Changes in Conformation and Enzymatic Activity of Stem Bromelain in Alkaline Media*

Takashi Murachi and Mitsue Yamazaki

ABSTRACT: The changes in conformation of stem bromelain in alkaline media have been studied by measuring optical rotatory dispersion parameters, viscosity, and sedimentation coefficient as functions of pH values. The enzymatic activity on Bz-L-ArgEt of the alkali-treated stem bromelain has also been examined. In the range pH 7–10, no significant change in physical parameters is observed. When the pH is increased from 10 to 12, optical rotatory dispersion parameters and viscosity undergo large changes with concomitant loss of the enzymatic activity. In the pH range between 12 and 13, optical rotatory dispersion parameters show small but significant changes with the pH while both viscosity and sedimentation coefficient change markedly. These results clearly indicate that no substantial change in conformation occurs up to pH 10 and beyond that pH the conformational change proceeds through at least two, mutually distinguishable, stages. This is in excellent agreement with what has previously been predicted to occur in stem bromelain from the observed abnormality of phenolic hydroxyl ionization with increasing pH values.

Earlier communications from this laboratory have shown that stem bromelain is a basic glycoprotein of mol wt 33,000 (Murachi, 1964; Murachi *et al.*, 1964, 1967). It contains 19 tyrosyl residues/mole of protein. A spectrophotometric titration study indicated that 9 out of the total 19 residues are in the "exposed" state while the rest are "buried" inside the molecule (Tachibana and Murachi, 1966). From the time-dependent features of ionization of these buried tyrosine hydroxyl groups at alkaline pH values and also from the change in reactivity of tyrosyl residues against cyanuric fluoride with pH, it was suggested that conformational change occurs when stem bromelain was exposed for hours to pH values higher than 10.3. At least two, probably three, different stages of conformational change could be distinguished over the pH range between 10 and 13.5. The presence of a denaturing agent like 4 M guanidine hydrochloride abolished such abnormal ionization behavior of phenolic groups, providing additional indirect evidence for the importance of secondary and tertiary structure of the protein in determining accessibility to the medium of ionizable groups of its amino acid residues.

The present investigation was undertaken to validate these earlier pieces of evidence by making different kinds of physical measurements on stem bromelain solutions at varying alkaline pH values. The choice of the measurements was made so that the data would give more direct evidence for conformational change occurring in alkaline media. Four different parameters in optical rotatory dispersion spectra were determined in addition to measurements of viscosity and sedimentation in ultracentrifuge. The data obtained were compared with each other and also with the change in enzymatic activity at each pH value studied. The circular dichroic spectra of stem bromelain were also studied in connection with the present investigation, the results of which will be described in an accompanying communication (Sakai *et al.*, 1970).

Materials and Methods

Stem Bromelain. The enzyme preparation used was isolated from the crude "Bromelain" (lot 184) from the Dole Corp., Honolulu, Hawaii,[1] according to the method of Murachi *et al.* (1964). Fraction 6 was employed for the present experiment. The molecular weight of the enzyme protein was assumed to be 33,000 (Murachi *et al.*, 1964). 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). 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 pH was measured on a Hitachi Model M4 pH meter. The sample solutions were allowed to stand after preparation at 25° for 3 hr to attain the ionization equilibrium at that pH value, and then used for the physical measurements and the enzyme assay.

Enzyme Assay. The hydrolysis of Bz-L-ArgEt was followed by titration with a Radiometer Model SBR2-SBU1-TTT1 Autotitrator at 25° and at pH 6.0 (Inagami and Murachi, 1963). The reaction mixture contained 0.005 M L-cysteine, 0.1 M KCl, 0.01 M Bz-L-ArgEt, and approximately 0.5×10^{-4} M stem bromelain in a total volume of 10 ml. The enzyme solution, which had been kept at a given pH value at 25° for 3 hr, was adjusted to pH 6.0 with 1 N acetic acid immediately before the assay. The latter pH adjustment caused

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[1] We are indebted to Dr. Ralph M. Heinicke for a generous supply of this material.

