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Leu-Enkephalin Generally Labeled with Tritium in Studying the Selank Inhibiting Effect on the Enkephalin-Degrading Enzymes of Human Blood Plasma

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Abstract—A method of analysis of enkephalinase activity in blood plasma based on the application of Leu-enkephalin generally labeled with tritium at all its amino acid residues was developed. The method allows the simultaneous estimation of activity of several peptidases in microquantities of tissues. [G-^{3}H]Leu-enkephalin was prepared by the method of solid phase catalytic isotope exchange (120 Ci/mmol) and subjected to proteolysis by the treatment with blood plasma. The resulting radioactive metabolites were separated by HPLC in the presence of the mixture of unlabeled fragments of Leu-enkephalin as internal standards. It was shown that aminopeptidases, dipeptidylaminopeptidases, and dipeptidylcarboxypeptidases respond for approximately 80%, 2%, and 10% of the total enzymatic activity, respectively. The new pathway of degradation of Leu-enkephalin by carboxypeptidase that provides for ~6% of the total enkephalin-degrading activity was discovered. Bestatin was shown to predominantly inhibit aminopeptidases and carboxypeptidases, whereas selank is more specific for carboxypeptidases and dicarboxypeptidases.

Key words: bestatin, biodegradation of peptides, blood serum enkephalins, enkephalins, inhibitors of proteases, selank

INTRODUCTION

Biologically active peptides regulate practically all of the vitally important functions of organism.[2] However, the lifetime of regulatory peptides is limited owing to a high rate of their hydrolysis by proteolytic enzymes (peptidases). For example, the half-life of enkephalins, opioid peptides, in blood is as short as several minutes; and a series of peptidases participate in the biodegradation of these pentapeptides. Inhibition of these enzymes results in an increase in the enkephalin lifetime, which may play an important role in the treatment of diseases that reduce the activity of endogenous opioid system [1].

It was previously demonstrated that selank, a new anxiolytic peptide, [2, 3] affects the anxious level of organism and significantly inhibits the enkephalin-degrading enzymes in human blood plasma [4]. It is possible that one of the mechanisms of its anxiolytic action is associated with this effect [5, 6]. The enkephalin-degrading enzymes can cleave many other biologically active regulatory peptides, and, therefore, the study of activity of these enzymes in norm and pathology and the search for their new inhibitors are an important problem in modern biology and medicine.

One of the new methodological approaches to the solution of this problem is connected with an application of peptides generally labeled by tritium. The incorporation of the label in practically all the amino acid residues enables the preparation of peptides with high specific radioactivities and the simultaneous identification of practically all possible products of their enzymatic hydrolysis. This feature allows the analysis of the activity ratio of the enzymes that cleave the studied peptide using microquantities of biological samples.

In this study, we investigated enkephalinases of human blood plasma with the use of Leu-enkephalin generally labeled by tritium and the effect of selank on these enzymes.

RESULTS AND DISCUSSION

The peptides with Tyr residues labeled by tritium were up to now used as radioactive substrates for studying the enzymatic hydrolysis of enkephalins [7]. As a result, only Tyr-containing fragments of enkephalin

