CHAPTER IV

DISCUSSION

Latex deproteinization by enzyme has been reported as the favorable method to produce DPNR. In the past, many kinds of protease have been used for removal of protein from latex, namely Alcalase, Superase (Chang et al.,1977), papain (Yapa, 1975, Visessanguan,1992), bromelain (Yapa et al., 1980) etc., but papain seems to be more effective than others because at optimized conditions it requires shorter time for latex deproteinization (Visessanguan, 1992). Papain treated rubber can also be stored for upto 2 years under normal factory storage condition without fungus infection (Anandan and Loganathan, 1984). However, the major cost of DPNR production is imported papain. The attempt of this research is firstly to reduce the production cost by replacing imported papain with locally produced papain and secondly to prepare immobilized papain and test the feasibily of using immobilized papain for latex deproteinization in order to understand the optimal conditions, efficiency of the process and the reutilization of immobilized papain.

1. The optimum conditions for papain immobilization

The physical adsorption method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carrier. This method was chosen for papain immobilization because it is the simplest method and caused little or no conformational change of enzyme protein or destruction of active site of enzyme (Chibata,

1978).

Chitin was chosen to be the carrier due to its advantages as described in Chapter I. Although there are limitted number of amine groups per unit weight of chitin available for binding, linkages with papain molecules on chitin were apparently detected after washing with 6 M HCl and 5 M KOH to improve its surface properties (Stanley, 1975). The optimal size and amount of pretreated chitin was determined. Although, it was found that papain immobilized on small mesh-size chitin (40-80 mesh) gave higher activity due to higher surface area, but too small mesh-size was found to have diffusional limitation as reported by Puvanakrishnan and Bose (1980). Besides the particle size of chitin, the adsorption of papain on chitin is dependent on other experiment variables such as papain concentration, pH and reaction time etc. (Kanasawud, 1990). It was found from the result that pH influenced the activity of immobilized papain. Since papain has isoelectric point (pI) of 8.75, papain dissolved in phosphate-cysteine-EDTA buffer pH 6.0 has a positive charge which can be attracted to amino groups of chitin by hydrogen bonds or Van der Waals forces. A major influence on the quantity of papain adsorbed on chitin was papain concentration exposed to the unit surface of chitin during the immobilization process. It was found that the activity of immobilized papain did not increase, although higher concentration of papain (more than 7 mg/ml) were used (Figure 5 b). That is because the activity of immobilized papain increases with the increment of enzyme concentration until it approaches to the saturation value (Kanasawud, 1990). This also agrees with a report published by Chiou and Beuchat (1987) which the activity of papain

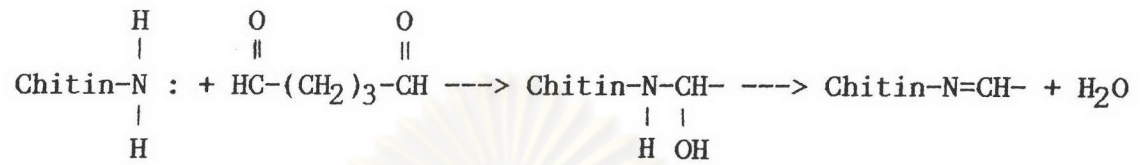
immobilized on Dowex MWA-1 increased substantially at concentration of 1-20 mg papain in phosphate buffer pH 12.5 and insignificantly increased between 20-80 mg papain.

To strengthen the binding forces, glutaraldehyde is commonly used as a coupling reagent to immobilized proteins through a formation of intermolecular linkage (Habeeb and Hiramoto, 1968). It was added at the optimum concentration (0.7 %) into the suspension of immobilized papain. Moreover, the reaction time is one of the important parameters for papain immobilization. It was found that the amount of bound papain in terms of % activity almost linearly increased with an increment of reaction time from 15 to 45 min, then sligthly decreased with an increment of time (Figure 5 f).

However, enzyme immobilization by the physical adsorption method has the major disadvantage that is the binding force between enzymes and the carrier is generally weak, so that adsorbed enzymes are usually released from the carrier during washing and utilization (Chiou and Beuchat,1987 and Chibata,1978). From the result as shown in Figure 5 g, it was found that about 65 % of the adsorbed papain was released during washing. This agrees with a report published by Chiou and Beuchat (1987) that papain immobilized on Dowex MWA-1 by physical adsorption method followed by intermolecular crosslinking with 1.0 % glutaraldehyde was washed out at least 48%.

