Optimization of Covalently Coupling Enzymes to Polymeric Membranes: EPR Studies of Papain

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SYNOPSIS

In an effort to better understand bioreactor systems, papain (EC 3.4.22.2) was covalently immobilized onto vinyl alcohol/vinyl butyral copolymer (PMB) membrane by means of glutaraldehyde (GA), 1,1'-carbonyldiimidazole (CDI), or 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP). Various kinetic and performance characteristics of the immobilized papain were evaluated. It was found that the characteristics of the membrane-bound papain depended on the immobilization methods. The CDI- and FMP-immobilized papain bioreactors showed better storage and thermal stability than did the GA-immobilized papain bioreactor, although the apparent Michaelis constant, K_m, of the GA-immobilized papain was closer to the free enzyme than to the corresponding CDI- and FMP-immobilized enzymes. In separate experiments, a 6-carbon spacer was inserted between the membrane surface and the covalently bound enzyme. It was found that the insertion of a spacer reduced the disturbance of the enzyme systems, resulting in K_m values intermediate between the free and directly bound enzymes for all three immobilization methods. Electron paramagnetic resonance spectroscopy was also used to investigate the conformational change and the active site structure of papain. It was found that the active site SH group of papain immobilized with a 6-carbon spacer had faster motion than that of directly bound enzyme, but slower motion than that of the free enzyme. With both direct-coupling and with a spacer, the SH group motion at the active site of papain by CDI and FMP immobilizations was similar, but slower than the corresponding GA immobilization. The conformational changes of the active site of papain upon immobilization with and without a spacer were in agreement with the functional properties of the enzyme. There was a good correlation between the motion of spin-labeled cysteine in the active site of papain and kinetic properties of this protease: As motion slowed, K_m increased and V_{max} decreased. Of the immobilization procedures used, GA immobilization with a spacer yielded kinetic and structural characteristics most similar to the free enzyme while providing increased stability and reusability relative to the latter. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

Considerable attention has been focused on the preparation of immobilized enzymes in the past decades, and many different kinds of support matrices and techniques for enzyme immobilization have been developed.[1-7] Immobilized enzyme systems can have a wide range of potential applications in enzyme engineering, among which are enzyme bioreactors and enzyme electrodes. Such systems have several advantages over the free enzyme including their ease of recoverability and reusability, operational simplicity, and thermal and storage stability.[1,4] Among the support matrices used, polymeric materials have been used extensively since they can have various functional groups and can be easily modified chemically.[4]

To advance knowledge about immobilized enzymes, greater understanding of the structure and function of the proteins upon immobilization is necessary. In this study, papain (EC 3.4.22.2) was co-

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valently immobilized onto the surface of a vinyl alcohol/vinyl butyral copolymer membrane, with or without a spacer, employing glutaraldehyde, 1,1'-carbonyldiimidazole, or 2-fluoro-1-methylpyridinium toluene-4-sulfonate. In this work, enzymatic activities and other properties of the immobilized papain and electron paramagnetic resonance (EPR) investigations of the influence of coupling chemistry on the conformational change and the active site structure of the immobilized papain are reported.

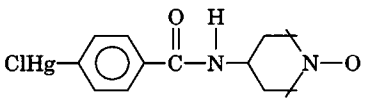
EXPERIMENTAL

Materials

Vinyl alcohol/vinyl butyral (in the ratio of 20:80) copolymer (PVB), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMC), and 6-aminocaproic acid (6-AA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Crude papaya latex, glutaraldehyde (GA) solution (25% v/v in water), triethylamine (TEA), 1,1'-carbonyldiimidazole (CDI), and 2-fluoro-1-methylpyridinium toluene-4-sulfonate (FMP) were purchased from Sigma Chemical Co. (St. Louis, MO). Bis(2-ethylhexyl) phthalate (DOP) was obtained from Fluka Chemie AG (Buchs, Switzerland), while casein was obtained from Eastman Kodak Co. (Rochester, NY). Other chemicals and solvents were reagent grade.

Enzyme Preparation and SL-PMB Synthesis

Papain was isolated and purified from crude papaya latex, and spin-labeled *p*-chloromercuribenzoate (SL-PMB) was synthesized as reported previously.[8] As described previously[8] and as shown below in Figure 11 (Results), this spin label reacts exclusively with the single SH group of papain. In this protease, this single SH group is located at the active site.[9,10]:



Membrane Preparation and Enzyme Immobilizations

The PVB membrane was prepared by dissolving 0.4 g of PVB powder and 0.5 mL of DOP in 8 mL tetrahydrofuran (THF) and cast onto a glass plate (44 cm^2) at room temperature. After 1.5 h for evaporation of THF, the membranes formed were soaked in deionized water for at least 2 h and, at most, overnight. A transparent membrane was obtained with a thickness of about 0.1 mm.

Immobilization of papain onto PVB membranes by GA was the same as reported previously.[11] Basically, for direct immobilization of papain to PVB membrane without a spacer, the procedure was carried out as follows: 5 mL of 5% GA solution was added to the membranes at room temperature and reacted for 24 h. Following this time, 3 mL of aqueous papain solution (2 mg/mL) was added and the mixture was incubated at 4°C for 48 h. The membranes were washed with deionized water to remove the unreacted papain and cut into 0.78 cm^2 circular pieces for further experiments. The immobilization of papain onto PVB membranes with a 6-carbon spacer was prepared via two steps: First, 0.596 g 6-AA dissolved in 5 mL deionized water was added to the membrane–GA system, incubated at room temperature for 24 h, and washed with deionized water. Then, 3 mL papain solution (2 mg/mL) was added to the membrane system and reacted for 1 h, and 0.2 g CMC in 3 mL 50 m*M* phosphate buffer was added. The mixture was incubated at 4°C for 48 h. Figure 1 shows the reaction schemes for the GA immobilization.

There are two processes involved in the enzyme immobilization by CDI.[12] The first requires the activation of the support to enable covalent binding

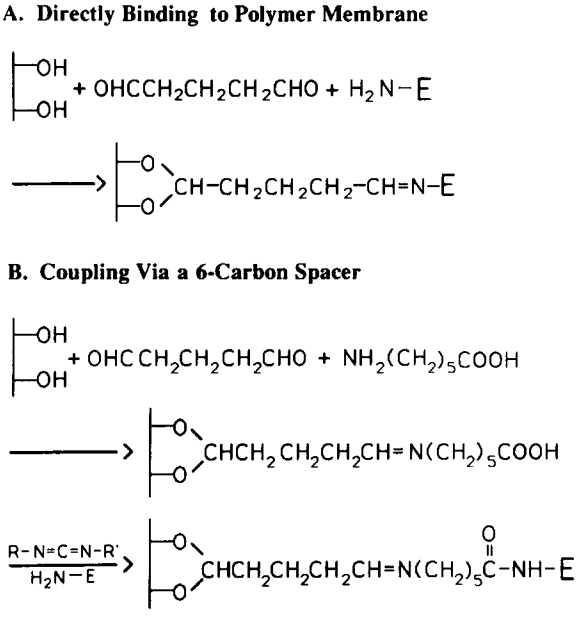


Figure 1 Reaction schemes for glutaraldehyde immobilization of an enzyme: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

of the enzyme. The second is the binding of the enzyme to the activated solid support. PVB membrane activation by CDI was carried out as follows: Dry PVB membrane was washed with 3 × 10 mL acetonitrile (dried over molecular sieves 4A). Then, 0.30 g CDI in 10 mL acetonitrile was added to the membrane system and reacted at room temperature for 2 h. The resulting adduct was then washed sequentially with a decreasing amount of acetonitrile in water (75 : 25, 50 : 50, 25 : 75 v/v acetonitrile: water) and, finally, with water and used immediately.

