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HIGHLY SENSITIVE METHOD FOR DETERMINATION OF ESTERASE ACTIVITY OF α-CHYMOTRYPSIN AND α-CHYMOTRYPSIN-LIKE ENZYMES USING MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KENJI KUROMIZU*, HIROSHI HIGUCHI, OKITOSHI ABE and YUKIKO SHIMOKAWA
Laboratory of Chemistry, Fukuoka Dental College, 700 Ta, Sawara-ku, Fukuoka 814-01 (Japan)
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SUMMARY

A new substrate, Dns-L-phenylalanine ethyl ester, with high UV absorption has been developed for the determination of the esterase activity of α-chymotrypsin and α-chymotrypsin-like enzymes. The product, generated by the enzyme action, Dns-L-phenylalanine, was clearly separated from the ester substrate by micro reversed-phase high-performance liquid chromatography. The substrate was highly stable under the enzyme assay conditions used. As little as 0.15 ng of α-chymotrypsin and 1.49 ng of subtilisin BPN′ could be detected when a long reaction time was employed. Hydrolyses of the substrate by α-chymotrypsin and α-chymotrypsin-like enzymes were blocked by specific inhibitors of the enzymes.

INTRODUCTION

High-performance liquid chromatography (HPLC) is one of the most powerful tools for the determination of the reactants and products in chemical reactions. HPLC has been applied to enzyme reactions such as adenosine deaminase, purine nucleotide phosphorylase in erythrocytes, and acid and alkaline phosphatase in serum[1–3]. Recently, HPLC has also been used for the determination of the esterase activity of papain in comparison with a conventional pH-stat method[4] and for the measurement of angiotensin-converting enzyme activity in biological samples[5].

During the isolation of proteases in seeds and green leaves of *Vicia angustifolia* L., a species of common vetch, we have found two proteases with esterase activity in both seeds and leaves (unpublished work), one of which possessed α-chymotrypsin-like activity and the other both α-chymotrypsin- and trypsin-like activities. The synthetic ester substrates commonly used, such as acetyl-L-tyrosine ethyl ester[6] and benzoyl-L-phenylalanine ethyl ester[7], were not sensitive so that most of the proteases were used up for the measurement of the activity during the isolation because their contents were so low. We therefore wished to develop a highly sensitive method for the determination of the esterase activity of the proteases. A relatively highly sensitive radial diffusion method using tosyl-L-arginine methyl ester and bromophenol

blue was introduced for the determination of esterase activity[8]. However, this method requires on overnight reaction and can detect only trypsin-like activity. In addition, no kinetic treatment could be applied. To overcome these shortcomings, we have developed a new sensitive substrate, Dns-L-phenylalanine ethyl ester (Dns-L-Phe-OEt), introducing a dansyl group with high ultraviolet absorption.

In this paper, we describe the enzyme assay, a micro-HPLC method, stability of the substrate and the pH dependence on the reaction of α-chymotrypsin with the new substrate. Kinetic parameters, the detection limit of the enzyme and the effects of the specific inhibitors are also described in the reaction of both α-chymotrypsin and subtilisin BPN′.

EXPERIMENTAL

Materials

α-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas was purchased from Boehringer (Mannheim, G.F.R.). The specific activity of the enzyme was 56.7 units/mg of active enzyme, where 1 unit is equal to the hydrolysis of 1.0 μmole of benzoyl-L-tyrosine ethyl ester per minute at pH 7.8 and 25°C[9]. The concentration of active enzyme in the stock solution was determined graphically by extrapolating the absorbance at 400 nm to zero time using *p*-nitrophenyl acetate as a titrant[10].

Subtilisin BPN′ (E.C. 3.4.4.16; subtilopeptidase C) from *Bacillus amyloliquefaciens*[11] and Kunitz pancreatic trypsin inhibitor (PTI) from bovine pancreas[12] were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

Protein concentration was determined by measuring the absorbance at 280 nm in a 1-cm light-path cell based on $E_{1\%} = 20.4$ at 280 nm for α-chymotrypsin[13], 11.7 for subtilisin BPN′[14] and 8.3 for PTI[15]. Molecular weights of 25,310 for α-chymotrypsin[16], 27,537 for subtilisin BPN′[17] and 6513 for PTI[15] were used throughout to elucidate the enzyme and inhibitor concentrations. An $E_{1\%}$ of 4.3 and a molecular weight of 7930 were used for a proteinase inhibitor (VAI) isolated from *Vicia angustifolia* L. by Abe *et al.*[18]. The molecular weight of 7930 was elucidated from the recently established complete amino acid sequence of the inhibitor.