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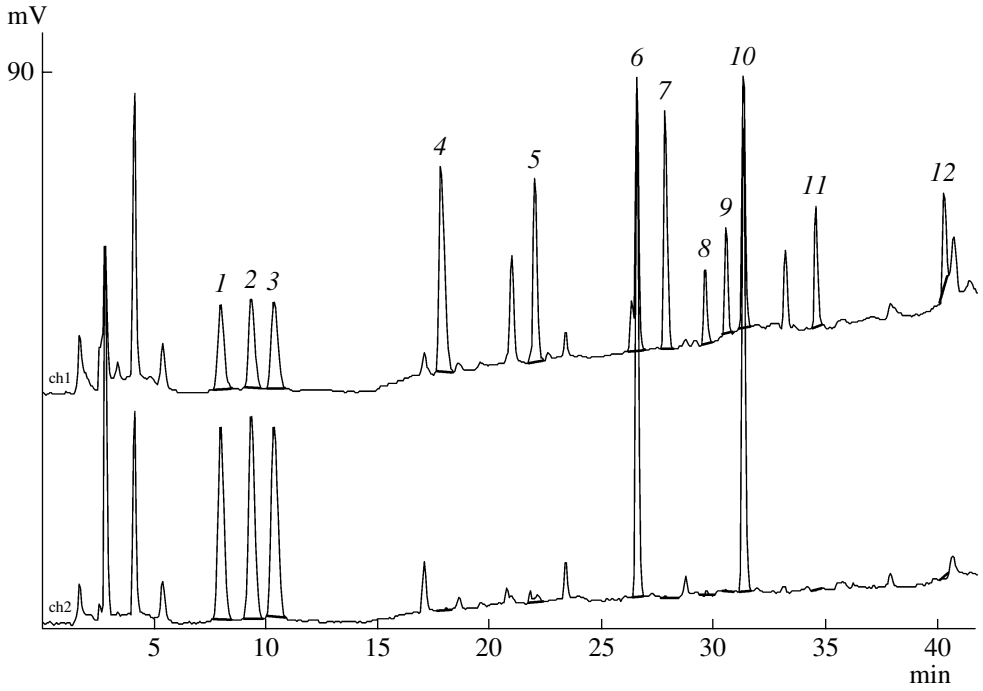
[2] Abbreviations: SPCIE, solid phase catalytic isotope exchange; TFA, trifluoroacetic acid.



The study of the Leu-enkephalin biodegradation in the presence of bestatin (500 μM) in the blood plasma for 30 min. The mixture of peptide fragments of Leu-enkephalin (5 μg of each fragment) and the peptide fraction extracted from the serum sample (1 μl) containing [G-^{3}H]Leu-enkephalin and bestatin were separated by HPLC under the conditions given in the Experimental section. The profiles obtained at 250 and 280 nm are designated as ch1 and ch2, respectively. The following peptides and amino acids were found: *1*, YGG; *2*, Y; *3*, YG; *4*, F; *5*, GF; *6*, YGGF; *7*, FL; *8*, GGFL; *9*, bestatin; *10*, YGGFL; *11*, GGF, and *12*, GFL.

were radioactive among the hydrolysis products. In this study, we used [G-^{3}H]Leu-enkephalin (120 Ci/mmol) generally labeled by tritium, which was obtained by SPCIE [8]. We chose the temperature of 160°C at which the SPCIE reaction proceeds with a high selectivity, and the substitution of protons at the asymmetric carbon atoms was not accompanied by racemization of either amino acids or peptides [9]. The substitution of isotopes for hydrogen atoms proceeds through the one-center mechanism in solid phase, and the configuration of asymmetric atoms is preserved [10]. The SPCIE reaction is successfully used for the introduction of tritium label into compounds of various classes, including proteins, and their intrinsic properties are completely retained [11, 12]. According to NMR, the tritium label was distributed in [G-^{3}H]Leu-enkephalin as follows: 28% in Tyr, 30.5% in Gly^2, 31.8% in Gly^3, 9.1% in Phe^4, and 0.61% in Leu^5 [12]. The incorporation of the label into all the amino acid residues increased the specific radioactivity of the peptide more than threefold in comparison with the Tyr-labeled enkephalin and allowed the study of all possible hydrolysis products of this peptide using a very low amounts of blood plasma (5 μl).

The hydrolysis products of Leu-enkephalin were separated by HPLC at 250 and 280 nm. The choice of wavelengths for the detection was determined by the necessity to enable the reliable identification of the Leu-enkephalin biodegradation products among the components of blood plasma. A typical chromatogram of the mixture of peptide fragments of Leu-enkephalin and the peptide fraction extracted from the plasma sample containing [G-^{3}H]Leu-enkephalin and bestatin is given in the figure. All the products of biodegradation of Leu-enkephalin are quantitatively separated under the chromatographic conditions chosen. This method allows the identification of all the fragments of Leu-enkephalin formed upon the cleavage by separate peptidases with high reliability and the determination of their concentrations on the basis of their molar radioactivity.

