Preparation and Chemical Properties of Purified Stem and Fruit Bromelains*

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The sulfhydryl-activated proteases of the stem and the fruit of the pineapple plant have been isolated in chromatographically purified form and some of the chemical properties of the preparations have been compared. One main proteolytically active component has been obtained from stem bromelain by gel filtration on Sephadex G-75 and chromatography on sulfoethyl-Sephadex at *p*H 6. The enzyme present in the pineapple fruit has been purified by acetone precipitation followed by chromatography on DEAE-cellulose. Both these preparations give single zones upon chromatography and electrophoresis. However, end-group analysis by the cyanate method indicates that the preparations are not homogeneous. The principal NH_2-terminal residue in the stem enzyme is valine (0.9 residue per molecule of mw 36,000), but fractional amounts of other NH_2-terminal residues (alanine 0.2, glycine 0.06) were also found. The principal NH_2-terminal residue in the fruit enzyme is alanine (0.9 residue per molecule of mw 30,000), but once again additional NH_2-terminal residues were noted (valine 0.3, serine 0.2, glycine 0.1). The use of phenylmercuric acetate as an inhibitor of autodigestion during the purification procedures led to the smallest quantities of additional NH_2-terminal residues. The stem enzyme is basic and amino acid analyses show a relatively high content of lysine and arginine. The enzyme from the fruit is an acidic protein. The carbohydrate analyses support the recent conclusion of Murachi and associates that the stem enzyme is a glycoprotein. The fruit enzyme also contains carbohydrate that cannot be removed by the procedures used thus far. The purified enzymes differ in their relative activities toward casein and benzoyl-L-argininamide.

The number of proteolytic enzymes in preparations of bromelain from the stem of the pineapple has not yet been definitely established. Two groups of investigators, Murachi and Neurath (1960) and El-Gharbawi and Whitaker (1963), have found several chromatographically separable, proteolytically active components in such preparations. On the other hand, Murachi *et al.* (1964)[1] have reported more recently that most of the activity could be obtained in a single component which gave a single zone upon electrophoresis and ultracentrifugation. In the work to be described in this communication, stem bromelain has been subjected to gel-filtration and ion-exchange chromatography and has yielded a single active product which has been characterized chemically. The data thus supplement the observations of Murachi *et al.* (1964) on the physical-chemical properties of the enzyme. For comparative purposes we have also prepared purified samples of the bromelain which is present in the fruit of the pineapple (see Ota *et al.*, 1961).

EXPERIMENTAL PROCEDURE

Starting Materials.—The stem enzyme was purified from commercial, powdered stem bromelain (lot 318) supplied by the Dole Corporation, Honolulu, Hawaii. The fruit enzyme was extracted from both green and ripe Hawaiian pineapple[2] by a procedure based upon the one described by Heinicke and Gortner (1957) for the preparation of commercial stem bromelain. The fresh fruit was freed of leaves and epithelium and the juice was expressed with a hydraulic press. The juice (*p*H 3.2–3.5) was cooled to 0–4° and one volume of cold acetone was added. The precipitate had low enzymatic activity and was discarded. The enzyme was precipitated by the addition of two more volumes of cold acetone and the precipitate was collected by centrifugation and dried under reduced pressure. The dried product was ground to a powder in a mortar; yield, 3.3–3.7 g of crude enzyme per liter of juice. The supernatant solution did not have detectable proteolytic activity.

Assay.—Proteolytic activity against casein was measured by the method of Kunitz (1947). The hydrolysis of benzoyl-L-argininamide (BAA)[3] was followed by application of the ninhydrin method (Moore and Stein, 1954). The activity against benzoyl-L-arginine-*p*-nitroanilide (BAPA) was determined at *p*H 6 and 25° by method II of Erlanger *et al.* (1961). To obtain maximum proteolytic activity, mercaptoethanol or cysteine plus EDTA were used as activating agents. The assays of the crude enzyme preparations were performed as follows:

Against Casein.—A total volume of 2 ml contained 200–400 μg of crude enzyme. The concentrations of reagents were mercaptoethanol, 0.005 M (or cysteine, 0.005 M plus EDTA, 0.001 M) and casein, 0.5% in *p*H 7.6, 0.1 M sodium phosphate buffer. After 20 minutes at 40°, 3 ml of 5% trichloroacetic acid was added and the absorbancy of the supernatant fluid was measured at 280 mμ in a 1-cm cell.

