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ISOLATION AND CHARACTERIZATION OF PROLYLENDOPEPTIDASE-INHIBITING PEPTIDES FROM BOVINE BRAIN

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Backgrounds:
Prolylendopeptidase [EC 3.4.21.26] (PEP), a member of prolyl oligopeptidase family of serine protease, is widely distributed in various organs, particularly in the brain. A similar enzyme has been purified from *Flavobacterium meningosepticum*. PEP, which specifically cleaves the peptide bond at the carboxyl side of proline residues of several biologically active peptides such as vasopressin, oxytocin, substance P, TRH, and neurotensin, is considered to play an important role in the regulation of these peptides. Among these peptides, vasopressin has been suggested to be concerned with the learning and memory processes. Various kinds of PEP inhibitor were synthesized and have been reported to have anti-amnesic effects in animal models. Endogenous inhibitors of PEP were also detected in porcine pancreas (Mr=6,500), rat brain (Mr=7,000) and sperm of ascidian (Mr=8,000), but their structures have not been clarified.

Objectives:
In the preliminary research for the utilization of meat by-products, a PEP-inhibiting activity was observed in the extract of bovine brain. In the present study, isolation of the PEP-inhibiting peptides from bovine brain and the brain PEP inhibiting activity of synthetic peptides related to the isolated peptides were described.

Methods:
During the course of purification of PEP-inhibiting peptides, the inhibiting activity was monitored with PEP produced by *Flavobacterium meningosepticum* (Seikagaku Corporation) [1]. A 40-μl portion of 0.2 mM Z-Gly-Pro-*p*NA in 40 % 1,4-dioxane, 80 μl of 0.1 M phosphate buffer, pH 7.0, and 40 μl of the inhibitor fraction were mixed and the mixture was incubated for 5 min at 30 ℃. The reaction was initiated by adding 40 μl of PEP of *F. meningosepticum* origin (0.01 unit/ml in 0.1 M phosphate buffer, pH 7.0). After incubation for 15 min at 30 ℃, the reaction was stopped by adding 200 μl of 1 N HCl. Then, 20 μl of the solution was applied onto a μ Bondasphere C_{18} column for HPLC and was eluted with 50 % CH_3CN containing 0.1 % trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The *p*-nitroaniline released was measured by monitoring the absorbance at 410 nm.
The inhibition of PEP (derived from bovine brain) was assayed in the following way [2]; A 40-μl portion of a peptide solution, 80 μl of a buffer solution (40 mM Tris-HCl buffer, pH 7.0, containing 20 mM EDTA and 20 mM 2-mercaptoethanol) and 40 μl of PEP from bovine brain (0.1 unit/ml in 20 mM Tris-HCl, pH 7.0, containing 10 mM EDTA and 10 mM 2-mercaptoethanol) were mixed and the mixture was incubated for 5 min at 37 ℃. The reaction was initiated by adding 40 μl of Z-Gly-Pro-*p*NA (1 or 2 mM). After incubation for 15 min at 37 ℃, the reaction was stopped by adding 200 μl of 1 N HCl. The *p*-nitroaniline released was measured as described above. PEP used in this experiment had been purified from a bovine brain extract by column chromatography on Q-Sepharose, PBE-94, and Hiload 16/60 Superdex 200.
Peptides were synthesized by the solid phase method with a 430A Peptide Synthesizer (Applied Biosystems, Inc.).

Results and disucussion:
Bovine brain was excised and frozen at –20 ℃ just after slaughter. The frozen brain was crashed, heated for 10 min at 80 ℃ in distilled water and homogenized. The homogenate was centrifuged for 60 min at 7,000 x g and the supernatant was centrifuged again for 30 min at 27,000 x g. The supernatant was ultrafiltered with a YM-10 membrane (Amicon). The filtrate was then applied to a Sephadex LH-20 column (2.6 x 92 cm) equilibrated with 20 % methanol. Two fractions showed PEP inhibiting activity and they were each applied onto a DEAE-TOYOPEARL 650M column (1.6 x 65 cm) equilibrated with distilled water and was eluted with a linear gradient of $HCOONH_4$ concentration (0 to 1 M). Seven active fractions were obtained. Each fraction was applied onto a μ Bondapak C_{18} column (3.9 x 300 mm) for HPLC and eluted with a linear gradient of CH_3CN concentration (0.7 to 63 %) in 0.1 % TFA. The

peaks showing PEP-inhibiting activity were applied onto a Shim-Pack PA-SP column (8 x 100 mm) for HPLC and eluted with a linear gradient of the concentration of Na-phosphate buffer (0 to 0.5 M), pH 6.5. The active fractions were each applied onto a μ Bondasphere C_{18} column for HPLC and eluted with a linear gradient of CH_3CN concentration (0.7 to 63 %) in 0.1 % TFA. Finally, PEP-inhibiting peptides were purified by HPLC on a μ Bondasphere C_{18} column repeated twice.

