Origins of Circular Dichroism Bands in Bowman-Birk Soybean Trypsin Inhibitor*

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ERNEST KAY

From the Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, California 90024

The spectral properties of Bowman-Birk soybean trypsin inhibitor (BBI) were investigated by analyzing difference absorption spectra and difference CD spectra and by comparing them with those of tyrosyl model compounds. The O-acetylation of tyrosyl side chains showed that the ultraviolet CD bands of BBI above 225 nm originate from disulfide side chains and tyrosyl phenolic groups; phenylalanyl residues do not give rise to detectable CD in BBI in this wavelength region. The results of the tyrosyl ionization experiment were consistent with this interpretation.

A broad negative CD band centered around 280 nm in BBI arises mainly from disulfide bonds \( (\epsilon_L - \epsilon_R = -0.83 \text{ M}^{-1} \text{ cm}^{-1} \text{ per disulfide}) \). Each of 2 tyrosyl residues gives rise to negative CD in this region; together they contribute approximately 10% of the total CD intensity at 277 nm \( (\epsilon_L - \epsilon_R = -0.36 \text{ M}^{-1} \text{ cm}^{-1} \text{ per tyrosyl}) \). Disulfide bonds in BBI also have a broad positive CD band centered around 240 nm \( (\epsilon_L - \epsilon_R = 0.9 \text{ M}^{-1} \text{ cm}^{-1} \text{ per disulfide}) \). Tyrosyl side chains give rise to a sharp positive peak at 231 nm, overlapping with the positive disulfide CD. Dimerization of monomeric BBI did not alter the CD profile.

One of two tyrosyl phenolic groups is relatively exposed and can be O-acetylated by 100- to 1500-fold molar excess of N-acetylimidazole. The other is inaccessible to the reagent even in the presence of 8 M urea, but can be acetylated in the presence of 6 M guanidine hydrochloride. Fully acetylated BBI has the near-ultraviolet CD of BBI and the far-ultraviolet polypeptide CD very similar to those of the native inhibitor, indicating that O-acetylation of two tyrosyl side chains did not induce much conformational change in BBI.

The near-ultraviolet CD of BBI was altered in the presence of 8 M urea or 6 M guanidine hydrochloride, with a greater change brought about by the latter. Dithiothreitol (20 mM) completely abolished the tyrosyl and disulfide CD in this region.

Bowman-Birk soybean trypsin inhibitor was first described by Bowman in 1944 as an antitryptic material which was soluble in alcohol and precipitated with acetone (1). Since then several trypsin inhibitors of different molecular weights but very similar amino acid compositions have been isolated from soybean (2-5). They were shown to be the same protein by Frattali (5) and the difference in molecular weight was explained by the concentration-dependent self-association of BBI (6, 7).

The amino acid composition of BBI is characterized by a high disulfide content (seven disulfides/M, = 7975) and the lack of tryptophan and glycine. Aromatic amino acids consist of 2 tyrosyl and 2 phenylalanyl residues (5, 8, 9). The lack of tryptophan makes spectral analyses of BBI in the near-ultraviolet region relatively simple. An earlier study of the protein using CD and ORD techniques revealed that BBI was nonhelical. Only 1 tyrosyl residue was considered to give rise to a large negative CD centered around 280 nm. A positive peak at 234 nm was attributed to tyrosyls. Aromatic amino acids (tyrosyls and phenylalanyls), or disulfide bonds, or both, were thought to cause the 245 nm shoulder (10). However, the 280 nm CD band reported is too large for a tyrosyl residue only and relatively intense CD above 300 nm is suggestive of a sizable disulfide contribution (11).

In the present study, the origins of the ultraviolet CD bands of BBI above 225 nm were identified, using model compounds to analyze the changes in absorption and CD spectra accompanying O-acetylation and ionization of tyrosyl phenolic groups in the protein.

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1The abbreviations used are: BBI, Bowman-Birk soybean trypsin inhibitor; \( \epsilon_L - \epsilon_R \), molar extinction coefficient for left circularly polarized light minus that for right circularly polarized light.

2Ikeda et al. (10) thought that BBI they had isolated had a molecular weight of 16,400. The 280 nm negative CD was attributed solely to 1 of 4 tyrosyls/16,400. Disulfide CD was poorly understood then and the possibility of its contribution to the near-ultraviolet region (~280 nm) was not considered in BBI.
EXPERIMENTAL PROCEDURE

Absorption spectra were recorded on a Cary model 15 spectrophotometer. CD spectra were measured with an instrument described elsewhere (12-14). To obtain difference CD spectra, each CD spectrum was stored in a signal averager and recorded separately before subtracting one spectrum from the other.

