CHAPTER III

RESULTS

1. Determination of activity and specific activity of crude papain

Crude papain was prepared as shown in the Method 1.1 and determined for its activity and protein content as described in Methods 4.2 and 5.2. The activity and specific activity of free crude papain were 1,090 ± 35 CDU/mg papain and 1,300 CDU/mg protein, respectively.

2. Papain immobilization

2.1 Optimal conditions for papain immobilization by physical adsorption method

Pretreated chitin at mesh size 40 - 80 showed the highest relative activity when used as carrier for papain adsorption as shown in Figure 5 a, while 10 - 40 mesh chitin showed 88.5 % relative activity (Figure 5 a), but commercial chitin contains of 10-40 mesh size as major quantity (50 - 55 % by weight), while the quantity of 40-80 mesh chitin, and more than 80 mesh chitin were only 30 - 35 % and 10-15 %, therefore the 10-40 mesh chitin was selected for further use as carrier for papain immobilization.

Crude papain at various concentration (1 - 10 mg/ml) was immobilized on chitin. It was found that papain at the concentration of 7 mg/ml (Figure 5b) which was dissolved in phosphate-cysteine-EDTA buffer pH 6.0 (Figure 5c) and having total activity about 12,300 CDU / g wet chitin gave the highest % relative activity. The

reaction time between chitin and papain solution was selected at 15 min due to its highest % activity (Figure 5d). To strengthen the binding force, glutaraldehyde was added without removing the prior solution at various concentration (0.1-1.0 % and 1.0-10.0 % (w/v)). Figure 5e and 5f showed the highest % activity when the 0.7 % of glutaraldehyde was used, and stirred with physical-adsorbed immobilized papain on chitin for 45 min, respectively. The activity of papain immobilized by physical adsorption method (PIP) was stable at 65-70 CDU/g chitin after 4 times washing with distilled water (Figure 3.1g) at specified optimal conditions (Figure 6).

2.2 Optimal conditions for papain immobilization on chitin by covalent-binding method

The highest % activity of covalently-immoblized papain on chitin was obtained from these immobilization conditions : pretreated chitin was crosslinked with 0.4% glutaraldehyde in Tris buffer pH 8.0 (Figure 7 a) by using the ratio of buffer : glutaraldehyde = 19 : 1 (Figure 7 b). Chitin was left in the solution for 30 min before removing all of the solution and, then, washed 2 times by distilled water to get rid of excess glutaraldehyde. Papain solution having total activity about 12,800 CDU / g wet chitin was obtained by dissolving papain at the optimal concentration, 7 mg/ml (Figure 7 c), in Tris-cysteine-EDTA buffer pH 8.0 (Figure 7 d) and stirred with glutaraldehyde-treated chitin for 45 min (Figure 7 e). The activity of covalently-immobilized papain (CIP) of 450 - 470 CDU / g chitin was stable after washing 4 times with distilled water (Figure 7 f) and the overall optimal conditions was shown in Figure 8.

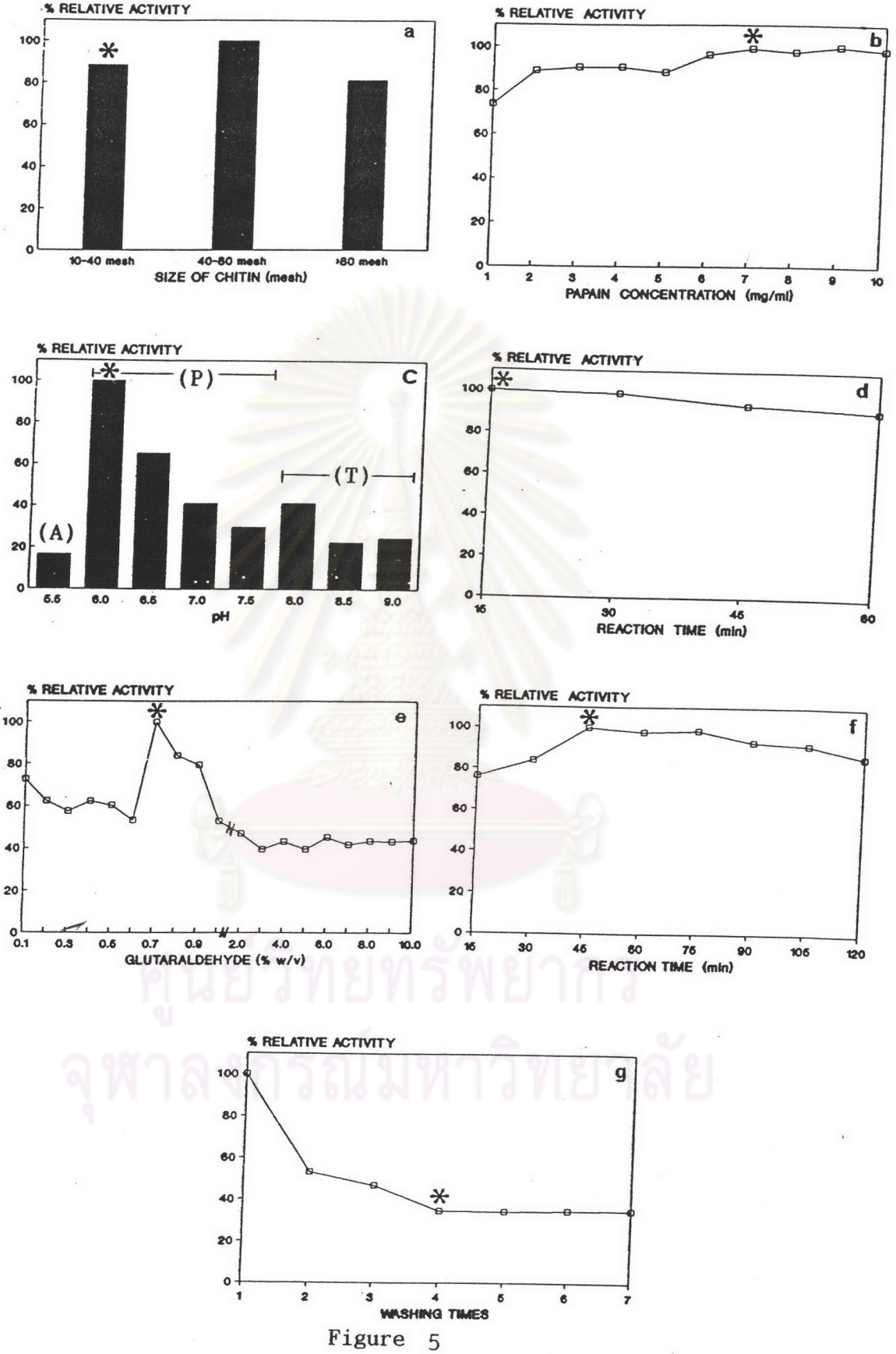

% RELATIVE ACTIVITY
a
10-40 mesh
40-80 mesh
>80 mesh
SIZE OF CHITIN (mesh)
% RELATIVE ACTIVITY
b
PAPAIN CONCENTRATION (mg/ml)
% RELATIVE ACTIVITY
C
(P)
(T)
(A)
pH
% RELATIVE ACTIVITY
d
REACTION TIME (min)
% RELATIVE ACTIVITY
e
GLUTARALDEHYDE (% w/v)
% RELATIVE ACTIVITY
f
REACTION TIME (min)
% RELATIVE ACTIVITY
g
WASHING TIMES

Figure 5

Figure 5 Physical adsorption method for immobilization of papain on chitin

To immobilize papain on chitin by physical adsorption method, the following conditions were investigated at room temperature. The optimal conditions were selected from the maximum % relative activity of immobilized papain.

a) The size of chitin

b) The papain concentration

c) The pH of buffer for papain immobilized on chitin
   1) 0.1 M acetate buffer (A)
   2) 0.1 M phosphate buffer (P)
   3) 0.1 M Tris buffer (T)

d) The reaction time between papain solution and chitin

e) The concentration of glutaraldehyde

f) The reaction time between papain-adsorbed chitin and glutaraldehyde solution

g) The optimal washing times

1 g dry Chitin 10-40 mesh

Pretreated in 6 M HCl, 5 M KOH, 1% NaCl and 1% acetic acid

+

10 ml papain 7 mg/ml

in phosphate-cysteine-EDTA buffer pH 6.0

↓ stir 15 min

add 1 ml glutaraldehyde 0.7 % (w/v) in 9 ml phosphate buffer

↓ stir 45 min

wash 4 times with distilled water

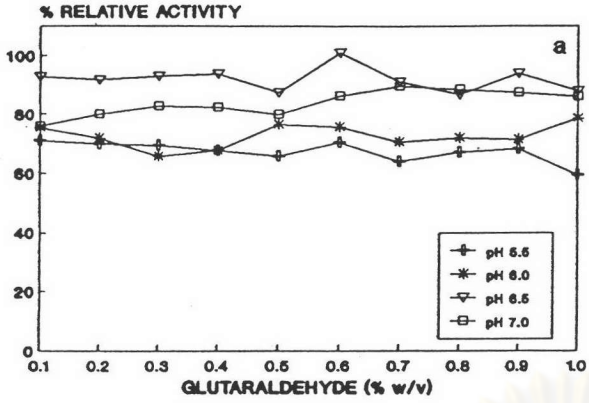
↓

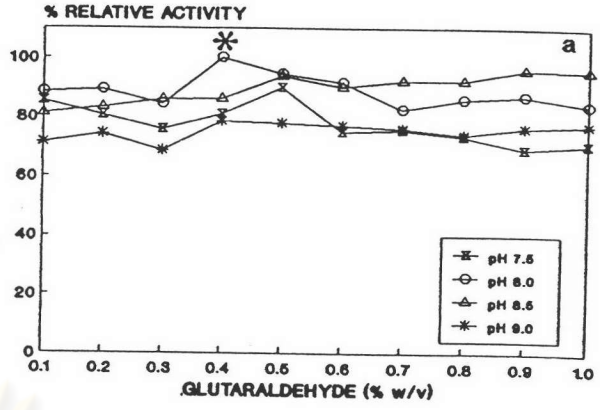
physically-adsorbed immobilized papain on chitin

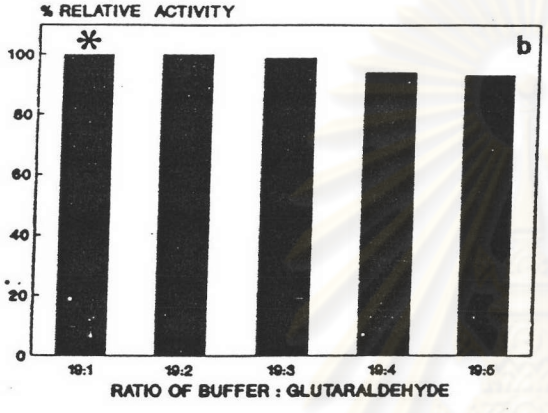
65-70 CDU/g chitin

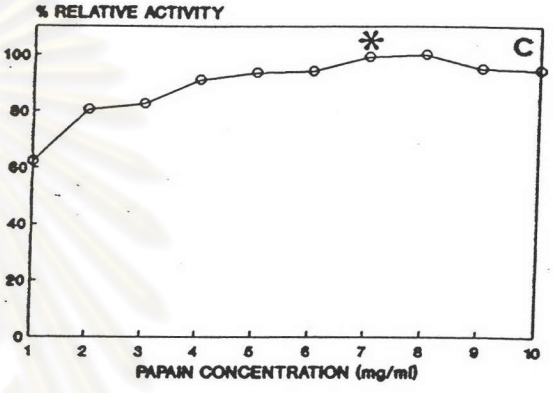
340 CDU/mg protein

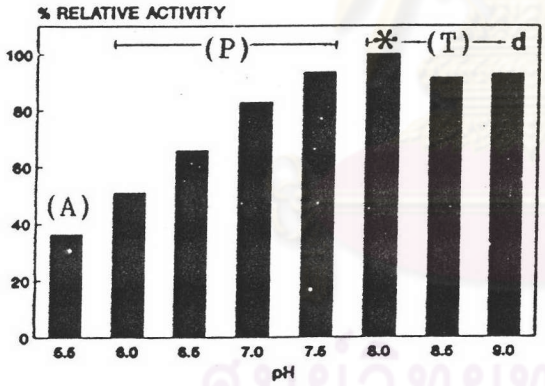
Figure 6 The optimal conditions for papain immobilization on chitin by physical adsorption method

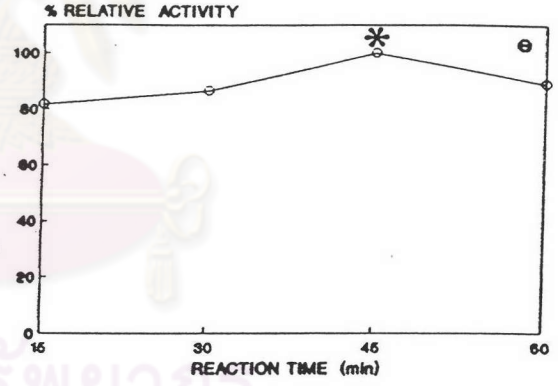












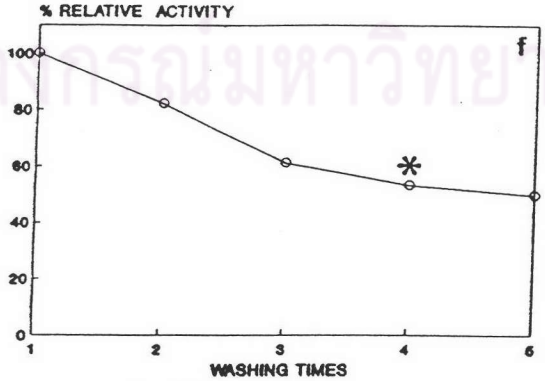


Figure 7

Figure 7 Covalent-binding method for immobilization of papain on chitin

To immobilize papain on chitin by covalent-binding method, the following conditions were investigated at room temperature. The optimal conditions were selected from the maximum % relative activity of immobilized papain.

