ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2011, Vol. 37, No. 4, pp. 421–427. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © K.V. Shevchenko, T.V. V'yunova, I.Yu. Nagaev, L.A. Andreeva, L.Yu. Alfeeva, N.F. Myasoedov, 2011, published in Bioorganicheskaya Khimiya, 2011, Vol. 37, No. 4, pp. 475–482.

Studies of the Aminopeptidase Proteolysis of Semax Analogues with Different *N*-Terminal Amino Acid Residues

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Received August 31, 2010; in final form, October 22, 2010

Abstract—Proteolysis of semax (Met-Glu-His-Phe-Pro-Gly-Pro, Sem) and its analogues with the substitution of Ala, Gly, Thr, or Trp for the *N*-terminal Met was studied. This substitution was shown to change the degradation rate of these peptides by leucine aminopeptidase (EC 3.4.11.2, Sigma, Type VI, 9.2 activity units/mg). [Ala[1]]Sem, [Gly[1]]Sem, and [Thr[1]]Sem (the semax analogues) proved to be more stable to the proteolysis than semax itself. It was demonstrated that the primary product of the proteolysis was His-Phe-Pro-Gly-Pro (Sem-5). In the case of [Trp[1]]Sem, the comparable amount of Glu-His-Phe-Pro-Gly-Pro (Sem-6) was found to be formed along with Sem-5. In was established that all the studied semax analogues could be used as inhibitors of its proteolysis.

Keywords: semax, aminopeptidase, rat brain membranes, semax analogues, peptide degradation

DOI: 10.1134/S1068162011040133

INTRODUCTION

Biodegradation of semax in vivo is known to result in the formation of a number of its shortened analogues that are often no less biologically active than the starting peptide [1]. It has been found that the main products of the Sem degradations in the rat brain are peptides that are formed by the cleavage by aminopeptidases. The number of Sem metabolites in the brain is continuously increased with time [1]. Moreover, it was reported [2] that sites of specific binding of some of these peptides (Sem-5 and Sem-3) are present in rat brain membranes. These peptides were found to be specifically bond in different regions of the rat brain [2]. Semax and Sem-5 were found on the membranes of hippocampus and cerebellum, whereas Sem-3 was localized on membranes of basal nuclei. Therefore, semax is a source of unique regulatory complex of the peptides.

Neurotropic activity of a number of the Sem analogues with the substitution of Gly, Ala, Thr, or Trp for Met was studied and their neurotropic effects were compared with those of semax in the paper [3]. The influence of [Trp[1]]Sem on NFR in an experiment of rat learning with the negative reinforcement was found to be the same as that in the control group of the Sem-treated rats. At the same time, the rest of the Sem analogues ([Gly[1]]Sem, [Ala[1]]Sem, and [Thr[1]]Sem) caused no significant changes in rat behavior in comparison with the control group of rats. NFR for these peptides was significantly lower than that for the group that was treated with semax. Thus, the replacement of Met1 by Ala, Gly, Thr, or Trp in semax limited spectrum of the nootropic action or resulted in a complete loss of the nootropic effect of the peptides. The authors of paper [3] proposed that modification of the *N*-terminal amino acid residue in the Sem molecule changed not only the rate but the pathway of proteolytic degradation of the starting peptide as well. As a result, these peptides formed a different set of metabolic products which had no neurotropic activity. Thus, kinetic studies of degradation of semax and its analogues by different aminopeptidases in vitro are important for understanding of cerebral processes in vivo.

The goal of this study was investigation of the degradation of semax and its analogues in the presence of aminopeptidase M (its former name was leucine aminopeptidase, EC 3.4.11.2, Type VI) and under the action of membrane enzymes of the rat brain in vitro.

RESULTS AND DISCUSSION

The microsomal aminopeptidase M (EC 3.4.11.2, Leucine Aminopeptidase Microsomal, Type VI; Sigma, United States) was used as an aminopeptidase for the studies of proteolysis of semax and its analogues. Experiments were performed in a phosphate-

Abbreviations: AU, activity unit; MERB, membrane enzymes of rat brain; NFR, the number of fulfilled reactions; Sem, semax; Sem-6, Glu-His-Phe-Pro-Gly-Pro; Sem-5, His-Phe-Pro-Gly-Pro; Sem-4, Phe-Pro-Gly-Pro; Sem-3, Pro-Gly-Pro; PBS, phosphate-salt buffer.

