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STABLE LYSOZYME–CELLO-OLIGOSACCHARIDE COMPLEXES

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ABSTRACT

Stable complexes that formed when [^{14}C]cello-oligosaccharides and lysozyme were incubated under various conditions were isolated chromatographically and characterized. Complexation occurred over the pH range of 3–9 but was favored at high pH; the extent of complexation was inversely proportional to temperature. The stoichiometry of the complex was 1:1 sugar–protein, but no radiolabeled peptides could be isolated from a tryptic digest. The minimum requirements for association were native enzyme and a substrate composed of three or more (1→4)-β-D-glucopyranosyl residues. Acceptors and competitive inhibitors of lysozyme inhibited complexation and stimulated dissociation of the complex. Lysozyme did not hydrolyze the cello-oligosaccharides nor did it use them as D-glucosyl donors. Ultracentrifugation, molecular-sieve chromatography, and light-scattering studies indicated that the precipitated complexes were large, heterogeneous aggregates of protein and oligosaccharide which conform to the following equilibrium: n(protein + oligosaccharide) ⇄ (protein–oligosaccharide)$_n$. Polymerization is a cooperative phenomenon.

INTRODUCTION

Although several authors have tended to favor a carbonium ion as the stable intermediate along the reaction pathway of lysozyme-catalyzed reactions[1], a covalent compound still remains a plausible candidate for the stable intermediate[1]. A non-covalent intermediate, a carbonium ion for example, might be subject to dissociation from the enzyme during catalysis to form a mixture of α and β anomers. A covalent intermediate has the distinct advantage of remaining stable to dissociation during the time required for the leaving group to depart from the enzyme and for the acceptor to bind to the enzyme[2]. This mechanism predicts ping-pong kinetics[3] for lysozyme-catalyzed saccharide transfer and is strongly implicated by X-ray crystallographic studies[4]. The original goal of this project was the isolation of a stable, covalent intermediate that might form when lysozyme was incubated with very poor substrates, such as the cello-oligosaccharides. These substrates were selected in the hope that the

transfer step to acceptor or water would be rate-limiting and that detectable amounts of covalent intermediates would thus build up. High cello-oligosaccharide–enzyme levels were used in this study because lysozyme has a low affinity for these saccharides[5], although it appears to hydrolyze them under forcing conditions[6].

Incubation of lysozyme with radiolabeled cello-oligosaccharides of three to six D-glucose residues for long time-periods did give rise to stable complexes. This paper describes the isolation and partial characterization of the protein–cellotriose complex.

EXPERIMENTAL

Materials. — The sources of materials and chemicals used in this project were: radiolabeled cellulose (tobacco-leaf residue 50 μCi/mg), Nuclear Chicago Corp; purified cellulose powder, *Micrococcus lysodeikticus* cells, horseradish peroxidase, α-chymotrypsin, ribonuclease and β-D-glucosidase, Nutritional Biochemical Corp.; lysozyme, α-amylase, Worthington Biochemical Corp.; lysozyme, Miles Laboratories; Sephadex G-50 (fine) and G-200 (superfine), Pharmacia Chemical Co.; Bio-Gel P-30 (100–200 mesh), P-100 (50–150 mesh), 2-acetamido-2-deoxy-D-glucose, D-glucose oxidase and trypsin, Calbiochem; Amberlite CG-50 (100–200 mesh) and Amberlite MB-3 (20–50 mesh) ion-exchange resins, Mallinkrodt; AG-50W-X4 (200–400 mesh) ion-exchange resin, Bio-Rad Laboratories; iodoacetamide, iodoacetic acid, Mann Research Corp.; 2,5-diphenyloxazole (POP), Eastman Organic Chem. Co.; 1,4-bis-2-(5-phenyloxazole)benzene (POPOP), Packard; and urea, Fisher Chemical Co. All chemicals used were reagent grade. *N,N'*-diacetylchitobiose was a generous gift from Dr. J. A. Rupley.

Preparation of radiolabeled cello-oligosaccharides. — Radiolabeled cellulose (40 mg) was diluted 1:10 with unlabeled, purified cellulose powder, and digested according to the procedure of Miller[7], neutralized, and fractionated on a 5 × 25-cm charcoal–Celite column at room temperature[8]. The cello-oligosaccharide fractions were purified by preparative chromatography on Whatman No. 3MM paper with a 58:34:23 1-butanol–pyridine–water (v/v) solvent system, with a minimum of three ascents. The chromatogram was then dried and placed on a sheet of Kodak No-screen Medical X-ray film (14 × 17 inches). The resulting radiogram was used to locate the radiolabeled materials on the paper chromatogram. The edge strips were cut from the paper and developed by the silver nitrate–sodium hydroxide method of Trevelyan *et al*[9]. The bands corresponding to the [^{14}C]cello-oligosaccharides of interest were cut from the chromatogram and eluted by descending irrigation with distilled water in a chromatography chamber. The aqueous eluates from each oligosaccharide were concentrated to about 2 ml under diminished pressure. Rechromatography of each oligosaccharide with nonlabeled standards, and radioautography, yielded chromatographically pure sugars.