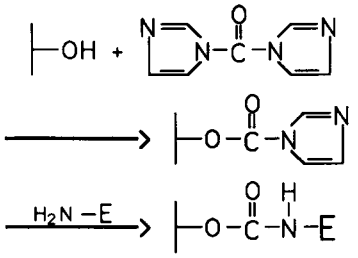
For direct coupling without a spacer, 6.0 mg papain in 7.5 mL 100 m*M* phosphate buffer, pH 7.0, was added to the CDI-activated membrane and incubated at 4°C for 24 h. The membrane was then washed to remove the unreacted papain and cut into round pieces of 1 cm diameter for further experiments. For coupling with a 6-carbon spacer, 0.596 g 6-AA in 7.5 mL of 100 m*M* phosphate buffer, pH 7.0, was added to the activated membrane, reacted at 4°C overnight, and washed with phosphate buffer. Then, the membrane was treated at 4°C with 6.0 mg papain and 0.2 g CMC in 10 mL of 50 m*M* phosphate buffer, pH 7.0, and maintained at 4°C for 24 h. The reaction schemes for CDI immobilization are presented in Figure 2.

The activation of PVB membrane by FMP was carried out as follows[13]: Dry PVB membrane was washed three times with acetonitrile (dried over molecular sieves 4A). Then, 0.20 g FMP and 0.20 mL TEA in 10 mL acetonitrile was added to the membrane and incubated at room temperature for 1 h. The resulting adduct was washed with a decreasing amount of acetonitrile in water (75 : 25, 50 : 50, 25 : 75 v/v acetonitrile:water) and then with deionized water and used immediately. TEA was added as base to neutralize the liberated acid.

For direct coupling without a spacer, 6.0 mg papain in 7.5 mL 100 m*M* phosphate buffer, pH 8.0, was added to FMP-activated membrane and reacted at 4°C for 24 h. The membrane was then washed with 100 m*M* phosphate buffer, pH 7.5, containing 150 m*M* NaCl. After that, the membrane was treated with 10 mL 100 m*M* Tris buffer, pH 8.0, for 1 h to deactivate any unreacted activated hydroxyl groups.

For coupling with a 6-carbon spacer, 0.596 g 6-AA dissolved in 10 mL 100 m*M* phosphate buffer, pH 8.0, was added to the activated membrane, incubated at 4°C for 24 h, and washed with 100 m*M* phosphate buffer, pH 7.5, containing 150 m*M* NaCl. The resulting membrane was then treated with 10 mL 100 m*M* Tris buffer, pH 8.0, for 1 h. Three milliliters of papain solution (2.0 mg/mL) was then

A. Directly Binding to Polymer Membrane



B. Coupling Via a 6-Carbon Spacer

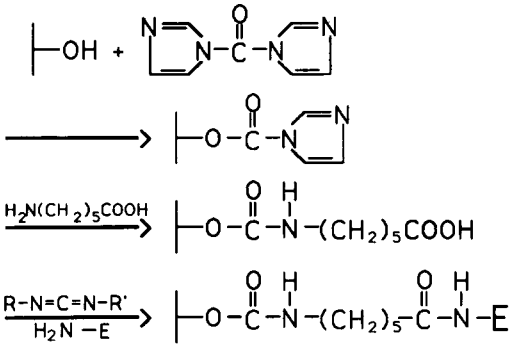


Figure 2 Reaction schemes for carbonylimmidazole immobilization of an enzyme: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

added to the membrane; after 1 h, 0.2 g CMC in 4.5 mL 50 m*M* phosphate buffer, pH 7.0, was added and incubated at 4°C for 24 h. Figure 3 gives the reaction schemes for FMP immobilization.

As shown in Figures 1–3, the nature of the chemistry involved in coupling the protein to the membrane indicates that the distance between the membrane surface and papain is shortest for the FMP method and longest for the GA method.

Enzyme Loading and Activity Determination

The amount of protein bound onto PVB membranes was determined by the Lowry method[14] indirectly from the difference between the initial total protein exposed to the membrane and the amount of protein recovered in the washes. Various parameters were used to characterize the membrane-immobilized enzyme system. According to Imai et al.,[3]

$$\text{Immobilization yield (\%)} = \frac{(A - B)}{A} \times 100 \quad (1)$$

$$\text{Activity yield (\%)} = \frac{C}{A} \times 100 \quad (2)$$

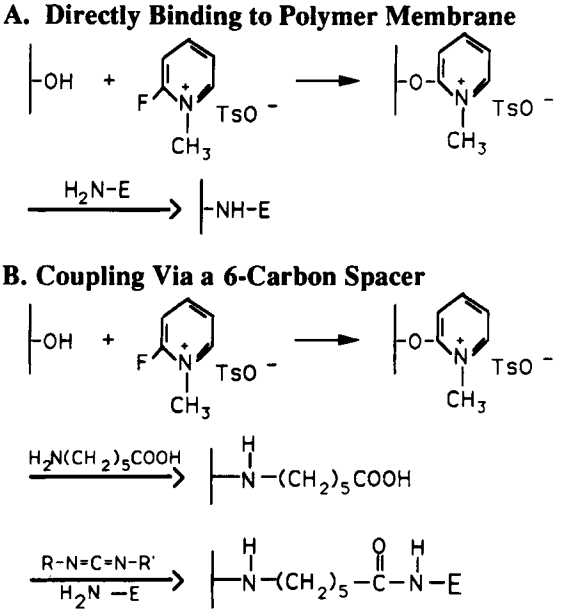


Figure 3 Reaction schemes for FMP immobilization of an enzyme: (A) direct binding to the membrane; (B) binding to the membrane via 6-carbon spacer.

$$\text{Activity ratio (\%)} = \frac{C}{(A - B)} \times 100 \quad (3)$$

where A is the total activity of enzyme added in the initial immobilization solution; B, the activity of the residual enzyme in the immobilization and washing solutions after the immobilization procedure; and C, the activity of the immobilized enzyme.

The proteolytic activity of the membrane-immobilized and free papain, which was used to compare the characteristics of the enzyme between the two states, was determined by the trichloroacetic acid precipitate method.[8] Casein was used as the substrate as previously described.[8] The reaction with the free enzyme was carried out in the mixture of 0.1 mL enzyme solution containing a given amount of enzyme, 0.54 mL of 0.5% casein solution, and 0.86 mL of 50 mM Tris buffer (pH 8.0). The reaction with the immobilized enzyme was carried out in the presence of 3×0.78 cm^2 pieces of membrane. After incubating the reaction mixture under shaking for 5 min at 37°C, the reaction was stopped by adding 5% trichloroacetic acid. The precipitate was removed by centrifugation and the absorbance of the supernatant read at 280 nm was used to calculate the activity of papain based on calibration curves. The molecular weight of casein was taken to be 30,000. Each property of the membrane-immobilized papain was studied by using at least two different membrane preparations, but most often three different preparations were used.

