Fractionation and Specificity Studies on Stem Bromelain*

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The presence, in the juice of pineapples, of a proteolytic enzyme or enzymes under the name of bromelin (2) has long been known, and some of the characteristics of this enzyme have been described (3-7). The juice of the stem of the pineapple plant was found by Heinicke and Gortner (8) to contain a similar enzyme. The acetone precipitate from the stem juice, which is called stem bromelain,† is now available in quantity, and this was used as the starting material in the present investigation.

The purpose of the present study was to purify and characterize stem bromelain to such an extent that further investigations could be carried out on the structure of the enzyme and the mechanism of its action. Such an analysis is of particular interest in view of recent studies on crystalline papain (10), since similarities of the pineapple enzyme to papain have been reported by previous investigators (3-5). Another objective of this investigation was to determine the possible usefulness of this enzyme for studies of the amino acid sequence of peptides and proteins.

EXPERIMENTAL

Materials

Stem bromelain. “Bromelain” No. 10 from the Hawaiian Pineapple Company, Honolulu, Hawaii, was used.† The light brown, dry powder could be fairly readily dissolved in water or in 1% NaCl to make a 10% solution.

Trypsin (lot No. T746F) was a product from Worthington Biochemical Corporation, Freehold, New Jersey. The enzyme was dissolved in 0.01 M HCl and dialyzed extensively in the cold against 0.001 M HCl before use.

Casein and hemoglobin were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. BAEE† and other synthetic substrates were prepared in this laboratory or purchased from Mann Research Laboratories, Inc. Crystalline glucagon lot No. 238-234B-54-2 was obtained from Eli Lilly and Company, Indianapolis, Indiana. A preparation of B chain of oxidized insulin was prepared by the method of Sanger and Thompson (11) from crystalline beef insulin, T-2842, obtained from Eli Lilly and Company.

Iont Exchange Resins. DEAE-cellulose was prepared in this laboratory from Solka-Floc BW200 according to the method described by Peterson and Sober (12). CM-cellulose was prepared from Whatman standard grade, ashless cellulose according to Kies and Simpson (13). Duolite CS101 resin was a product obtained from Bio-Rad Laboratories, Berkeley, California. Lot No. B413, 200 to 400 mesh, was used.

DFP was prepared in this laboratory by Mr. E. S. Awad according to the method of Saunders and Stacey (14).

Measurement of Enzymatic Activity

Proteolytic Activity—Hydrolysis of casein was measured according to the method of Kunits (15) and that of hemoglobin according to that of Anson (16), by use of a Beckman model DU spectrophotometer to determine the trichloroacetic acid soluble peptides at 280 mμ. The nitrogen content of the enzyme solution was determined by the Kjeldahl method. Specific activity was expressed as the change in absorbancy at 280 mμ per minute per mg enzyme N.

Peptidase Activity—The activities toward BAEE, ATEE, and hippuryl-dl-phenyllactate were measured by the esterase method (17). The assays were carried out by autotitration with 0.1 N NaOH in a “pH-stat” (18, 19), manufactured by International Instrument Company. The assembly was used with a temperature-controlled reaction vessel designed for small volumes (20). The hydrolysis of L-leucinamide was assayed also in a pH-stat with 0.1 N HCl, based on the calculation that at pH 8.05 the cleaving of 1 mole of leucinamide causes the uptake of 0.48 mole of acid. The hydrolysis of BAA was measured by determining ammonia liberation in a Conway unit, and that of CBZ-L-glutamyl-L-tyrosine by determining tyrosine liberation with the ninhydrin reagent (21). Specific activity was expressed in terms of μmoles substrate hydrolyzed per minute per mg enzyme N.

Enzymatic Anilide Synthesis—CBZ-glycinanilide synthesis was measured according to the method of Murachi (22). The activity was expressed in terms of the first order rate constant, k1, per mg enzyme N.

Column Chromatography

Column chromatography of the crude bromelain on Duolite CS101 was carried out essentially as described by Hirs (23) for the chromatography of proteins on Amberlite IRC-50 (XE-84).
All operations were conducted at cold room temperature (approximately 4°). One gram of crude bromelain was dissolved in 10 ml of water and dialyzed against two changes of 2 liters of 0.2 m potassium phosphate buffer, pH 6.05, overnight. After centrifugation, 10 ml of the supernatant fluid were applied to the resin column (1.8 x 40 cm) which had been equilibrated with the same buffer. A continuous flow of 0.2 m potassium phosphate buffer, pH 6.05, was begun and fractions of 10 ml were collected. The optical density of each fraction was measured at 280 nm.

