On the Molecular Weights of the Proteolytic Enzymes of Stem Bromelain*

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Each of the five proteolytically active components of stem bromelain, separated by the method of M. El-Gharbawi and J. R. Whitaker *Biochemistry 2*, 476, 1963), has been analyzed for molecular weights, amino acids, carbohydrate, tryptic peptides, and N-terminal amino acids. The molecular weights for components I through V (calculated from amino acid composition) were: 18,997; 19,650; 17,885; 18,020; and 20,011, respectively. Molecular weights determined by the Archibald method and by gel filtation were in agreement with these values. These results disagree with the molecular weight of approximately 33,000 reported by T. Murachi, M. Yasui, and Y. Yasuda *Biochemistry 3*, 49, 1964). The components contained two to five residues of carbohydrate per molecule (as glucose) and two to four residues of hexosamine. All five components gave valine as the major N-terminal amino acid by the 2,4-dinitrofluorobenzene method. Each component was unique in its tryptic peptide map.

Heterogeneity of stem bromelain has been reported by several workers (Heinicke and Gortner, 1957; Murachi and Neurath, 1960; Ota *et al.*, 1961; El-Gharbawi and Whitaker, 1963). El-Gharbawi and Whitaker (1963) reported the separation of five proteolytically active components from stem bromelain. The active components were partially characterized, mainly from the enzymatic standpoint, and were found to differ from each other in terms of *p*H optimum, heat stability, specific activity in hydrolyzing α-benzoyl-L-argininamide, inhibition by iodoacetamide, and electrophoretic movement on cellulose acetate.

More recently, Murachi *et al.* (1964) have reported that the majority of the proteolytic activity of stem bromelain can be purified as a single component with a molecular weight of approximately 33,000 as determined by ultracentrifugation. Quantitative N-terminal end-group analysis of a purified preparation of stem bromelain tends to support the molecular weight of 33,000 (Ota *et al.*, 1964).

The purpose of this research was to investigate more thoroughly the physical and chemical differences among the five proteases from stem bromelain. This has been done by determining amino acid composition, carbohydrate content, molecular weight, tryptic peptide fingerprints, and N-terminal amino acids of each of the five components.

Materials and Methods

"Bromelain" No. 15 from the Dole Corp., Honolulu, Hawaii was used.[1] It had a specific activity (change in absorbance per minute per mg protein) against casein at *p*H 7.0 and 35.0° of 0.77, and contained 3.20% water and 51.5% protein on a moisture-free basis by the biuret method (Layne, 1957).

Bio-Rex 70 (200–325 mesh, control No. DS 2572 B-1065) was from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-100 (140–400 mesh beads, lot To 1554, water regain of 10 ± 1) was from Pharmacia, Uppsala, Sweden. Whatman No. 4 and 3 MM filter papers were used. Casein, L-cysteine hydrochloride, ninhydrin, anthrone, D-tryptophan, and dinitrophenyl-amino acids were from Nutritional Biochemicals Corp., Cleveland, Ohio. *N*,*N*-Dinitrophenyl-L-lysine and trypsin (2 × crystallized, salt-free) were from Mann Research Laboratories, Inc., New York. 2,4-Dinitrofluorobenzene and Versene were from Eastman Organic Chemicals, Rochester, N.Y. *p*-Dimethylaminobenzaldehyde was from K and K Laboratories, Inc., Jamaica, N.Y. All other chemicals were reagent grade. Deionized water was used throughout this research.

Chromatographic separation and rechromatography of the proteolytically active components of stem bromelain were carried out at 2° as described by El-Gharbawi and Whitaker (1963). Proteolytic activity was measured by the method of Kunitz (1947) as previously described (El-Gharbawi and Whitaker, 1963). After rechromatography the peak fractions were precipitated with 95% saturated ammonium sulfate at 2° and centrifuged at 5800 × *g* for 20 minutes at 2°, and the precipitate was dissolved in a small quantity of water. The solution was dialyzed exhaustively against large volumes of water at 2°. After lyophilization, the dry powder was stored in a freezer. The preparations were free of ammonium sulfate.

Amino acid analyses (single determinations) were performed by Analytica Corp., New York. The samples were dried *in vacuo* over phosphorus pentoxide and hydrolyzed with constant-boiling hydrochloric acid in evacuated sealed Pyrex tubes at 115° for 16 hours. The hydrolysates were assayed for amino acids by the method of Spackman *et al.* (1958).

