Systematic Purification of Five Glycosidases from Streptococcus (Diplococcus) pneumoniae*

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Five of the six known glycosidases in the culture medium of Streptococcus pneumoniae have been purified from 600- to 55,000-fold by a systematic procedure of ion exchange and affinity chromatography. Following partial separation of the glycosidases on DEAE-Sephadex, the neuraminidase, the endo-α-N-acetylgalactosaminidase, the β-galactosidase, and the β-N-acetylgalactosaminidase were further purified on agarose affinity adsorbents with ligands derived, respectively, from ovine submaxillary mucin glycopeptides, antifreeze glycoprotein, p-aminophenyl-1-thio-β-D-galactoside, or p-aminophenyl-1-thio-β-D-N-acetylglucosaminide. The purified enzymes had specific activities from 25 to 48 μmol/min/mg. The endo-β-N-acetylgalactosaminidase was purified further by gel filtration and ion exchange chromatography and a persistent contaminant of β-N-acetylgalactosaminidase was removed by adsorption on p-aminophenyl-1-thio-β-D-N-acetylglucosaminide-agarose. Each glycosidase preparation was substantially free of contaminating glycosidic, hemolytic, and proteolytic activities. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single polypeptide species for the β-galactosidase, the endo-α-N-acetylgalactosaminidase, and for the β-N-acetylgalactosaminidase, corresponding to apparent molecular weights of 350,000, 190,000, and 180,000, respectively.

Rapid assay procedures for three of the glycosidases were developed. Substrates for neuraminidase and endo-β-galactosidase were synthesized by treatment of asialo-α,α-acid glycoprotein with specific glycosyltransferases to produce either [14C]NeuAcα2 → 6Gal, or [14C]GalNAcα1 → 3 (Fucα1 → 2)Gal at the nonreducing termini of the oligosaccharide chains. The only ovalbumin glycopeptide, (AsnGlcNAc3Man3), that served as a substrate for the S. pneumoniae endo-β-N-acetylgalactosaminidase was labeled in the terminal mannose residues by reduction with NaBH4, after mild periodate treatment.

Glycosidases are useful tools for the structural and functional analysis of oligosaccharides associated with glycoproteins and cell membranes. However, many glycosidases that have been described are of limited value because of low pH optima (1), activity only with low molecular weight substrates (2), or contaminating activities (3).

Streptococcus (Diplococcus) pneumoniae type I is a rich source for six extracellular glycosidases which are active at neutral pH on both low and high molecular weight substrates. Neuraminidase (5), endo-β-galactosidase (6), β-galactosidase (5), endo-α-N-acetylgalactosaminidase (7, 8), β-N-acetylgalactosaminidase (9), and endo-β-N-acetylgalactosaminidase (10), have been partially purified and characterized from this source although none has been purified to homogeneity.

The neuraminidase, the β-galactosidase, and the β-N-acetylgalactosaminidase are exoglycosidases which hydrolyze glycosidic bonds formed by sialic acid, galactose, and N-acetylgalcosamine, respectively, when these unsubstituted monosaccharides are at the nonreducing end of oligosaccharides (5, 9). The endo-α-N-acetylgalactosaminidase hydrolyzes glycosidic bonds formed by N-acetylgalactosamine and the hydroxyl group of either serine or threonine when in the sequence Galβ1 → 3GalNAcα-O-Ser/Thr (7, 8). The endo-β-N-acetylgalactosaminidase hydrolyzes the glycosidic bond between 2 N-acetylgalactosamine residues in the oligosaccharide with the following structure (11, 12).

\[
\begin{align*}
\text{Man}(\alpha1 \rightarrow 6) \\
\text{Man}β1 \rightarrow 4\text{GlcNAc}β1 \rightarrow 4\text{GlcNAc-Asn}
\end{align*}
\]

Other sugars may be substituted on the mannose linked α1 → 6 to mannose but the mannose linked α1 → 3 to mannose cannot be substituted (11, 12). Moreover, the N-acetylgalcosamine linked to asparagine may be substituted in α1 → 6 linkage with fucose. The endo-β-galactosidases hydrolyze oligosaccharides and A and B blood group types to release trisaccharides with the structures GalNAcα1 → 3 (Fucα1 → 2)Gal (A type) and Galα1 → 3 (Fucα1 → 2)Gal (B type) when the galactose is in a β1 → 4 linkage but not in a β1 → 3 linkage with N-acetylgalactosamine or N-acetylgalactosamine (6).