Further elution was carried out by application of a linear concentration gradient with respect to potassium ion, by the use of two reservoirs each containing 400 ml of 0.2 m potassium phosphate buffer but one of them, in addition, 2 m KCl. Those fractions which showed high activities toward casein were immediately combined, dialyzed overnight against three changes of 7 liters of distilled water, and lyophilized.

Tri-chromatographic runs of the crude bromelain were made also on DEAE-cellulose at pH 8.0 and on CM-cellulose at pH 6.0, according to the procedures described by Keller et al. (24), and by Ellis and Simpson (13), respectively.

![Fig. 1.](http://www.jbc.org/) Effect of cysteine, KCN, and EDTA on the casein digestion by crude stem bromelain. X, none; Δ, EDTA; □, KCN; ●, cysteine; ○, cysteine and EDTA. A total volume of 20 ml contained 0.023 mg enzyme protein N, 0.5% casein, 0.075 m potassium phosphate buffer, pH 7.2, and 0.005 m effectors. Incubation was at 35°. An aliquot of 2 ml was mixed with 3 ml of 5% trichloroacetic acid at given time intervals and the absorbancy of the supernatant fluid was measured at 280 μm.

![Fig. 2.](http://www.jbc.org/) pH-dependence of hydrolysis of BAE by crude stem bromelain. Reaction mixture contained 0.17 mg enzyme protein N, 0.02 m BAE, 0.01 m potassium phosphate buffer, 0.01 m cysteine, 0.002 m EDTA, and 0.1 m KCl in a total volume of 2.5 ml. The reaction was followed by autotitrination with 0.1 n NaOH in a pH-stat at 25°.

**RESULTS**

**Enzymatic Activity of Crude Stem Bromelain**

**Effect of Cysteine**—Casein was rapidly hydrolyzed by crude enzyme, as shown in Fig. 1. When cysteine was added to the reaction mixture, the enzyme retained activity through a longer period of incubation. Similar findings were obtained with BAEE as substrate. The addition of EDTA besides cysteine was not essential for full activity, in contrast to mercuripapain (10). As shown in Fig. 1, KCN also had an activating effect.

**Effect of HgCl₂**—Crude bromelain was strongly inhibited by mercuric ion. Thus, 0.79 x 10⁻⁴ m HgCl₂ caused complete inhibition of the enzymatic hydrolysis of casein when tested under the condition described for Fig. 1, but with three times as much of the enzyme. Inhibition of 50% was observed at 0.45 x 10⁻³ m HgCl₂. Inhibition was instantaneous and could be completely reversed by the addition of excess cysteine (0.001 m), suggesting that sulfhydryl groups of the enzyme protein are essential for its catalytic activity.

**pH Dependence**—The pH activity curve for the hydrolysis of BAEE by crude bromelain, presented in Fig. 2, showed a broad maximum near pH 7.0. A similar pattern of pH dependence was obtained with casein or hemoglobin as substrates.
Action on Various Substrates—As shown in Table I, the enzyme hydrolyzes casein and hemoglobin, and among synthetic substrates BAEE and BAA, which are trypsin substrates, but not ATEE, a substrate for chymotrypsin, or CBZ-L-glutamyl-L-tyrosine, a substrate for papain. The activities toward L-leucinamide, a leucinaminopeptidase substrate, and toward hippuryl-dl-β-phenyllactate, a carboxypeptidase-A substrate, were much less than that toward BAEE. The enzyme catalyses the synthesis of anilide from CBZ-glutamic acid and aniline as the fruit enzyme was reported to do (5).

As also shown in Table I, bromelain activity is comparable to that of papain. Both enzymes have similar activities toward casein, but toward hemoglobin bromelain is four times as active as papain, whereas toward synthetic trypsin substrates bromelain has only 1/4 or 1/6 of the activity of papain, suggesting that the specificities of the two enzymes are not the same.