Tryptophan analyses were carried out by the spectrophotometric method of Fraenkel-Conrat (1957) and by the colorimetric method of Udenfriend and Peterson (1957). Total carbohydrates were determined by a slight modification of the anthrone procedure of Trevelyan and Harrison (1952). Glucose was used for preparation of the standard curve.

Dinitrophenyl derivatives of the N-terminal amino acids of the five components were prepared by the method of Sanger (1945) as described by Porter (1957). One-dimensional descending chromatography of the DNP-amino acids was carried out as described by Blackburn and Lowther (1951) at constant temperature (23°) for 12 hours, in which time the solvent front had migrated about 30 cm. About 20–30 μg of each known DNP-amino acid was chromatographed on the same sheet of paper with the unknowns.

Peptide mapping of the components was done by a modification of the procedures described by Kimmel *et al.* (1962) and Ingram (1958). The enzyme was denatured in 0.01 N NaOH for 20 minutes at room temperature and the *p*H was adjusted to 7.9. Tryptic

* Taken from a master's thesis by Gad Feinstein, University of California, Davis, September, 1963.

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

digestion (substrate-trypsin = 25:1) was carried out in a *p*H-stat (International Instruments Co.) at 38° and *p*H 7.9 in the presence of a nitrogen atmosphere saturated with water. Hydrolysis was essentially complete after 2 hours. The hydrolyzed components were lyophilized and dissolved in water. Electrophoresis was carried out for 4 hours at 2° on Whatman 3 MM paper strips in a Spinco Model R paper electrophoresis cell at 400 v (amperage increased from 12 ma at the beginning to 30 ma at the end) in *p*H 6.4 pyridine-acetate buffer (Ingram, 1958). After drying in an oven at 60° for 20 minutes, the paper strips were sewed to sheets of Whatman 3 MM filter paper perpendicular to the direction of migration during electrophoresis. Descending chromatography was carried out at constant temperature (23°) for 18 hours using butanol–acetic acid saturated with water. The sheets were dried in an oven at 60°, sprayed with ninhydrin, and again dried.

Molecular-weight determinations on component II were carried out by the Archibald method as described by Schachman (1957). Fifteen mg of enzyme was dissolved in 1.5 ml of *p*H 3.9, 0.10 M acetate buffer which contained 0.02 M cysteine (Smith *et al.*, 1954). The enzyme solution was dialyzed overnight against the acetate buffer at 2°. A Spinco Model E ultracentrifuge equipped with a cylindrical-lens schleiren optical system was operated at 20,014 rpm and 22°. Molecular weight determinations on all five components were also carried out on a 1.4 × 197–cm Sephadex G-100 column at *p*H 6.0 as described by Whitaker (1963). Molecular weights were also calculated from the amino acid compositions. All *p*H measurements were made with a Beckman Model G *p*H meter.

Results

Carbohydrate Content—There appear to be two to five residues of carbohydrate (as glucose) per molecule in the five components (Table I). In addition, there

Table I
Carbohydrate Content of Components I–V of Stem Bromelain[a]

Component	I	II	III	IV	V
Grams carbohydrate/mole enzyme[b]	831	394	404	404	442
Moles carbohydrate/mole enzyme[c]	4.62	2.19	2.25	2.25	2.46

[a] Determined by the anthrone method of Travelyan and Harrison (1952). [b] Based on molecular weights calculated from amino acid composition (Table III). [c] As glucose units.

are two to four residues of hexosamine as found by amino acid analysis (Table III). Ota *et al.* (1964)[2] found 1.49% carbohydrate and three residues of glucosamine in their preparation.

Tryptophan Content.—The values of tryptophan obtained by the two methods are not in complete agreement with each other (Table II). However, repeated determinations by the two methods gave quite reproducible results. The average values from both methods were used in calculations of amino acid compositions and molecular weights. In the case of tyrosine the values obtained from the spectrophotometric determinations are close to those obtained by amino acid analysis (Tables II and III).

[2] We are indebted to Dr. Stanford Moore for his generosity in making this manuscript available to us before publication.

