Note

N-Lower-fatty-acyl Derivatives of Chitosan as Adsorbents for Lysozyme and Chitinase

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Since chitin, (1→4)-2-acetamido-2-deoxy-β-D-glucan, was used as an adsorbent for lysozyme (EC 3.2.1.17) in affinity chromatography,[1] various adsorbents for lysozyme and chitinase (EC 3.2.1.14) have been reported.[2-7] These adsorbents have several disadvantages including their hydrolytic degradation during chromatographic operation, low capacity for adsorption, and time-consuming preparation. Both lysozyme and chitinase have a similar broad specificity[8]: *N*-propionyl and *N*-butyryl derivatives were hydrolyzed at a lower rate than crab shell chitin, and *N*-pentanoyl derivative was little hydrolyzed.

We now report that *N*-lower fatty-acyl derivatives of chitosan are novel adsorbents for these enzyme based on their specificity for the *N*-acyl groups of the substrate.

Bacthwise method. Fifty mg (>120 mesh) of crab shell chitin, or *N*-formyl, *N*-acetyl, *N*-propionyl, *N*-butyryl, or *N*-pentanoyl derivatives (d.s. 0.9–1.0 for *N*-acyl) of chitosan[9] was suspended in 3 ml of 0.05 M potassium phosphate buffer solution (pH 6.2), and the mixture was degassed with stirring under reduced pressure at room temperature for 30 min. One mg of lysozyme (hen egg-white, grade III, Sigma) dissolved in 1.0 ml of the same buffer solution was added. The mixture was stirred at 4°C for 2 hr. After centrifuging at 2,800 × *g* at 4°C for 15 min,

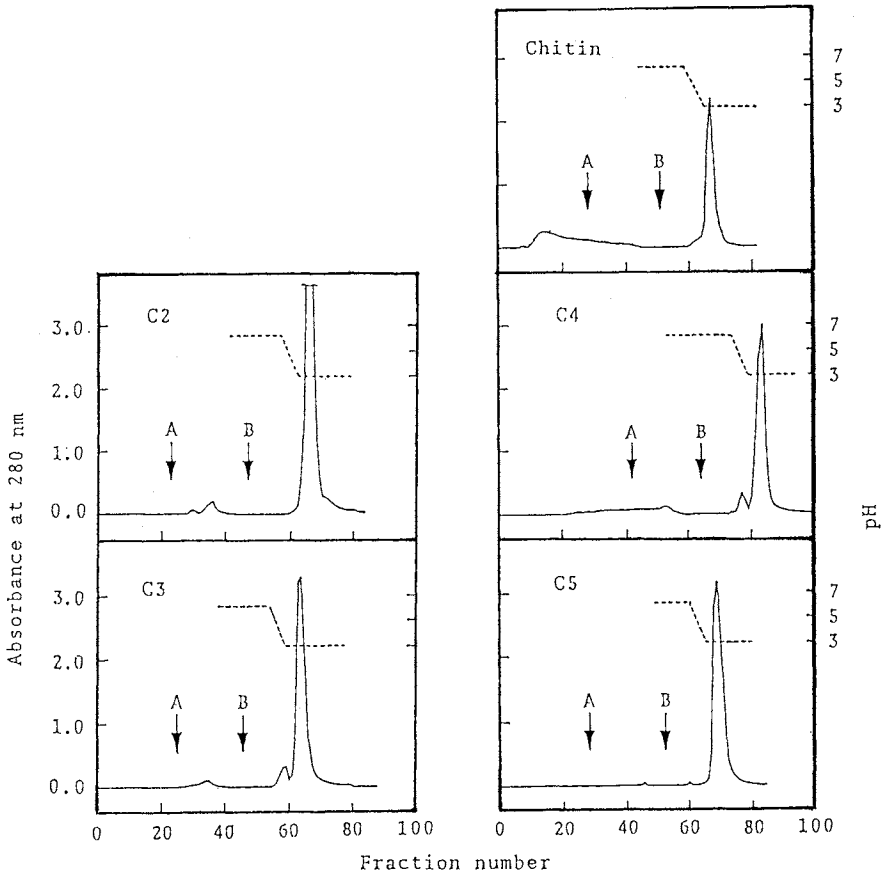

Fig. 1. Elution Profiles of the Affinity Chromatography of Lysozyme.

