Chemistry of Natural Compounds, Vol. 34, No. 6, 1998

INHIBITION OF ANGIOTENSIN-CONVERTING ENZYME BY PEPTIDES FROM THE VENOM OF THE VIPER *Echis multisquamatus*

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UDC 547.993

Angiotensin I-converting enzyme [dipeptidyl carboxypeptidase] (ACE, CE 3.4.15.1) isolated in the pure form from ox kidney, has been characterized physicochemically and kinetically. Inhibition of the enzyme by homogeneous peptides from the venom of the Central Asian viper Echis multisquamatus *Ch. has been investigated.*

The detection of peptide inhibitors of the angiotensin-converting enzyme (ACEI), their study, and the subsequent synthesis of structural analogs and their use as antihypertensive agents are considered by some authors as one of the most impressive discoveries in cardiology of the last decade [1]. At the same time, the synthetic analogs, together with an indisputable effect in the treatment of systemic arterial hypertension, exhibit a serious side effect [2], and this is stimulating the search for and the structural-functional investigation of new ACEIs from natural sources, the most suitable of which are snake venoms.

Eight peptide ACE inhibitors have previously been isolated from the venom of the Central Asian viper *Echis multisquamatus* and their bradykinin-potentiating activities have been characterized [3]. In the present paper we consider results from the study of the kinetics of the enzyme reaction using a specific chromogenic substrate of ACE, N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) and also the results of an analysis of the inhibition of the enzyme by pure peptides from the viper venom.

According to the results of gel filtration on Sephadex G-200, the molecular mass of the native angiotensin-converting enzyme from ox kidney is 180—190 kDa. The ACE preparation obtained was characterized by a homogeneous molecular mass distribution on electrophoresis under denaturing conditions. The results of different authors on the molecular mass of ACE from various sources are contradictory [4]. A detailed electrophoretic analysis has eliminated these contradictions. Thus, the molecular mass of the enzyme that had not been reduced with 2-mercaptoethanol but had been denatured by boiling in Na-DDS was 45.0 kDa (single band). Treatment with 2-mercaptoethanol led to the appearance of a single band with a mol. mass of 62.0 kDa (complete denaturation). The enzyme undenatured by boiling but reduced with 2-mercaptoethanol (partial denaturation) migrated in the form of two bands with mol. masses of 127.0 and 62.0 kDa. Thus, the ACE that we had obtained was a homogeneous protein apparently consisting of three subunits each with a molecular mass of 62.0 kDa. After comparing these results with those of gel filtration, in the kinetic calculations the mass of the enzyme was taken as 180 kDa.

To measure the ACE activity we used the continuous spectrophotometric method (in the visible region) proposed by B. Holmquist [5], which is based on the use of FAPGG as substrate. Figure 1 shows kinetic curves of the hydrolysis of FAPGG (50 μM) by the enzyme isolated. At a low concentration of the substrate (in the region $[S] < K_m$) the reaction kinetics are first-order with respect to the substrate. The upper curve presents the relationship for an enzyme concentration twice that for the lower curve. It can be seen that when the concentration of ACE was doubled the rate of the reaction increased twofold and, correspondingly, the rate constant of the first-order reaction also increased (the time of half-hydrolysis fell by a factor of two).

Institute of Biochemistry, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (371) 62 32 56. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 821—825, November-December, 1998. Original article submitted June 22, 1998.

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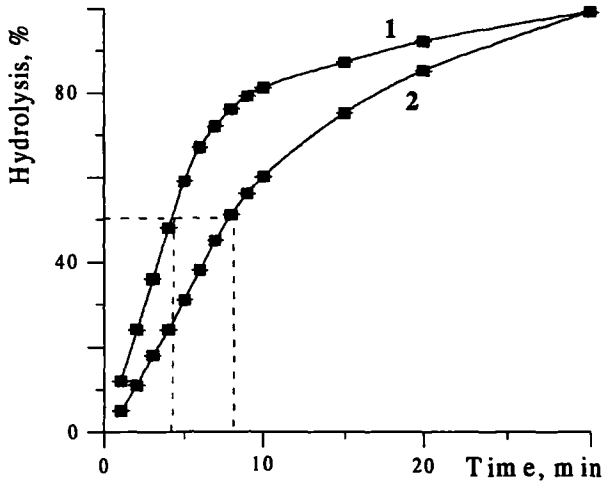