The effect of pH on the activity of papain was studied in solutions of various pH at 37°C. The pH buffers used were 50 mM acetate buffer for pH 3–6, 50 mM phosphate buffer for pH 6–8, and 50 mM Tris buffer for pH 8–10. The activity of papain in acetate or phosphate buffers at pH 6.0 or in phosphate or Tris buffers at pH 8.0 were, respectively, identical, implying that the nature of the buffer had no effect on the activity of this protease.

The effect of temperature on the enzymatic activity of papain was studied at various temperatures. The reaction mixtures minus casein were maintained at the desired temperature for 10 min to reach equilibrium. The casein solution, also at the desired temperature, was then added to the papain solution.

The operational stability of the immobilized papain was performed with a batch system. Six 0.78 cm^2 pieces of papain–PVB membranes were added and the reaction mixture was incubated for 10 min at 37°C. The membrane pieces were then removed and washed. These same membrane pieces were put into another reaction mixture and the activity was determined by the method described above. The above procedure was repeated 10 times.

Enzyme Spin-Labeling and EPR Measurements

Papain was spin-labeled exclusively at the active site with the SH-specific spin label SL-PMB according to the method described earlier.[8] Spin-labeled papain was then immobilized onto the PVB membranes by the methods used in this study. The EPR spectra were recorded on a Bruker ESP 300 spectrometer with a rectangular cavity at room temperature. Typical EPR parameters were the following: microwave frequency 9.7 GHz, microwave power 20 mW, modulation frequency 100 kHz, and modulation amplitude 0.32 G. For the immobilized papain samples, 100–200 spectra (5 s scan time) were accumulated by the computer-controlled spectrometer.

RESULTS

Immobilization of Papain

Effects of the amount of the added enzyme to the PVB membrane and of the time and pH of the coupling reaction in the immobilization on the resulting activity obtained were investigated first. The best conditions to obtain a high enzymatic activity with

economic usage of time and enzyme were those given in the Experimental section.

Various properties of the membrane-immobilized papain were compared with those of the free enzyme using casein as a substrate. Some characteristics of the immobilized papain under the experiment conditions used are summarized in Table I. The results in Table I indicate that papain is most extensively bound to the FMP-activated membrane, and least, to the GA-modified one. The enzyme loading by the FMP method is almost double of that by the GA method and is about 25% greater than the CDI method, whether using a spacer or not. But the FMP immobilization of papain resulted in the lowest activity ratio, while the GA immobilization had the highest activity ratio, probably due to the interactions of the enzyme with the membrane surface, since the distance between the enzyme and membrane surface is largest for the GA method and smallest for the FMP method (see Figs. 1–3). However, the effect of enzyme loading on the kinetic properties of immobilized enzyme could not be ruled out. Since the enzyme loadings by CDI and FMP immobilizations were higher than the corresponding GA method, the interaction between the enzyme molecules might decrease their enzymatic activity.

The activity yield for papain immobilized by the FMP method was about 60% larger with a spacer and 30% larger without a spacer than that of papain immobilized by the GA method, presumably because more protein was bound by the former procedure (see enzyme loading, Table I). Also, with all three immobilization methods, the activity yield and the activity ratio for the membrane-immobilized enzyme with a spacer were higher than those without a spacer, although the immobilization yield of the directly bound papain was higher. This last result suggests that direct immobilization leads to more protein bound to the membrane, but less of the enzyme is active than is the case for spacer-linked enzyme.

The catalytic properties of many enzymes can be described by Michaelis–Menten kinetics. The dependence of the reaction rate of papain on casein concentrations was studied in this research. The plot of the rate vs. the substrate concentration is similar to that obtained in Michaelis–Menten kinetics. Values of K_m and V_{max} obtained from the Lineweaver–Burk double-reciprocal plots of papain in solution and immobilized onto PVB membranes are summarized in Table II. Smaller K_m values reflect greater affinity of the substrate for papain. The K_m for free papain is the smallest determined.

The kinetic parameters for the membrane-immobilized papain depend on the immobilization method. It can be seen from Table II that for each immobilization procedure the value of K_m for the membrane-immobilized papain without a spacer is larger than that for the enzyme immobilized with a spacer. Also, the value of V_{max} is smallest for the directly immobilized enzyme. With both parameters, intermediate values between the free enzyme and directly immobilized papain were obtained for the protease immobilized via a 6-carbon spacer. For the GA immobilization, there is a large change in these kinetic parameters when comparing papain linked by a 6-carbon spacer to the directly immobilized papain. The Michaelis constants for the CDI- and FMP-immobilized papain are similar, and both are larger than that by the GA immobilization. This result suggests that of all the systems studied GA immobilization via a 6-carbon spacer leads to a system that most resembles the free enzyme with respect to kinetic properties.

Table I Characteristics of Membrane-Immobilized Papain by GA, CDI, and FMP Methods[a]

	Enzyme Loading ($\mu g/cm^2$)	Immobilization Yield (%)	Activity Yield (%)	Activity Ratio (%)
GA				
Via 6-carbon spacer	13.6 ± 0.6	10.0 ± 0.5	6.0 ± 0.3	60.0 ± 3.6
Directly bound	18.0 ± 0.5	13.2 ± 0.4	5.0 ± 0.2	37.3 ± 2.5
CDI				
Via 6-carbon spacer	20.5 ± 1.2	15.1 ± 0.9	7.8 ± 0.3	52.0 ± 1.7
Directly bound	27.7 ± 1.3	20.4 ± 1.0	6.5 ± 0.2	31.7 ± 1.5
FMP				
Via 6-carbon spacer	26.6 ± 1.6	19.6 ± 1.2	9.5 ± 0.8	48.3 ± 1.5
Directly bound	34.2 ± 1.7	25.2 ± 1.5	6.9 ± 0.6	27.5 ± 0.9

[a] Results present represent the mean and standard deviation of three different membrane preparations.

Effects of pH and Temperature on the Enzymatic Activity of Membrane-Immobilized Papain

The effects of variation in pH and temperature on the activity of membrane-immobilized papain were studied, and the results were compared with those for the free enzyme. Figure 4(A) shows the pH dependence of the enzymatic activity of free and directly bound papain by all three methods, and Figure 4(B) gives the data for free and membrane-immobilized enzyme with a 6-carbon spacer. As shown in Figure 4, the optimum pH value of reaction is about 7.0 under the experimental conditions for all cases, probably reflecting the ionization of histidine 159 in the active site.[9,10] The rise in activity in all cases around pH 4 may reflect the ionization of aspartic acid also in the active site cleft of papain.[9,10] However, at the extrema pH values, papain immobilized without a spacer by the CDI and FMP methods maintains the highest relative activity, while the free papain maintains the lowest. This result may reflect changes in the ionization or conformation of the key amino acid residues around the active site of papain upon immobilization. At pH 4, there is a relatively larger difference in the relative activity of papain directly immobilized by the CDI and FMP procedures compared to the spacer-linked enzyme than is the case for GA immobilization [comparing Figs. 4(A) and (B)]. In the latter case, behavior closer to the free enzyme is observed.