Effect of DFP—DFP, dissolved in n-propanol, was preincubated with crude bromelain at 25° for 1 hour at pH 7.2, and the enzymatic activity toward casein was subsequently measured in the presence and absence of 0.001 M cysteine, under the same conditions as described for Fig. 1. It was found that 0.001 M DFP neither inhibits the enzyme nor does it change its activation by cysteine, suggesting that, in contrast to certain other endopeptidases (28), no reaction with the active site had occurred. DFP also failed to inhibit the proteolytic activity of the chromatographically purified Fraction 5 of the enzyme preparation (see below). The present results are at variance with those recently reported by Heinicke (29), who suggested that DFP is a specific inhibitor of sulphydryl proteases.

Chromatographic Fractionation of Stem Bromelain

Electrophoresis of Crude Bromelain—It is apparent from Fig. 3 that the crude preparation contains at least two major electrophoretic components. A similar pattern was also observed in the presence of 0.001 M HgCl₂.

Column Chromatography—Attempts were made to separate the components of the crude preparation by means of column chromatography on ion exchange resins. When DEAE-cellulose was used in 0.005 M potassium phosphate buffer at pH 7.0, approximately 80% of the protein applied to the column appeared in the “break-through” peak. Incomplete resolution into two components was achieved by the use of CM-cellulose in 0.005 M potassium citrate buffer at pH 6.0 with subsequent application of a citrate concentration gradient at the same pH.

The most successful result was obtained with Duolite CS101 at pH 6.05. As shown in Fig. 4, the enzymatic activity was found largely in the protein contained in Peaks 2 and 5. Fraction 2 emerged soon after the “break-through” peak and Fraction 5 after application of a K⁺ concentration gradient. Chromatography on Duolite CS101 was found to be so sensitive to pH that at pH 5.9 no protein appeared after the “break-through” peak even after collecting 100 fractions, whereas at pH 6.2 more than 70% of the material emerged before the 50th fraction, resulting in poor resolution.

The fractions corresponding to the shaded area in Fig. 4 were combined, dialyzed, and lyophilized.

General Properties of Fractions 2 and 5—As shown in Table II, Fractions 2 and 5 have specific activities similar to one another toward casein and also toward BAEE. Both enzymes are similarly inhibited by either HgCl₂ or p-chloromercuribenzoate, inhibition being reversed by the addition of cysteine. It may be of interest to note in Table II that Fractions 2 and 5 have μν, or very low, activities toward L-leucinamide or hippuryl-dl-β-phenyllactate, whereas the crude preparation (Table I) showed appreciable hydrolysis of these substrates.

Without further purification, Fractions 2 and 5 showed reasonably high homogeneity with respect to electrophoresis and sedimentation. The calculated constants are shown in Table II.

Preparation of Crude Crystalline Bromelain

Attempts were made to obtain a crystalline enzyme preparation by fractionation of stem bromelain with ammonium sulfate and sodium chloride. The following is a preliminary description of the procedure which was found to yield crude crystalline material.

Ten grams of stem bromelain were dissolved in 50 ml of water, and 5 ml of 0.1 M HgCl₂ were added to obtain a mercury derivative of the enzyme. After adjustment to pH 7.5 with NaOH, the mixture was centrifuged for 30 minutes at 14,000 r.p.m.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Action of crude stem bromelain on various substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>pH</td>
</tr>
<tr>
<td>Casein</td>
<td>7.2</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>7.2</td>
</tr>
<tr>
<td>BAEE</td>
<td>7.2</td>
</tr>
<tr>
<td>BAA</td>
<td>7.2</td>
</tr>
<tr>
<td>L-Leucinamide</td>
<td>7.2</td>
</tr>
<tr>
<td>HPLA*</td>
<td>7.5</td>
</tr>
<tr>
<td>ATEE</td>
<td>7.2</td>
</tr>
<tr>
<td>CBZ-L-Glu-L-Tyr</td>
<td>4.0, 7.6</td>
</tr>
<tr>
<td>CBZ-Gly and aniline†</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Hippuryl-dl-β-phenyllactate.
† Synthesis of CBZ-L-glycinanilide was determined.

