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Fractionation and Partial Characterization of the Proteolytic Enzymes of Stem Bromelain*

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By modification of the procedure of Murachi and Neurath five proteolytically active components have been separated from crude stem bromelain by chromatography on Bio-Rex 70 at *p*H 6.10. Zone electrophoresis on Sephadex G-75 also gave five proteolytically active components. These components had similar absorbancy at 280 mμ and similar specific activities on casein at *p*H 7.0 and in clotting milk. They differed in chromatographic properties on Bio-Rex 70, electrophoretic properties on Sephadex G-75 and cellulose acetate, absorbancy at 260 and 292 mμ, stability to heat, inhibition by iodoacetamide, activity on α-benzoyl-L-argininamide, and variation in activity on casein at different *p*H values.

The presence of proteolytic activity in the juice of the pineapple fruit has long been known (Chittenden, 1892), and some of the properties of this activity have been described (Chittenden, 1892; Caldwell, 1905; Willstätter *et al.*, 1926; Maschmann, 1934; Bergmann and Fraenkel-Conrat, 1937; Berger and Asenjo, 1939; Bergmann *et al.*, 1937; Greenberg and Winnick, 1940; Balls *et al.*, 1941). A number of studies have indicated that there are at least two proteolytic enzymes in the juice of the fruit (Chittenden, 1894; Caldwell, 1905; Bergmann *et al.*, 1937). Heinicke and Gortner (1957) reported that the juice of the stem of the pineapple plant also contains proteolytic activity. On the basis of its action on different proteins at several *p*H values they concluded that stem bromelain is a mixture of four proteases. Ota *et al.* (1961) have reported work on the fractionation of bromelain. Murachi and Neurath (1960) separated two proteolytically active fractions from stem bromelain by ion exchange column chromatography on Duolite CS101 and reported some properties of these two fractions.

The purpose of the present study, which was well under way at the time the publication of Murachi and Neurath appeared, was to separate the several proteolytic enzymes of stem bromelain and to characterize these components partially. It is anticipated that the ready availability and commercial usefulness of stem bromelain will stimulate a lot of basic research on this group of enzymes. It will be shown in this report that stem bromelain can be fractionated, by a slight modification of the procedure of Murachi and Neurath, into five components which have proteolytic activity. Some of the properties of these components will be presented.

Materials and Methods

"Bromelain" No. 15 from the Dole Corporation, Honolulu, Hawaii was used.[1] The yellowish-brown powder was readily soluble in water and dilute salt solutions, had a specific activity against casein at *p*H 7.0 of 0.77, and contained 3.20% water and 51.5% protein on a moisture-free basis by the biuret method (Layne, 1957).

Amberlite IRC-50 (XE-64, 200–400 mesh, lot No. 124) was obtained from Rohm and Haas Co., Philadelphia, Pa. Bio-Rex 70 (200–325 mesh, control No. DS 2572 B-1065) was obtained from Bio-Rad Laboratories, Richmond, Calif. Bio-Rex 70 is prepared by grinding, sizing, and purifying standard-grade Duolite CS101 resin. DEAE-cellulose[2] was obtained from Eastman Kodak Company, Rochester, N. Y. CM-cellulose was prepared in this laboratory from Whatman coarse-grade, ashless cellulose (carefully sieved dry to give 100-mesh size) by the method of Peterson and Sober (1956). It had a titratable carboxyl content of 0.41 meq per g. Before use, it was washed with cysteine (1.25×10^{-2} M) to remove all traces of monochloroacetic acid. Sephadex G-75 (lot No. To 6386) was obtained from Pharmacia, Uppsala, Sweden. It was screened to obtain 100–150 mesh size (dry) for use. Purified potato starch (lot No. 91662) was from J. T. Baker Chemical Co., Phillipsburg, N. J. Hydrolyzed starch (lot No. 159) for gel electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto, Canada.

L-Cysteine hydrochloride, "Hammersten-quality" casein, ninhydrin, hydrindantin, *N*-ethyl maleimide, and iodoacetamide were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Versene was from Eastman Organic Chemicals, Rochester, N. Y. α-Benzoyl-L-argininamide hydrochloride monohydrate was obtained from Mann Research Laboratories, Inc., N. Y. Carnation instant nonfat dry milk was used in assaying milk-clotting activity. Deionized water was used throughout.

Proteolytic activity was measured by the method

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[1] We are indebted to Dr. Ralph Heinicke of Dole Corporation for this sample.

