Protease Immobilization onto Porous Chitosan Beads

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SYNOPSIS

Water-insoluble proteases were prepared by immobilizing papain, ficin, and bromelain onto the surface of porous chitosan beads with any length of spacer by covalently fixation. The activity of the immobilized proteases was found to be still high toward small ester substrate, *N*-benzyl-L-arginine ethyl ester (BAEE), but rather low toward casein, a high-molecular-weight substrate. The relative activity of the immobilized proteases with spacer gave an almost constant value for the substrate hydrolysis within the surface concentration region studied. The values of the Michaelis constant K_m and the maximum reaction velocity V_m for free and immobilized proteases on the porous chitosan beads are estimated. The apparent K_m values were larger for immobilized proteases than for the free ones, while V_m values were smaller for the immobilized proteases. The pH, thermal, and storage stability of the immobilized proteases were higher than those of the free ones. The initial enzymatic activity of the immobilized protease maintained almost unchanged without any elimination and inactivation of proteases, when the batch enzyme reaction was performed repeatedly, indicating the excellent durability.

INTRODUCTION

Since the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization,[1] which offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture, and adaptability to various engineering designs.[2-4] A concerted or sequential reaction of several enzymes is also obtainable by the use of mixed or stratified beds. Furthermore, the interest in the immobilized enzymes and their application to bioprocessing,[5,6] analytical system,[7] and enzyme therapy[8] has steadily grown in the past decade. Thus, many approaches to the preparation of water-insoluble enzymes have been explored in recent years[9-12] to study the enzyme reaction in biphasic systems similar to those existing *in vivo*.

However, effects of polymer supports on the activity of enzymes have not been studied in detail until now. In this study, papain, ficin, and bromelain are selected as hydrolytic enzymes and the polymer support employed is porous chitosan beads (ACW),[13] which have very narrow pore size distribution and carry a large number of activated ester groups with a different chain length reactive with the amino groups of proteins. The effect of spacer length on the hydrolytic activity of the immobilized proteases is studied. *N*-Benzyl-L-arginine ethyl ester (BAEE) and casein are selected as a low- and a high-molecular-weight substrate, respectively, for the enzyme reaction in this study. The stabilities and durabilities are also investigated for the immobilized proteases.

EXPERIMENTAL

Materials

Different sizes of porous chitosan beads (ACW; Chitopearl), which carry active groups with spacer, were kindly provided by Fuji Spinning Co. Ltd. (Tokyo, Japan). Figure 1 shows the scanning electron micrographs (SEM) of the ACW beads. Papain (3.5 m Anson μg/mg, Merck), ficin (No. DCP1402 Wako Pure Chem. Ind. Ltd.), bromelain (EC 3.4.22.4, No. B-2252, Sigma Chem. Co.), and BAEE

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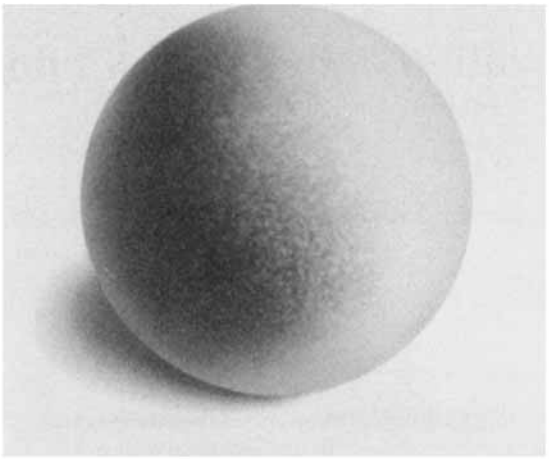