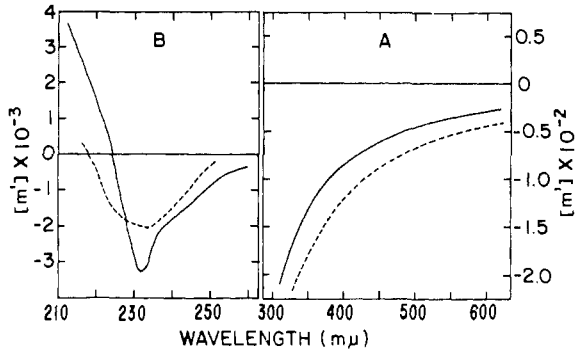

FIGURE 1: Optical rotatory dispersion spectra of stem bromelain. In part A, at pH 7.29 (solid line) and pH 12.59 (broken line). In part B, at pH 6.13 (solid line) and pH 12.67 (broken line).

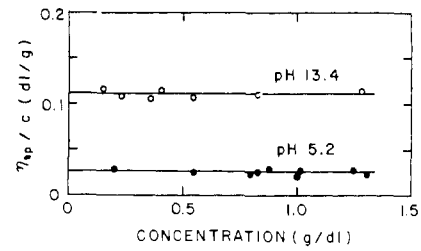

FIGURE 2: Reduced viscosity of stem bromelain as a function of protein concentration. The data at pH 5.2 are taken from Murachi *et al.* (1964).

turbidity when the enzyme protein had been exposed to higher pH values, but the assay was carried out without removing the insoluble protein. The activity was expressed in per cent, taking the specific activity of the native enzyme as 100%.

Optical Rotatory Dispersion Measurement. The measurements were made on a Jasco Model ORD-UV-5 spectropolarimeter. For the 300–600-mμ region a 5-cm cylindrical cell with quartz windows was used with 1.2–1.4 × 10^{-4} M stem bromelain, the scale setting across the 20-cm chart paper being ±0.5–1.0°. For the 225–260-mμ region a 1-cm quartz cell was used with 4.4–4.6 × 10^{-6} M stem bromelain, the scale setting being ±0.1°. In a few instances a 0.1-mm cell was used with 1.35 × 10^{-4} M stem bromelain; this permitted reliable measurements down to 210 mμ.[2] The temperature was close to 25°. The rotations measured were converted into values of the mean residue rotation, [*m'*], by the equation (Moffitt and Yang, 1956)

$$[m'] = \frac{3}{n^2+2} \times \frac{M_0}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (1)$$

where [α] is the specific rotation at a given wavelength, λ, M_0 is the mean residue molecular weight, n is the refractive index of the solvent, λ_0 is constant, and a_0 and b_0 are the parameters. The M_0 value for stem bromelain was assumed to be 110. This value was used in the previous report (Murachi *et al.*, 1964) and permitted direct comparison between present and previous data. A value of M_0 = 116 would be obtained if the molecular weight, 33,000 (Murachi *et al.*, 1965), were divided by the number of amino acid residues per mole, 285 (Murachi, 1964). The discrepancy between these two values is due to the fact that stem bromelain is a glycoprotein containing two *N*-acetylglucosamine residues and 2.1% neutral sugar per mole (Murachi, 1964; Yasuda *et al.*, 1970). An n value of 1.34 was used, since most of 0.5 ionic strength of sample solutions was contributed by KCl (Tachibana and Murachi, 1966). No correction in refractive index was made for different wavelengths. λ_0 was assumed to be 212 mμ (Urnes and Doty, 1961).

The optical rotatory dispersion patterns in the 300–600-mμ region were evaluated in terms of the parameters a_0 and b_0 of eq 1. The rotatory dispersion constant, λ_c, was also calculated from the slope of a plot of [α] *vs.* [α]λ^2 (Yang and Doty, 1957). The data in the 210–260-mμ region were mainly compared in terms of [*m'*] values at 233 mμ where the optical rotatory dispersion pattern for the native stem bromelain showed a negative maximum (see below).

