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Inhibition of Enkephalin Aminopeptidase by Synthetic Peptide Inhibitors

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Abstract: In an effort to increase effective action of enkephalins, several peptide inhibitors of enkephalin aminopeptidase which contain a zinc binding site at N-terminus and side chains of enkephalin were synthesized. Enkephalin aminopeptidase was purified from rat brain by $(NH_4)_2SO_4$ fractionation,DEAE-cellulose chromatography, gel-filtration and rechromatography on DEAE-cellulose. The enzyme hydrolyzes L-Tyr-β-naphthylamide with K_m value of 5.91×10^{-5} M. The peptide inhibitors are shown to be very strong competitive inhibitors of enkephalin aminopeptidase ($K_i=10^{-7}$ M).

Enkephalins (Tyr-Gly-Gly-Phe-Met or Leu) are of great interest because of their opiate-like properties (Hughes *et al.*, 1975) and their roles as neurotransmitters or neuromodulators (Fredrickson, 1977). It is well known that their physiological activity is very transitory due to their rapid degradation by serum and brain enzymes (Meek *et al.*, 1977; Erdos *et al.*, 1978). The principal inactivating enzymes are believed to be aminopeptidase (Knight *et al.*, 1978; Meek *et al.*, 1977). Most efforts directed at obtaining longer acting enkephalins have centered on the synthesis of enkephalin analogues resistant to enzymatic degradation. For example a simple substitution of Gly with D-Ala has resulted in a compound which is active via intracerebral or vascular administration (Pert *et al.*, 1976). Another approach to increase the effective action of enkephalins would be to limit their rate of degradation by blocking enzymatic pathways associated with their catabolism. This approach has proven to be of therapeutic value in treating diseases such as myasthenia gravis, where specific inhibitors of acethylcholinesterase are administered (Beeson *et al.*, 1975). Inhibition of the enkephalin-degrading enzymes should prolong enkephalin activity and may be expected to produce neuropharmacological effects *in vivo*.

In a continuing search for such inhibitors, we have synthesized several peptides. Several enkephalin degrading aminopeptidases have been purified and characterized (Hersh, 1981 Schnebli *et al.*, 1979). As shown in several other aminopeptidases, the enkephalin aminopeptidase contains zinc and is inhibited by 1,10-phenanthrolin. The natural aminopeptidase inhibitors, amastatin (Aoyagi *et al.*, 1978) and bestatin (Umezawa *et al.*, 1976) contain (2S,3R)-3-amino-2-hydroxy acid as N-terminal residue and also inhibit enkephalin aminopeptidase. Therefore, application of design priciple of inhibitors dictated the incorporation of 3-amino-2-hydroxy acid to the suitable peptides. Inhibitors which contain (2R, 3R)-3-amino-2-hydroxy acid as zinc binding site at N-terminus and side chains of enkephalin were synthesized. The inhibitory potency on enkephalin aminopeptidase which was purified from rat brain was measured. The peptides were found to be very strong inhibitors ($K_i=10^{-7}$ M) and to show competitive inhibition pattern.

Materials and Methods

Peptides, 1, 2, 3, (2S,3R)AHPBA-Gly-Gly-Phe-Leu, (2S,3R)AHPBA-Ala-Gly-Phe-Leu, (2S,3R)AHPBA-D-

Table 1. Physical constants for the protected peptides

No	Cmpd[a]	Mp. ℃	R_f	Formula	Elemental anal.
1	Boc-AHPBA-Gly-Gly-Phe-Leu-OMe	90~ 92	0.34[b]	$C_{28}H_{37}N_5O_7$	C, 60.74; H, 6.48; N, 12.66
2	Boc-AHPBA-Ala-Gly-Phe-Leu-OMe	101~104	0.35[b]	$C_{29}H_{39}N_5O_7$	C, 59.88; H, 6.45; N, 12.42
3	Boc-AHPBA-D-Ala-Gly-Phe-Leu-OMe	112~115	0.35[b]	$C_{29}H_{39}N_5O_7$	C, 60.04; H, 6.72; N, 12.09
4	Boc-AHpHBA-Gly-Gly-Phe	102~104	0.26[c]	$C_{28}H_{36}N_4O_9$	C, 58.98; H, 6.05; N, 9.88
5	Boc-AHpHBA-Gly-Gly-Phe-NH_2	[d]	0.28[c]	$C_{28}H_{37}N_5O_8$	C, 58.88; H, 6.78; N, 12.35

[a]All amino acids are of the L configuration. [b]5% Methanol in chloroform. [c]10% Methanol in chloroform. [d]Amorphous solid.

