

Amino acid sequence of a novel Kunitz-type chymotrypsin inhibitor from hemolymph of silkworm larvae, *Bombyx mori*

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The primary structure of the chymotrypsin inhibitor (SCI-III), from larvae of the silkworm, *Bombyx mori*, has been determined. This inhibitor seems to be a Kunitz-type inhibitor judging from the sequence homology with the bovine basic pancreatic trypsin inhibitor, but has one more amino acid in the half-cystine frame nearest the N-terminus. The amino acid at the deduced reactive site (P_1) is phenylalanine and this inhibitor is the first example of the Kunitz type that is assigned phenylalanine to its reactive site.

Proteinase inhibitor	Chymotrypsin	Amino acid sequence	Specificity	Binding region
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1. INTRODUCTION

It is well known that protein proteinase inhibitors are widely distributed among animals, plants and bacteria and they are thought to play an important role in regulating the activities of proteinases in vivo. Accumulation of primary structure data about these inhibitors has revealed the existence of several homologous structures between some inhibitors [1]. Kunitz-type inhibitor, one of these groups, is based on the frame structure of 6 half-cystine residues of bovine basic pancreatic trypsin inhibitor (BPTI) [2]. This group includes inhibitors isolated from bovine colostrum [3], snail [4], and several snake venoms [5–8]. We have reported the isolation and characterization of 3 low- M_r (7000) chymotrypsin inhibitors from hemolymph of the silkworm larvae, *Bombyx mori*. Two (SCI-I and -II) are basic, one (SCI-III) is an acidic protein and they have similar amino acid compositions except for charged amino acid contents. These inhibitors are rare ones isolated from insect and can inhibit only chymotrypsin at a 1:1 molar ratio. Elucidation of their covalent structure is necessary for comparison with those of other species and for estimation of the reactive site, or

the primary inhibitor-proteinase binding site. Here, the primary structure of SCI-III is described.

2. MATERIALS AND METHODS

The isolation procedure of SCI-III was as in [9] except for the use of SP-Sephadex C-25 at pH 3.0 instead of chymotrypsin-Sepharose 4B. The *S*-carboxymethylation was also according to [9]. Tryptic digestion of *S*-carboxymethylated SCI-III (SCM-SCI-III) was carried out at 100:1 (w/w) substrate:enzyme in 0.1 M NH_4HCO_3 (pH 8.0) at 35°C for 4 h. Peptides were separated on a Dowex 50-X2 column using a gradient of pyridine-acetate buffer from 0.2 M (pH 3.1) to 2 M (pH 5.0) [10]. Detection of peptides was performed by ninhydrin reaction after alkaline hydrolysis.

Chymotryptic digestion of SCM-SCI-III was carried out at 100:1 (w/w) substrate:enzyme in 0.1 M NH_4HCO_3 (pH 8.0) at 25°C for 30 min. Peptides were separated on DEAE-Sephadex A-25 using a linear gradient of NH_4HCO_3 from 0.01 to 1 M and were detected by the absorbance at 230 nm.

Digestion of SCM-SCI-III by *Staphylococcus aureus* V8 protease was carried out at 25:1 (w/w)

substrate:enzyme in 0.05 M NH_4HCO_3 (pH 7.8) at 35°C for 24 h. Peptides were separated by high-performance liquid chromatography (HPLC) with a Jasco Finepak SIL C_{18} column as in [11]. Chymotryptic subfragments were generated in 0.1 M NH_4HCO_3 (pH 8.0) (peptide:enzyme, 30:1) at 35°C for 2 h and peptides were also separated as in [11].

Amino acid analyses of peptides were performed with a Jeol JLC-8AH amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 22 h.

Automated sequence analyses were performed with a Jeol JAS-47K sequence analyzer according to the manufacturer's manual using 0.25 M Quadrol buffer as a coupling buffer. Manual sequence analyses were carried out as in [12]. Phenylthiohydantoin amino acid derivatives were identified by HPLC as in [13].

