STUDIES ON STEM BROMELAIN AND STEM STARCH FROM PINEAPPLE PLANTS[1]

YIH-MING CHEN[2] and HO-YUAN LIU[3]

Abstracts: The authors have made an attempt to develop a practical method for the separation of bromelain and starch from pineapple stems. The bromelain was obtained either by the acetone method or by the tannic acid method. Results showed that most bromelain was precipitated in the 40-50% acetone concentration, also that most bromelain was precipitated when 2-4 g of tannic acid were added per liter, under these condition the highest specific enzyme activity was obtained.

The biochemical properties of crude enzyme were: optimal temperature 57°C; optimal pH 7.0; while Zn^{++} inactivated and Mg^{++} activated it to the highest degree.

Crude bromelain was made to separate the components by means of disc-electrophoresis. Six bands can be recognized in the zymographs, whether the bromelain was obtained by the acetone method or tannic acid method.

The pineapple stem starches are spherical granules of about 11 μ in diameter. Although the granules are easily broken, a typical granule has a Y-shaped fissure in its central area. Using chloral hydrate technique to prepare amylose and amylopectin and direct scale readings of a mixture of amylose and amylopectin, the results indicated that the amylose content of pineapple stem starch is 36%.

INTRODUCTION

Bromelain is a proteolytic enzyme existing in pineapple plants (*Ananas comosus* Merr.). Isolation of the enzyme from pineapple fruit and its characteristics have been studied by various workers (Chittenden, 1894; Vienes, 1903; Caldwell, 1905; Bergmann, *et al.* 1937) since 1894. The enzyme has also been isolated from the juice of the pineapple stem and called stem bromelain (Heinicke & Gortner, 1957). Bromelain can be used as a meat tenderizer, chill-proofing reagent for beer, food additive, bating reagent of hides, and so forth. Besides, it has also been employed in the paint industry to improve the stability of protein emulsifiers used in latex paints. And recently, it was found that bromelain could be applied as an antiphologistic with less after-effects.

Taiwan is one of the important pineapple production areas in the world, which is cropping over 270,000 M. T. per year. But the useful portion of the pineapple is only 45% of the fruit, the other portions such as the fruit stalk and stem are considered as the waste. Because of this, attempts were made to develop a practical method for the separation of bromelain and starch from the pineapple stem. The

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biochemical properties of the enzyme and the starch have been examined, and the results obtained are summerized in this paper.

MATERIALS AND METHODS

Stems of 2- to 3-year-old pineapple plants grown in southern Taiwan were first peeled and then smashed in a special milling machine. The homogenate was suspended in 0.1 M acetate buffer solution (pH 5.0) at 1:3 (w/v). The enzyme solution and starch were separated through canvas and nylon cloth, one-layer each, by centrifugation. The crude extract containing the enzyme was used for the isolation of bromelain. The starch fraction retained in the innerside of the canvas bag was collected, resuspended in water and then centrifuged to obtain the partially purified starch. The starch was dried in an oven at 60°C and then stored in a desiccator for further study of its biochemical characteristics.

The bromelain was obtained either by the acetone method (Ota *et al.*, 1964) or by the tannic acid method (Hwang and Hsu, 1970) with little modification. The assay of the proteolytic enzyme activity and calculation of enzyme activity unit is as follows:

The enzyme solution was made by dissolving 0.1 g of enzyme powder in cysteine-EDTA solution (cysteine-HCl, 5.3 g and EDTA, 2.2 g in 1,000 ml of distilled water, pH 4.5). Proteolytic activity against casein was measured by the Anson's method (1938) and Hagiwara's method (1954). One milliliter of the enzyme solution was mixed with 5 ml of 0.6% casein phosphate buffer solution (pH 7.0) and allowed to react at 37°C for 10 minutes. The reaction mixture was treated with 5 ml protein precipitant (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid) to stop enzyme reaction and then kept at 37°C for 30 minutes and filtered to remove the precipitates. Optical density (O. D.) of the resulting filtrate and blank solution were measured at 275 mμ in a Beckman DU Spectrophotometer. A blank solution was prepared by mixing the enzyme solution with the protein precipitant before adding the casein solution. A linear relationship between the tyrosine concentration and the optical density value was obtained (Fig. 1).

