Purification and Properties of Soybean Protein Inhibitors of Proteolytic Enzymes

R. F. Steiner and Victor Frattali[1]

A number of structurally dissimilar globular proteins that can inhibit the activity of certain proteinases, such as trypsin and α-chymotrypsin, constitute part of the seed protein in the soybean plant. These "soybean trypsin inhibitors," which can be inactivated by moist heat, contribute to the antinutritional quality of raw meal. Two distinct factors, the Kunitz and the Bowman-Birk inhibitors, have been subjected to physical, chemical, biochemical, and nutritional study. The Kunitz and most other protein inhibitors isolated from plant or animal sources are very efficient in abolishing or reducing tryptic activity; some, like the Bowman-Birk inhibitor, are almost equally active against tryptic and α-chymotryptic activity. Much attention has been focused on the interaction between trypsin and the Kunitz inhibitor with the expectation of formulating a general mechanism of action for these and other natural inhibitors.

Trypsin inhibitors of plant origin, particularly from legumes, currently are receiving a great deal of attention. Apart from the obvious benefits to animal nutrition that derive from an understanding of these deleterious factors, other lucrative avenues of investigation are afforded those who undertake a study of these plant proteins. The enzymologist, for example, is presented a natural tool with which to probe the active centers of a number of proteinases. The physical biochemist can gain insight into structural features common to all proteins, in addition to features that account for protein-protein interactions. Recently, for example, several cases of an inhibitor involved in a self-association process have come to light.

One important area has received very little attention. Few investigations have been directed toward the physiological significance of the existence of these factors in the plant. There has been some speculation, but no single, unequivocal statement can be put forth. Unlike the large number of homologous proteins and enzymes that can be identified in widely separated animal species, only recently has there been some indication of similarity among these plant factors from members of the same species. The prime function of these factors remains a mystery, for it is widely believed that the ability to inhibit certain proteinases is a fortuitous circumstance.

In view of the volume of publications on these plant proteins, this article deals only with two soybean inhibitors that were recognized as distinct entities over 20 years ago. Since that time, the crystalline soybean trypsin inhibitor of Kunitz has retained the focus of attention, while interest in the other has been relatively slight. This, of course, was a direct result of the classical series of publications on the crystallization and characterization of the Kunitz inhibitor at a time when few pure proteins were available. The fact remains, however, that soybean trypsin inhibitors were recognized independently by Read and Haas (1938), Ham and Sandstedt (1944), and Bowman (1944) prior to the appearance of Kunitz' first report (Kunitz, 1945). Concurrently, Bowman (1946) realized that one factor was the same as that crystallized by Kunitz and the other, although crude, was altogether different. From the time following the appearance of Bowman's reports to the beginning of this decade, interest in the second inhibitor remained dormant until Birk (1961) resumed the investigation. One term currently used in reference to this protein is "Bowman-Birk inhibitor." The problem of nomenclature with this and other proteins is pressing and can lead to some confusion in the literature. Cumbersome descriptive terms have been used in reference to this protein: acetone-insoluble factor, purified inhibitor AA, and soybean trypsin and α-chymotrypsin inhibitor. If all the inhibitors behave like the Kunitz inhibitor, a nomenclature system involving the botanical name of the source of the inhibitor, the location of the split bond in the inhibitor, and the Enzyme Commission designation for the enzyme that causes the split, should prove adequate. All soybean inhibitors that are apparently distinct are listed in Table I.

