Isolation and Properties of Complexes of the Bowman-Birk Soybean Inhibitor with Trypsin and Chymotrypsin*

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SUMMARY

Complexes of the Bowman-Birk soybean inhibitor with either trypsin or chymotrypsin and with both of these enzymes were isolated by gel filtration. Amino acid analyses and molecular weight estimates by gel filtration were consistent with the formation of a 1:1 molar complex between inhibitor and trypsin or chymotrypsin and a 1:1:1 molar complex composed of inhibitor, trypsin, and chymotrypsin. The binary complexes were also isolated by affinity chromatography employing insoluble derivatives of trypsin and chymotrypsin. Determination of the residual enzymic activity in solutions of the isolated complexes permitted an evaluation of their dissociation constants.

Among the proteinase inhibitors which are widely distributed in nature (see reviews by Liener and Kakade (1) and Laskowski and Sealock (2)), those which have the unique capacity to inhibit both trypsin and chymotrypsin are of special interest. Examples of these so-called “double-headed” inhibitors are turkey ovomucoid (3), the lima bean inhibitor (4, 5), and the Bowman-Birk soybean inhibitor (6, 7). The trypsin-reactive and chymotrypsin reactive sites of the Bowman-Birk inhibitor, which will hereafter be referred to simply as the “inhibitor,” have recently been identified as lysine-17 and leucine-48, respectively (8, 9), and the amino acid sequences immediately surrounding these sites were found to bear a striking homology to the corresponding sites of the lima bean inhibitor (10, 11).

If trypsin and chymotrypsin do in fact react with the inhibitor at nonoverlapping sites it should be possible to isolate complexes involving the inhibitor with either trypsin or chymotrypsin as well as a complex consisting of the inhibitor, trypsin, and chymotrypsin. Birk (12) demonstrated the formation of complexes of the inhibitor-trypsin and -chymotrypsin by paper electrophoresis, but these complexes were not actually isolated and characterized. Krahn and Stevens (13) recently reported the isolation of binary complexes of the lima bean inhibitor with either trypsin or chymotrypsin and a ternary complex composed of this inhibitor and both enzymes. The aim of the present study was to isolate similar complexes of the inhibitor with trypsin and chymotrypsin, and, from their molecular weights and composition, to determine the stoichiometry of these interactions. The isolation of these complexes also provided a direct means for evaluating the dissociation constants of such systems.

EXPERIMENTAL PROCEDURE

Materials—The Bowman-Birk inhibitor was prepared as described previously (8). Cytochrome c (horse heart) and bovine serum albumin used as molecular weight markers were purchased from Mann Research Laboratories, New York. Chymotrypsinogen A was a product of Sigma Chemical Co., St. Louis, Mo. The insoluble derivatives of trypsin and chymotrypsin were prepared from crystalline enzymes (Worthington Biochemical Corp., Freehold, N. J.) by coupling with cyanogen bromide-activated Sepharose 4B as described by Kassell and Marciniszyn (14). Unless indicated otherwise, all other reagents and techniques used in this study were essentially the same as reported earlier (8).

Isolation of Complexes of Inhibitor with either Trypsin or Chymotrypsin by Gel Filtration—Binary complexes of inhibitor with either trypsin or chymotrypsin were prepared from mixtures of the inhibitor with the appropriate enzyme as described in the legend to Fig. 1. The inhibitor was present in 25% molar excess in both instances. The ternary complex of inhibitor with trypsin and chymotrypsin was prepared by adding an excess of the isolated binary complex of inhibitor and trypsin to chymotrypsin as described in Fig. 1. Following application to a column of Sephadex G-75, the column was eluted with 0.1 M \((\text{NH}_4)_2\text{CO}_3\). The column effluent was monitored at 280 nm, and aliquots of each fraction were tested for antitrypsin and antichymotrypsin activities as described below. Pooled fractions, after dialysis and lyophilisation, were subjected to amino acid analysis following hydrolysis with 6 N HCl for 22 hours at 110°C (15).
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1 The abbreviations used are: BAPA, benzoyl-1-arginine-p-nitroanilide; GPNA, N-glutaryl-n-phenylalanine-p-nitroanilide.
RESULTS

