Phenolic Hydroxyl Ionization in Stem Bromelain*

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ABSTRACT: The ionization of the phenolic groups of stem bromelain has been studied by spectrophotometric titrations at 295 and 244 mμ in the range between pH 7 and 13.6. Of 19 tyrosyl residues/mole of protein, nine are in the "exposed" state while the rest are "buried" in the interior of the native molecule. The ionization of the "exposed" tyrosyl residues is reversible with a pK_{app} of 9.84 and it shows no time dependence. The "buried" tyrosyl residues become accessible to the solvent only at pH values higher than 10.3 and the process of ionization is time dependent. From the shape of a forward titration curve, seven "buried" tyrosyl residues with a pK_{app} of 11.8–11.9, one with a pK_{app} greater than 12, and one with a pK_{app} greater than 13 can be distinguished. The abnormal ionization of these groups is evidently dependent upon the unfolding of the protein molecule at alkaline pH values, since a normalized titration curve with a pK_{app} of 10.07 is obtained in the presence of 4 M guanidine hydrochloride. The occurrence of two types of tyrosyl residues, "exposed" and "buried," has also been demonstrated by studying the reactivity of stem bromelain with cyanuric fluoride as a function of pH. The number of the "exposed" residues that are reactive with cyanuric fluoride at pH values between 8.8 and 10.2 is 7–8/mole of protein, a result consistent with that of the titration experiment. The ratio of tyrosyl to tryptophyl residues in stem bromelain has been determined spectrophotometrically and is in good agreement with that obtained by amino acid analysis.

Earlier communications from this laboratory described the purification and physical characterization of stem bromelain (Murachi *et al.*, 1964), the amino acid composition of the purified enzyme (Murachi, 1964), and some of the kinetic studies (Inagami and Murachi, 1963). The present investigation was undertaken to obtain information concerning the three-dimensional structure of this enzyme protein by studying the behavior of the phenolic hydroxyl groups in the molecule.

The amino acid analysis (Murachi, 1964) has shown that stem bromelain contains 19 tyrosyl residues/mole. It is inconceivable that all of the 19 tyrosyl residues are in the "exposed" state, being located on the outside surface of the protein molecule and freely accessible to the solvent. Some of them must be exposed, while others are in the "buried" state, being located in the interior of the molecule and capable of being ionized only slowly or after denaturation. The occurrence of at least two different types of tyrosyl residues, "exposed" and "buried,"[1] has been recognized for years with various proteins, and can be studied either by means of spectrophotometric titration over a wide range of pH or by means of applying chemical modification specific for these groups.

The spectrophotometric procedures are based on the fact that the pH difference spectrum for the ionization of tyrosine has two maxima, one at 295 mμ, the other at 242 mμ (Hermans, 1962). The measurement in the 295-mμ region was developed by Crammer and Neuberger (1943) and successfully applied to various proteins such as pancreatic ribonuclease (Tanford *et al.*, 1955), egg white lysozyme (Tanford and Wagner, 1954; Inada, 1961), trypsinogen (Smillie and Kay, 1961), and papain (Glazer and Smith, 1961). The measurement in the 245-mμ region was recommended by Hermans (1962) for studying normal and abnormal tyrosine side chains in various heme proteins, and also used by Donovan (1964) for the titration of the sulfhydryl and phenolic groups of aldolase. In the present investigation the pH difference spectrum for stem bromelain was measured both in the 295- and 245-mμ regions.

In order to correlate the spectrophotometric data obtained to the chemical reactivity of the tyrosyl residues, we have further investigated the reaction of cyanuric fluoride with stem bromelain. The usefulness of cyanuric fluoride as a modifying agent for tyrosyl residues of a protein was first demonstrated by Kurihara *et al.* (1963). These authors found that the absorption in the 295-mμ region of tyrosine at alkaline pH was lowered to practically zero after the treatment with

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[1] There are various terms in the literature to describe the types of tyrosyl residues of a protein molecule, *e.g.*, normal and abnormal (Hermans, 1962), free and bound (Inada, 1961), unburied and buried (Donovan, 1964), and embedded (Tanford *et al.*, 1955). In the present communication uses will be made of "exposed" and "buried" with respective implications as explained above.

cyanuric fluoride. By the use of this phenomenon they studied the reactivity with cyanuric fluoride of tyrosyl residues of insulin and lysozyme.

