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SPACER EFFECTS ON ENZYMATIC ACTIVITY OF BROMELAIN IMMOBILIZED ONTO POROUS CHITOSAN BEADS

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Abstract—Bromelain was covalently immobilized onto the surface of porous chitosan beads with and without alkyl chain spacers of different lengths. The relative activity of the immobilized bromelain was found to be high toward a small ester substrate, *N*-benzyl-L-arginine ethyl ester (BAEE), but rather low toward casein, a high molecular weight substrate. Bromelain immobilized with spacer gave an almost constant activity by varying the surface concentration in marked contrast with the immobilized bromelain without spacer whose activity monotonously decreased with decreasing surface concentration. The relative activity of the immobilized bromelain for hydrolysis of a high molecular weight substrate was a strong function of spacer length. The pH, thermal, and storage stabilities of the immobilized bromelain were higher than those of the free bromelain. The bromelain immobilized directly onto the surface of chitosan beads without any spacer gave a higher stability than those immobilized with spacers. The spacer effect on the activity could be explained in terms of flexibility of the immobilized bromelain molecule.

INTRODUCTION

The bioreactor is generally composed of biologically active proteins and their bioinert carriers, and it will probably be in the bioreactor system that research on bioreactors is shifting from a biological perspective to a materials perspective. A large number of works have been devoted to the polymeric carriers, especially to immobilization of the proteins onto the carriers [1]. Since the recovery yield and the reusability of free enzymes as industrial catalysts are quite limited, attention has been paid to enzyme immobilization which may offer advantages over free enzymes; for example, possibility of continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reactive mixture, and adaptability to various engineering designs [2, 3]. A concerted or sequential reaction of several enzymes is also feasible by the use of mixed or stratified beds. Furthermore, interest in immobilized enzymes and their application to bioprocessing [4, 5], analytical system [6], and enzyme therapy [7] has grown steadily in the past decade. Thus, many approaches to the preparation of water-insoluble enzymes have been explored to study the enzyme reaction in biphasic systems similar to those existing *in vivo* [8–11]. The same basic techniques that have been used to immobilize enzymes could also be used to immobilize other biologically active agents, such as antibodies, antigens, chloroplasts, or mitochondria. Thus, it may be useful to obtain basic knowledge about the immobilized enzymes, such as specific activities or stabilities, for designing biosensors and bio-materials for therapeutic applications.

In the present study, a plant thiol endopeptidase bromelain was selected as a commercially available analogue of cathepsin B released during the acute and chronic stages of the inflammatory response. Although bromelain is a general plant thiol protease, it has preference for peptide bonds where the amino acid residue of the carbonyl group is arginine, lysine, or glutamine, and where this amino acid is joined on either side by amino acids with hydrophobic side chains. The polymer support employed is porous chitosan beads (ChB) [12, 13] which have a 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. *N*-Benzyl-L-arginine ethyl ester (BAEE) is selected as a low molecular weight substrate and casein as a high molecular weight substrate for the enzyme reaction in this study. The stability and durability of the immobilized bromelain are also investigated.

EXPERIMENTAL PROCEDURES

Materials

Porous chitosan beads which carry active groups with different spacer sizes were synthesized by the method reported previously [13].

Figure 1 shows the scanning electron micrographs (SEM) of the ChB beads. Bromelain (EC. 3.4.22.4; from