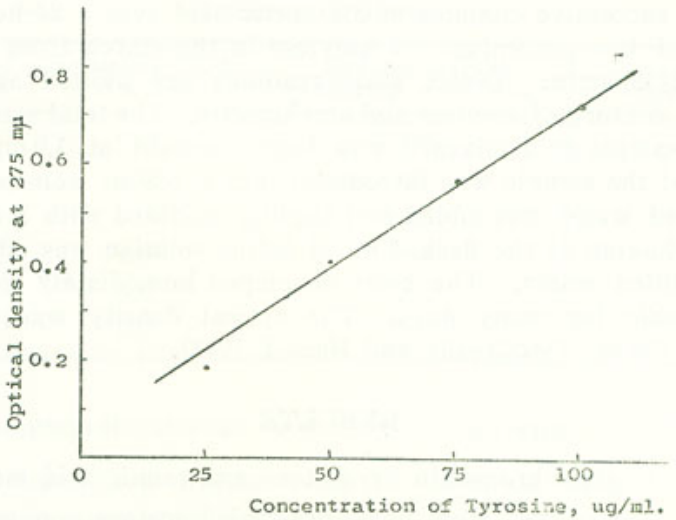

Fig. 1. The standard curve of tyrosine

Enzyme activity unit is expressed in tyrosine equivalent which is obtained from the standard curve. The calculation is made according to the following formula:

$$\text{Unit/mg bromelain} = \frac{Et - Eb}{Es} \times \frac{11}{10} \times \frac{V}{\text{mg of sample}}$$

where Et = The optical density was measured at 10 minutes.
Eb = The optical density was measured at 0 minutes before enzyme and substrate reaction.
Es = The optical density of 50 μg/ml of tyrosine was measured at 275 mμ.
V = Total volume of sample solution.

For the starch fractionation, the chloral hydrate technique (Muetgeert, 1961) and the Nussenbaum and Hassid's method (1952) were adapted. The chloral hydrate method was performed as follows: Dissolve 60 g of 100% chloral hydrate in 30 ml of distilled water and then add 4 g of sodium acetate dihydrate. With constant stirring, a suspension of 4 g of starch in 10 ml of distilled water was added to this chloral hydrate solution at room temperature. The solution was kept in the dark at 20°C for 72 hours, then was centrifuged at 6,000 xg for 10 minutes. The pellet was washed several times with 30% chloral hydrate solution. All the chloral hydrate solution, supernatant and aqueous washing, were collected and diluted with distilled water to make the final concentration of chloral hydrate solution between 5 and 8%, and then kept in the dark for 24 hours. The amylose was then precipitated and separated by centrifugation at 1,000 xg for 10 minutes. After washing with ethanol and ether, twice each, the amylose was dried at 60°C overnight and placed in a vacuum desiccator over phosphorus pentaoxide.

Acrylamide gel electrophoresis: For analyzing the components in the crude enzyme, 7% acrylamide gel columns of 7×0.7 cm were used for disc-electrophoresis in 0.05 M tris-glycine buffer system in the cold room. One milliliter of the sample solution, containing about 0.5 mg bromelain, was layered on the top of each gel column. Five mA of D.C. current per tube was applied for 40 minutes. The gel column was then removed gently from the glass tubes after rimming around the glass wall with a needle, and stained with 0.01% amino black in 3% acetic acid overnight in the cold room. The background stain in the gels were removed within the test tube by successive changes of 3% acetic acid over a 24-hours period.

