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Esterase Activity of Chymotrypsin on Oxygen-Substituted Tyrosine Substrates

Nakuleswar KUNDU, Sujata ROY, and Frederick MAENZA

Medical Foundation of Buffalo, Buffalo, New York

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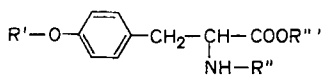
1. The replacement of the phenolic proton of tyrosine by alkyl, aryl or acyl groups completely abolishes the chymotryptic hydrolysis of tyrosine esters. Similarly, chymotrypsin fails to hydrolyze the tyrosyl bonds of dinitrophenyladrenocorticotropin.

2. Several model substrates have been synthesized and characterized. The action of chymotrypsin was followed potentiometrically in buffer containing some alcohol. The hydrolysis of the analogous compounds unsubstituted at the phenolic group was normal.

3. We conclude that inhibition is due, not to an inductive effect, but to a blocking effect which is markedly affected by replacement of the phenolic proton, even with a group as small as a methyl group.

In the course of our effort to develop an isotopic chemical method for the assay of adrenocorticotropin by dinitrophenylation with fluorodinitro-[^{3}H]-benzene and subsequent hydrolysis with chymotrypsin, it has been observed that no bis(dinitrophenyl)-seryltyrosine could be identified from the digested mixture. We inferred that the o-dinitrophenyl derivative of tyrosine may interfere with the action of chymotrypsin of tyrosyl peptide bonds [1]. The rates of hydrolysis of the ester bond of L-tyrosine derivatives having the general formula I with R′ = H are somewhat increased or decreased depending upon the nature of the substituents on the amino group [2], but very limited work has been done on the esterase activity of chymotrypsin when the phenolic group of tyrosine is blocked by alkylation [3] or acylation. In order to verify our hypothesis, model substrates of structure I were synthesized, and the esterase action of chymotrypsin on them was studied.



(I)

a) R′ = R″′ = CH_3; R″ = $COCH_3$
b) R′ = R″′ = C_2H_5; R″ = $COCH_3$
c) R′ = R″ = $COCH_3$; R″′ = C_2H_5
d) R′ = R″ = $COCH_2CH_3$; R″′ = C_2H_5
e) R′ = R″ = $(CH_3)_3C—CO$; R″′ = C_2H_5
f) R′ = $COCH_2CH_3$; R″ = $COCH_3$; R″′ = C_2H_5
g) R′ = $(CH_3)_3C—CO$; R″ = $COCH_3$; R″′—C_2H_5
h) R′ = $C_6H_3(NO_2)_2$; R″ = $COCH_3$; R″′ = C_2H_5
i) R′ = $C_6H_3(NO_2)_2$; R″ = $COCH = CH—COOH$; R″′ = C_2H_5
j) R′ = H; R″ = $COCH_3$; R″′ = C_2H_5
k) R′ = H; R″ = $COCH_2CH_3$; R″′ = C_2H_5
l) R′ = H; R″ = $(CH_3)_3C—CO$; R″′ = C_2H_5

MATERIALS AND METHODS

Melting points, specific rotations, $[\alpha]_D^{22°}$ ($c = 1$ in 1 N alcoholic HCl) and elemental analyses are given in Table 1. The purities of these substrates were also checked by gas chromatography (glass column, 6 ft × 4 mm chromosorb Q containing 1% OV-1, temperature 190 °C in all cases except. Ia, Ib and Ih where 170 °C was maintained); a single peak was observed in all cases. Retention times (t_R) are given in Table 1. Thin-layer chromatography (silica gel GF-254) in each instance showed a single fluorescent spot in two solvent systems (I & II), negative by Pauly's reagent. R_F values are given in Table 2. All these substrates were colorless crystalline solids, were not readily soluble in dilute sodium hydroxide solution and did not respond to Folin-Ciocalteau reagent.

SYNTHESIS OF SUBSTRATES

Preparation of Ia and Ib

These were synthesized from *N*-acetyl-L-tyrosine [4] with the appropriate alkyl iodide and silver oxide following the procedure of Synge *et al.* [5]. The puri-