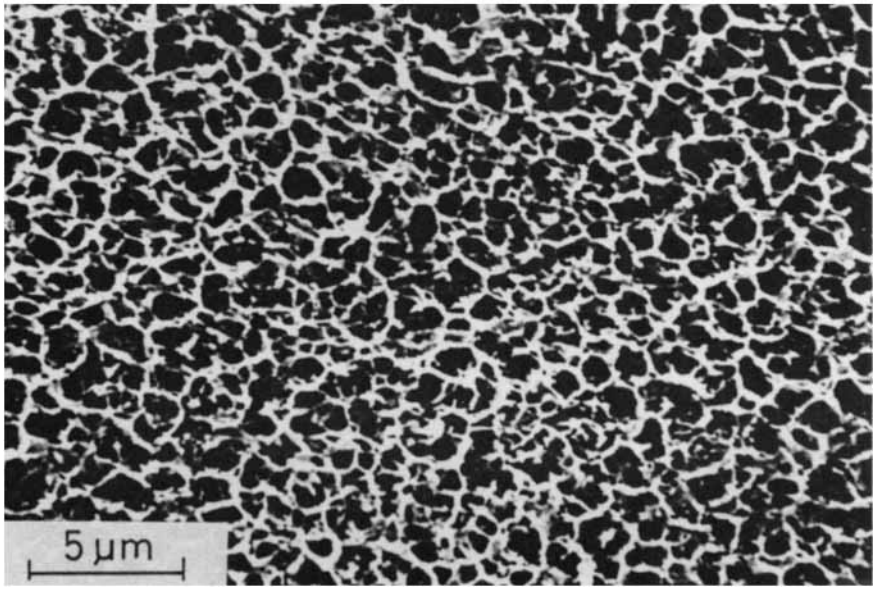

Figure 1 SEM of porous chitosan beads.

as a low-molecular-weight substrate, as well as other chemicals, were purchased from Nakarai Chem. Co. Ltd. Casein, as a high-molecular-weight substrate, was purchased from Wako Chem. Co. Ltd. (from Bovine Milk, Sigma C7891), being first heated in the 2% aqueous suspension adjusted to pH 8.0 for 30 min on a boiling water bath.

Immobilization of Proteases

Protease molecules were covalently immobilized on the porous chitosan beads through the reaction as shown in Figure 2. A typical immobilization procedure is as follows: 100 mg of the ACW beads was suspended in 5 mL 0.05 *M* phosphate buffer (PBS) at pH 7.4. To the suspension, a given amount of protease (papain, ficin, or bromelain) was added under stirring with a magnet in cold room, and the mixture was kept at 4°C for 16 h under stirring. The reaction mixture was then centrifuged at 6000 g at room temperature for 20 min after adding 5 mM $NaBH_4$ aqueous solution to reduce the remaining active groups. The water-insoluble product was suspended in 30 mL 0.05 *M* PBS at pH 7.4 and subjected to centrifugation under the same condition as described above. This procedure was repeated another two times. No residual protease could be detected in the last supernatant by UV spectrum measurement. The final product was stored at 4°C after lyophilization. The amount of proteases immobilized on the ACW beads was determined by the classical ninhydrin method after hydrolyzing the immobilized protease with 6 *N* HCl at 110°C for 1 h. The ACW beads were stable in 6 *N* HCl at 110°C. In all the

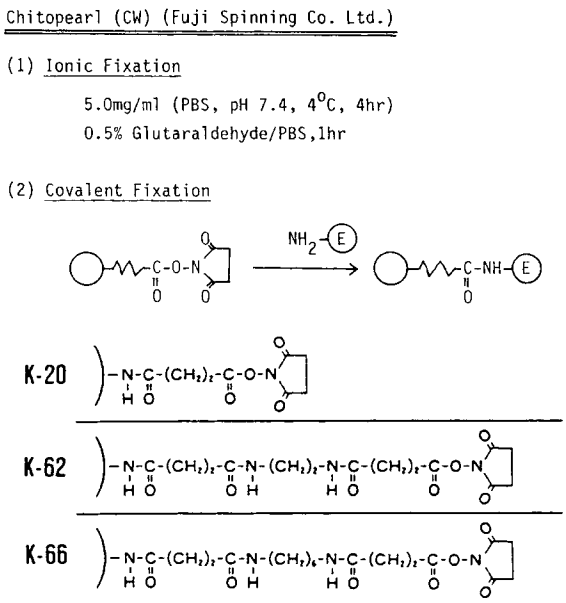

Figure 2 Scheme of the immobilization reactions.

experiments, the ACW beads without immobilized protease were used as "control" in determination of the amount of the immobilized protease.

