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Isolation and Characterization of Aminopeptidase (Jc-peptidase) from Japanese Cedar Pollen (*Cryptomeria japonica*)

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An aminopeptidase, Jc-peptidase, was purified from Japanese cedar pollen by seven steps, including precipitation with ammonium sulfate, ion-exchange chromatography, gel filtration, hydrophobic interaction chromatography on phenyl-agarose, and high-performance liquid chromatography. Purified Jc-peptidease has a molecular weight of 42 kDa and hydrolyzes the synthetic substrates of L-phenylalanyl-4-methylcoumaryl-7-amide (Phe-MCA) with $K_m = 5 \times 10^{-5}$ M, Tyr-MCA with $K_m = 7 \times 10^{-4}$ M, Leu-MCA with $K_m = 1 \times 10^{-3}$ M, and Met-MCA with $K_m = 1 \times 10^{-3}$ M. Other MCA analogues such as Arg-MCA or Glu-MCA failed to serve as its substrates. The activity was inhibited in the presence of phebestin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl]-L-phenylalanine, with $K_i = 4.7 \times 10^{-5}$ M, or bestatin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, with $K_i = 1.1 \times 10^{-4}$ M. According to amino acid sequence analysis, the N-terminal amino group seems to be blocked. The physiological function of the aminopeptidase (Jc-peptidase) has not been clarified in vivo.

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

INTRODUCTION

Japanese cedar (Sugi; *Cryptomeria japonica*) pollen contains a number of protein components, some of which are closely associated with allergic disease as allergens in Japan. Two major allergens of Japanese cedar pollen have already been reported. The first major allergen with molecular weight of 41−45 kDa, Cry j 1, was isolated and characterized by Yasueda et al. (*1*). Taniguchi et al. (*2*) reported that its amino acid sequence shows a significant homology to bacterial pectate lyase. The second major allergen with molecular weight of 37 kDa, Cry j 2, was isolated and characterized by Sakaguchi et al. (*3*). Ohtsuki et al. (*4*) found that Cry j 2 has polymethylgalacturonase activity.

In a previous study (*5*), we isolated a serine protease, Df-protease, from house dust mite (*Dermatophagoides farinae*) which is known as a major allergen for extrinsic asthma, allergic rhinitis, or atopic dermatitis. Df-protease was found to catalyze the activation of the kallikrein-kinin system in human plasma and to be closely associated with mite-induced allergy (*5*, *6*). Therefore, we have interest in proteases contained in Japanese cedar pollen that is one of the major causes of pollenosis in Japan. The present study deals with the isolation of a new type of aminopeptidase from Japanese cedar pollen, Jc-peptidase, together with its substrate specificity and its physicochemical properties.

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MATERIALS AND METHODS

Materials. Japanese cedar pollen was obtained from Kanagawa Prefecture Natural Environment Conservation Center (Atsugi, Japan). L-Tyrosyl-4-methylcoumaryl-7-amide (Tyr-MCA) and Glu-MCA were obtained from Bachem (Bubendorf, Switzerland), and other synthetic substrates such as Phe-MCA and Leu-MCA were purchased from Peptide Institute Inc. (Osaka, Japan). Amastatin, arphamenine A, arphamenine B, and phebestin were purchased from Peptide Institute Inc., bestatin was from Boehringer Mannheim Biochemicals Co. (Indianapolis, IN), and leuhistin, actinonin, TPCK and TLCK were from Sigma Chemical Co. (St. Louis, MO). Phenyl-agarose and Sephadex G-100 were purchased from Sigma and Pharmacia Biotech (Uppsala, Sweden), respectively. DEAE-Toyopearl 650C and TSK gel super SW 3000 were purchased from Tosoh Co. (Tokyo, Japan). Vivaspin 500 (VS0101) and polyoxyethylene lauryl ether (Brij 35) were purchased from Vivascience Ltd. (Lincoln, UK) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Polyvinyl difluoride (PVDF) membrane and molecular weight standard mixture (SDS−PAGE) were obtained from Nippon Genetics Co., Ltd. (Tokyo, Japan) and Bio-Rad Laboratories (Hercules, CA), respectively. Other reagents used were of analytical grade.

Collection of Japanese Cedar Pollen. At first, clusters of young male flowers on cedar trees were covered with paper bags for crossing. After 40 days, the bags containing pollen and mature male flowers were cut off the trees. The pollen was collected from the bags with an exclusive vacuum cleaner and was stored in a desiccator at 4 °C.

