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Mercapto-chitins: a new type of supports for effective immobilization of acid phosphatase

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Acid phosphatase was immobilized on two kinds of mercapto-chitins, 2-mercapto-chitin and 6-mercapto-chitin, and assayed with 4-nitrophenyl phosphate as the substrate. The optimal pH values for immobilization were 4.5 and 4.8, respectively. The resulting immobilized enzymes showed maximum activities at pH 6.0 and 5.5, almost the same as that for the soluble enzyme. 6-Mercapto-chitin/enzyme conjugate retained high activity even after repeated uses in batch systems, suggesting effective immobilization through covalent bond formation, while 2-mercapto-chitin/enzyme and chitin/enzyme conjugates showed decreases in activity after a few runs. © 1997 Elsevier Science Ltd

INTRODUCTION

Immobilization of biologically active species is becoming increasingly important as a tool for efficient production of useful compounds without unfavorable side reactions. In order to achieve high biological activities in the conjugate systems, choices of supports and immobilization methods are crucial. As supports, chitin and chitosan are considered to have high potential because of certain remarkable characteristics including the facile immobilization either by chemical reaction or physical adsorption, low bulk density, coarse porous structure, and low or no toxicity. Chitosan may be used more conveniently than chitin owing to the presence of free amino groups.

Immobilization of various enzymes on chitin and chitosan has been reviewed (Muzzarelli, 1977, 1980), and glutaraldehyde is used most widely to hold the enzymes. For example, lactase (Stanley *et al.*, 1975) and glucoamylase (Stanley *et al.*, 1978) immobilized on chitin with glutaraldehyde exhibited as high as 60–70% of the activities of the native enzymes. Porous beads of chitosan treated with glutaraldehyde were similarly utilized as supports for α-galactosidase, and the activity yield was 25–45% (Ohtakara *et al.*, 1987).

Although the glutaraldehyde method is convenient, there seem to be problems associated with the remaining aldehyde groups and possible elution of the aldehyde as a result of hydrolysis during operation. Without glutaraldehyde, enzymes can be immobilized on chitosan directly through ionic interaction in some cases, and α-chymotrypsin thus immobilized showed considerable activity (Muzzarelli *et al.*, 1976). Acid phosphatase could also be immobilized on chitosan in a similar manner (Muzzarelli *et al.*, 1976), though it was generally immobilized with glutaraldehyde on polyacrylamide beads (Weston *et al.*, 1971), chitin (Stanley *et al.*, 1975), or porous glass beads (Ohmiya *et al.*, 1983).

In order to retain high activity during extended use, immobilization with covalent bond formation under mild conditions would be desirable. For this purpose, insoluble materials having mercapto groups may be promising as supports, since mercapto groups readily form disulfide linkages with the mercapto or disulfide groups of enzymes. In this study, acid phosphatase was immobilized on two kinds of mercapto-chitins, 2-mercapto- and 6-mercapto-chitins, whose mercapto groups are readily accessible for reactants (Kurita *et al.*, 1996). 6-Mercapto-chitin proved to be particularly suitable in terms of the enzyme activity and durability of the resulting conjugate.

EXPERIMENTAL

General

Acid phosphatase from wheat germ was purchased from Sigma. The enzyme activity was determined with diso-

dium 4-nitrophenyl phosphate (phosphatase substrate from Sigma) as the substrate. A solution of 150 mg of the phosphate dissolved in 150 ml of deionized water was used as the substrate solution, and 4-nitrophenol formed by enzymatic hydrolysis was determined by UV spectroscopy with a Ubest-30 UV/VIS spectrophotometer (Jasco, Tokyo, Japan).

Chitin isolated from shrimp shells was a product of Katokichi Co. Ltd (Kagawa, Japan). It was treated with 1 mol/l aqueous sodium hydroxide at 100°C for 8 h in a nitrogen atmosphere and washed thoroughly with deionized water. The degree of acetylation per pyranose unit was 0.85 as determined by conductometric titration (Kurita *et al.*, 1992). Mercapto-chitins were prepared according to the methods described previously (Kurita *et al.*, 1993), and 2-mercapto-chitin with a degree of substitution (ds) of 0.30 and 6-mercapto-chitin with ds 0.51 were used as supports. Chitin and mercapto-chitins were finely pulverized to 100 mesh pass for immobilization experiments. All the chemicals were of reagent grade and used without further purification.

