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Characteristics of an Aminopeptidase from Japanese Cedar (*Cryptomeria japonica*) Pollen

HIROYUKI NAGATA, TSUTOMU TAKEI, RYOKO KOJIMA, YOH KODERA,*
HIROYUKI NISHIMURA, YUJI INADA, AND AYAKO MATSUSHIMA

Department of Biomedical Engineering, Toin University of Yokohama, Toin Human Science and Technology Center, 1614 Kurogane-cho, Aoba-ku, Yokohama 225-8502, Japan

A peptidase from Japanese cedar pollen, Jc-peptidase, was clarified to preferentially hydrolyze an MCA substrate of Phe-MCA (L-phenylalanyl-4-methylcoumaryl-7-amide). This study examined substrate specificities of Jc-peptidase using oligopeptides. Jc-peptidase hydrolyzed Phe-Phe and Tyr-Phe effectively and hydrolyzed Leu-Phe, Met-Phe, and Arg-Phe moderately. Other substrates such as Ala-Phe, Asp-Phe, and Pro-Phe were not hydrolyzed with the peptidase. Results obtained with a series of aminoacyl-Phe peptides were compatible with the facts obtained for MCA substrates except for Arg-MCA. Effects of amino acid residues in the P1′ position were also examined using Phe-amino acids. An N-terminal phenylalanine residue was actually released from bioactive peptides such as molluscan cardioexcitatory neuropeptide (FMRF-NH_2). Because the activity was inhibited with Zn^{2+} and EDTA, Jc-peptidase was inferred to belong to the metalloproteases. The N-terminal amino acid sequence was determined to be APIGVQLEIEENYVHMYNGF and an internal sequence to be EIFAATFNVDEETEA, but no homology with other proteins was found.

KEYWORDS: Japanese cedar pollen; *Cryptomeria japonica* pollen; aminopeptidase; metallopeptidase

INTRODUCTION

During the past decade, numerous studies have addressed the pathological consequences of extrinsic proteases and activation of host protease cascades triggered by bacterial infection or inhalation of allergens (*1*−*6*).

We are interested in proteases in Japanese cedar (*Cryptomeria japonica*) pollen, which is a major cause of pollenosis in Japan. The pollen contains many protein components, some of which are associated closely with allergic diseases as allergens in Japan (*7*, *8*). However, proteases or peptidases in Japanese cedar pollen have not been reported except in our studies: arginine-specific proteases, Jc-protease; and an aminopeptidase, Jc-peptidase (*9*, *10*). The latter, Jc-peptidase, was purified in a homogeneous state. It hydrolyzes MCA substrates with hydrophobic amino acid residues such as L-phenylalanyl-4-methylcoumaryl-7-amide (Phe-MCA), but no amino acid was released from other MCA substrates having basic, acidic, and some neutral amino acid residues or proline residue at the N terminus (*9*).

Aminopeptidases comprise is a large group of enzymes that encompasses myriad substrate specificities, physicochemical characteristics, and biological functions (*11*−*13*). With regard to substrate specificity, an aminopeptidase that hydrolyzes only hydrophobic amino acid residues in the N terminus has not been found, other than that revealed in our previous paper (*9*).

Matheson and Travis (*14*) purified an alanine-specific aminopeptidase from mesquite pollen. That study observed differences in substrate specificity between synthetic substrates and oligopeptides, with attention to metabolism of bioactive peptides regarding allergic diseases. A previous paper by the authors determined the substrate specificity of Jc-peptidase using only a series of MCA substrates. We failed to determine N-terminal amino acid sequencing and did not address participation of metal ions in enzymic activity as an activity was not inhibited with 0.5 mM EDTA. Therefore, the present paper examines substrate specificity using peptides, the effect of metal ions, and partial amino acid sequences.