Dns chloride, phenylmethylsulphonyl fluoride (PMSF) and *p*-toluenesulphonyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Nakarai Chemicals (Kyoto, Japan) and L-phenylalanine ethyl ester hydrochloride from Protein Research Foundation (Minoh, Osaka, Japan). Dns-DL-phenylalanine (Dns-DL-Phe-OH) from Sigma was recrystallized from ethanol–diethyl ether. All other chemicals were of reagent grade.

Water for HPLC was deionized and distilled, and then filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, U.S.A.) just before use.

Synthesis of Dns-L-phenylalanine ethyl ester

To a solution of L-phenylalanine ethyl ester hydrochloride (0.5 mmole) dissolved in ammonia-free 0.5 *M* sodium hydrogen carbonate solution (20 ml), dansyl chloride (0.5 mmole) in 10 ml of acetone was added in a nitrogen atomosphere. The reaction mixture was agitated for 4 h at 37°C. After removal of acetone by evaporation, the residue was extracted into two portions (20 ml each) of ethyl acetate and the

combined extracts were successively washed with 0.5 *M* sodium hydrogen carbonate solution, 1 *M* hydrochloric acid and saturated sodium chloride solution. The organic layer was separated, dried over anhydrous sodium sulphate and then evaporated to an oil. Treatment of the oil with diethyl ether–light petroleum gave an amorphous powder, 175 mg (82%).

Stock solution (33 m*M*) for enzyme assay was prepared by dissolving the powder in dimethyl sulphoxide (DMSO) without further purification. Homogeniety of the product was as certained by a single spot on thin-layer chromatography [R_F = 0.90 in chloroform–methanol (5:1) and 0.92 in chloroform–methanol–acetic acid (95:5:1)] and by a symmetrical single peak on HPLC (See Figs. 1 and 2a). The solution was stable for at least 4 months at 4°C.

Absorption spectrum

Three absorption maxima at 215, 248 and 332 nm were observed on the absorption spectrum of Dns-DL-Phe-OH in 70% methanol measured with a Hitachi Model 124 spectrophotometer. The absorption at 215 nm was the strongest of the three, but was accompanied by significant noise. The absorption intensity at 248 nm was relatively stable and about three times higher than that at 332 nm. Therefore, the UV detector was set at 248 nm for monitoring the column effluent from the micro-HPLC system. A molar absorptivity of 13,050 ± 150 $M^{-1} \cdot cm^{-1}$ at 248 nm was evaluated from absorption measurements at five different concentrations of Dns-DL-Phe-OH in 70% methanol.

Typical enzyme assay

To a solution of 10 μl of the stock substrate in 80 μl of 50 m*M* Tris · HCl (pH 8.0) containing 10 m*M* calcium chloride were added 10 μl of the enzyme dissolved in 1 m*M* hydrochloric acid containing 20 m*M* calcium chloride at a thermostatically controlled temperature of 37°C. After incubation for 10 min, the enzyme reaction was terminated by the addition of 10 μl of acetic acid and the solution was immediately subjected to HPLC for enzyme analysis. However, for the sake of convenience, the sample can be stored in a freezer because no further change in hydrolysis by the enzyme action could be detected even after 1 week when the sample was stored at −20°C.

Analysis of reaction product by micro-HPLC

The product, Dns-L-Phe-OH, was clearly separated from the substrate using a Jasco Familic-100 N micro-HPLC system (Japan Spectroscopic Co., Tokyo, Japan), equipped with an SIC-5000 E calculator (System Instruments, Dover, MA, U.S.A.). The column (180 × 0.5 mm I.D. PTFE tube) was packed by the slurry method with 10-μm octadecylsilane-coated particles (Fine-sil ODS-10) for reversed-phase HPLC. Elution was performed by the isocratic elution of 70% methanol at ambient temperature (about 23°C) at a flow-rate of 16 μl/min and the effluent was monitored with a UVIDEC-100 UV detector (Japan Spectroscopic Co.) using a 5-μl flow cell at 248 nm. A 0.6-μl volume of the sample was injected.

