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## Purification and Characterization of Chymotrypsin Inhibitor CI-3 from Hemolymph of Silkworm, *Bombyx mori*

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The chymotrypsin inhibitor 3 (CI-3) whose expression is controlled by *Ict-E* gene on the 22nd linkage group was purified from the larval hemolymph of silkworm *Bombyx mori* by combination of a series of column chromatography and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The molecular weight of CI-3 was 40 kDa and its isoelectric point was 5.5. The results of analysis on the protein properties revealed that the inhibitory activity of CI-3 against  $\alpha$ -chymotrypsin was remarkably stable at pH 6.9–10.6, but lost 30% and 70% of the inhibitory activity at pH 5.9 and pH 11.9, respectively. CI-3 was quite stable at the temperature between 0°C to 50°C and lost inhibitory activity completely at the temperatures higher than 60°C. CI-3 showed strong inhibitory activity toward  $\alpha$ -chymotrypsin and silkworm digestive juice (DJ) protease.

Key Words: *Bombyx mori*; hemolymph; chymotrypsin inhibitor; protein purification

### INTRODUCTION

Protease inhibitors are widely found in animals, plants and microbes (Sato and Murano, 1973; Yoshimoto and Laskowski, 1982; Brizin *et al.*, 1984; Taguchi *et al.*, 1994). They have recently been extensively studied since they involved in regulation of other functional proteins, protection tissues from harmful proteases and prevention of invading proteases (Kurioka *et al.*, 1999; Yoshimoto *et al.*, 1982; Narayanaswamy *et al.*, 1988).

According to the electrophoretic mobility in polyacrylamide gel, there are at least 16 chymotrypsin inhibitors (CIs) in the larval hemolymph of silkworm, *Bombyx mori*. These CIs are classified into three groups and are controlled by *Ict-A*, *Ict-D*, and *Ict-E* genes located on the 2nd, 19th and 22nd linkage group, respectively (Fujii *et al.*, 1989, 1996a; Deng *et al.*, 1990; Shinohara *et al.*, 1993). The first group including CI-9, -10, -13, -13', -1, -2, -2', -b1 and -b2, controlled by *Ict-A* gene, is Kunitz-type CIs with low molecular weight. While the *Ict-D* gene is responsible for the production of CI-6, -7, and -8 which are Serpin-type CIs with an oligosaccharide chain and molecular mass of 42 kDa. (Shirai *et al.*, 1997). These protease inhibitors have been purified, characterized, and the studies on their functions are in progress. It is worthwhile to note that the genomic structures of CI-13 and CI-b1 have been clarified and CI-b1 was confirmed as an immune-related gene (He *et al.*, 2003). In addition, it was found that CI-8, once secreted

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into hemolymph, was sequestered into the fat body again after the onset of spinning (Shirai *et al.*, 2000). Moreover, the receptors of CI-8, which were expressed in the midgut of silkworm during metamorphosis, were identified and purified in our laboratory (data unpublished). CIs referred to as CI-3 and CI-4 belonging to the third group are controlled by the *Ict-E* gene mapped to the starting position of the 22<sup>nd</sup> linkage group (Fujii *et al.*, 1996b). Our previous studies of 400 silkworm *B. mori* strains preserved in Kyushu University demonstrated that every strain contains CI-3 or CI-4 or both. It indicated that CI-3 and CI-4 are essential components and play important roles during the life of silkworm. In the present study, CI-3 was purified and characterized as a first step toward the elucidation of its physiological functions.

## MATERIALS AND METHODS

### Insects and hemolymph

Silkworm strain, *B. mori*, used in this study is e01 preserved in the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University. The larvae were reared on mulberry leaves. Hemolymph collected from larvae on day 4 of the 5<sup>th</sup> instar was centrifuged at 3,000 g for 10 minutes at 4 °C to remove hemocytes. The resultant hemolymph was mixed with a trace of phenylthiourea and stored at -20 °C until to be used.

