Immobilization of Invertase on Carboxymethylchitosan and Chitosan

Hideo KUSAOKE

Abstract

An immobilization method using carboxymethylchitosan prepared from chitosan as an insoluble carrier was investigated. Invertase was immobilized on carboxymethylchitosan using the bifunctional agent, glutaraldehyde. The most active preparation was based on the enzyme bound carboxymethylchitosan with low degree of substitution (d. s.) such as 0.41. Its activity yield was 24.1 %. The activity of the immobilized enzyme decreased as the d. s. of carboxymethyl groups increased. Compared with free invertase, immobilized enzyme exhibited similar pH optimum but more activity at the range of pH 5−7. Immobilized enzyme improved thermal stability. The immobilized enzyme retained *ca.* 70 % of the initial activity even after the repeated use of 10 times.

1. Introduction

Research on chitin and its derivatives has been developed rapidly, as witnessed by the publification of several books and reviews.[1-5] Chitin and chitosan have been reported to be available as supports for several enzymes; chitin has been recently used as a solid support to immobilize α−chymotrypsin,[6] α−galactosiadae,[7] β−galactosidase,[8] glucose isomerase,[9,10] glucoamylase,[11] α−amylase,[12] papain,[13] β−amylase,[14] and acid phosphatase[6] and invertase[15] and chitosan to immobilize trypsin,[16,17] protease,[18] β−glucosidase,[19] glucose isomerase,[17] glucose oxidase,[17] urease,[17] lysozyme,[20] pepsin,[21] and alkaline phosphatase.[21] Immobilization of those enzymes on chitin and chitosan can be accomplished by means of such procedures as simple adsorption,[20,22,23] cross-linking of enzyme and support with dialdehydes,[6-19] and enclosure in gel.[21] In these studies, the use of chitosan derivatives as support materials for enzymes have not been reported to be available.

In our previous papers, glycolchitosan,[24] partially N− and O−acylated chitosan gel,[25] and chitosan-aldehyde gel[26] have been effectively used as supports for immobilization of enzymes, using a bifunctional reagent, glutaraldehyde.

In this study, we prepared carboxymethylchitosan (CM−chitosan) by the introduction of carboxymethyl (CM) groups to chitosan molecule as an affinity site for enzyme, and immodilized invertase on CM−chitosan with glutaraldehyde. This paper describes the preparation and some properties of the immobilized invertase and compares enzyme activities on CM−chitosans with various degrees of substitution (d. s.) and chitosan.

2. Materials and methods

2. 1 Materials and reagents

Chitosan was supplied by Tokyo Kasei Kogyo Co., Ltd. and the degree of deacetylation of chitin was 83%, measured by potentiometric titrations.[1] Invertase derived from *Batillus* strain was purchased from SIGMA Chemical Industries, Ltd. Glutaraldehyde, monochloroacetic acid, and sucrose as a substrate were purchased from Wako Pure Chemicals Industries. Elemental analyses were performed at the Elemental Analysis Center of Research Laboratory of Resourses Utilization of Tokyo Institute of Technology, Tokyo.

2. 2 Preparation of CM−chitosans

CM−chitosan was prepared by the method of Trujillo with a little modification;[27] Chitosan (4 g) was mixed with dimethylsulfoxide (20 ml) overnight. The excess solvent was removed by filtration and chitosan cake was then washed with ethyl alcohol. Chitosan was added to 42% NaOH (50 ml), and the slurry was stirred for 1hr. The mixture was filtered, and the wet cake obtained by pressing.

To 2-propanol solution (50 ml) of chitosan cake, the corresponding amount of monochloroacetic acid was added in small portions. The reaction mixture was stirred at room temperature overnight. The product collected by filtration was added to water (200 ml), the pH of which was adjusted to neutrality with conc. HCl. The mixture was stirred to the viscous solution. The product was precipitated by pouring the solution into one liter of acetone, washed with ethanol, and dried at 50−60 °C in oven. The d. s. of CM groups in CM−chitosan was calculated from the ratio of carbon to nitrogen as found by elemental analyses (see Table 1). The infrared spectrum of CM−chitosan showed a strong absorption peak of C=O in a free acid form at 1736 cm^{-1}.

