Chitin Coated Cellulose as an Adsorbent of lysozyme-like Enzymes

Preparation and Properties

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As a new adsorbent of lysozyme-like enzymes, chitin coated (CC)-cellulose was prepared. CC-cellulose was stable and had good flow properties for use in column chromatography. Affinity chromatography with CC-cellulose showed that 3~5 mg of lysozyme/ml resin was adsorbed specifically and desorbed quantitatively under mild conditions. The utilities of the method of affinity chromatography with CC-cellulose are discussed.

Affinity chromatography is a good and simple way to collect a specific enzyme.¹⁾ Many enzymes have been isolated with this method.¹⁾ In particular, when the substrate for the enzyme is a polymer and insoluble in water, the polymer substrate was directly used as an adsorbent.^{2~4)} Chitin was used as a specific adsorbent of lysozyme^{5~7)} and used for the separation of photooxidized lysozyme.⁸⁾ To improve the holding capacity and reproducibility of the adsorbent, the carboxymethyl (CM)-chitin^{*9)} and deaminated chitin¹⁰⁾ were developed. It was found that CM-chitin binds lysozyme tightly at pH 7~9 under low ionic strength and over a much wider pH range under high ionic strength, and that the lysozyme bound could be eluted quantitatively with 0.2 M acetic acid.⁹⁾ The results were similar to those reported for chitin columns.^{5,6)}

CM-chitin is gelatinous and the physical properties make it difficult to manipulate it.⁹⁾ As an improved adsorbent of lysozyme-like enzymes, chitin coated (CC)-cellulose was prepared and its properties were examined.

MATERIALS AND METHODS

Materials. Chitin was prepared from shrimp shells following the method of Offer.¹¹⁾ Cellulose

* Abbreviations used are: CC-cellulose; chitin coated cellulose, CM-chitin; carboxymethyl chitin, Trp 62-oxindole-lysozyme; lysozyme whose Trp 62 is oxidized to oxindolealanine.

powder (100~200 mesh) was the product of Toyo Roshi Co., Ltd. Lysozyme and Trp 62-oxindole-lysozyme were prepared as described previously.¹²⁾ Five times recrystallized ovalbumin was obtained from hen egg white following the method of Kekwic and Cannan.¹³⁾ Glycol chitin was prepared as described before.¹⁴⁾ *M. lysodeikticus* cells were obtained from Worthington. Sephadex G-75 (medium) was purchased from Pharmacia and Bio-Rex 70 (100~200 mesh) was the product of Bio Rad Lab. All reagents used were analytical or reagent grade.

Equipments. A Hitachi 124 Spectrophotometer was used for optical absorption measurements. pH was measured with a Hitachi-Horiba pH meter F-5.

Preparation of chitin coated cellulose. Alkali chitin was obtained following the method reported before.⁹⁾ Starting with 2.5 g of chitin, *ca.* 40 ml of alkali chitin was obtained (the volume was adjusted with 14% NaOH). Five grams of cellulose powder was added to the alkali chitin and the mixture was well kneaded. Then 80 ml of cold water was added to the mixture in several portions under kneading. Finally, 300 ml of 2 M acetic acid was poured into the mixture. The total procedure after getting alkali chitin took about one hour. The residue thus obtained was crashed with a spatula and washed with water and kept as a water suspension. The resin gave a good flow rate in column chromatography.

CC-cellulose column chromatography. As sample solutions (pH 8) containing 1 M NaCl was applied on a CC-cellulose column equilibrated with a starting buffer (0.1 M phosphate buffer (pH 8) containing 1 M NaCl). The column was first eluted with the starting buffer and then with 0.1 M acetic acid. The flow rates were *ca.* 10 ml/hr for the starting buffer elution and *ca.* 3 ml/hr for 0.1 M acetic acid elution.

All the procedures were performed at room temperature (10~20°C).

Analytical Bio-Rex 70 column chromatography. A sample solution (*ca.* 0.05 M in salt concentration, pH 10) was applied on a Bio-Rex 70 column (1.2 × 80 cm) equilibrated with a starting buffer (0.05 M borate carbonate buffer (pH 10.0)). Elution was performed with a linear gradient formed between 500 ml of the starting buffer and 500 ml of the same buffer containing 0.08 M NaCl.

Activity assays. Lysozyme activity was measured in two ways. The assay using glycol chitin has been previously reported.¹⁵⁾ In this study the assay was performed at pH 5.5, because this pH was found to be closer to the optimum pH for lysozyme under this assay condition. Lytic activity was measured using *M. lysodeikticus* cells. A tenth ml of a sample solution was added to 3 ml of cell suspension (O.D.₄₅₀ = 0.7) in 0.05 M, phosphate buffer (pH 7.0). After incubation for 10 min at 40°C, the decrease in absorption at 450 nm was read and compared with the curve obtained with a standard lysozyme solution (O.D.₂₈₀ = 0~0.08).