Table 1. *Physical constants of the substrates*

Substrates	Formula	Melting point	$[\alpha]_D^{22}$	t_R	M_r	Analysis			
							C	H	N
		°C	degrees	min					
Ia	$C_{13}H_{17}O_4N$	104–105[a]	22.3	0.8	251.28		—	—	—
Ib	$C_{15}H_{21}O_4N$	92–93	18.3	1.2	279.33	Calcd	64.49	7.57	5.01
						Found	64.47	7.61	4.95
Ic	$C_{15}H_{19}O_5N$	89–90	9.7	1.2	293.31	Calcd	61.41	6.52	4.77
						Found	61.33	6.49	4.68
Id	$C_{17}H_{23}O_5N$	103–104	8.3	3.0	321.36	Calcd	63.53	7.21	4.36
						Found	63.85	7.08	4.45
Ie	$C_{21}H_{31}O_5N$	51–52	7.3	3.4	377.47	Calcd	66.81	8.28	3.71
						Found	66.85	8.34	3.69
If	$C_{16}H_{21}O_5N$	96–97	8.1	2.4	307.34	Calcd	62.42	6.88	4.55
						Found	62.02	6.76	4.27
Ig	$C_{18}H_{25}O_5N$	90–91	7.4	2.3	335.39	Calcd	64.45	7.51	4.17
						Found	64.60	7.53	4.14
Ih	$C_{19}H_{19}O_8N_3$	113–114	—	0.7	417.37	Calcd	54.67	4.58	10.06
						Found	54.46	4.44	9.88
Ii	$C_{21}H_{19}O_{10}N_3$	58–60 (d)	—	—	473.39	Calcd	53.28	4.62	8.87
						Found	53.03	4.40	8.43

[a] Literature values: 106-107 °C [5] and 104-105 °C [3].

Table 2. R_F *values of the substrates*
Solvent I was chloroform–iso-octane–ethanol (80:40:4, v/v/v) and solvent II was chloroform–acetone (80:20, v/v)

Substrates	R_F in solvent	
	I	II
Ia	0.31	0.60
Ib	0.37	0.73
Ic	0.26	0.65
Id	0.41	0.89
Ie	0.71	0.98
If	0.30	0.70
Ig	0.33	0.76
Ih	0.43	0.78
Ii	0.03	0.15
Ik	0.23	0.50
Il	0.35	0.62

fication of Ia was done by chromatography on florisil, instead of distillation, and finally by crystallization from ether containing a little petroleum ether. Ib was obtained as an oil which could be directly purified by crystallization from a mixture of benzene and petroleum ether.

The identity of Ia was confirmed by hydrolysis with 1 N NaOH solution at room temperature yielding the corresponding acid, crystallized from hot water, mp 153 °C (*cf.* [5] mp 151 °C).

Preparation of Ic, Id and Ie

A mixture of L-tyrosine ethyl ester hydrochloride (Aldrich Chemical Co.), the appropriate acid anhydrides, triethylamine and dry tetrahydrofuran, was warmed for an hour in an atmosphere of nitrogen. After removing the amine salt, Ic and Id became crystalline on standing overnight in the cold. They were purified by crystallization from a mixture of benzene and petroleum ether (charcoal) yielding colorless crystals. In the case of Ie, however, chromatography on florisil was required before crystallization from petroleum ether.

Preparation of If and Ig

These were prepared similarly from *N*-acetyl-L-tyrosine ethyl ester (Ij) (Aldrich Chemical Co.) with the appropriate acid anhydrides, triethylamine and tetrahydrofuran. The crude material (If) was purified by direct crystallization from a mixture of benzene and petroleum ether. The crude material (Ig) was purified by chromatography, followed by crystallization from petroleum ether.

Preparation of Ih

N-Acetyl-L-tyrosine ethyl ester (Ij) in absolute alcohol, and triethylamine solution was dinitrophenylated with a solution of fluorodinitrobenzene in ethanol. The solid obtained after removing the solvent under vacuum was dissolved in benzene (5 ml), purified by column chromatography with partially deactivated neutral alumina deactivated with water (5%). and finally recrystallized from aqueous ethanol yielding an almost colorless crystalline solid.

Preparation of Ii

L-Tyrosine ethyl ester in dry tetrahydrofuran was first maleylated with maleic anhydride. After work-up, the crude material could not be purified by crystallization or chromatography on florisil. Finally, the oily matter was purified by high-voltage electrophoresis in a buffer of pyridine—acetic acid—water (25:1:225, by vol.) pH 6.5 at a voltage of 2 kV for 1.5 h. The maleylated product was identified by acid treatment followed by ninhydrin spray. The pure material was obtained as a low-melting solid after elution from the paper. This material was then dinitrophenylated in the usual way. After acidification of the reaction mixture, a solid was obtained, which was filtered, dried and then washed with benzene followed by cold hexane until a colorless solid was obtained. Its mobility in high-voltage electrophoresis was the same as that of the starting material.

