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IMMOBILIZED α -AMYLASE WITH HIGH ACTIVITY*By Machiko Shimada, Fumiharu Kokubun and Yoshio Nakamura*(Department of Chemistry, Faculty of Technology,
Gunma University, Kiryu, Gunma 376)**Synopsis**

Acrylic acid grafted cellulose was used as insoluble carrier for immobilization of α -amylase. Amino group of enzyme was coupled with carboxyl group of acrylic acid grafted cellulose by water soluble carbodiimide. And the activity of immobilized α -amylase to soluble starch was compared with that of free enzyme. In order to obtain the immobilized enzyme with high activity, various methods were tried. One of them is three steps reaction; the first step is the formation of ionic bond between carboxyl group and amino group, the second step is the formation of covalent bond between the residual carboxyl group and amino group, and the third step is the removal of ionic bond. As the result, immobilized α -amylase with high activity was obtained. The activity was 65% of that of free α -amylase. The value is very high as compared with those by other methods as polymer was used as substrate.

INTRODUCTION

Many workers have reported on immobilization of enzyme.¹⁻⁵⁾ Some of them^{4,5)} used grafted polymers as insoluble carriers to water. And they have found that grafted polymers are useful as carriers.

We have carried out grafting reaction onto cellulose⁶⁻⁸⁾ and nylon^{6,9)} by γ -ray irradiation, and have found that grafting reaction is an excellent method to introduce new properties to trunk polymers without disturbing their original good properties. In this study, acrylic acid grafted cellulose was used as insoluble carrier for immobilization of α -amylase, for it has many carboxyl groups to couple with amino groups in enzyme, specific surface area is very large, and hydrophilicity increases with grafting reaction. In many experiments, enzymatic activity decreased after immobilization when polymer was used as substrate. In order to make the decrease minimum, protection of active site during immobilization reaction and efforts to make conformational change of enzyme minimum were attempted in this study.

EXPERIMENTAL**Materials**

Scoured cotton cellulosic fibers of the Acala variety was purified by extracting with hot ethanol-benzene mixture (1:1 volume ratio). Then the fibers were washed with methanol and water prior to drying.

Crystallized α -amylase from *Bacillus subtilis* was supplied by Nagase Biochemical, Ltd.

Acrylic acid was distilled under reduced pressure before use.

Other chemicals used were reagent grade and were used without further purification.

Graft Copolymerization

After cellulose was dried under reduced pressure at 50°C for 20 h, it was irradiated with ⁶⁰Co γ -rays at an exposure rate of 1.0×10^6 R/h for 1 h in nitrogen atmosphere at room temperature.

Graft copolymerization of acrylic acid onto the irradiated cellulose was carried out in the reaction system containing 10% acrylic acid and 90% water under nitrogen atmosphere at 30°C. The liquor to material ratio was 100. After graft copolymerization, the samples were extracted with hot water

to remove homopolymer.

Extent of grafting was expressed as weight percent increase by grafted polymer to original weight of cellulose.

Immobilization Reaction

Diethylaminopropyl carbodiimide hydrochloride (water soluble carbodiimide, WSC) was dissolved in 0.2 M acetate buffer solution at pH 4.7. In the solution, acrylic acid grafted cellulose was immersed for 2 min at room temperature. Then, α -amylase was added to the mixture and incubated at 10°C for 12 h. The extent of grafting of the samples was about 50%. The ratio of each component was as follows:

$$AA/WSC = 1/0.2 \text{ (mol/mol)}$$

$$AA/\alpha\text{-amylase} = 1/0.5 \text{ (wt/wt)}$$

where AA stands for acrylic acid in grafted cellulose.

After the immobilization reaction, samples were washed with water and immersed in 2 M acetate buffer solution at pH 4.4 for 6 h at 10°C to remove water soluble carbodiimide and ionically bonded α -amylase. For some samples, nitrogen analysis was carried out by using Yanagimoto MT 3 Element Analyzer.

Some of the reactions were carried out with three steps. The first step was the reaction of acrylic acid grafted cellulose with α -amylase in 0.2 M acetate buffer solution at 10°C for 2 h. In the second step, water soluble carbodiimide was added to the reaction mixture and stirred for 2 min, and then α -amylase was added and reacted for 10 h at 10°C. In the third step, the samples were immersed in 2 M acetate buffer solution at pH 4.4 for 5 h at 10°C.