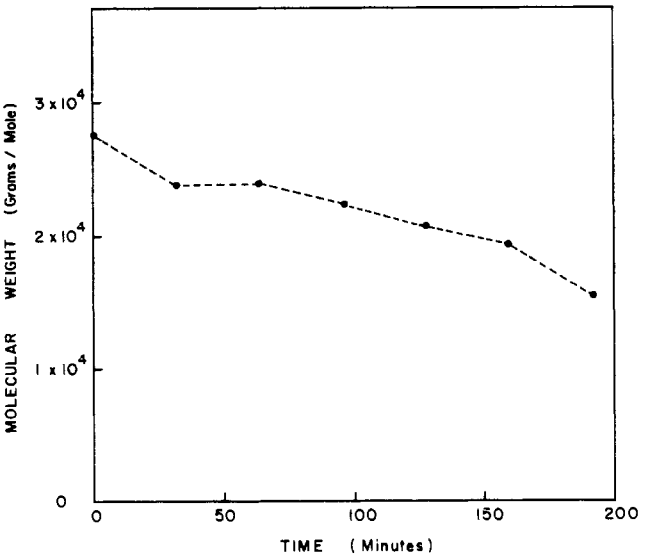

Fig. 1.—Molecular weight of component II of stem bromelain as determined by the Archibald method (Schachman, 1957). The enzyme was dissolved in 0.1 M *p*H 3.9 acetate buffer containing 0.02 M cysteine to give a 1.5% solution and dialyzed against the same buffer at 2°. A Spinco Model E ultracentrifuge with a synthetic-boundary cell and a schlieren optical system was operated at 20,014 rpm and 22°. Time was measured after the rotor had attained constant speed. Molecular weights were calculated by the method of Ehrenberg (1957).

Table II
Tryptophan and Tyrosine Content of Components I–V of Stem Bromelain

Component	Tyrosine[a]		Tryptophan[a]		
	Amino Acid[b] Analyzer	Spectrophotometric[c] Method	Colorimetric[d] Method	Spectrophotometric[e] Method	Average
I	11	9.7	5.4	6.2	6
II	11	10.1	5.7	5.2	5
III	10	9.2	7.3	5.1	6
IV	10	9.0	7.0	4.5	6
V	11	10.7	7.6	5.9	7

[a] Moles amino acid residue/mole enzyme. Based on molecular weight calculated from amino acid composition (Table III). [b] From Table III. [c] Fraenkel-Conrat (1957). [d] Udenfriend and Peterson (1957).

Amino Acid Composition.—With the exception of components I and V the known composition essentially accounted for the total weight. The percentage known composition was: I, 88.96; II, 105.52; III, 102.96; IV, 96.07; and V, 93.03. The molar amino acid compositions of the components are shown in Table III. The variation in amino acid composition among the components is particularly evident when one compares the amino acid residues which are present to the extent of less than ten residues per mole such as proline, half-cystine, leucine, phenylalanine, arginine, and tryptophan. The total number of amino acid residues per molecule varies from 162 residues for component III to 178 residues for component V.

The isoelectric points of all five components were reported by El-Gharbawi and Whitaker (1963) to be above *p*H 9.6. This basicity of the components is evident from Table IV in which the number of ionic groups per molecule is given. The greater number of cationic groups is in accord with the alkaline isoelectric points. Murachi *et al.* (1964) found the isoelectric point of their bromelain preparation to be at about *p*H 9.5.

TABLE III
MOLAR AMINO ACID COMPOSITION AND MOLECULAR WEIGHT OF COMPONENTS I–V OF STEM BROMELAIN

Amino Acid Residue	Number of Residues per Mole									
	I		II		III		IV		V	
	Calculated[a]	Integer	Calculated[a]	Integer	Calculated[a]	Integer	Calculated[a]	Integer	Calculated[a]	Integer
Aspartic acid	15.4	15	16.2	16	15.3	15	14.9	15	16.2	16
Threonine[b]	7.4	7	7.9	8	6.6	7	5.8	6	8.3	8
Serine[b]	15.9	16	15.8	16	15.6	16	16.7	17	16.0	16
Glutamic acid	12.3	12	12.3	12	11.0	11	11.0	11	13.0	13
Proline	9.1	9	8.4	8	7.4	7	7.3	7	6.4	6
Glycine	18.7	19	18.7	19	17.3	17	17.7	18	19.8	20
Alanine	18.0	18	20.4	20	17.7	18	19.1	19	18.1	18
Half-cystine	4.9	5	4.8	5	4.1	4	4.4	4	5.9	6
Valine	11.1	11	12.4	12	10.7	11	11.4	11	12.0	12
Methionine	1.9	2	2.1	2	2.0	2	1.9	2	2.1	2
Isoleucine	11.9	12	11.6	12	10.8	11	10.9	11	11.9	12
Leucine	5.1	5	5.0	5	4.4	4	5.1	5	6.8	7
Tyrosine[b]	10.8	11	11.2	11	10.3	10	10.1	10	11.0	11
Phenylalanine	5.2	5	4.7	5	4.9	5	4.1	4	5.1	5
Hexosamine[b]	3.4	3	4.2	4	2.8	3	2.2	2	4.0	4
Lysine	11.1	11	11.9	12	10.6	11	10.3	10	11.9	12
Histidine	0.7	1	0.9	1	1.2	1	1.1	1	0.9	1
Arginine	5.7	6	6.1	6	5.8	6	7.1	7	5.9	6
Tryptophan	5.8	6	5.3	5	6.2	6	5.7	6	6.7	7
Amide NH_3[c]	23.0	23	19.3	19	18.8	19	18.2	18	21.7	22
Total no. of residues		174		179		165		166		182
Molecular weight										
Calculated		18,997		19,650		17,885		18,020		20,011
Sephadex		17,800[d]		17,200[d]		17,200[d]		16,800[d]		16,500[d]
Ultracentrifugation[e]				22,200[d,f]						

