cytochrome c must react directly with cytochrome a (or a_3).

ACKNOWLEDGMENT

The skillful assistance of Miss Marjorie Krause is appreciated.

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Kinetic Studies of Bromelain Catalysis*

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Received April 8, 1963

Bromelain has been found to hydrolyze esters of L-arginine derivatives much faster than esters of other amino acids. Kinetics of the hydrolyses of the ethyl ester and amide of benzoyl-L-arginine have been compared over *p*H 3.2–9.6 and 15–35°. The apparent first-order rate constant for the ester is about 140 times higher than that for the amide, a situation conspicuously different from other SH-proteinases. The apparent Michaelis-Menten constants for the two substrates are also different to the same extent, hence the ratios of the two constants for the ester and the amide are very close and behave similarly as *p*H and temperature are changed. The two constants are more or less unchanged over a wide neutral *p*H range with both substrates. With the ester both constants decrease parallel toward acidic and alkaline *p*H values, while with the amide the apparent Michaelis-Menten constant increases and the apparent first-order rate constant decreases. With the ester the apparent Michaelis-Menten constant is practically constant from 15° to 35°, while other constants for both substrates increase with temperature. By comparing these features with papain and ficin the apparent kinetic parameters have been characterized in terms of individual rate constants and it has been concluded that bromelain involves a unique mechanism of catalysis among the SH-proteinases.

Stem bromelain, a proteolytic enzyme from pineapple stem, has been shown to be a sulfhydryl enzyme by Murachi and Neurath (1960). It hydrolyzes proteins like casein and hemoglobin at high rates, while a synthetic substrate of smaller molecular size, like BAEE,[1] has also been shown to be hydrolyzed at a moderate rate (Murachi and Neurath, 1960). A preliminary experiment in this laboratory indicated that arginine derivatives like BAEE and BAA are the best substrates for the enzyme among various amino acid esters and amides, respectively. These facts seemed to indicate a close similarity between bromelain and other sulfhydryl enzymes from plants like papain and ficin. However, an important difference has been noted in that the rate of the BAEE hydrolysis is conspicuously higher than the rate for its amide analog, BAA, by a factor of more than 100, while both papain (Stockell and Smith, 1957; Smith and Parker, 1958) and ficin (Hammond and Gutfreund, 1959) have been shown to hydrolyze an ester (BAEE) and a corresponding amide (BAA) at almost identical rates. Since the similarity of the rates of the ester and amide hydrolyses has been considered as one of the important features of the enzymes from plant with a sulfhydryl group at the active center, as contrasted from other proteolytic

* This work was supported in part by grants from the Ministry of Education, Japan, and the National Institutes of Health, U. S. Public Health Service (GM 08714).

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[1] The abbreviations used are: BAEE, benzoyl-L-arginine ethyl ester; BAME, benzoyl-L-arginine methyl ester; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; BAA, benzoyl-L-argininamide.

TABLE I

AMINO ACID ESTER HYDROLYSIS BY BROMELAIN[a]

Substrate	Rate × 10^3 (sec^{-1})	Relative Rate (%)
BAEE	29.4	100
BAME	91.7	312
TAME	14.8	50.3
L-Histidine ethyl ester	<0.1	—
L-Lysine ethyl ester	2.81	9.56
Glycine ethyl ester	5.40	18.4
Benzoylglycine ethyl ester	11.9	40.5
Benzoyl-DL-alanine ethyl ester	3.96	13.5
L-Leucine ethyl ester	5.48	18.6
L-Phenylalanine ethyl ester	8.60	29.3
L-Tyrosine ethyl ester	6.39	21.7

[a] The reaction mixture contained in a total volume of 10 ml 0.01 M ester substrate, 0.1 M KCl, 0.005 M L-cysteine, and 0.41 μmole of bromelain. The reaction was carried out at *p*H 6.0 and at 25°. The rate of hydrolysis was measured in a *p*H-stat assembly with 0.01–0.1 N NaOH as titrant.

enzymes like those of pancreatic origin, it seemed to be of interest to look into the mechanism of the bromelain action. In the present study the hydrolyses of BAEE and BAA catalyzed by bromelain have been kinetically studied and the results are compared with those of papain.

MATERIALS AND METHODS

Bromelain.—The enzyme preparation used was isolated from crude Bromelain No. 181 from the Hawaiian Pineapple Company, Honolulu, Hawaii,[2] by the method of Murachi *et al.* (1964). The preparation was shown to be homogeneous by such criteria as ultracentrifuge and free-boundary electrophoretic analyses, diffusion pattern, and behavior in ion-exchange chromatography. The molecular weight was calculated from sedimentation and diffusion experiments to be 33,000 (Murachi *et al.*, 1964). The protein concentration was determined by measuring absorbancy at 280 mμ with a molar extinction coefficient of 6.33×10^4.

