BBA 66416

SPECIFIC MODIFICATION BY 3-DIAZOQUINOLINE OF BOVINE TRYPSIN AT THREE LYSYL RESIDUES ACCOMPANYING ENHANCEMENT OF ACTIVITY TOWARD SOME SYNTHETIC SUBSTRATES*

MASAMICHI KANAZAWA, NOBUO YOSHIDA** AND SHIN-ICHI ISHII

*Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo***, and Department of Biophysics and Biochemistry, Faculty of Science, the University of Tokyo, Tokyo (Japan)*

(Received 24th May, 1971)

SUMMARY

1 Treatment of bovine trypsin (EC 3 4 4 4) with a 20-fold molar excess of 3-diazoquinoline fluoroborate at pH 8 yielded an azo-derivative which possessed intensified activities toward N^{α}-benzoylarginine *p*-nitroanilide (2 5 times) and methyl N^{α}-*p*-toluenesulfonyl-L-argininate (1 6 times) at low substrate concentrations, whereas no apparent difference in the activity toward ethyl N^{α}-benzoyl-L-arginate was noted

2 Steady-state kinetic studies revealed that increases in catalytic rate constants were primarily responsible for the intensified activities Substrate activation phenomena on these methyl ester and nitroanilide substrates were still preserved after the modification

3 Spectral and amino acid analyses indicated that the azo-coupling occurred exclusively at three lysyl residues of trypsin Protection of the active site with β-naphthamidine exerted no effect on this unusual modification reaction

4 Succinylation of the enzyme with a 700-fold molar excess of succinic anhydride in half-saturated sodium acetate gave an active and stable enzyme derivative, in which eleven residues of lysine were acylated The specific modification of the unacylated three lysyl residues in this succinyltrypsin was achieved by 3-diazoquinoline under the same conditions as those for native enzyme The resulting azo-succinyltrypsin, devoid of any free ε-amino groups, also had the intensified activity toward N^{α}-benzoyl-DL-arginine *p*-nitroanilide

Abbreviations BAPA, N^{α}-benzoylarginine *p*-nitroanilide, TAME, methyl N^{α}-*p*-toluenesulfonyl-L-argininate, BAEE, ethyl N^{α}-benzoyl-L-arginate, DQ, 3-diazoquinoline fluoroborate, QA-, (quinolin-3-yl)azo, TNBS sodium 2,4,6-trinitrobenzenesulfonate, Z-, benzyloxycarbonyl, Boc-, *tert*-butyloxycarbonyl, DNP-, 2,4-dinitrophenyl

* This work was presented in part before the 7th International Congress of Biochemistry, Aug 1967, Tokyo

** Present address Shionogi Research Laboratory, Shionogi and Co , Ltd , Fukushima-ku, Osaka Japan

*** Inquiries should be addressed to this faculty

INTRODUCTION

In the course of the comparative study of chemical modification of chymotrypsin (EC 3 4 4 5) and trypsin (EC 3 4 4 4) with a series of aromatic diazonium compounds, unusual effects of 3-diazoquinoline on the activities of both of these proteolytic enzymes were observed[1] Regardless of the types of substrates employed, namely with ethyl *N*-acetyl-L-tyrosinate or with *N*-benzoyl-L-tyrosine *p*-nitroanilide, the enzyme activity decreased almost to the equal level when chymotrypsin was treated with *α*- or *β*-diazonaphthalene fluoroborate On the other hand, treatment of chymotrypsin with 3-diazoquinoline fluoroborate (DQ) resulted in a significant loss of its activity with *N*-benzoyl-L-tyrosine *p*-nitroanilide compared with a slight loss of its activity with ethyl *N*-acetyl-L-tyrosinate In addition, it has been found that trypsin treated with DQ was 2 5 times as active as native trypsin with the specific nitroanilide substrate, N^{α}-benzoyl-DL-arginine *p*-nitroanilide (DL-BAPA)

In the present study, chemical and kinetical investigations of this 3-diazoquinoline-modified trypsin were pursued

EXPERIMENTAL PROCEDURE

Materials

Bovine trypsin was purchased from Worthington Biochemical Corp Lot TRL 6261 DQ and *β*-diazonaphthalene were prepared from 3-aminoquinoline and from *β*-naphthylamine, which were the products of Tokyo Kasei Industries, Ltd , Tokyo, according to the procedure of ROE[2] The reagents were stored at 4° in their crystalline state L-BAPA[3], DL-BAPA, methyl N^{α}-*p*-toluenesulfonyl-L-argininate (TAME) and ethyl N^{α}-benzoyl-L-argininate (BAEE) were products of the Foundation for Promotion of Protein Research in the Institute for Protein Research, Osaka University L-BAPA was further purified, a 1% solution of L-BAPA was made in slightly warmed water, then the remaining insoluble materials were removed by filtration and the clear filtrate was freeze-dried Casein (Hammarsten) was a product of E Merck and was used after purification by the method of NORMAN[4] *β*-Naphthamidine was a gift from Dr K Tanizawa N^{α}-Acetyl-L-histidine was purchased from Cyclo Chemical Corp and *N*-acetyl-DL-tyrosine was a gift from Mr T Takami of Ajinomoto Co

Other chemicals were of reagent grade unless otherwise specified

Determination of activity toward BAPA

The initial steady-state rates of hydrolyses of DL-BAPA were assessed by Method II of ERLANGER *et al* [5] with slight modifications The reaction conditions are given in the legend of Table I For the hydrolysis of L-BAPA, increase in absorbance at 410 nm was traced continuously with a Hitachi 356 recording spectrophotometer in a manner similar to that of Method I by ERLANGER *et al* [5] The reaction conditions are given in the legend of Fig 2

Determination of esterolytic activity

These assays were performed by potentiometric titration employing a Radiometer Model TTT1C automatic titrator and a SBR2C titrigraph using 0 02 M, 0 05 M or 0 1 M NaOH as titrant, depending upon the experimental conditions For routine

TABLE I

STEADY-STATE KINETIC PARAMETERS ACCORDING TO THE ASSUMED MICHAELIS–MENTEN EQUATION, FOR THE HYDROLYSES OF DL-BAPA CATALYZED BY TRYPSIN AND ITS DERIVATIVES AT 25 IN 50 mM Tris–HCl BUFFER (pH 8.2) CONTAINING 20 mM $CaCl_2$ AND 1% DIMETHYLSULFOXIDE

The initial concentration of DL-BAPA was from 0.49 to 1.96 mM. For the calculations of k_{cat} values, the enzyme concentrations in molarity were obtained from the absorbance at 280 nm for native trypsin and by the method of LOWRY *et al*.[9] for other trypsin derivatives. Normality titrations were also performed independently (see EXPERIMENTAL PROCEDURE).