The results of examination of the products of Leu-enkephalin hydrolysis by the enzymes of blood sera taken from three healthy donors are presented in Table 1. One can see that, despite some variability of the results, aminopeptidases provide more than 80% of the examined activity (the Tyr and Gly-Gly-Phe-Leu products). Dipeptidylaminopeptidases lead to approximately 2% of the activity, and dipeptidylcarboxypeptidases to less than 10% (the activity was evaluated according to accumulation of the labeled Tyr-Gly and Tyr-Gly-Gly peptides with higher molar radioactivities, respectively). The ratio of activities of these three groups of enzymes of the enkephalin degradation in blood was repeatedly described in literature [7], and our results qualitatively correspond to it. We found no products that can be formed only as a result of simultaneous action of sev-

Table 1. Enkephalin-degrading activity of the enzymes of human blood plasma according to the chromatographic analysis of [G-^{3}H]Leu-enkephalin and its metabolites*

Enzyme	Fragment	Molar radioactivity, Ci/mmol	Concentrations of fragments formed, nM		
			I	II	III
–	YGGFL (substrate)	120	435(53)	359(63)	270(62)
Aminopeptidase	Y	33.6	325	202	173
	GGFL	86.4	302(81)	184(88)	160(86)
Dipeptidylaminopeptidase	YG	70.2	8(2)	2(1)	5(3)
	GFL	49.8	6	1	6
Dipeptidylcarboxypeptidase	YGG	108.4	42(11)	18(9)	8(4)
	FL	11.6	50	20	11
Carboxypeptidase	YGGF	119.3	23(6)	6(3)	12(6)

Note: The results of examination of the blood plasma samples taken from three healthy donors (I, II, and III) are presented. Incubation time was 15 min. The percent content of Leu-enkephalin remaining in the medium (in the row substrate) or the content of fragments expressed as percentage of the amount of Leu-enkephalin subjected to the biodegradation with the formation of the corresponding product are given in parentheses.

Table 2. The effect of bestatin on the activity of enkephalin-degrading enzymes of the human blood plasma according to the chromatographic analysis of [G-^{3}H]Leu-enkephalin and its metabolites*

Enzyme	Fragment	Concentrations of fragments formed, nM			
		control	bestatin, 500 μM		
		30 min	30 min	90 min	120 min
–	YGGFL (substrate)	167(38)	376(90)	357(74)	336(67)
Aminopeptidase	Y	230	8	22	33
	GGFL	216(79)	10(26)	25(20)	40(24)
Dipeptidylaminopeptidase	YG	9(3)	11(28)	35(29)	46(28)
	GFL	7	8	30	40
Dipeptidylcarboxypeptidase	YGG	19(7)	15(39)	49(40)	61(37)
	FL	20	20	50	60
Carboxypeptidase	YGGF	30(11)	3(8)	13(11)	17(10)

Note: The percent content of Leu-enkephalin remaining in the medium (in the row substrate) or the content of fragments expressed as percentage of the amount of Leu-enkephalin subjected to the biodegradation with the formation of the corresponding product are given in the parentheses.

eral enzymes for 15 min (for example: Gly-Gly-Phe, Gly-Phe, and Phe).

About 6% of Tyr-Gly-Gly-Phe was found among the hydrolysis products of [G^3-H]Leu-enkephalin, which suggests that carboxypeptidases have a relatively large contribution into the degradation of this peptide. The exocarboxypeptidase pathway of the hydrolysis of Leu-enkephalin was not identified in blood until now. Our results can be explained by both the advantages of the separation method used in this study and other methodological peculiarities. For example, a number of authors who stated that Leu-enkephalin does not cleaved by carboxypeptidases used EDTA as anticoagulant [7] and, in such a manner, inactivated Ca-dependent enzymes, including carboxypeptidases [13].