3. RESULTS AND DISCUSSION

The complete amino acid sequence of silkworm chymotrypsin inhibitor (SCI-III) is shown in fig.1 together with the key peptides used for sequence elucidation. This inhibitor consists of 63 amino acid residues and has 6 half-cystine residues at positions 10, 20, 36, 44, 57 and 61 from the N-terminus. This arrangement closely resembles that of the so-called Kunitz-type inhibitor, but differs

in only one respect, i.e., the half-cystine frame nearest the N-terminus contains 9 amino acid residues instead of 8 (fig.2). Other highly conserved amino acids in Kunitz-type inhibitor, such as Gly-12, Tyr-23, Asn-24, Phe-33, Tyr-35, Gly-36, Gly-37, Asn-43 and Phe-45 (numbered according to BPTI), which are thought to be in contact with the amino acids of proteinase on binding [14], also exist in SCI-III at the same positions (fig.2). Though the arrangement of half-cystine defines the Kunitz-type inhibitor, we believe SCI-III to belong to this group because the arrangement of key amino acids to be preserved, other than that of the half-cystine nearest the N-terminus, agrees well with that of Kunitz-type inhibitor. Estimation of excessively inserted amino acid within the half-cystine frame nearest the N-terminus of SCI-III is not easy because the conservation of amino acids in this region is not so rigid in Kunitz-type inhibitor. The effect of insertion on inhibiting capacity is not obvious, but judging from the magnitude of the dissociation constant ($K_i \approx 10^{-8}$ M) [9] and from the contact region profile deduced from crystallographical analysis of BPTI-trypsin complex [14], a serious defect as an inhibitor does not seem to result from the insertion. In any case, this is the first example of finding the discrepancy of the half-cystine frame in Kunitz-type inhibitor.

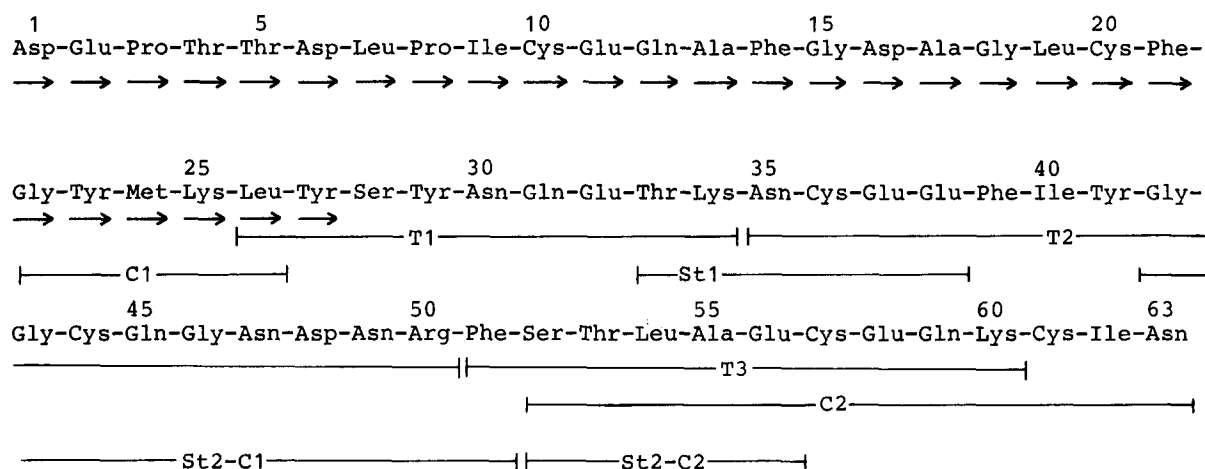


Fig.1. Amino acid sequence of silkworm chymotrypsin inhibitor, SCI-III: T1-T3, tryptic peptides of SCM-SCI-III; C1 and C2, chymotryptic peptides of SCM-SCI-III; St1 and St2, *S. aureus* V8 fragments of SCM-SCI-III; St2-C1 and St2-C2, chymotryptic peptides of St2; (→) sequence analyzer run of SCM-SCI-III. Only the overlapping key peptides are shown.

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