Calculation of the percentage of amylose in the starch from scale readings of amylose and amylopectin: Direct scale readings are plotted against percentage concentration of mixture of amylose and amylopectin. The total starch concentration, whether a pure sample or a mixture, was kept constant at 1.0 mg/ml of solution. Five milliliters of the sample was introduced into a 500 ml volumetric flask, about 100 ml of distilled water was added and slightly acidified with 3 drops of 6 N HCl. After vigorous shaking of the flask, 5 ml of iodine solution was added and made up 500 ml with distilled water. The color developed immediately to its full intensity and remained stable for many days. The optical density was read by using a Spectronic 20 at 630 mμ (McCready and Hassid, 1943).

RESULTS

A. Fractionation of stem bromelain by acetone and tannic acid method:

To the newly centrifuged stem juice, pre-cooled acetone was added slowly with constant stirring in several different concentrations of acetone. During the addition

of the acetone, the temperature of the solution was kept near 5C° in an ice-bath. The precipitates were dried in a vaccum desiccator, then crushed to powder and their enzyme activities were determined. The results obtained are given in Table 1. It showed that most bromelain precipitated at 40–50% acetone concentration, with the highest specific enzyme activity.

Table 1. The fractionation of bromelain by acetone from 600 grams of pineapple stems

Conc. of acetone (%)	Bromelain yield (in gram)	Enzyme activity (unit/mg of enzyme)	Percentage of total enzyme activity
0-25	0.88	31.06	0.79
25-40	2.36	163.35	10.05
40-50	2.60	848.29	63.88
50-60	1.51	477.53	20.07
60-70	1.00	108.70	3.14
70-80	0.83	21.35	0.51
80-90	0.44	23.29	0.29

The results of the fractionation of bromelain by tannic acid are given in Table 2. It is seen that most of the bromelain was precipitated by 2 to 4 g of tannic acid per liter. It is also obvious that the enzyme activity in these three fractions is the highest. This indicates that these enzyme powders are more pure than those precipitated at other ranges of the tannic acid concentration. Therefore the ranges between 2 to 4 g tannic acid per liter can be properly employed in bromelain preparation.

Table 2. The fractionation of bromelain by tannic acid from 1.7 kg of pineapple stems

Conc. of tannic acid (g/liter)	Bromelain yield (in gram)	Enzyme activity (units/mg of enzyme)	Percentage of total enzyme activity
1	1.11	139	5.05
2	1.58	652	37.33
3	1.04	803	30.26
4	0.83	679	20.42
5	0.31	278	3.12
6	0.51	178	3.27

B. Effect of low-temperature storage on the enzyme activity:

Raw materials, stored in a freezer (−20°C) for three weeks, were employed for extraction of stem bromelain by acetone method. As shown in the Table 3, the long-time storage at low-temperature caused a decline in enzyme activity.

Table 3. Effect of low-temperature storage on the yield and activity of bromelain extracted from 300 grams of pineapple stem

Bromelain extracted from	Yield (gram)	Bromelain activity (units/mg of enzyme)
Fresh material	1.65	671.5
Frozen material	2.35	126.7

C. Effect of temperature on enzyme activity:

The crude bromelain powder (0.1 g) was dissolved in cysteine-EDTA solution consisting of 5.3 g cysteine-HCl and 2.2 g EDTA in 1,000 ml of distilled water (pH 4.5). The solution was assayed at various temperatures by the method described in "Materials and Methods".

As shown in Fig. 2, the optimal temperature of bromelain was at 57°C. The bromelain activity dropped sharply above 67°C; this may be due to the inactivation of the enzyme by heat.

