Double-headed Protease Inhibitors from Black-eyed Peas

II. STRUCTURAL STUDIES BY OPTICAL ABSORPTION AND CIRCULAR DICHROISM*

(Received for publication, August 14, 1974)

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Two new double-headed protease inhibitors from black-eyed peas have amino acid compositions typical of the low molecular weight protease inhibitors from legume seeds. Black-eyed pea chymotrypsin and trypsin inhibitor (BEPCI) contains no tryptophan, 1 tyrosine, and 14 half-cystines out of 83 amino acid residues per monomer. Black-eyed pea trypsin inhibitor (BEPTI) contains no tryptophan, 1 tyrosine, and 14 half-cystines out of 75 residues per monomer. The molar extinctions at 260 nm are 2770 for BEPCI and 3440 for BEPTI. The single tyrosyl residue is very inaccessible to solvent in native BEPCI and BEPTI at neutral pH and titrates anomalously with an apparent pH - 12. Ionization of tyrosine is complete in 13 hours above pH 12. No heterogeneity of the local environment of the tyrosyl residues in different subunits can be detected spectrophotometrically. The large number of cystine residues leads to an intense and complex near-ultraviolet CD spectrum with cystine contributions in the regions of 248 and 280 nm and tyrosine contributions at 233 and 280 nm. An intact disulfide structure is required for appearance of the tyrosyl CD bands. The inhibitors are unusually resistant to denaturation when compared with similar low molecular weight proteins of high disulfide content. All observations are consistent with a far more rigid structure for BEPCI and BEPTI than for a typical protein.

The isolation and purification of two new protease inhibitors from black-eyed peas by affinity chromatography on trypsin- and chymotrypsin-Sepharose columns has been described (5). These new inhibitors share a special capacity of many of the legume protease inhibitors to inhibit 2 molecules of protease simultaneously. BEPCI is double-headed with respect to trypsin and chymotrypsin inhibition; BEPTI is double-headed for trypsin only, in the standard inhibitor assay. In this paper, we report the amino acid composition and investigate the optical properties of the two new inhibitors.

* This work was supported by Grant GM 14825 from the National Institutes of Health.

† National Institutes of Health Predoctoral Fellow (GM 47,233).

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The low molecular weight protease inhibitors from many different genera of legumes are quite similar (1). They all contain about 80 amino acid residues with a characteristic composition of little or no tryptophan and methionine, low tyrosine and phenylalanine, and high disulfide content. Thus, they are particularly interesting for absorbance and CD studies in the near ultraviolet region (2-4).

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Amino Acid Analyses—Samples of approximately 1 mg of reduced, carboxymethylated inhibitors were hydrolyzed at 110° in 6 M HCl and evacuated, sealed tubes for 48 hours. The amino acid compositions of the hydrolysates were determined using a Beckman amino acid analyzer. Tryptophan was determined separately on native and reduced, carboxymethylated inhibitor samples by spectrophotometric titration with N-bromosuccinimide as described by Patchornik et al. (6). Tyrosine, and 14 half-cystines out of 83 amino acid residues per monomer. 

** The abbreviations used are: BEPCI, black-eyed pea chymotrypsin and trypsin inhibitor; BEPTI, black-eyed pea trypsin inhibitor; LBI, lima bean trypsin inhibitor; PTI, pancreatic trypsin inhibitor; R x λm, measure of the sharpness of a spectral band; [β], molar ellipticity (degrees cm²/dmol); dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

EXPERIMENTAL PROCEDURES

Materials—N-Benzoxycarbonyl, dithiothreitol, and iodoacetic acid were obtained from Aldrich. n-10-Carboxybenzamidine was obtained from Moore-Kaufman. Guanidine HCl and urea were purchased from Mann. BEPCI and BEPTI were isolated and purified to homogeneity, as described earlier (5). Reduced carboxymethylcysteinyl inhibitors were prepared as described elsewhere (6).

