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"Chitin Leash": a polysaccharide heterobifunctional cross-linking agent which can be cleaved by lysozyme

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ABSTRACT

6-*O*-[(2-Hydroxyethyl)poly(2-oxyethyl)]chitosan ("glycolchitosan") was oxidatively cleaved with nitrous acid and then partly acetylated with acetic anhydride, reacted with bromoacetyl-*N*-hydroxysuccinimide, and reacted further with acetic anhydride. Conditions were selected, including fractionation by size-exclusion chromatography, so that the resulting "Chitin Leash" had an estimated, average molecular weight of 10 000 (dextran standards), corresponding to a length of ~40 sugar residues. It possessed 0.9 terminal aldehyde and 2.6 random (presumably) side-chain bromoacetyl reactive groups per chain (average values). As a model system, the Chitin Leash was used to crosslink staphylococcal nuclease (SNase) to ribonuclease A (RNase) with retention of 75 and 78%, respectively, of the starting enzyme activities. For this coupling, the Nase was first converted to a sulfhydryl SNase derivative which retained 74% of the activity of starting enzyme. The yields in this synthesis were: 13% Chitin Leash from glycolchitosan, 24% Chitin Leash-RNase from Chitin Leash and 45% SNase-Chitin Leash-RNase from the latter conjugate. The ratio of SNase to RNase in this conjugate was 1.0:0.94. In a second preparation, in which [^{14}C]acetic anhydride was used, a longer reaction time was employed for the coupling of Chitin Leash to RNase. This gave a 1.0:1.8:0.95 molar ratio of Nase: [^{14}C]Chitin Leash: RNase, revealing multiple attachment of the [^{14}C]Chitin Leash to RNase. The activity of the RNase in the final conjugate was 20%. The latter conjugate was ~70% hydrolyzed by diaminooctyl-succinyl-lysozyme, disconnecting the two enzymes while not affecting their activities.

INTRODUCTION

Cross-linking reagents are commonly used to establish a covalent linkage on or between molecules, especially macromolecules such as proteins and DNA. The resulting conjugates, depending on their composition, can then be used for therapeutic or diagnostic purposes including immunotherapy (e.g., antibody–toxin),

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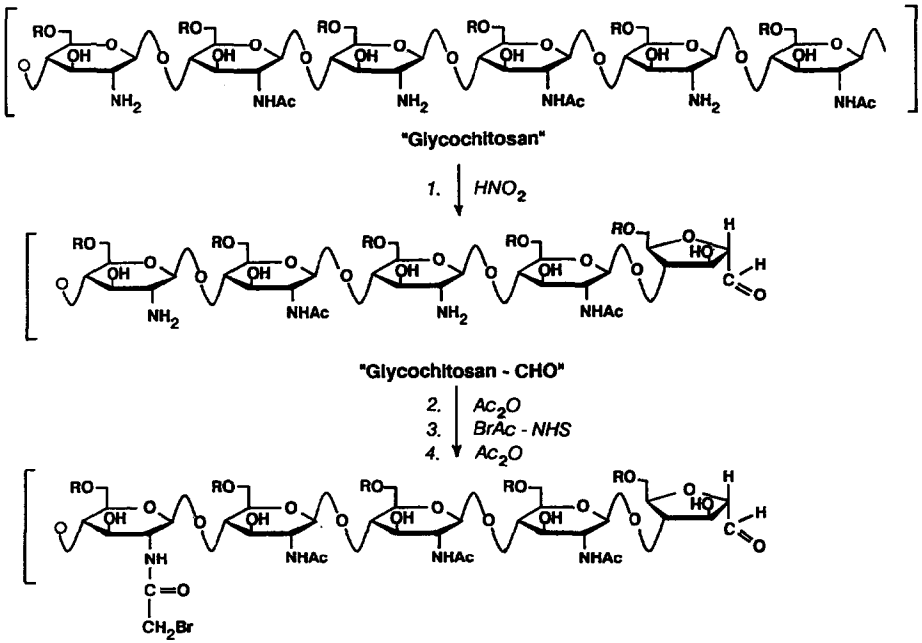
immunoassays (e.g., antibody–enzyme), and hybridization assays (e.g., DNA–enzyme as a DNA probe). Cross-linking reagents are also used to attach molecules to surfaces for chromatographic or assay purposes. A variety of cross-linking reagents are employed which differ basically in their length, reactive groups, polarity, and reversibility. The reversibility is achieved by either incorporating a cleavable group internally into the cross-linker, or attaching the cross-linker to a substrate via a cleavable bond. Usually the former technique is implied when a cross-linking agent is classified as reversible.

