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Determination of Pepstatin-Sensitive Carboxyl Proteases by Using Pepstatinyldansyldiaminopropane (Dansyl-Pepstatin) as an Active Site Titrant

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N-Pepstatinyl-N'-dansyldiaminopropane (dansyl-pepstatin) was prepared by the coupling of pepstatin A and N-dansyl-diaminopropane. The dansyl-pepstatin obtained strongly inhibited pepsin activity by forming a 1:1 complex. The fluorescence of the dansyl group (excitation at 320 nm, and emission near 520 nm) increased with the formation of the complex. The increase in fluorescence of dansyl-pepstatin solution was proportional to the amount of added pepsin, chymosin and cathepsin D until dansyl-pepstatin was saturated by these enzymes and at higher protease concentrations the fluorescence did not increase further. Therefore, the net amounts of active pepstatin-sensitive carboxyl proteases could be determined by detecting the inflection point of increased fluorescence upon addition of the protease to a dansyl-pepstatin solution of known concentration. Moreover, the protease concentrations of many samples were obtained easily by measurements of increased fluorescence compared with that caused by authentic protease solution. The minimum detectable amount of pepsin was about 20 pmol. On the other hand, the fluorescence did not increase upon mixing with inactivated pepsin, chymotrypsin, or trypsin. The K_i value of dansyl-pepstatin for pepsin was similar to that of pepstatin A. It was possible to determine the amount of chymosin contained in rennet by this method. The inactivation curve of pepsin in pH 6.5 buffer was also determined quickly and easily by the use of this method. This assay method for pepstatin-sensitive carboxyl proteases is very simple and easy, and it is possible to determine the net amounts of active pepstatin-sensitive carboxyl proteases even in crude mixtures.

Key words: cathepsin D, chymosin, dansyl-pepstatin, determination of carboxyl proteases, pepsin.

It is difficult to determine the amounts of active proteases because many proteases contain some denatured protein even in the crystalline state. Consequently, the amounts of active proteases can not be determined by measurements of their weight or their absorbance. Immunoassay is very sensitive and has a high specificity, but the antibodies may react with the inactive enzyme (*1*).

In the case of some serine proteases, the amounts of active enzymes can be determined by measurement of the amount of *p*-nitrophenol released at the burst by the use of *p*-nitrophenyl acetate as a substrate (*2*). Moreover, *p*-nitrophenyl ester derivatives of an aza-amino acid (Ac-Aphe-ONp) and some aza-peptides can be utilized as active site titrants for chymotrypsin-like enzymes (*3, 4*).

On the other hand, a good titrant for carboxyl proteases has not been reported. Pepstatin, an inhibitor from *Streptomyces*, strongly inhibits the activities of many carboxyl proteases by forming an equimolar protease-inhibitor complex (*5-9*). The amount of pepstatin-sensitive carboxyl proteases can be obtained by measurement of the amount of pepstatin needed for complete inhibition of the enzymes activities (*7*). This method is laborious and the errors are relatively large. Furthermore, measurement of the amount of active pepsin was achieved by the difference spectral technique (*9*). However the sensitivity was low (the pepsin concentration used was at the mg/ml level).

On the other hand, fluorometric methods are used to determine protease activities (*10, 11*). The fluorescence of the dansyl group increases when this group is transferred from an aqueous environment to a non polar environment or is bound to proteins (*12, 13*). It was reported that dansyl-amino acid derivatives bound with chymotrypsin and increased the fluorescence of the dansyl group (*14*). Furthermore, some dansyl-peptide substrates for pepsin were synthesized and the fluorescence of these substrates increased with the formation of enzyme-substrate complexes (*15*).

Therefore, a dansylated pepstatin might form a tightly bound equimolar inhibitor-enzyme complex with pepstatin-sensitive carboxyl proteases and the fluorescence of the dansyl group should increase with formation of the complex. Thus, we synthesized dansylated pepstatin and used it for the determination of the active form of some pepstatin-sensitive carboxyl proteases. By using this method, it was possible to determine the amounts of active

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Abbreviations: DMF, dimethyl formamide; DMSO, dimethyl sulfoxide; BOP reagent, benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate; TCA, trichloroacetic acid.

proteases easily and specifically with a high sensitivity. Moreover, we determined the rate of denaturation of pepsin in pH 6.5 buffer.