Enzyme	K_m (*mM* ± S.E.)	k_{cat} (sec^{-1} ± S.E.)
Trypsin*	1.34 ± 0.15	1.00 ± 0.06
	1.52 ± 0.08	0.848 ± 0.023
QA-trypsin*	1.00 ± 0.19	2.52 ± 0.22
	1.16 ± 0.13	2.18 ± 0.16
Succinyltrypsin	0.955 ± 0.080	1.57 ± 0.06
QA-succinyltrypsin	0.499 ± 0.055	2.07 ± 0.07

* Two separate experiments.

assays 10 mM BAEE was employed as substrate in 10 mM Tris–HCl buffer (pH 8.0) containing 0.1 M KCl and 20 mM $CaCl_2$ at 25°. In kinetic studies, where reaction mixtures in large quantities were treated, nitrogen was passed over the surface of the solutions to exclude CO_2.

Analysis of kinetic data

Calculations of all the kinetic parameters were performed on a FACOM 230-60 digital computer in the Hokkaido University Computing Center. The program of MARQUARDT's[6] method, which was kindly offered by I.B.M., Japan, was used for the analyses of substrate activation phenomena after modifications by Mr. H. Nakata of this laboratory to the form applicable to the FACOM 230-60.

Determination of caseinolytic activity

The method of KUNITZ[7] was used. The reaction conditions are given in the legend of Fig. 6.

Estimation of enzyme concentration

For native trypsin, concentration in molarity was obtained from the absorbance at 280 nm using an optical factor of 0.65 mg ml^{-1} A^{-1}, and a molecular weight of 23 800 (ref. 8) and was verified by amino acid analysis. For other trypsin derivatives, molar concentrations were obtained by the method of LOWRY *et al*.[9] using a standard curve made with native trypsin and were also verified by amino acid analyses. Solutions of trypsin and (quinolin-3-yl)azo (QA-)trypsin were made up in 1 mM HCl, kept at 4° and used within a week. Succinyltrypsin and QA-succinyltrypsin solutions were made up in appropriate buffers. The operational normality of a solution of trypsin and of QA-trypsin was, respectively, 52% and 54% of the molarity based on weight, when determined by active-site titration using *p*-nitrophenyl N^{α}-Z-L-lysinate which was synthesized according to BENDER *et al*.[10]

Preparation of QA-trypsin

A 40 μM solution of commercial bovine trypsin in 50 mM Tris–HCl buffer (pH

8 2) containing 20 mM $CaCl_2$ was placed in an ice bath A 20-fold molar excess of DQ in cold methanol was added with gentle stirring (final methanol concentration was 2%) After standing for 30 min at 4° the reaction was terminated by lowering the pH to about 2 with 3 M HCl The precipitation formed during the reaction was dissolved on acidification The solution was freed from excess reagent and by-products by gel-filtration through a column of Sephadex G-25 with 10 mM HCl containing 0 5 M KCl as eluant at 4° The protein fractions were pooled, dialyzed exhaustively against 1 mM HCl at 4° and freeze-dried

Preparation of succinyltrypsin

A 5% solution of trypsin in half-saturated sodium acetate containing 20 mM $CaCl_2$ was placed in an ice bath A 700-fold molar excess of pulverized succinic anhydride was added portion-wise After stirring for 1 5 h at 4° the solution was filtered, dialyzed against distilled water at 4° and freeze–dried

Preparation of QA-succinyltrypsin

The procedure was the same as that for QA-trypsin except for the following modifications which were necessary because of the low solubility of the succinylated trypsin in acidic media the reaction was terminated by adding sodium azide in a 100-fold molar excess over DQ and the gel filtration was carried out with 50 mM Tris–HCl buffer (pH 8 9) as eluant, followed by dialysis against distilled water at 4°

Estimation of free amino groups

The number of free amino groups remaining after succinylation was determined by the reaction of the modified protein with sodium 2,4,6-trinitrobenzene sulfonate (TNBS)[11] To 1 ml of protein solution in 0 1 M borate–HCl buffer (pH 8 8) was added 1 ml of 4% $NaHCO_3$ containing 15% Brij 35 After standing for 10 min at 24°, the solution was mixed with 1 ml of 0 1% TNBS This mixture was allowed to react at 40° for 1 h in the dark, then 1 ml of 1 M HCl was added The absorbance of the resulting solution at 340 nm was read against a blank prepared by treating 1 ml of the above-mentioned buffer instead of the protein solution by the same procedure Brij 35 was added as an effective denaturant to insure the complete trinitrophenylation of protein, to minimize its autolysis during the reaction and to prevent its precipitation on acidification

Amino acid analysis

This was performed on a Beckman Spinco Model MS or on a Hitachi KLA-3B automatic amino acid analyzer according to the procedure of SPACKMAN *et al* [12] Protein samples were hydrolyzed with redistilled, constant boiling HCl in evacuated sealed tubes at 110°for 22 h Tryptophan content was determined with alkaline hydrolyzates obtained with 4 M $Ba(OH)_2$ at 110° for 19 h

Analysis of synthesized azo-compound

The elemental analyses were performed at the Shionogi Research Laboratory and also at this department The molecular weight measurements by a Mechrolab Inc Model 301A vapor pressure osmometer with 95% methanol as solvent were performed at the above-mentioned laboratory

Synthesis of azo-amino acid

N^{ε}-(Quinolin-3-yl)azo-n-caproic acid DQ (486 mg, 2 mmoles) in 6 ml of cold methanol was added slowly over a period of 30 min to a solution cooled at 0° of ε-amino-*n*-caproic acid (117 mg, 1 mmole) in 15 ml of 20 mM carbonate–bicarbonate buffer (pH 10 5) The mixture was stirred at 4° for 1 h The pH of the medium was kept between 10 5 and 11 2 throughout the reaction with 1 M NaOH Then the pH was adjusted to about 2 with HCl The acidified mixture was extracted with five 20-ml portions of ethyl acetate and the combined organic phases were washed with 20 ml of H_2O, concentrated to small volume, and extracted with 2 20-ml portions of 5% Na_2CO_3 The aqueous phases were acidified to pH 2 with HCl and re-extracted with three 20-ml portions of ethyl acetate The organic layers were dried over Na_2SO_4 and evaporated to dryness, yield 97 mg (34%) The brownish residue was dissolved in hot methanol, treated with charcoal and crystallized The compound had m p 170–190° (decomp) (Found C, 62 99, H, 6 15, N, 19 86%, mol wt , 288 $C_{15}H_{18}O_2N_4$ requires C, 62 93, H, 6 30, N, 19 58%, mol wt , 286 14)

N^{ε}-(Quinolin-3-yl)azo-N^{α}-Z-L-lysine This compound was prepared from N^{α}-Z-L-lysine, which had been obtained by treating N^{α}-2-N^{ε}-Boc-L-lysine[13] with excess anhydrous HCl in dry ethyl acetate at room temperature for 60 min, by the same procedure as described above The compound had m p 80–90° (decomp) (Found - C, 63 16, H, 5 20, N, 15 96%, mol wt , 440 $C_{23}H_{25}O_4N_5$ requires C, 63 45, H, 5 75, N, 16 09%, mol wt , 435 49)