The conclusions of some recent reports of the effects of these factors on animal nutrition warrant mentioning. Two effects caused by raw soybean meal in animal diets are pancreatic hypertrophy and growth inhibition. Over 50 years ago, Osborne and Mendel (1917) recognized that these effects could be abolished simply with moist heat treatment of meal. Other heat-labile substances in soybean meal, in addition to trypsin inhibitors, possess physiological activity (Rackis, 1965). A large volume

Table I. Soybean Trypsin Inhibitors

Inhibitor	Mol. Wt. and/or Sedimentation Coefficient	Reference
Bowman-Birk inhibitor	8,000	Frattali, 1969; Millar *et al.* 1969
SBTI-A_1	14,300: 1.8	Rackis and Anderson, 1964
1.9S	16,400: 1.9	Yamamoto and Ikenaka, 1967
F_1	18,300	Frattali and Steiner, 1968a
Kunitz inhibitor	21,500 ± 800	Wu and Scheraga, 1962
Kunitz inhibitor	22,700: 2.30	Rackis and Anderson, 1964
Kunitz inhibitor (SBTI-A_2)	21,600: 2.29	Rackis and Anderson, 1964; Rackis *et al.*, 1962
F_3	23,400	Frattali and Steiner, 1968a
SBTI-B_1	4.07	Rackis and Anderson, 1964
SBTI-B_2	4.62	Rackis and Anderson, 1964

Laboratory of Physical Biochemistry, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md. 20014

[1] Present address, Laboratory of Metabolism, Naval Medical Research Institute, Bethesda, Md.

of the literature on plant proteinase inhibitors from leguminous meals deals directly with physiological and nutritional effects. Consequently, no attempt to present a comprehensive coverage will be made, especially since excellent reviews have appeared recently (Liener, 1962; Pusztai, 1967). Two pertinent publications deal directly with the physiological effects of soybean meal and of the Kunitz and Bowman-Birk inhibitors in the diets of chicks and rats (Gertler *et al.*, 1967; Rackis, 1965). From the results of a study on the physiological response of rats to raw or treated meal diets, Rackis (1965) concluded that the trypsin inhibitors are responsible for the pancreatic hypertrophic response and from 30 to 50% of the growth-inhibiting effect. In a somewhat similar study, which included the Bowman-Birk in addition to the Kunitz inhibitor, Gertler *et al.* (1967) affirm the pancreatic hypertrophic effect, but conclude that these inhibitors have a minor role in the growth depression of chicks and rats.

KUNITZ INHIBITOR

General Molecular Properties. Perhaps the most thoroughly characterized plant proteinase inhibitor is the soybean inhibitor which was isolated, crystallized, and studied by Kunitz (1945, 1946, 1947). This protein, which has been the subject of numerous investigations, is commercially available from several sources in crystalline form. However, these commercial preparations often contain significant quantities of several other inhibitors that can be removed by ion-exchange chromatography or preparative acrylamide gel electrophoresis (Frattali and Steiner, 1968a, 1969a).

The general molecular properties of the Kunitz inhibitor are now well established. The molecular weight is close to 21,500 (Wu and Scheraga, 1962). The molecule consists of a single polypeptide chain, crosslinked by two disulfide bridges (Steiner, 1965; Wu and Scheraga, 1962). No evidence for self-association has been reported. The solubility properties are those of a globulin, with a distinct minimum in solubility at the isoelectric point, pH 4.5 (Kunitz, 1947). The amino acid composition has no especially unusual features (Wu and Scheraga, 1962).

From measurements of intrinsic viscosity, fluorescence polarization, and frictional ratio, this inhibitor appears to be a typical globular protein of low molecular asymmetry and high internal rigidity (Edelhoch and Steiner, 1963; Laskowski and Laskowski, 1954; Steiner and Edelhoch, 1963). The α-helical content, as estimated from optical rotatory dispersion is small (Jirgensons, 1961).

The best known property of the Kunitz inhibitor is its ability to form at neutral pH a one-to-one stoichiometric complex with trypsin (Laskowski and Laskowski, 1954). The complex, which can be crystallized, is devoid of proteolytic activity. While appreciable interaction also occurs with chymotrypsin, the magnitude of the association constant does not approach that for trypsin (Laskowski and Laskowski, 1954).