Fig. 3. Ascending electrophoretic pattern of crude stem bromelain in 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M LiCl, after 301 minutes at 4.03 volts cm⁻¹. Approximately 1.5% solution of enzyme protein was used. The calculated mobility values were +0.70 × 10⁻⁵ cm² volt⁻¹ sec⁻¹ for component A, and −1.17 × 10⁻⁵ cm² volt⁻¹ sec⁻¹ for component B.
Fig. 4. Column chromatography of crude stem bromelain on Duolite C8101 resin. The conditions of the experiment are given in the text. Relative activity was determined with casein as substrate under standard assay conditions (Fig. 1). The activity is shown on an arbitrary scale.

Table II

General properties of Fractions 2 and 5 of stem bromelain

The conditions of assays of the enzymatic activities and the units of specific activities are the same as in Table I. Electrophoresis and ultracentrifugation were carried out in 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M LiCl.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Fraction 2</th>
<th>Fraction 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield of lyophilized material (%)</td>
<td>7.9</td>
<td>9.3</td>
</tr>
<tr>
<td>Activity toward casein:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without cysteine</td>
<td>2.51</td>
<td>2.31</td>
</tr>
<tr>
<td>With cysteine</td>
<td>9.35</td>
<td>11.8</td>
</tr>
<tr>
<td>With HgCl₂ (10⁻⁴ M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Activity toward BAEE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without cysteine</td>
<td>1.01</td>
<td>1.05</td>
</tr>
<tr>
<td>With p-Cl-Hg-benzoate (10⁻⁴ M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Activity toward L-leucinamide</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Activity toward HPLA*</td>
<td>&lt;0.04</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Electrophoretic mobility, †μ, pH 7.4</td>
<td>-1.85 × 10⁻⁵</td>
<td>+1.18 × 10⁻⁵</td>
</tr>
<tr>
<td>Sedimentation constant, s₂₀,₀, pH 7.4</td>
<td>2.73 S</td>
<td>2.76 S</td>
</tr>
</tbody>
</table>

* Hippuryl-dl-β-phenyllactate.
† In cm² volt⁻¹ sec⁻¹.

To the supernatant solution (55 ml) were added 12.5 g of ammonium sulfate at pH 7.5 at room temperature and after 1 hour the mixture was centrifuged. The precipitate was washed with a solution of 6.25 g of ammonium sulfate in 25 ml of water and the washed precipitate was dissolved in water to make a solution of 25 ml. At pH 7.5, 2.25 g of ammonium sulfate were added and the precipitate was collected by centrifugation. Electrophoresis of the precipitate in 0.04 M potassium phosphate buffer, pH 7.4, containing 0.1 M LiCl showed that the material consisted mainly of one component having a positive mobility at that pH, whereas the starting material, as mentioned above, contained at least two major components (see Fig. 3). The precipitate was dissolved in 100 ml of water, reprecipitated by adding 30 g of NaCl, washed with saturated NaCl solution, and then dissolved in 400 ml of 80% saturated sodium chloride solution by adjusting to pH 9.0 with NaOH. When 0.1 N acetic acid was added slowly to lower the pH to approximately 8, a faint turbidity due to crystalline material appeared, which did not increase substantially by standing but could be increased by further, cautious addition of acetic acid to a yield of about 5% of the starting material. Microscopic observation revealed the presence of materials appearing like twisted long threads, which did not change in habit upon recrystallization. The specific activity of the crude crystalline product thus obtained was the same as, or only slightly higher than, that of the starting material as assayed toward casein in the presence of 0.005 M cysteine. A similar type of crude crystalline material was also obtained when stem bromelain was treated in the same manner except that 0.01 M cysteine was employed throughout the procedure instead of converting the enzyme into the mercury derivative.

Action of Bromelain on Glucagon

In order to study further the specificity of bromelain, glucagon and the B chain of oxidized insulin were used as substrates. These substrates were incubated with the enzyme, and after a certain time period the digest was subjected to various techniques for determining which of the peptide bonds was hydrolyzed by the enzyme. A more extensive study was carried out with glucagon than with insulin, since the former was found to be a better substrate for this enzyme.