Against BAA.—The concentrations of reagents in a total volume of 2 ml were mercaptoethanol, 0.005 M and BAA, 0.01 M in *p*H 6.0, 0.1 M phosphate buffer. The quantity of crude enzyme was 200–400 μg. After 20 hours at 24–25°, an aliquot of 0.2 ml was diluted to 10 ml with distilled water and 1 ml was used for analysis by the ninhydrin method. The absorbancy was measured at 570 mμ.

The activities of the purified enzymes were expressed as defined in Table II, with the use of 3–6 hours of hydrolysis in the assay against BAA.

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[2] We should like to express our thanks to Dr. Ralph Heinicke who kindly supplied the samples of stem bromelain and the pineapple fruit used in this work.

[3] The abbreviations used are: BAA, benzoyl-L-argininamide; BAPA, benzoyl-L-arginine-*p*-nitroanilide; EDTA, ethylenediaminetetraacetate; SE-, sulfoethyl-; CM-, carboxymethyl-; DEAE-, diethylaminoethyl-.

Gel Filtration and Chromatography.—Columns (2 × 75 cm) of Sephadex G-75 (medium particle size, AB Pharmacia, Uppsala, Sweden) were prepared by the procedure of Crestfield *et al.*, (1963b). The column was equilibrated at 4° with a 0.02 M sodium phosphate buffer at *p*H 5.5, or at 25° with a 0.02 M sodium citrate buffer at *p*H 5.5, saturated with phenylmercuric acetate (less than 10^{-4} M).

Columns of sulfoethyl-Sephadex (C-25, fine mesh size, 2.0 meq/g, AB Pharmacia) were prepared in the manner described for the columns of Sephadex G-75. A 2 × 35–cm column was used for purification of the stem enzyme and a smaller one (0.9 × 35 cm) for rechromatography. Eluents were 0.3 M sodium phosphate buffer at *p*H 6.0 (without phenylmercuric acetate) at 4° or 0.3 M sodium citrate buffer at *p*H 6.0, 5 × 10^{-4} M in phenylmercuric acetate, at 25°.[4]

Columns (0.9 × 32 cm) of CM-cellulose (0.51 meq/g, Bio-Rad Laboratories, Richmond, Calif.) were prepared from samples of the exchanger that had been cycled through acid and base as described by Peterson and Sober (1962) and equilibrated with 0.1 M citrate buffer at *p*H 6.0, 5 × 10^{-4} M in phenylmercuric acetate. Gradient elution was employed with increasing molarity toward 0.3 M citrate buffer at *p*H 6.0.

DEAE-cellulose (0.96 meq/g, Selectacel Type 40, lot 1305, Schleicher and Schuell Co.) was cycled through acid and base (Peterson and Sober, 1962) and equilibrated with a 0.02 M sodium citrate buffer at *p*H 6.0 that contained 5 × 10^{-4} M phenylmercuric acetate. A 2 × 20–cm column was used for purification and a 0.9 × 20–cm column for rechromatography.

Columns (0.9 × 30 cm) of IRC-50 were prepared essentially as described by Hirs *et al.* (1953).

For the purification of stem bromelain a 200-mg sample was dissolved in 4.2 ml of cold 0.02 M sodium citrate buffer at *p*H 5.5. After insoluble material had been removed by centrifugation at 30,000 × *g* for 15 minutes, the supernatant solution (4.0 ml) was applied to a column of Sephadex G-75 (see Fig. 1A).

The crude fruit bromelain contained much more insoluble material and yielded a very viscous solution which gave broad zones and poor separation upon gel filtration. Hence an ion-exchange column was used in order to separate the bulk of the mucopolysaccharides from the enzymatically active proteins. In this step, 200 mg of the crude fruit enzyme was added to 10 ml of cold 0.02 M citrate buffer at *p*H 6.0 and, after centrifugation as above, 9 ml of the clear supernatant solution was applied to a column of DEAE-cellulose (see Fig. 2A).

In general, when phenylmercuric acetate was present in the eluents to minimize autodigestion, chromatography was carried out in a constant-temperature room at 24–25°.

Protein concentration in the effluent fractions was estimated by the ninhydrin method after alkaline hydrolysis (Crestfield *et al.*, 1962) and proteolytic activity was measured against casein. Free carbohydrate was determined with the anthrone reagent of Dreywood (1946) by the quantitative procedure of Scott and Melvin (1953), after deproteinization with 5% trichloroacetic acid (see Roe, 1955). The recoveries of protein and proteolytic activity were calculated by reference to the results obtained on analysis of aliquots of the sample originally added to the column.