Structural Transitions. At 25° the physical parameters of the Kunitz inhibitor in water, including optical rotation, ultraviolet absorbance, fluorescence polarization, and ultraviolet fluorescence intensity, are invariant between pH 6 and 9, indicating that no significant change in molecular conformation occurs in this range (Steiner and Edelhoch, 1963; Wu and Scheraga, 1962). At pH's acid to pH 5, the development of a small, but finite, difference spectrum, together with appreciable changes in ultraviolet fluorescence intensity and optical rotation, suggests that a conformational change may occur (Steiner and Edelhoch, 1963). There is some evidence of the occurrence of a second minor transition above pH 9 (Steiner and Edelhoch, 1963; Wu and Scheraga, 1962). Above pH 12 time-dependent changes in ultraviolet absorbance and fluorescence intensity indicate the presence of a further time-dependent structural alteration (Steiner and Edelhoch, 1963).

The urea denaturation of the Kunitz inhibitor has been studied in detail (Edelhoch and Steiner, 1963). Exposure to 9*M* urea at neutral pH and 25° does not appear to reduce the molecule to a completely structureless random coil, in view of the important further changes in physical properties occurring at alkaline pH and at elevated temperature in this solvent.

The kinetics of the urea-denaturation of the protein at neutral pH in the temperature range 30° to 60° can be monitored by observations of viscosity, difference spectra, ultraviolet fluorescence intensity, and optical rotation (Edelhoch and Steiner, 1963). Rate constants determined by all four criteria agree closely, as would be predicted for a "two-state" model for the denaturation process. The product of denaturation in 9*M* urea has undergone, by all the physical criteria, a very extensive loss of structure. However, removal of urea at neutral pH and 25° results in a quantitative recovery of inhibitory activity (Steiner, 1965, 1966a).

Reduction and Reoxidation of Disulfide Bonds. The two cystine crosslinks of the Kunitz inhibitor may be quantitatively reduced by treatment with excess β-mercaptoethanol in 9*M* urea at 50° and pH 7 to 8 (Steiner, 1965). A further increase in intrinsic viscosity and reduction in rotational relaxation time, as measured by fluorescence polarization, occur as compared with the denatured protein in 9*M* urea. The values of these parameters suggest that the reduced protein in 9*M* urea approaches the limiting state of a structureless random coil (Steiner, 1965).

The reduced protein, as isolated by removal of urea and reducing agent at pH 3, is devoid of inhibiting activity. If air-reoxidation of cysteine groups is allowed to proceed at pH 8 to 9 a partial recovery of activity is observed (Steiner, 1965). About 30 to 40% of the original activity is regained for a completely reoxidized preparation. In contrast to the reported behavior of several other proteins (Anfinsen, 1962), there is no major dependence of the extent of recovery upon concentration for concentrations in the range 0.8 to 0.1 gram per liter.

If the reoxidized material is fractionated with respect to molecular size by gel filtration, using Sephadex G-100, two fractions may be isolated (Steiner, 1965). One of these is homogeneous and equivalent in activity and in molecular weight to the native inhibitor. The second fraction consists of aggregates which retain a slight degree of inhibiting capacity. A comparison of the time-dependence of reoxidation and recovery of activity indicates, somewhat surprisingly, that the later stages of reoxidation are less efficient in promoting activity

recovery than the initial. From these results it appears that the processes competing effectively with reformation of the native molecule involve the formation of aggregates. For the monomeric species, the "correct" pairing of half-cystine residues to yield the active molecule is overwhelmingly favored thermodynamically. If reduced inhibitor is allowed to reoxidize under strongly denaturing conditions in 9*M* urea, the product contains a preponderance of inactive species, in which "incorrect" pairing of half-cystine residues has occurred. Treatment of this largely inactive material with the disulfide-interchange catalyzing enzyme of Anfinsen results in a further major increase in activity (Steiner *et al.*, 1965).

Chemical Modification. Studies upon the chemical modification of the Kunitz inhibitor have centered attention upon whether particular groups are essential for inhibitor activity and for the correct refolding of the denatured molecule. The criterion adopted as a test of the latter is the extent to which activity of the modified protein remains after a denaturation-renaturation cycle, consisting of treatment with 9*M* urea at 50°, followed by removal of urea at 25° (Steiner, 1966a).