The HPLC separation of hydrolysis products of the peptides generally labeled by tritium was also applied to studying the specificity of inhibitors in such a complicated enzymic system as blood plasma (Table 2). One can see from the table that the enkephalin degradation rate decreases more than sixfold in the presence of bestatin taken at a concentration of 500 μM, which is usually used for the most complete inhibition of these enzymes [14]. After 30-min action of the blood plasma enzymic system on Leu-enkephalin in the presence of bestatin, the concentration of products of its cleavage by aminopeptidases and carboxypeptidases decreased

Table 3. The effect of selank on the activity of enkephalin-degrading enzymes of the human blood plasms according to the chromatographic analysis of [G-^{3}H]Leu-enkephalin and its metabolites*

Enzyme	Fragment	Concentrations of fragments formed, nM			
		control	Selank, 15 μM		
		30 min	30 min	90 min	120 min
	YGGFL (substrate)	167(38)	297(72)	154(40)	146(33)
Aminopeptidase	Y	230	117	223	233
	GGFL	216(79)	110(95)	220(94)	228(93)
Dipeptidylaminopeptidase	YG	9(3)	4(4)	11(5)	14(5)
	GFL	7	3	8	10
Dipeptidylcarboxypeptidase	YGG	19(7)	1(1)	3(1)	5(2)
	FL	20	2	4	7
Carboxypeptidase	YGGF	30(11)	0(0)	0(0)	0(0)

* The percent content of Leu-enkephalin remaining in the medium (in the row substrate) or the content of fragments expressed as percentage of the amount of Leu-enkephalin subjected to the biodegradation with the formation of the corresponding product are given in parentheses.

20-fold and tenfold, respectively. At the same time, bestatin exerts no influence on the activity of dipeptidylaminopeptidase and dipeptidylcarboxypeptidase. In blood plasma, Leu-enkephalin is mainly hydrolyzed by dipeptidylpeptidases rather than by aminopeptidases in the presence of 500 μM bestatin. These results agree well with the fact that bestatin is an effective inhibitor of aminopeptidases and does not affect dipeptidylpeptidases [15]. It was also shown that bestatin can significantly inhibit the observed relatively low activity of carboxypeptidases of blood plasma toward Leu-enkephalin.

Table 3 illustrates the inhibiting effect of selank (a peptide anxiolytic Thr-Lys-Pro-Arg-Pro-Gly-Pro) on the main groups of enkephalin-degrading enzymes in blood plasma. One can see that selank completely inhibits carboxypeptidases, almost 20-fold decreases the activity of dipeptidylcarboxypeptidases, and only twofold reduces the accumulation of the products of *N*-terminal hydrolysis of Leu-enkephalin Tyr and Tyr-Gly at the concentration of 15 μM corresponding to its IC_{50} in this polyenzymic system [4]. Consequently, the inhibiting effect of selank toward aminopeptidases is one order of magnitude lower than that toward carboxypeptidases. In other words, selank can be considered as a relatively selective inhibitor of carboxy- and dipeptidylcarboxypeptidases of blood plasma that cleave Leu-enkephalin. In the presence of 15 μM selank, the Leu-enkephalin biodegradation by blood plasma proceeds more selectively than without it by the pathway associated with the action of aminopeptidases.

This study and the determination of the selank IC_{50} (15 μM) relative the total activity of enzymes hydrolyzing Leu-enkephalin [4] were carried out using the tenfold diluted blood plasma. The calculated IC_{50} of selank is expected to be one order of magnitude higher in the whole blood. On the other hand, an estimation indicates that the concentration of this peptide in blood is no higher than 1 μM when it is injected to animals intraperitoneally at doses of 100–400 μg/kg; these doses are known to exert the anxiolytic effect [3, 6]. This concentration is obviously insufficient for the inhibition of aminopeptidases that provide 80% of total enkephalinase activity. However, the activity of carboxypeptidases and dipeptidylcarboxypeptidases in the blood plasma are one order of magnitude lower (Table 1) and the degree of their inhibition by selank is one order of magnitude higher than that of aminopeptidases (Table 3). Therefore, we can presume that, upon the selank pharmacological action, its concentration in blood is quite sufficient for the inhibition of carboxypeptidases and dipeptidylcarboxypeptidases.