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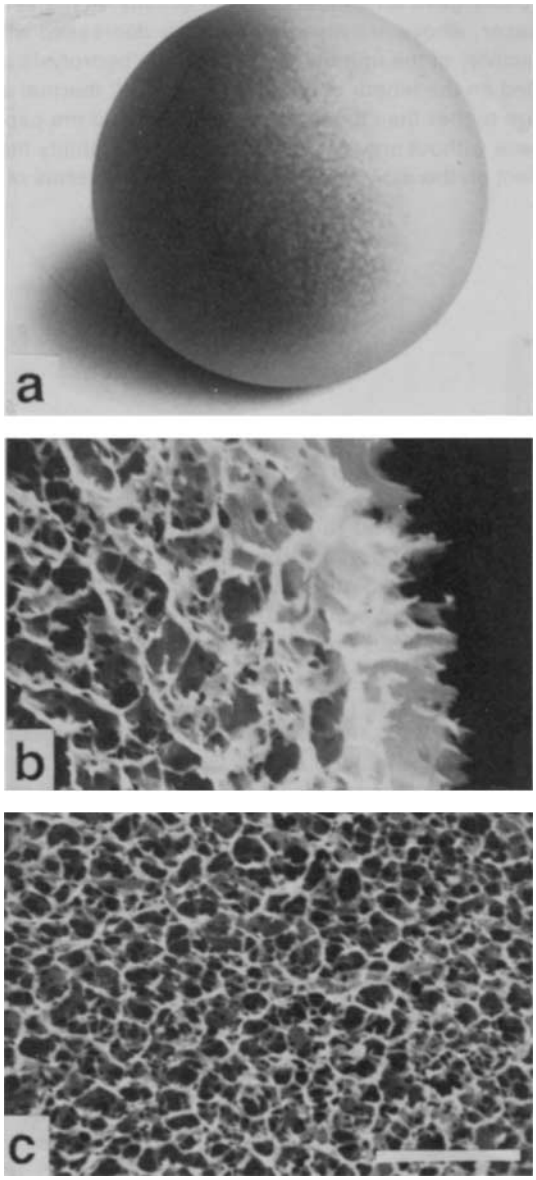

Fig. 1. SEMs of the surface of porous chitosan bead (ChB): (a) wholeshape (diameter is about 300 μm); (b) sectional appearance; (c) surface appearance of porous ChB.

pineapple stem; 800 units per mg protein; Amano Pharmaceutical Co., Ltd), BAEE, casein and other chemicals were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Immobilization of bromelain

Bromelain molecules were covalently immobilized onto carboxyl-activated ChB beads by reaction with *N*-hydroxysuccinimide as shown in Fig. 2. A typical immobilization procedure is performed as follows: 100 mg of the ChB beads were suspended in 5 ml of 0.05 M phosphate buffer (PBS) at pH 7.4. A given amount of bromelain was added to the stirred suspension and maintained at 4°C for 16 hr. The concentration of the remaining carboxyl-activated groups was then reduced by adding 5 mM $NaBH_4$ aqueous solution. The reaction mixture was then centrifuged at 6000 rpm for 20 min at room temperature. The water-insoluble product was suspended in 30 ml of 0.05 M PBS at pH 7.4 and subjected to centrifugation under the same conditions as described above. The resuspension was performed three times. The supernatant of the third resuspension was free of bromelain as determined by UV spectroscopy. The final product was stored at 4°C after lyophilization. The amount of bromelain immobilized on the ChB beads was determined by the classical ninhydrin method after hydrolysing the immobilized bromelain with 6 N HCl at 110°C for 1 hr. The ChB beads were stable under these conditions at 110°C. In all the experiments, the ChB beads without immobilized bromelain were used as "controls" in the immobilized bromelain quantification.

Activity measurements

The hydrolytic activity of free and immobilized bromelain was determined using BAEE as a low molecular weight substrate. The enzymatic activity was calculated from the initial rate of BAEE hydrolysis by determining KOH consumed within the given period of time [14].

The caseinolytic determinations were done essentially according to Bergmeyer [15], with minor modifications to overcome some problems encountered with the insoluble conjugates. The activities of free and immobilized bromelain were determined in the following way. The reaction mixture consisted of 2 ml of 0.01 M PBS at pH 8.0, 1.0 ml of the free bromelain solution or the immobilized bromelain suspension in 0.05 M PBS which contained 2 mM EDTA and 5 mM cystein and 1.0 ml of 2.0 wt-% casein solution. The reaction mixtures were vigorously stirred at 37°C for 20 min, followed by termination with trichloroacetic acid additions to a final concentration of 3.0 wt-%. The absorbance of the solution or the supernatant at 280 nm was plotted vs the enzyme weight to determine the enzymatic activity. The relative activity (RA) was defined as the ratio of the hydrolytic activity of the immobilized enzyme to that of the free enzyme, which was used to evaluate the activities of the immobilized bromelain.