At least 10 of the 11 lysine groups in the inhibitor molecule can be converted to homoarginine by treatment with methyl isourea without a significant loss of activity (Steiner, 1966a). Most of the activity survives a denaturation-renaturation cycle. The implication is that the lysines are not essential for correct refolding of the denatured molecule.

At least one of the three tryptophans may be oxidized by *N*-bromosuccinimide without a major loss of activity. The residual activity largely persists through a denaturation-renaturation cycle, suggesting that at least one tryptophan is nonessential. However, oxidation of all three tryptophans under strongly denaturing conditions results in complete and irreversible inactivation (Steiner, 1966a).

Two of the four tyrosines of the Kunitz inhibitor can be iodinated without loss of activity or of the ability to refold (Steiner, 1966a). Iodination of all four tyrosines under denaturing conditions results in a complete loss of activity, which is not restored by removal of the denaturant.

These results are too limited in scope to form an extensive pattern. However, in two instances, modification of readily available and reactive groups of a particular class has permitted retention of activity, while alteration of groups, which react only after disruption of the native structure, blocks reformation of the active molecule.

Interaction with Trypsin. The free energy of combination of the Kunitz inhibitor with trypsin at neutral pH is very high, of the order of −12 kcal. Below pH 6 the magnitude of the free energy of association decreases rapidly, until by pH 3 there is no detectable interaction (Steiner, 1954). Below pH 5 the combination is reflected by a release of bound hydrogen ions, which may be used to monitor the equilibria (Lebowitz and Laskowski, 1962).

Laskowski and coworkers recently demonstrated that the combination is accompanied by a limited proteolysis of the inhibitor (Finkenstadt and Laskowski, 1965, 1966; Ozawa and Laskowski, 1966). A single arginine-isoleucine bond is split reversibly, according to the mechanism:

$$T + I \rightleftarrows TI \rightleftarrows T + I^*$$

where T is trypsin, I is the original inhibitor, TI is the complex, and I* is the modified inhibitor. This capacity for limited proteolysis may prove to be a general property of protein inhibitors of trypsin.

The formation of the enzyme-inhibitor complex is accompanied by the development of a positive difference spectrum and by small changes in ultraviolet fluorescence intensity and optical rotation (Edelhoch and Steiner, 1965). The difference spectrum is indicative of an environmental change for both tryptophan and tyrosine groups and is in the direction expected for a change to a less polar environment. This could reflect either a conformational change upon combination or a masking of chromophores in proximity to the site of combination, or both. Complex formation also results in a diminished reactivity of tyrosines with iodine and of tryptophans with *N*-bromosuccinimide (Steiner, 1966b).

BOWMAN-BIRK INHIBITOR

Purification. Isolation of the Bowman-Birk inhibitor is based on Bowman's procedure (1946) which stemmed from an investigation of antitryptic factors (Bowman, 1944, 1945). The crude inhibitor, then referred to as an acetone-insoluble antitryptic factor, was satisfactorily shown to be distinct from an alcohol-insoluble factor. This latter factor is now recognized as Kunitz' soybean trypsin inhibitor. Later, Birk (1961) and Birk *et al.* (1963) resumed investigation of the acetone-insoluble inhibitor and succeeded in isolating a highly pure preparation. Essentially following the CM-cellulose chromatographic procedure of Birk *et al.* (1963), apparently identical inhibitor preparations were isolated from two different soybean variety meals, Lee and Hawkeye (Frattali, 1969). When further analyzed, these inhibitor preparations were found to contain several additional minor protein components. To remove these contaminants, a second chromatographic procedure involving DEAE-cellulose chromatography proved satisfactory.

This inhibitor, prepared according to Birk *et al.* (1963), is now commercially available. Although sufficiently pure for most studies, small amounts of minor protein contaminants have been observed in several samples. Adequate separation of these components from the inhibitor has been achieved on a preparative scale with a polyacrylamide gel electrophoresis procedure (Frattali and Steiner, 1969a). As a demonstration of homogeneity, electrophoretic migration patterns on an analytical-scale polyacrylamide gel for a few preparations are illustrated in Figure 1.