EXPERIMENTAL PROCEDURES

Materials—Pepsin (bovine), chymosin, and cathepsin D (bovine spleen) were purchased from Sigma Chemical Company, USA. Pepstatin A was purchased from Peptide Institute, Osaka. Pepstatin-Sepharose 4B was prepared in this laboratory. Concentrations of protease solutions were based on the weights of proteases. The proteases used were dried in a desiccator under reduced pressure for 5 h.

N-Pepstatinyl-N′-Dansyldiaminopropane (Dansyl-Pepstatin)—To a mixture of 1,3-diaminopropane (0.5 ml, 6 mmol) and acetonitrile (3 ml), a solution of dansyl chloride (100 mg, 0.37 mmol) in acetonitrile (2 ml) was added dropwise at room temperature. The mixture was stirred for 2 h. The precipitate was removed by filtration and the filtrate was evaporated. The resulting residue was washed successively with petroleum ether and dried *in vacuo*. The residue (mono-*N*-dansyldiaminopropane) was dissolved in a mixture of DMSO (3 ml) and DMF (0.6 ml), and pepstatin A (30 mg, 0.044 mmol) was added to it. Then, the solution was cooled at 0°C, and BOP reagent (40 mg, 0.09 mmol) was added. The solution was stirred for 1 h at 0°C and 24 h at room temperature. The solution was evaporated, then the residue was dissolved in ethanol and chromatographed on an LH-20 column (1.8×50 cm) at a flow rate of 8.1 ml/h; 3.1 ml fractions were collected. The absorbance at 400 nm was measured and fractions 14-18 were combined. The fractions were evaporated and rechromatographed under the same conditions. Fractions 14-19 were combined and evaporated, and the precipitate was obtained with the aid of ether; yield, 29 mg (67%); mp 237-239°C. Found: C, 60.18; H, 8.73; N, 11.35%. Calcd for $C_{49}H_{82}N_8O_{10}S_1$: C, 60.39; H, 8.50; N, 11.50%.

Inhibitory Activity of Dansyl-Pepstatin—Forty microliter portions of pepstatin A or dansyl-pepstatin solution (100 μM in ethanol) were added to 2 ml of a pepsin I solution (10 μM in 0.1 M citrate buffer pH 4.0) and the mixture was incubated for 2 min at room temperature. The remaining activity was measured by the modified Anson method (*16*). Five microliters of the reaction mixture was taken and added to 1 ml of heat-denatured hemoglobin solution (0.1 M citrate buffer pH 2.0). The mixture was incubated for 10 min at 37°C, then 2 ml of 5% TCA was added. After 30 min, the resulting precipitate was filtered off and 1 ml of the filtrate was added to 2 ml of 0.5 M NaOH. Then, 0.6 ml of phenol reagent was added to the solution and the absorbance at 650 nm was measured. The activity of chymosin was determined by the same procedures as in the case of pepsin, and that of cathepsin D was determined by the use of hemoglobin solution (pH 3.5) as a substrate.

Affinity Chromatography of Pepsin on a Pepstatin-Sepharose 4B Column—Five hundred microliters of pepsin I solution (100 μM in citrate buffer pH 4.0) was applied to a pepstatin-Sepharose 4B column (1×7 cm), which was washed with citrate buffer (pH 4.0), and then eluted with Tris-HCl buffer (pH 8.5, containing 1 M NaCl) at a flow rate of 7.3 ml/h; 2.6 ml fractions were collected and the absorbance at 280 nm was measured (*17*).

Determination of Pepsin-Dansyl-Pepstatin Complex—Five hundred microliters of pepsin I solution (100 μM in citrate buffer pH 4.0) and 50 μl of dansyl-pepstatin solution (1 mM) were mixed and after 5 min, 500 μl of the solution was applied to a Bio-Gel P-30 column (1.2×50 cm). This was eluted with citrate buffer (pH 4.0) at a flow rate of 10.2 ml/h and 2.6 ml fractions were collected. The absorbance at 280 nm and the fluorescence at 520 nm (excitation at 320 nm) were measured.

Measurement of Fluorescence—In a fluorescence cell, 3 ml of citrate buffer (pH 4.0) and 20-100 μl of dansyl-pepstatin solution (2-10 μM) were mixed and 20 μl of enzyme solution (1-20 μM in water) was added. After incubation for 2 min, the fluorescence spectrum was measured (excitation at 320 nm).