*N-Acetyl-(quinolin-3-yl)azo-*DL*-tyrosine* DQ (243 mg, 1 mmole) in 3 ml of cold methanol was added slowly over a period of 30 min to a solution cooled at 0° of *N*-acetyl-DL-tyrosine (223 mg, 1 mmole) in 12 ml of 10 mM borate buffer (pH 9 0) The mixture was stirred at 4° for 1 h The pH of the medium was kept between 9 0 and 9 2 with 1 M NaOH throughout the reaction Then the pH was adjusted to about 2 with HCl The resulting precipitate was collected by filtration and washed with cold water The yellow compound was dissolved in water by raising the pH to 9 and precipitated by acidification, yield 105 mg (28%) After crystallization from ethanol the compound had m p 280–290° (decomp) (Found C, 62 95, H, 4 82, N, 14 57% $C_{20}H_{25}O_4N_5$ requires C, 63 49, H, 4 76, N, 14 81%)

RESULTS

Reaction conditions

The reaction conditions for the azo-coupling of trypsin were selected to obtain a preparation which possessed the most enhanced activity toward DL-BAPA The pH dependence of the enhancement is shown in Fig 1 As generally expected in azo-coupling, the reaction proceeded more rapidly in an alkaline medium than in an acidic medium Although the enhancement reached a maximum after a 30-min reaction with a 20-fold molar excess of DQ at pH 8 9, the esterolytic activity was decreased to 37% by a 30-min reaction with a 40-fold molar excess of DQ at pH 8 9 The reaction at pH 8 2, however, resulted in no significant loss of the esterolytic activity, maintaining the activity toward DL-BAPA at a high level Therefore the conditions described in EXPERIMENTAL PROCEDURE were selected as milder and more specific ones The concentration dependence of the reaction was also noticed, the enhancement observed was maximal in an enzyme concentration range between 20 and 40 μM

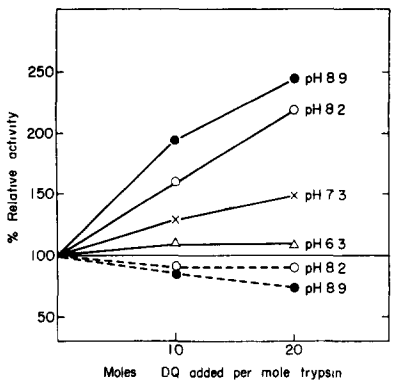

Fig 1 Effect of DQ on the activity of trypsin Enzyme, 20 μM in 50 mM Tris–HCl buffer, (pH 7 3, 8 2 or 8 9) containing 20 mM $CaCl_2$, or 50 mM phosphate buffer (pH 6 3), was allowed to react with DQ dissolved in methanol (final 2%, by vol) at 4° for 10 min Suitably diluted aliquots were assayed with DL-BAPA, 0 49 mM (solid lines) and with BAEE, 10 mM (broken lines) The activity is given relative to a control that contained no DQ

Hydrolysis of p-nitroanilide

Since the BAPA preparation used hitherto is a racemate and contains the D-isomer which behaves as a competitive inhibitor[5], the kinetics for the hydrolysis of the substrate cannot be analyzed simply by the Michaelis–Menten equation For comparison, however, the equation was assumed to be applicable to the hydrolysis of the racemate, and the steady-state kinetic parameters k_{cat} and K_m were calculated from the initial rate data by means of the statistical method of WILKINSON[14] The results are listed in Table I The difference in the catalytic rate constant is marked The overall catalytic rate constant of QA-trypsin and of succinylated trypsin was about twice that of unmodified trypsin The changes of K_m were insufficient to explain the intensified activities, except for that of QA-succinyltrypsin The apparent pH optimum of QA-trypsin-catalyzed hydrolysis of the substrate was identical with that of unmodified trypsin (*i e* pH 8 2)

In order to examine the effect of the D-isomer on the enhancement of activity, as well as to examine whether the substrate activation phenomenon recently reported by NAKATA AND ISHII[15] with this substrate is still preserved after the modification or not, the kinetics of trypsin- and of QA-trypsin-catalyzed reactions were investigated by using the L-BAPA preparation over a wide range of substrate concentration As represented in Fig 2 in the form of an EADIE[16] plot, the enhancement of activity in the modified trypsin was observed again with this optically homogeneous substrate It was further noticed that the plots did not afford straight lines, indicating that the enzymatic processes catalyzed by both trypsin and QA-trypsin deviated from the simple Michaelis–Menten equation in the substrate concentration range higher than 1 mM With the initial rate data obtained in the lower substrate concentration range, the values of k_{cat} and K_m shown in Table II were calculated as described above The increase in QA-trypsin of k_{cat}, the overall catalytic rate constant, is marked

It is still ambiguous[15] whether the mechanism of the substrate activation, indicated by the deviation at high substrate concentration, is the same as that assumed for the similar phenomenon observed in the trypsin-catalyzed hydrolysis of TAME expressed by Eqn 1 according to TROWBRIDGE *et al* [17] However, the propriety of the

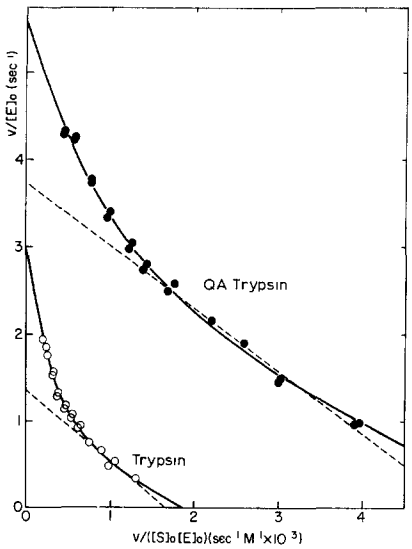

Fig 2 Eadie plots for the trypsin- (○) and QA-trypsin- (●) catalyzed hydrolyses of L-BAPA at 25° in 50 mM Tris–HCl buffer, (pH 8 2) containing 20 mM $CaCl_2$ and 1% dimethylformamide (spectro grade, Nakarai Chemicals) The initial substrate concentration was from 0 246 to 9 84 mM The broken straight lines were drawn according to the simple Michaelis–Menten equation with the values indicated in Table II for K_m and k_{cat} which were calculated from the data at the substrate concentrations lower than 1 mM by the weighted and non-linear regression method of WILKINSON[14], and the solid curves according to Eqn 1 with the values in Table III for the four parameters which were calculated from the all available data by the method of MARQUARDT[6]

equation to the experimental data shown in Fig 2 was examined by computer analysis[6,45] By the method of MARQUARDT for the least-squares estimation of

$$\frac{v}{[E]_0} = \frac{k_s[S]/K_s + k_{ss}[S]^2/K_sK_s'}{1 + [S]/K_s + [S]^2/K_sK_s'} \qquad \text{(Eqn 1)}$$

where $k_s = \dfrac{k_{ES}[ES] + k_{SE}[SE]}{[ES] + [SE]}$, $k_{ss} = k_{SES}$

$$K_s = \frac{[E][S]}{[ES] + [SE]}, \quad K_s' = \frac{([ES] + [SE])[S]}{[SES]}$$

non-linear parameters, the values of the four parameters in Eqn 1 were directly obtained as summarized in Table III By using these values, k_{ES} was also calculated (k_{SE} was assumed to be zero) Among the estimated values, increases in QA-trypsin of k_s and k_{ss}, namely 3 0 times and 2 0 times respectively of that of native trypsin, are marked But the ratio in QA-trypsin of k_{SES} to k_{ES}, which may be called the activation ratio, is smaller than that of native trypsin The smaller activation ratio may imply that the product-formation process from the activated ternary complex SES is not as accelerated by the chemical modification as that from the usual binary complex ES

Hydrolysis of ester

The substrate-activation study with the methyl ester substrate TAME was also

TABLE II

STEADY-STATE KINETIC PARAMETERS, ACCORDING TO THE MICHAELIS–MENTEN EQUATION, FOR THE TRYPSIN- AND THE QA-TRYPSIN-CATALYZED HYDROLYSES OF L-BAPA AND TAME

For the experimental conditions, see the legends of Figs. 2 and 3.