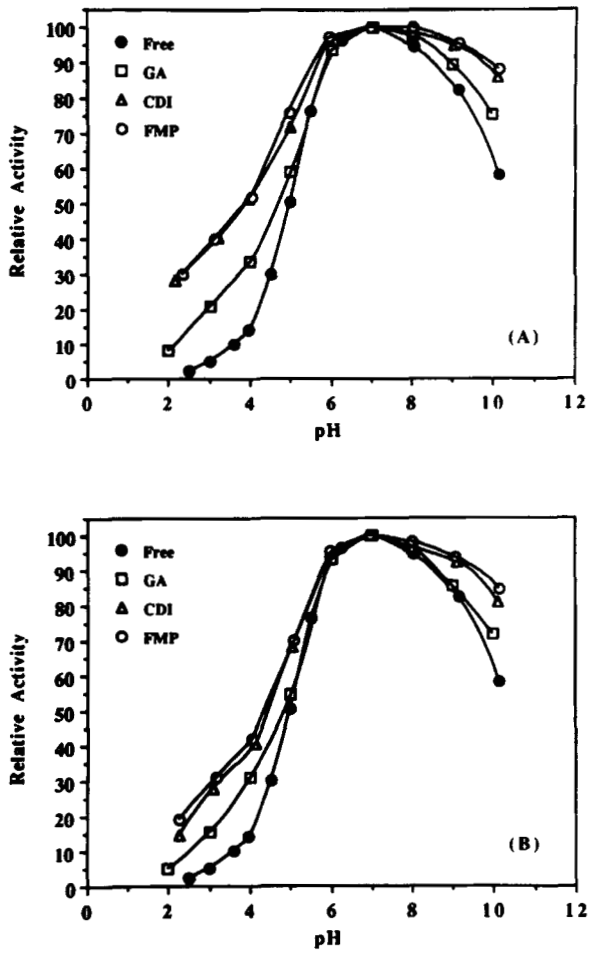


Figure 4 pH dependence of the enzymatic activity of free and membrane-immobilized papain. The activity assay was carried out at 37°C: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

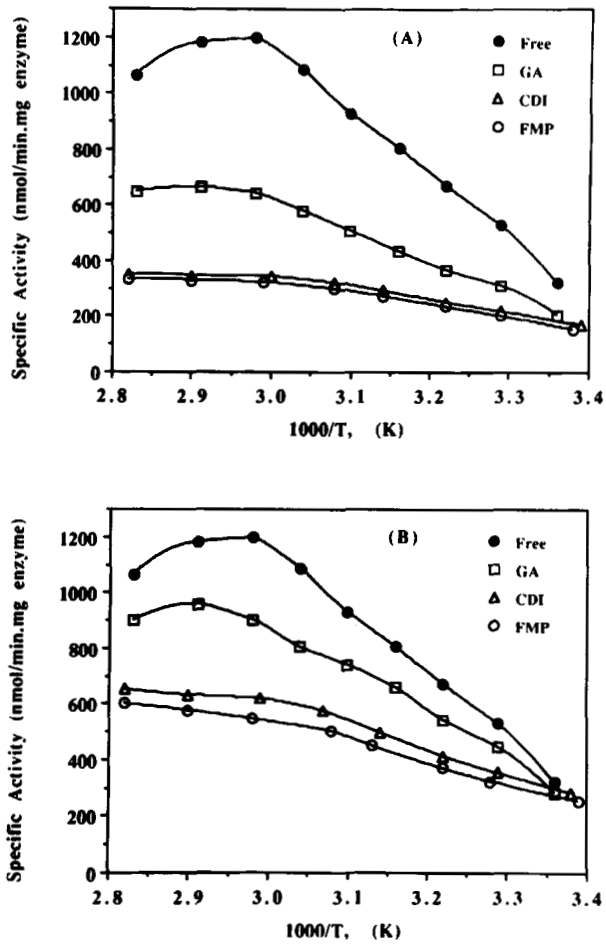


Figure 5 Temperature dependence of the enzyme activity of free and membrane-immobilized papain. Activity was measured in 50 m*M* Tris buffer: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

The temperature dependence of the specific activity of the membrane-immobilized papain was compared with that of the free enzyme (Fig. 5). The reactions were carried out at different temperatures

Table II Kinetic Parameters of Free and Immobilized Papain by GA, CDI, and FMP Methods[a]

	K_m (μM)	V_{max} (μmol/min mg enzyme)	E_a (kJ/mol)
Solution	26.3 ± 0.7	0.992 ± 0.024	30.1 ± 0.8
GA			
Via 6-carbon spacer	37.4 ± 1.9	0.631 ± 0.033	26.3 ± 1.5
Directly bound	79.0 ± 3.4	0.412 ± 0.025	24.8 ± 1.1
CDI			
Via 6-carbon spacer	70.9 ± 2.7	0.556 ± 0.029	21.4 ± 1.0
Directly bound	98.5 ± 5.2	0.334 ± 0.013	17.2 ± 0.9
FMP			
Via 6-carbon spacer	73.3 ± 4.8	0.415 ± 0.017	18.3 ± 1.0
Directly bound	99.8 ± 5.8	0.292 ± 0.007	15.8 ± 0.6

[a] Results present represent the mean and standard deviation of three different membrane preparations.

and pH 8.0. The temperature dependence for the free papain is sharper than for the membrane-immobilized enzyme. Temperature has the smaller effect on activity of papain immobilized by the CDI and FMP methods: At higher temperatures, the activity of papain immobilized onto PVB membrane by the CDI and FMP methods remains unchanged. The values of the activation energy, E_a, determined from the linear portion of the Arrhenius plots (not shown), are summarized in Table II and indicate that upon immobilization the activation energy of papain is reduced. The value of E_a for GA immobilization using a spacer most closely resembles that of the free enzyme (Table II).

Stability of Membrane-Immobilized Papain

Papain in solution has been reported to be remarkably stable under various conditions.[15] The stability of the enzyme is, however, affected by several other factors, e.g., temperature and denaturants. We wondered how the stability of papain would be affected by immobilization to a PVB membrane. The thermal stability of the enzyme was examined by heating the free and immobilized papain (in 50 mM Tris buffer, pH 8.0) at 80°C. Samples were withdrawn after different incubation times and the residual activity of papain was determined at pH 8.0 and 37°C. Figure 6(A) shows the results of free and directly bound enzyme by the GA, CDI, and FMP methods and Figure 6(B) gives the results for free and membrane-immobilized enzyme with a 6-carbon spacer. The results presented in Figure 6 show that the thermal stability of papain was improved by immobilization. The membrane-immobilized papain is more stable