Competition of Dansyl-Pepstatin and Pepstatin A for Binding with Pepsin—In a fluorescence cell, 3 ml of citrate buffer (pH 4.0), 50 μl of dansyl-pepstatin solution (100 μM) and various amounts of pepstatin A solution (100 μM) were mixed and 70 μl of pepsin I solution (100 μM) was added. After 2 min, the fluorescence spectrum was measured (excitation at 320 nm).

Interaction of Dansyl-Pepstatin with Inactivated Pepsin and Other Proteases—To 1.8 ml of Tris-HCl buffer (pH 7.5), 0.2 ml of pepsin I solution (100 μM in water) was added. The mixture was incubated for 24 h at 30°C. After determination of remaining activity, the increase in fluorescence was measured as described above. Chymotrypsin and trypsin were dissolved in Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ (10 μM) and the increases in fluorescence were measured in pH 7.5 buffer.

Determination of Inactivation of Pepsin in pH 6.5 Buffer—To 1.8 ml of citrate buffer (pH 6.5), 0.2 ml of pepsin I solution (100 μM in water) was added at 30°C. The mixture was incubated for 1, 2, 3, or 24 h, then 50 μl of the reaction mixture was taken. It was diluted with 3 ml of citrate buffer (pH 4.0) and 60 μl of dansyl-pepstatin solution (10 μM) was added. The increase in fluorescence was measured as described above. On the other hand, the reaction mixture was diluted to 0.2 μM with citrate buffer (pH 2.0) and the remaining activity was measured as described above.

RESULTS AND DISCUSSION

In studies of proteases, it is necessary to determine the net amounts of active enzymes. In the case of some serine proteases, good titrants have been synthesized (*3, 4*). Moreover, a fluorogenic active-site titrant for serine proteases was synthesized and it was shown that this titrant was very sensitive (*18*). On the other hand, carboxyl proteases do not form stable covalently bound acyl intermediates and a good titrant for carboxyl proteases has not been obtained (*19, 20*). For example, Tomasselli *et al.* synthesized some pepstatin analogs and indicated that these inhibitors were useful for the titration of HIV-1 protease (*21*). However this method was essentially the same as the titration by pepstatin. We synthesized dansyl-pepstatin as a candidate fluorogenic titrant for pepstatin-sensitive carboxyl proteases.

Matthews *et al.* synthesized biotin-labeled pepstatin as a probe for cathepsin D and investigated the subcellular location of cathepsin D (*22*). They synthesized biotin-labeled pepstatin by the coupling of *N*-pepstatinyl-1,2-diamino-

ethane and biotin-*N*-hydroxysuccinimide. We tried to synthesize dansyl-pepstatin by the coupling of *N*-pepstatinyl-1,3-diaminopropane and dansyl chloride, but the yield was very low (below 5%). Instead, dansyl-pepstatin was obtained by the coupling of pepstatin A and monodansyldiaminopropane in moderate yield. The obtained dansyl-pepstatin contained no unreacted pepstatin A as judged from HPLC assays (Fig. 1).

As shown in Fig. 2, dansyl-pepstatin inhibited pepsin activity with similar potency to pepstatin A. Similarly chymosin and cathepsin D were inhibited by both inhibitors with essentially the same potencies (data not shown). The activity of one molar pepsin I was almost wholly inhibited by 0.7-0.8 molar inhibitor. This indicated that the pepsin I used in this experiment, contained about 70-80% of active enzyme. Similarly, the chymosin and cathepsin D contained about 80-90 and 20-30% of active enzyme, respectively.