Activity Measurement and Protein Quantification. The activity of peptidase from pollen in each purification step (described below in Results) was measured using Phe-MCA as a substrate. To 1.19 mL of 50 mM Tris−HCl (pH 7.3) containing 0.04 mM synthetic substrate and 0.01% Brij 35 was added 10 μL of the diluted protein preparation

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((0.1−2) × 10^{-3} unit), and the hydrolytic rate was fluorometrically determined at 37 °C with a Hitachi fluorometric spectrophotometer F-3000 (Tokyo, Japan). One unit is defined to release 1 μmol of 7-amino-4-methylcoumarine (AMC) in 1 min. The substrate specificity of the enzyme was measured as follows. To the various kinds of substrates (0.04 mM) was added the enzyme preparation obtained by purification Step 6 (1.5 × 10^{-3} unit) as the same procedure described above. The Km value was determined by measuring the enzymic activity using 0.01−0.1 mM of corresponding substrate. The inhibitory effect was measured by the same procedure described above in the presence and the absence of a inhibitor (0.5 mM).

A concentration of protein at each purification step was determined by the Bradford method (*7*) using a rapid protein quantification kit (Dojindo Laboratories, Kumamoto, Japan).

Electrophoresis and Blotting. Electrophoresis of the enzyme obtained by Step 6 was performed in 10% polyacrylamide gel at pH 8.8 in the presence of SDS and 2-mercaptoethanol by the method of Laemmli (*8*). The gel was stained with Coomassie brilliant blue R-250.

Further purified peptidase obtained by Step 7 was also applied to SDS−PAGE as described above to determine the sequence. The peptidase (about 10 pmol) on the gel was blotted electrophoretically to PVDF membrane, and stained with Coomassie brilliant blue R-250. The amino terminal sequence was analyzed at APLO Life Science Institute (Tokushima, Japan) using a Hewlett-Packard protein sequencing system model G1005A (Palo Alto, CA).

RESULTS AND DISCUSSION

Purification of Aminopeptidase. Purification of aminopeptidase, Jc-peptidase, from Japanese cedar pollen was carried out as follows.

Step 1. Extraction and Precipitation with Ammonium Sulfate. Pollen (50 g) was added to 1000 mL of 125 mM ammonium bicarbonate containing 0.01% polyoxyethylene lauryl ether (Brij 35) and stirred at 4 °C for 5 h. After centrifuging the sample, proteins in the supernatant (700 mL) were precipitated by adding ammonium sulfate with 90% saturation at 4 °C. The precipitate thus formed was collected by centrifugation at 14000*g* and was dissolved in 50% glycerol solution containing 85 mM NaCl and 0.01% Brij 35. The solution was dialyzed against 1 mM phosphate buffer (pH 7.3) containing 15 mM NaCl and 0.01% Brij 35 (Buffer A).

Step 2. Adsorption with Anion-Exchange Resin. The dialyzed solution was centrifuged (14000*g* for 20 min) to remove a small amount of precipitate, and the supernatant was passed through a glass filter (6.6 × 60 cm) containing 45 mL of DEAE-Toyopearl 650C equilibrated with Buffer A. After washing the resin with Buffer A, the adsorbed materials were eluted with 0.3 M NaCl dissolved in Buffer A. The eluate having the aminopeptidase activity was collected.

Step 3. Gel Filtration on Sephadex G-100 (coarse). The eluate was put on a column of Sephadex G-100 (5.0 × 100 cm) equilibrated with 50 mM citrate buffer (pH 6.0) containing 0.1 M NaCl and 0.01% Brij 35, and was fractionated by 11 mL/tube. Fractions having aminopeptidase activity were collected and dialyzed against Buffer A.

Step 4. Anion-Exchange Chromatography on DEAE-Toyopearl 650C. The protein solution was put on a column of DEAE-Toyopearl 650C (1.5 × 7.5 cm) equilibrated with Buffer A. After the column had been washed with Buffer A (30 mL) to remove unadsorbed materials, protein fractions with aminopeptidase activity were eluted with Buffer A with a linear gradient concentration of NaCl solution (0−0.35 M).

Step 5. Gel Filtration on Sephadex G-100 (fine). The eluate was put on a column of Sephadex G-100 (2.6 × 100 cm) in the same manner as Step 3 and was fractionated by 4 mL/tube. Fractions having aminopeptidase activity were collected and dialized against Buffer A.

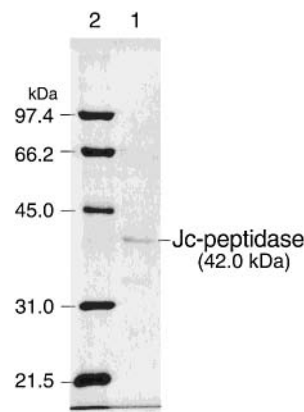

Figure 1. SDS–PAGE pattern of purified Jc-peptidase from Japanese Cedar. Photograph of SDS–PAGE pattern in 10% polyacrylamide gel containing 5% of 2-mercaptoethanol. Lane 1: Jc-peptidase at Step 6. Lane 2: Molecular weight markers (Bio-Rad Laboratories) containing phosphorylase b (MW 97,400), serum albumin (MW 66,200), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), and trypsin inhibitor (MW 21,500).

