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Photoregulation of Deacylation Rate of Acyl Trypsin Derived from Photoresponsive Inverse Substrate

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Abstract—The acyl trypsin was prepared by use of an inverse substrate, which is comprise of a photoresponsive 4-phenylazobenzoyl moiety. The acyl group in acyl trypsin has been shown to isomerize from *trans*-form (**4t**-trypsin) to *cis*-form (**4c**-trypsin)/from *cis*-form to *trans*-form by irradiation of UV–vis light. The deacylation rate of the *cis*-form (**4c**-trypsin) has been shown to be 18.6 times faster than that of the *trans*-form (**4t**-trypsin).

Development of useful methods for photoregulation of the activity of naturally occurring proteins and enzymes has been major target in the research field of recent protein engineering.[1,2] From the standpoint of clinical application, regulation of enzyme activity will be a promising technique. For example, photoregulation of trypsin-like enzymes such as thrombin and plasmin is connected to the development of coagulants and anticoagulants.[3,4]

The methods of modification of enzymes by covalent attachment of photoresponsive components are classified into three categories. One of them is the specific modification of the enzyme active site by the use of photoisomerizable inhibitors. Pioneering work by Erlanger is known to be classified in this category, but it is very difficult to design such residues that are photoisomerizable and are involved in covalent modification at the same time.[5] The second one is the direct random introduction of photoresponsive components into the enzyme molecule. In this case, the multi-site modification of enzynes will occur. Thus, the products are less informative on the structure–function relationship of the emzyme.[6] The third method is the site-specific incorporation of photoresponsive components into an enzyme.[7–9]

Previously, we reported that inverse substrates such as *p*-amidino- and *p*-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes and allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site.[10,12] The esters provided a novel method for the specific introduction of the non-specific structure into a trypsin active site. In this respect, inverse substrates are expected to be a useful tool as the carrier of a photoresponsive molecule into trypsin and trypsin-like enzymes. Herein, we report the synthesis and properties of a photoresponsive inverse substrate.

We designed 4′-phenylazobenzoic acid 4-guanidinophenyl ester methanesulfonate (**4**) as an inverse substrate. Synthetic procedures are shown in Scheme 1.

Condensation of 4-phenylazobenzoic acid (**1**) with 4-[*N*′,*N*″bis(*Z*)guanidino]phenol (**2**)[13] by using benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent in DMF afforded 4′-phenylazobenzoic acid 4-[*N*′,*N*″bis(*Z*)guanidino]phenyl ester (**3**) in 84% yield. Deprotection was carried out by methansulfonic acid to give 4′-phenylazobenzoic acid 4-guanidinophenyl ester (*trans*-Azo-OGu) (**4t**) in 73% yield. The structure was determined by IR, UV, ^{1}H NMR spectra and elemental analysis.[14]

Compound (**4t**) exhibits reversible photoisomerization. Upon irradiation of UV light at around 365 nm, the *trans*-form (**4t**) isomerizes to the *cis*-form (**4c**). Irradiation of **4c**

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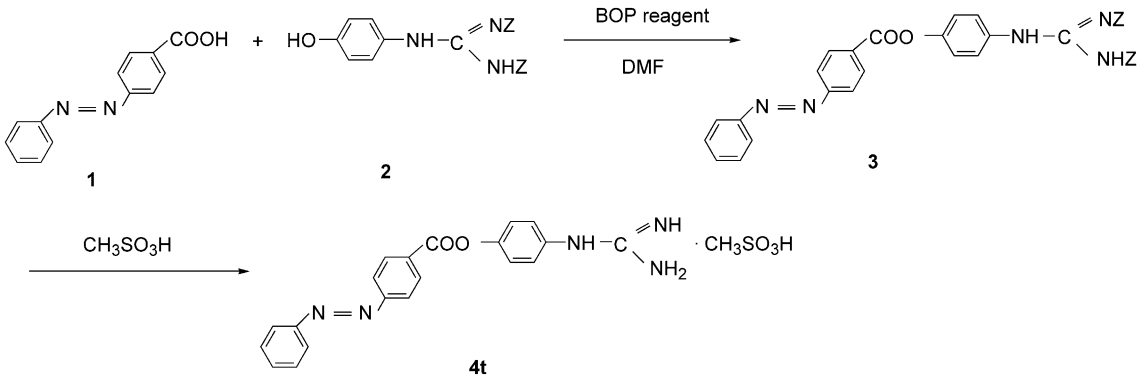


