The Partial Amino Acid Sequence of Trypsin Inhibitor II from Garden Bean, Phaseolus vulgaris, with Location of the Trypsin and Elastase-reactive Sites

(Received for publication, July 22, 1974)

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The amino acid sequences of garden bean (Phaseolus vulgaris) trypsin inhibitor II and a related molecular species II' have been examined. The entire sequence of II' has been determined with the exception of five internal residues, positions 24 to 28. Inhibitor II' appears to be derived from II by loss of eight NH₂-terminal residues. The garden bean inhibitors are highly homologous to the Bowman-Birk soybean inhibitor and lima bean trypsin inhibitor IV. The trypsin-reactive site has been located in the second half of the molecule, while the first reactive site has been found to be directed against elastase. Garden bean inhibitor II (and IV) is thus a double-headed inhibitor, simultaneously inhibiting 1 molecule of trypsin and 1 of elastase.

The isolation of three isoinhibitors of trypsin from the garden bean, Phaseolus vulgaris, var. great northern, has been previously reported (1). These inhibitors closely resemble other low molecular weight inhibitors from legumes such as lima beans (2, 3), mung bean (4), navy and kidney beans (5, 6), and the Bowman-Birk inhibitor of soybean (7). They have in common low molecular weights (approximately 8,000) and amino acid compositions characterized by high contents of serine, aspartic acid, and half-cystine, and the absence of tryptophan and carbohydrate.

While all three inhibitors from Phaseolus vulgaris strongly inhibit bovine trypsin, they vary greatly in their inhibition of bovine a-chymotrypsin. Inhibitor IIIb simultaneously and independently inhibits both trypsin and chymotrypsin. It therefore resembles the double-headed inhibitors typified by the Bowman-Birk soybean and lima bean IV inhibitors (3, 8-11). However, both inhibitors I and II are essentially inactive toward chymotrypsin and inhibit only 1 mol of trypsin per mol of inhibitor. This suggests that the second reactive site, normally directed against chymotrypsin, may have been changed during the course of evolution to a nonreactive form.

In the hope of elucidating the structural changes responsible for the loss of activity at this reactive site, we have determined the amino acid sequence of one of the inhibitors. Inhibitor II was chosen for several reasons. It is a major, if not the major, inhibitor species in the bean. Second, its action against trypsin varies significantly from that of the other two inhibitors. As previously noted, the dissociation constant of II with bovine β-trypsin is two to three orders of magnitude higher than those of inhibitors I or IIIb (1). We thus also hoped to obtain from the primary structure some indication of the basis for this significant difference in reactivity.

This paper presents a partial sequence of isoinhibitor II' and most of the sequence of isoinhibitor II, and describes the location of the two independent reactive sites directed against elastase and trypsin.

EXPERIMENTAL PROCEDURE

Materials—Garden bean inhibitor II was the same preparation described previously (1). A second preparation, inhibitor II', was isolated from garden beans, var. great northern, obtained from Chas. C. Hart Seed Co., Wethersfield, Connecticut. This material behaved identically to II on Sephadex G-50, and on gradient elution from DEAE-cellulose and SP-Sephadex C-25. However, it eluted earlier upon equilibrium chromatography on SP-Sephadex. The amino acid composition was similar to that of II (Table I),† with the loss of 8 amino acid residues (2 histidyl, 3 aspartyl, 2 seryl, and 1 glutamyl residue). The tryptic peptide map of performic acid-oxidized II' was identical with that of II (Fig. 12 of Ref. 1) with the exception that the most acidic weakly staining peptide was more mobile.

Crystalline salt-free bovine trypsin, 3 times crystallized bovine a-chymotrypsin, leucine aminopeptidase, elastase, carboxypeptidase A, and carboxypeptidase B (Lots TL8AF, CD10BK, LACP2FB, ESFF3CB, COADEFP6FC, and COBDFP6EA, respectively) were obtained from Worthington Biochemicals, Freehold, New Jersey.