Materials and Methods

Stem Bromelain. The enzyme preparation used was isolated from the crude "Bromelain" (lot 184) from the Hawaiian Pineapple Co., Honolulu, Hawaii,[2] 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.*, 1964).

Cyanuric Fluoride. Cyanuric fluoride, $(CNF)_3$, was prepared from cyanuric chloride by the method of Maxwell *et al.* (1958).[3] The product was purified by redistillation twice; bp 72–74°.

Spectral Observation. Measurements were made on a Hitachi-Perkin-Elmer Model UV-VIS-139 spectrophotometer; 10-mm quartz cells were used. The temperature was 25 ± 0.5°. The protein solutions of a given alkaline pH and of pH 7.0 were compared for the spectral changes in the region 230–320 mμ. The titration of phenolic hydroxyl groups was carried out either at 295 or 244 mμ by directly measuring the difference in absorbance between the alkaline and the neutral solutions. At zero time, 2 ml of an aqueous solution of the protein was mixed with 2 ml of a buffer solution: 0.02 M Tris buffer containing 1.0 M KCl for pH 7–9, 0.02 M carbonate–bicarbonate buffer containing 1.0 M KCl for pH 9–10.5, and mixtures of 1.0 M KOH and 1.0 M KCl with varying mutual ratios for pH 10.5–13.6. In all instances, KCl was added to maintain the ionic strength of the sample and the reference solutions fairly constant at $\mu = 0.5$. The protein concentrations used for the titration were in a range of $0.2–2.0 \times 10^{-5}$ M. The first optical measurement was made 20 sec after the preparation of the alkaline sample, and the subsequent measurements were continued usually for 3 hr. Symbols used are: A, absorbancy; ϵ, molar absorbancy.

Titration in 4 M Guanidine Hydrochloride. Guanidine hydrochloride was recrystallized from methanol. For the titration, 2 ml of a protein solution in 8 M guanidine hydrochloride at pH 7.0 was mixed with 2 ml of a mixture of 1.0 M KOH and 1.0 M KCl, giving a desired pH value.

Evaluation of Data for Spectrophotometric Titration. In order to evaluate the data for titration in any pH range a plot of $[H^+]\Delta\epsilon$ *vs.* $\Delta\epsilon$ was made. This plot was based on the following equation

$$[H^+]\Delta\epsilon = \Delta\epsilon_{max}K - \Delta\epsilon K \quad (1)$$

where $\Delta\epsilon_{max}$ is a difference in molar absorbancy attainable after a complete ionization and K is the apparent dissociation constant. Theoretically, $\Delta\epsilon_{max}$ must be an integral multiple of $\Delta\epsilon$ for a single ionizable group. A straight line was drawn to provide the best fit with the experimental data. The intercept on the abscissa gave an $\Delta\epsilon_{max}$ value, and from the slope an apparent pK value, pK_{app}, was calculated. Since the isoelectric point of stem bromelain is pH 9.55 (Murachi *et al.*, 1964), the electrostatic interaction factor will be small in moderately alkaline media. The present method is the same in principle as that of Hermans (1962), but a straight plot seems to give greater accuracy. For calculating the number of ionized tyrosyl residues of protein, use was made of values $\Delta\epsilon_{295\,m\mu}$ 2.33×10^3 and $\Delta\epsilon_{244m\mu}$ 1.10×10^4 for the ionization of a single phenolic group. The justification of the use of these values are given below (see Results).

Reaction of Tyrosyl Residues with Cyanuric Fluoride. The reactivity of the tyrosyl residues of stem bromelain with cyanuric fluoride was studied according to the method of Kurihara *et al.* (1963). A buffered solution of the enzyme was prepared by mixing 3 ml of 4.14×10^{-5} M stem bromelain with 6 ml of the buffer of a desired pH value; 1.0 M Tris buffer was used for pH 7–9, 1.0 M sodium carbonate–bicarbonate buffer for pH 9–10.5, and 1.0 M sodium carbonate plus appropriate amount of 1.0 M KOH for pH values above 10.5. The mixture was allowed to stand for 3 hr at room temperature to attain complete ionization equilibrium at each pH. Cyanuric fluoride (0.2 M, 1 ml) in dioxane was added to the buffered sample solution and the mixture was left standing at room temperature for 3 hr, during which the reaction proceeded to completion and cyanuric fluoride present in excess underwent hydrolysis yielding cyanuric acid. The mixture was then divided into two halves. One of them was brought to pH 12.8 by the addition of KOH solution, while the other half was adjusted to pH 7.0. Correction was made for a small difference in volume between the two halves. After 1 hr at room temperature, the absorbance of the neutral solution was read against that of the alkaline solution; the ΔA obtained represents the amount of the ionizable tyrosine hydroxyl groups that have remained unaffected after the treatment with cyanuric fluoride. ΔA_0, *i.e.*, the ΔA value of the protein that had not been treated with cyanuric fluoride, was obtained by a control run using 1 ml of pure dioxane in place of 1 ml of cyanuric fluoride in dioxane. The calculated difference, $\Delta A^* = \Delta A_0 - \Delta A$, may thus represent the amount of the phenolic groups that have been reacted with cyanuric fluoride at that pH. The use of symbol ΔA^* was chosen for the conformity to the previous report (Kurihara *et al.*, 1963).