Optimal pH for immobilization of acid phosphatase

A mixture of 50 mg of a mercapto-chitin and 10 mg of the acid phosphatase in 50 ml of a 0.1 mol/l buffer solution, acetate buffer for pH 3.5–6.3 or Tris buffer for pH 6.5–8.5, was shaken at 10°C for 24 h, and the supernatant was removed by centrifugation. The solid portion was washed with acetate buffer of pH 4.8 and centrifuged. The washing and centrifugation procedure was repeated three times, and the resulting immobilized enzyme was assayed without drying to elucidate the optimal pH for immobilization.

Assay of the immobilized enzymes

A mercapto-chitin/enzyme conjugate obtained above was suspended in 10 ml of deionized water and 35 ml of acetate buffer of pH 5.5, and the mixture was shaken at 37°C for 5 min. The 4-nitrophenyl phosphate solution (10 ml) was added, and the mixture shaken at 37°C for 10 min. To 5.5 ml of the supernatant were added 0.5 ml of 0.5 mol/l calcium chloride and 2.0 ml of 0.5 mol/l sodium hydroxide to give a light yellow solution. It was filtered, and the absorbance at 400 nm due to the 4-nitrophenoxide in the filtrate was measured.

Optimal pH of the immobilized enzymes

The acid phosphatase (10 mg) was treated with 2-mercapto- and 6-mercapto-chitins (50 mg) in 50 ml of acetate buffer of pH 4.5 and 4.8, respectively, at 10°C for 24 h, and the products were washed three times with acetate buffer of pH 4.8. Without drying, they were suspended in 10 ml of deionized water and diluted with 35 ml of 0.1 mol/l acetate or Tris buffer of pH 3.5–8.5. The 4-nitrophenyl phosphate solution (10 ml) was added, and the hydrolysis behavior was followed by the method described above.

Amount of enzyme immobilized on the supports

2-Mercapto- and 6-mercapto-chitins (50 mg) were suspended in 50 ml of acetate buffer of pH 4.5 and 4.8, respectively, and 10 mg of the acid phosphatase was added. The mixtures were shaken at 10°C for 24 h, and the amounts of immobilized enzyme were estimated from the amounts of enzyme in the supernatants. The activities of the supernatants were measured with the 4-nitrophenyl phosphate solution similarly, and the amounts of enzyme were determined with a calibration line obtained on the basis of the activities of solutions containing various amounts of the soluble enzyme.

RESULTS AND DISCUSSION

Mercapto-chitins

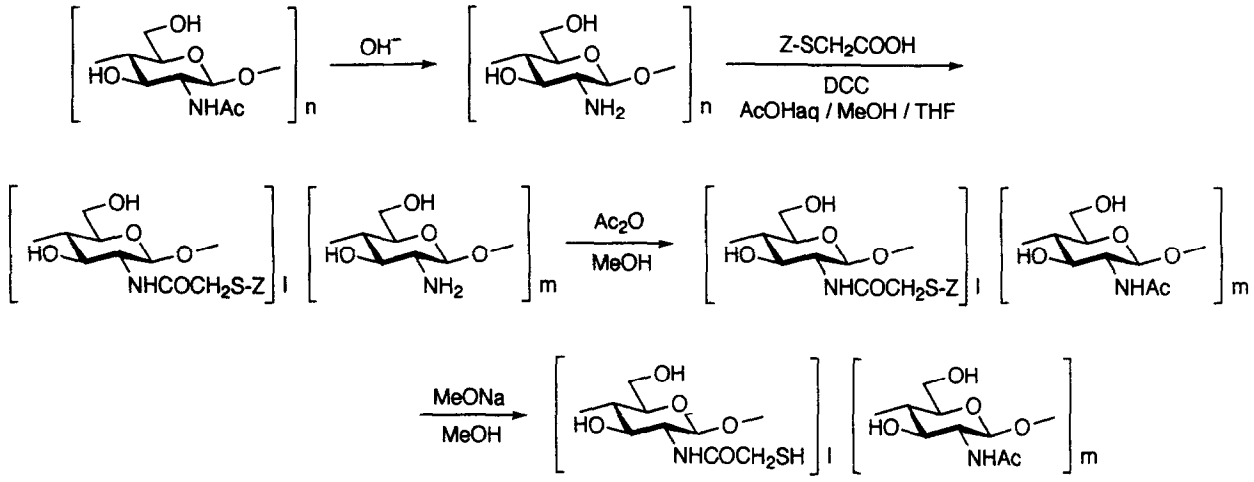
Mercapto-chitins were prepared by series of modification reactions starting from chitin and chitosan (Kurita *et al.*, 1993). Chitosan was N-acylated with *S*-(benzyloxycarbonyl)mercaptoacetic acid, and the remaining free amino groups were selectively acetylated with acetic anhydride. S-Deprotection of the product gave rise to 2-mercapto-chitin (Scheme 1). To prepare 6-mercapto-chitin, chitin was first tosylated to give tosyl-chitin. It was subjected to thioacetylation with potassium thioacetate followed by S-deacetylation (Scheme 2).