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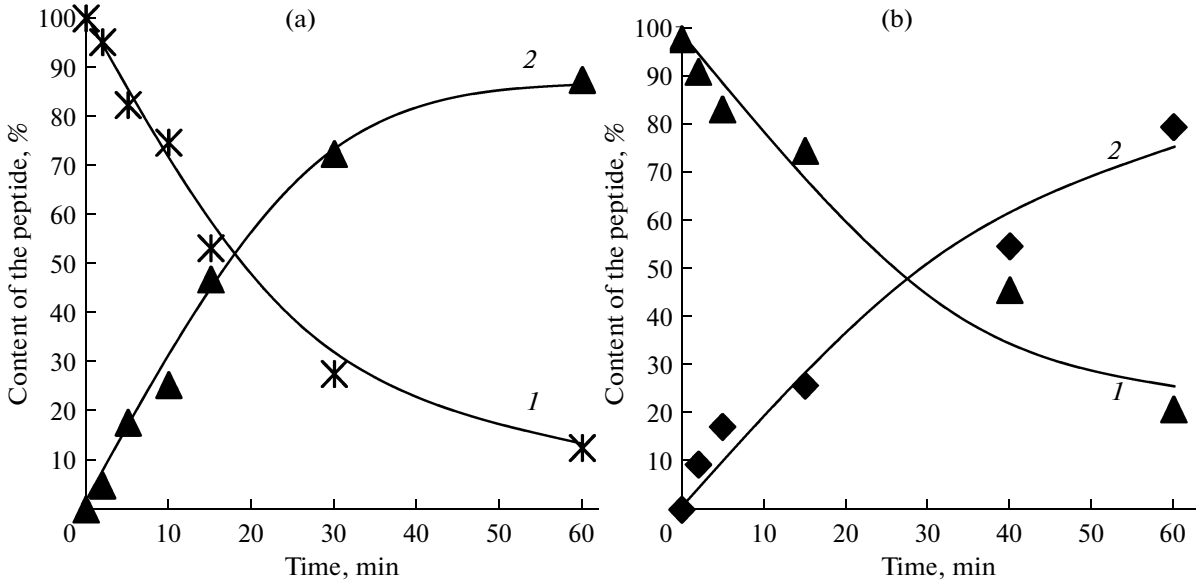

Fig. 1. Kinetics of semax degradation (a): (*1*), Sem and (*2*), Sem-5; (b): (*1*), Sem-5 and (*2*), Sem-4 by aminopeptidase M.

salt buffer (PBS, pH 7.4). The proteolytic conditions were chosen during the Sem degradation at different ratios of the substrate and the enzyme. The optimum ratio for these studies proved to be 10.3 μmol of the peptide per 1 AU of the peptidase. Under these conditions, semax was cleaved by more than 90% within 60 min.

The rate of the Sem cleavage by aminopeptidase M was analyzed in the Lineweaver–Burk coordinates at different concentrations of the substrate. The maximum rate of the Sem proteolysis (V_{max} = 58 μM/min) and the Michaelis constant (K_m = 200 μM) were calculated from the Michaelis–Menten equation on the basis of the obtained data.

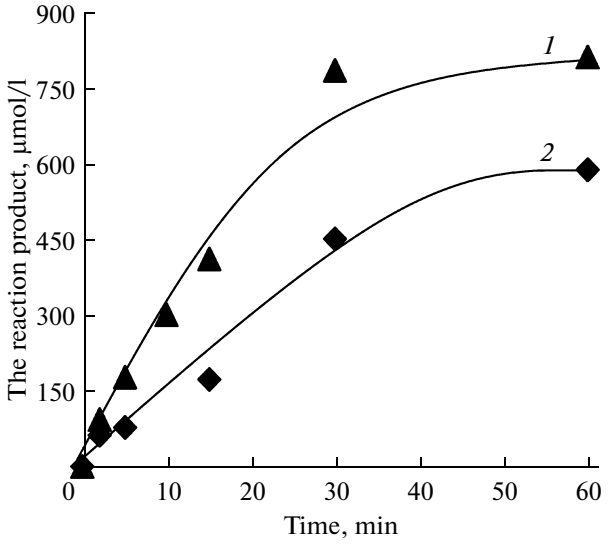

Fig. 2. Kinetics of the formation of (*1*) Sem-5 from semax and (*2*) Sem-4 from Sem-5 by the action of aminopeptidase M.

Analysis of the composition of proteolytic metabolites demonstrated that semax was cleaved to only Sem-5 and the *N*-terminal Met-Glu dipeptide at all of the used substrate–enzyme ratios (Fig. 1a).