Internal-cleavage cross-linking agents have incorporated a variety of labile groups; e.g., an acid-sensitive group such as an ortho ester[1], carboxyl ester[2,3] acetal[1], or ketal[1]; a thiol-sensitive disulfide[4]; a periodate-sensitive vic-glycol[5,6]; an oxidation-sensitive selenide[7] or olefin[6]; a cyanogen bromide sensitive amide[8]; a dithionite sensitive azo group[9]; and a nuclease-sensitive ribonucleic acid[10,11]. It is not always desirable for a cross-linker to be reversible, and some of these same groups have been used to achieve cross-linking where the conditions or structure of the cross-linker have been selected to minimize reversibility, e.g., the use of a sterically-hindered disulfide [12]. Both reversible and nonreversible cross-linking reagents have been reviewed (e.g., [13,16]).

Towards a goal of developing internal-cleavage cross-linking agents which can be cleaved biospecifically, we have selected glycolchitosan for study, as reported here. "Glycolchitosan" (6-*O*-[(2-hydroxyethyl)poly(2-oxyethyl)]chitosan) is derived from chitin, a polymer of 2-acetamido-2-deoxy-D-glucose (typically including minor amounts of 2-amino-2-deoxy-D-glucose), by first removing a significant fraction of the acetyl groups by hydrolysis (yielding chitosan), followed by alkylation of the C-6 hydroxyl groups with 2-chloroethanol[17]. In contrast to chitin, glycolchitosan is soluble in water. Chitosan can be oxidatively cleaved by nitrous acid to form fragments terminating at one end in an aldehyde-substituted tetrahydro furan moiety[18,19]. Taking these observations into account, and the fact that glycolchitin (6-*O*-[(2-hydroxyethyl)poly(2-oxyethyl)]chitin, obtained by re-acetylating glycolchitosan[20]); is susceptible to cleavage by lysozyme[21], we reasoned that nitrous acid oxidation of glycolchitosan should form a soluble polysaccharide product that could be useful as a precursor for lysozyme-cleavable cross-linking reagents. Some promising results towards this goal are reported here.

RESULTS AND DISCUSSION

Chitin Leash.—Our design for preparing a "Chitin Leash" is shown in Scheme 1. Glycolchitosan is the starting material. With this objective, we determined that 52% of the amino groups on the starting glycolchitosan were free (deacetylated). This was done by conducting a known assay[17] for this purpose, in which the glycolchitosan was first reacted exhaustively with nitrous acid, and then with MBTH–Fe^{3+} to form a colored complex. This assay technique was useful to us throughout the project for characterizing the derivatives. We also used this assay



Scheme 1. The preparation of the Chitin Leash. R = H or $(CH_2CH_2O)_x$-H; Ac = acetyl. The pattern of the substitutents (H, Ac, $COCH_2Br$) on the Chitin Leash is not defined.

(or the $MBTH^1$–Fe^{3+} part) to similarly characterize the derivatives of glycolchitosan that we prepared.

To prepare the Chitin Leash, the glycolchitosan was first reacted with a limited amount of nitrous acid in order to oxidatively cleave at just some of the 2-amino-2-deoxy-D-glucose sites. After some preliminary, scouting experiments were done (data not shown), three conditions were tested in more detail: 0.20, 0.25, and 0.30 mole of sodium nitrite per sugar residue (2-amino-2-deoxy-D-glucose-2-acetamido-2-deoxy-D-glucose). Since the cleavage products contained both residual amino groups and a terminal aldehyde, which potentially can react with each other, we immediately quenched the reaction with excess acetic anhydride to convert the 2-amino-2-deoxy-D-glucose residues into 2-acetamido-2-deoxy-D-glucose.

These three, apparently fully acetylated products, that we call "Glycolchitin-CHO", were each characterized in three ways. By size-exclusion chromatography on a P-60 column, which also achieved purification, the estimated molecular weights of the three Glycolchitin-CHO preparations were 16 000, 10 000, and 7 000, respectively, from the three sodium nitrite ratios cited above. The column was calibrated with dextran size standards. A representative separation on this column, for the $NaNO_2$: sugar ratio of 0.3, is shown in Fig. 1. The final product (peak A') was obtained by collecting the center part of the initial broad peak A, and passing this collected fraction through the column a second time.