Substrate	Enzyme	K_m (mM ± S.E.)	k_{cat} (sec^{-1} ± S.E.)
L-BAPA	Trypsin	0.823 ± 0.083	1.35 ± 0.07
	QA-trypsin	0.719 ± 0.039	3.73 ± 0.11
	Enzyme	$K_{m\,(app.)}$ ($M \times 10^6$ ± S.E.)	k_{cat} (sec^{-1} ± S.E.)
TAME	Trypsin	8.87 ± 1.29	71.2 ± 4.8
	QA-trypsin	8.99 ± 1.78	107 ± 10

carried out in connection with the study of the difference in the rate determining step of the enzymatic process. Fig. 3 shows the dependence of the initial velocities of trypsin- and QA-trypsin-catalyzed hydrolyses of TAME upon the substrate concentration at pH 8.0 for the entire substrate concentration range studied. Enhancement of activity by the chemical modification was observed in the lower substrate concentration range (*e.g.* 1.6 times at 1 mM), which was explained in terms of k_{cat} as shown

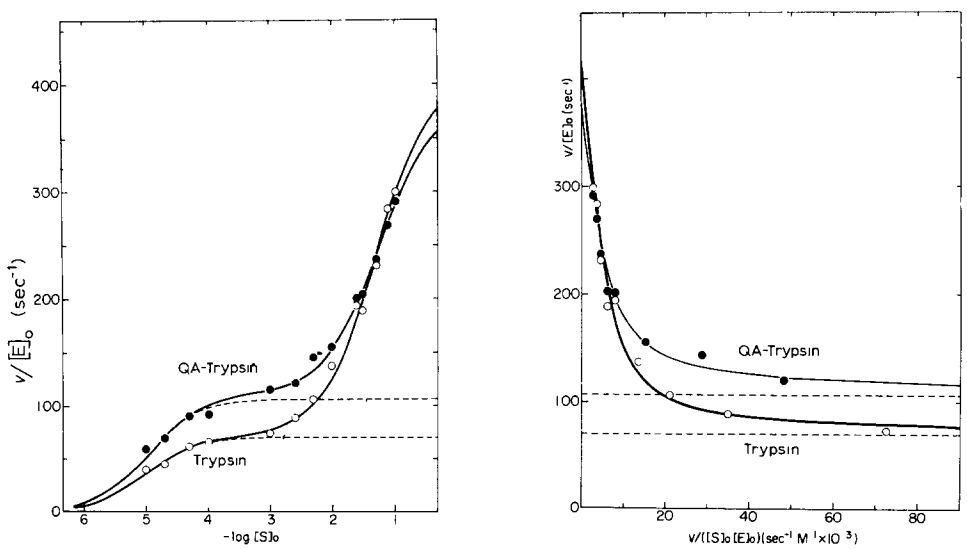

Fig. 3. The substrate-concentration dependence of the initial velocities for the trypsin- (○) and the QA-trypsin- (●) catalyzed hydrolyses of TAME at 25° in 40 mM KCl (pH 8.0) containing 10 mM $CaCl_2$. The initial substrate concentration was from 10 μM to 0.1 M. The broken curves were drawn according to the simple Michaelis–Menten equation with the values indicated in Table II for $K_{m(app.)}$ and k_{cat} which were calculated from the data at the substrate concentrations lower than 1 mM, and the solid curves according to Eqn. 1 with the values in Table III for the four parameters which were calculated from all the available data by the method of MARQUARDT[6].

Fig. 4. Eadie plots for the trypsin- (○) and the QA-trypsin- (●) catalyzed hydrolyses of TAME. Broken lines and solid curves were drawn in the same ways as those in Fig. 2.

TABLE III

STEADY-STATE KINETIC PARAMETERS FOR THE SUBSTRATE ACTIVATIONS OF THE TRYPSIN- AND THE QA-TRYPSIN-CATALYZED HYDROLYSES OF L-BAPA AND TAME

For the experimental conditions see the legends of Figs 2 and 3

Substrate	*Enzyme*	K_s	K'_s	k_s	k_{sb}	k_{ES}	*Activation ratio* (k_{SES}/k_{ES})
		(mM ± S.E.)	*(mM ± S.E.)*	*(sec^{-1} ± S.E.)*	*(sec^{-1} ± S.E.)*	*(sec^{-1})*	
L-BAPA	Trypsin	0.420 ± 0.101	7.47 ± 1.51	0.773 ± 0.119	2.88 ± 0.13	0.821	3.5
	QA-Trypsin	0.385 ± 0.120	5.20 ± 2.07	2.30 ± 0.48	5.59 ± 0.30	2.52	2.2
		($M \times 10^6$ ± S.E.)	*($M \times 10^2$ ± S.E.)*	*(sec^{-1} ± S.E.)*	*(sec^{-1} ± S.E.)*	*(sec^{-1})*	
TAME	Trypsin	9.96 ± 3.58	4.95 ± 0.88	72.3 ± 4.9	41.1 ± 2.5	72.3	5.7
	QA-Trypsin	10.9 ± 2.1	5.42 ± 1.04	11.1 ± 4	38.4 ± 2.3	11.1	3.5

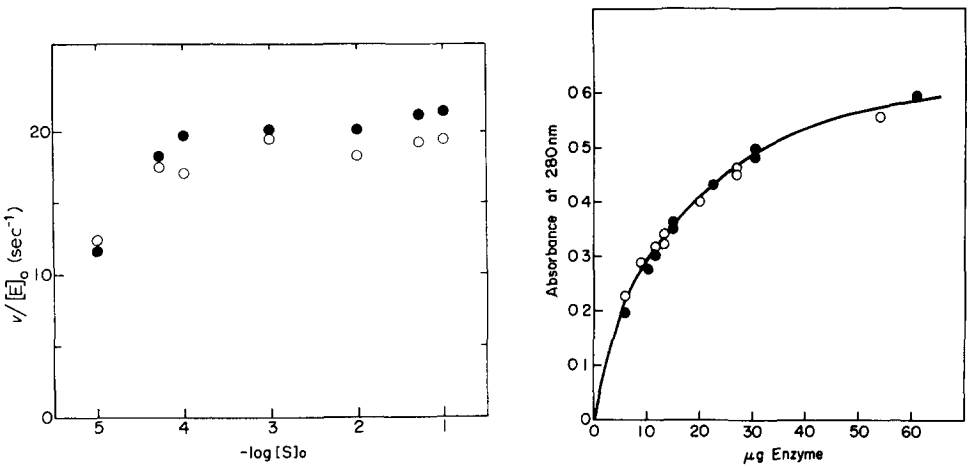

Fig 5 The substrate-concentration dependence of the initial velocities for the trypsin- (○) and the QA-trypsin- (●) catalyzed hydrolyses of BAEE Experimental conditions were the same as used for the determination of the activity toward TAME

