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ARTICLE

Purification and Characterization of a New Rhizopuspepsin from *Rhizopus oryzae* NBRC 4749

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A secretory aspartic protease (also termed as rhizopuspepsin) was purified from *Rhizopus oryzae* NBRC 4749 by ion exchange chromatography with a yield of 45%. The enzyme was a nonglycoprotein with a molecular mass of 37 kDa as determined by SDS−PAGE analysis. N-terminal sequence and LC-MS/MS analyses revealed that this rhizopuspepsin corresponded to the hypothetical protein RO3G_12822.1 in the *R. oryzae* genome database. Comparison of genomic and cDNA genes demonstrated that the rhizopuspepsin contained two introns, whereas only one intron was reported in other rhizopuspepsin genes. Phylogenetic analysis also indicated that this rhizopuspepsin was distinct from other rhizopuspepsins. The temperature and pH optima for the purified rhizopuspepsin were 50 °C and pH 3.0, respectively, and a half-life of about 3.5 h was observed at 40 °C. The enzyme preferentially cleaved the peptides with hydrophobic and basic amino acids in the P1 site but had no activity for the Glu, Pro, Trp, and aliphatic amino acids containing the β-branch side chain.

KEYWORDS: ***Rhizopus oryzae*; rhizopuspepsin; purification; cleavage specificity; phylogeny; intron**

INTRODUCTION

Rhizopus oryzae, a filamentous fungus, is widely used in the production of fermented foods and alcoholic beverages in Southeast Asia, China, and Japan (*1*). In recent years, it has also been employed in the production of oriental-style yogurt (*2*, *3*). *R. oryzae* is isolated frequently from patients suffering from zygomycosis disease (*4*). In zygomycosis, both the penetration of vessel walls by hyphae and procoagulatory activity were observed in human beings that may be associated with the extracellular proteolytic activity of *Rhizopus* concerned (*5*). Aspartic proteases are one of the dominant secretory proteases from *Rhizopus* spp. (*6*) and are referred to as rhizopuspepsins (EC 3.4.23.21). To the best of our knowledge, only four rhizopuspepsins have been purified and characterized from *Rhizopus microsporus* var. *rhizopodiformis* (*5*), *Rhizopus chinensis* Saito (*7*), *Rhizopus hangchow* No.3545 (*8*), and *R. oryzae* MTCC 3690 (*9*). Among them, *R. chinensis* rhizopuspepsin has been well studied in terms of cleavage specificity (*10*−*12*) and crystal structure (*13*). The rhizopuspepsin exhibits cleavage specificity to the peptides with hydrophobic amino acids in the P1 and P1′ sites, but its action is less stringent than that of pepsin, and it has trypsin-like ability to cleave substrates with Lys in the P1 site (*10*−*12*). However, the cleavage specificities of rhizopuspepsins from *R. oryzae* have not been characterized yet.

Secretory aspartic protease (Sap) is often encoded by a single-copy gene in the fungi such as *Aspergillus fumigatus* (*14*), *Coccidioides immitis* (*15*), and *Glomerella cingulata* (*16*). However, the Saps secreted from *Rhizopus niveus* Yamazaki IFO 4810 (*17*) and *Candida albicans* (*18*) were reported to be the products of multigene families. Recently, the uncharacterized genome of *R. oryzae* 99−880 had become available in a database (http://www.broad.mit.edu/tools/data/seq.html), in which the rhizopuspepsin gene family is proposed to contain five members showing 76−92% identity in their amino acid sequence and one intron in each gene. Expression of genes in this gene family had been investigated at the transcriptional level (*19*). However, only one aspartic protease has been purified and characterized from *R. oryzae* MTCC 3690 (*9*). Moreover, the identity of the purified enzyme corresponding to the members of rhizopuspepsin gene family has not been experimentally determined. In this study, we report the purification and characterization of a new member of rhizopuspepsin corresponding to the hypothetical protein RO3G_12822.1 found in the *R. oryzae* 99−880 genome.

