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BINDING OF *STREPTOMYCES* PEPSIN INHIBITOR (ACETYL-PEPSTATIN) WITH CHYMOSIN (RENNIN)

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Summary

Chymosin (Rennin) was effectively purified using an AH-Sepharose 4B column. Binding of *Streptomyces* pepsin inhibitor (acetyl-pepstatin) with chymosin was studied spectroscopically. The binding caused ultraviolet difference and CD spectral changes suggesting microenvironmental changes around tryptophan and/or tyrosine residue(s) in chymosin. The fluorescence intensity of a hydrophobic probe, 2-*p*-toluidinylnaphthalene-6-sulfonate, increased in the presence of chymosin and was further amplified when *Streptomyces* pepsin inhibitor was added to the chymosin-2-*p*-toluidinylnaphthalene-6-sulfonate solution. The binding and dissociation-rate constants between chymosin and the inhibitor were determined using 2-*p*-toluidinylnaphthalene-6-sulfonate as a probe. The binding constant was determined from the binding and dissociation-rate constants, to be $3.1 \cdot 10^7\ M^{-1}$ at 25°C, pH 5.5.

Introduction

Streptomyces pepsin inhibitor is a strong inhibitor of pepsin and many acid proteases of microbial origin [1,2]. It binds to the active site of the acid proteases sometimes overlapping the catalytic site [3]. On pepsin, a conformational change has been suggested upon binding with the substrate or the inhibitor [4]. Chymosin is an animal acid protease like pepsin, but its substrate specificities are different from each other [5]. In this paper, spectroscopic studies concerning microenvironmental changes around chromophore groups and the hydrophobic site of chymosin are reported. The results are compared with those of pepsin which we have reported in the previous paper [6].

Abbreviation: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate.

Experimental

Materials

Chymosin was purified with AH-Sepharose 4B from commercial crude extract. Dry powder of chymosin, as a crude extract (Rennin NF XI), was purchased from Difco Laboratories (Detroit, MI, U.S.A.). 3-times recrystallized porcine pepsin was purchased from Miles. *Streptomyces* pepsin inhibitor was a gift from Professor S. Murao and Dr. K. Oda of the University of Osaka Prefecture. A fluorescence probe, TNS (2-*p*-toluidinylnaphthalene-6-sulfonate) was purchased from Sigma and purified by recrystallization from distilled water. The concentration of stock solutions of chymosin and pepsin was determined spectrophotometrically, using $E_{1\,cm}^{1\,mg/ml} = 1.53$ at 277.5 nm and the molecular weight of 30 700 for chymosin [5] and the molar absorption coefficient $\epsilon = 50\,990\ cm^{-1} \cdot M^{-1}$ at 278 nm for pepsin [7]. The TNS concentration was determined using $\epsilon = 19\,300\ cm^{-1} \cdot M^{-1}$ at 366 nm [8].

Methods

The enzyme activity for chymosin was measured through milk clotting activity with 10% skim milk powder in 0.01 M $CaCl_2$ at 35 °C. The amount of enzyme in 5.5 ml which clots the milk in 1 min was defined to contain 400 units of activity [9]. Measurements of ultraviolet difference and CD spectra were carried out using a spectrophotometer Union-Giken SM 401 and a Jovin-Ivon-Dichro Graph Mark-III J, respectively. The experimental procedures for the spectroscopic studies were the same as our previous paper on pepsin [6]. Fluorescence spectra were measured with a fluorescence spectrophotometer Union-Giken SF 401. Kinetic study of fast fluorescence change was carried out with a stopped-flow apparatus (Union-Giken SF-70). Spectroscopic and kinetic measurements were made at 25 °C, pH 5.5 (0.01 M acetate buffer).

Results

Purification of chymosin

AH-Sepharose 4B was found to be useful for the purification of chymosin. AH-Sepharose 4B was swollen in 0.01 M acetate buffer (pH 5.5) and packed in a 5 ml syringe. Total volume of swollen AH-Sepharose 4B was 3 ml. Pure chymosin was obtained from crude material by a one-step purification procedure. 10 g of commercially available chymosin as dry powder was dissolved in 250 ml 0.01 M acetate buffer (pH 5.5) and centrifuged at 4 °C and 7000 × *g* for 20 min. The supernatant was charged on the AH-Sepharose 4B column at 4 °C. After charging, the column was washed with 0.16 M NaCl in 0.01 M acetate buffer (pH 5.5). Chymosin was eluted with 0.5 M NaCl in 0.01 M acetate buffer (pH 5.5). The elution pattern is shown in Fig. 1. The recovered enzyme activity measured by the clotting method was 60% of the total activity of charged enzyme. Purified enzyme showed a single band in disc electrophoresis and the activity per absorbance of 1.0 of purified enzyme solution was six times that of the original supernatant solution.