Titrimetric Determination of Protease Activity

The hydrolytic activity of free protease and the immobilized one was determined using BAEE as a low-molecular-weight substrate. In 0.05 *M* PBS at pH 8.0, 2.5 mL aqueous BAEE solution was added to 2.5 mL free protease solution or 2.5 mL of the immobilized protease suspension. To activate each protease, 2 mM EDTA and 5 mM cystein were added in PBS.[14]

The final reaction mixture had 2.92×10^{-3} M as the substrate concentration. The reaction mixture was maintained at 37.0°C under constant stirring, pH being kept at 8.0 by an addition of 0.05 *N* KOH using a microburet titration. After a predetermined period of time, the enzymatic activity was calculated from the initial rate of BAEE hydrolysis by counting the amount of KOH consumed within the given period of time. To obtain reproducible activity values, vigorous stirring was necessary, especially for the immobilized protease suspensions. Correction was made for nonenzymatic hydrolysis. Three or four different amounts of free protease and the immobilized protease were used in each activity determination. The activity of the immobilized enzymes was expressed as the relative activity in percent based on that of free enzymes.

Caseinolytic Activity Determination

The caseinolytic determinations were done essentially according to Bergmeyer,[15] with minor modifications to overcome some special problems encountered with the insoluble conjugates. The activities of free and immobilized proteases were determined in the following way. The reaction mixture consisted of 2 mL 0.01 *M* PBS at pH 8.0, 1.0 mL of the free enzyme solution or the immobilized enzyme suspension in 0.05 *M* PBS, which contained 2 mM EDTA and 5 mM cystein, and 1.0 mL 2.0 wt % casein solution. The reaction mixtures were vigorously stirred at 37.0°C for 20 min, followed by termination with trichloroacetic acid additions to have a concentration of 3.0 wt %. The absorbance of the solution or the supernatant at 280 nm were plotted against the enzyme weight to evaluate the enzymatic activity.

Stability Measurements

The thermal stability of the immobilized proteases was evaluated by measuring the residual activity (ZA) of proteases exposed to various temperatures in 0.05 *M* PBS of pH 7.4 for various periods of time. After heating, the samples were quickly cooled and assayed for its enzymatic activity at 37.0°C immediately or after storage at 4°C. Storage before the assay (30 min to 48 h) did not alter the measured activities practically. The remaining activities were related to the original activities (assayed at 37.0°C without heating). The kinetics and thermoinactivation were investigated by determining the residual activity of the free and immobilized proteases after incubation at various temperature. The first-order inactivation rate constants, k_i, were estimated by the equation

$$\ln A = \ln A_0 - k_i t, \qquad (1)$$

where A_0 is the initial activity and A is the activity after t min of temperature effect.[16]

To determine the pH stability, the free and immobilized proteases were incubated in BAEE–PBS at 37.0°C and various pH regions for 20 min.

To evaluate durabilities of the immobilized proteases when repeatedly used, the dried immobilized proteases were washed in 0.05 *M* PBS two times and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This cycle was repeated on the same sample. To check the possibility of any leakage of protease molecules under washing, the amount of the immobilized protease

was determined after the last batch test. The storage stability of the free and immobilized proteases was evaluated by placing the proteases in 0.05 *M* PBS of pH 7.4 at 25°C for various periods of time and the activity was assayed using the above-mentioned techniques.