Stability measurements

The thermal stability of the immobilized bromelain was evaluated by measuring the residual activity (ZA) of the enzyme exposed to various temperatures in 0.05 M PBS at pH 7.4 for various periods of time. After heating, the samples were quickly cooled and assayed for their enzymatic activity at 37.0°C immediately. Some samples were treated after storage at 4°C. Storage before the assay (30 min to 48 hr) did not alter the measured activities significantly.

The remaining activities were related to the initial activities (assayed at 37.0°C without heating). The kinetics and thermal inactivation were investigated by determining $ZA(=A/A_o)$ of the free and immobilized bromelain after incubation at various temperatures. The first-order inactivation rate constants, k_i, were estimated by the equation:

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

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

To determine the pH stability, the free and immobilized bromelain were incubated with a definite amount of BAEE in PBS at various PHS at 37.0°C for 20 min.

To evaluate the durability of the immobilized bromelain when reused, the dried immobilized bromelain was washed in 0.05 M PBS twice and then resuspended in a fresh reaction mixture. The enzymatic activity was then measured. This process was repeated 10 times on the same sample. The amount of immobilized bromelain was determined after the last batch test to check the possibility of any leakage of bromelain molecules under washing. The storage stability of the free and immobilized bromelain was evaluated by placing bromelain in 0.05 M PBS, pH 7.4, at 25°C for various periods of time and assaying for the activity.

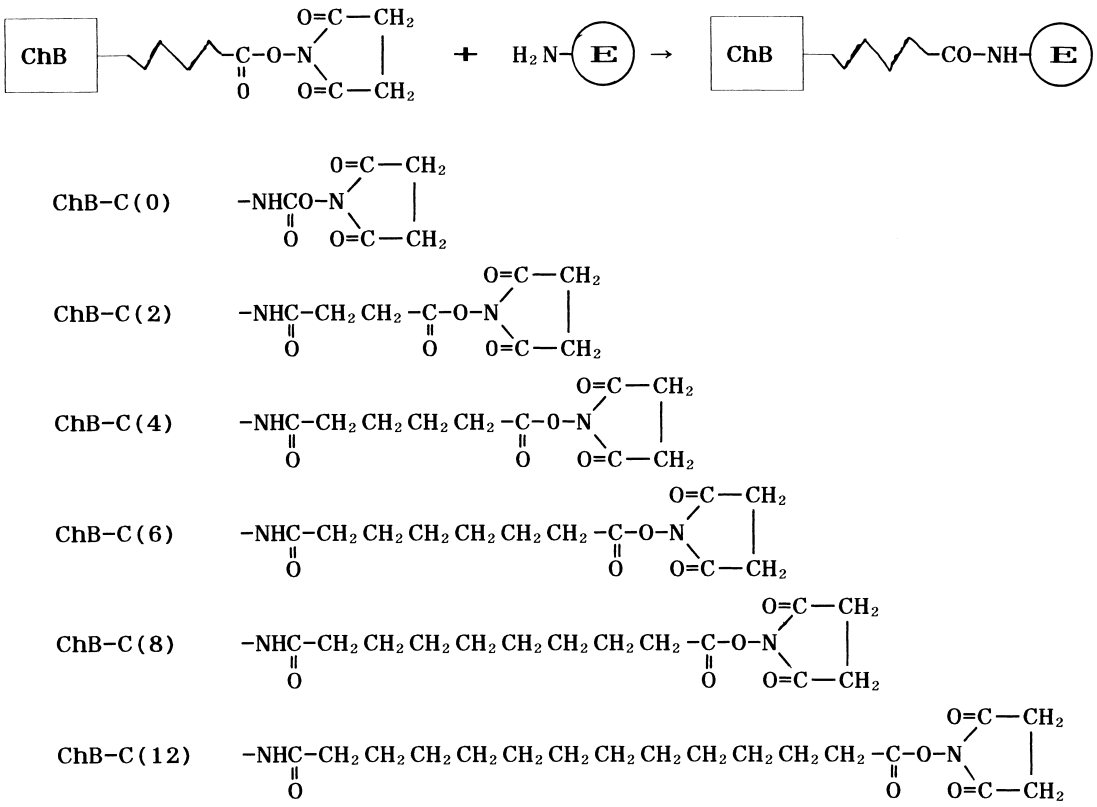

Fig. 2. Scheme of immobilization reactions.