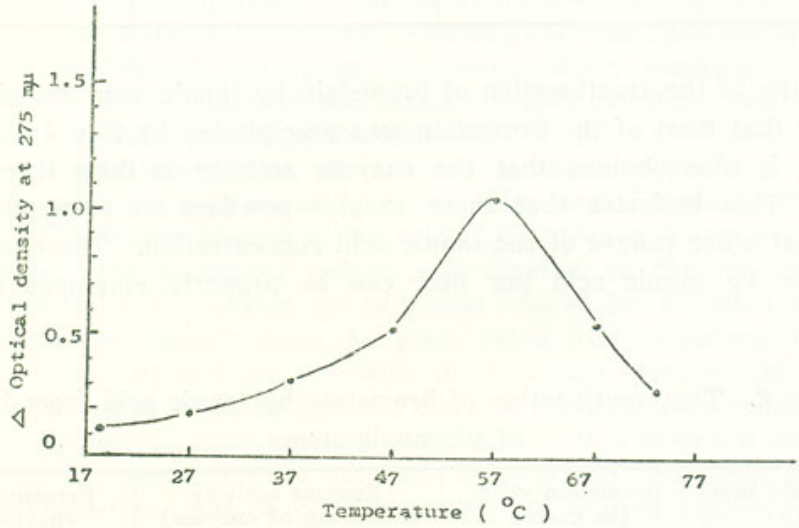

Fig. 2. Effect of reaction temperature on bromelain activity

D. Effect of pH on enzyme activity:

Casein-buffer solutions (0.6%) of various pH values were prepared for reacting with the bromelain solution under the conditions of above-mentioned assay method. The curve in Fig. 3 showed that the enzyme preparation possessed three pH optimum values, namely pH 7.0, pH 9.0, and pH 11. It is therefore possible that the bromelain preparation contained at least three components.

E. Effect of metallic ions on enzyme activity:

The degree of activation or inhibition was expressed in each case as the ratio of the activity of the treated enzyme to the untreated enzyme solution. As shown in Table 4, Zn^{++} inactivated and Mg^{++} activated it to the highest degree.

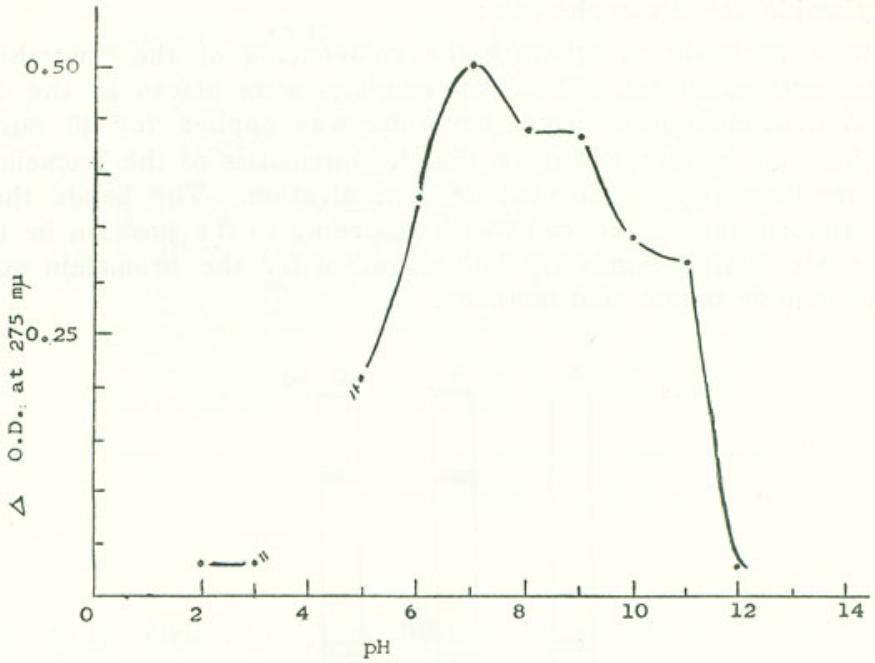

Fig. 3. Effect of reaction pH on bromelain activity

Note: The breakage of the curve is due to the fact that casein does not dissolve in this pH range.