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Ultraviolet Spectroscopy—Absorption spectra were obtained using a Cary 14 spectrophotometer with slit width 0.2 mm at the slowest scan rate. Protein concentrations were determined from absorbance at 280 nm. Derivative spectra were hand-calculated by measuring Aλ at Aλ intervals of 1 nm. The ΔA/Δλ values were confirmed at 0.5-nm intervals in the region of extrema. The resulting derivative spectra were expressed as -dA/dλ. Contributions from cystine were removed substantially by subtraction of the derivative spectrum of 7 M cystine from the inhibitor derivative spectra as described by Brads and Kaplan (2).

Spectrophotometric Titration of Inhibitor Tyrosine—Titration studies were performed by ultraviolet and by CD spectroscopy in the pH range 8.5 to 13 using 0.05 M glycine-KOH buffers adjusted to an ionic strength of 0.2 by addition of KCl. The ultraviolet titration was...
were made at room temperature on a Cary model 60 spectropolarimeter equipped with a model 6001 circular dichroism accessory calibrated with \( \beta \)-10-camphor sulfonic acid. An optical pathlength of 1 cm and maximum optical densities of about 0.8 were used routinely. Blank spectral curves were obtained after every sample scan for each solvent used. The CD data are reported as molar ellipticities \([\Theta]\) based on the monomer molecular weights of the inhibitors.

**RESULTS AND RELATED DISCUSSION**

**Amino Acid Analysis**—The amino acid compositions of BEPCI and BEPTI are shown in Table I. They show the characteristic features of the low molecular weight protease inhibitors from legume seeds, including high cystine (greater than 15% by weight), serine, and aspartic plus glutamic acid content, and little or no tryptophan, tyrosine, phenylalanine, and methionine.

The principal differences in the amino acid compositions of the black-eyed pea iso-inhibitors can be seen in the ratio of basic to potentially acidic residues, 7/19 for BEPCI versus 8/16 for BEPTI, and the presence of the single methionine in BEPTI and the second phenylalanine of BEPCI. The minimum molecular weights calculated from the amino acid analysis, 8950 for BEPCI and 8200 for BEPTI, agree with results from sodium dodecyl sulfate gel electrophoresis, which indicated monomer molecular weights under 10,000, with BEPCI slightly larger than BEPTI (9). BEPCI and BEPTI differ more from each other than from pure isoinhibitors found in the garden bean (8). BEPCI is amazingly similar to garden bean isoinhibitor IIIb in amino acid composition and number; both are chymotrypsin inhibitors with double-headed activity. BEPTI strongly resembles garden bean isoinhibitor I in size and amino acid composition; both inhibit trypsin, but not chymotrypsin.

**Circular Dichroism Studies**—Circular dichroism measurements were made at room temperature on a Cary model 60 spectropolarimeter equipped with a model 6001 circular dichroism accessory calibrated with \( \beta \)-10-camphor sulfonic acid. An optical pathlength of 1 cm and maximum optical densities of about 0.8 were used routinely. Blank spectral curves were obtained after every sample scan for each solvent used. The CD data are reported as molar ellipticities \([\Theta]\) based on the monomer molecular weights of the inhibitors.

