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Studies on the reactive site of the cystatin superfamily using recombinant cystatin A mutants

Evidence that the QVVAG region is not essential for cysteine proteinase inhibitory activities

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For study of the inhibition mechanism of the cystatin superfamily, cystatin A artificial mutants were obtained in which a well-conserved QVVAG region in the cystatin superfamily was changed to KVVAG or QVTAG and these mutants were then expressed in *E. coli*. For this, genes with these sequences were synthesized enzymatically from 11 oligodeoxynucleotides and expressed under the tac promoter gene of the *E. coli* plasmids. The products expressed were then purified on Sephadex G-50 and HPLC DEAE-5PW columns. The substitutions in cystatin A were confirmed by the amino acid compositions, N-terminal amino acid sequences and elution positions on ion-exchange chromatography of the products. The K_i values of these products for the cysteine proteinases, papain and cathepsins B, H and L, were determined in comparison with those of wild type recombinant cystatin A. Results showed that the cystatin A mutants had similar inhibitory activities to those of wild type recombinant cystatin A. Namely replacement of amino acids in the QVVAG sequence of cystatin A did not significantly affect the inhibitory activities on these proteinases. The results suggest that the QVVAG region is less important than the N-terminal region of cystatin for inhibitory activities on cysteine proteinases.

Cystatin A; Cystatin superfamily; Cysteine proteinase inhibitor; Cystatin A mutant

1. INTRODUCTION

Recently many endogenous cysteine proteinase inhibitors have been purified from various animal tissues. In our laboratory cystatin α and β were purified from rat liver and these amino acid sequences were determined [1,2]. Then amino acid sequences of human cystatin A and B were determined by Green and Ritonja et al. [3,4]. Several lines of evidence suggest that endogenous inhibitors of cysteine proteinases play a significant role in control of intracellular proteolysis. There are two well-conserved regions in the amino acid sequences of the cystatin superfamily: glycine[4] in the N-terminal region and the $Q^{46}V^{47}V^{48}A^{49}G^{50}$ sequence in the middle portion of cystatin A (amino acid sequence numbers of cystatin A). These sites have been presumed to be important from the following reported findings. On formation of a mixed disulfide between cysteine[3] and glutathione cystatin β lost its inhibitory activities [5]. Chicken cystatin lost its inhibitory activities on removal of its N-terminal region [6]. The truncated c-*Ha-ras* product, p21, containing the QVVAG sequence is an effective inhibitor of cathepsin L [7]. Recently, the three-dimensional structure of chicken cystatin

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Abbreviations: MW, molecular weight; SDS-PAGE, SDS-polyacrylamide electrophoresis; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; Z, benzyloxycarbonyl; OMe, methyl ester

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was determined by X-ray crystallographic analysis, and results suggested that the N-terminus of chicken cystatin is adjacent to the QVVAG sequence and that N-terminal glycine is complementary to the active site of papain [8]. But there is no direct proof that the QVVAG sequence is important for the inhibitory activity.

Cystatin A (12 kDa) is a member of the cystatin superfamily [9] and is mainly located in the epidermis and in leukocytes [10,11]. In this study, cystatin A and its artificial mutants in which QVVAG was replaced by KVVAG or QVTAG could be expressed in *E. coli* by a similar method to that used in the case of cystatin α [12]. Then the products expressed were purified and their inhibitory activities on the cysteine proteinases, papain and cathepsins B, H and L were examined. Results showed that the artificial cystatin A mutants showed similar inhibitory activities to those of wild type recombinant cystatin A. These results suggest that the QVVAG region is less important than the N-terminal region for these inhibitory activities. The QVVAG region might be involved in stabilization of the interaction between cysteine proteinases and their inhibitors.

2. MATERIALS AND METHODS

2.1. *Materials*

E. coli strains MC 1061 and HB 101 were used for transformation with cloning and expression of plasmids, respectively. Plasmid pBR322 was used for cloning the synthetic gene fragments. Plasmid pKK223-3 for expression of the synthetic gene was obtained commercially. Rat cathepsins B, H and L were purified as described [13–15]. Papain (EC 3.4.22.2) was purchased from Sigma. Aminomethylcoumarin substrates were purchased from the Peptide Institute (Osaka). Sodium ampicillin was purchased from Meiji Seika Kaisha.

