Circular Dichroism of Stem Bromelain*

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ABSTRACT: Studies of circular dichroism are reported on stem bromelain in the spectral region between 210 and 320 mμ.

At neutral pH values the observed circular dichroism spectrum exhibits a negative band maximum at 300 mμ and a broad positive band at 280 mμ. In the far ultraviolet, two negative circular dichroism bands are observed at 214 and 222 mμ with molecular ellipticity values of −4200 and −3900 deg $dmol^{-1}$ cm^2, respectively. The circular dichroism spectra above 260 mμ undergo large changes in 50% 2-chloroethanol and in 7.2 M urea at pH 2.8, whereas the spectra change to a much smaller extent in 80% ethylene glycol. In alkaline media, the circular dichroism spectra above 240 mμ change gradually as the pH value increases. The appearance of a positive band at 250 mμ is characteristic in alkaline media and the increase in molecular ellipticity shows a fairly linear relationship to the number of tyrosyl residues per mole of stem bromelain at given pH values. The data on the far-ultraviolet circular dichroism spectra at neutral and alkaline pH values, together with the data on the optical rotatory dispersion spectra (Murachi, T., and Yamazaki, M. (1970), *Biochemistry 9*, 1935), suggest the simultaneous presence of α helix, β structure, and the less ordered structure in a molecule of native stem bromelain.

The circular dichroism spectra in the ultraviolet region have recently received increasing attention in studying the fine structure of polypeptides and proteins (see, for example, Beychok, 1967). This paper describes circular dichroism spectra of stem bromelain, a proteolytic enzyme from pineapple tissue, in aqueous media at neutral and alkaline pH values, and in 80% ethylene glycol, 50% 2-chloroethanol, and 7.2 M urea. The measurements were made over the wavelength range from 210 to 320 mμ.

Tachibana and Murachi (1966) studied the ionization of tyrosine hydroxyl groups of stem bromelain in alkaline media, and obtained indirect evidence for conformational changes occurring at pH values higher than 10.3. More direct evidence for this has become available from recent experiments on optical rotatory dispersion, viscosity, and ultracentrifugal sedimentation at varying pH values (Murachi and Yamazaki, 1970). In correlation with these studies, emphasis has been made, in the present experiments, upon the change in circular dichroism spectra that accompanies phenolic hydroxyl ionization occurring with and without concomitant change in conformation.

Materials and Methods

Stem Bromelain. The purified enzyme (fraction 6) was prepared as previously described (Murachi *et al.*, 1964).[1] The protein concentration was determined by measuring the absorbance at 280 mμ, using a molar absorbancy of 6.68×10^4 (Murachi *et al.*, 1965).

Optical Measurements. Optical rotatory dispersion and circular dichroism measurements were performed with a Jasco Model ORD–UV-5 spectropolarimeter equipped with a circular dichroism attachment. A 10-mm quartz cell was used for optical rotatory dispersion measurements between 230 and 290 mμ and for circular dichroism spectra between 240 and 320 mμ both with $0.2–0.3 \times 10^{-4}$ M stem bromelain. For circular dichroism spectra between 210 and 240 mμ a 0.1-mm cell was used with 1.35×10^{-4} M stem bromelain. The scale setting across the 20-cm chart paper for optical rotatory dispersion was 0.1°. Temperature was close to 20°.

Numerical Conversion of Data. The rotations measured were converted to values of the mean residue rotation, $[m']$,

$$[m'] = \frac{3}{n^2 + 2} \times \frac{M_0}{100}[\alpha]$$

where $[\alpha]$ is the specific rotation at a given wavelength, M_0 is the mean residue molecular weight which was assumed to be 110 (Murachi and Yamazaki, 1970), and n is the refractive index of the solvent which was taken from compilation by Fasman (1963). Instrument performance of the circular dichroism attachment was checked by reproducing reported circular dichroic spectra of camphor and poly-α-L-glutamic acid. Molecular ellipticity, $[\theta]$, was obtained by using $[\theta] = 3300(\epsilon_L - \epsilon_R)$, where $\epsilon_L - \epsilon_R$ is the difference between the molar extinction coefficients for the left and right circularly polarized light. In the calculation of $(\epsilon_L - \epsilon_R)$, average residue weight was used instead of molecular weight. The units of $[\theta]$ are degrees $dmole^{-1}$ cm^2.