Substrates.—BAEE hydrochloride, BAME hydrochloride, TAME hydrochloride, BAA hydrochloride monohydrate, L-histidine ethyl ester dihydrochloride, L-lysine ethyl ester dihydrochloride, glycine ethyl ester, L-leucine ethyl ester, ethyl hippurate, benzoyl-DL-alanine ethyl ester, L-phenylalanine ethyl ester hydrochloride, and L-tyrosine ethyl ester hydrochloride were purchased from Mann Research Laboratories, New York, and used as substrates without further purification. Since BAEE, BAME, and TAME are too hygroscopic to be weighed accurately the concentrations of these solutions were determined by titrating acids liberated quantitatively by the action of trypsin.

Assay of the Rate of Catalysis.—The rate of the hydrolysis of an ester was measured by using a Radiometer Model SBR2/SBU1/TTT1 titration equipment with 0.1 N NaOH as titrant. The reaction solution was 10 ml in volume containing 0.005 M L-cysteine, 0.1 M KCl, 0.41 μmole of enzyme, and the ester substrate. It was placed in a thermostated water bath and kept under a nitrogen stream. The reaction solution for the amide hydrolysis contained 0.03 M buffer, 0.005 M L-cysteine, 0.1 M KCl, 0.51 μmole of enzyme, and the amide substrate in a total volume of 5 ml. The buffers used were acetic acid–sodium acetate with *p*H values between 3.7 and 5.6, potassium phosphate between *p*H 6.1 and 7.0, and Tris-HCl between *p*H 7.9 and 8.3. An aliquot of 0.5 ml was withdrawn at 10-minute intervals and the ammonia was determined by a combination of the microdiffusion technique and the indophenol method with sodium nitroprusside as catalyst (Chaney and Marbach, 1962).

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

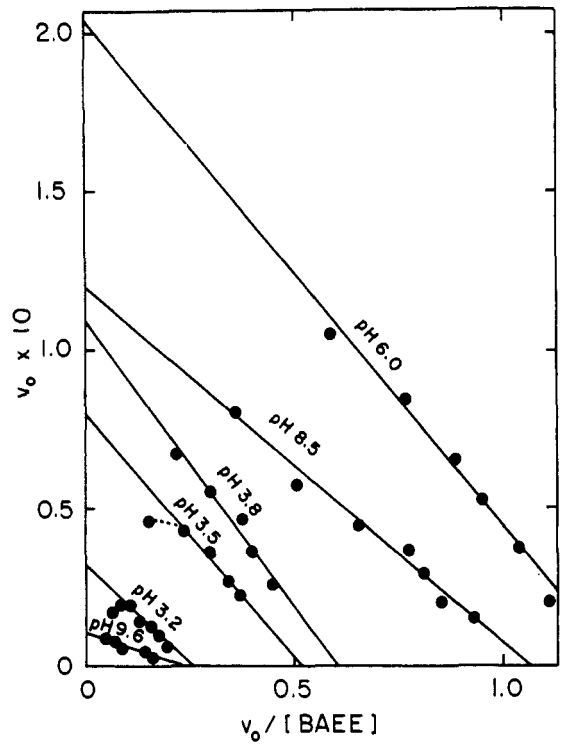

FIG. 1.—Examples of Eadie plots for BAEE hydrolysis by bromelain at 25° and at various *p*H values: v_0, initial rate of hydrolysis in μmoles BAEE hydrolyzed/sec; [BAEE], molar concentration of BAEE. The reaction mixture contained 0.005 M L-cysteine, 0.1 M KCl, 0.41 μmole of enzyme, and BAEE in a total volume of 10 ml. The rate of hydrolysis was measured in a *p*H-stat assembly with 0.1 N NaOH as titrant.

The rates of the hydrolyses, which were practically constant for more than 10 minutes in the case of BAEE hydrolysis and more than 40 minutes with BAA, were plotted against the rates divided by the concentrations of a substrate according to the method of Eadie (1942) to obtain the apparent Michaelis-Menten constant, $K_{m(app)}$, and the apparent first-order rate constant, $k_{3(app)}$. At *p*H values above 8 the observed rate of the BAEE hydrolysis had to be corrected for the hydroxide ion–catalyzed hydrolysis, but the reaction condition has been such that the rate of the latter catalysis did not amount to 20% of the rate of the bromelain catalysis even at *p*H 9.6. With BAA a similar correction for a nonenzymatic hydrolysis was made at *p*H below 4. The rate of this reaction was less than 5% of the total observed rate. Satisfactory plots were obtained by choosing the concentration range 0.01–0.2 M for BAEE and 0.004–0.005 M for BAA.