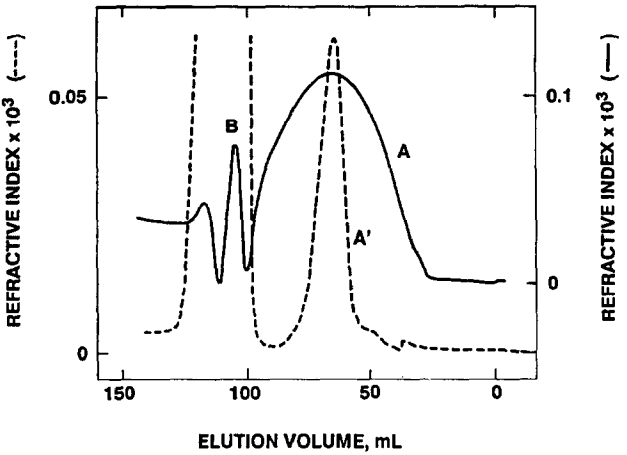


Fig. 1. Size exclusion chromatography of Glycolchitin-CHO. The latter derived from a reaction sequence in which 0.30 mol of $NaNO_2$ per mol of monomer was reacted initially with glycolchitosan followed by exhaustive acetylation. Peak A (solid line), crude product. Peaks B (solid and dashed lines), salts. Peak A' (dashed line; purified product), reloaded peak fractions 66–72 mL from peak A. Column, P-60, 1.2×100 cm; mobile phase, 25 mM HEPES, 25 mM NaCl, 0.01% NaN_3, pH 7.0; flow rate, 10 ml/h; detection, refractive index.

Each of the three products was 100% active as a substrate for lysozyme in comparison with exhaustively acetylated glycolchitin as a reference substrate. Based on the MBTH–Fe^{3+} colorimetric reaction, and taking into account the molecular weight, the number of aldehyde groups per chain was 1.1, 0.94, and 1.0, respectively.

In order to establish a minimal substrate activity of the anticipated Chitin Leash (see Fig. 1) towards hydrolysis by lysozyme, we decided to partly acetylate the Glycolchitosan-CHO prior to reaction with bromoacetyl-NHS, and then finally cap any residual amino groups by further reaction with acetic anhydride. This strategy comprises steps 2–4 of Scheme 1. Based on our observation that 40, 60 and 100% of the amino groups present on glycolchitosan are acetylated by a 0.50, 1.0, and 3.0 molar excess (relative to the amino groups) of acetic anhydride, respectively, we reacted Glycolchitosan-CHO (derived from a $NaNO_2$: sugar molar ratio of 0.25) with a 0.50 molar excess of acetic anhydride. (The amino groups were monitored with the NO_2H–MBTH–Fe^{3+} technique as indicated above.) This was followed by a 0.25 similar molar excess of bromoacetyl-NHS (selected based on preliminary experiments), and then additional acetic anhydride.

The subsequent Chitin Leash was purified by size exclusion chromatography, and the same average molecular (10 000) was observed as for the Glycolchitin-CHO derived from the corresponding parent Glycolchitosan-CHO. The Chitin Leash was 80% active (see above) as a substrate for lysozyme, and possessed 2.6 bromoacetyl groups per molecule (as an average number). The latter value was obtained by measuring the loss in amino groups using the NO_2H–MBTH–Fe^{3+}

assay before and after the partly acetylated Glycolchitosan-CHO was reacted with bromoacetyl-NHS and then acetic anhydride (steps 3 and 4 in Scheme 1). The number of aldehyde groups per Chitin Leash (measured as above) was 0.9.

Chitin Leash-RNase.—To demonstrate the ability of the Chitin Leash to function as a reversible cross-linker between two different proteins, it was used to link ribonuclease A (RNase) to staphylococcal nuclease (SNase). These two enzymes were selected as model proteins for this purpose, largely because of ongoing work with them in other projects in our laboratory. First the Chitin Leash was attached to RNase via its aldehyde group by incubation in the presence of $NaCNBH_3$. The molar ratio of Chitin Leash to RNase in the reaction was 1 : 1.4. A control reaction was conducted in which the Chitin Leash was reacted with excess glycine in the presence of $NaCNBH_3$ prior to incubation with RNase, to estimate the degree of background binding of RNase to Chitin Leash.

Purification of the Chitin Leash-RNase, and the product from the control reaction, by size exclusion chromatography gave the chromatograms shown in Fig. 2. The curves were obtained by collecting the fractions and conducting a BCA assay to measure total protein. Two peaks are observed from each reaction. The earlier peak, A, (estimated $M_r = 24\,000$ using dextran standards) is Chitin Leash-RNase (see below). The second, later-eluting peak is unconjugated RNase as well as unreacted (but invisible by BCA) Chitin Leash. It is apparent, from the relative peak areas, that 17% of the RNase has undergone coupling to the limiting amount of Chitin Leash. This corresponds to a 24% yield of Chitin Leash-RNase from