RESULTS AND DISCUSSION

Analysis of the product of the enzyme reaction

As a preliminary experiment, the hydrolysis of Dns-L-Phe-OEt by α-chymotrypsin was first followed by thin-layer chromatography. A typical thin-layer chromatogram is shown in Fig. 1. It was found that the substrate was easily hydrolysed by α-chymotrypsin and that the reaction could be qualitatively analysed by chromatographing the reaction mixture on a thin-layer plate and by irradiation with long-wave UV light (mainly at 365 nm). For the quantitative analysis of the enzyme reaction, HPLC was introduced. Solvents other than methanol, for example acetonitrile, *n*-propanol and dioxane, gave less effective separation and/or a broader peak on the ester substrate. In addition, the ester was not eluted, not only with methanol at a concentration of 60% or less, but also at 80% or higher concentrations. The reason for the latter phenomenon is unclear. A 70% solution of methanol in water was chosen as the solvent for isocratic elution in HPLC.

Fig. 2 illustrates a typical elution pattern showing that the product, Dns-L-Phe-OH, was clearly separated from the ester substrate and other contaminants.

Calibration graph of Dns-L-phenylalanine

A stock solution of purified Dns-DL-Phe-OH (10 m*M*) in absolute methanol was prepared and was successively diluted to appropriate concentrations. Each 0.6-μl portion of the diluted solution was injected into the micro-HPLC system and the peak area was integrated using an SIC-5000 E calculator. The result is shown in Fig. 3. A linear relationship between the integrated peak area and the amount of Dns-DL-Phe-OH injected was obtained over a relatively wide range from 5 to 500 pmole. Further, accuracy of the motor-drived injection of 0.6 μl was ascertained from five separate runs at every concentration.

Stability of the substrate

The stability of the substrate under the assay conditions used was examined. A

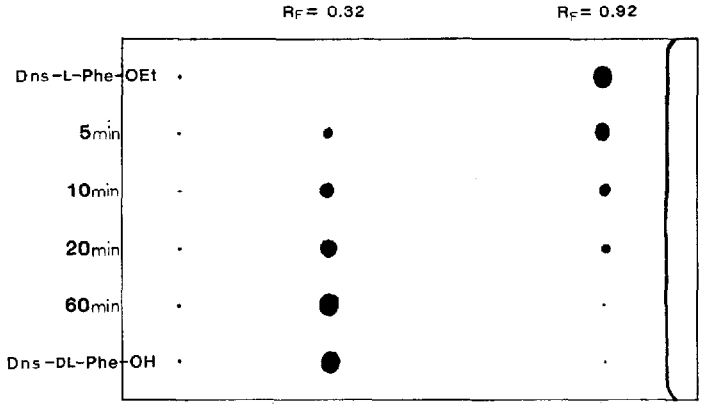

Fig. 1. Thin-layer chromatogram of the reaction product of Dns-L-Phe-OEt by α-chymotrypsin. Aliquots of the incubation mixture of the substrate ($3.3 \cdot 10^{-3}$ *M*) and bovine α-chymotrypsin ($6.7 \cdot 10^{-7}$ *M*) were applied to a thin-layer plate (silica gel G) at the indicated intervals of time. After chromatography with chloroform–methanol–acetic acid (95:5:1), fluorogenic spots on the plate were revealed by irradiation with long-wave UV light.