Angiotensin-converting enzyme and neutral endopeptidase belong to the aforementioned enzymes. In addition to enkephalin, they can hydrolyze a number of other regulatory peptides. For example, these enzymes catalyze the conversion of angiotensin I into angiotensin II, cleave substance P, neurotensin, natriuretic peptide, bradykinin, etc. [16]. Accordingly, the mechanism of biological action of selank can be associated not only with its effect on the opioid system, but also on a number of other systems of regulatory peptides. There is a reason to search for new areas for selank application, such as regulation of blood pressure and other functions of cardiovascular system and the correction of processes associated with memory and learning. The corresponding peptides generally labeled by tritium can also be used in such studies.

In this work, we used the Leu-enkephalin generally labeled with tritium for the discovery of a previously unknown way of cleavage of this peptide by carboxypeptidases, the activity of which is approximately 6%

of the total enkephalin-degrading activity of the enzymes of human blood plasma. Moreover, selank was shown to exhibit the effect toward carboxypeptidases and dipeptidylcarboxypeptidases one order of magnitude more pronounced in comparison with that toward aminopeptidases and dipeptidylaminopeptidases.

Our procedure provides for the content in the chromatographic fraction of more than 95% of radioactivity of the starting [G-^{3}H]Leu-enkephalin at zero time of its interaction with the blood plasma with regard to its dilution. The chromatographic profiles of the starting plasma sample to which the model mixture of the unlabeled peptides was added, the same samples in the presence of [G-^{3}H]Leu-enkephalin obtained at various biodegradation times, and the samples obtained in the presence of selank (15 μl) completely coincided at UV detection. They differ only in the distribution of radioactivity in chromatographic fractions of the corresponding fragments. In the experiments carried out in the presence of bestatin (500 μM), the peak of quantitatively extracted bestatin can also be observed in the chromatogram simultaneously with the products of the [G-^{3}H]Leu-enkephalin biodegradation. Thus, our procedure based on the use of peptides generally labeled with tritium allows one to study the biodegradation of physiologically active peptides under the action of several enzymatic systems on microsamples of tissues of any organism with a high sensitivity.

EXPERIMENTAL

The following instruments and devices were used in this study: an Ultraspec 4050 spectrophotometer (LKB, Sweden), a Varian UNITY-600 NMR spectrometer (United States), an Model 116 UV detector for HPLC (Gilson, France), an OBT-1 semiindustrial installation for the substitution of tritium for hydrogen (Russia), a Σ960 plate spectrophotometer (Metertech, Taiwan), an RZhS-20 scintillation counter (Russia), a 5414 centrifuge (Eppendorf, Germany), K-23D and K-70D centrifuges (MLM, Germany), an UH 4 liquid thermostat (MLM, Germany), a PBO-64 vacuum rotary evaporator (Microtechna, Czech Republic), and a Chinisto Alfa-5 Lyophilic Drier (Medizinischer Apparatebau, Germany). The following HPLC columns were also used: a Kromasil C18 column (10 × 250 mm, Elsiko, Russia) and a Nucleosil C18 column (4 × 250 column, Macherey-Nagel, Germany).

The heptapeptide selank and Leu-enkephalin and its metabolites were prepared in the Institute of Molecular Genetics, Russian Academy of Sciences.

The blood plasma was obtained from five healthy donors with the use of heparin (20 U per ml of venous blood) as anticoagulant. The blood was centrifuged at 1000 *g* for 10 min at 4°C. The plasma samples were frozen and stored at –20°C.

