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Oryzacystatin-II, a Cystatin from Rice (*Oryza sativa* L. *japonica*), Is a Dimeric Protein: Possible Involvement of the Interconversion between Dimer and Monomer in the Regulation of the Reactivity of Oryzacystatin-II

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We examined the biochemical and structural properties of oryzacystatin-II, a phytocystatin in rice (*Oryza sativa* L. *japonica*), under heat-stress conditions. The enzyme inhibitory reactivity of oryzacystatin-II was enhanced by heating in a temperature-dependent manner and reached a maximum level by heating at 65 °C for 10 min. Size-exclusion chromatography showed that oryzacystatin-II forms a homodimer at ambient temperature and that the enhancement of inhibitory reactivity is due to the conversion of the dimeric to a monomeric form. The monomeric form of oryzacystatin-II reverted to the dimer during storage at 4 °C, suggesting that dimerization is an intrinsic property of oryzacystatin-II. The affinity of the monomer for cysteine proteinases was significantly higher than that of the dimer. This is the first paper to describe the noncovalent dimerization for a cystatin under nonstress conditions.

KEYWORDS: Oryzacystatin-II; cystatin; dimerization; cysteine proteinase inhibitor

INTRODUCTION

The cystatin superfamily of proteins is a class of evolutionarily related inhibitors of cysteine proteinases and is widely distributed among vertebrates, invertebrates, and plants. These inhibitors play important roles in homeostasis and the defense mechanisms against infection via the regulation of endogenous/exogenous cysteine proteinases (*1*, *2*). The mammalian members have been classified into three families (*3*). Family 1 (stefin family) consists of proteins lacking disulfide bonds with molecular mass of ca. 11 kDa. Family 2 is called cystatin family and is composed of proteins having two intramolecular disulfide loops with the molecular mass of 13−14 kDa. The third family is the kininogen family. The members of this family have the highest molecular weights (ca. 60−120 kDa) and contain three repeats of the cystatin domain. In contrast, while the cystatins of plant origin, known as phytocystatins, show a high degree of sequence similarity to family 2 cystatins, they resemble the stefin family by virtue of the absence of disulfide bonds and cysteine residues. The phytocystatins also possess a specific consensus sequence [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N found in the region corresponding to a predicted N-terminal α-helix. These features differentiate the phytocystatins from mammalian members of the cystatin superfamily (*4*).

The cystatins are generally expressed as monomeric proteins, with the exception of human cystatin F, which is known to be produced as an inactive dimer with an intermolecular disulfide bond and which is reduction-dependently activated (*5*, *6*). However, some members of the cystatin superfamily have been reported to form noncovalent homodimers under stress conditions. The wild-type molecular species of human cystatin C dimerizes in vitro at elevated temperature, at low pH, and with mild chemical denaturation. The dimerization is accompanied by a complete loss of reactivity as a cysteine proteinase inhibitor (*7*). NMR and crystallographic studies indicate that cystatin C forms a dimer via a mechanism of three-dimensional domain swapping, in which there is a crossing-over of the β-strands from one monomer to the other (*8*−*10*). An L68Q substitution in human cystatin C, which leads to extensive amyloid formation in patients with hereditary cystatin C amyloid angiopathy, destabilizes the protein and enhances the dimerization and the oligomerization (*11*, *12*). Another example of the dimerization of cystatins is oryzacystatin-I (OC-I), a phytocystatin from rice, which forms a homodimer when incubated at high-temperature (*13*). However, the dimeric form of OC-I, in contrast to human cystatin C, shows affinity for papain identical to that of the

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monomeric form, even though the overall three-dimensional structure of OC-I resembles that of family 2 cystatins, which includes human cystatin C (*14*).

In this study, we first reassigned the identity of OC-III (*15*), a cystatin recently identified in the calli of rice, to OC-II proposed by Martínez et al. (*16*). Second, we examined whether thermal treatments could influence the properties of recombinant OC-II (i.e., the molecule previously reported as OC-III), in a manner similar to human cystatin C and OC-I. Surprisingly, thermal treatment enhanced the inhibitory reactivity of OC-II, and this enhancement correlated with the conversion of dimer to monomer. Our results indicate that OC-II forms a homodimer at ambient temperature and that the monomeric molecule has reactivity higher than that of the dimer. To our knowledge, OC-II is the first example of noncovalent dimerization of the cystatins under nonstress conditions.

MATERIALS AND METHODS

Database Analysis. Homology analysis of oryzacystatins was performed by searching databases representing the entire rice genome (Rice Annotation Project Database [http://rapdb.lab.nig.ac.jp] and TIGR Rice Genome Browser [http://www.tigr.org/tigr-scripts/osa1_web/gbrowse/rice/]).