Identification of the Reaction Products.—Ascending paper chromatography was carried out with Toyo No. 51 paper. The solvent systems used in experiments with BAEE were *n*-butanol–acetic acid–water (4:1:5), 80% (v/v) *n*-propanol, and *n*-butanol-pyridine-water (1:1:1). In experiments with BAA only the *n*-butanol–acetic acid–water system was used. High-voltage paper electrophoresis was also carried out with Toyo

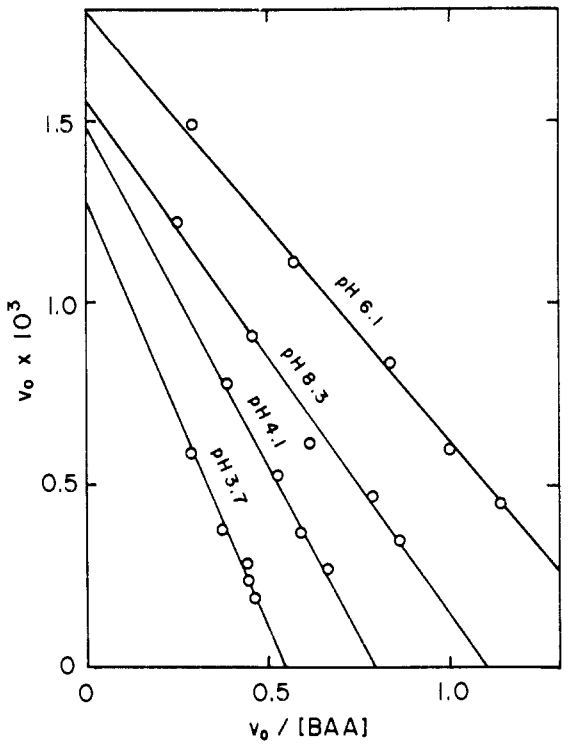

FIG. 2.—Examples of Eadie plots for BAA hydrolysis by bromelain at 25° and at various *p*H values: v_0, initial rate of hydrolysis in μmoles NH_3 liberated/sec; [BAA], molar concentration of BAA. The incubation mixture contained 0.005 M L-cysteine, 0.1 M KCl, 0.51 μmole of enzyme, and BAA in a total volume of 5 ml. The *p*H values were maintained by the use of buffers (see text). The rate of hydrolysis was measured by determining NH_3 in 0.5-ml aliquots withdrawn at 10-minute intervals.

No. 50 paper at *p*H 6.5 (pyridine–acetic acid–water, 10:0.4:90) and 4,000 v, for 15 minutes.

After certain periods of incubation, aliquots of the reaction mixture initially containing 0.25 μmole of substrate were applied to paper strips with authentic samples of BAEE, BAA, benzoyl-L-arginine, and L-cysteine as references. The papers were stained with Sakaguchi and ninhydrin reagents.

RESULTS

Specificity of Bromelain.—In order to obtain some idea about the pattern of specificity of the enzyme with respect to the amino acid residues, esters of amino acids and amino acid derivatives were screened for susceptibility to the catalytic action of bromelain. The assays were carried out at *p*H 6, where both Michaelis-Menten constant and the first-order reaction rate may be considered to be at the maximum level. The concentrations of the esters were uniformly 0.01 M. The results obtained are shown in Table I. As was anticipated from the previous observation (Murachi and Neurath, 1960), esters of L-arginine derivatives have been found to be the best substrates. Among the three esters examined BAME is the most preferred substrate, followed by BAEE. TAME is hydrolyzed only at one-half the rate of BAEE, which is a marked difference from the pattern of the specificity of trypsin (Schwert *et al.*, 1948; Schwert and Eisenberg, 1949) and suggests a difference in the mechanism of catalysis between the two enzymes.

Attempts were made to determine the two kinetic constants, $k_{3(app)}$ and $K_{m(app)}$, for L-leucine ethyl ester hydrolysis by plotting the rate against ratio of the rate to the concentration of the ester. However, the ratio did not start to decrease as the concentration of the substrate was increased up to 0.2 M, indicating that the $K_{m(app)}$ value is much higher.