MATERIALS AND METHODS

Strains, Plasmid, and Growth Conditions. *R. oryzae* NBRC 4749, obtained from the Japan Society for Culture Collections, was grown in glucose−casein medium (GC medium, pH 5.4) consisting of 0.1% KH_2PO_4, 0.2% KCl, 0.05% $MgCl_2$, 0.001% $FeCl_3$, 0.001% $ZnCl_2$, 2% glucose, and 1% casein. Spores suspended in GC medium (6.5×10^4 spores/mL) were cultivated under aerobic conditions at 30 °C for 36 h. *Syncephalastrum racemosum* BCRC 31638, obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), was grown in Potato Dextrose Broth (Difco, Detroit, MI) under aerobic conditions at 24 °C for 24 h. Plasmid pUC1918 (*20*) was used for gene cloning in *Escherichia coli*. The *E. coli* NovaBlue carrying the recombinant plasmids was grown aerobically at 37 °C in Luria−Bertani medium. Ampicillin (100 μg/mL) was added to the medium wherever necessary.

DNA Manipulations. Genomic DNA was prepared from mycelia of *R. oryzae* and *S. racemosum* according to the method described previously (*21*). DNA amplification, purification, and cloning were performed according to the standard protocols (*22*). The cDNA was

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prepared from *R. oryzae* and *S. racemosum* using RNeasy and OneStep RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The location of intron in the genes was determined by comparing the DNA sequences between cDNA and genomic DNA for rhizopuspepsin and syncephapepsin genes.

Enzyme Activity Assay. Proteolytic activity was measured using bovine serum albumin (BSA) (Bio-Rad Laboratories, Hercules, CA) as substrate. Reaction mixture (800 μL) containing 0.1 μg enzyme, 10 mg BSA, and 100 mM glycine-HCl buffer (pH 3.0) was incubated at 37 °C for 30 min. The reaction was stopped by adding 200 μL of 2 M perchloric acid (Sigma, St. Louis, MO). Precipitated protein was removed by centrifugation at 14,000*g* for 5 min. One enzyme unit was defined as the amount of enzyme that increases 1.0 of absorbance at 280 nm per min under the assay conditions (*18*).

Milk-clotting activity was determined as described by Arima et al. (*23*). A 10% suspension of skim-milk powder in 10 mM $CaCl_2$ was used as the substrate. The unit of milk-clotting activity is defined as the amount of enzyme capable of clotting 1.0 mL of substrate in 40 min at 37 °C.

Purification of Proteases. The culture broth was centrifuged at 8000*g* and 4 °C for 20 min to remove mycelia. Fast protein liquid chromatography (FPLC) analysis was performed using an AKTA purifier (GE Healthcare Biosciences, Uppsala, Sweden). The supernatant was filtered through a 0.45 μm membrane filter (Millipore, Milford, MA) and injected into a HiTrap Q Fast Flow column (GE Healthcare Biosciences), previously equilibrated with 40 mM citric acid−Na_2HPO_4 buffer (pH 5.0). The column was eluted with a flow rate of 0.3 mL/min for 20 mL using the equilibration buffer and then with a NaCl stepwise gradient from 0 to 47 mM for 5 mL, at 47 mM for 20 mL, 47 to 113 mM for 5 mL, and at 47 mM for 10 mL. Fractions were collected, and proteins in each fraction were separated on 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) according to the standard protocols (*22*). The gel was stained with 0.1% silver nitrate. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories) using BSA as the standard.

Determination of Molecular Mass. The molecular mass of the proteins was determined using TotalLab software (Nonlinear, Durham, NC) on the polyacrylamide gel following manufacturer's instructions. In addition, the protein sample (0.5 μL) was spotted onto the MALDI target and allowed to air-dry. Then, 0.5 μL of matrix solution [saturated solution of sinapinic acid in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid] was applied to the dried sample and dried again. The analysis of molecular mass was performed using Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in linear mode with an accelerating voltage of 20 kV.

