RAT SERUM HYDROLASES CLEAVING N-ACETYL-L-TYROSINE ETHYL ESTER

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Two protein fractions (I and II) cleaving a synthetic substrate, N-acetyl-L-tyrosine ethyl ester, were found in rat serum; they differ in the size of their molecules, in their surface electric charge and in the value of apparent activation energy. The character of serum fraction I is that of a peptidase; it is activated by bivalent metals and partly inhibited by the polyvalent proteinase inhibitors Trasylol and Antilysin. This fraction cleaves in addition to N-acetyl-L-tyrosine ethyl ester also *p*-toluenesulfonyl-L-arginine methyl ester and L-cysteinyl methyl ester, and certain amide bonds of synthetic substrates. Serum fraction II belongs according to its substrate specificity (it cleaves exclusively ester bonds, preferentially bonds in the neighborhood of the aromatic ring) and sensitivity to esterase inhibitors physostigmine and quinine to the group of esterases. Both enzymes are inhibited by fatty acids, aliphatic alcohols, and by higher concentrations of substrates. Their values of Michaelis constants of the cleavage of N-acetyl-L-tyrosine ethyl ester and their pH-optima are similar.

The enzyme cleaving the ester bond of the synthetic substrate N-acetyl-L-tyrosine ethyl ester (ATEE) and thus releasing ethyl alcohol, has been studied predominantly in relation to the so-called C'l esterase of human serum[1-5]. The enzyme hydrolyzing ATEE and present in large quantities in rat serum was investigated by Hanson[6] and Kleine[7,8]. Hladovec and Rybák[9] reported on the increase of the quantity of this enzyme in the inflamed tissue of rat paws in which the inflammation had been induced by a suspension of kaolin. Similar observations published later also Domenjoz and coworkers[10] who studied inflammations induced in rats by local application of dextran, formalin, or trypsin. Nothing was known about the character of this enzyme at that time. We have reported in our earlier papers some of our data on the occurence of the enzyme in rat serum and tissues[11], and on the inhibitory effects of certain low molecular weight compounds on the enzyme[12,13]. We found in subsequent studies that the ATEE-hydrolyzing activity present in rat serum was not uniform. The aim of this study was to characterize the proteins hydrolyzing ATEE.

EXPERIMENTAL

Material. N-Acetyl-L-tyrosine ethyl ester (ATEE), N-acetyl-L-phenylalanine methyl ester (AcPheOMe), N,O-diacetyl-L-tyrosine ethyl ester (DATEE), L-cysteine methyl ester (CysOMe), N-acetyl-L-tyrosine amide ($AcTyrNH_2$), N-α-benzoyl-D,L-arginine *p*-nitroanilide

(BAPA), N-α-*p*-toluenesulfonyl-L-arginine *p*-nitroanilide (TAPA), and L-leucine amide ($LeuNH_2$) were prepared by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry, Prague. N-α-*p*-Toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Fluka AG, Buchs 56, α-naphthyl acetate (α-NA) and β-naphthyl acetate (β-NA) were products of Lachema, Brno. The rat serum was obtained from the blood of decapitated rats after the separation of the blood coagulum by centrifugation. Cytochrome c was a commercial preparation of Koch-Light, human thyreoglobulin was prepared in the laboratories of the 3rd Clinic of Internal Medicine, Prague, and kindly provided by Dr J. Šrámková. Human γ-globulin and albumin and bovine hemoglobin were prepared in this Institute. The proteinase inhibitor Antilysin was a preparation of the Research Institute for Pharmacy and Biochemistry, Prague, kindly provided by Dr V. Mansfeld, Trasylol was a product of Bayer.

Analytical methods. The colorimetric determination of ATEE was carried out by the method of Rybák and coworkers[14]. The latter involves the diazo coupling reaction of N-acetyl-L-tyrosine and of its alkyl esters with *p*-nitroaniline and subsequent separation of their diazo derivatives based on their different solubility in water and in an organic solvent. The determination of methyl alcohol liberated by the enzymatic cleavage of the substrates TAME, CysOMe, and AcPheOMe was effected by the method of Siegelman and coworkers[15]. The determination of α- and β-naphthol liberated from the naphthyl acetate substrates was carried out by the diazo coupling reaction with sodium nitrite and sulfanilic acid according to Bray[16]. The quantity of *p*-nitroaniline, released from BAPA and TAPA by the enzymatic cleavage, was established by the colorimetric method based on the diazotation of *p*-nitroaniline and on the coupling of the diazo compound with sodium phenolate[17]. The degree of hydrolysis of N-acetyl-L-tyrosine amide was assayed by paper chromatography in the system n-butanol–acetic acid–water (3 : 1 : 1). The N-acetyl-L-tyrosine liberated was detected by the diazo coupling reaction with sodium nitrite and *p*-nitroaniline according to Bray[16]. The results were evaluated only qualitatively. The hydrolysis of $LeuNH_2$ was evaluated by the ninhydrin method[18]. The protein content was established by the method of Lowry and coworkers[19].