RESULTS AND DISCUSSION

Effect of surface concentration on activity

The effect of the initial concentration of bromelain on the saturated surface concentration of the immobilized bromelain was studied using the ChB-C(6) bead. The adsorption time for these experiments was 3 hr. As is seen in Fig. 3, the amount of immobilized bromelain increased with the initial bromelain concentration in the low concentration region, below 3.0 mg/ml. A constant value of surface concentration is approached for concentration levels greater than 4.0 mg/ml. Thus, in all the following experiments, the initial bromelain concentration was 4.0 mg/ml, unless otherwise mentioned. The maximum amount of immobilized bromelain onto ChB-C(6) beads was 0.80 wt-%. Figure 4 illustrates the effect of the surface concentration (SC wt%) of the immobilized bromelain onto ChB-C(6) beads on the relative activity (RA%) of BAEE hydrolysis. It is clearly seen that the RA of immobi-

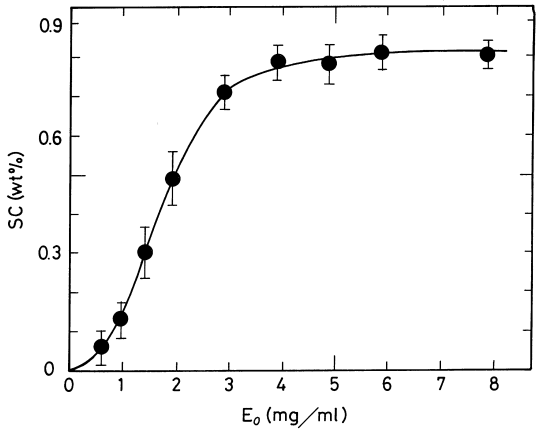

Fig. 3. Effect of the initial concentration (E_o) of bromelain on the surface concentration (SC wt%) of bromelain immobilized onto ChB(6) beads.

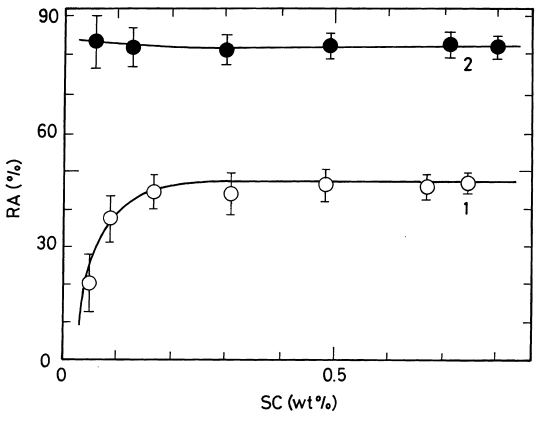

Fig. 4. Effect of the surface concentration (SC) of immobilized bromelain on the relative activity (RA) in the hydrolysis reaction of BAEE at pH 8.0 and 37.0°C: (1) ChB-C(0)–bromelain (○) and (2) ChB-C(6)–bromelain (●).

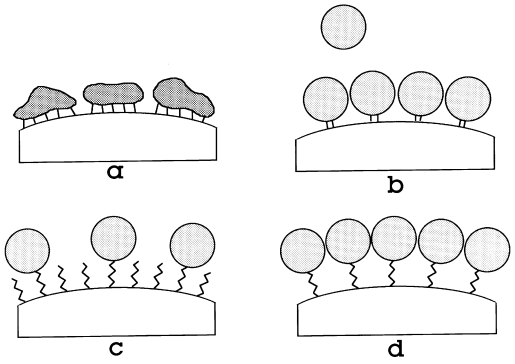

Fig. 5. Schematic representation of the molecular state of enzymes immobilized on the surface of ChBs: (a) and (b) denote that of ChB-C(0)–enzyme, while (c) and (d) denote that of ChB-C(*n*)–enzyme. (a) Sparse immobilization without spacer; (b) dense immobilization without spacer; (c) sparse immobilization with spacer; (d) dense immobilization with spacer.