Others. The pH of solutions was measured at 20° with a Radiometer Model 4 pH meter. 2-Chloroethanol was purified by distillation. Since some hydrochloride impurities could not be completely removed by distillation (Hamaguchi

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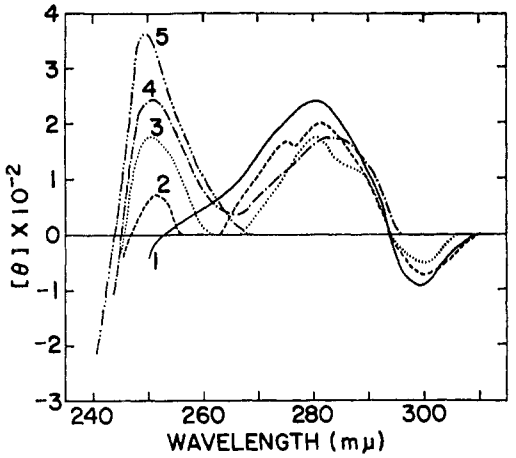

FIGURE 1: Circular dichroism spectra of stem bromelain from 240 to 320 mμ in aqueous media. Curves 1, at pH 7.0; 2, pH 9.6; 3, pH 10.2; 4, pH 11.0; 5, pH 12.8.

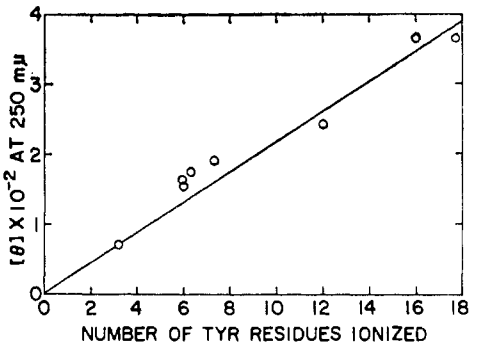

FIGURE 2: Correlation of positive ellipticity in circular dichroism spectra of stem bromelain at 250 mμ to the number of tyrosyl residues at alkaline pH values.

and Kurono, 1963), the apparent pH of the aqueous 2-chloroethanol solution of stem bromelain varied from 3.6 to 2.0 depending upon the concentrations of 2-chloroethanol from 8 to 70%. Ethylene glycol was purified by distillation. Concentrations of organic solvents used are described in per cent (v/v). Urea was recrystallized from 70% ethanol.

Results

Circular Dichroism Spectra in the 240–320-mμ Region. The enzyme protein was dissolved in a solution of 0.5 ionic strength at desired pH values as previously described (Tachibana and Murachi, 1966). The sample solutions were allowed to stand after preparation at 20° for 2 hr to attain the ionization equilibrium at that pH value. The circular dichroism spectra were recorded between 240 and 320 mμ at varying pH values. Figure 1 illustrates some of the representative results obtained. The spectrum at pH 7.0 exhibits a negative band maximum at 300 mμ and a broad positive band at 280 mμ. The diphasic character of the circular dichroism spectrum has some analogy to the circular dichroism pattern of chymotrypsin or chymotrypsinogen (Fasman *et al.*, 1966), but is quite different from circular dichroism patterns of hen egg-white lysozyme (Ikeda *et al.*, 1967) and carbonic anhydrases B and C (Beychok *et al.*, 1966). The spectrum at pH 8.20 (not shown in the figure) was essentially the same as that at pH 7.0. As the pH further increases, the negative band at 300 mμ becomes shallower and disappears at pH 11.0. The positive ellipticity in the 270–290-mμ region persists up to pH 11.0, but it finally disappears at pH 12.8. The most striking feature of the changes in circular dichroism spectra at alkaline pH values is that a positive band with a maximum at 250 mμ appears and markedly increases with pH. The observation on stem bromelain well agrees with what we have observed on egg-white lysozymes of hen and duck (Ikeda *et al.*, 1967; Ikeda and Hamaguchi, 1969) and on trypsin inhibitors (Baba *et al.*, 1969).

The spectrophotometric titration data for stem bromelain reported in the previous communication (Tachibana and Murachi, 1966) provide precise information concerning the number of ionized tyrosyl residues per mole of protein at any given pH value. When the $[\theta]$ values for circular dichroism bands at 250 mμ obtained at various pH values are plotted against the number of ionized tyrosyl residues, a fairly linear relationship is noted as shown in Figure 2. The same type of linear relationship has been obtained with lysozymes (Ikeda *et al.*, 1967; Ikeda and Hamaguchi, 1969). These observations clearly indicate that the positive circular dichroism band at 250 mμ is due to the ionized tyrosyl residues of the molecule.

Effect of Ethylene Glycol, 2-Chloroethanol, and Urea on Optical Rotatory Dispersion and Circular Dichroism Spectra. An aqueous solution of stem bromelain was mixed with a given amount of ethylene glycol, 2-chloroethanol, or 8 M urea in 0.1 N HCl. The mixture was allowed to stand at least 2 hr at 20° prior to optical measurements. The addition of acidic urea solution to make a final concentration of 7.2 M gave an apparent pH value of 2.8, causing a considerable degree of denaturation of enzyme protein.