Fig 6 A comparison of the caseinolytic activity between trypsin (○) and QA-trypsin (●) at 37° in 0 1 M phosphate buffer (pH 7 6) The substrate concentration was 0 5% (w/v)

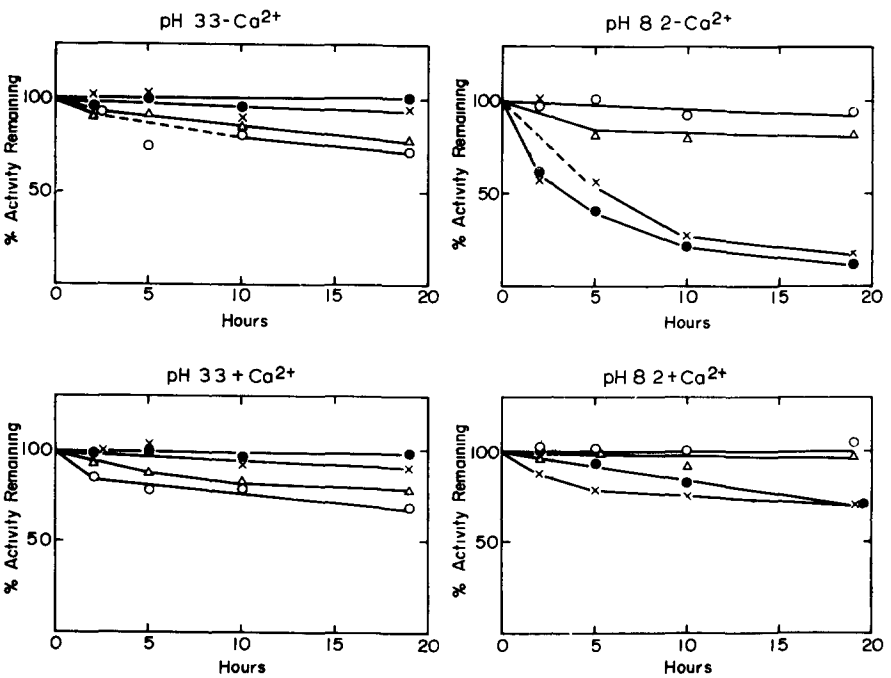

Fig 7 Stability of trypsin and its derivatives The loss of enzyme activity during the incubation of 0 05% solutions of trypsin and its derivatives in sodium veronal (36 mM)–sodium acetate (36 mM)–HCl buffer containing 20 mM $CaCl_2$ or not, at 25°, was followed by determining the activity of aliquots taken at timed intervals with DL-BAPA as substrate ●, trypsin, × QA-trypsin, △, succinyltrypsin, ○, QA-succinyltrypsin

TABLE IV

AMINO ACID COMPOSITIONS OF THE 22-h ACID HYDROLYZATES OF TRYPSIN AND ITS DERIVATIVES

Analytical values are related to 14 moles of alanine

Residue	*Trypsin*	*QA-trypsin*	*Succinyltrypsin*	*QA-succinyl-trypsin*	*DQ-modified trypsin in the presence of β-naphthamidine*	*β-Diazonaphthalene-modified trypsin*	*Theory for trypsin*[21]
Lys	13.2	*10.3*	13.8	*11.1*	*10.0*	15.2	14
His	2.85	3.00	3.03	3.39	3.07	2.94	3
Arg	1.81	*	2.05	*	*	1.81	2
Asp	21.6	22.6	23.5	22.5	21.7	22.4	22
Thr	9.18	9.62	9.95	9.21	8.93	9.14	10
Ser	28.9	29.9	31.4	26.7	30.9	28.2	34
Glu	14.2	14.0	15.5	15.9	13.2	15.0	14
Pro	8.13	8.43	7.98	8.15	9.10	8.10	8
Gly	24.8	24.9	25.9	25.5	24.9	24.6	25
Ala	14	14	14	14	14	14	14
Cys	9.69	11.0	9.41	12.5	10.9	9.42	12
Val	15.0	14.7	15.1	16.7	13.8	14.7	17
Met	1.93	2.02	2.01	1.86	1.62	1.88	2
Ile	13.6	13.6**	13.8	14.2	13.8	14.1	15
Leu	13.7	13.7	14.2	13.8	14.1	13.4	14
Tyr	9.48	9.51	10.0	9.40	9.55	*8.73*	10
Phe	3.01	2.98	2.85	3.07	3.00	2.95	3
Trp***	4.32	4.17					4

* Not determined because of the overlap of arginine peak with QA-lysine peak

** N-terminal isoleucyl residue was found intact after the modification because DNP-isoleucine could be determined as methyl ester by gas chromatography from the modified protein after dinitrophenylation and acid hydrolysis (M KANAZAWA, unpublished)

*** Alkaline hydrolysis as described in EXPERIMENTAL PROCEDURE

in Table II Only a slight change of activity was detected, however, in the high substrate concentration range where the substrate activation was observed The computer analysis carried out in the same way as for L-BAPA revealed the increase in QA-trypsin of k_s to 1 5 times of that of unmodified trypsin and also the decrease of the activation ratio as was the case of L-BAPA (Table III) Eadie plots shown in Fig 4 cover only part of the substrate concentration range studied

On the contrary, no apparent change of activity was observed in the hydrolysis of BAEE (Fig 5), whose structure differed from TAME in the substituent on amino function and differed from BAPA in that on carboxyl function The effect of an α-amino-protecting group in synthetic substrates on trypsin-catalyzed hydrolyses has been discussed in some articles[18,19]

Hydrolysis of casein

As shown in Fig 6, no difference in caseinolytic activity was observed after the chemical modification