† This research was supported by Grant HL-15892 from the National Heart and Lung Institute and Grant PRP-30 from the American Cancer Society. A portion of this work was taken from a thesis by K. A. W. submitted to the Roswell Park Division of the Graduate School of Cancer Society. A portion of this work was taken from a thesis by K. A. W. submitted to the Roswell Park Division of the Graduate School of Cancer Society. A portion of this work was taken from a thesis by K. A. W. submitted to the Roswell Park Division of the Graduate School of Cancer Society.

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tained from Worthington. Carboxypeptidase C (lot 12-73) was from Röhm and Hass, Darmstadt, Germany, and thermolysin, 3 times crystallized (Lot 84) from Calbiochem. Conzo red elastin was from Sigma. TPCK-trypsin was prepared according to Wang and Carpenter (12). Dansyl chloride and Sequanal-grade phenylisothiocyanate and trifluoroacetic acid were obtained from Pierce. Pyridine was purified by distillation twice from ninhydrin. Polyamide thin layer sheets (Chen Ching Trading Co., Ltd., Taipei, Taiwan) were obtained from Gallard-Schlesinger, New York. Sephadex G-10, G-25, and G-50 (sodium form) were from Pharmacia. Iodoacetamide (Sigma) was recrystallized immediately from hot water before use. Urea solutions were deionized before use by passage through a mixed bed of Dowex 50-X8 and Dowex 1-8.

All other chemicals were reagent grade or better. Glass-distilled water was used throughout.

Enzymatic Digests—Inhibitor II was reduced and carboxamidomethylated with iodoacetamide in a manner analogous to that described by Hirs (13), with a 12-hour reduction. The desalted RCAM-II solution from Sephadex G-10 was then lyophilized for storage.

Tryptic digestion of RCAM-II (22.3 mg) was carried out with 225 μg of TPCK-trypsin at 37° in 0.1 M ammonium bicarbonate, pH 7.85. After 1.5 hours, another 225 μg of trypsin were added and the incubation continued for an additional 1.5 hours. The reaction was then terminated by adjusting to pH 4 with glacial acetic acid. Complete tryptic digests of RCAM-II (19 mg) were carried out in a similar manner, with a total digestion time of 2 hours. Tryptic-modified inhibitor was prepared by incubating 35 mg of inhibitor II with 3 mg of TPCK-trypsin (3 mol %) in 0.05 M sodium formate, pH 3.75. After 4 days at room temperature, the trypsin was removed and the modified inhibitor recovered by gel filtration of a column (2.5 x 90 cm) of Sephadex G-50 in 0.1 M HCl. The resulting inhibitor II was purified and carboxamidomethylated as described above.

Fractionation of Peptides—Tryptic and chymotryptic digests were initially fractionated by gel filtration on columns (2.5 x 90 cm) of Sephadex G-25 in either 10% acetic acid or 0.01 M HCl. Peptides were further purified from the column fractions by high voltage paper electrophoresis as described by Katz et al. (14). Electrophoresis was carried out at 2 V/cm at pH 3.6 in pyridine-acetic acid-water (1:10:300) for 100 to 120 min in a Gilson model D Electrophorator. Peptides were visualized with 0.2% ninhydrin in acetone. Mobilities are given relative to the position of the peptide in the complete sequence, numbering from the N-terminus.

Determination of Inhibitor Activity—Inhibitor activity against porcine pancreatic elastase was determined by a modification of the Congo red-elastin assay developed by Naughton and Sanger (17) and described by Shotton (18). Elastase, approximately 120 μg, and inhibitor, 36 to 50 μg, were incubated in 0.6 ml of 0.07 M sodium borate, pH 8.5, for 20 min at room temperature. Congo red-elastin suspension (1 mg/ml in 0.02 M sodium borate, pH 8.8), 3.5 ml, was then added with vigorous mixing. At intervals of approximately 10 min the reaction mixture was briefly centrifuged, and the absorbance of the supernatant at 450 nm was recorded. Also run at the same time was an elastase control, containing elastase and elastin, but no inhibitor, and a blank containing only elastin. From absorbance data, the times for 50% release of the Congo red into solution were calculated (18), and thus the amount of elastase inhibited in the enzyme-inhibitor reaction mixture. A unit of inhibitor is defined as that amount of inhibitor which will inhibit the enzymatic activity of 1 mg of active elastase (or trypsin).

