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THE PROTEOLYTIC ACTION OF PAPAIN ON PROTEINS IN *HEVEA* LATEX

By

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SUMMARY

Treatment of natural rubber latex with papain serves two purposes. First it acts as a coagulant, second it reduces the nitrogen content of the final product substantially, the reduction generally being in the range of 30—50%. *Papain breaks down* Hevea *proteins to the amino acids, this is reflected in the increase in the quantities of several amino acids namely glutamic acid, serine, αalanine, tryptophan, phenylalanine, valine and leucines present in the serum after enzymic action. Papain appears to be more effective at a neutral than at an alkaline pH though the latter gave a rubber of a better colour. Temperatures higher than the ambient temperature were relatively favourable for the proteolytic effect. Of the chemical activators examined only cysteine and thiourea appeared to have any desirable effect on deproteinization. Inhibitory effect of hydroxylamine hydrochloride was so small that it can be used in combination with papain in the manufacture of CV rubber with a low nitrogen content. The slight increase in the ash content is, apparently, the only disadvantage of the papain treatment which may be of some consequence.*

Organic acids such as formic, acetic and oxalic acid are the commonly used coagulants for natural rubber latex. The use of mineral acids such as hydrochloric acid has been re-assessed recently (Karunaratne & Piyadasa, 1973). Biological methods of coagulation have also been reported by several workers. (John, 1966; John & Pillai, 1971: Satchuthananthavale & Satchuthananthavale, 1971).

When organic acids are used they not only agglomerate the rubber particles by bringing down the pH of the latex to near that of the isoelectric point of proteins which protect the rubber particles, but also precipitate serum proteins which become occluded in the rubber coagulam. These serum proteins, the presence of which is reflected in the high nitrogen content, apparently confers hardly any advantage to the final rubber. The high nitrogen content is a problem of considerable concern, particularly in skim rubber which usually contains over 1% nitrogen; it makes the rubber fast curing and scorchy.

Methods such as alkaline hydrolysis have been tried out to bring down the high nitrogen content in rubber. Another method is to subject the latex to the action of enzymes which attack proteins and render them soluble.

Proteolytic enzymes from both vegetable (*e.g.* papain, ficin and bromelin) and animal sources (*e.g.* pepsin, trypsin) have already been tried out on skim as well as field latex by a number of workers (De Vries, 1920; Wentworth, 1939; as quoted by Morris, 1954; Baker, 1940; Nadarajah *et al.*, 1973). Baker (1940) has reported that trypsin is the most effective of the three enzymes, trypsin, pepsin and papain, he examined at a temperature of 30°C but trypsin being an animal enzyme is most unlikely to be available as cheaply and conveniently as an enzyme from a vegetables source, like papain.

It was felt desirable to explore the possibilities of using papain in the manufacture of rubber with a low nitrogen content. This is of particular interest in view of the fact that papain is a locally available raw material. This paper reports the results of the studies on some factors affecting papain treatment.

MATERIALS AND METHODS

Of the two grades of papain (white and brown) available, oven dried papain which is white in color was used in all the experiments reported in this paper. It was added as a slurry in a small volume of water. Latex obtained from Dartonfield Group was diluted (1:1), prior to papain treatment, with water in all experiments.

The semi-micro Kjeldahl method was used in all the nitrogen determinations. Ash content was determined by the B.S. method. Amino acids were analysed by two dimensional paper chromatography on Whatman No. 1 paper using n-Butanol-acetic acid-water (120:30:50 v/v) and phenol-ammonia as the solvent systems.

RESULTS

Amino acid analysis : Papain at a concentration of 0.05% w/v was found to coagulate latex overnight and the pH of the resulting clear serum was in the range of 6.2—6.4. The time taken for coagulation decreased with increasing concentrations of the enzyme. It has been reported by several workers that papain does not digest proteins to the level of amino acids (Bondi & Birk, 1954; Winnick, 1944). On the contrary, in this study, an increase in the amount of several amino acids namely glutamic acid, serine, α-alanine, tryptophan, phenyl-alanine, valine and leucines was observed (Table 1); with increasing concentrations of papain, nitrogen contents of rubber, obtained from this treatment decreased correspondingly (Table 1).