Assay of α -Amylase Activity

Free α -amylase and immobilized α -amylase were added to the solution containing 0.1 M

acetate buffer solution and 10% soluble starch, and incubated for 5 min at 38°C. The values of pH were 5.9 and 7.0 for free and immobilized α -amylase, respectively.

Before checking reducing end groups, next procedure was carried out to avoid the influence of protein. After the hydrolysis, 1 ml of the solution was poured into the test tube containing 0.5 ml of 0.3 N ZnSO₄ solution. And immediately, 0.5 ml of Ba(OH)₂ solution was added and shaken, and then the tube was centrifuged for 5 min at 5000 rpm. The supernatant was used to check the amount of reducing end group by Nerson-Somogyi method.^{10,11)}

RESULTS AND DISCUSSION

The reactions of acrylic acid grafted cellulose (Cell-COOH) with water soluble carbodiimide (WSC) and α -amylase were carried out. After the reactions, the samples were washed with distilled water and weighed. The results are shown in Table I. Weight increases by the reactions mean that acrylic acid grafted cellulose reacted with water soluble carbodiimide and α -amylase independently. After weighing, the samples were immersed in 2 M acetate buffer solution, washed with distilled water, dried, and weighed. For some samples, nitrogen contents were analyzed. The results are shown in Table I. Water soluble carbodiimide and α -amylase which reacted independently with acrylic acid grafted cellulose could easily be removed by immersion in 2 M acetate buffer solution. But weight of the sample which reacted with water soluble carbodiimide and α -amylase at the same time did not decrease to the original weight after immersion in buffer solution. And it was also clarified that grafted cellulose reacted with α -amylase to yield im-

Table I. Reactions of acrylic acid grafted cellulose with water soluble carbodiimide and α -amylase.

W_0 (g)	Reacted with	W_1 (g)	(W_1/W_0) (%)	W_2 (g)	(W_2/W_0) (%)	N content (%)
0.1669	WSC	0.1709	(2.4)	0.1661	(0)	0
0.4096	α -amylase	0.4316	(5.4)	0.4076	(0)	0
0.4424	WSC + α -amylase	0.4649	(5.1)	0.4571	(3.3)	—

W_0 : Initial weight of acrylic acid grafted cellulose.

W_1 : Weight of sample after reaction in 0.2 M acetate buffer solution at pH 4.7.

W_2 : Weight of sample after immersion in 2 M acetate buffer solution at pH 4.4.

mobilized enzyme after reaction with water soluble carbodiimide and drying. Therefore, next mechanism is inferred as the immobilization process.

It is also clear that water soluble carbodiimide and α -amylase ionically bonded are easily removed from grafted cellulose by immersion in 2 M acetate buffer solution. And α -amylase covalently bonded is left on cellulose. The enzymatic activities of immobilized α -amylase with ionic bond and covalent bond were 60% and 20% of free enzyme, respectively. Protein was detected by biuret reaction during hydrolysis of soluble starch owing to α -amylase ionically bonded. But for α -amylase covalently bonded, no protein was found during hydrolysis of starch. As α -amylase ionically bonded is easy to leave from carboxyl group during hydrolysis of starch, the residual activity is rather

high as compared with that of α -amylase covalently bonded. Therefore, the decrease of activity by immobilization is elucidated by the change of conformation of enzyme. In order to obtain immobilized enzyme with high activity, conformational change by immobilization should be minimized.

The molecular weight of α -amylase is reported to be 45,000–59,000.¹²⁾ And molecular weight of α -amylase per free amino group was found to be 4,000 by measuring free amino groups in α -amylase. Therefore, there are about 10–15 free amino groups in one α -amylase molecule. According to Matsuura et al¹³⁾, histidine, lysine, aspartic acid, and tyrosine are distributed in active site of amylase. Therefore, many amino groups in amylase react with carboxyl groups in acrylic acid grafted cellulose. It is expected that the more the reaction sites the immobilized enzyme has, the less the residual activity is.

As shown in Table I, ionic bond is formed easier than covalent bond, and easily removed by 2 M acetate buffer solution. Considering these properties, various trials were carried out to obtain immobilized enzyme with high residual activity. Weight increase and relative activity of immobilized enzyme compared with that of free enzyme are shown in Table II. The samples (reaction number 1 and 2) were immobilized with the ordinary method; that means acrylic acid grafted cellulose reacted with water soluble carbodiimide for 2 min and then reacted with α -amylase. Three samples (reaction number 3–5) were immobilized by three

Table II. Immobilization of α -amylase on acrylic acid grafted cellulose by various methods.