Figure 3 shows the effects of ethylene glycol, 2-chloroethanol, and urea on circular dichroism spectra. In the presence of 80% ethylene glycol the circular dichroism spectrum in the 295–310-mμ region is almost unchanged, while decrease and splitting of the positive band are observed at wavelengths shorter than 295 mμ. The latter changes, however, are much less pronounced as compared to the change of hen egg-white lysozyme. Lysozyme was found to undergo an increase of circular dichroism band at 288 mμ in the presence of ethylene glycol (Glazer and Simmons, 1966; Ikeda and Hamaguchi, 1969); 50% 2-chloroethanol gives a circular dichroism spectrum entirely different from that of the native enzyme. As shown in Figure 3, in 2-chloroethanol and also in 7.2 M urea at pH 2.8 the band near 300 mμ disappears while the negative ellipticity with a maximum near 274 mμ appears. However, when the optical rotatory dispersion patterns were compared, these two solvents were found to give quite different effects. Thus, as shown in Figure 4, the optical rotatory dispersion in 7.2 M urea at pH 2.8 represents a simple dispersion in this region without having a trough at 233 mμ, whereas the pattern in 50% 2-chloroethanol shows a typical trough at 233 mμ with $[m'] = -5500°$. The $[m']_{233\,m\mu}$ in 7.2 M urea is $-2300°$, a value close to that for a randomly coiled polypeptide chain. Since the native stem bromelain has an $[m']_{233\,m\mu}$ value of

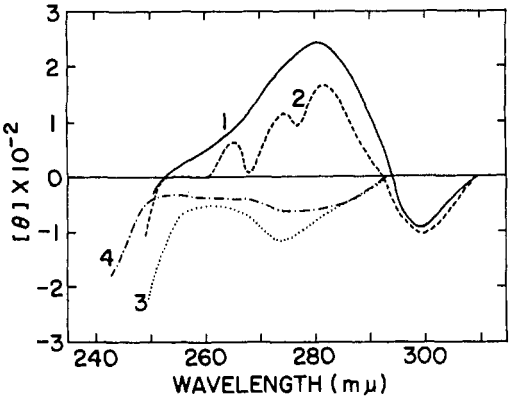

FIGURE 3: Effects of organic solvents and urea on the circular dichroism spectrum of stem bromelain from 240 to 320 mμ. Curves 1, in aqueous medium at pH 7.0 (control); 2, in 80% ethylene glycol; 3, in 50% 2-chloroethanol; 4, in 7.2 M urea at pH 2.8.

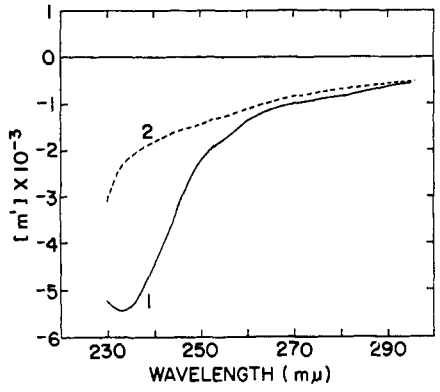

FIGURE 4: Effects of organic solvent and urea on the optical rotatory dispersion spectrum of stem bromelain from 230 to 300 mμ. Curves 1, in 50% 2-chloroethanol; 2, in 7.2 M urea at pH 2.8.

−3450° (Murachi and Yamazaki, 1970), the value obtained in 50% 2-chloroethanol implies a considerable increase in helical content.

Spectra in the 210–240-mμ Region. As shown in Figure 5, the circular dichroism spectrum at pH 6.1 has two negative maxima at 214 and 222 mμ with [θ] values of −4200 and −3900, respectively. When the pH of the solution is brought to 12.7 the circular dichroism band near 222 mμ disappears, while that near 214 mμ remains almost unchanged.

Discussion

Earlier studies on the phenolic hydroxyl ionization in stem bromelain indicated that 8–9 out of the total 19 tyrosyl residues/mole of protein are in the "exposed" state while the rest are "buried" inside the molecule (Tachibana and Murachi, 1966). It was suggested that those "buried" tyrosyl residues become accessible to the solvent only after the enzyme protein has undergone a conformational change by being exposed to alkali of pH values higher than 10.3. The occurrence of such conformational changes in alkaline media has been proved more directly by measuring optical rotatory dispersion parameters, viscosity, and sedimentation coefficients (Murachi and Yamazaki, 1970). The data have indicated that the native conformation persists up to about pH 10.3 while further increase in pH gives rise to irreversible changes in conformation. On the other hand, the results of the present experiments show that a positive circular dichroism band near 250 mμ appears as the pH of the medium increases, and that the increment of the [θ] value is almost linearly proportional to the number of the ionized tyrosyl residues per mole of protein over a wide range of pH values, 7.0–13.3 (Figures 1 and 2). These earlier and present observations together suggest that the positive circular dichroism band near 250 mμ is due to the ionized tyrosyl residues but less directly reflects the conformational change of the molecule.