Preparation of N-Propionyl-L-tyrosine Ethyl Ester (Ik)

This was prepared by acetylation of L-tyrosine following the method described in literature [6]. It was then esterified at 0 °C with absolute ethanol saturated with dry HCl gas. The solvent was removed under vacuum at 40 °C, and the residual oil was dried under vacuum over P_2O_5 and solid KOH. Thin-layer chromatography (solvent system II) indicated a minor contamination with Id. After chromatography on florisil, the fraction obtained by elution with 2% ethanolic benzene gave a single spot on thin-layer chromatography with both solvent systems. Initially, this fraction was an oil which gradually crystallized. It was recrystallized from a mixture of benzene and hexane, m.p. 85—86 °C. It does not respond to ninhydrin, but it is positive with Folin-Ciocalteau reagent. The identity of the material was proved by further acylation with propionic anhydride under the condition described before to yield *N,O*-dipropionyl-L-tyrosine ethyl ester (Id).

The same material can also be prepared from Id by hydrolysis with dilute sodium hydroxide and subsequent esterification.

Preparation of N-Pivaloyl-L-tyrosine Ethyl Ester (Il)

N-Pivaloyl-L-tyrosine could not be prepared from L-tyrosine following the previous method. Under the same conditions used for the preparation of Ik, 90% of the starting material remained unreacted. Il was, however, made from Ie by mild hydrolysis with dilute sodium hydroxide solution and subsequent esterification with absolute ethyl alcohol saturated with dry HCl gas. The material after chromatography on florisil was obtained as an oil. Comparison of the mobility with Ie on thin-layer chromatography in two solvent systems (I and II) as well as conversion to Ie by further acylation confirmed the identity of the material.

RESULTS

Effect of Ethanol on the Activity of Chymotrypsin

Solutions of *N*-acetyl-L-tyrosine ethyl ester (Ij) were made in phosphate buffer containing various concentrations of ethanol. The kinetics of the esterase activity of chymotrypsin in these solutions was measured potentiometrically by the addition of 25 mM potassium hydroxide solution. The results (Fig. 1) show that alcohol concentrations up to 20% had only minimal effect on the rate of hydrolysis. The kinetics of the hydrolysis of *N*-propionyl-L-tyrosine ethyl ester with chymotrypsin in various concentrations of alcoholic buffer as well as that of *N*-pivaloyl-L-tyrosine ethyl ester in 40% alcoholic buffer were similar to those of Ij. The results are shown in Fig. 2.

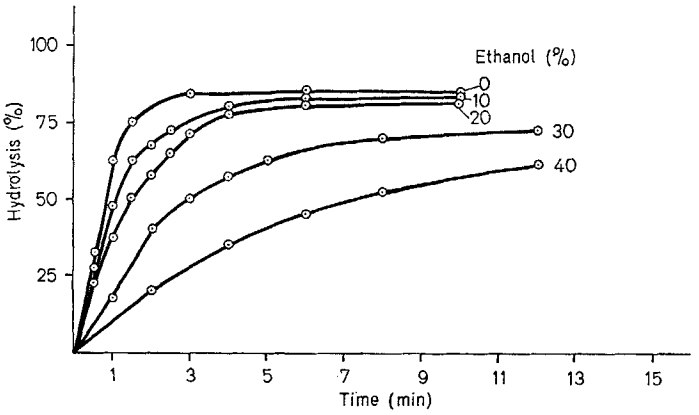


Fig. 1. *Comparison of the esterase action of chymotrypsin (0.5 mg/0.5 ml) on 0.01 M* N*-acetyl-L-tyrosine ethyl ester in 10 ml 0.01 M phosphate buffer pH 7.8 containing various concentrations of ethanol at 25 °C*

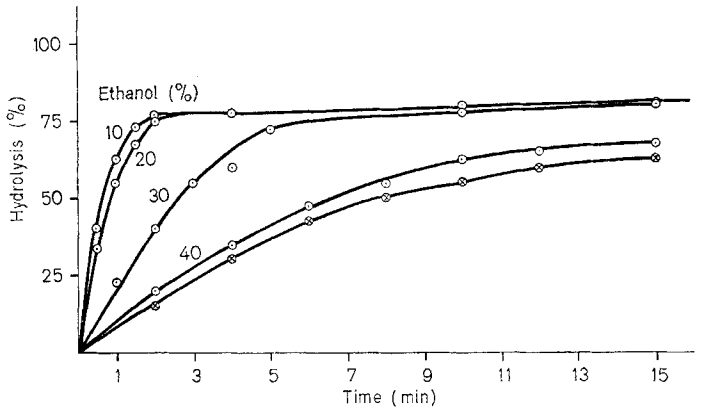


Fig. 2. *Comparison of the esterase action of chymotrypsin (0.5 mg/0.5 ml) on 0.01 M* N-*propionyl-*L-*tyrosine ethyl ester (⊙) in 10 ml 0.01 M phosphate buffer pH 7.8 containing various concentrations of ethanol and on 0.01 M* N-*pivaloyl-*L-*tyrosine ethyl ester (⊗) in 40% alcoholic buffer solution both at 25 °C*