Table 4. Effect of metallic ions on bromelain activity after 10 minutes incubation at 37°C

Reagents	Relative activity
$SnCl_2 \cdot H_2O$	14.4
$CdSO_4$	28.3
$Pb(CH_3COO)_2 \cdot 3H_2O$	104.3
$CuSO_4$	75.1
$FeCl_3$	41.1
$Ca(H_2PO_4)_2$	113.5
$HgCl_2$	74.0
$ZnSO_4$	7.6
$NiSO_4$	51.6
$MgSO_4$	110.0
$AgNO_3$	83.7
$FeSO_4$	50.2
Uranyl acetate	21.8
Control	100.0

* Reagent concentration: 2×10^{-3} M.

Enzyme concentration: 0.1 g of bromelain dissolved in 1,500 ml of cysteine-EDTA solution.

F. Polyacrylamide gel electrophoresis:

Attempts were made to separate the components of the crude bromelain by means of disc-electrophoresis. The crude enzymes were placed at the cathodes of each tube. A D.C. current of 5 mA per tube was applied for 40 minutes. The electrophoretograms are indicated in Fig. 4. Intensities of the bromelain bands in zymograms are indicative of the enzyme concentration. The bands therefore are artificially grouped into three categories according to the position in the electrophoretic field, six distinct bands can be recognized for the bromelain extraction by either the acetone or tannic acid methods.

Fig. 4. Schematic zymographs of stem bromelain fractionated by polyacrylamide gel electrophoresis.
A: A commercial preparation of bromelain from Sankyo Co., B: Extract by acetone method; C: Extract by tannic acid.

G. Starch grains from pineapple stem:

The granules of starch extracted from pineapple stems were examined under a microscope. Their characteric appearance were spherical granules of about 11 μ in diameter (Fig. 5). Although the granules are easily broken, a typical granule has a Y-shaped fissure in its central area.

H. Amylopectin and amylose fractionation from pineapple stem starch:

Using the chloral hydrate technique, the yield of amylose and amyopectin obtained from 6.88 g of dried pineapple stem starch was shown in Table 5. The amylose fraction contained 35.2% of total recovery starch.

I. Calculation of the percentage of amylose in pineapple stem starch from direct scale readings of a mixture of amylose and amylopectin:

Direct scale readings are plotted against percentage concentration of mixture of amylose and amylopectin. The total starch concentration, whether a pure sample or a mixture, is kept constant at 1.0 mg/ml of solution. As shown in Fig. 6, an artificial mixture of amylose and amylopectin (20, 40, 50, 60, and 80% amylose) falls

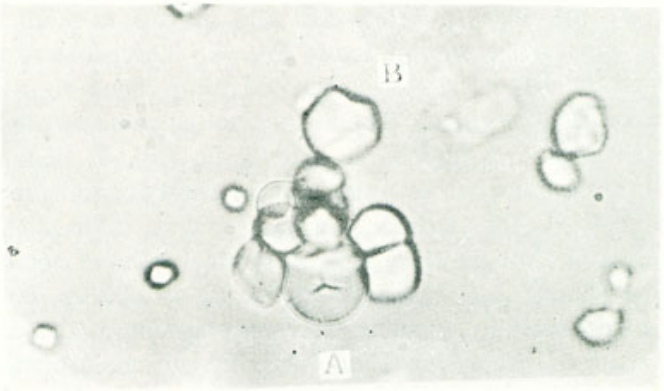

Fig. 5. Starch granules from pineapple stem, ×1,000
A. Typical starch granule; B. Broken starch granule.