Preparation of Recombinant Oryzacystatin-I and -II. The expression and purification of recombinant OC-I and -II was performed as described previously (*15*). Briefly, the OCs were expressed in *Escherichia coli* (*E. coli*) BL21 (GE Healthcare Bio-Science Corp., Piscataway, NJ) as the fusion protein of GST (glutathione-*S*-transferase) and OC. Each OC was obtained by digesting the GST-OC fusion with factor Xa (Novagen, Madison, WI), following the purification of the fusion protein on a GSTrap HP column (GE Healthcare Bio-Science). The N-terminal amino acid sequence of the recombinant OC was confirmed on a PPSQ-21 protein sequencer (Shimadzu Co., Kyoto, Japan).

Enzyme Inhibitory Assay. Enzyme activities were measured using fluorogenic substrates purchased from Peptide Institute Inc., Osaka, Japan. Ficin (0.23 nM; Sigma-Aldrich), papain (0.14 nM; Sigma-Aldrich), and cathepsin B (0.27 nM; from human liver; Calbiochem) were preincubated for 5 min at 40 °C with various concentrations of OC-I/-II in a total volume of 2.0 mL of assay buffer. The assay buffer consisted of 0.1 M sodium phosphate buffer (pH6.8)−1 mM ethylenediaminetetraacetic acid (EDTA)−4 mM dithiothreitol (DTT)−0.05% Brij35 for ficin and papain, and 0.1 M 2-(*N*-morpholino)ethanesulfonate (MES) (pH6.0)−1 mM EDTA−4 mM DTT−0.05% Brij35 for cathepsin B. After preincubation, the fluorogenic substrate was added to the reaction mixture to a final concentration of 20−50 μM, after which it was incubated for 1−5 min at 40 °C. Carbobenzoxy (*Z*)-L-Phe-L-Arg-4-methyl-coumaryl-7-amide (MCA) was used as the substrate for ficin and papain, and *Z*-L-Arg-L-Arg-MCA was used for cathepsin B. The release of 7-amino-4-methylcoumarin (AMC) was monitored with a thermostated RF-5300PC spectrofluorophotometer (excitation, 380 nm; emission, 440 nm; Shimadzu). Active concentrations of papain and ficin were determined by titration with E-64 (l-*trans*-epoxysuccinylleucylamido[4-guanidino]butane) (Peptide Institute) using *Z*-Phe-Arg-MCA as the substrate according to the method of Barrett and Kirschke (*17*). Inhibition constant (K_i) values were calculated from residual enzyme activities, using Henderson's equation (*18*). One unit of inhibition against the proteinase was defined as the inhibition of the liberation of 1 μmol of AMC from the fluorogenic substrate per minute.

Analyses. Protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. Denaturing PAGE (polyacrylamide gel electrophoresis) was performed using 16% Tris glycine gels (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. Nondenaturing PAGE was carried out in 15% acrylamide gels using a Tris/glycine buffer system described by Laemmli (*19*). The proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad). Size exclusion chromatography (SEC) was performed at 5 °C with SMART system (GE Healthcare Bio-Science) equipped with a Superdex 75 PC 3.2/30 column (3.2 × 300 mm, flow rate 50 μL/min). The column was equilibrated in 50 mM HEPES (*N*-2-hydroxyethypiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0) containing 0.15 M NaCl. Blue dextran 2000, bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (25.0 kDa), and ribonuclease A (13.7 kDa) (low molecular weight gel filtration calibration kit; GE Healthcare Bio-Science) were used for calibrating the column.

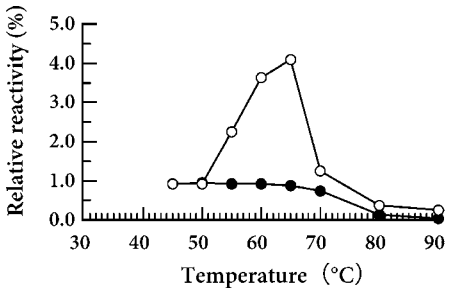

Figure 1. Effect of thermal treatment on the inhibitory reactivity of oryzacystatin (OC)-I and -II against ficin. The 50 μM solutions of recombinant OC-I (closed circle) or OC-II (open circle) were incubated at the indicated temperature in 0.1 M Na_2HPO_4–NaH_2PO_4 (pH 6.8) containing 1 mM EDTA, 4 mM DTT, and 0.05% Brij35 for 10 min. After the incubation, the inhibitory reactivity against ficin was measured, and the relative reactivity was calculated as a percentage from the ratio of inhibitory reactivity of the incubated OC to that of nonincubated OC.