Amino Acid and Carbohydrate Analyses.—The fractions containing the purified enzymes to be analyzed were pooled and desalted by gel filtration on a column of Sephadex G-25 with 5% acetic acid as the eluent (Crestfield *et al.*, 1963b). Lyophilized samples were weighed out for analysis.

Acid hydrolysis was carried out with 6 N HCl for 20 hours at 110° in an evacuated sealed tube (Moore and Stein, 1963; Crestfield *et al.*, 1963a). The amino acid and hexosamine contents of the hydrolysates were determined by the method of Spackman *et al.* (1958). Performic acid oxidation for the determination of half-cystine as cysteic acid was performed by the method of Moore (1963). An approximate value for tryptophan was obtained after alkaline hydrolysis. A sample (2 mg) was hydrolyzed with 5 N NaOH containing 2.5% prehydrolyzed starch (see Drèze, 1956) at 110° for 16 hours in an evacuated sealed tube. The sample was acidified with HCl and *p*H 2.2 buffer was added. Aliquots of the supernatant solutions were chromatographed on the 50-cm column of the amino acid analyzer with *p*H 5.28, 0.35 M citrate buffer as eluent. The amide-NH_3 value was determined with the use of Conway distillation by a modification of the procedure used by Hirs *et al.* (1954). The neutral and acidic amino-terminal residues were estimated by the cyanate method of Stark and Smyth (1963).

To determine the carbohydrate content of the enzymes, samples (1–2 mg) in 0.5 N HCl were heated in evacuated sealed tubes at 100° for 3 hours. The hydrolysates were evaporated and redissolved in 1 ml of water. In order to remove amino acids, peptides, and glucosamine from the partial hydrolysate (see Nolan and Smith, 1962) prior to carbohydrate analysis, the solution was transferred, with the aid of three 1-ml portions of water, onto a column of Dowex 50-X2 (0.9 × 10 cm, 200–400 dry mesh, Bio-Rad Laboratories, Richmond, Calif.). Before each use the column was washed with, in turn, 1 N NaOH, water, 6 N HCl, and finally water. The height of the column should be 10 cm after the final water wash, measured under 10 psi of air pressure. The column was developed at 20–25° with water at about 100 ml/hour; the first 30 ml of the effluent, including the water displaced by the sample, was collected. These solutions were concentrated to dryness, the residue was dissolved in 1 ml of water, and 0.5-ml samples were used for analysis by the anthrone method.

Results

Enzymatic Activities of Crude Bromelain Preparations.—Against casein the absorbancies at 280 mμ per mg of preparation were 7.4 for stem bromelain, 4.0 for the crude enzyme from green fruit, and 3.0 for the crude enzyme from ripe fruit. Against BAA the absorbancies at 570 mμ per mg of preparation were 3.7, 9.1, and 7.2, respectively, for the three samples. Thus the crude stem enzyme is more active against casein and less active against benzoylargininamide than the crude enzyme from the fruit. Neither crude preparation showed detectable ability to hydrolyze L-leucinamide, carbobenzoxy-L-glutamyl-L-phenylalanine, carbobenzoxyglycyl-L-phenylalanine, or glycyl-L-phenylalaninamide.[5] Thus, both were active only against trypsin substrates. Without the addition of mercaptoethanol or cysteine plus EDTA, the crude enzymes showed about 25% of their maximum activity against casein.

Purification of the Stem Enzyme.—Columns of polymethacrylic acid resin (Amberlite IRC-50, Duolite

[4] When an attempt was made to use a phosphate buffer containing phenylmercuric acetate, the recoveries of enzyme from sulfoethyl-Sephadex were unexplainably low.

[5] The identity of each substrate was checked by analyses for C, H, and N.