Isolation of Complexes by Gel Filtration—Fig. 1 shows the isolation of complexes of the inhibitor with trypsin and/or chymotrypsin by gel filtration. A mixture of the inhibitor with trypsin produced the pattern shown in Fig. 1A. The peak labeled Tr-BBI exhibited antichymotryptic activity but only a trace of antitryptic activity, the properties to be expected of a complex comprised of the inhibitor and trypsin. When the inhibitor was mixed with chymotrypsin, the major component shown in Fig. 1B, Peak Ch-BBI, was judged to be a complex of the inhibitor and chymotrypsin since it displayed strong antitryptic activity but was virtually devoid of inhibitory activity toward chymotrypsin. When chymotrypsin was added to an excess of the isolated complex of the inhibitor and trypsin, the two peaks shown in Fig. 1C were obtained. The inability of the first peak, Tr-BBI-Ch, to inhibit either enzyme serves to identify this fraction as a complex of the inhibitor with both enzymes. The second peak was located in the same position and had the same inhibitory properties as Tr-BBI in Fig. 1A. When Peaks Tr-BBI-Ch and Tr-BBI were treated with trichloroacetic acid, the filtrates exhibited antitryptic activity, presumably due to the liberation of free inhibitor from the respective complexes.

When trypsin was added to an excess of the isolated complex of the inhibitor and chymotrypsin, a pattern similar to that of Fig. 1C was obtained. The first peak had the properties of trypsin-inhibitor-chymotrypsin, and the second peak represented that portion of the inhibitor-chymotrypsin complex which had not combined with trypsin.

Amino Acid Composition—Each of the complexes shown in Fig. 1 was subjected to amino acid analysis with the results shown in Table I. The amino acid composition of these complexes agreed, within experimental error, with theoretical values based on the assumption that the inhibitor forms a 1:1 molar complex with either trypsin or chymotrypsin and a 1:1:1 molar complex with both enzymes.

Molecular Weight Determinations—The Sephadex G-75 column which had been employed for the isolation of the binary and ternary complexes was calibrated for the estimation of molecular weights with proteins of known molecular weight. From the curve shown in Fig. 2, the molecular weight of the binary complexes of the inhibitor with either trypsin or chymotrypsin was estimated to be 32,000, and the ternary complex of the inhibitor with both of these enzymes was estimated to have a molecular weight of 55,000. Based on molecular weight values of 8,000 for the Bowman-Birk inhibitor (8, 21), 24,000 for trypsin (22), and 25,000 chymotrypsin (23), the experimental values for the molecular weight of these complexes are in excellent agreement with theoretical predictions.

The position of excess inhibitor shown in Fig. 1, A and B, corresponded to a molecular weight of 15,000, which is approximately twice the expected value for the monomeric form of the Bowman-Birk inhibitor. This anomalous behavior is most likely due to the fact that this inhibitor is known to undergo concentration-dependent polymerization in solution (24). The application of more dilute solutions of the inhibitor to the column, however, failed to give values corresponding to the molecular weight of its monomeric form.

Isolation of Binary Complexes by Affinity Chromatography—

### Table I

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Bowman-Birk Inhibitor</th>
<th>Trypsin</th>
<th>Chymotrypsin</th>
<th>Tr-BBI</th>
<th>Ch-BBI</th>
<th>Tr-BBI-Ch</th>
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<td>Theory</td>
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<tr>
<td>Half-cystine</td>
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<td>Valine</td>
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</table>

* Theoretical values taken from Reference 8.
* Theoretical values taken from Reference 22.
* Theoretical values taken from Reference 23.
* Based on sum of theoretical values for enzyme and inhibitor assuming molar combining ratios of 1:1 for trypsin-inhibitor and chymotrypsin-inhibitor and 1:1:1 for trypsin-inhibitor-chymotrypsin.
* Values obtained by analysis as described in text but uncorrected for losses incurred during acid hydrolysis (22 hours at 110°).
plied to same column and eluted under the same conditions as described in Fig. 1. A certain amount of α-chymotrypsin calculated to be in stoichiometric dextran; BBI (15,000), and trypsin-inhibitor-chymotrypsin (TV-BBZ-Ch), denoted by open circles. Positions of Bowman-Birk inhibitor and reference proteins, with molecular weight enclosed in parentheses, are taken from Fig. 1, and molecular weights are values taken from curve established by reference proteins. Fig. 3 depicts an experiment in which the inhibitor was first bound to trypsin which had been covalently attached to Sepharose. The column was then charged with a solution containing an amount of α-chymotrypsin calculated to be in stoichiometric excess of the inhibitor adsorbed to the column. When no further chymotryptic activity could be detected in the effluent, the column was eluted with 0.01 M HCl. The peak eluted under these conditions possessed a high level of antitryptic activity but displayed only slight chymotrypsin-inhibitor activity. Amino acid analysis of this fraction confirmed its identity as a binary complex of the inhibitor and chymotrypsin. A similar experiment was conducted in which the inhibitor was first attached to insoluble chymotrypsin, the column charged with an excess of trypsin, and finally eluted with dilute acid. The peak in this instance was inhibitory toward chymotrypsin but only slightly so toward trypsin. The amino acid composition of this peak corresponded to what one would expect from a binary complex of the inhibitor and trypsin.