Fig. 1. Kinetic curves of the hydrolysis of FAPGG (50 μM) by the angiotensin-converting enzyme from ox kidney. The reaction was monitored at 328 nm. *1*) 40 μl of ACE in a sample with a volume of 2 ml; *2*) 20 μl of the ACE preparation per sample.

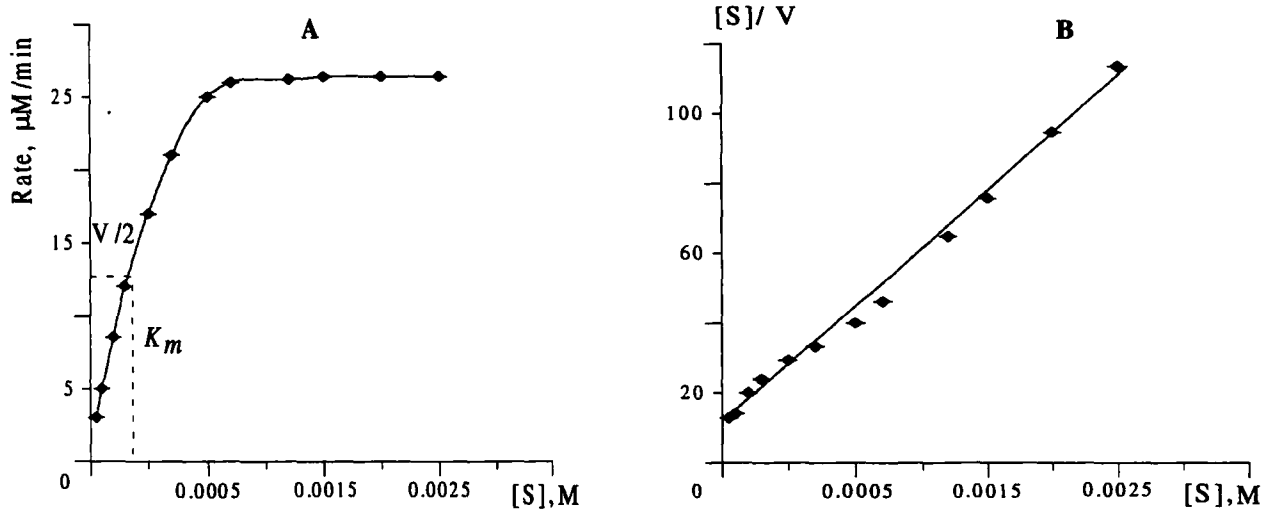

Fig. 2. Graphs of the dependence of the rate of hydrolysis of FAPGG by angiotensin-converting enzyme on the substrate concentration: K_m is the Michaelis constant; V the maximum rate of the reaction; and B the regression curve from these results.

Such behavior of the kinetic curves with a doubling of the concentration of the enzyme shows that under the given conditions ($[E] << [S]$) only the enzyme concentration determines the rate of the enzymatic reaction; i.e., its true catalytic activity is measured.

Figure 2 shows the complete kinetic curve of the hydrolysis of FAPGG by the angiotensin I-converting enzyme from ox kidney, plotted by measuring the initial reaction rates (first 10% of hydrolysis). The values of K_m and k_{cat} for the ox kidney ACE calculated from the results obtained are, respectively, 3.4×10^{-4} M and 26,400 min^{-1}. It is interesting to compare the values obtained for the angiotensin-converting enzymes from other sources. Thus, K_m and k_{cat} for the ACE from rabbit lungs are 3.0×10^{-4} M and 19,000 min^{-1} [5], and for the enzyme from bovine lungs 6.4×10^{-4} M and 15,840 min^{-1} [4].