Table 5. Yield of amylose and amylopectin obtained from 6.88 g dried pineapple stem starch

Sample	Dry wt. (g)	Iodine color intensity (% of stem starch)	% of total recovery
8 g (air dried)	6.88	100	
(A) Amylose	2.33	215	35.2
(B) Amylopectin	4.28	38.9	64.8
A+B	6.61		100.0

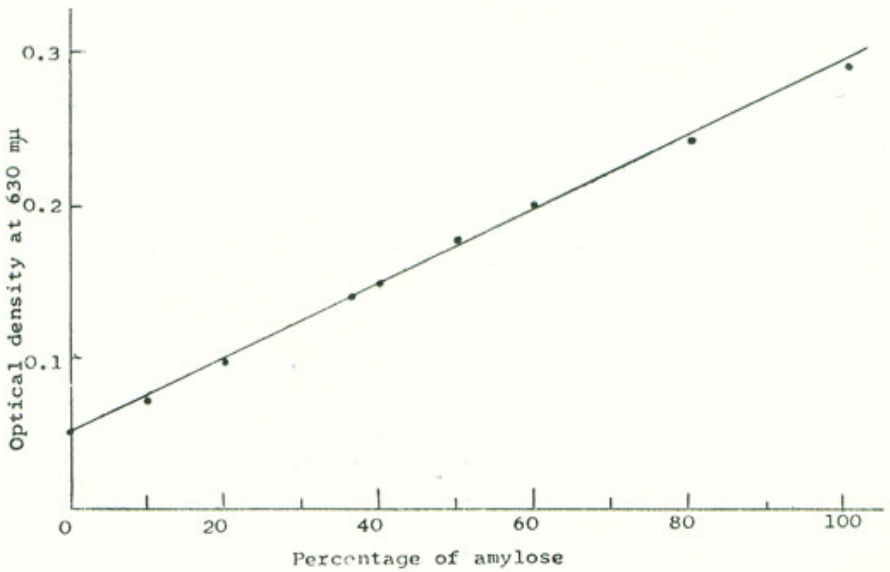

Fig. 6. Direct scale readings are plotted against percentage concentration of mixture of amylose and amylopectin

in a straight line connecting pure amylopectin and pure amylose. Referring the absorption spectra of amylose, amylopectin and pineapple stem starch with iodine complex, the 36% of amylose obtained from Fig. 6. This data agrees well with the value of 35.2% found by direct determination of total recovery of amylose obtained by analyzing the separate fractions. The absorption spectra of amylose, amylopectin, and stem starch with iodine complex is shown in Fig. 7. Staining with iodine, amylose shows an intense blue while amylopectin shows faint reddish-purple. The light absorption curve of the iodine complexes was measured over the visible spectrum. Fig. 6 indicates that amylose and amylopectin have remarkable differences in absorption spectra, where λ_{max} of amylopectin is at 560 to 600 mμ and that of amylose is at 630 mμ, respectively. Quantitative expression of the differing intensities has been made in terms of the "Blue Value" (B. V.) which is a measure of the light absorption at 680 mμ of 0.01% solution of the polymers in iodine (Bourne *et al.* 1948). B. V. of amylose and amylopectin are 0.255 and 0.03, respectively.