Scheme 1. Synthesis of inverse substrate.

at longer wavelength than 400 nm results in the reverse isomerization. This isomerization rate is considerably slow. Figure 1 shows the spectral changes associated with the photoisomerization of **5** as a model of acyl trypsin from inverse substrate **4**. Yields of the *cis*-form (**5c**) from the *trans*-form (**5t**) by irradiation at around 365 nm were determined by the absorption spectra. The data are in good agreement with the yield calculated on the basis of the peak intensity of HPLC and ^{1}H NMR spectra, respectively.

The kinetic constants for the trypsin-catalyzed hydrolysis were analyzed on the basis of the following scheme.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

In this scheme, the following symbols are used: E, enzyme; S, substrate; ES, enzyme–substrate complex; EA, acyl enzyme; P_1, alcohol component of the substrate; P_2, acid component of the substrate; *K*s, dissociation constant of enzyme–substrate complex; k_2, rate constant of acylation step; k_3, rate constant of deacylation step. Analysis of kinetic parameters for trypsin-catalyzed hydrolysis of **4t** was carried out by the thionine displacement method,[11,15] and the values are listed in Table 1. Compound **4t** was shown to have strong binding affinity and to promote efficient acylation. The values for k_2/K_s (specificity index) are nearly equal within **4t** and **4c**. The difference between **4t** and **4c** is emphasized at their deacylation steps. These data suggest that both isomers are a specific substrate of trypsin to give acyl trypsin, and the acyl trypsin from **4t** is more stable.

The kinetic parameters for **4t** were directly determined from the rate assay and those for **4c** were calculated from the apparent data of the mixture of **4t** and **4c**. Figure 2 exemplifies the apparent enhancement of deacylation rates constant which were proportional to the ratio of the *cis*-form (**4c**-trypsin). The other parameters of **4c** were also calculated from the apparent data resulted from the *cis–trans* mixture.

Acyl trypsin was successfully obtained according to our previous paper as follows.[16] Trypsin was incubated with a

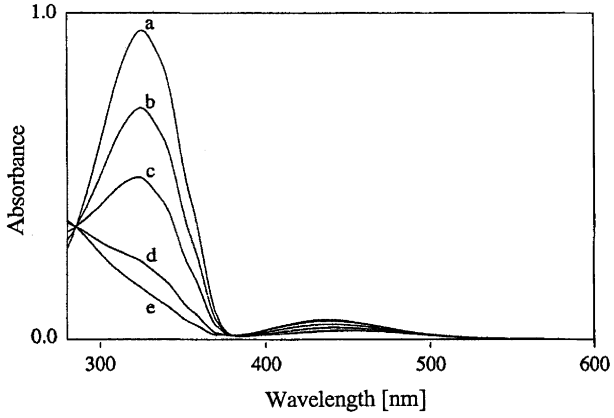


Figure 1. Photochromic attribute of acyl enzyme model containing azobenzene group **5** at 4×10^{-5} M in $CHCl_3$: (a) 100% **5t** (no irradiation of **5t**, and or irradiation of **5c** at longer wavelength than 400 nm for 10 h); (b) 20% **5c** (irradiation of **5t** at around 365 nm for 30 s); (c) 50% **5c** (irradiation of **5t**: 60 s); (d) 90% **5c** (irradiation of **5t**: 120 s); (e) 100% **5c** (irradiation of **5t**: 240 s).

Table 1. Kinetic parameters for trypsin-catalyzed hydrolysis of inverse substrates

Substrate	K_s (M)	k_2 (s^{-1})	k_3 (s^{-1})	k_2/K_s (M^{-1} s^{-1})
trans-form (**4t**)	1.06×10^{-5}	1.06	3.78×10^{-4}	1.51×10^{5}
cis-form (**4c**)	4.73×10^{-5}	7.75	7.02×10^{-3}	1.64×10^{5}