a) The effect of glutaraldehyde concentration and pH of buffer
   pH 5.5 of 0.1 M acetate buffer
   pH 6.0-7.5 of 0.1 M phosphate buffer
   pH 8.0-9.0 of 0.1 M Tris buffer

b) The ratio of buffer and glutaraldehyde solution

c) The papain concentration

d) The pH of buffer (crude papain was dissolved in theses buffer which were prepared into various pH)
   1) 0.1 M acetate buffer (A)
   2) 0.1 M phosphate buufer (P)
   3) 0.1 M Tris buffer (T)

e) The reaction time between glutaraldehyde-treated chitin and papain solution

f) The optimal washing times

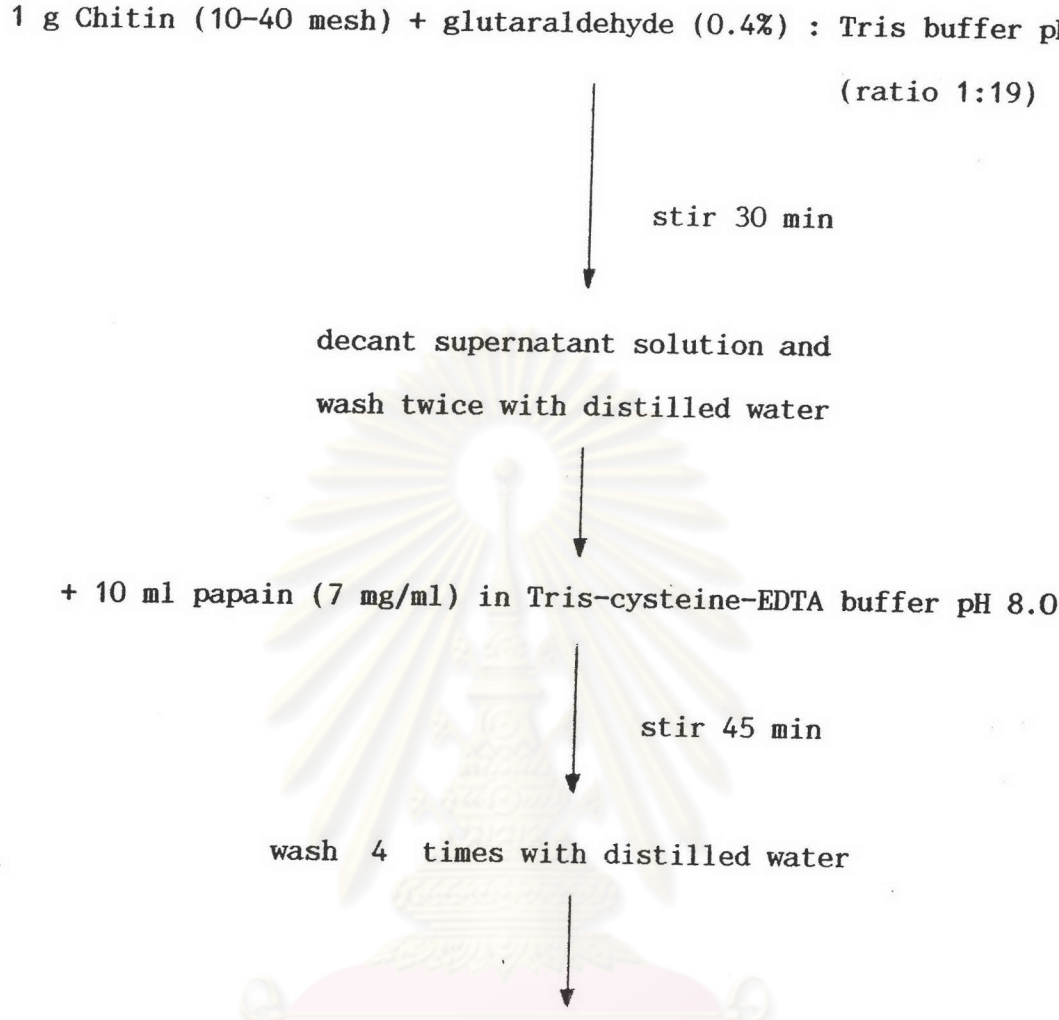


Figure 8 The optimal conditions for papain immobilization on chitin by covalent-binding method

2.3 Comparison of the papain immobilization methods between physical adsorption method and covalent-binding method

To evaluate the papain immobilization method, the specific activity and yield of immobilization were compared and shown in Table 5 (the calculation was shown in Appendix 2).

Table 5 Comparison of papain immobilization by physical adsorption and covalent-binding method.

Immobilization method	Total activity of free papain added (CDU/g)*	Immobilized papain		% yield
		Activity (CDU/g)*	Specific activity (CDU/mg protein)**	
Physical adsorption	12300	65-70	340	1.67 %
Covalent binding	12800	450-470	1200	23.00 %

* CDU/g = casein digestion unit per gram wet weight of chitin

**CDU/mg protein = casein digestion unit per mg of protein which was adsorbed on chitin

Table 5 showed that CIP gave higher yield (10-times : approximated by 23/1.67) and specific activity (4-times :approximated by 1200/340) than PIP, therefore, CIP seems to be more suitable for

the latex deproteinization. However, to confirm this, the reaction of immobilized papains with various substrates was compared with that of free papain (FP).

3 Properties of immobilized papain on chitin by both methods comparing to free papain

3.1 Effect of pH and temperature on papain activity (working pH and working temperature)

When casein was used as substrate at various pH (pH 5.5-9.0), PIP and FP showed the maximum %relative activity at the pH range 5.5-9.0 at 40 ^{o}C, whereas CIP showed the maximum %relative activity at pH range 5.5-6.5 at 40 ^{o}C (as shown in Figure 9 a).

In case of the effect of temperature on the activity (working temperature), Figure 9 b shows that PIP has the maximum % relative activity at temperature range 70-80 ^{o}C whereas CIP and FP have higher % relative activity at every temperature and wider maximum % relative activity at temperature range of 50-80 ^{o}C.

3.2 Effect of pH and temperature on stability of immobilized papain and free papain

Both immobilized papains and free papain were incubated in different pH of buffer (acetate buffer pH 5.5, phosphate buffer pH 6.0-7.5 and Tris buffer pH 8.0-9.0) at 40 ^{o}C for 3 hours. After that the activity of immobilized papains and free papain were assayed with casein (method 4.2). Figure 10a indicates that CIP is stable at pH range 5.5-9.0 while free papain and PIP show stability at narrower pH range 7-8 and 7.5-9 at 40 ^{o}C, respectively.

The temperature stability of immobilized papains and free

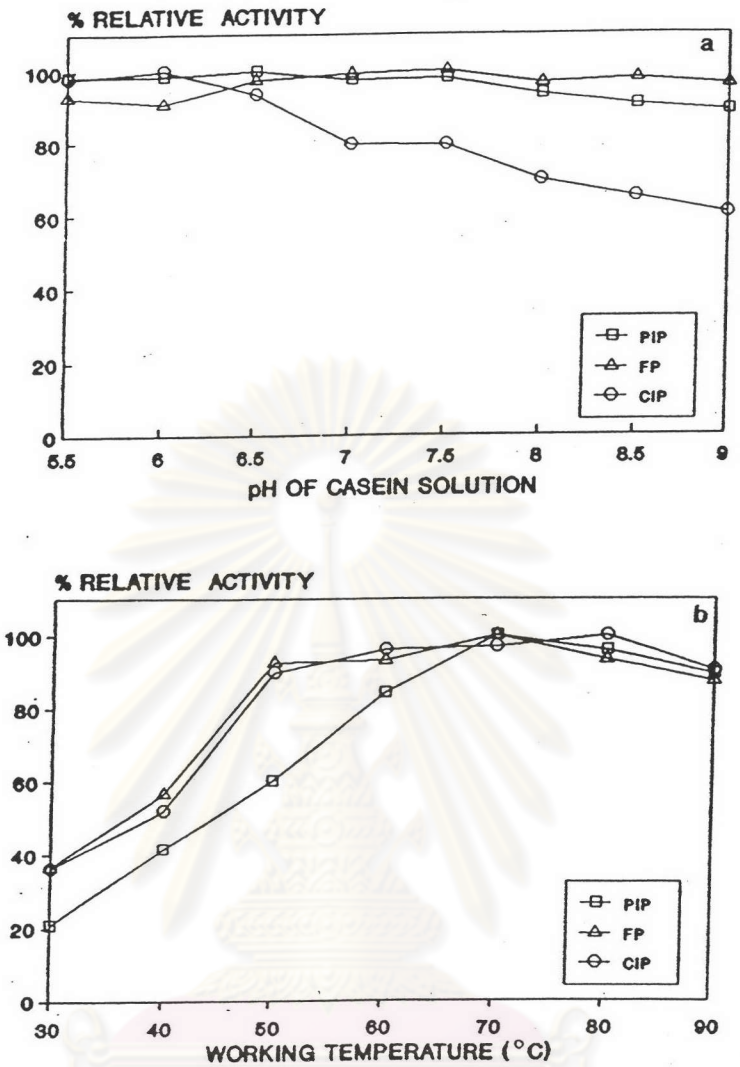


Figure 9 The effect of pH and temperature on the activity of immobilized papain and free papain.

(a) 1% casein in acetate buffer(pH 5.5), phosphate buffer (pH 6.0-7.5) and Tris buffer (pH 8.0-9.0) were used as substrate to determine papain activity at 40 ^{o}C.

(b) 1 % casein in phosphate buffer pH 6.0 was used as substrate to determine papain activity at various temperature (30-90^{o}C).

papain shown in Figure 10 b were obtained by incubating immobilized papain and free papain at different temperature, 30 - 90°C for 3 hrs. The result showed that both immobilized papains and free papain were stable at the same temperature range of 30-50 °C.

3.3 Effect of substrate concentration on enzymatic activity

(Kinetic expression)

Immobilized papain by both methods and the corresponding free papain were assayed by using casein and ovalbumin as substrate at 40 °C. For CIP and free papain, proteins in rubber latex were also used as substrate. Each substrate was prepared at various concentration (S) as shown in Method 7.3 and the activity of immobilized papain and free papain (V) were determined. From Lineweaver-Burk plot shown in Figure 11 (a-e), K_m (Michealis-Menten constant) and V_{max} (maximum velocity) were calculated and shown in Table 6 and 7. The K_m of both immobilized papains and free papain were lower when casein was used as substrate than when ovalbumin and proteins in rubber latex were used indicating that both immobilized papains and free papain have more affinity with casein than the other two substrates. The V_{max} of PIP was not significantly different from that of FP when casein and ovalbumin were used as substrate. On the contrary, V_{max} of CIP was higher than that of FP when casein and rubber latex were used and lower than that of FP when ovalbumin was used as substrate, which means that CIP was more efficient than FP when casein and rubber latex were used as substrates.

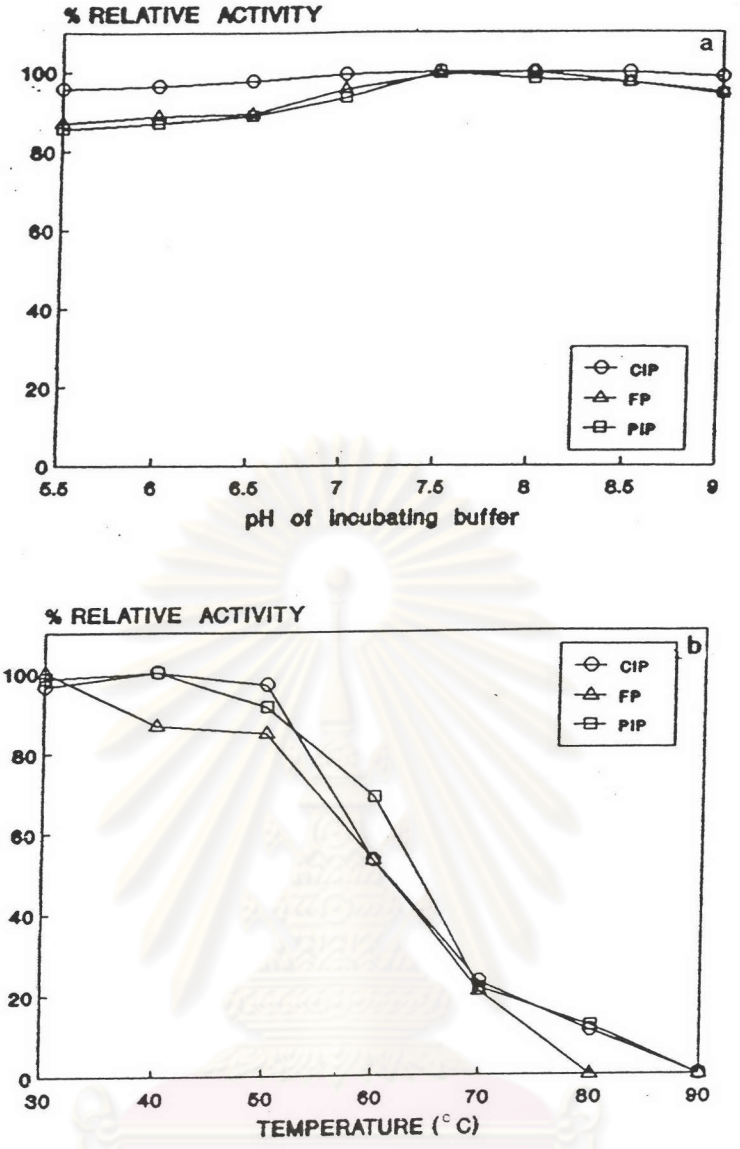


Figure 10 The effect of pH and temperature on the stability of immobilized papain and free papain.

(a) Immobilized papain and free papain were incubated in different pH of cysteine-EDTA buffer at 40°C for 3 hrs before determining the activity (Method 4.2).

(b) Immobilized papain and free papain were incubated in phosphate cysteine-EDTA buffer pH 7.5 at various temperature for 3 hrs before determining the activity (Method 4.2).

Table 6 K_m and V_{max} from Lineweaver-Burk plot of PIP and FP.

Substrate#	K_m (%substrate concentration,g/100 ml)		V_{max} (CDU/mg protein)	
	PIP	FP	PIP	FP
casein	0.932	0.764	1049.32	1055.97
ovalbumin	1.656	1.035	530.78	576.04

casein and ovalbumin in phosphate buffer pH 6.0 were used as substrate at 40°C.

Table 7 K_m and V_{max} from Lineweaver-Burk plot of CIP and FP.

Substrate #	K_m (%substrate concentration,g/100 ml)		V_{max} (CDU/mg protein)	
	CIP	FP	CIP	FP
casein	0.725	0.704	2,164.50	2,026.75
ovalbumin	1.051	0.912	864.30	1,000.40
rubber latex	27.850	10.620	616.90*	236.19*

Note * Unit of V_{max} when rubber latex was used as substrate is % retention protein.

casein, ovalbumin in phosphate buffer pH 6.0 and rubber latex at pH 7.5 were used as substrate at 40°C.

