

Amino Acid Sequence and Secondary Structural Analysis of the Corn Inhibitor of Trypsin and Activated Hageman Factor*

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The amino acid sequence of a corn inhibitor for trypsin and activated Hageman Factor (Factor XIIa) was determined by automated Edman degradation from the intact inhibitor and two fragments generated by specific cleavage of the inhibitor. The 112-residue sequence is unique at each position except 91, where both Ala and Glu were found. The structural heterogeneity suggests the occurrence of two genes (possibly allelic) for the inhibitor. Based on analysis of fragments produced by the interaction of the inhibitor with trypsin-agarose, the reactive site peptide bond is identified as Arg 36-Leu 37. There is no strong similarity between the sequence of the corn inhibitor and the sequences published for other serine protease inhibitors. Thus, the corn inhibitor represents a new family of protease inhibitors. Circular dichroism measurements and a theoretical prediction of secondary structure indicate that the inhibitor has helix and β sheet contents of approximately 40 and 20%, respectively.

Corn seeds contain a small protein that is a potent inhibitor (1) of mammalian trypsins (2, 3). For reasons that are not understood, seeds of a high lysine corn cultivar, opaque-2 (4), have an elevated trypsin inhibitor level (3, 5). We have previously purified the inhibitor from opaque-2 corn and reported some of the physicochemical properties of the protein (6). More recently, it has been reported (7, 8) that corn trypsin inhibitor is also a specific inhibitor of activated Hageman Factor (Factor XIIa of the intrinsic blood clotting process).

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Of the many serine proteases tested, only trypsin and Factor XIIa are inhibited by the corn protein (6-8). In order to accomplish a detailed analysis of the inhibitor's interactions with activated Hageman Factor and with trypsin, it is necessary to know the inhibitor's structure. An amino acid sequence has been proposed (9) for a trypsin inhibitor from an unspecified strain of maize (corn). However, certain of the opaque-2 inhibitor's properties, particularly its molecular weight and amino acid composition (6), are quite different from the properties of the maize inhibitor (9). The basis for these differences does not appear to be differences in source material, since inhibitors indistinguishable from the opaque-2 inhibitor have been found in genetically diverse types of corn (5) and even in a different species (10). Furthermore, it appears that corn seeds contain only one major trypsin inhibitor (10) which may consist of two or more closely related variants (7).

Whether or not the maize protein studied by other workers is a different inhibitor from the opaque-2 inhibitor, it is clear that the sequence proposed for the maize inhibitor (9) cannot correspond to the opaque-2 corn inhibitor of trypsin (6) and activated Hageman Factor (7). We therefore undertook sequence determination of the inhibitor from opaque-2 corn seeds. In addition to reporting that sequence, we also provide experimental and theoretical estimates of secondary structure in the protein.

MATERIALS AND METHODS AND RESULTS¹

DISCUSSION

The corn inhibitor of trypsin and activated Hageman Factor consists of 112 amino acid residues and has a calculated molecular weight of 12,028 (Figs. 1 and 4). This is in good agreement with the molecular weight of 12,500 that we had

¹ Portions of this paper (including "Materials and Methods," "Results," Figs. 2, 3, 5, and 6, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-1759, cite the authors, and include a check or money order for \$4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

determined by sedimentation equilibrium in the analytical ultracentrifuge (6). The amino acid sequence reported in this paper is also in good accord with the amino acid composition that we had determined for the corn inhibitor (6). On the other hand, there is little similarity between our sequence and that reported by other workers (9) for a trypsin inhibitor from maize. The 8 NH₂-terminal residues are identical in the two sequences as are the first 2 residues of the COOH-terminal tryptic fragment. No other correspondence occurs between these two sequences. There is similarity in the relative sizes of the proposed tryptic fragments, but a large discrepancy in the overall length of the proteins and the previously reported sequence (9) contains only 65 residues.

Laskowski has formulated a "standard mechanism" of action for protein inhibitors of serine proteases (11). In that mechanism, two forms of an inhibitor exist in association with the protease at equilibrium: a single-chain (virgin) form and a two-chain (modified) form. Each form is fully inhibitory, and the same equilibrium mixture is obtained by combining either form of the inhibitor with the protease.