[2] The following abbreviations are used: DEAE-cellulose, diethylaminoethyl cellulose; CM-cellulose, carboxymethylcellulose; BAA, α-benzoyl-L-argininamide.

of Kunitz (1947) in the presence of 1% casein and 1.25×10^{-2} M each cysteine and Versene at *p*H 7.0 (except for *p*H-activity experiments) and 35.0°. Relationship between change in optical density and enzyme concentration was established over the range used. Protein content of the purified enzyme solutions was measured by the Lowry method (Layne, 1957). Specific activity was expressed as the change in absorbency at 280 mμ per minute per mg enzyme protein. The activity toward BAA was measured by the ninhydrin method (Moore and Stein, 1954) in the presence of 0.02 M BAA and 1.0×10^{-3} M each of Versene and mercaptoethanol at *p*H 7.2 (phosphate buffer containing NaCl, $\mu = 0.35$) and 35.0°. *N*-Ethyl maleimide was used to prevent interference of the mercaptoethanol with the ninhydrin reaction (Whitaker, 1961). Specific activity was expressed in terms of μmoles BAA hydrolyzed per minute per mg enzyme protein. Milk-clotting activity was determined by the method of Balls and Hoover (1937) as modified by Whitaker (1959). One unit of enzyme activity was defined as that amount which would clot 1 ml milk in 1 minute at 35.0°. Specific activity was expressed as units activity per mg enzyme protein.

Amberlite IRC-50 (XE-64) was purified by the method of Hirs (1955). Columns (1.8 × 40 cm) were prepared in 0.2 M *p*H 5.0 acetate and 0.2 M *p*H 6.0 and 7.0 phosphate buffers. Salt elution with a logarithmic gradient was used. DEAE-cellulose columns (1.8 × 40 cm) were prepared in 0.001 M buffers at *p*H 4.0 (acetate), *p*H 8.0 (phosphate), and *p*H 9.0 (borate; 2.35 × 20.5 cm column used). Salt elution with a logarithmic gradient was used. CM-cellulose columns (1.8 × 40 cm) were prepared in 0.01 M sodium phosphate buffers at *p*H 6.1 and 7.0. Salt elution with a linear gradient was used, with 500 ml of 0.01 M phosphate in the mixing chamber and 500 ml of 1.37 M NaCl in the 0.01 M buffer in the second vessel.

Chromatography on Bio-Rex 70 was carried out essentially as described by Murachi and Neurath (1960) for chromatography of stem bromelain on Duolite CS101. The resin was suspended in 0.25 N NaCl–0.25 N HCl and stirred well. It was then washed with water until free of chloride ions and suspended in 0.1 M potassium phosphate buffer, *p*H 6.10. The *p*H was adjusted with KOH over a period of time. The resin was filtered, suspended in new buffer, and allowed to stand for at least 4 hours before the column was packed. The *p*H must remain at the desired value. The columns (1.8 × 40 cm) were poured in one segment by use of an extension tube. All subsequent operations were carried out in a cold room (approximately 2°). The column was washed overnight with 500 ml of the 0.1 M *p*H 6.10 buffer. One gram of crude bromelain was dissolved in 10 ml of water and dialyzed against two changes of 2 liters of 0.1 M potassium phosphate, *p*H 6.10, overnight. After centrifugation, 5 ml of the supernatant fluid was added to the top of the column. Elution was started with 0.1 M *p*H 6.10 potassium phosphate buffer, and 5-ml fractions were collected at a rate of 0.5 ml/minute. Optical density was measured on all fractions at 280 mμ. After the breakthrough, further elution was carried out by successive application of two linear salt gradients. The first gradient, started at fraction No. 51, had 500 ml of 0.1 M *p*H 6.10 potassium phosphate buffer in the mixing vessel and 500 ml of 0.6 M KCl in the 0.1 M buffer in the second vessel. The second gradient, started at tube No. 101, had 500 ml of 0.3 M KCl in 0.1 M *p*H 6.10 potassium phosphate buffer in the mixing vessel and 500 ml of 2 M KCl in the 0.1 M buffer in the second. Activity against casein was measured on every second tube. Fractions of each component with high activity were combined, frozen rapidly, and stored at −30°. Fractions were collected and pooled from several columns. For rechromatography, 10 g (wet weight) Bio-Rex 70, prepared as for a column, were added to the pooled components, and the slurry was transferred to a dialysis bag and dialyzed overnight against 12 liters of 0.1 M *p*H 6.10 potassium phosphate buffer. The resin was filtered on a small Buchner funnel and contained over 90% of the protein originally in the solution. The resin-enzyme was transferred to the top of a 1.8 × 35 cm Bio-Rex 70 column to give a total height of 40 cm. Chromatography was then carried out as described above.