To strengthen the linkages between papain and chitin,covalent -binding method was considered. The method used in this research was carrier-binding with bifunctional reagent, glutaraldehyde. This method is based on the formation of crosslinks between the amino groups of the carrier or the amino groups of enzyme protein and the

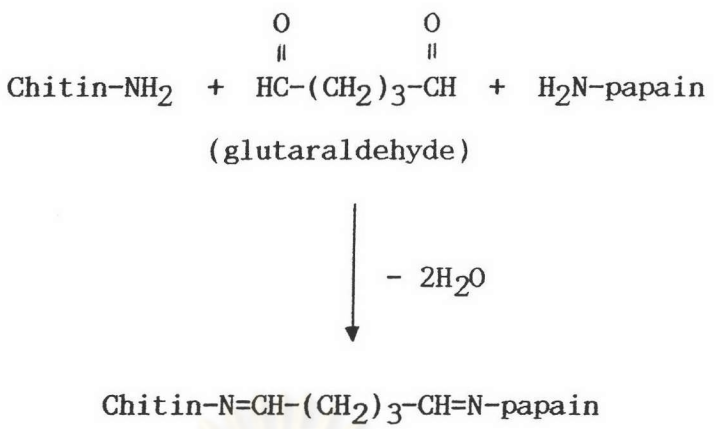
aldehyde groups of glutaraldehyde (Chibata,1978). As shown in Figure 6 a, the optimal concentration of 0.4 % glutaraldehyde in 0.1 M Tris buffer was obtained. The reaction between chitin and glutaraldehyde in buffer pH 8.0 can be explained and represented as equation below :



(glutaraldehyde)

Chitin was reacted with glutaraldehyde in basic solution. Then, the product containing a carbon-nitrogen double bond resulting from elimination of a molecule of water was obtained (Morrison and Boyd, 1987). Since the crosslinks of immobilized enzymes with glutaraldehyde bring about a significant loss of the activity as a result of denaturation of enzyme proteins such as immobilized lactase ,immobilized glucose isomerase etc. (Synowiecki et al., 1982), the amount of glutaraldehyde dissolved in buffer must be controlled to the minimum and as shown in Figure 6 b, the optimum ratio between buffer and 0.4 % glutaraldehyde is 19:1.

In case of papain solution used for immobilization, the optimal concentration of 7 mg/ml of papain was dissolved in Tris-cysteine-EDTA buffer pH 8.0 and the same reaction as shown above can occur. The reaction between papain and glutaraldehyde-treated chitin in basic solution, named as Schiff base reaction, can be represented as :



After 4 times washing,immobilized papain on chitin by covalent binding method has 23 % yield. Comparison to the activity yield of papain immobilized on Dowex MWA-1 by the same method which had 9 % yield (Ohmiya,1978), it was found that papain covalently-immobilized on chitin in this research gave better result of 2.5 times of activity yield.

2. Properties of immobilized papain on chitin

Information on the changes of enzymatic properties caused by the immobilization of enzymes is useful not only for the application of immobilized system but also for the elucidation of structure-function relationships and the mechanism of enzyme reaction. Changes of enzymatic properties are considered to be caused by the following two factors. One is the change of enzyme itself, and the other is due to the physical and chemical properties of the carriers used for immobilization. The former involves the modification of amino acid residues in the active center of the enzyme, the conformational changes of the enzyme protein, and the changes of charge on enzyme, while the latter involves the formation of diffusion layers around

immobilized enzymes (Chibata, 1978).

As enzymes are proteins, the catalytic activity is markedly affected by environmental conditions, especially the pH of aqueous medium. The effect of pH on enzyme activity means the effect of pH on the ionization of prototropic group in the active site of enzyme that may cause the changes of three dimensional conformation and substrate binding. Figure 9 a showed the same optimal pH range,5.5-9.0,of PIP and FP at 40^{o}C, indicating that there is no conformational changes of papain immobilized on chitin by physical adsorption method. While CIP shows the lower and narrower optimal pH range between 5.5 - 6.5. The pH shift towards the acidic side after immobilization of papain on chitin by cavalent-binding method may alter the conformational structure of enzyme resulting in the changes of substrate specificity (Chibata, 1978 ; Kanasawud, 1987).