Solid phase isotope exchange of tritium for hydrogen in Leu-enkephalin. Aluminum oxide (100 mg, Alusorb A 075, Chemapol) was mixed with Leu-enkephalin (5.0 mg, Bachem) in an aqueous solution (1 ml). Water was removed in a vacuum at 20°C. Aluminum oxide with the applied peptide was mixed with Rh/Al_2O_3 catalyst (10 mg, Fluka). This solid mixture containing Leu-enkephalin (0.5 mg) was placed in an ampoule of 10 ml volume. The ampoule was evacuated, filled with gaseous tritium up to the pressure of 250 torr, and kept for 20 min at 160°C. The ampoule was cooled, evacuated, and hydrogen was passed. The peptide was desorbed with 20% aqueous ethanol and twice solved in 20% aqueous ethanol and evaporated in a vacuum in order to remove easily exchanging tritium. The resulting [G-^{3}H]Leu-enkephalin was purified by successive HPLC on a Kromasil C18 and a Nucleosil C18 column; this procedure allowed the removal of the autolysis products and diastereomers. The quantity of peptide was determined according to the data of UV-detection, and its molar radioactivity from the results of liquid radioactivity counting. The total radioactivity of [G-^{3}H]Leu-enkephalin was 25 mCi and its molar radioactivity 120 Ci/mmol. The homogeneity of the labeled peptide was confirmed by TLC on Silufol plates in 2 : 2.4 : 1 : 1 2-butanone–*tert*-butanol–ammonia–water mixture. The preservation of the native peptide structure was confirmed by ^{1}H NMR, and the tritium distribution in it was determined by ^{3}H NMR.

The enkephalinase activity was determined according to the accumulation rate of the products of enzymatic degradation of [G-^{3}H]Leu-enkephalin by the method [17]. The incubation mixture had the final volume of 250 ml and contained [G-^{3}H]Leu-enkephalin with the total and specific radioactivities of 15 μCi and 120 Ci/mmol, respectively, the tenfold diluted blood plasma, 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 500 nM [G-^{3}H]Leu-enkephalin, and the studied inhibitors at the concentrations listed in Tables 1 and 2. The incubation was carried out at 37°C, aliquots of 50 μl each were taken, and the biodegradation was stopped by the addition of 0.2 M HCl (5 μl). The samples were diluted by acetonitrile (200 μl) and cooled to –20°C.

Peptides were extracted from the biological samples with organic solvents and purified by HPLC for the determination of [G-^{3}H]Leu-enkephalin and its peptide fragments. The mixture containing 80% acetonitrile was centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was taken and dried on a rotary evaporator at a reduced pressure. The residue was dissolved in two portions of methanol (500 μl each), placed in a plastic centrifuge tube of 1.5 ml volume, and centrifuged under the conditions described above. The supernatant was dried on a rotary evaporator at a reduced pressure. The dry residue was dissolved in 0.1% TFA (100 μl).

Chromatographic analysis of the products of [G-^{3}H]Leu-enkephalin biodegradation. The mixture

of peptide fragments and the products of [G-^{3}H]Leu-enkephalin biodegradation was separated by HPLC on a Kromasil C18 column at 20°C. A Beckman 165 spectrophotometer (Altex, United States) was used for the simultaneous detection at 250 and 280 nm. The resulting samples of the peptide extracts from the blood plasma (20 μl each) were mixed with the solution (30 μl) of Leu-enkephalin and its metabolites (5 μg of the each peptide), applied onto the HPLC column, and eluted with a gradient of 80% acetonitrile in 0.1% TFA (10 min at 4% of 80% acetonitrile and, then, from 4 to 58% within 40 min) at a flow rate of 1 ml/min (figure). The YGGFL and its fragments were isolated. The radioactivity of every fraction was measured by the liquid scintillation counter. The molar radioactivity of the peptide fragments of Leu-enkephalin (Table 1) was calculated on the basis of ^{3}H NMR data of distribution of tritium label in the peptide [^{3}H]YGGFL [12]. The concentrations of [G-^{3}H]Leu-enkephalin and its fragments in the plasma were determined on the basis of radioactivity of the peptide fraction of the corresponding metabolite and its molar radioactivity.

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