MATERIALS AND METHODS

Materials. Japanese cedar (*C. japonica*) pollen was collected in late March from mature male flowers at the Kanagawa Prefecture Natural Environment Conservation Center (Atsugi, Japan). Aminopeptidase (Jc-peptidase) was prepared from Japanese cedar pollen stored at −80 °C according to the procedure described in a previous paper (*9*). Phe-Met-D-Arg-Phe-NH_2 and dipeptides used in this study were purchased from Bachem AG (Bubendorf, Switzerland). Oligopeptides such as molluscan cardioexcitatory neuropeptide (Phe-Met-Arg-Phe-NH_2, FMRF-NH_2) and proadrenomedulin, PAMP-12 (FRKKWQKWALSR-NH_2), were purchased from the Peptide Institute, Inc. (Osaka, Japan). Waters AccQ·Fluor reagent kit and AccQ·Tag amino acid analysis column (3.9 × 150 mm) were purchased from Nihon Waters Corp. (Tokyo, Japan). Amino acid Standard H solution was obtained from Pierce Biotechnology, Inc. (Rockford, IL). Polyvinylidene difluoride (PVDF) membrane and a molecular weight standard mixture for SDS-PAGE were obtained from Nippon Genetics Co., Ltd. (Tokyo, Japan) and Bio-Rad Laboratories Inc. (Hercules, CA), respectively. Other reagents used were of analytical grade.

Procedure. *Activity.* Peptide hydrolytic activity of Jc-peptidase was measured as follows. To peptide substrates (30 μmol) dissolved in 90

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Table 1. Hydrolysis of Xaa-Phe[a] and D-Phe-D-Phe by Jc-Peptidase

dipeptide	hydrolysis of dipeptides[b] (μmol/min/mg)	relative activity (%)
Phe-Phe	2.6	100
Tyr-Phe	1.6	62
Leu-Phe	0.74	28
Met-Phe	0.57	22
Arg-Phe	0.35	13
Ala-Phe	0.28	11
Gly-Phe, Pro-Phe, Asp-Phe, D-Phe-D-Phe	∼0	∼0

[a] Xaa-Phe, aminoacyl-phenylalanine. [b] Reaction time: 10 min.

μL of 10 mM phosphate buffer (pH 7.5) was added 0.6 milliunits of Jc-peptidase dissolved in 10 μL of 10 mM phosphate-buffered 140 mM saline (PBS), of which 1 unit is defined as releasing 1 μmol of AMC per minute. Subsequently, we incubated it at 37 °C for the desired time and then added 400 μL of acetonitrile to stop the reaction, followed by standing at 4 °C for 10 min to precipitate the enzyme and salts. After 10 min, the mixture was centrifuged (10000 rpm, 10 min), and 17 μL of the supernatant was dried in a vacuum using a Waters Pico·Tag workstation. The resultant amino acid was then labeled with *N*-hydroxysuccinimidyl-6-aminoquinolyl carbamate and analyzed according to the method of the AccQ·Tag amino acid analysis system (Waters Corp.). The amount of each amino acid released was calculated from the chromatogram using the amino acid standard labeled in the same manner.

Amino Acid Sequences. Electrophoresis of the enzyme was performed in 8% polyacrylamide gel at pH 8.8 in the presence of 0.1% SDS and 2-mercaptoethanol according to the method of Laemmli (*15*). Proteins on the gel were blotted electrophoretically to a PVDF membrane and stained with Ponceau S. The amino-terminal sequence was analyzed using the band corresponding to 43 kDa using a protein sequencer (PPSQ-21; Shimadzu Corp., Kyoto, Japan). An internal sequence of Jc-peptidase was analyzed at the APLO Life Science Institute (Tokushima, Japan) as follows. The stained membrane was destained to remove Ponceau S; then the 43 kDa protein was digested with lysylendopeptidase at 35 °C overnight, and the mixture was separated by an HPLC method (TSKgel ODS-80Ts, 2.0 × 250 mm; Tosoh Corp.). The highest peak on the chromatogram was examined using a protein sequencing system (G1005A; Hewlett-Packard Co., Palo Alto, CA).

