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EXTRACTION AND ACTIVITY OF BROMELAIN FROM PINEAPPLE

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RINGKASAN

Bromelain (EC.3.4.22.4) ialah satu enzim proteolitik yang terdapat didalam spesi-spesi dari keluarga "Bromeliaceae".

Disebabkan Malaysia menghasilkan lebih 250.000 ton nenas tiap-tiap tahun, maka tindakan-tindakan untuk menjadikan nenas ini sebagai sumber bagi enzim proteolitik sedang dititikberatkan.

Kajian-kajian cubaan telah dijalankan untuk mengekstrak bromelain dari daun, tangkai, kulit, buah dan batang nenas. Aktibiti dari ekstrak bromelain dilakukan dengan menggunakan kaedah pembekuan susu. Kesan garam dan pH terhadap aktibiti di atas telah dikaji.

Hasil-hasil menunjukkan yang bromelain memang terdapat di dalam buah, tangkai, kulit, daun dan batang nenas. Kadar penghasilan bromelain dari buah, tangkai, kulit dan batang masing-masing ialah 0.08%, 0.06%, 0.075% dan 0.1 0.6%. Terlampau sedikit bromelain yang diekstrak dari daun.

Pada pH = 4.6, aktibiti susu dari bromelain batang (1000 - 5000 M.C.U.) adalah lebih tinggi dari aktibiti bromelain buah (500 - 900 M.C.U.). pH juga mempunyai kesan terhadap aktibiti pembekuan susu di mana terlalu rendah (terlalu asidik) atau terlalu tinggi (terlalu basik) akan mengakibatkan aktibitinya rendah. pH optimum ialah 3.8. Garam juga didapati mempunyai kesan penekanan terhadap aktibiti pembekuan susu oleh bromelain.

INTRODUCTION

In 1976 the total production of fresh pineapple for canning was 224,956 metric tons by both the estates and the smallholders. Of the total pineapple acreages in Johore, approximately 28,000 acres are estimated to be old stands of pineapple. The Malayan Pineapple Industry Board is undertaking a replanting scheme of the old pineapple areas. The acreage to be replanted under the Third Malaysia Plan is expected to be 18,000 acres, at the replanting rate of about 3,500 acres per year. During the replanting the normal practice is to kill the plants by chemical spray followed by burning (Malayan Pineapple Industry Board, Tampoi).

BALLS, THOMPSON and KIES (1941) reported that the pineapple plant contains a reasonable amount of proteolytic enzyme (bromelain). The Pineapple Research Institute of Hawaii in 1959 started a study on the proteolytic enzymes of the pineapple plant. They found that not only all varieties of commercial pineapple contain protease, but all species of the genera "Bromeliaceae" contain similar, but probably slightly different protease. Furthermore, they found that the protease of the fruit, leaves and stem represented different mixtures of proteases. To avoid coining hundreds of new plant protease, HEINICKE and GORTNER (1957) suggested that the name "Bromelain" should represent any proteolytic enzyme obtained from any member of the family *"Bromeliaceae"*.

If the possibility of extracting and utilising the enzyme is economical, the pineapple that are rejected and the wastes from the fruits and stems could be utilised as another source of proteolytic enzyme.

There are conflicting reports on the protease activity in pineapple fruit at different stages of development and ripening. BALLS, *et al.*, (1941) reported that green, immature pineapple fruit have less protease than fully ripe ones. Another worker, DE SOUZA (1948) found little protease in market-ripe fruits but appreciable quantities in green fruit. While HEINICKE (1954) as cited by GORTNER and SINGLETON (1965) found that the bromelain concentration in pineapple flesh did not change appreciably as the fruit developed, but at maturity, when the fully expanded fruit begins to ripen, the protease content increases. However, GORTNER and SINGLETON (1965) also observed that a marked drop in protease activity during the final ripening occurred and this was not accompanied by a corresponding change in protein concentration, as it is speculated that bromelain is transformed to another protein which may have a different metabolic role, such as flavour producing enzyme.