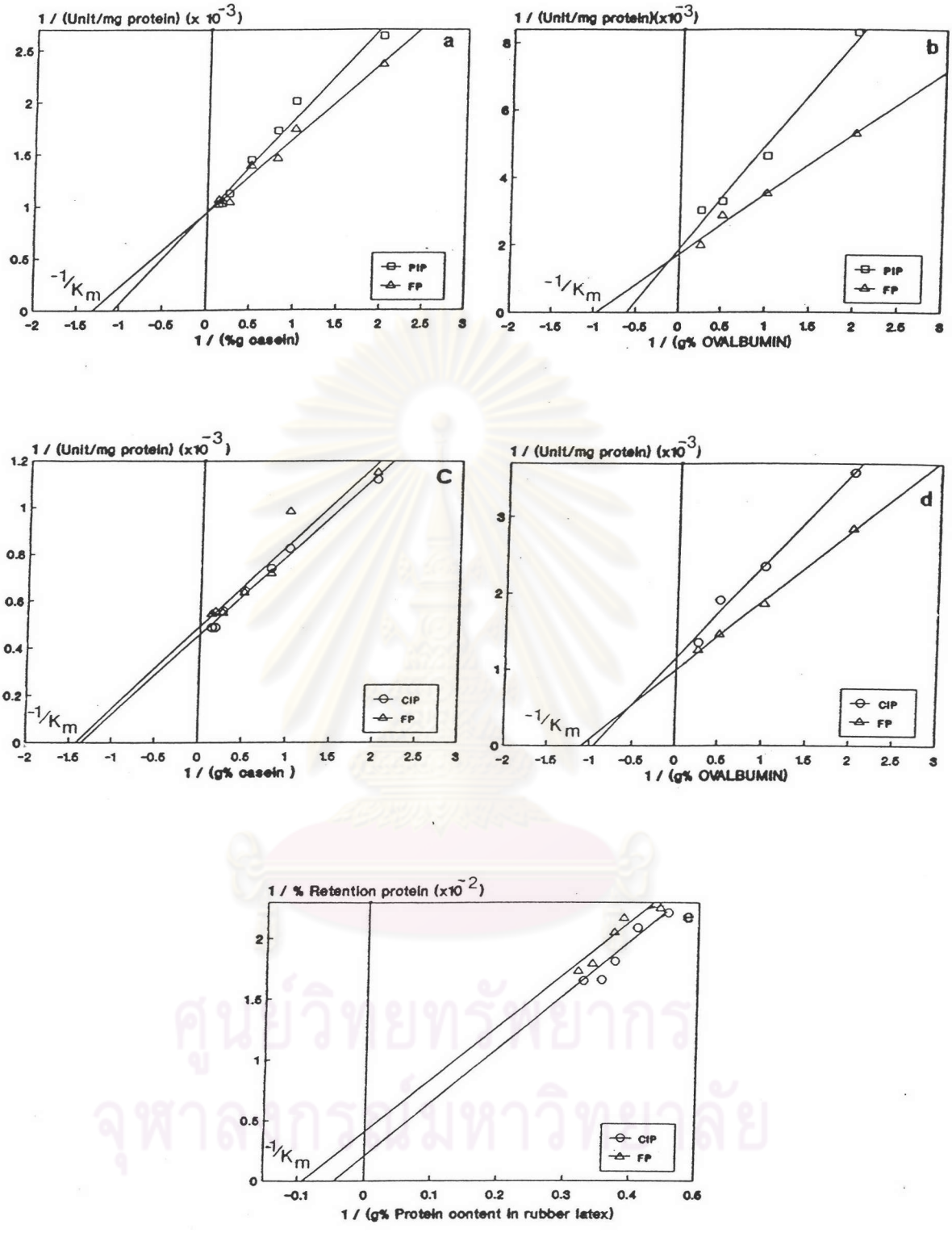


Figure 11

Figure 11 Lineweaver-Burk plot of immobilized papain and free papain when casein, ovalbumin and rubber latex were used as substrate.

Casein and ovalbumin at pH 6.0, 40 ^{o}C were used at various concentrations as substrate of PIP and its corresponding FP (a,b) and of CIP and its corresponding FP (c,d). The enzyme activities were calculated as the velocities of enzyme.

When rubber latex,pH 7-8,was used as substrate of CIP at 40^{o}C and of FP at 50^{o}C (Visessanguan, 1992), % retention protein in rubber was calculated as the velocity of enzyme (e).

a) Lineweaver-Burk plot of PIP and corresponding FP when casein was used as substrate.

b) Lineweaver-Burk plot of PIP and corresponding FP when ovalbumin was used as substrate.

c) Lineweaver-Burk plot of CIP and corresponding FP when casein was used as substrate.

d) Lineweaver-Burk plot of CIP and corresponding FP when ovalbumin was used as substrate.

e) Lineweaver-Burk plot of CIP and corresponding FP when rubber latex was used as substrate.

4. Effect of temperature on storage stability

Storage of aqueous suspensions of PIP and CIP in phosphate - cysteine-EDTA buffer pH 7.5 at room temperature for 48 hrs indicate that stability of CIP is the best as evident by the % retention activity (68.53%) comparing to PIP (41.84%) and free papain which lost all its activity after storage for 19 hrs (Figure 12).

The long-term storage stability of immobilized papain comparing to free papain was also performed at 4 ^{o}C for 3 months. Figure 13 demonstrates clearly that both PIP and CIP are more stable than FP showing % retention activity of 69.77 % and 81.96 %, respectively, where free papain can be stored only 4-5 days at 4 ^{o}C, or twice longer than storage at room temperature.

5. Continuous operation stability of covalently-immobilized papain

On the continuous operation of covalently-immobilized papain on chitin, the immobilized papain on chitin was packed into a temperature-controled glass column (2.6x35 cm) and 1% casein solution pH 6.0 was then continuously loaded onto the column at the flow rate of 6 ml/hr at 40 ^{o}C for 7 days. The result shown in Figure 3.14 indicated that the initial rate of CIP packed in the column increased exponential during the first 10 hrs. When it reached the equilibrium, after 11 hrs, the activity of CIP was stable for almost 4 days before the activity gradually decreased to about 64 % of the maximum activity. Small amount of released papain was found in the eluent since the operation started until the column reached its equilibrium at 11 hrs after starting continuous operation.

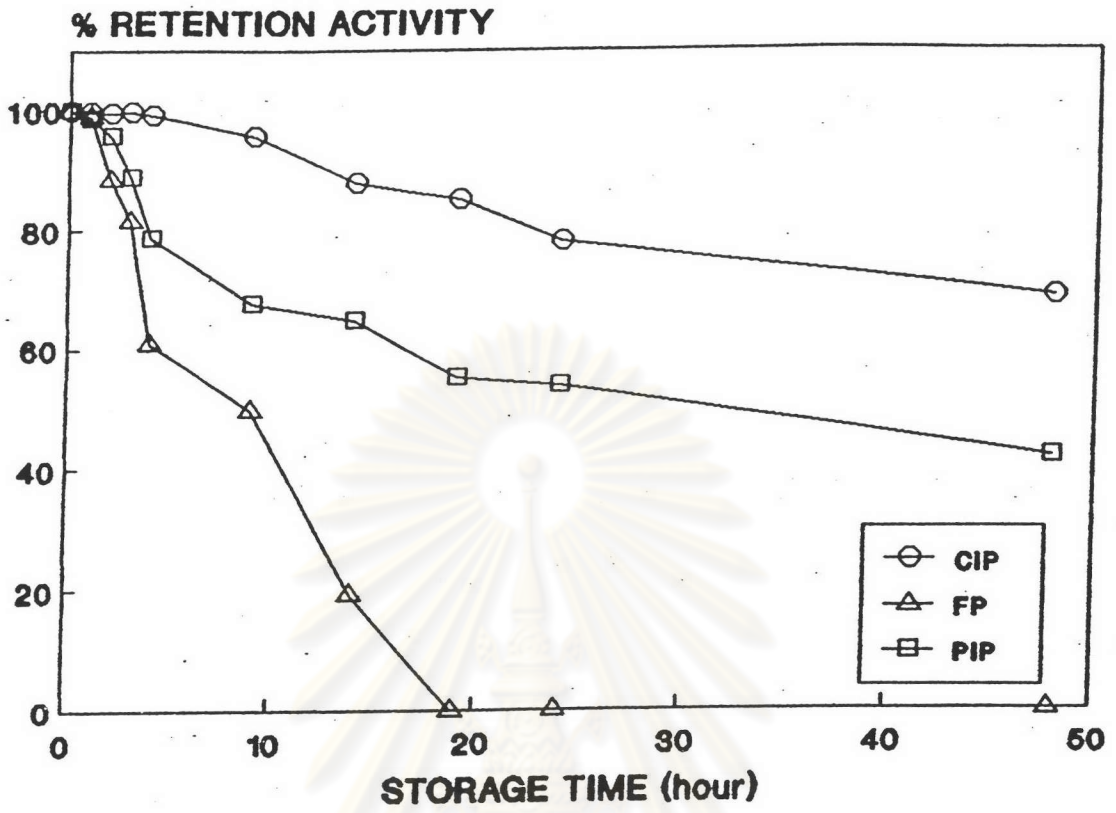


Figure 12 Storage stability of immobilized papain and free papain at room temperature.

Immobilized papain on chitin and free papain were stored in phosphate-cysteine-EDTA buffer pH 7.5 at room temperature for 48 hours. At time intervals, the activity of immobilized papain and free papain were determined as described in Method 4.2.

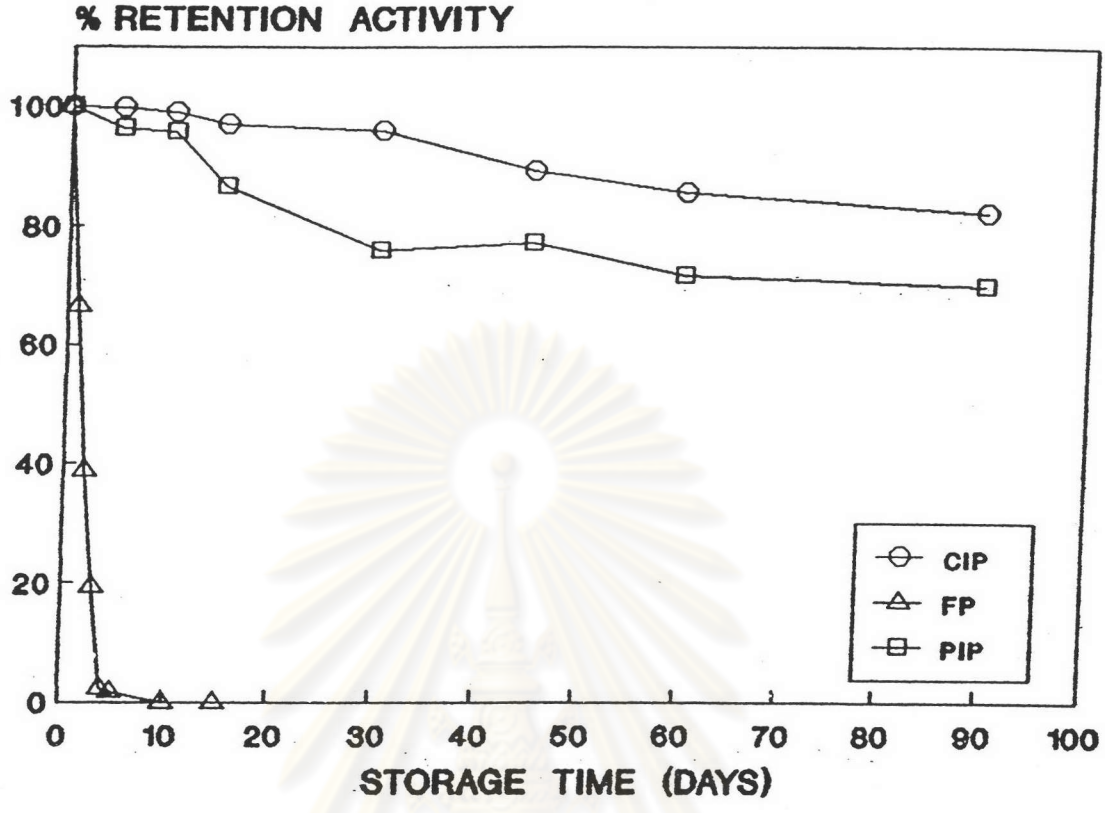


Figure 13 Storage stability of immobilized papain and free papain at 4°C.

Immobilized papain on chitin and free papain were stored in phosphate-cysteine-EDTA buffer pH 7.5 at 4 °C for 3 months. At time intervals, the activity of immobilized papain and free papain were determined as described in Method 4.2.

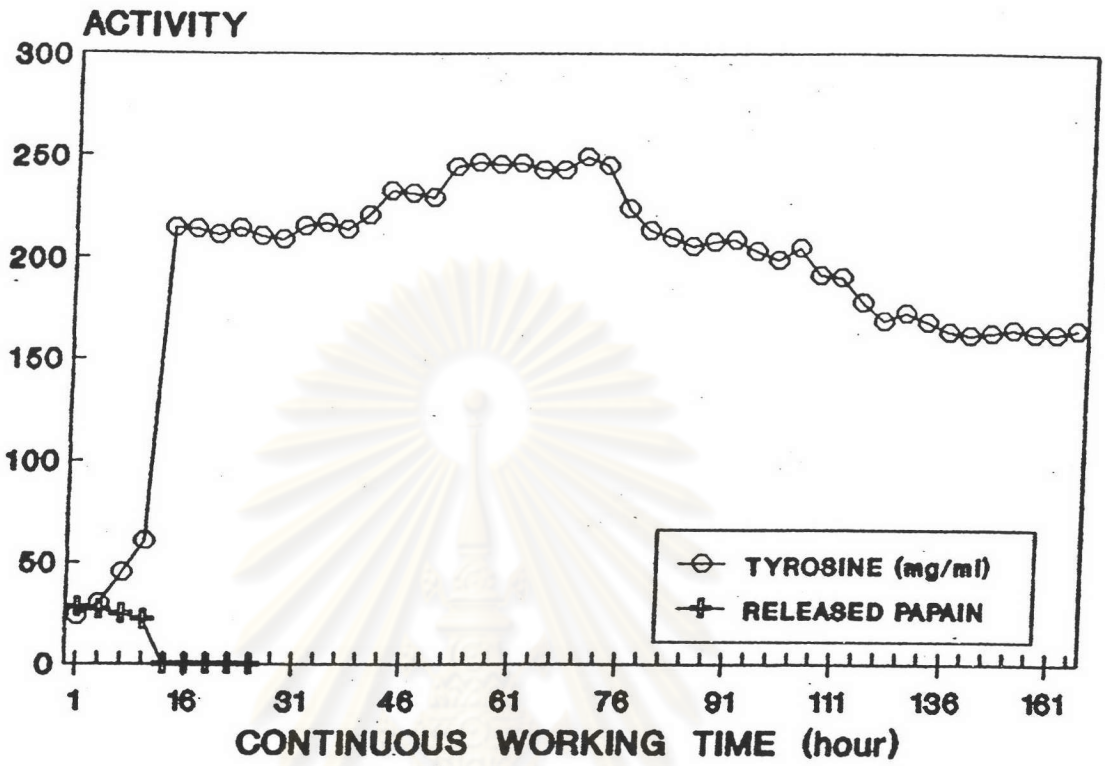


Figure 14 Continuous operation stability of CIP

1 % casein in phosphate buffer pH 6.0 was continuously loaded onto CIP packed in a temperature-controled column at the flow rate 6 ml/hr at 40°C for 7 days. The eluent was determined for tyrosine liberated by CIP as described in Method 4.2.

From all the previous results, immobilization of papain by covalent-binding method shows advantage than physical adsorption method becuase CIP shows 7-fold higher activity, 4-fold higher specific activity and 10-fold higher yield. Moreover, CIP can be stored longer after preparation and more stable in wider range of temperature and pH (50-80 ^{o}C and pH 5-9). Although the working pH range of CIP is slightly narrower than PIP,but due to more advantages previously described, CIP was chosen for further studies of latex deproteinization.