RESULTS AND DISCUSSION

Effect of Surface Concentration on the Activity

Figure 3 shows the surface concentration of papain immobilized onto a ACW-K-20 beads at different enzyme concentrations. It is seen that 6 h is sufficient for the reaction to level off, independently of the concentration of papain, although the saturated surface concentration of the immobilized papain apparently depends on the initial papain concentration of the reaction mixture. The effect of the initial concentration of papain on the saturated surface concentration of papain immobilized was also studied using the ACW-K-66 beads and allowing the reaction to proceed for 12 h. As is seen in Figure 4, the amount of immobilized papain is almost proportional to the initial papain concentration, at least, in the low concentration level below about 5.0 mg/mL. In all the following experiments, the initial papain concentration was kept to 5.0 mg/mL unless otherwise mentioned. The maximum amount of immobilized papain onto ACW-K-20 beads was 0.78 wt %. Figure 5 illustrates the effect of the surface concentration of papain immobilized onto ACW-K-20 beads on the relative activity of BAEE hydrolysis. It is clearly shown that the relative activity of the immobilized papain is independent on the surface concentration and gives a constant value in the whole concentration region studied.

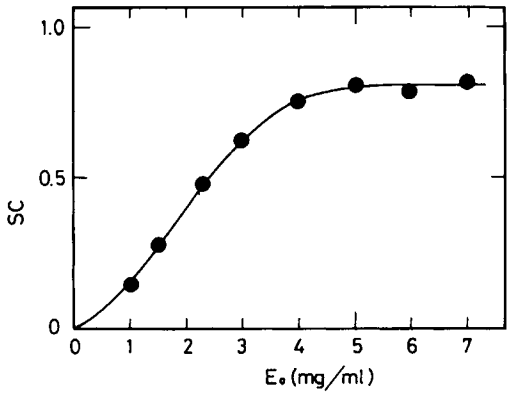

Figure 4 Effect of papain concentration (E_0) on the amount of the immobilized papain on the surface of ACW beads for 16 h.

Determination of Michaelis Constant and Maximum Reaction Velocity

All enzymatic hydrolysis reactions yield data that can be analyzed in the framework of the Michaelis–Menten mechanism: The rate of hydrolysis was expected to be the first order in enzyme concentration.

It was necessary to confirm this since the enzyme concentration varied over a wide range so that the

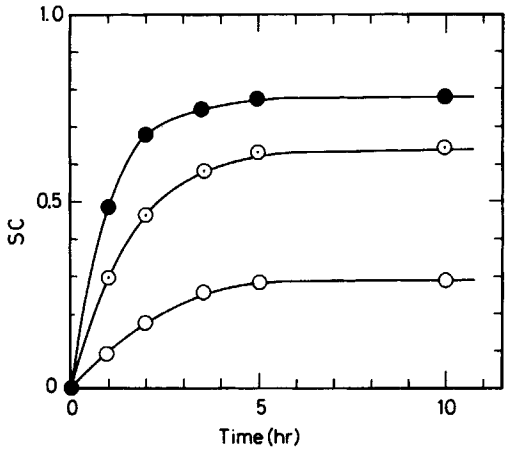

Figure 3 Immobilization of papain on ACW-K-20 beads at different papain concentrations, pH 7.4 and 25°C under slow constant stirring. (●), 5.0 mg/mL; (⊙), 3.0 mg/mL; (○), 1.5 mg/mL.

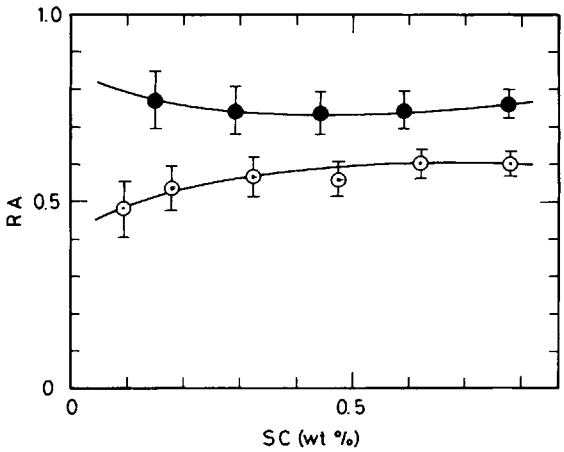

Figure 5 Effect of the surface concentration of the immobilized papain on the relative activities (RA). Hydrolysis: BAEE, pH 8.0 and 37.0°C. (⊙), ACW-K-20-papain; (●), ACW-K-66-papain.