The catalytic activity of an enzyme is also dependent on temperature, as in the case of ordinary chemical catalyst, but the activity is lost at high temperature due to thermal denaturation of enzyme (Messing, 1975). CIP on chitin and FP had the same optimal temperature range at 50-80 ^{o}C while PIP had the narrower range of the optimal temperature at 70-80 ^{o}C (Figure 9 b).

The stabilization of enzyme is an important characteristic in determining the feasibility of application of immobilized enzyme. The enzyme stability depends on pH and temperature during storage and operational condition (Kaul and Mattiasson, 1990). Figure 10 a shows that CIP is stable in wider range of pH than PIP and FP whereas the temperature stability are the same in all cases i.e. they were stable at the temperature range of 30-50 ^{o}C (Figure 10 b).

The storage stability is another important factor in application of immobilized enzyme, both of PIP and CIP are more stable than FP when stored at room temperature for 48 hrs (Figure 12). The stability of immobilized papain was considered to be caused by a reduction of autolysis of protease (Chibata,1978). Longer storage stability of immobilized papain and free papain were observed when they were stored at 4 ^{o}C for 3 months, where more than 80 % of enzyme activity can be retained in CIP (Figure 13). Weetal (1970) also reported that papain covalently coupled to cellulose by the azide method remained 68 % activity when stored at 5 ^{o}C in distilled water for 58 days.

Besides the storage stability, the operational stability of immobilized enzyme is one of the most important factor affecting the success of industrialization of an immobilized system. A column packed with CIP can be continuously used with casein for more than 3 days at 40 ^{o}C (Figure 14). However, this operation stability is shorter when comparing with papain immobilized on porous glass which can be operated for 35 days at 45 ^{o}C continuously (Weetal and Mason, 1973) due to the degradation of chitin.

As already mentioned, when enzymes were immobilized, the enzyme activities and substrate specificity are usually changed. The values of Michaelis constant, K_m and the maximum reaction velocity, V_{max} of FP, PIP and CIP were estimated from Figure 11 (a-e) and tabulated in Table 6 and 7. Normally, if an enzyme has a small value of K_m,it achieves maximal catalytic efficiency at low substrate concentration because K_m is the substrate concentration at which the reaction velocity is half maximal (Voet, 1990). From the results,

using crude papain,there are detectable changes in Michealis constant (K_m), which reflect the affinity between enzyme and different substrates. It was found that the K_m value of immobilized papain and free papain were higher when ovalbumin and rubber latex were used as substrate than when casein was used as substrate. These results suggest that the K_m and V_{max} are influenced by the change in diffusion rate of the immobilized enzyme and substrate. An increment of the molecular weight seems to diminish the diffusion (Ohmiya, 1978). Since the molecular weight of casein is about 20,000-24,000 while the molecular weight of ovalbumin is 45,000 (Stecher, 1968) and rubber particles are $7.60 \times 10^5 - 8.86 \times 10^5$, the diffusion rate of three substrates seemed to be discriminated. From this reason, different K_m values were obtained. Table 7 showed the highest K_m value of CIP while rubber latex was used as substrate due to the diffusional limitation (Kanasawud, 1990) of high Mw of the rubber molecule. The same result was reported by Shingo and Yashuhiro (1990) that K_m value of papain immobilized on copoly (ethylene/acrylic acid) fibre is 4.5 times higher than that of FP when BAEE (N- α -Benzoyl-L-arginine ethyl ester) was used as substrate at pH 8.0 at 37^oC.

The maximal velocity of a reaction, Vmax, occurs when enzyme is saturated i.e. when it is entirely in the enzyme-substrate complex form (Voet, 1990). The Vmax value shows the reaction efficiency of enzyme to decompose the complex to products hence this value can be used in the comparison of enzyme efficiency (ปราณี อ่านเปรื่อง, 2535). From the results, it was found that the Vmax value of PIP was not significantly different from the values of FP. While the Vmax value of CIP was higher than that of FP when casein and rubber latex were

used as substrates and lower than that of FP when ovalbumin was used as substrate. These results might be because of the conformational changes of papain caused by covalent - binding of papain on chitin.