RESULTS AND DISCUSSION

Reassignment of Oryzacystatin-III to Oryzacystatin-II. We recently reported a novel phytocystatin in rice (*Oryza sativa* L. *japonica* (*O. sativa* L. *japonica*)) (*15*). The amino acid sequence of this protein was similar to that of OC-I and OC-II previously reported in rice (*20*, *21*). In particular, this protein showed higher homology to OC-II with the sequence identity of 89 and 98% at amino acid and nucleotide levels, respectively. We inferred that this protein was a third member of the rice cystatins and was distinct from the other two molecules and designated it OC-III (*15*). In this study, we confirmed the presence of the OC-III gene in the genome of *O. sativa* L. *japonica* cultivar Nipponbare but could not find the coding sequence corresponding to OC-II through searching the databases representing the entire rice genome. The differences between the nucleotide sequences of OC-II and -III represent only three deletions and one transition change (*15*), and the difference in amino acid sequences between these phytocystatins can be explained by the frameshift (UniProt Knowledgebase: http://www.pir.uniprot.org/database/knowledgebase.shtml). Furthermore, Martínez et al. identified 12 genes (OC-I to -XII) encoding putative cystatins in the rice genome database (*16*). In their study, the sequence of OC-III was assigned to OC-II (locus ID: Os05g41460), and the gene corresponding to OC-II which had been previously identified has not been found. These findings indicate that the sequence of OC-II previously reported is probably due to misreading of the nucleotide sequence and OC-III is the "true" OC-II. Therefore, henceforth, the previously reported OC-III should be called OC-II.

Effect of Heating on the Inhibitory Reactivity of Oryzacystatin-I and -II. We examined whether thermal treatment could affect the inhibitory reactivity of OC-I and -II (i.e., OC-III previously reported) against ficin (**Figure 1**). The inhibitory reactivity of OC-I was not influenced by incubation at ≤70 °C and decreased when incubated at further high temperature. In contrast, the reactivity of OC-II was drastically enhanced by incubation at elevated temperature in the range of 55−65 °C, and the reactivity reached a maximum by incubating at 65 °C.

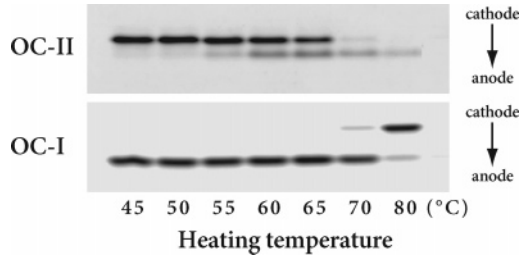

Figure 2. Non-denaturing polyacrylamide gel electrophoresis of oryzacystatin (OC)-I and -II incubated at various temperatures. The 50 μM solutions of recombinant OC-I or OC-II were incubated at the indicated temperature in 50 mM HEPES (pH 7.0)–0.15 M NaCl for 10 min. The arrows on the right indicate the direction of the electric field.

However, by heating at ≥70 °C, the inhibitory reactivity of OC-II declined and the reactivity was lower than that of the nonheated molecule when incubated at 80 or 90 °C.

Structural Changes by Incubating Oryzacystatins at High Temperatures. The structural changes produced by heating were evaluated by electrophoretic analysis. In analysis by non-denaturing PAGE (**Figure 2**), OC-I demonstrated a band shift at 80 °C (and 70 °C slightly), corresponding with the decline of inhibitory reactivity. The protein band showing slower mobility at 70 and 80 °C most likely corresponds to the dimeric form of OC-I, because Nagata et al. (*13*) reported that OC-I forms a homodimer by incubation at high temperature. This was confirmed by SEC analysis of OC-I (**Figure 3A**). The first and second peaks in the SEC chromatogram represent molecules with the molecular sizes of 37 and 17 kDa, respectively, as estimated from retention time. The values of molecular size are not too far from the calculated molecular weights (based on the amino acid sequence) of dimeric (22.8 kDa) and monomeric (11.4 kDa) OC-I. Nagata et al. (*13*) also indicated that OC-I formed a multimer larger than dimer by incubation at 95 °C, and that the dissociation constant of the dimer for papain was indistinguishable from that of the monomer. Thus, the decline of the inhibitory reactivity of OC-I by incubation at 80 and 90 °C might be due to a decrease of the active fraction of the protein through thermal denaturation, although significant aggregation or precipitation of OC-I could not be observed by non-denaturating PAGE and SEC.