[a] Calculated on the basis of each residue involved except histidine and hexosamine. We assumed two residues of methionine initially and worked from amino acids with lowest number of residues involved upward in order. Histidine was not used in the calculation as Smith and Kimmel (1960) reported papain gave little more than one residue histidine per mole by chromatographic amino acid analysis but two by enzymatic assay. [b] Corrected approximately for decomposition during acid hydrolysis. The factors applied were: serine, 0.90; threonine, 0.95; tyrosine, 0.95 (Moore and Stein, 1963); hexosamine, 0.34 (Plummer and Hirs, 1963). [c] Corrected for ammonia from decomposition of threonine, serine, tyrosine, and hexosamine. Not included in total of residues. [d] ±10%. [e] Archibald method as described by Schachman (1957). [f] Average of molecular weight at 32, 64, 96, 128, and 160 minutes (Fig. 1).

TABLE IV
IONIC GROUPS OF COMPONENTS I–V OF STEM BROMELAIN

Type of Group	Component Number				
	I	II	III	IV	V
	(no. of groups per molecule of enzyme)				
Aspartic acid	15	16	15	15	16
Glutamic acid	12	12	11	11	13
Terminal α-carboxyl[a]	1	1	1	1	1
	28	29	27	27	30
Total amide groups	23	19	19	18	22
Total anionic groups	5	10	8	9	8
Lysine	11	12	11	10	12
Histidine	1	1	1	1	1
Arginine	6	6	6	7	6
Terminal α-amino	1	1	1	1	1
Total cationic groups	19	20	19	19	20
No. of positive charges per molecule	14	10	11	10	12

[a] Assumed value on basis of one terminal α-amino group found.

The partial specific volume of component II, calculated from its amino acid composition by the method described by Schachman (1957), was 0.730 cc/g. The amide groups were assigned approximately equally as glutaminyl and asparaginyl residues. The partial specific volume reported by Murachi *et al.* (1964) for a single active component was 0.743 ml/g.

Molecular Weights.—The molecular weights of all five components were calculated from the amino acid composition and determined by Sephadex-gel filtration. The results are shown in Table III. The molecular weight of component II was also determined by ultracentrifugation. From amino acid composition, the molecular weights were found to range from 17,885 for component III to 20,011 for component V. By gel filtration, the molecular weights ranged from 16,500 for component V to 17,800 for component I. There is good agreement between the two methods for components I, III, and IV. The molecular weight of crystalline papain determined by gel filtration is 17,600 (unpublished data), while the accepted value is 20,700. Despite the use of cysteine in the solution (Smith *et al.*, 1954), the molecular weight of component II was found to be dependent upon the time of centrifugation under the conditions used (Fig. 1). This appears to indicate either heterogeneity or depolymerization. If one ignores the initial and final values (Ehrenberg, 1957), the average molecular weight of component II was found to be 22,200 ± 2000. Therefore the molecular weights by all three methods are in reasonable agreement.