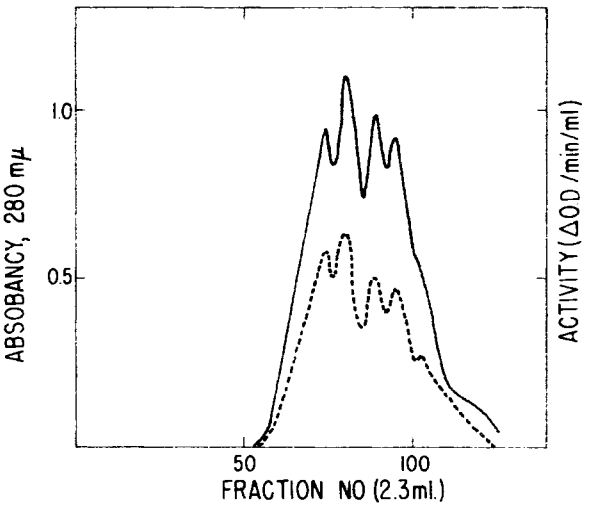

FIG. 1.—Electrophoretic separation of CM-cellulose-purified bromelain on Sephadex G-75. Crude bromelain was purified on CM-cellulose at *p*H 7.0 (Fig. 2), and the active fraction was precipitated with ammonium sulfate and dialyzed against 0.05 M *p*H 5.0 acetate buffer containing 0.1 N NaCl and 0.2 g applied to a Sephadex G-75 column (3.0 × 50 cm) packed in the same buffer. Electrophoresis was carried out for 38 hours at 2°, 100 ma and 100 volts. The material was then eluted with the acetate buffer. The solid line represents the absorbancy at 280 mμ, the dashed line the activity on casein at *p*H 7.0.

Separation of crude bromelain on a Sephadex G-75 column (4.0 × 35 cm) was carried out at 2° with water as the eluent and at a flow rate of 1–2 ml/minute.

Electrophoresis on starch and Sephadex G-75 columns (3.0 × 50 cm) at 2° and *p*H 5.0 in 0.05 M sodium acetate containing 0.1 M NaCl was carried out essentially as described by Flodin and Porath (1954). After 38 hours at 100 ma and 100 volts electrophoresis was stopped and the columns eluted with the buffer.

Free boundary electrophoresis was carried out in a Tiselius electrophoresis apparatus, Perkin-Elmer Model 38. All runs were performed at 4° with a 2-ml cell assembly and silver-silver chloride electrodes. Zone electrophoresis on paper (2.5-cm wide S and S 2043A) was carried out at 2° for 20 hours at 0.3 to 1.0 ma/strip and 200–400 volts with a Spinco Model R Durram-type unit. Zone electrophoresis on cellulose acetate was performed on 1.0 × 12.5 cm strips held horizontally for 1 hour at 23° and 1–2 ma per strip and 300 volts. Protein was stained with Amido 10B. Starch gel electrophoresis was carried out at *p*H 5.0, 7.0, and 8.5 as described by Smithies (1955).

Heat stability of the enzymes was determined on solutions containing 0.3 mg protein/ml in 0.1 M *p*H 6.10 potassium phosphate buffer containing 0.5 M KCl. Iodoacetamide inhibition of the enzymes was