The specific activities of each of the cello-oligosaccharide fractions were determined by means of the phenol–sulfuric acid assay for carbohydrate[10] coupled with liquid scintillation-counting of ^{14}C activity.

In order to characterize the cello-oligosaccharides further, an aliquot of [^{14}C]cellotriose was subjected to hydrolysis by β-D-glucosidase. [^{14}C]Cellotriose (50 μl) was added to 0.1M acetate buffer (0.25 ml, pH 5.0) containing 0.5 mg of β-D-glucosidase, and allowed to react for 8 h at room temperature. Aliquots (5 μl) were removed at various time-intervals and spotted onto Whatman No. 1 chromatography paper. Standards of nonlabeled cello-oligosaccharides were spotted at both edges. The paper was irrigated with 2 ascents, radio-autographed, and edge strips containing the standards were developed as already described. The products of the hydrolysis migrated with R_F values equal to those of standards of D-glucose and cellobiose.

Scintillation counting — ^{14}C activity was measured by spotting small aliquots (50–200 μl) of the radiolabeled samples on a 2.5-cm disc of Whatman No. 42 filter paper. The discs were dried with hot air and transferred to counting vials. Scintillator (5 ml) was added to the counting vials. The scintillator consisted of 4 g of PPO and 50 mg of POPOP per liter of toluene. The vials were counted for ^{14}C activity in a Beckman liquid-scintillation spectophotometer model DPM-100.

Chromatography. — Ion-exchange fractionation of labeled and unlabeled enzyme was conducted on a 1 × 3-cm column of Amberlite CG-50 at room temperature or at 3°. The columns were equilibrated with 0.1M phosphate buffer (pH 7.2), loaded with 0.5 ml of enzyme solution, and eluted with 25 ml of 0.1M phosphate buffer and then with 0.2M citrate buffer at pH 7.2.

O-(Carboxymethyl)chitin, prepared according to the procedure of Imoto *et al.*[11], was packed into a 1 × 8-cm column and equilibrated with 0.1M phosphate buffer of pH 8.0. A 2-ml aliquot of labeled enzyme, freed from excess cello-oligosaccharide, was applied to the column. The column was eluted with 20 ml of the pH 8.0 buffer prior to changing the elution liquid to 0.2M acetic acid. The 1-ml fractions were assayed for protein and radioactivity.

Columns (0.9 × 60 cm) of Bio-Gel P-30, and P-100 were calibrated with proteins of known molecular weight and used to determine the molecular weight of the lysozyme–cello-oligosaccharide complex. An aliquot of the complex (500 μl), freed of excess cello-oligosaccharide, was placed on a column and eluted with 0.1M citrate buffer (pH 7.5). Fractions (1 ml) were collected and assayed for protein, radioactivity, and carbohydrate as described previously.

Lysozyme–cello-oligosaccharide complex. — To establish conditions for incorporation, incubations were performed with cello-oligosaccharides (3.72mM) and lysozyme (1.13mM) at pH values of 3.0, 4.5, 6.0, 7.0, and 8.0 in 0.1M sodium citrate buffer and 0.1M phosphate buffer for 210 h at 37°. Cello-oligosaccharide–lysozyme incubations with molar ratios 2:1 and 5:1 were performed in 0.1M sodium citrate buffer (pH 7.5). Control incubations were performed by using denatured lysozyme (boiled in sodium borohydride solution) for the protein and D-glucose, cellobiose, maltotriose, or methyl α-maltotetraoside as the substrate analogs.

The complex was routinely prepared by incubating 1.6mM lysozyme with a five-fold molar excess of [^{14}C]cello-oligosaccharide (8mM) at 25 ±2°, in 0.1M sodium

citrate buffer (pH 7.5). To determine the extent of ^{14}C incorporation, aliquots (100 μl) were removed from the incubation mixture at various time-intervals and applied to a column of Sephadex G-50 (see later) to remove excess cello-oligosaccharide. The complex used for the characterization studies was prepared by incubating the cellotriose–lysozyme mixture for at least 5 days at 25 $\pm$2°, and then removing the excess cellotriose by fractionation on the Amberlite CG-50 column. These experiments were repeated in the presence of a known competitive inhibitor of lysozyme, 2-acetamido-2-deoxy-D-glucose.