Ultraviolet difference and CD spectral changes

Qualitative evidence that *Streptomyces* pepsin inhibitor may bind to the

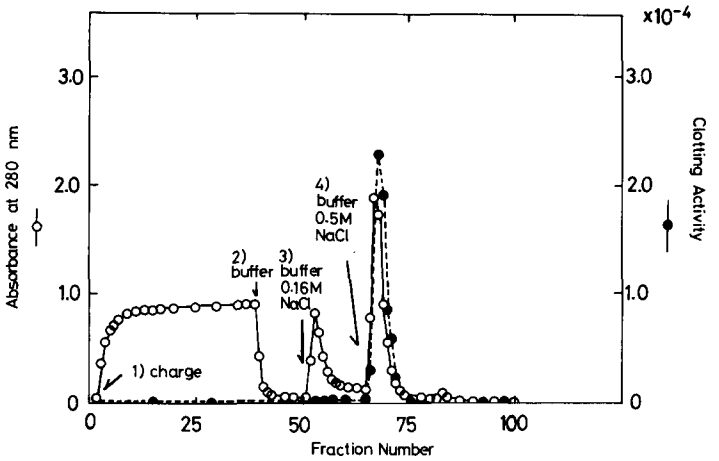


Fig. 1. Chromatography of chymosin on an AH-Sepharose 4B column, collecting a 5.0 ml fraction at a flow rate of 15 ml/h. (1) Charging crude chymosin in 0.01 M acetate buffer (pH 5.5). (2) Washing with 0.01 M acetate buffer (pH 5.5). (3) Washing with 0.16 M NaCl in 0.01 M acetate buffer (pH 5.5). (4) Effusing purified chymosin with 0.5 M NaCl in 0.01 M acetate buffer (pH 5.5).

active site of chymosin is its inhibition of the clotting activity of chymosin; the clotting time was delayed up to 10-times depending on the inhibitor concentration when the inhibitor was added to the enzyme-milk solution. Further investigation of the chymosin-inhibitor interaction was made with spectrophotometric methods.

A characteristic ultraviolet difference spectrum was observed upon the binding of chymosin with the inhibitor (Fig. 2a). The shape of the difference spectrum was similar to that of the pepsin-inhibitor system, suggesting microenvironmental change of tryptophan and/or tyrosine residue(s). A trough around 300 nm has been interpreted as being due to a microenvironmental change around the tryptophan residue(s) [6,10].

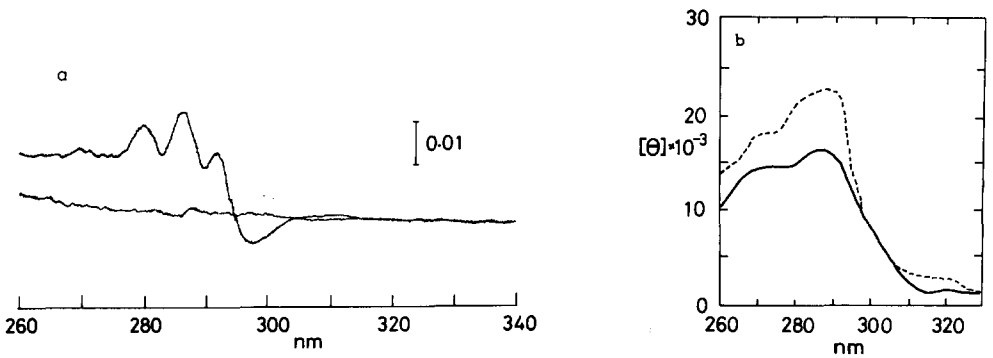
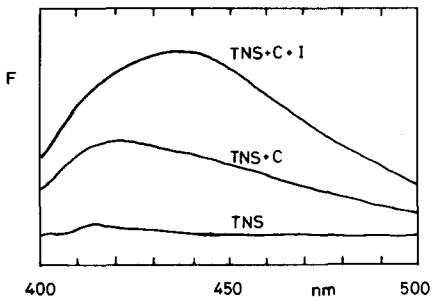


Fig. 2. Ultraviolet difference spectrum (a) and CD spectral change (b) of chymosin upon binding with *Streptomyces* pepsin inhibitor. (a) Concentration of chymosin and the inhibitor are 16.6 μM and 25 μM, respectively, at pH 5.5 and 25°C. The horizontal line is an enzyme-enzyme baseline. (b) Concentrations of chymosin and the inhibitor are 22 μM and 50 μM, respectively, at pH 5.5 and 25°C. (———) chymosin, (- - - - -) chymosin-inhibitor complex.