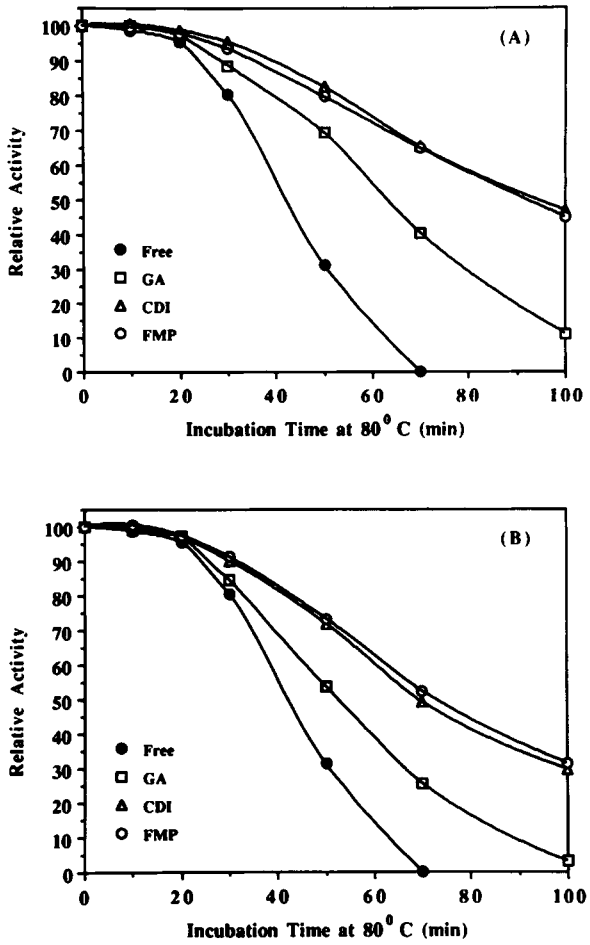


Figure 6 Time dependence of thermal stability (as determined by relative enzymatic activity) of free and membrane-immobilized papain. Samples were incubated in 50 mM Tris buffer (pH 8.0) and 80°C for various time periods: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

than is the free papain, and the enzyme immobilized by the CDI and FMP methods behave similarly and is more stable than that by the GA method. In all cases, directly bound enzyme without a spacer was more stable to temperature. This observation may be due to the difficulty of changing the resulting enzyme conformation after binding papain onto the membrane surface,[3] a possibility consistent with the modest temperature dependence of enzymatic activity of papain immobilized to the PVB membrane by the CDI and FMP methods (Figs. 5 and 6).

The storage stability of the immobilized membrane preparation was measured by storing the free and the membrane-immobilized papain in 50 m*M* Tris buffer, pH 8.0, at room temperature for different periods of time. The residual activity was measured at these different storage times. Figure 7(A) shows the results of free and directly bound enzyme by GA, CDI, and FMP methods, and Figure 7(B) gives the results for free and membrane-immobilized enzyme with a 6-carbon spacer. The relative activity of the free papain fell sharply to zero in less than 1 week, whereas the GA-immobilized papain with and without a spacer remains 50 and 70% active, respectively, after 2 week's storage. For CDI-immobilized papain with and without a spacer, activity remains at 75 and 85%, respectively, after 2 weeks, and the results for the FMP immobilization are close to those for CDI immobilization. Again, the enzyme immobilized by the CDI and FMP methods shows better stability than that immobilized by the GA method or for the free enzyme. Also, when the immobilized enzyme was stored in buffer solution, no active enzyme could be detected, indicating that once bound no enzyme is leached from the membrane. This result was found for all three immobilization methods.

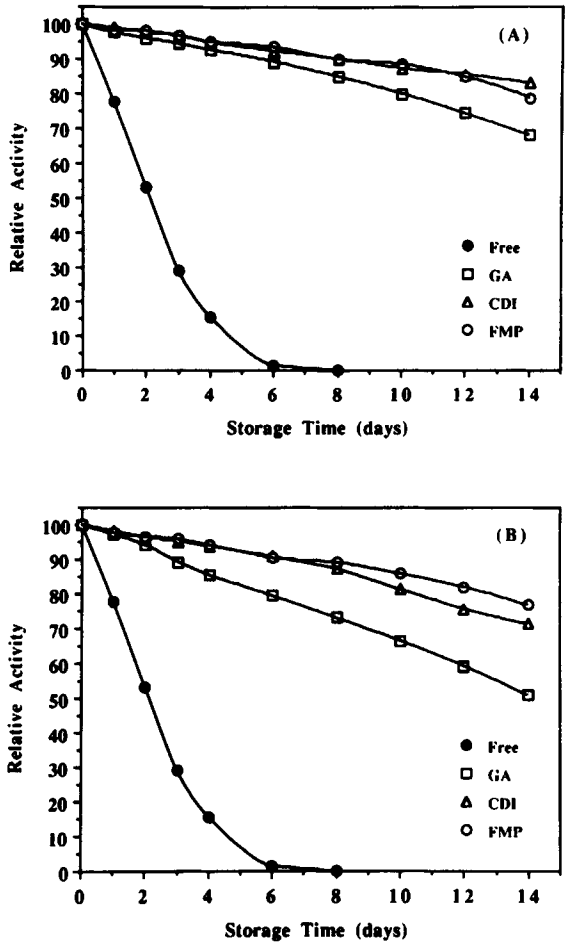


Figure 7 Dependence of relative activity of free and membrane-immobilized papain during storage in 50 m*M* Tris buffer (pH 8.0) at room temperature: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

The stability of the free and the membrane-immobilized papain to denaturants was studied by measuring the residual activities of the enzyme kept in the solutions with different concentrations of denaturants at room temperature for 2 h before the activity measurement. The results for urea are shown in Figure 8(A) for free and directly bound enzyme and in Figure 8(B) for free and membrane-immobilized enzyme with a 6-carbon spacer. The results for guanidine hydrochloride are shown in Figure 9(A) for free and directly bound enzyme and in Figure 9(B) for free and membrane-immobilized enzyme with a 6-carbon spacer. Somewhat enhanced stability toward these two denaturants has been obtained by immobilization. Papain immobilized by the CDI and FMP methods appears to be slightly more stable than that immobilized by the GA method. Further, there is a hint that papain bound by direct immobilization has greater resistance to either denaturant than in the case of immobilization with a spacer, although this hint is only a suggestion. In addition, it should be noted that the stronger denaturing effect of guanidine hydrochloride and the relative resistance to urea denaturation of the membrane-immobilized papain parallels the same results found for papain in solution.[8]

One of the potential advantages of immobilized enzyme over free enzyme is repeated usage. The reusability of the membrane-immobilized papain is shown in Figure 10(A) for directly bound enzyme and in Figure 10(B) for membrane-immobilized enzyme with a 6-carbon spacer. The relative activities of GA-, CDI-, and FMP-immobilized papain under the experimental conditions used decreased, respec-

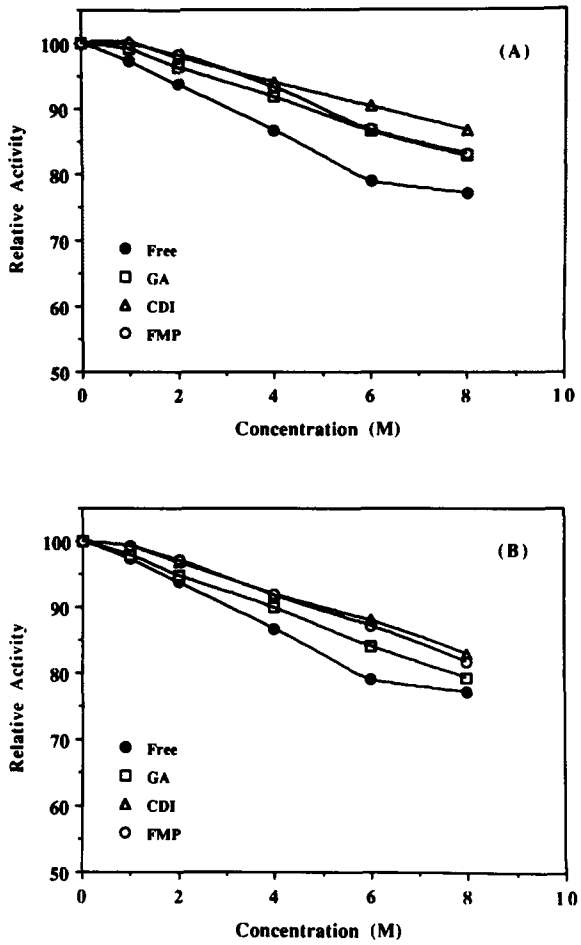


Figure 8 Stability of free and membrane-immobilized papain toward urea. Activity was measured at pH 8.0 and 37°C: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

tively, to about 90, 95, and 93% initially with repeated cycles and then were almost kept constant, suggesting that the membrane bioreactor is highly functional over at least 10 cycles. The results here suggest that papain immobilized by the CDI and FMP methods may be slightly more stable than that immobilized by the GA method.