**TABLE I**

| Amino acid composition of black-eyed pea inhibitors | BEPCI | BEPTI |
|--------------------------------------------------|-------|-------|
| Residues per molecule | Integer | Residues per molecule | Integer |
| Lysine | 4.2 | 4 | 4.6 | 6 |
| Histidine | 4.0 | 3 | 3.4 | 3 |
| Arginine | 3.4 | 3 | 3.2 | 3 |
| Carboxymethylcysteine | 14.0 | 14 | 13.8 | 14 |
| Aspartic acid | 11.4 | 11 | 9.4 | 9 |
| Threonine | 3.8 | 4 | 3.4 | 3 |
| Serine | 12.4 | 12 | 9.9 | 10 |
| Glutamic acid | 8.0 | 8 | 7.0 | 7 |
| Proline | 5.0 | 6 | 5.8 | 6 |
| Glycine | 3.0 | 3 | 2.2 | 2 |
| Alanine | 4.8 | 5 | 2.8 | 3 |
| Valine | 1.5 | 1 | 0.8 | 1 |
| Methionine | 0 | 0 | 0.6 | 1 |
| Isoleucine | 3.4 | 3 | 4.2 | 4 |
| Leucine | 2.0 | 2 | 2.1 | 2 |
| Tyrosine | 0.8 | 1 | 0.9 | 1 |
| Phenylalanine | 2.2 | 2 | 1.0 | 1 |
| Tryptophan* | 0 | 0 | 0 | 0 |
| Total | 83 | 75 |
| Molecular weight | 8950 | 8200 |

* Determined independently with N-bromosuccinimide by the method of Patchornik et al. (7)

Fig. 1 (left). Near-ultraviolet absorption spectra of BEPCI and BEPTI in water at room temperature. The individual spectra remain completely unchanged in \( 10^{-3} \text{ M HCl} \), pH 3.0, and in 0.1 M Tris-HCl, pH 8.0.

Fig. 2 (right). Derivative spectra of BEPCI and BEPTI after correction for contributions from cystine.
and BEPTI after correction for cystine contributions, as described by Brandts and Kaplan (2). The crossover points, corresponding to the extinction maxima of the conventional spectra of BEPCI and BEPTI, are 278.7 and 278.3 nm respectively. Brandts and Kaplan (2) have found that this parameter, $\lambda_{\text{max}}$, is related linearly to the degree of accessibility of tyrosyl chromophores to the solvent. The calculated linear relationship of Brandts and Kaplan (2) is shown as the solid line in Fig. 3. Using this, the average accessibility of the solvent-accessible tyrosines of lima bean isoinhibitors ranges from 0.001 to 0.014, with 2 buried and 2 accessible tyrosines (LBI VII) and RNase (the three accessible plus the three inaccessible tyrosines of ribonuclease), and pancreatic trypsin inhibitor, with 2 buried and 2 accessible tyrosines (PTI) are taken from Brandts and Kaplan (2).

Fig. 3 (left). Relationship of accessibility of the tyrosine hydroxyl to the solvent and the wavelength of maximum extinction of the tyrosine residue in a protein. The calculated dependence of accessibility on $\lambda_{\text{max}}$ (solid line) and the values for lima bean isoinhibitors (LBI VII and VI) and RNase (the three "inaccessible" tyrosines of ribonuclease) are taken from Brandts and Kaplan (2).

Fig. 4 (center). Approximate degree of spectral heterogeneity of protein tyrosyl chromophores. $R \times \Delta \lambda_v$ values calculated for BEPCI and BEPTI relative to values reported by Brandts and Kaplan (2) for proteins and the model compound acetyltirosine ethyl ester. It can be seen that BEPCI and BEPTI have a degree of heterogeneity almost as low as the lowest value found by Brandts and Kaplan (2).

In proteins containing more than one tyrosine, heterogeneity in the tyrosyl environments is reflected in decreased sharpness of the derivative spectra. Brandts and Kaplan used the parameter $R \times \Delta \lambda_v$ to characterize band sharpness (2), where $R$ is the ratio of the intensity of the major derivative peak to that of the minor peak and $\Delta \lambda_v$ is the half-width of the major band. Both of these quantities increase when there is spectral heterogeneity due to multiple chromophores. Fig. 4 shows $R \times \Delta \lambda_v$ values calculated for BEPCI and BEPTI relative to values reported by Brandts and Kaplan (2) for proteins and the model compound acetyltirosine ethyl ester. It can be seen that BEPCI and BEPTI have a degree of heterogeneity almost as low as the lowest value found by Brandts and Kaplan (2).