The kinetics of degradation of Sem-5 by aminopeptidase M was studied in order to determine possibility of cleavage of Sem-5 by this enzyme (Fig. 1b). As follows from Fig. 1b, Sem-5 is rapidly cleaved in the absence of semax, but the rate of conversion of semax into Sem-5 is approximately 2.4 times higher than that of Sem-5 into Sem-4 (Fig. 2). Therefore, proteolysis of the pentapeptide is probably impossible if both Sem-7 and Sem-5 are present in the reaction mixture. This fact is explained by two reasons: competition of Sem-7 and Sem-5 for active sites of the enzyme and the higher rate of conversion of Sem-7 into Sem-5 in comparison with that of Sem-5 into Sem-4.

The similar experiments with Sem-4 demonstrated that there is no further degradation of Sem-4 in the presence of this enzyme.

In order to investigate the influence of the Met-Glu dipeptide and free amino acids (methionine, alanine, glycine, threonine, and tryptophan), the dipeptide or one of the aforementioned amino acids were added to the reaction mixture in the molar ratios of 1 : 1, 10 : 1, and 100 : 1 relative to the semax quantity. All the above substances were found to have no effect on the semax degradation.

Kinetics of the degradation of [Ala^1]Sem, [Gly^1]Sem, [Thr^1]Sem, and [Trp^1]Sem by aminopeptidase M was studied for evaluation of the effect of modification of the *N*-terminal amino acid residue of semax on proteolysis of these peptides. The pro-

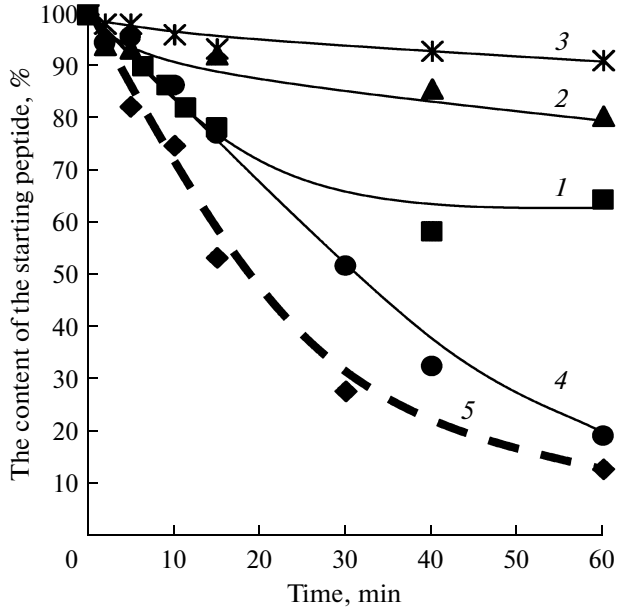

Fig. 3. Kinetics of the degradation of (*1*) [Ala^1]Sem, (*2*) [Gly^1]Sem, (*3*) [Thr^1]Sem, (*4*) [Trp^1]Sem, and (*5*) semax by aminopeptidase M.

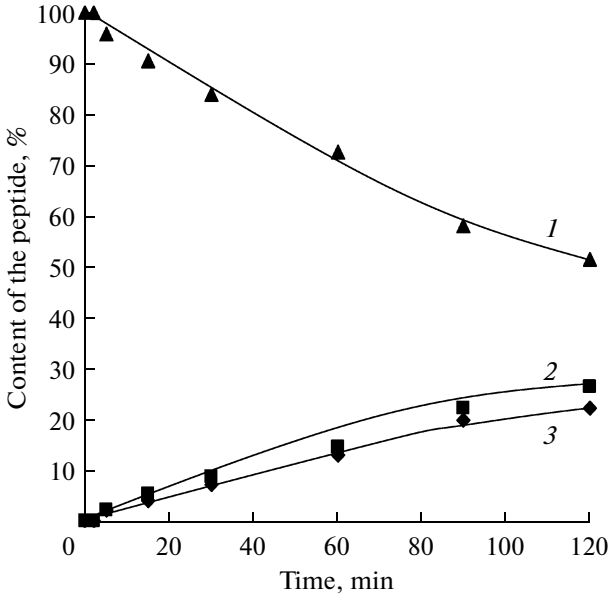

Fig. 4. Kinetics of the degradation of [Trp^1]Sem by aminopeptidase M; (*1*) [Trp^1]Sem, (*2*) Sem-6, and (*3*) Sem-5.

teolytic reaction was performed under the same conditions as those of the semax proteolysis (Fig. 3).

