Note

Some Properties of a Cysteine Proteinase Inhibitor from Corn Endosperm

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Though a number of proteinaceous cysteine proteinase inhibitors (cystatins) of animal origin have long been studied in detail,¹⁾ little is well-defined about plant cystatins except for oryzacystatins from rice seeds.^{2,3)} Preceding the studies on oryzacystatins, however, we purified a cysteine proteinase inhibitor from corn.^{4,5)} In this study we carried out amino acid analysis and kinetic studies on this inhibitor as a corn cystatin and also tried to compare the results of the studies with those obtained with oryzacystatins.

Inhibitory activities against cysteine proteinases were measured either by the method using benzoylarginine *p*-nitroanilide (BAPA)⁵⁾ or by that using benzoylarginine β -naphthylamide (BANA). Using the BANA method, inhibited proteinase activity was measured as follows: 0.1 ml of 0.5 M sodium phosphate buffer (pH 6.0) containing 10 mM EDTA, 0.1 ml of 50 mM 2-mercaptoethanol, 0.4 ml of proteinase solution, and 0.2 ml of inhibitor solution were mixed and incubated at 37°C for 10 min. The reaction was started by addition of 0.2 ml of 1 mM BANA. After incubation at 37°C for 20 min, 1 ml of 2% HCl in ethanol and 1 ml of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol solution were added to the reaction mixture to stop the reaction for development of an orange color. After it was left to stand at room temperature for 30 min, the reaction mixture was measured as to its absorbance at 540 nm.

Purification of corn cystatin CI-4a⁵⁾ was carried out; the method used for the purification was basically the same as reported previously.⁵⁾ The obtained protein was further purified by a HPLC system using an ODS column (Senshu Pak, 4.6 ϕ $\times$ 250 mm). This treatment was effective in removing some small protein contaminants and in highly purifying the CI-4a fraction.

The amino acid composition of CI-4a was analyzed with a Derivatizer/Analyzer System (ABI). We calculated the residue number based on the assumption that the residue number of methionine, whose content is the lowest of all, is one. The results were as follows: aspartic acid + asparagine, 9; threonine, 4; serine, 5; glutamic acid + glutamine, 8–9; proline, 3; glycine, 9; alanine, 8–9; valine, 8; methionine, 1; isoleucine, 2; leucine, 4; tyrosine, 2–3; phenylalanine, 3; histidine, 2; lysine, 2; and arginine, 5. The contents of cysteine and tryptophan were not measured. The sum of the number of all amino acid residues except for cysteine and tryptophan is 75–78, which agrees with the relative molecular mass of CI-4a, 9,200 daltons, estimated by SDS-polyacrylamide gel electrophoresis.⁵⁾

For analysis of the N-terminal sequence we put CI-4a directly into a protein sequencer (ABI Model 470A/120A), with the result that its N-terminal amino acid residue was probably masked. We then partially hydrolyzed CI-4a using trypsin. Approximately 75 μ g of CI-4a was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 2 M urea and 1 mM DTT. To this solution was added 3.5 μ g of trypsin (TPCK treated, Sigma) and the mixture kept at 37°C for

Table I. *K_i* VALUES OF THE INHIBITORY REACTIONS BY CI-4a AND TWO ORYZACYSTATINS AGAINST VARIOUS CYSTEINE PROTEINASES

Cysteine proteinase	<i>K_i</i> value (M)		
	Corn CI-4a	Oryzacystatin-I ³⁾	Oryzacystatin-II ³⁾
Papain	2.3×10^{-8}	3.02×10^{-8}	0.83×10^{-6}
Ficin	8.3×10^{-8}	—	—
Chymopapain	9.2×10^{-8}	—	—
Cathepsin B1	1.6×10^{-5}	—	—
Cathepsin H	2.3×10^{-8}	0.79×10^{-6}	1.00×10^{-8}

Fig. 1. Partial Amino Acid Sequence of CI-4a Inhibitor Compared with Those of Oryzacystatin-I and II. Identical amino acid residues are boxed.

72 hr. The resulting tryptic hydrolyzate was put into a HPLC system with an ODS column (Senshu Pak, $4.6\phi \times 250$ mm) and separated by TFA/ CH_3CN solvent system. The peptide fragments obtained were lyophilized and their amino acids were sequenced. A clear sequence was obtained from the analysis of fragment P-12 (Fig. 1). There was a significant degree of amino acid sequence similarities between P-12 and both oryzacystatin-I and oryzacystatin-II. Particularly, the sequence Ala-Val-Thr-Glu-His was found both in CI-4a and in oryzacystatin-I.

CI-4a was compared with two oryzacystatins with respect to inhibition profiles against various kinds of cysteine proteinases. For the comparison, K_i values were obtained from Lineweaver-Burk plots.⁶⁾ As Table I shows, the K_i of the inhibition of papain by CI-4a was almost the same order as for oryzacystatin-I but different from oryzacystatin-II. Interestingly, the K_i of CI-4a against cathepsin H is the same as that of this inhibitor against papain, while oryzacystatin-I and oryzacystatin-II show greatly different inhibition constants between cathepsin H and papain.³⁾

It is thus likely that CI-4a, one of the corn cystatins, partly resembles both oryzacystatin-I and oryzacystatin-II in amino acid sequence and inhibition spectrum. Further information on CI-4a, especially its complete amino acid sequence, will be provided in the near future.

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