Gel filtration on Sephadex G-200. The rat serum (4—5 ml) was applied to a 2·5 . 100 cm column maintained at 14°C by tap water. The column was eluted by 0·1M Tris-HCl buffer in 0·2M-NaCl at pH 8·0 and 5 ml fractions were collected at a rate of 15—20 ml/h into test tubes placed in an automatic fraction collector. The protein content of the fractions was determined spectrophotometrically at 280 nm in Unicam SP 500 Spectrophotometer and their enzymatic activity was assayed. The molecular weights of the enzymatically active fractions were determined by gel filtration[20] on a Sephadex G-200 column calibrated by the following protein standards: human thyreoglobulin (mol. wt. 670 000), human γ-globulin (mol. wt. 150000), human albumin (mol. wt. 70000), bovine hemoglobin (mol. wt. 64500), and cytochrome c (mol. wt. 12000).

Chromatography on DEAE-*Sephadex* A-50. Active fractions, obtained by gel filtration on Sephadex G-200 and dialyzed against the starting buffer, were applied to the column (2·5 . 46 cm) cooled at 14°C by tap water. The column was eluted first by 0·1M Tris-HCl buffer in 0·14M-NaCl at pH 7·8, then by a linear gradient developed in a three-chamber mixing device from 50 ml portions of buffers of increasing molarity and decreasing pH: 0·1M Tris-HCl in 0·14M-NaCl at pH 7·8, 0·15M Tris-HCl in 0·14M-NaCl at pH 6·5 and 0·5M Tris-HCl in 0·14M-NaCl at pH 4·5. The last portions of the material were eluted by the final buffer of the gradient. Fractions 3 ml in volume were collected at a rate of 15—18 ml/h. The rechromatography of the samples on the DEAE-Sephadex A-50 column was effected by buffers of lower ionic strength and more alkaline pH of the starting buffer. The composition of the elution gradient was the following: 50 ml of 0·1M Tris-HCl in 0·12M-NaCl at pH 8·5, 50 ml of 0·15M Tris-HCl in 0·12M-NaCl at pH 6·5, and 50 ml of 0·5M Tris-HCl in 0·12M-NaCl at pH 4·5.

RESULTS AND DISCUSSION

The ATEE-hydrolyzing activity of rat serum is not uniform as shown in experiments with gel filtration on Sephadex G-200 (Fig. 1). Two protein fractions (designated I and II) cleaving ATEE were found. The major part of the serum activity is involved in serum fraction II which has a lower molecular volume. Both fractions were purified by ion-exchange chromatography on DEAE-Sephadex and rechromatographed on the same sorbent. Serum fraction I is bound more firmly to DEAE-Sephadex under identical conditions of elution. This indicates its higher surface electric charge. None of the serum fractions could be isolated perfectly. The immunoelectropherograms of serum fraction I and II showed the presence of two and two to three precipitation lines, respectively. The electrophoretic mobility of the two fractions is roughly the same. The approximate molecular weights of the two fractions were determined by gel filtration on a Sephadex G-200 column. Their values were read off the graph shown in Fig. 2. The molecular weight of serum fraction I is 320 000, of serum fraction II 75000.

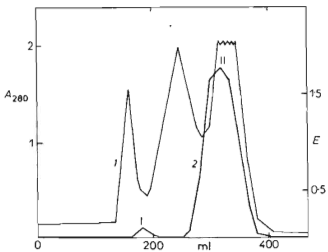

FIG. 1

Gel Filtration of Rat Serum on Sephadex G-200 Column

1 Absorbance at 280 nm; *2* ATEE-cleaving activity (fraction I and II). E, Enzymatic activity in u/ml; ml, elution volume in ml; A_{280}, absorbance at 280 nm.

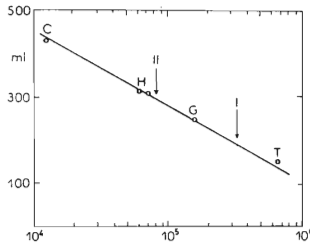

FIG. 2

Dependance of Elution Volumes of Protein Standards on their Molecular Weights on Gel Filtration on Sephadex G-200

T, human thyreoglobulin; G, human γ-globulin; A, (point next to H) human albumin; H, bovine hemoglobin; C, cytochrome c. The arrows designate the elution volumes of serum fractions I and II. mol. wt., molecular weights of protein standards; ml, elution volumes in ml.