Time Course of Glucagon Hydrolysis—Fig. 5 shows the progress curves of glucagon hydrolysis by fraction 5 as measured in the pH-stat. After 10 to 15 minutes of incubation the alkali uptake due to hydrolysis reached a plateau indicating the splitting of a
limited number of peptide bonds. Thereafter, no appreciable alkali uptake was observed even upon the addition of additional enzyme. In Table III are presented the amounts of alkali uptake in terms of the mole ratio NaOH to glucagon, at various pH values. The data obtained with Fractions 2 and 5 are in good agreement with each other except at pH 8.6. From such data the number of peptide bonds split and the average pK of the amino group formed can be calculated by use of the Henderson-Hasselbalch equation (30, 31). In the present case it was alternatively assumed that one or two peptide bonds were split, since, according to the plots shown in Fig. 5, it seemed unlikely that more than two peptide bonds were hydrolyzed. Omitting the values obtained at pH 8.6, where a considerable drift was observed during the autotitration, it appears from the data presented in Table III that only one equivalent of peptide bond was hydrolyzed per mole of glucagon, the pK of the ionizing group being 7.3; this value was remarkably constant within the pH range studied. In these measurements and calculations the pH was operationally defined as the value selected on the autotitrator. It will be shown below that independent experimental evidence, based on quantitative end group analysis, also indicated that only one equivalent of peptide bond per mole of glucagon was hydrolyzed by bromelain.

**NH₂-Terminal Analysis of the Digest**—A 20-minute enzymatic digest of 2 µmoles of glucagon at pH 8.0 was subjected to FDB treatment to detect any new amino terminal residues. Some of the paper chromatograms obtained are traced in Fig. 6, and the quantitative results are shown in Table IV. Experiments with trypsin as the degrading enzyme were carried out as a reference, and the results obtained were in good agreement with those reported by Bromer et al. (32), indicating that the peptide bonds Lys-Tyr, Arg-Arg, and Arg-Ala of glucagon were susceptible to tryptic action. DNP-histidine (α-N, imidazol-N-disubstituted) was not determined because of incomplete extraction of this derivative into the ether phase (25) and also because of indistinguishable coincidence on the chromatogram with unknown amounts of dinitrophenol. The presence of a small amount of free alanine in the digest was detected by chromatography of the ether-extractable material from the FDB-treated digest before acid hydrolysis of DNP-peptides. The identification was confirmed by eluting the spot and hydrolyzing the eluted material.

![Figure 5. Hydrolysis of glucagon by Fraction 5 of bromelain.](image)

The reaction mixture contained 2 µmoles of glucagon, 0.014 mg of enzyme protein N, and 0.005 M potassium phosphate buffer in a total volume of 2.5 ml. The reaction was followed by autotitration with 0.1 N NaOH in a pH-stat at 25°.

**TABLE III**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH 7.4</th>
<th>NaOH per mole glucagon</th>
<th>pKₐ calculated from:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>0.50</td>
<td>7.40, 6.92</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>0.72</td>
<td>7.30, 7.45</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.82</td>
<td>7.35, 7.84</td>
</tr>
<tr>
<td></td>
<td>8.3</td>
<td>0.90</td>
<td>7.34, 8.21</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>0.97</td>
<td>7.16, 8.57</td>
</tr>
<tr>
<td>Fraction 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>0.54</td>
<td>7.33, 6.97</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>0.71</td>
<td>7.32, 7.44</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0.82</td>
<td>7.34, 7.84</td>
</tr>
<tr>
<td></td>
<td>8.3</td>
<td>0.91</td>
<td>7.32, 8.22</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>0.75</td>
<td>8.12, 8.38</td>
</tr>
</tbody>
</table>

The conditions of experiments are given in Fig. 5. For calculation of pKₐ, see the text.

![Figure 6. Tracing of a descending chromatogram of DNP-amino acids from glucagon on Whatman No. 1 paper in tert-amyl alcohol-phthalate (pH 6.0) at room temperature for 24 hours.](image)

