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Isolation, Partial Purification, Characterization, and Tissue Distribution of Phenylmethylsulfonyl Fluoride-Inhibited Feline Carboxypeptidase

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Abstract—Phenylmethylsulfonyl fluoride (PMSF)-inhibited carboxypeptidase from cat liver was purified 148-fold by chromatography on CM- and DEAE-cellulose with 27.3% yield. Molecular weight of the enzyme is 100-110 kD as determined by gel filtration on Sephadex G-150. The enzyme has maximum activity at pH 5.50-5.75; its activity is completely inhibited by PMSF or *p*-chloromercuribenzoate and partially inhibited by iodoacetamide. EDTA, 2-mercaptoethanol, N-ethylmaleimide, Co^{2+} and Ca^{2+}, basic carboxypeptidase inhibitor guanidinoethylmercaptosuccinic acid, and angiotensin-converting enzyme inhibitor captopril do not influence its activity. The enzyme cleaves arginine from enkephalin-Leu5-Arg6 and dansyl-Phe-Leu-Arg to form enkephalin-Leu5 and dansyl-Phe-Leu, respectively, and very slowly cleaves leucine from carbobenzoxy-Gly-Leu. Further cleavage of either enkephalin-Leu5 or dansyl-Phe-Leu was not detected. The highest activity of this enzyme was found in adrenal glands and testicles; this activity was 30% lower in hypophysis, and still lower in liver and kidney. The PMSF-inhibited carboxypeptidase activity in brain was about 6-16 times lower than that in adrenal gland. In brain regions, the highest activity was detected in gray matter of cerebral hemispheres and cerebellum, and slightly lower activity was found in thalamus/hypothalamus, striatum, and hippocampus. The lowest activity was found in quadrigeminal bodies, medulla oblongata, and white matter of cerebral hemispheres. The enzyme exists mainly in soluble form; the activity of membrane-associated enzyme is 7-25% of soluble enzyme activity depending on tissue type. We consider here a possible involvement of PMSF-inhibited carboxypeptidase in the metabolism of biologically active peptides.

Key words: PMSF-inhibited carboxypeptidase, purification, liver, neuropeptides

Carboxypeptidases eliminating arginine and lysine residues from peptide C-ends play an important role in metabolism of neuropeptides. These enzymes form biologically active neuropeptides from their inactive precursors [1, 2]. About ten enzymes expressing the mentioned substrate specificity have been described to date. All of them but lysosomal carboxypeptidase B are zinc-dependent metalloenzymes belonging to the same family of carboxypeptidase B-like enzymes [2, 3]. Recently we reported about a novel carboxypeptidase from cat brain that eliminates arginine residues from the C-end of dansyl-Phe-Leu-Arg [4]. This enzyme expresses its activity at pH values corresponding that in secretory vesicles and has a molecular weight about 100-110 kD; inhibitors of metal-dependent basic carboxypeptidases did not inhibit its activity. The enzyme was inhibited by PMSF and was named PMSF-inhibited carboxypeptidase [4]. It is involved in some physiological processes accompanied by significant changes in the level of biologically active peptides [5, 6]. Previously reported data [4-6] suggest an involvement of this enzyme in neuropeptide metabolism. So it is of some interest to examine the physicochemical properties and substrate specificity of the purified enzyme.

The goal of this study was to partially purify the PMSF-inhibited carboxypeptidase from cat liver, characterize the partially purified enzyme, and evaluate its tissue distribution.

MATERIALS AND METHODS

The following chemicals and materials were used: CM-cellulose, DEAE-cellulose, enkephalin-Leu5, enkephalin-Leu5-Arg6, N-ethylmaleimide, iodoacetamide, *p*-chloromercuribenzoic acid, and bovine serum albumin (Serva, Germany); Sephadex G-150 and molecular weight markers for gel filtration chromatography (Pharmacia, Sweden); phenylmethylsulfonyl fluoride, guanidinoethylmercaptosuccinic acid, and captopril

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(Sigma, USA); 2-mercaptoethanol (Merck, Germany); and Silufol UV-254 pre-coated thin-layer chromatography (TLC) plates (Reanal, Hungary). Dansyl-Phe-Leu-Arg was synthesized by Dr. Kalikhevich as described elsewhere [7]. All other chemicals (chemical purity or analytical purity grade) were manufactured in Russia.