2.2. *Chemical synthesis of the oligomers*

The 11 oligomers were synthesized by the phosphoramidite

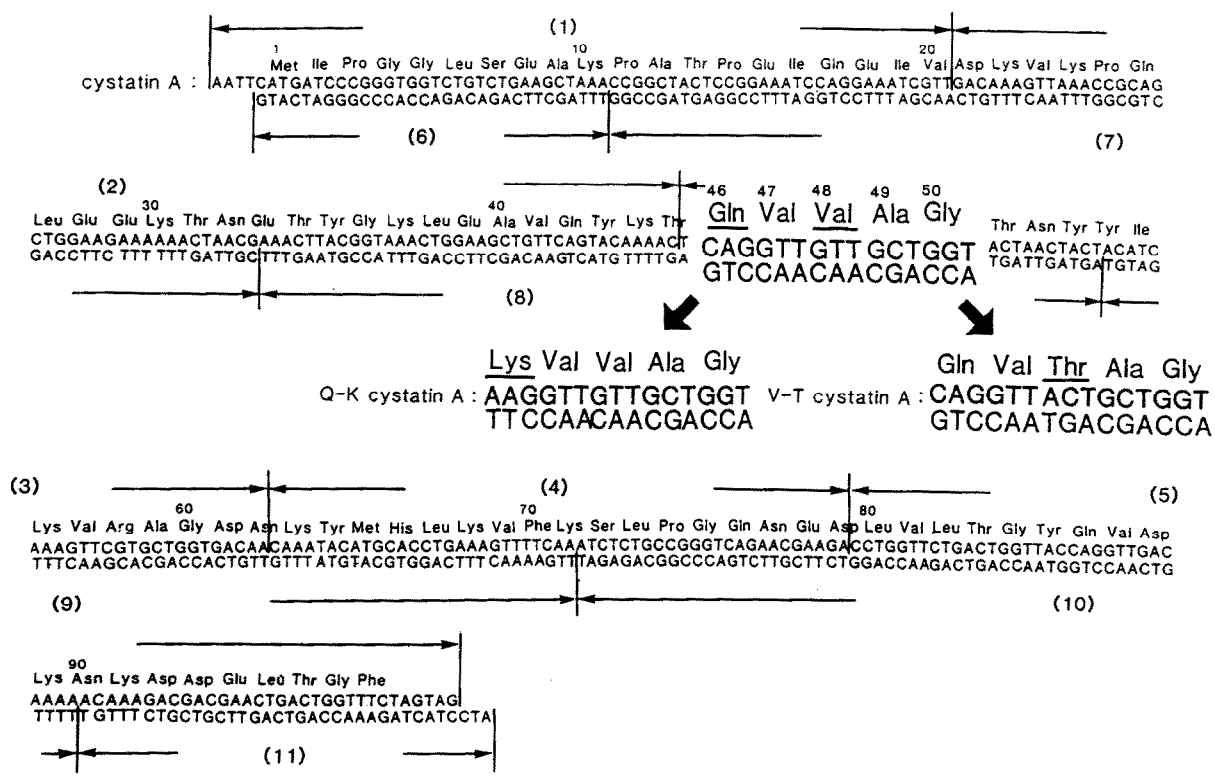

Fig.1. Design of synthetic gene fragments based on the amino acid sequence of cystatin A and its mutants. The recognition sites of *Eco*RI and *Bam*HI were placed at the 5′- and 3′-ends of the gene, respectively. A tandem translation stop codon was inserted at the 3′-end. The DNA fragment length is 74 to 32 bases, average 61 bases (upper strand) and 51 bases (lower strand). The individual oligodeoxyribonucleotides synthesized chemically are named fragments 1–11. Enzymatic joining points are indicated by lines. Large letters show the QVVAG amino acid sequence of cystatin A and the mutated sequences KVVAG (Q-K cystatin A) and QVTAG (V-T cystatin A).

method [16] in an automatic DNA synthesizer (Applied Biosystems, Inc.). The product was removed from the solid support and purified by PAGE. The main product was extracted from the gel and precipitated by ethanol.

