HUMAN ERYTHROCYTIC PROLYL ENDOPEPTIDASE II HYDROLYZING TEPROTIDE, AN INHIBITOR OF SNAKE VENOM PEPTIDYL DIPEPTIDASE

L. P. Alekseenko and V. N. Orekhovich

UDC 612.111.1.015.1:577.152.34]:
014.46:615.919:598.12

KEY WORDS: thiol prolyl endopeptidases; teprotide; peptidyl dipeptidase A; human erythrocytes.

Intracellular thiol [2, 11] prolyl endopeptidases (PE), which hydrolyze natural low-molecular-weight (3-14 residues) regulator peptides at prolyl bonds, are found in virtually all animal tissues [5] and also in leukocytes and erythrocytes [1, 4]. PE *in vitro* specifically hydrolyze neurohypophyseal and hyopthalamic hormones, vasoactive peptides of the renin and kinin systems, and some other regulator peptides [3, 13, 14].

The molecule of the nine-member peptide teprotide (BPP_{9a}, SQ-20881), a specific inhibitor of peptidyl dipeptidase A (PDP-A), isolated from snake venom, has three prolyl peptide bonds which may also be hydrolyzed by PE. PDP-A (carboxycathepsin, angiotensin-converting enzyme) [15] is the key enzyme [6] of the renin-angiotensin system responsible for regulating vascular tone *in vivo*. The peptide effector angiotensin II generated by it has a powerful pressor action, realized not only through receptors of arterial smooth muscle cells, but also through mobilization of aldosterone and central stimulation of the hypothalamo-hypophyseal axis, leading to vasopressin secretion. PDP-A also potentiates the pressor response through inactivation of the hypotensive peptide bradykinin [6]. Teprotide is a powerful competitive inhibitor of PDP-A ($K_i = 1 \cdot 10^{-7}$ M) [12]. Experiments on animals and clinical trials have shown that it sharply depresses the blood pressure (BP) in hypertensives but causes virtually no change in BP in normotensives [10], and it also has a favorable action on the heart during acute hypoxia and prevents ventricular hypertrophy in chronic alveolar hypoxia. Administration of teprotide 17 min after clinical death to experimental dogs in a combination of resuscitation measures led to significant improvement of the peripheral blood supply and to rapid and more complete recovery of CNS functions [7].

According to one view teprotide is resistant to the action of tissue proteinases [10]. Data on its degradation by PE from different sources are contradictory. It has been shown that PE from bovine adenohypophysis hydrolyzes teprotide [3], whereas PE from lamb kidney [13] hydrolyzes a 13-member peptide similar in structure to teprotide. Later, however, reports appeared that teprotide is a competitive inhibitor of PE from rabbit brain — endo-oligopeptidase B (EO-B) [12, 14]. During bioassay of inhibition of the rate of bradykinin hydrolysis $K_i = 3 \cdot 10^{-6}$ M [14].

Since teprotide is a "true antihypertensive agent" [10] and in the very near future may find extensive clinical applications, it was necessary to demonstrate the possible pathways of its degradation in the body and to study the action of human erythrocytic PE [1, 4] on it, more especially because another PDP-A inhibitor, captopril, is inactivated by erythrocytic thiol methyl transferase. In the present study hydrolysis of teprotide by human erythrocytic prolyl endopeptidase II (PE-E) and dipeptidyl D,L-alanine-peptidylhydrolyase, and by PE from bovine adenohypophysis (PE-A) was investigated. The properties of these enzymes were compared.

EXPERIMENTAL METHOD

Proteins with PE activity were extracted by adsorption on DEAE-Sephadex A50 (pH 7.5, 0.05 M NaCl) from a hemolysate of human erythrocytes (3-day packed red cells from a blood transfusion station) and from extract of adenohypophysis obtained from an abattoir, and after

Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 3, pp. 308-311, March, 1985. Original article submitted June 15, 1984.