As shown in Fig. 3, pepsin I contained some pepstatin-Sepharose 4B-unbound protein (21% of total A_{280}), and the content of active pepsin was calculated as 74% of the amount obtained by weight (as shown in Table I, the content of pepsin I obtained by measurement of the absorbance was 94% of the weight). When pepsin I and dansyl-pepstatin were mixed in a 1:1 molar ratio and subjected to gel

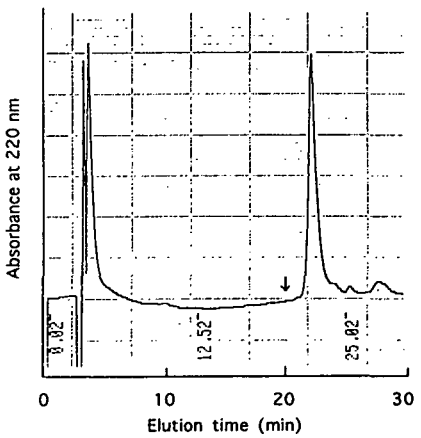

Fig. 1. **RP-HPLC analysis of dansyl-pepstatin.** Dansyl-pepstatin was applied to a C_{18} reversed-phase column (SPHERI-5 RP-18, 100×4.6 mm, Applied Biosystems) and eluted with a linear gradient starting from 80% solvent A and 20% solvent B to 10% solvent A and 90% solvent B at a flow rate of 0.5 ml·min^{-1}. Solvent A, 0.1% TFA; solvent B, 2-propanol/acetonitrile/TFA (70 : 30 : 0.07, v/v). The arrow indicates the elution time of pepstatin A.

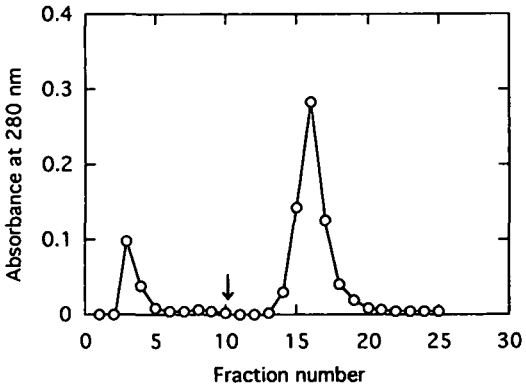

Fig. 3. **Affinity chromatography of pepsin I on a pepstatin-Sepharose 4B column.** Five hundred microliters of pepsin I solution (100 μM in citrate buffer pH 4.0) was applied to a pepstatin-Sepharose 4B column (1×7 cm). This was washed with citrate buffer (pH 4.0), and then eluted with Tris-HCl buffer (pH 8.5, containing 1 M NaCl) as described in "EXPERIMENTAL PROCEDURES." The arrow indicates the change of buffer.

TABLE I. **Amounts of active enzymes contained in commercial carboxyl proteases.**

Enzyme	Weight	Absorbance[a]	Fluorescence[b]
Pepsin I	10 μM	9.4 μM	7.3±0.3 μM
Pepsin II	10 μM	9.1 μM	8.0±0.4 μM
Chymosin I	10 μM	9.0 μM	8.1±0.5 μM
Chymosin II	10 μM	9.2 μM	9.0±0.5 μM
Cathepsin D[c]	10 μM	–	2.7±0.1 μM
Rennet	10 mg/ml		9.7±0.5 μM

[a]For the absorption coefficients of pepsin and chymosin, $E^{1\%}_{280}=14.85$ and $E^{1\%}_{275.5}=15.3$ were used (*26, 27*). [b]Data are presented as mean± SD from five determinations. [c]The purity of this enzyme described in the catalog was about 40%.

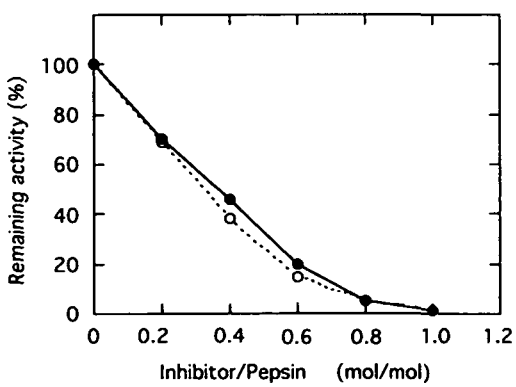

Fig. 2. **Inhibition of pepsin I with pepstatin A and dansyl-pepstatin.** Two milliliters of pepsin I solutions (100 μM) were incubated with various amounts of pepstatin A and dansyl-pepstatin for 2 min, and remaining activities were measured by the modified Anson method as described in "EXPERIMENTAL PROCEDURES." (●) Pepstatin A; (○) dansyl-pepstatin.