Crude BBI was extracted from defatted soybean flour (Nutrisoy 7-B) (Archer Daniels Midland, Pales Verdes, Calif.) with ethanol and precipitated with acetone according to procedure of Bowman (15) as modified by Birk (16). The crude inhibitor was chromatographed on a CM-cellulose column (2.8 x 32 cm) (2), then on an anion exchange column (1.9 x 9 cm) and eluted with an exponential gradient of sodium acetate, pH 5.0, from 0.15 to 0.25 M (1 liter each) (4). DEAE-Sephadex A-50 substituted for DEAE-cellulose. The inhibitor was further purified by gel filtration on a Sephadex G-100 column (3 x 43 cm) (4). The purified BBI was dialyzed against cold water, lyophilized, and stored at 4°C until used. The final yield was 367 mg of BBI from 1.5 kg of defatted soybean flour. Homogeneity of the purified preparation was ascertained by polyacrylamide gel disc electrophoresis (17).

Acetylation of BBI (18, 19) was performed as described previously (20). Reaction media were dialyzed against cold water to terminate the acetylation and to remove reagents. Spectrapor 3 membrane (Spectramed, Los Angeles) with an approximate molecular weight cutoff of 3500 was used for the dialysis so that acetylated BBI could be recovered quantitatively.

The concentration of native BBI was estimated spectrophotometrically using the values of $M_r = 7975$ (5) and $A_{280} = 4.4$ (4, 5).

Trypsin (EC 3.4.4.4.) (Worthington) activity was assayed by mixing BBI with trypsin in a reaction buffer consisting of 40 mM Tris-Cl, pH 8.1/10 mM CaCl$_2$. Trypsin inhibitor activity was assayed by mixing BBI with trypsin in the above buffer and incubated for 1 min before adding the substrate.

Other chemicals used were of analytical grade. Double-distilled water was used throughout.

RESULTS

Absorption Spectra of Acetylated BBI—Native BBI has an absorption maximum at 274.5 nm, a shoulder around 282 nm, and fine structure at 268, 264, and 257 nm (Fig. 1A). The 282 nm shoulder and 275 nm peak arise, respectively, from 0-0 and 0-0 vibronic transitions of tyrosyl phenolic groups (23, 24). The fine structure can be assigned to phenylalanyl transitions (25). The other two traces in Fig. 1 show the decrease in near-ultraviolet absorption upon acetylation of tyrosyl side chains with N-acetylimidazole. After 2 hours at room temperature, acetylation was terminated by chromatography on a Sephadex G-10 column (1.5 x 55 cm) with water. Molar extinction coefficients of N-acetyl-L-tyrosine and N,O-diacetyl-vtyrosine (18, 22) were used for quantitative determination.

When acetylating in the presence of 6 M guanidine hydrochloride, estimated by ex-}

![CD Spectra of Acetylated BBI](http://www.jbc.org/)

**Fig. 1.** Absorption spectra of BBI, acetylated BBI, and deacetylated BBI in 50 mM sodium phosphate, pH 7.0. —, native or acetylated and deacetylated BBI; ——, acetylated with 1500 molar excess of N-acetylimidazole in 50 mM sodium borate, pH 8.3, for 2 hours; ——, acetylated with 1500 molar excess of N-acetylimidazole in 6 M guanidine hydrochloride for 2 hours; ——, extrapolated disulfide absorption. B, difference absorption spectra of BBI produced by subtracting the spectrum of fully acetylated derivative (——) from native BBI spectrum (——, A).

The wavelength profiles of the difference absorption spectrum between the native and fully acetylated BBI (Fig. 1B) and that between N-acetyl-L-tyrosinamide and N,O-diacetyl-L-tyrosinamide (Fig. 2) are very similar. When both tyrosyls in BBI were fully acetylated, the decrease in $e$ was 2500 at 276 nm and 8000 at 228 nm, values comparable to the loss of $e$ when N-acetyl-L-tyrosinamide was O-acetylated, 1300 at 274 nm and 5000 at 226 nm. Thus, the loss of the near-ultraviolet absorption upon acetylation BBI can be accounted for by the absorption change in the tyrosyl side chain transitions; a blue-shifting and hypochromism of $I_0$ vibronic bands and a blue-shifting of $I_1$ transition.