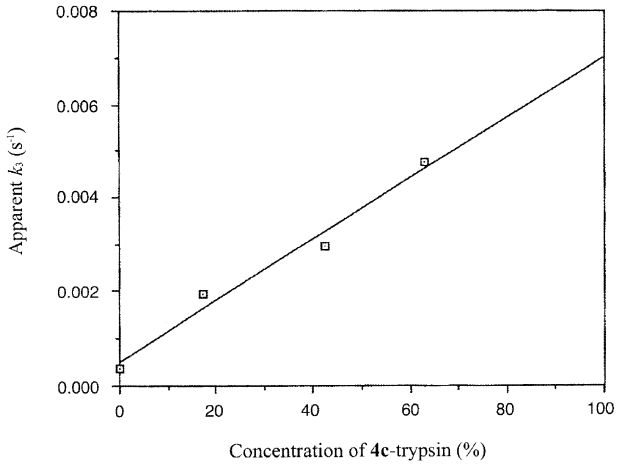
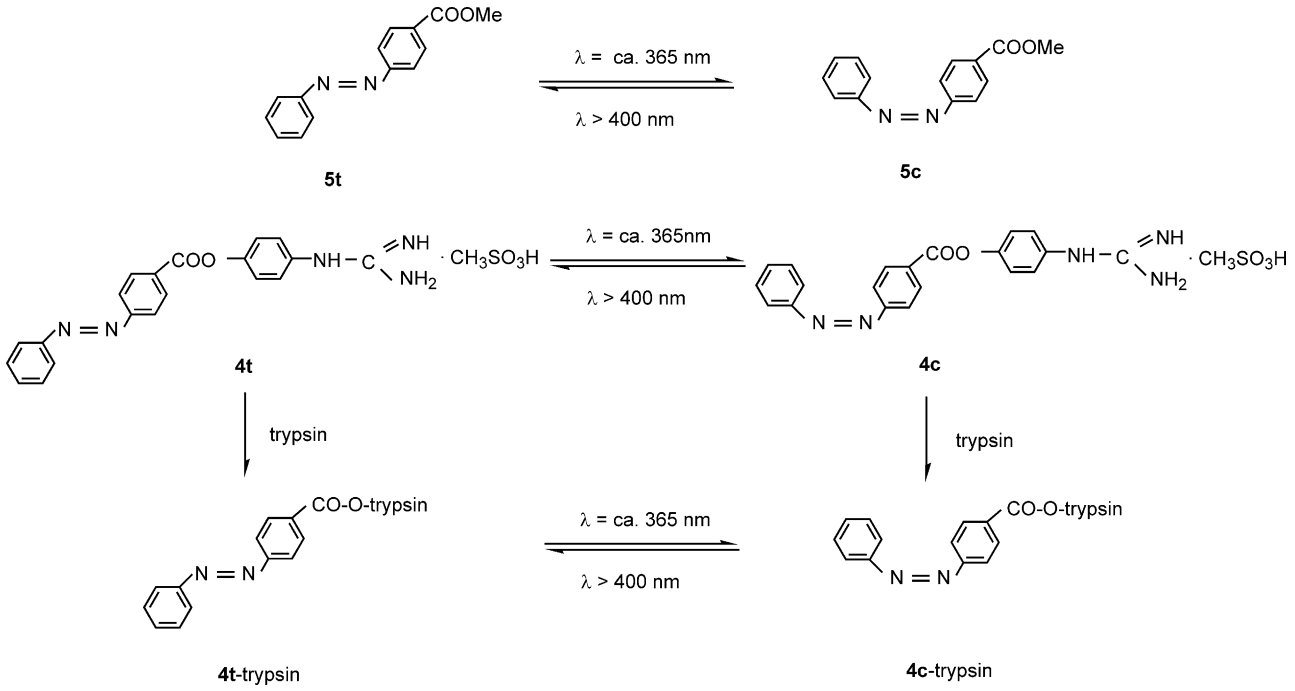


Figure 2. Apparent deacylation rate constants.



Scheme 2. Photoisomerization.

15–20 molar excess of *trans*-Azo-OGu (**4t**), or a mixture of *trans*-**4t** (45%) and *cis*-Azo-OGu (**4c**) (55%) for 2 min in 50 mM Tris–HCl buffer (containing 25 mM $CaCl_2$, pH 8.0) at 25 °C. After the pH was adjusted to 3.0 by addition of 1 M HCl, the reaction mixture was gel-filtered and lyophilized. Both resulted preparations were completely inactive as a result of acylation at the active site, while the enzymatic activity was completely recovered after incubation for 2.5 h at pH 8.0 as a result of deacylation (Scheme 2).