TABLE 1

EFFECT OF INCREASING CONCENTRATIONS OF PAPAIN ON THE AMINO ACID CONTENT IN THE SERUM

AMINO ACID	CONCENTRATION OF PAPAIN (% ON LATEX)					
	Control acid	0.025	0.05	0.1	0.2	0.4
Aspartic	+	+	—	—	+	—
Glutamic	+++++	+++++	+++++	—	—	++++++++
Serine	++	+++	+++	++++	++++	+++++
α-alanine	++++	+++++	++++++	+++++++	+++++++	++++++++
Glutamine	++	—	—	—	—	—
Tryptophan	—	+	+	++	+++	+++
Ph.alanine	++	+++	+++++	++++++	+++++++	++++++++
Valine	++	++	+++	++	+++	++++
Leucines	+	++	+++	++++	++++	++++++++
Nitrogen % (resulting rubber)	0.550	0.546	0.492	0.488	0.439	0.395

(In the visual estimation, intensity of + is equivalent to 0.5 x 10^{-6} g of each amino acid).

The increase in amino acid content was particularly marked in the case of tryptophan and leucines. It is possible that the free amino acids in dried papaw milk, papain, may also have contributed to the increase of some of these amino acids (Table 2). When latex was treated with a papain sample precipitated by ethanol, there was still an increase of the five amino acids namely glutamic acid, glycine, £-alanine, valine and phenylalanine compared with acid coagulation (Table 2). This suggests that they have resulted from a hydrolysis of latex proteins. Glutamine was found usually in the acid coagulated serum. However it was interesting that no glutamine was found in the serum after coagulation with crude papain. On the other hand it was present in the serum after treatment with pure papain.

TABLE 2

AMINO ACIDS IN THE SERUM OBTAINED FROM COAGULATION OF FIELD LATEX

Amino acid	Coagulated with			
	Acid	Crude papain	Pure papain	Free amino acids in raw papaw milk
Aspartic	—	+	—	?
Glutamic	+++	+++++	++++	+
Glycine	+	++++	++	+++
Serine	—	—	—	+
α Alanine	++	+++++	+++	++
Glutamine	++	—	++	—
Valine	++	+++	++++	++
Tryptophan	—	+	—	—
Phenylalanine	—	+++	+	++
Leucines	—	++++	—	—

(In visual estimation, intensity of + is equivalent to 0.5 x 10^{-6}g of each amino acid).

Results of amino acid analysis of serum obtained from skim latex are given in Table 3. No increase was noticed in aspartic acid, glutamic acid, serine, and αalanine contents, after papain treatment. Tryptophan and valine, which were not found in the acid coagulated serum, were present. Considerable increases in as partic acid, glutamic acid and serine were noticed when the skim latex was adjusted to pH 7.0 with dil. H_2SO_4 This seems to support the reports that papain is more effective at a neutral pH than at an alkaline pH.

TABLE 3

AMINO ACIDS IN THE SERUM OBTAINED FROM COAGULATION OF SKIM LATEX

Amino acid	Treatment		
	Acid	Papain	Acid/Papain
Aspartic acid	+	+	+++
Glutamic acid	+	+	+++
Serine	+	+	+++
α Alanine	+++++	+++++	++++++
Glutamine	—	—	++
Tryptophan	—	+++	++
Valine	—	++	++

(In the visual estimation, intensity of one + is equivalent to 0.5 x 10^{-6} g of each amino acid).

Clonal variation : Comparatively lower nitrogen contents than that of bulk latex of the factory was obtained when the papain treatment was carried out on the latex of individual clones (Table 4).

TABLE 4

COMPARISON OF PROPERTIES OF PAPAIN AND ACID COAGULATED RUBBER OF SOME OF THE POPULAR CLONES

Clone	Nitrogen %		Reduction in nitrogen content from acid coagulation %	Po		PRI		Ash %	
	acid	papain		acid	papain	acid	papain	acid	papain
RRIC 89	0.39	0.20	50	52	57	35	65	0.10	0.17
RRIC 28/59	0.30	0.19	35	44	45	61	58	0.16	0.18
RRIC 7	0.50	0.20	61	48	57	68	75	0.12	0.23
PB 86	0.41	0.18	57	45	65	64	65	0.07	0.27
RRIC 52	0.45	0.18	59	45	61	67	66	0.08	0.22
RRIC 45	0.46	0.21	55	34	49	62	61	0.13	0.27

Nearly all the clones examined had a higher Initial Wallace Plasticity (P_0) after papain treatment. No marked change in the Plasticity Retention Index (PRI) was observed with the exception of clone RRIC 89. It has been reported that papain treated rubber has a slightly higher ash content (Wentworth, 1939; Nadarajah *et al.*, 1973). This was found to be true with all the clones investigated. However, when papain, precipitated from raw papaw milk with ethanol was used, interestingly, no such increase was observed (Table 5).