The presence of two negative circular dichroism bands at wavelengths shorter than 230 mμ has been known in a number of proteins including myoglobin, lysozyme, and ribonuclease (Greenfield and Fasman, 1969). The bands at 208 and 222 mμ are attributed to α helix. It must be noted that at pH 6.1 stem bromelain also gives two negative circular dichroism bands in this wavelength region, but one of these bands is at 214 mμ, a wavelength significantly longer than the usual value, 208 mμ (Figure 5). This discrepancy has made it more impractical to estimate the conformation of stem bromelain from the computed curves based on reference conformations of poly-L-lysine (Greenfield and Fasman, 1969). Stem bromelain contains two *N*-acetylglucosamine residues per mole which constitute the proximal part of the heterooligosaccharide prosthetic group of the molecule (Yasuda *et al.*, 1970). It has been reported that some oligosaccharides containing *N*-acetylglucosamine show negative circular dichroism bands at wavelengths near 214 mμ (Kabat *et al.*, 1969). Further investigations will be needed to determine whether the band at 214 mμ, instead of at 208 mμ, for stem bromelain reflects such effects of the carbohydrate moiety or other, yet unclarified, effects of the side-chain chromophores and disulfide bridges (Greenfield and Fasman, 1969).

The $[\theta]_{222\ m\mu}$ value obtained for stem bromelain (Figure 5) is far less than the values reported for 100% helical polyamino acids: 39,800 for poly-L-lysine (Townend *et al.*, 1966) and −38,000 to −39,000 for poly-L-glutamic acid (Cassim and Yang, 1967). This is in accord with a shallow optical rotatory dispersion trough at 233 mμ of the same sample (Murachi and Yamazaki, 1970) and also with the

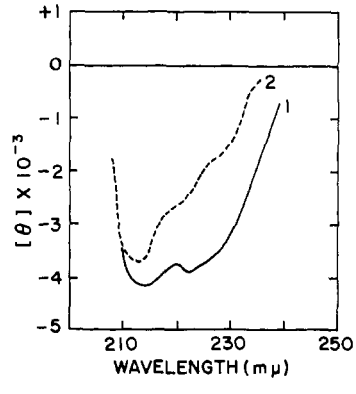

FIGURE 5: Circular dichroism spectra of stem bromelain from 210 to 240 mμ. Curves 1, at pH 6.13; 2, pH 12.67.

earlier report that helical content of stem bromelain was calculated from the Moffitt parameter b_0 to be 13% (Murachi *et al.*, 1964). The question then arises as to the nature of the conformation for the remaining, nonhelical part of the molecule. If one simply assumes that the nonhelical part is of randomly coiled conformation, the resultant circular dichroism spectrum would have a deeply negative band at 202 mμ with much shallower band at 222 mμ, since it was reported that randomly coiled poly-L-glutamate shows practically no ellipticity at 222 mμ while the $[\theta]$ value at 202 mμ is as low as $-50{,}000$ (Holzwarth and Doty, 1965; Iizuka and Yang, 1966). Such a pattern would be entirely different from the observed circular dichroism spectrum that have two bands of comparative magnitude near 222 and 214 mμ. One must then assume the presence in a molecule of stem bromelain of some structure other than α helix and random coil. The most probable one would be the β structure. The presence of the latter in place of a part of random coil would effectively diminish the band at 202 mμ, since the circular dichroism spectrum of β-conformed poly-L-lysine was reported to show a negative band at 217 mμ and a positive ellipticity at wavelengths shorter than 207 mμ (Townend *et al.*, 1966). The presence of β structure has been demonstrated in Bence-Jones proteins (Ikeda *et al.*, 1968), immunoglobulins (Sarker and Doty, 1966), and β-lactoglobulin (Townend *et al.*, 1967). The circular dichroism spectrum at pH 12.7 (Figure 5) can be similarly explained in terms that only α helix has been disrupted by alkali while β structure remains. This explanation is consistent with the finding described in the preceding communication (Murachi and Yamazaki, 1970) that at pH values higher than 13 the Moffitt parameter b_0 for stem bromelain is 0° while a_0 is $-350°$. The fact that the obtained a_0 value is much higher than $a_0 = -600°$ for a completely random coil structure may indicate the presence of undisrupted β structure, since the β structure would have made a positive contribution to a_0 value. It may be clear from the foregoing discussion that all the data so far available are compatible with the simultaneous presence of α helix, β structure, and the less ordered structure in a molecule of native stem bromelain. However, the information is still insufficient to assign each of these structures an exact size of fraction of the whole molecule.

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