lized bromelain without any spacer decreases gradually with decreasing surface concentration of the immobilized bromelain, especially at a surface concentration below 0.1 wt%, whereas the immobilized bromelain with spacer gives an almost constant RA value even at low surface concentration, which is markedly higher than that without spacer. We suggest a possible hypothesis in terms of structural deformation of the immobilized bromelain molecules as illustrated in Fig. 5. The covalently immobilized bromelain without spacer should undergo strong deformation in the lower surface concentration region, due to the multipoint attachment of bromelain molecules to the surface of the ChB bead as shown in Fig. 5(a). On the other hand, higher surface concentration of enzyme molecules may be ascribed to a reduced interaction with the substrate undergoing weak deformation (Fig. 5(b)), whereas the immobilized bromelain molecule with spacer must be protected from heavy structural deformation even in the lower surface concentration region owing to the spacer effect. The low RA of bromelain immobilized without spacer but having a high surface concentration may be ascribed to a reduced interaction with the substrate, as widely accepted.

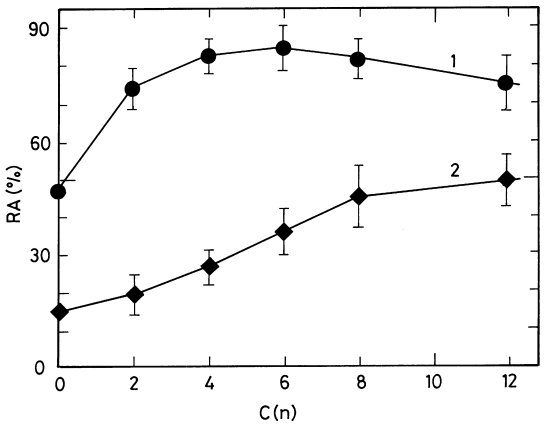

Fig. 6. Effect of the spacer length, *C*(*n*), of immobilized bromelain on the relative activity (RA) at pH 8.0 and 37.0°C for (1) BAEE (●) and (2) casein (◆).

Effect of spacer on the relative activity

The effect of the spacer length of the immobilized bromelain on the RA of substrate hydrolysis was investigated. Figure 6 illustrates the experimental results of the effect of the spacer length *C*(*n*) of the immobilized bromelain on RA, which shows that the immobilized bromelain is rather active in hydrolysis toward BAEE, but less active toward casein. The low activity toward casein probably reflects the difficult approach of a high molecular weight substrate to the active site of the enzyme because of steric hindrance caused by enzyme immobilization and the large size of the macromolecular substrate. In addition, it is apparent in Fig. 6 that an optimum spacer length exists for the immobilized bromelain toward the hydrolysis of the low molecular weight substrate. The highest activity was obtained with ChB-C(6)–bromelain. On the other hand, RA toward the high molecular weight substrate increased by increasing the length of the spacer at least in the length range examined. This fact indicates that the addition of spacer to the carrier surface probably reduces steric interference with the substrate binding process, especially toward high molecular weight substrates.

Effect of pH on the activity

Figure 7 presents the pH effect on RA of immobilized and free bromelain for BAEE hydrolysis in PBS at 37°C. Immobilized bromelain has the same pH optimum as the free bromelain, but the pH profile is considerably widened. Immobilized bromelain displays a greater stability, especially at lower pH regions.

Thermal stability of the immobilized enzymes

The thermal stability of immobilized enzymes is one of the most important criteria considered before their utilization in applications. The immobilized

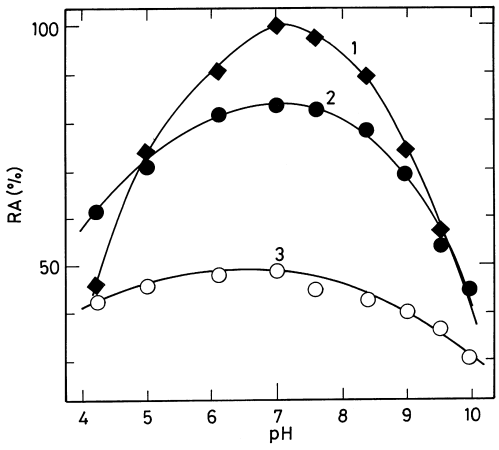

Fig. 7. Effect of pH of the reaction medium on the relative activity (RA) of BAEE hydrolysis at 37.0°C: (1) free bromelain (◆), (2) ChB-C(6)–bromelain (●), and (3) ChB-C(0)–bromelain (○).