EPR Measurements

To obtain a better understanding of the effect of immobilization on the activity of the enzyme, it is necessary to study the active site structure of the immobilized papain. Electron paramagnetic resonance (EPR) spectroscopy has been used to study biological systems (reviewed in Refs. 16–18), including proteins.[19] EPR has many advantags over other methods of examining the structure of biological samples including its extreme sensitivity and the influence of motion and polarity on the spectra.[18,20] Clark et al.[21,22] successfully applied the spin-label EPR technique to enzymes immobilized to polymer beads. More recently, Asakura et al.[23] used EPR methods to study an enzyme entrapped rather than covalently bound in a silk fibroin membrane.

In this study, EPR spectroscopy has been used to study how the active site structure of the enzyme depends on the immobilization method employed. As reported earlier,[8] SL–PMB binds exclusively to the single SH group of papain. This SH group is located in the active site of this protease.[9,10] This exclusivity of SL–PMB binding was demonstrated by the absence of an EPR spectrum of papain that had been previously treated with *p*-chloromercuri-

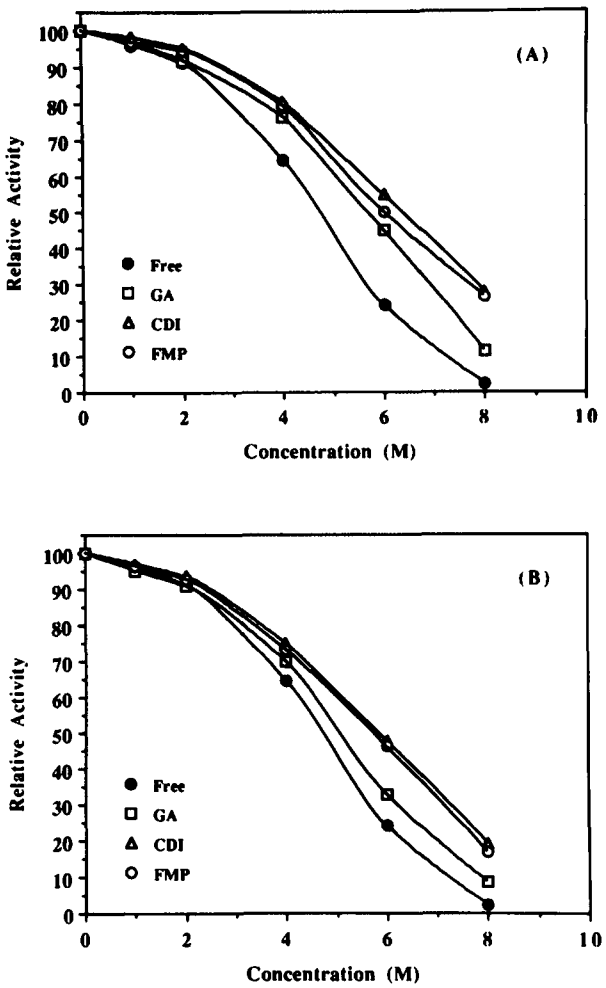


Figure 9 Stability of free and membrane-immobilized papain toward guanidine hydrochloride. Activity was measured at pH 8.0 and 37°C: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

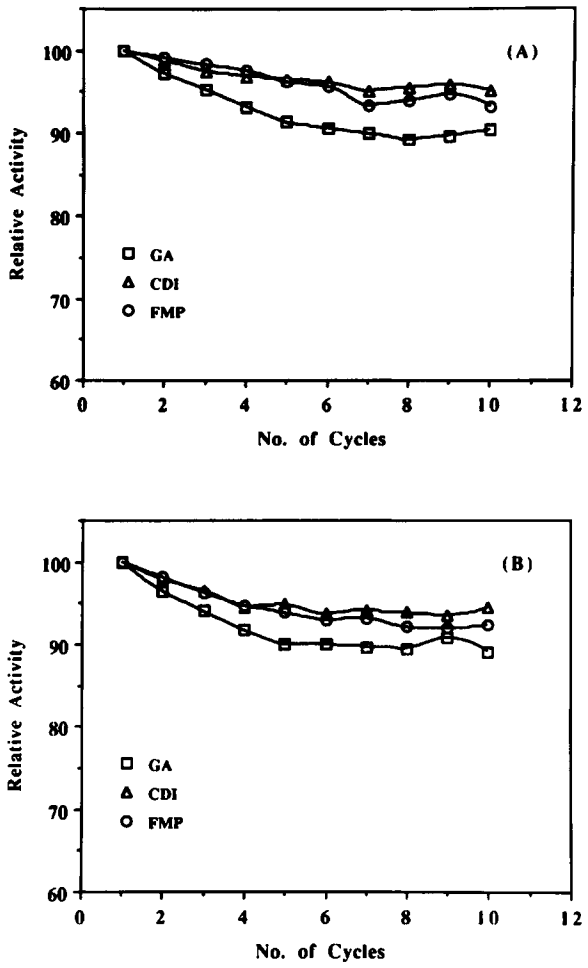


Figure 10 Reusability of PVB membrane-immobilized papain. Relative enzyme activity was measured at pH 8.0 and 37°C: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

benzoate, a reagent that reacts only with SH groups (Fig. 11).

The EPR spectra for the spin-labeled, membrane-immobilized papain without a spacer are shown in Figure 12(A) and those with a 6-carbon spacer are shown in Figure 12(B). The three-line pattern of the EPR spectrum is due to the hyperfine interaction between the nitroxyl electron magnetic moment and the magnetic moment of the ^{14}N nucleus in the nitroxyl group. The EPR spectra of the spin-labeled papain immobilized via a 6-carbon spacer show a characteristic asymmetric broadening of the high-field line with a loss in amplitude due to the influence of the protein structure on the rotational motion of the attached spin label.

The EPR spectrum of the spin-labeled papain immobilized by the GA method with a spacer also shows that the peak-to-peak amplitude of the low-field line is higher than that of the middle-field line, similar to that of free papain (Fig. 11). Such unusual EPR spectra are probably due to the rapid anisotropic motion of the piperidine moiety of the spin-labeled molecule with respect to the enzyme and the benzoic acid ring.[8,11,24,25] The EPR spectra of the spin-labeled papain immobilized by the CDI and FMP methods with a spacer shows the disappearance of such an unusual spectrum [Fig. 12(B)], indicating that the active site structure of papain is different in these cases relative to GA immobilization using a spacer.