N-*Terminal Amino Acids.*—All five components consistently gave two DNP-amino acids after treatment with 2,4-dinitrofluorobenzene and acid hydrolysis. These were identified as valine and threonine. Quantitatively, it was observed that DNP-threonine was approximately 20 ± 5% of the amount of DNP-valine formed from each component by treatment with 2,4-dinitrofluorobenzene. Ota *et al.* (1964) found valine

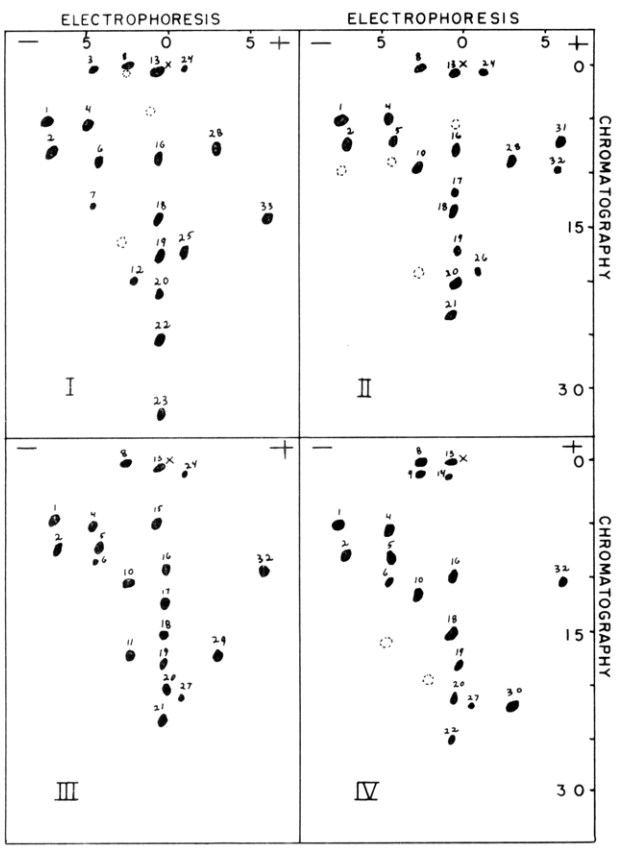

FIG. 2.—Tryptic peptides of components I–IV of stem bromelain. The experimental procedures are described in the text. The peptides indicated by the solid lines were found in all separations; those indicated by the dashed lines were found in only a few separations.

to be the major N-terminal amino acid in their preparation. In addition to these two amino acids, component II gave trace amounts of glutamic acid and serine.

Peptide Mapping.—The results of the electrophoretic and chromatographic separation of the peptides obtained from the tryptic digestion of components I–IV are shown in Figure 2. (All of component V was lost before peptide mapping could be performed.) It is reassuring to find that the number of peptides obtained is in reasonable agreement with the number expected from the amino acid composition and molecular weights and the known specificity of trypsin.

At *p*H 6.4 the majority of the peptides in all four components migrated toward the cathode (Fig. 3). While the migration distances of the peptides were very similar, the quantitative amounts of peptides from the four components were different. There are significant differences among the four components after chromatography perpendicular to the direction of movement by electrophoresis. Of eighteen to nineteen peptides, there are ten which appear to be identical in all four components. There are some peptides which were found in only two or three components and still others which were found only in a single component.

DISCUSSION

The data on amino acid composition, gel filtration, ultracentrifugation, and number of peptides formed on tryptic hydrolysis all indicate that the molecular weights of the five components are in the range of 18,000–22,000. Molecular weights considerably different from this should have resulted in a considerably different number of peptides being formed by hydrolysis with trypsin.

However, it has recently been reported that the majority of the proteolytic activity can be obtained as

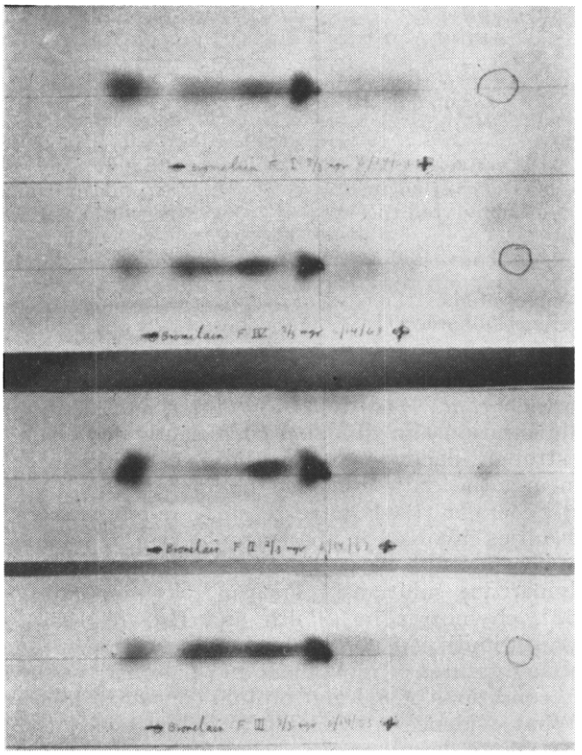

FIG. 3.—Paper electrophoresis of tryptic digests of components I–IV of stem bromelain. Electrophoresis was carried out on Whatman 3 MM paper at 2° and 400 v for 4 hours in a Spinco Model R electrophoresis cell using *p*H 6.4 pyridine-acetate buffer. The paper strips were sprayed with 0.2% ninhydrin in methanol. Location of the peptides at extreme right is indicated with circles as the color faded soon after spraying.