Concentrations of trypsin, chymotrypsin, elastase, and inhibitor II solutions were determined spectrophotometrically with optical factors (reciprocals of 0.1 x extinction coefficient E(280) of 0.67, 0.40, 0.54, and 2.35, respectively, to convert absorbance at 280 nm to milligrams of protein per ml.

RESULTS

Tryptic Peptides of RCAM-II—The results of the gel filtration of the tryptic digest of RCAM-II are shown in Fig. 1. Six peptide fractions were obtained. These were submitted to analytical and preparative high voltage paper electrophoresis as illustrated in Fig. 2. Peak A (not shown) contained only one weakly staining species at the origin. Peak B contained a single major, weakly staining peptide, while Peak C had one strongly staining peptide. The latter two species were subject to further preparative electrophoresis and obtained in pure form at this stage. The amino acid compositions of the resulting peptides are shown in Table 1 and the sequence data in Table II. Peptide T-I by composition consists of T-II plus T-III. Like intact RCAM-II it appears to be blocked at the NH2 terminus, precluding any degradation from this end. Digestion by carboxypeptidase B yielded the terminal arginine, with no further degradation occurring with carboxypeptidase A. T-II similarly appears to be blocked and undoubtedly comprises the NH2 terminus of both RCAM-II and T-1. Digestion with carboxypeptidase A revealed the terminal sequence -Val-Cys, past the pyrrolidonecarboxylic acid residue, allowing further degradation of the tryptic digest of T-III proceeded until the glutamine residue at position 7 was exposed. Cyclization of this residue to pyrrolidonecarboxylic acid hampered further degradation. Treatment of the resulting peptide with 0.1 M NaOH for 72 hours at room temperature partially hydrolyzed the pyrrolidonecarboxylic acid residue, allowing further degradation to establish -Gln-Cys-. Insufficient material was available for further degradation. Digestion by carboxypeptidase B established the COOH-terminal arginine.

Peptide T-Va appears to be derived from T-V by a non-tryptic like cleavage at the S-carboxamidomethylcysteinyl-threonyl bond.

Chymotryptic Peptides—The gel filtration of the chymotryptic digests of RCAM-II is shown in Fig. 3. Examination of the following designations for peptides are employed: T-, tryptic peptides; C-, chymotryptic peptides; Th-, thermolysin peptides of T-II; the Roman numerals following the enzyme designation indicate the position of the peptide in the complete sequence, numbering from the NH2 terminus. Primed (') peptides are derived from inhibitor II, unprimed are derived from II. T-L and T-S refer to the long and short peptides obtained from reduced and carboxamidomethylated trypsin-modified inhibitor II.
Fig. 1. Gel filtration of tryptic peptides of RCAM-II. The tryptic digest of 22.3 mg of RCAM-II was applied to a column (2.5 x 90 cm) of Sephadex G-25. The column was eluted with 10% acetic acid at 25 ml per hour, with 4-ml fractions collected. Fractions were pooled as indicated. ---, absorbance at 244 nm; ---, absorbance at 275 nm.

Fig. 2. Analytical high voltage paper electrophoresis of tryptic peptide pools from Fig. 1. Electrophoresis was at pH 3.6 at 2 kV for 100 min as described in the text. The capital letters near the origin correspond to the pools in Fig. 1. Roman numerals to the right of each peptide indicate the corresponding tryptic peptide. The major staining peptides in each pool are cross-hatched; minor peptides are unhatched. Mobilities are relative to arginine. Pool A contained only a weakly staining peptide at the origin and is not shown.