Purification of PMSF-inhibited carboxypeptidase. The enzyme was purified from soluble liver fraction of adult cats with body weight 2.5-3.5 kg. Liver was perfused with cool (4°C) 0.25 M sucrose and homogenized in 50 mM sodium acetate buffer containing 50 mM NaCl and 100 mM EDTA, pH 4.0, in the ratio of 1 : 4 (w/v). The homogenate was centrifuged for 60 min at 20,000*g*. The supernatant was adjusted to pH 3.5 with acetic acid. The extract was applied onto a column with CM-cellulose (100 ml) pre-equilibrated with 50 mM sodium acetate buffer containing 50 mM NaCl, pH 3.5 (flow rate 100 ml/h). Then the column was washed with 300 ml of the same buffer. The enzyme was eluted with 50 mM sodium acetate buffer containing 50 mM NaCl, pH 6.0, and 20-ml fractions were collected. Ultraviolet absorption at 280 nm and enzymatic activity were measured in these fractions. The fractions containing PMSF-inhibited activity were pooled and adjusted with NaOH to pH 6.0. The resulting preparation was applied onto a column with DEAE-cellulose (20 ml) equilibrated with 50 mM sodium acetate buffer containing 50 mM NaCl, pH 6.0 (flow rate 20 ml/h). The column was washed with 60 ml of the initial buffer. Elution was performed with 20 mM sodium acetate buffer containing 50 mM NaCl, pH 3.5, and 10-ml fractions were collected. The fractions exhibiting the enzymatic activity were pooled and used in experiments on the properties of PMSF-inhibited carboxypeptidase.

Determination of PMSF-inhibited carboxypeptidase activity. Enzymatic activity was determined by the elimination of dansyl-Phe-Leu from dansyl-Phe-Leu-Arg inhibited by 1 mM PMSF at pH 5.6 [4]. Aliquots containing 140 μl of 50 mM sodium acetate buffer with 50 mM NaCl, pH 5.6, were mixed with 50 μl of enzyme preparation and 10 μl of 25 mM PMSF solution in ethanol. PMSF solution was added into the reaction mixture after the enzyme preparation. Control samples contained 150 μl of specified buffer and 50 μl of enzyme. Samples were pre-incubated at 37°C for 8 min, and 50 μl of 210 μM dansyl-Phe-Leu-Arg was then added (its solution in distilled water was pre-warmed at 37°C). The samples were incubated for 60 min at 37°C, and reaction was terminated with 50 μl 1 M HCl. Extraction of the reaction product, dansyl-Phe-Leu, with chloroform and measurement of fluorescence in chloroform phase (λ_{ex} = 360 nm, λ_{em} = 530 nm) were performed as described [7]. Enzymatic activity was determined from the difference between increments of fluorescence in samples containing and not containing PMSF and expressed as nmol dansyl-Phe-Leu produced during 1 min of incubation per 1 mg protein.

To determine pH optimum, 0.5 M acetate-phosphate buffers (0.5 M sodium acetate adjusted with 0.5 M phosphoric acid) were used. Reactions with group-specific reagents were conducted for 30 min at 37°C.

Determination of molecular weight of the enzyme. The enzyme preparation after chromatography on DEAE-cellulose was applied onto a column (120 ml) with Sephadex G-150 (flow rate 7 ml/h per 1 cm^2) equilibrated with 20 mM sodium acetate buffer, pH 5.6, 100 mM NaCl. Fractions (3.5 ml each) were collected, and PMSF-inhibited carboxypeptidase activity was determined in these fractions. The column was calibrated using dextran blue (2000 kD), catalase (232 kD), aldolase (158 kD), BSA (67 kD), ovalbumin (43 kD), and cytochrome *c* (12.3 kD) as standards.

Studies on substrate specificity of PMSF-inhibited carboxypeptidase. Partially purified enzyme preparation was incubated with dansyl-Phe-Leu-Arg (final concentration 42 μM), enkephalin-Leu5-Arg6 (42 μM), and carbobenzoxy-Gly-Leu (42 μM and 3.5 mM) for 60 min at 37°C and pH 5.5. Hydrolysis products were separated by two-dimensional thin-layer chromatography on Silufol UV-254 plates. Separation in the first direction was performed in the solvent system ethyl acetate–propan-2-ol–CH_3COOH–H_2O (40 : 40 : 1 : 19 v/v) and in the second direction in the system butan-2-ol–CH_3COOH–H_2O (4.5 : 1.5 : 6.0 v/v, upper phase).

