Rapid Communication *J. Biochem.* 123, 568-570 (1998)

Crystallization and Preliminary X-Ray Diffraction Studies of a Rice Cysteine Proteinase Inhibitor, Oryzacystatin-I

Norio Kudo,* Makoto Nishiyama,* Hiroshi Sasaki,* Keiko Abe,† Soichi Arai,† and Masaru Tanokura*,[1]

**Biotechnology Research Center and* †*Department of Applied Biological Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657*

Received for publication, February 16, 1998

Oryzacystatin-I from rice seeds was overexpressed in *Escherichia coli*, purified, and crystallized by the sitting-drop vapor diffusion method. Crystals obtained with 2-methyl-2,4-pentanediol as a precipitant exhibited space group $I4_122$, with unit cell parameters of $a=b=100.0$ Å, $c=54.2$ Å, and diffracted up to 2.8 Å resolution at 100 K. The crystals have one molecule per asymmetric unit.

Key words: crystallization, cysteine proteinase inhibitor, oryzacystatin, rice seed, X-ray crystallography.

Cystatins are a group of proteins which bind to cysteine proteinases and inhibit their activities (*1*). All the proteins belonging to the cystatin superfamily exhibit similarities in their amino acid sequences and functions, and have been classified into three distinct families, stefins, cystatins, and kininogens, based on their primary sequences (*1*). Several biochemical studies have shown that the N-terminal region of cystatins, especially a conserved Gly in the region, is important for the inhibitory activity (*2-4*). Previously, the three-dimensional structures of some cystatins were determined; that of chicken cystatin (cystatin family) by X-ray crystallography (*3, 5*), and those of human stefin A and cystatin A (stefin family) by NMR (*6, 7*). Crystallographic analysis of a papain-stefin B complex revealed that the N-terminal region of cystatin masks the catalytic cleft of papain (*8*), which strongly supported the above assumption.

Cystatins from plants are distinguishable from those from other sources, because they are single-chain proteins and contain no cysteine residues to form disulfide bonds. This suggests that plant cystatins can be classified into a new family of cystatins, phytocystatins (*9, 10*). Phytocystatins share only about 30% sequence homology with other cystatin families.

OC-I is a phytocystatin from rice seeds (*Oryza sativa*) (*11*). The cDNA and genomic DNA of OC-I have been cloned into *Escherichia coli*, and its expression system has been established in *E. coli* cells (*12, 13*). Using the expressed protein, the functional region of OC-I has been analyzed (*14, 15*). Interestingly, an OC-I mutant lacking the N-terminal region containing the conserved Gly has been found to inhibit papain (*14*). This suggested that OC-I inhibits the proteinase in a manner different from in the cases of other cystatins. To understand the inhibitory activity of OC-I, we have crystallized OC-I and collected preliminary X-ray diffraction data. This is the first report of the crystallization of a phytocystatin family protein.

In order to obtain the large quantities of OC-I required for screening of the crystallization conditions, a new expression system involving the T7 promoter was constructed in *E. coli* (*16*). A fragment of the OC-I cDNA was amplified by the polymerase chain reaction using pOC26 as a template (*12*) and oligonucleotides (5′-CC CAT ATG TCG AGC GAC GGA GGG CCG-3′ and 5′-CC GAA TTC TTA GGC ATT TGC ACT GGC ATC-3′) as primers. The former oligonucleotide contains the translational initiation codon, ATG, of OC-I, and the latter includes the stop codon, TAA. After digestion with *Nde*I and *Eco*RI, the resulting DNA fragment was ligated with a pET26b plasmid digested at which restriction sites, and the introduced into *E. coli* BL21 (DE3). The plasmid thus constructed was termed pOC-I-w.

A large amount of OC-I was produced as an active molecule in *E. coli* cells and purified by the procedure reported by Arai *et al.* (*15*) with a minor modification. An overnight preculture (15 ml) of *E. coli* BL21 (DE3) carrying pOC-I-w was transferred to 1.5 liters of Terrific Broth medium (*17*) supplemented with 50 μg/ml kanamycin, followed by cultivation at 37°C for 3 h. IPTG was then added to a final concentration of 1 mM. At 15 h after the IPTG addition, the cells were harvested by centrifugation (10,000×*g*, 5 min), suspended in 25 mM Tris-HCl, pH 7.5 (buffer A), and the lysed by sonication. After centrifugation (20,000×*g*, 20 min) of the sonicate, ammonium sulfate was added to the supernatant to obtain 75% saturation. The precipitant was dissolved in buffer A and dialyzed against the same buffer. The resulting solution was applied to a Q-Sepharose Fast Flow column (25×150 mm, Pharmacia), equilibrated with buffer A, and eluted with a linear gradient of 0 to 1.0 M NaCl. The fractions containing papain-inhibitory activity were collected and applied to a Superdex 200 FPLC gel filtration column (Pharmacia), equilibrated with buffer A containing 150 mM NaCl, at the flow rate of

[1] To whom correspondence should be addressed. E-mail: utanok@hongo.ecc.u-tokyo.ac.jp

Abbreviations: MPD, 2-methyl-2,4-pentanediol; OC-I, oryzacystatin-I.