Step 6. Hydrophobic Interaction Chromatography on Phenyl-Agarose. To the eluate was added ammonium sulfate (final concentration 20%), and the mixture was applied to the column (bed volume 1 mL) equilibrated with 20% ammonium sulfate in Buffer A without Brij 35. After washing with 20% ammonium sulfate, followed by 10% ammonium sulfate dissolved in Buffer A without Brij 35 to remove proteins other than aminopeptidase, the solution with aminopeptidase activity was eluted with 5% ammonium sulfate in Buffer A. An aliquot of the active eluate was applied to SDS−PAGE (**Figure 1**). A major band at 42 kDa was accompanied by two minor bands.

Step 7. Gel Filtration on High-Performance Liquid Chromatography (HPLC). After the sample solution was concentrated 5 times by ultrafiltration with Vivaspin 500, an aliquot of the sample was put on a column TSK gel super SW 3000 (7.6 mm × 30 cm) equilibrated with 50 mM Tris−HCl pH 7.3 (flow rate 0.3 mL/min). Three fractions were obtained; one fraction had the Phe-MCA hydrolytic activity, but the other fractions did not have any activities to hydrolyze MCA substrates. Protein in the active fraction was named Jc-peptidase. Its molecular weight was estimated to be 43 kDa, which is in good agreement with that obtained by SDS−PAGE.

The amino-terminal sequence of Jc-peptidase on Coomassie-stained PVDF membrane was analyzed, but no N-terminal amino acid could be identified. The N-terminal amino group of intact Jc-peptidase is probably blocked, though there is a possibility that the N-terminus was protected during the processing such as electrophoresis and/or transfer system.

In a preliminary experiment, we found that the aminopeptidase in Japanese cedar was unstable. An aqueous solution of the aminopeptidase, however, was stable for a few days at 4 °C between pH 6 and 9 in the presence of Brij 35. Therefore, all purification processes except affinity chromatography (Step 6) and HPLC (Step 7) were carried out in the presence of 0.01% Brij 35. The aminopeptidase showed the optimum activity at pH 7.3. Collecting the fractions with the aminopeptidase activity, purified aminopeptidase was obtained, which is summarized in **Table 1**. The aminopeptidase was purified about 96-fold with 0.57% recovery and its specific activity was 6.7 units/mg (37 °C, pH 7.3).

Table 1. Purification of Jc-Peptidase

	step of purification	volume (mL)	protein (mg)	specific activity (unit/mg protein)	purification (times)	recovery (%)
	crude extract	700	290	0.070	1.0	100
1	ammonium sulfate precipitation	440	240	0.080	1.1	98
2	anion-exchange resin (filtrate)	58	38	0.43	6.1	81
3	gel filtration chromatography (Sephadex G-100, 5.0 × 100 cm)	140	8.3	1.2	18	50
4	anion-exchange chromatography (DEAE-Toyopearl 650C)	13	1.6	2.8	40	23
5	gel filtration chromatography (Sephadex G-100, 2.6 × 100 cm)	41	1.2	3.2	46	19
6	phenyl-agarose	4.0	0.19	3.7	53	3.5
7	HPLC	1.5	0.017	6.7	96	0.57

Table 2. Aminopeptidase Activities and K_m Values of Jc-Peptidase[a]

substrate	K_m	relative activity
monoamino acid		
Phe-MCA	5×10^{-5} (M)	100 (%)
Tyr-MCA	7×10^{-4}	55
Leu-MCA	1×10^{-3}	22
Met-MCA	1×10^{-3}	19
Cys(Bzl)-MCA		4
Ala-MCA, Arg-MCA, Lys-MCA, Glu-MCA, Pyr-MCA		1 ∼ 0
dipetide		
Gly-Phe-MCA, Lys-Ala-MCA, Gly-Pro-MCA		0
tripeptide		
Ala-Ala-Phe-MCA		0
N-blocked peptide		
Glt-Phe-MCA, Suc-Ala-Ala-MCA, Z-Leu-Leu-Leu-MCA, Suc-Ile-Ile-Trp-MCA, Boc-Gln-Gly-Arg-MCA, Glt-Ala-Ala-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Ala-Ala-Pro-Phe-MCA		0

[a] MCA: 4-Methylcoumaryl-7-amide (final 0.04 mM). Aminopeptidase activity measured with Phe-MCA as the substrate was taken as 100%.

