

PROTEOLYTIC ACTION OF BROMELAIN ON *HEVEA* LATEX

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SUMMARY

The proteolytic action of purified bromelain and unfractionated juice of pineapple –PAJ– (*Ananas comosus*), on *Hevea* proteins was studied. Both were found to be effective in hydrolysing *Hevea* proteins to yield a low protein rubber which meets specifications for deproteinized natural rubber (DPNR). It showed a temperature optimum around 45°C and a broad pH max. near 8.0. The highest deproteinization (63.15%) of latex was observed with 0.08% w/v bromelain. PAJ treatment of latex is simple and economically feasible and therefore of great commercial importance.

INTRODUCTION

Enzyme deproteinization of *Hevea* latex has recently been studied by several workers recently with a view to producing a rubber with improved dynamic properties (see John *et al*, 1977 and Yapa *et al*, 1980). Removal of proteins has also been found to be essential in cyclized rubber manufacture from NR by using the p-toluene sulphonic acid method. The effect of 25 proteolytic enzymes of different origin on skim latex obtained from *Hevea* was studied by Morris (1954). In recent years, an enzyme of bacterial origin, namely superase, has been investigated extensively in Malaysia for the manufacture of DPNR, whilst in Sri Lanka papain has been used for the production of DPNR which has been successfully used in the production of cyclized rubber. However, studies carried out in our laboratory showed that pineapple juice (PAJ) treated rubber could be cyclized readily and completely by the p-toluene sulphonic acid method. This present investigation was undertaken to study the action of purified bromelain (the proteolytic enzyme present in pineapple juice), on *Hevea* latex and also to explore the possibilities of using crude pineapple juice in DPNR manufacture.

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EXPERIMENTAL

General

Fresh pineapple juice obtained from fresh fruits of *A. comosus* was used in experiments with PAJ. In the study on the effect of concentration, temperature and pH, purified Bromelain (2 x crystallized) obtained from pineapple stem Koch Light, UK) was added to fresh *Hevea* latex obtained from the Rubber Research Institute estate at Dartonfield and diluted 1:1 with water.

Effect on proteins associated with rubber

All serum proteins and amino acids were removed by double centrifugation at 12,000 rpm. The rubber cream obtained after the first centrifugation was resuspended in distilled water and centrifuged again at 12 000 rpm for 20 min. Rubber cream was separated and redissolved in distilled water at 15% dry rubber content. Conditions of enzyme treatments are indicated in the results section. Bromelain was added to latex at 0.01% w/v and left for 24h at room temperature. Latex was then coagulated with Mg ions. The water clear serum was used for amino acid analysis on paper by using n-butanol-acetic acid-water (120:30:50 v/v) and phenol-ammonia solvent systems. Nitrogen content of the rubber fraction was determined by micro-Kjeldahl method.

Electrophoresis of PAJ

This was carried out by polyacrylamide gel electrophoresis; acrylamide at 7.5% with Tris glycine buffer, pH 8.5. Running time was 55 min. Gels were fixed in 12.5% trichloroacetic acid for 2 hours and stained with Coomassie Brilliant Blue for 48 hours as described by Chrambach *et al* (1967).

RESULTS

Effect of concentration

The effect of bromelain concentration on deproteinization is indicated in Fig. 1. Of the different concentrations tried out, 0.08% (w/v) bromelain showed the highest proteolytic effect on *Hevea* proteins; the nitrogen content obtained by this

concentration was 0.14%. The proteolytic action decreased with higher concentrations presumably due to faster gelling of latex forming a coagulam which affected the enzyme action.

Effect of Temperature

The range of temperatures tried in this study was 25°C – 75°C at 10°C intervals. The effect of temperature on bromelain treatment of latex is given in Fig 2. The highest deproteinization action was observed at 45°C which gave a nitrogen content of 0.12%. Proteolytic action of bromelain on latex proteins decreases gradually with increasing temperatures, above 45°C. Higher temperatures also affected the coagulation of latex forming a thin film of rubber at the surface of latex.

Effect of pH

The effect of pH on bromelain treatment is shown in Fig 3 indicates a broad max near pH 8 for the hydrolysis of latex proteins by bromelain. However, the higher pH values affected the coagulation, often resulting in milky serum. Table 1 gives the gelling time at different pH values; the gelling time was found to be noticeably long, extending into several hours, when the pH of the latex was above 8.