Stability of modified trypsin

While it is well known that trypsin easily undergoes inactivation due to auto-

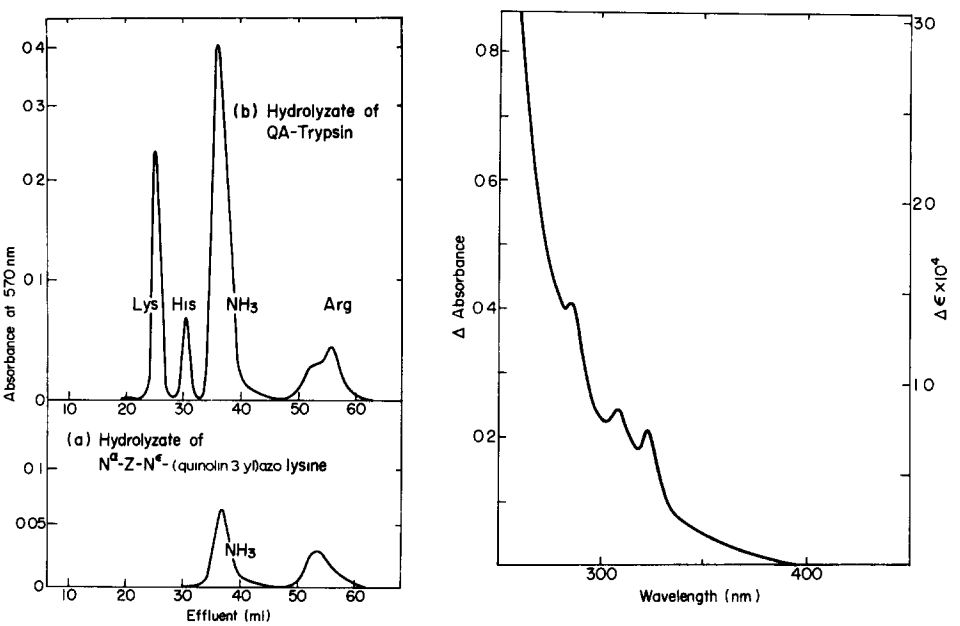

Fig 8 Identification of N^{ε}-QA-lysine by ion-exchange chromatography (a) Acid hydrolyzate of N^{α}-Z-N^{ε}-QA-L-lysine (b) Acid hydrolyzate of QA-trypsin Chromatograms were obtained on a short column of Amberlite IR-120 (0 9 cm × 8 cm) eluted by the standard method of SPACKMAN *et al* [12] A control experiment showed that the ammonia peak was derived from the dilution buffer

Fig 9 Difference spectrum of QA-trypsin *versus* unmodified trypsin In the sample cuvette of a Cary 14 spectrophotometer the solution of QA-trypsin in 0 1 M NaOH was placed and in the reference cuvette, the solution of trypsin, the reference solution was diluted gradually with 0 1 M NaOH until the spectrum gave a good agreement with that of N^{ε}-QA-N^{α}-Z-L-lysine The protein concentrations of the solutions in the cuvettes were determined thereafter by the method of LOWRY *et al* [9]

lysis at neutral pH, succinyl-trypsin was highly stable as previously reported[20] As Fig 7 shows, however, QA-trypsin was not stabilized by the azo-coupling and was also susceptible to the protective effect of Ca^{2+} against autolysis At pH 3 3, only a slight inactivation was observed with QA-trypsin

Kinds and numbers of modified residues

Amino acid analyses of 22-h acid hydrolyzates indicated that QA-trypsin lost about three lysyl residues upon the modification (Table IV) The content of tyrosyl, histidyl and tryptophanyl residues, which are generally expected to be azo-coupled, remained unchanged The loss of the three lysyl residues was also observed with the 12-, 17- and 45-h acid hydrolyzates GUNDLACH *et al* [22] reported that azo-derivatives of amino acids did not regenerate their parent amino acids on acid hydrolysis, and the findings allowed direct estimation of the number of azo-groups introduced onto a protein from the comparison of amino acid compositions This was confirmed in the present study by using the acid hydrolyzate of N^{α}-Z-N^{ε}-QA-lysine as a model compound As shown in Fig 8a, no peak corresponding to lysine was detected but a new peak appeared after the ammonia peak instead The same peak was observed as a shoulder just ahead of the arginine peak in a chromatogram of the hydrolyzate of QA-trypsin (Fig 8b) The peak was also observed with the alkaline hydrolyzates of both QA-trypsin and N^{α}-Z-N^{ε}-QA-lysine, and with the latter compound after the specific removal of the benzyloxycarbonyl group with HBr in acetic acid Neither acid hydrolyzate of N^{ε}-QA-amino-*n*-caproic acid* nor that of *N*-acetyl-QA-tyrosine gave any peak in the chromatograms covering the usual amino acids

Fig 9 shows the difference spectrum of QA-trypsin *versus* unmodified trypsin in 0 1 M NaOH The spectrum agreed well with that of N^{α}-Z-N^{ε}-QA-lysine and of N^{ε}-QA-amino-*n*-caproic acid Calculation of the number of the azo-lysine residues in the QA-trypsin by using the molar absorptivity of the former compound at 322 nm (Table V) gave the value of 3 0, which was compatible with that obtained by amino

TABLE V

SPECTRAL PROPERTIES OF AZO AMINO ACIDS IN 0 1 M NaOH

Wavelengths of maximal absorption (λ_{max}) and molar extinction coefficients (ε)

Derivative	λ_{max} *(nm)*	$\varepsilon(M^{-1}\ cm^{-1})$
N^{α}-QA-N^{ε}-Z-L-lysine	308	3 000
	322	2 500
N^{ε}-QA-amino-*n*-caproic acid*	308	2 600
	322	2 200
N^{α}-Acetyl-QA-DL-tyrosine	335	16 000
	495	12 400
N^{α}-Acetyl-QA-L-histidine	445	17 200**

* Absorption spectrum of N^{ε}-QA-amino-*n*-caproic acid was identical with that of N^{ε}-QA-N^{α}-Z-L-lysine except for the value of the molar extinction coefficient which did not change at all after the treatment of acid hydrolysis

** For the calculation, 100% purity and complete reaction of DQ were assumed

* The R_F value in thin-layer chromatography of this compound on silica gel with the solvent system of chloroform–methanol–acetic acid (90 10 1, by vol) did not change at all after the treatment of acid hydrolysis

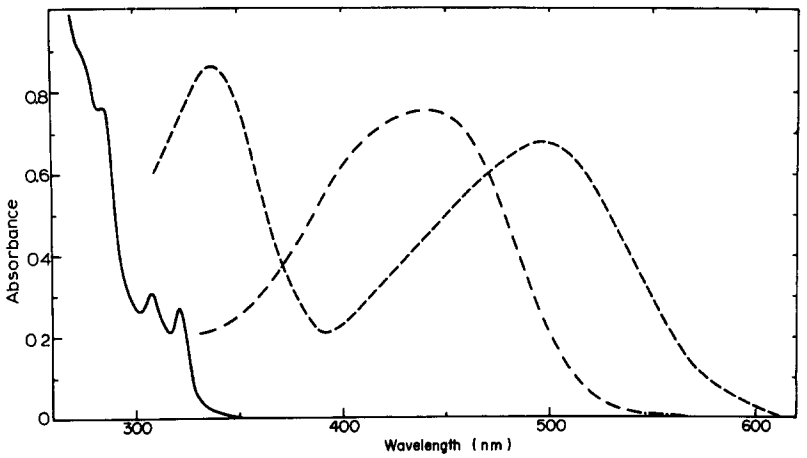

Fig 10 Absorption spectra of azo amino acids in 0 1 M NaOH The solution of N^{α}-acetyl-QA-histidine was obtained by treating 0 5 mM DQ in 10 mM borate buffer (pH 9 5) with a 20-fold molar excess of N^{α}-acetylhistidine for 3 5 h at 4° to give the maximun absorption, and the solution was suitably diluted with 0 1 M NaOH for the spectrum measurement — — —, N-acetyl-mono-QA-tyrosine, 0 054 mM, — —, N^{α}-acetyl-monohistidine, 0 044 mM, ———, N^{ε}-mono-QA-N^{α}-Z-lysine, 0 105 mM

acid analysis Hence, the enhancement of activity observed can be ascribed solely to the modification at three lysyl residues