Determination of Dissociation Constants—During the course of these studies it was consistently noted that control tubes containing solutions of the various complexes to which no enzyme had been added displayed a low but measurable level of activity toward synthetic substrates or casein. Advantage was taken of this observation to determine in a direct fashion the concentration of free trypsin or chymotrypsin present in an equilibrium mixture of the complex, free enzyme, and free inhibitor.

In the case of the ternary complex, casein was not suitable as a substrate since it does not serve to distinguish between trypsin and chymotryptic activity. Dissociation constants calculated according to the equations given under "Experimental Procedure." Other Observations—The inhibitor-trypsin complex was capable of inhibiting chymotryptic activity with either GPNA or casein as substrates, whereas the inhibitor-chymotryptin complex inhibited the action of trypsin on BAPA but not on casein. Similar observations have been reported by Birk (12). At the moment we have no explanation for this phenomenon.

Complex formation between the inhibitor and chymotrypsinogen A at pH 8.6 could be demonstrated by electrophoresis on

![Fig. 2. Estimation of molecular weight of inhibitor complexes with trypsin and/or chymotrypsin by gel filtration. Reference proteins, 2 to 5 mg dissolved in 1 ml of 0.1 M (NH₄)₂CO₃, were applied to same column and eluted under the same conditions as described in Fig. 1. Vₑ is the void volume as measured with blue dextran; Vₑ is the elution volume for each protein sample. Reference proteins, with molecular weight enclosed in parentheses, are denoted by open circles. Positions of Bowman-Birk inhibitor (BBI), trypsin-inhibitor (Tr-BBI), chymotrypsin-inhibitor (Ch-BBI), and trypsin-inhibitor-chymotrypsin (Tr-BBI-Ch), denoted by triangles, are taken from Fig. 1, and molecular weights are values taken from curve established by reference proteins.](http://www.jbc.org/)

![Fig. 3. Isolation of binary complex of Bowman-Birk inhibitor (BBI) and chymotrypsin (Ch) by affinity chromatography on Sepharose-trypsin; 8 mg of inhibitor in 1 ml of 0.1 M borate buffer containing 0.3 M NaCl and 0.01 M CaCl₂, pH 7.6, were applied to column (1.0 X 10 cm), equilibrated, and subsequently eluted with same buffer. Flow rate, 8 ml per hour; volume of fractions, 1 ml. At point indicated by second vertical arrow, 30 mg of α-chymotrypsin were applied to the column and elution continued with borate buffer. First peak shown in figure was excess chymotrypsin not retained by the column. When no further chymotrypsin activity could be detected in effluent, 0.01 M HCl, to which had been added 0.3 M NaCl and 0.01 M CaCl₂, was introduced into the column where indicated by third vertical arrow. Ch-BBI denotes complex of chymotrypsin and inhibitor displaced by the dilute acid. Solid curve denotes absorbance at 280 nm; ---, chymotrypsin-inhibitor activity with casein as substrate; -----, trypsin-inhibitor activity with BAPA as substrate.](http://www.jbc.org/)
cellulose acetate. Complex formation, however, was extremely slow and appeared to reach a maximum in 24 hours at which time the complex precipitated. Identification of the precipitate as a stoichiometric 1:1 complex of the inhibitor and chymotrypsinogen was established by amino acid analysis. Attempts to isolate the inhibitor-zymogen complex by gel filtration were unsuccessful; the application of a mixture of the inhibitor and chymotrypsinogen to Sephadex G-75 yielded two peaks corresponding to each of these two components.