6. Optimal conditions for deproteinization of field latex covalently-immobilized papain

Fresh field latex was reported having the variation of % DRC from 20-40 %,depending on several factors such as rubber clone,age of rubber tree, day of tapping, temperature, humidity, etc. so that the %DRC of each lot must be examined and adjusted to 25% DRC before use. The advantage of using field latex as starting material is that the pH of field latex is in the range of 6.0 - 6.5 which is easier to adjust the pH of latex to the optimal pH of immobilized papain. The disadvantage of using field latex is that its nitrogen content is high (0.4 % - 0.7 %) and there are so many soluble organic compounds in the serum and lutoid fraction which might interfere the enzyme acivity so that higher concentration of enzyme could be required.

By using field latex, clone RRIM 600, the highest % nitrogen reduction was obtained at pH 7-8 (Figure 15 a). In case of the optimal temperature, although the result from Figure 15 b indicated that the highest % nitrogen reduction was obtained at 50 ^{o}C, it was

found that at this temperature the latex was not stable and was coagulated with immobilized papain which caused the loss of rubber and immobilized papain; therefore, the temperature at 40°C was chosen as the optimal temperature for latex deproteinization.

The concentration of immobilized papain was determined by varying from 10-40 p.h.r. It was found that the latex was coagulated after incubating with 30 p.h.r. and 40 p.h.r. of immobilized papain so no further study with these two concentrations. The optimal concentration of immobilized papain, 20 p.h.r. (1 g wet immobilized papain on chitin per 5 g dry rubber) was obtained (Figure 15 c) (total activity of added papain was about 10,000 unit).

According to Figure 15 d, using speed of shaking at 120, 150 and 200 rpm, the maximum % nitrogen reduction were obtained without significant different. However, the latex was more coagulated when speed of shaking at 150 rpm and 200 rpm were used. Consequently, the maximum 60 % nitrogen reduction was observed after 6 hours at the optimal latex-deproteinization conditions that was the latex, pH 7-8, was reacted with 20 p.h.r. of immobilized papain at 40°C at 120 rpm shaking.

7. Effect of viscosity-stabilizer, reducing agent and nonionic detergent

Hydroxylamine hydrochloride ($NH_2OH.HCl$) was added into field latex as viscosity-stabilizer and mild bactericide. It was found from Figure 16 a that the % nitrogen reduction of control and field latex previously added with 0.10 p.h.r. and 0.15 p.h.r. of hydroxylamine hydrochloride are not significantly different. From this result, it

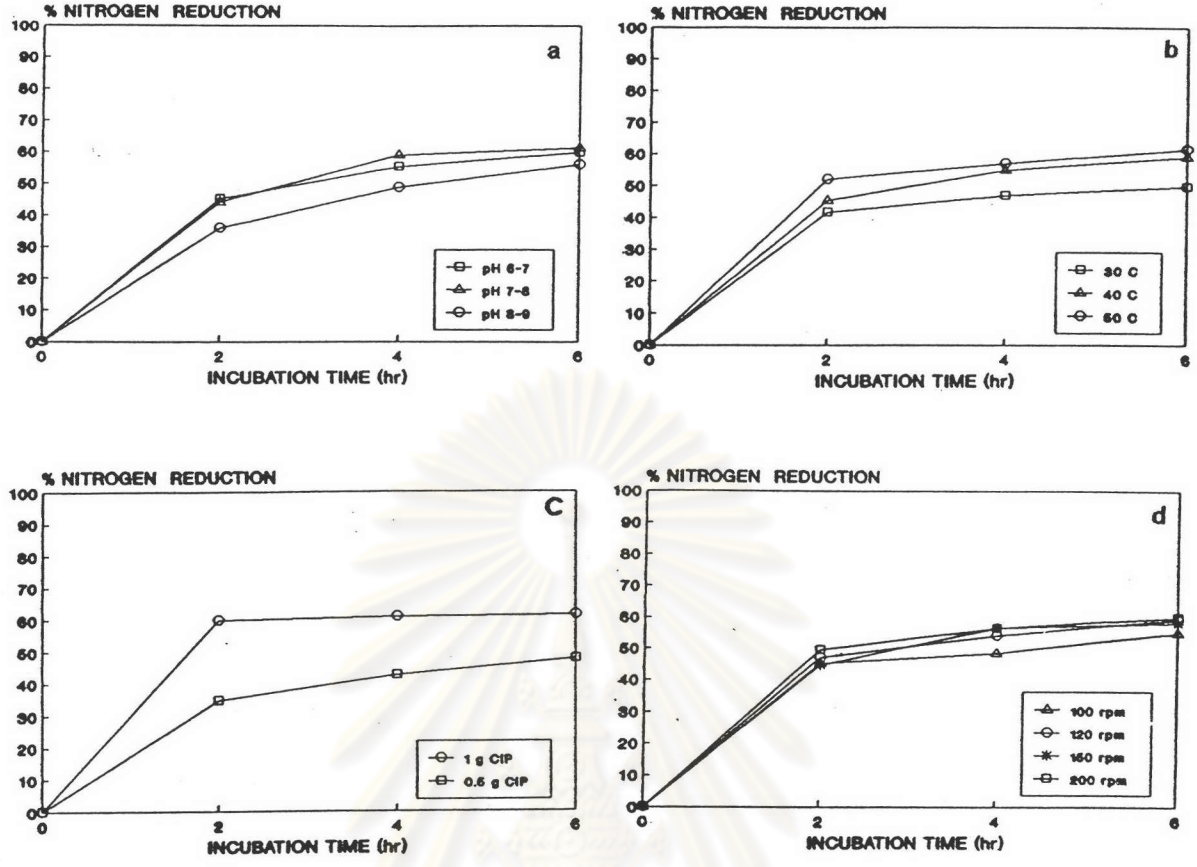


Figure 15 Optimization of fresh field latex deproteinization by immobilized papain on chitin

Fresh field latex was prepared to 25 % DRC and treated with CIP at verifying conditions (a-d). The digested latex was separated from CIP and coagulated,dried and determined for %nitrogen reduction.

a) The effect of pH on latex deproteinization

b) The effect of temperature on latex deproteinization

c) The effect of the concentration of immobilized papain on latex deproteinization

d) The effect of speed of shaking on latex deproteinization

can be concluded that adding hydroxylamine hydrochloride into field latex as the latex viscosity-stabilizer has no influence on the latex deproteinization by CIP and increasing of % nitrogen reduction to 63% can be obtained at sixth hour.

In order to prevent latex discoloration, sodium metabisulfite must be added into field latex. As shown in Figure 16 b, adding 0.05 p.h.r. of sodium metabisulfite has a small influence on latex deproteinization by immobilized papain as evident by 63 % nitrogen reduction was obtained and higher than the control.

Nonionic detergent, Triton X-100, must be added in order to solubilize proteins and also stabilize the colloidal state of rubber particles. The criteria used for selecting optimal concentration of Triton X-100 are ; prolonged coagulation during immobilized papain treatment, but completely coagulation under microwave or steam coagulation and maximum % nitrogen reduction. The final % nitrogen reduction of 52-53 % was obtained as shown in Figure 16 c. This result showed that % nitrogen reduction of latex after addition of Triton X-100 in every concentration was less than the control. The results suggested that Triton X-100 may act as the inhibitor of immobilized papain on latex deproteinization. However, adding Triton X-100 is essential because of its advantages as described so that the minimum concentration of Triton X-100 which can be used under the prior criteria must be determined in each lot of field latex.

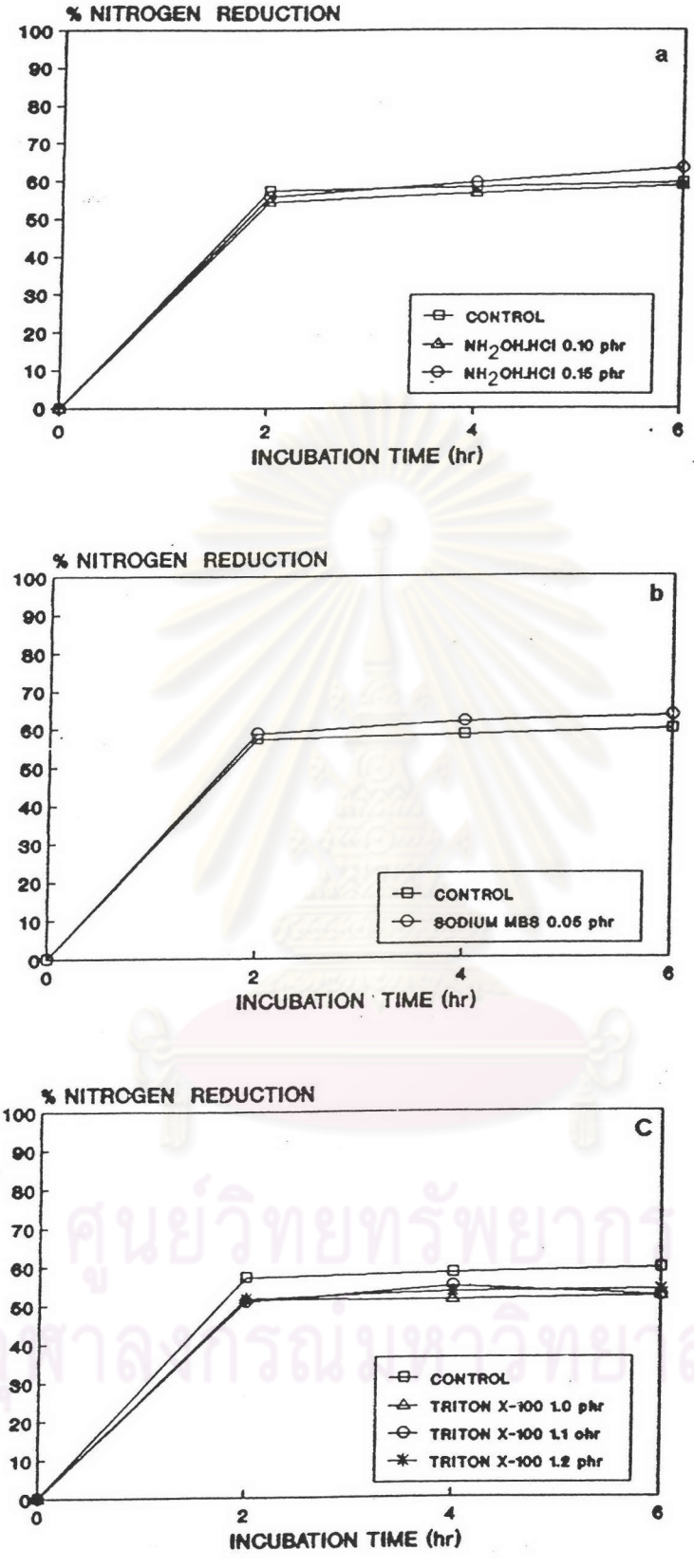

% NITROGEN REDUCTION
a
CONTROL
NH2OH.HCl 0.10 phr
NH2OH.HCl 0.15 phr
INCUBATION TIME (hr)
% NITROGEN REDUCTION
b
CONTROL
SODIUM MBS 0.05 phr
INCUBATION TIME (hr)
% NITROGEN REDUCTION
C
CONTROL
TRITON X-100 1.0 phr
TRITON X-100 1.1 phr
TRITON X-100 1.2 phr
INCUBATION TIME (hr)

Figure 16

Figure 16 The effect of hydroxylamine hydrochloride, sodium metabisulfite and Triton X-100 on latex deproteinization

The 25 % DRC latex was added with various concentration of hydroxylamine hydrochloride or sodium metabisulfite or Triton X-100 and incubated with CIP for 6 hours. The digested latex was coagulated, dried and the % nitrogen reduction was calculated.

a) The effect of the viscosity-stabilizer,hydroxylamine hydrochloride, on latex deproteinization

b) The effect of reducing agent, sodium metabisulfite, on latex deproteinization

c) The effect of Nonionic detergent,Triton X-100, on latex deproteinization

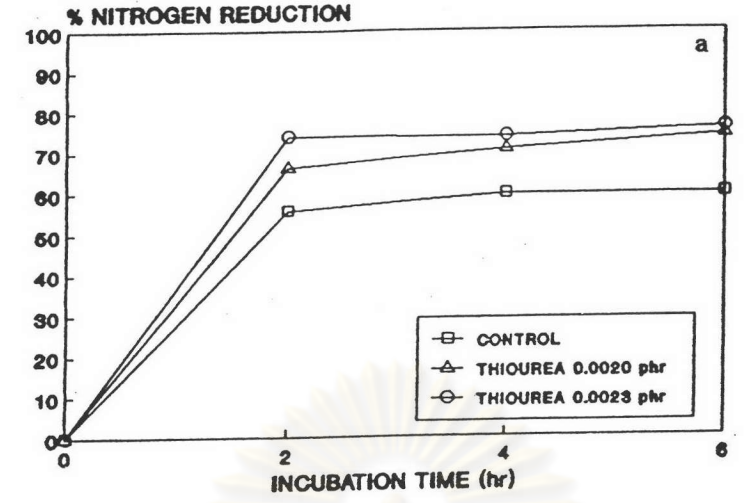
8. Adding the activators and metal-chelating agent of papain

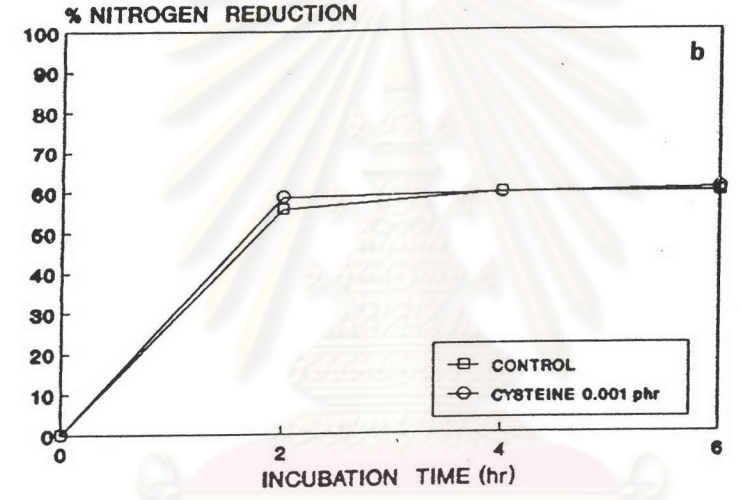
In order to increase % nitrogen reduction, the activators of papain were considered. Cysteine and thiourea were reported to enhance the proteolytic action of free papain ;thus,they were studied in case of immobilized papain.

1 g (wet weight) of immobilized papain was added with 0.0020 p.h.r. or 0.0023 p.h.r. of thiourea into field latex. After six hours, 75 % nitrogen reduction was obtained from the digested latex previously added with 0.0023 p.h.r. of thiourea whereas the latex with 0.0020 p.h.r. and control gave 74 % and 60 % nitrogen reduction, respectively (Figure 17 a). This results indicates that thiourea enhances the proteolytic action of immobilized papain which causes the increase of latex deproteinization. However, there was previous report that high concentration of thiourea can inhibit the deproteinization process so that no further investigation has been performed with higher concentration of thiourea.