Following published procedures it has been difficult to purify each enzyme free of residual contamination of the

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† Streptococcus pneumoniae type I is often designated in the literature as Diplococcus pneumoniae. Although the organism was earlier classified by the latter name, S. pneumoniae is now preferred (4).
other glycosidases (3). This paper presents a systematic method of purification for five of these glycosidases free of contaminating glycosidic, proteolytic, or hemolytic activities. Four of the enzymes have specific activities from 75 to 2000 times those reported previously. The β-galactosidase, the endo-α-N-acetylgalactosaminidase, and the β-N-acetylglucosaminidase appear homogenous as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. A preliminary report of this work has been presented (13).

**EXPERIMENTAL PROCEDURES**

### Materials

- p-Nitrophenyl β-D-galactoside and p-nitrophenyl β-D-N-acetylgalactosaminide were obtained from Sigma Chemical Co.
- p-Aminophenyl-1-thio-β-D-galactoside was obtained from Sigma Chemical Co.
- p-Nitrophenyl-β-D-galactoside and p-nitrophenyl β-D-N-acetylgalactosaminide were obtained from Bachem Inc. (Marina Del Ray, Calif.).
- CMP-[14C]NeuAc, UDP-[14C]GalNAc, GDP-[U-14C]Fuc, and NaBH₄, were obtained from New England Nuclear. Ovalbumin was purchased from Sigma Chemical Co.
- GDP-fucose (14) and oligosaccharides were obtained from P. A. L. DeVries (University of Illinois) (17).
- Dr. K. Schmid (Boston University School of Medicine) supplied a generous gift of α-galactosidase. Streptococcus pneumoniae was obtained by Dr. G. Ashwell (National Institutes of Health).

#### Analytical Methods

Free sialic acid was estimated by the thiobarbituric acid procedure (18) and total sialic acid by the Svennerholm (19) or the periodate resorcinol methods (20). N-Acetylglucosamine and Galβ1→3GalNAc were assayed by the Morgan-Elson reaction (21). The galactose content in acid hydrolysates of oligosaccharides was determined with galactose dehydrogenase (22-25). Protein was determined by the Lowry method (25) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed as previously described (26) and molecular weights were estimated by this method employing a cross-linked protein with known molecular weights (53,000 to 218,000) obtained from Gsell-Ehinger Biochemical Mfg. Corp. Hexosaminidase activity was determined by incubation of the sample (20 μl) with 0.5 ml of human erythrocytes (50% v/v) in 25 mM sodium barbitol, pH 7.1, 0.9% sodium chloride at 37°C for 30 min. Cells were removed by centrifugation and the absorbance of the diluted supernatants determined. Each sample was compared to a control in which the cells were lysed with denaturating water. Proteolytic activity was assayed by the method of Lin (27).

#### Preparation of Enzymes

α-N-Acetylgalactosaminidase was obtained from Clostridium perfringens (15). Partially purified α-fucosidase from C. perfringens was prepared by a procedure employing chromatography of the cell-free growth medium on Bio-Gel P-100 and DEAE-cellulose as described for the α-N-acetylgalactosaminidase. C. perfringens neuraminidase from Worthington Biochemical Co. was freed of protease activity as described previously (28). β-D-Galactoside α2→6 sialyltransferase (29) and β-D-fucosyl α1→2galactoside α1→3 β-N-acetylgalactosaminyltransferase (30) were prepared as previously described. β-Galactoside α1→2 fucosyltransferase was prepared from a Triton X-100 extract of porcine submaxillary glands by chromatography on Sephadex G-25 and GDP-agarse.

### Preparation of α,β-Galactosyltransferases

The disaccharide Galβ1→3GalNAc was obtained by digestion of antifreeze glycoproteins with pure endo-α-N-acetylgalactosaminidase and isolated by gel filtration on a column (1.5 x 33 cm) of Sephadex G-25 (superfine) equilibrated with water. The disaccharide contained only galactose and N-acetylgalactosamine in a 1:1 molar ratio and gave a single reducing sugar spot (31) on descending paper chromatography (Whatman No. 3MM, ethyl acetate/pyridine/water, 2:1:2).