HEINICKE and GORTNER (1957) in their work on stem bromelain found that the concentration of the enzyme increased with the maturity of the plant. The central portion or the stele contain more enzyme than the outer portion, i.e. the cortex.

The number of proteolytic enzymes present in pineapple plant bromelain has not yet been definitely ascertained. HEINICKE and GORTNER (1957) showed that crude bromelain could be separated electrophoretically at pH 6.5 into four distinct components with different proteolytic activity. Several proteolytic active components have also been separated from commercial powdered stem bromelain by chromatography on cation exchange resin at pH 6.1 (GREENBERG and WINNICK, 1940, MURACHI and NEURATH, 1960). The preparation of homogenous stem bromelain, accounting for a major part of the total proteolytic activity has been reported by OTA, MOORE and STEIN (1964) and MURACHI, YASHI and YASUDA (1964). OTA, *et al.*, (1964) also purified bromelain from the stem as well as from both the immature and ripe fruit. No differences were detected in the enzyme obtained from these two sources. However, the fruit enzyme contained much less lysine, arginine and histidine than the stem bromelain.

Many research workers (CHITTENDEN, 1892; WILLSTATTER, GRASSMAN and AMBROS, 1926; GREENBERG and WINNICK, 1949) have prepared bromelain from filtered pineapple juice. These workers have used either sodium chloride, ammonium sulphate, acetone or alcohol to precipitate the enzyme from the juice. The viscosity of the juice, combined with low yield of enzyme, the high cost of recovery and the value of mill juice for other byproducts have effectively prevented any commercial exploitation of fruit juice for enzyme production.

BALLS, *et al.*, (1941) proposed that the only economic advantage in the manufacture of bromelain over papain is by utilising material from byproducts of another pineapple industry. Raw material must be from factory waste since other products are too valuable. The amount of enzyme is too small and it would not pay to destroy the sugar or citric acid. They further proposed that the bromelain may be precipitated by alcohol and recovered later. The residual juice would thereafter be available for the usual alcoholic fermentation as no sugar is lost.

MATERIALS AND METHODS

Studies were carried out on the extraction of enzyme from the following parts of the pineapple plant which were obtained from the Jalan Kebun, MARDI station.

1. Whole fruit (ripe and unripe)
2. Flesh only
3. Skin only
4. Stalk
5. Stem
6. Leaves

The recovery rate was calculated and the enzyme activity was tested using the milk clotting method.

i. Extraction of bromelain from fruit and skin

Extraction of the enzyme was carried out using GREENBERG (1940) method. The fruits sample were cut into small pieces, macerated and weighed. The juice was pressed and filtered through cheesecloth. The pH of the juice was adjusted to pH 6.0. Ammonium sulphate was added until saturation to precipitate the enzyme. Partial purification was carried out by redissolving the crude enzyme in sodium cyanide and repeatedly precipitating it, firstly with 0.6 percent saturated Ammonium sulphate and then with acetone. The precipitate was thoroughly washed with acetone and ether and then dried in a vacuum oven.

ii. Extraction of enzyme from stem

The extraction of enzyme from stem was carried out by a method based on that of HEINICKE and GORTNER (1957). The raw materials for the extraction of the enzyme were matured pineapple stem. These were collected after the final fruit harvest and were freed of leaves and sucker stems. The stems were weighed and then placed in a scraping machine. The juice was pressed and filtered through cheesecloth. Acetone was added to the stem juice in two stages. The first step was the addition of two volumes of acetone to two volumes of stem juice. The precipitated form was discarded as it has low enzymatic activity, poor colour and poor stability. Addition of another volume of acetone precipitated the main enzymatic fraction. This was collected by centrifugation (20,000 g for 25 minutes) and washed with acetone and di-ethyl-ether, and then dried in vacuum oven. The acetone was recovered from the supernatant solution by distillation.

iii. Assay of the enzyme

The enzyme extracted was tested for its activity using the milk clotting method (BALLS and HOOVER, 1937). Experiments were also carried out to see the effect of pH and salt on the milk clotting activity of the enzyme.

iv. Definition of activity of bromelain

The activity of bromelain was based on its milk clotting property. One milk clotting unit (MCU) is defined as the amount of enzyme which clots the milk in one test tube (under the conditions described) in one minute.