All available evidence indicates that the corn inhibitor obeys the standard mechanism in its interaction with trypsin

and, presumably, with Factor XIIa. If isolated by chromatography on immobilized trypsin, the corn inhibitor is recovered as a mixture of single-chain and two-chain forms (6). Each form is inhibitory, and the two forms are indistinguishable in molecular weight and amino acid composition (6). When the single-chain form is separated from the two-chain form (by DEAE-cellulose chromatography) and then chromatographed on trypsin-agarose, a mixture of single- and two-chain forms is eluted (6). Furthermore, purified two-chain inhibitor generates a mixture of single-chain and two-chain forms upon chromatography on trypsin-agarose.² These results are entirely consistent with single- and two-chain forms of corn inhibitor obtained by chromatography on trypsin-agarose being the virgin and modified forms of a protein that obeys the standard mechanism of inhibition.

Other evidence further strengthens this conclusion. Corn inhibitor isolated by chromatography on an immunoabsorbent (rather than on trypsin-agarose) is a single-chain protein that we assume to be the virgin form of the inhibitor (10). In this paper, we have reported that the NH₂-terminal sequence of the immunoabsorbent-purified inhibitor is the same as that of the single-chain form eluted from trypsin-agarose. This information coupled with our previously published results on the properties of the immunoabsorbent-purified inhibitor (10) leads to the conclusion that the single-chain inhibitor eluted from trypsin-agarose is the same protein as the immunoabsorbent-purified inhibitor. Hence, we can identify the single-chain inhibitor eluted from trypsin-agarose as the virgin form of the corn inhibitor. Furthermore, we conclude from the results of chromatography of the two-chain form on trypsin-agarose (see above), that the two-chain inhibitor eluted from trypsin-agarose is the modified form of the corn inhibitor; i.e. that only one bond (the reactive site bond) is cleaved during chromatography of the corn inhibitor on trypsin-agarose. That reactive site bond is identified in this paper as Arg 36-Leu 37.

The reactive site region of the corn inhibitor is notable in its high content of proline (4 of the 8 residues immediately surrounding the reactive site bond are prolines). It is unusual for proline to occur in the active site regions of serine protease inhibitors (11). The occurrence of 4 proline residues in this short stretch of polypeptide would greatly restrict the chain's flexibility. The prolines presumably help maintain the reactive site region in a conformation that is particularly well suited to bind to the active sites of trypsin and of Factor XIIa and less well suited to bind to the active sites of other trypsin-like enzymes of blood plasma.

The corn inhibitor is similar to other protease inhibitors in having a high content of cysteine. All 10 cysteine residues in this protein are involved in disulfide bonds (6), but we have not determined the pairings. A peculiarity of the corn inhibitor is its three tryptophan residues. Tryptophan is usually absent in the plant protease inhibitors whose sequences have been published.

Laskowski and Kato (11) introduced the term "family" to denote, in its technical sense, a set of protease inhibitors that are closely related to each other in amino acid sequence. Members of a family are sufficiently similar in sequence that they can be aligned readily by simple visual inspection. Laskowski and Kato identified eight such families (11). Our visual inspection of the corn inhibitor sequence and other protease inhibitors whose sequences have been reported revealed no clear similarities between the corn inhibitor and other inhibitors. This lack of strong similarity was confirmed by a

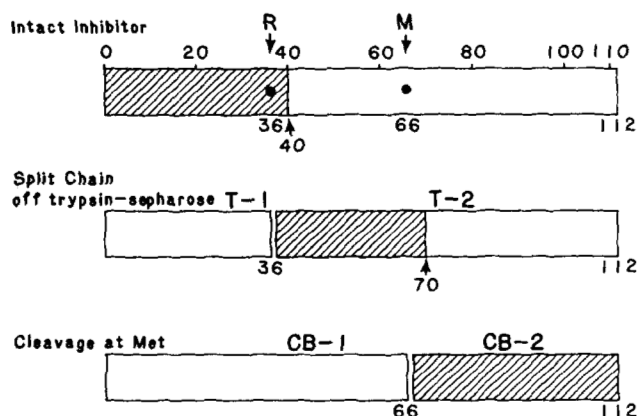


FIG. 1. Summary of the sequencing strategy. Hatched areas denote those portions of the intact inhibitor and selected fragments that were degraded. Standard, one-letter abbreviations are used to designate residues of importance to the production of appropriate fragments.