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Neuraminidase—Assay mixtures (75 µl) contained 2 µmol of sodium cacodylate, pH 6.0, 1 mg of α,α-dicyclohexylcarbodiimide, and 7.5 µg of bovine serum albumin. Reactions were initiated by the addition of enzyme and were incubated at 37°C for 10 min. The sialic acid released was estimated by the thiobarbituric acid procedure (51). One unit of activity will release 1 µmol of sialic acid/min under these assay conditions.

An alternate assay used to locate neuraminidase in column eluates employed [14C]NeuAc,α,α-dicyclohexylcarbodiimide. Incubation mixtures (55 µl) contained [14C]NeuAc,α,α-dicyclohexylcarbodiimide (12,000 cpmp), 0.55 µmol of sodium cacodylate, pH 6.0, and 7.5 µg of bovine serum albumin. Enzyme (2 µl) was added and the reaction incubated for 6 min at 37°C and stopped with 1 ml of 50 mM sodium citrate, pH 3.0. The mixture was applied to a column (1 ml) of Dowex 50-X2 (200 to 400 mesh) in a Pasteur pipette followed by 1.5 ml of 50 mM sodium citrate, pH 3.0. The eluate, which contained the [14C]sialic acid, was collected directly into scintillation vials and counted. The assay was linear with time and enzyme concentration when less than 25% of the total counts were released.

Endo-β-galactosidase—Assay mixtures (50 µl) contained [14C](Galβ1→3/Fucα1→2)Gai,α,α-dicyclohexylcarbodiimide (12,200 cpmp), 0.5 µmol of sodium cacodylate, pH 6.0, and 1.5 µg of bovine serum albumin. Enzyme was added to initiate the reaction and the mixtures were incubated at 37°C for 20 min. Reaction was stopped with 1.0 ml of sodium citrate, pH 3.0, and the free [14C](Galβ1→3/Fucα1→2)Gai was separated from the [14C]-protein substrate on a column of Dowex 50-X2 (200 to 400 mesh) as described above for the neuraminidase assay. Activity is expressed as counts per min released under the assay condition.

β-Galactosidase—Assay mixtures (50 µl) contained 0.2 µmol of p-nitrophenyl-p-N-acetylglucosaminide (pNPG) and 0.2 µmol of p-nitrophenyl-p-N-acetylglucosaminide (pNPG) as substrate for the endo-α-N-acetylglucosaminidase and p-nitrophenyl-p-N-acetylglucosaminide-Sepharose 4B—Either (Galβ1→3GalNAc)-α,α-dicyclohexylcarbodiimide was added to an equal volume of the same buffer containing ovine serum albumin. Enzyme (2 to 10 µl) was added to initiate the reaction, and after incubation at 37°C for 5 to 15 min, the reaction was stopped by adding 1 ml of 0.5 M sodium carbonate and the absorbance at 400 nm was measured (ε400 nm = 17.7). One unit of activity will hydrolyze 1 µmol/min under these assay conditions.

Endo-α-N-acetylgalactosaminidase—Either (Galβ1→3GalNAc)-porcine submaxillary mucin or antifreeze glycoprotein was used as substrate for the endo-α-N-acetylgalactosaminidase. Enzyme (2 µl) was added to 100 µl of substrate (0.2 µmol in 50 mM sodium cacodylate, pH 6.0, and 10 µg of bovine serum albumin). Enzyme (2 to 10 µl) was added to initiate the reaction, and after incubation at 37°C for 5 to 30 min, the reaction was stopped with the addition of 1 ml of 0.5 M sodium carbonate and the absorbance at 400 nm was measured (ε400 nm = 17.7). One unit of activity will hydrolyze 1 µmol/min under these assay conditions.

β-N-Acetylgalactosaminidase—β-N-Acetylgalactosaminidase was assayed as described for the β-galactosidase with p-nitrophenyl-p-N-acetylglucosaminide as substrate.

Endo-β-N-acetylglucosaminidase—The enzyme was assayed by a modification of the method of Tarentino and Maley (54). Emulsion (2 µl) was added to 50 µl of 20 mM sodium cacodylate, pH 6.0, and incubated at 37°C for 20 min. The reaction was stopped with the addition of 50 µl of 0.8 M potassium borate, pH 9.1, and the released disaccharide measured with the Morgan-Elson method for N-acetylamino sugars (21). Galβ1→3GalNAc gives 110% of the color yield of free GalNAc. Units of activity of the enzyme are defined as micromoles of Galβ1→3GalNAc released per min under the assay conditions. The assay with antifreeze glycoprotein was linear with time and enzyme concentration up to a final absorbance at 585 nm of 0.25.

