CLEAVAGE OF SOME SUBSTRATES DERIVED FROM N-ACETYL-L-TYROSINE ETHYL ESTER BY THE ENZYME FROM RAT SERUM. EFFECT OF LIPOPHILIC CHARACTER OF SUBSTRATE

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Some tyrosine methyl esters were tested as possible substrates of the enzyme from rat serum which cleaves the ester bond of N-acetyl-L-tyrosine ethyl ester. The optimum pH of the esterolysis and the Michaelis constants for the individual substrates were determined. The R_F-quotients of the tyrosine derivatives were determined by paper chromatography and the dependence of the Michaelis constants on the lipophility of the substrates was examined.

The enzyme cleaving the ester bond of the synthetic substrate, N-acetyl-L-tyrosine ethyl ester, and thus liberating ethyl alcohol, has been studied predominantly in relation to the so-called C'_1 esterase of human blood serum[1-5]. According to certain authors this complement component increases vascular permeability[6,7], similarly to the enzyme of rat serum[8] whose relation to inflammatory processes[9] has also been studied.

The occurrence of the enzyme from rat serum in the rat serum and tissues[8] and the inhibitory effect of certain low molecular weight compounds[10,11] on this enzyme have been studied in our previous experiments. We found that aliphatic alcohols and fatty acids act as competitive inhibitors of the reaction and that the inhibitory activity depends on the lipophilic character of the inhibitor. In this paper, which reports on studies on some L-tyrosine methyl esters as substrates of the investigated enzyme, we have tried to utilize this finding as a criterion of the effect of the lipophility on the affinity of the substrate and to deduce certain rules governing the susceptibility of the substrate to cleavage by the enzyme.

EXPERIMENTAL

Chemicals. A series of L-tyrosine methyl esters was employed as substrates for the investigated enzyme from rat serum. The following compounds were available: L-tyrosine methyl ester hydrochloride (TyrOMe.HCl), L-O-methyltyrosine methyl ester hydrochloride (Tyr(Me)OMe.HCl), N-acetyl-L-tyrosine methyl ester (AcTyrOMe), N-acetyl-L-O-methyltyrosine methyl ester (AcTyr.(Me)OMe), N-acetyl-L-O-ethyltyrosine methyl ester (AcTyr(Et)OMe), and N-succinyl-L-O-methyltyrosine methyl ester (SucTyr(Me)OMe). These compounds, including N-acetyl-L-tyrosine ethyl ester (ATEE) were synthetized[12] in the Research Institute for Pharmacy and Biochemistry, Prague. The substrates were used as 0·25—25 mM solutions in the mixture acetone–water, 1 : 4, unless they were soluble in water (in the form of hydrochlorides).

The determination of the hydrolysis of L-tyrosine methyl esters was effected by colorimetric measurement of the enzymatically liberated methanol by the method of Siegelman and coworkers[13]. The hydrolysis of ATEE was evaluated both colorimetrically according to Rybák and coworkers[14] and also by the spectrophotometric method described in the preceding report[10]. One unit of activity was defined as the quantity of enzyme liberating 1 μmol of N-acetyl-L-tyrosine during 20 min of incubation. The determination of protein content in individual steps of purification of the enzyme was carried out by the method of Lowry and coworkers[15].

Chromatography on a column of Sephadex G-200. The rat serum (5 ml) was applied to a jacketed column (2·5 . 100 cm) cooled by water. Fractions 5 ml in volume were eluted by 0·1M Tris-HCl buffer in 0·2M-NaCl at pH 8·0 and collected in test tubes at a rate 15 ml/h. The protein content of the fractions was measured at 280 nm in Unicam SP 500 spectrophotometer. The esterolytic activity of the fractions was determined with respect to the hydrolysis of ATEE and the remaining substrates.

Chromatography on a column of DEAE-Sephadex A-50. Gel filtration on Sephadex G-200 afforded two active fractions. The more active fraction of lower molecular weight was lyophilized and then purified on a 2·5 . 46 cm column of DEAE-Sephadex cooled by water. Before being applied to the column, the sample (4 ml of 5% solution) was equilibrated with 0·05M Tris-HCl buffer in 0·1M-NaCl at pH 8·0 and eluted by the same buffer, followed by a linear gradient. The latter was developed in a three-chamber mixing device (50 ml of 0·05M Tris-HCl buffer in 0·1M NaCl at pH 8·0, 50 ml of 0·15M Tris-HCl buffer in 0·1M NaCl at pH 7·0, and 50 ml of 0·25M Tris-HCl buffer in 0·1M NaCl at pH 5·0). The last amounts were eluted by the final buffer component of the gradient. Fractions 3 ml in volume were collected at a rate of 18 ml/h and their protein content at 280 nm and esterolytic activity against the substrates mentioned were determined.