Release of ^{14}C from the lysozyme–cellotriose complex. — The release of ^{14}C from the enzyme was studied by incubating [^{14}C]cellotriose-labeled lysozyme in 0.1M citrate buffer (pH 7.2) at 37°. Aliquots (200 μl) were withdrawn at various time-intervals and fractionated on Sephadex G-50 or Amberlite CG-50. Radioactive enzyme-samples were pooled and counted. The foregoing experiment was repeated in the presence of 10mM *N,N'*-diacetylchitobiose. This experiment was also repeated with *Micrococcus lysodeikticus* cell walls, except that an aliquot was withdrawn after an incubation period of 1 min and fractionated on an Amberlite CG-50 column.

In order to identify the saccharide released from the complex, the [^{14}C]-cellotriose–lysozyme complex was incubated in 0.1M citrate buffer (pH 7.2) for 12 h at 60°. After the 12-h delabeling period, the mixture was streaked onto Whatman No. 1 paper with nonlabeled cello-oligosaccharide standards at each side. The chromatogram was developed with the 1-butanol–pyridine–water solvent system mentioned earlier. The cello-oligosaccharides were then located by radioautography and by the silver nitrate reducing-sugar assay of Trevelyan *et al.*[9].

The effect of pH on delabeling of the complex was studied in citrate and pyrophosphate buffers. Aliquots of the cello-oligosaccharide–lysozyme complex were incubated with 0.5M citrate (pH 3.0, 5.0, 7.0, and 9.0) and 0.1M pyrophosphate buffers at 37°. Aliquots (0.2 ml) were removed at various time-intervals and fractionated on an Amberlite CG-50 column. Radioactive protein samples were pooled and counted.

Action of lysozyme on cellotriose and cellotetraose. — Lysozyme (100 mg) and cellotriose (40 mg) were placed in a tube containing 1.0 ml of 0.1M citrate buffer (pH 7.3) and incubated at 33 $\pm$3°. The D-glucose oxidase assay[14] was employed to detect any production of D-glucose resulting from the lysozyme-catalyzed hydrolysis of the cello-oligosaccharides. Aliquots (1 ml) were tested for production of D-glucose after various times. Controls of cellotriose and cellotetraose were conducted without lysozyme to check for any uncatalyzed degradation. These high concentrations of lysozyme did not interfere with the D-glucose oxidase assay.

Tryptic digestion. — The [^{14}C]cellotriose–lysozyme complex (17 mg in 10 ml of 0.2M sodium citrate buffer, pH 7.2) was denatured with urea (4.8 mg), and the disulfide bonds were reduced with sodium borohydride (17 mg), following the procedures of Canfield[12]. After the digest had reacted for 60 min at 60°, a 0.1-ml aliquot was assayed to insure inactivation of the enzyme. The digest was then cooled to 24°, and the pH was adjusted to 8.5 with 2.0M hydrochloric acid by using a Beckman Zeromatic pH meter. Iodoacetamide (510 mg) was added to the mixture at

pH 8.5, and the reaction was allowed to proceed for 20 min at room temperature. Water (10 ml at 4°) was added to the reaction mixture, which was then dialyzed for 48 h at 6° against 1 liter of distilled water and 10 g of Amberlite MB-3 ion-exchange resin.

The dialyzate suspension was brought to pH 10 with 0.09M ammonium hydroxide and warmed to 60° to increase susceptibility to tryptic digestion. The dialyzate was cooled to 37°, and then placed in a water bath at 37°. Trypsin (8 mg) was added, and the mixture was incubated for 16 h. Aliquots taken after 15 and 30 min, and 16 h reacted with ninhydrin and gave the same absorbance, indicating completion of hydrolysis after 15 min. The peptides were fractionated as already described.

Ultracentifugation of the lysozyme–cellotriose complex. —The sample of complex used for ultracentrifugation was prepared as follows: a 0.5-ml aliquot of the lysozyme–cellotriose incubation mixture was fractionated on a column of Bio-Gel P-100. The column was eluted with 0.1M citrate buffer (pH 7.5) at 4°, and 1-ml samples were collected. The first peak from the column corresponded to the complex, and was used for ultracentrifugation studies. The centrifugation was performed on a Beckman Model E analytical ultracentrifuge at 4°, operating at rotor speeds of 12,000, 30,000, and 56,000 r.p.m.

Protein analysis and activity. — The concentration of lysozyme was determined from the absorbance[15] at 280 nm on a Zeiss PMQ-II spectrophotometer by using $\varepsilon_{280\,nm} = 3.54 \times 10^4\ M^{-1}\ cm^{-1}$. The enzymic activity was assayed by the standard lysis procedure as described by Shugar[16]. Elution of the protein standards from the columns was monitored by absorbance at 280 nm.