### Zymogram for CIs

In the detection of CIs activity by zymogram, samples were electrophoretically separated. Gels were then incubated with  $\alpha$ -chymotrypsin solution, and stained by protease substrate *N*-acetyl-D, L-phenylalanine- $\beta$ -naphthylester dissolved in N, N'-dimethylformamide and tetrazotized orthodiamisidine (Sigma, Inc.).

### Purification of CI-3

Hemolymph was fractionated by salting out with ammonium sulfate solution between 30 to 85% saturation. Precipitates were dissolved in 50 mM Tris-HCl (pH 7.0) and dialyzed overnight against the same buffer with 20% saturation of ammonium sulfate. Dialysate was loaded onto a Butyl Toyopearl 650 M column (2.0×35 cm) equilibrated with the same buffer. The column was firstly washed at 4 °C to remove the non-adsorbed proteins, then followed by linear gradient elution with ammonium sulfate from 20% to 0% to dissolve the adsorbed proteins. Inhibitor-containing fractions of target proteins were collected and applied to chromatography on a DEAE Sepharose column (1.5×40 cm) that have been equilibrated with 50 mM Tris-HCl (pH 7.0). The adsorbed proteins were eluted with a linear gradient of sodium chloride from 0 to 3 M. Active fractions were combined and dialyzed overnight against 1 mM phosphate buffer (PB). Samples were then loaded into a hydroxylapatite column and eluted with PB of increasing concentration from 1 mM to 0.5 M to dissolve the adsorbed proteins. CI activity was monitored by native-PAGE and followed by zymogram throughout the purification steps.

### Electrophoresis and recovery of target protein

The above protein sample was loaded onto 12% polyacrylamide gel in the presence of sodium dodecyl sulfate for electrophoresis (Laemmli, 1970). The pieces of gel with the

target protein were cut down and the proteins were eluted by an electro-eluter. Protein concentration was determined by the Lowry's method using bovine serum albumin as a standard (Hayashi, 1983).

### Determination of isoelectric point and molecular mass

Two-dimensional polyacrylamide gel electrophoresis (2D/PAGE) was carried out for determination of isoelectric point and molecular mass. The immobilized electrolyte used in the first isoelectric focusing was Immobiline™ Drystrip of pH 3–10 and 18 cm in size. The second electrophoresis was done in a 15% SDS-PAGE. For isoelectric point (pI) calibration, a mixture of proteins with a pI value from 5.1 to 7.1 was used as markers. The molecular mass markers used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.4 kDa) purchased from Amersham Pharmacia Biotech Co. Ltd.

### Assay of inhibitory activity of CI-3

Inhibitory activity against chymotrypsin was measured by the method using casein as substrate, described by Fujii (Fujii *et al.*, 1989). One unit of inhibitory activity was defined as the amount of inhibitor required for the complete inhibition of 400 pmoles  $\alpha$ -chymotrypsin. The effects of temperature and pH on the inhibitory activity were also examined by the method previously reported (Fujii *et al.*, 1989). In each inhibitory reaction for effects of pH on the stability of CI-3, 100  $\mu$ l CI-3 solution containing 2.5 U of inhibitory activity was incubated with 40  $\mu$ l buffers of different pH. Citrate-phosphate buffer was used for pH 3.0 to pH 7.0, Tris-HCl buffer for 7.0 to 9.0 and glycine-NaOH buffer for 9.0 to 11.0. The residual inhibitory activities of CI-3 were measured after incubating for 24 hours at 4°C in the indicated buffers. For effects of temperature on the stability of CI-3, in each reaction 2.5 U CI-3 solution was mixed with 40  $\mu$ l 50 mM Tris-HCl (pH 7.4) and kept under different temperature for 30 minutes before measurement of the residual inhibitory activity. Specificity of CI-3 against various proteases was investigated by the method previously reported (Fujii *et al.*, 1989). The proteases used in this study were  $\alpha$ -chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), ficin (EC 3.4.22.3), carboxypeptidase A (EC 3.4.17.1), V8 proteinase (EC 3.4.21.19), and *Serratia* peptidase from *Serratia* sp. All of these proteases were obtained from Sigma (Sigma, Inc.). Digested juice was collected from the 5th instar larvae.