Other Methods. The pH was measured with a Hitachi Model 4 glass-electrode pH meter equipped with a temperature compensator. The ratio of tyrosyl to tryptophyl residues of proteins was determined spectrophotometrically by the following three different

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

[3] We wish to thank Drs. Kazuo Shibata and Hiroo Horinishi for their kind advice concerning the preparation of cyanuric fluoride.

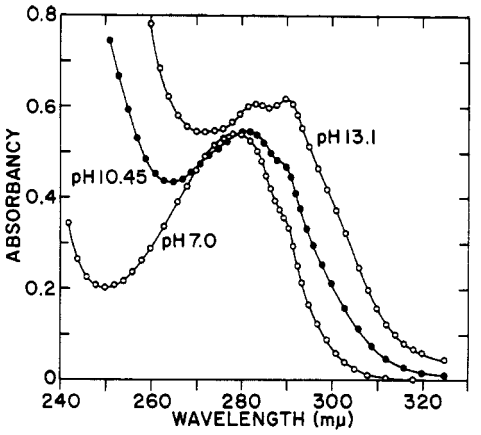

FIGURE 1: Ultraviolet absorption spectra of stem bromelain (0.81×10^{-5} M).

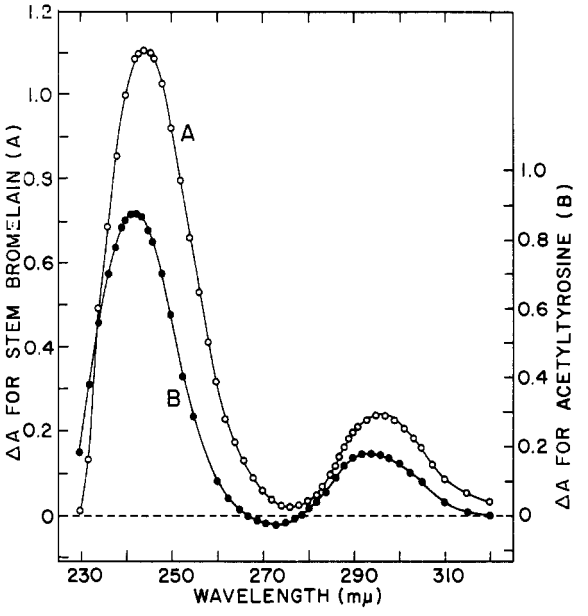

FIGURE 2: Difference spectra of stem bromelain (A) and of *N*-acetyl-L-tyrosine (B). The sample compound was dissolved in 0.5 N NaOH and allowed to stand at 25° for 3 hr before determination. The pH of the reference solution was 7.0. The spectra were determined at a concentration of 0.53×10^{-5} M for stem bromelain and of 0.60×10^{-4} M for acetyltyrosine.

methods. In the method of Bencze and Schmid (1957), the absorbance in the range between 278 and 293 mμ was measured with 0.6×10^{-5} M stem bromelain in 0.1 N NaOH. The ratio was calculated from the slope drawn tangentially to the two characteristic absorption maxima. In the method of Brown *et al.* (1963) the difference in absorbance at 294 mμ was measured between 0.9×10^{-5} M stem bromelain solutions in 0.2 N NaOH and 0.1 N HCl, respectively. A more classical method of Goodwin and Morton (1946) was also employed, whereby the measurements were made at 280 and 294.4 mμ with $0.8\text{–}1.5 \times 10^{-5}$ M stem bromelain solutions in 1.0 N NaOH. In these experiments crystalline pepsin (Worthington Biochemical Corp., Freehold, N. J.) and five-times-recrystallized lysozyme of hen egg white, prepared in this laboratory (Alderton, 1950), were used as reference compounds.