RESULTS

Formation and fractionation of the complex. — Long-term incubation of lysozyme with a five-fold molar excess of cellotriose, cellotetraose, and cellohexaose in citrate buffer produced lysozyme–cello-oligosaccharide complexes (Fig. 1). Under these incorporation conditions, citrate was an order of magnitude more effective in promoting complex formation than phosphate. Labeled and unlabeled lysozyme could be separated from excess [^{14}C]cello-oligosaccharides by fractionation on Sephadex G-50 or Amberlite CG-50. The protein and excess substrate were incompletely fractionated on the first pass through a Sephadex G-50 column. However, concentration of the fraction from the first peak and rechromatography on Sephadex G-50 achieved complete resolution (Fig. 2) of the complex from excess cello-oligosaccharide.

One pass of the incubation mixture through the Amberlite CG-50 column effected complete resolution of the protein and complex from excess cello-oligosaccharide (Fig. 3). Rechromatography of the material from the second peak resulted in a single radioactive peak, which was a mixture of labeled and unlabeled lysozyme. As no radioactivity was associated with the protein peak from aliquots chromato-

graphed at zero time, we ruled out the possibility that the elution of ^{14}C activity with the protein was an artifact. Both chromatographic procedures showed that approximately 10–15% of the enzyme had been incorporated into the complex in about 120–150 h.

Specificity of complexation. — Incorporation of ^{14}C activity into the protein fraction as a function of time with various substrates is shown in Fig. 1. Cellobiose was the only cello-oligosaccharide used that failed to display significant incorporation into lysozyme during the 5-day incubation period. Denatured enzyme also failed to form radiolabeled complexes. The inhibitor of lysozyme, 2-acetamido-2-deoxy-D-glucose, also prevented incorporation of label into the enzyme.

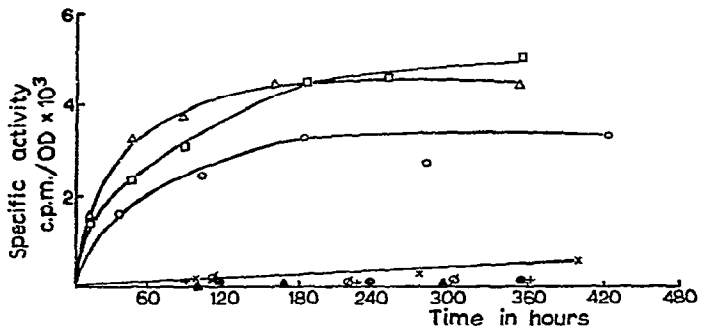

Fig. 1. Formation of lysozyme–cello-oligosaccharide complexes at 25 ±2°, pH 7.5, 0.2M citrate buffer. ×, cellobiose; □, cellotriose; △, cellotetraose; ○, cellohexaose; ∅, denatured enzyme; ▲, methyl α-maltotetraoside; ●, maltotriose; +, cellotriose in presence of 2-acetamido-2-deoxy-D-glucose.

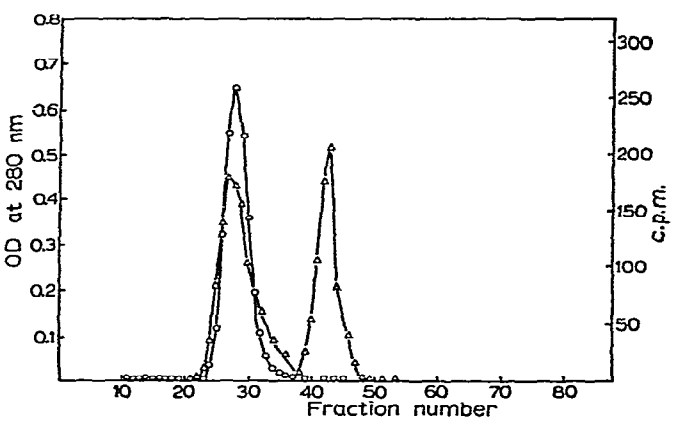

Fig. 2. Chromatography of lysozyme–[^{14}C]cello-oligosaccharide incubation-mixture; room temp., eluant, 0.2M citrate pH 7.5, fractions of 1 ml; column (1.4 × 65 cm), Sephadex G-50 (fine). First peak, labeled and unlabeled lysozyme, second peak, excess of labeled cello-oligosaccharide. Rechromatography of pooled and concentrated enzyme fraction from the first separation; ○, optical density at 280 nm; △, counts.min^{-1} of ^{14}C activity.