Band sharpness as reflected by $R \times \lambda \Delta \lambda_v$ may be due to weakened interactions between the chromophore and the local environment, such as repression of hydrogen bonding due to inaccessibility of the solvent or lack of a hydrogen-bonding partner in the protein interior, or to decreased mobility of the local environment so that structural fluctuations of the solvent are damped. From the results of Figs. 3 and 4, one can conclude that the single tyrosine of BEPCI or BEPTI is extremely inaccessible to the solvent at neutral pH. Its hydroxyl group may be situated in a position of restricted mobility and limited hydrogen bonding. Note that this very low heterogeneity is observed for an aqueous solution in which (as will be shown later) isolated protein monomers represent only about 12% of the total oligomer population (18). Thus, the multiple tyrosines of the inhibitor oligomers appear to be in equivalent environments in their individual subunits.

Alkaline titration of the single tyrosine of BEPCI, as monitored by absorbance at 295 nm, is shown in Fig. 5. The behavior of BEPTI (not shown) was almost identical. Between pH 8.5 and 11.5 the response of the system to base was rapid. Above pH 11.5 a slow, time-dependent increase in absorbance was observed over a 13-hour period. No further changes occurred after 13 hours up to 31 hours. The full extent of the slow spectral change at pH 13 corresponded to the ionization of about 1 mol of tyrosine/mol of monomer BEPCI, with an apparent $pK_a = 12.0$, based on the molar extinction at 295 nm of the tyrosinate anion of 2400 (19). At short times, the extent of ionization was less than 30% and was reversible. This suggests that the tyrosyl residue in the native inhibitor is mostly inaccessible to the solvent. Some kind of change in structure is required for complete ionization of the phenolic hydroxyl. Similar abnormal ionization behavior has been seen in the Kunitz soybean inhibitor (20) and in the Brazilian black-eyed pea inhibitor (21).

The explanation for abnormal tyrosine titration is embedded in the phenomenon of hydroxyl in the protein interior so that it is inaccessible to the solvent (22). This is consistent with the low accessibility of the phenolic hydroxyl at neutral pH calculated from the derivative spectra of the native inhibitors and the band sharpness seen in the inhibitor derivative spectra.

**Circular Dichroism Studies of Native BEPCI and BEPTI**

—CD spectra of native BEPCI and BEPTI are shown as a function of increasing pH in Fig. 6. Samples were allowed to
The spectra of BEPCI and BEPTI are unchanged in distilled water or in buffer from pH 3.0 to 8.0. At pH 8.0, BEPCI has a negative band at 280 with monomer molar ellipticity $\theta$ of $-90,000$ and positive bands at 248 and 233 nm with $\theta$ at 17,000 and 12,000, respectively. A crossover point occurs at exactly 260 nm. The spectrum of BEPTI is slightly, but reproducibly, different. It has a negative band at 280 nm with $\theta$ of $-18,500$ and positive bands at 247.5 and 234 with $\theta$ of 16,000 and 16,000, respectively. A crossover point occurs at 262.5 nm.

The behavior of the CD near 280 nm upon increasing the pH from 8.0 to 13.0 is qualitatively the same for BEPCI and BEPTI. The negative band at 280 nm decreases and with an apparent isosbestic point at 293 nm for both inhibitors. Larger differences are seen in the positive bands of the two inhibitors when the pH is raised from 8.0 to 13.0. The 248 nm band of BEPCI increases X-fold while the positive band at 234 nm disappears at pH 12.5, analogous to the behavior of BEPTI. Apparently, the band at 233 nm in BEPTI is more difficult to assign. Both protonated tyrosine and disulfide transitions are involved (27). A similar positive CD band is seen in bovine neurophysin-II (4) near 245 nm. This band is assigned unequivocally to disulfide transitions because it is unaltered even when the single tyrosine is 70% ionized (4). The entire CD spectrum of neurophysin-II is remarkably similar to that of BEPCI. (From the admittedly limited viewpoint of amino acid composition, disulfide content, molecular weight, and self-assembly properties, it is remarkable that the neurophysins are not protease inhibitors.)