These EPR spectra can be characterized by the relative height ratio of the high-field line H to the middle-field line M. As described previously,[8] Berliner[26] used the parameter $(M/H)^{1/2}$ to indicate the change in the molecular motional rate of the spin label. As spin-label motion increases, $(M/H)^{1/2}$ decreases. As shown in Figure 12, immobilization of the protease has a large effect on the motional freedom of the spin label bound to the active site of papain. For the membrane-immobilized papain with a 6-carbon spacer, the EPR spectrum shows a slower motion of the spin label bound to the single SH group than that for the spin label bound to the single SH group of free papain (Fig. 11), as indicated by the increased value of $(M/H)^{1/2}$. For the directly membrane-immobilized papain labeled with SL–PMB, the most striking characteristic of the EPR spectra is that the spectrum changes to a rigid powder pattern. The lineshape of these kinds of spectra reflects severely restricted spin label mobility.[16–18,20] Comparison of Figures 11 and 12 suggests that spin label bound to immobilized papain with a 6-carbon spacer has slower motion than that bound to free papain, but faster motion than the spin label bound in the active site of the directly bound enzyme. Clearly, the conformation of the active site of papain is different when immobilized by these methods. As judged by EPR, relative to CDI and FMP methods, the active site structure of GA-immobilized papain using a spacer is closer to that of the free enzyme, similar to the kinetic findings (Table II).

The spectra for SL–PMB-labeled papain directly immobilized to the PVB membrane by the CDI and FMP methods are similar, but show a greater separation of outer hyperfine extrema than does the corresponding spectrum of GA-immobilized papain. Greater extrema separation reflects slower motion of the spin label. A role for increased polarity of the spin-label microenvironment for the CDI-direct immobilized case, which would have the effect of increasing extrema separation,[16–18] cannot be ex-

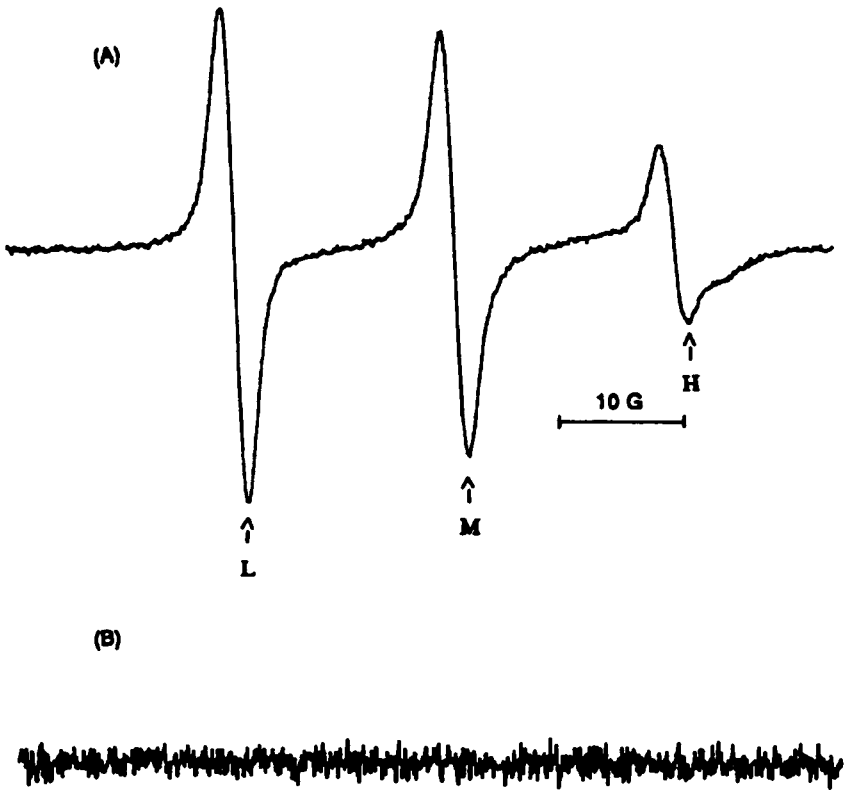


Figure 11 EPR spectra of SL–PMB-labeled papain in 50 m*M* phosphate buffer, pH 7.0: (A) untreated with PMB; (B) after prior PMB treatment. L, M, and H refer to the low-, middle-, and high-field EPR lines, respectively.

cluded. However, given that the spin label is bound to the active site of papain and is, thus, already exposed to buffer, this possibility seems less likely than does a motional-decreased basis of the increased extrema separation.

In the powder spectra of Figure 12(A), it is difficult to accurately calculate the M/H ratio, whereas in Figure 12(B), more confidence can be placed in the calculations. Nevertheless, this parameter provides a reasonably good correlation of molecular motion at the active site of papain with its kinetic properties as a function of coupling chemistry (Fig. 13). As motion of the single cysteine in the active site of papain decreases [$(M/H)^{1/2}$ increases], K_m increases and V_{max} decreases. These correlations are nearly linear (Fig. 13).

DISCUSSION

In this report, the kinetic and structural properties of PVB membrane–papain systems were investigated. GA, CDI, and FMP were used as the immobilization chemistry to form a stable linkage between papain and the PVB membranes. Specifically, the immobilized papain is more stable than is the free enzyme under a number of experimental conditions. Especially good membrane storage stability and reusability, which are considered to be advantageous for continuous operations, were noted. The papain–PVB membrane retained at least about 90% of its original activity after 10 batch reactions. However, the Michaelis constant of the immobilized enzyme was higher than that of the free enzyme. The results of this study also suggested that CDI and FMP immobilizations yielded a bioreactor of greater stability than those produced by GA immobilization, but of less specific activity. Indeed, the kinetic and structural features of free papain are most nearly mimicked using the GA-immobilization method and employing a spacer.

EPR was used to investigate the effect of immobilization on the structure of papain and to use this information to better understand the kinetic behavior of papain and properties of the bioreactor. As noted above, the CDI and FMP directly bound and spacer-linked enzyme has a different structure of the active site from those of GA-immobilized pa-