Cysteine, another activator of free papain, was studied in case of immobilized papain. Figure 17 b shows that there is no significant change in the % nitrogen reduction between control and cysteine added latex. Consequently, it is not essential to add cysteine into latex in deproteinization process.

EDTA was added into the latex as the metal - chelating agent. From Figure 17 c, there was no significant effect of EDTA on the reduction of nitrogen content. On the contrary, it seemed to have a slightly inhibitory action. Therefore, EDTA must not be added into the latex deproteinization process.


% NITROGEN REDUCTION
a
CONTROL
THIOUREA 0.0020 phr
THIOUREA 0.0023 phr
INCUBATION TIME (hr)


% NITROGEN REDUCTION
b
CONTROL
CYSTEINE 0.001 phr
INCUBATION TIME (hr)

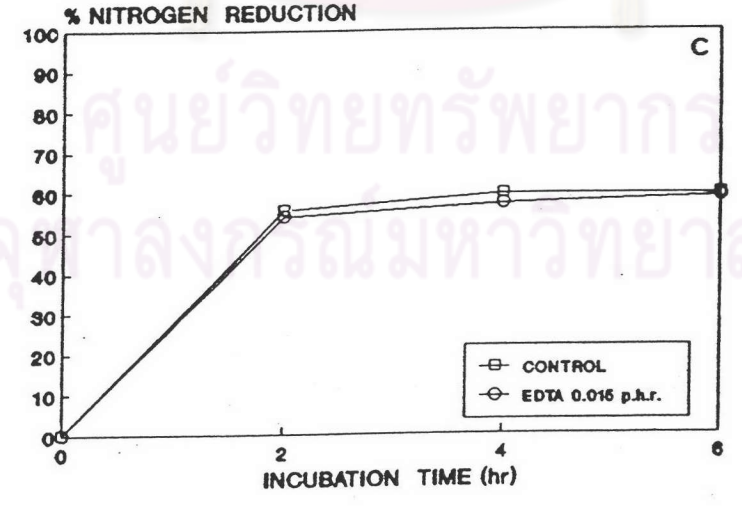

% NITROGEN REDUCTION
C
CONTROL
EDTA 0.015 p.h.r.
INCUBATION TIME (hr)

Figure 17

Figure 17 The effect of thiourea, cysteine and EDTA on latex deproteinization

The 25 %DRC latex was added with various concentration of thiourea or cysteine or EDTA and incubated with CIP at for 6 hrs. The digested latex was coagulated, dried and the % nitrogen reduction was calculated.

a) The effect of the papain activator, thiourea, on latex deproteinization

b) The effect of the papain activator, cysteine, on latex deproteinization

c) The effect of the metal-chelating, EDTA, on latex deproteinization

9. Effect of latex dilution on latex deproteinization

It was reported that nitrogen content could be decreased by dilution of latex prior to the enzyme treatment; therefore, the suitable dilution volume of water for the deproteinization by immobilized papain was studied. Figure 18 a shows that the dilution of latex to 20 % DRC before use as starting material was necessary in the latex deproteinization process to obtain the minimum of %nitrogen retention of raw rubber i.e. the maximum 77 % nitrogen reduction was obtained after 4 hrs. Dilution of latex into 10 % DRC was attempted, but it caused the incomplete coagulation.

Dilution of latex after enzyme treatment can also increase % nitrogen reduction because latex proteins have been hydrolyzed into small oligopeptides and soluble amino acids which are readily soluble in water and easily removed from rubber particles after coagulation. Figure 18 b indicates that the dilution ratio of latex:water = 1:0.5 was suitable for obtaining the maximum 78 % nitrogen reduction of raw rubber. Dilution larger than the ratio of 1 : 0.5 was not attempted as it appeared to be impracticable and also because of the loss of rubber due to incomplete coagulation.

10. Overall optimal conditions for rubber latex deproteinization

According to the results from 9. , it was found that % nitrogen reduction of digested latex previously diluted to 20 % DRC before papain treatment and % nitrogen reduction of digested latex which diluted with the ratio of 1:0.5 after papain treatment were not significantly different. Thus, to be assured for the optimal conditions for latex deproteinization, fresh field latex was

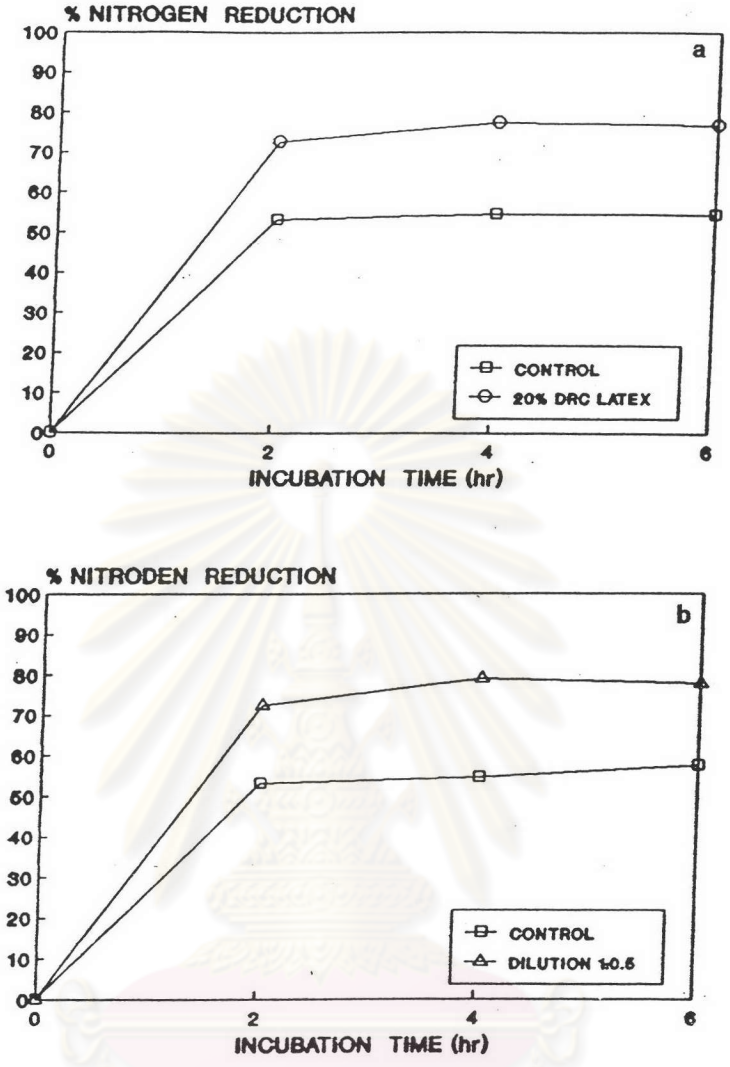


Figure 18 The effect of latex dilution on latex deproteinization

Field latex was diluted with water to be 10 % DRC, 20 % DRC and 25 % DRC before adjusting to the optimal conditions and adding of CIP for the study of effect of latex dilution (Method 12.4).

a) The effect of latex dilution before enzyme treatment on latex deproteinization

b) The effect of latex dilution after enzyme treatment on latex deproteinization

previously prepared to be 20 % DRC and 25 % DRC and deproteinized by using the optimal conditions (Figure 15 a-d) with the adding of selected chemicals (Figure 16 a-c, 17 a). Latex sample was collected and coagulated. The digested latex (25 % DRC) sample must be diluted with water at the ratio 1 : 0.5 before the microwave coagulation. Figure 19 shows that the 77 % nitrogen reduction can be obtained at the third hour from the 20% DRC latex whereas 71% and 70% of the nitrogen reduction were obtained from latex diluted after CIP treatment and control, respectively. It is therefore concluded that 20 % DRC latex adjusted pH to 7.5 in 0.15 p.h.r. hydroxylamine hydrochloride, 0.05 p.h.r. sodium metabisulfite, 1.2 p.h.r. Triton X-100, 0.0023 p.h.r.thiourea, and deproteinized with 20 p.h.r. CIP with shaking at 120 rpm at 40 ^{o}C for 3 hrs are the selected optimal conditions as summarized in Figure 20 .

11. Batch reusability of immobilized papain on chitin for latex deproteinization

Since one of the advantages of immobilized enzyme is that it can be reused so there was attempt to reuse immobilized papain on chitin for the deproteinization of latex. As shown in Figure 21 , immobilized papain can be used to deproteinize the latex for 2 times. However, it was found that only 68 % and 20 % nitrogen reduction of latex were obtained in the second and the third use of immobilized papain, respectively . Moreover, there was % DRC lost during the deproteinization process caused by the coagulation of latex with immobilized papain on chitin.

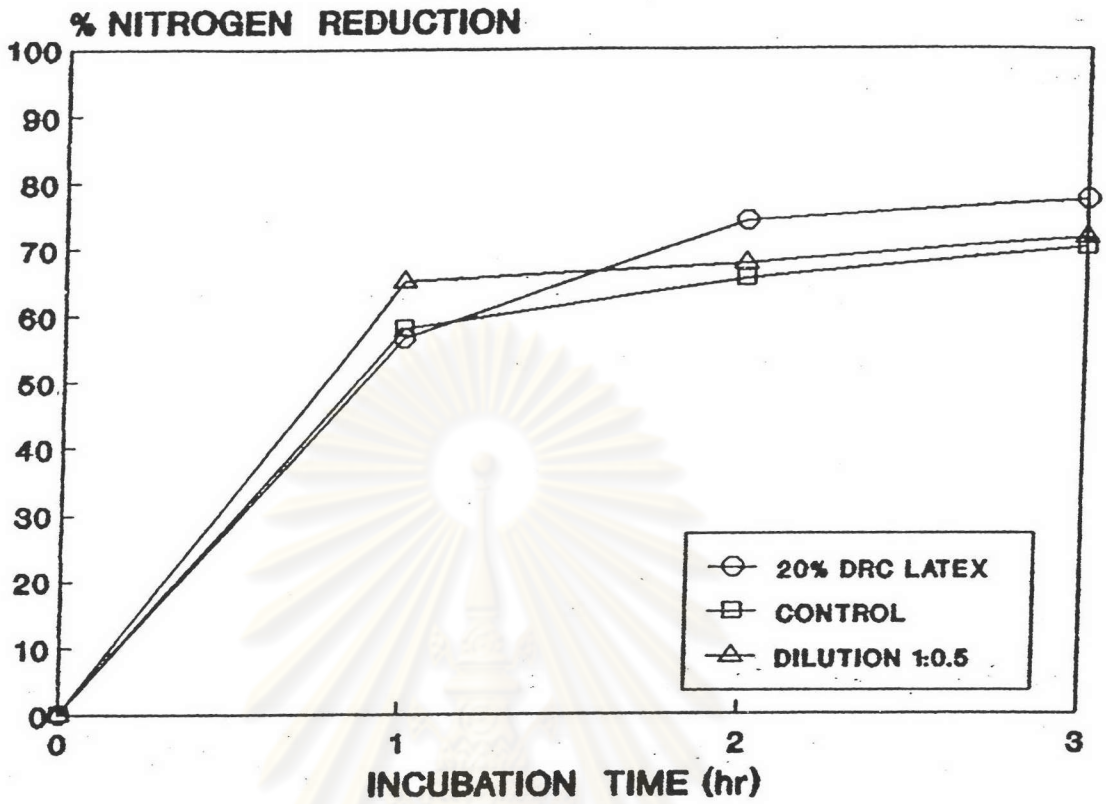


Figure 19 Profile of rubber latex deproteinization by immobilized papain on chitin

The 25 % DRC latex was prepared under optimal conditions (Figure 15) and used as the control. Another flask with 25 % DRC latex was diluted with water (ratio 1:0.5) after enzyme treatment where the flask starting with 20 % DRC was not diluted. The % nitrogen reduction was determined and compared with control which was 25 % DRC latex without any dilution.

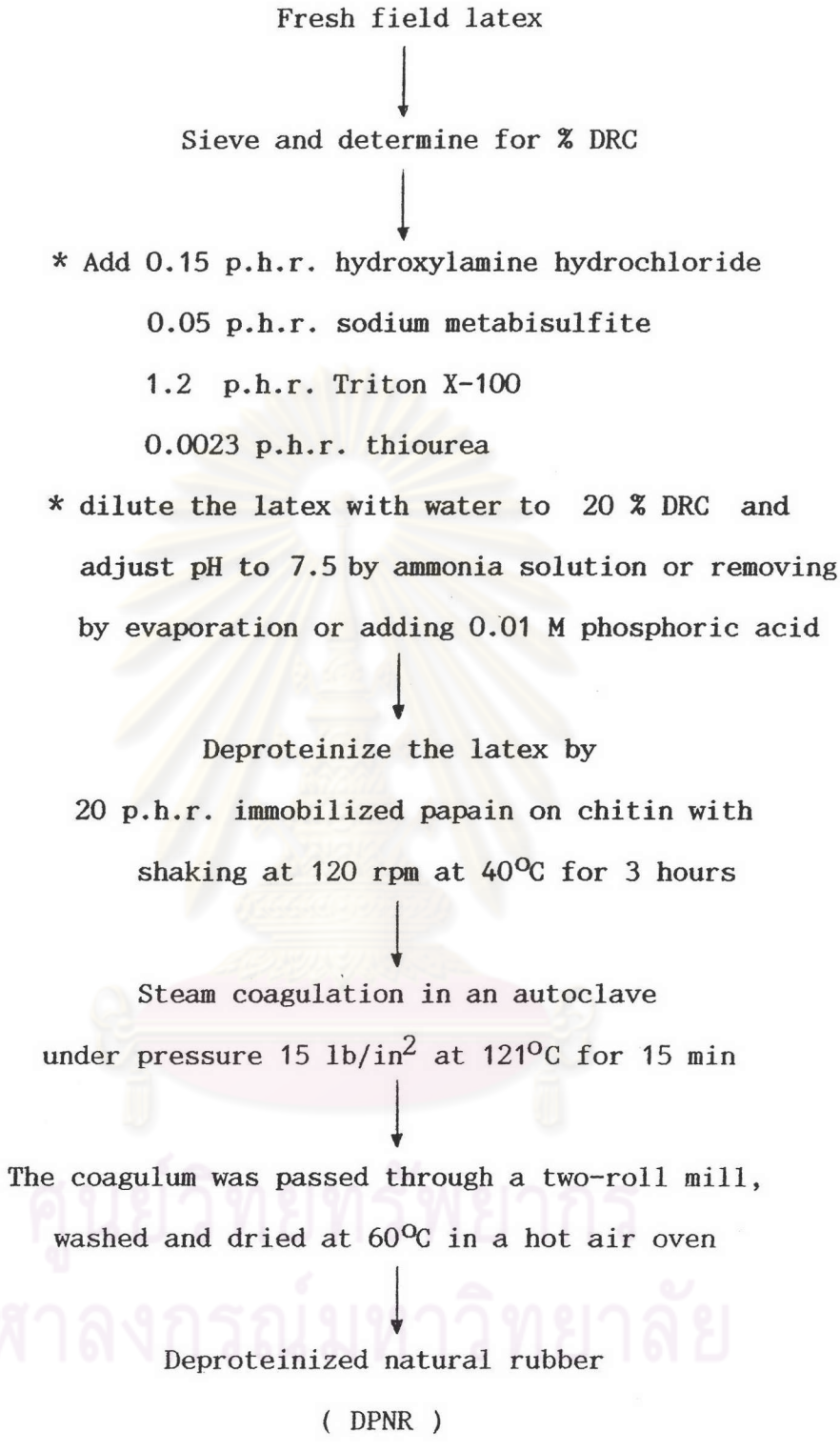


Figure 20 The optimal conditions for latex deproteinization by immobilized papain on chitin

