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Specificity of a Membrane-Bound Neutral Endopeptidase from Rat Kidney[1)]

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The substrate specificity of a purified kidney neutral endopeptidase was studied. The endopeptidase hydrolyzed a variety of biologically active peptides, such as angiotensins (angiotensins I, II and III), bradykinins (bradykinin, Lys-bradykinin, Met–Lys–bradykinin and des-^{9}Arg–bradykinin), enkephalins (Leu–enkephalin and Met–enkephalin), neurotensin and substance P, and was found to cleave only the bonds at the amino side of hydrophobic amino acids in the peptides. However, when a hydrophobic amino acid was present at the C-terminus or at the position adjacent to the N-terminus, the bond of the hydrophobic residue was not cleaved. In further studies on the degradation of a series of homo-oligopeptides, the enzyme appeared to hydrolyze those consisting of at least a tetrapeptide unit of hydrophobic amino acids such as Ala and Phe. The specificity of the membrane-bound neutral endopeptidase from rat kidney indicated by these results can be summarized as follows: the enzyme requires at least a tetrapeptide unit for hydrolysis, and cleavage occurs only at the amino side of a hydrophobic amino acid, when one is present at the third position of the tetrapeptide unit.

Keywords——neutral endopeptidase; substrate specificity; biologically active peptide; homo-oligopeptide; kidney membrane-bound enzyme

Introduction

The proteases associated with cell membranes were surveyed, and the peptidases so far identified in kidney membranes are one neutral endopeptidase and four arylamidases.[2)] The biological function of the membrane-bound neutral endopeptidase is supposed to be proteolysis in cells and subcellular organelles, and the enzyme may have important roles in the modification of biologically active peptides and in the turnover of some peptides.

Recently, we reported the purification from rat kidney microsomal membranes of an enzyme which hydrolyzes succinyl trialanine *p*-nitroanilide (Suc–$(Ala)_3$–*p*NA) to succinyl dialanine and Ala–*p*NA.[3)] Suc–$(Ala)_3$–*p*NA has been used as a chromogenic substrate for elastase.[4)] However, the membrane-bound neutral endopeptidase obtained from kidney was found to differ from elastase.[3,5)] The purified enzyme obtained from rat kidney had a molecular weight of about 92000 and was inhibited by metal-chelating agents and phosphoramidon.[3)] These findings indicate that the enzyme is essentially similar to kidney neutral metallo-endopeptidase (EC 3.4.21.11).[6)]

In the present study, several biologically active peptides were digested with the neutral endopeptidase and the degradation products were analyzed to determine the substrate specificity of the enzyme. The action of the enzyme toward various peptides prompted us to propose that the enzyme plays a role in the degradation of many peptide hormones.

To elucidate the substrate specificity of this enzyme further, we employed homo-oligopeptides as substrate, based on the results for known biologically active peptides, and found a new feature of the enzyme action.

Experimental

Materials——A purified neutral endopeptidase was prepared from rat kidney microsomal membranes as described previously.[3] Homo-oligopeptides, Suc–$(Ala)_2$–Val–*p*NA, $(Ala)_2$–Phe–*p*NA and leucine aminopeptidase (type IV from porcine kidney microsomes) were purchased from Sigma Chem. Co. (St. Louis). Angiotensins I, II and III, bradykinin, Lys-bradykinin, Met-Lys-bradykinin, des-^{9}Arg-bradykinin, Leu-enkephalin, Met-enkephalin, substance P, neurotensin, Suc–$(Ala)_3$–*p*NA, Suc–$(Ala)_2$–*p*NA and Suc–Ala–*p*NA were obtained from the Protein Research Foundation (Osaka).

Determination of Enzyme Activity——Enzyme activity was determined with Suc–$(Ala)_3$–*p*NA by an indirect enzyme assay method, in the presence of excess aminopeptidase.[3] Under the assay conditions, Suc–$(Ala)_3$–*p*NA is cleaved into succinyl dialanine and Ala–*p*NA by the neutral endopeptidase, and then the resulting Ala–*p*NA is hydrolyzed into Ala and *p*NA by the aminopeptidase. One unit of the enzyme activity is defined as the amount of enzyme that liberated 1 μmol of *p*NA from Suc–$(Ala)_3$–*p*NA per minute.

Determination of the Cleavage Sites in Peptides——Peptides (1 mM) were incubated with the purified enzyme (6×10^{-4} units) in a final volume of 50 μl of Tris–HCl, pH 8.0 (50 mM), at 25 °C for 60 min. The reaction was stopped by adding 10 μl of 30% perchloric acid. Each mixture was centrifuged and 10 μl of the supernatant was subjected to high-performance liquid chromatography (HPLC) with a Shimadzu HPLC system equipped with a TSK-GEL ODS-120A column (Toyo Soda). The sample was eluted with a mixture of acetonitrile and phosphate buffer (pH 2.3). The amounts of peptides were monitored by measuring the ultraviolet (UV) absorbance at 210 nm. The solvent systems were acetonitrile–10 mM phosphate buffer (pH 2.3) (3.5 : 96.5, v/v) for homo-oligopeptides and a stepwise gradient of acetonitrile, 10, 15 and 20%, for biologically active peptides.