Studies on tissue distribution of PMSF-inhibited carboxypeptidase. Weighed samples of tissues were homogenized in 20 mM sodium acetate buffer, pH 5.6, containing 50 mM NaCl (1 : 50 w/v). To obtain soluble and membrane-associated enzyme fractions, the homogenate was centrifuged for 60 min at 30,000*g* and 4°C. The pellet was resuspended in the initial volume of homogenization medium and was taken as membrane fraction, and the supernatant was taken as the soluble fraction. PMSF-inhibited carboxypeptidase activity and protein concentration by Lowry [8] were determined in each fraction.

RESULTS

Purification of PMSF-inhibited carboxypeptidase. We purified the enzyme from the soluble fraction of cat liver by ion-exchange chromatography on CM-cellulose followed by chromatography on DEAE-cellulose. A typical purification protocol is given in the Table 1. The purification started from the extraction of enzyme from homogenate. Maximum extraction level was observed at pH 4.0 in the presence of 100 mM EDTA.

During chromatography on CM-cellulose, PMSF-inhibited carboxypeptidase was eluted as a single narrow symmetric peak (Fig. 1). Fractions expressing its activity (620-680 ml) were pooled and further purified on a column with DEAE-cellulose (Fig. 2). Again, as in the preceding step, the enzyme was eluted as a single symmetric

Table 1. Purification of PMSF-inhibited carboxypeptidase from cat liver

Purification step	Volume, ml	Protein, mg/ml	Specific activity, nmol/min per mg protein	Yield, %
Homogenate	350	27.5	0.280	100
Extract	300	23.4	0.378	98
CM-cellulose chromatography	50	7.58	2.36	33.2
DEAE-cellulose chromatography	9.9	1.32	56.1	27.3

Table 2. Effects of group-specific reagents on the PMSF-inhibited carboxypeptidase activity

Reagent	Concentration, mM	Activity
Control		100
Co^{2+}	1	99
PMSF	1	2
Guanidinoethylmercaptosuccinic acid	0.001	103
Captopril	0.01	110
EDTA	1	100
2-Mercaptoethanol	1	96
N-Ethylmaleimide	1	93
Iodoacetamide	1	63
p-Chloromercuribenzoate	0.01	10

peak. As a result, the enzyme was 148-fold purified with 27% yield (Table 1).

Physicochemical properties of PMSF-inhibited carboxypeptidase. The partially purified enzyme has molecular weight 100-110 kD, as evident from the gel-filtration experiment on Sephadex G-150, and was not contaminated by other basic carboxypeptidases (Fig. 3). Maximum enzymatic activity was observed at pH 5.50-5.75 (Fig. 4).

PMSF and *p*-chloromercuribenzoate completely inhibit activity of the enzyme, and iodoacetamide inhibits about half its activity (Table 2). EDTA, 2-mercaptoethanol, N-ethylmaleimide, Co^{2+} and Ca^{2+}, the basic metallocarboxypeptidase inhibitor guanidinoethylmercaptosuccinic acid, and the angiotensin-converting enzyme inhibitor captopril do not affect its activity. These data suggest the enzyme not to be a metal-dependent carboxypeptidase.

Substrate specificity of PMSF-inhibited carboxypeptidase. The partially purified enzyme, as it is evident from TLC, eliminates arginine from both enkephalin-Leu5-Arg6 and dansyl-Phe-Leu-Arg to form enkephalin-Leu5 and dansyl-Phe-Leu, respectively. Further stages of hydrolysis were not revealed. Being incubated with 42 μM carbobenzoxy-Gly-Leu, the enzyme does not hydrolyze this substrate; nevertheless, an extremely faint spot of leucine was observed with 3.5 mM carbobenzoxy-Gly-Leu. In all cases PMSF completely inhibited the substrate hydrolysis.

Thus, the substrate specificity of PMSF-inhibited carboxypeptidase is indicative for its basic nature, that is, the enzyme cleaves arginine and lysine residues from peptide C-ends. It can also cleave substrates of carboxypeptidase A, but with far less activity and affinity.