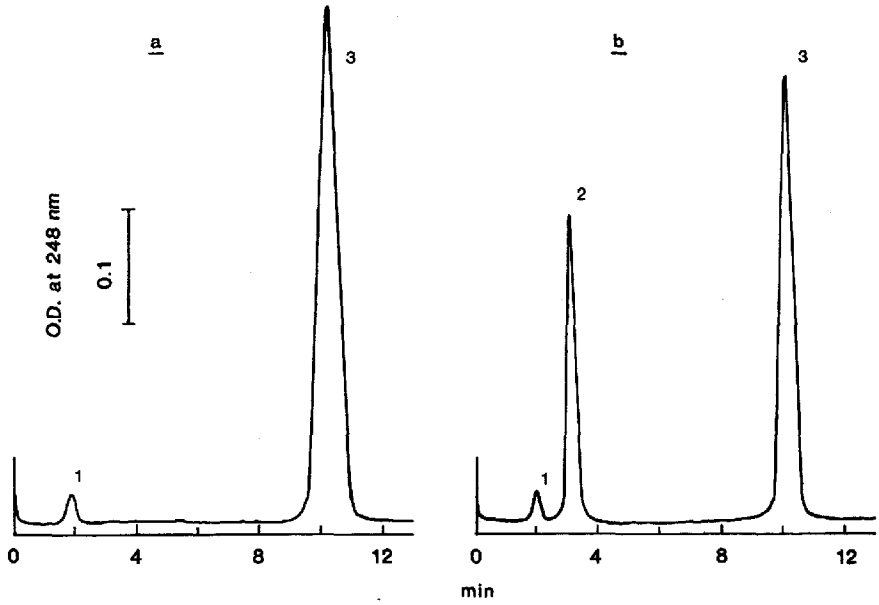

Fig. 2. Typical elution pattern of the enzyme reaction mixture obtained by micro-HPLC. The mixture contained $3.3 \cdot 10^{-3}$ *M* Dns-L-Phe-OEt and α-chymotrypsin ($3.3 \cdot 10^{-8}$ *M*) in 50 m*M* Tris · HCl (pH 8.0) and 10 m*M* $CaCl_2$, and was allowed to stand for 10 min at 37°C. The reaction was terminated by the addition of 10 μl of acetic acid. A portion (0.6 μl) of the mixture was injected and analysed by micro-HPLC. (a) Blank run without enzyme; (b) reaction mixture. Peaks: 1, contaminants, mainly Dns-NH_2; 2, Dns-L-Phe-OH; 3, Dns-L-Phe-OEt.

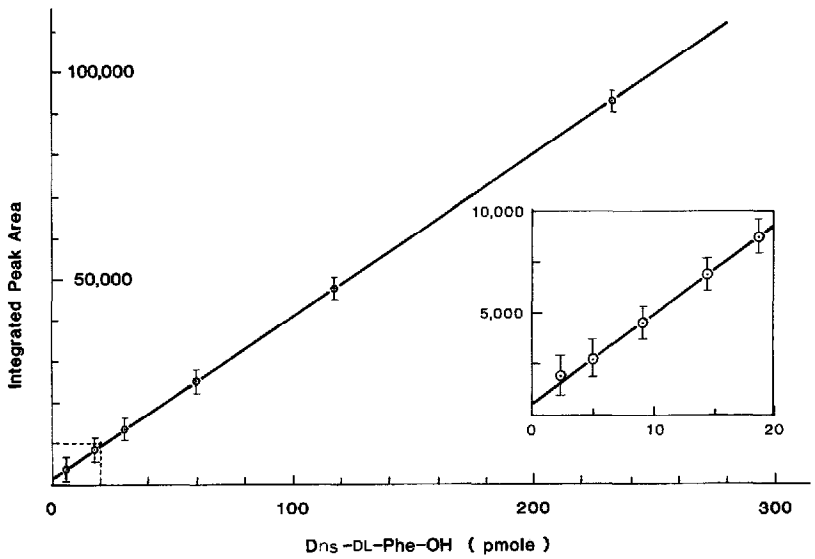

Fig. 3. Calibration graph of Dns-DL-Phe-OH. A portion (0.6 μl) of Dns-DL-Phe-OH in absolute methanol diluted to an appropriate concentration was analysed by micro-HPLC. The peak area was integrated with an SIC-5000 E calculator. The points and bars represent the mean ± S.D. from the five separate runs at the indicated concentrations. The insert shows the dotted area on an enlarged scale.

mixture of 10 μl of the stock substrate (33 m*M* in dimethyl sulphoxide) and 90 μl of 50 m*M* Tris · HCl (pH 8.0) containing 10 m*M* calcium chloride was allowed to stand at 37°C for appropriate intervals of time. An aliquot (0.6 μl) of the mixture was withdrawn and analysed by micro-HPLC. Less than 0.3% of the ester substrate was hydrolysed to the acid on standing for 22 h. No decomposition could be observed under the assay conditions used.