2.3. *Design of the gene*

Chemical synthesis of the cystatin A gene and its expression in *E. coli* have been reported by Kaji et al. [17]. But we designed 11 long length overlapping oligodeoxynucleotide fragments based on the amino acid sequence of cystatin A [18] according to the codon usage of *E. coli* [19] as shown in fig.1. The DNA fragment length was 74 to 32 bases, average 61 bases (upper strand) and 51 bases (lower strand). The recognition sites of *Eco*RI and *Bam*HI were placed at the 5′- and 3′-end of the gene, respectively. As the N-terminal amino acid of cystatin A is methionine, an additional methionine codon was not necessary for initiation. A tandem translation stop codon was incorporated at the C-terminal.

2.4. *Assembly of gene fragments and cloning in* E. coli

The assembly was performed as shown in fig.2. Fragments 1, 6 and 7 were mixed, phosphorylated and annealed. Similarly, fragments 2 and 8 were mixed, phosphorylated and annealed. These two annealed mixtures were then ligated to make segment

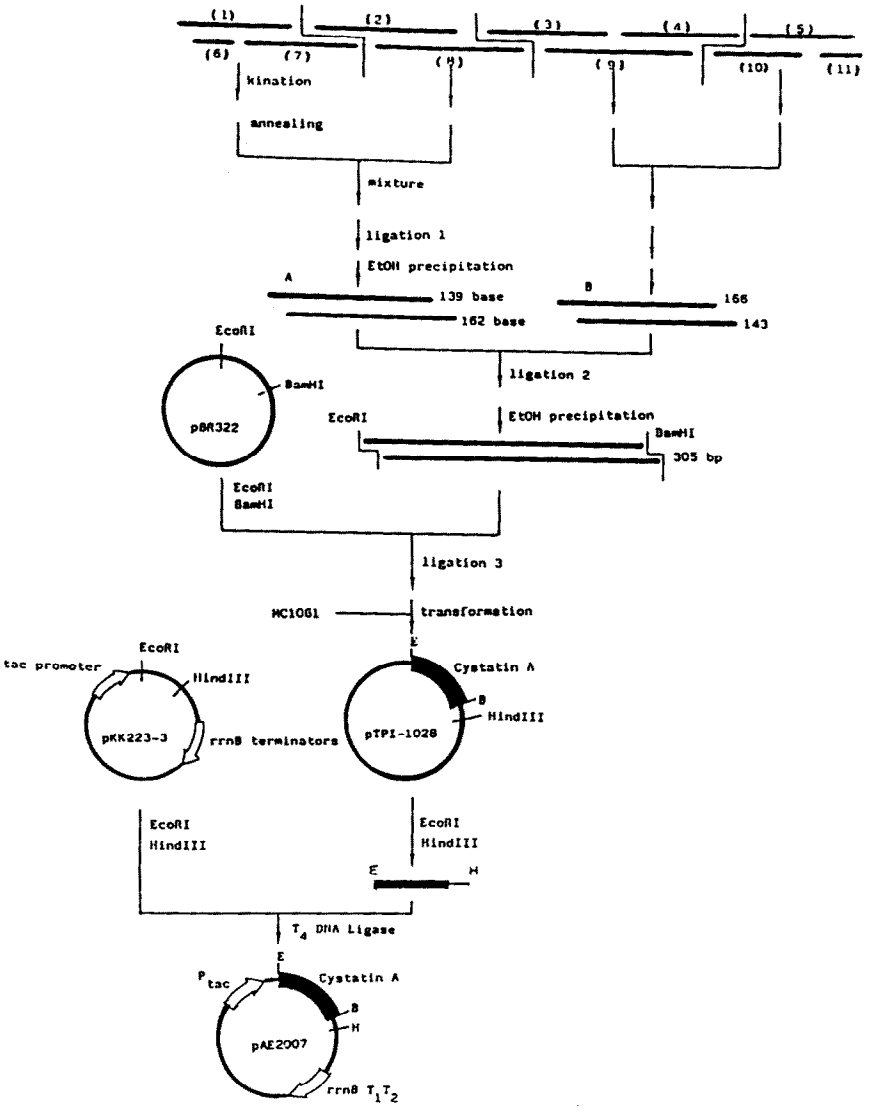

Fig.2. Assembly of the chemically synthesized DNA fragments and construction of the expression plasmid for the cystatin A gene under tac promoter control. The fragments were phosphorylated, annealed and ligated to make a cystatin A gene. This synthesized cystatin A gene was introduced between the *Eco*RI and *Bam*HI sites of pBR322 which had been digested with these restriction enzymes. The *Eco*RI-*Hin*dIII fragment of this plasmid containing the cystatin A gene was introduced into expression plasmid pKK223-3.