Ser-Ala-Gly-Thr-Ser-Cys-Val-Pro-Gly-Trp-Ala-Ile-Pro-His-Asn-	15
Pro-Leu-Pro-Ser-Cys-Cys-Trp-Tyr-Val-Thr-Ser-Arg-Arg-Cys-Gly-	30
Ile-Gly-Pro-Arg-Pro-Arg-Leu-Pro-Trp-Pro-Glu-Leu-Lys-Arg-Arg-	45
Cys-Cys-Arg-Glu-Leu-Ala-Asp-Ile-Pro-Ala-Tyr-Cys-Arg-Cys-Thr-	60
Ala-Leu-Ser-Ile-Leu-Met-Asp-Gly-Ala-Ile-Pro-Pro-Gly-Pro-Asp-	75
Ala-Gln-Leu-Glu-Gly-Ala-Leu-Glu-Asp-Leu-Pro-Gly-Cys-Pro-Arg-	90
Ala-Val-Gln-Gln-Gly-Phe-Ala-Ala-Thr-Leu-Val-Thr-Glu-Ala-Glu	105
Glu	
Cys-Asn-Leu-Glu-Thr-Ile-Ser-(112)	112

FIG. 4. The complete amino acid sequence of the corn trypsin/activated Hageman Factor inhibitor. The arrow indicates the reactive site bond.

² M.-G. Lei and G. R. Reeck, unpublished observations.

computerized search of the Protein Data Base of the National Biomedical Research Foundation's *Atlas of Protein Sequence and Structure*. (That data base contained the sequence of at least one representative of each of the eight families of Laskowski and Kato.) Thus, the corn inhibitor is the first documented representative of a family of inhibitors for which no reliable sequence information has been available.

The corn inhibitor is not only distinct from the other known inhibitors in amino acid sequence, it appears to be different in its secondary structure from at least those inhibitors whose structures have been elucidated by x-ray crystallography (12). In each of those three inhibitors, the content of β -pleated sheet is substantially greater than the content of α -helix. Thus, pancreatic trypsin inhibitor (Kunitz) was found to contain no helix and a good deal of structure resembling β -pleated sheet (12). Soybean trypsin inhibitor (Kunitz) was reported to contain about 25% of its residues in a helical configuration and about 50% in β -sheet (13). The subtilisin inhibitor contains about 17% helix and 35% pleated sheet (14). On the other hand, our estimates from admittedly less reliable methods (see "Results" in the Miniprint) suggest that the corn inhibitor contains substantially more helix (about 40%) than it does β -sheet (about 20%).

The structural heterogeneity identified at residue 91 presumably accounts for two of the isoelectric variants of the corn inhibitor that were first described by Hojima *et al.* (7). That heterogeneity may also account for the apparent chromatographic heterogeneity noted in our earlier work (6). An interesting question is the genetic basis of the heterogeneity. From the data currently available, it is not possible to know whether the different genes for the inhibitor are allelic or if multiple loci exist. We hope to address that question by isolating and sequencing the genes and surrounding DNA sequences.

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SUPPLEMENTARY MATERIAL TO AMINO ACID SEQUENCE AND
SECONDARY STRUCTURAL ANALYSIS OF THE CORN INHIBITOR
OF TRYPSIN AND ACTIVATED HAGEMAN FACTOR

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MATERIALS AND METHODS

ISOLATION OF THE INHIBITOR. Seeds of opaque-2 corn (*Zea Mays*) were harvested in the agronomy fields of Kansas State University and generously provided by Professor Clyde A. Wasson. Nearly all of the inhibitor used in the current work was isolated by chromatography of extracts of corn seeds over a trypsin-agarose affinity matrix as described previously (6). The inhibitor obtained from a trypsin-agarose column is recovered as a mixture of single-chain and split-chain proteins. These two forms of the inhibitor were separated by chromatography on DEAE-cellulose (6). Intact inhibitor was also isolated using an alternative approach (10), which consisted of chromatography on an immunoabsorbent constructed by immobilizing affinity-purified antibodies against the inhibitor on cyanogen bromide-activated agarose.

PREPARATION AND ISOLATION OF INHIBITOR FRAGMENTS. Following reduction and S-pyridylethylation (15) the two fragments of the split-chain form of the inhibitor were separated by gel filtration. An additional 15 mg of the S-pyridylethyl single-chain inhibitor was cleaved at the single methionyl residue by cyanogen bromide (16). The resulting products were isolated by high-performance liquid chromatography using the 0.1% trifluoroacetic acid acid buffer system of Mahoney and Hermodson (17) and large-pore (300 Å) reversed-phase supports (18,19).

AMINO ACID ANALYSIS. In all cases, fragments were recovered by lyophilization and characterized by analysis on a Beckman 6300 amino acid analyzer after hydrolysis in vacuo for 24 hours in glass-distilled, 6N HCl at 110°C. Amino acid analyses of the intact inhibitor were carried out after 24, 48, 72, and 120 hours of hydrolysis. The color value for S-(pyridylethyl) cysteine, which is eluted as a discrete peak between lysine and ammonia, is 1.2 times that for leucine (20). Tryptophan content was determined by amino acid analysis as the Trp/His ratio following base hydrolysis using corn starch as an antioxidant (21).