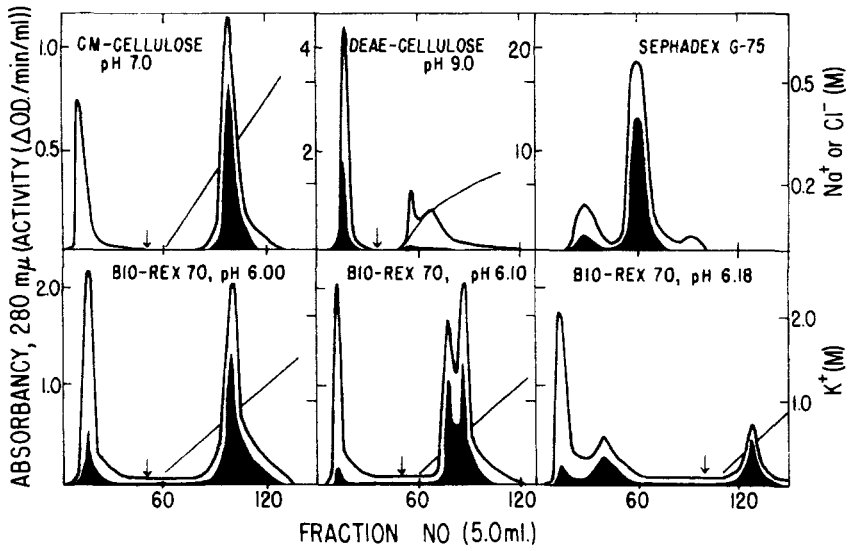

FIG. 2.—Chromatography of crude stem bromelain on CM-cellulose, DEAE-cellulose, Sephadex G-75, and Bio-Rex 70. Dialyzed solutions containing 0.2 g enzyme were applied to CM- and DEAE-cellulose columns, 1.8 g to Sephadex G-75 column, and 0.5 g to the Bio-Rex 70 columns. For chromatography on Bio-Rex 70 the starting buffer was 0.2 M potassium phosphate; for gradient elution, the mixing bottle contained 500 ml of 0.2 M potassium phosphate buffer and the second bottle 500 ml of 2 M KCl in 0.2 M potassium phosphate buffer. All other conditions were as described in the text. The line curves represent absorbancy at 280 mμ, the shaded areas activity on casein at *p*H 7.0.

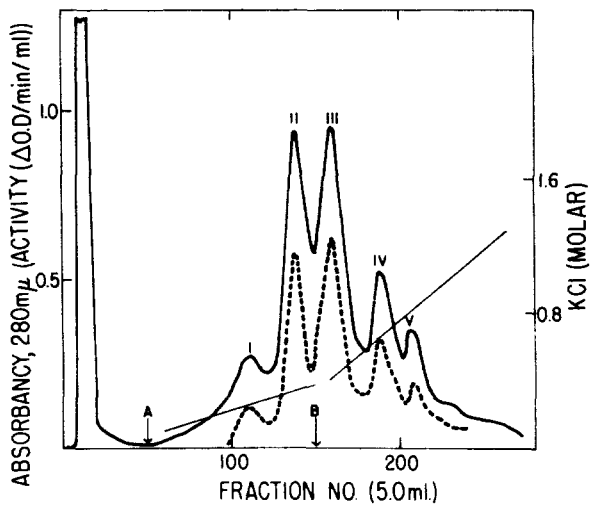

FIG. 3.—Chromatography of crude stem bromelain on Bio-Rex 70 at *p*H 6.10. Five ml of a dialyzed solution of bromelain (0.5 g) was applied to a 1.8 × 40 cm column. Starting buffer was 0.1 M *p*H 6.10 potassium phosphate. Gradient A, started at fraction No. 51, had 500 ml of 0.1 M *p*H 6.10 potassium phosphate buffer in the mixing bottle and 500 ml 0.6 M KCl in the 0.1 M buffer in the second bottle. Gradient B, started at fraction No. 101, had 500 ml of 0.3 M KCl in 0.1 M *p*H 6.10 potassium phosphate buffer in the mixing bottle and 500 ml of 2 M KCl in the 0.1 M buffer in the second bottle. Solid line represents absorbancy at 280 mμ, the dashed line activity on casein at *p*H 7.0 Per cent recovery: activity, 76.4%; protein, 91.5%. OD of breakthrough peak was 1.85.

carried out at room temperature (approximately 23°) for 1 hour on solutions containing 0.3 mg protein/ml in 0.1 M *p*H 6.10 potassium phosphate buffer containing 0.5 M KCl. Residual activity of a 0.1-ml aliquot was determined on casein with 1.25×10^{-2} M cysteine in the reaction mixture.

All *p*H measurements were made with a Beckman Model G *p*H meter.