### Sequencing of N-terminal amino acid residues of CI-3

After electrophoresis on 10% SDS-polyacrylamide gel, CI-3 protein was transferred to a PVDF membrane. The pieces corresponding to CI-3 were prepared and then submitted to analysis. Sequences of N-terminal amino acid residues were determined by automated Edman degradation (Edman and Begg, 1967).

## RESULTS AND DISCUSSION

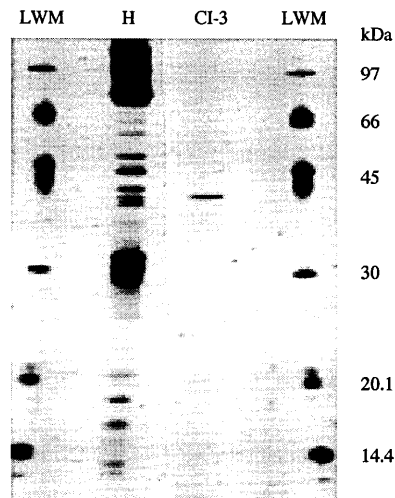
According to the previous research, the most poorly understood chymotrypsin inhibitors presented in silkworm hemolymph are CI-3 and CI-4. In this research, we have

used the e01 strain of *B. mori* which has CIs-3, 6, 7, 8, and 13 in the hemolymph to isolate CI-3. Through four-step procedure as described in Materials and Methods, the final preparation showed one protein band on SDS-PAGE followed by silver staining (Fig. 1). This result indicated that CI-3 was highly purified. The proteins were combined, lyophilized and stored at  $-20^{\circ}\text{C}$  as purified CI-3 for consequent analysis.

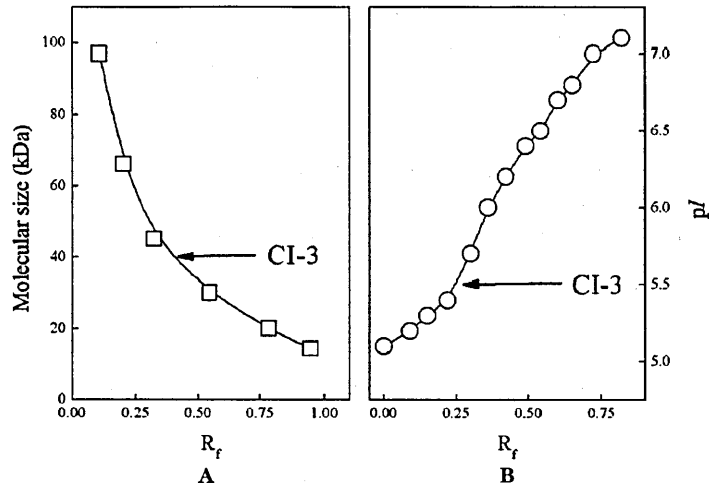
2-D electrophoresis was carried out to determine the molecular weight and isoelectric point of CI-3. As shown in Fig. 2A and 2B, the molecular weight and pI of CI-3 have been analyzed and compared with those of several markers. From the two curves, CI-3 was estimated to be a protein of approximately 40 kDa and pI of 5.5.

Purified CI-3 was stable below  $37^{\circ}\text{C}$  in neutral pH buffer (Fig. 3A). CI-3 retained nearly 60% of its antichymotrypsin activity after 30 min of heat treatment at  $50^{\circ}\text{C}$ , whereas this inhibitor lost most of its antichymotrypsin activity after heat treatment at the temperatures higher than  $60^{\circ}\text{C}$ . Among the purified chymotrypsin inhibitors presented in silkworm hemolymph, CI-3 showed similar thermal stability to CI-8 and lower stability than Kunitz-type CIs such as CI-13 and CI-b1 (Shirai *et al.*, 1997; Fujii *et al.*, 1989). The effects of pH were also investigated at  $4^{\circ}\text{C}$ . As shown in Fig. 3B, CI-3 was remarkably stable at pH of 6.9 to 10.6. However, it retained 70 and 30% of its antichymotrypsin activity at pH 5.9 and 11.9, respectively. This result indicated that CI-3 were easily inactivated in alkaline pH as well as in acidic pH.