**CD Spectra of Acetylated BBI** —Fig. 3, A and B, presents the CD spectra corresponding to the absorption spectra shown in Fig. 1. The near-ultraviolet CD spectra of BBI is dominated by a broad intense negative band centered around 280 nm, a sharp positive peak at 291 nm and a pronounced shoulder around 245
Acetylating one or two tyrosyl side chains caused only a slight decrease in the intensity of the 280 nm CD band. The positive peak at 231 nm, however, diminished greatly upon acetylation. The shoulder at 245 nm was relatively unaffected by this treatment. Deacetylation of the modified BBI fully restored the native CD spectrum.

The spectrum profile of difference CD between the native BBI and the fully acetylated derivative (Fig. 3B) matches very well with that of difference absorption spectra shown in Figs. 1B and 2. Double peaks at 284 and 277 nm are characteristic of tyrosyl \( \varepsilon_r \) transition and the positive peak at 231 nm corresponds well with the \( \varepsilon_r \) band of the tyrosyl absorption spectrum.

The assignment of the lost CD in acetylated BBI to tyrosyl side chains was reinforced by the CD of N-acetyl-L-tyrosinamide and its O-acetylated derivative. As in the absorption change (Fig. 2), the modification resulted in a drastic reduction in \( \varepsilon_r \) CD accompanied by a hypochromic shift of about 10 nm (Fig. 4A). The change observed in \( \varepsilon_r \) band was less drastic, about 15% reduction of intensity and 6 to 7 nm blue shift at the maximum.

A good matching of the difference CD spectrum profiles between the proteins (Fig. 2B) and the model compounds (Fig. 4B) suggests that the observed CD changes in acetylated BBI are due mainly to O-acetylation of tyrosyl phenolic groups.

Origins of BBI CD above 230 nm—Based on the above analysis, the origins of BBI CD above 231 nm are assigned to disulfide bonds and tyrosyl phenolic groups. The broad negative CD centered around 280 nm is largely due to cystinyl, with approximately 10% of the intensity at 277.5 nm arising from the \( \varepsilon_r \) transition of tyrosyl side chains. Disulfide bonds also give rise to a broad positive band around 240 nm. The intensity of this CD at its maximum is approximately the same or slightly larger than that of the 280 nm band. The tyrosyl \( \varepsilon_r \) transition gives rise to a sharp positive CD at 231.5 nm, overlapping with the broad disulfide band.

CD Spectra of Ionized BBI—As the pH of BBI solutions was raised progressively from neutrality, the 231 nm positive peak decreased and disappeared completely when tyrosyl phenolic groups were fully ionized (Fig. 5). The intensity of the 245 nm shoulder decreased with the partial ionization, then increased with the full ionization of tyrosyl side chains. The 280 nm negative CD band decreased slightly with rising alkalinity. At pH where both tyrosyls were fully ionized (pH 12.8), approximately 20% reduction in the CD intensity at 275 nm was observed.

The disappearance of the 231 nm peak and the increase in the 245 nm CD can be accounted for by the bathochromic shift...
CD of Bowman-Birk Inhibitor

in the tyrosyl \( \lambda_{a} \) band by about 15 nm (Figs. 1B, 6 and 7). The positive CD at 245 nm in the ionized BBI, therefore, must be the sum of contributions from disulfide and ionized tyrosyl side chains.

The decrease of 245 nm CD at an intermediate pH (pH 10) indicates that the alkali condition also affects disulfide CD. A noticeable reduction of CD intensity above 300 nm at pH 12.8 cannot be accounted for by the change of tyrosyl CD and must be attributed to an alteration in disulfide CD because tyrosyl side chains do not have CD in this region either under neutral or alkaline conditions (Fig. 7).

Effect of Urea, Guanidine Hydrochloride, and Dithiothreitol on BBI—Urea (8 M) elicited some change in tyrosyl CD without much affecting the disulfide CD (Fig. 8). Guanidine hydrochloride (6 M) caused a greater change in tyrosyl CD as well as some alteration in disulfide CD. This is consistent with the result of acetylation experiments where complete acetylation of tyrosyl side chains by 1500-fold molar excess of N-acetylimidazole was achieved only in the presence of 6 M guanidine hydrochloride (Fig. 1A). Only 1 tyrosyl residue could be acetylated by the reagent alone or with 8 M urea.