Effects of pH, time and α-chymotrypsin concentration on the enzyme reaction

Changes of the initial velocity at different pH values was examined using 50 m*M* each of Tris · HCl, sodium veronal and sodium phosphate buffers in the presence of 10 m*M* calcium chloride. Both at acidic pH and at pH higher than 9 a slow enzyme reaction was observed, as expected. The low rate of hydrolysis in phosphate buffer can probably be attributed to the lower stability of the enzyme owing to the consumption of Ca^{2+} ion. The optimum pH was 8.4 in both the Tris · HCl and veronal buffers, as shown in Fig. 4.

A time-course experiment was carried out in the standard assay medium using $3.3 \cdot 10^{-8}$ *M* bovine α-chymotrypsin. At appropriate intervals of time, a sample of the mixture was withdrawn and analysed by micro-HPLC. Linearity between the rate of hydrolysis of the substrate and time was observed for at least 45 min under the conditions used. An incubation time of 10 min was employed for the general enzyme assay.

α-Chymotrypsin of various concentrations was incubated with $3.3 \cdot 10^{-3}$ *M* Dns-L-Phe-OEt for 10 min in 50 m*M* Tris · HCl (pH 8.0) containing 10 m*M* calcium chloride. The reaction mixture was analysed by micro-HPLC as described above. The

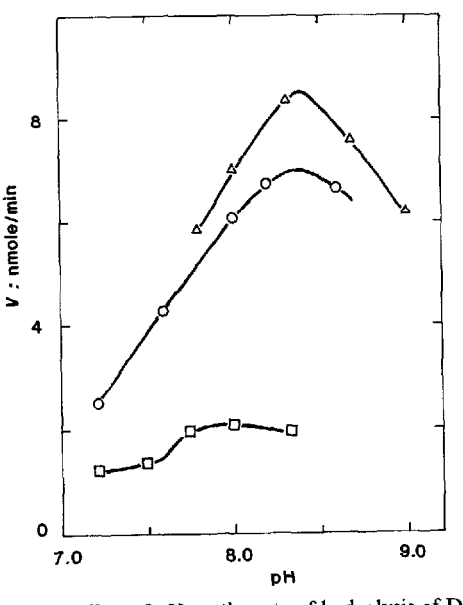

Fig. 4. Effect of pH on the rate of hydrolysis of Dns-L-Phe-OEt ($3.3 \cdot 10^{-3}$ *M*) by α-chymotrypsin. Buffers with the indicated pH values were 50 m*M* Tris · HCl (○), 50 m*M* sodium phosphate (□) and 50 m*M* sodium veronal (△), and all contained 10 m*M* $CaCl_2$. Enzyme, $3.3 \cdot 10^{-8}$ *M*; incubation, 10 min at 37°C; *v*, rate of hydrolysis of Dns-L-Phe-OEt (nmole/min).

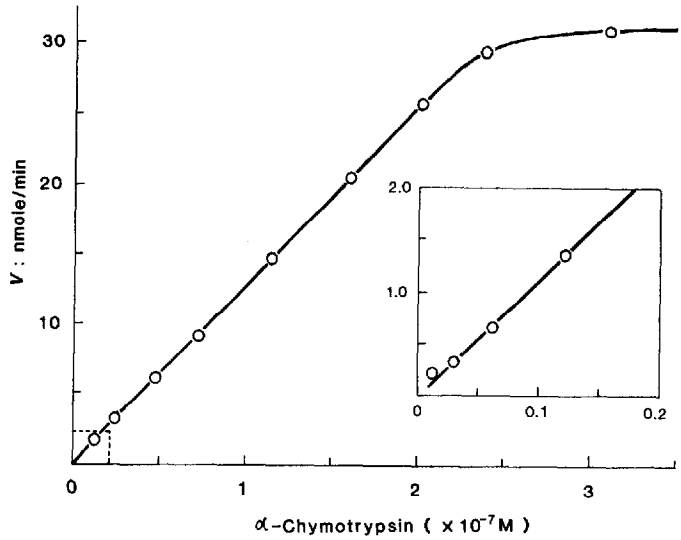

Fig. 5. Rate of hydrolysis of Dns-L-Phe-OEt as a function of enzyme concentration. The substrate ($3.3 \cdot 10^{-3}$ *M*) in 50 m*M* Tris · HCl (pH 8.0) containing 10 m*M* $CaCl_2$ was incubated with various concentration of α-chymotrypsin ($1.17 \cdot 10^{-9}$–$4.68 \cdot 10^{-7}$ *M*) at 37°C for 10 min. Analyses of Dns-L-Phe-OH generated by the enzyme action were performed by micro-HPLC. The insert shows the dotted area on an enlarged scale.

initial rate of hydrolysis of Dns-L-Phe-OEt by α-chymotrypsin was proportional to the enzyme concentration between $2.5 \cdot 10^{-9}$ and $2.0 \cdot 10^{-7}$ *M*, as shown in Fig. 5.

