycerated trypsin solution was added to the substrate solutions of lysozyme or $\alpha$-lactalbumin (enzyme: substrate = 1:20, w/w) and incubated for 2, 4, 6, 8, and 10 h at 37°C. Enzyme activity was stopped with addition of SDS-PAGE sample buffer solution, and each substrate solution was kept at -20°C. The effects of glycation on proteolytic activities of trypsin were studied by the comparison of SDS-PAGE patterns of either lysozyme or $\alpha$-lactalbumin treated with native and glycerated trypsin.

Measurement of free amino group content. The amino group content of the glycerated trypsin was measured by fluorometric methods using fluorescamine (Roche Co., Ltd.) by the method of Böhlen et al. (1973).15) The emission fluorescence at 475 nm was measured as described previously.16)

Amino acid analysis. Native and glycerated trypsins were hydrolyzed in evacuated sealed tubes by 6 N HCl at 110°C for 24 h. The content of fructosyl-lysine (Amadori compound) was measured from the furosine content by the method of Bujard and Finot (1978).17)

Differential scanning calorimetry. Differential scanning calorimetry (DSC) thermograms were recorded on a Daini Seikocho Model SSC/560 thermal analyzer with a DSC cell programmed at the temperature increase rate of 2°C/min. Samples of 50 $\mu$l of 5% protein solution dissolved in phosphate buffer (I = 0.1, pH 7.0) were pressure-sealed in silver pans weighing approximately 1.47 g. A sealed pan that contained a volume of the buffer equal to that of the sample was used as a reference. The de-naturation temperature was defined as the temperature of the endothermic peak.

Electrophoresis. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)
(12.5% and 15% acrylamides) was done by the method of Laemmli.17)

Results and Discussion

Increase in apparent trypic activity upon modification with glucose

The catalytic activity toward BAPA substrate was measured for trypsins reacted with glucose for various periods (0 to 8 days). Figure 1 shows the increase in activity and the decrease in free amino groups of trypsin during the 8-day reaction with glucose. The activity rose gradually with increases in the reaction time for 4 days, and then decreased slowly in the subsequent reaction time. The trypsin reacted with glucose for 4 days showed the highest apparent activity, which was about 1.6 times high as that of native trypsin. Trypsin treated in the same manner without glucose showed no activity change during the 8-day treatment. Thus trypsin was not inactivated by incubating it at 50°C for 8 days, and the apparent activity was rather raised by the reaction with glucose through the Maillard reaction.

The free amino group content of trypsin reacted with glucose decreased to 40% of the native one after the 2-day reaction with glucose, and this value kept constant until the end of the reaction period (8th day). On the other hand, trypsin treated without glucose did not lose the free amino groups during the 8-day treatment. This indicates that about 60% of lysine residues of a trypsin molecule (8.4 mol/mol) were modified with glucose within the 2-day reaction. The apparent activity of trypsin still rose after the 2-day reaction, suggesting that the high activity of glycated trypsin was not simply due to blocking of the free amino groups.

The content of Schiff base and the Amadori rearrangement compound, fructosyl-lysine, in the trypsin glycated for the 4-day reaction was estimated by amino acid analysis. The contents of Amadori rearrangement compounds in native and glycated trypsins were compared in Table I. About 30% of lysine residues of a trypsin molecule was converted to fructosyl-lysine (Amadori rearrangement compound), and about 15% of lysines were the Schiff base form. Free lysine was about 40%, which was in good agreement of the results from free amino group measurement (Fig. 1). The remaining 15 to 20% of lysines might be converted into further degradation products through the advanced steps of the Maillard reaction. Arginine is reactable with glucose in the amino-carbonyl reaction, but no detectable loss of arginine residues was observed for the trypsin reacted with glucose in the 4-day treatment. Loss of lysine and arginine residues was not observed in the trypsin treated without glucose.

SDS-PAGE patterns of native and glycated trypsin after the 4-day reaction are shown in Fig. 2. The mobility of glycated trypsin was a little lower than that of native trypsin. Although the glycated protein band was rather broad, no polymerized protein bands could be detected. In our previous work,11 we found that the amount of polymerized protein increased greatly in longer reaction times during dry storage with glucose. The reason why the trypsin activity of glycated protein decreased after longer reaction times might be due to the formation of polymerized proteins.