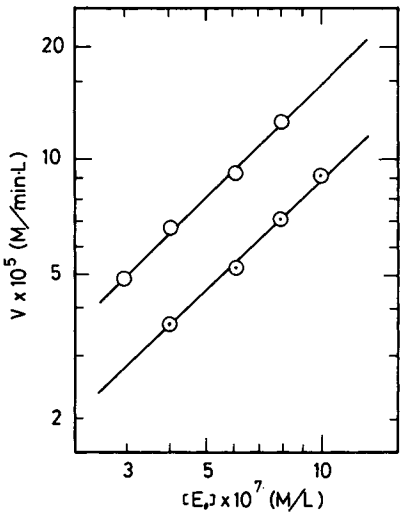

Figure 6 Effect of the papain concentration on the reaction velocity. Hydrolysis: BAEE, pH 8.0 and 37.0°C. (○), free papain; (⊙), ACW-K-20-papain.

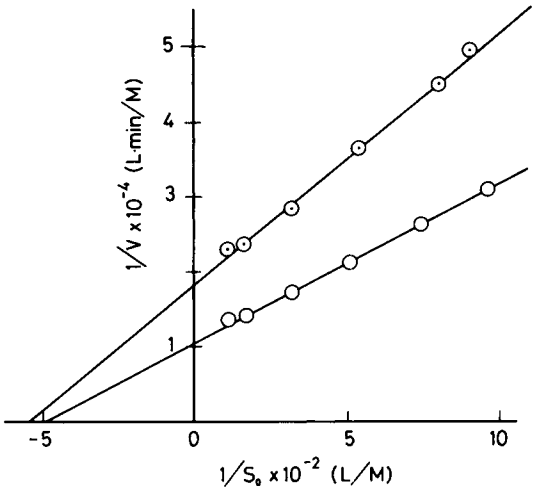

Figure 7 Lineweaver–Burk plots of 1/V vs. 1/S. (○), free papain; (⊙), ACW-K-20-papain.

rate could always be measured. Figure 6 shows the expected experimental results of BAEE hydrolysis by the free and the immobilized papain; they indicate the first-order behavior with the papain concentration.

Initial reaction rates were determined at different initial BAEE concentrations ranging from 1.00–10.0 mM. Figure 7 shows Lineweaver–Burk plots for the free and the immobilized papain. The values of the Michaelis constant K_m and the maximum reaction velocity V_m for the free and the immobilized papain on ACW beads are estimated from Figure 7 and tabulated in Table I with the experimental results for ficin and bromelain.

The apparent K_m values of the immobilized proteases were higher than that of the free one. It may be due to the limitation of diffusion resistance. On the other hand, the V_m values of the immobilized proteases were lower than that of the free one, suggesting the relative activity of the immobilized protease decreased in the course of the covalent fixation, especially with the case of shorter spacer.

Effect of Spacer on the Activity

The effect of the spacer length on the relative activity of BAEE hydrolysis was investigated at the almost same surface concentration of the immobilized proteases. Table II summarizes the results, which show that the immobilized proteases are still active in hydrolysis toward the low-molecular-weight substrate, BAEE, but less active toward casein, a high-molecular-weight substrate. The low activity toward casein probably reflects the difficult approach of casein to the active site of the enzymes because of steric hindrance caused by the enzyme immobilization and the large size of the macromolecular substrate.