RESULTS AND DISCUSSION

Substrate Specificity and Inhibitors. A preliminary experiment found that the Jc-peptidase released the N-terminal phenylalanine residue from bioactive peptides such as molluscan cardioexcitatory neuropeptide (FMRF-NH_2) and proadrenomedulin peptide (PAMP-12, FRKKWQKWALSR-NH_2) (data not shown). The release rates of phenylalanine differed from each other. Assuming that the reaction rate is affected by the type of amino acid residue in the P1′ position and the length of peptides, we examined substrate specificity of Jc-peptidase using phenylalanine-containing peptides because Phe-MCA was the most effective MCA substrate of Jc-peptidase (*9*). Amino acid residues in substrates are numbered, respectively, as P3, P2, P1, etc., toward the N terminus from the cleavage site and P1′, P2′, P3′, etc., toward the C terminus (*16*).

Table 1 summarizes the effects of N-terminal residues on the hydrolysis of aminoacyl-phenylalanine with Jc-peptidase: among the substrates tested, Phe-Phe and Tyr-Phe were suitable substrates. Other hydrophobic aminoacyl-phenylalanines, such as Leu-Phe and Met-Phe, as well as a basic aminoacyl-phenylalanine, Arg-Phe, were also hydrolyzed with the aminopeptidase. In contrast, no amino acid was released from other dipeptides having neutral and acidic amino acid residues and proline residue. These results were compatible with facts obtained in a previous study using MCA substrates, except those of Arg-MCA (*9*). Jc-peptidase did not hydrolyze Arg-MCA but did hydrolyze Arg-Phe. Nevertheless, the latter hydrolyzation was slow. The authors' previous study surveyed inhibitors of Jc-peptidase and revealed that 3-chloro-4-pivaroyloxyphenyl *p*-guanidinobenzoate acetate, which has a guanidyl group, inhibited the enzyme with the K_i value of 20 μM (unpublished data). The value was the lowest among tested inhibitors and was a half or a fifth of the K_i value obtained for phebestin or bestatin (*9*), a typical aminopeptidase inhibitor, respectively. Oddly, Jc-peptidase recognized only hydrophobic MCA substrates. Considering the specificity for peptide substrates, Jc-peptidase may recognize not only hydrophobic amino acid residues but also arginine residues.

Table 2. Hydrolysis of Phe-Xaa[a] Dipeptides by Jc-Peptidase

dipeptide	hydrolysis of dipeptides[b] (μmol/min/mg)	relative activity (%)
Phe-Phe	2.6	100
Phe-Tyr	2.4	92
Phe-Met	2.0	77
Phe-Ala	1.8	69
Phe-Arg	1.3	50
Phe-Trp	1.0	38
Phe-Leu	0.87	33
Phe-Ile	0.38	15
Phe-Gly, Phe-Asp Phe-Pro, Phe-Glu Phe-Val, Phe-Ser	∼0	∼0
Phe-Met-Arg-Phe-NH_2	0.08	
Phe-Met-D-Arg-Phe-NH_2	0.34	

[a] Phe-Xaa, phenylalanyl-peptides. [b] Reaction time: 10 min for dipeptides, 60 min for tetrapeptides.

Table 2 shows effects of P1′ residues on dipeptide hydrolysis. Some dipeptides containing a hydrophobic amino acid residue in the P1′ position (Phe-Phe, Phe-Tyr, or Phe-Met) and a basic amino acid residue in the P1′ position (Phe-Arg) were suitable substrates. Other dipeptides with hydrophobic or hydrophilic amino acid in the P1′ position, such as Phe-Val or Phe-Glu, were unsuitable as substrates. These results indicate that the hydrolysis rate of phenylalanyl-peptides by Jc-peptidase is also affected by amino acid residues in the P1′ position.

The influence of the P2′ position of peptides on Jc-peptidase was examined next. The bottom of **Table 2** shows that the phenylalanine-releasing velocity for Phe-Met-Arg-Phe-NH_2 with Jc-peptidase was slower than that for Phe-Met-D-Arg-Phe-NH_2, suggesting that the enzyme also recognizes the P2′ position.