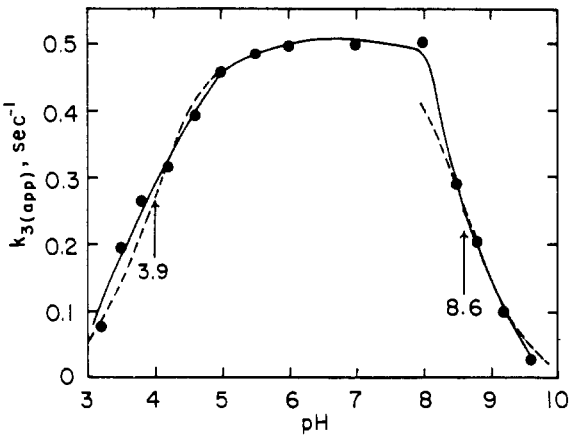

FIG. 3.—*p*H-dependence at 25° of the apparent first-order rate constant $k_{3(app)}$ of the bromelain-catalyzed hydrolysis of BAEE in 0.1 M KCl and 0.005 M L-cysteine. The broken lines illustrate calculated titration curves of the indicated *pK* values.

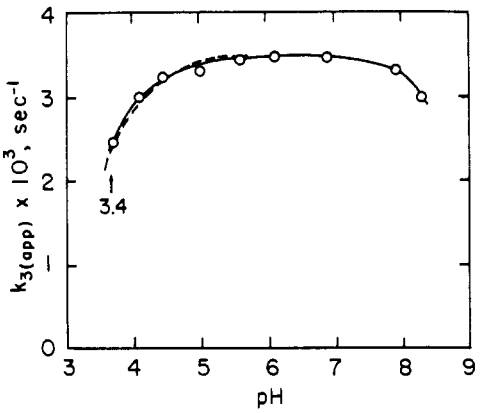

FIG. 4.—*p*H-dependence at 25° of the apparent first-order rate constant $k_{3(app)}$ of the bromelain-catalyzed hydrolysis of BAA in 0.1 M KCl, 0.005 M L-cysteine, and 0.03 M buffers. The broken line is a calculated titration curve of *pK* 3.4.

Stability of the Enzyme.—As a preliminary experiment the stability of the enzyme was tested over a wide range of *p*H from 4 to 10. The *p*H values of the enzyme solution were adjusted to the desired levels with various buffers, and the solutions were kept at 5°. After 2, 24, and 48 hours the activity on casein was tested with and without 0.005 M L-cysteine by a modified Kunitz method (Hagihara *et al.*, 1958). Only a slight decrease in activity was recognized after 48 hours, while practically no loss occurred after 24 hours over the entire range of *p*H.

*p*H-*Profiles of* $k_{3(app)}$.—Eadie plots were made for BAEE hydrolysis over a range between *p*H 3.2 and 9.6 at 25°. As shown from the examples in Figure 1, substrate inhibition was observed at BAEE concentrations higher than 0.2 M at low *p*H values, while at higher *p*H values the inhibition did not seem to start up to this concentration of the substrate. Examples of BAA hydrolysis are shown in Figure 2, in which no sign of the substrate inhibition is observable within the range of substrate concentrations employed.

The $k_{3(app)}$ values are practically constant and highest between *p*H 5.5 and 8 with both BAEE and BAA, as shown in Figures 3 and 4. With the amide

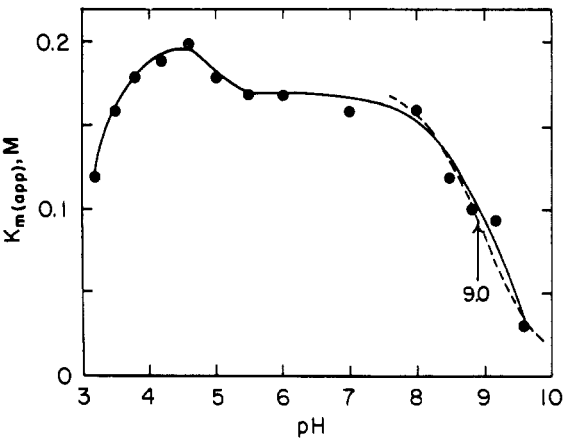

FIG. 5.—*p*H dependence at 25° of the apparent Michaelis-Menten constant $K_{m(app)}$ of the bromelain-catalyzed hydrolysis of BAEE in 0.1 M KCl and 0.005 M L-cysteine. The broken line is a calculated titration curve of *pK* 9.0.

substrate not much data could be obtained beyond the flat region. Although the behaviors of the $k_{3(app)}$ values for the two substrates have many features in common, the absolute values for the two substrates are different by a factor of more than 100, i.e., $k_{3(app)}$ for BAEE is 0.50 sec^{-1} at *p*H 6.0 and 25° while that for BAA is 3.5×10^{-3} sec^{-1} under the same condition. This feature makes bromelain unique among other SH-proteinases, although these enzymes have many other common features.