Optimal pH for immobilization

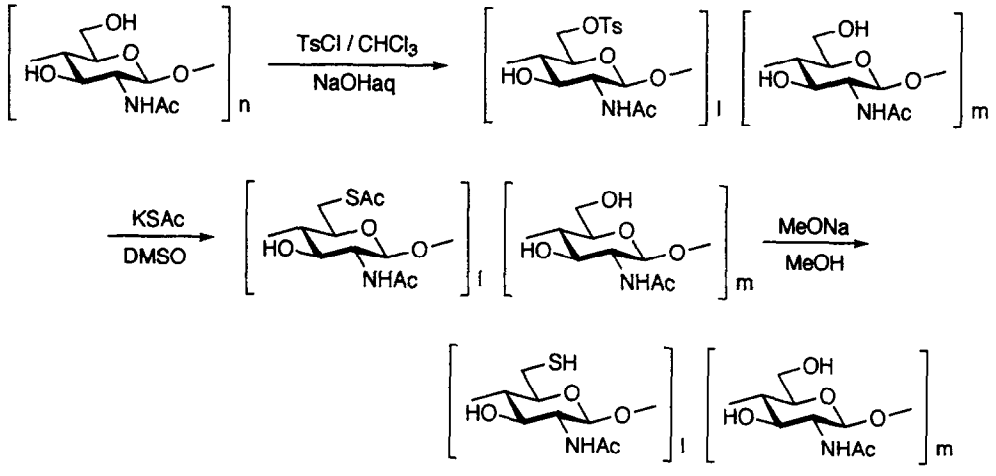
The acid phosphatase was immobilized on 2-mercapto- and 6-mercapto-chitins at 10°C in acetate or Tris buffer of various pH values to determine the pH optima for immobilization. The resulting mercapto-chitin/enzyme conjugates were assayed with 4-nitrophenyl phosphate by UV spectroscopy. The optimal pH corresponding to the highest activity was 4.5 for the immobilization on 2-mercapto-chitin. The other activities were thus evaluated as values relative to this highest activity, and the data are illustrated in Fig. 1. 6-Mercapto-chitin/enzyme was found to show lower activity than 2-mercapto-chitin/enzyme. It showed the highest activity when prepared at pH 4.8, and close to pH 4.5 for 2-mercapto-chitin/enzyme.

Amount of immobilized enzyme

2-Mercapto- and 6-mercapto-chitins were therefore treated with the acid phosphatase in acetate buffer of pH 4.5 and 4.8, respectively, and the supernatants were



Scheme 1



Scheme 2

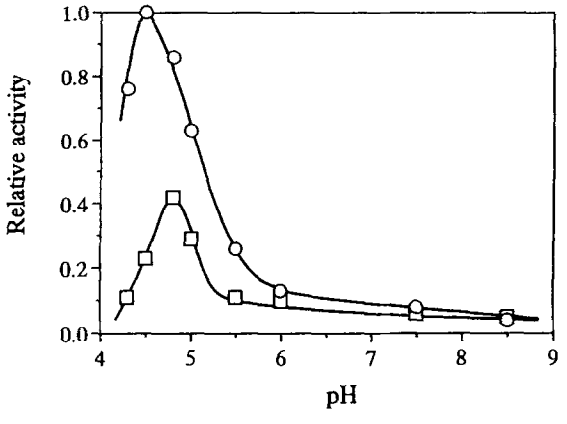


Fig. 1. Effect of pH on the immobilization of acid phosphatase on mercapto-chitins: (○) 2-mercapto-chitin; (□) 6-mercapto-chitin.

assayed with 4-nitrophenyl phosphate. On the basis of the activities of the supernatants, the amounts of enzyme immobilized on the mercapto-chitins were determined. For 50 mg of the supports, the amounts of enzyme immobilized on 2-mercapto- and 6-mercapto-chitins were 4.5 and 2.0 mg, respectively.

Optimal pH for the immobilized enzymes

In order to assess the activity profiles of the immobilized enzymes, the effect of pH values on the activity of the soluble acid phosphatase was first examined, and the results are shown in Fig. 2. The native enzyme exhibited the highest activity at pH 5.5.