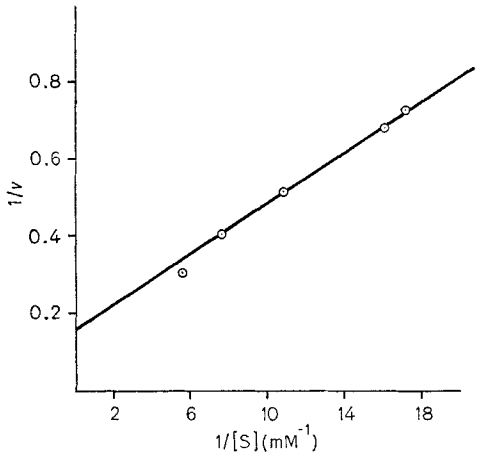


Fig. 3. *Determination of* K_m *value of* N-*maleyl-*L-*tyrosine ethyl ester at 25 °C.* The K_m value was found to be 28.3 mM

Chymotryptic Digestion of N-*Maleyl-*L-*tyrosine Ethyl Ester*

Five different concentrations of the substrate (13.1 mM, 11.3 mM, 9.1 mM, 6.2 mM, 5.8 mM) were made in phosphate buffer pH 7.8. The same enzyme concentration (0.086 mg/ml) was used in each case. The rate of hydrolysis was determined potentiometrically. K_m was calculated to be 28.3 mM (Fig. 3).

Chymotryptic Digestion of the Substrates Ia—Ii

Solutions (0.01 M) of Ia, Ib, Ic, Id, If and Ih were made in 10 ml phosphate buffer pH 7.8, containing 20% ethanol; solutions of Ie and Ig were prepared in buffer containing 40% ethanol and Ii in buffer alone. The same amount of chymotrypsin (0.5 mg/0.5 ml) was added and the reaction was followed potentiometrically under the same conditions as in the oxygen-unsubstituted substrates. No change in pH was observed during three hours of incubation. At this time, the incubation mixtures yielded unchanged substrates by thin-layer chromatography with solvent systems I and II.

DISCUSSION

All the prepared *N,O*-disubstituted substrates were sparingly soluble in phosphate buffer pH 7.8, with the exception of Ii which was soluble by virtue of its *N*-maleyl group. Solubility studies indicated that most of them were soluble in 20% alcoholic buffer with the exception of Ie and Ig which required 40% alcohol. Hence, the kinetics of hydrolysis of these substrates were determined in the solvent systems containing the least amount of alcohol compatible with a 0.01 M solution of the substrate. In order to compare these results with those having a phenolic proton, it was necessary to determine the kinetics of the latter at the same alcohol concentration. The presence of ethanol in the buffer inhibited the hydrolysis of the oxygen-unsubstituted substrates but not to a great extent until 30% or 40% alcohol concentration was employed. Considering the initial slope of the curves, it has been observed that the presence of 10% alcohol in the buffer inhibits the hydrolysis by only 15%, but the presence of 30% to 40% alcohol inhibited by 80%—85%. This is true irrespective of the nature of the substituent (acetyl, propionyl or pivaloyl) on the amino group. The rates of hydrolysis of *N*-acetyl, *N*-propionyl, and *N*-pivaloyl-L-tyrosine ethyl ester in the presence of 40% alcohol are almost identical. But in the cases of *N,O*-disubstituted substrates, no hydrolysis could be detected even after three hours and at this time, the unchanged substrates were recovered from the incubation mixtures. Peterson *et al.* [3], however, reported that *O*-methyl tyrosine ester derivative (Ia)

could be hydrolyzed by chymotrypsin in aqueous solution contrary to our observation. We were unable to prepare a homogenous solution of the stated concentration for kinetics study unless alcohol was used.

It was obvious from these results that the spatial requirement of chymotrypsin at the *para*-phenyl position is rather stringent. While removal of the hydroxyl group is well tolerated [7], enlargement is not, since replacement of the phenolic proton by either methyl or acetyl (as well as larger) groups completely abolishes esterase activity. The assumption that peptidase activity is also lost has support in the initial observation that chymotrypsin can not hydrolyze generally dinitrophenylated adrenocorticotropin at the tyrosyl bonds.

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N. Kundu's present address:
Endocrine Laboratories, Children's Hospital of Buffalo
219 Bryant Street, Buffalo, New York 14222, U.S.A.

S. Roy and F. Maenza
Medical Foundation of Buffalo
Buffalo, New York 14203, U.S.A.