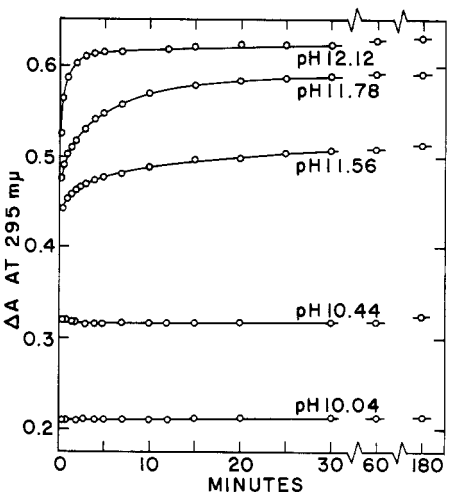

FIGURE 3: Time dependence of the ultraviolet absorption at 295 mμ of stem bromelain (1.63×10^{-5} M).

Results

Ultraviolet Absorption Spectra. The ultraviolet absorption spectra of stem bromelain at three different pH values are shown in Figure 1. The spectrum obtained at pH 7.0 was found to be identical with the one at pH 5.2 that had been reported previously (Murachi *et al.*, 1964). The pattern of the spectral shift at alkaline pH's shown in Figure 1 is closely similar to that of papain (Glazer and Smith, 1961). An alkali difference spectrum of stem bromelain is shown in Figure 2, in comparison with that of *N*-acetyl-L-tyrosine. A few discrepancies in pattern are noted between these spectra: (1) the two absorption maxima for stem bromelain are at 244 and 295 mμ, while those for acetyltyrosine are at 242 and 293.5 mμ; (2) a negative difference found with acetyltyrosine between 267 and 277 mμ is not seen with stem bromelain; and (3) a small positive difference is still present at 320 mμ with stem bromelain.

From curve B in Figure 2, a $\Delta\epsilon_{295\ m\mu}$ of 2.33×10^3 and a $\Delta\epsilon_{244m\mu}$ of 1.10×10^4 were obtained for the ionization of a tyrosine phenolic group. These values are in good agreement with those previously reported (Crammer and Neuberger, 1943; Beaven and Holiday, 1952; Donovan, 1964). By the use of these values, the number of ionized phenolic groups in stem bromelain was calculated from the data shown as curve A in Figure 2. Thus, the calculation from the data at 295 mμ gave a value of 18.6 tyrosyl residues/mole of protein. The good agreement of this value with the result

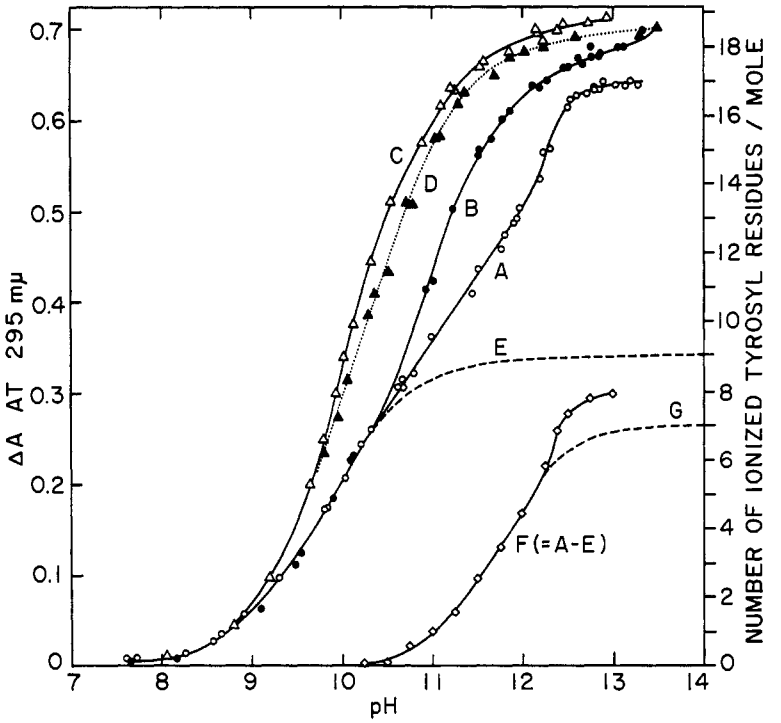

FIGURE 4: Spectrophotometric titration curve of stem bromelain ($1-2 \times 10^{-5}$ M). A, (○), forward titration 20 sec after preparation of alkaline sample solutions; B, (●), forward titration 3 hr after preparation of alkaline sample solutions; C, (△), forward titration in 4 M guanidine hydrochloride; D, (▲), reverse titration, reversed after exposure to pH 13.6 for 3 hr; E, theoretical ionization curve of 9 tyrosyl residues/mole with a pK_{app} of 9.84; F, (◇), calculated difference between curves A and E; G, theoretical ionization curve of 7 tyrosyl residues per mole with a pK_{app} of 11.8.

