PROLYLENDOPEPTIDASE ACTIVITY IN VARIOUS ANIMAL TISSUES

L. P. Alekseenko, N. N. Zolotov, V. F. Pozdnev, and V. N. Orekhovich

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Prolylendopeptidases (PE) of animals and man are soluble intracellular enzymes which hydrolyze peptide, amide, and ester bonds formed by the carboxyl group of proline in synthetic and natural peptides [3-5, 7, 9-11, 13]. All PE now known belong to two types of proteinases: "serine," as the "postprolinehydrolyzing" enzyme, and "cysteine," as the prolyl-D,L-alanine peptidylhydrolase discovered by the writers in the adenohypophysis [12]. Thiol-dependent PE were found recently by the writers in hemolysates of human erythrocytes also [1, 2]. Hydrolysis of biologically active peptides such as angiotensins, bradykinin, neurotensin, substance P, carboxycathepsin inhibitors from snake venoms, the immunogenic peptide tuftsin, and so on, by tissue PE *in vitro* suggests that PE also participate *in vivo* in the inactivation and cascade conversions of various polypeptide bioregulators, thereby performing a highly important function in the system of neurohumoral regulation.

Having isolated a prolyl-specific enzyme from the adenohypophysis in a homogeneous state [3], and having partially purified PE from human erythrocytes, the next stage was to study the distribution of PE activity in the tissues of various animal organs. Additional information on tissue PE could be obtained by determination of their molecular weight by gel-filtration [6] and also by the action of specific inhibitors on PE activity. The task was facilitated by the fact that all PE already characterized have a molecular weight of about 70 kilodaltons or about 100 kilodaltons and they are readily separable by gel-filtration under appropriate conditions. Activity of "cysteine" PE is specifically inhibited by p-chloromercuribenzoate (PCMB) [3].

EXPERIMENTAL METHOD

Homogenates of various bovine organs: spleen, liver, skeletal muscles, small intestine, adenohypophysis, thyroid gland, cerebral cortex, heart, lungs, testes, pancreas, and kidneys, were extracted with twice the volume of water. The residue was separated by high-speed centrifugation and the extract used in the work. The protein content was determined spectrophotometrically [8]. The substrate for PE (which we synthesized by the carbodi-imide method), namely N-carbobenzoxy-glycyl-prolyl-p-nitroanilide (Z-Gly-Pro-pNa), was chromatographically homogeneous.

Determination of PE Activity. The enzyme preparation (100 µl) was incubated with 100 µl of 0.25 mM substrate solution in 0.3 M borate buffer, pH 8.2, containing 0.1 mM dithiothreitol and 1% Triton X-100 for 2-3 h at 37°C. The reaction was stopped by addition of 0.5 ml of 0.3 M NaOH and 0.5 ml of a 5% solution of zinc sulfate. Optical density of the samples after removal of the residue was determined at 382 nm and the concentration of liberated p-nitroaniline was calculated. The coefficient of molar extinction for it under these conditions was 12,800 $M^{-1} \cdot cm$/liter. Specific activity was expressed in micromoles p-nitroaniline liberated/mg protein/min.

Determination of Inhibition by PCMB. A solution of the enzyme preparation (50 µl) was incubated with 50 µl of a solution of the inhibitor in 0.02 M Tris-HCl, pH 7.5, for 30 min at 20°C. The final PCMB concentrations were 5×10^{-5} and 10^{-3} M. PE activity was determined as described above and the results were expressed as percentages of PE activity in samples not containing PCMB. The molecular weights of the PE in the extracts were determined by gel-fil-

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TABLE 1. PE Activity in Extracts of Various Bovine Organs

Organ	Specific activity, nmoles/mg protein/min	Activity of native tissue, nmoles/g tissue/min	Protein concentration in extract, mg/ml
Pituitary (anterior lobe)	1,18	95,58	20,25
Liver	1,73	350,81	109,39
Kidneys	1,53	157,53	51,48
Heart	2,55	124,90	24,49
Skeletal muscles	3,82	163,42	21,39
Lungs	1,82	230,19	63,24
Spleen	0,82	114,30	69,87
Testes	1,17	67,17	28,70
Pancreas	5,35	961,82	89,89
Thyroid gland	2,80	577,92	103,02
Cerebral cortex	17,12	616,42	18,00
Small intestine	2,79	293,12	52,53
Blood plasma	0	—	—

tration [6] on an Ultragel AcA-44 column (2.6 × 96 cm), equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.1 mM dithiothreitol. The free volume of the column was determined by glycogen filtration. The column was calibrated by filtration of different individual proteins with mol. wt. from 12.4 to 240 kilodaltons. The ratio of their elution volumes to the total void volume of the column (V_e/V_o) was calculated and dependence of this ratio on mol. wt. was plotted graphically. The extract (1 ml, 30-50 mg protein) was applied to the column and optical density at 280 nm was recorded continuously in the eluate (Uvicord II, from LKB, Sweden). Fractions measuring 3.4 ml were collected over a period of 6 min. PE activity was determined in all fractions of eluate containing protein. The molecular weight of the proteins with PE activity was determined from their V_e/V_o ratio on the graph.