Gu and Willing (*17*) showed that tomato, porcine, and *Escherichia coli* leucine aminopeptidases (LAPs) rapidly hydrolyzed peptide substrates with bulky nonpolar (Leu, Val, Ile, and Ala), basic (Arg), and sulfur-containing (Met) residues at the P1 position, whereas P1 Asp or Gly residues were not hydrolyzed efficiently. On the other hand, those enzymes hydrolyzed aromatic residues in the P1 position at different rates (*17*, *18*). Moreover, they also indicated that Pro, Asp, Lys, and Gly in the P1′ position and Arg in the P2′ position slowed the hydrolysis rates of these enzymes.

The results shown in the tables imply that Jc-peptidase prefers hydrophobic amino acid residues at the P1 and P1′ positions of substrates. Its specificity seems to resemble that of LAPs. Moreover, P2′ recognition by Jc-peptidase is quite similar to that by LAPs: Arg at the P2′ position may also suppress the reaction rate in the case of Jc-peptidase. On the other hand, an enzyme that hydrolyzes an N-terminal phenylalanine residue

Table 3. Effect of EDTA and Divalent Metal Ions

inhibitor	residual activity (%)
–inhibitor	100
Zn^{2+}	0.1
Cu^{2+}	0.8
Co^{2+}	74
Mn^{2+}	94
Mg^{2+}	97
Ca^{2+}	101
EDTA[b]	13

[a] Enzyme, aminopeptidase, 2 nM; inhibitor, 0.5 mM ([b], EDTA, 10 mM); substrate, Phe-MCA (K_m = 0.05 mM), 0.04 mM; buffer, 50 mM Tris-HCl, pH 7.3; reaction condition, preincubation at 37 °C for 30 min without substrate, reaction at 37 °C for 20 min.

more quickly has not been found: Jc-peptidase may belong to a class of leucine aminopeptidases with novel substrate specificity.

A previous paper by the authors (*9*) reported that Jc-peptidase was not inhibited with 0.5 mM EDTA. Most aminopeptidases are known to be metalloenzymes (*19*), and divalent metal cations, usually zinc but sometimes other cations such as cobalt or manganese, putatively activate the water molecule in the catalytic site (*19*). We reexamined the effect of EDTA and metal ions. **Table 3** shows that enzyme activity was inhibited with 10 mM EDTA or 0.5 mM Zn^{2+} or Cu^{2+}. Vogt (*20*) and Stirling (*21*) obtained a similar result in aminopeptidase from *E. coli* having Mn^{2+} or Mg^{2+} in its active site. According to the kinetic study using a Lineweaver–Burk plot, Zn^{2+} was a competitive inhibitor (K_i = 20 μM) but Cu^{2+} was a noncompetitive one. Those results imply that the Jc-peptidase may be a metalloaminopeptidase of which the active site contains metal ion(s) other than Zn^{2+}.

Amino Acid Sequencing Analysis. The N-terminal sequence and an internal sequence were

NH_2-APIGVQLEIEENYVHMYNGF...

(K)EIFAATFNVDEETEA...

These sequences were compared with known sequences available in GenBank and SwissProt using the Basic Local Alignment Search Tool (BLAST) program (*22*), but no homology with other proteins was found. Sequences of the LAPs from different kingdoms (animal, plant, or bacteria) are highly conserved in the C-terminal domain, where the metal-binding and catalysis sites are located (*23*). For example, Tu et al. (*18*) purified two LAPs having similar substrate specificities from tomato and found homologies between them only in the central and C-terminal regions. Therefore, it may be natural that we found no homology with other LAPs using the N-terminal sequence because Jc-peptidase has a new substrate specificity.

To clarify the whole sequence of Jc-peptidase mentioned above, we tried RT-PCR to determine the sequence of cDNA for the enzyme. Unfortunately, we were unable to obtain any mRNA for Jc-peptidase from pollen extract; however, we detected a mRNA for actin as a positive control.

This is the first report for pollen aminopeptidase with metal ion(s) in its active site. Although its roles in pollen and in patients suffering from pollenosis are unknown, we infer that Jc-peptidase may participate in cedar pollenosis with cooperative action of proteinase and peptidase in pollen and the host.

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