Composition. The amino acid composition of the Bowman-Birk and two other inhibitors, isolated from soybeans and lima beans, are given in Table II. Common features shown by these three inhibitors include a high proportion of sulfur-containing amino acids and an absence of glycine residues. This lack of glycine in the two soybean inhibitors is humorous in light of the botanical name for the soybean plant, *Glycine max*. The compositional similarity between these two inhibitors is striking. This, coupled with other similar traits

Table II. Amino Acid Composition

Amino Acid	G. Amino Acid/ 100 G. Protein[a]		Residues/ BBI Molecule	Integer	
	BBI[b]	1.9S[c]		BBI	LBI[d]
Asp	19.12	18.40	11.69	12	14
Thr	2.84	2.86	1.94	2	3
Ser	11.56	11.46	8.96	9	12
Glu	12.67	12.70	7.01	7	5
Pro	8.18	8.95	5.79	6	6
Gly	0.06	0	0.07	0	0
Ala	4.55	4.42	4.16	4	3
Cys-1/2	20.80	19.10	13.98	14	14
Val	1.32	1.43	0.92	1	1
Met	1.67	2.11	0.91	1	0
Ile	3.04	3.19	1.89	2	4
Leu	3.14	3.16	1.95	2	3
Tyr	4.47	4.42	2.01	2	1
Phe	4.02	4.09	1.98	2	1
Lys	8.73	8.75	4.86	5	4
His	1.76	1.82	0.92	1	3
Arg	4.28	4.21	2.00	2	2
NH_3	1.28	1.27	6.12	6	5
Trp	—	0.74	—	0	0
Total	113.49	112.58			
Mol. wt.				7,975	8,291

[a] Average or extrapolated values obtained after 24, 48, and 72 hours of hydrolysis.
[b] Bowman-Birk inhibitor (Frattali, 1969).
[c] 1.9S soybean inhibitor (Yamamoto and Ikenaka, 1967).
[d] Lima bean inhibitor, component 2 (Jones *et al.*, 1963).

(Yamamoto and Ikenaka, 1967), raises the distinct possibility that the two are the same. However, considering that families of closely related inhibitors have been shown to exist (Haynes and Feeney, 1967; Jones *et al.*, 1963), the status of the relationship between the two will depend on further study.

Physical Characteristics. Table II shows that the minimum molecular weight value for the Bowman-Birk inhibitor is very close to 8000. On the basis of a series of ultracentrifuge experiments, this figure has been found to be the true molecular weight (Millar *et al.*, 1969). Of all the soybean inhibitors characterized to date, this is the smallest (Table I). Nevertheless, the distinction of being the smallest known proteinase inhibitor belongs to the 3000 to 4000 molecular weight carboxypeptidase B inhibitor isolated from potatoes (Rancour and Ryan, 1968). In any event, the low value for the soybean inhibitor puts it in a size class comparable to a family of lima bean inhibitors (Haynes and Feeney, 1967; Jones *et al.*, 1963), inhibitors from kidney bean (Pusztai, 1968) and *Phaseolus aureus* (Chu *et al.*, 1964), and, interestingly, two pancreatic proteinase inhibitors (Greene *et al.*, 1966; Kassell *et al.*, 1965). From the standpoint of genetic relationships or taxonomic classification, investigation of structural homologies of the plant inhibitors could prove of some value.