Distribution of PMSF-inhibited carboxypeptidase activity among cat tissues and brain structures. Table 3 shows the distribution of the enzyme activity among var-

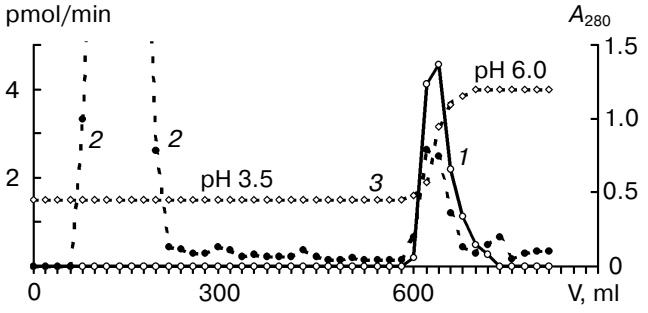

Fig. 1. Ion-exchange chromatography of liver extract on CM-cellulose: *1*) PMSF-inhibited carboxypeptidase activity; *2*) protein; *3*) pH.

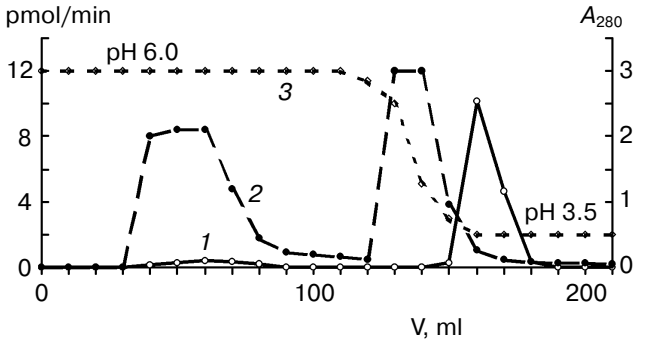

Fig. 2. Ion-exchange chromatography of partially purified PMSF-inhibited carboxypeptidase on DEAE-cellulose: *1*) enzymatic activity; *2*) protein; *3*) pH.

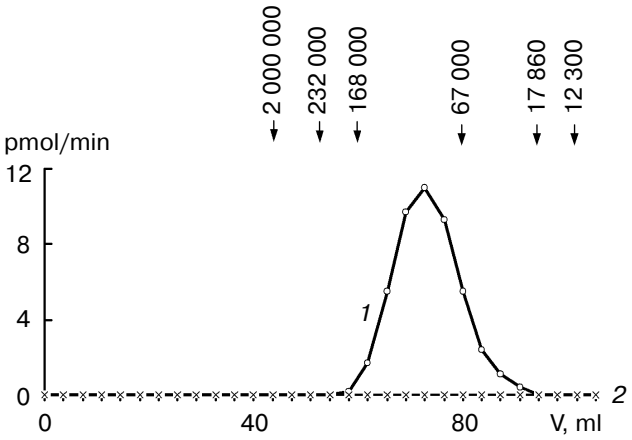

Fig. 3. Gel-filtration of partially purified PMSF-inhibited carboxypeptidase from cat liver on Sephadex G-150: *1*) PMSF-inhibited carboxypeptidase activity; *2*) activity not inhibited by PMSF.

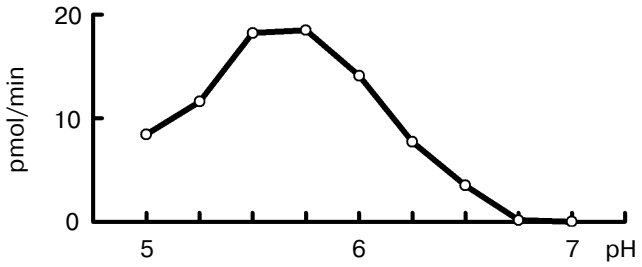

Fig. 4. Effect of pH on the activity of the PMSF-inhibited carboxypeptidase.

ious cat tissues and brain structures. The maximum enzyme activity was found in adrenal glands and testicles, about 30% lower level was found in hypophysis. The enzyme activity in liver and kidney was 42 and 23%, respectively, of those observed in adrenal glands. The PMSF-inhibited carboxypeptidase activity in brain structures was about 6-16 times lower than that in adrenal glands. In brain regions, the highest activity was detected in cerebellum and gray matter of the cerebral hemispheres, and slightly lower activity was found in thalamus/hypothalamus, striatum, and hippocampus. The lowest activity was found in quadrigeminal bodies, medulla oblongata, and white matter of the cerebral hemispheres, in which it was two- to three-fold lower than in gray matter of the cerebral hemispheres. The studied carboxypeptidase activity in spinal cord structures was about 1.5-fold lower than in corresponding structures of brain. So, the distribution of PMSF-inhibited carboxypeptidase activity among tissues and structures of the