Identification of Proteases. Each protein band of interest in SDS−PAGE was excised from the gel for in-gel digestion according to methods described previously (*24*, *25*). The gel pieces were washed twice with 50 μL of 50% acetonitrile and then dried for 5 min in a SpeedVac (Eppendorf, Hamburg, Germany). An equivalent volume of 50 mM ammonium bicarbonate solution containing 20 ng/μL of trypsin (Promega, Madison, WI) was added, and the gel pieces were incubated at 37 °C for 16 h. Nanoscale capillary LC-MS/MS analysis of the trypsin-cleaved materials (*26*) was performed to identify the proteins from *R. oryzae* NBRC 4749 using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTAR^{XL} quadrupole-time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems, Foster City, CA). The nanoscale capillary LC separation was performed on a RP C18 column (15 cm, 75 μm i.d.) with a flow rate of 200 nL/min and a 70 min linear gradient of 5−50% buffer B. Buffer A contained 0.1% formic acid in 2% aqueous acetonitrile; buffer B contained 0.1% formic acid in 98% aqueous acetonitrile. Data acquisition was performed by automatic Information Dependent Acquisition (Applied Biosystems). The product ion spectra generated by nanoLC-MS/MS were searched using Mascot Server (http://www.matrixscience.com) or Pro ID program (version 1.1, Applied Biosystems). The mass tolerance of both precursor ion and fragment ions was set to 0.3 Da.

To determine the N-terminal amino acid sequence of protease, the purified enzyme on the polyacrylamide gel was transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting in 3-(cyclohexylamino) propanesulfonic acid buffer at pH 11.0 and stained with Coomassie blue R-250. The stained portion was excised and used for N-terminal sequencing. The N-terminal sequence was assayed using a 492 cLC protein sequencer (Applied Biosystems).

Glycoprotein Staining. Purified proteins were denatured in a solution of 2% SDS and 5% β-mercaptoethanol for 5 min at 100 °C. Subsequently, the denatured proteins were resolved by SDS−PAGE and subjected to carbohydrate staining using an improved periodic acid−Schiff (PAS) reaction (*27*). The polyacrylamide gel was soaked for 16 h in solution A (10% acetic acid and 25% isopropanol) and then placed in solution B (0.2% periodic acid) for 1 h. After rinsing with distilled water four times, the gel was soaked in solution C (21% NaOH, 1.4% ammonium hydroxide, and 0.8% silver nitrate) for 10 min. Finally, the polyacrylamide gel was washed in four changes of distilled water and then incubated in staining solution (0.05% citric acid, 0.1% formaldehyde, and 15% methanol) for 5−10 min. The glycoprotein of *Aspergillus sojae* leucine aminopeptidase (LAP) (*28*) was used as a positive control in the glycoprotein analysis.

Phylogenetic Analysis. Amino acid sequence alignments were performed with Clustal W (*29*). The evolutionary history was inferred by using the Minimum Evolution method (*30*). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (*31*). The evolutionary distances were computed using the JTT matrix-based method (*32*) and expressed as the units of the number of amino acid substitutions per site. The Minimum Evolution tree was searched using the Close-Neighbor-Interchange algorithm (*33*) at a search level of 1. The Neighbor-Joining algorithm (*34*) was used to generate the initial tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Phylogenetic analyses were conducted in MEGA4 (*35*). Sap of *S. racemosum*, also termed as syncephapepsin (*36*), was used as an outgroup in this study.

Enzyme Characterization. The protease activity was assayed against BSA at different pH values using 100 mM buffers of glycine-HCl (pH 2.2 to 3.6) and citric−Na_2HPO_4 (pH 2.6 to 6.0). The effect of temperature on protease activity was determined in 100 mM glycine-HCl buffer (pH 3.0) at temperatures ranging from 20 to 90 °C. Thermostability was assayed by incubating the purified enzyme in 100 mM glycine-HCl buffer at pH 3.0 and 40 °C using BSA as substrate or in 100 mM K_2HPO_4−KH_2PO_4 buffer at pH 6.5 and 60 °C using casein as substrate.

Chromogenic peptides were synthesized from Kelowna International Scientific Inc. (Taiwan). The chromogenic peptides, Lys-Pro-Ala-Lys-Phe (P1)-Nph-Arg-Leu (Nph, *p*-nitrophenylalanine), with substitutions in P1 site (Arg, Asp, Cys, Glu, Ile, Leu, Lys, Phe, Pro, Trp, Tyr, and Val), were used to analyze the cleavage specificity of the enzyme. Kinetic analysis was performed at 37 °C and pH 3.0 in 100 mM glycine-HCl buffer containing substrates at various concentrations between 10 and 60 μM. The decrease in absorbance at 300 nm was monitored using Spectrophotometer DU-800 (Beckman Instruments, Palo Alto, CA). K_m and k_{cat} values were obtained using nonlinear regression fitting to the Michaelis−Menten equation.