Determination of R_F-values of substrates. The R_F-values of the substrates were determined by ascending chromatography on Whatman No 1 in the system n-amyl alcohol–water and n-hexanol–water, which had been prepared by shaking of equal parts of both components for 1 h at room temperature. The spots were detected[16] by the Millon reagent (in the case of tyrosine methyl esters) or by the diazo reaction according to Bray (in the case of ATEE).

RESULTS AND DISCUSSION

Two active fractions (I + II), hydrolyzing ATEE and the remaining substrates, were obtained by gel filtration on the Sephadex G-200 column (Fig. 1). The more active, low molecular weight fraction II was purified further by fractionation on DEAE-Sephadex (Fig. 2). In order to make the graphs more clear only the activity of the enzyme hydrolyzing ATEE is shown in the figures. The maximums of activities hydrolyzing the remaining substrates lie in the same peak. The data on the individual fractionation steps and on the achieved purification degree are summarized in Table I. The enzyme purified by this procedure was lyophilized and used for the determination of the pH-optimum of the cleavage, of the time dependence of the esterolysis, and of the Michaelis constants. The determination of the pH-optimum of cleavage of the substrates was performed in Britton–Robinson buffers in the range 5·9 – 9·4. The obtained value was the same for all substrates in the pH-range 8·0 – 8·5. This result is in agreement with the found value of pH-optimum of cleavage of ATEE by the enzyme from rat serum and from rat liver homogenate[8]. Kleine[17] reports a pH-opti-

mum of 7·6–8·6 for the hydrolysis of ATEE and 8·0–9·0 for L-tyrosine ethyl ester by the unpurified enzyme from rat serum.

The cleavage of the substrate as a function of time follows the kinetics of zero order within relatively narrow time limits and at low concentrations of the substrates. At higher concentrations substrate inhibition can be observed. We determined the Michaelis constants for the individual substrates by the method of Lineweaver and Burke[18] with respect to this finding. We used four different substrate concentrations ($6{\cdot}2 \cdot 10^{-4}$, $1{\cdot}25 \cdot 10^{-3}$, $1{\cdot}87 \cdot 10^{-3}$, and $2{\cdot}5 \cdot 10^{-3}$M) and the samples were incubated 10 min at 37°C. The K_m-values are given in Table II.

When we follow how the individual substituents of hydrogen atoms on the functional groups of tyrosine affect the size of the Michaelis constant, we arrive at these conclusions: Both the substrates with a free amino group and substrates in which the hydrogen atom of the amino group has been replaced by an acetyl or succinyl group are cleaved. The replacement of the hydrogen atom by an acetyl group decreases somewhat the value of the Michaelis constant (pairs of substrates TyrOMe and

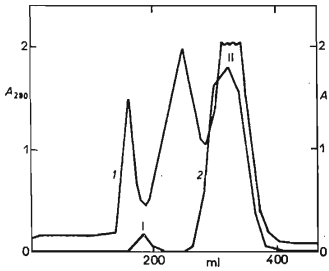

FIG. 1

Gel Filtration of Rat Serum on a Column of Sephadex G-200

1 Absorbance at 280 nm (A_{280}); *2* ATEE-esterase activity (fraction I and II). *A* Enzymic activity expressed in units/ml.

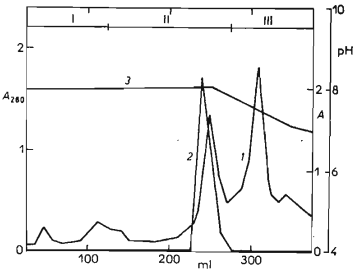

FIG. 2

Chromatography of Fraction II on a Column of DEAE-Sephadex A-50

1 Absorbance at 280 nm (A_{280}); *2* ATEE-esterase activity, *3* pH gradient. *A* Enzymic activity expressed in units/ml. I elution by 0·05M Tris-HCl buffer in 0·1M-NaCl at pH 8·0; II elution gradient (composition: 50 ml of 0·05M Tris-HCl in 0·1M-NaCl at pH 8·0, 50 ml of 0·15M Tris-HCl in 0·1M-NaCl at pH 7·0, and 50 ml of 0·25M Tris-HCl in 0·1M-NaCl at pH 5·0), and III elution by 0·25M Tris-HCl in 0·1M-NaCl at pH 5·0.

TABLE I

Purification of Enzyme

The given enzymic activity was determined with respect to the hydrolysis of ATEE.

Fraction	Volume ml	Protein mg/ml	Act. units/ml	Spec. act. units/mg protein	Purification
Rat serum	5	71	31·40	0·44	1
Sephadex G-200[a]	65	1·1	1·68	1·53	3·5
DEAE-Sephadex A-50	15	0·2	1·82	9·1	20·7

[a] Fraction II.

