**Inhibition by Equine \( \alpha_2 \)-Macroglobulin of an \( N \)-Succinyl-L-
Trialanine \( p \)-Nitroanilide-Hydrolyzing Protease
Purified from Pronase**

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The binding of an \( N \)-succinyl-L-trialanine \( p \)-nitroanilide-hydrolyzing protease (STA-protease) purified from pronase to equine \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M) was investigated in comparison with that of trypsin. The \( \alpha_2 \)M subunits (about 90000 daltons), which were electrophoretically detected in the reaction mixture of \( \alpha_2 \)M and trypsin, were undetectable in that of \( \alpha_2 \)M and STA-protease. The binding molar ratios of enzyme to \( \alpha_2 \)M were estimated from the inhibition curves of caseinolytic activity to be 1.5:1 for native and acetylated STA-protease and 2:1 for native and acetylated trypsin. The finding of greater incorporation of monodansylcadaverine into \( \alpha_2 \)M reacted with acetylated enzymes than into that reacted with the native enzymes suggests that free amino groups in the enzymes are involved at least partly in the formation of the \( \alpha_2 \)M-proteinase complexes. The numbers of thiol groups generated in \( \alpha_2 \)M bound to STA-protease and in \( \alpha_2 \)M bound to trypsin were both estimated to be approximately 4 mol per mol of \( \alpha_2 \)M by the use of thiol-directed fluorescent probes, though there were slight differences in the microenvironments of thiol groups generated in the two \( \alpha_2 \)M-proteinase complexes. The values of \( K_{\text{cat}}/K_m \) were one-half (\( \alpha_2 \)M–STA-protease complex) and one-sixth (\( \alpha_2 \)M–trypsin complex) of those of the uninhibited enzymes.

These results suggest that STA-protease binds to \( \alpha_2 \)M both covalently and noncovalently, as does trypsin, and its hydrolytic activities towards casein and low-molecular-weight substrates are inhibited to various extents.

**Keywords**—pronase; elastase-like protease; trypsin; equine \( \alpha_2 \)-macroglobulin; thiol-directed fluorescent probe; monodansylcadaverine

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Blood plasma of humans and many other vertebrates contains several proteinase inhibitors, and active proteinases administered *in vivo* are immediately trapped by \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M) and other inhibitors to form complexes.\( \text{\textsuperscript{11}} \) \( \alpha_2 \)M forms complexes with many kinds of proteinases, such as serine, thiol, carboxyl, and metallo enzymes. These \( \alpha_2 \)M–proteinase complexes retain hydrolytic activities towards low molecular weight substrates, but their proteolytic activities towards large protein substrates are decreased or eliminated. When the \( \alpha_2 \)M–proteinase complex is formed, limited proteolysis occurs and triggers a conformational change in \( \alpha_2 \)M which traps the enzyme molecule.\( \text{\textsuperscript{2}} \) The complex shows electrophoretically a fast form (F-form) which is distinct from a slow form (S-form) corresponding to native \( \alpha_2 \)M.\( \text{\textsuperscript{3}} \) Furthermore, thiol groups and active glutamyl residues appear as a result of cleavage of the internal \( \beta \)-cysteinyl-\( \gamma \)-glutamyl thiol esters at the binding site.\( \text{\textsuperscript{4-7}} \) However, the mechanism is not understood in detail.

We previously reported on the *in vivo* effect of an \( N \)-succinyl-L-trialanine \( p \)-nitroanilide-hydrolyzing protease (STA-protease) purified from Pronase on glucose metabolism in the
mouse. The enzyme suppressed the production of hyperglycemia by epinephrine and the conversion of pyruvate into blood glucose; the maximum decrease in blood sugar level was seen at 2 h after intravenous injection into fasted mice, in contrast to the case of insulin. Native proteinases injected will be trapped by $z_2M$ and other proteinase inhibitors, mainly transferred to the liver, and subsequently degraded. If the degradation products affect glucose metabolism, hypoglycemia may result.

In the present paper, the binding of STA-protease and trypsin to equine $z_2M$ was investigated by gel electrophoresis and by the determination of thiol groups and active glutamyl residues using thiol-directed fluorescent probes and monodansylcadaverine (MDNESC).