SEQUENCE ANALYSES. Intact S-pyridylethylated inhibitor and the fragments were degraded in a Beckman Model 890D sequencer according to the procedure of Edman and Begg (22) as modified by Hermodson *et al.* (23), using the "peptide program" of Hermodson *et al.* (24). Prior to the addition of the peptide to the sequencer cup, 2 mg of Polybrene were dissolved in 0.7 ml of 50% acetic acid and applied to the cup of the sequencer, dried under vacuum, and subjected to three complete cycles of automated Edman degradation. The peptide under investigation was then introduced into the cup and sequentially degraded. The amino acid derivatives were converted to the PTH-amino acids as previously described (15). PTH-amino acid derivatives were identified by high-performance liquid chromatography using a 0.46 X 25 cm Ultrasphere ODS 5µm column (Beckman) (25,26) and a Varian Model 5060 equipped with a Model 8000 autosampler, a Varian UV 100 variable wavelength detector and a Hewlett-Packard 3390 A recorder/integrator. The detector was operated at 269 nm throughout except for a 3 min. interval, from 15.5 min. to 18.5 min., when the wavelength was automatically changed to 313 nm to facilitate the identification of the DTE adduct of dehydrothreonine.

From the yields of those PTH-amino acids that can be quantitated by HPLC (15,26), it was calculated that at least 70% of the amount of each derivative expected was recovered after each cycle. In no case were identifications attempted with a signal to noise ratio of less than 3. In all cases, only a single sequence was observed. Repetitive yields ranged from 94-97% throughout.

CIRCULAR DICHROISM MEASUREMENTS. Far ultraviolet circular dichroism spectra of single- and two-chain inhibitors were obtained in the laboratory of Dr. John R. Cann, University of Colorado Medical Center, on a Cary 60 spectropolarimeter with a circular dichroism attachment. Spectra were obtained on solutions of the single- and two-chain proteins at 0.48 and 0.46 mg/ml, respectively. The solvent was 0.15 M NaCl/10 mM Tris-Cl, pH 8.0. A cell with a 1 mm pathlength and a full scale setting of 0.04° were used.

RESULTS

SEQUENCE DETERMINATION. The strategy used in defining the sequence of the corn trypsin/activated Hageman Factor inhibitor is shown in Figure 1. The first 40 residues of the intact inhibitor were identified by stepwise degradation of the intact-inhibitor. Following the isolation of the split-chain components by gel filtration (Fig. 2) three peptides were identified by sequence analysis: intact chain, residues 1-36 (T-1), and residues 37-112 (T-2). Edman degradation of peptide T-2 placed residues 37-76 and provided a 4 residue overlap with the sequence obtained from the degradation of the intact inhibitor. Peptide CB-2, isolated as shown in figure 3 after cyanogen bromide cleavage, allowed placement of residues 67-112.

The overlapping portions of the partial sequences allowed unambiguous positioning of all fragments and completion of the inhibitor sequence as presented in Figure 4. Good agreement between the sequence and amino acid analyses (Table I) indicates that the sequence as presented is correct and complete. A summary of the representative stepwise yields, as estimated by high-performance liquid chromatography, appears in Table II.

Each cycle of the Edman degradation yielded only one unit residue per cycle with a single exception, residue 91. At this site both Ala and Glu with a ratio of 2:1, respectively, were identified.

CIRCULAR DICHROISM MEASUREMENTS. The far ultraviolet circular dichroism spectrum of the intact inhibitor is shown in Figure 5. The circular dichroism spectrum of the split-chain inhibitor was indistinguishable (not shown) from that of the intact inhibitor. To estimate contents of helix and beta sheet we used a least-squares program that forced a summation to 100% of the estimates for helical, beta sheet and irregular structures. With poly-lysine reference spectra (27), we obtained estimates of 34% helix and 24% beta sheet for the corn inhibitor. With the reference spectra of Chen *et al.* (28), the estimates were 46% helix and 20% beta sheet.

PREDICTED SECONDARY STRUCTURE. The rules of Chou and Fasman (29) were used to predict secondary structure, including beta turns, in the corn inhibitor (Figure 6). Forty-four residues (39%) are predicted to occur in helices and 19 residues (17%) in beta sheets. The predicted helices and sheets are largely limited to the C-terminal two-thirds of the protein sequence. Several beta turns are predicted, including one near the reactive site bond.

