274 (ε 8.300, 9.800). *Anal.* Calcd. for $C_9H_{13}O_5N_3$: C, 44.45; H, 5.39; N, 17.28. Found: C, 44.39; H, 5.57; N, 17.05.

Method B (from II)——To a solution of O^2,2′-cyclocytidine hydrochloride (II) (1 g) in water (40 ml) was added 2N KOH (3.8 ml) and the solution was allowed to stand for 4 hours at room temperature with stirring. The reaction mixture was treated with Diaion SK-1B (ammonium form, 16 ml) in batch and the resin was removed by filtration. The filtrate was concentrated to dryness *in vacuo* to give white prisms (0.76 g) in 82.0% yield. Recrystallized compound was identical with arabinosylcytosine obtained by the method A by all criteria.

Method C (from I)——A suspension of cytidine (35.0 g) in ethyl acetate (3.5 liter) was refluxed with partially hydrolyzed phosphorus oxychloride (316 ml, $POCl_3/H_2O=1$ mole/mole) for 2 hours. The reaction mixture was cooled and poured into ice water (3.0 liter) with stirring to hydrolyze completely phosphorus oxychloride. The acidic solution, after ethyl acetate was removed by evaporation from it, was subjected to the column of Diaion SK-1B (acid form, 2.45 liter) followed by washing with water (16.6 liter) and elution with chilled 2N KOH solution (4.0 liter). In order to make the elution effective the alkaline solution was circulated through the column. The eluate, after removing potassium ion by Diaion SK-1B (ammonium form, 8.5 liter), was passed through the column of Diaion SA-11B (borate form, 0.42 liter) to remove unreacted cytidine and the resin was washed with water. The effluent containing III and washings were combined and it was concentrated to a small volume to give white prisms (22.0 g).

Recrystallization from minimum amount of water gave pure material which was identical with arabinosylcytosine obtained by the method A.

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Proteolytic Enzymes. V.[1] Further Application of *p*-Nitrophenyl *p*-Amidinobenzoate as a Titrant of Trypsin[2]

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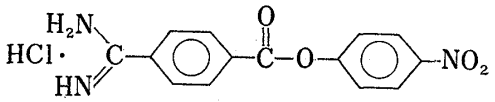
The purity of an enzyme preparation and hence the operational normality of an enzyme solution cannot be fully determined by catalytic rate assay alone. For this purpose "titration" methods using a specific reagent which reacts stoichiometrically with enzyme active sites have been described.[4] *p*-Nitrophenyl *p*-amidinobenzoate hydrochloride (NPAB) **1** was proposed to be a good reagent for active site titration of trypsin (EC 3.4.4.4)[5] in connection with our studies of synthetic trypsin inhibitors.[6] Only fifty to



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seventy % molecules were observed to have the active site in solutions of commercial trypsin preparation,[5] whose total protein concentrations had been determined based on absorbancy as usual. The result was consistent with those obtained by other titrants.[4,7] The present note describes two examples of further application of NPAB as a titrant of trypsin.

Experimental

Materials——Highly purified soybean trypsin inhibitor (STI) was prepared from soybean flakes by the method described previously.[8] Trypsin was a Worthington, TRL-7FA and benzoyl-L-arginine ethyl ester hydrochloride (L-BAE) was a gift from Ajinomoto Co. Ltd.

Methods——The enzyme and STI were mixed and incubated at the concentration of 7.70×10^{-8} and $0-7.65\times10^{-8}$M, respectively, at 25° for 5 min[9] in 50 ml of 0.02M $CaCl_2$, 0.1M KCl (pH 8.2), and then the substrate was added to the final concentration of 4.9×10^{-4}M. The rate of hydrolysis at pH 8.2 was measured by a Radiometer pH-stat titrator Model TTTlc at 25° with 0.02N NaOH under nitrogen stream. Trypsin and STI concentrations were calculated based on the molar absorbancy coefficient at 280 mμ to be 39000 and 21600,[8] respectively.

Kinetic parameters were determined from Lineweaver-Burk plots. The hydrolysis of L-BAE by trypsin was carried out in 0.025M $CaCl_2$ or 0.02M KCl at 25°; pH 8.0.