 0007-4888/85/9903-0302$09.50

TABLE 1. Properties of Thiol PE from Human Erythrocytes and Bovine Adenohypophysis and of EO-B from Rabbit Brain

Properties	PE-E	PE-A	EO-B	Literature source
Mol. wt.	66 000	68 000	68 000	[2, 4, 5, 9]
Isoelectric point, pH	4,70	4,76	4,55	[2, 5, 9]
pH-optimum of activity on different substrates	7,5—8,1	7,8—8,2	8,3—8,5	[1, 3, 12, 14]
Residual activity in absence of activators of thiol groups	0	1 %	3 %	[12]
Action of inhibitors, % inhibition of activity:				
PCMB				
10^{-5} M	70	60	—	
$5 \cdot 10^{-5}$ M	100	100	—	
DFP				
$5 \cdot 10^{-4}$ M	15	100	—	
10^{-3} M	100		—	
TPCK, $5 \cdot 10^{-4}$ M	90	90	—	
TLCK, $2 \cdot 10^{-4}$ M	20	0	—	
Pepstatin, 12 μg/ml	0	0	—	
Leupeptin, 15 μg/ml	2	—	—	
EDTA-Na_2, 10^{-3} M	0	0	—	
o-Phenanthroline, 10^{-3} M	0	0	—	
Human blood serum in dilutions of 1:1 and 1:5	0	0	—	

Legend. Reaction performed: hydrolysis of prolyl-peptide, amide, and ester bond in presence of thiol group activators (2-mercaptoethanol, dithiothreitol, cysteine, glutathione) [2-5, 8, 9-11, 14].

elution with sorbent in 0.15 M NaCl they were salted out with $(NH_4)_2SO_4$ at 75% saturation. Erythrocytic proteins (the PE-E preparation) were fractionated by gel-filtration on Sephadex G-100 in 0.02 M Tris-HCl buffer, pH 7.5, with 0.15 M NaCl, 1 mM dithiothreitol, and 1 mM EDTA-Na_2. The column measured 2.5 × 100 cm. Proteins from the adenohypophysis (PE-A preparation) were fractionated by covalent chromatography on sepharoyl-4-aminophenyl mercuriacetate, gel-filtration on Sephadex G-75, adsorption chromatography on DEAE-Sephadex A-25, and isoelectric focusing in a sucrose density gradient, pH 4.0-6.0 [2]. PE activity was determined by the ninhydrin method based on hydrolysis of 0.01 M L-Gly-Pro-Ala-OH, or fluorometrically based on hydrolysis of $8 \cdot 10^{-6}$ M of L-Gly-Pro-7-(4-methyl)-coumarinamide (from Opton, West Germany; λ_{ex} = 380 nm, λ_{em} = 460 mn) in 0.2 M borate buffer, pH 8.1, or in 0.2 M Tris-HCl buffer, pH 7.5, in the presence of 1 mM of 2-mercaptoethanol. To study the action of inhibitors — 10^{-6}-10^{-3} M diisopropylfluorophosphate (DFP), paracholoromercuribenzoate (PCMB), tosyl-phenylalanine-chloromethylketone (TPCK), tosyl-lysine-chloromethylketone (TLCK), o-phenanthroline, EDTA-Na_2 — 3-12 mg/ml of pepstatin and 5-15 mg/ml of leupeptin were incubated with the enzymes for 3 and 18 h and then with the substrate for 1 h in the present of 1 mM 2-mercaptoethanol. Hydrolysis of teprotide (SQ-20881, from Serva, West Germany): 5-8 μg/ml of the enzymes was incubated with 0.33 mg/ml of peptide in 0.1 M borate buffer, pH 8.1, in the presence of 1 mM 2-mercaptoethanol for 3 and 18 h at 37°C. The hydrolysis products were revealed by chromatography on Whatman No. 1 paper in a system of solvents: 1) N-butanol—acetic acid—pyridine—water (30:6:20:24 by volume); 2) N-butanol—acetic acid—water (10:1:3 by volume). Specific reactions for detection were as follows: ninhydrin for peptides with free α-NH_2-groups, Sakaguchi's for arginine residues, and Ehrlich's for tryptophan residues [1]. Amino acid analysis of the hydrolysis products: peptides were eluted from chromatograms with 0.1 M NH_4OH, hydrolyzed with 6 N HCl, and chromatographed on paper in system 1 of solvents, using the corresponding amino acids as reference substances.