Amino Acid Analysis——Fractions corresponding to discrete peaks were manually collected and evaporated under reduced pressure, and then each residue was hydrolyzed in 6 N HCl for 22 h at 110 °C. The hydrolysates were subjected to amino acid analysis using an amino acid analysis system (Shimadzu).

Results

Neutral Endopeptidase-Catalyzed Hydrolysis of Synthetic Substrates

Table I summarizes the apparent rates of hydrolysis of some synthetic substrates. The enzyme cleaved the bonds between the second and third amino acid residues of the tripeptidyl-*p*NA substrates. Upon comparison of the specific activities, the cleavage of an Ala–Ala bond was found to be 8.83- and 6.63-fold slower than in the case of Ala–Val and Ala–Phe bonds, respectively. Thus, the enzyme has higher activity for peptide bonds involving the amino group of a long-chain aliphatic amino acid or an aromatic amino acid.

Hydrolysis of Some Biologically Active Peptides by the Neutral Endopeptidase

HPLC and amino acid analysis were performed to identify the peptide products derived from biologically active peptides as substrates. The action of the enzyme toward angiotensins I, II and III is summarized in Table II. Incubation of the enzyme with angiotensin I gave seven peptide products. Three cleavage sites were identified, the peptide fragments being

TABLE I. Synthetic Substrate Specificity of the Neutral Endopeptidase

Substrate	Rate of hydrolysis (μmol/min/mg protein)
Suc–Ala–Ala↓Ala–*p*NA	1.67
Suc–Ala–Ala↓Val–*p*NA	14.17
Ala–Ala↓Phe–*p*NA	11.21
Suc–Ala–Ala–*p*NA	No hydrolysis
Suc–Ala–*p*NA	No hydrolysis

Enzyme was added to each reaction mixture (0.25 ml) containing 1 mM substrate in 50 mM Tris–HCl, pH 8.0. After incubation for 60 min at 25 °C, the amount of hydrolyzed products was quantified by HPLC with a TSK-GEL ODS-120A column. Elution was carried out with 5 mM phosphate buffer (pH 3.9)–50% methanol and the eluate was monitored at 340 nm. Downward pointing arrows indicate the sites of cleavage.

TABLE II. Sites of Cleavage of Angiotensins I, II and III by the Neutral Endopeptidase

Substrate	Structure	Products found
Angiotensin I	Asp–Arg↓Val–Tyr↓Ile–His–Pro↓Phe–His–Leu	Asp–Arg (1), Ile–His–Pro (2.8), Asp–Arg–Val–Tyr (1.8), Val–Tyr (0.8), Asp–Arg–Val–Tyr–Ile–His–Pro (2.6), Phe–His–Leu (3.4), Ile–His–Pro–Phe–His–Leu (1.6)
Angiotensin II	Asp–Arg↓Val–Tyr↓Ile–His–Pro–Phe	Asp–Arg (1), Asp–Arg–Val–Tyr (2.2), Val–Tyr (0.7), Ile–His–Pro–Phe (3.6)
Angiotensin III	Arg–Val–Tyr↓Ile–His–Pro–Phe	Arg–Val–Tyr (1), Ile–His–Pro–Phe (1.1)

Angiotensins (1 mM) were incubated with 6×10^{-4} unit of enzyme for 60 min at 25 °C, the reaction was terminated by adding perchloric acid, and the soluble materials were applied to a TSK-GEL ODS-120A column. Elution was performed with a stepwise gradient of acetonitrile (0—15 min, 10%; 15—40 min, 15%; and 40—70 min, 20%). Hydrolyzed products are listed in the order of increasing retention time. Yields of hydrolyzed peptides are expressed (in parenthesis) relative to the first peptide eluted on HPLC analysis. Under the assay conditions, percentages of substrates cleaved were: angiotensin I, 54%; angiotensin II, 51%; and angiotensin III, 49%. Downward pointing arrows indicate the sites of cleavage.