EXPERIMENTAL RESULTS

Comparison of PE activity in extracts of various bovine organs (Table 1) showed that it was highest in the cerebral cortex, where it was 10 times higher than PE activity in the liver, about three times higher than in the pancreas, four times higher than in skeletal muscles, and 21 times higher than in the spleen. Calculation of the values per gram wet weight of tissue gave a different distribution of PE activity in the organs. Highest activity in this case was found in the pancreas; it was about 1.5 times lower in the cerebral cortex and thyroid gland, and 2.7 times lower in the liver than in the pancreas. Lowest activity was observed in the testes, where it was about 1/14 of the PE activity in the pancreas. As Table 1 shows, on extraction of organ homogenates with water, different quantities of proteins passed into solution, and where comparatively little protein was extracted (brain, skeletal muscles) the extracts had high PE activity. PE activity in organs of other species of animals differs in its distribution. In extracts of human organs highest PE activity is observed in skeletal muscles, testes, kidneys, and salivary glands, lowest activity in the aorta, mesentery, and heart [9]. Highest activity in rabbit organs is found in the small intestine, cerebral cortex, lungs, and liver [13]. Highest PE activity in rat organs was found in the testes and liver, lowest in the small intestine and pancreas [14]. PE activity in the blood plasma was very low and none could be detected by hydrolysis of Z-Gly-Pro-pNa. In human blood serum PE activity was found on hydrolysis of succinyl-glycyl-prolyl-4-methylcoumarin-7-amide [9]. By the action of serum on the substrate a product is formed on average at the rate of 3.7 nmoles/ml/min (units), equivalent to 0.3% of PE activity in the liver. PE activity in rat blood serum based on hydrolysis of the same substrate was 7 units, 100 times lower than PE activity in rat liver [14]. PE activity in human sperm and saliva, based on hydrolysis of carbobenzoxy-glycyl-prolyl-4-methylcoumarin-7-amide was 3 and 0.9 units, respectively. No PE activity could be found in human urine [14]. The study of dependence of PE activity on protein concentration in extracts of adenohypophysis, liver, kidneys, pancreas, spleen, and skeletal muscles showed that an increase in protein concentration in the extracts from 0.5 to 4 mg/ml leads to a reduction of specific PE activity by 40-50%. In the writers' view this decrease is due to the presence of PE inhibitors in the extracts, and their action is reduced by dilution of the extract. A PE inhibitor was recently found in pig pancreas and partially purified [15].

