Note

Two Distinct Species of Corn Cystatin in Corn Kernels†

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Besides corn cystatin I (CC-I) we found, by cloning, another molecular species of cystatin, named corn cystatin II (CC-II), which was found by screening with CC-1 cDNA as a probe. The dissimilarity in amino acid sequence between CC-I and CC-II was distinct around the N-terminal region. Enzymologically, CC-II protein expressed in Escherichia coli was a stronger inhibitor of cathepsin L than CC-I.

Cystatins as proteinaceous inhibitors of cysteine proteinases are known to occur in animal organs and many have been well characterized.1) However, little information had been available on plant cystatins until oryzacystatins were found in rice seeds and characterized.2,3) Subsequently, we isolated a clone for a cystatin from a cDNA library of corn kernels by using a mixture of cDNA inserts for oryzacystatin I and II and designated this cystatin as corn cystatin I (CC-I).4) We also expressed CC-I in Escherichia coli and found it to be indeed an inhibitor of cysteine proteinases.4) The occurrence of this inhibitor in corn kernels was then confirmed by the Western blotting using an anti-CC-I antibody.5) However, we have detected several isoforms of cystatin by protein chemistry studies. To detect other species of cystatin for clarification of their physiological roles in corn, we attempted to obtain information on the variety of cystatins occurring. In this paper we report on the results of screening of clones for cystatins by using an insert of CC-1 cDNA as a probe.

The construction of a λgt10 library of immature corn kernel DNA and the screening using CC-1 cDNA insert were done as described previously.4) Positive clones were amplified by PCR using λgt10 primers and subcloned into a pUC18 plasmid vector treated with HincII after being filled in with T4 DNA polymerase. The nucleotide sequences of the obtained subclones were analyzed for both strands by an ABI 373A DNA Sequencer, and cDNA inserts of nine clones were found to contain a characteristic sequence generally conserved in all known cystatins. From the deduced amino acid sequences it was found that these nine clones had structural variations and it appeared that they could be classified into four groups (Fig. 1). Since, however, the variation between the sequences of isoforms 1 and 2 and that between the sequences of isoforms 3 and 4 are very small, it may be reasonable to think that the corn cystatin can be composed of two major species, CC-I with isoform 1 or 2 and CC-II with isoform 3 or 4. CC-I being the cystatin we have already reported,4) CC-II is a novel corn cystatin. The nucleotide sequences and deduced amino acid sequences of both corn cystatins are aligned in Fig. 2. CC-II differs from CC-I in that the 17–19 amino acid residues are not identical and also in that the deletion of one amino acid residue in CC-II in the position corresponding to Ala-21 in CC-I. The similarity of the amino acid sequence between CC-I and CC-II was low (52%) in the 21st to 41st amino acid residue region following the signal sequence, which is probably the N-terminal region of the mature protein. In the central or C-terminal region, the similarity of the amino acid sequence was very high and the sequences of both cystatins coincided almost completely.

To compare the properties of CC-I and CC-II as cysteine proteinase inhibitors, the expression of both cystatins was done with Escherichia coli. For the expression of CC-I, the plasmid pCC7H was used8) and the expressed protein was purified as described before.9) In the case of CC-II, the expression plasmid was prepared by amplifying λZC37 in the first place. Subsequently, the amplified DNA was digested with HinfI. The resulting DNA fragments were treated with the Klenow fragment to produce blunt ends, and then digested with Spal. The fragment containing the coding region of CC-II was inserted into pUC18 plasmid which

| isoform 1 | MRKHKVLVQSVRVSQIVQIYMTVQQM |
| isoform 2 | --------A--------------X-----N-NA-ED-N---T---T---V---Q---E---H-- |
| isoform 3 | QELARFVWENQKANALLGFQKVHVAKTQVGAHMYLTIEVVDGVEKLYEAKV |
| isoform 4 | ------------K-----D------------- |
| isoform 5 | ------------D--K------------- |

Fig. 1. Amino Acid Sequences of the Isolated Clones Encoding Cystatins.
Amino acids are shown by the one-letter code in the top row only. In the other rows, identical amino acids are represented by bars. The points where amino acid deletion has occurred are indicated by X.

† The nucleotide sequence data for corn cystatin II reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D38130.
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Fig. 2. Comparison of CC-I and CC-II for Nucleotide and Deduced Amino Acid Sequences.

Table Inhibition of Cysteine Proteinases by Corn Cystatin I and II (CC-I and CC-II)

<table>
<thead>
<tr>
<th>Cystatin</th>
<th>Papain</th>
<th>Cathespin B</th>
<th>Cathespin H</th>
<th>Cathespin L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-I</td>
<td>$5.7 \times 10^{-8}$</td>
<td>$2.9 \times 10^{-7}$</td>
<td>$5.7 \times 10^{-8}$</td>
<td>$1.7 \times 10^{-8}$</td>
</tr>
<tr>
<td>CC-II</td>
<td>$6.6 \times 10^{-6}$</td>
<td>$1.3 \times 10^{-6}$</td>
<td>$1.1 \times 10^{-6}$</td>
<td>$1.1 \times 10^{-10}$</td>
</tr>
</tbody>
</table>

$a$ $K_i$ values were found from Dixon plots.$^8$

Proteolytic activities were each measured by the method described in ref. 5 using the following substrates for respective enzymes: N-benzoyl-L-arginine-2-naphthylamide for papain and cathespin B, L-arginine-2-naphthylamide for cathespin H, and N-carboxybenzoyl-L-phenylalanyl-L-arginine-7-(4-methyl) coumarylaldehyde for cathespin L. The cysteine proteinases used here were obtained as described in ref. 5.

had been filled in after digestion with EcoRI and then digested with SphI. The expression plasmid pCC37 was thus obtained. CC-II protein was expressed in E. coli YAL21 by the same method as in the case of CC-I protein. The expressed CC-II protein was purified by gel filtration on Sephadex G-50, ion-exchange HPLC on Shin-pack PA-DEAE (Shimadzu), and reversed-phase HPLC on Capcell Pak C8 (Shiseido).

The inhibition profiles of CC-I and CC-II against various kinds of cysteine proteinases are shown in Table. Both cystatins were strong inhibitors of papain, cathespin H, and cathespin L, but weak inhibitors against cathespin B. A characteristic difference was observed between the two corn cystatins for the inhibition of cathespin L. For the inhibition of this lysozymal cysteine proteinase, CC-II had a lower $K_i$ ($1.1 \times 10^{-10}$) than CC-I ($1.7 \times 10^{-8}$). In this experiment, both corn cystatins were expressed as fusion proteins consisting of a polylinker-originated polypeptide and a truncated corn cystatin lacking the amino acid residues corresponding to the signal peptide, respectively. In the inhibition of papain, no distinct difference of $K_i$ values was observed between oryzacystatin expressed as a fusion protein in E. coli and native oryzacystatin occurring in rice. Therefore, the influence of polylinker-originated polypeptide in cystatins expressed as fusion proteins on the inhibitory activity seems to be negligible. The lower $K_i$ for the inhibition of cathespin L by CC-II
may be due to the substitution of some amino acids around the probable N-terminal region of the mature CC-I and this suggests the possibility that protein engineering could be applied to re-modelling of corn cystatin for the purpose of strongly inhibiting cathepsin L, which is a physiologically undesirable factor.8,9

References