EXPERIMENTAL RESULTS

Homogeneous preparations of PE-A purified 15,000 times and PE-E purified 1200 times, containing aminopeptidase as an impurity, were used. Table 1 gives some of their properties compared with those of EO-B from rabbit brain. The enzymes had very close physicochemical characteristics. The isoelectric point of EO-B was shifted a little into the acid zone compared with the other forms of PE. Activity was inhibited by both PCMB and DFP, which is characteristic of proteolytic enzymes of this type. Human blood serum evidently does not contain PE inhibitors.

Teprotide was hydrolyzed by both PE preparations with the formation of four-five products (Fig. 1). The product was found in both 3-h and 18-h digests, was revealed by ninhydrin, and reacted for arginine. It was identified as Pro-Arg-OH. Product 1*a* was present

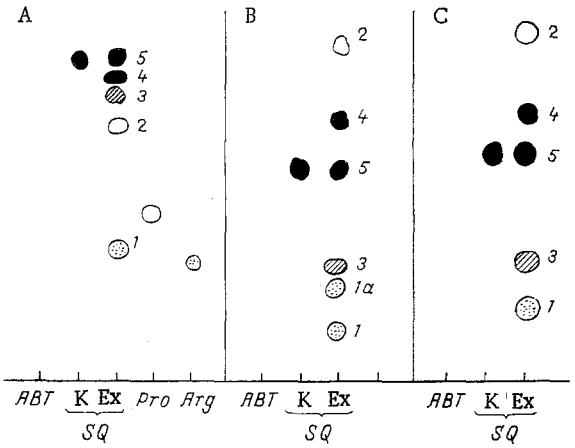

Fig. 1. Schemes of chromatograms of hydrolysis products of teprotide (PE-E) from human erythrocytes and PE-A from bovine adenohypophysis. A) Scheme of chromatograms in solvent system 1 showing R_f values of products separated in this system: 1) 0.37 (NS); 2) 0.70 (N); 3) 0.78 (E); 4) 0.82 (SE); 5) 0.86 (SE) unhydrolyzed teprotide; B, C) schemes of chromatograms in solvent system 2. Solvent passed through twice for 20 h each time. R_f values (second front) of products separated in this system; 1) 0.06 (NS); 1a) 0.12 (NS); 2) 0.44 (N); 3) 0.14 (E); 4) 0.35 (SE); 5) 0.29 (SE) unhydrolyzed teprotide; A, C) PE digest; B) PE-A digest. Schemes B and C shown on twice the scale of scheme A. Letters in parentheses after R_f values indicate color reactions given by product: N) ninhydrin, E) Ehrlich's for tryptophan, S) Sakaguchi's for arginine. K) Control; Ex) experiment.

only in 3-h digests, it reacted for arginine and was detected by ninhydrin. It was identified as Arg-Pro-Gln-Ile-Pro-Pro-OH. Product 2 was detected only by ninhydrin a few hours after treatment of the chromatograms with the reagent. It was identified as Gln-Ile-Pro-Pro-OH. Product 3 was detected only by the reaction for tryptophan. It was identified as pGlu-Trp-Pro-OH. Product 4 was detected by ninhydrin and gave reactions for tryptophan and arginine. It was identified as pGlu-Trp-Pro-Arg-Pro-OH. Free proline was not found in the digests. The two preparations, PE-E and PE-A, thus hydrolyze teprotide pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-OH at the Pro^3-, Arg^4-, and Pro^5-Gln^6-bonds. Peptides corresponding in structure to products 1-3 have been shown to have virtually no inhibitory action on PDP-A activity [10], whereas those corresponding to products 4 and 1*a* have an inhibitory action weaker by 14 and 32 times respectively.