Table I Michaelis Parameters K_m and V_m at pH 8.0, 37°C

Sample Code	[E] (M/L)	K_m (M/L)	V_m (M/min, L)
Papain	6.0×10^{-7}	2.0×10^{-3}	9.4×10^{-5}
ACW-K-20-Pap.	6.0×10^{-7}	1.8×10^{-3}	5.5×10^{-5}
Ficin	6.0×10^{-7}	18.0×10^{-3}	4.3×10^{-5}
ACW-K-20-Fic.	6.0×10^{-7}	9.8×10^{-3}	2.2×10^{-5}
Bromelain	8.0×10^{-7}	11.2×10^{-2}	2.4×10^{-5}
ACW-K-20-Bro.	8.0×10^{-7}	7.8×10^{-2}	1.8×10^{-5}

Table II Effect of the Length of the Spacer on the Relative Activity of the Immobilized Enzyme

	Papain			Ficin			Bromelain		
ACW	wt %	BAEE	Cas.	wt %	BAEE	Cas.	wt %	BAEE	Cas.
K-20-E	0.78	0.60	0.25	0.66	0.56	0.18	0.80	0.75	0.35
K-62-E	0.80	0.79	0.37	0.68	0.64	0.28	0.72	0.79	0.43
K-66-E	0.77	0.75	0.40	0.62	0.68	0.31	0.68	0.72	0.48

In addition, it is apparent in Table II that the optimum spacer length exists for all of the immobilized proteases toward the hydrolysis of BAEE, a low-molecular-weight substrate. The highest activity was obtained with ACW-K-62-protease series. On the other hand, the enzymatic activity toward the high-molecular-weight substrate increased as the spacer becomes longer, at least in the length range examined. This fact indicates that the addition of spacer to the carrier surface probably reduces the steric interference with the substrate binding process, especially toward high-molecular-weight substrates.

Thermal Stability of the Immobilized Proteases

The thermal stability of immobilized enzymes is one of the most important criteria of their application. As is well known, the activity of the preparation of immobilized enzymes, especially in a covalently bound system, is more resistant against heat and denaturing agents than that of the soluble form.[17] The effect of temperature on the stability of the immobilized papain in PBS is shown in Figure 8. The immobilized papain are more stable than the free papain in the range of higher temperatures. The immobilized papain at 70°C after 60 min effect exhibited activity 2.8–3.4 times that of the free one. The kinetic curve of thermal inactivation of the case of ACW-K-66-papain at 75°C reveals a two-stage process characterized by the following constants: $k_1 = 3.83 \times 10^{-2}$ min^{-1} and $k_2 = 0.93 \times 10^{-2}$ min^{-1} (Fig. 9). The free papain loses 90% of its initial activity at 75°C for 45 min.

The immobilized papain onto ACW-K-66 beads is slightly less stable than that onto ACW-K-20 beads (Fig. 8). This result suggests that the immobilization of papain onto ACW-K-20 beads, which carry a shorter spacer, stabilized the papain molecule due to the multipoint attachment of the papain molecule to the end of the spacer chain of the ACW beads through reduction in molecular mobility, compared to the case of the longer spacer chain.

The pH effect on the activity of the immobilized and free papain for BAEE hydrolysis was studied

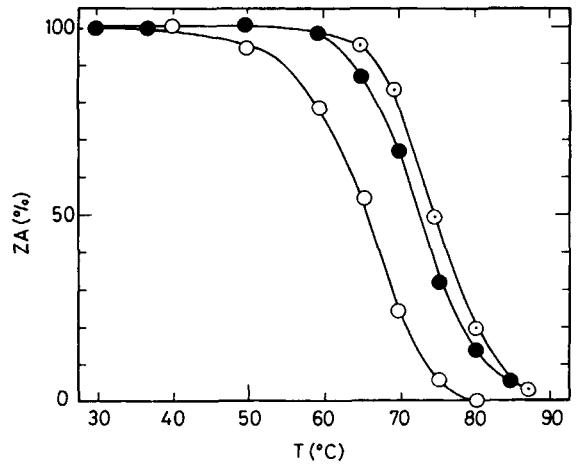

Figure 8 Effect of the heat treatment at the given temperature and pH 8.0 for 1 h on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (●), ACW-K-66-papain; (⊙), ACW-K-20-papain.