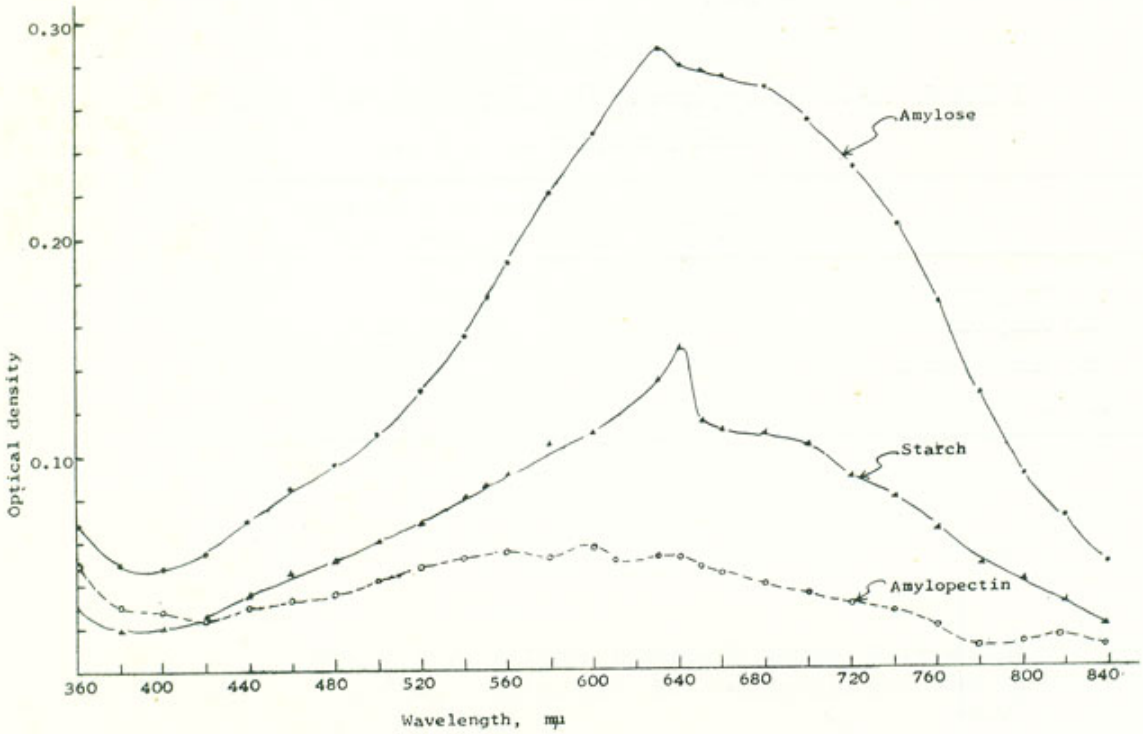

Fig. 7. The absorption spectra of amylose, amylopectin and pineapple stem starch with iodine complex.

DISCUSSION

Several organic solvents, such as acetone and ethanol, have been used for extraction of bromelain (Heinicke and Gortner, 1957; Su *et al.* 1967). The using of tannic acid to separate bromelain from stem extract was recently reported by Hwang and Hsu. In order to obtain higher yields of enzymes from pineapple stems, some hydraulic press methods, such as freezing and thawing followed by oil press under 20,000 lb/sq. in. (Hwang and Hsu, 1970), had been devised. However, some difficulties were encountered as only a small amount of pineapple juice was

obtained by this method. Thus, in this study, a special designed masher was employed to crush up the stems and these were then extracted by acetate buffer.

The number of proteolytic enzymes in preparations of bromelain from the pineapple stem has not yet been definitely established. Two groups of investigators, Murachi & Neurath (1960) and El-Gharbawi & Whitaker (1963), have reported several chromatographically separable, proteolytically active components in such preparations. On the other hand, Murachi *et al.* (1964) and Ota *et al.* (1964) have reported more recently that most of the activity obtained is in a single component which gives a single zone upon electrophoresis and ultracentrifugation. In the present study, the crude stem bromelain, either extracted by acetone or by tannic acid, was run by acrylamide electrophoresis to yield six bands. This is necessary to prepare the pure form of bromelain for further studies.

Starch in the pineapple stem is found abundantly. By using a special centrifuge apparatus, it can be easily collected. Very few references have been found in literature to the nature of pineapple stem starch. It has long been recognized that starch can be separated into two fractions based upon their physical properties. These two fractions are designated as amylose and amylopectin. Using chloral hydrate technique to separate amylose and amylopectin, the results obtained indicate that the amylose content of pineapple stem starch is 36%, which is much higher than that in most plants, most starches usually contain from 15–25% amylose (Whelan, 1958). A higher amylose content is found in the Easter Lily (32%) (Lansky and Kooi, 1949), in specially bred varieties of maize (up to 77%), and also in wrinkled peas (up to 72%).

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