Viscosity. The measurements were made with an Ostwald viscometer with an outflow time of 140 sec with 7 ml of water at 25°. The protein concentration used was 1.2–1.4 × 10^{-4} M. A parallel stem pycnometer of approximately 3-ml capacity was used for density measurements.

Sedimentation in Ultracentrifuge. The analyses were performed with a Hitachi Model UCA-1 ultracentrifuge. All runs were made at 20–25°, and the obtained sedimentation coefficients were converted into s_{20} in Sevedberg units. The protein concentration used was approximately 2 × 10^{-4} M. An alkali-resistant cell of conventional size was used for runs at high pH values.

Results

Optical Rotatory Dispersion in the 300–600-mμ Region. The rotatory dispersion patterns illustrated in Figure 1A are tracings of the representative data at neutral and alkaline pH values. As with most proteins, the levo rotation of stem bromelain in this wavelength region increases in alkaline media. The rotatory dispersion constant, λ_c, as calculated from the pattern shown in Figure 1A is 241 mμ at pH 7.29, a value identical with the value reported at pH 7.4 (Murachi *et al.*, 1964). A λ_c value of 212 mμ is obtained from the pattern in Figure 1A at pH 12.59.

Optical Rotatory Dispersion in the 210–260-mμ Region. Figure 1B shows tracings of the data obtained at two different pH values. The dispersion curve at pH 6.13, obtained with 0.1-mm cell, is similar to the reported optical rotatory dispersion patterns of various globular proteins, *e.g.*, hen egg-white lysozyme and myoglobin (Greenfield *et al.*, 1967). The trough is at 233 mμ, [*m'*] = −3450. The dispersion curve at pH 7.12, obtained with a 10-mm cell, is identical with the one at pH 6.13 shown in the figure over the wavelength region 225–260 mμ. As the pH increases, however, the trough becomes shallower and broader. The optical rotatory dispersion spectrum of the native stem bromelain has a crossover point at 224 mμ, while the alkali-denatured bromelain shows negative [*m'*] values at wavelengths shorter than 218 mμ.

Viscosity. The viscosity measurements were carried out at various protein concentrations with alkali-denatured

[2] These measurements were performed by Dr. Kozo Hamaguchi.