As already mentioned, EO-B from rabbit brain is very similar in its properties to PE-A and PE-E, and it is unlikely that teprotide, which is hydrolyzed by these enzymes, could be a specific competitive inhibitor for EO-B, as it is for PDP-A. The conclusions drawn by Camargo's group [12, 14] regarding teprotide as an inhibitor of EO-A are evidently the result of technical error, because the velocity of hydrolysis of the substrates (angiotensins and bradykinin) in the presence of teprotide was measured by them biologically, and the reaction products were not identified. "Competitive inhibition" of hydrolysis of the above-mentioned vasoactive peptides evidently signifies only that teprotide is a more suitable substrate for EO-B than angiotensins and bradykinin.

The investigation showed that human erythrocytes possess proteolytic enzymes capable of hydrolyzing teprotide and peptides similar to it in structure at physiological pH values and, consequently, can completely or largely detoxicate these substances when they enter the blood stream as a result of therapeutic administration or snake bite.

At the same time, the results demand that data on physiological activity of angiotensin I [10] obtained *in vivo* after administration of high doses of teprotide to prevent angiotensin II formation from angiotensin I by the action of PDP-A be treated with great caution. Since the body possesses a PE capable of hydrolyzing the inhibitor, data on agiontensin I activity may be unsound. This may also apply to investigations of bradykinin hydrolysis *in vivo* following administration of teprotide to inhibit PDP-A activity.

LITERATURE CITED

1. L. P. Alekseenko, O. A. Gol'dina, and V. N. Orekhovich, Dokl. Akad. Nauk SSSR, 268, 234 (1983).
2. L. P. Alekseenko, N. N. Zolotov, and V. N. Orekhovich, Dokl. Akad. Nauk SSSR, 250, 233 (1980).
3. L. P. Alekseenko, N. N. Zolotov, V. N. Orekhovich, et al., Dokl. Akad. Nauk SSSR, 245, 1001 (1979).
4. L. P. Alekseenko, N. N. Zolotov, V. N. Orekhovich, et al., Dokl. Akad. Nauk SSSR, 263, 1258 (1982).
5. L. P. Alekseenko, N. N. Zolotov, N. F. Pozdnev, et al., Byull. Éksp. Biol. Med., No. 11, 24 (1983).
6. Yu. E. Eliseeva, V. N. Orekhovich, L. V. Pavlikhina, et al., Vopr. Med. Khimii, 16, 646 (1970).
7. V. N. Orekhovich, Yu. E. Eliseeva, L. V. Pavlikhina, et al., Dokl. Akad. Nauk SSSR, 250, 253 (1980).
8. A. C. M. Camargo, H. Caldo, and M. R. Reis, J. Biol. Chem., 254, 5304 (1979).
9. K. M. Carvalho and A. C. M. Camargo, Biochemistry (Washington), 20, 7082 (1981).
10. D. W. Cuchman and M. A. Ondetti, Prog. Med. Chem., 17, 41 (1980).
11. G. D. J. Green and E. Shaw, Arch. Biochem., 225, 331 (1983).
12. L. J. Greene, C. C. Spandaro, A. P. Martins, et al., Hypertension, 4, 178 (1982).
13. M. Koida and R. Walter, J. Biol. Chem., 251, 7593 (1976).
14. A. R. Martins, H. Caldo, H. L. L. Coelho, et al., J. Neurochem., 34, 100 (1980).
15. "Nomenclature Committee of the International Union of Biochemistry (NC-IUB)," Europ. J. Biochem., 116, 423 (1981).