Nucleotide Sequence Accession Numbers. The newly identified sequences for rhizopuspepsin from *R. oryzae* NBRC 4749 and syncephapepsin from *S. racemosum* BCRC 31638 have been deposited in GenBank under accession numbers FJ539002 and FJ539001, respectively.

RESULTS AND DISCUSSION

Purification of Proteases from *R. oryzae*. Proteins have been used as the sole nitrogen source in the culture media for the induction of protease production (*19*). When casein was used as the sole nitrogen source in GC medium for *R. oryzae* NBRC 4749, protease activity in the culture broth reached the maximal level in 36 h (data not shown). The culture broth was centrifuged to remove the pellets, and the supernatant was subjected to chromatography on a HiTrap Q Fast Flow column. The bound proteins were eluted using stepwise gradients of 0 to 47 mM and 47 to 113 mM NaCl. The resulting elution profile showed two separate peaks, A and B (**Figure 1A**). The fractions of the two peaks were assayed for protease activity and homogeneity. Two proteases, designated as protease A and B, corresponding to peaks A and B, respectively, were purified and appeared as electrophoretically homogeneous by one-step column chromatography

Table 1. Purification of Proteases from *R. oryzae* NBRC 4749

			proteolytic activity [a]			milk-clotting activity [b]		
procedure	total volume (mL)	total protein (mg)	total activity (U)	specific activity (U/mg)	yield (%)	total activity (U)	specific activity (U/mg)	yield (%)
supernatant	50	3.12	5.59	1.79	100	157.15	50.37	100
Q sepharose								
peak A	13	0.10	2.52	25.18	45	111.75	1117.47	71
peak B	10	0.08	2.18	27.25	39	25.54	319.25	16

[a] Enzyme activity was determined using BSA as substrate. [b] Enzyme activity was determined using skim milk as substrate.

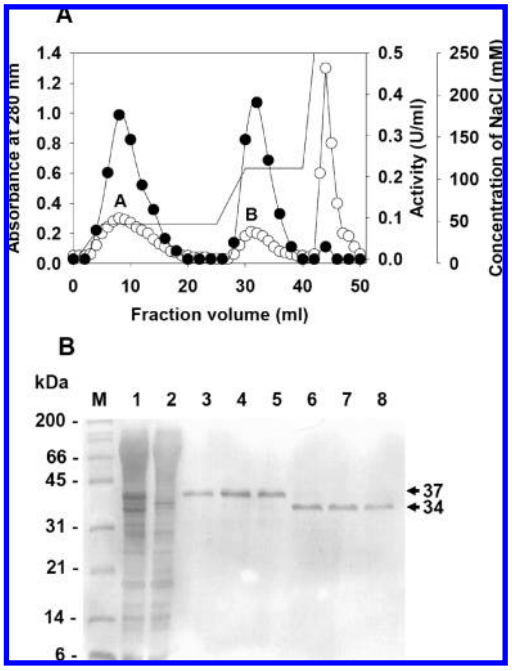

Figure 1. Elution profile of FPLC (**A**) and SDS−PAGE analyses (**B**) of the proteases from *R. oryzae* NBRC 4749. (●), proteolytic activity; (○), absorbance at 280 nm; (—), NaCl gradient. Lane M, molecular weight markers; lane 1, culture supernatant; lane 2, flowthrough; lanes 3−5, eluted fractions from peak A; lanes 6−8, eluted fractions from peak B.

(**Figure** 1**B**). The molecular masses of proteases A and B estimated by SDS−PAGE were 37 and 34 kDa, respectively. The molecular mass of protease A is close to the rhizopuspepsin from *R. hangchow* No.3545 (37 kDa) (*8*) and that of protease B is close to the rhizopuspepsins from *R. oryzae* MTCC 3690 (34 kDa) (*9*), *R. microsporus* var. *rhizopodiformis* (34.5 kDa) (*5*), and *R. chinensis* Saito (35 kDa) (*7*). The proteolytic activities of proteases A and B were 25.18 and 27.25 U/mg, respectively. The yield of proteases A and B were 45% and 39%, respectively (**Table** 1). The purification of proteases from *R. oryzae* NBRC 4749 only involved one-step column chromatography, whereas three to four steps were required for the proteases from *R. chinensis* Saito (*7*), *R. oryzae* MTCC 3690 (*9*), and *R. hangchow* No.3545 (*8*).