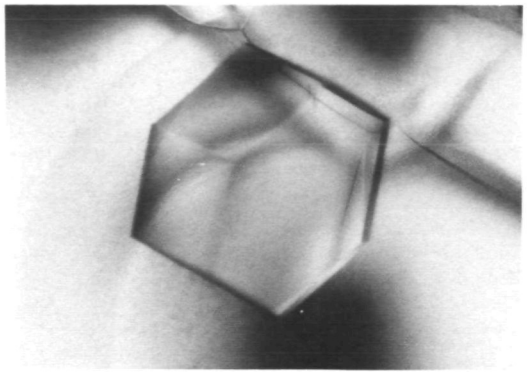

Fig. 1. **A typical crystal of oryzacystatin-I.** The approximate size of the crystal is $0.5 \times 0.5 \times 0.5$ mm^3.

TABLE I. **Strategy and statistics for data collected.**

Detector	Rigaku R-AXIS IIc (imaging-plate, 200×200 mm)
Crystal used	1
Cell dimensions	$a=b=100.0$ Å, $c=54.2$ Å
Detector distance	130 mm
Collected reflections	12,556
Unique reflections	6,551
Resolution	2.8Å
R-sym	4.5%
Completeness	88.6%

2.5 ml/min. The active fractions were dialyzed against 25 mM Tris-HCl, pH 8.0, and then applied to a Mono-Q column (Pharmacia) equilibrated with the same buffer. Elution was carried out with a linear gradient of 0 to 0.3 M NaCl. The active fractions were dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and then concentrated in Centriprep 3 ultrafiltration tubes (Amicon). Purity was analyzed by SDS-polyacrylamide gel electrophoresis (*18*).

Crystallization trials were performed with the hanging-drop vapor diffusion method using a crystallization screening kit (Hampton Research). Plate-like crystals appeared in the presence of MPD as a precipitant. After refinement of the crystallization conditions, crystals suitable for X-ray analysis were obtained by mixing 10 μl of the protein solution (50 mg/ml in 10 mM Tris-HCl, pH 7.5), and 10 μl of a reservoir solution consisting of 50–60% MPD, 100 mM magnesium chloride, and 0.1 M HEPES-NaOH, pH 7.5. A drop was set against 750 μl of the reservoir solution at 5–20°C by the sitting-drop vapor diffusion method. Crystals were obtained in 5 days. Figure 1 shows a typical crystal ($0.5 \times 0.5 \times 0.5$ mm^3) suitable for X-ray diffraction measurements. The X-ray diffraction patterns were recorded using a Rigaku imaging-plate detector system, R-AXIS IIc, with double mirror-focused CuKα radiation from a Rigaku RU-200R X-ray generator. A cooling device was used to keep the crystals at 100 K (Rigaku CN2364B2). The reflection intensities were evaluated with the program package, PROCESS (Rigaku). Crystals mounted on a hair-loop were subjected to flash-cooling to 100 K in a cold nitrogen stream. Because MPD is a good cryo-protective agent, no further treatment was required for cryo-crystallography. Three-dimensional data sets to 2.8 Å were collected. The crystals belong to space group $I4_n22$ with cell

TABLE II. **Summary of X-ray data collection statistics for OC-I.**

Resolution (Å)	Average intensity (I)	Average $I/\sigma(I)$	No. of *R*-factor	No. of Reflections	No. of Completeness
-15.0	184.0	19.2	0.082	40	0.645
15.0-10.0	199.4	27.3	0.062	94	0.752
10.0-7.5	139.2	19.8	0.080	182	0.798
7.5-5.0	56.3	15.6	0.084	769	0.861
5.0-3.5	48.2	13.4	0.089	2,140	0.907
3.5-3.0	10.5	4.8	0.126	1,804	0.902
3.0-2.8	3.9	2.4	0.162	1,522	0.878
All reflections	34.0	11.7	0.091	6,551	0.886

R-factor $=\Sigma_h\Sigma_i|I(h)_i-\langle I(h)\rangle|/\Sigma_h\Sigma_i I(h)_i$, where $\langle I(h)\rangle$ is the average intensity of i observation of reflection h.

dimensions of $a=b=100.0$ Å, and $c=54.2$ Å. The systematic absence of reflections ($hkl=00l$, $l=4n$) indicated that the space group was $I4_122$. Assuming that there is one molecule per asymmetric unit, the crystal volume per unit molecular mass (V_m) and the solvent volume fraction (V_{sol}) were estimated to be 2.98 Da/Å^3 and 0.587, respectively. These values are within the ranges of those of typical protein crystals (*19*). Among 12,556 recorded observations, 6,551 reflections were unique. This represented more than 88.6% of the possible reflections (7,400) at 2.8 Å. The average $I/\sigma I$ was greater than 2.4 in the highest resolution range. Tables I and II summarize the strategy and statistics for the data collected, respectively. Since OC-I is homologous with other cystatins, whose structures have been solved (*3, 5–8*), we are now in the process of determining the structure of OC-I using molecular replacement methods.