Detection limit of enzyme

α-Chymotrypsin was simply diluted with 1 m*M* hydrochloric acid in the pres-

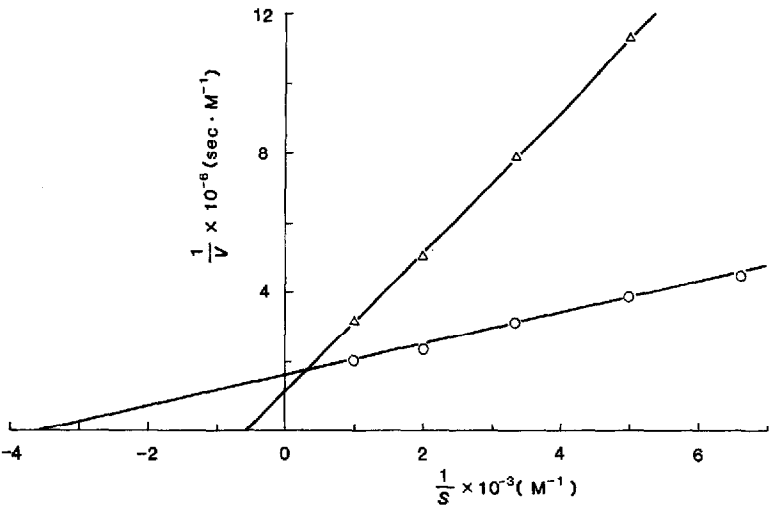

Fig. 6. Lineweaver–Burk plot for the hydrolysis of Dns-L-Phe-OEt by bovine α-chymotrypsin (○) and subtilisin BPN′ (△). α-Chymotrypsin ($2.34 \cdot 10^{-8}$ *M*) and subtilisin BPN′ ($5.4 \cdot 10^{-8}$ *M*) were incubated with a series of substrate concentrations ($1.5 \cdot 10^{-4}$–$1.0 \cdot 10^{-3}$ *M*) using the standard procedure. The activity was analysed by micro-HPLC. Points indicate the average of three separate runs.

TABLE I

KINETIC CONSTANTS FOR HYDROLYSIS OF DNS-L-Phe-OEt BY BOVINE α-CHYMOTRYPSIN AND SUBTILISIN BPN'

The constants are given together with those of the other ethyl ester substrates for the purpose of comparison. All the k_0/K_m values were calculated from the values of k_0 and K_m reported in the literature cited.

Substrate	*α-Chymotrypsin*				*Subtilisin BPN'*			
	K_m *(M)*	k_0 *(sec^{-1})*	k_0/K_m *($M^{-1}\ sec^{-1}$)*	*Ref.*	K_m *(M)*	k_0 *(sec^{-1})*	k_0/K_m *($M^{-1}\ sec^{-1}$)*	*Ref.*
Dns-L-Phe-OEt	$2.85 \cdot 10^{-4}$	27.3	95,790	This work	$1.89 \cdot 10^{-3}$	15.2	8042	This work
Ac-L-Phe-OE	$2.5 \cdot 10^{-3}$	61.1	24,400	20	$1.66 \cdot 10^{-2}$	30.6	1800	20
	$8.8 \cdot 10^{-4}$	63.1	71,700	21				
	$1.2 \cdot 10^{-3}$	173	144,160	22				
Bz-L-Phe-OEt	$2.8 \cdot 10^{-3}$	14.4	5140	23	—*	–	–	
Ac-L-Tyr-OEt	$1.3 \cdot 10^{-3}$	154	118,500	20	$2.22 \cdot 10^{-2}$	383.3	17,300	20
	$7 \cdot 10^{-4}$	193	275,710	6				
Bz-L-Tyr-OEt	$2.6 \cdot 10^{-3}$	43.0	16,540	23	—*	–	–	