### Materials and Methods

**Chemicals**—Pronase (from Streptomyces griseus, type E, 70 PUK/mg) was a gift from Kaken Chemical Co., Tokyo. Trypsin treated with $N$-tosyl-$L$-phenylalanine chloromethyl ketone (from bovine pancreas, 10000 BAEE U/mg), acetylated trypsin (from bovine pancreas, 10000 BAEE U/mg), and MDNESC were purchased from Sigma Chemicals Co., St. Louis. Polyethylene glycol (PEG) 6000, 2,4,6-trinitrobenzenesulfonic acid (TNBS), $N$-(1-anilinonaphthyl-4)-maleimide (ANM), $N$-(dimethylaminoo-4-methyl-3-coumarinyl)maleimide (DACM), and $N$-(3-fluoranthonyl)maleimide (FAM) were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Chelating Sepharose 6B, CM-Sepharose 6B and Sepharose 4B were from Pharmacia Fine Chemicals Co., Uppsala, and a TSK-Gel 4000 SW column was from Toyo Soda Manufacturing Co., Ltd., Tokyo. N-Succinyl-$L$-triaxilane $p$-nitroanilide (Suc-(Ala)$_2$pNA) and benzoyl-$L$-arginine $p$-nitroanilide (Bz-Arg-pNA) were purchased from the Protein Research Foundation, Osaka. All other chemicals used were of analytical grade.

**Purification of STA-Protease from Pronase**—STA-protease was purified from pronase, according to the method of Ueki et al. Acetylation of STA-protease—Acetylation of STA-protease was carried out with acetic anhydride under the conditions reported by Siegel and Awad. Acetic anhydride (15 μl) was added seven times at 7 min intervals to a stirred solution of 5 mg of the enzyme in 5 ml of 5 mM sodium acetate and calcium acetate buffer, pH 6.7, containing 20% glycerol. This pH value was maintained by the addition of 2 N NaOH during the acetylation. After the removal of glycerol by gel filtration on Sephadex G-25, the acetylated enzyme was separated from the native enzyme on a CM-Sepharose column (1.5 × 15 cm) with the buffer, pH 5.0. The number of free amino groups remaining in the acetylated enzyme was determined with TNBS by the method of Okuyama and Satake.

**Determination of Enzymatic Activities**—The hydrolytic activity towards Suc-(Ala)$_2$pNA was determined in 0.05 M Tris-HCl buffer, pH 8.5. The rate of hydrolysis of Bz-Arg-pNA was determined in the buffer, pH 8.0, containing 5 mM CaCl$_2$.

**Purification of Equine $z_2M$ and $z_1$-Antitrypsin**—Equine $z_2M$ was purified by a modification of the method of Kurecki et al. and the inhibitory activity was determined by the bound trypsin activity assay method of Ganrot. PEG 6000 was added to equine serum at a concentration of 5.5%. The suspension was allowed to stand for 60 min and then centrifuged at 10 000 × g for 15 min. Further PEG 6000 was added to the supernatant to give a final concentration of 12.5%. The resulting suspension was allowed to stand for 60 min and then centrifuged again. The precipitate was dissolved in 0.02 M phosphate buffer, pH 6.0, containing 0.15 M NaCl, and the solution was dialyzed at 4°C for 24 h against the buffer. The dialysate was applied to a zinc-chelated Sepharose 6B column (2.5 × 20 cm) equilibrated with the buffer. After being washed with the buffer, the column was eluted with 0.02 M cacodylate buffer, pH 5.0, containing 0.15 M NaCl. An active protein fraction was collected and concentrated in a collodion bag. Finally, gel filtration of the concentrated fraction was performed on a Sepharose 4B column (2.5 × 45 cm) with 0.02 M phosphate buffer, pH 7.5, containing 0.15 M NaCl. The first peak was collected, concentrated, and stored at 4°C.

**Electrophoresis**—Samples were pretreated with 1 mM phenylmethanesulfonyl fluoride at 37°C for 30 min to inactivate enzymes.

Polyacrylamide gel electrophoresis (PAGE) was carried out using 5% gel in the discontinuous Tris–borate buffer system of Van Leuven et al. This system gave adequate separation of the F- and S-forms of $z_2M$.

For sodium dodecyl sulfate (SDS)—PAGE, samples were reduced and denatured in 0.0625 M Tris–HCl buffer, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol at 37°C for 30 min. The run was done in 10% gel, according to the method of Laemmli. The gel was stained for protein with Coomassie Brilliant Blue R-250 and destained by diffusion overnight.