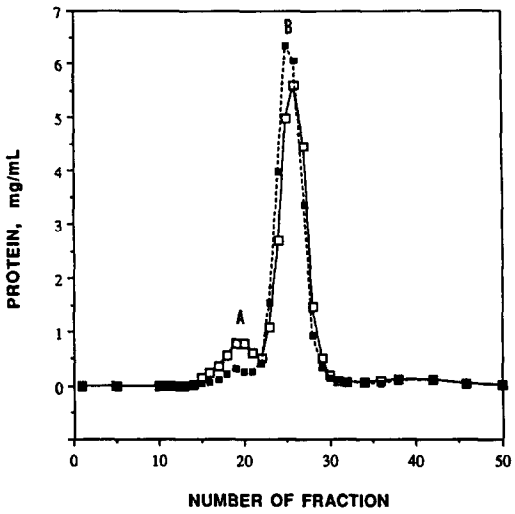


Fig. 2. Size exclusion chromatography of mixtures derived from Chitin Leash. Peak A, □, Chitin Leash-RNase from the reaction; ■, Chitin Leash-RNase from the control reaction. Peaks B, RNase similarly from the reaction (□) and control reaction (■). Column P-60, 1×30 cm; mobile phase 50 mM Tris·HCl, 0.15 M NaCl, pH 7.0, 6 mL/min; detection, fraction collection and then BCA assay for protein.

Chitin Leash. The presence of some Chitin Leash-RNase in the control reaction (7% yield) is apparently due to nonspecific binding, incomplete quenching of the aldehyde in the glycine–$NaCNBH_3$ reaction, or reaction of the RNase onto the bromoacetyl sites of the Chitin Leash. Primarily, however, the small amount of Chitin Leash-RNase in the control reaction indicates that Chitin Leash-RNase had formed, as intended, as a covalent conjugate via a reductive amination reaction. The RNase activity of fraction 20 (central fraction) of peak A (Fig. 2) of Chitin Leash-RNase was 78%, taking into account the amount of RNase protein (BCA assay) in this fraction.

SNase-Chitin Leash-RNase.—The bromoacetyl moiety is attractive for protein conjugation reactions since it reacts preferentially with sulfhydryl groups. SNase was converted to a sulfhydryl SNase derivative by reacting it with SPDP and then mercaptoethanol. Based on UV measurement of the 2-thiopyridine released when the SPDP substituent on SNase was activated with mercaptoethanol, this sulfhydryl SNase contained 1.0 sulfhydrul group per molecule. The enzymatic activity of the sulfhydryl-SNase, using DNA as a substrate, was 74% relative to that of SNase.

Reaction of sulfhydryl SNase with Chitin Leash-RNase, in a molar ratio of 1.6:1, gave SNase-Chitin Leash-RNase. This conjugate was purified by size exclusion chromatography on a P-100 column, giving the chromatogram shown as a solid line in Fig. 3. The early-eluting zone A contained the desired conjugate (see below). For a control reaction (dashed line in Fig. 3), comprising the reaction of NEM-quenched sulhydryl SNase (to mask the sulfhydryl group) with Chitin Leash-RNase, no significant peaks were observed in the early-eluting zone A.

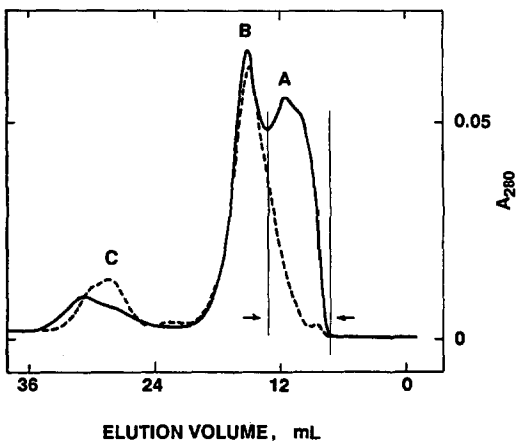


Fig. 3. Size exclusion chromatography of mixtures derived from Chitin Leash-RNase. Solid line, reaction of this conjugate with sulfhydryl SNase; dashed line, control reaction (sulfhydryl SNase capped with NEM before reaction with Chitin Leash-RNase). A, SNase-Chitin Leash-RNase; B, Chitin Leash-RNase; C, salts. Column, P-100, 1.0×30 cm; mobile phase, 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0, 6.2 mL/h. Fractions 12–22 (0.67 mL each) were combined to constitute the product.