A, which was purified by ethanol precipitation. Segment B was prepared in the same manner. Segments A and B were then ligated followed by the total cystatin A gene and this gene was purified by ethanol precipitation. The chemically and biochemically synthesized cystatin A gene was then ligated to pBR322 which had been digested with *Eco*RI and *Bam*HI. The plasmid was introduced into *E. coli* MC1061 and the resulting ampicillin-resistant transformants were screened by colony hybridization with radiolabeled oligomer fragment 1 and 11 as probes. Restriction enzyme analysis revealed that 10 of the 12 hybridization positive transformants contained the desired *Eco*RI-*Bam*HI fragment corresponding to the synthetic gene of cystatin A. The nucleotide sequence of the *Eco*RI-*Bam*HI fragment was confirmed by the method of Maxam and Gilbert [20].

2.5. *Construction of the expression plasmid for cystatin A*

The *Eco*RI-*Hin*dIII fragment of pTPI-1028 containing the *Eco*RI-*Bam*HI fragment was ligated to the *E. coli* tac expression plasmid (pKK223-3) at the *Eco*RI and *Hin*dIII sites by a one-step reaction with T4-DNA ligase (fig.2) [21]. This plasmid was transferred to *E. coli* HB101 and the resulting transformants were screened by the same method as described above. One of the positive transformants, pAE2007, was employed for purification of cystatin A.

2.6. *Modification of cystatin A amino acid*

We changed two amino acids in the QVVAG region of cystatin A to hydrophilic amino acids, Q^{46} to K (Q-K cystatin A) and V^{48} to T (V-T cystatin A) by assembly and cloning of the synthesized DNA fragments as shown in figs 1 and 2. The nucleotide sequences of the *Eco*RI-*Bam*HI fragments from these two kinds of plasmids were confirmed by the method of Maxam and Gilbert. In addition two kinds of expression plasmids of the modified cystatin A gene were constructed in the same way as in fig.2 [expression plasmid pAαE8 (Q-K cystatin A) and pAβE2 (V-T cystatin A)].

2.7. *Purification and characterization of recombinant cystatin A and its mutants expressed by* E. coli

The expression plasmid, pAE2007 (wild type recombinant cystatin A) was grown at 37°C for 16 h in L-broth (1% trypsin, 0.5% yeast extract (Difco), 1% NaCl and 40 μg/ml ampicillin). The cells were harvested by centrifugation at 10000 × *g* for 30 min, frozen and thawed twice and suspended in 5 vols of 25 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. This suspension was shaken in a vortex mixer operated at full speed for 1 min and centrifuged at 20000 × *g* for 15 min at 4°C. The supernatant was applied to a Sephadex G-50 column equilibrated with the same Tris-HCl buffer. Fractions showing inhibitory activities on papain were pooled. After concentration by ultrafiltration using a YM-10 membrane (Diaflo), they were dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.5. The dialysates were further purified by HPLC on DEAE-5PW (Toyo Soda Inc.).

The other expression plasmids, pAαE8 (Q-K cystatin A) and pAβE2 (V-T cystatin A), were cultured and purified in the same way as the expression plasmid pAE2007.

The inhibitory activities of recombinant cystatin A and its mutants on the cysteine proteinases, papain and cathepsins B, H and L were assayed fluorometrically using aminomethylcoumarin substrates according to the method of Green et al. [3]. Fluorescence was monitored in a fluorescence spectrometer (Hitachi 650-10Ms). Emission at 460 nm was measured with excitation at 370 nm. The K_i values of inhibitors were determined from Lineweaver-Burk plots.

Amino acid analysis was performed with a Hitachi 835 amino acid analyzer after hydrolysis with 6 N HCl in a sealed tube at 110°C for 24 h. N-terminal amino acid sequence analysis of recombinant cystatin A and its mutants were performed with an automated gas-phase sequencer (Applied Biosystems, Model 470A) after desalting by reverse-phase HPLC on COSMOSIL 5C4-300 (Jasco). PTH-amino acids produced in the sequencer were analyzed in an Applied Biosystems, Model 120A analyzer.