Leu-enkephalin	Tyr–Gly–Gly↓Phe–Leu
Met-enkephalin	Tyr–Gly–Gly↓Phe–Met
bradykinin	Arg–Pro–Pro–Gly↓Phe–Ser–Pro↓Phe–Arg
Lys-bradykinin	Lys–Arg–Pro–Pro–Gly↓Phe–Ser–Pro↓Phe–Arg
Met-Lys-bradykinin	Met–Lys–Arg–Pro–Pro–Gly↓Phe–Ser–Pro↓Phe–Arg
Des-^{9}Arg-bradykinin	Arg–Pro–Pro–Gly↓Phe–Ser–Pro–Phe
neurotensin	Pyr–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr↓Ile–Leu
substance P	Arg–Pro–Lys–Pro–Gln–Gln↓Phe↓Phe–Gly↓Leu–Met–NH_2

Fig. 1. Sites of Cleavage by the Neutral Endopeptidase in Biologically Active Peptides

The cleavage sites were assigned on the basis of the known primary structures of the peptides and the amino acid compositions of the hydrolyzed products. Downward pointing arrows indicate the sites of cleavage.

formed by cleavage at the ^{2}Arg–^{3}Val, ^{4}Tyr–^{5}Ile and ^{7}Pro–^{8}Phe bonds. Incubation of the enzyme with angiotensin II gave four peptide products with cleavage at the ^{2}Arg–^{3}Val and ^{4}Tyr–^{5}Ile bonds. The ^{7}Pro–^{8}Phe bond, which was cleaved in the case of angiotensin I, however, was not cleaved. The enzyme cleaved angiotensin III into two peptide fragments with cleavage at the ^{3}Tyr–^{4}Ile bond. The enzyme hydrolyzed the substrates stoichiometrically into the respective products. The disappearance of substrate and appearance of hydrolyzed products were proportional, even on prolonged incubation. This suggests that there was no other cleavage site.

Figure 1 summarizes the cleavage sites in other biologically active peptides. The enzyme cleaved both Leu-enkephalin and Met-enkephalin at the ^{3}Gly–^{4}Phe bond. This is the same site as previously reported for enkephalinase. Incubation of the enzyme with bradykinin led to the formation of five products, which are formed by cleavage at the ^{4}Gly–^{5}Phe and ^{7}Pro–^{8}Phe bonds. Lys-bradykinin and Met-Lys-bradykinin were cleaved at the same sites. Deletion of the C-terminal Arg residue (des-^{9}Arg-bradykinin) resulted in no hydrolysis at the ^{7}Pro–^{8}Phe bond. Neurotensin was cleaved at the ^{11}Tyr–^{12}Ile bond. Seven peptides were formed from substance P, being produced by cleavage at the ^{6}Gln–^{7}Phe, ^{7}Phe–^{8}Phe and ^{9}Gly–^{10}Phe bonds.

Action of the Enzyme toward Homo-Oligopeptides

Following incubation of the enzyme with several homo-oligopeptides, the liberated

TABLE III. Products of Endopeptidase Digestion of Several Homo-Oligopeptides

Substrate	Degradation products	Possible cleavage sites
$(Ala)_2$	No hydrolysis	—
$(Ala)_3$	No hydrolysis	—
$(Ala)_4$	$(Ala)_2$,	Ala–Ala↓Ala–Ala
$(Ala)_5$	$(Ala)_2$, $(Ala)_3$	Ala–Ala↓Ala↓Ala–Ala
$(Ala)_6$	$(Ala)_2$, $(Ala)_3$, $(Ala)_4$	Ala–Ala↓Ala↓Ala↓Ala–Ala
$(Phe)_2$	No hydrolysis	—
$(Phe)_3$	No hydrolysis	—
$(Phe)_4$	$(Phe)_2$	Phe–Phe↓Phe–Phe
$(Phe)_5$	$(Phe)_2$, $(Phe)_3$	Phe–Phe↓Phe↓Phe–Phe
$(Phe)_6$	$(Phe)_2$, $(Phe)_3$, $(Phe)_4$	Phe–Phe↓Phe↓Phe↓Phe–Phe
$(Gly)_2$	No hydrolysis	—
$(Gly)_3$	No hydrolysis	—
$(Gly)_4$	No hydrolysis	—
$(Gly)_5$	No hydrolysis	—
$(Gly)_6$	No hydrolysis	—

Each homo-oligopeptide (1 mM) was incubated with 6×10^{-4} unit of enzyme. After incubation of the reaction mixture for 60 min at 25 °C, perchloric acid-soluble materials were applied to a TSK-GEL ODS-120A column. Elution was carried out with acetonitrile and 10 mM phosphate buffer (pH 2.3) (3.5 : 96.5, v/v) and the peptide-containing peaks were monitored at 210 nm. In the case of a series of Phe-oligopeptides, the substrates were dissolved in 1-methyl-2-pyrrolidinone, since long-chain, Phe-oligopeptides are insoluble in H_2O.