The trypsin glycated by the 4-day reaction was used for the following enzymatic and physicochemical experiments.

Glycated trypic activity toward protein substrates

The effects of glycation on proteolytic activities of trypsin were further studied by the comparison of SDS-

![Fig. 1. Changes of Trypsin Activity and Free Amino Groups in Trypsin upon Glycation.](image)

Increases in catalytic activity (A) and decreases in free amino groups (O) of trypsin reacted with glucose for various periods (0–8 days) at 50°C and 65% R.H. Trypsin activity (Δ) and amino groups (O) treated similarly without glucose were also analyzed for comparison.

Table I. Modification Rate of Lysine in Trypsin Incubated with Glucose through Amino-carbonyl Reaction (mol/mol protein)

<table>
<thead>
<tr>
<th></th>
<th>Try-O</th>
<th>Try-G-4</th>
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</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>14.0</td>
<td>9.5</td>
</tr>
<tr>
<td>Furosine</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Reactive lysine</td>
<td>14.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Free lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schiff base</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructosyllysine</td>
<td>0.0</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* Measured by amino acid analysis.
* \[ \text{[reactive lysine]} = \text{[free lysine]} + \text{[Schiff base]} = \text{[lysine]} - 0.5(\text{fructosyllysine}) \]
* \[ \text{[fructosyllysine]} = 4.9(\text{furosine}) \]

The fructosyllysine, Schiff base, and free lysine contents were calculated as below by the method of Bujard and Finot (1978).16

![Fig. 2. SDS-PAGE Patterns of Native Trypsin and Glycated Trypsin.](image)

Lane A, native trypsin; B, trypsin glucose mixture; C, trypsin reacted with glucose for 4 days.
Physicochemical and Enzymatic Properties of Glycated Trypsin

Fig. 3. SDS-PAGE Patterns of Protein Substrates Hydrolyzed with Trypsin and Glycated Trypsin.
Lysozyme (upper) was treated with native and glycated trypsins for 2, 4, and 6 h at 37°C. α-Lactalbumin (lower) treated with native and glycated trypsin for 2, 4, 6, 8, and 10 h at 37°C. Glycated trypsin was prepared by a 4-day reaction.

PAGE patterns of either lysozyme or α-lactalbumin treated with native and glycated trypsin (Fig. 3). Some cleavage product bands were observed in the lysozyme sample treated with native trypsin, but a broad cleavage product band was observed in the lysozyme sample treated with glycated trypsin. Moreover, a sharp monomer band remained in the lysozyme sample treated with native trypsin, but the monomer band became much weaker in the lysozyme sample treated with glycated trypsin. When α-lactalbumin was used as a substrate, the difference between native and glycated trypsin was much smaller. However, in 10 h of incubation, the monomer band of α-lactalbumin treated with glycated trypsin became much weaker than that with native trypsin.

Enzymatic properties of glycated trypsin

Figure 4 shows the pH dependence of catalytic activity of trypsin and glycated trypsin toward BAPA. The maximum catalytic activity was observed at pH 9.0 for both trypsins with and without glycation, and there was no great difference in the pH dependency profile between the two trypsins. Thus, glycation had no strong influence on the pH dependency of trypsic activity, i.e., the apparent trypsic activity was raised by the glycation in the whole pH region between 6 to 11.

An earlier study showed that acetylation of trypsin increased the enzyme's activity toward both p-toluensulfonylarginine amide (TAA) and benzamidine, and that the enhanced activity of acetyl trypsin relative to trypsin is due to a combination of decrease in $K_m$ and an increase in $k_{cat}$ for the modified enzyme.

Fig. 4. Effects of pH on the Activity of Trypsin and Glycated Trypsin. ○, native trypsin; ●, glycated trypsin. The glycated trypsin was prepared by the 4-day reaction. The buffer solutions of pH 3 to 6, 7 to 9, and 8 to 13 were made with sodium acetate buffer, Tris-HCl buffer, and glycine-NaOH buffer (pH 0.1), respectively. The activity was shown in values (%) relative to the maximum activity of each sample.

Fig. 5. Lineweaver–Burk Plots of Trypsin and Glycated Trypsin. ○, native trypsin; ●, glycated trypsin. The glycated trypsin was prepared by the 4-day reaction.