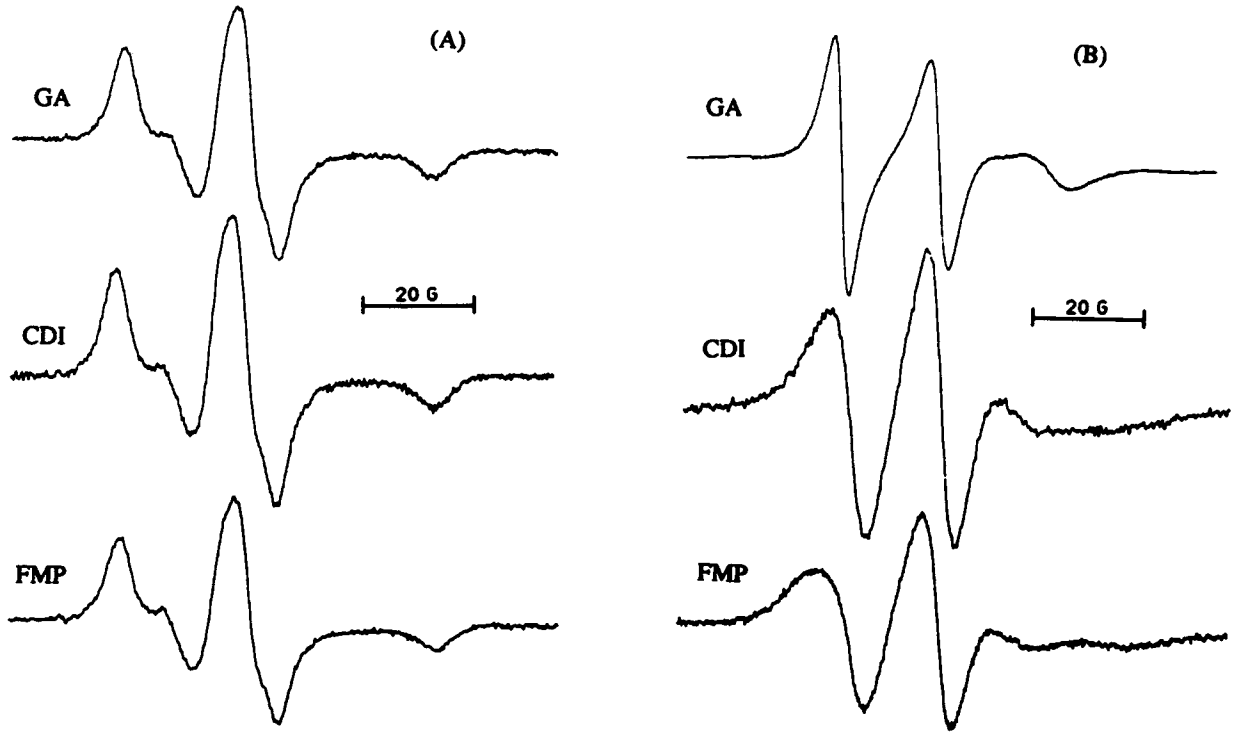


Figure 12 EPR spectra of SL-PMB-labeled papain at room temperature: (A) direct binding to the membrane; (B) binding to the membrane via a 6-carbon spacer.

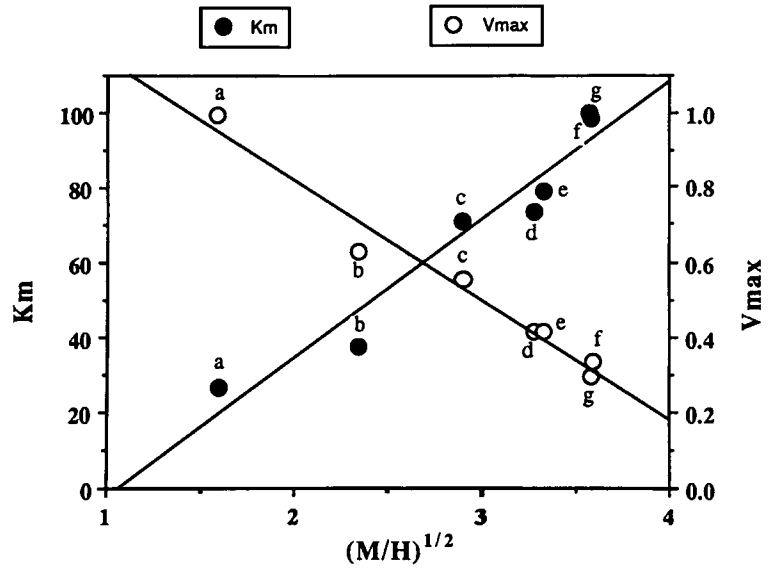


Figure 13 Correlation between the motion of SL-PMB covalently bound to the single cysteine residue of papain located in the active site of the enzyme $(M/H)^{1/2}$ with (●) K_m and (○) V_{max} as a function of coupling chemistry and use of a spacer: a, free enzyme; b, GA with a spacer; c, CDI with a spacer; d, FMP with a spacer; e, GA directly bound; f, CDI directly bound; g, FMP directly bound. Correlation coefficients: K_m vs. $(M/H)^{1/2}$ = 0.933; V_{max} vs. $(M/H)^{1/2}$ = −0.971.

pain as judged by the EPR spectra. For the bound papain with a spacer, the label is apparently undergoing isotropic motion, albeit slowly [$(M/H)^{1/2}$ is greater], in the former cases in contrast to an apparently anisotropically oriented label in the latter case. Also, for the directly immobilized enzyme, EPR spectra for CDI and FMP immobilizations give similar outer hyperfine separations, but the separation is greater than that of the GA immobilization. These results suggest that the active site structure of papain under CDI and FMP immobilizations are very close, but different from that of GA immobilization, consistent with the kinetic and performance properties of these systems. EPR spectra of SL–PMB-labeled papain indicate that the active site conformation of papain depends on immobilization conditions. CDI and FMP immobilizations, with or without a spacer, lead to systems with slower motion of the active site SH group than was the case of GA immobilization. In addition, the EPR spectra of spin-labeled papain directly bound to the PVB membrane indicate that the spin label is highly immobilized, suggesting a less spacious active site conformation, whereas introduction of a 6-carbon spacer increases the motion of SL–PMB spin label at the active site of papain.

Consistent with these findings, introducing a spacer between the polymer support and the enzyme improves the retention of the activity of the bound enzyme. The insertion of a spacer between the support and enzyme reduces the disturbance for substrate–enzyme complex formation since the enzyme bound with a spacer is further separated from the membrane surface and may have a higher mobility.[27] Also consistent with the EPR results, the spacer has a large effect on apparent K_m values of immobilized papain. The K_m value of directly bound papain by the GA method (79.0 μM) was three times higher than that of the free enzyme, but the K_m value of the papain bound by the GA method via a 6-carbon spacer was only 1.4 times higher than free papain. With the CDI- and FMP-immobilized enzyme, K_m of the spacer-linked system was not as close to the free enzyme; rather, the K_m value was closer to that of the directly immobilized enzyme. (The distance between the enzyme and the polymeric membrane, even with a spacer, was shorter with CDI and FMP immobilizations than with GA immobilization with or without a spacer, respectively.) This trend of the dependence of K_m on the immobilization condition may be due to conformational changes of enzyme because of the binding itself or this trend may reflect diffusion limitations. Since the molecular size of casein is relatively large, this polypeptide is difficult to diffuse onto the membrane, and the concentrations of casein on the membrane surface and in the bulk phase are likely not identical. This putative diffusion limitation could lead to the increased apparent K_m and the apparent temperature independence of reaction rate at higher temperatures.[28] The conformation change of the active site of papain upon immobilization may also play an important role. As detected by EPR, immobilization of papain onto the PVB membrane resulted in a more constricted active site conformation than for the free enzyme or papain immobilized via the 6-carbon spacer. Alteration of this active site structure is consistent with the higher values of Michaelis constant K_m for the directly immobilized enzymes.

The results presented here indicated that the PVB membrane could be used as a satisfactory support for the immobilization of papain. The activity retention by the immobilized enzyme can be improved by the insertion of a spacer between support and enzyme. This study also showed that the properties of the bound enzyme were related to the structure of the bound enzyme. The behavior of the membrane-immobilized papain can be explained by the active-site conformation of papain. Our results could be helpful in providing insight into a membrane-immobilized enzyme system design with optimal properties, and of the conditions employed in this study, GA immobilization with a spacer gave the best mix of enhanced stability/reusability with minimal confomation alterations of the enzyme active site.

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