As one can see from Fig. 3, [Ala^1]Sem, [Gly^1]Sem, [Thr^1]Sem, and [Trp^1]Sem are less effectively cleaved by aminopeptidase M than semax itself under the same conditions. Thus, our data confirm the proposal [3] that the change in biological activity of the Sem analogues after modification of its *N*-terminal part can be associated with the change in the rate of proteolysis of the modified peptides. Therefore, we can propose several explanations of preservation of the nootropic activity of [Trp^1]Sem. First, the rate of its proteolysis is higher than that of the rest of the Sem analogues, and the completeness of its hydrolysis within 60 min differs by less than 10% from that of semax. Second, a comparable amount of Sem-6, which is known to exhibit high nootropic activity [4], is formed along with Sem-5 in the course of proteolysis of [Trp^1]Sem distinct from the other semax analogues. Sem-5 and Sem-6 arise in the ratio of 1 : 1 5 min after the beginning of incubation of [Trp^1]Sem (see Fig. 4). The content of Sem-6 in the reaction mixture proved to be more than 25% in 120 min.

The low biological activity of the aforementioned semax analogues and their stability to aminopeptidase M (EC 3.4.11.2) give an idea of using these peptides as inhibitors of the semax proteolysis. This assumption was checked in the studies of the semax stability towards aminopeptidase M in the presence of its analogues in the incubation medium (Table 1).

As one can see from Table 1, the semax stability to aminopeptidase M increases in the presence of the Sem analogues. This observation is particularly evident after prolonged incubation with amino peptidase M. Incubation of semax for 60 min in the presence of its analogues ([Ala^1]Sem, [Gly^1]Sem, [Thr^1]Sem, and [Trp^1]Sem) at the Sem/analogue ratio of 1 : 5 resulted in the 6, 6.4, 4.8, and 7.5 increase in the semax stability, respectively.

Table 1. Influence of the semax analogues on the proteolysis of semax by leucime aminopeptidase

Added analogue*	Sem/analogue, mol/mol	Reaction time, min			
		15	30	60	120
–	–	69.80	54.50	12.60	3.50
[Ala^1]Sem	1 : 0.5	55.93	48.37	29.40	1.70
	1 : 3			53.30	
	1 : 5			75.72	
[Gly^1]Sem	1 : 0.5	89.60	68.81	31.50	2.80
	1 : 3			57.58	
	1 : 5			80.34	
[Thr^1]Sem	1 : 0.5	83.21	75.00	25.90	1.00
	1 : 3			55.12	
	1 : 5			59.88	
[Trp^1]Sem	1 : 0.5	86.6	82.0	71.2	58.4
	1 : 3	96.0	93.6	88.4	74.3
	1 : 5	98.9	97.9	94.6	85.7

* The reaction conditions were: 10.3 μmol of Sem per one AU of aminopeptidase M, 30°C. The residual content of semax in the incubation mixture was given in % of the starting amount.

Table 2. Content of Sem-6 in the reaction mixture of proteolysis of [Thr[1]]Sem by aminopeptidase M* depending on the content of the added semax

Sem : [Thr[1]]Sem, mol/mol	Reaction time, min				
	15	30	60	90	120
0 : 1	15.8	30.3	53.9	71.1	100.0
1 : 0.5	0.0	0.0	5.3	5.3	9.2
1 : 1	0.0	2.6	7.9	11.8	17.1
1 : 3	0.0	7.9	15.6	22.4	31.6
1 : 5	3.9	9.2	15.8	23.7	36.8

* The reaction conditions were: 10.3 μmol of [Trp[1]]Sem per one AU of aminopeptidase M, 30°C. The content of Sem-6 in the incubation mixture was given in percent of its amount that was formed under the analogous conditions without the addition of semax.

Hence, the maximum effect is observed, as expected, in the case of [Trp[1]]Sem and decreased in the row [Gly[1]]Sem > [Ala[1]]Sem > [Thr[1]]Sem. It is interesting that the inhibition of the Sem proteolysis by [Gly[1]]Sem, [Ala[1]]Sem, and [Thr[1]]Sem at the ratio of 1 : 0.5 is completely disappeared after 120-min incubation. At the same time, the inhibiting effect of [Trp[1]]Sem on the Sem proteolysis is preserved after 120 min (the semax stability is increased by more than one order).

We can investigate the change in stability of [Trp[1]]Sem in the presence of semax, because Sem-6 is formed only from [Trp[1]]Sem during the incubation of the mixture of semax and [Trp[1]]Sem (Table 2, Fig. 5).

As follows from our data, the formation of Sem-6 decreased by 2.7 times in comparison with the experiment without semax, even if the [Trp[1]]Sem/Sem ratio was as high as 5 : 1. If the semax content in the incubation medium is two times higher, the formation of Sem-6 decreases by approximately elevenfold. We conclude on the basis of these results that affinity of semax to the active site of the enzyme is much higher than that of [Trp[1]]Sem.

Proteolysis of semax and its analogues was studied in the presence of MERB in vitro. We choose the MERB concentration (0.195 μmol of semax per 1 mg of the protein) so that semax in the analogous concentrations was cleaved within 60 min approximately in the same degree as with the use of aminopeptidase M.