Besides hydrolytic activity, rhizopuspepsins from *R. oryzae* also have milk clotting activity, which is important in yogurt and cheese production (*2*, *9*). We found that the milk-clotting activity of proteases A and B were 1117.47 and 319.25 U/mg, respectively (**Table** 1). The purified protease A possessed 71% of total milk-clotting activity and contributed to the major milk-clotting activity in a culture supernatant of *R. oryzae* NBRC 4749.

Identification of the Purified Proteases. The tryptic peptides of protease A determined from LC-MS/MS analysis had no similarity to proteins in the NCBI GenBank database but were identified as the hypothetical protein RO3G_12822.1 in the *R. oryzae* 99−880 genome database (http://www.broad.mit.edu/tools/data/seq.html) with 44.9% sequence coverage (data not shown). The N-terminal amino acid sequence (GSGSVPVTDEGNDVE) of protease A was also identical to the amino acid sequence at position 68−82 of the hypothetical protein RO3G_12822.1. This protein not only displayed 67−72% identity to rhizopuspepsins 1 to 5 from *R. oryzae* 99−880, rhizopuspepsins 1 to 5 from *R. niveus* Yamazaki IFO 4810 (*17*), rhizopuspepsin from *R. microsporus* var. *rhizopodiformis* (*5*), and rhizopuspepsin from *R. chinensis* Saito (*37*, *38*) but also exhibited 60% identity to syncephapepsin from *S. racemosum* No.11 (*36*). Although the molecular mass of rhizopuspepsin from *R. hangchow* No.3545 is the same as that of protease A from *R. oryzae* NBRC 4749, differences in N-terminal amino acid sequence indicated that these two proteins are encoded by different genes. Analysis of amino acid sequence revealed that the conserved residues of hydrophobic−hydrophobic-Asp-Thr/Ser-Gly, hydrophobic−hydrophobic-Gly, and Tyr-Gly-X-Gly in the catalytic regions of aspartic protease (*39*, *40*) were also found in the hypothetical protein RO3G_12822.1. Taken together, the hypothetical protein RO3G_12822.1 was a new member of rhizopuspepsin in *R. oryzae* and designated as rhizopuspepsin 6.

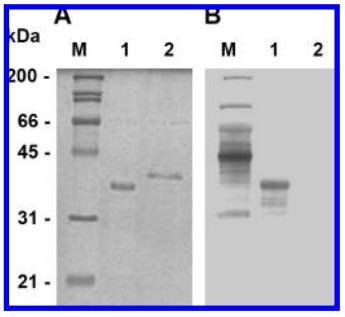

Figure 2. SDS−PAGE analysis of rhizopuspepsin 6 with Coomassie blue staining (**A**) and PAS carbohydrate staining (**B**). Lane M, molecular weight markers; lane 1, *A. sojae* LAP; lane 2, rhizopuspepsin 6.

The deduced amino acid sequence of the rhizopuspepsin 6 comprised 393 residues with the expected molecular mass of 40,986 Da. The data of the N-terminal amino acid sequence of purified rhizopuspepsin 6 suggested that 67 residues corresponding to the prepro region were removed during maturation of the enzyme. Since rhizopuspepsin 6 was not glycosylated (**Figure** 2), the calculated molecular mass of mature rhizopuspepsin 6 would be 33,924 Da, which is in accordance with 33,935 Da obtained from MALDI-TOF mass spectrum analysis (**Figure** 3).

LC-MS/MS analysis revealed that the protease B was a mixture containing the proteases corresponding to rhizopuspepsins 2, 4, and 5 in the *R. oryzae* 99−880 genome database with sequence coverages of 44%, 22%, and 7%, respectively (data not shown). These findings indicated that rhizopuspepsins 2, 4, and 5 were also produced in our culture conditions.