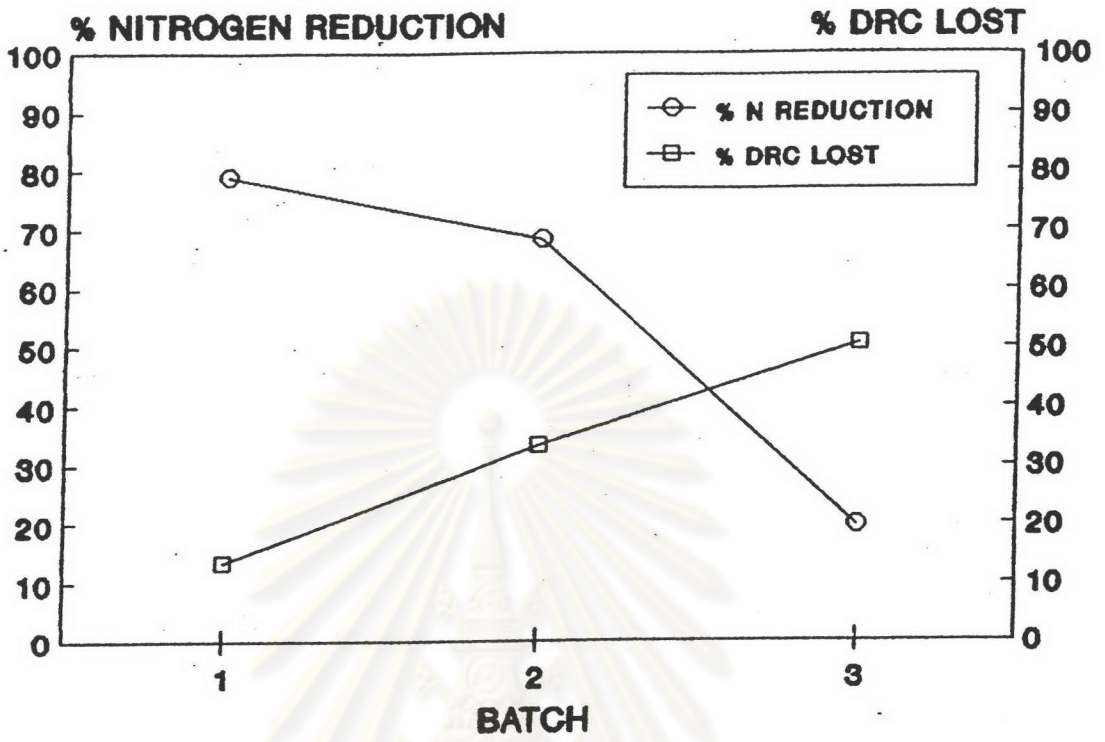


Figure 21 Batch reusability of immobilized papain on chitin for rubber latex deproteinization

12. Effect of deproteinization on the molecular weight distribution

Since weight average molecular weight ($\bar{M}w$) and the molecular weight distribution (MWD) of natural rubber are important criteria of rubber quality, it is necessary to make sure that there is no degradation of the rubber molecules under the conditions used for deproteinization. Figure 22 shows the comparative study of MWD of DPNR produced from immobilized papain, DPNR produced from free papain and the control, non-deproteinized rubber.

The MWD of solid dry rubber obtained from Hevea latex shows a bimodal distribution of 2 peaks (Figure 22). The weight average molecular weight ($\bar{M}w$) of DPNR produced from CIP and FP were lower than that of control as shown in Table 8. The number average molecular weight ($\bar{M}n$) of DPNR produced from CIP and FP were observed to be higher than the high protein control rubber. The molecular weight distribution characteristics of rubber can be expressed by polydispersity ($\bar{M}w/\bar{M}n$). Both DPNR produced from CIP and FP had narrower molecular weight ranges with low polydispersity (< 5) of 4.39 and 3.76, respectively while control showed the wider range of molecular weight and had high polydispersity (5-9) of 6.56 (Table 8). Deproteinization of rubber latex by CIP and FP have no drastic effect on the molecular weight distribution as compared to non deproteinized control rubber.

Table 8 Weight average molecular weight ($\bar{M}w$), number average molecular weight ($\bar{M}n$) and molecular weight distribution (MWD) or polydispersity of DPNR produced from CIP and FP and control.

	DPNR from CIP	DPNR from FP	Control
$\bar{M}n \times 10^{-5}$	1.73	1.71	1.34
$\bar{M}w \times 10^{-5}$	7.60	6.47	8.86
Polydispersity	4.39	3.76	6.56

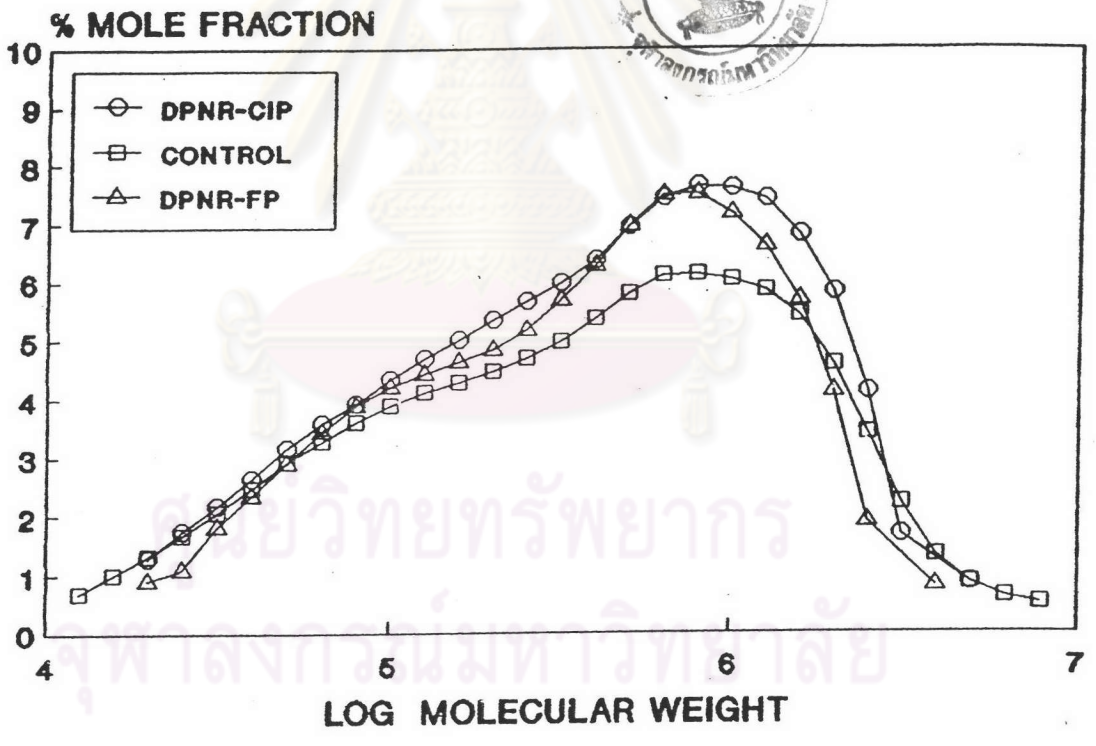


Figure 22 The comparative study of MWD between the DPNR produced from CIP and FP with the control.

13 Physical properties of raw rubber

The physical properties that determine the quality of raw rubber of DPNR produced from CIP, DPNR produced from FP and the control were compared. Figure 23 a shows the significant reduction in nitrogen content from 0.492-0.504 to 0.077-0.110 or 80.54-77.91 % by using CIP and FP, respectively. Moreover, it was found that DPNR produced from CIP contained lower total nitrogen content than DPNR produced from FP significantly, however, both DPNR contained total nitrogen content lower than DPNR specification (% N < 0.12 %).

Apart from the decrease in nitrogen content, the volatile matter of both DPNR were obviously decreased lower than control and RRIM specification significantly (Figure 23 b). The ash content and dirt content of DPNR produced by CIP and FP were 0.183 - 0.205 and 0.017-0.018, respectively and lower than those of control but higher than proposed specification (ash content 0.13 g% and dirt content 0.015 g%, respectively) (Figure 23 c and d).

Deproteinization improves the rubber color, as evident by the color index of both DPNR were lower than 4 and DPNR produced from CIP had lower color index than DPNR produced from FP (Figure 23 e and Figure 24).

As for the Mooney viscosity and other resilient properties namely the initial plasticity (Po), plasticity retention index (PRI) and storage hardening (ΔP), Figure 25 a showed a slight increase of Po about 4 units of DPNR produced from CIP while DPNR produced from FP was not significantly different comparing to control sample. On the other hand, the PRI of both DPNRs were about 80%, which was lower than that of the control but higher than the acceptable value of 60

(Figure 25 b). When the rubber samples were kept over P_2O_5 at 60 ^oC for 24 hours for storage hardening test (Δ P), Δ P lower than 7 can be acheived in both DPNRs. The increase of Δ P of control sample was higher than DPNR samples about 8 - 11 units and also higher than the acceptable value about 4 units which indicated that DPNRs should be able to withstand storage hardening better than non-deproteinized rubber (Figure 25 c) .

Mooney viscosity of both DPNRs were lower than its control, non deproteinized natural rubber samples about 6 - 9 units and the Mooney viscosity of DPNR produced from CIP was higher than the DPNR produced from FP (Figure 25 d). Moreover,to confirm that the Mooney viscosity of rubber can be stabilized during storage, the rubber samples were kept at room temperature for 5 months. Figure 26 shows that the Mooney viscosity of all rubber samples were not significantly different during the test period.

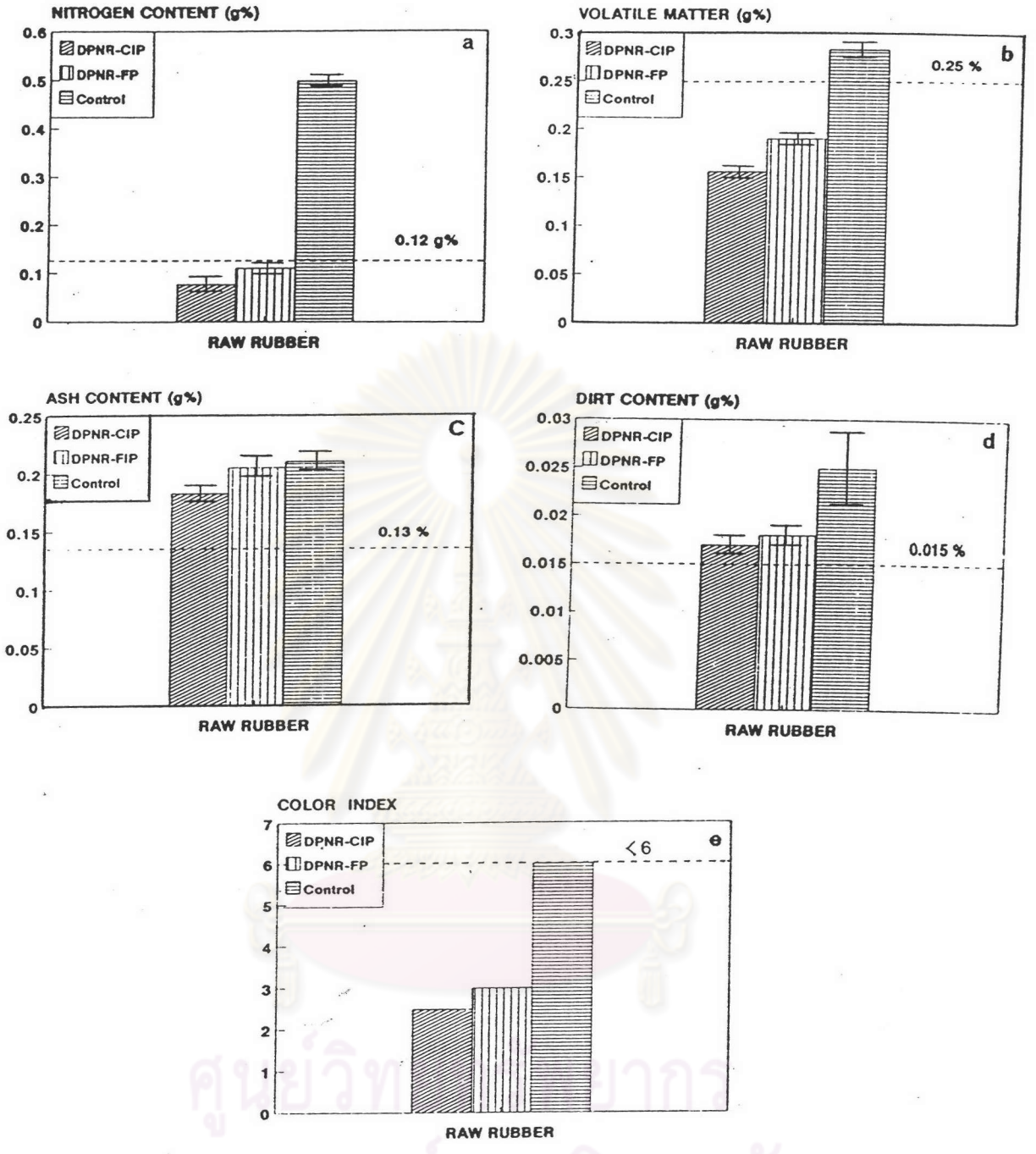


Figure 23 Comparison of raw rubber properties among DPNR produced from CIP and DPNR produced from FP and the control.

a) % nitrogen content (n=4) b) the volatile matter (n=3)

c) ash content (n=3) d) dirt content (n=3)

e) color index (n=3)

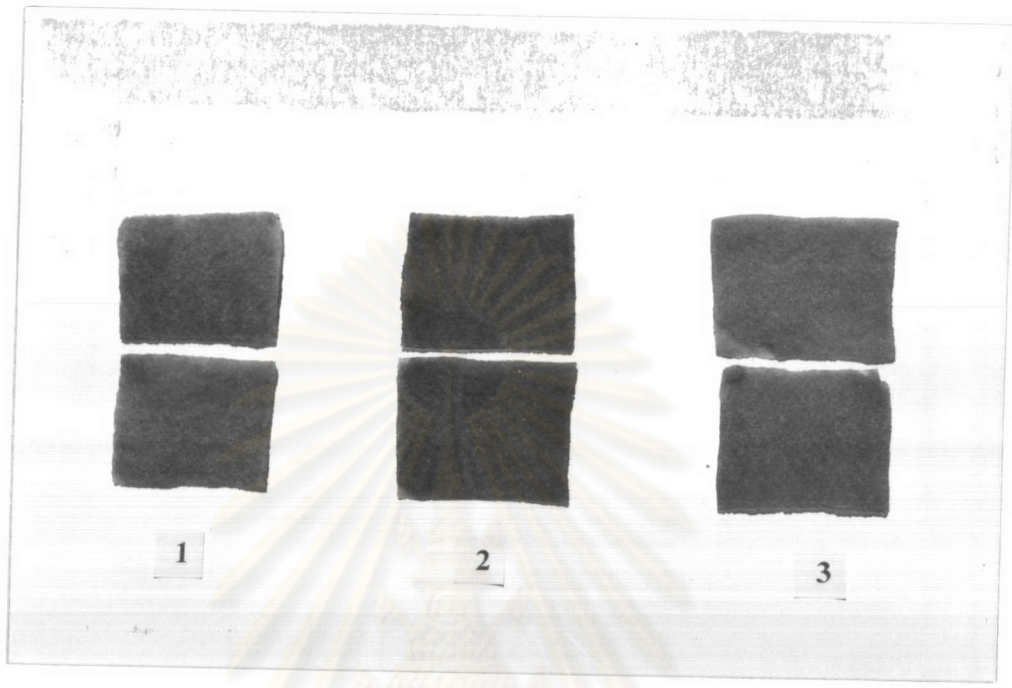


Figure 24 Comparison of raw rubber color between both DPNR and the control.