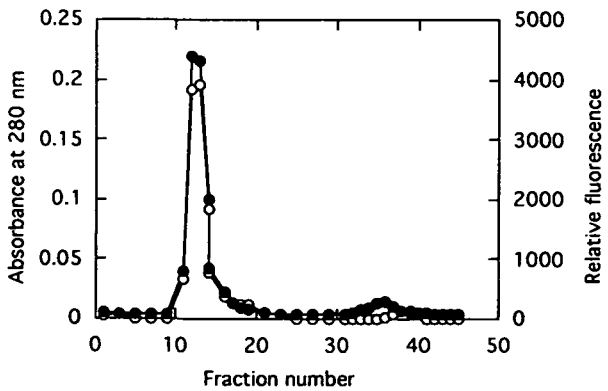

Fig. 4. **Determination of pepsin-dansyl-pepstatin complex.** Five hundred microliters of pepsin I solution (100 μM in citrate buffer pH 4.0) and 50 μl of dansyl-pepstatin solution (1 mM) were mixed and 500 μl of the mixture was applied to a Bio-Gel P-30 column (1.2×50 cm) as described in "EXPERIMENTAL PROCEDURES." (○) Absorbance at 280 nm; (●) relative fluorescence at 520 nm.

filtration, most of the dansyl-pepstatin was eluted with pepsin (Fig. 4). This indicated that dansyl-pepstatin and pepsin formed a tightly bound complex. The recovery of dansyl-pepstatin eluted with pepsin was 71%, so pepsin and dansyl-pepstatin formed a complex with a molar ratio of 1:0.96. Thus, pepsin and dansyl-pepstatin form a tightly bound 1:1 complex, as is the case with pepstatin (*7*). The formation of the complex occurred even at pH 1.5 or 5.5 (data not shown). The fluorescence of the dansyl group was increased by the addition of pepsin. The maximum increase in fluorescence near to 520 nm became progressively greater as the pH of the buffers increased, because the intensity of fluorescence of the dansyl group is small at pH 1.5-2 (Fig. 5). However, the fluorescence became unstable above pH 5, and the determination of pepsin was carried out at pH 4.0.

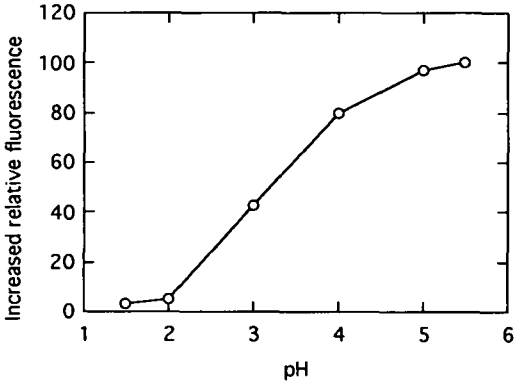

Fig. 5. **Effect of pH on increase in fluorescence.** In a fluorescence cell, 3 ml of citrate buffer of various pHs and 40 μl of dansyl-pepstatin solution (10 μM) were mixed and 60 μl of pepsin solution (10 μM in water) was added. The increase in fluorescence near 520 nm was measured as described in "EXPERIMENTAL PROCEDURES."

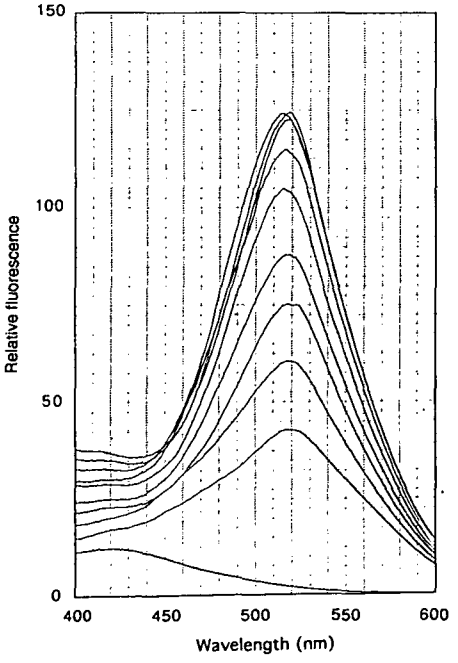

Fig. 6. **Fluorescence spectrum of dansyl-pepstatin solution with addition of pepsin solution.** In a fluorescence cell, 3 ml of citrate buffer (pH 4.0) and 40 μl of a dansyl-pepstatin solution (10 μM) were mixed and 20 μl portions of pepsin I solution (5 μM in water) were added. The fluorescence spectrum was measured (excitation at 320 nm). First (bottom) curve, citrate buffer; second curve, 40 μl of dansyl-pepstatin solution (10 μM in ethanol) was added; third curve, 20 μl of pepsin solution was added to the dansyl-pepstatin solution; higher curves, 20 μl portions of pepsin solution were added.