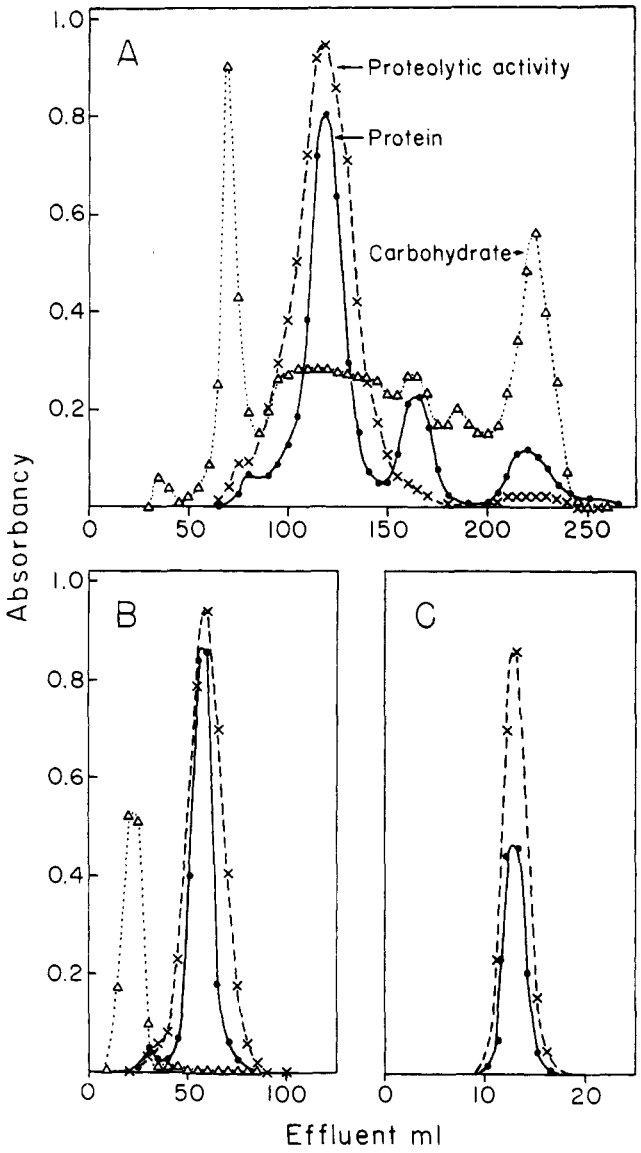

FIG. 1.—Chromatography of stem bromelain. (A) Sephadex G-75, 2 × 75-cm column, *p*H 5.5, 0.02 M sodium citrate buffer saturated with phenylmercuric acetate, 10 ml/hour, 5-ml fractions, 0.025-ml aliquots were analyzed. Sample: 200 mg of crude enzyme. (B) SE-Sephadex, 2 × 35-cm column, *p*H 6.0, 0.3 M sodium citrate buffer containing 5 × 10^{-4} M phenylmercuric acetate, 10 ml/hour, 5-ml fractions, 0.025-ml aliquots were analyzed. The proteolytically active fraction eluted between 105 and 130 ml in (A) was applied to the column. (C) Rechromatography of (B) on SE-Sephadex, 0.9 × 35-cm column, *p*H 6.0, 0.3 M sodium citrate buffer containing 5 × 10^{-4} M phenylmercuric acetate, 3 ml/hour, 1-ml fractions, 0.5-ml aliquots were analyzed. A 0.1-ml aliquot from the fraction eluted between 50 and 65 ml in (B) was diluted with 2 ml of water and applied to the column. ●——●, protein (absorbancy at 570 mμ from 0.025-ml aliquots determined by the ninhydrin method after alkaline hydrolysis); ×- - - - -×, activity (absorbancy from 0.025-ml aliquots at 280 mμ on casein assay); Δ- - - - -Δ, carbohydrate (absorbancy at 625 mμ from 0.06-ml aliquot by the anthrone method after precipitation of protein with trichloroacetic acid).

CS101, or Bio-Rex 70) were used by Murachi and Neurath (1960) and by El-Gharbawi and Whitaker (1963) to separate several active components from crude stem bromelain. In the present experiments, however, only one active zone could be obtained from columns of IRC-50. It emerged when the molarity of the buffer at *p*H 6.0 was increased from 0.1 M toward 0.3 M. The peak was asymmetric and only 30–50% of the proteolytic activity was recovered. Better conditions for purification were afforded by a combination of gel filtration on Sephadex G-75 and ion-exchange chromatography on sulfoethyl-Sephadex. The results are illustrated in Figure 1. In the first step, elution from Sephadex, essentially all the activity appeared in one retarded zone which was separated from faster- and slower-moving carbohydrate- and protein-containing components of the mixture (Fig. 1A). The several effluent fractions that had high proteolytic activity were pooled and added directly to a column of sulfoethyl-Sephadex equilibrated with 0.3 M buffer at *p*H 6.0. Bromelain has a finite distribution coefficient in this system, hence there is no need to employ gradient elution (Fig. 1B). Once again activity is found in a single zone. Free carbohydrate is eluted in advance of this zone and none is detectable in the fractions containing the enzyme. The recovery of protein and proteolytic activity is more than 90% of that applied to the column. Rechromatography of the main component on SE-Sephadex yields a single symmetrical peak at the same effluent volume (Fig. 1C). The enzyme thus prepared migrated as a single band upon electrophoresis on cellulose acetate (*p*H 6.0, 0.02 M citrate buffer, 40 v/cm, 5-cm path). Nor could further fractionation be obtained by passage of the protein over columns of CM-cellulose or DEAE-cellulose (see El-Gharbawi and Whitaker, 1963). Desalting was effected on a column of Sephadex G-25 (5% acetic acid as eluent), as indicated in the experimental section.