Traces of yellowish or brownish spots are indicated with dotted lines. 1, Intact glucagon; 2 and 3, the digest of glucagon by Fraction 5 of bromelain; 4, the digest by Fraction 2; 5 and 6, the digest by trypsin; 7, mixture of known DNP-amino acids. 1, 3, and 4, were from the ethereal phase after acid hydrolysis of DNP-peptides; 2 and 5 were from the ethereal phase before acid hydrolysis. The digestion by bromelain was carried out in a pH-stat with 2 µmoles of glucagon and 0.014 mg of N enzyme in 2.5 ml of solution containing 0.005 M potassium phosphate buffer, pH 8.0, for 20 minutes at 25°. The digest by trypsin was prepared by incubating in a pH-stat 2 µmoles of glucagon with 0.009 mg of N trypsin in 2.5 ml of solution containing 0.005 M Tris buffer, pH 8.0, and 0.05 M CaCl₂ for 20 minutes at 25°.

\[ pH = pKₐ + \log \left( \frac{[A]}{[HA]} \right) \]

where A denotes a conjugate base. Pechere and Neurath (31), however, made their calculation by the erroneous use of the equation, \[ pH = pKₐ - \log \left( \frac{[A]}{[HA]} \right) \].
Table IV

Yields of DNP-amino acids from digest of glucagon by bromelain and trypsin

The conditions of enzymatic digestion were the same as those described for Fig. 6. The figures in the table are moles DNP-amino acid per mole glucagon.

<table>
<thead>
<tr>
<th>DNP-amino acid</th>
<th>Fraction 2</th>
<th>Fraction 5</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free DNP-alanine*</td>
<td>0.04</td>
<td>0.06</td>
<td>None</td>
</tr>
<tr>
<td>DNP-alanine</td>
<td>0.55</td>
<td>0.53</td>
<td>0.18</td>
</tr>
<tr>
<td>DNP-tyrosine</td>
<td>None or trace</td>
<td>None or trace</td>
<td>1.08</td>
</tr>
<tr>
<td>DNP-glutamic acid</td>
<td>0.45</td>
<td>0.43</td>
<td>None</td>
</tr>
<tr>
<td>ε-DNP-lysine</td>
<td>0.57</td>
<td>0.57</td>
<td>0.63</td>
</tr>
<tr>
<td>DNP-arginine</td>
<td>None</td>
<td>None</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* Before acid hydrolysis of DNP-peptides.

in 6 N HCl for 16 hours, which yielded only DNP-alanine. The presence of free alanine was confirmed also by carboxypeptidase-A action on the digest (see below).

The results shown in Fig. 6 and Table IV indicate that from 1 mole of glucagon, approximately ½ mole of DNP-alanine and ½ mole of DNP-glutamic acid appear as N-terminal groups, in addition to histidine which is the N-terminus of the substrate. It follows from the amino acid sequence of glucagon (33),

\[
\]

that the appearance of DNP-alanine is the result of hydrolysis of the Arg-Ala bond (18, 19), whereas the appearance of DNP-glutamic acid could be due to the splitting of any one of the three glutamine bonds (in positions 8, 20, and 24). In order to decide among these, the following series of experiments were undertaken.

Separation of Basic Peptides from Digest—When an aliquot of the 20-minute digest of glucagon was subjected to high voltage electrophoresis on paper at pH 3.6, one spot corresponding to one or more basic peptides was obtained after spraying with ninhydrin reagent, as shown in Fig. 7. The same was found after electrophoresis at pH 6.5, except that the spot of basic peptides was broader and looked as though it consisted of two closely adjacent spots.

The amino acid composition of the eluted spot of the basic peptide at pH 3.6 is given in Table V and corresponds to that of a peptide containing the sequence 1 to 18 (His through the second Arg) of glucagon plus one-half equivalent alanine, suggesting that cleavage at the Arg-Ala bond occurred.

Separation of Acid-insoluble Peptides from the Digest—It splitting had occurred at the Arg-Ala and Ala-Glu(NH\(_2\)) bonds had occurred.

Separation of Acid-insoluble Peptides from the Digest—It splitting had occurred at the Arg-Ala and Ala-Glu(NH\(_2\)) sites, two peptides, 19 to 29 (Ala through Thr) and 20 to 29 (Glu(NH\(_2\)) through Thr), would be expected to be present in the digest and, according to the work of Bromer et al. (32), these two peptides would be acid-insoluble. To prove this, the digest of 0.4 µmole of glucagon was lyophilized and the residue was extracted three times with 1 ml of 0.001 M acetic acid. The amino acid composition of the acid-insoluble material thus obtained was determined. The data in Table VI may be most reasonably explained on the basis that the acid-insoluble material is approximately an equi-
TABLE VI
Amino acid composition of acid-insoluble peptide from digest of glucagon by Fraction 5