RESULTS

Electrophoresis.—Unless otherwise indicated the experiments reported here were performed on the active fraction obtained from the chromatography of crude bromelain on CM-cellulose at *p*H 7.0 (Fig. 2). Zone electrophoresis on paper strips was carried out at *p*H 5.0 and 6.0 (0.1 M acetate) and 6.0 and 7.0 (0.1 M phosphate). The protein remained at the origin. Zone electrophoresis on cellulose acetate strips (*p*H 7.0 0.02 M sodium phosphate and *p*H 9.6 0.025 M each Veronal–sodium acetate) gave two components which moved quite rapidly (approximately 1.5 cm/hour) and with only a little separation. Zone electrophoresis on starch columns (*p*H 5.0 0.05 M acetate and *p*H 7.0 0.05 M phosphate, both of which contained 0.1 M NaCl) gave only one component. Free boundary electrophoresis (*p*H 5.0, 5.5, and 6.0 0.05 M acetate, *p*H 7.0 and 7.5 0.05 M phosphate, and *p*H 8.0 and 8.7 0.05 M glycine buffers, all containing 0.1 M NaCl) for 3 hours at 20 ma and 100 volts gave two components which moved as cations at all *p*H values used. Our crude stem bromelain, under the conditions Murachi and Neurath used except that NaCl was used in place of LiCl, gave two main components which moved as cations. Zone electrophoresis on Sephadex G-75 at *p*H 5.0 gave five components, all with proteolytic activity (Fig. 1). Subsequent experiments have shown that there is a small amount of ion exchange of bromelain on Sephadex G-75 under the conditions used here.

Chromatography.—Chromatography of crude bromelain on Amberlite IRC-50 at *p*H 5.0, 6.0, and 7.0 failed to separate the material. At *p*H 5.0, the protein was so strongly adsorbed it was not removed with a salt gradient. At *p*H 6.0 and 7.0 all the material came off with the starting buffer. Chromatography of crude bromelain on CM-cellulose at *p*H 6.10 and 7.00 gave two components having an absorbancy at 280 mμ: an inert component, which constituted approximately 33% of the material, and a second component, eluted by the salt gradient, which contained all the proteolytic activity (for results at *p*H 7.0 see Fig. 2). The peak fraction had a specific activity on casein of 1.14. Starting buffer elution of component 2 with 0.15 M NaCl in 0.01 M *p*H 7.0 phosphate buffer removed

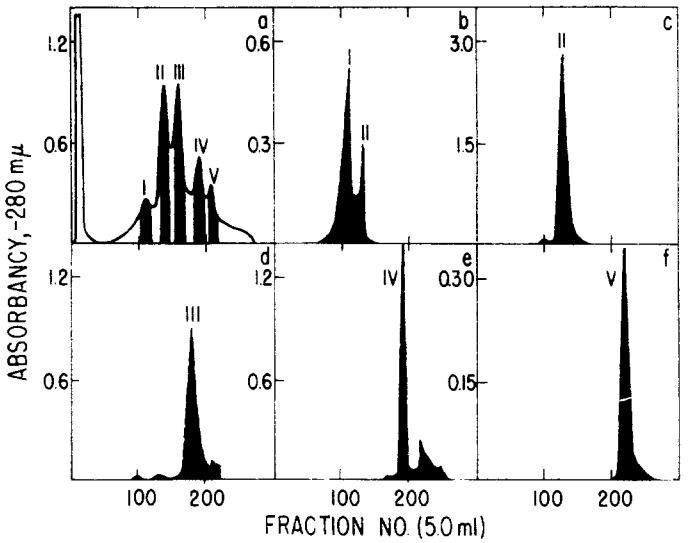

FIG. 4.—Rechromatography of the five proteolytically active components on Bio-Rex 70 at *p*H 6.10. Part *a* shows the portion of each component pooled from the chromatography of crude bromelain (shaded areas). For rechromatography, the protein from the pooled fractions of several columns was adsorbed onto 10 g Bio-Rex 70 at *p*H 6.10 by dialysis against 0.1 M *p*H 6.10 potassium phosphate buffer. The resin-enzyme was removed by filtration and applied to the top of a 1.8 × 35 cm Bio-Rex 70 column. Rechromatography was carried out as described in Fig. 3.

all the material but gave a nonsymmetrical peak which indicated the presence of more than one component. On chromatography of crude bromelain on DEAE-cellulose at *p*H 4.0, 8.0, and 9.0, better than 80% of the active material came off in the breakthrough (for results at *p*H 9.0 see Fig. 2). Fractions on the frontal side of the breakthrough peak at *p*H 9.0 had specific activities as high as 2.0. However, at this *p*H only 30% of the activity added to the column was recovered although most of the protein came off. Chromatography on Sephadex G-75 gave two active components (Fig. 2). The first component contained 10% of the total activity with a maximum specific activity on casein of 0.50. The peak fraction of component 2 had a specific activity of 1.12. This may indicate a difference in molecular weights among the active components of bromelain.