By the procedure outlined above, which includes some losses since only the center fractions of the active zones were pooled, the main isolated component accounts for 42% of the total protein and 60% of the proteolytic activity in the original crude stem bromelain.

Purification of the Fruit Enzyme.—The most effective purification procedure proved to be passage of a solution of the crude enzyme over DEAE-cellulose (Fig. 2A). Carbohydrate passed straight through the column in 0.02 M buffer at *p*H 6.0 and was quantitatively removed together with some protein and a small amount of proteolytic activity. A change to 0.5 M buffer eluted the adsorbed enzyme which then contained no detectable free carbohydrate. Upon rechromatography the active component emerged at the same relative effluent volume and in good yield (Fig. 2B). A single band was obtained by electrophoresis of this material on cellulose acetate at *p*H 6.0 (see above).

The active fraction isolated from the crude bromelain of the green fruit by this procedure accounted for 43% of the total protein and 88% of the proteolytic activity originally present. The same procedure was applied to the ripe fruit with the same results except that yields were a little lower (32% of the total protein and 87% of the proteolytic activity).

Amino-terminal Residues.—The purity of the bromelain preparations was further examined by the sensitive and quantitative cyanate procedure for NH_2-terminal residues described by Stark and Smyth (1963). Two analyses of stem bromelain gave the following neutral and acidic NH_2-terminal residues per molecule of mw 35,730: valine, 0.96 and 0.90; alanine, 0.25 and 0.20; glycine, 0.06 and 0.05.[6] On the basis of an assumed molecular weight of 30,000, the fruit enzyme gave: alanine, 0.90 and 0.83; valine, 0.36 and 0.34; serine, 0.22 and 0.17; glycine, 0.13 and 0.05; glutamic

[6] The basic NH_2-terminal residues of the stem enzyme were determined and found to be negligible.