The acid-insoluble peptide was the residue from acetic acid extractions of the lyophilized digest (see text).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Found</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>0.643</td>
<td>2.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.650</td>
<td>2.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.018</td>
<td>0.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.042</td>
<td>0.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.091</td>
<td>1.2</td>
</tr>
<tr>
<td>Serine</td>
<td>0.110</td>
<td>1.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.029</td>
<td>0.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.397</td>
<td>0.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.385</td>
<td>0.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.056</td>
<td>0.6</td>
</tr>
<tr>
<td>Methionine†</td>
<td>0.317</td>
<td>1.0</td>
</tr>
<tr>
<td>Valine</td>
<td>0.303</td>
<td>1.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.327</td>
<td>1.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.335</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* The average of values for methionine and valine was taken as unity.
† As methionine sulfone.

molar mixture of peptides 19 to 20 and 20 to 29 containing intact glucagon as an impurity. FDB treatment of the acid-insoluble material showed the occurrence of DNP-alanine and DNP-glutamic acid approximately in 1:1 ratio. These results favor the view that cleavage of the Arg-Ala and Ala-Glu(NH₂) bonds took place.

**Action of Carboxypeptidase-A on Digest**—If the bond Ala-Glu(NH₂) were one of the points of attack, the resulting C-terminal alanine should be released by the action of carboxypeptidase-A. This was found to be the actual case, as shown in Fig. 8. Carboxypeptidase-A liberated from intact glucagon, threonine, asparagine, methionine, leucine, tryptophan, threonine, valine, and phenylalanine, a result which is consistent with the observations of Bromer et al. (34). From the 20-minute digest of glucagon with bromelain, alanine was liberated in addition to these eight amino acids. It is worthy of note that the rate of liberation of alanine was very high and the amount liberated was approximately 0.5 that of threonine.

From these lines of evidence, it may be concluded that both Arg-Ala (18-19) and Ala-Glu(NH₂) (19-20) bonds are susceptible to bromelain and that both bonds are alternatively but not simultaneously cleaved. This is in agreement with the previously stated view that only one equivalent bond is cleaved per mole of substrate.

**Action of Bromelain on B Chain of Oxidized Insulin**

When the B chain of insulin was incubated with either Fraction 2 or Fraction 5, only a small uptake of alkali was recorded on the pH-stat. By use of 5 times the amount of enzyme employed for the digestion of glucagon, approximately 4 mol of NaOH was taken up per mole of B chain after 40 minutes of incubation at pH 8.5.

The 40-minute digest was treated with FDB and the DNP-amino acids were determined. The results are shown in Table VII. Although a reference experiment carried out with trypsin as the enzyme gave results which are consistent with the known amino acid sequence of the B chain (35, 36) and with the specificity of trypsin, the experiments with bromelain resulted in detection of only a small amount of DNP-glutamic acid with a trace amount of DNP-glycine. The absence of free alanine indicates the resistance of the C-terminal Lys-Ala bond to this

![Fig. 8. Liberation of amino acids from glucagon by the action of carboxypeptidase-A. A, Action on intact glucagon; B, action on the digest of glucagon by Fraction 5 of bromelain. Plots for asparagine, methionine, leucine, and glutamine, which were similar to those for tryptophan and valine, were omitted to avoid crowded figures. For A, 2 μmoles of glucagon in 4.5 ml solution were mixed with 0.5 ml of 0.25% solution of carboxypeptidase-A in 1.0 M ammonium acetate, pH 8.5, containing 0.001 M DFP. For B, 20-minute digest (2.5 ml) of 2 μmoles of glucagon, the same as in Fig. 6, was acidified with 0.3 ml of 0.1 N HCl and treated at 90° for 2 minutes. The solution was neutralized, diluted to 4.5 ml, and mixed with 0.6 ml of carboxypeptidase-A solution as above. At given time intervals each 0.5-ml aliquot was withdrawn for amino acid analysis. Zero time values were obtained by a separate experiment without the addition of carboxypeptidase-A.](http://www.jbc.org/)
enzyme. Because of the limited scope of this specific investigation, no further conclusions can be drawn concerning further possible sites of cleavage.