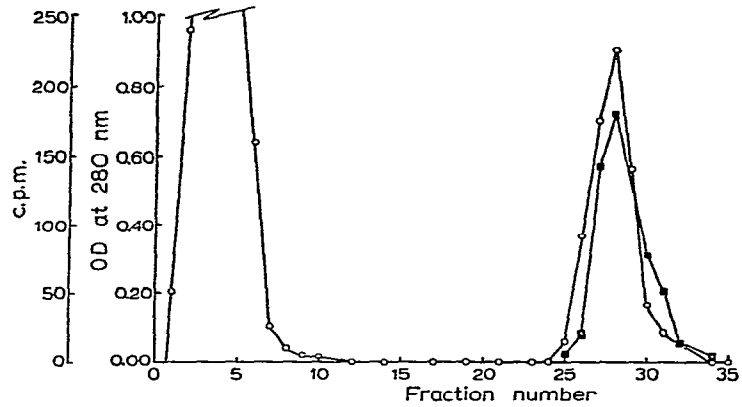

Fig. 3. Fractionation of lysozyme–cellotriose incubation-mixture. Column (1 × 3 cm) of Amberlite CG-50 (200–400 mesh), room temp., pH 7.2. Unreacted cellotriose washed off with 25 ml of 0.1M phosphate, enzyme eluted with 0.2M citrate, 1-ml fractions collected; □, optical density at 280 nm; ○, counts per min. First peak, excess of labeled cellotriose, second peak, labeled and unlabeled lysozyme.

In order to examine the anomeric specificity of the reaction, methyl α-[^{14}C]-maltotetraose and [^{14}C]maltotriose were incubated with lysozyme. No complex resulted with either substrate.

A precipitate usually appeared in the lysozyme–cello-oligosaccharide mixture. This precipitate increased upon cold storage of the mixture but dissolved readily in buffer upon dilution. This precipitate never appeared in the lysozyme control-incubation.

As expected when the ratio of cello-oligosaccharide to enzyme was raised, the fraction of enzyme forming the complex increased. Because of the difficulty in

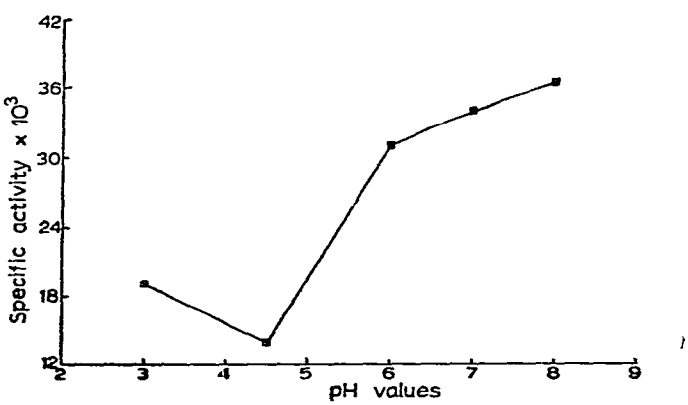

Fig. 4. Effect of pH on the incorporation of cellotriose into the complex. Incorporation studies in 0.1M citrate buffer at 37°.

preparing radioactive substrate, most incorporation experiments were performed with a cello-oligosaccharide–lysozyme ratio of 5:1. The extent of complex formation between lysozyme and cellotriose as a function of pH at 37° is reported in Fig. 4. Complexation was moderately dependent upon pH, with maximum incorporation occuring in the range of pH 7–8. We selected pH 7.5 as a suitable value for further studies.

Temperature dependence of complexation. — After 5 days of incubation (at 25°) the incorporation of ^{14}C activity appeared to reach a steady state. The extent of aggregation was extremely temperature-dependent. When cello-pentaose was incubated at 3 ±3° for 6 weeks, approximately 85% of the protein became associated in the complex. At 40°, only a few percent of the protein reacted, whereas at 60°, appreciable amounts of the complex did not accumulate. A temperature of 25° was selected as a good compromise between the rate and the extent of complexation.

Isolation, characterization, and stoichiometry of the complex. — The incubation mixture was fractionated on calibrated P-100 columns. The labeled protein was eluted in the void volume of the column (Fig. 5). The protein:cello-oligosaccharide ratio of the first peak from the Bio-Gel P-100 column was 1.1 ±0.1. From the elution volume, the minimum molecular weight of the complex was estimated to be 80,000 daltons.