TABLE 5

EFFECT OF OTHER CONSTITUENTS IN PAPAIN ON PROPERTIES OF RAW RUBBER OBTAINED BY PAPAIN COAGULATION

Coagulant	P_0	PRI	Ash %	Nitrogen %	Reduction in nitrogen from acid coagulation %
Crude papain (dried papaw milk)	45	60	0.21	0.22	56.2
Pure papain (Ethanol precipitated)	42	64	0.14	0.32	36.8
Acid/formic acid 2%	48	75	0.16	0.51	—

Effect of activators : There was no very significant effect from potassium cyanide, urea, EDTA and sodium sulphide on the reduction of nitrogen content. On the contrary they seem to have a slightly inhibitory action (Table 6). Cysteine and thiourea were found to enhance the proteolytic action of papain. Neither P_0 nor ash content showed a profound change by addition with papain, of any of these compounds to latex. There was hardly any significant change in the PRI with the exception of those treated with EDTA which increased the PRI by more than 10 units.

TABLE 6

EFFECT OF SOME OF THE WELL KNOWN ACTIVATORS OF PAPAIN (0.05% ON LATEX) ON RAW RUBBER PROPERTIES

Treatment	P_0	PRI	Ash %	Nitrogen %	Reduction of nitrogen content from acid coagulation %
Acid coagulation	59	59	0.16	0.46	—
Papain coagulation	58	60	0.28	0.25	46
Papain + Cysteine (0.01M)	58	60	0.32	0.22	51
Papain + thiourea (0.01M)	60	58	0.30	0.22	52
Papain + KCN (0.005M)	52	64	0.28	0.26	43
Papain + urea (0.01M)	58	57	0.30	0.25	45
Papain + EDTA (0.01M)	55	71	0.25	0.28	39
Papain + Na_2S (0.01M)	54	57	0.28	0.32	31

High concentrations of thiourea were found to be inhibitory on the deproteinization process although such concentrations improved the PRI considerably (Table 7).

TABLE 7

EFFECT OF USE OF INCREASING CONCENTRATIONS OF THIOUREA WITH PAPAIN (0.05% ON LATEX) ON THE RAW RUBBER PROPERTIES

Thiourea concentration/mole	P_0	PRI	Ash %	Nitrogen %	Reduction in nitrogen from acid coagulation %
0.0 (papain only)	45	51	0.22	0.25	41.0
0.025	46	61	0.24	0.23	44.8
0.05	47	83	0.24	0.26	29.8
0.1	44	84	0.18	0.29	15.8
0.2	44	84	0.18	0.36	15.8
0.4	40	93	0.20	0.42	11.7

Hydroxylamine hydrochloride($NH_2OH.HCl$) which is used in the manufacture of viscosity- stabilized rubber has been reported to act as an inhibitor of papain (Anon, 1972). It was found in this study that hydroxylamine hydrochloride has no more than a slight inhibitory action on papain (Table 8). Preliminary studies have shown that papain has no influence on the viscosity stabilization by NH_2 OH.HCl.

TABLE 8

EFFECT OF HYDROXYLAMINE HYDROCHLORIDE ON THE PROTEOLYTIC ACTION OF PAPAIN

Treatment	Nitrogen content %	Reduction in nitrogen content from acid coagulation %
1) Acid coagulation (2% HCOOH)	0.349	—
2) Papain (0.05%)	0.261	25.2
3) Hydroxylamine hydrochloride 0.4%	0.434	—
4) Papain 0.05%+Hydroxylamine hydrochloride 0.4% (1:1)	0.264	24.3

Effect of temperature : Table 9 summarizes the results of an experiment carried out to study the effect of papain treatment at different temperatures. Although there was no apparently distinct pattern in reduction of the nitrogen content it appears that papain is satisfactorily effective even at high temperatures as has been reported by many previous workers. PRI showed a significant decrease at higher temperatures whilst the color of the rubber had improved considerably.

TABLE 9

EFFECT OF PAPAIN TREATMENT AT DIFFERENT TEMPERATURES ON SOME RAW RUBBER PROPERTIES

Treated at temperatures	Properties				Colour of the rubber
	P_0	PRI	Ash %	Nitrogen %	
4°C	49	67	0.21	0.43	discolored
30°C	55	67	0.24	0.21	discolored but slightly better than at 4°C
40°C	48	49	0.24	0.32	no discoloration except for some brownish spots
70°C	55	55	0.26	0.26	no discoloration/ white
100°C	52	42	0.42	0.22	no discoloration except for a few brownish spots
Control/acid coagulation	48	75	0.16	0.51	white

Effect of pH : The reduction in the nitrogen content by papain treatment at different pH values did not show a distinct pattern (Table 10). PRI seems to decrease in alkaline conditions. The colour of the rubber improved gradually with the increasing pH; at pH 9 the colour was almost white whilst at pH 5.5 it was brownish.