the resulting fractions by high voltage paper electrophoresis revealed over 40 distinct peptides, most in low yields. This apparently was caused by the susceptibility of bonds involving S-carboxamidomethylcysteinyl residues to chymotryptic cleavage. Several useful peptides were obtained, as presented in Tables II and III. Attention is at this point drawn to peptides C-II and C-III, which establish the sequence -Thr-Thr-Asx- in T-L. The limit tryptic peptides in T-L may thus be deduced as shown in Table IV, assuming approximately 10% contamination, presumably with intact inhibitor. T-II and T-III have already been ordered by T-I, and may be placed at the NH$_2$-terminus of T-L due to the blocked NH$_2$-terminus of T-II. Carboxypeptidase B digestion of T-L reveals a COOH-terminal arginine. This is consistent with the previously observed presence of arginine at the reactive site of inhibitor II (1). Carboxypeptidase A digestion following carboxypeptidase B treatment rapidly released threonine, S-carboxamidomethylcysteine, and methionine, indicating T-V to be COOH-terminal in T-L. The limit tryptic peptides in T-L may thus be ordered: (T-II)-(T-III)(T-IV)-(T-V). T-L may be placed at the NH$_2$-terminus of intact RCAM II, both due to the nature of the trypsin modification reaction that produced it, and the presence of the blocked NH$_2$-terminus. T-S may be placed at the COOH terminus of RCAM-II. This follows from the specificity of the modifying cleavage with exposure of a new NH$_2$-terminus and the resistance of both T-S and RCAM-II to carboxypeptidases A and B. The order (T-L)-(T-S) is thus established, and therefore the ordering of all of the limit tryptic peptides in RCAM-II.

**Tryptic Peptides of RCAM-II'**—Tryptic hydrolysis of RCAM-II' was carried out and the peptides fractionated in a manner identical with that with RCAM-II. Peptides identical with T-III, IV, V, VI, VII, VIII, IX, and X were found in RCAM-II'. Peptides T-I' and T-II', compared to the analogous peptides in RCAM-II, lacked the same 8 amino acid residues (2 histidyl, 3 aspartyl, 2 seryl, and 1 glutamyl) lacking in intact RCAM-II' (Table V). Unlike RCAM-II, the NH$_2$-terminus of RCAM-II' was reactive in the Edman reaction. Degradation in this manner directly places T-I and T-II' at the NH$_2$-terminal position (Table VI).

To elucidate the structure of T-II', the peptide was further fragmented with thermolysin. T-II', 167 nmol, was incubated with 13 mg of thermolysin in 0.01 M Tris-Cl + 0.002 M CaCl$_2$, pH 8.0, at 40°. After 4 hours the reaction was stopped by freezing. Preparative high voltage paper electrophoresis yielded two major components, Th-A (mobility 0.02 compared with the mobility of 0.27 of T-II') and Th-B (mobility 0.26). Together they account for the composition of T-II' (Table V). Unlike RCAM-II, the NH$_2$-terminus of RCAM-II' was reactive in the Edman reaction. Degradation in this manner directly places T-I' and T-II' at the NH$_2$-terminal position (Table VI).

**Protease Reactive Sites**—The deduced amino acid sequences of RCAM-II and II' are shown in Fig. 5. The trypsin reactive site bond in II is located at Arg 52-Ser 53 (44-45 in II') by the results from the modified inhibitor II. It is of interest to note that this position corresponds to the second chymotrypsin-reactive site in both Bowman-Birk soybean inhibitor (8, 9) and lima bean inhibitor (10). The question then remains as to what
Amino acid compositions and properties of tryptic peptides of RCAM-II

| Peptide | Amino Acid |
|---------|------------|
| T-I     | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-II    | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-III   | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-IV    | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-V     | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-Va    | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-VI    | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-VII   | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-VIII  | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-IX    | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| T-X     | Lys, Arg, Pro, Thr, Ser, Gly, Ala |
| Sum     | RCAM-II, RCAM-II' |

**Table II**

Sequences of tryptic and chymotryptic peptides of RCAM-II

Arrows pointed to the right below the sequence are residues determined by the Edman method; those above by leucine aminopeptidase. Arrows pointed to the left above the sequence are the residues determined by carboxypeptidase A and/or B digestion. The sequence of residues within a parenthesis is not determinable by data from the individual peptides.