Succinyltrypsin was found to be acylated in 75% of its amino groups as determined by the TNBS method, by comparing the color value at 340 nm with that of unmodified trypsin This suggests that about 11 out of 14 ε-amino groups of lysyl residues in trypsin are succinylated, since the α-amino group of N-terminal isoleucyl

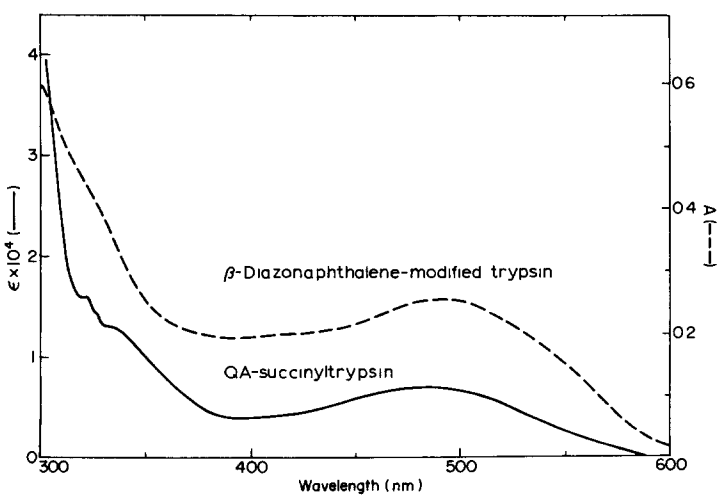

Fig 11 Absorption spectra of QA-succinyltrypsin and the β-diazonaphthalene-modified trypsin in 0 1 M NaOH The left ordinate is for QA-succinyltrypsin, and the right ordinate for β-diazonaphthalene-modified trypsin The protein concentration of the latter was not determined

residue has been reported not to be reactive to an acylating reagent such as acetic anhydride[23,*]

The specific modification of the remaining three lysyl residues in succinyltrypsin could be performed by DQ under the same conditions as used in the direct azo-coupling reaction of native trypsin (Table IV, QA-succinyltrypsin), but was accompanied by a slight loss of tyrosyl residues Spectrophotometric investigation showed incorporation of 0 55 mole of azoquinolyl groups to tyrosyl residues per mole of succinyltrypsin (Fig 11) The characteristic absorption bands near 310 nm due to QA-lysyl residues were not observed with this trypsin derivative, hindered by the large absorption of azo-tyrosyl residues

Table IV also gives the amino acid composition of a trypsin derivative prepared by the reaction with DQ under the same conditions except for the presence of 6 mM of β-naphthamidine, a powerful competitive inhibitor[24,25] The enzyme derivative had the same enhanced activity toward DL-BAPA at a fixed substrate concentration (1 e 0 5 mM)

Treatment of trypsin with a 20-fold molar excess of β-diazonaphthalene fluoroborate, which had a close similarity to the molecular structure with DQ, under the same conditions as used for the preparation of QA-trypsin, did not modify lysyl residues Amino acid analysis of the resulted trypsin derivative indicated that azo-coupling occurred at about one tyrosyl residue In Fig 11, the absorption spectrum of the derivative is shown It resembles that of *N*-acetyl-QA-tyrosine The activity of this azo-derivative toward DL-BAPA was slightly decreased (78% at the substrate concentration of 0 5 mM)

DISCUSSION

Aromatic diazonium compounds are known to couple readily with tyrosyl and histidyl residues of proteins, HORINISHI *et al* [26] described diazonium-1H-tetrazole as a useful reagent for the determination of the reactivity of histidyl residues Thus, aromatic diazonium compounds have been widely employed for the modification of enzyme proteins, to study the structure–activity relationship[22,27], and for specific labelling of antibody active-sites[28]

It is shown by the present experiments that with a 20-fold molar excess of 3-diazoquinoline azo-coupling occurred exclusively at three lysyl residues of trypsin, contrary to the expectation that the coupling would occur preferentially at tyrosyl and/or histidyl residues The finding that after 11 of 14 lysyl residues has been succinylated the remaining three lysyl residues were readily modified with DQ strongly suggests that these two types of lysyl residues are distinguishable from each other by their susceptibility to succinylation and DQ azo-coupling

The possibility that ε-amino groups of proteins would react with diazonium compounds in one to two stoichiometry yielding pentazdiene derivatives[29] has been

* In separate experiments (S ISHII AND C YUI, unpublished), the succinylation of eleven lysyl residues was confirmed by dinitrophenylation of the trypsin derivative in 5 M guanidine HCl at pH 8 followed by the quantitative determination of the regenerated lysine and ε-DNP-lysine in the acid hydrolyzate of the DNP-protein with an amino acid analyzer The fact that the N-terminal isoleucyl residue remained unacylated in the same trypsin derivative was also checked by Stark's cyanate method The enzymatic properties of the succinylated trypsin will soon be described elsewhere from this laboratory

pointed out by several authors[30–33] The results of the elemental analyses of the two model QA-amino compounds prepared in the present study, however, agreed well with the triazene (1,3-disubstituted) derivatives[34] Molecular weight measurements by a vapor pressure osmometer gave values of 440 and 288 for N^{α}-Z-N^{ε}-QA-lysine and N^{ε}-QA-amino-*n*-caproic acid respectively, also suggesting the existence of a single QA-group in both of the compounds This was confirmed with the latter compound by means of mass spectrometry (*m*/e 284) and NMR (one to one existence of quinoline nucleus and caproyl moiety)* (M KANAZAWA, unpublished) It may be worth noting that the reactions of bovine insulin and hen egg white lysozyme with DQ resulted also in the preferential loss of some lysyl residues in both proteins (M KANAZAWA, unpublished), implying an unusual property of this diazonium compound

The enhancement of activity toward BAPA and TAME due to the DQ modification is now known to be explainable primarily in terms of k_{cat}, the rate constant of a rate-limiting step in the sequential process of trypsin catalysis It may be emphasized that the enhancement may possibly occur in both of the major two steps in the catalytic reaction because the rate-limiting step corresponds to the acylation for BAPA and to the deacylation for TAME It should be also pointed out that activity enhancement by modification was observed only in the substrates which showed the pronounced substrate activation No enhancement was detected in the activities toward BAEE and N^{α}-benzoyl-L-arginine amide (by preliminary experiments), both of which had been reported not to exhibit the apparent substrate-activation phenomenon[35,36] There might be some common mechanism between the phenomena of the activity enhancement due to the DQ modification and the substrate activation TRENHOLM *et al* [37,38] have reported that the acetylation of trypsin with *N*-acetylimidazole induces the increased catalytic rate constant toward TAME only at high substrate concentrations where the substrate activation phenomenon is distinct This forms a striking contrast to the present findings which indicated that the activity enhancement occurs mainly at the low concentrations of TAME and of BAPA as indicated by the smaller value of the activation ratio