Rechromatography of zone A from the reaction of sulfhydryl SNase with Chitin Leash-RNase on the same column gave essentially a single peak (BCA assay for total protein; data not shown, but a peak for the ^{14}C version of this conjugate is shown later in Fig. 4). The SNase activity in the corresponding collection from the control reaction was negligible. For the peak from the reaction, we determined the total protein in this sample (BCA assay), and the amount of RNase protein (Ellman's reagent: RNase has four disulfide bonds but SNase has none). This gave the amount of SNase protein by subtraction, and established that the molar ratio of the SNase to RNase protein in the conjugate was 1.0:0.94. This data also established that the yield of SNase-Chitin Leash-RNase was 45% from the starting Chitin Leash-RNase, the limiting reagent. The SNase and RNase moieties in this conjugate retained (average values) 75 and 78%, respectively, of their activities relative to that of their native forms. (EDTA was present in the assay for RNase in order to inactivate SNase, since the latter possesses ribonuclease activity.) Thus, within experimental error, sulfhydryl SNase (74% active) lost no activity (the 1% gain is within experimental error) upon attachment to Chitin Leash-RNase, and the activity of RNase was uchanged in going from Chitin Leash-RNase to SNase-Chitin Leash-RNase.

Encouraged by these results, we repeated the entire procedure, but used [^{14}C]acetic anhydride for step 2 in Fig. 1. All of the characteristics of the [^{14}C]Chitin Leash were the same as those of the Chitin Leash. We increased the time for the coupling of [^{14}C]Chitin Leash to RNase from the previous 42 h for the Chitin Leash to 90 h with the intent of increasing the yield of [^{14}C]Chitin Leash-RNase to above 24%. However, there was no change in the yield of this step. The final, molar stoichiometry of the SNase-[^{14}C]Chitin Leash-RNase in this new conjugate was 1:.0:1.8:0.95. Thus, the increased time for the coupling of [^{14}C]Chitin Leash to RNase led to the attachment of nearly two Chitin Leash chains, on the average, to RNase. Perhaps this included some coupling due to reaction of the bromoacetyl moieties with RNase. Making measurements as above, we found that the SNase and RNase moieties in this radiolabeled conjugate were 82 and 20% active, respectively. The increased attachment of the [^{14}C]Chitin Leash to RNase apparently was the reason for the significant drop (78% previously, 20% now) in the activity of this latter enzyme upon incorporation into the conjugate.

Cleavage of SNase-[^{14}C]-Chitin Leash-RNase by lysozyme.—In addition to lysozyme, a diaminooctyl-succinyl-lysozyme (DAO-succinyl-lysozyme) was also available in our laboratory from work on another project. The latter conjugate is prepared by exhaustively reacting lysozyme with succinic anhydride, followed by coupling with 1,8-diaminooctane in the presence of a water soluble carbodiimide. The amount of DAO has been optimized so that an average of 1.0 DAO chains is attached to each succinyl-lysozyme molecule. The lysozyme moiety in the DAO-succinyl-lysozyme conjugate retains 99% activity on a chitin substrate relative to

native lysozyme at pH 7.0. (The succinyl-lysozyme retains only 17% activity, a curious result.)

Since the DAO-succinyl-lysozyme is more acidic than lysozyme (due to exhaustive succinylation), we considered that, of the two, the former enzyme should provide a faster rate of hydrolysis of the basic conjugate (both SNase and RNase are basic proteins), SNase-[^{14}C]Chitin Leash-RNase. The hydrolysis of the latter by lysozyme and DAO-succinyl-lysozyme was monitored by size exclusion chromatography (data not shown). Based on the decreasing intensity with time of the peak for SNase-[^{14}C]Chitin Leash-RNase, along with the accompanying appearance of a later-eluting, major peak for the hydrolysis products, the hydrolysis rate, as anticipated, was faster with DAO-succinyl-lysozyme than with lysozyme. For example, ~ 70% of the conjugate was hydrolyzed with DAO-succinyl-lysozyme after 3 h in comparison with ~ 20% hydrolysis by lysozyme (15% by succinyl-lysozyme) under the same conditions.