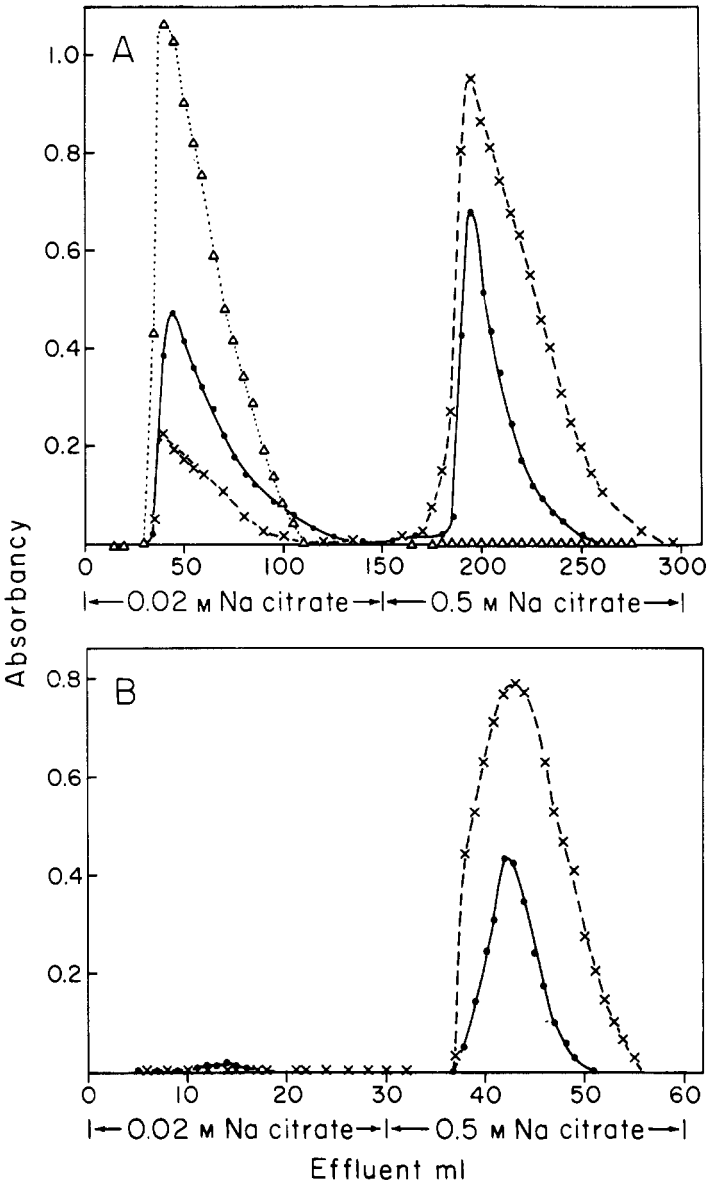

Fig. 2.—Chromatography of fruit bromelain. (A) DEAE-Cellulose, 2 × 20–cm column, *p*H 6.0, 0.02 M → 0.5 M sodium citrate buffer containing 5×10^{-4} M phenylmercuric acetate, 15 ml/hour, 5-ml fractions, 0.1-ml aliquots were analyzed. Sample: 200 mg of crude enzyme. (B) Rechromatography of (A). DEAE-Cellulose, 0.9 × 20–cm column, *p*H 6.0, 0.02 M → 0.5 M sodium citrate buffer containing 5×10^{-4} M phenylmercuric acetate, 3 ml/hour, 1-ml fractions, 0.2-ml aliquots were analyzed. A 1-ml aliquot from the fraction eluted between 190 and 210 ml in (A) was applied to the column after dialysis against water. ●——●, protein (absorbancy at 570 mμ from 0.1-ml aliquots determined by the ninhydrin method after alkaline hydrolysis); ×- - - - -×, activity (absorbancy at 280 mμ from 0.1-ml aliquots on casein assay); Δ- - - - -Δ, carbohydrate (absorbancy at 625 mμ from 0.03-ml aliquots by the anthrone method).

acid, 0.07 and 0.09. These results are corrected for blank values which were determined as recommended by Stark and Smyth. The special procedures required for the determination of NH_2-terminal cysteine and tryptophan were not carried out.

These results were obtained on samples of the enzymes that had been in contact with phenylmercuric acetate during the chromatographic purification. Autodigestion during preparation thus was minimized. When phenylmercuric acetate was omitted, the amounts of extraneous NH_2-terminal residues in the final preparations were definitely increased. For example, stem bromelain prepared without phenylmercuric acetate gave the following end groups per molecule of mw 35,000: valine, 0.62; alanine, 0.28; glycine, 0.28; serine, 0.69; glutamic acid, 0.08. The same trend was observed when autodigestion was deliberately allowed to occur at 40°, *p*H 7.7 (0.1 M Tris buffer containing mercaptoethanol) for 15 hours. Under such circumstances the NH_2-terminal alanine content of the stem enzyme rose 7-fold and the NH_2-terminal glycine content rose 10-fold. In addition, 0.5–1.0 equivalent of NH_2-terminal serine, aspartic acid, glutamic acid, and tyrosine, and lesser amounts of NH_2-terminal methionine, isoleucine, and leucine could be detected. A very similar kind of change occurred when the fruit enzyme was allowed to autodigest.

Amino Acid and Carbohydrate Composition.—The results obtained upon analysis of the purified stem and fruit enzymes are given in Table I. The values for stem bromelain are expressed as residues per molecule of mw 35,730. This is the figure which best fits the data, assuming two residues of histidine and five of methionine per molecule. This value is in fair accord with the results of Murachi *et al.* (1964)

TABLE I

AMINO ACID COMPOSITION OF STEM AND FRUIT BROMELAINS[a]