Table 1. *Effect of pH on gelling time*

pH	Gelling time (min)	Serum
5.0	5.2	Clear
6.0	9.4	Clear
7.0	21.0	Clear
8.0	45.0	Clear
9.0	259.0	Clear
10.0	Over 7 hours	Milky

* Bromelain at 0.08% w/v

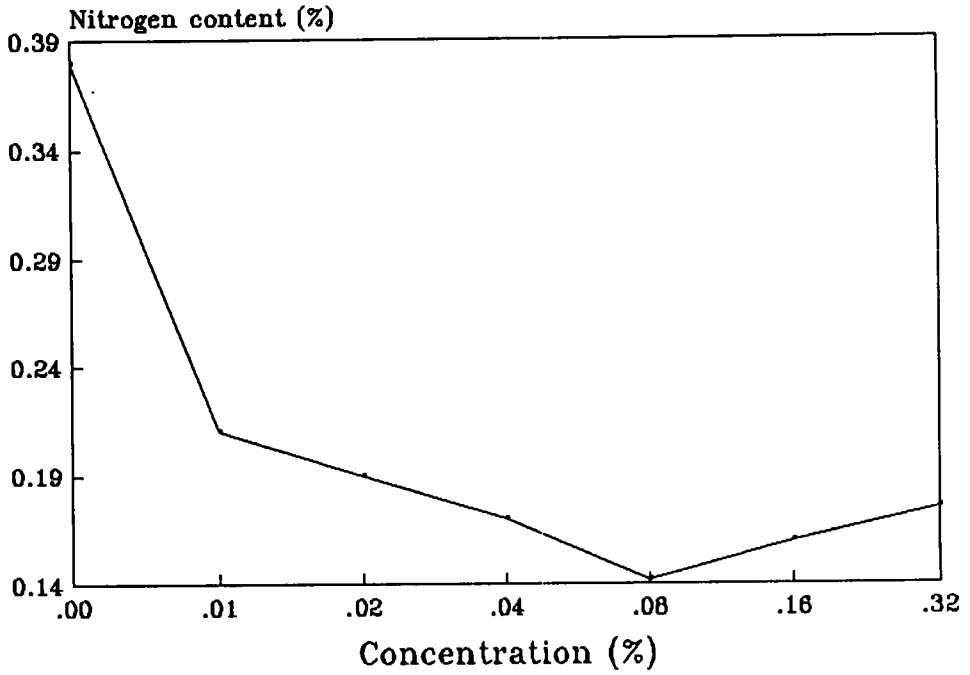


Fig. 1 Effect of bromelain concentration on deproteinization

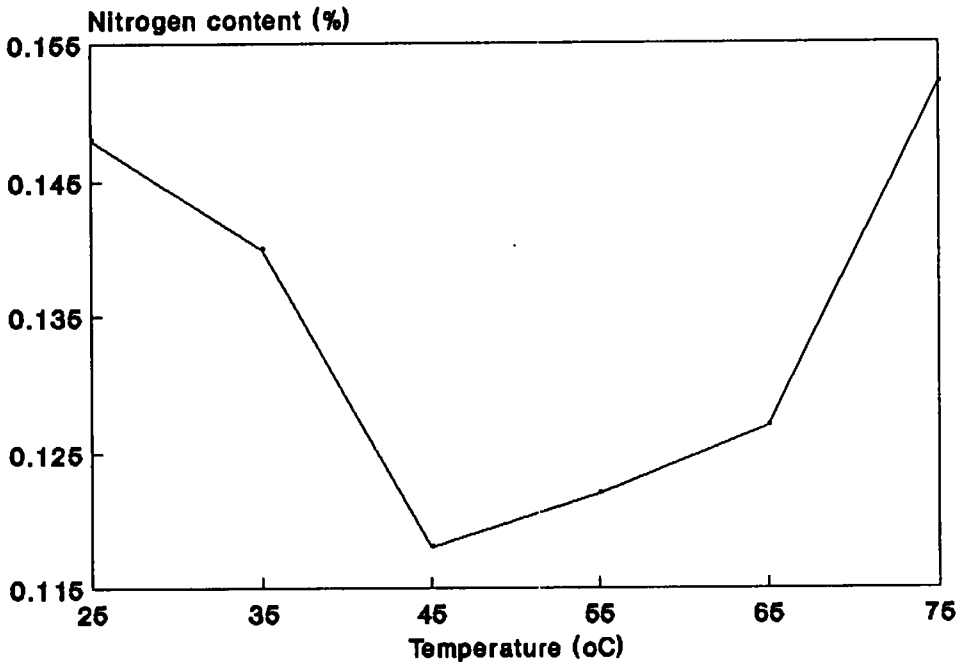


Fig. 2 Effect of temperature on bromelain treatment of *Hevea* latex

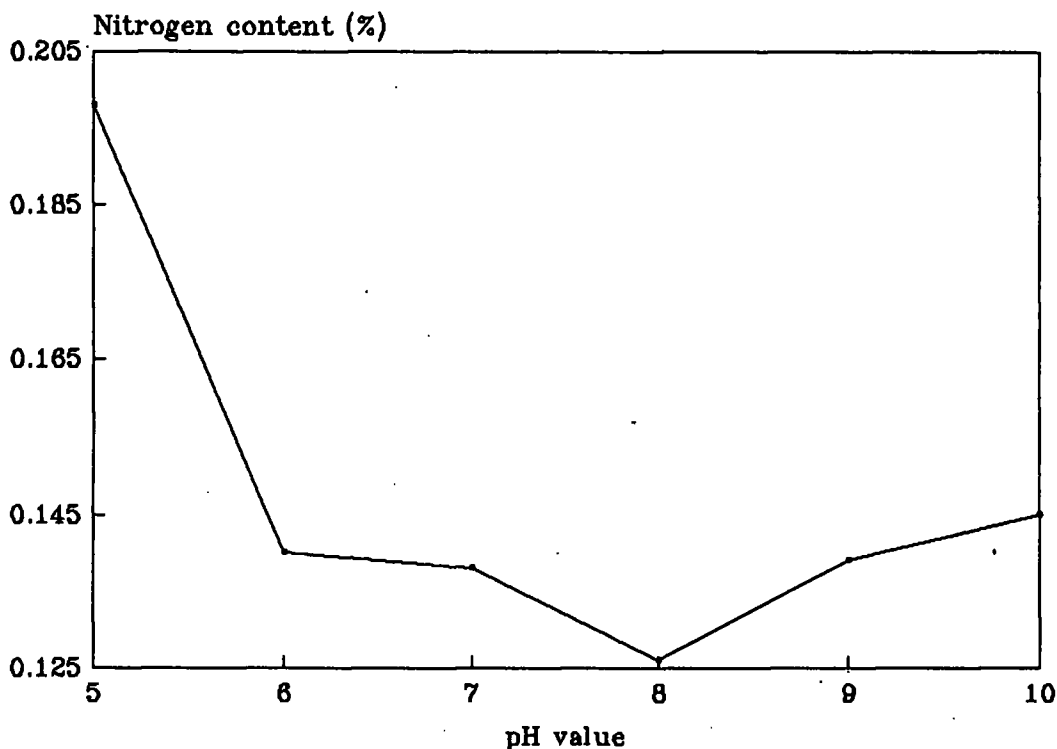


Fig. 3 Effect of pH on bromelain treatment of *Hevea* latex

Pineapple juice

In view of the possible practical implications of using purified bromelain in large scale manufacture of low protein rubber, the possibility of using fresh PAJ without any additional treatments was investigated. The effect of different concentrations of PAJ on deproteinization (Table 2) shows that low protein rubber can be obtained by PAJ treatment of latex. However, increasing concentrations seem to have little effect on further removal of proteins; the degree of deproteinization up to a concentration of 8% v/v was found to be the same and concentrations above this were found to have an inhibiting effect on hydrolysis. Gelling time decreased with increasing concentrations of PAJ. The pH of the resulting serum too decreased with increasing concentrations of PAJ.

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Table 2. *Effect of PAJ treatment on deproteinization and gelling time*

PAJ concentration (% v/v)	Gelling time (min)	pH of serum	Nitrogen content of rubber % w/w
1	33.0	5.72	0.18
2	16.0	5.10	0.18
4	12.0	4.50	0.18
8	9.1	4.20	0.18
16	6.0	4.15	0.20
Control (HCOOH)	22.0	4.81	0.41

Electrophoresis

Disc gel electrophoresis of PAJ (crude) obtained from ripe fruits of *A. comosus* showed 13 bands on acrylamide gel (Fig 4). Bands 1,2,3 and 4 were very prominent. Purified bromelain obtained from pineapple stem (Koch Light product) showed a diffuse single band in the same area. Buffered PAJ (with Tris-glycine buffer, pH 7.5) and PAJ with NaSHO₃ (added as a preservative) also gave 13 bands similar to those of crude PAJ.