20 g of dried whole milk were diluted to 100 ml with acetate buffer of pH 4.6. The solution of bromelain were prepared by grinding the crude enzyme to a smooth paste with a small amount of distilled water in a mortar. The paste was diluted to the volume required. 1 ml of the various dilutions of this solution was incubated in a test tube at 40°C with 10 ml of milk preparation. The enzyme was first activated using sodium cyanide solution (0.02M) for 30 to 60 minutes. The time taken for the formation of precipitate through the tube was recorded. The reciprocal of time taken to reach incipient clotting was taken as a measure of the rate of the reaction. The activity of the enzyme was calculated based on the formula below:

$$E = K/t$$

where

E = Enzyme concentration

K = Kinetics

t = time (min)

Et = K

when

t = I

E = K

$\therefore$ the enzyme activity (I/K) = I/E when t = I

The effect of pH on the milk clotting activity was carried out based on the same procedure except that in this case the pH were varied (3.5, 3.8, 4.6, 5.8) while other factors were kept constant.

To see the effect of salt on milk clotting, the enzyme was dissolved in different concentration of salt solution (5%, 10%), while other factors were kept constant. The activity was similarly calculated.

RESULTS AND DISCUSSION

Recovery Rate

As shown in *Table 1* the concentration of enzyme was found to be greater in ripe fruits compared to immature fruits. This is similarly reported by BALLS, *et al.*, (1941) who found that green immature pineapple fruit have less protease than fully ripe ones.

Bromelain is also present in leaves, stalk and stem. However, it was found most abundantly in the stem (0.1–0.6%) especially matured ones. Leaves too contain enzyme but yield poor recovery. A byproduct of stem bromelain extraction was starch (10–15% on fresh weight basis) which is separated from the juice before the extraction of bromelain is carried out.

From *Table 2*, the milk clotting activity of stem bromelain is observed to be higher than that of fruit or skin bromelain. This is similar to the finding by HEINICKE and GORTNER (1957) who reported that the stem bromelain has MCU/g of 2000 – 5000 while that of fruit

TABLE 1. BROMELAIN CONTENT OF THE PINEAPPLE PLANT

	Part of the Plant	Recovery Rate (%)
1.	Whole fruit (ripe)	0.06 – 0.08
2.	Flesh only (ripe)	0.08 – 0.125
3.	Skin only	0.05 – 0.075
4.	Stalk	0.04 – 0.06
5.	Stem	0.10 – 0.6
6.	Whole fruit (immature)	0.04 – 0.06
7.	Flesh (immature)	0.05 – 0.07

TABLE 2. ACTIVITY OF BROMELAIN AT pH 4.6 FROM PINEAPPLE PLANT

	Part of the Plant	MCU/g
1.	Whole fruit (ripe)	500 – 900
2.	Flesh (ripe) of fruit	500 1000
3.	Skin of fruit	200 400
4.	Stem :	
	Stem bromelain divided into :	
	a. First precipitation	50 – 150
	b. Second precipitation	1000 5000

bromelain to be 800 – 2500. The activity of the skin bromelain is the lowest (200 – 400 MCU/g).

However, the milk clotting activity cannot be compared with the result of other workers as the use of different types of milk, technique or reagents can lead to different milk clotting values.

In *Table 3,* it is shown that pH has an effect on the milk clotting activity of bromelain.

The milk clotting activity was reduced when the pH is too acidic or slightly acidic. When the pH is greater than 6, the activity is similarly reduced. It was observed that the optimum pH for milk clotting activity is about 3.8. This is comparable to that reported by BALLS, *et al.,* (1941) who observed that the optimum pH for milk clotting activity is about 3.5.