The large negative CD band centered at 280 nm in BEPCI and BEPTI is more difficult to assign. Both protonated tyrosine and disulfides can contribute to CD at 280 nm. The decrease in $\theta_{280}$ and increase in $\theta_{290}$ with increasing pH reveal typical tyrosine titration behavior and indicate some tyrosine contribution. However, the magnitude of the pH effects on the 280 nm band are much smaller than the effects near 245 nm. Moreover, analysis of the ultraviolet spectra of BEPCI and BEPTI showed an anomalously high disulfide contribution to $\epsilon_{280}$ nm. Thus, the negative band at 280 nm probably results from both tyrosyl and disulfide transitions. The behavior of the 280 nm band in the lima inhibitors is exactly like that of BEPCI and BEPTI (26). Acetylation of the single tyrosine of one lima bean inhibitor only slightly decreased 280 nm CD at pH 7.5, suggesting that both tyrosine and disulfide transitions are involved (27). In neurophysin-II,
the negative band at 280 nm is attributed solely to disulfide transitions, because ionization of the single tyrosine has no effect on it (4).

**CD Studies on Partially Denatured BEPCI**—BEPCI was subjected to 0.02 M dithiothreitol at various temperatures. It seemed reasonable to expect that these conditions would break disulfide bonds but could leave a substantial fraction of the structure intact. All samples were kept under a nitrogen atmosphere except where noted. A 3-hour exposure to dithiothreitol at room temperature had no effect on the CD spectrum. Ten additional hours at 37° led to substantial changes in the spectrum. An additional hour at 60° resulted in more dramatic changes. These results are shown in Fig. 7. Slow reoxidation was carried out after nitrogen removal by dialysis at 4° for 80 to 106 hours. This resulted in no changes whatsoever in the CD spectrum. In contrast to the CD alterations induced by dithiothreitol treatment, the ultraviolet spectrum of BEPCI remained completely unchanged throughout.

Information about the degree of disulfide modification can be inferred from the decrease in CD at 248 nm, since at pH 8.0 only disulfide transitions contribute here. The 248 nm band is unchanged after 3 hours at room temperature, about one-half its original value after incubation at 37°, and very small after incubation at 60°. The effect of dithiothreitol on the tyrosyl band at 233 nm is obscured by the rapidly decreasing single/ noise ratio below 240 nm due to absorbance by dithiothreitol, but this band apparently disappears.

The recalcitrance to reduction exhibited by the disulfide bonds of BEPCI may best be appreciated by comparison with neurophysin-II, which has an equally high disulfide content (4). For neurophysin-II, 30 min at room temperature in 0.02 M dithiothreitol is sufficient to cause a 60% decrease in [θ]_{222} and a 73% decrease in [θ]_{208}, while 90 min at room temperature entirely abolishes both bands (4). Thus, reducing conditions which completely remove the optically active disulfide transitions of neurophysin-II have no effect on the disulfides of BEPCI. The behavior of BEPTI is entirely similar to that of BEPCI. The only protein for which even more refractory disulfide bonds are reported is neocarzinostatin (28). This low molecular weight protein is similar to the trypsin inhibitors in many respects.