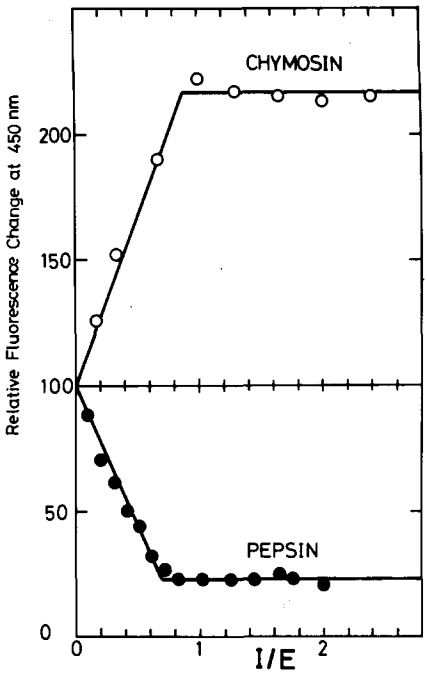


Fig. 3. Fluorescence spectra of TNS in the presence of chymosin or chymosin-*Streptomyces* pepsin inhibitor complex. The vertical coordinate (F) shows fluorescence intensity in arbitrary unit. The concentration of chymosin (C), the inhibitor (I) and TNS are 16, 25 and 26 μM, respectively. pH 5.5, 25°C.

Fig. 4. Titration of chymosin and pepsin by *Streptomyces* pepsin inhibitor using TNS as a probe. I/E shows the ratio between the inhibitor and the enzyme. The concentration of chymosin, pepsin and TNS are 15, 24 and 26 μM, respectively. The vertical scale shows the relative fluorescence change at 450 nm.

Further evidence of the microenvironmental change of tryptophan and/or tyrosine residue(s) of chymosin was suggested by CD spectral change. The intensity of the positive CD spectrum of chymosin between 270 and 300 nm increased by about 30% upon binding with the inhibitor (Fig. 2b). The behaviour was again the same as that of pepsin [6].

Interaction with TNS

Fluorescence intensity of TNS increased in the presence of chymosin. Such a phenomenon was the same as that of the pepsin-TNS system [11]. Addition of *Streptomyces* pepsin inhibitor to the chymosin-TNS system caused further enhancement of the fluorescence intensity as shown in Fig. 3. In contrast to the chymosin-TNS system, the fluorescence intensity of the pepsin-TNS system decreased upon addition of the inhibitor. Fluorescence titration using TNS as a probe showed stoichiometry of approx. 1 : 1 for both chymosin- and pepsin-inhibitor complexes (Fig. 4). Slight deviation from stoichiometry of 1 : 1 may be due to the existence of inactive enzyme.

Kinetic studies of the inhibitor binding and dissociation

The process of the inhibitor binding with chymosin was studied by the

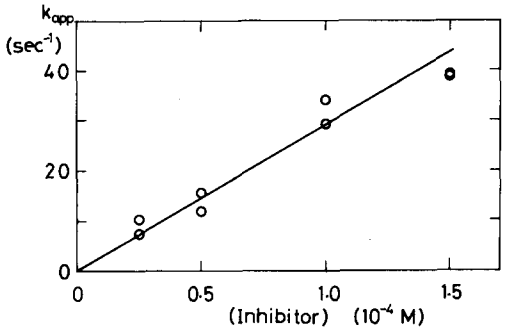


Fig. 5. Concentration dependence of apparent first-order rate constant between chymosin and *Streptomyces* pepsin inhibitor measured with the fluorescence stopped-flow method in the presence of TNS (50 μM). The horizontal scale shows the initial concentration of the inhibitor which is much excess over that of chymosin.

fluorescence stopped-flow method using TNS as a probe. The solution containing chymosin and TNS was mixed with the solution containing the inhibitor and TNS. The fluorescence emission was observed through a cut-off filter (transmitting above 430 nm) from the right angle to the excitation beam (366 nm). The time course of the fluorescence increase obeyed pseudo-first-order kinetics under the conditions that initial concentration of the inhibitor was much in excess of that of chymosin. Fig. 5 shows the plot of the apparent first-order rate constant vs. the inhibitor concentration, from which the second-order rate constant was determined to be $2.9 \cdot 10^5$ $M^{-1} \cdot s^{-1}$ at 25 °C, pH 5.5.