3. The condition of latex deproteinization by CIP

The optimal conditions for fresh field latex deproteinization by CIP was shown in Figure 20. The deproteinization of latex depends on the pH of latex. Since the isoelectric point (pI) of the protein which protects the natural rubber particles, mainly α-globulin, is approximately 4.3-4.6 (Nadarajah et al., 1973), the latex having pH range of 6-9 is negatively charged. The more negative charge of latex, the less % N reduction can be obtained(Figure 15 a) because it was over the pH optimum range of CIP. Furthermore, the rate of nitrogen reduction is directly related to temperature until a certain temperature is reached but at high temperature, the rate is decreased due to the denaturation of papain and the coagulation of latex (Koosakul, 1994).

Under the condition used for deproteinization, there is no inhibitory action of hydroxylamine hydrochloride (Figure 16 a). Yapa and Balasingham (1974) and Yapa (1975) have also reported that papain and hydroxylamine hydrochloride can be combined suitably for the manufacture of low nitrogen-CV rubber. Sodium metabisulfite, the reducing agent, and thiourea were found to enhance the proteolytic action of immobilized papain on chitin. However, high concentration of thiourea (> 0.05 M) was found to inhibit the deproteinization process (Yapa and Balasingham, 1974), thus the amount of thiourea using must be controlled. Although cysteine was reported as the

papain activator (Yapa and Balasingham, 1974), it was considered not to use due to its high price and low activating potential. EDTA, the metal-chelating agent, was also omitted because it does not increase CIP activity (Yapa and Balasingham, 1974). Although Koosakul (1994) reported that 1.1 p.h.r.Triton X-100 enhanced the removal of proteins from field latex by free papain but Figure 16 c shows that higher concentration of Triton X-100 (1.2 p.h.r.) inhibited the activity of immobilized papain on chitin resulting in the decrease of % nitrogen reduction.

The dilution of latex before and after enzyme treatment were found to give a greater reduction in % nitrogen contents as reported by Nadarajah (1973); Yapa (1980) and Chang (1977), respectively.

Figure 19 indicated that the DPNR production by CIP in this research required less reaction time (3 hrs) and the removal of nitrogen content was higher (0.077 g % of the retention N content or 80-84 % nitrogen reduction) when comparing with the previous work of Chang et al. (1977) which 0.12 g % of retention N content was obtained by using Alcalase with centrifuged field latex for 24 hrs before acid coagulation and John (1977) which 0.2 g % of retention nitrogen content was obtained by using free papain with 10% DRC field latex for 20 hrs before acid coagulation. Moreover, the % nitrogen reduction of this research was also higher than the results of Visessanguan (1992) which 0.11 g %N or 70-75 % nitrogen reduction was obtained by using free papain at 50 ^{o}C for 2 hrs but the longer time was used. However, when these results are comparing with the results of Koosakul (1994) in which 0.070 g % N was obtained by using free papain at 50 ^{o}C in 50 min, it was found that DPNR from CIP required

longer reaction time and the removal of nitrogen content is about the same, and not better.

4. Effect of deproteinization on molecular weight distribution (MWD)

The bimodal distribution (type 2) of the molecular weight distribution (MWD) profiles were observed from either of the control or DPNR samples produced from CIP and FP. These results confirm that the deproteinization of latex by CIP and FP do not have any significant effect on the type of MWD profile. Eng et al. (1993) reported that the broad MWD of rubber is associated with branching and cross-linking between rubber molecules and gel phase within the rubber particles. Proteins which held or chemically bonded to the rubber have been postulated to be responsible for the occurrence of branching and cross-linking. Hence, when proteins are digested, the MWD profile slightly shifted from high molecular weight to low molecular weight. Furthermore, since the weight average molecular weight (Mw) decreases with the decrement of nitrogen content(Ichikawa et al., 1993), it is in good correlation with the narrower molecular weight range (low polydispersity, $\bar{M}w$ / $\bar{M}n$) was observed from DPNR produced by CIP as well as from DPNR produced by FP.

5. The properties of DPNR

The deproteinization of natural rubber latex by using immobilized papain and free papain theoretically follows that enzyme treatment decreases the proteinaceous non-rubbers by breaking them down to amino acids which are dissolved and washed out during dewatering (Chang et al., 1977). The remaining nitrogen content of

DPNR produced by CIP was less than that of DPNR produced by FP. However, both of them were lower than that of the control and that of RRIM specification (1977) which the nitrogen content must be below 0.12 g%.