Ala-Gly-Phe-Leu and 4, 5, (2S,3R)AHpHBA-Gly-Gly-Phe and (2S,3R)AHpHBA-Gly-Gly-Phe-NH_2 were synthesized in solution by chain elongation from C-terminal end with pure (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoic acid and (2S,3R)-3-amino-2-hydroxy-4 (4'-hydroxyphenyl)butanoic acid using DCC/HOBt as condensing agents. The physical properties of the protected peptides are presented in Table 1. The synthesis of these peptides were reported separately (Moon *et al.*, 1991). L-Tyrosine-β-naphthylamide was obtained from Sigma Chemical Co.

Purification of enzyme

Fresh rat brains were homogenized briefly in a Waring Blendor with 10 vols. cooled, deaerated 0.01 M Tris-HCl (pH 7.4)/1 mM dithiothreitol and then homogenized by hand using a glass homogenizer with Teflon pestle. The homogenate was centrifuged at 20,000 ×g for 30 min to give a postmitochondrial supernatant. Ammonium sulfate precipitation, dialysis, and chromatography on Sephadex G-100 and DEAE-cellulose were performed using a modification of the reported procedure (Schnebli *et al.*, 1979).

Protein concentration

Protein was determined by the measuring absorbance at 280 nm using serum albumin.

Enzyme assays

The enzyme activity was measured by colorimetric determination of the hydrolysis of L-Tyr-β-naphthylamide after converting the liberated naphthylamine to a red dye. Using Tyr-β-naphthylamide as a substrate, a 10 mM stock solution of the substrate in dimethylsulfoxide was diluted 50-fold into 0.1 M Tris-HCl buffer (pH 7.4 final substrate concentration, 0.2 mM); to 2 ml diluted substrate, 20 μl enzyme was added to initiate the reaction; after 15 min, the reaction was stopped by adding 1 ml freshly prepared solution of Fast Garnet GBC salt(1 mg/ml in 1 M sodium acetate (pH 4.2) 10% Tween 20). After 15 min, the resulting color was measured at 530 nm, using β-naphthylamine as standard.

Inhibition of enkephalin aminopeptidase

Inhibition experiments were carried out by adding enzyme to mixture of substrate and inhibitor. After 5 min, the enzymatic hydrolysis was stopped by adding solution of Fast Garnet GBC salt and absorbance at 530 nm, using β-naphthlyamine as standard. K_i's were first determined by fitting the kinetic data to the Michaelis-Menten equation and to Lineweaver-Burk plots. The kinetic data were then subjected to computer analysis and fit to the followings for linear competitive and noncompetitive inhibition according to Cleland's iterative least-squares method (Cleland, 1967).

Competitive

$$V=\frac{VS}{K_m(1+I/K_{is})}+S$$

Noncompetitive

$$V=\frac{VS}{K_m(1+I/K_{is})+S(1+I/K_{ii})}$$

Results and Discussions

Purification of rat brain aminopeptidase

By use of a reported procedure (Barclay *et al.*, 1978), an enzyme which hydrolyzes both enkephalin

Table 2. Purification of rat brain aminopeptidase

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min·mg)
Postmitochondrial supernatant	5,500	26,300	4.8
40~70% $(NH_4)_2SO_4$	518	22,900	44
First DEAE-cellulose	14	18,600	1329
Sephadex G-100	1.9	13,400	7053
Second DEAE-cellulose	0.8	6,900	8625

*Starting material : 20 rat brains (38 g wet weight). Total protein was calculated from A_{280}, using bovine serum albumin as standard. For enzyme actity L-Tyr-β-naphthylamide was used as substrate.