Interaction of semax with MERB for 15 min resulted in its 45% degradation distinct from the in vivo experiments [5] in which the Sem concentration in the rat brain decreased fivefold during the same time period (Fig. 6). Thus, the Sem proteolysis in the presence of MERB was more slow in comparison with that in vivo, but faster in comparison with its cleavage by aminopeptidase M (Fig. 3).

Sem-5 is mainly generated during the interaction of semax with MERB, and total concentration of other products of the degradation begins to predominate over the Sem-5 concentration only after one hour (Fig. 6). The main part of semax was found to convert into Sem-5 within approximately 40 min, whereas concentrations of Sem-3 and Sem-4, at that time, proved to be about 11 and 4%, respectively. After the next 20 min, the content of Sem-5 decreased to 43%, and that of Sem-3 and Sem-4 achieved 32 and 18%, respectively; i.e., the rate of Sem-5 degradation increased.

We proposed the following explanation for this effect. The shorter peptides are evidently formed from Sem-5 under the action of the MERB enzymes, similarly to the reaction with aminopeptidase M. MERB is a complex aggregate of membrane-bound enzymes. Sem-5 and semax can differ in their influence on its functioning. Sem-5 that is formed during the incubation reacts with MERB and somehow corrects functioning of this enzymatic complex. As a result, the pro-

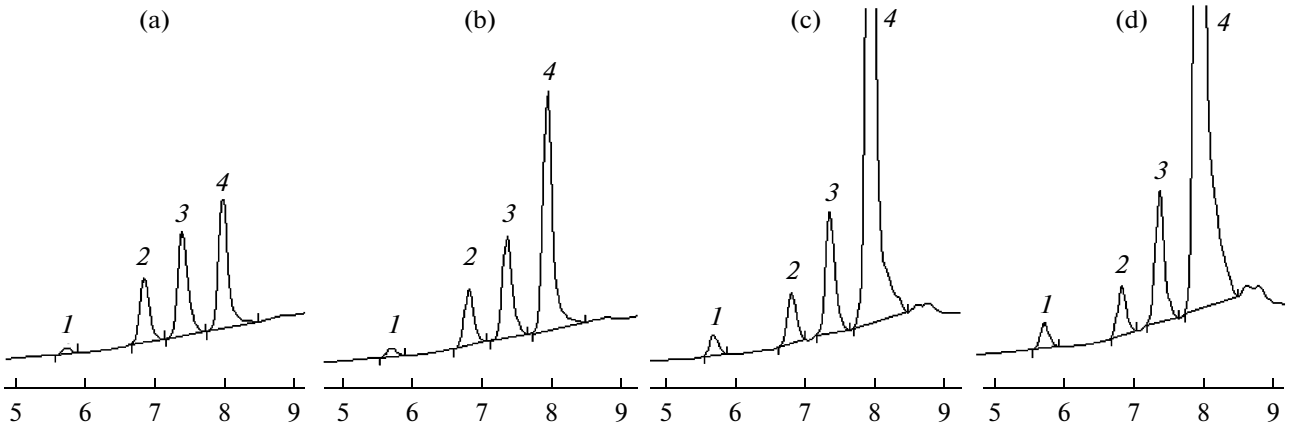

Fig. 5. HPLC of the reaction mixture of proteolysis of the mixtures of Sem and [Trp[1]]Sem in the following ratios: (a) 1 : 0.5, (b) 1 : 1, (c) 1 : 3, (d) 1 : 5, by aminopeptidase M 60 min after the start of the reaction. Peaks *1*, *2*, *3*, and *4* corresponded to Sem-6, Sem-5, Sem, and [Trp[1]]Sem, respectively.