of amino acid analysis, *i.e.*, 19 tyrosines per molecule (Murachi, 1964), may indicate an almost complete unfolding of the protein molecule after the latter was exposed for 3 hr at 25° in 0.5 N NaOH. With the data at 244 mμ one must take into consideration a contribution by the sulfhydryl group of stem bromelain, since the enzyme protein was known to contain one reactive cysteinyl residue/mole (Murachi and Yasui, 1965). Donovan (1964) reported $\Delta\epsilon_{243\ m\mu}$ of the sulfhydryl group of aldolase in water to be 3.8×10^3. After subtracting the latter value from the observed $\Delta\epsilon_{244\ m\mu}$ for stem bromelain (curve A in Figure 2), a value of 18.9 ionized phenolic groups/mole was obtained as calculated by using 1.10×10^4 for $\Delta\epsilon_{244\ m\mu}$ of acetyltyrosine. The obtained value is again in excellent agreement with the value calculated from the data at 295 mμ.

The ionization of phenolic groups of stem bromelain was found to be a time-dependent process. Figure 3 illustrates a few of the typical results obtained. At pH 10.04, the difference spectrum at 295 mμ appeared instantaneously after a neutral solution was adjusted to that pH, and it remained practically unchanged for several hours. The same was true over the range between pH 7 and 10.3. At pH values higher than 10.3, a time-dependent ionization was observed. As shown in Figure 3, the change in $\Delta\epsilon_{295\ m\mu}$ with time is gradually increased as the pH increases. In these instances, an ionization equilibrium seems to be attained within 3 hr.

Titration Curves. In Figure 4, the values of the difference in absorbance at 295 mμ between neutral and alkaline solutions of stem bromelain, which have been converted to values of molar absorbancy, are plotted *vs.* pH. Curves A and B represent the ionization of phenolic groups 20 sec and 3 hr, respectively, after the preparation of the alkaline sample. When the sample solution was brought to pH 10.2 for 2 min and then quickly reversed to pH 7.0, the difference spectrum disappeared instantaneously and completely. At lower pH values from 8.5 to 10.3, where no time dependence of ionization was observed, the portions of both curves A and B coincide, conforming to a first-order sigmoid curve. As shown in Figure 5A the data can be best fitted to a straight line with a pK_{app} of 9.84. The number of phenolic groups concerned can be calculated from the obtained value of $\Delta\epsilon_{max}$ to be 9 groups/mole of protein. Curve E in Figure 4 represents a theoretical ionization curve for nine phenolic groups with a pK_{app} of 9.84. Subtraction of curve E from the experimental points for curve A provides data F which can be partly fitted to a theoretical ionization curve G for seven phenolic groups with a pK_{app} of 11.8. The apparent deviation of plot F from curve G at pH values higher than 12 may indicate the occurrence of one additional phenolic group which can be ionized only at very high pH values, although no sufficiently

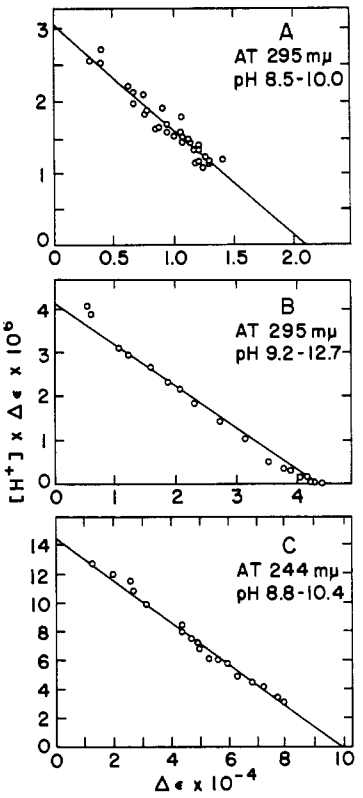

FIGURE 5: Evaluation of titration curves of stem bromelain according to $[H^+]\Delta\epsilon = \Delta\epsilon_{max}K - \Delta\epsilon K$. For details, see text. A, titration at 295 mμ between pH 8.5 and 10.0; B, titration in 4 M guanidine hydrochloride at 295 mμ between pH 9.2 and 12.7; C, titration at 244 mμ between pH 8.8 and 10.4.

