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On the transglycosylase activity of lysozyme*

Sharon and Seifter[1] were the first to show that the incubation of a tetrasaccharide, obtained from bacterial cell-wall with egg-white lysozyme, produced a disaccharide in addition to compounds having a low R_F value. On the basis of this observation, they concluded that lysozyme catalyzes transglycosylation as well as hydrolyzes the bacterial cell-wall oligosaccharides. Maksimov *et al.*[2] also observed the formation of an insoluble chitin-like product during the incubation of chitin oligosaccharides with lysozyme. In the preceding papers[3,4] of this series, it was reported that *p*-nitrophenyl β-glycosides of chitin oligosaccharides were hydrolyzed by lysozyme to liberate *p*-nitrophenol which can be assayed colorimetrically, whereas *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside is not susceptible to lysozyme. In this paper, a clear demonstration of the transglycosylase activity of lysozyme was attempted by incubating *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside[5] (**1**), as the glycosyl acceptor, with lysozyme in the presence of a chitin oligosaccharide, as the glycosyl donor. Fig. 1 shows the liberation of *p*-nitrophenol during a typical experiment. In the case of tetra-*N*-acetylchitotetraose as the glycosyl donor, a smooth liberation of *p*-nitrophenol was observed. Paper chromatography of the reaction mixture after catalytic hydrogenation, which enables to detect the resulting *p*-aminophenyl glycosides with the diazotized *p*-aminobenzenesulfonic acid reagent[6], revealed an intense spot ($R_{compound\ 1}$ 0.55) at the area expected for glycosides of chitin oligosaccharides. The spot was also detected with the benzidine–periodic acid reagent[7], and it was, therefore, assumed to be one of the *p*-aminophenyl glycosides of chitin oligosaccharides produced by transglycosylase activity of lysozyme and subsequent catalytic hydrogenation. Tri-*N*-acetylchitotriose induced the liberation of *p*-nitrophenol only after a short induction period, and at a rate much slower than that of

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the tetraose. Since tri-*N*-acetylchitotriose reacts slowly, the transglycosylation was probably accelerated by the formation of small amounts of higher oligosaccharides during the induction period. In contrast to the cases of the trimer and tetramer, the incubation of di-*N*-acetylchitobiose and **1** with lysozyme did not cause the liberation of any detectable amounts of *p*-nitrophenol, even after 100 h at 40°. This result clearly indicates that lysozyme can catalyze neither hydrolysis nor polymerization of di-*N*-acetylchitobiose under the present experimental conditions.

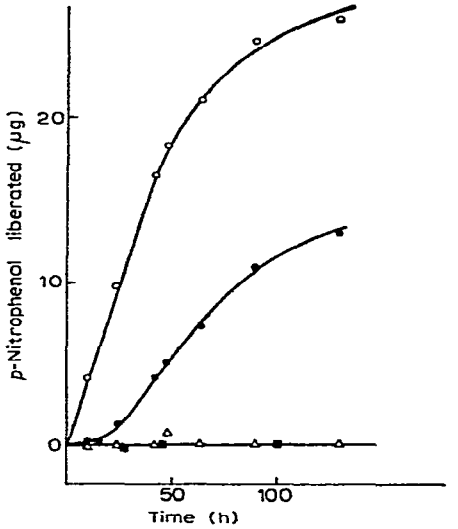
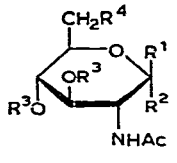


Fig. 1. The lysozyme-catalyzed liberation of *p*-nitrophenol from **1** in the presence of tetra-*N*-acetylchitotetraose (○), tri-*N*-acetylchitotriose (●), and di-*N*-acetylchitobiose (△), and from **4** in the presence of tetra-*N*-acetylchitotetraose (■). Experimental details are given in the text.

In order to elucidate the structural specificity requirements of the sugar residue whose glycosidic bond undergoes attack by lysozyme (residue D in the schema proposed by Blake *et al.*[9]), the transglycosylation reaction was applied to *p*-nitrophenyl 2-acetamido-2,6-dideoxy-β-D-glucopyranoside (**4**). Compound **4** was synthesized by



	R^1	R^2	R^3	R^4
(1)	$OC_6H_4NO_2$-*p*	H	H	OH
(2)	H	Cl	Ac	H
(3)	$OC_6H_4NO_2$-*p*	H	Ac	H
(4)	$OC_6H_4NO_2$-*p*	H	H	H

formation of the glycosyl chloride from 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-β-D-glucopyranose[8], followed by condensation with *p*-nitrophenol, and subsequent deacetylation. Although incubation of **4** with lysozyme in the presence of tetra-*N*-acetylchitotetraose did not liberate any detectable amounts of *p*-nitrophenol, as shown in Fig. 1, examination of the reaction product by paper chromatography, after catalytic hydrogenation, revealed the presence of *p*-aminophenyl glycosides of chitin

oligosaccharides ($R_{compound\ 4}$ 0.33, 0.49). This result suggests that the hydroxyl group at C-5 of "the residue D" may be essential for the attack by lysozyme of the glycosidic bond of the residue. This assumption is in agreement with the three-dimensional lysozyme–substrate model[9] which suggests interaction between lysozyme and the hydroxyl group at C-5 of the distorted "sugar residue D". Final evidence will have to await the isolation of transfer product derived from **4** or the synthesis of the *p*-nitrophenyl β-glycoside of chitin oligosaccharides whose hydroxyl group at C-6 of "the residue D" is reduced. Work is now in progress along these lines.