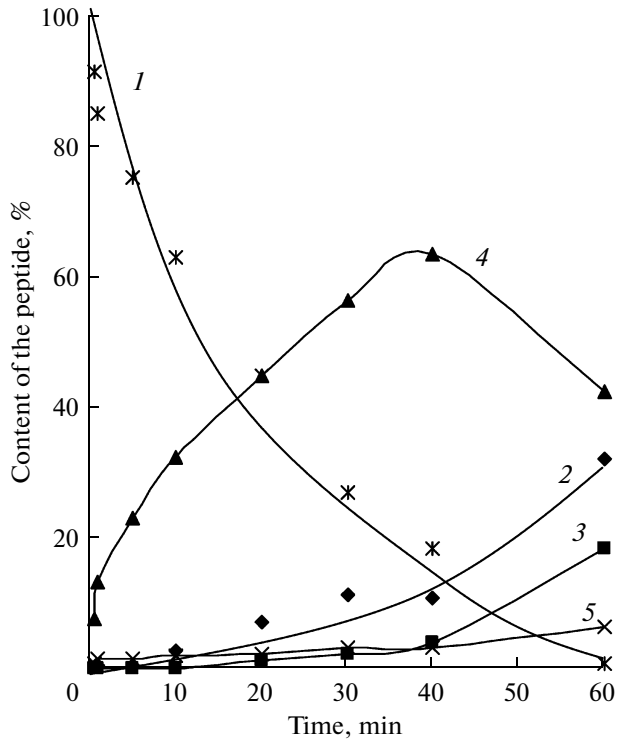

Fig. 6. Kinetics of (*1*) the Sem degradation in the presence of MERB and accumulation of the following products of this reaction: (*2*) Sem-3, (*3*) Sem-4, (*4*) Sem-5, and (*5*) Sem-6.

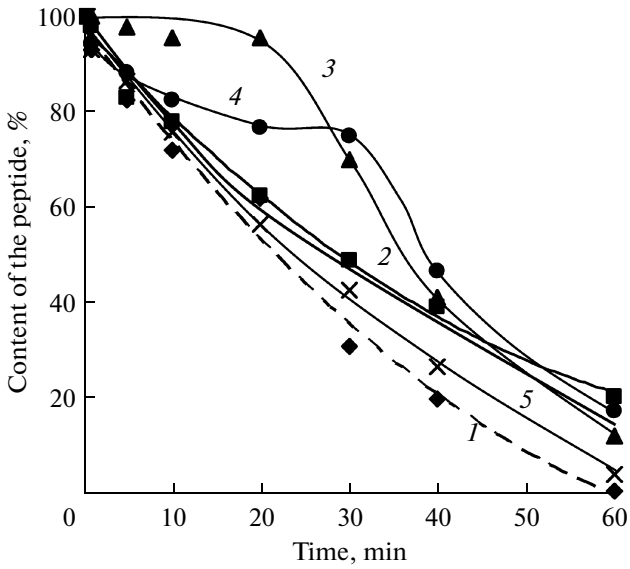

Fig. 7. Kinetics of proteolysis of (*1*) semax and its analogues: (*2*) [Ala^1]Sem, (*3*) [Gly^1]Sem, (*4*) [Thr^1]Sem, and (*5*) [Trp^1]Sem in the presence of MERB.

teolysis of Sem-5 is accelerated, and we can propose that the substrate nature strongly affects functioning of the MERB enzymatic complex.

Thus, the main product of the semax proteolysis by both aminopeptidase M and MERB is Sem-5. However, MERB is a more complex enzymatic aggregate, and its enzymatic activity can be significantly changed with variations in the composition of the incubation medium. If this proposal is true, the semax analogues will differently interact with MERB, and the kinetics of proteolysis of each analogue will be distinguished from that of proteolysis by the aminopeptidase M in a different degree. In other words, the similar effects will possibly be observed as a result of changes in the content of [Gly^1]Sem, [Ala^1]Sem, [Trp^1]Sem, and [Thr^1]Sem in the course of the incubation with MERB, as it took place during the formation of Sem-5 from semax under the analogous conditions (Fig. 2b).

The experiments demonstrated that:

(1) The proteolysis rate of [Gly^1]Sem, [Ala^1]Sem, [Trp^1]Sem, and [Thr^1]Sem by MERB proved to be higher than that by the leucine aminopeptidase M, as in the case of semax itself (Fig. 3, 7).

(2) [Gly^1]Sem and [Thr^1]Sem were most slowly cleaved by both MERB and aminopeptidase M, whereas [Ala^1]Sem was hydrolyzed more effectively, and proteolysis of [Trp^1]Sem was comparable with that of semax.

(3) The pronounced kinetic effects were observed in the case of [Gly^1]Sem and [Thr^1]Sem. The curve of time dependence of the peptide content at the beginning of the reaction (the first 20–30 min) looked like that for the proteolysis of these peptides by aminopeptidase M. Then, the rate of proteolysis of these peptides by MERB considerably increased.

(4) The similar time dependence of the proteolysis rate could also be observed for the other aforementioned peptides, but the second stage of the process was ensured several minutes after the beginning of the reaction, and the picture was indistinct.

Thus, these data confirm our proposal that the substrate nature strongly affects functioning of the MERB enzymatic complex.

Analysis of the products of proteolysis of semax and its analogues demonstrated that the *N*-terminal proteolysis is characteristic for most of the peptides (Fig. 8).