When the enzyme was dissolved in salt (sodium chloride) solution, the milk clotting activity was also affected. When the salt concentration is increased the activity was reduced.

This is shown in *Table 4.* The effect of salt was studied since salt is added to meat during cooking. This is similar to the finding of THOMPSON, *et al.*, (1973) who worked on the protease extracted from ginger.

TABLE 3. THE EFFECT OF pH ON MILK CLOTTING ACTIVITY OF BROMELAIN

pH	MCU/g	
	Stem	Fruit
3.4	840	147
3.8	8.000	1.515
4.6	2.150	350
5.8	130	50

MCU — Milk Clotting Unit

TABLE 4. EFFECT OF SALT (SODIUM CHLORIDE) ON THE CLOTTING ACTIVITY OF FRUIT BROMELAIN

Salt (sodium chloride) concentration	MCU/g
0%	380
5%	180
10%	150

MCU — Milk Clotting Unit

CONCLUSION

The experiment shows that the possibility of extracting bromelain from the pineapple plant is great, especially from the stem where the recovery rate (0.1 — 0.6%) is higher than that of fruits (0.06 — 0.08%). The stem is available in abundance after harvesting of fruits and more so during replanting time. At present, the stem is either burned down or left to rot. The extraction of bromelain from fruit is more economical during the peak season when greater rejection of undersized and over-ripe pineapple occur.

In the market, the cheapest source of proteolytic enzyme is papain. For bromelain to be competitive with papain, the raw materials used should be that of factory waste as other products of pineapple industry are very valuable. It will be more appropriate, if bromelain extraction is a byproduct activity of the pineapple industry. Pineapple canning, animal feed milling and bromelain extraction should be integrated activities as they are interrelated.

From the experiment carried out by another worker.(En. Mat Isa Awang – personal communication) on the prqteolytic activity of the bromelain preparation, it was found that our enzyme preparation is of about the same strength as bromelain purchased from Sigma Chemical Co. The activity of stem bromelain was higher (1.97 U Cas./min. mg solid) than that of fruit bromelain (1.83 U Cas./min/mg solid) but the specific activity of fruit bromelain (3.16 U Cas./min/mg. enz. protein) is more superior than stem bromelain (3.01 U Cas./min/mg. enz. protein). In terms of milk clotting activity, the stem bromelain is much more superior (1000 to 5000 MCU) compared to that of fruit bromelain (500 – 900 MCU) at pH 4.6.

Production of stem bromelain yields several other byproducts such as starch and cattle feed. While none of these by itself is valuable enough to warrant harvesting stumps, they help to defray part of the operating costs.

SUMMARY

Bromelain (Ec. 3.4.22.4) is a proteolytic enzyme found in all species of genera "Bromeliaceae".

As Malaysia produces greater than 250,000 tons of fresh pineapple annually, the possibility of utilising these pineapples as a source of proteolytic enzyme is being looked into.

Trials were carried out on the extraction of bromelain from leaves, stalk, skin, fruit and stem. Activity of the extracted bromelain was measured using milk clotting method. The effect of pH and salt on milk clotting activity was also studied.

The result indicated that bromelain is present in fruit, stalk, leaves, skin and stem. The recovery rates of bromelain from fruit, stalk, skin and stem are 0.08%, 0.06%, 0.075% and 0.1 – 0.6% respectively. Very little bromelain was recovered from the leaves.

Milk clotting activity of the stem bromelain (1000 – 5000 MCU) was higher than that of fruit bromelain (500 – 900 MCU) at pH 4.6. pH has an effect on the milk clotting activity. Too acidic or slightly acidic resulted in a low milk clotting activity. The optimum pH for milk clotting activity is about 3.8. Salt was found to have an inhibitory effect on milk clotting activity of bromelain.

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