*p*H-*Profiles of* $K_{m(app)}$.—The changes of the $K_{m(app)}$ values for both BAEE and BAA are not appreciable over the wide range of *p*H tested as shown in Figures 5 and 6. Unlike the behaviors of $k_{3(app)}$ there is a remarkable difference in the *p*H profiles of $K_{m(app)}$ between the ester and the amide substrates. With the ester $K_{m(app)}$ decreases on both sides of the flat region which extends between *p*H 5.5 and *p*H 8.0, although there is a small hump at around *p*H 4.5 before the value starts to fall as *p*H is decreased. With the amide substrate, on the other hand, the value seems to increase on both sides of the minimum which is more or less flat within the accuracy of experimental error and again lies between *p*H 5.5 and 8. Here again it must be noted that the $K_{m(app)}$ for BAEE is much higher than that for BAA. The value for the latter over the flat minimum region is 1.2×10^{-3} M, while that for BAEE over the plateau region is 0.17 M, or 142 times the value for BAA. This factor is practically identical with the corresponding ratio, 140,

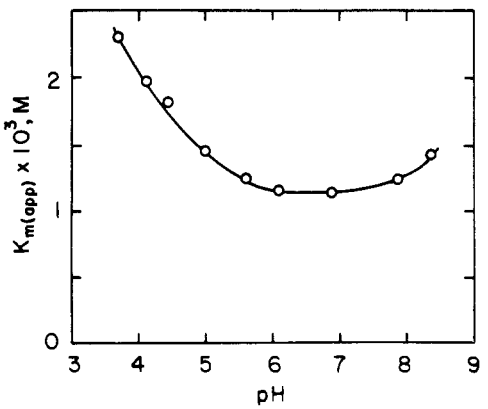

FIG. 6.—*p*H-dependence at 25° of the apparent Michaelis-Menten constant $K_{m(app)}$ of the bromelain-catalyzed hydrolysis of BAA in 0.1 M KCl, 0.005 M L-cysteine, and 0.03 M buffers.

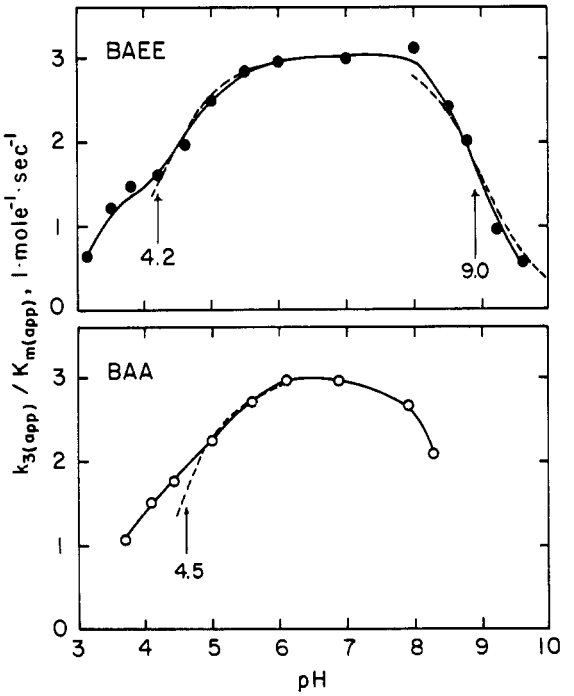

FIG. 7.—*p*H-dependence at 25° of the ratio, $k_{3(app)}/K_{m(app)}$, of the bromelain-catalyzed hydrolysis of BAEE (top figure, filled circles) and BAA (bottom figure, open circles). The broken lines illustrate calculated titration curves of the indicated *pK* values.

for the $k_{3(app)}$ values for the two substrates, suggesting that these two constants are related with a common proportionality factor.

*p*H-*Profiles of the Ratio,* $k_{3(app)}/K_{m(app)}$.—In the case of papain several evidences have been presented by Stockell and Smith (1957) and Smith and Parker (1958) which suggest that the ratio $k_{3(app)}/K_{m(app)}$ represents a single rate constant, probably the second-order rate constant of the initial complex formation k_1. In the case of bromelain the proportionality between $k_{3(app)}$ and $K_{m(app)}$ values for BAEE and BAA indicates that the ratios $k_{3(app)}/K_{m(app)}$ for the two substrates should be very close. Figure 7 shows that this similarity holds not only over a neutral *p*H region, the value being 2.9, but over a very wide *p*H range. The *p*H-profiles of the ratios for the two substrates are bell-shaped with a rather broad plateau between *p*H 6 and 8. The good coincidence of the two curves is remarkable in view of the fact that absolute values of $k_{3(app)}$ and $K_{m(app)}$ are vastly different for the two substrates and furthermore the *p*H-profiles of $K_{m(app)}$ are in opposite directions, one being concave upward, the other concave downward.