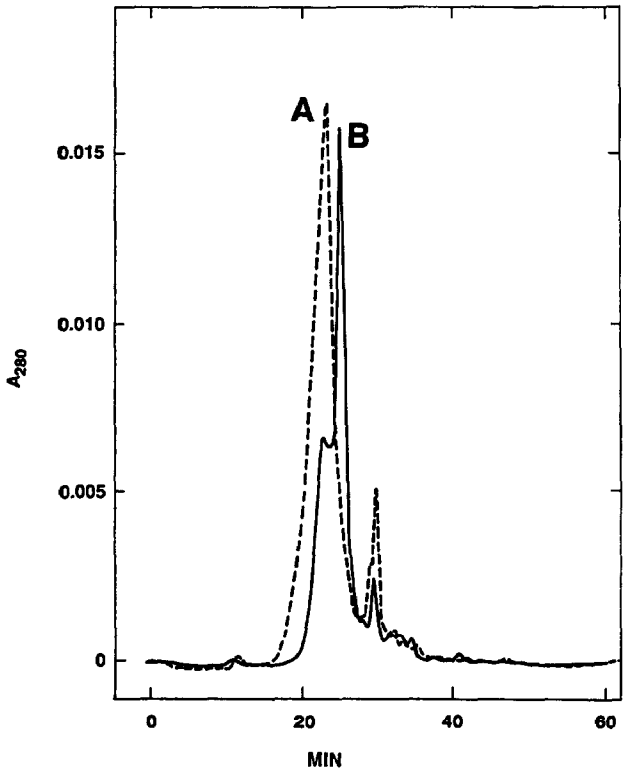


Fig. 4. Size exclusion chromatography of SNase-[^{14}C]Chitin Leash-RNase before (dashed line) and after (solid line) hydrolysis (42 h, 37°C) by DAO-succinyl-lysozyme. Column, Superose 12; mobile phase, 0.1 M HEPES, 0.5 M NaCl, 1 mM EDTA, pH 7.2 at 0.6 mL/min. Samples injected, 10 μL containing 15 μg of SNase-[^{14}C]Chitin Leash-RNase protein. Peak A, starting conjugate; peak B, liberated enzymes.

DAO-succinyl-lysozyme was therefore utilized for exhaustive hydrolysis of SNase-[^{14}C]Chitin Leash-RNase. The appearance of the sample by size exclusion chromatography before and after such hydrolysis is shown in Fig. 4. The major new peak B at 24.5 min corresponds to the elution position of the native, free enzymes. We estimate that ~80% of the conjugate was hydrolyzed (42 h 37°C). Fractions were collected, and protein concentrations and enzymatic activities were measured for both SNase and RNase, as above, in the fraction corresponding to the maximum for peak B (hydrolysis products) in Fig. 4. The released SNase and RNase products were 79 and 24% active relative to the native forms of these enzymes. These values are essentially the same as in the parent, radiolabeled conjugate.

CONCLUSION

A new type of molecular leash has been developed that is derived from chitin and is ~40 sugars long. The leash possesses two kinds of functional groups: an aldehyde at one end and 2.6 (average value) bromoacetyl groups at presumably random positions along its length. It is low in cost, uncharged but polar, and cleaved by lysozyme. Further, a family of related leashes could potentially be similarly prepared with variation in their length and also the number and types of functional groups. Analogs can be anticipated as well that are not susceptible to cleavage by lysozyme. Applications in such areas as biomolecular probes, immunotherapy, affinity chromatography, and intramolecular cross-linking are anticipated for such a family of chitin-derived leashes.

EXPERIMENTAL PROCEDURES

Reagents and supplies.—The cross-linking reagents, *N*-succinimidyl-3-(2-pyridyldithio)propanoate (SPDP), bromoacetyl-*N*-hydroxysuccinimide ester (bromoacetyl-NHS), 5,5′-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), *N*-ethylmaleimide (NEM), glycolchitosan (degree of polymerization ≥ 400), egg-white lysozyme, ribonuclease A (RNase), acetic anhydride, *N*,*N*-dimethylformamide (DMF), succinic anhydride, dextrans (9.4 K, 19.5 K, and 40.2 K), 1,8-diaminooctane (DAO), and Tris base were obtained from Sigma (St. Louis, MO). [14C]Acetic anhydride (50 mCi, 185 mg) was purchased from Dupont NEN Research Products (Boston, MA). Ready-Solv MP liquid scintillation cocktail (5 mL was used for each 50 μL aqueous ^{14}C sample) was from Beckman Instruments (Fullerton, CA). PD-10 desalting columns were obtained from Pharmacia (Piscataway, NJ). BCA protein assay reagent was purchased from Pierce (Rockford, IL).

Centricon 10 and 30 Concentrators were purchased from Amicon (Danvers, MA). Staphylococcal nuclease (SNase) was prepared and purified as described[22]. All other chemicals were reagent or HPLC grade unless indicated otherwise.

Equipment.—High performance liquid chromatography (HPLC) was done with a FPLC system (Pharmacia) using a Superose 12 column from Pharmacia and a Refracto IV refractive index detector from Milton Roy (Storrs, CT).

Absorbance measurements, including kinetic assays of enzyme activity, were performed on a Lambda 3B UV/VIS spectrophotometer connected to a 3600 Data Station computer (Perkin–Elmer, Wellesley, MA). Sample and culture incubations were done in a Model 50 shaking water bath from Precision Scientific (Chicago, IL), and centrifugations were performed using a Microfuge B microcentrifuge from Beckman (Carisbad, CA), and an IEC Model CRU-5000 centrifuge from Fisher (Medford, MA).