Unlike any other of the soybean inhibitors which have been studied in detail, the Bowman-Birk inhibitor reversibly self-associates in aqueous solution. This fact, in conjunction with a partial specific volume value of 0.69 calculated from the amino acid analysis, can partially explain why a value, triple that given here, was first reported (Birk *et al.*, 1963). A detailed account of the association process has been prepared (Millar *et al.*, 1969). Briefly, it was determined that this phenomenon, manifested as a variation of the inhibitor's apparent molecular weight with concentration, is consistent with a theoretical model that involves equilibria among monomer, dimer, and trimer species. From the trend of the experimental data, it became obvious that the molecular weight value approached at infinite dilution was consistent with the minimal value arrived at from the amino acid analysis. Further assessment of the data indicated that, at a total protein concentration of 10 mg. per ml., approximately equal mole fractions of monomeric and dimeric species exist in equilibrium with a small fraction, $\sim$ 7%, of trimer. Two other reports have appeared which suggest that other inhibitors self-associate. Specifically, Haynes and Feeney (1967) indicate that amino acid analysis and inhibition data for one of the lima bean inhibitors yield a molecular weight of $\sim$ 8000, while sedimentation velocity experiments give a value of 16,200. Pusztai (1968) reported that a kidney bean inhibitor exhibits a variable molecular weight in the range 10,000 to 15,000; 10,000 was given as the most probable value.

Trypsin- and Chymotrypsin-Inhibiting Capacity. The Bowman-Birk inhibitor, though potent, cannot be classified with the Kunitz inhibitor as a stoichiometric inhib-

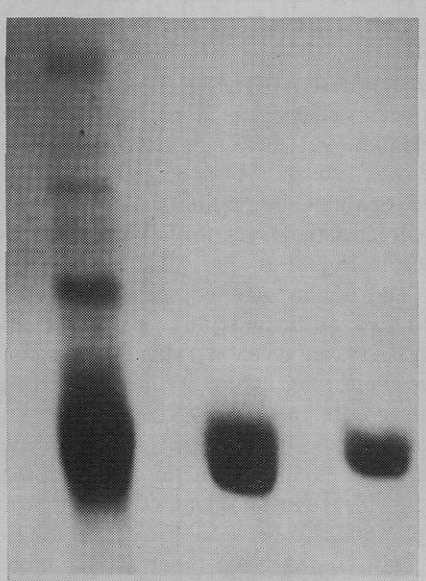

Figure 1. Polyacrylamide gel electrophoretic migration patterns for several Bowman-Birk inhibitor preparations

Left to right. Commercially available material, and CM- and DEAE-cellulose purified preparations from Hawkeye and Lee variety soybean meal

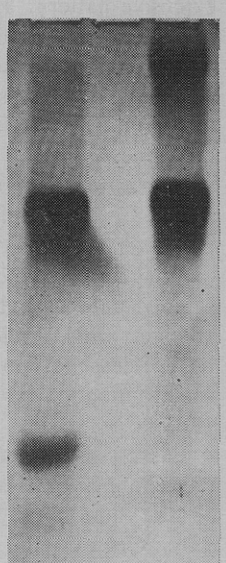

Figure 2. Polyacrylamide gel electrophoretic migration patterns for chymotrypsin-inhibitor complex

Amount of inhibitor in both samples held constant at 0.049 mg. Enzyme, 0.13 mg. (left) and 0.26 mg. (right). Two bands in pattern on left are for complex and excess inhibitor; two bands on right show complex and excess enzyme

itor of trypsin. With a synthetic substrate, no linear relationship exists between degree of inhibition and inhibitor concentration when enzyme concentration is maintained constant (Frattali, 1969). This is in contrast to the Kunitz inhibitor which forms a stable complex with trypsin. However, if a similar comparison between the two inhibitors is made with chymotrypsin, the Bowman-Birk is more effective than the Kunitz inhibitor. Specifically, with an enzyme concentration of the order of 1 μg. per ml., the Bowman-Birk inhibitor is approximately twice as effective as the Kunitz inhibitor at a mole ratio of enzyme to inhibitor of 1 to 1 (Frattali, 1969; Frattali and Steiner, 1968a). Nevertheless, the Bowman-Birk inhibitor does not have the capacity to abolish chymotryptic activity completely at reasonable concentrations.