Reducing disulfide bonds with dithiothreitol completely eliminated the disulfide and tyrosyl CD bands. In addition a change in polypeptide CD in the far-ultraviolet region was observed, as evidenced by the onset of an intense negative CD above 240 nm.

Effect of BBI Polymerization on CD—BBI in solution has been shown to exist in monomer, dimer, and perhaps, trimer forms (5-7). High protein concentration and high ionic strength promote self-aggregation. The aggregation is also more favorable at pH 5 than pH 7 (6, 7). Because of this property, CD of BBI at 0.09 and 1.63 mg/ml was measured in three different buffer systems. Buffer A, 50 mM sodium phosphate, pH 7.0; Buffer B, 50 mM sodium phosphate, pH 7.0/100 mM NaCl; Buffer C, 50 mM sodium acetate, pH 5.0/100 mM NaCl. Estimated average molecular weight is 8,250 for 0.09 mg/ml of protein at pH 7 and 12,500 for 1.63 mg/ml at pH 5 (7). Since BBI has molecular weight of 7975 (5), the monomeric form is predominant in the former, while it is intensively polymerized in the latter. CD spectra recorded under these diverse conditions were essentially identical and are represented in Fig. 3A (---). Apparently, self-association of BBI does not induce conformational change and tyrosyls and...
weight for the chondroitin sulfate chains in the proteoglycan and would not contain hexuronic acid (42-44). The digestion since they would be smaller than the chondroitin sulfate elution pattern of the chondroitin sulfate, with a shoulder indicating that keratan sulfate chains were possibly present in the profile of the chondroitin sulfate chains. The shoulder indicated by the shading in b was recovered and analyzed for hexosamines.

![Chromatogram of proteoglycan fraction](image)

**Fig. 5.** Column elution profiles for a, the monomer proteoglycan (Gu-D1) on Sepharose 2B; b, a chondroitinase ABC digest of Gu-D1 on Sepharose 4B; c, a papain digest of Gu-D1 on Sepharose 6B. The fraction indicated by the shading in b was recovered and analyzed for hexosamines.

**TABLE I**

Incorporation of 35S radioactivity into proteoglycans

<table>
<thead>
<tr>
<th></th>
<th>Hexuronic acid [dpm/μg]</th>
<th>Hexuronic acid [dpm/μg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gu-residue</td>
<td>0.52 (21%)</td>
<td>19.6 (11%)</td>
</tr>
<tr>
<td>Gu-extract</td>
<td>1.98 (79%)</td>
<td>154 (89%)</td>
</tr>
<tr>
<td>Mg-residue</td>
<td>0.56 (21%)</td>
<td>16.8 (9%)</td>
</tr>
<tr>
<td>Mg-extract</td>
<td>2.05 (79%)</td>
<td>169 (91%)</td>
</tr>
<tr>
<td>Associative gradients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gu-gel</td>
<td>0.36 (32%)</td>
<td>45.5 (34%)</td>
</tr>
<tr>
<td>Gu-Al</td>
<td>1.14 (64%)</td>
<td>80.5 (62%)</td>
</tr>
<tr>
<td>Mg-gel</td>
<td>0.62 (34%)</td>
<td>51.2 (34%)</td>
</tr>
<tr>
<td>Mg-Al</td>
<td>1.15 (64%)</td>
<td>97.0 (65%)</td>
</tr>
<tr>
<td>Dissociative gradient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gu-D1</td>
<td>1.68 (65%)</td>
<td>140 (66%)</td>
</tr>
</tbody>
</table>

Values are average values per 100-mm culture plates of 8-day cultures.

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**TABLE II**

Composition of Gu-D1 proteoglycan fraction

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Residues/1000</th>
<th>mmol/mol of glucuronic acid</th>
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</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>Histidine</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Arginine</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>Threonine</td>
<td>88</td>
<td>41</td>
</tr>
<tr>
<td>Serine</td>
<td>115</td>
<td>53</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>172</td>
<td>80</td>
</tr>
<tr>
<td>Proline</td>
<td>61</td>
<td>29</td>
</tr>
<tr>
<td>Glycine</td>
<td>103</td>
<td>48</td>
</tr>
<tr>
<td>Alanine</td>
<td>81</td>
<td>37</td>
</tr>
<tr>
<td>1/2 cystine</td>
<td>/</td>
<td>3</td>
</tr>
<tr>
<td>Valine</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>Methionine</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>47</td>
<td>22</td>
</tr>
<tr>
<td>Leucine</td>
<td>63</td>
<td>29</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>21</td>
<td>10</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>35</td>
<td>16</td>
</tr>
<tr>
<td>Glucosamine</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>Galactosamine</td>
<td></td>
<td>803</td>
</tr>
</tbody>
</table>