β-N-Acetylgalactosaminidase—β-N-Acetylgalactosaminidase was assayed as described for the β-galactosidase with p-nitrophenyl-p-N-acetylglucosaminide as substrate.

Preparation of Affinity Adorbents

All adsorbents were prepared by reacting cyanogen bromide-activated Sepharose 4B (29, 35) with the appropriate ligand. Adsorbents were stored in 0.1% sodium azide.

Ovine Submaxillary Mucin-Sepharose 4B—Glycopeptides of ovine submaxillary mucin were prepared by digestion with thermolysin. Mucin (10 mg/ml) dissolved in 0.1 M Tris/HCl, pH 8.0, containing 10 mM calcium chloride, was incubated with thermolysin (30 µg/ml) overnight at 37°C, dialyzed against deionized water, and lyophilized. Cyanogen bromide-activated Sepharose 4B was washed with 10 volumes of 0.2 M sodium pyrophosphate, pH 8.5, and then added to an equal volume of the same buffer containing ovine submaxillary mucin glycopeptides (10 mg/ml). After 2 h at room temperature, the gel was washed in a sintered glass funnel, and the filtrate retained for quantitation of uncoupled protein (ε280 nm = 1.3). About 50% of the antifreeze glycoprotein was coupled giving 12 µmol of Galβ1→3GalNAc of settled gel.

p-Aminophenyl-1-thio-β-galactoside-Sepharose 4B—Cyanogen bromide-activated gel was washed with 0.2 M sodium pyrophosphate, pH 8.5, and added to an equal volume of the same buffer containing antifreeze glycoprotein (14 mg/ml). The reaction was allowed to proceed at 4°C for 5 min and then for an additional 2 h at room temperature. The gel was washed with 1.0 M NaCl in a sintered glass funnel and the filtrate retained for quantitation of uncoupled protein (ε280 nm = 1.3). About 50% of the antifreeze glycoprotein was coupled giving 12 µmol of Galβ1→3GalNAc of settled gel.

p-Aminophenyl-1-thio-β-N-acetylgalactosaminide-Sepharose 4B—The ligand was coupled as described for the p-nitrophenyl-1-thio-β-galactoside to yield 3 µmol of p-nitrophenyl-1-thio-β-N-acetylgalactosaminide/ml of settled gel. Before use the gel was treated with an equal volume of 1 M ethanolamine, pH 8.5, at room temperature for 2 h and washed exhaustively with deionized water.

Purification of S. pneumoniae Glycosidases

Step 1: Culture Supernatant—A strain of S. pneumoniae, type 1, was grown for 72 h at 37°C in 200 ml of tryptone soja broth (TSB) supematant (55). The cells were collected by centrifugation at 7200 × g for 20 min and discarded. Further procedures were performed at 4°C unless otherwise noted.

Step 2: First Ammonium Sulfate Precipitation—Ammonium sulfate (301 g/liter) was added with stirring to the cell-free growth medium, the solution stirred overnight, and then centrifuged at 7200 × g for 30 min. The resulting sticky brown precipitate was dissolved in a minimum volume of water and dialyzed overnight against distilled water.

Step 3: Second Ammonium Sulfate Precipitation—Ammonium sulfate (175 g/liter) was added to the dialyzed fraction from Step 2, stirred for 2 h, and the precipitate removed by centrifugation at 7200 × g for 40 min and discarded. Additional ammonium sulfate (310 g/liter) was added to the supernatant and after 1 h, the precipitate containing the glycosidases was collected by centrifugation (7200 × g for 40 min), dissolved in a minimum amount of water, and dialyzed against 10 mM Tris-HCl, pH 7.5.