The maximum increase in fluorescence near to 520 nm was proportional to the amount of added pepsin until dansyl-pepstatin was saturated by pepsin (Figs. 6 and 7a). Above the saturating pepsin concentration, the fluorescence did not increase further. The amount of active pepsin was determined by detecting the inflection point of increased fluorescence. The amount of active enzyme could be obtained easily from measurement of the fluorescence intensity by the use of a standard curve, as shown in Fig. 7a. At low concentrations of pepsin, the inflection point became unclear. The minimum detectable amount of pepsin in this method was about 20 pmol with a standard deviation of 7.3%. On the other hand, inactivated pepsin, chymotrypsin, and trypsin did not increase the fluorescence. This result showed that dansyl-pepstatin bound only with active pepsin. As shown in Fig. 8, dansyl-pepstatin and pepstatin A bound with pepsin competitively, and the K_i value of dansyl-pepstatin was 0.8 times that of pepstatin A. The K_i value of pepstatin A was 4.6×10^{-11} M (*23*), so the K_i value of dansyl-pepstatin was about 3.7×10^{-11} M. The theoretical curves of amount of resulting EI complex (inhibitor

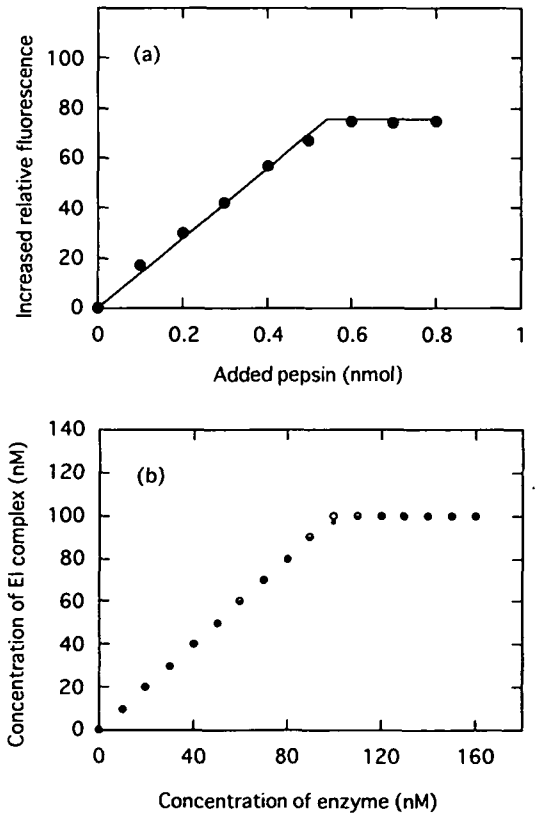

Fig. 7. **(a) Increase in fluorescence of dansyl-pepstatin solution by addition of pepsin solution.** The maximum increases in fluorescence shown in Fig. 6 were plotted. **(b) Theoretical curve of amount of resulting EI complex.** When enzyme solution was added to inhibitor solution (100 nM), the concentration of the resulting EI complex was calculated. (●) $K_i=10^{-2}$ M; (○) $K_i=10^{-4}$ M.