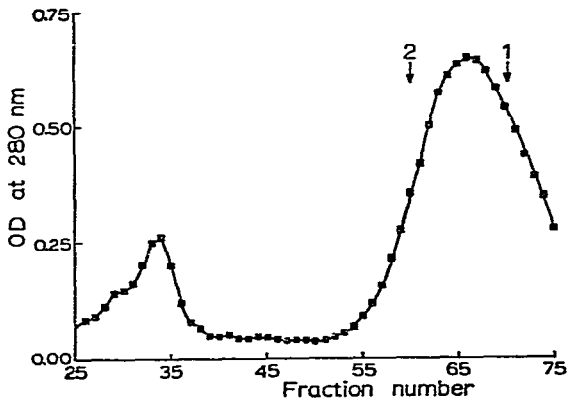

Fig. 5. Fractionation on a calibrated Bio-Gel P-100 column of the lysozyme–cellotriose incubation-mixture. Column (0.9 × 60 cm) with pH 7.5, 0.1M citrate buffer as eluant. First peak, lysozyme complex, second peak, unmodified lysozyme. Fraction size, 1 ml; flow rate 3 ml.h^{-1}. $\frac{1}{1}$, fraction 71, elution volume of lysozyme monomer; $\frac{2}{1}$, fraction 61, estimated elution volume of lysozyme dimer assuming that it behaves as a typical globular protein.

The results of affinity chromatography are shown in Fig. 6. The lysozyme–cello-oligosaccharide complex behaved chromatographically as the unmodified enzyme.

Although others[17] have successfully resolved native and modified lysozyme mixtures by ion-exchange chromatography, all attempts by ion-exchange chromato-

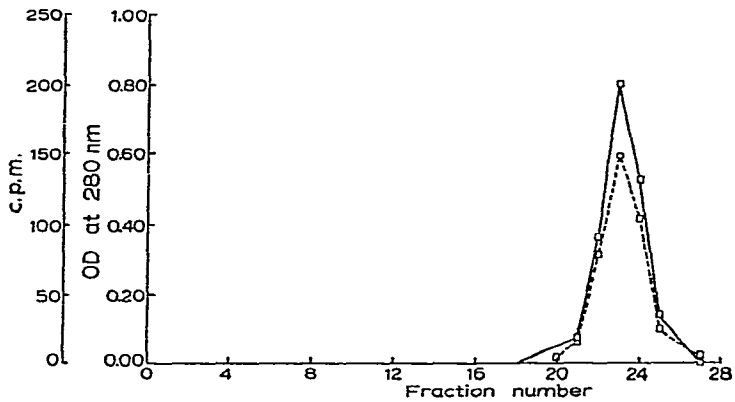

Fig. 6. Affinity chromatography of mixture of lysozyme and lysozyme–cello-oligosaccharide complex on a modified chitin column at room temp. Column washed with 20 ml of 0.1M phosphate buffer (pH 8.0) and then 0.2M acetic acid; fractions of 1 ml; ———, optical density at 280 nm; — — —, counts per min.

graphy, with numerous combinations of ion-exchange resins, pH and salt gradients, at various temperatures, invariably led to decomposition of the complex.

Light scattering of the complex. — The time course of complexation was monitored by measuring the light scattering of the developing aggregate. The absorbancy at 450 nm in a 1-cm cuvette was used as an index of light scattering. The incubation conditions were identical to the standard conditions used for incorporation of radioactive cello-oligosaccharides into the complex. Before each measurement, the tube containing the complex was shaken vigorously to disperse and partially disaggregate the precipitate.

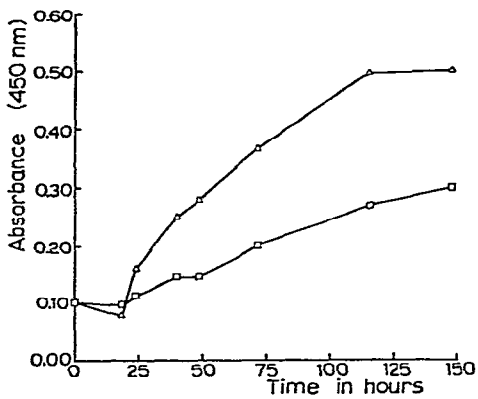

Fig. 7. Time course of protein aggregation, as measured by light scattering. Incubation performed at 25°, pH 7.5, in 0.1M citrate buffer. Tubes were shaken vigorously before measuring absorbance at 450 nm. ▲, with cellotetraose; □, without cellotetraose.

The time course of complexation, as measured by light scattering at pH 7.5, is recorded in Fig. 7. Protein aggregation is obviously stimulated by the cello-oligosaccharides. The control sample containing only lysozyme was just barely turbid to the eye, whereas tubes containing the cello-oligosaccharides always displayed a noticeable precipitate. Vigorous shaking of the precipitate dispersed it to a colloidal state, and it dissolved readily upon dilution in citrate buffer.

Search for covalent saccharide–peptide fragments. — When the complex was denatured at 60° in urea and sodium borohydride, approximately 30–50% of the radioactivity was retained in the complex. After exhaustive dialysis, about 10% of the radioactivity remained with the precipitate. To test for a covalent saccharide–protein compound, the mixture of protein was subjected to tryptic hydrolysis and ion-exchange chromatography after denaturation and dialysis. All of the radioactivity was eluted in the void volume prior to the appearance of peptides; no [^{14}C] peptides could be detected.