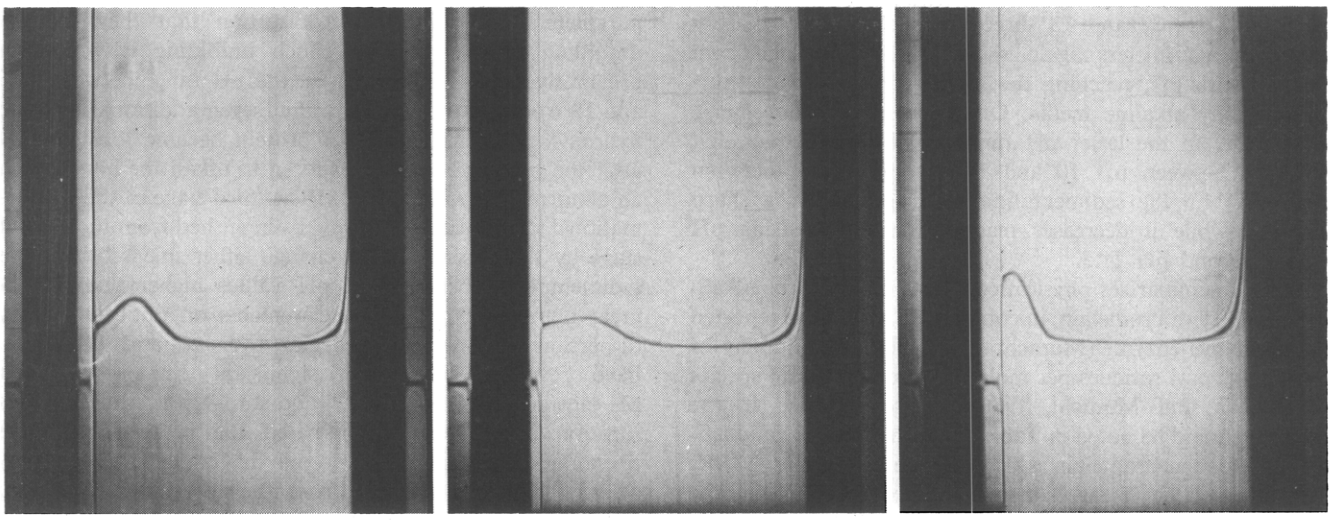

FIGURE 3: Sedimentation velocity pattern of stem bromelain at pH 9.87 (A), pH 11.65 (B), and pH 13.3 (C). In each case, the exposure shown was made 50 min after the top speed was reached.

stem bromelain. The results obtained are shown in Figure 2 in comparison with the earlier data obtained at pH 5.2 (Murachi *et al.*, 1964). There is practically no concentration dependence of the reduced viscosity, η_{sp}/c, at pH 13.40 as at pH 5.2. Based on this observation, the viscosity measurement at each pH value between 7.12 and 13.40 was made only at one single concentration of the protein, the reduced viscosity calculated, and the value regarded as having a physical significance equivalent to that of the intrinsic viscosity of the sample protein.

Sedimentation in Ultracentrifuge. Figure 3 shows sedimentation patterns of native and alkali-denatured stem bromelains. The broad peak in the 60-min picture at pH 11.65 may indicate polydispersity of the solute. However, no indication was obtained at any alkaline pH values studied for the occurrence of oligomers that gave distinct splits in the Schlieren patterns as was reported by Smith *et al.* (1954) for crystalline papain in alkaline media.

Variation of Physical Parameters and Enzymatic Activity at Alkaline pH Values. In Figure 4 are summarized the data obtained at various pH values. All of the physical parameters studied remain practically unchanged when the pH is increased from 7 to 10, and this is paralleled with only slight decrease in enzymatic activity within that pH range. The parallelism is also observed in the range pH 10–12 where optical rotatory dispersion parameters and viscosity undergo large changes with concomitant loss of the enzymatic activity. In the pH

TABLE I: Physical Properties of Native and Alkali-Denatured Stem Bromelain.[a]

Property	Native	Denatured
"Exposed" Tyr (moles/mole)	8–9[b]	19[b]
Optical rotatory dispersion		
a_0 (deg)	−190[c]	−350
b_0 (deg)	−78[c]	0
λ_c (mμ)	241[c]	212
$[m']_{233\ m\mu}$ (deg)	−3450	−1800
Intrinsic viscosity		
$[\eta]$ (dl/g)	0.039[c]	0.11
Sedimentation coefficient		
s_{20} (S)	2.73[c]	1.03

[a] Native enzyme refers to the enzyme in solution between pH 5.2 and 7.4. Alkali-denatured enzyme refers to the enzyme that has been allowed to stand in 0.5 N KOH at 25° for 3 hr. [b] From Tachibana and Murachi (1966). [c] From Murachi *et al.* (1964).