The electrophoretic patterns of OC-II indicated the band shift opposite to those of OC-I (**Figure 2**). The incubation at ≥55 °C induced the occurrence of a protein band showing faster mobility, and the band intensity reached maximum by heating at 65 °C. However, the incubation of OC-II at 70 and 80 °C resulted in the decrease of the intensities of the protein bands. Under denaturing PAGE conditions, the band pattern of heated OC-II was the same for temperatures between 45 and 90 °C (data not shown). These results suggest that the elimination of the bands shown in non-denaturing PAGE is due to the formation of OC-II aggregates which could be dissolved by treatment with detergent (i.e., sodium dodecyl sulfate). The aggregation and/or the precipitation of OC-II caused by incubating at high temperature is also demonstrated by the SEC chromatogram, in which the peak observed after incubation at 75 °C is very small compared to that before heating (**Figure 3B**).

SEC demonstrated another noteworthy difference between OC-I and -II, corresponding to the results in non-denaturing PAGE. The chromatogram of OC-II before heating showed the main and the minor peaks, and the molecular mass of the main peak component was estimated to be 35 kDa on the basis of the retention time, compared to the calculated molecular mass (24.2 kDa) of the OC-II dimer on the basis of the amino acid sequence. The chromatogram of OC-II incubated at 50 °C was similar to that before heating, but incubation at 65 °C induced an increase in the abundance of the minor peak observed before heating. The molecular mass of this peak component was estimated to be 16 kDa from the data of SEC. This value is quite close to the value (12.1 kDa) of the calculated molecular weight of monomeric OC-II on the basis of the amino acid sequence. These results suggest that OC-II exists as a dimeric protein at ambient temperature in contrast to OC-I, which exists as a monomer.

Interconversion between Dimeric and Monomeric Forms of Oryzacystatin-II. To clarify whether the dimerization is an intrinsic property of OC-II, we examined the reversibility of the temperature-dependent change (**Figure 4**). The inhibitory reactivity of unheated OC-II against ficin was constant during storage at 4 °C. In contrast, when the OC-II solution incubated at 65 °C for 10 min was stored at 4 °C, the specific inhibitory reactivity gradually decreased to the level of unheated OC-II. This change corresponded with a decline in the abundance of monomer. The sum of the amounts of dimer and monomer was almost invariable during the storage as judged by the peak areas in SEC (data not shown), indicating the reversible conversion of the monomeric form of OC-II to the dimeric form. These results suggest that the dimerization is an intrinsic property of OC-II and not due to misfolding during the expression of recombinant protein in *E. coli*.

Enzyme Inhibition by Dimeric and Monomeric Oryzacystatin-II. The results in **Figure 4** also imply that the temperature-dependent enhancement of inhibitory reactivity of OC-II results from the conversion of the dimer to the monomer. To investigate this possibility, we purified these two molecular species of OC-II by SEC and estimated the K_i values for several cysteine proteinases (**Table 1**). The reversion from monomer to dimer was slow at low concentration (ca. 3 μM) enough to evaluate the affinities of the monomeric OC-II for enzymes (data not shown). The K_i value of the monomer for papain (80% active) and ficin (70% active) was 23-fold and 30-fold, respectively, lower than that of the dimer. The monomeric OC-II inhibited the activity of cathepsin B with a K_i value of 1.11×10^{-9}, while the K_i value of the dimer could not be estimated due to the weakness of the inhibition. These results indicate that monomeric OC-II has considerably higher affinity for cysteine proteinases than the dimeric form, suggesting that the increase in inhibitory reactivity of OC-II observed in this study is due to the conversion of dimer to monomer.

In this study, we demonstrate that OC-II noncovalently forms a dimer under nonstress conditions and that the dimer is converted to a monomer, which has higher affinity for cysteine proteinases, by thermal treatment. The considerable difference of the affinities for enzymes between the dimeric and the monomeric forms may indicate some involvement of this conversion in the regulation of OC-II in vivo. Until now, transcriptional regulation of the phytocystatins has been reported as the response to environmental stresses (including cold-, heat-, and drought-stress), wounding, and fungal infection (*22*−*26*). Compared with the regulation of gene expression, regulation of activity based on the structural conversion should be advantageous with regard to the rapidity of the response to the stress and the physiological alterations. One example of this type of regulation is human cystatin F. Cystatin F is produced as a dimer, which is inactive as a cathepsin inhibitor, with an intermolecular disulfide bond (*5*). The dimer can be activated