2. 3 Immobilization of invertase on CM−chitosan

The dried CM−chitosan (0.5 g) was dissolved or swollen in 10% acetic acid (20 ml) and the mixture was adjusted to pH 6 with 4 N NaOH. Invertase (10 mg) in 1 ml of 0.05 M

Table 1 Preparation of CM−chitosans

Sample No.	Monochloroacetic acid / Hexosaminide residue	Carbon / Nitrogen (w/w)	D. s.[1)]
1	1.2	5.44	0.61
2	2.4	5.84	0.41
3	5.2	7.75	1.52
4	8.4	6.44	0.76

1) The degree of substitution of carboxymethyl groups in CM−chitosan was calculated from the carbon / nitrogen values.

acetate buffer (pH 5) was added to the mixture in ice-water bath. An aliquot of 25 % glutaraldehyde was then added so that the final concentration of glutaraldehyde was approximatey 2 %.[8)] The mixture was kept for one hour in ice-water bath with occational stirring, and a light-brown precipiate of immobilized enzyme formed stored overnight at 4 °C.

The resinous product was collected by filtration, and washed with distilled water (50 ml), soaked with 3 M NaCl (25 ml), 0.1 M acetate buffer at pH 7 (25 ml) and 0.1 M acetate buffer at pH 4 (25 ml) and finally washed with distilled water (50 ml). This treatment was repeated twice. The immobilized enzyme on CM−chitosan was lyophilized to give a powdered sample for enzyme assays.

Immobilization of enzyme on chitosan was carried out on the basis of the method of Tsumura et al.[17)]

2. 4 Assay of invertase activities

Soluble invertase activity was determined by incubating aliquots containing 0.5 mg of enzyme in solution which was 0.3 M sucrose (1 ml), 0.04 M acetate buffer at pH 4.2 (5 ml), and distilled water (4 ml). After incubation for 20 min at 40 °C, the enzyme was inactivated by heating in boiling water for 5 min.

In the assay of CM−chitosan bound enzyme activity, 1/20 of total amount of immobilized enzyme prepared was weighed accurately and used instead of 0.5 mg of enzyme. The reaction mixture was incubated for 20 min at 40 °C with stirring. The reaction was stopped by heating in boiling water for 5 min. The mixture was filtered, and glucose liberated in filtrate was determined by the glucose oxidase-chromogen (Glucostat) procedure (kits supplied by the Boehringer. Mannheim).

Table 2　Immobilization of Invertase on CM－chitosan

Supports	d. s.	Immobilized enzyme activity (Units)	Activity yield (%)
CM－chitosan	0.41	83	24.1
CM－chitosan	0.61	63	18.3
CM－chitosan	0.76	37	10.8
CM－chitosan	1.52	72	20.9
Chitosan		93	27.0

The activity yield was expressed as the percentage of immobilized enzyme activity to added enzyme (344 units).

3. Results and Discussion

3. 1 Activity yield of invertase immobilized on CM－chitosan

Activity yields of invertase immobilized on CM－chitosan with various d. s. of CM groups and chitosan are shown in Table 2. Activity yields of CM－chitosan bound enzyme were 10－24 %, and its maximum activity yield, which was 24.1 % in the treatment with CM－chitosan with low d. s. of 0.41, showed a slight decrease in comparison with that of chitosan (27 %). The remarkable effect of introduction of CM groups to chitosan molecule as functional groups on the activity of the immobilized enzyme was not found.

The effects of d. s. of CM groups in CM－chitosan on the activity and water contents of the immobilized enzyme prepared from 0.5 g of CM－chitosan are shown in Fig. 1. The wet weight of immobilized enzyme increased with the decrease of d. s. of CM groups in CM－chitosan. The activity of the immobilized enzyme decreased as the d. s. of CM groups increased. Chitosan, one of supports tested, showed the highest values of both wet weight (*ca.* 16 g) and activity of immobilized invertase (93 units). From these results, the amounts of water in wet support proved to affect the activity of immobi-

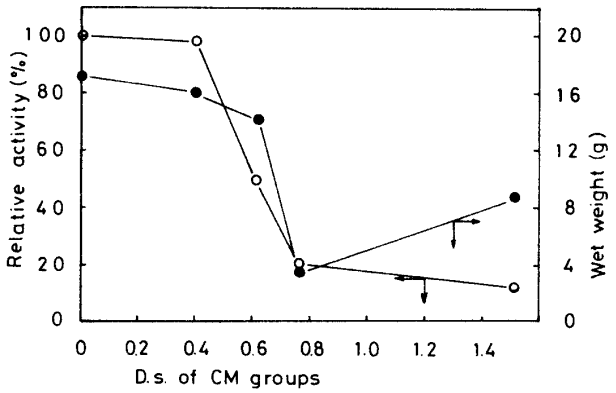

Fig. 1　Effect of D. s. of CM Groups in CM－chitosan on Activity and Water Contents of Immobilized Enzyme

The activity of enzyme immobilized on CM－chitosan with various d. s. of CM groups with glutaraldehyde was based on 20－min assays at 40 °C using 0.05 M acetate buffer of pH 4.2. Water amounts were measured as the wet weight of immobilized enzyme prepared from 0.5 g of CM－chitosan.