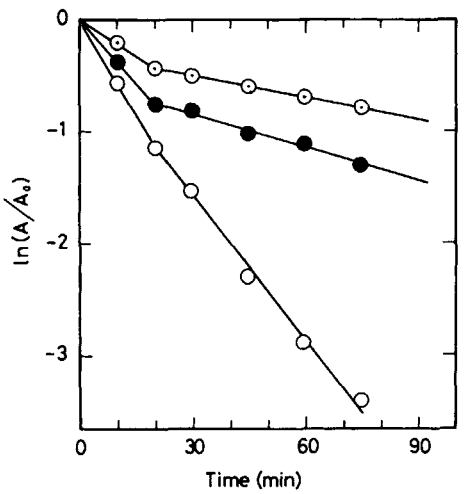

Figure 9 Kinetics of temperature inactivation at 75°C of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (●), ACW-K-66-papain; (⊙), ACW-K-20-papain.

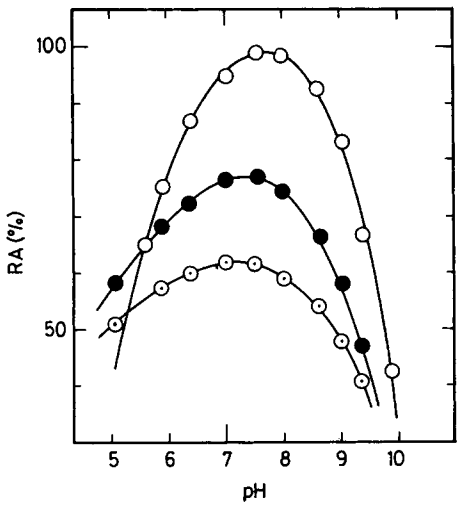

Figure 10 Effect of pH of the reaction medium on the relative activities of BAEE hydrolysis at 37.0°C. (○), free papain; (●), ACW-K-66-papain; (⊙), ACW-K-20-papain.

in PBS at 37.0°C in various pH regions, and is presented in Figure 10. The immobilized papain has the same pH optimum as the free one (pH 8.0), but the pH profile is considerably widened due to diffusional limitations.[18] Immobilized papain displays a greater stability at higher pH values.

Durability for Repeated Use

The durability of the immobilized papain is also very important in applications because they are subjected to repeated hydrolysis reactions. Figure 11 illustrates the effect of repeated use on the residual activity of BAEE hydrolysis by the immobilized papain. The activity is seen to be retained without any definite loss, irrespective of the spacer interposition, even if the batch reaction is repeated at least 10 times.

It was found that the amount of the immobilized papain after the last batch was equivalent to the original one within the experimental error in each cases, suggesting that no leakage of the immobilized papain occurred under the repeated washing. This high stability is in marked contrast with the rather poor durability of the papain that was immobilized by ionic adsorbance on the porous chitosan beads (see Fig. 11).[19]

Storage Stability

Aqueous suspensions of the immobilized papain could be stored at 4°C for 6 months without a significant loss of activity, whereas the corresponding free papain lost more than 30% of their initial activity under the same condition. The higher stability of the immobilized papain can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of papain molecules on the surface of ACW beads. However, it is often pointed out that lyophilization of enzymes directly from the water suspensions is normally accompanied by loss of the enzymatic activity. The enzymatic activity retained after lyophilization of the immobilized and free proteases was determined. Very high residual activities are observed for the immobilized proteases for BAEE hydrolysis, that is, 90% for ACW-K-20-Papain and 84% for ACW-K-66-Papain, respectively, despite only 70% for native papain. It is of interest to point out that there is a similarity between the thermal and storage stabilities to lyophilization. These findings can be accomodated in a general framework by considering the state of the covalent fixation between the carrier material and the enzyme molecules. It is reported that hydrophilic carriers such as Sephadex, Sepharose, and polyacrylamide yield enzyme derivatives of high lyophilization and thermal stabilities.[20-22] The chitosan beads belong to the hydrophilic carrier.