In an effort to characterize the active fractions differing in molecular weight and surface electric charge, we studied the effect of temperature, pH, concentration of substrates, bivalent metals, and certain inhibitors on the rate of cleavage of ATEE catalyzed by serum fractions I and II. The profile of the curves characterizing the temperature dependance of the cleavage is different for the two fractions, as apparent from Fig. 3. Serum fraction II shows a linear profile of the log *V versus* $1/T$ plot over the temperature range 20–50°C and a temperature optimum lying around 50°C. The slope of the curve characterizing serum fraction I is less steep and is linear over the range 20–45°C. The optimum temperature is around 45°C. The apparent activation energy was calculated from the Arrhenius plot of reaction rate *versus* temperature; its values for both fractions are given in Table I. As can be seen in Fig. 3, the rate of cleavage of ATEE catalyzed by serum fractions I and II sharply decreases at higher temperatures. Both fractions are thermolabile and are inactivated after 30 min of incubation at 55°C.

The effect of pH on the cleavage of ATEE was investigated in Britton–Robinson buffers in the range pH 3–12. The curves characterizing both fractions are bell shaped. The pH-optimum of fraction I lies in the range 8·0–8·5. The optimum activity of fraction II is slightly shifted toward neutral pH. The dependance of the rate of cleavage on the concentration of the substrate is expressed by a hyperbolic

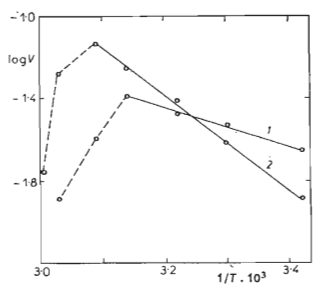

FIG. 3

Effect of Temperature on Hydrolysis of ATEE

1 Serum fraction I; *2* serum fraction II. *T*, Absolute temperature; *V*, maximum reaction rate.

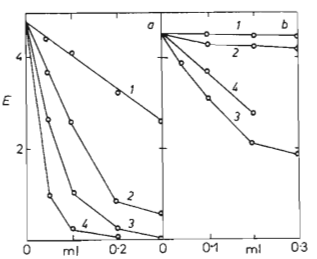

FIG. 4

Inhibition of Hydrolysis of ATEE by Antiinflammatory Drugs

1 Sodium salicylate; *2* antipyrine; *3* phenacetin; *4* trimethazone. *E* enzymatic activity in u/ml; ml, volume of 0·1M inhibitor solutions. *a* Serum fraction II, *b* serum fraction I.

TABLE I

Values of Activation Energies Calculated from the Arrhenius Plot

μ, apparent activation energy in cal/mol; t_1, t_2 limiting temperatures of the linear course in 0°C.

Fraction	μ	$t_1 - t_2$
I	4 300	20—45
II	14 850	20—50

TABLE II

Activation Effect of Mn^{2+}, Co^{2+} and Ca^{2+}-ions on Serum Fraction I and Inhibitory Effect of Zn^{2+}-ions on Serum Fraction II

The serum fractions were pre-incubated 10 min at 37°C with the activator or inhibitor and then incubated with 0·025M ATEE for 20 min at the same temperature.

Concentration mM	Activity, %			
	$MnCl_2$	$CoCl_2$	$CaCl_2$	$ZnCl_2$
0	100	100	100	100
0·5	—	—	—	88·5
1·0	131	142	164	82·4
3·0	160	171	193	58·2
5·0	—	—	—	45·5

TABLE III

Inhibitory Effect of Trasylol and Antilysin on Serum Fraction I

Inhibitor Units in 1 ml	Activity, %	
	trasylol	antilysin
0	100	100
1 000	100	90
2 000	93	83
4 000	70	76

saturation curve; higher concentrations of the substrate inhibit the hydrolysis of ATEE both by serum fraction I and by serum fraction II. The Michaelis constants of the cleavage of ATEE are K_M $3{\cdot}3 \cdot 10^{-3}$ ($\pm 0{\cdot}3$)M for serum fraction II and K_M $4{\cdot}1 \cdot 10^{-3}$ ($\pm 0{\cdot}3$)M for serum fraction I.

The behavior of the two fractions toward bivalent metals is diametrically different. Manganese, calcium, and cobalt chloride activate serum fraction I, zinc and magnesium chloride are without any effect. By contrast, serum fraction II is inhibited by zinc chloride (Table II) whereas the remaining metals are without any effect even if present in maximum concentration (5mM). In experiments investigating the inhibitory effect of zinc, the nonspecific effect of high ionic strength was eliminated first by using comparable concentrations of sodium chloride.