The ash content represents as minimal figure for the amount of mineral matter present in the rubber. While the dirt content is an obvious contaminant in raw rubber. They were decreased after enzyme treatment. Steam coagulation normally yields higher level of nitrogen and ash content than acid coagulation (John and Sin, 1977). The dilution of latex before coagulation can remove some ash and nitrogen content in the latex (Nadarajah et al., 1973).

The volatile matter which includes moisture and short chain fatty acids is decreased by latex deproteinization. Since proteins are polar and have hydrophilic characteristics (Smith, 1974), they enhance the absorption of water in natural rubber. Associated with the decrement of nitrogen level is a lower moisture content and the decrement of the volatile matter.

Rubber with light color is required in the production of light colored rubber product, which requires a color limit of six unit or less on Lovibond color scale (as quoted by Koosakul, 1994). The color indexes of DPNR produced by CIP and FP were 2.5 and 3.0, respectively. Both of them were lighter than the color index of the control. There are several factors causing discoloration of raw rubber. As noted by Hasma and Subramaniam (1986), the color of rubber depends on its clonal origin of rubber, process of latex after tapping etc. However, the most important factor is usually due to the polyphenol oxidase in latex which catalyses the oxidation of

phenols to ortho-quinones which can react with naturally occurring amino acids and proteins present in latex to give colored product (De Haan-Homans,1949). Discoloration is also caused by non-enzymatic reaction between carbonyl and amino groups to give an unsaturated carbonyl amino derivative, showing brown or black color (Rinderknecht and Jurd,1958). Thus, the removal of proteins improves the color of raw rubber.

An important specification for raw rubber is the plasticity retention index (PRI) which is a measured value of resistance of raw rubber to oxidative degradation by a short time ageing (Bateman and Sekhar, 1966). High PRI value corresponds to good ageing resistance. The PRI values obtained from both DPNR were not significantly different. However, these values were lower than that of control significantly. Kasinathan et al. (1971) explained that the PRI value is dependent on antioxidants such as amino acid present in the latex of natural rubber, the PRI value increased from 106.0 to 116.8 and 117.1 when 0.1 % and 0.2 % tyrosine were added to the latex. The removal of proteins and washing will leach out naturally occuring antioxidants which are dominantly tocotrienol (80%), amines and amino acids (20%) (Morimoto, 1985) resulting in the decrement of the PRI value. Moreover, PRI value was also decreased with latex dilution. Sivabalasunderam and Nadarajah (1965) showed the results that PRI value was decreased from 108.5 to 105.9 and 101.2 when latex was diluted with water at the ratio 10:1 and 10:4, respectively.

Mooney viscosity is a rubber characteristic which is roughly proprotional to the weight average molecular weight ($\bar{M}w$) and MWD (Nielsen,1977). It also depends on the branching of the polymer chain

including linkages to some cross-linking non-ruber molecules. The molecular weight of the branches seem to be more important than their number (Long et al., 1964). Rubbers with high molecular weight show high Mooney viscosity (Table 8 and Figure 25 d). Removal of proteins in the latex reduced the cross-linking non-rubber molecules in the rubber. Consequently, the reduction of Mooney viscosity was obtained. As previously noted by Visessanguan (1992) and Koosakul (1994), the 3-5 unit drop can be observed from their investigation.

Mooney viscosity of rubber can be changed during storage resulting in hardening (Yip, 1990). Hardening is a slow crosslink reaction between the rubber molecules involving the aldehyde or carbonyl groups which are incorporated on the main rubber chain and amino acids present among the non-rubber constituents (Roberts,1990). Sekhar (1960) reported that the rate of hardening change is greater at zero humidity and elevated temperatures. Therefore, a convenient rapid laboratory assessment of the susceptability to hardening is provided by stroage the rubber at 60 ^{o}C for 24 hrs over P_2O_5 and this method is known as accelerate storage hardening test (ASHT) which confirms the ability of the rubber in stabilizing its viscosity versus the storage time. Constant viscosity (CV) grades of either NR or DPNR in which the hardening is inhibited can be produced by adding hydroxylamine hydrochloride to react with the carbonyl groups (Roberts, 1990). Thus, the hardening of DPNR produced from CIP and FP are less than the untreated rubber.