**DISCUSSION**

Although several lines of evidence (6-9) have strongly suggested that the Bowman-Birk inhibitor must have independent binding sites for trypsin and chymotrypsin, the isolation of binary complexes of the inhibitor with either of these two enzymes and of a ternary complex of the inhibitor with both enzymes constitutes the most telling proof for the existence of independent binding sites. The amino acid composition and molecular weight of these complexes further established the fact that the monomeric form of the inhibitor (mol wt 8000) forms a 1:1 molar complex with trypsin or chymotrypsin and a 1:1:1 molar complex with both of these proteases.

Despite what would appear to be an obvious example of a stoichiometric interaction, determination of the inhibition of the activity of trypsin or chymotrypsin as a function of inhibitor to protease ratio led Frattali (21) to conclude that the association between Bowman Birk inhibitor and these enzymes was non-stoichiometric. He attributed this nonstoichiometric inhibition to the relatively high dissociation constants of the enzyme-inhibitor complexes, although no experimentally determined values for this parameter were reported. As pointed out by Laskowski and Sealock (2), the determination of a dissociation constant from titrimetric data is subject to inaccuracies since it depends on the extent of deviation from linearity in the region of the equivalence point where inhibition is nearly complete. Not only is it difficult to measure the curvature of the plot and the equivalence point with a high degree of precision, but one is also limited to measuring low levels of activity.

The isolation of pure complexes of inhibitor and enzyme permits an alternative approach to the determination of dissociation constants which does not suffer from many of the shortcomings of the titrimetric assay. Since these complexes are isolated from mixtures in which the inhibitor is present in excess, any demonstrable enzymatic activity can be attributed solely to the dissociation of the complex. The level of enzyme activity to be measured can be made to accommodate the limits of the assay system by simply controlling the amount of complex placed into solution. Furthermore, there is no ambiguity regarding the purity of the enzyme and inhibitor in the complex since the very manner whereby the latter was isolated insure that only active enzyme and active inhibitor are present in the complex. After our studies had been completed, our attention was directed to a paper by Berezin et al. (25) who used a similar approach to measure the dissociation constant of a complex of trypsin with bovine pancreatic inhibitor. These authors point out, however, that adding substrate to the purified complex may cause a shift in the equilibrium in the direction of free enzyme and inhibitor. This perturbation of the equilibrium by the substrate would tend to increase the dissociation constant so that the experimental values shown in Table II may be somewhat biased in that direction.

Using synthetic substrates for measuring enzymic activity, the dissociation constant (Kd) for the inhibitor-trypsin complex (2.8 x 10^{-8} M) was much lower than the value obtained with the inhibitor-chymotrypsin complex (2.9 x 10^{-4} M). With casein as the substrate, the Kd for the inhibitor-chymotrypsin complex was comparable to that obtained on the synthetic substrate. Unfortunately, it was not possible to calculate a reliable value for the Kd of the inhibitor-trypsin complex on casein because of the sigmoid nature of the activity curve. The reason for this anomalous behavior is not clear. Using casein as the substrate, Birk (12) has reported dissociation constants of 5.6 x 10^{-4} M and 5.0 x 10^{-4} M for the inhibition of trypsin and chymotrypsin, respectively. Although at variance in absolute terms, both sets of data are in substantial agreement that the association of the Bowman-Birk inhibitor with trypsin is stronger than its association with chymotrypsin.

The Kd of the ternary complex determined with the trypsin substrate, BAPA, was identical with the value obtained with the binary complex of the inhibitor and trypsin. This would suggest that trypsin binds equally well to the inhibitor in the presence or absence of chymotrypsin, a further indication of the truly independent nature of the binding sites. In view of this, it was surprising to note that the Kd of the ternary complex determined on the chymotrypsin substrate (4.3 x 10^{-8} M) was in fact somewhat lower than the Kd of the binary complex of the inhibitor and chymotrypsin. This could be strictly interpreted to mean that when the inhibitor is complexed with trypsin its affinity for chymotrypsin is actually enhanced. This conclusion, however, must be tempered by the realization that these observations may be complicated by the perturbing effects of the substrate on the equilibrium between complex, free enzyme, and free inhibitor. Further study is obviously necessary to clarify this issue.

**REFERENCES**


*We are grateful to Dr. M. Laskowski, Jr., for calling this paper to our attention.*
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