The 2-mercapto-chitin/enzyme and 6-mercapto-chitin/enzyme conjugates prepared at pH 4.5 and 4.8, respectively, were then assayed at various pH values to establish the pH optima, and the results are shown in Fig. 2. The activities of the two kinds of conjugates were calculated to be relative to the highest value of the soluble enzyme of the same amount. In Fig. 2, the highest activities were observed at pH 6.0 and 5.5 for 2-mercapto-chitin/enzyme and 6-mercapto-chitin/enzyme, respectively, indicating that the optimal pH values were not changed much on immobilization, particularly with 6-mercapto-chitin. This is noteworthy when compared

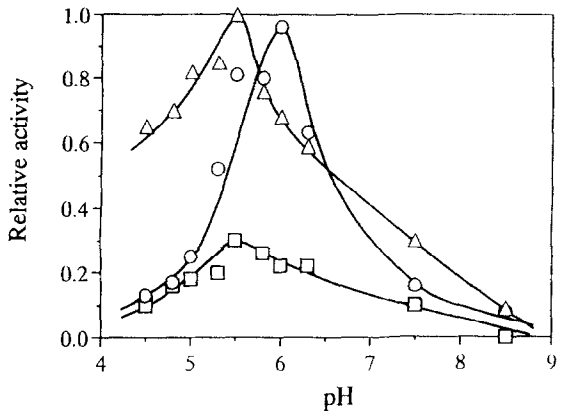


Fig. 2. pH dependences of acid phosphatase activity: (△) soluble enzyme; (○) enzyme on 2-mercapto-chitin; (□) enzyme on 6-mercapto-chitin.

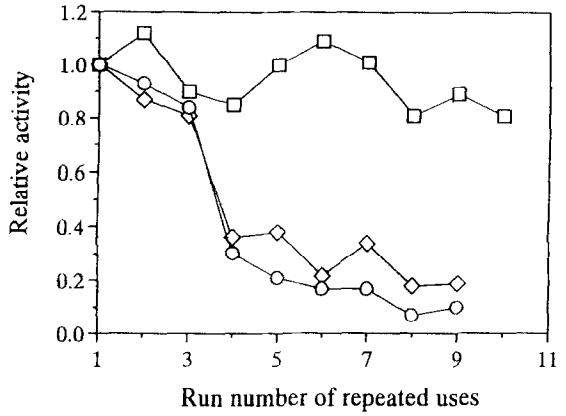


Fig. 3. Changes in the activity of immobilized acid phosphatases in repeated uses: (○) enzyme on 2-mercapto-chitin; (□) enzyme on 6-mercapto-chitin; (◇) enzyme on chitin.

with the results thus far reported; the optimal pH of the immobilized acid phosphatase shifted to the acid side. 4.8 for the immobilized enzyme on chitin with glutaraldehyde (Stanley *et al.*, 1975), 5.0 for that on chitosan (Muzzarelli *et al.*, 1976), and 4.5 for that on glass beads with glutaraldehyde (Ohmiya *et al.*, 1983).

As indicated in Fig. 2, the enzyme immobilized on 2-mercapto-chitin and 6-mercapto-chitin retained 96 and 30% of the activity of the soluble enzyme at optimal pH values.

Repeated uses of the immobilized enzymes

2-Mercapto-chitin/enzyme and 6-mercapto-chitin/enzyme were used repeatedly for the hydrolysis of 4-nitrophenyl phosphate in batch systems to elucidate the effectiveness of the immobilization on these mercapto-chitins. For comparison, chitin was treated with the acid phosphatase under the same conditions to effect possible immobilization by adsorption, and the resulting conjugate was also assayed for comparison.

Changes in the activity in repeated uses of the three kinds of conjugates are illustrated in Fig. 3 where the activity values are shown as relative to those of the corresponding first runs. 2-Mercapto-chitin/enzyme showed a marked reduction in the activity after a few runs, and the behavior is quite similar to that of chitin/enzyme. This implies that the immobilization on 2-mercapto-chitin was not due to covalent bond formation as in the immobilization on chitin. In sharp contrast, 6-mercapto-chitin/enzyme exhibited 80% of the initial activity even in the 10th run, indicating high durability.

CONCLUSION

Both the mercapto-chitins appear to immobilize the acid phosphatase. Despite the high initial activity of the 2-mercapto-chitin/enzyme, the conjugate showed a sharp reduction of the activity in extended use, suggesting that the enzyme was fixed only loosely, probably by adsorption. A remarkable difference was observed for the conjugate with 6-mercapto-chitin, which retained high activity even after repeated uses. The activity yield of the 6-mercapto-chitin/enzyme was 30% based on the activity of the soluble enzyme as shown in Fig. 2, while the reported activities were 20% for the enzyme on chitin with glutaraldehyde (Stanley *et al.*, 1975b) and 65% for that on chitosan without any intermediate reagent (Muzzarelli *et al.*, 1976). These results indicate that 6-mercapto-chitin is a promising support for effective immobilization of certain enzymes.

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