**DISCUSSION**

The ordering of the tryptic peptides in inhibitor II was accomplished by use of several chymotryptic peptides and the isolation of the two fragments from the trypsin modified inhibitor. While these two fragments were not obtained in pure form, we are confident that the ordering data obtained are correct. The degree of contamination present would not be sufficient to invalidate the results of the analytical digests of T-L and T-S.

The complete amino acid sequence of inhibitor II' had been established. The absence of a reactive NH₂ terminus in intact inhibitor II allowed the unambiguous identification of the new terminus as Ser-Ile-Pro-. The position of the elastase reactive site was thus established.

is located at the first reactive site, which in the low molecular weight legume inhibitors is typically the trypsin reactive site.

In the garden bean inhibitor II this site is Ala 25-Ser 26, (17/18 in II') suggestive of an elastase reactive site. Indeed, inhibitor II (and II') is a potent inhibitor of porcine elastase, with a specific activity of 1.9 units/mg (compared to 2.65 against bovine trypsin). Further, both trypsin and elastase are inhibited simultaneously and independently; i.e. II is a true double-headed inhibitor. Garden bean inhibitors I and IIIb (1) were much weaker inhibitors of elastase, having specific activities 4- to 5-fold lower (0.4 unit/mg).

Proof of the location of the elastase reactive site at Ala 25-Ser 26 was afforded by the following experiment. Inhibitor II, 7 nmol, was reacted with 7 mol% porcine elastase in 0.2 M sodium succinate at pH 5.0. After 48 hours at room temperature, the modified inhibitor was subjected to Edman degradation. The absence of a reactive NH₂ terminus in intact inhibitor II allowed the unambiguous identification of the new terminus as Ser-Ile-Pro-. The position of the elastase reactive site was thus established.
Amino acid compositions and properties of chymotryptic peptides of RCAM-II

All values are from 20-hour hydrolysis, with no correction for destruction of threonine, serine, tyrosine, or Cmc (S-carboxymethylcysteine). Amino acids present at less than 0.1 residue are not reported. Numbers in parentheses are integer residue values.

| Amino Acid | C-T | C-I | C-II | C-IV | L-V | C-VI |
|------------|-----|-----|------|------|-----|------|
| Lys        | 0.5(1) | --  | --   | 1.0(2) | 0.9(2) | 0.7(1) |
| Thr        | 0.6(2) | --  | --   | 1.0(2) | 0.8(2) | 0.6(2) |
| Gla        | --   | 0.3(3) | 0.7(1) | 0.5(2) | 0.5(2) | 0.3(1) |
| Leu        | 1.3(1) | --  | --   | 0.7(1) | 0.7(1) | 0.7(1) |
| Met        | 0.2(1) | 2.0(2) | 2.0(2) | 2.0(2) | 2.0(2) | 2.0(2) |

* Purification steps: (a) electrophoresis at pH 3.6; (b) paper chromatography.

Numbers in parentheses refer to peaks in Fig. 3.

Comparison of the sequences of Bowman-Birk soybean inhibitor (8), lima bean inhibitor IV (19, 20), and the garden bean iso-inhibitors II and II' is made in Fig. 5. It is apparent that a high degree of homology exists among the four inhibitors. This is especially striking if both the NH₂- and COOH-terminal regions, which appear to be highly variable, are neglected. Thus, comparing residues 4 to 65 of garden bean II' with the corresponding region of Bowman-Birk inhibitor (omitting the unsequenced region 24 to 28 of II', and assuming the same amide assignments), one finds 39 (68%) identical residues. Of the 18 nonidentical residues, 13 can be explained by single base changes in the codons specifying these residues. A similar comparison exists between lima bean and garden bean inhibitors II and II'.