EXPERIMENTAL

*General** — Descending paper chromatography was performed on Toyo Roshi No. 53 paper in butyl alcohol–pyridine–water (6:4:3). Di-*N*-acetylchitobiose, m.p. 260–262° (dec.), $[\alpha]_D^{26}$ +17° (*c* 0.42, water, after 4 h); tri-*N*-acetylchitotriose, m.p. 304–306°, $[\alpha]_D^{28}$ +2° (*c* 0.94, water, after 2 h); and tetra-*N*-acetylchitotetraose, $[\alpha]_D^{26}$ −3° (*c* 0.52, water, after 2 h) were prepared according to the procedure of Rupley[10]. Egg-white lysozyme (crystallized 4 times, lot No. 4103) was kindly provided by Seikagaku Kogyo Co.

*2-Acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-α-*D*-glucopyranosyl chloride* (**2**). — A solution of 2-acetamido-1,3,4-tri-*O*-acetyl-2,6-dideoxy-β-D-glucopyranose[8] (1.50 g), in glacial acetic acid (20 ml), saturated at 0° with hydrochloric acid, was kept for 48 h at room temperature. After dilution with chloroform (50 ml), the solution was washed once with water, twice with an ice-cold saturated solution of sodium hydrogen carbonate, and finally with water. The solution was dried with sodium sulfate, and the crystalline residue, obtained after evaporation of the solvent, was recrystallized from ethyl acetate–hexane to give white needles (1.00 g, 71%), m.p. 145° (dec.), $[\alpha]_D^{25}$ +153° (*c* 0.88, chloroform).

Anal. Calc. for $C_{12}H_{18}NO_6Cl$: C, 46.83; H, 5.89; N, 4.56. Found: C, 46.74; H, 5.92; N, 4.55.

p-*Nitrophenyl 2-acetamido-3,4-di-*O*-acetyl-2,6-dideoxy-β-*D*-glucopyranoside* (**3**). — To a solution of **2** (830 mg) and *p*-nitrophenol (535 mg) in acetone (12 ml) was added 1.0M sodium hydroxide (3.88 ml). The mixture was kept for 16 h at 5°. After evaporation of the acetone, the crystals formed were collected, washed with ice-cold water until the washings were colorless, and dried, white needles (370 mg, 34%), m.p. 239–240° (dec.). Recrystallization from methanol–chloroform raised the m.p. to 245° (dec.), $[\alpha]_D^{25}$ −8° (*c* 0.61, chloroform).

Anal. Calc. for $C_{18}H_{22}N_2O_9$: C, 52.68; H, 5.40; N, 6.83. Found: C, 52.53; H, 5.47; N, 6.76.

p-*Nitrophenyl 2-acetamido-2,6-dideoxy-β-*D*-glucopyranoside* (**4**). — A suspension of **3** (273 mg) in dry methanol (6.5 ml) was warmed to 40°, 1.0M sodium methoxide (0.26 ml) was added, and the mixture was shaken until complete dissolution. After

*For conditions, see reference 3.

a further heating for 10 min at 40°, the mixture was kept overnight at 5°. The solution was evaporated, and the residue was recrystallized from water–methanol to give white needles (140 mg, 62%), m.p. 216–217° (dec.), $[\alpha]_D^{25}$ −11° (*c* 0.54, water).

Anal. Calc. for $C_{14}H_{18}N_2O_8$: C, 51.53; H, 5.56; N, 8.59. Found: C, 51.64; H, 5.73; N, 8.24.

Transglycosylation reaction. — All enzymic experiments shown in Fig. 1 were performed as follows: a 2.5×10^{-3}M solution of glycoside (**1** or **4**) in a 0.04M sodium citrate buffer (pH 5.1), containing 5 mg lysozyme per ml and a drop of toluene to inhibit bacterial growth, was incubated at 40° in the presence of an equimolar concentration of chitin oligosaccharide. A blank solution was prepared in the same way but without chitin oligosaccharide. At various time intervals, aliquots (1 ml) were withdrawn, and the reaction was interrupted by addition of 0.2M borate buffer (pH 9.8, 2.0 ml) according to Woollen *et al.*[11]. The color was measured at 400 nm, and the values obtained were corrected for the blank. The amount of *p*-nitrophenol liberated by the enzyme was determined from the molar extinction coefficient(1.8×10^{-2}) of the chromophore at this wavelength. At the end of the incubation period, the reaction mixture was transferred into a Visking cellophane tube and dialyzed thoroughly at 5° against several changes of distilled water. The combined dialyzates were concentrated, and the residue was dissolved in aqueous methanol. The solution was then hydrogenated at room temperature and normal pressure in the presence of 10% palladium on charcoal. After the rapid absorption of hydrogen had ceased, the mixture was filtered, the filtrate was evaporated, and the residue was dissolved in distilled water. A sample of the solution was chromatographed (descending) on a sheet of Toyo Roshi No. 53 paper for 12 h at 20°. Aminophenyl glycosides were detected with benzidine–periodic acid[7] and diazotized *p*-aminobenzenesulfonic acid[6].

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