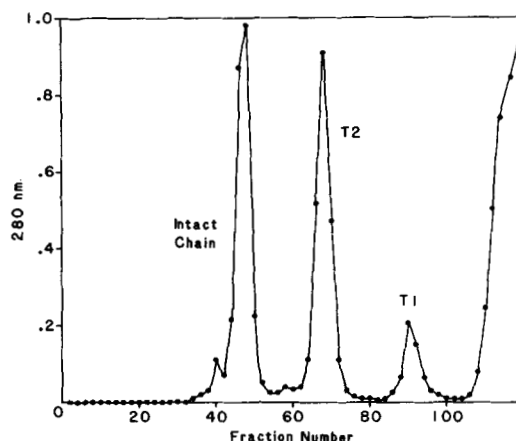


FIGURE 2. Elution profile produced by gel filtration of a mixture of S-pyridylethyl single-chain and two chain inhibitors. The mixture of inhibitors has been obtained by chromatography of an extract of corn seeds on a trypsin agarose column.

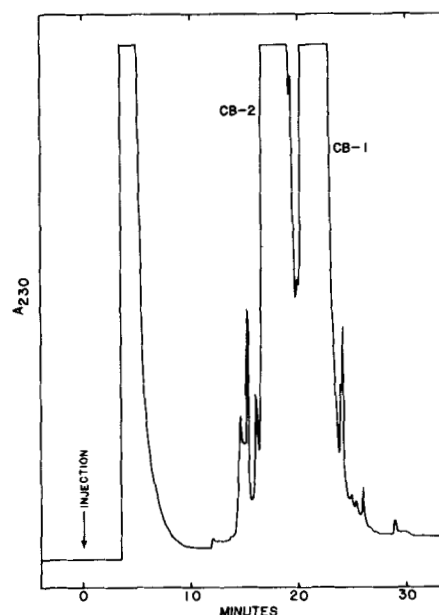


FIGURE 3. Chromatogram illustrating the isolation of fragment CB-2 generated by cleavage with cyanogen bromide. Fragment was isolated by application of a n-propanol gradient, from 0 to 60% over 30 min. Chart speed, 1 cm/min; pressure 88 atm; temperature, 23°C.

Table I

Amino Acid Analysis

Whole Chain(6)	Residues 1-36	Residues 37-66	Residues 67-112	Residues 37-112
(Seq) ³ (Comp)	(Seq) ³ (Comp)	(Seq) ³ (Comp)	(Seq) ³ (Comp)	(Seq) ³ (Comp)
Asp 6 6.4	1 1.0	1 1.1	4 4.0	5 4.5
Thr 6 6.2(4)	2 1.8	1 1.0	3 3.5	4 3.2
Ser 6 5.6(4)	4 3.6	1 1.0	1 0.8	2 1.9
Glu 10.3 10.4	- 0.2	2 2.2	8.3 8.2	10.3 10.1
Pro 14 13.5	6 5.7	3 3.9	5 4.6	8 8.1
Gly 9 9.6	4 4.1	0 0.1	5 4.8	5 5.2
Ala 11.7 11.6	2 2.0	3 3.0	6.7 6.3	9.7 9.2
Val 4 4.0(1)	2 1.7	-	2 1.9	2 2.0
Met 1 1.3	-	1 0.6	0 0.3	1 0.8
Ile 6 6.3	2 1.7	2 1.8	2 1.7	4 3.8
Leu 11 11.4	1 0.8	5 4.7	5 4.7	10 9.5
Tyr 2 1.8	1 0.9	1 0.9	-	1 0.5
Phe 1 1.0	-	-	1 0.2	1 0.6
His 1 1.0	1 1.0	-	-	-
Lys 1 1.1	-	1 0.9	-	0.2 1 0.6
PE-Cys 10 9.9	4 3.9	4 3.9	2 1.8	6 6.0
Arg 9 9.2	4 3.9	4 3.9	1 0.9	5 4.9
Trp 3 3.3(2)	2 ND	1 ND	-	1 ND

- (1) Averages of duplicate 120hr hydrolyses.
- (2) Determined after base hydrolysis in starch and expressed as the ratio of His/Trp.
- (3) Values in sequence columns indicate compositions determined from the sequence.
- (4) Determined by extrapolation to zero hr of hydrolysis.
- (5) ND, not determined.
- (6) Averages of duplicate 24-, 48-, 72-, and 120- hr hydrolyses, except where otherwise noted.