TABLE V
COMPARISON OF THE AMINO ACID COMPOSITION OF STEM BROMELAIN WITH THAT REPORTED BY OTA *et al.*[a]

Amino Acid Residue	Mixture of Components I–V[b] (moles/ 19,000 g)	Ota *et al.*[a] (moles/ 19,000 g)
Aspartic acid	15.8	15.8
Threonine	7.2	7.2
Serine	16.2	15.0
Glutamic acid	11.9	12.2
Proline	8.0	7.6
Glycine	18.4	18.4
Alanine	18.9	18.8
Half-cystine	4.7	5.6
Valine	11.6	11.5
Methionine	2.0	2.7
Isoleucine	11.5	11.3
Leucine	5.1	5.3
Tyrosine	10.8	11.1
Phenylalanine	4.8	4.7
Lysine	11.3	12.2
Histidine	1.0	1.0
Arginine	6.2	6.1
Tryptophan	6.0	4.3
Amide-NH_3	20.0	22.2

[a] Ota *et al.*, 1964. [b] Moles amino acid per 19,000 g mixture based on the composition of I, 16.8%; II, 27.6%; III, 30.4%; IV, 15.2%; and V, 10.1% as reported by El-Gharbawi and Whitaker (1963) and the data of Table III.

TABLE VI
AMINO ACID AND TRYPTIC PEPTIDE RELATIONSHIPS AMONG THE FIVE COMPONENTS OF STEM BROMELAIN[a]

Components Compared	Amino Acid Difference	Ambiguous	Peptides[b] Total Combined	Peptides[b] Different
V converted to II	glu,gly,$^1/_2$-cys,leu$_2$,try$_2$	pro$_2$,ala$_2$		
II converted to I	asp,thr,ala$_2$,val,lys	pro,try	37	15
I converted to IV	thr,glu,pro$_2$,gly,$^1/_2$-cys, isoleu,tyr,phe,lys	ser,ala,arg	38	14
IV converted to III	ser,gly,ala,leu,arg	thr,phe,lys	39	9

[a] Compared on the basis of postulated conversion of component V → II → I → IV → III as indicated by molecular weight differences (Table III). [b] Only those tryptic peptides found in all experiments are included here (see Fig. 2).

a single component with a molecular weight of 33,000 (Murachi *et al.*, 1964) to 35,730 (Ota *et al.*, 1964). The sedimentation and diffusion coefficients were found to be strongly dependent upon the bromelain concentration and the data indicated polymerization. Winzor and Scheraga (1964) have recently re-emphasized the difficulties involved in the extrapolation of sedimentation coefficients to zero protein concentration for polymerizing solutions. Trypsin (Cunningham *et al.*, 1953), chymotrypsin (Smith and Brown, 1952) and papain (Smith *et al.*, 1954) have all been shown to form mobile mixtures of monomers and polymers under certain conditions of *p*H and protein concentration.

What evidence is available to indicate that the five components described here were formed from a single parent molecule of molecular weight 33,000–36,000? Table V presents a comparison between the amino acid composition of a mixture of the five components and the purified stem bromelain of Ota *et al.* (1964). With the exception of lysine and one half-cystine, methionine and tryptophan (determined by different methods by the two groups of workers), and serine (which involved correction for decomposition during acid hydrolysis), the agreement between the two results is perfect. In order to accept the above hypothesis, one must conclude that the part of the molecule lost during the autolysis (approximately 45%) has the same amino acid composition as the active fragments left. This would appear to be highly unlikely.

Table VI summarizes the amino acid and tryptic peptide differences among the five components studied here. As a first approximation, the amino acid data would lead to the conclusion that the five components are identical or differ by only a few amino acids perhaps as the result of autodigestion. However, the tryptic peptide data do not support such a conclusion. Ota *et al.* (1964) found there were nonstoichiometric amounts of several N-terminal amino acids in their preparation; however, they were not able to demonstrate any fragmentation of the original material.

From the data presented one cannot speculate on the number of proteolytically active components in a single pineapple plant as the material used here represented pooled material.

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