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Note

A convenient synthesis of glycolchitin, a substrate of lysozyme

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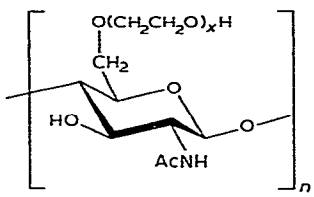
The activity of lysozyme has been mainly determined by lysis of such bacteria as *Micrococcus lysodeikticus*. Because of the complicated nature of this assay, it does not reflect the true enzymic activity[1,2]. Recently, an alternative method that uses glycolchitin (**1**) as the substrate has been developed[3–6], but this method has not yet been widely used, because of the difficulty of preparing **1** if oxirane (ethylene oxide) is used for the glycolation of chitin[7]. Consequently, we have developed a convenient method for the synthesis of **1** by using 2-chloroethanol (ethylene chlorohydrin) instead of ethylene oxide, and have now revealed the relationship between the degree of glycolation of chitin and the ability of **1** to act as a substrate of lysozyme.



1

$x = 0$ or 1

A suspension of powdered chitin in aq. NaOH solution was kept for 4 h at room temperature under ~20 mmHg (in order to promote penetration of the alkali into the chitin particle), and then filtered. The alkaline chitin thus obtained was mixed with crushed ice, and kept for 20 to 30 min, giving a highly viscous, alkaline chitin solution which was diluted with aq. NaOH solution. Treatment of this alkaline chitin solution with the appropriate amount of ethylene chlorohydrin gave water-soluble glycolchitin. If the structure of glycolchitin as obtained here was assumed to be **1**, like that obtained by the method of Senzu and Okimasu[7] (wherein ethylene oxide was used, instead of ethylene chlorohydrin), the degree of glycolation could be calculated from the ratio of C to N as found by elemental analysis. Although a

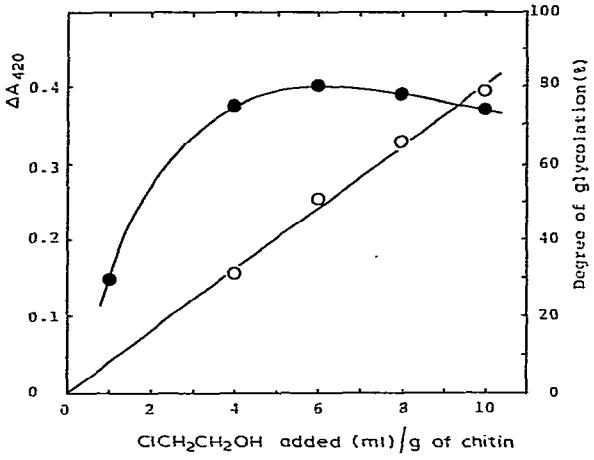


Fig. 1. Degree of glycolation of chitin (○), and ability of glycolchitin to act as a substrate of lysozyme (●), against amount of ethylene chlorohydrin added per g of chitin. (For details, see the text.)

trace of moisture could not be removed from the sample, that did not interfere with calculation of the degree of glycolation. As shown in Fig. 1, the degree of glycolation of chitin was proportional to the proportion of ethylene chlorohydrin used under the conditions employed.

Hydrolysis of glycolchitin catalyzed by lysozyme was tested by measuring the increase in reducing power according to the method of Imoto and Yagishita[6]. As may be seen in Fig. 1, 30 to 80%-glycolated chitin was hydrolyzed by lysozyme with almost equal efficiency, and this was practically the same as that observed in the case of glycolchitin prepared by use of ethylene oxide[6]; but, at a low degree of glycolation, the glycolchitin was much less efficiently hydrolyzed, indicating that it was not a suitable substrate for measurement of the activity of lysozyme.

In the original preparation of glycolchitin, ethylene oxide was used for glycolation of chitin. Ethylene chlorohydrin is not so volatile as, and is more readily handled than, ethylene oxide, for which a pressure bottle is needed for the reaction. Thus, the method described here makes glycolchitin widely available for activity measurements of lysozymes and chitinases. The alkaline chitin solution obtained in the procedure may also be used for the preparation of chitin-coated cellulose, which is an adsorbent employed in the affinity chromatography of lysozymes and chitinases[8,9]. Moreover, the present method should be applicable to the solubilization of other polysaccharides, such as cellulose.

EXPERIMENTAL

Materials. — Chitin powder was obtained from Nakarai Chemical, Ltd. Hen egg-white lysozyme (five times recrystallized) was a gift from Eizai Co. All chemical compounds used were of the highest purity available.

Synthesis of glycolchitin. — Powdered chitin (5 g) was suspended in 42% (w/w) NaOH solution (100 mL), and the suspension was kept for 4 h at room temperature under diminished pressure (~20 mmHg), with occasional swirling. (When <42% (w/w) NaOH solution was used, the yield of water-soluble glycolchitin was markedly lessened.) The alkaline chitin thus obtained was filtered off, washed with 42% (w/w) NaOH solution, and pressed well (to remove most of the excess of NaOH solution), until the weight of the cake of alkaline chitin was < 15 g. The cake was transferred to a beaker, and vigorously mixed with finely crushed ice (~50–70 g, precooled in a freezer before use). After mixing for 20 to 30 min, a highly viscous, alkaline chitin solution was obtained. The solution was diluted to 250 mL with NaOH solution, and the concentration of NaOH was adjusted to 14% (w/w) (At this stage, a gel was sometimes formed, and this prevented glycolation of the chitin; in this case, the gel was cooled below 0°, and mixed well, to give a homogeneous solution.) The alkaline chitin-solution was cooled in an ice bath, and an appropriate amount of ethylene chlorohydrin was added dropwise, with mixing, during 30 min. The ice bath was then removed, and the mixture was allowed to stand overnight at room temperature. The mixture was recooled in an ice bath, and acetic anhydride (10 mL) was added dropwise, with stirring. (If this step was omitted, an appreciable amount of deacetylated glycolchitin was obtained, and this was not efficiently hydrolyzed by lysozyme.) After being stirred for 30 min, the mixture, in an ice bath, was made neutral with acetic acid, and dialyzed against running water for 3 days, and then exhaustively against distilled water. The dialyzate was centrifuged, and the supernatant liquor was lyophilized, to give ~4 g of glycolchitin as a fibrous material.

Anal. Found: C, 42.78–44.90; N, 5.79–5.47.

Hydrolysis of **1**, *catalyzed by lysozyme.* — To 0.05% glycolchitin in 0.1M acetate buffer, pH 5.5 (1 mL) was added 13 μg of lysozyme in 0.1 mL of the same buffer, and the mixture was incubated for 30 min at 40°. After the reaction, 2 mL of the color reagent (0.5 g of potassium ferricyanide in 1 L of 0.5M sodium carbonate) was added, and the mixture was immediately heated to the boil and kept boiling for 15 min. (A control solution contained no enzyme.) After cooling, the optical absorbance at 420 nm was measured.

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