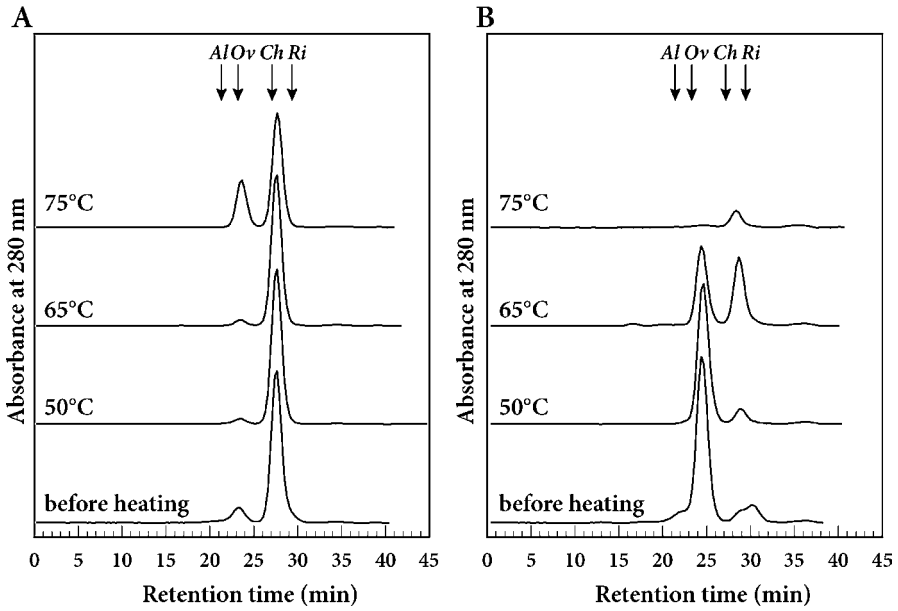

Figure 3. Size exclusion chromatography of oryzacystatin (OC)-I and -II incubated at various temperatures. The 20 μM solutions of OC-I (**A**) and -II (**B**) were incubated for 10 min at 50, 65, or 75 °C in 50 mM HEPES (pH 7.0)–0.15 M NaCl, before chromatography. The retention times for the calibration standards are indicated: Al, bovine serum albumin; Ov, ovalbumin; Ch, chymotrypsinogen A; Ri, ribonuclease A.

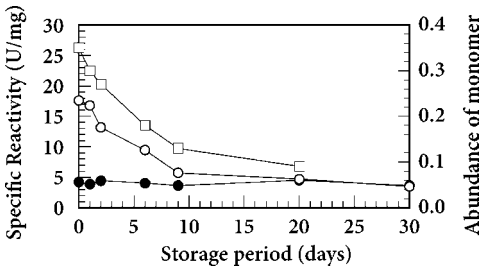

Figure 4. Stability of oryzacystatin (OC)-II after incubation at 65 °C for 10 min. A 50 μM solution of recombinant OC-II was incubated at 65 °C for 10 min, in 0.1 M Na_2HPO_4–NaH_2PO_4 (pH6.8) containing 1 mM EDTA, 4 mM DTT, and 0.05% Brij35, and then stored at 4 °C. The 50 μM OC-II (in the same buffer) without the thermal treatment was also stored at 4 °C. The inhibitory assay against ficin and the size exclusion chromatography (SEC) were performed for the incubated and the nonincubated OC-II at the indicated times. Abundance of monomer was calculated from the SEC data using the following formula: the peak area of OC-II monomer divided by the sum of the peak areas of OC-II monomer and dimer. Symbols: specific reactivity of OC-II incubated (open circle) and nonincubated (closed circle); the abundance of monomer for incubated OC-II (open square).

Table 1. Enzyme Inhibition by the Dimeric and Monomeric Forms of Oryzacystatin-II

	K_i value (M)		
	papain	ficin	cathepsin B
dimer	1.69×10^{-9}	2.00×10^{-9}	a
monomer	7.07×10^{-11}	6.57×10^{-11}	1.11×10^{-9}

[a] K_i value could not be determined due to weak inhibition.

in a reducing environment, and this activation is accompanied by a shift from the dimeric to the monomeric form (*6*, *27*). In another example, dimerization and parallel inactivation has been suggested to have a role in the physiological regulation of human cystatin C in acidified compartments such as lysosome in vivo (*7*). To our knowledge, OC-II is the first example of noncovalent dimerization of a cystatin under nonstress conditions. It remains to be clarified if the dimerization and the conversion to the monomer have any physiological implication in rice. The structural basis of the interconversion between the dimeric and the monomeric forms must also be elucidated to understand the molecular mechanism of the activation of OC-II via conversion to the monomeric form. These issues are now under investigation.

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