3. RESULTS

3.1. *Western blotting analysis*

The crude preparations of recombinant cystatin A and its mutants were subjected to 15% SDS-PAGE followed by a Sartoblott-II immunoblotting system (Sartorius), using rabbit antiserum against rat skin cystatin α. The recombinant cystatin A and its mutants were visualized with goat anti-IgG Fab′ conjugated peroxidase (MDL) and Konica Immunostain Kits. The expressed products showed mobilities in 15% SDS-PAGE corresponding to about 12 kDa as predicted from their genes (data not shown).

3.2. *Analysis by SDS gel electrophoresis*

Samples of 3 μg of the purified cystatins were subjected to 15% SDS-PAGE analysis. Each gave a single band with a mobility corresponding to 12 kDa which was stained with Coomassie brilliant blue (fig.3). The yield of purified product by our procedure was about 0.5 mg of protein per gram wet weight of cells.

3.2. *Chromatographic separation of the three kinds of recombinant cystatin As*

The amino acid compositions of the cystatin A mutants are compared with that of the wild type recombinant cystatin A in table 1. Substitutions of amino acids, Q^{46} to K and V^{48} to T, were detected. Wild type recombinant cystatin A, Q-K cystatin A and V-T cystatin A were separated at different positions in 75 mM, 37.5 mM and 55 mM NaCl on HPLC on a DEAE-5PW column (fig.4). Moreover, we confirmed that the sequence of their 10 N-terminal amino acids was MIPGGLSEAK.

3.3. *Inhibition profiles of the three kinds of recombinant cystatin As*

The K_i values of recombinant cystatin A and its

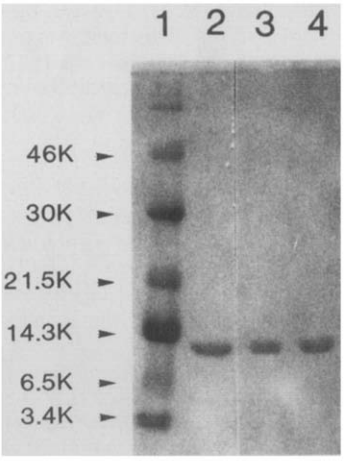

Fig.3. SDS-electrophoresis of purified recombinant cystatin A and its artificial mutants. Samples of 3 μg of purified recombinant cystatin A and its mutants were subjected to 15% SDS-PAGE. They gave a single band of 12 kDa when stained with Coomassie brilliant blue (lanes: 1, marker; 2, recombinant cystatin A; 3, Q-K cystatin A; 4, V-T cystatin A).

Table 1

Amino acid composition of wild type recombinant cystatin A and its artificial mutants

	Wild type cystatin A	Q-K cystatin A	V-T cystatin A	R[a]
Asx	11.0 (11)[b]	11.1 (11)	11.2 (11)	11
Thr	6.5 (7)	6.6 (7)	7.7 (8)↑[c]	7
Ser	2.1 (2)	1.9 (2)	2.0 (2)	2
Glx	14.7 (15)	13.8 (14)↓[c]	14.6 (15)	15
Gly	8.4 (8)	8.4 (8)	8.4 (8)	8
Ala	5.6 (6)	5.5 (6)	5.5 (6)	5
Cys	0.2 (0)	0.1 (0)	0.1 (0)	0
Val	8.7 (9)	8.5 (9)	7.6 (8)↓[c]	9
Met	1.3 (1)	1.1 (1)	1.8 (2)	2
Ile	3.8 (4)	3.9 (4)	3.8 (4)	4
Leu	8.0 (8)	8.1 (8)	8.6 (9)	8
Tyr	3.7 (4)	5.5 (6)	6.0 (6)	6
Phe	2.2 (2)	2.1 (2)	2.4 (2)	2
Lys	12.0 (12)	12.9 (13)↑[c]	12.0 (12)	12
His	1.3 (1)	1.1 (1)	1.1 (1)	1
Arg	1.1 (1)	1.1 (1)	1.0 (1)	1
Pro	4.8 (5)	4.9 (5)	4.8 (5)	5
Total	96	98	100	98

[a] Taken from the data presented in [15]
[b] Values in parentheses are nearest integers
[c] Upward and downward arrows show differences due to amino acid substitutions in cystatin A mutants