**Determination of MDNESC and Thiol-Directed Fluorescent Probes Bound to $z_2M$**—The reaction mixture of $z_2M$ and enzyme was incubated with MDNESC or a thiol-directed fluorescent probe and subjected to high-performance
liquid chromatography (HPLC, Shimadzu LC-4A) on a TSK-Gel 4000 SW column (7.5 × 600 mm) with 0.1 M phosphate buffer, pH 6.95, containing 0.2 M NaCl and 5% isopropanol or the buffer containing 0.2 M NaCl. The fluorescence intensity of the fraction of α₂M separated was determined. The number of thiol groups generated was calculated by the method of Ellman using 1 mM 5,5'-dithiobis(2-nitrobenzoic acid).¹⁸)

**Estimation of Proteins**—Protein concentrations were determined either by the method of Lowry et al.¹⁹) or from the absorbance at 280 nm.

**Results**

**Gel Electrophoresis of Reaction Mixtures of α₂M and Proteinases**

Reaction mixtures prepared at various molar ratios of enzymes to α₂M were investigated by gel electrophoresis as shown in Figs. 1 and 2. Patterns of SDS–PAGE were distinctly different between reaction mixtures of α₂M and trypsin, and α₂M and STA-protease (Fig. 1). The 180000 daltons α₂M subunits were progressively converted to 90000 daltons species as increasing amounts of trypsin were added to a constant amount of α₂M. In the case of mixtures of α₂M–STA-protease, a small amount of the 90000 daltons α₂M species was detected in the mixture of 1:0.3 (inhibitor:enzyme), but it completely disappeared in the

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**Fig. 1. SDS–PAGE of Reaction Mixtures of α₂M and Proteinases**

Equine α₂M (1 μM) was incubated with a proteinase (0—4 μM) in 50 mM Tris–HCl buffer, pH 8.0, containing 5 mM CaCl₂ at 37°C for 5 min. Then, SDS–PAGE was carried out as described in Materials and Methods.

- a) Molar ratios of STA-protease to α₂M.
- b) Marker proteins: rabbit muscle phosphorylase b (M, 94000), bovine serum albumin (67000), ovalbumin (43000), bovine erythrocyte carbonic anhydrase (30000), soybean trypsin inhibitor (20100), and bovine β-lactalbumin (14400).
- c) Molar ratios of trypsin to α₂M.

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**Fig. 2. PAGE of α₂M–Proteinase Complexes**

PAGE was carried out with the samples prepared as described in the legend to Fig. 1.

- a) Molar ratios of STA-protease to α₂M.
- b) Molar ratios of trypsin to α₂M.
mixtures of 1:0.5 or more. The conversion of S-form into F-form was observed by PAGE of $\alpha_2$M reacted with STA-protease or with trypsin (Fig. 2).

**Inhibitions of Native and Acetylated Proteinases by $\alpha_2$M**

Table I shows the change in enzymatic activity caused by acetylation of free amino groups in the enzymes. Hydrolytic activities of STA-protease towards casein and Suc-(Ala)$_3$-pNA were decreased to 75 and 67% of the original levels by the acetylation. The activity of the acetylated trypsin towards Bz-Arg-pNA was 5.7 times higher than that of the native enzyme, though the caseinolytic activity was decreased to 62% of that of the native enzyme. Free amino groups were decreased from 2 to 1 on STA-protease and from 15 to 4 on trypsin by the

<table>
<thead>
<tr>
<th></th>
<th>Number of free amino groups</th>
<th>Relative activity (%)</th>
<th></th>
<th>SuC-(Ala)$_3$-pNA</th>
<th>or</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA-protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>2</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylated</td>
<td>1</td>
<td>75</td>
<td>67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylated</td>
<td>4</td>
<td>62</td>
<td>570</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hydrolytic activities of STA-protease and trypsin were determined with Suc-(Ala)$_3$-pNA and Bz-Arg-pNA, respectively, under the conditions described in Materials and Methods.

![Fig. 3. Inhibitory Effect of $\alpha_2$M on Caseinolytic Activities of Native and Acetylated Proteinases](image)

![Fig. 4. Inhibitory Effect of $\alpha_2$M on Hydrolytic Activities of Native and Acetylated Proteinases towards Low-Molecular-Weight Substrates](image)
acetylation. Figure 3 shows the inhibitory effects of $\alpha_2$M on the caseinolytic activities of the native and acetylated enzymes. When the molar ratios of $\alpha_2$M to enzyme were 0.75:1 for STA-protease and 0.5:1 for trypsin, the caseinolytic activities were decreased to 14 (native STA-protease), 27 (acetylated enzyme), 5 (native trypsin), and 2% (acetylated trypsin) of the original levels. No further change in the relative activity was observed on addition of more $\alpha_2$M. The binding amounts of enzyme per mol of $\alpha_2$M were estimated to be 1.5 mol for native and acetylated STA-protease and 2 mol for native and acetylated trypsin. There was no difference between the binding ratios of native and acetylated enzymes to $\alpha_2$M.