peptides were analyzed by HPLC. Table III summarizes the cleavage of Ala-, Phe- and Gly-homopeptides by the enzyme. When $(Ala)_4$ was used as substrate, only $(Ala)_2$, not $(Ala)_3$, was formed. $(Ala)_5$ was also cleaved, and $(Ala)_2$ and $(Ala)_3$ were produced. $(Ala)_2$, $(Ala)_3$ and $(Ala)_4$ were produced from $(Ala)_6$, but $(Ala)_4$ was eliminated on prolonged incubation. Amino acid analysis of the hydrolyzed products revealed that a free amino acid was not liberated from any of the substrate peptides. A series of Phe-homopeptides showed the same hydrolysis patterns as seen for Ala-homopeptides. The enzyme was completely inert toward a series of Gly-homopeptides. On the basis of these results, the enzyme seems to require a length of four amino acids as a minimum unit and cleavage occurs between the second and third amino acid residues of the tetrapeptide unit. Furthermore, based on the results of experiments with biologically active peptides, a hydrophobic amino acid is required for hydrolysis at the third position of the tetrapeptide units.

Discussion

There is a close resemblance between the so-called enkephalinase and the kidney membrane-bound neutral endopeptidase which hydrolyzes the substrate, Suc–$(Ala)_3$–*p*NA, to succinyl dialanine and Ala–*p*NA. Both enzymes showed the highest specific activity in kidney, and the purified enzymes had similar molecular weights. Furthermore, phosphoramidon and metal-chelating agents (*o*-phenanthroline, ethylenediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid (EDTA), *etc.*) are potent inhibitors of both enzymes. As reported in this communication, the Suc–$(Ala)_3$–*p*NA–hydrolyzing enzyme specifically cleaved the ^{3}Gly–^{4}Phe bond of enkephalins. This result raises a question as to the cellular roles of this membrane-bound neutral endopeptidase. As a first step to answering this question, we attempted to determine the specificity for synthetic substrates of the enzyme and found that the Suc–$(Ala)_3$–*p*NA used in

the purification procedures was a rather poor substrate for this enzyme (Table I).

Enkephalinase has been purified from bovine pituitaries,[7] rat brain,[8] and rat,[9] rabbit[10] and human[11] kidneys. On the other hand, immunological experiments by Relton *et al.* showed that the kidney neutral endopeptidase (EC 3.4.21.11) is identical in activity with the so-called enkephalinase.[12] Matsas *et al.* also reported that kidney brush-border neutral endopeptidase was identical with the enkephalinase and cleaved both Leu-enkephalin and substance P.[6d] Several other authors have also reported that the kidney membrane-bound neutral endopeptidase can degrade not only enkephalins but also a variety of biologically active peptides such as angiotensins,[5c,e,10] bradykinin,[5e,10] insulin B chain,[5c-e,6a] and dynorphin.[9]

In the present study, in order to confirm the sites of cleavage by rat kidney neutral endopeptidase, we checked its action on several biologically active peptides, including those investigated by others. The enzyme has a rather broad specificity for the bond at the amino side of a hydrophobic amino acid. Typical results were obtained with a series of angiotensins I, II and III. The ^{2}Arg–^{3}Val, ^{4}Tyr–^{5}Ile and ^{7}Pro–^{8}Phe bonds were cleaved in angiotensin I. However, ^{9}His–^{10}Leu (Leu is the C-terminal hydrophobic amino acid) in angiotensin I was inert to the enzyme. Angiotensin II has two cleavage sites at the ^{2}Arg–^{3}Val and ^{4}Tyr–^{5}Ile bonds, but a third possible cleavage site in angiotensin I (^{7}Pro–^{8}Phe) is not hydrolyzed by the enzyme. This failure to hydrolyze certain bonds indicated that C-terminal His–Leu (probably His only) is necessary for the enzyme action toward the ^{7}Pro–^{8}Phe bond. Angiotensin III has only one cleavage site, at the ^{3}Tyr–^{4}Ile bond. These results suggest that an additional peptide bond on each side of the cleavage site is a primary requirement for the enzyme attack. Other biologically active peptides are also degraded through cleavage of similar bonds. Thus, this enzyme seemed to degrade various peptide hormones in similar ways.

In the experiments on homo-oligopeptides ranging in length from two to six amino acid residues, the enzyme preferentially cleaved tetra- or longer peptides consisting of Ala and Phe. Analysis of the hydrolyzed products revealed that the enzyme recognized an amino acid sequence unit of four hydrophobic amino acids and cleaved the bond between the second and third amino acid residues of the tetrapeptide unit. From this point of view, it can be considered that the C-terminal *p*NA in the synthetic substrates acts as an amino acid analogue.

In conclusion, we confirmed that a sequence unit of four amino acids (X–X–Y–X: Y is a hydrophobic amino acid) is involved in the recognition of substrates of this enzyme for cleavage of the X–Y bond.

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