Chemically unpreserved PAJ kept at 4°C for one week was subjected to electrophoresis on acrylamide gel. Only six bands which were also mostly in a diffuse state as shown in Fig 4 were obtained. Some of these diffuse bands appeared to be formed from a combination of several bands. Buffered PAJ showed a better band pattern than unbuffered PAJ; here the bands 1,2,3,4 and 5 were clear enough although slightly affected, bands 8,9 and 10 were also fairly clear, but bands 6,7,11,12 and 13 were not clear.

PAJ sample preserved with NaSHO₃ also showed a streaking effect similar to unpreserved PAJ. There was no change in the electrophoretic pattern of purified bromelain which again gave a single diffuse band after standing at ambient temperature for one week.

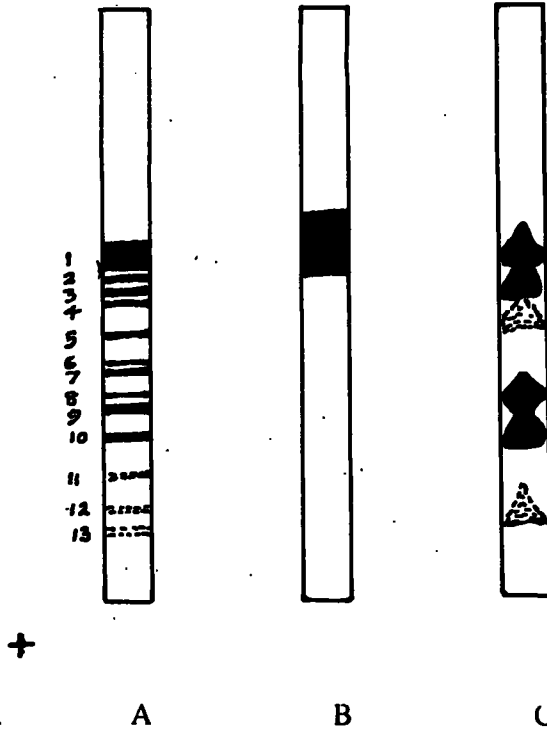


Fig. 4 Polyacrylamide gel electrophoresis of (A) Crude PAJ, (B) Bromelain (stem) and (C) Preserved PAJ 13 bands were observed in A, one in B and 6 in C

Amino acid analysis

The serum obtained after enzyme treatment followed by coagulation, was analyzed for amino acids by paper chromatography and the results are shown in Figs 5 and 6. Seven amino acids were detected in the serum obtained from bromelain treatment (Fig 5) whilst five amino acids were detected after PAJ treatment of resuspended rubber particles (Fig 6). Eleven amino acids were detected in the serum obtained from bromelain treatment of field latex whilst 10 amino acids were detected after PAJ treatment of field latex. Although no attempt was made to do a quantitative