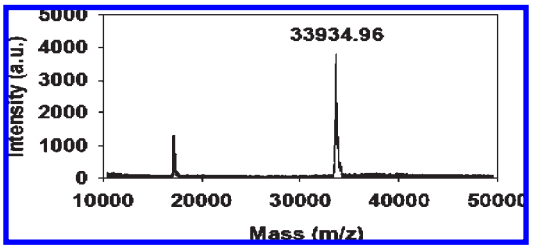

Figure 3. MALDI-TOF mass spectrum of purified rhizopuspepsin 6. The single charged molecular ion is labeled.

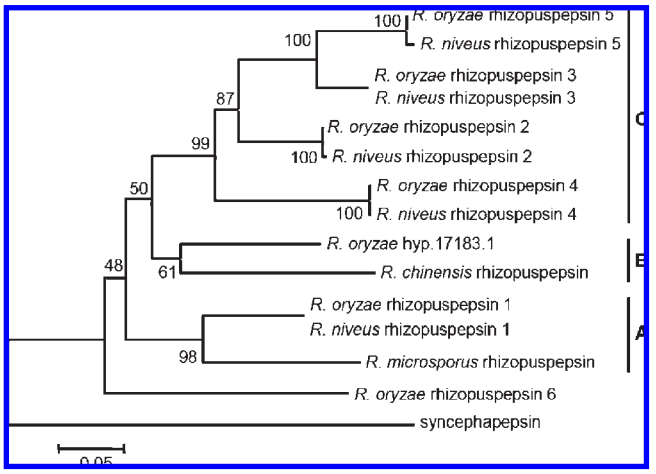

Figure 4. Minimum Evolution phylogenetic tree inferred from the amino acid sequences of rhizopuspepsins. The numbers at the nodes represent the bootstrap values for 1000 replications. The scale bar corresponds to the number of amino acid substitutions per site. Clades A, B, and C described in the text are indicated by A, B, and C, respectively. Amino acid sequence accession numbers at Broad Institute are as follows: *R. oryzae* rhizopuspepsin 1, RO3G_02201.1; *R. oryzae* rhizopuspepsin 2, RO3G_01762.1; *R. oryzae* rhizopuspepsin 3, RO3G_13330.1; *R. oryzae* rhizopuspepsin 4, RO3G_07001.1; *R. oryzae* rhizopuspepsin 5, RO3G_01236.1; *R. oryzae* rhizopuspepsin 6, RO3G_12822.1; *R. oryzae* hypothetical protein, RO3G_17183.1. Amino acid sequence accession numbers at NCBI are as follows: *R. niveus* rhizopuspepsin 1, P10602; *R. niveus* rhizopuspepsin 2, P43231; *R. niveus* rhizopuspepsin 3, Q03699; *R. niveus* rhizopuspepsin 4, Q03700; *R. niveus* rhizopuspepsin 5, P43232; *R. chinensis* rhizopuspepsin, P06026; *R. microspores* rhizopuspepsin, CAA72511; *S. racemosum* syncephapepsin, P81214.

Phylogeny and Gene Structure of Rhizopuspepsins. To ascertain the relationship among the rhizopuspepsins, a phylogenetic tree was constructed using syncephapepsin as an outgroup. The rhizopuspepsins could be separated into 3 clades (**Figure** 4). *R. oryzae* 99−880 rhizopuspepsin 1, *R. niveus* Yamazaki IFO 4810 rhizopuspepsin 1, and *R. microsporus* var. *rhizopodiformis* rhizopuspepsin were clustered into clade A. *R. oryzae* 99−880 hypothetical protein RO3G_17183.1 and *R. chinensis* Saito rhizopuspepsin formed clade B. Rhizopuspepsins 2 to 5 from *R. oryzae* 99−880 and *R. niveus* Yamazaki IFO 4810 were grouped into clade C. Interestingly, rhizopuspepsin 6, identified in this study, was placed outside of these three clades. Similar phylogenetic topology was also obtained from the analysis using the Neighbor-Joining method (*34*) (data not shown).