Thus, on the basis of a study of the kinetics of the hydrolysis of FAPGG by the angiotensin-converting enzyme obtained we have determined the range of substrate concentration within which the kinetics are described by the equation of a first-order reaction ($[S] < K_m$) and have also measured the main kinetic parameters of enzymatic hydrolysis and have thereby selected conditions for testing the inhibitory activity of viper venom peptides.

TABLE 1. Inhibitory Properties of Peptides from Viper Venom with Respect to the Angiotensin-Converting Enzyme

Peptide	Concentration in the experiment, μM	Time of half-hydrolysis of FAPGG, min	k, min^{-1}*
-	-	11.4	0.0608
É-IIIa-1-1-1	0.48	19.5	0.0355
É-IIIa-1-2-1	0.59	20.0	0.0347
É-IIIa-1-3-1	0.63	22.5	0.0308
É-IIIa-2-1	0.83	26.3	0.0263
É-IIIa-2-2	8.30	57.5	0.0121
	0.83	42.5	0.0163
É-IIIa-2-3	8.30	58.5	0.0118
	0.083	29.5	0.0235
	0.0083	13.2	0.0525
É-IIIa-2-4	8.3	43.4	0.0160
	0.083	30.0	0.0231
É-IIIb-2	0.14	15.5	0.0447

*Observed first-order reaction rate constant in the presence of the inhibitor.

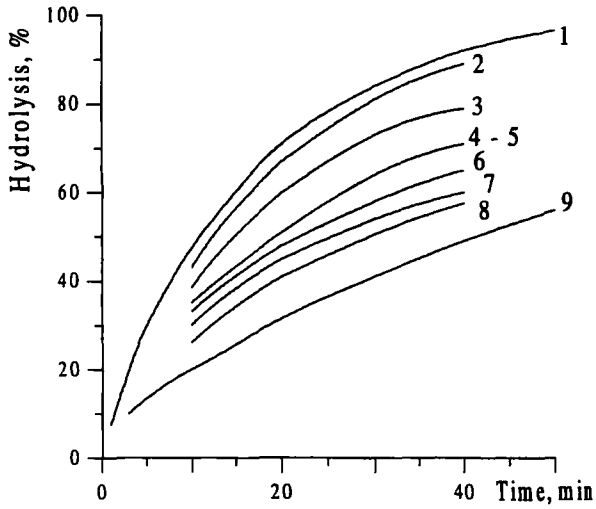

Fig. 3. Kinetic curves of the hydrolysis of FAPGG by the angiotensin-converting enzyme: *1*) in the absence of an inhibitor; *2*) in the presence of the peptide É-IIIa-2-3 in a concentration of 0.0083 μM; *3*) in the presence of É-IIIb-2, 0.14 μM; *4*) in the presence of 0.48 μM É-IIIa-1-1-1; *5*) in the presence of É-IIIa-1-2-1, 0.59 μM; *6*) in the presence of É-IIIa-1-3-1, 0.63 μM; *7*) in the presence of É-IIIa-2-1, 0.83 μM; *8*) in the presence of É-IIIa-2-4, 0.083 μM; *9*) in the presence of É-IIIa-2-2, 0.83 μM.

The activity of an inhibitor was measured by varying the concentration of the peptide in experiments performed at a low (50 μM) concentration of the substrate. In preliminary control experiments it was shown that the observed first-order rate constant of the reaction depends linearly on the concentration of inhibitor. The results obtained are given in Fig. 3 and in Table 1.

According to the equation $d[A]/dt = k[A]$, where $[A]$ is the concentration of the reactant (in the present case the substrate FAPGG), the rate constant of a first-order reaction has the dimensions of min^{-1}. The physical sense of such dimensions is the number of molecules of substrate hydrolyzed by the enzyme in 1 min. On the basis of the integral form of the equation, the rate constant is determined by measuring the time of hydrolysis of half the substrate. The figures in the table, thus, reflect how

the rate constant changes in the presence of a given concentration of an inhibitor.