Chitin Leash.—To 5% acetic acid (15 mL) containing glycolchitosan, (0.5 g), was added NO_2Na (18.75 mg, 0.125 mol per mol of sugar monomer) in water (0.2 mL). The solution was stirred at rt for 15 min and this step was repeated. The solution was adjusted to pH 7–8 with NaOH (10 N) and a 0.5 molar excess of Ac_2O (105 μL) was added. One mL of the stirred solution was removed 15 min later for amino group measurement (see below), and 0.25 molar excess of bromoacetyl-NHS (132 mg) in DMF 400 μL was added to the remaining sample. Another 1.0 mL of the solution was removed after 20 min. Additional Ac_2O (840 μL) was added followed by stirring for 15 min. During the bromoacetylation and acetylation reactions the pH was kept at 7–8 with NaOH (10 N). The product was eluted with water and collected as fraction 90–180 mL from a 5.6 × 15 cm P-6 column. It was then lyophilized, redissolved in water (5 mL), and fractionated on a P-60 column (1.6 × 100 cm; calibrated with dextrans from Sigma) in 25 mM HEPES, 25 mM NaCl, 0.01% NaN_3, pH 7.0 with refractive index detection. Product fractions (50–60 mL) having an average estimated M_r of 10 000 were pooled, and dialyzed against running water (distilled and deionized) for 24 h. Lyophilization gave 65 mg of a white, cotton-like solid which was stored at rt in a vacuum desiccator.

Each of the above two 1.0 mL aliquots were transferred to a separate 5 mL reaction vial containing $NaBH_4$ (20 mg). After stirring at rt overnight and then extensive dialysis against water, the samples were lyophilized and stored in a vacuum desiccator until their primary amino groups were measured (see below).

[14C]Chitin Leash.—This was prepared in a 4-fold larger scale, following the above procedure except that before bromoacetylation with bromoacetyl-NHS, [14C]Ac_2O (50 μCi, 1.85 MBq; 185 mg, 171 μL) and Ac_2O (249 μL) were added to the solution.

MBTH assay.—A known procedure utilizing NO_2H oxidation followed by reaction with MBTH, and then $FeCl_3$ was followed to measure the number of free amino groups (equivalent to the number of 2-amino-2-deoxy-D-glucose residues) in the glycolchitin and derived products[23]. The assay was standardized with 2-amino-2-deoxy-D-glucose on a weight basis. Also the MBTH–Fe^{3+} stage of this proce-

dure was used to measure the number of aldehyde groups, similarly relying on standardization with 2-amino-2-deoxy-D-glucose treated with nitrous acid.

Enzyme activity.—Known methods were used to measure the activity of lysozyme (glycolchitin substrate[24]) and SNase (hyperchromic shift[25]). To measure ribonuclease A activity, ribonuclease A (50 μL) standard solutions (0, 2, 4, 6, 8, and 10 ng/mL) in 0.05% BSA, 0.5 M Tris · HCl, 5 mM EDTA, pH 7.5, or sample, diluted with buffer as necessary, was added to a RNA solution (0.1 mL of 7 mg/mL highly polymerized yeast RNA in water; Calbiochem, San Diego, CA) in a 12 × 75 mm V-shaped plastic test tube. After incubation at 37°C for 20 min, the solution was combined with ice-cold 14 mmol/L solution of lanthanum acetate (50 μL) in 24% perchloric acid, placed in an ice bath for 5 min, and centrifuged at 1 700*g* for 15 min at 4°C. A 0.1-mL aliquot of the supernatant was diluted with water (1.0 mL) for an absorbance reading at 260 nm of the acid-soluble RNA.

RNase-Chitin Leash-Nase.—Chitin Leash (10 mg, M_r = 10 K) was dissolved in 0.1 M NaOAc (2 mL), pH 5.5 and combined with RNase solution (0.2 mL of a 77.8 mg/mL, $E^{0.1\%} = 0.72$; ref 26) prepared with the same buffer. $NaCNBH_3$ (0.62 mg in 20 μL of water) was added to reach a final concentration of 25 mM. After stirring at rt for 42 h, the sample was purified over a P-60 column (1.0 × 30 cm) in 50 mM Tris · HCl, 0.15 M NaCl, pH 7.0 at 6.4 mL/h. Protein concentration in each fraction (0.64 mL) was quantitated by the BCA assay. Fraction No. 20 was analyzed for RNase activity. Fractions 15–22 were pooled and centrifuge-concentrated with a Centricon 10 concentrator to a volume of 230 μL having a protein concentration of 10.38 mg/mL, giving the product.