Substrate Specificity. Because of the low recovery in the purification steps in the absence of Brij 35, the partially purified Jc-peptidase (Step 6) was tested for substrate specificity (**Table 2**). Among the substrates tested, Phe-MCA was found to be the most effective, and the Km value was calculated to be 5×10^{-5} M. Synthetic substrates, Tyr-, Leu-, and Met-MCAs, containing hydrophobic amino acid as well as Phe-MCA, were also hydrolyzed with the aminopeptidase with Km = 7×10^{-4} M, Km = 1×10^{-3} M, and Km = 1×10^{-3} M, respectively. The other synthetic substrates having acidic and basic amino acids in the N-terminal site could not be hydrolyzed with Jc-peptidase. In the case of dipeptidyl- and tripeptidyl-MCAs such as Gly-Phe-, Lys-Ala-, Gly-Pro-, and Ala-Ala-Phe-MCAs, the release of 7-amino-4-methylcoumarine (AMC) as a hydrolytic product of the above substrates could not be observed. Furthermore, N-blocked peptides shown in **Table 2** were not served as the substrate at all. It will be concluded that the aminopeptidase, Jc-peptidase, has a monopeptidyl aminopeptidase activity toward aromatic and hydrophobic amino acids.

Travis et al. (*9*−*11*) isolated peptidases from pollens of mesquite (*Prosopis veltina*) (*9*, *10*) and ragweed (*Ambrosia artermisiifolia*) (*11*), and their substrate specificities were, however, quite different from those for Jc-peptidase isolated by us.

Inhibition. A next series of experiments is concerned with the inhibition of the aminopeptidase activity of Jc-peptidase with various kinds of inhibitors using Phe-MCA as the substrate, which is shown in **Table 3**. Phebestin and bestatin inhibited the enzymic activity relatively among the inhibitors tested with

Table 3. Effect of Inhibitors on the Aminopepridase Activity of Jc-Peptidase with Phe-MCA as a Substrate[a]

Inhibitor	Relative activity (%)	Structure[b]
Phebestin	27	
Bestatin	39	
Amastatin	74	
Leuhistin	91	
Arphamenine A	91	
Arphamenine B	93	
Actinonin	98	

[a] K_i value: phebestin (4.7×10^{-5} M), bestatin (1.1×10^{-4} M). [b] Structure of side chain of N-terminal amino acid analogue.

the K_i values of 4.7×10^{-5} M and 1.1×10^{-4} M, respectively. Both inhibitors are phenylalanine analogues, having a benzyl group near the α-amino group in the molecule, which are known as competitive inhibitors of aminopeptidases M and N. Inhibition of Jc-peptidase with bestatin is, however, not so strong (K_i = 1.1×10^{-4} M) in comparison with aminopeptidases M (leucine aminopeptidase) from swine kidney with K_i value of 2×10^{-8} M (*12*). Although Jc-peptidase was slightly inhibited with amastatin, an inhibitor for aminopeptidase A, other aminopeptidase inhibitors such as leuhistin, arphamenine A, arphamenine B, and actinonin scarcely inhibited the enzymic activity. Other protease inhibitors such as EDTA, monoiodoacetic acid, TPCK, and TLCK could not inhibit the Jc-peptidase activity at all. Jc-peptidase may recognize the hydrophobic groups including a phenyl or alkyl group near an amino group in the inhibitor molecules.

It was reported that Df-protease from house dust mite (*Dermatophagoides farinae*) catalyzed the activation of the kallikrein-kinin system in human plasma and is closely associated with mite-induced allergy (*6*). Therefore, the aminopeptidase isolated from Japanese cedar pollen, Jc-peptidase, is expected to associate with some allergic reactions. However, we have no evidence for any relation between them. Jc-peptidase could not release kinin from normal human plasma. The radioallergosorbent test (RAST) of sera from 10 pollenosis patients revealed that the IgE-titer toward Jc-peptidase was much lower than those toward Cry j 1 or Cry j 2 (Data not shown).

From the results obtained above, Jc-peptidase is a novel aminopeptidase with unique substrate specificity but is not a major antigen. Now we are exploring the physiological role of Jc-peptidase in the inflammation of pollenosis.

ABBREVIATIONS USED

Actinonin, (3-[[1-[[2-(hydroxymethyl)-1-pyrrolidonyl]carbonyl]-2-methylpropyl]-carbamoyl]octanohydroxamic acid; amastatin, [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl]-L-valyl-L-valyl-L-aspartic acid; arphamenine A, (2*R*,5*S*)-5-amino-8-guanidino-4-oxo-2-phenylmethyloctanoic acid; arphamenine B, (2*R*,5*S*)-5-amino-8-guanidino-4-oxo-2-*p*-hydroxyphenylmethyloctanoic acid; bestatin, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine; leuhistin, (2*R*,3*S*)-3-amino-2-hydroxy-2-(1H-imidazol-4-ylmethyl)-5-methylhexanoic acid; phebestin, (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-valyl-L-phenylalanine; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone; TLCK, N^{a}-tosyl-L-lysyl chloromethyl ketone.

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