Temperature Dependence.—Values for $k_{3(app)}$ and $K_{m(app)}$ have been determined for the BAA hydrolysis at 15°, 20°, 25°, and 30° and for the BAEE hydrolysis at 15°, 20°, 25°, 30°, and 35° as shown in Table II. The $K_{m(app)}$ of the BAEE hydrolysis differs from other parameters in that it remains practically constant from 15° to 35° while others increase, though to varying degrees, as temperature is raised. The ratios, $k_{3(app)}/K_{m(app)}$, for BAEE and BAA at temperatures other than 25° are not identical but remain fairly close. Arrhenius plots based on these data are shown in Figures 8 and 9. The plots are on reasonably straight lines except for $k_{3(app)}$ and $k_{3(app)}/K_{m(app)}$ for the BAA hydrolysis at 15°. Apparent activation energies were estimated from the slope of the plots as shown in the bottom row of Table II.

Products of BAEE and BAA Hydrolysis.—The

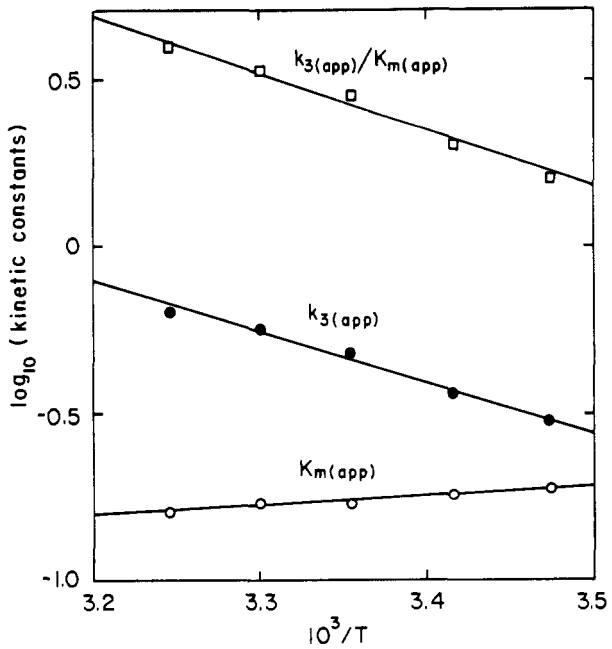

FIG. 8.—Arrhenius plots of the apparent kinetic constants $k_{3(app)}$, $K_{m(app)}$, and $k_{3(app)}/K_{m(app)}$ of the bromelain-catalyzed hydrolysis of BAEE determined at 15°, 20°, 25°, 30°, and 35° at *p*H 6.0.

production of benzoylarginine from both BAEE and BAA when these substrates were incubated with the enzyme was always demonstrated by paper chromatography with three different solvent systems and also by high-voltage paper electrophoresis described above. No Sakaguchi-positive spots were revealed other than the two strongly positive spots corresponding to the unchanged substrate and benzoylarginine, respectively, and a faint one at the origin where the enzyme protein remains. Ninhydrin stainings also reveal no products other than those found in the chromatogram for the incubation mixture at time zero. These results eliminate the possibility of transfer of benzoylarginyl moiety of BAEE or BAA to cysteine added as activator under the conditions employed.

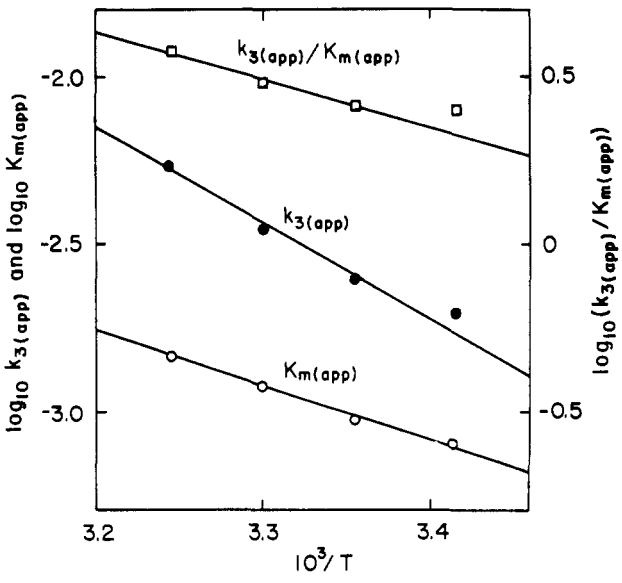

FIG. 9.—Arrhenius plots of the apparent kinetic constants, $k_{3(app)}$, $K_{m(app)}$, and $k_{3(app)}/K_{m(app)}$, of the bromelain-catalyzed hydrolysis of BAA determined at 15°, 20°, 25°, and 30° at *p*H 6.0.