TABLE II

Values of Michaelis Constants of Substrates Compared to R_F-Values

The determined R_F-quotients are average values from three determinations in the system n-amyl alcohol–water.

Substrate	$K_m \cdot 10^3$ M	R_F	Substrate	$K_m \cdot 10^3$ M	R_F
TyrOMe	11·0	0·726	AcTyr(Me)OMe	3·8	0·908
SucTyr(Me)OMe	6·2	0·794	AcTyr(Et)OMe	3·4	0·923
Tyr(Me)OMe	5·2	0·848	ATEE	3·3	0·914
AcTyrOMe	4·3	0·862			

AcTyrOMe, Tyr(Me)OMe, and AcTyr(Me)OMe). On the other hand, the succinyl group, which introduces into the substrate molecule a free carboxyl group, decreases the affinity of the enzyme for the substrate (SucTyr(Me)OMe and Tyr(Me)OMe, AcTyr(Me)OMe). The enzyme does not require the free OH-group of tyrosine. Derivatives with a methoxyl and ethoxyl group show a K_m-value lower than the substrates with unsubstituted hydrogen: TyrOMe and Tyr(Me)OMe, AcTyrOMe and AcTyr(Me)OMe (or, alternatively AcTyr(Et)OMe). A slight increase of the affinity of the enzyme for the substrate was also observed after the replacement of methyl by ethyl in the carboxyl group (AcTyrOMe.AcTyrOEt).

We have shown[10,11] that the competitive inhibition of the ATEE-hydrolyzing enzyme by fatty acids and aliphatic alcohols increases with the increasing lipophilic character of the inhibitor. This conclusion led us to investigate an analogous dependence of the affinity of the enzyme for the substrate on the lipophilic character of the substrates. To characterize their lipophilic character, we determined the R_F-

values of the substrates by ascending chromatography in a system containing amyl alcohol and water. The average R_F-values are given in Table II. A comparison of these values with the K_m-values shows that the substrate with the lowest R_F-value shows the highest Michaelis constant and that the K_m-values decrease with increasing R_F-values. Similar results were obtained with the system n-hexanol–water. The graphical plot of the relation of the Michaelis constants of the substrates to their lipophilic character as expressed by the R_F-quotient indicate an almost linear dependence in a certain R_F-range (0·794 – 0·923) (Fig. 3). Similarly, the correlation coefficient of this relation, calculated for the given set of substrates in this R_F-range (r_{xy} 0·97) confirms the dependence of both values. A substrate which does not fall into this series and was therefore not considered in the calculation of the correlation coefficient, is TyrOMe, the only one of all substrates tested which has both functional groups free. This substrate is cleaved by the enzyme only very little. The nature

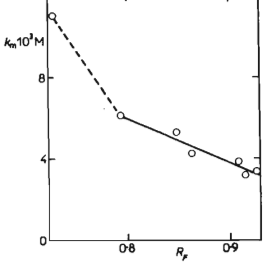

FIG. 3

Relation between K_m and R_F-Quotient of Substrates

The K_m- and R_F-values are given in Table II.

of the relation between K_m and R_F for $R_F < 0·794$ could not be expressed for the lack of substrates of the appropriate type.

In spite of the fact that the number of compounds tested was small, we can draw certain conclusions on the appropriateness of the substrates for the ATEE-hydrolyzing enzyme of rat serum. The tyrosine derivative with both functional groups free (TyrOMe) was found to be the least appropriate substrate. It appears that the radical which introduces into the molecule a free hydrophilic group (SucTyr(Me)OMe) is not appropriate as substituent. On the contrary, the K_m-values of the substrates increase after the substitution of the hydrogen atom of both functional groups of tyrosine by an alkyl or, alternatively, aryl. Generally it can be said that the Michaelis constant of tyrosine derivatives is positively influenced above all by substituents decreasing its hydrophilic character. On the contrary, the affinity of the enzyme for the substrate is decreased by groups bearing an electric charge. It can be assumed that

the substitution of the functional groups of tyrosine by more hydrophobic alkyls or aryls could lead to a substrate with a higher affinity for the investigated enzyme than that of ATEE used so far.

Studies on the relation between the structure of the substrate and its affinity for the enzyme have been performed, *e.g.* with chymotrypsin[19]. The existence of a relation between the structure of the substrate and its effect on the kinetics of cleavage by chymotrypsin has been reported by Bender and Kezdy[20,21]. Recently, Pliška and Barth[22] observed the dependence of the binding of α-chymotrypsin to a number of substrates of varying peptide chain length on the increasing lipophility of the substrate. Our results obtained with tyrosine derivatives as substrates for the esterase from rat serum point to an analogous dependence of the binding between the enzyme and the substrate on its lipophilic character and thus confirm the more general validity of these relations.

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