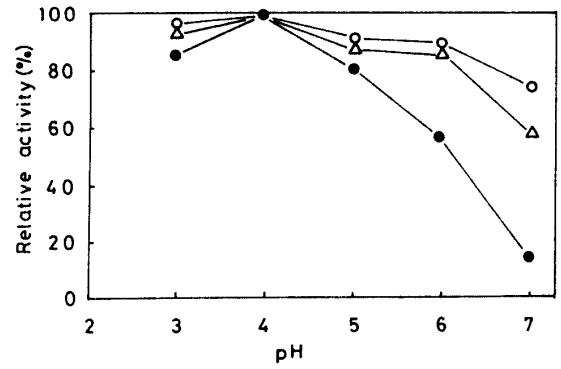

Fig. 2 Effect of pH on Invertase Activity.

Activity was based on 20-min assays at 40 °C in acetate buffer. Symbols: (●); free enzyme, (○); enzyme immobilized on CM-chitosan with d. s. of CM groups of 0.41, and (△); enzyme immobilized on chitosan.

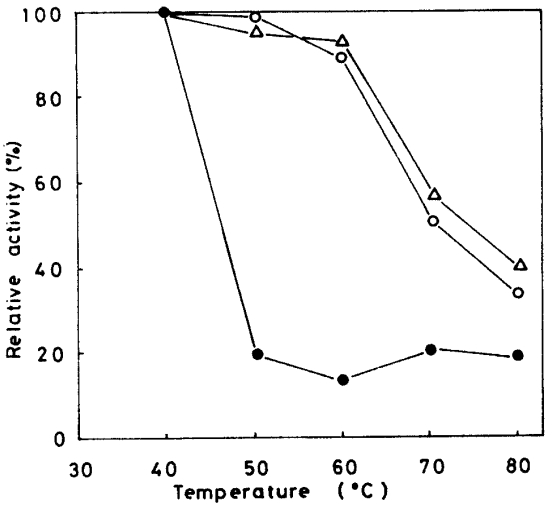

Fig. 3 Effect of Temperature on Invertase Activity.

Activity was based on 20-min assays at pH 4.2 in acetate buffer. Symbols: (●); free enzyme, (○); enzyme immobilized on CM-chitosan with d. s. of CM groups of 0.41, (△); enzyme immobilized on chitosan.

lized enzyme.

3. 2 Properties of immobilized invertase

The effects of pH on the activity of invertase are shown in Fig. 2, in comparison with those of native enzyme. The pH optimum for both the immobilized and free form of the enzyme was approximately pH 4 at the acidic range of pH from 3.0 to 7.0. However, the immobilized enzyme showed greater activity at higher pH than pH 4.

The effects of temperature on the stability of the enzyme are shown in Fig. 3. Immobilized enzyme nearly retained its original activity below 60 °C, but native enzyme showed the steep drop of activity at about 50 °C. Immobilized enzyme proved to be more thermostable than the native enzyme.

3. 3 Repeating use of immobilized invertase

The stability of invertase immobilized on CM-chitosan (d. s. of CM groups of 0.41) or chitosan on repeated use was tested by measuring the activity for the formation of glucose by hydrolysis of sucrose. The solution of 0.3 M sucrose (1 ml) in 0.1 M acetate buffer (pH 4.2) was added to the mixture of 9 ml of a suspension containing 150 mg as wet weight of immobilized enzyme (83 units on CM-chitosan or 93 units on chitosan) and the reaction mixture was incubated for 20 min at 40 °C with stirring, as described in the experimental section. At the end of the reaction, the immobilized enzyme was collected by filtration, washed with water and resuspended in a reaction mixture of 1 ml of 0.3 M sucrose in 0.1 M acetate buffer at pH 4.2, 4 ml of 0.05 M acetate buffer (pH 4.2) and 5 ml of water. The enzyme

assay was repeated 10 times in the same reaction. As shown in Fig. 4, enzymes immobilized on both CM−chitosan and chitosan retained about 70 % of the original enzyme activity after the repeated use for 10 times. These data would be of advantage in the hydrolysis of sucrose. This will assure the available application as the supports for the bioreactor.