* Not determined.

ence of 20 m*M* calcium chloride and used for the general enzyme assay. The incubation time was extended to 60 min and the reaction mixture was analysed by micro-HPLC using isocratic elution of 70 % methanol. The peak area was integrated with an SIC-5000 E calculator. The detection limit of the enzyme was as little as 0.15 ng (about 6 fmole) at a signal-to-noise ratio of 5. In a similar procedure but using 10 m*M* sodium phosphate (pH 7.2) for the enzyme dilution, as little as 1.49 ng (54 fmole) of subtilisin BPN′ could be detected. As the substrate is completely stable for at least 22 h under the conditions used, more sensitive detection for the esterase activity would be possible if a more stable condition of the enzyme at high dilution could be achieved. Indeed, considerable inactivation of the enzyme action was observed in the reaction of subtilisin BPN′ under the assay conditions used.

Kinetic constants

The initial rate of hydrolysis of the substrate by bovine α-chymotrypsin and subtilisin BPN′ was measured at various concentrations of the substrate between $1.5 \cdot 10^{-4}$ and $1.0 \cdot 10^{-3}$ *M* using the standard procedure. A double reciprocal plot of the initial rate of hydrolysis and the substrate concentration is shown in Fig. 6. The Michaelis constant (K_m) and molecular activity (k_0) were elucidated to be $2.85 \cdot 10^{-4}$ *M* and 27.3 sec^{-1} for α-chymotrypsin and $1.89 \cdot 10^{-3}$ *M* and 15.2 sec^{-1} for subtilisin BPN′. These are summarized in Table I with other ethyl ester substrates. A relatively high k_0/K_m value for Dns-L-Phe-OEt in comparison with the other substrates revealed that this was a reasonably good substrate for α-chymotrypsin and the related enzymes.

Inhibition of the enzyme activity by specific inhibitors

The enzyme activity of α-chymotrypsin and subtilisin BPN′ was inhibited by their specific inhibitors, as shown in Table II. Almost complete inhibition of α-chy-

TABLE II

INHIBITION OF THE HYDROLYSIS OF DNS-L-Phe-OEt BY SPECIFIC INHIBITORS

Bovine α-chymotrypsin ($3.3 \cdot 10^{-8}$ *M*) and subtilisin BPN′ ($5.4 \cdot 10^{-8}$ *M*) were pre-incubated with and without the inhibitors for the indicated time in 50 m*M* Tris · HCl (pH 8.0) containing 10 m*M* $CaCl_2$. After the addition of the substrate ($3.3 \cdot 10^{-3}$ *M*), the remaining enzyme activity was measured by analysis of Dns-L-Phe-OH by micro-HPLC. The activity is expressed as the rate of hydrolysis of Dns-L-Phe-OEt in the assay medium (100 μl). Parentheses indicate the relative ratio of the inhibitory activity as a percentage. Abbreviations: PMSF, phenylmethylsulphonyl fluoride; TPCK, *p*-toluenesulphonyl-L-phenylalanine chloromethyl ketone; PTI, bovine pancreatic trypsin inhibitor (Kunitz); VAI, proteinase inhibitor isolated from *Vicia angustifolia* L. by Abe *et al.*[18].

Inhibitor	*Concentration of inhibitor (M)*	*Pre-incubation time (min)*	*Activity (nmole/min)*	
			α-Chymotrypsin	*Subtilisin BPN′*
None	0	15	4.12 (0%)	2.46 (0%)
PMSF	$1 \cdot 10^{-4}$	15	0.91 (78%)	0.34 (86%)
TPCK	$1 \cdot 10^{-4}$	15	1.12 (73%)	2.35 (4.5%)
PTI	$1 \cdot 10^{-6}$	5	0.411 (90%)	–*
VAI	$1 \cdot 10^{-6}$	5	0.165 (96%)	–*

* Not determined.

motrypsin activity by the proteinase inhibitors PTI and VAI was observed in a 5-min pre-incubation. Subtilisin BPN′ activity is not affected by TPCK, which is a potent inhibitor of α-chymotrypsin, while PMSF strongly inhibits both enzyme activities.

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