Table II. Kinetic Parameters of Glycated Trypsin with BAPA as a Substrate

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mm)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (1/mm s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.932</td>
<td>0.798</td>
<td>0.856</td>
</tr>
<tr>
<td>Glycated trypsin</td>
<td>0.428</td>
<td>0.634</td>
<td>1.481</td>
</tr>
</tbody>
</table>

Hence, the apparent $K_m$ and $k_{cat}$ for BAPA of trypsin and glycated trypsin were calculated from the Lineweaver–Burk plots (Fig. 5 and Table II). The apparent $K_m$ of trypsin (0.932) agreed well with the $K_m$ (0.939) reported by Robbins and Summara, while the $K_m$ of glycated trypsin was almost half that of native trypsin. The $k_{cat}$ of glycated trypsin was slight lower than that of native trypsin. Consequently, the $k_{cat}/K_m$ value, which reflects the
specific activity for BAPA, of glycated trypsin was higher than that of native trypsin. Although the value of \(k_{cat}\) for hydrolysis of BAPA was decreased slightly by the glycation, the glycation increased the affinity of trypsin for the BAPA substrate, resulting in the increase in the \(k_{cat}/K_m\) value by about 1.7 times.

The enzymatic stability of trypsin and glycated trypsin was examined at 37°C in Tris buffer, pH 8.0. The samples were incubated at 37°C for 72 h, and the trypsin activity was measured periodically during that time (Fig. 6). The glycated trypsin almost completely retained its original activity even after the 72-h incubation at 37°C. On the other hand, native trypsin lost its activity rapidly, i.e., the activity decreased to about half of original one after the initial 24-h incubation. It has been reported that loss of trypsin activity by autolytic degradation is prevented also by attachment of carbohydrate.\(^{22}\) The lactosylated L-asparaginase showed increased thermal stability and resistance to proteolytic cleavage.\(^{23}\) The protective effects of glycation on the autolytic inactivation would be partly due to the fact that the glycation through the Maillard reaction blocked lysine residues, which are the polypeptide cleavage site for trypsin hydrolysis.

**Heat-stability of glycated trypsin**

The activity of trypsin and glycated trypsin toward BAPA was measured under various thermal conditions between 30 and 80°C. As shown in Fig. 7, the apparent activity of both trypsin and glycated trypsin increased with a rise in temperature below 60°C. The activity was then lowered sharply over 60 and 70°C for trypsin and glycated trypsin, respectively. The activity ratios of glycated trypsin/tryptin were 1.5, 1.7, and 2.7 at 30, 60, and 70°C, respectively. The higher ratio (2.7) at 70°C suggested that the glycation stabilized trypsin molecules against thermal denaturation.

The residual activity of glycated trypsin after the heat treatment was compared with that of native trypsin (Fig. 8). The residual activity after heat-treatment was expressed as a percentage to their original activity. Although there were no big differences in the residual activity between the samples heated at 60°C, heat-treatment at 65°C made a
clear difference, i.e., the residual activities of trypsin and glycosylated trypsin were about 40% and 65%, respectively. An actual residual activity of the glycosylated trypsin heated at 65°C for 1 min was about 1.5-fold the activity of trypsin heated similarly. Such a heat stability of the glycosylated trypsin was also observed for samples heated at 70°C.

Thermal unfolding of the glycosylated trypsin molecule was analyzed by DSC, and compared with that of trypsin. Figure 9 shows differential scanning calorimetric patterns of trypsin and glycosylated trypsin. Trypsin showed a sharp endothermic peak at 60.5°C, which reflects its denaturation temperature. On the other hand, the glycosylated trypsin showed the denaturation temperature of 64.5°C, with a slightly broad endothermic peak. The extent of glycation would be slightly different from molecule to molecule, and this might result in the broad endothermic peak of glycosylated trypsin.

Thus, it was demonstrated that the glycosylated trypsin was more heat-stable than trypsin in the activity at high temperature, the residual activity after heating and the heat-induced unfolding temperature. It is still uncertain why glycation makes proteins stable, though several studies showed that l-asparaginase, ovalbumin, and β-lactoglobulin were stabilized by glycation and that heat stability of a glycoprotein, ovomucoid, was decreased by deglycation.

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References