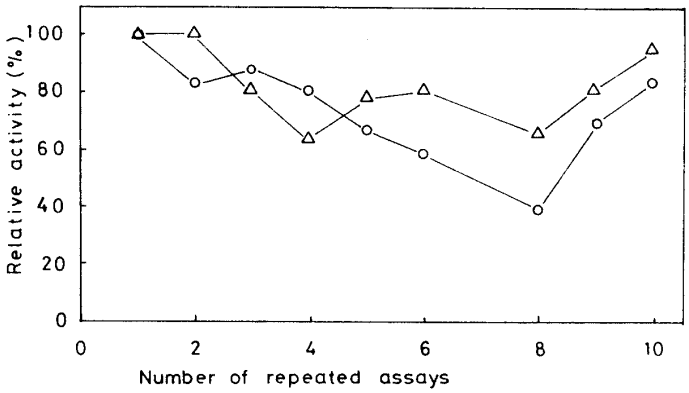

Fig. 4　Effect of Repeated Assays on Activity of immobilized Invertase

The reaction condition is described in the text. At the end of the reaction, the mixture was filtered and the filtrate was boiled for 5 min. Invertase activity of immobilized enzyme was assayed by measuring the amount of glucose released. The precipitate was repeatedly used for activity assays of immobilized invertase.

Symbols: (○); enzyme immobilized on CM−chitosan with d. s. of CM groups of 0.41, (△); enzyme immobilized on chitosan.

Refferences

1) R. A. A. Muzzarelli, "CHITIN", Pregamon Press, London, 1977.
2) J. P. Zikakis, "CHITIN CHITOSAN AND ELATED ENZYMES", Academic Press, New York, 1984.
3) R. A. A. Muzzarelli, *Carbohydrate Polymers*, **3**, 53 (1983).
4) S. Hirano, Protein, *Nucleic acid, and Enzyme*, **22**, 59 (1977).
5) S. Tokura, *Kobunshi Kako*, **32**,18 (1983).
6) R. A. A. Muzzarelli G. Barontini, R. Rocchetti, *Biotechnol. Bioeng.*, **18**, 1445 (1976).
7) M. Mitsutomi, Y. Uchida, and A. Ohtakara, *J. Ferment. Technol.*, **63**, 325 (1985).
8) W. L. Stanley, G. G. Watters, B. Chan, and J. M. Mercer, Biotechnol. *Biotechnol. Bioeng.*, **17**, 315 (1975).
9) W. L. Stanley, G. G. Watters, S. H. Kelley, B. Chan, J. A. Garibaldi, and J. E. Shade, *ibid.*, **18**, 439 (1976).
10) F. Chen, H. Weng, *ibid.*, **25**, 725 (1983).
11) W. L. Stanley, G. G. Watters, S. H. Kelley, A. C. Olson, *ibid.*, **20**, 135 (1978).
12) P. O. Flor and S. Hayashida, *ibid.*, **25**, 1973 (1983).
13) J. W. Finley, W. L. Stanley, G. G. Watters, *ibid.*, **19**, 1985 (1977).
14) J. Synowiecki, Z. E. Sikorski, M. Naczk, H. Piotrkowaska, *ibid.*, **24**, 1871 (1982).
15) J. Synowiecki, Z. E. Sikorski, and M. Naczk, *ibid.*, **23**, 231 (1981).
16) Y. Nozawa, T. Matsushita, K. Yamashina, and F. Higashide, *ibid.*, **24**, 753 (1982).
17) T. Kasumi, M. Tsuji, K. Hayashi, and N. Tsumura, *Agric. Biol. Chem.*, **41**, 1865 (1977).
18) J. Leuba, and F. Widmer, *Biotechnol. Letters*, **1**, 109 (1979).
19) F. Bissett and D. Sterberg, *Appl. Environ. Microbiol.*, **35**, 750 (1978).
20) R. Rocchetti, *Biotechnol. Bioeng.*, **20**, 87 (1978).
21) S. Hirano and O. Miura, *ibid.*, **21**, 711 (1979).
22) J. F. Kennedy and C. E. Doyle, *Carbohydr. Res.*, **28**, 89 (1973).
23) R. Yamaguchi, Y. Arai, S. Hirano, and I. Ito, *Agric. Biol. Chem.*, **42**, 1297 (1978).

24) H. Kusaoke, K. Kasaba, and T. Sakurai, *Sen-i Gakkaishi*, **43**, 495 (1987).
25) H. Kusaoke, S. Iso, K. Hirose, T. Sakurai, and K. Kimura, *Sen-i Gakkaishi*, in press.
26) H. Kusaoke, T. Nihei, H. Kusano, K. Kimura, *Sen-i Gakkaishi*, in press.
27) R. Trujillo, *Carbohydr. Res.*, **7**, 483 (1968).