To examine the enzymatic stability in the continuous reaction system under a rather drastic condition, effects of the storage in PBS of pH 7.4 at 37.0°C were studied for the immobilized papain. The residual activity at BAEE hydrolysis is given in Figure 12. It is apparent that the immobilized papain is much more stable than the free one. Again, the immobilized papain with a shorter spacer shows a

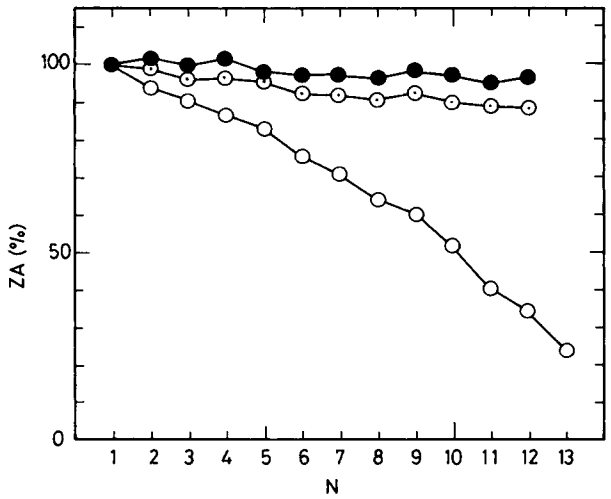

Figure 11 Effect of the repeated use on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. (⊙), ACW-K-20-papain; (●), ACW-K-66-papain; (○), Chitosan-papain.

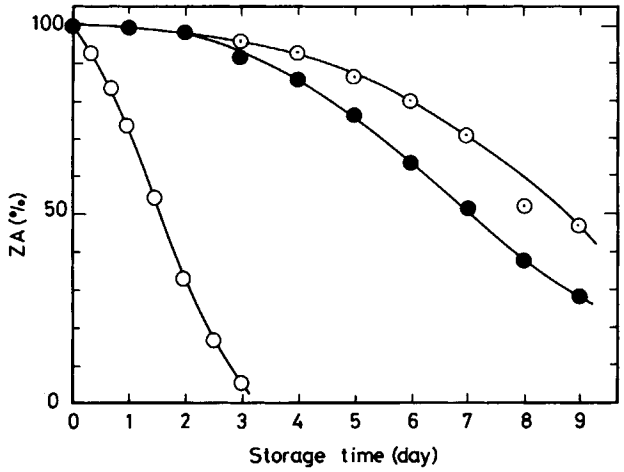

Figure 12 Effect of the storage in PBS at pH 7.4 and 37.0°C on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. (○), free papain; (●), ACW-K-66-papain; (⊙), ACW-K-20-papain.

more stable activity than that with a longer space in spite of the initial lower activity.

The similar behaviors are observed with cases of ficin and bromelain.

CONCLUSION

The immobilized proteases, such as papain, ficin, and bromelain, onto the surface of porous chitosan beads with any length of spacer by covalent fixation gave rather high activity toward the small ester substrate, *N*-benzyl-L-arginine ethyl ester (BAEE), but lower activity toward casein, a high-molecular-weight substrate. The relative activity of the immobilized proteases with spacer gave an almost constant value for the substrate hydrolysis. The apparent K_m values were larger for immobilized proteases than for the free ones, while V_m values were smaller for the immobilized proteases.

The pH, thermal, and storage stability of the immobilized proteases were higher than those of the free ones. The initial enzymatic activity of the immobilized proteases maintained almost unchanged without any elimination and inactivation of proteases, indicating the excellent durability.

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