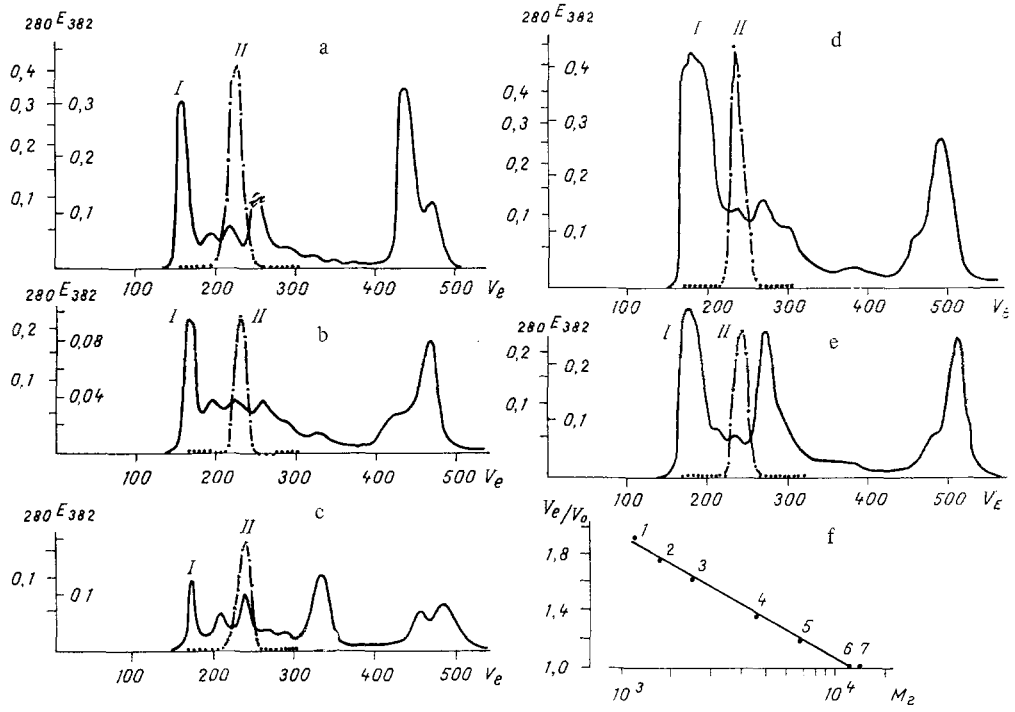

Fig. 1. Gel-filtration of extracts of adenohypophysis (a), kidneys (b), muscles (c), liver (d), and spleen (e) through a column of Ultragel AcA-44. I) Absorption curve of proteins at 280 nm; II) absorption curve as a result of enzymic reaction of p-nitroaniline at 382 nm; f) molecular weight of known proteins as a function of ratio of their elution volume to void volume of column (V_e/V_o): 1) cytochrome C (124 kilodaltons); 2) myoglobin (1.78 kilodaltons); 3) chymotrypsinogen A (25 kilodaltons); 4) ovalbumin (45 kilodaltons); 5) serum albumin, monomer (67 kilodaltons); 6) aldolase (147 kilodaltons); 7) catalase (240 kilodaltons).

PCMB, an inhibitor of "cysteine" proteinases, inhibits PE activity differently in extracts of different bovine organs. In the adenohypophysis it inhibits PE activity by 70% in a concentration of 5×10^{-5} M and completely inhibits its activity in a concentration of 10^{-3} M. Inhibition of activity in extracts of other organs was incomplete, even with this high concentration of inhibitor: it reached an average level of 85% in extracts of spleen, muscles, and liver. Inhibition of activity in extract of the kidneys was particularly weak. In the presence of PCMB in a concentration of 5×10^{-5} M it amounted to about 20%, and with 10^{-3} M it was 65%. The results for inhibition of PE activity by PCMB in the extracts can be correlated to some extent with data on inhibition of activity of highly purified PE from adenohypophysis and kidneys. For instance, activity of the "postproline hydrolyzing" enzyme from the kidneys is inhibited only partially even in the presence of a considerable excess of PCMB, whereas prolyl-D,L-alanine peptidylhydrolase from the adenohypophysis is completely inhibited by a concentration of 2.5×10^{-5} M.

Gel-filtration of extracts of five organs on a column of Ultragel AcA-44 (Fig. 1) showed that PE activity is associated with proteins with mol. wt. of 57 (extract of spleen), 60 (extracts of muscles and liver), and 66 kilodaltons (extracts of adenohypophysis and kidneys). PE activity associated with proteins with mol. wt. of about 115 kilodaltons could not be detected in any of the extracts studied, as was demonstrated initially for the "postproline hydrolyzing" enzyme [11]. Previously, during isolation of highly purified PE from extract of adenohypophysis it was shown that this gland contains only one enzyme [5, 10] with PE activity, with a molecular weight of 70 ± 5 kilodaltons. The extract of adenohypophysis containing a well characterized enzyme could thus be used as a standard for comparison of gel filtration curves of PE extracts from other organs. Kato et al. [9], by gel-filtration on Sephadex G-100, found only one enzyme with mol. wt. of 70 kilodaltons in brain extract, but in human kidney extract, besides the main component (70 kilodaltons) with PE activity, they observed weak activity in the zone of proteins with mol. wt. of 100 kilodaltons. Activity of the high-mo-

lecular-weight component was weakly inhibited by PCMB. However, we were unable to detect PE activity in the high-molecular-weight protein fraction in bovine kidney extract. Using hydrolysis of Z-Gly-Pro-pNa, we were unable to find PE activity in bovine blood plasma on gel-filtration, but Kato et al. [9], using hydrolysis of succinyl-glycyl-prolyl-4-methylcoumarin-7-amide, observed very low PE activity in human blood serum, associated only with proteins with mol. wt. of about 100 kilodaltons.

The present investigation thus showed that all bovine organs tested contain PE activity, hydrolyzing Z-Gly-Pro-pNa as substrate. PE activity in extracts of adenohypophysis, liver, kidneys, spleen, and skeletal muscles is exhibited by proteins with mol. wt. of about 60 ± 6 kilodaltons. High-molecular-weight proteins with PE activity were not found in these extracts. PE activity in the extracts is inhibited to different degrees by PCMB. Total inhibition of PE activity by this inhibitor was observed only in extract of adenohypophysis. PE activity in bovine blood plasma was not found by means of hydrolysis of Z-Gly-Pro-pNa.

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