TABLE II
TEMPERATURE DEPENDENCE OF KINETIC PARAMETERS AND ENERGIES OF ACTIVATION OF BROMELAIN CATALYSIS AT *p*H 6.0

Temperature (°C)	$k_{3(app)} \times 10^2$ (sec^{-1})		$K_{m(app)} \times 10^2$ (M)		$k_{3(app)}/K_{m(app)}$ (l × mole^{-1} × sec^{-1})	
	BAEE	BAA	BAEE	BAA	BAEE	BAA
15	30	0.20	19	0.080	1.6	2.5
20	36	0.25	18	0.093	2.0	2.6
25	48	0.35	17	0.12	2.9	2.9
30	56	0.55	17	0.14	3.3	3.8
35	63		16		3.9	
Apparent Energy of Activation (kcal mole^{-1})						
	7.3	12.8	−1.1	7.7	7.7	6.4

DISCUSSION

Among proteinases of plant origin, papain and ficin have so far received careful kinetic studies and have been found to share many unique features in common (Stockell and Smith, 1957; Smith and Parker, 1958; Bernhard and Gutfreund, 1956; Hammond and Gutfreund, 1959). Both of these require a sulfhydryl group, which is believed to be in the active center, in the reduced state for the catalytic activity. Although the specificity of these enzymes does not seem to be as strict as with trypsin, peptides of positively charged amino acids, particularly arginine, seem to be most preferred. The two enzymes also hydrolyze esters and amides of these amino acids, and, as cited in Table III, the apparent first-order rate constants, $k_{3(app)}$, of the hydrolyses of ethyl ester and amide of benzoyl-L-arginine are practically identical, while the apparent Michaelis-Menten constant, $K_{m(app)}$, for the amide is approximately twice as high as that for the ester. This similarity of the kinetics of ester and amide hydrolyses seems to be unique to the two plant enzymes in contrast with pancreatic enzymes, for example, where an ester usually undergoes hydrolysis faster than the corresponding amide by a factor of more than 100 (Zerner and Bender, 1963; Schwert and Eisenberg, 1949; Neurath and Schwert, 1950). The present finding that the rate of BAEE hydrolysis is 140 times higher than that of BAA hydrolysis indicates that the above cited feature is not a common characteristic of all the sulfhydryl proteinases. This is particularly interesting in view of the apparent similarity between bromelain and the other two in many other respects, such as occurrence, substrate specificity, and presence of the sulfhydryl group in the active center.

The kinetic features of papain have been explained by a two-step mechanism (Smith and Parker, 1958) in which the hydrolysis step of an acylated enzyme

TABLE III
COMPARISON OF BAEE AND BAA HYDROLYSES CATALYZED BY SH-PROTEINASES

Enzyme	Substrate	$K_{m(app)}$ (M)	$k_{3(app)}$ (sec^{-1})
Bromelain[a]	BAEE	0.17	0.50
	BAA	0.0012	0.0035
Papain	BAEE[b]	0.0020	12
	BAA[c]	0.040	10
Ficin[d]	BAEE	0.025	1.0[e]
	BAA	0.048	0.9[e]

[a] *p*H 6.0, 25°. [b] *p*H 5.6, 25° (Smith and Parker, 1958). [c] *p*H 5.6, 25° (Stockell and Smith, 1957). [d] *p*H 6.06, 25° (Hammond and Gutfreund, 1959). [e] Relative values taking $k_{3(app)}$ of ficin with BAEE as unity.

is rate limiting because it is the only step common to BAEE and BAA. In the case of bromelain, however, the 140-fold difference of $k_{3(app)}$ between BAEE and BAA indicates that the process common to the two substrates cannot be rate limiting for both. It also seems to be improbable that the difference of the rate of initial binding of BAEE and BAA is large enough to explain the difference in $k_{3(app)}$ in view of the similarity of the size and gross structure of the two molecules. Therefore a mechanism involving three or more steps, like the one proposed for chymotrypsin by Gutfreund and Sturtevant (1956)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} \underset{+}{ES_2} \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad P_1$$

$$\frac{d[P_2]}{dt} = \frac{k_{3(app)}[E]_0[S]}{[S] + K_{m(app)}}$$

where $[E]_0$ is the total concentration of enzyme and $k_{3(app)}$ and $K_{m(app)}$ are

$$k_{3(app)} = \frac{k_2k_3}{k_2 + k_3}$$

$$K_{m(app)} = \frac{k_{-1} + k_2}{k_1}\frac{k_3}{k_2 + k_3}$$

will be required. The observed difference of $k_{3(app)}$ between BAEE and BAA may be explained if the rate-limiting step for BAEE is the third one and that for BAA is the second.