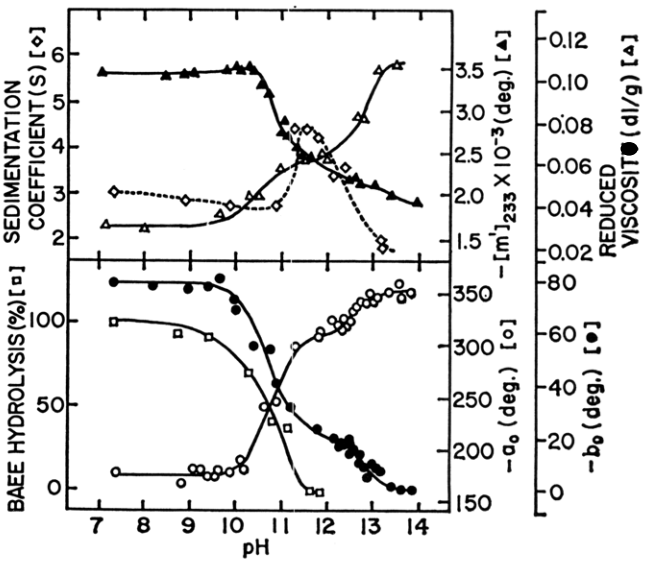

FIGURE 4: Changes in physical parameters and enzymatic activity of stem bromelain in alkaline media. Experimental details are described in text. BAEE is Bz-L-ArgEt.

range between 12 and 13 three kinds of optical rotatory dispersion parameters again show smaller but significant changes with pH, reaching the highest or the lowest value in extremely alkaline media. On the contrary, the change in viscosity in the latter pH range is still larger than that observed between pH 10 and 12. At pH values between 10.8 and 11.5 the sedimentation coefficient shows a sharp increase, while it decreases markedly with increasing pH values beyond pH 11.5.

Table I summarizes physical constants obtained for alkali-denatured stem bromelain, in comparison with those reported for the native enzyme (Murachi *et al.*, 1964). The number of exposed tyrosyl residues per molecule of each protein species (Tachibana and Murachi, 1966) is also included in the table. It should be noted in Table I that the b_0 value of alkali-denatured stem bromelain is 0° while the a_0 value is −350°. The latter value is far higher than the reported a_0 value of approximately −600° for most of the completely denatured proteins. The implication will be discussed in conjunction with the results of circular dichroism measurements in an accompanying communication (Sakai *et al.*, 1970).

Discussion

The principal objective of the present investigation was to see whether various physical measurements on stem bromelain molecule in alkaline media could indicate the occurrence of conformational changes of the protein, which had been predicted in an earlier communication (Tachibana and Murachi, 1966). Our particular interest was focused on whether these physical measurements could provide data which should agree with the prediction that the conformational changes were to occur only at pH values higher than 10.3 with such characteristic features as being time dependent and proceeding through a few more stages that were distinguishable from one another. The data actually obtained have shown that our objective was satisfactorily attained. The data in Figure 4 clearly indicate that stem bromelain molecule, when exposed to increasing alkalinity, exhibits conformational response through at least three different stages which are represented by the pH profile of any one of the physical parameters shown in the figure.

Thus, in the first stage, *i.e.*, from pH 7 to 10, the protein conformation must remain almost unchanged, since no significant change in physical parameters is detected in this pH region. The earlier titration data showed that nine tyrosine hydroxyl groups per mole of protein were accessible to the medium in this pH range, the ionization being nontime dependent, reversible, and with a normal pK value of 9.84 (Tachibana and Murachi, 1966). In the second stage, *i.e.*, from pH 10 to 12, a large change in conformation is indicated by the observed values for optical rotatory dispersion parameters as well as viscosity. The changes in these physical parameters occur in such a direction that they indicate unfolding of the molecule. Such unfolding is what was previously suggested to occur in the pH range between 10.3 and 12 from the titration data that seven additional tyrosine hydroxyl groups per mole of protein became ionized only after the protein had been exposed to alkali for hours, with an abnormal pK value of 11.8. The third stage of the conformational change can be readily distinguished from the second stage by the observed large change either in viscosity or in sedimentation coefficient at pH values higher than 12. A great number of careful measurements on optical rotatory dispersion parameters, particularly on a_0 and b_0 values, have revealed small but significant humps in their pH profiles between pH 12 and 13. Again, the previous titration experiment demonstrated the presence of three phenolic groups per mole of protein that could be titrated only at very high pH values. From the foregoing discussion, it is apparent that stem bromelain, when exposed to alkali, loosens gradually the internal folding of the molecule between pH 10 and 12, and undergoes further change to an extensively denatured form above pH 12. An excellent agreement of the present findings with what were previously predicted is quite evident.

Acknowledgment

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