Acyl enzyme (**4t**-trypsin) obtained using **4t** exhibited reversible photoisomerizable properties. Irradiation of the *trans*-form (**4t**-trypsin) at around 365 nm resulted in formation of the *cis*-form (**4c**-trypsin). Irradiation of **4c**-trypsin at longer wavelength than 400 nm resulted in formation of **4t**-trypsin. Figure 3 illustrates the spectral changes associated with the photoisomerization of **4**-trypsin. This behavior was almost the same as that of **5** shown in Figure 1.

As shown in Table 1, it is evident that the deacylation rate of the photoisomerized *cis*-form (**4c**-trypsin) is 18.6 times faster than that of the *trans*-form (**4t**-trypsin), although K_s and k_2 values of **4c** are 4.46- and 7.31-fold larger than those of **4t**. The difference in deacylation rates of **4t** and **4c** enables photoregulation of the enzyme activity by an external light source. Figure 4

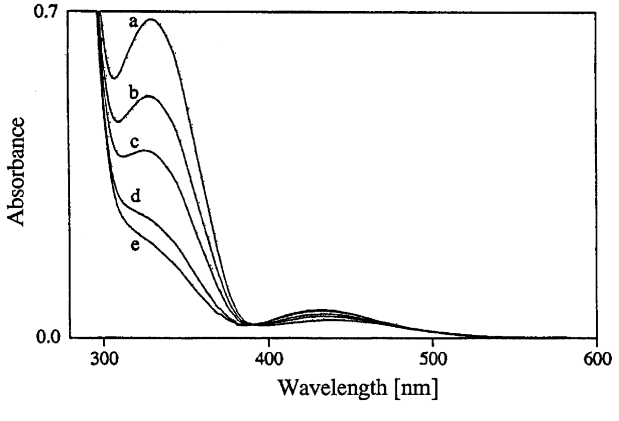


Figure 3. Photochromic attribute of acyl enzyme (**4**-trypsin) at 5.6×10^{-5} M: (a) 100% **4t**-trypsin (no irradiation of **4t**-trypsin); (b) 10% **4c**-trypsin (irradiation of **4t**-trypsin at around 365 nm for 30 s); (c) 30% **4c**-trypsin (irradiation of **4t**-trypsin: 60 s); (d) 55% **4c**-trypsin (irradiation of **4t**-trypsin: 120 s); (e) 65% **4c**-trypsin (irradiation of **4t**-trypsin: 120 s). Absorption spectra were obtained in a medium composed of I mM HCl.

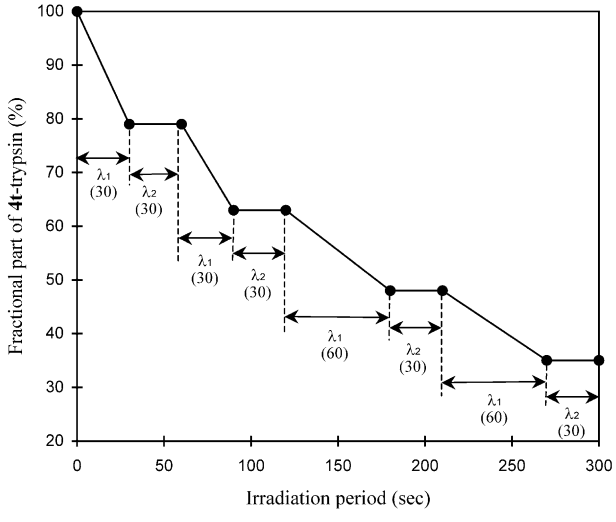


Figure 4. Photoresponsive on–off experiment using acyl trypsin (**4t**-trypsin) (5.6×10^{-5} M in 1 mM HCl). Alternate irradiation at around 365 nm (λ_1) and at longer wavelength than 400 nm (λ_2). The value in parentheses is irradiation time (s).

exemplifies the interconversion of **4t**-trypsin to **4c**-trypsin by the sequential on–off and off–on switching of **4t**-trypsin.[17]

In conclusion, chemical modification of the active site of the trypsin by use of an inverse substrate carrying azobenzene photoresponsive groups have been applied to photoregulation of the enzyme activity.

Thus, inverse substrates will provide a useful means to regulate the activity of trypsin and trypsin-like enzymes.

Acknowledgements

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