Table 3. PMSF-inhibited carboxypeptidase activity among cat tissues and brain structures (nmol dansyl-Phe-Leu formed in 1 min per 1 mg protein; mean ± SD, n = 5-6)

Tissues and brain structures	Activity		
	homogenate	soluble fraction	membrane-associated fraction
Adrenal glands	3.74 ± 0.31	8.58 ± 1.60	2.03 ± 0.26
Kidney	0.85 ± 0.07	2.34 ± 0.29	0.59 ± 0.06
Liver	1.59 ± 0.16	3.65 ± 0.42	0.45 ± 0.03
Testicles	3.58 ± 0.17	6.16 ± 0.81	1.07 ± 0.09
Brain			
hypophysis	2.51 ± 0.41	3.63 ± 0.49	0.63 ± 0.15
cerebellum	0.61 ± 0.03	2.36 ± 0.22	0.26 ± 0.05
thalamus/hypothalamus	0.51 ± 0.06	2.28 ± 0.12	0.16 ± 0.01
hippocampus	0.44 ± 0.05	2.06 ± 0.22	0.20 ± 0.03
striatum	0.44 ± 0.06	1.26 ± 0.12	0.17 ± 0.02
quadrigeminal bodies	0.29 ± 0.03	2.02 ± 0.25	0.24 ± 0.04
medulla oblongata	0.26 ± 0.03	1.61 ± 0.22	0.20 ± 0.03
Cerebral hemispheres			
gray matter	0.64 ± 0.05	2.25 ± 0.27	0.25 ± 0.01
white matter	0.23 ± 0.03	1.96 ± 0.24	0.15 ± 0.01
Spinal cord			
gray matter	0.43 ± 0.03	n. d.	n. d.
white matter	0.14 ± 0.02	n. d.	n. d.
roots	0.12 ± 0.01	n. d.	n. d.

Note: n. d., activity was not determined.

feline central nervous system essentially matches that of neuropeptides [9]. Note also that the highest activity of the enzyme in brain is in neuron body-rich structures.

The enzyme activity distribution in both soluble and membrane-associated fractions corresponds (with a few exceptions) to that in homogenates of tissues and brain structures (Table 3). However, some differences were found. Maximum enzymatic activity among homogenates (in adrenal glands) was almost 16 times higher than minimum enzymatic activity (in white matter of cerebral hemispheres), whereas this difference among soluble fractions was less than seven times, and among membrane-associated fractions it was 13.5 times. Besides, the ratio between activities in soluble and membrane-associated fractions varied depending on tissue or brain structure. In particular, the ratio between soluble and membrane-associated fractions was 4 : 1 in adrenal glands and kidney, 6 : 1 in hypophysis and testicles, 8 : 1 in liver, 8 : 1 to 11 : 1 in most brain structures achieving 14 : 1 in thalamus/hypothalamus. This is, for example, why the minimum enzymatic activity among brain homogenates was found in white matter of hemispheres, and the minimum enzymatic activity among soluble fractions was found in striatum, whereas among membrane-associated fractions it was minimal in white matter of cerebral hemispheres, thalamus/hypothalamus, and striatum. Hence, the enzyme exists mainly in soluble form with the ratio between its activities in soluble and membrane-associated forms depending significantly on tissue and brain structure. This fact suggests different functions of soluble and membrane-associated enzyme forms *in vivo*.