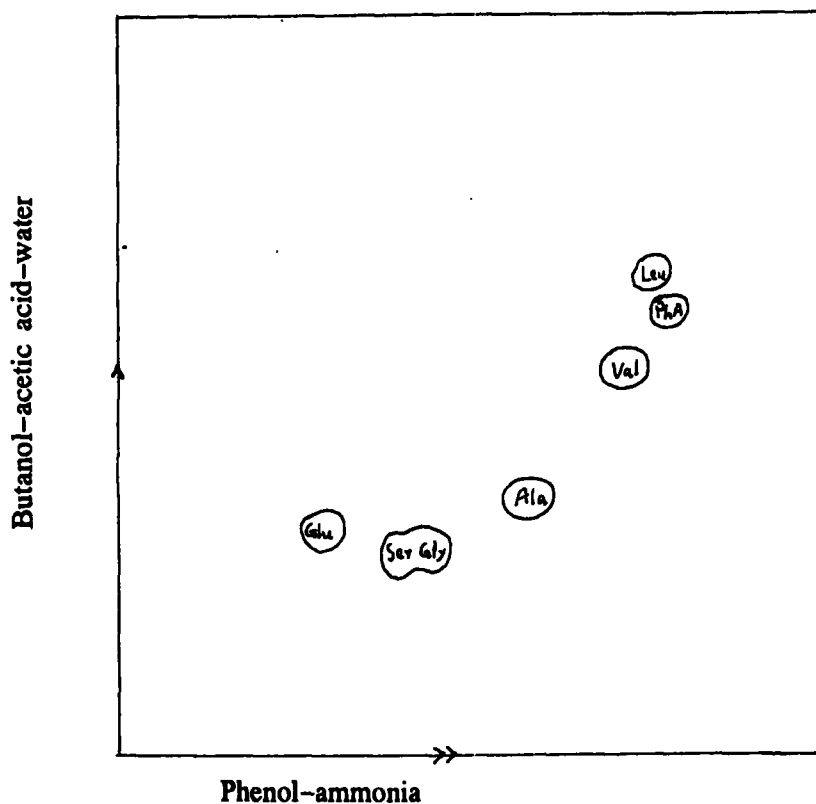


Fig. 5 Chromatographic analysis of rubber serum obtained after treatment with Bromelain; seven amino acids were detected with ninhydrin

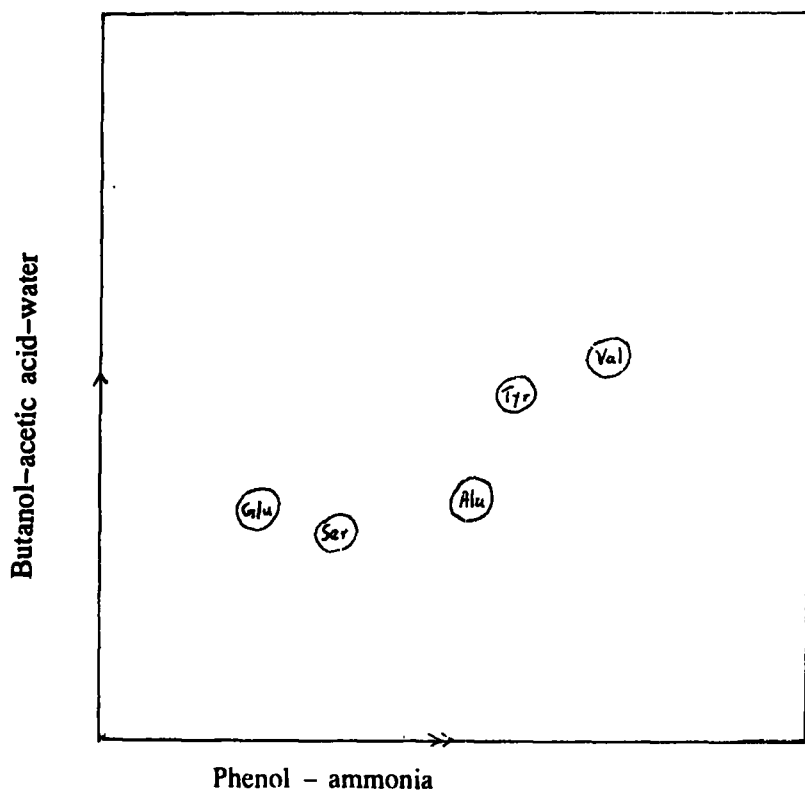


Fig. 6 Chromatographic analysis of rubber serum obtained after treatment with crude PAJ, for amino acids; five amino acids were detected with ninhydrin

analysis, it appeared, judging by the intensity of spots, that bromelain treatment had yielded a higher amount of amino acids than PAJ treatment. This was further supported by the protein content of the resulting rubbers; the protein content of bromelain treated rubber was 0.20% whilst that of PAJ treated rubber was 0.30% indicating a higher degree of hydrolysis in the former.

DISCUSSION

Bromelain is one of the four pineapple enzyme systems, (others being IAA oxidase, peroxidase and phosphatase) that has been extensively studied using the pineapple plant stem as source material (Heinicke and Gortner, 1957 as quoted by Dull, 1971; Gortner and Kent, 1953 and 1958). Bromelain extracted from pineapple stem was used in majority of the investigations of these studies. However, the use of raw juice obtained from the pineapple fruit was also investigated by us because of its obvious practical importance, particularly in view of the fact that it has been reported by Pieris *et al* (1978) that the commercial extraction of bromelain would not be economically feasible. It has also been shown that PAJ is as effective as papain in hydrolysing *Hevea* proteins and that the resulting rubber could be successfully cyclized by using the p-toluene sulphonic acid method (Yapa and Lionel, 1980).