Step 4: Chromatography on DEAE-Sephadex A-25—The dialyzed fraction was applied to a column of DEAE-Sephadex A-25 (5.0 × 120 cm) equilibrated in 10 mM Tris-HCl, pH 7.5 (50). The column was washed with 3 liters of equilibration buffer followed by a linear gradient of sodium chloride formed with 12.5 liters of 10 mM Tris-HCl and 12.5 liters of 10 mM Tris-HCl containing 0.5 M sodium chloride. Fractions (15 ml) were collected at a flow rate of 5.5 ml/min. Peaks of glycosidase activity were pooled as they eluted from the column to give fractions enriched in neuraminidase and endo-β-galactosidase (A), β-galactosidase (B), endo-α-N-acetylgalactosaminidase (C), β-N-acetylgalactosaminidase (D), and endo-β-N-acetylgalactosaminidase (E).

Step 5 (A to E): Concentration of Glycosidase Fractions—Each fraction (A to E) was stirred with ammonium sulfate (510 g/liter) 1 h, centrifuged at 7200 × g for 30 min, and the precipitate containing the glycosidases was dissolved in a minimum amount of water and dialyzed against 0.1 M cacodylate, pH 6. Fraction A (60 ml) was further concentrated to 10 ml by ultrafiltration over a PM-30 membrane (Amicon Corp.).

Step 6A: Chromatography of Neuraminidase on Ovine Submaxillary Mucin-Sepharose 4B—Concentrated neuraminidase from step 5 (Fraction A, 10 ml) was applied to a column (9 × 4 cm) of step
submaxillary mucin-Sepharose 4B equilibrated with 0.1 M sodium cacodylate, pH 6.0 at 4°C, and the column was then washed with 400 ml of 20 mM sodium cacodylate, pH 6.0, containing 2.0 M sodium chloride. The column was then immersed in a constant temperature bath (37°C) and neuraminidase was eluted by the immediate application of 30 ml of 20 mM sodium cacodylate, pH 6.0, containing 1.0 M sodium chloride. Fractions were collected in an ice bath, adjusted to pH ~7.5 with 1 M cacodylic acid, and those containing neuraminidase were pooled. A flow rate of 12 ml/min was maintained throughout the column development.

**Step 6B: Chromatography of β-Galactosidase on p-Aminophenyl-thio-β-D-galactoside-Sepharose 4B** — The concentrated galactosidase solution from Step 5 (Fraction B, 61 ml in 10 mM sodium cacodylate, pH 6.0) was made 0.15 M in sodium chloride by addition of the solid salt and applied to a column (1.5 x 4 cm) of p-aminophenylthio-β-D-galactoside-agarose equilibrated with 0.1 M sodium cacodylate, pH 6.0. A flow rate of about 1.5 ml/min was maintained throughout the column development. The column was washed with 60 ml of 1 M sodium chloride in 0.1 M sodium cacodylate, pH 6.0. Elution of the β-galactosidase was achieved with 15 mM p-aminophenyl-thio-β-D-galactoside in 50 mM sodium cacodylate, pH 6.0, in the following manner. One bed volume (8 ml) was applied at 4°C, the column was immersed in a 37°C constant temperature bath and elution immediately resumed. Fractions were moved to an ice bath soon after collection. Active fractions were pooled and dialyzed exhaustively against 25 mM sodium cacodylate, pH 6.0. Elution of the β-galactosidase could also be achieved by using 20% ethylene glycol (v/v) instead of the 15 mM p-aminophenyl-thio-β-D-galactoside in the elution buffer.

**Step 6C: Chromatography of Endo-α-N-acetylgalactosaminidase on Antifreeze Glycopeptide-Sepharose 4B** — The concentrated solution of endo-α-N-acetylgalactosaminidase from Step 5 (Fraction C, 85 ml) in 0.1 M sodium cacodylate, pH 6.0, was made 0.05 M in NaCl by adding the solid salt, and applied at 4°C to a column (0.6 x 3 cm) of antifreeze glycopeptide-agarose at a flow rate of 1 ml/min. The column was washed with 60 ml of 2 M NaCl in 20 mM sodium cacodylate, pH 6.0. The column was brought to 37°C and elution continued with the same buffer. Fractions of 2 ml were collected and those containing the endo-α-N-acetylgalactosaminidase were pooled.