The existence of nitrogen content in natural rubber affects the cure behavior of rubber compound. DPNR produced by CIP and FP exhibit shorter scorch time, cure time and cure rate but higher

torque rise than control. A similar evidence was observed by Visessanguan (1992) for fresh field latex deproteinization, and John (1977) for skim rubber deproteinization. From the results, it is hypothesized that, the removal of proteins in rubber latex to the minimum and consistent amount by deproteinization process should improve the homogeneity of mixing and chemical dispersion in rubber resulting in homogeneous vulcanization.

The homogeneous vulcanization of DPNR compound caused by the removal of proteins resulting in the improvement of stress-strain properties. The removal of protein increased tensile strength and % elongation at break but decreased hardness. The similar result was observed by Visessanguan (1992). However, 300 % modulus of DPNR and control showed no significant differences (Figure 30 a-d). The hydrophilic proteins influence the vulcanizate properties due to its enhancement of water absorption. The water absorption causes the replacement of some of the protein-protein hydrogen bonds by protein-water hydrogen bonds (Roberts,1990). The removal of protein decreased water content in the rubber; therefore,the mastication efficiency was increased (Perera and Siriwardena, 1985). Knight and Tan (1975) reported that the proteinaceous matters can act as a reinforcing filler. The elimination of proteins in rubber causes a reduction of stiffening action of rubber and reduction of modulus and hardness.

Since protein plays important role in heat resistance of rubber compounds (Morimoto, 1985), the elimination of protein causes the changes on heat-ageing properties. The formation of more cross-links can be occured during heat-ageing (Blow, 1984), it was found that when the rubber vulcanizates were aged at 70 ^{o}C for 7 days,

hardness and 300 % modulus of DPNR and control were increased (Figure 30 d,e). Tensile strength of DPNR was insignificantly increased while tensile strength of control was increased significantly. On the contrary, it was found that %elongation at break of aged vulcanizated was decreased (Figure 30 a-c). The same results were reported by Ichikawa (1993) that 500 % modulus of DPNR produced by Alcalase 2.0T and three times centrifugations increased from 1.6 MPa to 1.7 MPa after ageing at 70°C for 96 hrs. where %elongation at break decreased from 880% to 840% after ageing. In contrary, tensile strength of DPNR produced by Alcalase 2.0T and three time centrifugations increased from 24.8 MPa to 25.6 MPa after ageing at 70°C for 96 hrs.

6. Estimated the production cost of DPNR produced by covalently-immobilized papain

The cost per kilogram of DPNR production based on a laboratory scale production was shown in Table 9. The cost of DPNR produced by CIP which is about 96.77 Baht/kg is higher than the cost of DPNR produced by free papain that is 77.90 Baht/kg (Koosakul, 1994). The major cost for DPNR production is chitin, the carrier of immobilized papain The cost can be reduced by replacing chitin with other cheaper carrier. An inorganic carrier such as sand will be considered to use due to its superior operation stability and thermal stability (Weetall, 1970) and continuous operation for latex deproteinization.

7. Suggestion for further research

Although the superior quality of DPNR is required to fulfil the objectives of rubber goods producer. The cost of DPNR is another important factor to determine the consumer acceptance. From this research, it was found that imported chitin (Sigma, product code number C 3387), the carrier of immobilized papain is a mojor cost for DPNR production. To reduce the cost of DPNR production, local chitin should be used.

Moreover, an immobilized enzyme system has distinct advantage for industrial use. The major concern is the reactor. The author is suggesting that a basic reactor type which is a stirred tank reactor can be modified by using immobilized papain enclosed in mesh containers attached to the impeller blades (Figure 34) to give adequate agitation with minimal attrition.

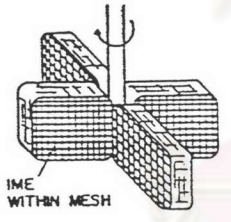


Figure 34 A stirrer which the immobilized papain is enclosed in mesh containers.

Since this research is based on a laboratory scale using 250 ml shake flask at 120 rpm,the proper speed of agitation should be investigated in a scale-up reactor to improve the efficiency of immobilized papain for latex deproteinization. Loss of enzyme activity due to latex coagulation should be prevented by suitable reactor design and slower agitation speed. In case of free papain, Koosakul (1994) could increase the deproteinization efficiency by using optimal agitation speed(60 rpm) of a two-flat blade paddle in a 41 cm reactor to obtain a decrease in total nitrogen to 0.07 g% within 50 min.