preparation isolated from the cultured chick limb bud chondrocytes would be about 20,000 (45). Another aliquot of the papain digest from Gu-D1 was fractionated on cetylpyridinium chloride cellulose columns (35). The fraction eluted with 1% cetylpyridinium chloride contained essentially all the glucosamine present in the digest which indicates that the glucosamine in the original preparation was present primarily either in keratan sulfate or glycopeptides (35, 46) and not in hyaluronic acid or heparan sulfate. As was shown previously (8), the bulk of the material was recovered in the 0.75 M MgCl₂ fraction which is typical for chondroitin sulfate (35).

The elution profile observed on Sepharose 4B for a chondroitinase ADC digest of Gu-D1 is shown in Fig. 5b. A symmetrical, included peak with a Kᵥ of 0.32 was observed. The peak of hexuronic acid in the column total volume represents oligosaccharide digestion products. The peak in the excluded volume probably indicates that the preparation contained a trace amount of hyaluronic acid to which core molecules bind. Similar elution patterns have been observed for chondroitinase digests of bovine nasal proteoglycans (13, 22). The included peak indicated in Fig. 5b was recovered and a portion analyzed for its hexosamine content. The ratio of galactosamine:glucosamine was 1.5, which indicates that the chondroitinase digestion removed more than 90% of the chondroitin sulfate from the macromolecules. The result also indicates that the glucosamine component is present within the core structure of the proteoglycan molecules as would be expected for keratan sulfate (44). The distribution of the disaccharide digestion products recovered from the chondroitinase digest is shown in Table III. Absorption at 232 nm of the disaccharides eluted from paper chromatographs (27) indicated that there were 11%, 31%, and 58% of the nonsulfated, 4-sulfated, and 6-sulfated disaccharides, respectively, in the Gu-D1 preparation. The distribution of radioactivity in the 4- and 6-sulfated disaccharides was about the same, Table III.

aminoacylglucosamine chains. The elution profile observed when the digest was chromatographed on Sepharose 6B is shown in Fig. 5c. The hexuronic acid analyses revealed a single, symmetrical peak whereas the radioactivity exhibited a slight shoulder near an elution volume of 60 ml. The major peak shows the elution profile of the chondroitin sulfate chains. The shoulder indicates that keratan sulfate chains were possibly present in the digest since they would be smaller than the chondroitin sulfate chains and would not contain hexuronic acid (42-44). The elution pattern of the chondroitin sulfate, with a Kᵥ of 0.51, is similar to those observed for chondroitin sulfate isolated by papain digestion of proteoglycans isolated from bovine nasal septum (Kᵥ = 0.55) and the Swarm rat chondrosarcoma (Kᵥ = 0.48) on the same column (26). Thus the average molecular weight for the chondroitin sulfate chains in the proteoglycan preparation isolated from the cultured chick limb bud chondrocytes would be about 20,000 (45). Another aliquot of the papain digest from Gu-D1 was fractionated on cetylpyridinium chloride cellulose columns (35). The fraction eluted with 1% cetylpyridinium chloride contained essentially all the glucosamine present in the digest which indicates that the glucosamine in the original preparation was present primarily either in keratan sulfate or glycopeptides (35, 46) and not in hyaluronic acid or heparan sulfate. As was shown previously (8), the bulk of the material was recovered in the 0.75 M MgCl₂ fraction which is typical for chondroitin sulfate (35).
chains, expected around 250 to 280 nm (Fig. 4), also does not have measurable CD in this region (Fig. 3).

The CD spectrum of BBI in 6 M guanidine hydrochloride indicates that there is a considerable alteration in disulfide CD (Fig. 8). However, the CD profile of fully acetylated BBI differ only slightly from that of native BBI above 280 and from 255 to 240 nm where only cystinyls have CD in either protein (Fig. 3A). Apparently, on removing guanidine hydrochloride, disulfide bonds reverted to their native configuration and very little, if any, conformational change was induced by the O-acetylation of tyrosyl side chains in BBI. The virtual identity of the far-ultraviolet CD between the native and fully acetylated BBI (Fig. 9) is also consistent with this observation.

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REFERENCES