**Step 6D: Chromatography of β-N-Acetylgalactosaminidase on p-Aminophenylthio-β-N-acetylgalactosamine-Sepharose 4B** — The concentrated N-acetylgalactosaminidase from Step 5 (Fraction D, 60 ml) was mixed with 6 ml of 1 M sodium chloride containing 0.1 M sodium cacodylate, pH 6.0, and applied to a column (1.5 x 8.5 cm) of p-aminophenylthio-β-N-acetylgalactosamine-agarose equilibrated with 0.05 M NaCl in 0.1 M sodium cacodylate, pH 6.0. The column was washed with 100 ml of 0.5 M NaCl, 0.05 M sodium cacodylate, pH 6.0, and the β-N-acetylgalactosaminidase then eluted with 1 M NaCl, 50 mM Tris-HCl, pH 6.4, at a flow rate of 1 ml/min. Pooled active fractions were adjusted to pH 6.5 to 7.0 with 1 M cacodylic acid and dialyzed overnight against 50 mM sodium cacodylate, pH 6.0.

**Step 6E: Chromatography of Endo-β-N-acetylglucosaminidase on Sephacryl G-200** — A column (5 x 88 cm) of Sephacryl G-200 was equilibrated with 0.01 M Tris-HCl, pH 7.6, containing 0.1 M NaCl. Endo-β-N-acetylglucosaminidase concentrated to 85 ml in Step 6D was applied to the column. The exclusion volume of the column containing the enzyme activity was pooled.

**Step 7E: Chromatography of Endo-β-N-acetylglucosaminidase on DEAE-Sephadex A-25 and p-Aminophenyl-1-thio-β-N-acetylglucosaminide-agarose** — The concentrated β-N-acetylglucosaminidase was further purified on DEAE-Sephadex A-25 as previously described (10). To remove the substantial β-N-acetylglucosaminidase activity that remained, the enzyme was adjusted to pH 6.0 with 1 M sodium citrate and applied to an 8-ml column of p-aminophenyl-1-thio-β-N-acetylglucosaminide-agarose. The unretarded endo-β-N-acetylglucosaminidase activity was pooled and stored at -20°C.

## RESULTS

**Purification of Streptococcus pneumoniae Glycosidases** — Glycosidases were prepared from 15 liters of S. pneumoniae culture filtrate. The enzymes were initially fractionated on DEAE-Sephadex A-25 as shown in Fig. 1, and each partially pure glycosidase was further purified to remove contaminating glycosidases. A summary of the purification is given in Table I, which lists the steps common for all enzymes through chromatography on DEAE-Sephadex A-25, and the further purification of each enzyme separately.

### Purification of Neuraminidase

The neuraminidase from Step 5 was contaminated with endo-β-galactosidase and β-galactosidase. Two affinity adsorbents that have been used in the purification of other neuraminidases, N-(p-aminophenyl)-oxamic acid-agarose (36) and α1-acid glycoprotein-agarose (38), were found unsuitable for further purification since the other enzymatic activities also bound these adsorbents and eluted with the neuraminidase. One problem with the α1-acid glycoprotein-agarose is that degradation during chromatography may produce terminal galactose residues that serve as potential binding sites for the β-galactosidase. For this reason, ovine submaxillary mucin-agarose containing the disaccharide NeuAcα2 → 6GalNAc was examined and found to be effective. An example of the purification of 145 units of neuraminidase obtained on ovine submaxillary mucin-agarose is shown in Fig. 2. The neuraminidase was eluted substantially free of other glycosidases with variable yields of 27 to 80%. The final specific activity (45 units/mg of protein) is about 400 times higher than reported previously for this enzyme (5). The purified enzyme was found to be unstable, but with the addition of 0.25 mg of bovine serum albumin/ml, no loss of activity was observed over 2 months when stored at -20°C.

The column size and the time spent on the affinity adsorbent were critical factors for optimal purification. Loading of excess enzyme or prolonging the time of development of the column resulted in column failure. Loading of more enzyme enhanced the purification of the column, allowing for enzymatic cleavage of the sialic acid and premature elution of the enzyme. Several test columns showed that the enzyme activity was observed over 1 month when stored at -20°C.

### Purification of β-Galactosidase

The purification of the β-galactosidase is summarized in Table I. An example of the purification of β-galactosidase obtained on the affinity adsorb-
TABLE 1

Purification of glycosidases from *Streptococcus pneumoniae*

Results are shown for the preparation of five glycosidases from 15 liters of cell-free supernatant. Details of the purification scheme are given under "Experimental Procedures." Examples of the purification at different stages are given in Fig. 1 (Step 4A-E), Fig. 2 (Step 6A), Fig. 3 (Step 6B), Fig. 4 (Step 6C), and Fig. 5 (Step 6D).