(1) DPNR produced from CIP

(2) DPNR produced from FP

(3) The control

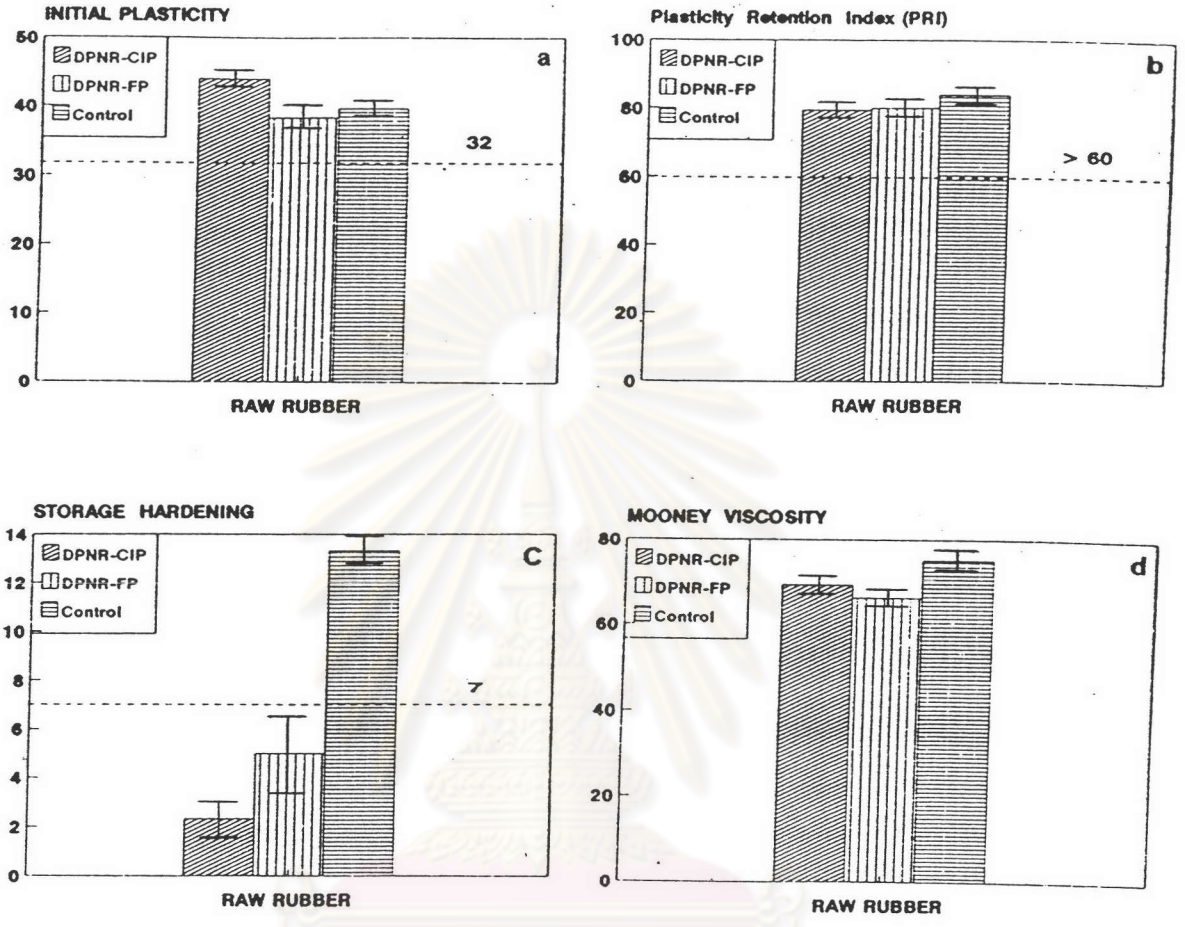


Figure 25 Comparison of the physical properties of raw rubber among DPNR produced by CIP and FP and control.

a) Initial plasticity (Po) (n=3)

b) plasticity Retention Index (PRI) (n=3)

c) Storage hardening (Δ P)(n=3)

d) Mooney viscosity (n=3)

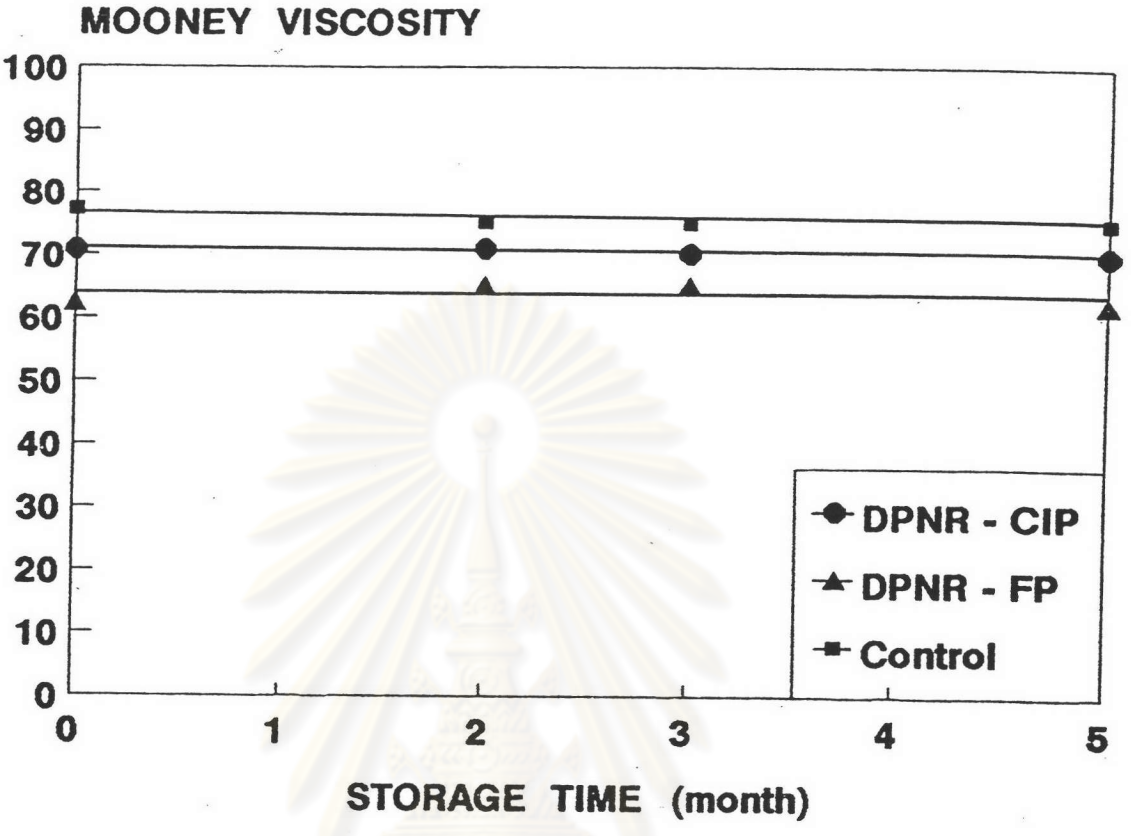


Figure 26 Mooney viscostity before and after storage

14. Cure characteristics

DPNR produced from CIP,DPNR produced from FP and control were compounded with compound additives on a two-roll mill at room temperature, left for 24 hours and, then, their cure characteristics were studied using EEKORNER Rheometer and shown in Figure 27.

Cure characteristics such as scorch time, cure time,cure rate and torque rise were compared as shown in Figure 28 (a-d). Both DPNRs had decrease scorch time (t_s) significantly from 104 $\pm$ 1 sec to 68 $\pm$ 1 and 83 $\pm$ 1 sec. The cure time (t_{90}) and cure rate (t_{90}-t_s) of DPNRs also decreased. The torque rise (M_H-M_L) of both DPNRs increased significantly but the torque rise between DPNR produced from CIP and FP were not significantly different.

The color of compounded DPNR produced from CIP had lighter color than DPNR produced from FP and the control rubber(Figure 29).

15. Properties of unaged and aged vulcanized rubber

Technological properties of the vulcanized DPNR produced from immobilized papain were compared with the vulcanized DPNR produced from free papain and the control, high protein rubber sample as shown in Figure 30 (a-e). After removal of most proteins, an increase in tensile strength and % elongation at break can be observed but hardness was decreased whereas 300 % modulus of all samples were not different significantly. The specific gravity of both DPNRs and control were similar.

After ageing at 70°C for 7 days,the value of tensile strength increased insignificantly, 300 % modulus and hardness of all rubber samples increased significantly from unaged rubber's values while

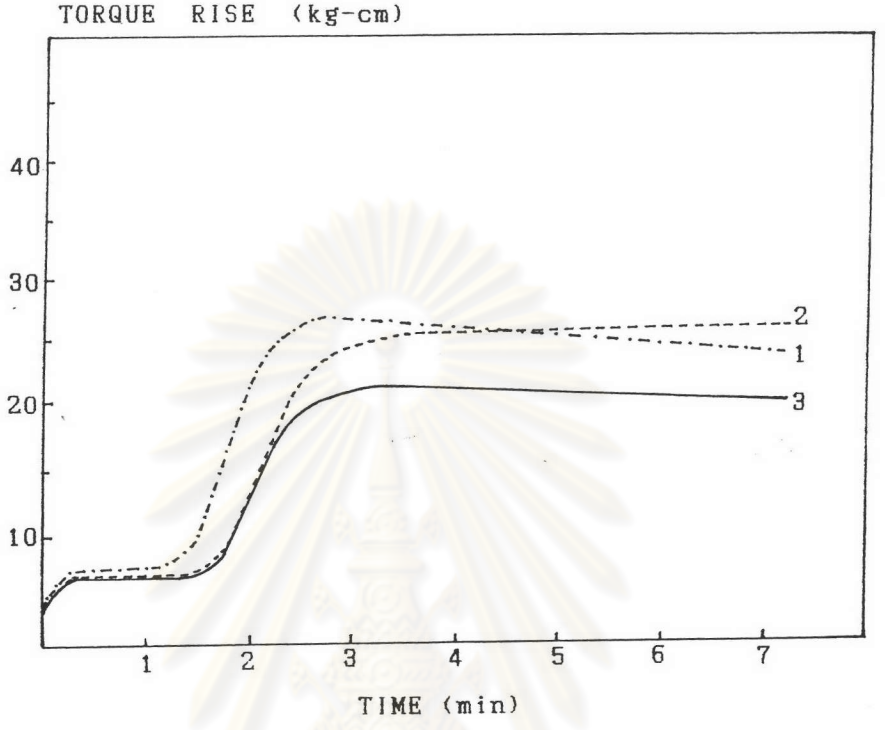


Figure 27 Rheograph of compounded rubber and DPNR

(1) -·-·-·- Compounded DPNR-CIP

(2) ------- Compounded DPNR-FP

(3) ——— Compounded control

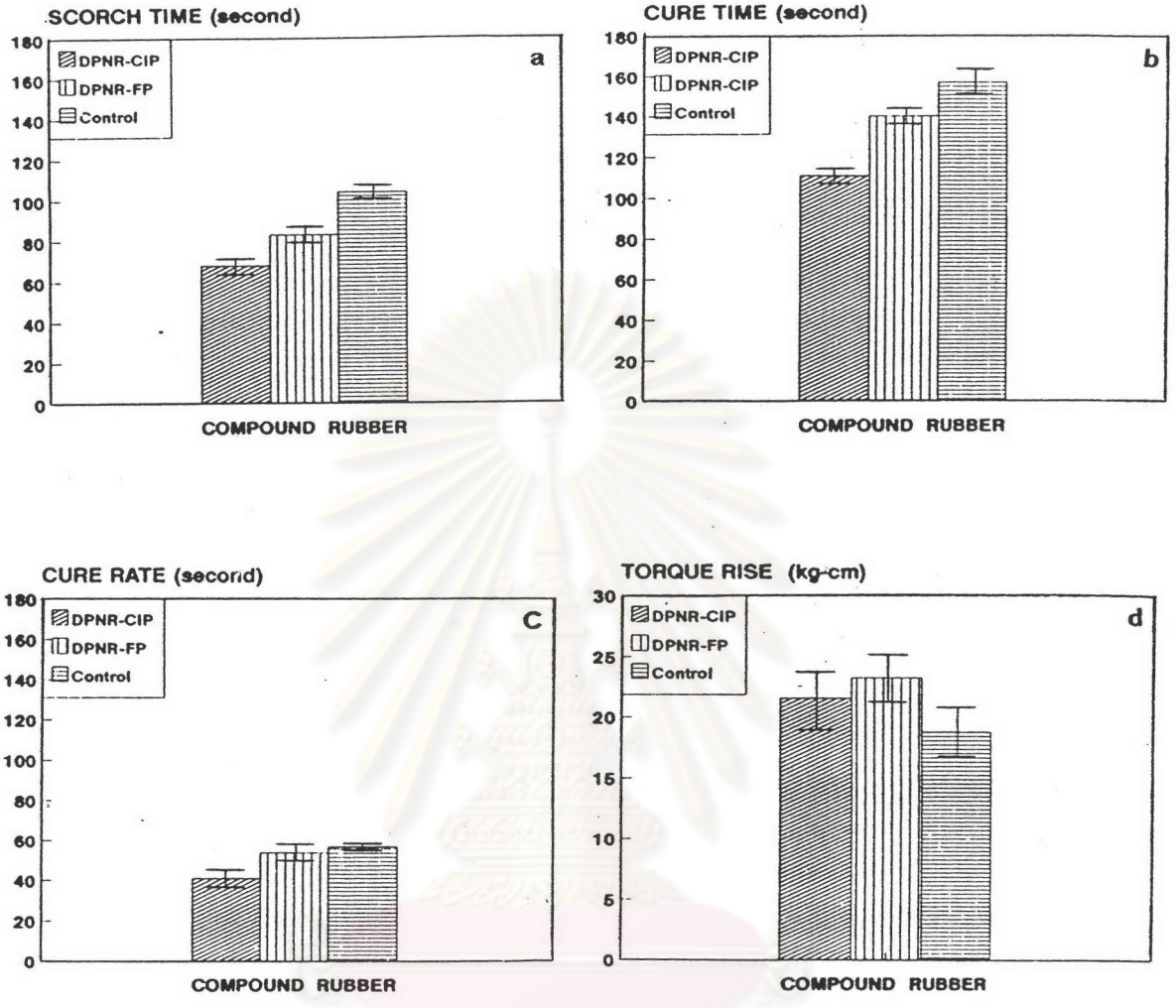


Figure 28 Comparison of cure characteristics between both DPNR and the control.