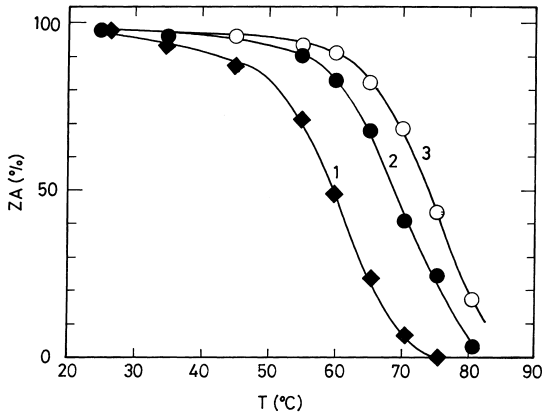

Fig. 8. Effect of 1 hr pretreatment at the given temperature and pH 8.0 in PBS on the residual activity (ZA) of bromelain on BAEE hydrolysis at pH 8.0 and 37.0°C: (1) free bromelain (◆), (2) ChB-C(6)–bromelain (●), and (3) ChB-C(0)–bromelain (○).

enzyme, especially in a covalently bound system, is more resistant to heat and denaturing agents than that of the soluble form [17]. The effect of temperature on the stability of the immobilized bromelain in PBS is shown in Fig. 8. Immobilized bromelain is more stable than free bromelain in the high temperature region. Immobilized bromelain treated at 65°C for 60 min exhibits an activity 3–4 times as high as that of the free bromelain. As shown in Fig. 9, the kinetic curve of thermal inactivation of the immobilized bromelain at 70°C reveals a two-stage process characterized by the two constants as listed in Table 1.

The free bromelain loses 90% of its initial activity after heat treatment at 70°C for 45 min. The higher stability of immobilized bromelain without spacer than with spacer shown in Fig. 8 is probably ascribed to stabilization of the bromelain molecule due to the multipoint attachment of the bromelain molecule to the surface of the ChB bead if no spacer is used, leading to a reduction in molecular

Table 1. The rate constant of k_i of thermal inactivation for immobilized bromelain at 70°C

Sample code	$_1$ (min^{-1}) × 10^2	k_2 (min^{-1}) × 10^2
ChB-C(0)–bromelain	1.5	0.6
ChB-C(6)–bromelain	2.8	0.8
Free bromelain	5.3	

mobility that is a common principle of enzyme stabilization [18], although the RA of immobilized bromelain without any spacer is lower than that of the immobilized bromelain with spacer.

Storage stability

Aqueous suspensions of the immobilized bromelain were stored at 4°C for 12 months without a significant loss of activity, whereas the corresponding free bromelain lost more than 50% of its initial activity under the same conditions. The higher stability of the immobilized bromelain can be attributed to the prevention of autodigestion and thermal denaturation as a result of the fixation of bromelain molecules on the surface of ChB beads. However, it is often pointed out that lyophilization of the enzyme aqueous solution is normally accompanied by loss of enzymatic activity. The ZA after lyophilization of the immobilized and free enzymes was determined. Very high ZA is observed for the immobilized bromelain for BAEE hydrolysis; that is, 95% for ChB-C(0)–bromelain and 90% for ChB-C(8)–bromelain, respectively, in contrast with only 70% for free bromelain. It is of interest to point out that there is a similarity between the thermal and storage stabilities to lyophilization. These findings can be accommodated in a general framework by considering the state of 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 [19–21]. ChB beads are hydrophilic carriers, so high lyophilization and thermal stabilities are expected.