The similarity of the ratio $k_{3(app)}/K_{m(app)}$, its pH-profile, and its temperature dependence (see below) between BAEE and BAA is a further noteworthy characteristic of the bromelain catalysis. In the above three-step mechanism the ratio is represented by

$$k_{3(app)}/K_{m(app)} = k_1k_2/(k_{-1} + k_2)$$

As the k_2 values for the two substrates differ by a factor of at least 140, the contribution of k_2 to the ratio should vanish in order that the ratios for the two substrates may be identical. This condition may be obtained if k_2 is much larger than k_{-1}, and if the ratio is reduced to k_1. It is interesting to note that essentially an equivalent conclusion has been obtained in the case of papain by an entirely different course of reasoning (Stockell and Smith, 1957). Thus, the $k_1 = k_{3(app)}/K_{m(app)}$ turns out to be 2.9 l $\times$ mole^{-1} $\times$ sec^{-1} which is much smaller than 500 l $\times$ mole^{-1} $\times$ sec^{-1} of ficin (Gutfreund, 1955) or 250 $\backsim$ 600 l $\times$ mole^{-1} $\times$ sec^{-1} of papain (Stockell and Smith, 1957; Smith and Parker, 1958).

As shown in Figure 5, the pH-profile of $K_{m(app)}$ of the BAEE hydrolysis is concave downward, whereas with BAA (Fig. 6) it is concave upward. On the other hand, with papain the pH-profiles of $K_{m(app)}$ are invariably concave upward either with BAEE or BAA (Smith and Parker, 1958; Stockell and Smith, 1957). Analyses of the slopes on the alkaline side of $k_{3(app)}$, $K_{m(app)}$, and $k_{3(app)}/K_{m(app)}$ for BAEE have indicated reasonably good coincidences with single-group titration curves of pK 8.6, 9.0, and 9.0, respectively, which are shown by the broken lines in Figures 3, 5, and 7. On the acidic side, however, the profiles are complex. Although $k_{3(app)}$ for the BAEE hydrolysis seems to be represented by a titration curve of pK 3.9 (broken line, Fig. 3), $k_{3(app)}/K_{m(app)}$ can be fitted only partly by a single-group titration curve of pK 4.2 between pH 4 and 6 (broken line, Fig. 7, top). The deviation below pH 4 suggests involvement of additional functional groups of lower pK values. The hump in the profile of $K_{m(app)}$ in Figure 5 also points to the complex nature in the acidic region. With BAA the sloping parts in either acidic or alkaline region is not sufficiently extensive to allow a reasonable analysis, but the slope of $k_{3(app)}$ at low pH values seems to be fitted by a titration curve of pK 3.4 (broken line, Fig. 4) and the plots of $k_{3(app)}/K_{m(app)}$ between pH 5 and 6 seem to coincide with a titration curve of pK 4.5 (broken line, Fig. 7 bottom). Deviation of points in a further acidic region from these curves again suggests that more than a single group, which dissociate in the acidic region, participate in the catalysis. Although the temperature-dependence of the pK values was not studied, it seems to be reasonable to infer that at least one carboxylate group and either an ϵ-ammonium, an undissociated sulfhydryl, or a tyrosyl group of bromelain are involved in the catalysis.

Since the bromelain catalysis seems to involve three or more steps, $k_{3(app)}$, $K_{m(app)}$ and $k_{3(app)}/K_{m(app)}$ may generally be complexes of several rate constants. Therefore the slopes of the Arrhenius plots shown in Figures 8 and 9 may not provide energy of activation of a single reaction step except for a fortuitous case where the complex can be approximated by a single rate constant. The apparent energy of activation given at the bottom of Table II may be a complex function of several activation energies. Although there are significant differences between the values for BAEE and BAA with both $k_{3(app)}$ and $K_{m(app)}$, it is noteworthy that the values for $k_{3(app)}/M_{m(app)}$ are very close. This fact seems to substantiate the above proposal that this ratio is of identical form with BAEE and BAA and that its dominant term is not affected by whether the substrate is an ester or an amide and probably is k_1. That $K_{m(app)}$ for BAEE remains practically unchanged between 15° and 35° may be explained by assuming equal changes of the denominator and the numerator. A similar indifference to temperature of $K_{m(app)}$ for the BAEE hydrolysis has been reported in the case of papain while $k_{3(app)}$ increases with temperature to the same extent as with bromelain (Smith and Parker, 1958).

Acknowledgment

The authors express appreciation to Miss Ayako Suzuki for her able technical assistance.

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