As indicated by Murachi and Neurath (1960), chromatography on Bio-Rex 70 (Duolite CS101) is very sensitive to *p*H (Fig. 2). At *p*H 6.00 most of the activity was found in a single component eluted by the salt gradient. The peak fraction had a specific activity against casein of 0.99. At *p*H 6.10 most of the activity was found in two components eluted by the salt gradient. The first peak fraction had a specific activity of 0.97, the second 0.96. At *p*H 6.18 most of the activity was obtained in two peaks, one of which came off before the salt gradient. The specific activity of the first peak fraction was 0.83, the second 1.13. The results obtained here at *p*H 6.18 correspond very closely to those obtained at *p*H 6.05 by Murachi and Neurath (1960). This indicates that one must determine the best *p*H to use with different batches of resin and then must control that *p*H very precisely in order to get reproducible results.

The results obtained here with Bio-Rex 70 indicated *p*H 6.10 to be the best *p*H to use for fractionation of crude bromelain. By the use of 0.1 M *p*H 6.10 potassium phosphate as the starting buffer and elution with two successive salt gradients, crude bromelain can be resolved into five components with proteolytic activity (Fig. 3). Further modification of the gradient has failed to indicate the presence of other components. Recently, the use of 0.5 M KCl in place of 0.6 M KCl in the second vessel of the first gradient has given better separation of components II and III. The approximate composition of crude bromelain was found to be 32.6% inert breakthrough material, and component I, 11.3%; II, 18.6%; III, 20.5%; IV, 10.2%; and V, 6.8%.

A number of column separations were performed under the above conditions, and the central fractions of each component were pooled, adsorbed on Bio-Rex 70, and then rechromatographed under the same conditions previously used in their separation (Fig. 4). Each component was eluted from the column in nearly the same elution volume as used for the crude material. On rechromatography, component II came off a little early (9 fractions) and component III a little late (20 fractions). This probably is a reflection of the degree of interaction among these two main components of crude bromelain. The data substantiate the conclusion that each of the components is chromatographically a separate entity. It is interesting to note that there appears to be very little tailing of components on this column of Bio-Rex 70. We have found tailing to be quite severe on CM-cellulose columns.

Properties of Components I–V.—All results reported here on components I-V were obtained on the rechromatographed material which was stored frozen. The effect of *p*H on the activity on casein of crude bromelain and components I-V is shown in Figure 5. With the exception of component I, all had maximum activity at *p*H 8.5. Component I had maximum activity at *p*H 6.0, although it had a second peak of activity at *p*H 8.5. Component IV also had a second peak of activity at *p*H 6.0, while component II had a second peak of activity at *p*H 7.0. All five components had approximately the same specific activity on casein at *p*H 7.0 (see also Table II). It is not clear why some of the components should have two *p*H optima. Presence of more than one *p*H optimum is usually considered to be due to the presence of more than one enzyme. Schwimmer (1962) has recently discussed the theory of double *p*H optima of enzymes and has offered three situations which could result in double *p*H optima. It would appear that a fourth situation could result when an enzyme is acting on as heterogene-

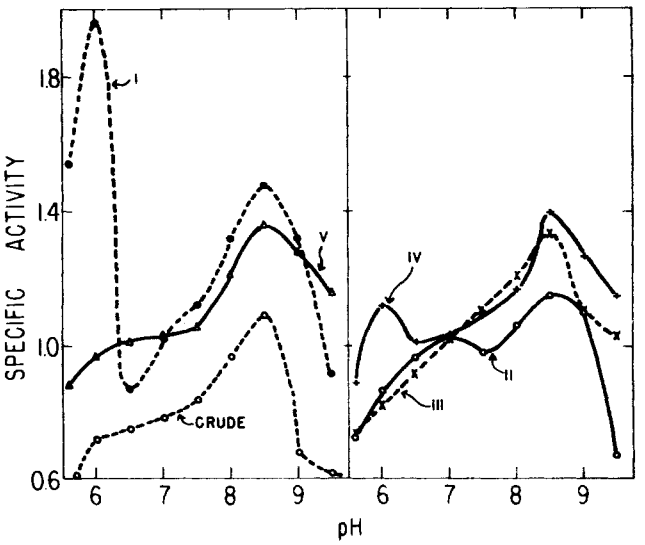

FIG. 5.—Effect of *p*H on the activity of crude stem bromelain and components I–V on casein at 35°. Activity was determined by the method of Kunitz (1947) in the presence of 1% casein, 0.2 M buffers and 1.25×10^{-2} M each Versene and cysteine. Acetate (*p*H 5.6–6.0), phosphate (*p*H 6.0–8.0), and borate (*p*H 8.0–9.5) buffers were used.