As a further check for the formation of a cellobiosyl- or cellotriosyl-lysozyme complex, the enzyme was incubated with cellotriose and cellotetraose and aliquots were examined for the appearance of D-glucose by chromatography and by D-glucose oxidase at various time intervals. Even though the D-glucose oxidase assay was sensitive enough to detect D-glucose resulting from only 0.025% of hydrolysis, no D-glucose was found. Paper chromatography and radioautography revealed that the only ^{14}C material in the reaction digest after 400 h of incubation (following denaturation of enzyme), was the starting cello-oligosaccharide.

Release of radioactivity. — Incubation of the complex with *N,N'*-diacetylchitobiose, an acceptor for lysozyme, only stimulated release of the cellotriose and never gave rise to transfer products. This experiment supports our earlier conclusion that lysozyme does not act on the cello-oligosaccharides under the conditions used

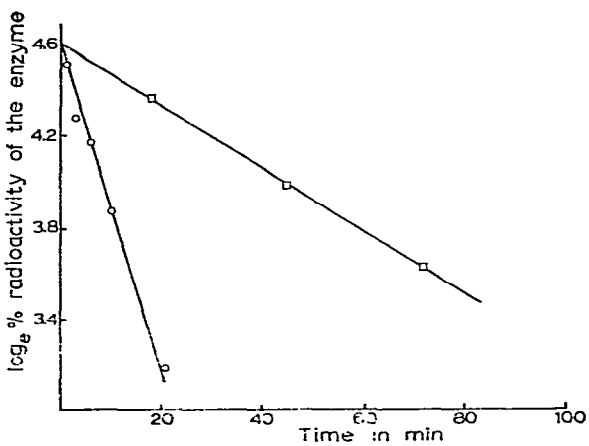

Fig. 8. Release of ^{14}C activity from the complex in the presence and absence of *N,N'*-diacetylchitobiose. □, release of radioactivity at 37° in pH 7.5 citrate buffer 0.2M; ○, release of radioactivity in the presence of 0.1M *N,N'*-diacetylchitobiose, 37°, pH 7.5 citrate buffer, 0.2M.

here. The stimulatory power of *N,N'*-diacetylchitobiose for release of label is shown in Fig. 8. The spontaneous decay of the complex had a half life of 52 min, but the half life was decreased to 10 min in the presence of 0.01M *N,N'* diacetylchitobiose at 37°.

Effects of pH on decomposition of the complex. — The experiments on decomposition of the complex were conducted in citrate buffer at pH values of 3, 5, 7, and 9 and the rates of decomposition were observed to be constant to ±25%.

Time course of polymerization. — In order to determine whether an indefinite association of lysozyme led to collodial aggregates, aliquots of lysozyme–cellotriose or –cellotetraose mixtures were chromatographed at 24-h intervals on columns of Bio-Gel P-100. A typical elution-profile of the incubation mixture is shown in Fig. 5. The first peak, the aggregrate, was eluted with the void columne and the second, larger peak was unpolymerized protein. At zero time, a single monomer peak was found, and this peaked in fraction 71.

The noteworthy features of these elution profiles were the growth of the leading peak with respect to time, the absence of significant quantities of oligomers, the decrease in elution volume of the monomer with time, and the failure of the optical density to return to the base line between the peaks.

DISCUSSION

Incorporation of radiolabel required the simultaneous presence of native enzyme and a substrate analog containing a minimum of three (1→4)-β-D-glucopyranosyl residues. Known competitive inhibitors[18] of lysozyme prevented incorporation of radioactive cello-oligosaccharides into the complex, and known acceptors[19] stimulated their release. The tempting interpretation of these results is that cellotriose–cellohexaose are acting as affinity labels of lysozyme and that the interaction at the active site is competitively inhibited by substrates.

However, further study convincingly excluded the possibility that the complex was a covalent intermediate and proved that it was a very tight association-complex. The more convincing data are the absence of the anticipated pH-dependence[18,20] for either incorporation or release of radioactive analogs, the failure of the enzyme to promote either hydrolysis or transfer reactions with the substrate analogs, the recovery of unmodified cello-oligosaccharides from the complex, the failure of affinity chromatography to fractionate the complex from unreacted protein, and the failure of the complex to yield radioactive peptides after denaturation and proteolysis.

Other workers have reported the formation of other stable enzyme–product[21,22] and antibody–hapten[23] complexes. Hayashi and coworkers[24] reported the production of lysozyme–product complexes formed by perfusion of competitive inhibitors into lysozyme crystals.