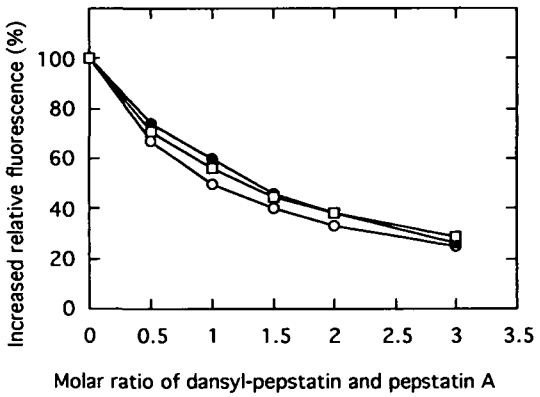

Fig. 8. **Competition for binding of dansyl-pepstatin and pepstatin A with pepsin.** In a fluorescence cell, 3 ml of citrate buffer (pH 4.0), 50 μl of dansyl-pepstatin solution (100 μM) and various amounts of pepstatin A solution (100 μM) were mixed and 70 μl of pepsin solution (100 μM) was added. After 2 min, the fluorescence spectrum was measured (excitation at 320 nm). (●) Increased relative fluorescence; (○) theoretical curve (K_i of pepstatin A and dansyl-pepstatin = 1:1); (□) theoretical curve (K_i = 1:0.8).

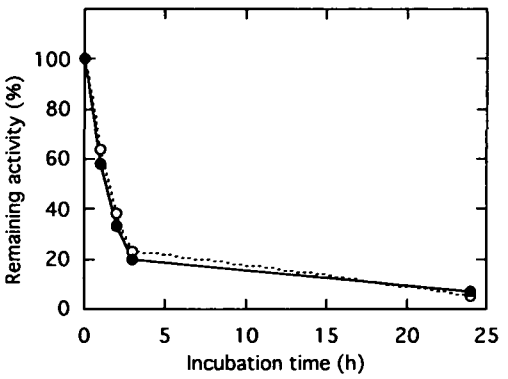

Fig. 9. **Rate of inactivation of pepsin in pH 6.5 buffer.** To a 1.8 ml of citrate buffer (pH 6.5), 0.2 ml of pepsin solution (100 μM in water) was added at 30°C. After incubation for 1, 2, 3, or 24 h, the increase in fluorescence by dansyl-pepstatin and the remaining activity were measured as described in "EXPERIMENTAL PROCEDURES." (●) Activity; (○) fluorescence.

concentration, 100 nM) are shown in Fig. 7b. Both curves ($K_i = 1 \times 10^{-2}$ and 1×10^{-4} M) are essentially the same, and it seems that the determination of protease is possible even if the affinity is relatively low. The dissociation constants of pepstatin A and pepstatin-sensitive carboxyl proteases are sufficiently small (*24, 25*), so the amounts of pepstatin-sensitive carboxyl proteases can be measured precisely. In practice, the forms of the curves obtained in titration (Fig. 7a) and that obtained theoretically (Fig. 7b) were essentially the same, within experimental error. The increase in fluorescence was very rapid, being completed within 5 s after mixing, so the rate of complex formation could not be measured. As shown in Fig. 7a, the increase in fluorescence stopped at the molar ratio of 1.33 for pepsin I and dansyl-pepstatin. This indicated that the pepsin I used contained about 75% of active pepsin.

Then, we determined the amounts of active enzymes contained in various commercially available carboxyl proteases. As shown in Table I, all samples contained some contaminants and/or inactive form. Based on the absorbances, commercial pepsin contained more than 90% of the enzyme, but the active pepsin contents were only 73 (pepsin I) and 80% (pepsin II) by our method. The pepsin I was purchased about 5 years ago, so a part of the enzyme could have been inactivated during storage, even at −20°C. The commercial chymosin contained 81 and 90% of active enzyme. The content of active cathepsin D obtained by our method was low (27%). The determination of absorbance was impossible, because the amount of cathepsin D in the purchased vial was to small. The purity of this enzyme described in the catalog was about 40%, and this is consistent with our result. Moreover, it was possible to determine the amount of active chymosin contained in rennet. This shows that the amounts of active pepstatin-sensitive enzymes can be determined even in crude extracts.

In pH 4.0 buffer, a part of the dansyl-pepstatin formed a nonspecifically bound complex with pepsinogen and caused an increase in fluorescence (about 70% of that with pepsin), however, in suitable conditions, it could be possible to reduce the increase in fluorescence with pepsinogen. In those conditions, the increase in fluorescence with pepsin was not affected. We are studying the activation of pepsinogen used by this method.

As an application of our method, the rate of inactivation of pepsin in pH 6.5 buffer was determined (Fig. 9). The inactivation curve obtained by this method and by activity measurement were good agreement, indicating that our assay method is reliable. The determination of carboxyl proteases used by dansyl-pepstatin is very convenient and should be useful for the determination of many pepstatin-sensitive carboxyl proteases.

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