REFERENCES

1. Barrett, A.J., Rawlings, N.D., Davies, M.E., Machleidt, W., Salvesen, G., and Turk, V. (1986) Cysteine proteinase inhibitors of the cystatin superfamily in *Proteinase Inhibitors* (Barrett, A.J. and Salvesen, G., eds.) pp. 515–569, Elsevier, Amsterdam
2. Abrahamson, M., Ritonja, A., Brown, M.A., Grubb, A., Machleidt, W., and Barrett, A.J. (1987) Identification of the probable inhibitory reactive sites of the cysteine proteinase inhibitors human cystatin C and chicken cystatin. *J. Biol. Chem.* **262**, 9688–9694
3. Bode, W., Engh, R., Musil, D., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J., and Turk, V. (1988) The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *EMBO J.* **7**, 2593–2599
4. Machleidt, W., Thiele, U., Laber, B., Assfalg-Machleidt, I., Esterl, A., Wiegand, G., Kos, J., Turk, V., and Bode, W. (1989) Mechanism of inhibition of papain by chicken egg white cystatin. *FEBS Lett.* **243**, 234–238
5. Dieckmann, T., Mitschang, L., Hofmann, M., Kos, J., Turk, V., Auerswald, E.A., Jaenicke, R., and Oschkinat, H. (1993) The structures of native phosphorylated chicken cystatin and of a recombinant unphosphorylated variant in solution. *J. Mol. Biol.* **234**, 1048–1059
6. Martin, J.R., Craven, C.J., Jerala, R., Kroon-Žitko, L., Žerovnik, E., Truk, V., and Waltho, J.P. (1995) The three-dimensional solution structure of human stefin A. *J. Mol. Biol.* **246**, 331–343
7. Tate, S., Ushioda, T., Utsunomiya-Tate, N., Shibuya, K., Ohyama, Y., Nakano, Y., Kaji, H., Inagaki, F., Samejima, T., and Kainosho, M. (1995) Solution structure of a human cystatin A variant, cystatin A$^{2\text{-}98}$ M65L, by NMR spectroscopy. A possible role of the interactions between the N- and C-termini to maintain the inhibitory active form of cystatin A. *Biochemistry* **34**, 14637–14648

8. Stubbs, M.T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarčič, B., and Turk, V. (1990) The refined 2.4 Å X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. *EMBO J.* **9**, 1939-1947
9. Kondo, H., Abe, K., Emori, Y., and Arai, S. (1991) Gene organization of oryzacystatin-II, a new cystatin superfamily member of plant origin, is closely related to that of oryzacystatin-I but different from those of the animal cystatins. *FEBS Lett.* **278**, 87-90
10. Abe, K., Kondo, H., Watanabe, H., Emori, Y., and Arai, S. (1991) Oryzacystatins as the first well-defined cystatins of plant origin and their target proteinase in rice seeds. *Biomed. Biochim. Acta* **50**, 637-641
11. Abe, K., Kondo, H., and Arai, S. (1987) Purification and characterization of a rice cysteine proteinase inhibitor. *Agric. Biol. Chem.* **51**, 2763-2768
12. Abe, K., Emori, Y., Kondo, H., Suzuki, K., and Arai, S. (1987) Molecular cloning of a cysteine proteinase inhibitor of rice (oryzacystatin). Homology with animal cystatins and transient expression in the ripening process of rice seeds. *J. Biol. Chem.* **262**, 16793-16797
13. Kondo, H., Emori, Y., Abe, K., Suzuki, K., and Arai, S. (1989) Cloning and sequence analysis of the genomic DNA fragment encoding oryzacystatin. *Gene* **81**, 259-265
14. Abe, K., Emori, Y., Kondo, H., Arai, S., and Suzuki, K. (1988) The NH_2-terminal 21 amino acid residues are not essential for the papain-inhibitory activity of oryzacystatin, a member of the cystatin superfamily. Expression of oryzacystatin cDNA and its truncated fragments in *Escherichia coli*. *J. Biol. Chem.* **263**, 7655-7659
15. Arai, S., Watanabe, H., Kondo, H., Emori, Y., and Abe, K. (1991) Papain-inhibitory activity of oryzacystatin, a rice seed cysteine proteinase inhibitor, depends on the central Gln-Val-Val-Ala-Gly region conserved among cystatin superfamily members. *J. Biochem.* **109**, 294-298
16. Studier, F.W., Rosenburg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60-89
17. Tartof, K.D. and Hobbs, C.A. (1987) Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus* **9**, 12
18. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
19. Matthews, B.W. (1986) Solvent contents of protein crystals. *J. Mol. Biol.* **33**, 491-497