a) scorch time (n=3) b) cure time (n=3)

c) cure rate (n=3) d) torque rise (n=3)

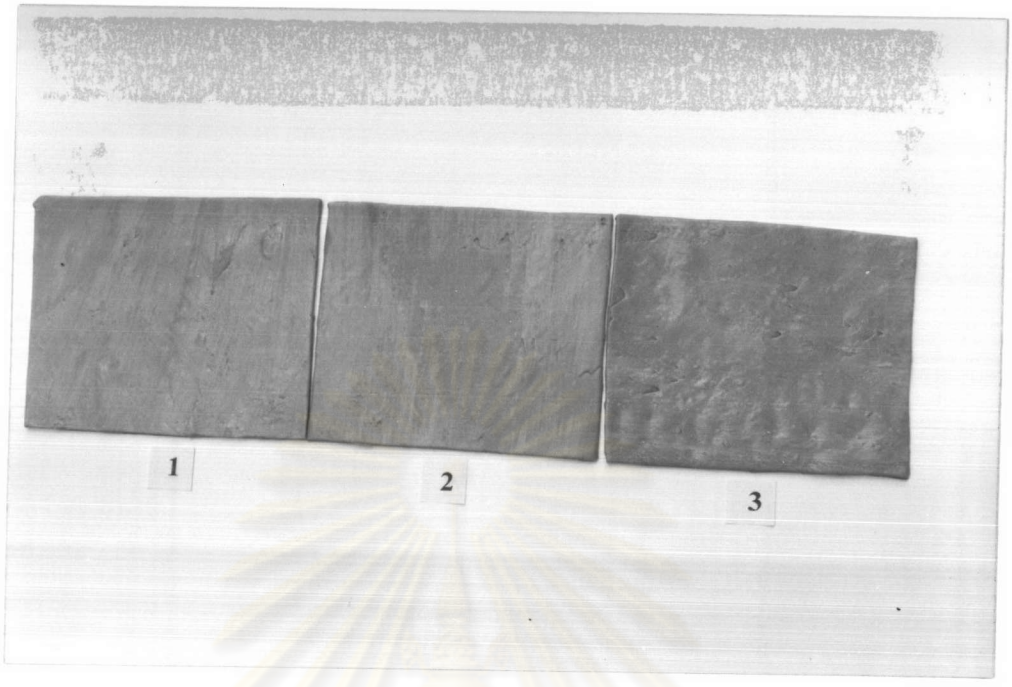


Figure 29 Comparison of compound rubber color among both DPNR and the control.

1) DPNR produced from CIP

2) DPNR produced from FP

3) the control

% elongation at break slightly decreased (Figure 3.26a-d). The effect of ageing on the physical properties of DPNR is less pronounced than high-proteins rubber, especially tensile strength and 300 % modulus (Figure 30 a-e).

The color of the vulcanized DPNR produced from immobilized papain was lighter than the color of DPNR produced from free papain and the control as shown in Figure 31 .

16. Estimated cost of production of DPNR produced from CIP

The cost of DPNR production from CIP based on laboratory scale production was shown in Table 9. The cost of DPNR produced by CIP was about 153.67 Baht/kg. The major cost for DPNR production was Tris, chitin, cysteine and Triton X-100. These expense can be reduced by replacing Tris and cysteine with phosphate and sodium bisulfite, respectively (Figure 32 and 33). It was observed that papain activity was increased when sodium bisulfite was used as papain activator instead of cysteine,so papain concentration used and papain cost were decreased from 2.9 Baht/kg to 1.4 Baht/kg. Consequently, the reduced cost of DPNR produced from CIP was about 96.77 Baht/kg.

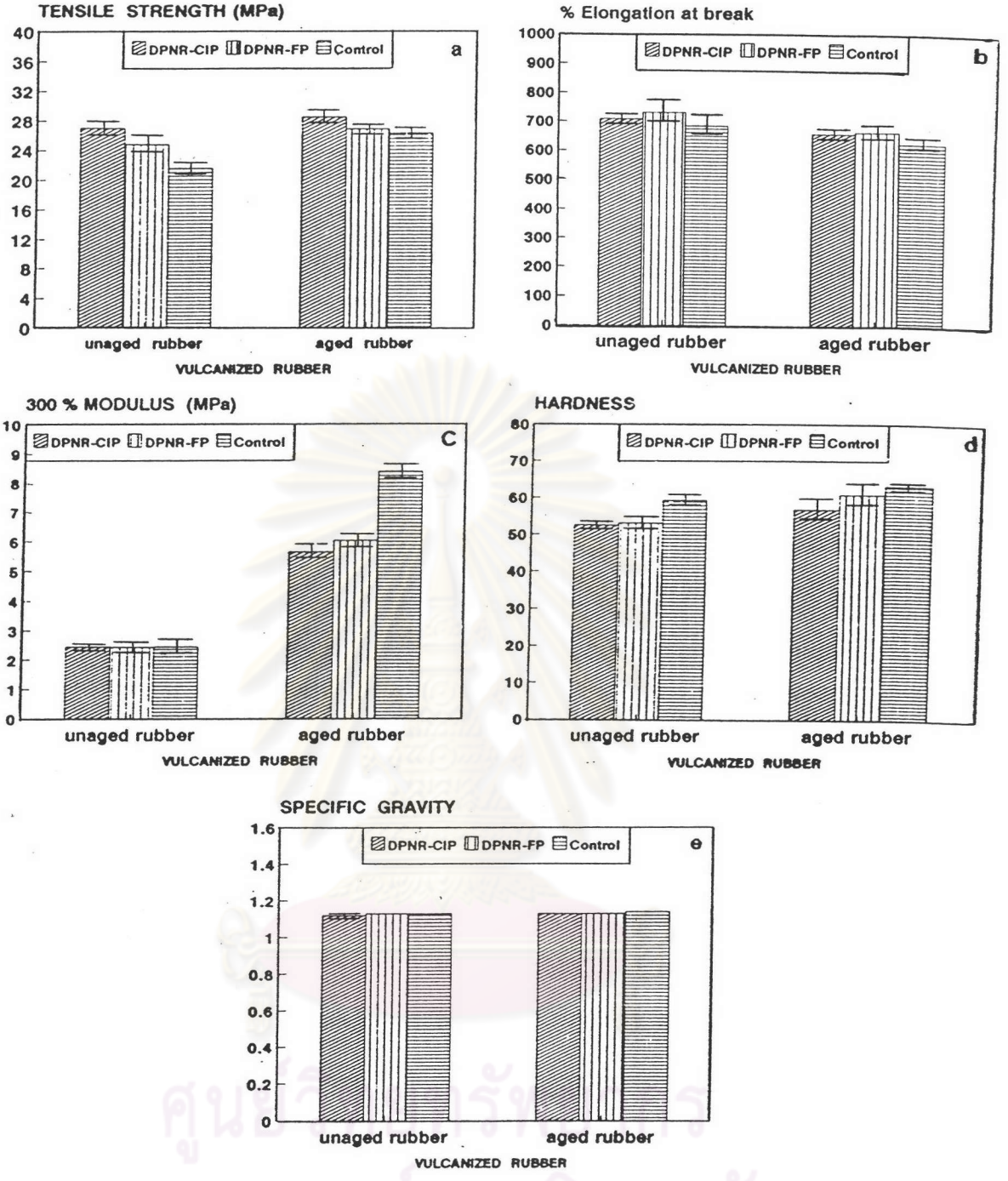


Figure 30 Comparison of vulcanizate properties between both DPNR and the control.

a) tensile strength (n=6) b) % elongation at break (n=6)

c) 300% modulus (n=6) d) hardness (n=3)

e) specific gravity (n=3)

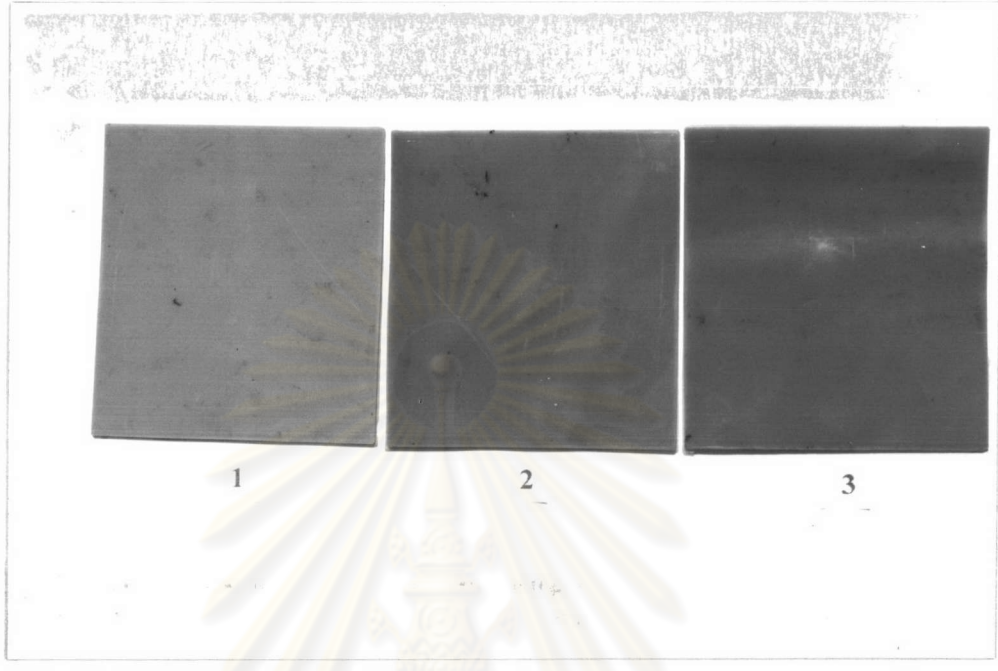


Figure 31 Comparison of vulcanizate rubber color among both DPNR and the control.

(1) DPNR produced from CIP

(2) DPNR produced from FP

(3) the control.

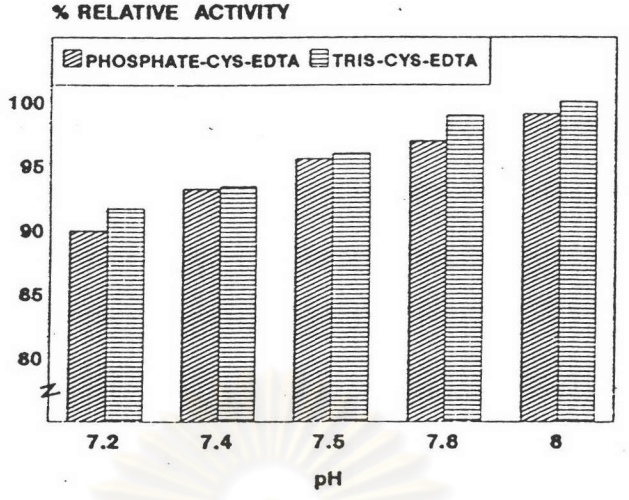


Figure 32 Comparison of the activity of immobilized papain when phosphate-cysteine-EDTA buffer and Tris-Cysteine-EDTA were used in the immobilization process.

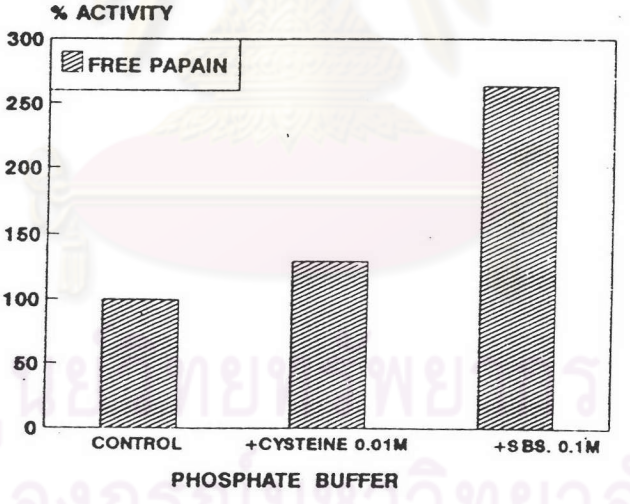


Figure 33 Comparison of the activity of papain solution when cysteine and sodium bisulfite were used as papain activator in the immobilization process.

Table 9 Production cost of DPNR

The cost of DPNR production was shown in column A and was decreased when Tris and cysteine was replaced by phosphate and sodium bisulfite. The reduced cost was shown in column B.

			Column A	Column B
	UNIT COST (Baht / unit)	CONSUMPTION (unit)	DPNR (Baht /unit)	DPNR (Baht /unit)
Rubber	12 15	1 kg	12	12
Chemicals				
papain	1000 / 1 kg	2.9 g	**2.90**	**1.40**
Tris 0.1 M	3612.5 / 1 kg	14.48 g	52.31	
cysteine 0.010 M	**515 / 1 kg**	0.72 g	**14.83**	
Phosphate 0.1 M	1365 / 1 kg			**11.59**
Sodium bisulfite 0.01 M	273 / 1 kg			**0.15**
EDTA 0.038 M	1443.75 / 1 kg	5.8 g	8.37	8.37
Glutaraldehyde 25%	775 / 1 litre	0.66 ml	0.51	0.51
chitin	545 / 1 kg	41.36 g	22.54	22.54
Ammonia	280 / 2.5 litre	10 ml	1.12	1.12
Hydroxylamine.HCl	2400 / 500 g	1.5 g	7.20	7.20
Sodium metabisulfite	550 / 500 g	0.5 g	0.55	0.55
Triton X-100	1150 / 1 litre	12.52 ml	14.39	14.39
Thiourea 0.0023 phr	619 / 1 kg	0.023 g	0.01	0.01
Utilities	2 / KW-h			
magnetic stirrer(19W)		1 h	0.04	0.04
shaker (1.1W)		3 h	6.60	6.60
Two roll-mill (1.5W)		0.5 h	1.50	1.50
Autoclave (2 kW)		1 h	4.00	4.00
Hot air oven (0.8 kW)		6 h	4.80	4.80
total cost (Baht/kg)			153.67	96.77