RESULTS

Specificity of adsorbent

Five mg of lysozyme was completely adsorbed on a 0.7 × 8 cm CC-cellulose column (3 ml resin) under the condition of pH 8 and 1 M NaCl. The adsorption under this condition was very strong and lysozyme was not

eluted even with 75 ml (25 times the bed volume) of elution with the starting buffer (0.1 M phosphate buffer (pH 8) containing 1 M NaCl). When 0.1 M acetic acid was employed as an eluent, lysozyme was eluted quantitatively (Fig. 1-a). Trp 62-oxindole-lysozyme showed much less affinity to the column (Fig. 1-b) which is in accord with its weak binding to the substrate.^{16, 17)} On the other hand, ovalbumin was eluted with the starting buffer in 6 ml. All ovalbumin was eluted in 12 ml and this is equivalent to 4 times the bed volume. No protein was eluted with 0.1 M acetic acid (Fig. 1-c).

Capacity and stability of CC-cellulose

An excess amount of lysozyme was applied on a CC-cellulose column (0.7 × 8 cm, 3 ml resin), and the column was washed with 8 times the bed volume of the starting buffer which is sufficient to remove noninteracting proteins. Then the column was eluted with 0.1 M acetic acid and *ca.* 15 mg of lysozyme was eluted. This procedure was repeated with the same column to examine the reproducibility and stability of the resin. The same amount of lysozyme was held in the second cycle. The chromatography shown in Fig. 1-a was repeated 3 times using the same

FIG. 1. CC-cellulose Column Chromatograph.

Five mg of lysozyme (a), 2 mg of Trp 62-oxindole-lysozyme (b) and 5 mg of ovalbumin (c) were applied on a CC-cellulose column (0.7 × 8 cm) and eluted with 0.1 M phosphate buffer (pH 8) containing 1 M NaCl and then with 0.1 M acetic acid. Arrows indicate the point of eluent change. The size of each fraction was 3 ml.

column and very reproducible results were obtained.

On the other hand, when 10 mg of lysozyme was applied on a column (0.7×8 cm) of the

FIG. 2. CC-cellulose Column Chromatograph of 10 mg of Lysozyme.

The Conditions were as in Fig. 1. Dotted line, on ten times expanded scale.

saturation amount of lysozyme was applied. Thus excess washing with the starting buffer should be avoided.

These results show that the capacity of CC-cellulose is 3 to 5 mg lysozyme/ml resin and that the resin is stable.

Isolation of lysozyme from hen egg white

Hen egg white was homogenized and 1 ml of the homogenate was diluted with 4 ml of 0.1 M phosphate buffer (pH 8.0). After the addition of NaCl to make 1 M, the solution was filtered and the filtrate (pH 8.3) was applied on a CC-cellulose column (1×8 cm) and chromatographed as described above. The result is shown in Fig. 3-a. The tailing part of the first peak (#6) showed an albumin rich protein spectrum but the latter peak (#15) showed a tryptophan rich protein spectrum (Fig. 3-b) which is characteristic for lysozyme. Fractions 6 and 9 showed little activity (less than 10%) against glycol chitin and bacterial substrates. Fraction 15 showed 182* and

FIG. 3. CC-cellulose Column Chromatograph of Hen Egg White and Absorption Spectra of the Fractions.

(a) One ml of hen egg white was applied on the column (1×8 cm) after dilution and eluted first with 0.1 M phosphate buffer (pH 8) containing 1 M NaCl and then with 0.1 M acetic acid. Arrow indicates eluent change. The size of each fraction was 3 ml. (b) The absorption spectrum of #6 is shown with dotted line and that of #15 with solid line.

same size, only 6% of lysozyme was eluted with the starting buffer after elution of 8 times the bed volume. As shown in Fig. 2, a gradual elution of lysozyme was observed when a near

95% activities against glycol chitin and the bacterial substrates, respectively.

* These samples showed usually high activities. The reason for this is under investigation.

Purity of the lysozyme isolated with CC-cellulose column

The purity of the lysozyme isolated with CC-cellulose column chromatography was examined with Sephadex G-75 column chromatography. Lysozyme is eluted from Sephadex columns much slower than usual proteins owing to its small molecular weight and specific affinity to Sephadex.^{17,18)} Sephadex column chromatography gives a good criterion of purity of lysozyme. Two ml of the latter peak (#15 in Fig. 3) were directly applied on a Sephadex G-75 column (1 × 103 cm) and eluted with 0.1 M acetate buffer (pH 5.0). The result is shown in Fig. 4. The

FIG. 4. Sephadex G-75 Column Chromatograph of Lysozyme Isolated with CC-Cellulose.