DISCUSSION

Although ion exchange column chromatography on Duolite CS101 proved to be a simple and reproducible procedure for the fractionation and purification of stem bromelain, it is of interest that little, if any, increase in specific activity accompanied chromatographic fractionation. This observation is in agreement with the finding that the crude crystalline bromelain obtained by fractionation with ammonium sulfate and subsequently with sodium chloride, did not show a higher specific activity than the crude enzyme.

Fractions 2 and 5 isolated by chromatography appear to be indistinguishable from each other in their activities toward various substrates and in their behavior toward activation and inhibition. Each of these two fractions appears to be homogeneous with respect to chromatography, electrophoresis and sedimentation in the ultracentrifuge, and the only significant difference between them relates to their electrophoretic mobility at pH 7.4 (Table II). The possibility that one of the two fractions is the dimer of the other seems unlikely since their sedimentation constants are so nearly alike (Table II). Although the values for electrophoretic mobility of Fractions 2 and 5 shown in Table II are not identical with those of components B and A, respectively, shown in Fig. 3, it may be reasonable to suppose that the two chromatographic fractions represent the respective two major electrophoretic components.

Although BAEE was found to be the best substrate for stem bromelain, a relatively broad specificity range was found when glucagon and the B chain of oxidized insulin were used as substrates. In this connection, two findings are of particular interest: (a) the enzyme splits the Arg-Ala bond of glucagon and yet leaves intact the Arg-Arg and the Lys-Tyr bonds which are readily susceptible to tryptic hydrolysis; and (b) the enzyme hydrolyzes with comparable rates the Ala-Glu(NH₂) and the Arg-Ala bonds. The failure of bromelain to split any of the lysyl bonds in either glucagon or the B chain of oxidized insulin suggests the possibility that this enzyme may be inert toward lysyl bonds in proteins in general and thus may be useful in sequence studies for the purpose of differential enzymatic hydrolysis of peptide bonds contributed by basic side chains. However, since the enzyme did not hydrolyze every arginyl bond in the two polypeptide substrates which have been tested but at the same time hydrolyzed the Ala-Glu(NH₂) bond in glucagon, the possibility exists that the preparation used consisted of a mixture of two or more different enzymes. Although this appears unlikely, in view of the reasonably high homogeneity of the preparation and the similarity in specificity of Fractions 2 and 5, it is clear that the final test concerning the specificity and usefulness of this enzyme must await further studies with other peptide and protein substrates.

Although a certain similarity in behavior and specificity of stem bromelain and papain is apparent from the data which have been presented, it is clear that significant differences exist with respect to their substrate specificities, the conditions of maximum activation, and their physical-chemical properties.

SUMMARY

1. The crude preparation of stem bromelain has proteolytic activity toward casein and hemoglobin. Among various synthetic substrates tested benzoyl-L-arginine ethyl ester was found to be most rapidly hydrolyzed. The enzyme was activated by cysteine or by cyanide, and strongly inhibited by mercuric ion, inhibition being completely reversed by an excess of cysteine. The enzyme showed a broad pH maximum around pH 7. Disopropylphosphorofluoridate had no specific effect on the enzymatic action.

2. Ion exchange column chromatography of the crude preparation on Duolite CS101 at pH 6.05 resulted in good resolution of two protein components, i.e. Fractions 2 and 5, which appear to correspond to two major components found by electrophoresis of the crude material at pH 7.4. Fractions 2 and 5 are remarkably similar to one another in their activities toward various protein, polypeptide, and synthetic substrates. Both have similar sedimentation constants and distinctly different electrophoretic mobility values at pH 7.4.

3. The action of purified preparations of bromelain on glucagon and the B chain of oxidized beef insulin was studied. An extensive study of the digestion of glucagon has revealed the fact that bromelain rapidly cleaves glucagon at either the arginylalanoyl or alanyl-glutamminyl bond, while it leaves intact lysyltyrosyl and arginyl-arginyl bonds. The B chain of oxidized insulin was found to be a relatively poor substrate for bromelain.

4. Crude crystalline enzyme was obtained by fractionation with ammonium sulfate and subsequently with sodium chloride.

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Takashi Murachi and Hans Neurath


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