Reaction number	W_0 (g)	A_1	Washing after first step	A_2	W_1 (g)	$(W_1/W_0 (\%))$	W_2 (g)	$(W_2/W_0 (\%))$	Relative* activity (%)
1	0.4424	—	—	0.50	0.4649	(5.1)	0.4571	(3.3)	24
2	0.2286	—	—	1.00	0.2432	(6.4)	0.2362	(3.3)	33
3	0.2597	0.10	—	0.50	0.2802	(7.9)	0.2767	(6.6)	54
4	0.2428	0.25	—	0.50	0.2596	(6.9)	0.2545	(4.8)	56
5	0.2570	0.50	—	0.50	0.2760	(7.4)	0.2700	(5.0)	54
6	0.2120	0.50	water	0	0.2230	(5.2)	0.2184	(3.0)	65

W_0 : Initial weight of acrylic acid grafted cellulose.

A_1 : Weight of α -amylase used in the first step/weight of acrylic acid in grafted cellulose.

A_2 : Weight of α -amylase used in the second step/weight of acrylic acid in grafted cellulose.

W_1 : Weight of sample after the second step.

W_2 : Weight of sample after the third step.

*: Specific activity of immobilized amylase at pH 7/specific activity of free amylase at pH 5.9.

These pH values are the values where each enzyme shows maximum activity.

steps; the first step is the fixation of enzyme by ionic bond, the second step is the formation of covalent bond, and the third step is the removal of ionic bond. From these results, it is clear that three steps immobilization method is useful to increase the residual activity. But it is obscure that α -amylase bound ionically and/or free enzyme react with acrylic acid grafted cellulose to generate immobilized α -amylase with covalent bond in the second step. So one sample (reaction number 6) was washed with water to remove unreacted free α -amylase after the first step, and then reacted with water soluble carbodiimide. The result shows that the free amino group in α -amylase bonded ionically reacts with carboxyl group in acrylic acid grafted cellulose to form covalent bond in the presence of water soluble carbodiimide. The relative activity of the sample (reaction number 6) was 65% as compared to that of free enzyme. The value is very high as compared with other methods.

From the results mentioned above, mechanism of immobilization reaction is inferred as shown in Scheme 1. The more the ionic bond in the first step, the more the possibility for amino group in active site to react with carboxyl group. That means amino group in active site is protected from formation of covalent bond in the second step, and little amino group reacts with carboxyl group in the second step. Therefore, conformational change will be minimized and the immobilized enzyme shows high activity.

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Scheme 1. Insoluble carrier, acrylic acid grafted cellulose reacts with α -amylase in 0.2 M acetate buffer solution at pH 4.7 at 10°C for 2 h in the first step to form ionic bond. In the second step, residual carboxyl group and amino group react to form covalent bond in the presence of water soluble carbodiimide. In the third step ionic bonds are removed by immersing the sample in 2 M acetate buffer solution. And highly active α -amylase bonded covalently is left.

- (1) Bound α -amylase with ionic bond
  - (2) Bound α -amylase with ionic and covalent bonds
  - (3) Highly active immobilized α -amylase with covalent bond
- : ionic bond, $-\text{COO}^- \text{NH}_3^+ -$
 ~~~~~: covalent bond, $-\text{CONH}-$

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高活性を示す固定化 α -アミラーゼ

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アクリル酸グラフトセルロースを担体として α -アミラーゼの固定化を試みた。酵素のアミノ基とアクリル酸グラフトセルロースのカルボキシル基を水溶性カルボジイミドで反応させ、得られた固定化酵素の可溶性デンプンに対する活性をもとの酵素と比較した。高活性を示す固定化酵素を得るために種々の方法が試みられたが、そのうちで次の方法は非常に有効であった。すなわち、第一

段階でアクリル酸グラフトセルロースのカルボキシル基と酵素のアミノ基とをイオン結合で結合させ、第二段階で残存するカルボキシル基とアミノ基を共有結合で結合させ、第三段階でイオン結合を除去し、共有結合のみを残す方法である。その結果、もとの酵素の65%の活性を示す固定化酵素が得られた。これは高分子基質を用いた場合、非常に高い値である。