Column: 1.6 × 2.7 cm. Adsorbents: *N*-acetyl (C2), *N*-propionyl (C3), *N*-butyryl (C4), *N*-pentanoyl (C5) derivatives and crab shell chitin. See the text for the experimental details.

a 2-ml portion was withdrawn from the supernatant solution for analyzing non-adsorbed enzyme protein. Enzymic activity and protein were analyzed as reported previously.[9] The powdery products (>120 mesh) of these *N*-acyl derivatives of chitosan were stable in both aqueous acidic and alkaline solutions, and gave a good eluting rate as an adsorbent for chromatography. By the batchwise method, 1.0 mg each of crab shell chitin, and the *N*-formyl, *N*-acetyl, *N*-propionyl, *N*-butyryl, and *N*-pentanoyl derivatives of chitosan adsorbed 14, 14, 26, 23, 20, and 20 μg of the intact lysozyme protein, respectively. By the chromatographic method, the adsorbed enzyme protein was recovered by eluting these columns with aqueous 0.1 M acetic acid (pH 3).[6] The recovery of protein was 91, 83, 74, 77, and 46% by chromatography on *N*-acetyl, *N*-propionyl, *N*-butyryl and *N*-pentanoyl derivatives and crab shell chitin, respectively. The capacity for adsorption of the intact lysozyme decreased with an increase in the carbon number of *N*-acyl group. The *N*-stearoyl derivative did not adsorb the intact lysozyme.

Chromatographic method. Each of the suspended mixtures of the *N*-acyl derivatives and crab shell chitin in the buffer solution was packed into a glass column (1.6 × 2.7 cm). A solution of lysozyme (7.0 mg) in 5.0 ml of the buffer solution was put on the column, and the columns were washed with the buffer solution, followed by eluting at 9 ml/30 min with the buffer solution containing 1.0 M NaCl (arrow A in Fig. 1) and finally with aqeuous 0.1 M acetic acid (arrow B in Fig. 1). One ml-fractions were collected, and both the enzyme activity and the optical absorption at 280 nm were analyzed.

A denatured lysozyme protein, which was prepared by heating at 100°C for 60 min, showed two peaks by chromatography on *N*-propionyl derivative: the pass-through fraction had no enzyme activity, and the adsorbed peak had enzyme activity. This indicates a regeneration of the lysozyme protein denatured by heating. By chromatography on *N*-propionylchitosan, a solution of lysozyme–CM-chitin (1 : 1, w/w) or lysozyme–*N*-acetylchitotrisaccharide (1 : 0.1, w/w) had only an active peak, which was adsorbed on the derivative and eluted with 0.1 M aqueous acetic acid. However, a solution of lysozyme–an excess CM-chitin (1 : 40, w/w) had two active peaks, which were not adsorbed as an enzyme–CM-chitosan complex and adsorbed as lysozyme on the derivative. These data strongly indicate that the enzymatically active protein adsorbs on these *N*-fatty acyl derivatives (C2–C6) in the form of enzyme–substrate complex, and is present in an equilibrium with substrate.

Isolation and partial purification of enzymes. A crude enzyme extract (245 ml, total protein 10,500 mg, total lysozyme activity 8,100 mU, specific activity 0.8 mU/mg protein) was prepared from hen egg-white by salting out at 20–80% saturation of ammonium sulfate. A crude enzyme extract (250 ml, total protein 775 mg, total activity 6,100 mU, specific activity 7.9 mU/mg protein) was prepared from fresh cabbage leaves (488 g). The crude enzyme extract (0.77 mU/mg) of hen egg-white was chromatographed on each of these columns to give partially purified fractions (7.8, 7.3, 6.8, 5.2, and 4.6 mU/mg protein from *N*-acetyl, *N*-propionyl, *N*-butyryl, and *N*-pentanoyl derivatives and crab shell chitin, respectively) of lysozyme. The crude extract (7.9 mU/mg protein) of cabbage leaves was also chromatographed to give partially purified fractions (32–24 mU/mg protein).

In conclusion, *N*-propionyl, *N*-butyryl and *N*-pentanoyl derivatives of chitosan are easy to prepare, have relatively large binding capacity for both lysozyme and chitinase, and are resistant to enzymatic hydrolysis. The results indicate their suitability as adsorbents for these enzymes.

References

1) I. F. Pryme, P. E. Joner and H. B. Jensen, *Biochem. Biophys. Res. Commun.*, **36**, 676 (1969).
2) Y. Imoto and K. Yagishita, *Agric. Biol. Chem.*, **37**, 465 (1973).
3) T. Yoshimoto and D. Tsuru, *J. Biochem.* (Tokyo), **76**, 887 (1974).
4) E. Junowicz and S. E. Charm, *FEBS Lett.*, **57**, 219 (1975).
5) D. A. Cornelius, W. H. Brown, A. F. Sharake and J. A. Rupley, *Method in Enzymol.*, **34**, 639 (1974).
6) N. Yamasaki and T. Ito, *Agric. Biol. Chem.*, **45**, 2939 (1981).
7) T. Imoto, K. Hayashi and K. Funatsu, *J. Biochem.* (Tokyo), **64**, 387 (1968).
8) S. Hirano and Y. Yagi, *Carbohydr. Res.*, **83**, 103 (1980).
9) S. Hirano, T. Yamamoto, M. Hayashi, T. Nishida and H. Inui, *Agric. Biol. Chem.*, **54**, 2719 (1990).