The beginning of the proteolysis for the majority of the Sem analogues is determined by the activity of diaminopeptidases (and monoaminopeptidases in the case of [Trp^1]Sem). The shorter peptides are probably formed from Sem-5 by the action of diaminopeptidases (Sem-3) or by the histidine cleavage (Sem-4). In distinction of the in vivo results [1], the amount of Sem-3 is always significantly lower than that of Sem-4 when the peptides interact with MERB. In some cases ([Gly^1]Sem and [Thr^1]Sem), no Sem-3 is even formed.

Therefore, [Gly^1]Sem, [Ala^1]Sem, [Trp^1]Sem, and [Thr^1]Sem were shown to be more stable to the proteolysis by aminopeptidases in comparison with semax itself. Substitution for the *N*-terminal residue of semax could influence the rate of the peptide degradation by

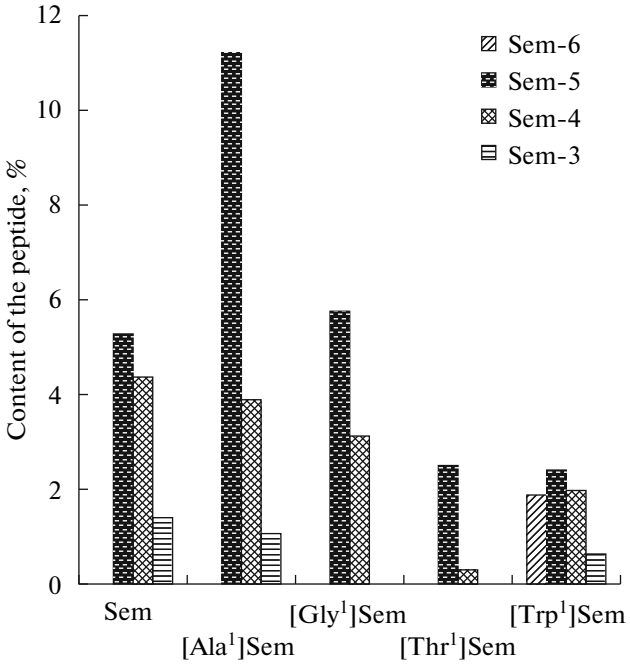

Fig. 8. The content of the products of *N*-terminal proteolysis (for 10 min) of semax and its analogues that were formed in the reaction mixture in the presence of MERB. The values were given in % of the total amount of the compounds.

aminopeptidases. Moreover, the rate of semax proteolysis was shown to decrease after the addition of its analogues to the incubation mixture; i.e., the competitive inhibition of the Sem proteolysis by Sem analogues was observed.

Different peptides were shown to differently affect the activity of the MERB enzymatic complex. The different activity of the enzymatic complex of cellular membranes in vivo during its interaction with semax or its analogues could probably be one of the reasons for the decrease in the spectrum of nootropic action (up to its complete loss) of the semax analogues with modified *N*-terminal amino acid residue [3].

EXPERIMENTAL

Aminopeptidase M (its previous name was leucine aminopeptidase, EC 3.4.11.2, Sigma, Type VI, microsomal, from pig kidney, 9.2 AU/mg) and necessary commercial products were used in this study. MERB (8.6 mg of the protein/ml) were obtained from the whole brain of the Wistar rats (males with the body weight of 200 g). H-Glu(OBu^t)-His-Phe-Pro-Gly-Pro-OH was synthesized in the Institute of Molecular Genetics of the Russian Academy of Sciences [6].

The reaction mixtures were analyzed by HPLC on a Milikhrom A-02 chromatograph equipped with a ProntoSIL-120-5-C_{18} AQ DB-2003 column (2 × 75 mm, the particle size of 5 μm) in a methanol gradient (from 5 to 100%) in the buffer (0.2 M $LiClO_4$ + 0.005 M $HClO_4$, pH 2.24) within 12 min at 35°C. The peptides were detected at 210 nm.

Semax, [Gly^1]Sem, [Ala^1]Sem, [Trp^1]Sem, and [Thr^1]Sem were synthesized by the method described in [7].

The prepared compounds were analyzed by TLC on silica gel plates (Silufol, Czech Republic) and detected by spraying the plates by the ninhydrin solution. Chromatographic mobility (R_f) was determined in the following chromatographic systems: (A) butanol–acetic acid–water (4 : 1 : 1), (B) butanol–acetic acid–pyridine–water (30 : 6 : 20 : 24), (C) chloroform–methanol–ammonium hydrate (6 : 4 : 1), (D) chloroform–methanol–ammonium hydrate (4 : 4.5 : 1.5), and (E) butanol–acetic acid–pyridine–water (30 : 6 : 20 : 24).