Akin to the primary bond scission that occurs in the Kunitz inhibitor upon exposure to trypsin, the Bowman-Birk inhibitor undergoes some alteration at acid pH in the presence of a low level of enzyme (Birk *et al.*, 1967). In addition, low levels of chymotrypsin under the same conditions also have an effect on the inhibitor. Whereas the chymotrypsin-inhibiting capacity of chymotrypsin-modified inhibitor was greatly reduced, about a 70% loss occurring over a 24-hour period at pH 3.75, the trypsin-inhibiting capacity of trypsin-modified inhibitor reportedly was unaffected. Cross-interactions between trypsin and chymotrypsin-modified inhibitor, and between chymotrypsin and trypsin-modified inhibitor, were also unaffected. From this and other evidence, it was deduced that the Bowman-Birk inhibitor, like other inhibitors (Haynes and Feeney, 1967; Pusztai, 1968), has two independent loci for attachment of trypsin and chymotrypsin. Repetition and extension of the experiments of Birk *et al.* (1967) have led us to essentially the same conclusion (Frattali and Steiner, 1969b). However, we find that the inhibiting capacity of either modified form towards the modifying enzyme is considerably less than that of native inhibitor against either enzyme. Trypsin-modified inhibitor differs from chymotrypsin-modified inhibitor in that the former rapidly recovers trypsin-inhibiting capacity when exposed to a near-stoichiometric level of trypsin at pH 8, whereas recovery of chymotrypsin-inhibiting capacity by the latter in the presence of high levels of chymotrypsin proceeds at a much slower rate. Birk *et al.* (1967) have suggested that interaction between trypsin and inhibitor at acid pH probably results in the formation of a new lysine or arginine carboxyl terminal group. In view of the similar behavior of the two modified forms (Frattali and Steiner, 1969b), it is also possible that chymotrypsin-modified inhibitor possesses a new tyrosyl or phenylalanyl carboxyl terminal group.

Polyacrylamide gel electrophoresis has been instrumental in identifying the chymotrypsin-inhibitor complex (Figure 2). By extrapolation of the stoichiometry involved in the two runs illustrated in Figure 2, it was calculated that one mole of chymotrypsin can complex more than 4500 but less than 9000 grams of inhibitor. Assuming a low dissociation constant for the complex, it can be concluded that this inhibitor possesses only one chymotrypsin-binding site.

Conformational Aspects of Inhibitor and Enzyme-Inhibitor Complexes. Several optical techniques, including ultraviolet absorption and luminescence and near- and far-ultraviolet circular dichroism, have been used to probe the structural characteristics of both the free and the enzyme-complexed inhibitor in solution. When pieced together, all the bits of information provide a picture, albeit incomplete, of the higher order structural aspects of the protein systems involved. The ultraviolet absorption technique has been heavily relied upon, since spectral perturbations can be interpreted in terms of the environmental states of certain chromophoric residues. In the 270- to 290-nm. region, any change that affects either tyrosyl or tryptophyl residues can usually be detected. Analysis can then be extended as a reflection of the physical state of a protein in solution.

The absorption spectrum and the fluorescence and phosphorescence spectrum for the Bowman-Birk inhibitor are indicative of the tyrosine residues in the protein (Frattali and Steiner, 1968b). These confirm the absence of tryptophan as a structural constituent in the inhibitor (Birk *et al.*, 1963). Spectral titration curves for the ionization of the phenolic hydroxyl group of tyrosine at alkaline pH have been used to categorize tyrosine residues in a number of proteins. Results of this technique with the Bowman-Birk inhibitor are consistent with titration of two tyrosyl residues (Frattali and Steiner, 1968b). A slight inflection in the curve at about pH 11.5 may be an indication that one of the two residues is more readily titrated than the other. A definite decision in this regard cannot be made without difficulty for a number of reasons. First and foremost, electrostatic effects on the ionization process have not been evaluated. Second, the difference spectrum for the protein in an ethylene glycol–water medium indicates that the two residues are almost completely accessible (Frattali and Steiner, 1968b). Further, neither 8*M* urea nor 6*M* guanidine · HCl causes any change commensurate with exposure of buried residues. Although these results do not warrant a conclusion as to the distinguishability of the tyrosine residues, the two are fairly accessible to solvent. The ability to differentiate the two groups would be desirable since, in all probability, one is involved in the chymotrypsin-binding site.