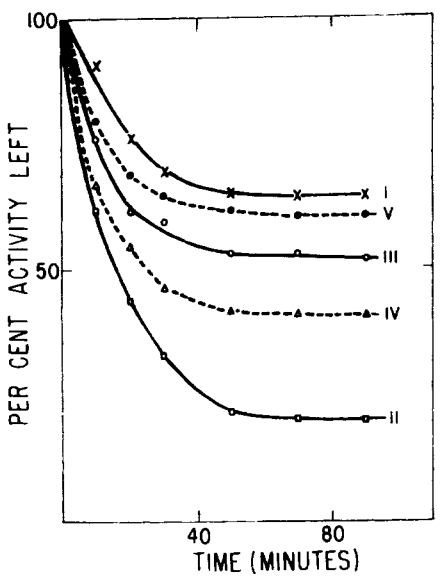

FIG. 6.—Heat stability of components I–V of bromelain. Solutions containing 0.3 mg/ml of enzyme protein in 0.1 M *p*H 6.10 potassium phosphate buffer containing 0.5 M KCl were held at 55.0°. At intervals samples were removed and their activity on casein at *p*H 7.0 was determined as described in Fig. 5.

ous a substrate as a protein and that multiple *p*H optima could be a reflection of the relative rates at which certain selected bonds in the substrate are split at different *p*H values.

The stability of the different components when held at 55° is shown in Figure 6. There was quite a marked difference in the degree of heat stability of the components, with stability decreasing in the order I, V, III, IV, and II.

The effect of iodoacetamide on the five components is shown in Figure 7. Components I and V were markedly inhibited by iodoacetamide. Components III, IV, and II, with I_{50} values of approximately 8.0×10^{-4} M, 1.0×10^{-3} M, and 2.2×10^{-3} M, are not inhibited by iodoacetamide to the same degree as components I and V.

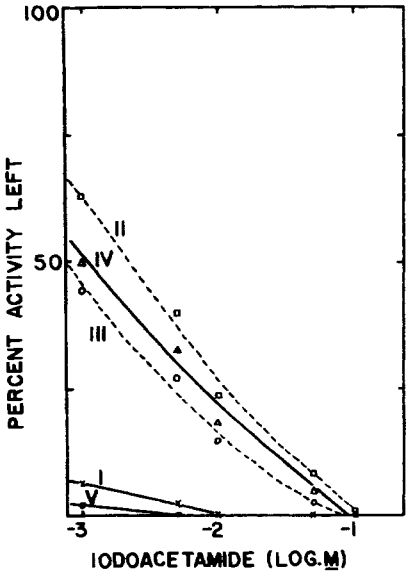

FIG. 7.—Inhibition of components I–V of bromelain by iodoacetamide. Solutions containing 0.3 mg/ml enzyme protein in 0.1 M *p*H 6.10 potassium phosphate buffer containing 0.5 M KCl were incubated at room temperature (approximately 23°) with iodoacetamide. After one hour, the activity of a 0.1-ml aliquot was determined on casein at *p*H 7.0 as described in Fig. 5.

The absorbancy at 260, 280, and 292 mμ of 1% solutions ($E_{1\text{ cm}}^{1\%}$) of the different components is given in Table I. At 280 mμ the values are remarkably constant, but at 260 and 292 mμ there are significant differences among the components. The protein concentration of the solutions was determined by the Lowry method (Layne, 1957).

TABLE I
EXTINCTION COEFFICIENTS ($E_{1\text{ cm}}^{1\%}$) OF THE RECHROMATOGRAPHED COMPONENTS AT 260, 280 AND 292 mμ

Component	$E_{1\text{ cm}}^{1\%}$ 280	260	292	280/260	280/292
I	16.1	8.32	7.45	1.94	2.16
II	16.1	7.75	7.61	2.08	2.12
III	16.1	8.86	8.72	1.82	1.85
IV	16.4	8.86	8.70	1.85	1.89
V	15.8	8.29	8.29	1.91	1.91

The activities of the different components on casein, milk, and BAA are given in Table II. Casein digesting and milk-clotting specific activities were constant among the components. Components II and V appeared to have approximately one half and three fourths, respectively, as much specific activity on BAA as the other three components

Electrophoresis of the five lyophilized components on cellulose acetate strips at *p*H 9.62 (0.025 M each Veronal–sodium acetate, specific resistance at 24.9° of 279 ohm cm) gave the following movements toward the cathode: I, 1.71 cm; II, 2.28 cm; III, 2.42 cm; IV, 2.22 cm; and V, 2.14 cm. Thus it would appear that the isoelectric points of all these components are above 9.6.