DISCUSSION

PMSF-inhibited carboxypeptidase was isolated from cat liver and 148-fold purified with 27% yield. The purified enzyme resembles previously described PMSF-inhibited carboxypeptidase from gray matter of cat cerebral hemispheres [4] in its physicochemical properties. PMSF-inhibited activity cleaving basic amino acid residues probably belongs to the same enzyme in brain and liver. The purified enzyme cleaves arginine residues from both enkephalin-Leu5-Arg6 and dansyl-Phe-Leu-Arg to form enkephalin-Leu5 and dansyl-Phe-Leu, respectively. The enzyme also hydrolyses, though to a considerable lesser degree, carbobenzoxy-Gly-Leu, a substrate of carboxypeptidase A. Note, however, that many basic carboxypeptidases (namely carboxypeptidases cleaving C-terminal arginine and lysine residues) express minor carboxypeptidase A-like activity [10-12]. Thus, the purified enzyme by its substrate specificity belongs apparently to basic carboxypeptidases. However, the PMSF-inhibited carboxypeptidase differs from all known basic metal-dependent carboxypeptidases and lysosomal carboxypeptidase B (EC 3.4.18.1) [1-3, 10] by the number of its physicochemical properties (molecular weight 100-110 kD, inhibition by PMSF, insensitivity to the basic metal-dependent carboxypeptidase inhibitor guanidinoethylmercaptosuccinic acid, EDTA, and Co^{2+}). The enzyme may be a serine carboxypeptidase based on its inhibition by PMSF.

Two serine carboxypeptidases are found in mammalian tissues: lysosomal carboxypeptidase A (cathepsin A, lysosomal carboxypeptidase L, EC 3.4.16.1) and carboxypeptidase C (angiotensinase C, prolylcarboxypeptidase, EC 3.4.12.4). Lysosomal carboxypeptidase C, molecular weight of 170-210 kD, is composed of 58-kD subunits and displays maximum activity at pH 5.6; its activity is inhibited by PMSF and it does not cleave C-terminal arginine residues [1, 10, 13]. Thus, PMSF-inhibited carboxypeptidase differs from carboxypeptidase C by its molecular weight and substrate specificity.

Lysosomal carboxypeptidase A comes in several forms of various molecular weights, 1270, 680, and 98 kD, the form of lower molecular weight is composed in turn of two ~47-kD subunits, each consisting of two polypeptides (19-20 and 31-32 kD) [10, 14, 15]. This 98-kD form accounts for 60-70 to 100% of the total enzymatic activity in various tissues. The enzyme displays maximum activity at pH 5.0-5.8, its activity is inhibited by PMSF, diisopropyl fluorophosphate, iodoacetamide, *p*-chloromercuribenzoate, and $HgCl_2$, whereas EDTA does not influence its activity [10, 14-17]. The enzyme cleaves C-terminal hydrophobic acid residues; the rate of cleavage of arginine is 20-50 times lower than that of leucine [10, 17]. The enzymatic activity in kidney and liver is more than one order higher than that in brain [10, 18]. Thus, the PMSF-inhibited carboxypeptidase by its molecular weight, pH optimum, and spectrum of inhibitors is similar to lysosomal carboxypeptidase A, but differs from that by substrate specificity and tissue localization.

Thus, the PMSF-inhibited carboxypeptidase differs from all the known carboxypeptidases found in animal tissues by its physicochemical and catalytic features, when put together and, possibly, is a new (not previously known) enzyme. The enzyme might be involved in the processing of neuropeptide precursors considering its ability to cleave enkephalin-Leu5-Arg6 *in vitro* to form enkephalin-Leu5, its maximum activity at pH values typical for secretory vesicles, its high activity in tissues with high level of biologically active peptides (adrenal glands, hypophysis, and testicles). Note, however, that the enzyme activity distribution in brain slightly differs from that of biologically active peptides in general [9]. It is not inconceivable that the PMSF-inhibited carboxypeptidase may be preferentially involved in metabolism of some distinct neuropeptides. Distribution of the enzyme activity among brain tissues and structures corresponds well to that of diazepam binding inhibitor and its processing

products with the maximum level in adrenal glands, testes, and ovaries, and the medium level in brain structures such as hemispheric gray matter, cerebellum, and hypothalamus [19, 20]. Note that the involvement in regulation of steroid hormone genesis is taken to be one of the main functions of diazepam binding inhibitor and similar peptides [19, 21]. Maximum activity of the enzyme is found in endocrine tissues synthesizing steroid hormones: ovaries, adrenal glands, and testes [22]. Moreover, PMSF-inhibited carboxypeptidase was shown to be involved in the response to exogenous steroid hormones [6] to much more extent than carboxypeptidase H (EC 3.4.17.10), a basic carboxypeptidase participating in the processing of various neuropeptide precursors [2, 3]. Hence, PMSF-inhibited carboxypeptidase may participate in the processing of biologically active peptide precursors, in particular, diazepam binding inhibitor, and biologically active products of its cleavage.

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