Figure 4 shows the activity profiles of the $\alpha_2$M-proteinase complexes obtained by the determination with Suc-(Ala)$_3$-pNA and Bz-Arg-pNA. Free enzyme remaining in the reaction mixture was inactivated by the addition of $\alpha_1$-antitrypsin. The transition points of the activity curves were found when the molar ratio of $\alpha_2$M to STA-protease and trypsin was 0.5:1, and the relative activities at these points were 25 (native STA-protease), 30 (acetylated enzyme), 30 (native trypsin), and 7% (acetylated trypsin).

Table II shows the kinetic parameters of $\alpha_2$M–proteinase complexes when the hydrolytic

| Table II. Kinetic Parameters of Proteinases and $\alpha_2$M–Proteinase Complexes |
|---------------------------------|--------|---------|-----------------|
| STA-protease                    | 19.6   | 0.53    | 27              |
| $\alpha_2$M–STA-protease complex | 41.7   | 0.55    | 13              |
| Trypsin                         | 0.6    | 0.70    | 1139            |
| $\alpha_2$M–trypsin complex     | 2.1    | 0.41    | 193             |

Each enzyme (1 mol) was incubated with 2 mol of $\alpha_2$M in 0.05 M Tris–HCl buffer, pH 8.0, containing 5 mm CaCl$_2$ at 37°C for 5 min. The $\alpha_2$M-proteinase complexes formed were separated from free enzymes by gel filtration on a Sepharose 4B column (2.5 × 45 cm) with 0.05 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl. Hydrolytic activities were determined with 2–10 mm Suc-(Ala)$_3$-pNA for STA-protease and with 0.1–0.5 mm Bz-Arg-pNA for trypsin as described in Materials and Methods. Kinetic parameters were calculated from the Lineweaver–Burk plots.

![Fig. 5. Binding of MDNSC to $\alpha_2$M Reacted with Native and Acetylated Proteinases](image-url)

$\alpha_2$M (1.72 μM) and enzyme (6.88 μM) were incubated with MDNSC (0–4 μM) in 200 μl of 0.1 M phosphate buffer, pH 7.5 containing 0.15 M NaCl at 37°C for 5 min. The fluorescence intensity of the MDNSC-binding $\alpha_2$M fraction separated by HPLC was determined with excitation at 350 nm and emission at 555 nm.  

- •, native STA-protease; ○, acetylated STA-protease; □, native trypsin; □, acetylated trypsin.
activities of STA-protease and trypsin were determined with Suc-(Ala)$_3$-pNA and Bz-Arg-pNA, respectively. Values of $K_{cat}/K_m$ were one-half ($\alpha_2$M–STA-protease complex) and one-sixth ($\alpha_2$M–trypsin complex) of those of the uninhibited enzyme.

**Cleavage of Thioester Bonds in $\alpha_2$M**

The cleavage of thioester bonds by the interaction of $\alpha_2$M with STA-protease or trypsin was investigated by using MDNSC and the thiol-directed fluorescent probes.

The binding profiles of MDNSC to active glutamyl residues generated by the cleavage of thioester bonds are shown in Fig. 5. Binding molar ratios of MDNSC progressively increased with increasing concentration of MDNSC, and the maximum ratio for $\alpha_2$M reacted with STA-protease or trypsin was 0.9 or 1.1 mol, respectively, per mol of $\alpha_2$M. In the case of $\alpha_2$M reacted with acetylated STA-protease or acetylated trypsin, this ratio was increased to 1.5 or 2.2 mol, respectively.

Generation of thiol groups in $\alpha_2$M reacted with STA-protease and trypsin was investigated by the reaction with FAM (Fig. 6). The fluorescence intensity of FAM-binding $\alpha_2$M increased as increasing amounts of enzymes were added to a constant amount of $\alpha_2$M. When the molar ratio of both enzymes to $\alpha_2$M was 2 : 1, the numbers of thiol groups generated in $\alpha_2$M reacted with STA-protease and trypsin were calculated to be 3.9 and 3.6 mol, respectively, per mol of $\alpha_2$M based on determination by using 5,5’-dithiobis(2-nitrobenzoic acid). Thus, four thioester bonds in $\alpha_2$M are considered to be cleaved by the interaction with STA-protease as well as trypsin.