The patterns of gene structure have been widely used to get insight into the evolution of multigene families (*41*). The exon−intron organization is commonly conserved in genes belonging to the same family (*42*). Comparison of genomic and cDNA genes revealed that rhizopuspepsin 6 from *R. oryzae* NBRC 4749 and

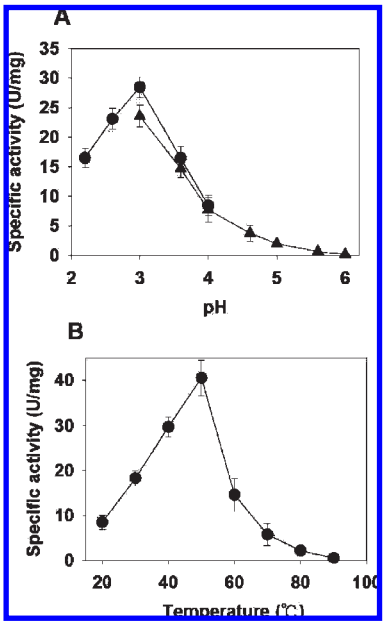

Figure 5. Effect of pH (**A**) and temperature (**B**) on protease activity. (**A**) Protease activity was measured at 37 °C in 100 mM glycine-HCl buffer (●) and 100 mM citric-Na_2HPO_4 buffer (▲). (**B**) Protease activity was measured in 100 mM glycine-HCl buffer at pH 3.0. Data are the means of three independent experiments. Bars indicate standard deviation (SD).

syncephapepsin from *S. racemosum* BCRC 31638 contained two and three introns, respectively, whereas other reported rhizopuspepsin genes from *R. oryzae* 99−880, *R. niveus* Yamazaki IFO 4810 (*17*), and *R. chinensis* Saito (*37*, *38*) possessed only one intron. Decoding analysis indicated that the introns in rhizopuspepsin and syncephapepsin genes belonged tothe phase 1 intron in which the exon is asymmetric (data not shown). Both phylogenetic and exon−intron structure analyses revealed that the rhizopuspepsin 6 gene is distinct from other reported rhizopuspepsin genes. Since the locations of introns 1 and 2 in the genes encoding rhizopuspepsin 6 and syncephapepsin were similar, it is likely that the two genes evolved from a common ancestor.

Enzymatic Properties of Rhizopuspepsin 6. The optimal pH of rhizopuspepsin 6 is pH 3.0 (**Figure** 5**A**), which is similar to the rhizopuspepsins from *R. chinensis* Saito (*7*) and *R. microsporus* var. *rhizopodiformis* (*5*). As shown in **Figure** 5**B**, hydrolytic activity of rhizopuspepsin 6 was increased with increase in temperature, reaching a maximum at 50 °C, which is lower than that of rhizopuspepsins (60 °C) from *R. chinensis* Saito (*7*) and *R. oryzae* MTCC 3690 (*9*). Thermostability of rhizopuspepsin 6 is shown in **Figure** 6. The enzyme exhibited a half-life of about 10 min and 3.5 h at 60 and 40 °C, respectively. However, the rhizopuspepsin reported from *R. oryzae* MTCC 3690 exhibited a half-life of about 20 min at 60 °C and remained fully active after incubation at 40 °C for 60 min (*9*). The data indicated that the gene encoding rhizopuspepsin 6 is different from that encoding *R. oryzae* MTCC 3690 rhizopuspepsin.

Cleavage Specificity of Rhizopuspepsin 6. Previous studies have reported that the rhizopuspepsin from *R. chinensis* Saito cleaved the peptides with Ala, Leu, Lys, Phe, and Tyr in the P1 site but had no significant activity to those with Glu, Gly, Ile, Ser, and Val (*10*−*12*). The cleavage specificity of purified rhizopuspepsin 6 was determined using a series of chromogenic peptides with amino acid variation in the P1 site (**Table** 2). We found that rhizopuspepsin 6 could hydrolyze the peptides with Arg, Asp, Cys, Leu, Lys, Phe, and Tyr in the P1 site and exhibited k_{cat}/K_m values of 113.61, 35.26, 73.29, 283.05, 87, 235.48, and