Addition of pepsin to the chymosin-inhibitor complex system containing TNS caused a time-dependent decrease of the fluorescence intensity due to transfer of the inhibitor from chymosin to pepsin (see Fig. 4). A typical time course determined with the fluorescence spectrophotometer is shown in Fig. 6.

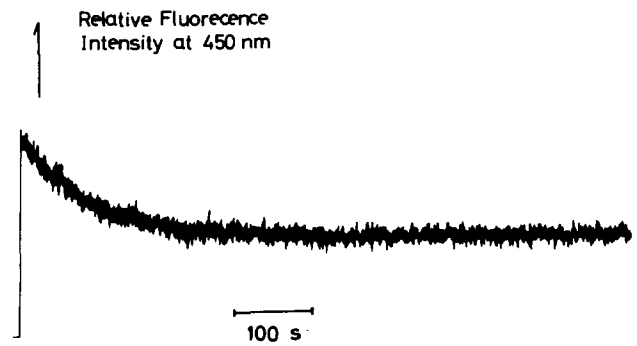


Fig. 6. A time course of dissociation reaction of the chymosin-inhibitor complex caused by pepsin using TNS as a probe. The vertical coordinate shows fluorescence intensity at 450 nm. The concentrations of chymosin, pepsin, the inhibitor and TNS are 6.6, 86, 25 and 100 μM, respectively. pH 5.5, 25°C.

The overall reaction scheme is as follows,

$$CI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C + I \quad (1)$$

$$P + I \overset{k_1'}{\rightarrow} PI \quad (2)$$

where C, P and I are chymosin, pepsin and the inhibitor, respectively. The dissociation reaction of the PI complex was assumed to be negligible, because the overall reaction for this system was virtually irreversible; the dissociation reaction of the PI complex could not be observed under present conditions. When the initial concentration of pepsin is much in excess of that of chymosin and the inhibitor, and the steady-state condition about the free inhibitor is assumed, i.e.,

$$\frac{d[I]}{dt} = 0 \quad (3)$$

the rate equation on the decrease of the chymosin-inhibitor complex concentration, [CI], is given as follows,

$$-\frac{d[CI]}{dt} = k_{-1}[CI]\left\{1 - \frac{k_1[C]}{k_1'[P]_0 + k_1[C]}\right\} \quad (4)$$

where [C] and $[P]_0$ are concentration of chymosin and initial concentration of pepsin. Since the value of k_1' was determined to be $1.0 \cdot 10^6\ M^{-1} \cdot s^{-1}$ by the stopped-flow method, the condition $k_1'[P]_0 >> k_1[C]$ was satisfied in the condition $[P]_0 >>$ [C]. Then, Eqn. 4 reduces to

$$-\frac{d[CI]}{dt} = k_{-1}[CI] \quad (5)$$

The apparent first-order rate constant of the fluorescence decrease can be regarded as the dissociation rate constant of the chymosin-inhibitor complex, k_{-1}. The dissociation rate constant ($9.5 \cdot 10^{-3}\ s^{-1}$) was almost independent (within 20%) of pepsin concentration between $8.6 \cdot 10^{-5}$ M to $3.0 \cdot 10^{-4}$ M. The binding constant between chymosin and the inhibitor was determined from both binding and dissociation rate constants to be $k_1/k_{-1} = 3.1 \cdot 10^7\ M^{-1}$.

Discussion

Pure chymosin could be obtained using an AH-Sepharose 4B column. The yield (60%) seems to be higher than that of the recrystallization method (28%) [12]. Our preliminary experiment showed that porcine pepsin from the commercial crude product could be purified with almost the same procedure.

Spectroscopic studies on the interaction between pepsin or chymosin and *Streptomyces* pepsin inhibitor suggest that microenvironmental change around the tryptophan and/or tyrosine residue(s) near the inhibitor binding site is of the same type for both enzyme.

The fluorescence measurement using TNS as a probe suggested formation of a ternary complex, chymosin-TNS-inhibitor. The binding site for TNS in

protein is thought to be hydrophobic [13]. The further fluorescence enhancement of TNS upon formation of the chymosin-inhibitor complex suggests inhibitor-induced microenvironmental change of chymosin. The identical example, about this point, is reported in pepsin; the fluorescence intensity of TNS bound to pepsin is further enhanced by the binding of the peptide fragment from pepsinogen [11].

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