It has been reported[25] that lysozyme undergoes a pH-induced head-to-tail dimerization governed by a group having a pK of ~6. The possibility of a sandwich-type complex containing two enzyme and one cello-oligosaccharide was therefore

checked. The elution profiles from molecular sieves, the sedimentation behavior of the complex in the ultracentrifuge, and the appearance of a precipitate, indicate that the cello-oligosaccharides induce polymerization of lysozyme. Molecular-exclusion chromatography on calibrated columns indicates that the aggregates contain a minimum of seven lysozyme monomers, and furthermore, light scattering and the sedimentation coefficient indicate very extensive polymerization. This anomalous result was certainly unexpected because substrates and acceptors are reported to shift the monomer–polymer equilibrium in the direction of the monomer[25]. Our observations further emphasize the importance of the *N*-acetyl group on the substrate in controlling lysozyme self-interactions[5].

It is well known that dimerization of lysozyme depends upon the enzyme concentration, the temperature, the ionic strength, and probably ionization of Glu-35 (see ref. 1 for pertinent references). The unexpected result of our experiments is that the cello-oligosaccharides induce polymerization below pH 5 and that attainment of the polymerization equilibrium is very slow. Although self-association of lysozyme in the pH range of 5–9 has been primarily interpreted in terms of a monomer–dimer equilibrium[25,26], several authors have pointed out that dimerization cannot be distinguished from indefinite association[27–29].

Fractionation of lysozyme–cello-oligosaccharide incubation-mixtures as a function of incubation time was very revealing. The elution profiles exhibit only two major components for all incubation periods in excess of 24 h (Fig. 5). The shape of the elution profile is characteristic of a system containing two slowly interconverting components[30–32], which may be described by Equation *1*.

$$n(\text{lysozyme} + \text{oligosaccharide}) \rightleftarrows (\text{lysozyme–oligosaccharide})_n \qquad (1)$$

As n is at least seven and is probably much larger, and since significant quantities of aggregated polymers are not detected by gel chromatography, the polymerization must be a cooperative phenomenon stimulated by the cello-oligosaccharide and high ionic strength. A simple, but unsupported, explanation of this observation would be that the cello-oligosaccharides induce a conformational change in the enzyme upon binding which in turn promotes aggregation. Partial equilibration of the slow migrating, monomeric lysozyme with the rapidly migrating aggregrate decreases the elution volume of the monomer. Fractionation requires 24 h and provides ample opportunity for interconversion of the species and for the aggregrate to "pull" the monomer down the column.

The 1:1 stoichiometry between protein and saccharide and the specificity for complexation suggest the active site of the enzyme is the point of sorption of the saccharide, although our results do not preclude adsorption of the saccharides at another site. The X-ray structure of the enzyme makes the latter possibility remote. In a very large aggregate of lysozyme, most active sites would be buried but a few would probably be exposed.

An aggregate of this type would be expected to have a stoichometry near 1:1 yet still bind to the affinity column. This type of aggregate is also consistent with the

inhibition of cello-oligosaccharide incorporation into the complex and the stimulation of its release by 2-acetamido-2-deoxy-D-glucose and *N,N'*-diacetylchitobiose. It concurs with earlier reports that *N*-acetylated chitosaccharides repress self-association of lysozyme[25,29].

In citrate, the spontaneous decomposition and the *N,N'*-diacetylchitobiose stimulated decomposition of the complex are strictly first-order with respect to time. We interpret this observation as meaning that small molecules diffuse rapidly into buried binding sites, where they stimulate slow disaggregation of the complex. The failure of the derivatized chitin columns to stimulate breakdown of the complex would imply that decomposition is not initiated at the peripheral binding-sites. Bacterial cell walls were enormously effective in catalyzing the break-up of the complex. The half-life of the complex under our assay conditions was only several min.

The formation and decomposition of the lysozyme–cello-oligosaccharide aggregate also depends on ionic strength[29] and buffer composition. Citrate is much more effective than phosphate at the same ionic strength. The elution profile of the complex from short ion-exchange columns was strongly dependent upon buffer composition, at constant pH and ionic strength. Sodium citrate was very effective, and sodium phosphate was of intermediate effectiveness, whereas potassium chloride was ineffective. All attempts to fractionate the complex on long ion-exchange columns invariably leads to decomposition of the complex.

The interesting picture that has emerged from this work is that citrate and cello-oligosaccharides are unusually effective in stimulating the polymerization of lysozyme. The aggregrate that forms may contain head-to-tail complexes of protein molecules sandwiching the cello-oligosaccharides between them. It is also possible that the cello-oligosaccharide molecules may act as a bridge by simultaneous binding to portions of two active sites. In either event, the exact positioning of the saccharides on the enzyme is unknown. The aggregates that are eventually precipitated from solution on long standing or cooling may be an interesting subject for X-ray crystallographic investigation.

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