With increasing modification of disulfide bonds, both 280 and 248 nm CD bands decrease in intensity at essentially equal rates. However, the most significant feature of the spectrum is the decrease in separation between these two bands as denaturation proceeds. Since the 280 nm CD band of BEPCI shifts toward shorter wavelength with denaturation, it must contain an appreciable disulfide component. An isolated tyrosine band would not be expected to shift. The simplest hypothesis is that the disulfide component of the 280 nm band is centered at longer wavelengths than the tyrosine component. Partial denaturation preferentially eliminates the disulfide band causing the apparent shift. If the actual disulfide contribution is intense and is located at such long wavelengths, the dihedral angles of at least some of the cystines in the native inhibitor must be fairly small (16, 17). An alternative, more speculative, hypothesis is that the observed shift results from a relaxation of the dihedral angles of some disulfides after the first few have been broken. A 30 to 40 nm red shift could result from increases in dihedral angles (16, 17).
measured immediately at pH 8.0 and after 24 hours of exposure to air at 4°C, pH 8.0 or pH 12.5 (Fig. 8). All of the near-ultraviolet CD bands of native BEPTI have disappeared in the pH 8.0 spectrum of the Method 1 sample. This indicates that all of the optically active disulfides of native BEPTI have been cleaved by treatment with urea and dithiothreitol. Attempts at air oxidation led to no recovery of optical activity suggesting that the denaturation is irreversible at pH 8.0. Method 1 denaturation also eliminates the tyrosyl CD band at 234 nm.

Air oxidation of the Method 1 sample for 24 hours at pH 12.5 results in a new CD band not seen in the Method 1 sample at pH 8.0 or in native BEPTI at pH 12.5. The new positive band has \([\theta]_{280}^{1}\) of 16,000. Optically active transitions of tyrosine have never been observed near 260 nm (14, 15, 25). Such large ellipticity for a single phenylalanine near 280 nm is out of the spectrum of the Method 1 sample. This indicates that all of the never been observed near 260 nm (14, 15, 25). Such large optically active disulfides of native BEPTI have been cleaved by treatment with urea and dithiothreitol. Attempts at air oxidation also eliminates the tyrosyl CD band at 234 nm. The CD spectrum of extensively denatured BEPTI at pH 12.5 apparently permits the recovery of one or more optically active disulfides. From the location of the CD band one can infer that the dihedral angle of these disulfides is near 90° (15, 16). Whatever structure reforms is fairly different from native BEPTI at alkaline pH.

The Method 2 sample has no near-ultraviolet CD bands. A 24-hour air oxidation at either pH 8.0 or pH 12.5 has no effect on the CD spectrum since carbamidomethylation effectively blocks re-formation of cleaved disulfides. The disulfide-dependent tyrosyl optical activity is eliminated in the Method 2 sample, as was observed in the Method 1 sample. Above 240 nm, the CD spectra at pH 8.0 of the Method 1 and Method 2 samples are identical. Below 240 nm, the two samples reveal a dramatic difference. The CD spectrum of the Method 1 sample, like that of native BEPTI, decreases continuously from 230 nm to below 210 nm with no shoulders or fine structure. This is interpreted as the absence of any typical secondary structure (no \(\alpha\), \(\beta\), or random coil) by the criteria of Greenfield and Fasman (31) and Chen et al. (32). It may represent an anomalous chain conformation imposed by some of the disulfides. In contrast, the CD spectrum of the Method 2 sample shows a distinct shoulder (\([\theta]_{280}^{1} \sim -77,000\)) and resembles the calculated CD spectrum for poly(L-lysine) with 20% \(\alpha\)-helix, 80% random coil (31). Apparently, cleavage and carbamidomethylation of all seven disulfide bonds is required to enable extensive unfolding of the peptide chain. The ineffectiveness of denaturation by Method 1 compared to carbamidomethylation was also seen by sodium dodecyl sulfate gel electrophoresis (5).

The general conclusion from all of these studies is that the large number of cystine residues in BEPCI and BEPTI leads to an intense and complex near-ultraviolet CD spectrum. The CD of the single tyrosine is dependent on the intactness of the disulfide network. The protein is remarkably resistant to cleavage of the disulfide bonds and alkaline titration of the tyrosine in the native inhibitor also proceeds very reluctantly. All of these findings suggest that the structure of these two protease inhibitors is unusual and is far more rigid than that of a typical globular protein.

Acknowledgment—We are grateful to Dr. Molly Pflumm for performing the amino acid analyses.

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Double-headed protease inhibitors from black-eyed peas. II. Structural studies by optical absorption and circular dichroism.
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J. Biol. Chem. 1976, 251:741-746.

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