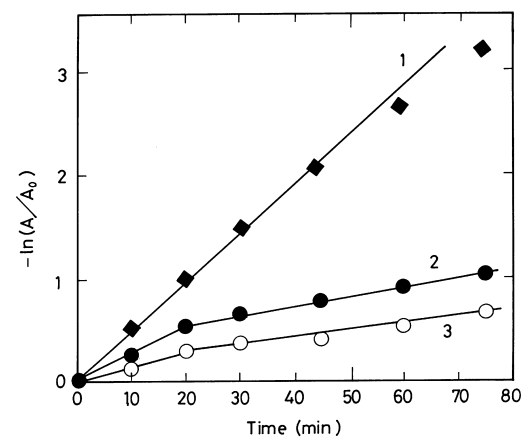

Fig. 9. Kinetics of thermal inactivation at 75°C for bromelain on BAEE hydrolysis at pH 8.0 and 37.0°C: (1) free bromelain (◆), (2) ChB-C(6)–bromelain (●), and (3) ChB-C(0)–bromelain (○).

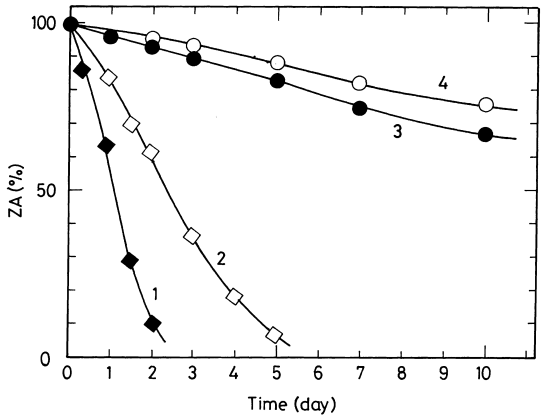

Fig. 10. Effect of storage time in PBS, at pH 7.4 and 37.0°C on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C: (1) free bromelain (◆), (2) bromelain adsorbed on ChB (◇), (3) ChB-C(6)–bromelain (●), and (4) ChB-C(0)–bromelain (○).

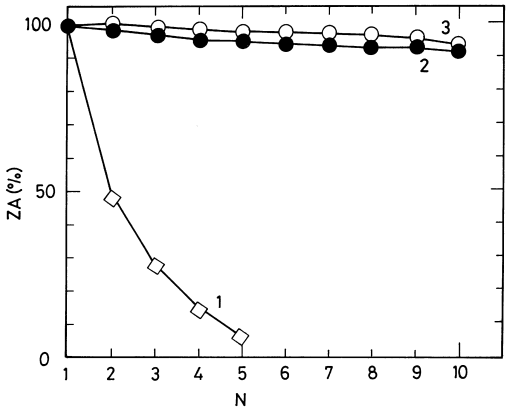

Fig. 11. Effect of repeated use on the residual activity (ZA) of BAEE hydrolysis at pH 8.0 and 37.0°C. *N* denotes number of batch reactions. (1) Bromelain adsorbed on ChB (◇), (2) ChB-C(6)–bromelain (●), and (3) ChB-C(0)–bromelain (○).

To examine the enzymatic stability in the continuous reaction system under rather harsh conditions, effects of storage in pH 7.4 PBS at 37.0°C were studied for immobilized bromelain. The RA of BAEE hydrolysis is given in Fig. 10. It is apparent that immobilized bromelain is much more stable than the free bromelain. Again, immobilized bromelain with a shorter spacer shows more stable activity than that with a longer spacer in spite of the initial lower activity.

Durability of repeated use

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

It was found that the amount of immobilized bromelain after the last batch was equivalent to the original one within experimental error in each case, suggesting that no leakage of immobilized bromelains occurred under repeated washing. This high stability is in marked contrast to the rather poor durability of bromelain immobilized by ionic adsorption on ChB beads.

CONCLUSIONS

Immobilized bromelain on the surface of ChB with any length of spacer by covalent fixation gave a rather high activity toward small substrates, whereas a low activity was observed for casein a high molecular weight substrate. The RA of immobilized bromelain without spacer decreased gradually with decreasing surface concentration of the immobilized bromelain. On the other hand, the immobilized bromelain with spacer gave an almost constant activity for substrate hydrolysis in the surface concentration region studied.

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

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