Amino Acid	Residues: Stem Bromelain Found	Stem Bromelain Expressed to Nearest Integer	Fruit Bromelain Leucine Set at 10 Residues: Green Fruit	Fruit Bromelain Leucine Set at 10 Residues: Ripe Fruit	Papain (Smith and Kimmel, 1960)
Aspartic acid	29.4	29	29.8	29.8	17
Glutamic acid	23.0	23	23.2	23.4	17
Glycine	34.6	35	32.6	32.2	23
Alanine	35.4	35	23.8	24.4	13
Valine	21.6	22	19.8	20.1	15
Leucine	10.0	10	10.0	10.0	10
Isoleucine	21.2	21	16.4	16.2	10
Serine[b]	28.2	28	32.2	32.0	11
Threonine[b]	13.6	14	13.5	13.8	7
Half-cystine[c]	10.5	10	10.0	10.0	6
Methionine[c]	5.1	5	6.0	5.8	0
Proline	14.2	14	11.6	12.0	9
Phenylalanine	8.8	9	7.6	8.0	4
Tyrosine[b]	20.8	21	22.4	22.2	17
Tryptophan[d]	8.1	8	5.6		5
Histidine	1.9	2	1.4	1.3	2
Lysine	22.9	23	7.8	8.3	9
Arginine	11.5	12	8.6	9.1	10
Amide ammonia[e]	41.6	42	43.0	43.4	19
Glucosamine[b]	5.8	6	<0.2	<0.2	
Carbohydrate (%)	1.46		3.2	3.3	

[a] Determined by ion-exchange chromatography (Spackman *et al.*, 1958). The amino acids, ammonia, and glucosamine recovered accounted for 100–103% of the nitrogen of the samples. The results for stem bromelain (average of two determinations) are expressed as the calculated number of residues for a molecule of mw 35,730 (see Murachi *et al.*, 1964). For comparison, data for the fruit bromelains, each the result of a single analysis, are expressed as mole ratios with leucine set at 10. [b] Corrected approximately for decomposition during 20 hr of acid hydrolysis. The factors applied were: serine, 0.90; threonine, 0.95; tyrosine, 0.95 (see Moore and Stein, 1963); glucosamine, 0.34 (see Plummer and Hirs, 1963). [c] Measured as cysteic acid or methionine sulfone after performic acid oxidation (Moore, 1963). [d] The values for tryptophan after alkaline hydrolysis (see text) are minimum values; further research on the method is needed in order to ascertain whether the hydrolytic condition gives a quantitative yield of the amino acid. [e] Determined with the use of Conway distillation (see Hirs *et al.*, 1954).

who determined the molecular weight to be 33,000 by physical methods.

The fruit enzyme is similar in amino acid composition to stem bromelain with the notable exception that it contains much less lysine, arginine, and histidine. This difference is reflected in the isoelectric points of the two proteins. The stem enzyme is basic and has an isoelectric point at about *p*H 9.5 (Murachi *et al.*, 1964), while the fruit enzyme is acidic and is strongly adsorbed by DEAE-cellulose at *p*H 6. The presence of glucosamine in the stem enzyme and its absence in the fruit enzyme is another difference between the two.

Murachi *et al.* (1964) were the first to observe that stem bromelain appears to be a glycoprotein. We have confirmed this observation for there is 1.5% carbohydrate, exclusive of glucosamine, in our purified preparation. The purified fruit enzyme, although devoid of glucosamine, contains about 3% carbohydrate.

Activities of the Purified Preparations.—The relative activities of the two purified enzymes are given in Table II, together with data on papain and α-chymotrypsin for comparison. Toward casein the fruit enzyme has about the same activity as papain and chymotrypsin, while the stem enzyme is about half as active. The fruit enzyme is relatively much more active against BAA than the enzyme from the stem.

TABLE II

RELATIVE ACTIVITIES OF PURIFIED STEM AND FRUIT BROMELAINS ON CASEIN, BENZOYL-L-ARGININAMIDE (BAA) AND DL-BENZOYLARGININE-*p*-NITROANILIDE (BAPA)[a]

Substrate	Relative Activities: Stem Bromelain	Fruit Bromelain: Green Fruit	Fruit Bromelain: Ripe Fruit	Papain	α-Chymotrypsin
Casein	9.2	25.3	23.7	24.2	23.8
BAA	0.14	7.3	6.3		
BAPA	0.9	14.3	14.7		

[a] The conditions of experiments with casein and BAA are the same as those given in the text for the crude enzymes, except that diluted solutions of purified enzymes were used (1–6 μg of enzyme N/ml). Activities are expressed as the changes in absorbancy at 280 mμ on casein, at 570 mμ on BAA, and at 410 mμ on BAPA, respectively, per minute per mg enzyme N. Crystalline papain and α-chymotrypsin were products from Worthington Biochemical Corp., Freehold, N. J., and were used without further purification.