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<td></td>
<td>(ml)</td>
<td>(mg)</td>
<td>(units)</td>
<td>(units/mg)</td>
<td>(%)</td>
<td></td>
</tr>
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<td>1.</td>
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<td>1,134</td>
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<td>13,900</td>
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### Neuraminidase

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<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<tr>
<td>4A.</td>
<td>DEAE-A25</td>
<td>3,200</td>
<td>2,016</td>
<td>563</td>
<td>0.28</td>
<td>50</td>
</tr>
<tr>
<td>5A.</td>
<td>Concentration</td>
<td>10</td>
<td>811</td>
<td>328</td>
<td>0.4</td>
<td>29</td>
</tr>
<tr>
<td>6A.</td>
<td>OSM-agarose</td>
<td>165</td>
<td>3.24</td>
<td>145</td>
<td>45</td>
<td>13</td>
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### B-Galactosidase

<table>
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<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<tr>
<td>4B.</td>
<td>DEAE-A25</td>
<td>2,100</td>
<td>651</td>
<td>49</td>
<td>0.075</td>
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<tr>
<td>5B.</td>
<td>Concentration</td>
<td>61</td>
<td>603</td>
<td>59</td>
<td>0.098</td>
<td>56</td>
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<tr>
<td>6B.</td>
<td>Gal-agarose</td>
<td>17</td>
<td>0.5</td>
<td>12.4</td>
<td>25</td>
<td>12</td>
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### Endo-a-N-Acetylgalactosaminidase

<table>
<thead>
<tr>
<th>Step</th>
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<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
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<tr>
<td>4C.</td>
<td>DEAE-A25</td>
<td>2,250</td>
<td>945</td>
<td>105</td>
<td>0.12</td>
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<tr>
<td>5C.</td>
<td>Concentration</td>
<td>85</td>
<td>935</td>
<td>59</td>
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<tr>
<td>6C.</td>
<td>Antifreeze-Glycoprotein-agarose</td>
<td>18</td>
<td>1.5</td>
<td>40</td>
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### B-N-Acetylglucosaminidase

<table>
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<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
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<tr>
<td>4D.</td>
<td>DEAE-A25</td>
<td>3,200</td>
<td>1,000</td>
<td>363</td>
<td>0.216</td>
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<tr>
<td>5D.</td>
<td>Concentration</td>
<td>64</td>
<td>975</td>
<td>254</td>
<td>0.261</td>
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<tr>
<td>6D.</td>
<td>GlcNAc-agarose</td>
<td>34</td>
<td>3.6</td>
<td>176</td>
<td>48.0</td>
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### Endo-p-N-Acetylglucosaminidase

<table>
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<tr>
<th>Step</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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</thead>
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<tr>
<td>5E.</td>
<td>DEAE-25 and Concentration</td>
<td>85</td>
<td>1,138</td>
<td>482</td>
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<td>6E.</td>
<td>G-200 Sepharcll</td>
<td>80</td>
<td>352</td>
<td>4.4</td>
<td>32</td>
<td>913</td>
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<td>7E.</td>
<td>DEAE-A25, GlcNAc-agarose</td>
<td>10</td>
<td>30</td>
<td>3.1</td>
<td>3</td>
<td>623</td>
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</tbody>
</table>

Abbreviations used are a-NeuAc-ase for neuraminidase, B-Gal-ase for B-galactosidase, Endo-a-GalNAc-ase for endo-α-N-acetylgalactosaminidase, B-GlcNAc-ase for B-N-acetylglucosaminidase and Endo-β-GlcNAc-ase for endo-β-N-acetylglucosaminidase. A unit of activity is defined as 1 μmol of product formed/min determined by the standard assay.