The luminescence spectrum for the Bowman-Birk inhibitor is characteristic of the two tyrosine chromophores; that for the Kunitz inhibitor reflects only tryptophan (Frattali and Steiner, 1968b). This latter occurrence is not unusual in a protein containing both tyrosyl and tryptophyl residues. Phosphorescence decay curves for the two inhibitors are consistent with the behavior expected for the predominant chromophores in either protein. Sole phosphorescent lifetime for the Bowman-Birk inhibitor, $\tau = 2.0$ seconds, is in agreement with a similar value for tyrosine (Steiner and Kolinski, 1968).

Interaction of either trypsin or chymotrypsin with inhibitor produces characteristic differences in ultraviolet spectra. A time-dependent spectrum was observed with chymotrypsin (Frattali and Steiner, 1968b). A positive spectral shift with either enzyme in the 283- to 286-nm. region reflects an environmental change for tyrosine residues. Since both enzyme and inhibitor contain such residues, it is impossible to separate what, very likely, are contributions from the two proteins in a complex. On the other hand, peaks at 290 to 294 nm. arise from a perturbation of tryptophyl residues, and,

consequently, can only reflect the status of these groups in the enzyme.

Inhibitor complex formation involving either trypsin or chymotrypsin produces an enhanced tryptophan fluorescence at ambient temperature (Frattali and Steiner, 1968b). The greatest change was obtained with the chymotrypsin-inhibitor complex at pH 5; however, the magnitude of the change decreases over a relatively short period of time. No such decrease was observed at either pH 3 or 7 over the same time period.

These observations are consistent with spectral and inhibiting capacity data. Prolonged exposure of chymotrypsin to inhibitor at acid pH decreases inhibiting capacity (Birk *et al.*, 1967). Since it is likely that there is little affinity between the two proteins at pH 3, prolonged periods of exposure are necessary to observe a decrease in inhibiting ability. At pH 5, affinity and enzyme activity increase, resulting in an accelerated decrease in inhibiting capacity. At neutral pH, the affinity is at or near its maximum, but either the inhibiting capacity of an altered inhibitor is stabilized at this pH, or the inhibitor becomes refractory to the action of the enzyme.

The near- and far-ultraviolet optical activity for this inhibitor has been examined with circular dichroism spectra (Frattali and Steiner, 1968b). Comparatively, the far-ultraviolet spectrum bears no resemblance to those proteins or amino acid copolymers that possess helical character, but is very similar to the spectrum for the random coil conformation of poly-L-glutamic acid (Holzworth and Doty, 1965). For this homogeneous polypeptide, a deep negative trough at 202 nm. and a weak positive peak in the range 210 to 235 nm. were observed (Holzworth and Doty, 1965). Regardless of a close parallel observed with the Bowman-Birk inhibitor (Frattali and Steiner, 1968b), it would be premature to consider the inhibitor as a structureless entity, since the apparently large number of disulfide bonds, about one for every 10 amino acid residues, must provide a good deal of molecular constraint. The only conclusion that can be made is that the inhibitor does not possess much α-helical character.

Examination of circular dichroism spectra of enzyme-inhibitor complexes yields interesting results. In the near-ultraviolet region where tyrosine residues contribute to the *CD* spectrum, a positive difference results when the summed spectra for chymotrypsin and inhibitor are compared with that for the complex; similar treatment with trypsin does not produce a comparable effect. On the other hand, in the far-ultraviolet region, where the principal absorber is the amide bond, a negative difference is produced with trypsin whereas the chymotrypsin-inhibitor complex shows very little change. The chymotrypsin result could be indicative of the involvement of one of the inhibitor's tyrosine residues in the binding site for the enzyme. The effect with trypsin appears to be evidence of an actual structural change when enzyme and inhibitor combine.

In summary, the Bowman-Birk inhibitor is a low molecular weight protein which self-associates in solution. Its α-helical content appears to be low. It strongly inhibits trypsin and α-chymotrypsin, although not in a stoichiometric fashion. Additionally, the inhibitor apparently undergoes alteration in the presence of either enzyme.

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