This similarity between the three legume inhibitors extends to the level of internal homology. Tan and Stevens (19) first noted the location of the protease reactive sites in lima bean inhibitor in two homologous regions of the molecule. This observation was later extended to the Bowman-Birk soybean inhibitor (8). A similar situation exists in the garden bean inhibitor II'. Comparing the residues around Ala 17, the elastase-reactive site (residues 10 to 23) and Arg 44, the trypsin-reactive site (residues 37 to 50), this internal homology is apparent. Of the 14 residues in each region, 8 are identical

Amino acid compositions of peptides of trypsin modified II

All values are after 20-hour hydrolysis. No corrections have been made for the destruction of threonine, serine, tyrosine, or Cmc (S-carboxymethylcysteine). Numbers in parentheses are the compositions predicted for T-L containing tryptic peptides T-II, III, IV and V, and T-S containing T-VI, VII, VIII, IX and X.

| Amino Acid | T-L | T-S | II |
|------------|-----|-----|----|
| Lys        | 0.3(1) | 0.4(2) | 0.4(1) |
| Arg        | 1.0(1) | 0.9(2) | 0.9(1) |
| Cmc        | 0.5(1) | 0.5(2) | 0.5(1) |
| Thr        | 2.0(2) | 1.9(2) | 1.9(2) |
| Ser        | 0.6(1) | 0.5(2) | 0.5(1) |
| Gla        | 0.3(1) | 0.3(2) | 0.3(1) |
| Gly        | 0.2(1) | 0.2(2) | 0.2(1) |
| Pro        | --   | --   | --   |
| Val        | 0.1(1) | 0.1(2) | 0.1(1) |

Comparison of the sequences of Bowman-Birk soybean inhibitor (8), lima bean inhibitor IV (19, 20), and the garden bean iso-inhibitors II and II' in Fig. 5. It is apparent that a high degree of homology exists among the four inhibitors. This is especially striking if both the NH₂- and COOH-terminal regions, which appear to be highly variable, are neglected. Thus, comparing residues 4 to 65 of garden bean II' with the corresponding region of Bowman-Birk inhibitor (omitting the unsequenced region 24 to 28 of II', and assuming the same amide assignments), one finds 39 (68%) identical residues. Of the 18 nonidentical residues, 13 can be explained by single base changes in the codons specifying these residues. A similar comparison exists between lima bean and garden bean inhibitors II and II'.

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(including 4 half-cystine residues) and 5 are related by a single base change in their codons.

While the garden bean inhibitor in many ways resembles the soybean and lima bean inhibitors, it does differ in several quite significant respects. As noted in the introduction, this investigation was undertaken with the hope of elucidating the structure of the second, apparently inactive, reactive site of an inhibitor that was presumed should be double-headed. In fact, the sequence suggested, and experiment has shown, that inhibitors II and II' are double-headed toward trypsin and elastase. While elastase inhibition by legume extracts and inhibitor preparations has been noted (21, 22), this is believed to be the first instance in which such an inhibitor has been isolated and characterized. This is also believed to be the first elastase inhibitor reactive site to be sequenced.

While inhibitors II and II' have now been eliminated as having a nonfunctional reactive site, the same can not be said of inhibitor I. Of the proteases tested, this species inhibits only trypsin significantly, and indeed either has an inactive reactive site or possesses activity toward an as yet unidentified protease.

A second significant difference between the garden bean and the lima and soybean inhibitors is the location of the trypsin-reactive site. As previously noted, in both the lima and soybean inhibitors the first reactive site is directed against trypsin, while the second is reactive to chymotrypsin (8-11). In garden bean inhibitors II and II' it is the second site that is directed against trypsin. The following speculative explanation is proposed. These inhibitors presumably initially arose as a single-headed inhibitor which through gene doubling and condensation gave rise to a double-headed inhibitor. The first double-headed inhibitor would presumably have both sites directed against the same enzyme. For the sake of simplicity, that enzyme can be assumed to be trypsin. At the present time, there are examples of both of these archetypes, the peanut (23) and mung bean (24) inhibitors, respectively. With the presence of two equivalent reaction sites, one site is free to further evolve without loss of the molecule’s original function. If it is the second site that changes, e.g. to a chymotrypsin reactive site, one obtains the configuration present in the lima and soybean inhibitors. If, on the other hand, it is the first reactive site that evolves, e.g. to an elastase reactive site, one obtains the configuration in garden bean inhibitors II and II'.