The chemical modifications of tyrosyl residues of several proteolytic enzymes have been reported to intensify their activities toward synthetic substrates As to zinc metalloproteases, the enhanced activities of both acetylated and succinylated carboxypeptidase A[39,40], and also of both nitrated and iodinated neutral subtilopeptidase amylosaccharilticus are reported[41] Acetylation of pepsin also caused enhancement of its activity[42] Acetylation of trypsin is known to induce its intensified activity toward DL-BAPA or TAME as summarized in the paper by HOUSTON AND WALSH[43] These intensified activities are almost in every case ascribed to the modification of the reactive tyrosyl residues, as concluded from the facts that the intensified activities are reversed to the original level by the treatment with hydroxylamine and from other chemical evidence This may form another contrast to the present findings, which indicated that the activity enhancement is brought about by the exclusive azo-coupling at three lysyl residues

It is known that commercial trypsin preparations are mixtures of α-, β- and other enzymatic species of different activities[44,45], and also contain inactive species

* The molecular formulae of the two mono QA-amino compounds have not yet been established The structure study is now in progress at this laboratory

For precise understanding of details further investigations using a fully-active trypsin preparation consisting of a single molecular species may be needed

ACKNOWLEDGEMENTS

The authors are grateful to Professor T ANDO in whose laboratory the early stage of this work has been accomplished for his continuous interest in this work They are also indebted to Mr H NAKATA for his generous offer of the computer programs

REFERENCES

1 N YOSHIDA AND S ISHII, *J Biochem Tokyo*, in the press
2 A ROE, in R ADAMS, *Organic Reactions*, Vol 5, Wiley, New York, 1962, p 193
3 N NISHI, S TOKURA AND J NOGUCHI, *Bull Chem Soc Japan*, 43 (1970) 2900
4 P S NORMAN, *J Exp Med*, 106 (1957) 423
5 B F ERLANGER, N KOKOWSKY AND W COHEN, *Arch Biochem Biophys*, 95 (1961) 271
6 D W MARQUARDT, *J Soc Ind Appl Math* 11 (1963) 431, IBM share library, No 3094 (1964)
7 M KUNITZ, *J Gen Physiol*, 30 (1947) 291
8 *Worthington Descriptive Manual*, 1967
9 O H LOWRY, N J ROSEBROUGH, A L FARR AND R J RANDALL, *J Biol Chem*, 193 (1951) 265
10 M L BENDER, J V KILHEFFER, Jr AND R W ROESKE, *Biochem Biophys Res Commun*, 19 (1965) 161
11 T OKUYAMA AND K SATAKE, *J Biochem Tokyo*, 47 (1960) 654
12 D H SPACKMAN, W H STEIN AND S MOORE, *Anal Chem*, 30 (1958) 1190
13 R SCHWYZER AND W RITTEL, *Helv Chim Acta*, 44 (1961) 164
14 G N WILKINSON, *Biochem J*, 80 (1961) 324
15 H NAKATA AND S ISHII, *Biochem Biophys Res Commun*, 41 (1970) 393
16 G S EADIE, *J Biol Chem*, 146 (1942) 85
17 C G TROWBRIDGE, A KREHBIEL AND M LASKOWSKI, Jr, *Biochemistry*, 2 (1963) 843
18 B R BAKER, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, Wiley, New York, 1967, p 61
19 V KALLEN-TRUMMER, W HOFMANN AND M ROTTENBERG, *Biochemistry*, 9 (1970) 3580
20 J F RIORDAN, M BIER AND F F NORD, *Arch Biochem Biophys*, 90 (1960) 125
21 O MIKES, V HOLEYSOVSKY, V TOMASEK AND F ŠORM, *Biochem Biophys Res Commun*, 24 (1966) 346
22 G GUNDLACH, C KOHNE AND F TURBA, *Biochem Z*, 336 (1962) 215
23 J LABOUESSE AND M GERVAIS, *Eur J Biochem*, 2 (1967) 215
24 K TANIZAWA, S ISHII, K HAMAGUCHI AND Y KANAOKA, *J Biochem Tokyo*, 69 (1971) 893
25 M MARES-GUIA, *Arch Biochem Biophys*, 127 (1968) 317
26 H HORINISHI, Y HACHIMORI, K KURIHARA AND K SHIBATA, *Biochim Biophys Acta*, 86 (1964) 477
27 H FRAENKEL-CONRAT, R S BEAN AND H LINEWEAVER, *J Biol Chem*, 177 (1949) 385
28 S J SINGER, *Science*, 153 (1966) 13
29 C SULING, in E MULLER, *Methoden der Organischen Chemie*, Vol X/3, Georg Thieme Verlag, Stuttgart, 4th ed, 1965, p 736
30 A N HOWARD AND F WILD, *Biochem J*, 65 (1957) 651
31 H G HIGGINS AND K J HARRINGTON, *Arch Biochem Biophys*, 85 (1959) 409
32 M TABACHNICK AND H SOBOTKA, *J Biol Chem*, 234 (1959) 1726
33 J H PHILLIPS, Jr, S A ROBRISH AND C BATES, *J Biol Chem*, 240 (1965) 699
34 C SULING, in E MULLER, *Methoden der Organischen Chemie*, Vol X/3, Georg Thieme Verlag, Stuttgart, 4th ed, 1965, p 695
35 J H CHEVALLIER AND J YON, *Biochim Biophys Acta*, 122 (1966) 116
36 S-S WANG AND F H CARPENTER, *J Biol Chem*, 243 (1968) 2943
37 H L TRENHOLM, W E SPOMER AND J F WOOTTON, *J Am Chem Soc*, 88 (1966) 4281
38 H L TRENHOLM, W E SPOMER AND J F WOOTTON, *Biochemistry*, 8 (1969) 1741
39 J F RIORDAN AND B L VALLEE, *Biochemistry*, 3 (1964) 1768

40 R T SIMPSON, J F RIORDAN AND B L VALLEE, *Biochemistry*, 2 (1963) 616
41 D TSURU, T YOSHIDA, T HIROSE, T YOSHIMOTO ADN J FUKUMOTO, *Int J Protein Res*, 2 (1970) 257
41 G E PERLMANN, *J Biol Chem*, 241 (1966) 153
43 L L HOUSTON AND K A WALSH, *Biochemistry*, 9 (1970) 156
44 D D SCHROEDER AND E SHAW, *J Biol Chem*, 243 (1968) 2943
45 R L SMITH AND E SHAW, *J Biol Chem*, 244 (1969) 4704
46 S A ARIHOOD AND C G TROWBRIDGE, *Arch Biochem Biophys*, 141 (1970) 131