Serum fraction I was partly inhibited by trypsin and kallikrein inhibitors Antilysin and Trasylol (Table III). The given concentrations of these inhibitors were without any effect on the cleavage of ATEE catalyzed by serum fraction II. The esterase inhibitors physostigmine and quinine showed inhibitory effect exclusively on serum fraction II (Table IV). Both serum enzymes are inhibited by antiinflammatory drugs, such as phenacetin, trimethazone, and antipyrine, serum fraction I to a lesser degree. The latter fraction is resistant also to sodium salicylate, whereas serum fraction II is inhibited by this compound under identical experimental conditions (Fig. 4). Fatty acids (C_3, C_4, C_5, C_8, C_{10}, C_{12}) and aliphatic alcohols (C_3, C_4, C_5) also inhibit the two fractions; the inhibition of serum fraction II has a competitive character[12,13].

Both serum enzymes differ in substrate specificity, as shown in Table V. Serum fraction II did not cleave the amide bond of the substrates tested in any of the experiments. This enzyme cleaves exclusively ester bonds, preferentially those in the neighborhood of the aromatic ring. It shows also a weak activity when tested with tosyl-L-arginine methyl ester as substrate. The specific, ATEE-hydrolyzing activity of serum fraction I is very low compared to the activity of fraction II. This enzyme cleaves more readily the ester bond of tosyl-L-arginine and L-cysteine methyl esters. It also hydrolyzes amide bonds to a low degree and a weak proteolysis of casein was observed too.

A comparison of the properties of fraction I and fraction II leads us to conclude that the ability to cleave ATEE is a characteristic common to two enzymes of rat serum which differ in the size of their molecules, in surface electric charge and in the value of apparent activation energy. From the data on substrate specificity, effect of bivalent ions, and the inhibitory effect of Trasylol and Antilysin, serum fraction I appears to be an enzyme of peptidase character. This enzyme was also shown to release from plasmatic kininogens active compounds which bring about the contraction of isolated rat uterus[21]. In view of this ability the enzyme belongs to peptidases of the kallikrein type. Since serum fraction II shows an affinity for substrates of the ester type and is inhibited by esterase inhibitors physostigmine and quinine, the enzyme falls into the group of typical esterases. Kleine[8] regards this

enzyme as an carboxyl ester hydrolase (B-esterase) for which phenyl butyrate is the optimum substrate. Our findings of the optimum temperature around 50°C, inactivation at 55°C, resistance to trypsin kallikrein inhibitors and on the contrary, of a sensitivity to esterase inhibitors, are in accordance with Kleine's findings. We obtained identical results also in experiments with the cleavage of DATEE, AcPheOMe, and TAME and found an identical molecular weight, *i.e.* approximately 70000. The values of the Michaelis constant obtained by us differ from those found by Kleine[8].

The results of the experiments with the hydrolysis of ATEE point to a similar active center of both enzymes, which can be inhibited by fatty acids and aliphatic

TABLE IV

Inhibitory Effect of Physostigmine and Quinine on Serum Fraction II

The concentration of physostigmine is given in M . 10^{-5}, of quinine in M . 10^{-4}.

Inhibitor concentration	Activity, %	
	physostigmine	quinine
0	100	100
1·0	85·0	65·4
2·0	78·9	56·3
4·0	56·6	43·6

TABLE V

Cleavage of Synthetic Substrates by Serum Fractions I and II

The specific activity of the serum fractions is given in units per mg of protein. The serum fractions were incubated in the presence of 0·025M substrates, 20 min at 37°C, except for the substrates marked *a* which were incubated 2 h at 37°C.

Substrate	Fraction I u/mg	Fraction II u/mg	Substrate	Fraction I u/mg	Fraction II u/mg
ATEE	0·9	9·1	AcPheOMe	—	3·1
α-Na	0	1·3	$AcTyrNH_2$	traces	0
β-Na	0	4·7	$LeuNH_2$	0·26	0
DATEE	0	0·68	BAPA[a]	0·04	0
TAME	3·5	0·01	TAPA[a]	0·02	0
CysOMe	3·3	0			

alcohols and also by the substrate, show a similar pH-optimum and similar values of Michaelis constants of ATEE hydrolysis. The data obtained in preceding studies on the inhibition of serum enzyme II by fatty acids[12] and aliphatic alcohols[13] and on the effect of the lipophilic character of the substrate on the values of Michaelis constants[22] show that the character of this center is hydrophobic.

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