On comparing the values of K_i calculated from these results (where K_i is the dissociation constant of the enzyme-inhibitor complex) with those already known, it is possible to draw the following conclusions. For example, for peptides É-IIIa-2-3, É-IIIb-2, and É-IIIa-2-4, values of the inhibition constants of 0.0013, 0.05, and 0.14 μM, respectively, were obtained. In other words, among the peptides isolated there were inhibitors exceeding in efficacy by an order of magnitude the widely used synthetic analog Captopril (K_i = 0.023 μM) and comparable with the most powerful of the known synthetic inhibitors *D*-Cys-*L*-Pro (K_i = 0.0055 μM) [6]. Another important conclusion, which can so far be drawn only in preliminary fashion, is that the angiotensin-converting enzyme has, in addition to a dipeptidyl dipeptidase activity, some specificity in relation to the amino acid composition of the dipeptide split out from the substrate. In support of this statement are reports of the inhibition of the enzyme on the use as substrate of bradykinin (C-terminal sequence — -Pro-Phe-Arg) [3] and other substrates [4].

Unfortunately, the approaches adopted for evaluating inhibition [5] do not permit competitive inhibition to be distinguished from the noncompetitive mechanism, which makes further investigations in this direction necessary.

EXPERIMENTAL

We used the venom of a viper (*Echis multisquamatus* Ch.) obtained from the Central Asian Zonal Zoological Combine and dried over calcium chloride; Sephadexes (Pharmacia, Sweden); TDK gels (Toyo-Soda, Japan); reagents for electrophoresis (Serva, Germany); and the specific chromogenic substrate of ACE, N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) (Sigma, USA).

Isolation of the peptides from the viper venom was achieved by a known method [3].

Isolation of ACE from ox kidney was performed as in [7], the purity of the enzyme being monitored by electrophoresis under denaturing conditions.

ACE activity was measured by using 50 μM FAPGG in 0.05 M Tris-HCl, pH 7.5, containing 0.3 M NaCl and 1 μM zinc chloride. The decrease in absorption at 328 nm was followed with the aid of a SF-46 spectrophotometer. In a typical case, the enzyme (20—40 μl) was added in a final concentration of 1—10 nM to 2 ml of thermostated (25°C) substrate in the spectrophotometer cell. As the unit of activity we took that amount of enzyme which catalyzed the hydrolysis of 1 μmole of substrate in 1 min under the conditions given above.

At the chosen substrate concentration, the reaction kinetics were first-order with respect to the substrate ($[S] < K_m$), and hydrolysis reached 98% in 10—30 min.

The rate constants for a first-order reaction were calculated from points on a graph of $\ln C_0/(C_0 - C)$ against time (t), where C_0 is the initial substrate concentration and C is the concentration of the hydrolysis product at time t. In practice, the constants were determined by measuring the time of hydrolysis of 50% of the substrate, since this follows from the rate equation of a first-order reaction.

The Michaelis constant for FAPGG was determined by measuring the initial reaction rates at various substrate concentrations and conditions under which the degree of hydrolysis of the substrate did not exceed 10%. In order to cover the region of suitable concentrations of the substrate (below and above K_m) the measuremts were performed at three different wavelengths (328, 345, and 352 nm) using a control consisting of a single concentration of the substrate that could be measured at two wavelengths.

The catalytic constant (k_{cat}) for the hydrolysis of the substrate FAPGG was calculated acording to [5]. In the calculation of the kinetic parameters we used the method of least squares.

The inhibitor constants (K_i) for the peptides from the viper venom were measured by varying the concentrations of the peptides in experiments conducted at a low substrate concentration (50 μM) at which the rate of the reaction obeyed a first-order equation. Inhibition constants were calculated from the equation $K_i = [I] \times (k_0/k_i - 1)$, where k_0 and k_i are the observed rate constants for a first-order reaction in the absence and in the presence of the inhibitor, respectively [5].

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