Result and Discussion

Stoichiometry of Interaction between Trypsin and Soybean Trypsin Inhibitor

Soybean trypsin inhibitor is a well known protein with its inhibitor effect.[10] It was recently demonstrated that the inactivation is accompanied by the formation of a stable complex between the enzyme and the inhibitor.[11] Ozawa and Laskowski[12] presented evidence that the interaction of trypsin with STI results in the proteolytic splitting of a labile peptide bond in the inhibitor and that the "active serine" of trypsin participates in the formation of a stable acyl-enzyme bond. However, the stoichiometry of this inactivation has not been precisely defined due to impurity of trypsin samples,[9] although Kunitz earlier proposed the one to one binding of the enzyme with the inhibitor.[10,13] By means of potentiometric measurement, Laskowski, *et al.* showed that, assuming the 100% purity of STI, an active portion of trypsin associates with the inhibitor.[14] Purification of commercial trypsin was tried in relation to STI-binding capacity.[15] Chromatographic purification of the enzyme has been advanced using a "small-molecule" titrant.[7] Shaw, *et al.* recently revealed that commercial bovine trypsin contains several types of limited autodigestion products, whose activities are more or less different from that of the native species.[16]

In order to confirm the stoichiometry of the inhibitory action of STI definitely in terms of the concentration of the active site of trypsin, the use of NPAB seemed preferable because it can well be employed as a titrant at pH range around 8, where usual binding experiments of STI are performed. Thus the rate of tryptic hydrolysis of L-BAE was examined as a function of STI *vs.* trypsin mole ratio at pH 8.2. Fig. 1 presents the stoichiometry of the inhibitory action of STI obtained in this manner. The extrapolation of the remaining activity to zero gave 0.51 as $[I]/[E_0]$ value for complete inactivation. This result indicates that one mole of

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trypsin is inactivated by 0.51 mole of STI on absorbancy basis. This stoichiometric value is in substantial agreement with the normality value, 55%, of the stock solution of trypsin obtained by the NPAB titration at pH 8.2 in the same manner as described previously.[5] Apparently the inactive species to NPAB in the trypsin sample does not interact with STI. The above results obtained at the same pH may therefore add a chemical evidence on the normality basis that the inactivation of trypsin by STI takes place with one to one stoichiometry of the active species of the enzyme and the inhibitor.[17]

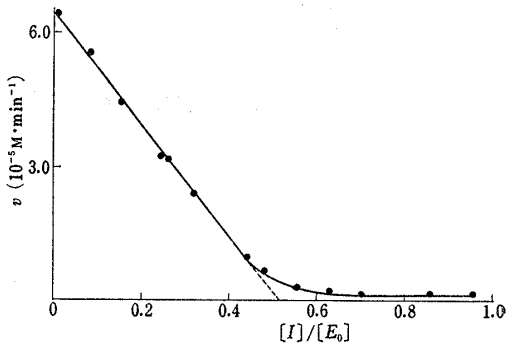

Fig. 1. Stoichiometry of STI Inactivation

Tryptic hydrolysis of benzoyl-L arginine ethyl ester hydrochloride (L-BAE) as a function of STI *vs.* trypsin mole ratio at pH 8.2, 25°.

Real Turnover Number of Trypsin aginst BAE as a Substrate

Kinetic parameters of trypsin substrates have usually been calculated from apparent enzyme concentrations used, which are determined based on absorbancy coefficient or on nitrogen centent[18]. For reasonable estimation of these parameters as a real measure of enzymatic activities, it is desirable to calculate them based on real concentrations of the active enzyme. Table I lists the kinetic parameters derived from the Lineweaver–Burk plots of the tryptic hydrolysis of L-BAE. Definition of these parameters are that described previously.[19] The values of turnover number, k_{cat}, were obtained by dividing observed V_{max} by trypsin concentration, which was determined based on the titration with NPAB. Thus these real k_{cat} values are nearly twice greater than conventional values as shown in Table I.

TABLE I. Kinetic Parameters for Tryptic Hydrolysis at pH 8.0, 25°

Medium	k_{cat} (sec^{-1})	K_m (M)
0.025M $CaCl_2$	31.1	3.6×10^{-6}
	14.6[a]	4.3×10^{-6}[a]
0.2M KCl	19.3	7.8×10^{-6}
	8.4[a]	1.0×10^{-5}[a]

[S]: 1.84×10^{-5}—6.98×10^{-4}M [E]: 2.70×10^{-9}M
a) lit. 18

Studies on further application of this reagent as a titrant of trypsin and the related enzymes are currently under way.

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