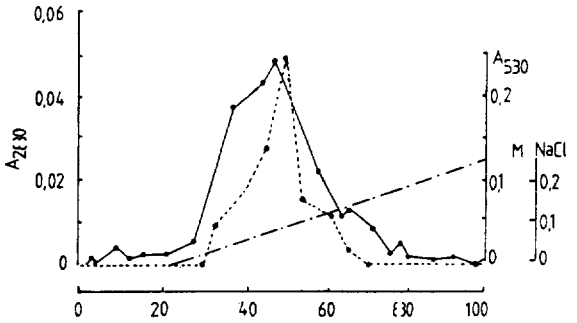

Fig. 1. Rechromatography of rat brain aminopeptidase on DEAE-cellulose. The active fractions from the Sephadex column were concentrated and dialyzed against 50 mM phosphate buffer (pH 7.0)/1 mM dithiothreitol. The material was applied to a DEAE-cellulose column (1×5 cm) equilibrated with the same buffer. The enzyme was eluted in 3 ml fractions with a linear salt gradient (0~0.25 M NaCl in the same buffer, 160 ml total). The enzyme was assayed with L-Tyr-β-naphthylamide as substrate (——, A_{280}; -----, A_{530}).

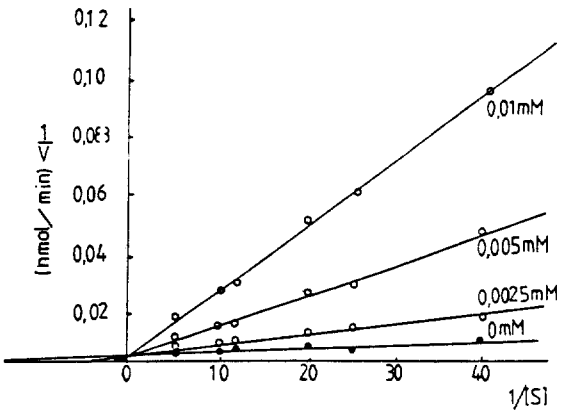

Fig. 2. Lineweaver-Burk plot of inhibition for enkephalin aminopeptidase by (2S,3R)AHPBA-Gly-Gly-Phe-Leu.

Table 3. Inhibition of enkephalin aminopeptidase by inhibitors

No	Compounds	$K_i \times 10^{-7}$ M	Pattern
1	(2S,3R) AHPBA-Gly-Gly-Phe-Leu	9.77	Comp.
2	(2S,3R) AHPBA-Ala-Gly-Phe-Leu	7.15	Comp.
3	(2S,3R) AHPBA-D-Ala-Gly-Phe-Leu	4.54	Comp.
4	(2S,3R) AHpHBA-Gly-Gly-Phe	5.12	Comp.
5	(2S,3R) AHpHBA-Gly-Gly-Phe-NH_2	4.30	Comp.

and some aminoacylarylamides were purified as summarized in Table 2. The procedure involved $(NH_4)_2SO_4$ fractionation, chromatography on DEAE-cellulose, molecular sieving on Sephadex G-100, and rechromatorgaphy on DEAE-cellulose (Fig. 1). The purification procedure was resulted in a 1800-fold enrichment of enzyme activity over the soluble brain extrcat used as the starting material.

Inhibition of enkephalin aminopeptidase

Peptides synthesized were tested as potent inhibitors of enkephalin aminopeptidase. The data are collected in Table 3. Lineweaver-Burk plot of the kinetics of the peptide 1 inhibition showed it to be a competitive inhibitor (Fig. 2). Peptide 2, 3, 4 and 5 also showed to be competitive inhibitors (not shown). These results were substantiated by fitting the data to computer programs for both competitive and noncompetitive inhibiton. This procedure avoids the inherent errors of reciprocal analysis of enzyme kinetics described by Dowd and Riggs (1965).

Inhibitor 1 which contains (2S,3R)-3-amino-2-hydroxy acid instead of tyrosine at N-terminus of Leu-enkephalin showed that the inhibition was competitive

with K_i of 5.62×10^{-7} M. Considering structure similarity of inhibitor 1 to enkephalin, inhibitor 1 would appear to interact with the enzyme inside of active site. Two bacterial peptide amastatin and bestatin which have been known to be potent inhibitors of aminopeptidase A, aminopeptidase B, leucine aminopeptidase, and aminopeptidase M (Rich *et al.*, 1984), have been reported to be noncompetitive inhibitors of enkephalin aminopeptidase (Barclay *et al.*, 1980). Even though peptides synthesized for this study and amastatin and bestatin have similar N-terminal residue, 3-amino-2-hydroxy acids, they appeared to interact with the enzyme with different mechanism of inhibition. It is probably due to different side chains of remaining residues.