The preparation of a control for RNase-Chitin was derived from the Chitin Leash (5 mg), glycine (3.75 mg; 100 fold excess over the Chitin Leash), and of $NaCNBH_3$ (0.314 mg) in a total volume of 110 μL. The solution was stirred at rt for 18 h prior to the addition of the RNase solution (0.1 mL, 77.8 mg/mL) and was stirred for an additional 30 h. The purification and assay procedures were followed as above.

SNase (7.67 mg in 0.3 mL of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5) was treated with a 2-fold molar excess over SNase of SPDP (0.287 mg) in DMF (0.1 mL) for 2.5 h at rt. To generate sulfhydryl SNase, mercaptoethanol (1 μL) was added. The resulting sample was centrifuge-purified on a PD-10 column, and 0.3 mL was combined with 160 μL of Chitin Leash-RNase solution, bubbled with N_2 for 2 min, and stirred at rt for 48 h in the dark. The purification of the resulting SNase-Chitin Leash-RNase was carried out on a P-100 column (1.0 × 30 cm) in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0 at a flow rate of 6.2 mL/h. Fractions 8–15 mL were pooled for rechromatography under the same conditions. Protein quantitation by BCA, enzyme activity assays, and disulfide content were done on each elution fraction (0.67 mL). Ellman's reagent was used for the latter measurement[27].

A control for the preparation of SNase-Chitin Leash-RNase was derived from sulfhydryl SNase (0.1 mL) that was quenched with a 30-fold molar excess over the

sulfhydryl SNase of NEM (144 μg) in DMF (12 μL) for 10 min at rt, followed by the addition of the Chitin Leash-RNase solution (50 μL). The sample was subjected to the same reaction and purification procedures.

SNase-[14C]Chitin Leash-RNase.—The above procedure was followed with the following exceptions: (*1*) The scale was 4-fold larger; (*2*) 50 mM $NaCNBH_3$, 0.1% NaN_3, and 90 h incubation were used to prepare [14C]Chitin Leash-RNase; and (*3*) the reaction period for the preparation of SNase-[14C]Chitin Leash-RNase was 96 h. The purified SNase-[14C]Chitin Leash-RNase (1.89 mg/mL) was stored at −20°C in aliquots.

Succinyl-lysozyme.—Sodium phosphate (70 mL, 0.2 M), pH 7.4, containing lysozyme (800 mg) (determined by an absorbance measurement at 281.5 nm, $E^{0.1\%} = 2.64$; ref 23) was combined with a 50-fold molar excess of succinic anhydride (278 mg) and stirred at rt for 30 min, followed by more succinic anhydride (278 mg). After 2.5 h, the product was dialyzed overnight against running distilled water, filtered through a paper filter (size 4, Whatman, UK) on a glass funnel, and lyophilized. The product, which lacked primary amine groups, based on the TNBS test, was stored at −20°C.

DAO-succinyl-lysozyme. Succinyl-lysozyme (413 mg) was dissolved in 1 M DAO (15 mL). After the pH was adjusted to 5.0 with 1 N HCl, a 2-fold molar excess over succinyl-lysozyme of EDC (11 mg) was added. After stirring at rt for 80 min, the solution was dialyzed for 48 h against running distilled water. To redissolve most of the precipitate formed during the dialysis, 4 M K_2HPO_4 (0.4 mL) was added to the dialysate, giving pH ~ 8.0, followed by filtration and lyophilization. The product was stored at −20°C.

Hydrolysis of SNase-[14C]Chitin Leash-RNase. SNase-[14C]Chitin Leash-RNase (0.2 mL, 1.89 mg/mL) was treated with lysozyme (50 μg) or DAO-succinyl lysozyme in 50 mM sodium phosphate (50 μL), 0.1 M NaCl, 1 mM EDTA, pH 7.0 in a 12 × 75 mm plastic test tube. After incubation at 37°C, 10 μL-aliquots were injected into a Superose 12 column as a function of time (mobile phase: 0.1 M HEPES, 0.5 M NaCl, 1 mM EDTA, pH 7.2 at 0.6 mL/min) with monitoring by UV (280 mm). Fractions were collected and analyzed for protein concentration (BCA assay), and also RNase and SNase activities.

Composition of RNase-[^{14}C]Chitin Leash-SNase.—The radioactivity defined the concentration of the [^{14}C]Chitin Leash. (The specific activity of the unconjugated [^{14}C]Chitin Leash was previously determined by counting a known weight of this substance.) Total protein was determined by the BCA method, and RNase with Ellman's reagent (SNase lacks disulfides). Subtraction of the concentration of RNase from the total protein concentration gave the concentration of SNase.

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