TABLE 10

EFFECT OF PAPAIN TREATMENT AT DIFFERENT pH VALUES ON SOME RAW RUBBER PROPERTIES

Treated at pH	Properties				
	P_0	PRI	Ash %	Nitrogen %	Colour of rubber
5.5	53	74	0.24	0.21	discolored-brownwish
6.0	52	73	0.21	0.25	discolored but better than at pH 5.5
7.0	52	78	0.20	0.24	discolored but better than at pH 6.0
8.0	56	71	0.31	0.29	discolored but better than at pH 7.0
9.0	55	70	0.26	0.24	discolored but better than at pH 8.0
Control/acid coagulation	48	75	0.16	0.51	white

Effect of soaking : This experiment was carried out to see if the nitrogen content of papain coagulated rubber could be further reduced by soaking the rubber in a suitable form in papain.

Coagula obtained by:

1) acid coagulation at room temperature

2) papain coagulation at room temperature

and 3) papain coagulation at 70 °C

were milled into thin laces and soaked in papain solutions for 24h under the same conditions used for coagulation. These laces after the 24h in soaking treatment were remilled and soaked again for another 24h in a fresh papain solution. Results are presented in Figs. 1 and 2. It is seen from Fig. 1 that nitrogen content has been reduced considerably by the soaking treatments. A reduction of 64% in the nitrogen content from acid coagulation was achieved, compared with the usual reduction of approximately 50%. The only disadvantage of the soaking treatment appeared to be the reduction of the PRI (Fig. 2).

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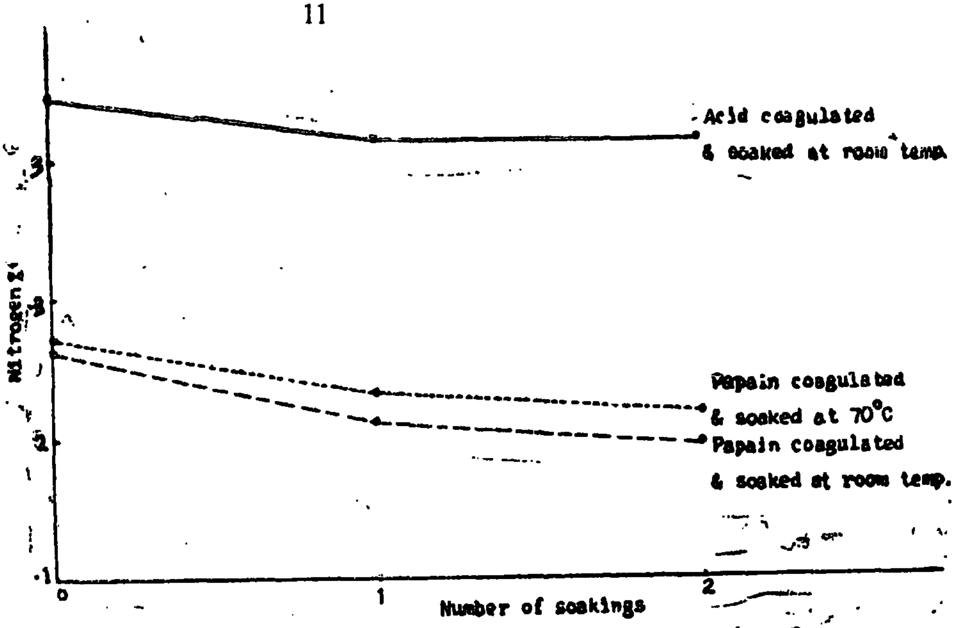

Fig. 1 Effect of soaking in papain of granulated coagulam on the nitrogen content.