Two ml of #15 in Fig. 3 were directly applied on a Sephadex G-75 column (1 × 103 cm) and eluted with 0.1 M acetate buffer (pH 5.0). Broken line denotes the position of native lysozyme. The size of each fraction was 3 ml.

main peak was eluted at the position of native lysozyme (45 ml) and a minor peak was seen at around 30 ml position. The relative area of the main peak was 95%.

The purity of the lysozyme isolated with CC-cellulose column chromatography was also examined using analytical Bio-Rex 70 column chromatography. In this chromatography, the difference in one net charge of lysozyme shifts the position of elution by more than 200 ml to the either side of native lysozyme.** Following the same procedure as

** unpublished observation.

in Fig. 3, lysozyme was isolated from 5 ml of a homogenate of hen egg white. After adjusting to pH 10, the main fraction of the latter peak was applied on a Bio-Rex 70 column (1.2 × 80 cm) directly and chromatographed as described above. The elution pattern is shown in Fig. 5. A symmetrical

FIG. 5. Bio-Rex 70 Column Chromatograph of Lysozyme Isolated with CC-Cellulose.

After adjustment to pH 10, the lysozyme fraction isolated with CC-cellulose was directly applied on a Bio-Rex 70 column (1.2 × 80 cm) equilibrated with a starting buffer (0.05 M borate carbonate buffer (pH 10.0)) and eluted with a linear gradient formed between 500 ml of the starting buffer and 500 ml of the same buffer containing 0.08 M NaCl. Broken line denotes the position of native lysozyme. The size of each fraction was 5 ml.

main peak was eluted at the native lysozyme position and showed 140% activity against glycol chitin. A small amount (5%) of impurity was eluted in void volume. This result also showed that the purity of the lysozyme isolated with CC-cellulose was 95%.

DISCUSSION

Important requirements for the adsorbent of affinity chromatography are specificity, a good flow rate and a high capacity. High capacity includes firm adsorption and quantitative desorption under mild conditions. The stability of the adsorbent may be required, if a column is used repeatedly.

The specificity of CC-cellulose was examined

by passing three proteins through a CC-cellulose column. Lysozyme showed a strong affinity to the column and Trp 62-oxindole-lysozyme which has much weaker binding to the substrate showed a weak affinity to the column. All lysozyme adsorbed was desorbed quantitatively with 0.1 M acetic acid. On the other hand, ovalbumin which should have no interaction with the column was eluted in 2 times the bed volume. All ovalbumin was eluted in 4 times the bed volume and the elution of 6~8 times the bed volume should be quite sufficient to remove all foreign proteins. Ovalbumin which has the low isoelectric point is one of the good proteins to check against nonspecific adsorption to the column, because amino groups which could be produced by the deacetylation of N-acetyl groups are the only charged group expected on CC-cellulose.

Lysozyme isolated from hen egg white with CC-cellulose showed 95% of purity in both gel and ionexchange chromatographies. This is a good proof of specificity of CC-cellulose.

CC-cellulose showed a good flow rate (at least 50 ml/hr) when packed in a column (0.7×8 cm). This is a sufficient rate to perform column chromatography on a large scale.

The capacity of CC-cellulose was found to be 3~5 mg lysozyme/ml resin. This amount is considerable but not large. The reasons may be that the chitin micell is not destroyed well and that the resin contains twice the amount of filtrant (cellulose). Owing to its good flow rate, this defect can be compensated by using more resin in column chromatography.

As the adsorption of the enzyme to the resin is a result of binding equilibrium, a small amount of lysozyme was eluted with a starting buffer even under a strong binding condition (pH 8, 1 M in NaCl). After applying a sample, the amount of the washing eluent should be minimized.

The stability of CC-cellulose was demonstrated and a CC-cellulose column can be used repeatedly.

Thus CC-cellulose was found to have a considerable capacity to adsorb lysozyme specifically and be stable. All enzyme adsorbed could be eluted under mild conditions. CC-cellulose is superior to CM-chitin owing to its better physical properties and being devoid of carboxyl groups. CC-cellulose column chromatography is a good method to study the binding of lysozyme to its substrate or to separate modified lysozyme. Also it can be used to collect lysozyme-like enzymes from various sources and this is described in the following paper.¹⁹⁾

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