Finally, the question of the lower affinity (pKd 8.5) of inhibitors II and II' for trypsin as compared to the other garden bean inhibitors remains. The sequence around the trypsin reactive site of II' is quite similar to that of the Bowman-Birk inhibitor (pKd 10, Ref. 25). It is not immediately obvious how the exchange of methionine for alanine or asparagine could account for the large difference in complex stability. The change from a lysine-type to an arginine-type reactive site also seems unlikely to cause the large change in behavior. The
enzymatic mutation of arginine-type Kunitz soybean inhibitor to a lysine-type inhibitor (26) causes essentially no change in the trypsin inhibitor complex stability. It is possible that differences in secondary or higher order structure are responsible.

Acknowledgments—We would like to thank Dr. M. Laskowski, Jr. for suggesting the elastase experiments and for useful discussion on evolutionary aspects.

REFERENCES
1. Wilson, K. A., and Laskowski, M., Sr. (1973) J. Biol. Chem. 248, 756-762
2. Jones, G., Moore, S., and Stein, W. H. (1963) Biochemistry 2, 66-71
3. Haynes, R., and Feeney, R. E. (1967) J. Biol. Chem. 242, 5378-5385
4. Chu, H.-M., and Chi, C.-W. (1965) Sci. Sinica 14, 1441-1453
5. Wagner, L. P., and Riehm, J. B. (1967) Arch. Biochem. Biophys. 121, 672-677
6. Pusztai, A. (1966) Biochem. J. 101, 379-384
7. Birk, Y., Gertler, A., and Khalef, S. (1963) Biochem. J. 87, 281-284
8. Odani, S., and Ikenaka, T. (1972) J. Biochem. 71, 839-848
9. Seidl, D. S., and Ziener, I. E. (1972) Biochim. Biophys. Acta 258, 303-309
10. Krahn, J., and Stevens, F. C. (1970) Biochemistry 9, 9646-9659
11. Krahn, J., and Stevens, F. C. (1972) Biochemistry 11, 1804-1808
12. Wang, S. S., and Carpenter, F. H. (1969) J. Biol. Chem. 244, 1619-1625
13. Hirs, C. H. W. (1967) Methods Enzymol. 11, 199-203
14. Katz, A. M., Preyer, W. J., and Anfinsen, C. B. (1959) J. Biol. Chem. 234, 2987-2990
15. Gray, W. R. (1972) Methods Enzymol. 25, 333-344
16. Woods, K. R., and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 389-370
17. Noughton, M. A., and Sanger, F. (1961) Biochem. J. 166 163
18. Shotton, D. M. (1970) Methods Enzymol. 19, 113-140
19. Tan, C. G. L., and Stevens, F. C. (1971) Eur. J. Biochem. 18, 515-523
20. Stevens, F. C., Wuerz, S. T., and Krahn, J. in Proceedings of the Second International Research Conference on Proteinase Inhibitors (Bayer Symposium V), Springer Verlag, Berlin, 1974, pp. 344-354
21. Vogel, R., Trautschold, L., and Werle, E. (1968) Natural Proteinase Inhibitors, Academic Press, New York
22. Bieth, J., and Frechin, J. C. in Proceedings of the Second International Research Conference on Proteinase Inhibitors (Bayer Symposium V), Springer Verlag, Berlin, 1974, pp. 291-304
23. Hochstrasser, K., Illichman, K., and Werle, E. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1503-1512
24. Chu, H.-M., and Chi, C.-W. (1965) Acta Biochem. Biophys. Sin. 5, 528
25. Seidl, D. S., and Liener, I. E. (1972) J. Biol. Chem. 247, 3533-3538
26. Sealock, R. W., and Laskowski, M., Jr. (1969) Biochemistry 8, 3703-3710
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*J. Biol. Chem.* 1975, 250:4261-4267.

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