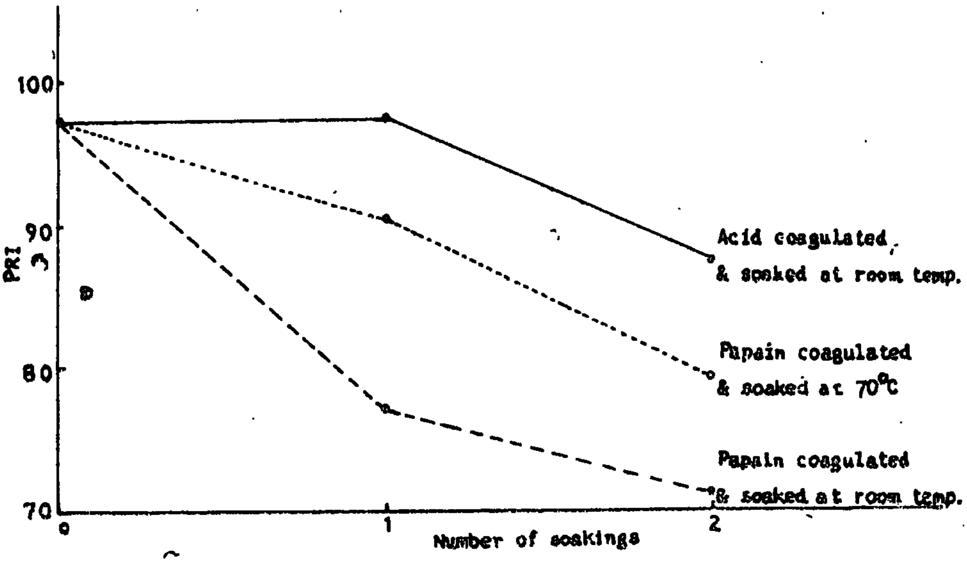

Fig 2 Effect of soaking in papain of granulated coagulam on the PRI.

DISCUSSION

The results of this investigation are broadly complementary to the reports of previous workers that papain can be used as a coagulant of natural rubber latex. The exact mechanism of action of papain in coagulating latex is not known although it can be assumed that it is initiated by an action of the enzyme on latex proteins which may in turn affect the colloidal stability of latex. It is also possible that organic acids present in dried papaw milk may also play a role in coagulation and papaw extracts have been shown to contain such acids (Chan *et al.*, 1971). This possibility is supported by the finding that coagulation with crude papain was faster than that with papain, precipitated and washed with ethanol. However, the reduction in nitrogen content, which obviously is the most important outcome of the papain treatment, is caused by the proteolytic enzyme by acting upon latex proteins. The nitrogen content was reduced by an average of about 40%. Reduction in nitrogen content was accompanied by an increase in amino acid content in the serum although it has been reported by several workers (Winnick, 1944; Bondi & Birk, 1958) that hydrolysis by papain does not extend to the amino acid level.

The PRI was not markedly affected by papain treatment except for an occasional reduction which may be attributed to the removal of some proteinaceous material acting as antioxidants. Ash content of papain treated rubber was slightly higher than that of acid coagulated rubber. The finding that pure papain has no influence on the ash content seems to suggest that a non proteinaceous material is responsible for this increase. The increase in ash content did not seem to require any special attention since it remained well within the specified limits for standard Sri Lanka Rubber (SLR). Nevertheless it has been suggested that this can be overcome to an appreciable extent by dilution of latex. (Nadarajah *et al.*, 1973). A disadvantage of the dilution method is that it could lead to a rubber with a considerably lower PRI, due to leaching out of naturally occurring antioxidants.

Discoloration of the rubber by papain treatment is another disadvantage. No discoloration was observed when papain precipitated with ethanol was used. This points to the possibility that this discoloration may have been initiated by some phenolic compounds present in papain. Treatment at an alkaline pH, seemed to improve the colour. This may be due to the formation of soluble phenates in latex when dilute Na OH is added to raise the pH to the desired level.

Cysteine, thiourea, potassium cyanide, sodium sulphide, and EDTA have all been reported to activate papain (Byers, 1967). Of these, only cysteine and thiourea were found to be effective. It was rather surprising that, KCN, which has been reported by a number of workers as an activator, did not produce this effect. Papain precipitated from ethanol was less effective in reducing the nitrogen content than crude papain. This may probably be attributed to the removal by ethanol of some naturaly occurring activators in papain such as thiols.

Two of the findings of this study that the degree of inhibition by $NH_2OH.HCl$ is negligible and papain has no effect on ability of $NH_2OH.HCl$. to stabilize viscosity are of obvious importance in view of the possibility that a combined treatment of NR latex with papain and $NH_2OH.HCl$ might lead to a rubber with two distinct advantages, stabilized viscosity and low nitrogen content. Preliminary investigations towards this objective have shown fairly encouraging results.

Papain by coagulation of latex creates a barrier for its own proteolytic action. When the coagulum is soaked in papain in the form of thin laces, it improves the chances of the enzyme acting on latex proteins by providing a considerably increased, exposed surface area. Results show that successive soaking and milling processes could satisfactorily be employed to obtain a rubber with a substantially low nitrogen content. The additional milling and soaking stages required, will obviously make it relatively, more expensive.

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