In order to obtain more potent inhibitors of the enzyme, several inhibitors were synthesized and tested. Since structure activity relationships for enkephalin analogs showed that replacement of Gly^2 with Ala or D-Ala resulted in drastic change in activity of enkephalin (Fredrickson, 1977), peptide 2 in which Gly^2 of inhibitor 1 was replaced by Ala, and peptide 3, in which Gly^2 was replaced by D-Ala were synthesized. Peptide 2 and 3 also showed slightly stronger inhibition than inhibitor 1. These results were found to closely parallel with structure-activity relationships of enkephalin analogs. Peptide 4, and 5, in which (2S,3 R)AHPBA was replaced by (2S,3R) AHpHBA and Leu was deleted, were found to be about 2-fold stronger inhibitors than inhibitor 1. Since the 4-hydroxybenzyl side chain of N-terminus of peptide 4 and 5 is the side chain of N-terminal residue of enkephalin, thus it is expected that the replacement of the benzyl side chain of P1 in the inhibitor 1 with the 4-hydroxybenzyl group could resulted in a slight increment in inhibitory potency. A reaction mechanism for enkephalin aminopeptidase has not been known yet. Wagner *et al.*, reported that enkephalin aminopeptidase contains 1 mol of Zn^{2+} per 1 mol of enzyme as the only metal component and enzyme activity was inhibited by metal chelators (Wagner *et al.*, 1981). The reaction mechanism of enkephalin aminopeptidase might be closely related to that of leucine aminopeptidase (Bryce *et al.*, 1964) based on the dependence of zinc ion for activity and the inhibition by amastatin and bestatin. Accordingly, the inhibitory mechanism

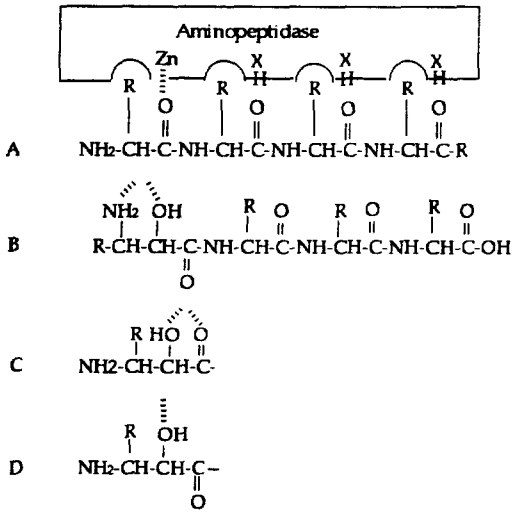

Fig. 3. Schematic models for binding of substrate (A), inhibitors (B, C, D) to aminopeptidase; (B) chelation of the active site zinc by the amino and hydroxyl groups; (C) chelation by the hydroxyl and carbonyl groups ; (D) inhibitor binds as the transition state analog.

of the peptide inhibitors on enkephalin aminopeptidase might be closely related to that on leucine aminopeptidase by amastatin and bestatin (Rich *et al.*, 1984). It, therfore, is proposed that the inhibition of the inhibitor 1~5 proceed by chelation of 2(S)-hydroxyl and 3-amino groups in the AHPBA or AHpHBA moiety in peptide to the zinc ion in the enzyme active site. Alternatively, 2(S)-hydroxyl group and the carbonyl group are zinc ligands. Both models are based on fact that a pair of adjacent hydroxy and amino or carbonyl groups has metal complexing properties. Other mechanism in which inhibitors are analogs of the transition state or tetrahedral intermediate for amide bond hydrolysis formed via coordination to the active site zinc ion also connot be excluded (Fig. 3).

Acknowledgment

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초록 : 합성한 펩티드 저해물에 의한 엔케파린 아미노펩티다제의 저해

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엔케파린의 작용시간을 증가시키기 위한 연구로서 엔케파린 아미노펩티다제의저해물을 합성하였다. 이들은 효소의 활성자리에 있는 zinc와 결합할 수 있는 결합자리를 가지고 엔케파린의 결사슬을 가진 저해물이다. 엔케파린 아미노펩티다제는 쥐로부터$(NH_4)_2SO_4$ 분별침전, DEAE-Cellulose 크로마토그래피, Sephadex G-100 겔 크로마토그래피 방법을 이용하여 정제하였다. 정제한 아미노펩티다제는 L-Tyr-β-naphthylamide를 5.19×10^{-5} M의 K_m값으로 분해시켰다. 합성한 펩티드성 저해물들은 모두 강한 경쟁적 저해작용을 나타내었다($K_i=10^{-7}$ M).