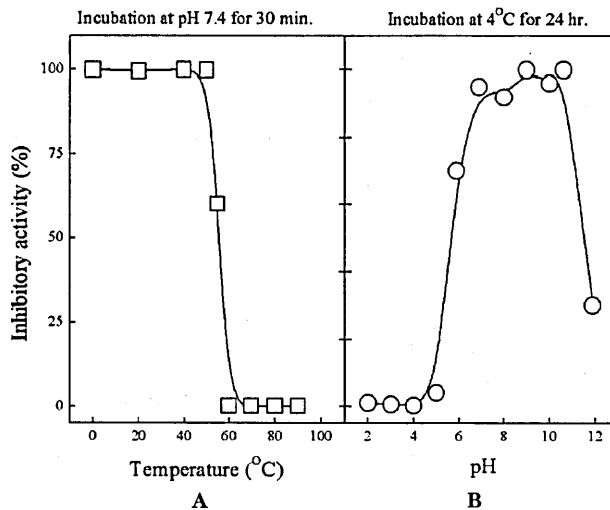
The purified CI-3 was tested against some serine proteases and the crude protease isolated from larval digestive juice, as listed in Table 1. The CI-3 presented an inhibitory activity towards  $\alpha$ -chymotrypsin, proteases from digestive juice, and very weak activity against ficin. Contrast to these, it can not inhibit trypsin, carboxypeptidase A, V8 pro-



**Fig. 1.** SDS-PAGE of the purified CI-3. H represents the hemolymph and LWM is the size markers.



**Fig. 2.** Determination of molecular weight and pI of CI-3. (A) Phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and  $\alpha$ -lactalbumin (14.4 kDa) were used as size standards. (B) A mixture of proteins with a pI value from 5.1 to 7.1 was used as isoelectric point markers. The arrows indicate the molecular weight and pI of CI-3.



**Fig. 3.** Effects of temperature and pH on the stability of the purified CI-3. (A) Effects of temperature on the stability of CI-3. For each test, 2.5 units of CI-3 was used. Results were expressed as the percentages of the activity at 4°C. (B) Effects of pH on the stability of CI-3. For each test, 2.5 units of CI-3 was used. CI activity was measured after incubating for 24 hours at 4°C at the indicated pHs.

teinase, and *Serratia* peptidase from *Serratia* *sp.*

The N-terminal amino acid sequences of CI-3 has been determined and resulted in a sequence up to 20 residues, 'AVTNLSNVLKNGNDNFTARM'<sup>20</sup>. We compared these partial sequences of CI-3 with those of other protease inhibitors available in Database. The search result showed that the N-terminal amino acid sequences of CI-3 are identical with those of antitrypsin and antichymotrypsin, and demonstrated that CI-3 might belong to the serpin family (Sasaki, 1991). Since CI-3 can inhibit  $\alpha$ -chymotrypsin specifically, it seems to show that CI-3 is a counterpart of antichymotrypsin. Future studies will involve cloning the gene of CI-3 that can allow us to identify the total sequence of this chymotrypsin inhibitor.

**Table 1.** Specificity of CI-3 against various proteases. 2.5 units of CI-3 were used for each measurement. The inhibitory activity was expressed in percentages of inhibitory activity against chymotrypsin.

Protease	Relative inhibitory activity (%)
$\alpha$ -chymotrypsin	100.0
trypsin	0.0
ficin	2.9
Carboxypeptidase A	0.0
V8 proteinase	0.0
<i>Serratia</i> peptidase	0.0
<i>B. mori</i> digestive juice protease	94.1

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