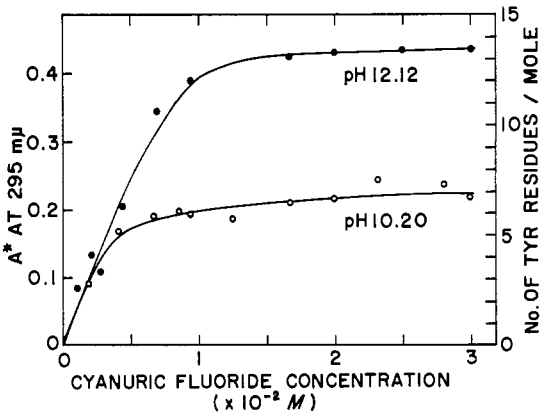

FIGURE 6: Reactivity of cyanuric fluoride with 1.38×10^{-5} M stem bromelain as a function of the concentration of cyanuric fluoride. ΔA^* is the difference in absorbancy before and after the treatment with cyanuric fluoride at 25° for 3 hr. Details of the experimental procedures are described in text.

accurate measurements could have been made in these pH regions due to a very rapid initial increase in $\Delta\epsilon_{295\ m\mu}$ with time (Figure 1). At a very high pH value, a further ionization was observed when the protein solution was left standing for a longer period of time. The rise in $\Delta\epsilon_{295\ m\mu}$ is a slower process, reaching after a few hours to a maximal value as shown at the upper end of curve B in Figure 4.

Curve C in Figure 4 shows the ionization curve obtained in the presence of 4 M guanidine hydrochloride. The ionization was found to be instantaneous at any pH value, no time dependence of the increase in $\Delta\epsilon_{295\ m\mu}$ being observed even after 20 hr of standing. From $\Delta\epsilon_{295\ m\mu}$ obtained at pH 12.8, a value of 18.9 tyrosyl residues/mole of protein was calculated. As shown in Figure 5B the data obtained between pH 9.2 and 12.7 conform to an ionization of 19 phenolic groups with a pK_{app} of 10.07.

The reverse titration curve for stem bromelain is shown in Figure 4, curve D. The data were obtained by titrating the protein solution which had been kept at pH 13.5 for 4 hr at 25° to attain complete ionization equilibrium. By the latter treatment the protein was irreversibly denatured and it became insoluble at pH values below 9. Consequently, the reverse titration could be continued only down to pH 9. The data of the reverse titration are fairly close to those of the forward titration in the presence of 4 M guanidine hydrochloride (curve C), suggesting an unfolding of the protein molecule to an almost equal extent in either one of these two cases.

When the pH difference spectrum was read at 244 mμ in a range of pH 7–13.5, a pattern of a titration curve almost identical with that of curve A was obtained (not shown). Again, no time-dependent increase in absorbance was observed up to pH 10.4, while beyond pH 10.4 higher values of $\Delta\epsilon_{244\ m\mu}$ were obtained after 3 hr of standing than those after 20 sec. The data obtained for the pH range 8.8–10.4 are plotted in Figure 5C, after a correction for ionization of one sulfhydryl group has been made by assuming a pK_{app} of the sulfhydryl group to be 9.8. The latter value was reported by Benesch and Benesch (1959) for the sulfhydryl groups in thiolated gelatin in aqueous solution. The plot in Figure 5C shows that the data represent the ionization of 9 tyrosyl groups/mole of protein with a pK_{app} of 9.84. The results are in excellent agreement with those of $\Delta\epsilon_{295\ m\mu}$ (Figure 5A). A further treatment of the experimental data for $\Delta\epsilon_{244\ m\mu}$ at pH values between 10.5 and 12 revealed that this portion of the titration curve can be best fitted with a theoretical ionization curve for seven tyrosyl groups with a pK_{app} of 11.9.

Reaction with Cyanuric Fluoride. The reactivity of cyanuric fluoride with stem bromelain was examined as a function of the concentration of cyanuric fluoride at pH 10.20 and 12.12. The results obtained are shown in Figure 6. With increasing cyanuric fluoride concentration the value of ΔA^* increases and reaches a plateau at each pH value, indicating that 2.0×10^{-2} M cyanuric fluoride is sufficient to modify apparently all the "ex-

TABLE I: Tyrosine–Tryptophan Ratio in Stem Bromelain, Pepsin, and Lysozyme.