Characteristics of the prepared peptides:

HCl·Met-Glu-His-Phe-Pro-Gly-Pro-OH: *M* 848.5; mp 115–117°C (decomposition); $[\alpha]_D^{22}$ –65.5° (*c* 1, CH_3OH), R_f 0.08 (A), 0.42 (B), 0.38 (C);

HCl · Ala-Glu-His-Phe-Pro-Gly-Pro-OH: *M* 788.5; mp113–115°C, $[\alpha]_D^{20}$ – 64.55° (*c* 1, methanol); R_f 0.234 (C), 0.484 (D), 0.162 (E);

HCl·Gly-Glu-His-Phe-Pro-Gly-Pro-OH: *M* 774.5; mp 110–111°C; $[\alpha]_D^{20}$ –69.66° (*c* 1, methanol); R_f 0.172 (C), 0.438 (D); 0.163 (E);

HCl·Thr-Glu-His-Phe-Pro-Gly-Pro-OH: *M* 818.5; mp 121–123°C, $[\alpha]_D^{20}$ –62.28° (*c* 1, methanol), R_f 0.313 (C), 0.860 (D), 0.175 (E);

HCl·Trp-Glu-His-Phe-Pro-Gly-Pro-OH: *M* 903.5; mp 119–122°C, $[\alpha]_D^{20}$ –61.45° (*c* 1, methanol), R_f 0.305 (C), 0.785 (D), 0165 (E).

Preparation of the membrane fraction of the rat brain. All the operations of the membrane isolation were performed at 4°C. Adult male rats of the Wistar line were decapitated. Their forebrain was isolated, washed with cold phosphate-salt buffer (10 mM sodium phosphate and 150 mM sodium chloride, pH 7.4), and homogenized in 10 mM Tris-HCl buffer (pH 7.4, 10 volumes) containing 0.32 M saccharose, 1 mM EDTA (buffer A), 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in a Teflon-glass homogenizer. The homogenate was centrifuged for 20 min at 1000 *g*. The precipitate was removed, and the supernatant was repeatedly centrifuged for 30 min at 24000 *g*. The dense mitochondria-rich brown precipitate on a bottom of the tube was discarded, and the less dense light precipitate of membranes was suspended in buffer A, replaced in a clean tube, and washed with the same buffer two times with the subsequent centrifugation (as described above) for precipitation of the membranes. The precipitate was suspended in 10 mM Trs-HCl buffer (pH 7.4) containing 0.22 M saccharose and stored in liquid nitro-

gen [8]. The protein concentration in the membrane samples was determined by the Lowry method [9]; it proved to be 8.6 mg/ml.

Proteolysis by aminopeptidase M and MERB. The proteolytic conditions of cleavage by aminopeptidase M were found in the experiments with degradation of semax at different ratios of the substrate and the enzyme. Aminopeptidase M (30 μl, 0.30 μg, 1.942×10^{-6} AU) in the phosphate-salt buffer (27.4 mM NaCl, 0.4 mM KCl, 2 mM Na_3PO_4 in 100 ml of water, pH 7.4) was added to the solution of the corresponding peptide (150 μg, 0.18 μmol) in the same buffer (290 μl). The amount of semax was varied from 2.6 to 82 μmol per one unit of the enzyme, and the reaction was continued up to 120 min. Semax degradation of more than 90% was achieved at the ratio of semax and amino peptidase M of about 10 : 1 (μmol/AU) for 60 min.

Degradation of the aforementioned semax analogues (Fig. 3) and the semax stability depending on the content of these analogues in the reaction mixture (Table 1) were studied under the conditions that were found for semax.

In the case of the experiments with MERB, the solution of MERB (107 μl, 8.6 mg of the protein/ml) was added to the same solution. The incubation mixture was stirred at a temperature of 30°C, and aliquots (20 μl) were taken in 0.5, 2, 5, 10, 20, 30, 40, and 60 min. The proteolysis was stopped by the addition of the equal volume of methanol to the samples. After the proteolysis of the peptides in the presence of MERB, the reaction mixture was purified by the solid-phase extraction on the reversed phase. The peptide mixture was applied onto a cartridge with a reversed phase, eluted with the mixture of methanol and 0.1% aqueous TFA (4 : 1), evaporated, and dissolved in the 200 μl of methanol–water mixture (10 : 90).

The samples which were obtained with the use of aminopeptidase M and MERB were analyzed by HPLC.

ACKNOWLEDGMENTS

This study was partially supported by the Program "Molecular and Cellular Biology" for Fundamental Studies of Presidium of the Russian Academy of Sciences, by the project "Scientific Schools," no. NSh-3626.2008.4, and by the project of the state support of young Russian scientists of the President of Russian Federation, no. MK-2375.2009.4 (under the direction of Academician N.F. Myasoedov).

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