The relative quantum yields obtained with ANM, DACM, and FAM were 0.71, 0.87, and 1.14, respectively, as shown in Table III. These results show that ANM and DACM

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**Table III. Relative Quantum Yields of Thiol-Directed Fluorescent Probes Bound to $\alpha_2$M-Proteinase Complexes**

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Excitation</th>
<th>Emission</th>
<th>Absorbance</th>
<th>Fluorescence$^a$</th>
<th>Absorbance$^a$</th>
<th>Quantum yield$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANM</td>
<td>350</td>
<td>445</td>
<td>350</td>
<td>0.66</td>
<td>0.93</td>
<td>0.71</td>
</tr>
<tr>
<td>DACM</td>
<td>400</td>
<td>469</td>
<td>400</td>
<td>0.81</td>
<td>0.93</td>
<td>0.87</td>
</tr>
<tr>
<td>FAM</td>
<td>346</td>
<td>472</td>
<td>346</td>
<td>0.82</td>
<td>0.72</td>
<td>1.14</td>
</tr>
</tbody>
</table>

$\alpha_2$M (1.5 $\mu$m) was reacted with enzyme (6 $\mu$m) in 0.1 M phosphate buffer, pH 8.0, containing 1 mM EDTA at 37 °C for 5 min, then incubated with a thiol-directed fluorescent probe (30 $\mu$m) for a further 10 min. After HPLC, the fluorescence intensity and the absorbance of each probe in the $\alpha_2$M fraction were determined.$^{21}$

$^a$ The ratio of the bound thiol-directed fluorescent probes on the $\alpha_2$M-STA-protease complex to those on the $\alpha_2$M-trypsin complex.

$^b$ The ratio of the fluorescence to the absorbance.
reacted with the $\alpha_2$M–trypsin complex give higher quantum yields than those reacted with the $\alpha_2$M–STA-protease complex. Thus, slight differences in the microenvironments of the thiol groups generated may exist.

**Discussion**

Several workers have carried out gel electrophoresis of the reaction mixture of human $\alpha_2$M and trypsin in the presence of SDS and 2-mercaptoethanol. When the amounts of trypsin added were lower than those required to saturate human $\alpha_2$M, two fragments of 111000 and 98000 daltons, in addition to the 185000 daltons subunit, were detected in some systems. On the other hand, essentially a single main fragment of 85000 daltons was detected in reaction mixture containing amounts of trypsin higher than that required to saturate $\alpha_2$M. Similar results have been reported with chymotrypsin and *Serratia* protease. On electrophoresis of the reaction mixture of $\alpha_2$M and STA-protease, many fragments smaller than 90000 daltons were detected. The $\alpha_2$M appears to be cleaved much more extensively by STA-protease than by trypsin. Conversion of the S-form into the F-form was observed with the $\alpha_2$M reacted with STA-protease as well as trypsin, however.

Feldman and Pizzo reported that the circular dichroic spectra of bovine, chicken, and frog $\alpha_2$M and ovomacroglobulin changed similarly after reaction with trypsin. Moreover, human and bovine $\alpha_2$M were reported to undergo identical conversion of the S-form into the F-form after reaction with trypsin. Equine $\alpha_2$M showed characteristics similar to those of bovine $\alpha_2$M after reaction with trypsin.

Salvensen et al. reported that the covalent binding of trypsin to human $\alpha_2$M was at least partly dependent on the presence of unblocked lysine side chains on the protein. The amount of covalent binding of radioactive trypsin to human $\alpha_2$M was decreased to two-thirds with acetylated trypsin (the number of free amino groups was decreased from 15 to 4). We found that the binding ratios calculated from inhibition curves of caseinolytic activities were not different for native and acetylated enzymes. However, free amino groups in enzymes may be involved at least partly in the formation of the $\alpha_2$M–proteinase complexes, as indicated by the greater incorporation of MDNSC into $\alpha_2$M reacted with acetylated enzymes than into that reacted with native enzymes.

The thiol-directed fluorescent probes used in the present study give higher fluorescence intensity in hydrophobic environments in the following order: ANM > DACM > FAM. Therefore, the relative quantum yields obtained suggest that the environment of thiol groups generated in the $\alpha_2$M–trypsin complex is more hydrophobic than that in the $\alpha_2$M–STA-protease complex. The change in the values of $K_{\text{cat}}/K_m$ of the $\alpha_2$M–proteinase complexes may be at least partly due to the different hydrophobicity, as described above.

In conclusion, although equine $\alpha_2$M is subjected to much more extensive cleavage by STA-protease than by trypsin, STA-protease is trapped both covalently and noncovalently by $\alpha_2$M, as in the case of human $\alpha_2$M–trypsin complex, and its hydrolytic activities towards casein and low-molecular-weight substrates are inhibited to various extents.

**References**