Protein	Ratio Calcd from Amino Acid Compn (Tyr/Try)	Ratio Found[a]		
		Method of Bencze and Schmid (1957)	Method of Brown *et al.* (1963) (hr)	Method of Goodwin and Morton (1946) (hr)
Stem bromelain	2.38 (19/8)[b]	1.99 (10 min) 2.35 (20 hr)	1.78 (1) [1]	2.15 (1) [2] 2.32 (2) [4]
Pepsin	3.00 (18/6)[c]	2.50 (10 min) 2.86 (20 hr)		2.76 (2) [2] 3.11 (20) [2]
Lysozyme	0.500 (3/6)[d]	0.423 (15 hr)	0.468 (1) [2]	0.683 (1) [3] 0.664 (3) [3]

[a] Before determination, the sample protein was exposed to alkali for indicated period of time. Number of determinations in brackets. [b] Murachi (1964). [c] Blumenfeld and Perlmann (1959). [d] Canfield (1963).

posed" tyrosyl residues of stem bromelain at a concentration of 1.38×10^{-5} M. A similar type of concentration dependence of the reaction was reported by Kurihara *et al.* (1963) with tyrosine, insulin, and lysozyme.

Using 2.0×10^{-2} M cyanuric fluoride and 1.38×10^{-5} M stem bromelain, the pH dependence of the reaction was studied. The results obtained are shown in Figure 7, curve A, in comparison with the data for the reaction with L-tyrosine, curve B. As was already reported by Kurihara *et al.* (1963), a simple pH dependence pattern was obtained with tyrosine; the ΔA^* value was constant between pH 9 and 12.5 and decreased outside these pH limits. A pattern of more complex nature was obtained with stem bromelain. As shown in Figure 7, besides the plot falling off at outside both pH 8.8 and 12.5, it has an apparently horizontal part between pH 8.8 and 10.2. A similarly stepwise dependence of ΔA^* values on pH was reported to occur with lysozyme and insulin (Kurihara *et al.*, 1963). From a fairly constant level of ΔA^* between pH 8.8 and 10.2 for stem bromelain, the number of tyrosyl residues that had been reacted with cyanuric fluoride at these pH values was calculated to be 7–8 residues/mole of protein. It is interesting to note that a significant rise of the ΔA^* value for stem bromelain begins after the pH value exceeds 10.2 and the latter pH value is close to that, beyond which a time-dependent ionization of tyrosyl residues has been encountered in the titration experiment (Figure 4).

Determination of the Ratio of Tyrosyl to Tryptophyl Residues. The results obtained by three different spectrophotometric methods are listed in Table I. Determinations by the methods of Benzce and Schmid (1957) and of Goodwin and Morton (1946) gave, after a longer exposure to alkali, a ratio which agrees satisfactorily with the result of amino acid analysis (Murachi, 1964). The method of Brown *et al.* (1963) gave a lower value for stem bromelain, although it gave a fairly accurate result with lysozyme.

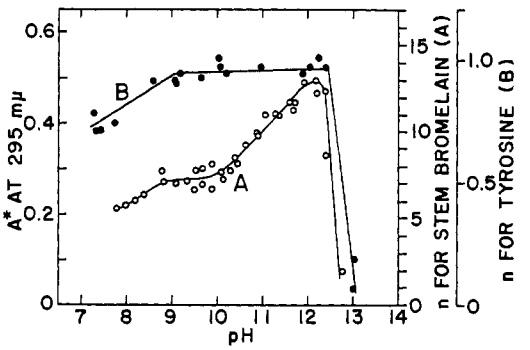

FIGURE 7: Reactivity of cyanuric fluoride with stem bromelain and with L-tyrosine as a function of pH. A, 1.38×10^{-5} M stem bromelain plus 2.0×10^{-2} M cyanuric fluoride; B, 2.32×10^{-4} M L-tyrosine plus 3.0×10^{-2} M cyanuric fluoride. ΔA^* is the difference in absorbancy before and after the treatment with cyanuric fluoride at 25° for 3 hr. *n* is the number of ionized tyrosyl residues per mole.