Fruit enzymes have not been investigated as thoroughly as the stem enzymes. Purification and characterization of fruit bromelain has been reported by Ota *et al*, 1964, as quoted by Dull, 1971) and more recently by Pieris *et al* (1978), from pineapple waste. The results of this study have shown that PAJ exhibits sufficient proteolytic activity to be used in DPNR manufacture from field latex. Gortner (1965) has reported that half of the protein in the fruit showed protease activity. Pieris *et al* (1978) too have reported that the highest bromelain activity is found in the edible portion. All these findings points to higher protease activity in PAJ extracted from pineapple fruits. A reduction in the latex protein content by 56% was obtained by action of PAJ at 4% v/v and this reduction is comparable to that of papain treatment of *Hevea* latex (Yapa & Balasingham, 1974). Higher PAJ concentrations of 16% v/v showed reduced proteolytic action (Table 2) presumably due to quick coagulation of latex by the higher organic acid content, which reduced the chances of bromelain acting on latex proteins in the liquid phase. The higher the amount of PAJ added to latex. The higher is the amount of organic acids associated with it and this is evidenced in the low pH of the resulting serum; at 16% v/v on latex, it is even lower than that of acid coagulated serum; pH of PAJ serum was 4.15 and that of acid serum was 4.81. The pH of fresh PAJ is around 3.5, depending on the variety and maturity of the fruit. PAJ therefore possesses like papain, the advantageous dual action property on *Hevea* latex of coagulation and protein digestion.

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The results of this study have shown that the highest deproteinization of latex is obtained by using a bromelain concentration around 0.085 w/v; the nitrogen content of the resulting rubber was 0.14%. Perera & Silva (1978) using similar concentrations have obtained rubbers with a N content of 0.20%. The reduced proteolytic action reported in their work could be due either to loss of activity in bromelain preparation or higher pH of latex. When the enzyme treatment is carried out on deammoniated latex, an incomplete deammoniation of latex would normally result in a higher alkalinity which would retard the protease action of bromelain. The present study shows that conditions over pH 8 retard the proteolytic action of bromelain. When bromelain has been used on field latex, a reduction in N content of 53.36% was obtained by Perera & Silva (1978) with 0.1% bromelain - which is comparable to results obtained by us. This rules out the possibility of a loss of activity in the bromelain preparation and lends further support for our finding that bromelain is less active at higher pH values.

Electrophoretic separation of proteins in PAJ showed 13 bands, of which a large number were acidic. It has been reported that fruit bromelain is acidic (Ota *et al*, 1964 as quoted by Dull, 1971), but no attempt was made to detect the actual bromelain band amongst the acidic bands. The basic proteins, the bands of which were comparatively more prominent than acidic proteins in the acidic region, showed similar electrophoretic behaviour to stem bromelain which gave a diffused band. It has been reported by Heinscke & Gortner, (1957 as quoted by Dull, 1971) that stem bromelain is basic. When potassium ferricyanide was omitted from the gel medium, the diffused band of stem bromelain gave two fairly clear bands.

One of the major considerations of this investigation was to see whether PAJ loses its proteolytic activity on storage even after the addition of chemical preservatives. Pieris *et al* (1978) have reported that bromelain is autodigested itself and is consequently highly unstable in solution. However we observed that NaHSO₃ - preserved PAJ, stored at 4°C, is fairly effective at least for two weeks in hydrolysing *Hevea* proteins. Electrophoretic separation of proteins in one week old NaHSO₃ - added PAJ showed an almost similar band pattern to that of unpreserved crude fresh PAJ; a slight streaking effect was the only noticeable difference observed.

The results of the present study have shown that PAJ treatment can be used in the manufacture of low protein rubber from field latex. Perera & Silva (1978) have recommended the use of pure bromelain on field latex as this has been found by them to be more effective than PAJ. However, we have consistently observed that fresh PAJ is as effective as bromelain (stem bromelain) on field latex and therefore can be used successfully in low protein rubber manufacture (Yapa & Lionel, 1980). The use of pure bromelain has the disadvantage that it is more expensive than crude PAJ. Pieris *et al* (1978) concluded that the commercial extraction of bromelain would not be economically feasible due to several reasons. One area that requires further study is

enzyme activation; it has been reported that bromelain is activated by cysteine or by cyanide and strongly inhibited by mercuric ions, inhibition being completely reversed by an excess of cysteine (Gortner & Kent, 1958). It would be interesting to see if such activation occurs in latex system as well. One of the reasons for PAJ being as effective as purified bromelain, may be due to the presence of naturally occurring activators in fresh unfractionated pineapple juice.

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