Values were calculated as numbers of residues per molecule

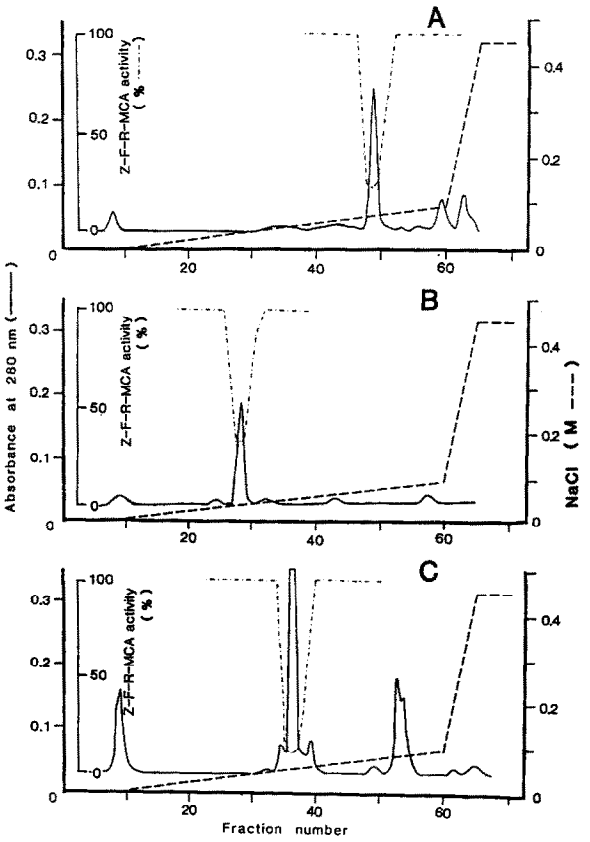

Fig.4. Purification of recombinant cystatin A and its artificial mutants by HPLC on DEAE-5PW. When applied to a HPLC DEAE-5PW column, recombinant cystatin A (A), Q-K cystatin A (B) and V-T cystatin A (C) were eluted with 75 mM, 37.5 mM and 55 mM NaCl, respectively.

Table 2

Comparison of K_i[a] values of wild type recombinant cystatin A and its artificial mutants for various cysteine proteinases

Proteinase	Substrate	K_i (nM)		
		Wild type cystatin A	Q-K cystatin A	V-T cystatin A
Papain	Z-Phe-Arg-MCA (pH 7.0)	5.4	2.3	5.7
Cathepsin B[b]	Z-Phe-Arg-MCA (pH 6.0)	140	226	143
Cathepsin H[b]	Arg-MCA (pH 7.0)	40	165	31
Cathepsin L[b]	Z-Phe-Arg-MCA (pH 5.5)	1.6	0.6	5.6

[a] K_i values were determined from Lineweaver-Burk plots for noncompetitive inhibition
[b] Rat liver cathepsins

mutants for various cysteine proteinases are compared in table 2. No significant differences were found in the K_i values of these three cystatin As. These results indicated that substitutions in the QVVAG region did not affect their inhibitory activities appreciably.

4. DISCUSSION

We used long fragments of 74 to 32 bases for designing the cystatin A gene and ligated the fragments containing the cystatin A gene into the *E. coli* tac expression plasmid by a one-step reaction. So the yield of purified recombinant cystatin A was about 0.5 mg per gram wet weight of cells.

Some previous evidence suggested that the QVVAG region might be important for the inhibitory activities of cysteine proteinase. For example, the QVVAG region in the cystatin superfamily is well-conserved in the one-dimensional structure. The smallest peptide, Z-Q-V-V-A-G-OMe showed some inhibitory activity on papain and cathepsin B [22]. Moreover N-truncated oryzacystatin lacking G^5 and retaining QVVAG inhibited papain as efficiently as the full-length oryzacystatin [23]. But there have been no direct studies on whether substitution of amino acids in this region caused significant changes in the activities. We found that replacement of amino acids in the QVVAG sequence of cystatin A did not significantly affect the inhibitory activity on papain or cathepsin B, H or L. Judging from Bode's docking model of a chicken cystatin-papain complex based on the X-ray crystal structure [8] and inhibition of papain by N-terminally truncated forms of chicken cystatin [6], the QVVAG region might play a role in stabilizing the complex between papain and chicken cystatin, while the N-terminal region should be essential for inhibitory activity. Our results provide evidence in support of this model. We presumed that the substitutions QVVAG to KVVAG and QVTAG would not change the hairpin loop-like structure, but that other substitutions that would change the hairpin loop-like structure might decrease the inhibitory activity. We conclude from this study that the QVVAG region is less important than the N-terminal region for cysteine proteinase inhibitory activities.

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