Discussion

The occurrence of two or more types of tyrosyl residues in a protein molecule has been known with various proteins. For example, three of six phenolic groups of ribonuclease ionize instantaneously and reversibly between pH 9 and 11.5; the other three ionize slowly and irreversibly above pH 11.5 (Tanford *et al.*, 1955). For tyrosyl residues of lysozyme, the occurrence of two types was demonstrated by studying the reactivity with cyanuric fluoride (Kurihara *et al.*, 1963), while a more careful experiment has revealed three different stages of ionization.[4] Chymotrypsin had been shown to contain four different types of tyrosyl residues (Inada *et al.*, 1964). The results of the present experi-

[4] K. Hamaguchi, unpublished observation.

TABLE II: Ionization of Tyrosine Hydroxyl Groups in Stem Bromelain.

Experimental Methods (mμ)	Total No. of Residues Found	"Exposed" Residues		"Buried" Residues	
		No.	pK_{app}	No.	pK_{app}
Forward titration (295)	18.6	9	9.84	10{7	11.8
				10{1	>12
				10{2	>13
Forward titration (244)	18.9	9	9.84	10{7	11.9
				10{3	>12
Forward titration (295) in 4 M guanidine·HCl	18.9	19	10.07	None	
Reverse titration (295)	—	19	10.11	None	
Reaction with cyanuric fluoride	—	7–8		11–12	

ment also indicate a complex nature of the ionization of 19 tyrosyl groups in stem bromelain. Thus, four different stages of ionization may be distinguishable in a forward titration of the protein solution with alkali (Figure 4). In stage 1, from neutrality to approximately pH 10.3, only the ionization of 9 "exposed" tyrosyl residues/mole of protein occurs with no time dependence, while in further stages the ionization of the "buried" tyrosyl residues are also involved. Stage 2, from approximately pH 10.3 to 12, represents a pH region where a time-dependent ionization of seven "buried" tyrosyl groups occurs, and stage 3, at pH values higher than 12, a very rapid ionization of probably one additional tyrosyl group ensues. A further slow process of ionization of the remaining two residues is encountered in stage 4, *i.e.*, while leaving the protein solution at a pH value of 13.6 or higher for several hours.

The calculated pK_{app} values of phenolic groups pertaining to each of these stages of ionization are listed in Table II. Numerical data obtained by other methods of experiments are also included in the table for comparison. The measurements at 244 mμ have given results almost identical with those obtained at 295 mμ. The obtained pK_{app} value, 9.84, for the "exposed" tyrosyl residues in stem bromelain is significantly lower than the apparent pK values reported for other proteins: *e.g.*, 10.3 for 11–12 tyrosyl residues in papain (Glazer and Smith, 1961), 10.5 for two residues in lysozyme (Inada, 1961), 10.2 for one residue in α-chymotrypsin (Inada *et al.*, 1964). The pK_{app} value, 10.07, obtained in the presence of 4 M guanidine hydrochloride is in good agreement with the reported pK_{app} value for tyrosine (Inada, 1961), indicating that all the phenolic groups of the denatured stem bromelain become freely accessible to the solvent.

As shown in Table II, the number of the "exposed" tyrosyl residues as determined by the use of cyanuric fluoride is lower than that obtained by the spectrophotometric titration by 1–2 residues/mole. The implication of this discrepancy must await future elucidation, although the difference seems to be within the magnitude of experimental errors. So far, the use of cyanuric fluoride has been limited to the cases in which the sample protein contains only 4 or less tyrosyl residues/mole (Kurihara *et al.*, 1963; Hachimori *et al.*, 1965). More information will be needed concerning the accuracy of the method as applied to a protein that contains a large number of tyrosyl residues. Nevertheless, the results of the experiment with cyanuric fluoride are consistent with those of the spectrophotometric titration in demonstrating that the "buried" tyrosyl residues begin to become accessible to the solvent as the pH rises up to 10.2–10.3 (Figure 7).

It is evident that the ionization of the "buried" residues is dependent on a composite process of unfolding of the protein molecule, because once the sample protein has been completely denatured either by 4 M guanidine hydrochloride or by a longer exposure to strong alkali, a normalized titration curve is obtained which no longer shows a time dependence. The unfolding of stem bromelain molecule at alkaline pH values is also being studied in this laboratory by some other means. Available data of rotatory dispersion experiments[5] have indicated that the conformation change is almost undetectable up to pH 10, whereas a large change occurs between pH 10 and 12 with a concomitant loss of the proteinase activity.These findings seem to be compatible with the results of the present experiments.

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[5] T. Murachi and M. Kanda, unpublished observation.

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