The purified peptides were analyzed for amino acid sequence by the Edman procedure with a 477A protein sequencer (Applied Biosystems), and the sequence determined were subjected to homology search (Table 1.). One of these peptides, EPPPPEPPPI, proved to be a new peptide. These peptides except for the one, corresponding to the N-terminal sequence of FK506-binding protein, were synthesized and their HPLC profiles were compared with those of the purified ones. Each of them showed the same retention time as that of the purified one. The amount of the N-terminal peptide of the FK506-binding protein was not enough to determine for the full sequence, so two peptides with possible sequences were synthesized and used in the following experiments.

These peptides were determined for the PEP-inhibiting activity with synthetic peptides and PEP purified from bovine brain. In these peptides, MPPPLPARVDFSLAGALN, corresponding to the sequence 38 to 55 of glial fibrillary acidic protein (GFAP(38-55)), showed the highest inhibitory activity against brain PEP (K_i value; 8.6 μM) (Table 1.). The mode of inhibition of bovine brain PEP was competitive. This peptide showed an K_i value nearly the same as that of the inhibitor purified from rat brain (K_i value; 2.6 μM). This is the first endogenous peptide having a PEP-inhibiting activity, of which the structure and K_i value were determined.

Homologous peptides of human type and mouse type to GFAP(38-55) also inhibited brain PEP with a similar K_i Value (Table 2.). Prolongation of the preincubation period of GFAP(38-55) with brain PEP to 60 min showed no effect on inhibiting activity. During the prolonged preincubation period, 10 % of GFAP(38-55) was cleaved to MPPPLP and ARVDFSLAGALN. The released peptide MPPPLP was not hydrolyzed further with brain PEP and showed nearly the same inhibiting activity to brain PEP (Table 2.), but a homologous peptide, MTPPLP (mouse type), was less effective. The triproline arrangement within the sequence may be essential to inhibiting activity, though which amino acid residue of MPPPLP binds to the S_1 site of brain PEP is unclear.

The PEP-inhibiting activities of the peptides were rather low, compared with such synthetic inhibitors as Z-Pro-prolinal (K_i value; 3.4 nM), Z-Thiopro-thioprolinal (K_i value; 0.01 nM) and Y-29794 (K_i value; 0.95 nM). However, Yoshimoto *et al* reported that such a relatively weak inhibitor of PEP as Z-Gly-Pro-CH_2Cl (K_i value; 70 μM) had also strong anti-amnesic effect, and Z-Gly-Pro (K_i value; 620 μM) and Z-Val-Pro (Ki value; 120 μM) showed the same tendency in animal models. Compared with these inhibitors, GFAP(38-55) and MPPPLP showed a relatively high potency of PEP inhibition. Thus, these peptides are expected to show an anti-amnesic effect to some extent, though in vivo stability of the peptide has remained to be clarified. The distribution and physiological property of the peptides obtained here are now under investigation.

Conclusion:

The peptides inhibiting prolylendopeptidase were isolated from bovine brain. The amino acid sequence of the peptide having the highest inhibitory activity (K_i value; 8.6 μM) was determined as MPPPLPARVDFSLAGALN (GFAP(38-55)). Homologous peptides of human type and mouse type to GFAP(38-55) and N-terminal peptide MPPPLP also inhibited brain PEP with a similar K_i Value.

Literature:

1) T. Ohmori, T. Nakagami, H. Tanaka and S. Maruyama, *Biochem. Biophys. Res. Commmun.*, **202**, 809-815, 1994.

2) S. Maruyama, T. Ohmori and T. Nakagami, *Biosci. Biotech. Biochem.*, **60**, 358-359, 1996.

Table 1. Brain PEP inhibiting activity of synthetic peptides.

peptide	K_i (μM)
MPPPLPARVDFSLAGALN[a]	8.6
GVQVETISPGDGRTFPK[b]	150
GVQVETISPGDGR[c]	162
EPPPPEPPPI	1400
APPA	12300

a, GFAP(38-55)

b and c, corresponding to the sequence 1-17 and 1-13 of FK506-binding protein, respectively.

Table 2. Brain PEP inhibiting activity of GFAP(38-55) related peptides.

peptide	K_i (μM)
MPPPLPTRVDFSLAGALN (Human)	4.6
MTPPLPARVDFSLAGALN (Mouse)	8.3
MPPPLP	8.8
MTPPLP	52.3