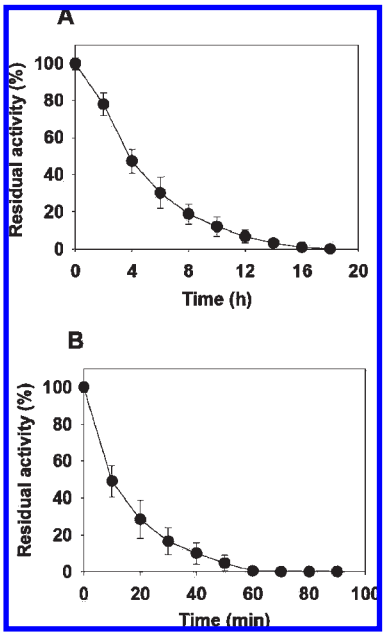

Figure 6. Thermostability of rhizopuspepsin 6. (**A**) Enzyme solution was incubated at pH 3.0 and 40 °C for 18 h prior to assaying the activity using BSA as the substrate. (**B**) Enzyme solution was incubated at pH 6.5 and 60 °C for 90 min prior to assaying the activity using casein as the substrate. Data are the means of three independent experiments. Bars indicate SD.

Table 2. Kinetic Parameters of Purified Rhizopuspepsin 6 for Substrate Series with Systematic Substitutions in the P1 Site[a]

P1 site[b]	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Arg	16.22 ± 2.09	143 ± 22	113.61
Asp	5.39 ± 2.56	154 ± 79	35.26
Cys	6.52 ± 1.24	90 ± 22	73.29
Glu	-[c]	ND[c]	ND
Ile	-	ND	ND
Leu	25.83 ± 3.99	92 ± 17	283.05
Lys	16.98 ± 3.47	196 ± 44	87
Phe	28.75 ± 4.93	122 ± 23	235.48
Pro	-	ND	ND
Trp	-	ND	ND
Tyr	16.99 ± 1.68	213 ± 22	79.7
Val	-	ND	ND

[a] Enzyme assays were performed in 100 mM glycine-HCl buffer at 37 °C (pH 3.0). [b] Chromogenic peptides Lys-Pro-Ala-Lys-Phe(P1)-Nph-Arg-Leu have different substitution in the P1 site. [c] Symbol: -, no detectable activity observed under assay conditions; ND, not determined.

79.7 mM^{-1} s^{-1}, respectively, whereas no significant activity was found for the P1 site with Glu, Ile, Pro, Trp, and Val. The preferential cleavage sites for rhizopuspepsin 6 were similar to those of *R. chinensis* Saito rhizopuspepsin (*10*). However, the preferential order was different in the two enzymes. For instance, rhizopuspepsin 6 had the highest activity to the peptides with Leu in the P1 site, whereas *R. chinensis* Saito rhizopuspepsin was most active to Phe.

We found that rhizopuspepsin 6 preferentially cleaved the peptides with hydrophobic amino acids in the P1 site (**Table** 2). However, this enzyme had no significant activity to the amino acids containing a β-branch side chain, such as Val and Ile, in the P1 site. This specificity seems to be a general feature of Saps from *C. albicans* (*43*), *S. racemosum* No.11 (*36*), *R. hangchow* No.3545 (*8*), *R. chinensis* Saito, *Mucor miehei*, and *Cladosporium* sp. (*11*). Although Trp is referred as a hydrophobic amino acid, rhizopuspepsin 6 had no significant activity to the peptide with Trp in the P1 site. Similar catalytic properties were found for the Saps from *C. albicans* (*43*) but not for *S. racemosum* No.11 Sap (*36*), which was active to the peptide with Trp in the P1 site.

The diversity of cleavage specificity to the peptides with charge amino acids in the P1 site was found in fungal Saps, such as *C. albicans*. Sap2 preferentially cleaved at Lys but exhibited no significant activity to Arg, Asp, and Glu; Sap3 preferentially cleaved at all charge amino acids; Sap1 and Sap6 did not have significant activity to charge amino acids (*43*). Rhizopuspepsin 6 was able to cleave the peptides with Arg, Asp, and Lys in the P1 site but showed no significant activity to Glu, unlike *R. hangchow* No.3545 rhizopuspepsin showing no significant activity to Arg, Glu, and Lys (*8*).

In conclusion, our study provides experimental evidence of the hypothetical protein RO3G_12822.1 belonging as a new member of the rhizopuspepsin gene family. This enzyme could easily be purified by one-step column chromatography from the culture broth of *R. oryzae* NBRC 4749. The purified enzyme possessed high milk-clotting activity and exhibited considerable differences in cleavage specificity, optimal reaction temperature, and thermostability from those of previously reported rhizopuspepsins. Further work on the overexpression of this enzyme is needed to evaluate its applications in the food industry.

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