DISCUSSION

By modification of the procedure of Murachi and Neurath (1960) crude stem bromelain has been separated into five components with proteolytic activity. Rechromatography of these components under the

TABLE II
ACTIVITY OF COMPONENTS I-V ON CASEIN, MILK, AND BAA

Component	Casein Digestion[a]	Milk Clotting[b]	BAA[c] ($\times 10^3$)	Casein Digestion / Milk-Clotting	Casein Digestion / BAA ($\times 10^{-1}$)
I	1.02	6.30	16.4	0.16	6.2
II	1.03	6.10	8.5	0.17	12.1
III	1.02	6.11	16.1	0.17	6.3
IV	1.03	6.10	15.7	0.17	6.6
V	1.03	6.31	11.5	0.16	9.0

[a] Proteolytic activity was measured by the method of Kunitz (1947) in the presence of 1% casein and 1.25×10^{-2} M each cysteine and Versene at *p*H 7.0 and 35.0°. Specific activity was expressed as the change in absorbancy at 280 mμ per minute per mg enzyme protein. [b] Milk-clotting activity was determined at 35.0° by the method of Balls and Hoover (1937) as modified by Whitaker (1959). Specific activity was defined as units activity per mg enzyme protein (one unit activity will clot 1 ml milk in one minute). [c] BAA activity was measured by the ninhydrin method (Moore and Stein, 1954) in the presence of 0.02 M BAA and 1.0×10^{-2} M each Versene and mercaptoethanol at *p*H 7.2 (phosphate buffer containing NaCl, $\mu = 0.35$) and 35.0°. Specific activity was expressed in terms of μmoles BAA hydrolyzed per minute per mg enzyme protein. Protein was determined by the Lowry method (Layne, 1957).

same conditions indicated that they were chromatographically separate entities and could be obtained in reasonable purity (by this criterion) by one passage through the column. Chromatography on CM-cellulose failed to separate these components. It is suggested that strong complexation occurs in crude bromelain, mainly owing to the large amount of acidic polysaccharides present (Heinicke and Gortner, 1957) and that separation can be achieved on Bio-Rex 70, and not on CM-cellulose, because of the high ionic strength that can be employed. Separation into five components was also achieved by electrophoresis on Sephadex G-75 columns but not on starch columns. This difference could be due to the ability of the Sephadex G-75 to separate the polysaccharides from the proteins.

In agreement with the results of Murachi and Neurath, very little increase in specific activity was obtained by the fractionation. The components, after rechromatography on Bio-Rex 70, had an increased specific activity of approximately 33% over that of the crude material. DEAE-cellulose chromatography gave a small amount of material with a specific activity on casein as high as 2.0, but because of the instability of bromelain at *p*H 9.0 this was not investigated further.

The five components separated by chromatography had similar absorbancy at 280 mμ and similar specific activities on casein at *p*H 7.0 and in clotting milk. However, there were differences in their heat stabilities, electrophoretic movement on cellulose acetate at *p*H 9.6, inhibition by iodoacetamide, absorbancy at 260 and 292 mμ, and specific activities on BAA and in the effect of *p*H on the activity of these components on casein. Obviously, they differ in their chromatographic properties under the conditions used in this study. Murachi and Neurath (1960) reported that the only differences between their two components were in electrophoretic mobility and chromatographic behavior.

A more complete understanding of the similarities and differences among these components must await the results of work on the molecular weights, amino acid composition, and electrophoretic properties and of detailed specificity studies, "active center" studies, and "fingerprinting" of the different components. It is hoped this report will stimulate such studies. One must caution against the acceptance of the "apparent" homogeneity of each of the five components without a great deal of additional proof. Murachi and Neurath (1960) found their two fractions to be homogeneous with respect to chromatography (under their conditions), electrophoresis, and sedimentation in the ultracentrifuge. It would be desirable to find other conditions of chromatography (different resins and *p*H) by which the purity of the components obtained on Bio-Rex 70 could be checked. So far, we have not had any success along these lines.

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