DISCUSSION

The preparation in pure form of a proteolytic enzyme that is capable of autodigestion has always proved difficult, and the bromelains appear to be no exceptions. The earlier preparations of the stem enzyme studied by Murachi and Neurath (1960) and El-Gharbawi and Whitaker (1963) were shown to be resolvable into several components by chromatographic procedures. The most recent preparation of Murachi *et al.* (1964) appears to be chromatographically and electrophoretically homogeneous, as does the material described in this communication. When a sensitive and accurate end-group method is applied to our preparation, however, it can be shown to contain small but significant amounts of extraneous end groups. Since the number of end groups increases if no attempt is made to minimize autodigestion during isolation, it is reasonable to consider that the extraneous end groups might arise as a result of a small amount of autodigestion that occurs despite the presence of the specific inhibitor, phenylmercuric acetate. Particularly is this the case since the same kinds of end groups arise if autodigestion of the purified preparation is deliberately allowed to take place. If autodigestion were the cause of the extraneous end groups, fragmentation of the molecule should be demonstrable by other means. In an attempt to demonstrate such fragmentation, 15 mg of purified stem bromelain was reduced with mercaptoethanol in 8 M urea and *p*H 8.6 Tris buffer, and carboxymethylated with iodoacetate according to the procedure described by Crestfield *et al.* (1963a). Had autodigestion cleaved the peptide chain, the splitting of the —S—S— cross-links should have yielded smaller fragments which would be separable by gel filtration on Sephadex G-75. However, when the reduced carboxymethylated sample was applied to a 2 × 65-cm column of Sephadex G-75 equilibrated with 50% acetic acid, only a single, high molecular weight component was obtained. End-group analysis of this component gave the same result obtained before reduction, carboxymethylation, and gel filtration, namely, 0.96 residue of NH_2-terminal valine, and 0.25 and 0.1 residues of NH_2-terminal alanine and glycine, respectively.

This experiment, therefore, does not support the hypothesis that bromelain isolated in the presence of phenylmercuric acetate has undergone autodigestion. There remains the possibility that the results of the end-group analyses are related to the presence of the carbohydrate moiety. It is clear that only further work can clarify the nature of the heterogeneity that gives the analytical data obtained with purified preparations of bromelains.

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The Stereochemistry of Decarboxylation of Isocitrate by Isocitric Acid Dehydrogenase*

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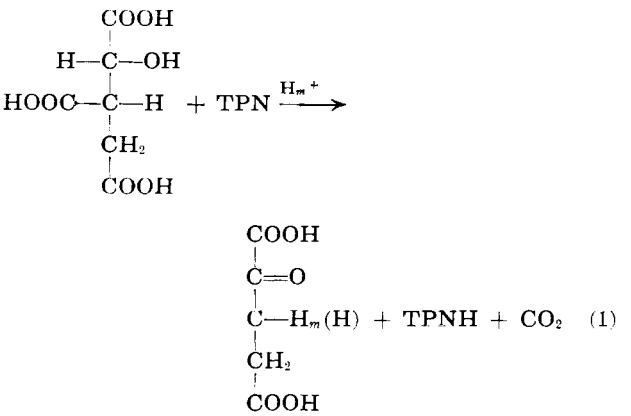
Stereospecifically β-tritiated α-ketoglutarate prepared by the oxidative decarboxylation of *threo*-D_s-isocitric acid with isocitric dehydrogenase in tritiated water undergoes complete exchange of its tritium with the medium hydrogen upon treatment with isocitric dehydrogenase under the conditions for the β-hydrogen exchange reaction. Therefore isocitric dehydrogenase acts upon the same β-hydrogen of α-ketoglutarate in both the oxidative decarboxylation and exchange reactions. The absolute configuration of stereospecifically β-tritiated α-ketoglutarate, which was prepared by the enzymic detritiation of randomly β-tritiated α-ketoglutarate, was determined from the known stereochemistries of the fumarase and aspartase reactions in the following way. Carbons 1–4 of this α-ketoglutarate were converted by a series of enzymic and chemical steps to β-tritiated L-aspartic and L-malic acids, which upon treatment with aspartase and fumarase, respectively, retained about 90% of their tritium. Comparison of the absolute configuration of the β-tritiated α-ketoglutarate with that of *threo*-D_s-isocitric acid revealed that in the oxidative decarboxylation of isocitric acid by isocitric dehydrogenase the replacement of the carboxyl group by a proton occurs with retention of configuration.

In the oxidative decarboxylation of *threo*-D_s-isocitric acid with TPN by isocitric dehydrogenase, a proton from the medium (H_m) takes the place of $-CO_2H$ in the β-position of α-ketoglutarate:



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