- A pmol of product formed/min/mg of protein.
- a-1-Acid glycoprotein was used as substrate in the standard assay.
- Antifreeze glycoprotein was used as substrate in the standard assay.
- OSM-agarose refers to ovine submaxillary mucin-Sepharose-4B.
- Gal-agarose refers to p-aminophenyl-1-thio-B-β-galactoside-Sepharose-4B.
- GlcNAc-agarose refers to p-aminophenyl-1-thio-B-β-N-acetylglucosaminide-Sepharose-4B.
FIG. 2. Purification of neuraminidase on ovine submaxillary mucin-agarose. The concentrated neuraminidase fraction (10 ml in 10 mM sodium cacodylate) from Step 5A was applied to a column of ovine submaxillary mucin-agarose (9 x 4 cm). The column was washed at 4°C with 2.0 M sodium chloride in 0.02 M sodium cacodylate, pH 6.0 (A) and elution of the neuraminidase was initiated by bringing the column to 37°C and washing with 1.0 M sodium chloride in 0.05 M sodium borate, pH 9.0 (B). Details of the column conditions are given in Step 6A of "Experimental Procedures." Symbols refer to protein (O), neuraminidase (Δ, 1 = 200 cpm in standard ([14C]NeuAcα-3GalNAcβ-3(Fucoseβ-2Gal)α–acid glycoprotein assay), endo-β-galactosidase (▪, 1 = 200 cpm in standard (14C)[GalNAcβ-3(Fucoseβ-2Gal)α–acid glycoprotein assay) and β-galactosidase (□, 1 = 0.001 unit/ml).

FIG. 3. Purification of β-galactosidase on p-aminophenyl-1-thio-β-D-galactoside-agarose. The concentrated β-galactosidase fraction obtained from Step 5B (61 ml in 10 mM sodium cacodylate, pH 6.0, 0.15 M sodium chloride) was applied to a column (1.5 x 4 cm) of p-aminophenyl-1-thio-β-D-galactoside-agarose. The column was washed with 0.1 M sodium cacodylate, pH 6.0, 1.0 M sodium chloride (A) and the enzyme eluted with 15 mM p-aminophenyl-1-thio-β-D-galactoside in 50 mM sodium cacodylate, pH 6.0 (B). Details of column conditions are given in Step 6B of "Experimental Procedures." Symbols refer to protein (O), neuraminidase (Δ, 1 = 360 cpm in standard assay), endo-β-N-acetylglucosaminidase (▪, 1 = 0.25 unit/ml), endo-α-N-acetylglucosaminidase (□, 1 = 0.025 unit/ml), and endo-β-galactosidase (□, 1 = 40 cpm in standard assay).

ent, p-aminophenyl-1-thio-β-D-galactoside-agarose, is shown in Fig. 3. The majority of the protein and the contaminating glycosidases present in the galactosidase (Fraction B) from the DEAE-step are unretarded on the column to yield a highly purified β-galactosidase substantially free of other glycosidases. The specific activity (25 units/mg of protein) was about 75 times that reported previously (5). Variable yields of 20 to 80% were obtained at this step. Although the basis for the occasional low yields has not been systematically examined, better yields were obtained when 20% ethylene glycol was used to elute the enzyme instead of p-aminophenyl-1-thio-β-D-galactoside. The enzyme lost 10 to 15% activity in 30 days when stored at either 4°C or 30°C at a concentration of 30 μg/ml in 50 mM sodium cacodylate, pH 6.0. No loss of activity was observed when the enzyme was stored at 4°C or −20°C in bovine serum albumin (0.5 mg/ml).

Purification of Endo-α-N-acetylglucosaminidase - The purification of the endo-α-N-acetylglucosaminidase on antifreeze glycoprotein-agarose is shown in Fig. 4. The enzyme was eluted free of other enzymatic activities with a 65% step yield to give a specific activity (27 units/mg of protein) over 2000 times that obtained by conventional purification procedures (7). The β-galactosidase does not bind presumably because of its strict substrate specificity for Galβ1→4R linkage. The enzyme can be stored for at least 2 months at −20°C with no loss of activity. Limitations on column capacity and chromatography time have not been observed but significant changes in the method used could cause hydrolysis of the affinity ligand from the agarose.

Purification of β-N-Acetylglucosaminidase - Chromatography of the β-N-acetylglucosaminidase on p-aminophenyl-1-thio-β-D-acetylglucosaminide-agarose is shown in Fig. 5. The enzyme is obtained substantially free of glycosidases with a high specific activity (48 μmol/min/mg of protein) which is about 200 times that obtained previously (9). Although this enzyme was obtained in low overall yield, it is relatively abundant in the culture filtrate. As with the endo-α-N-acetylglucosaminidase, 50% of the enzyme was lost in the first ammonium sulfate precipitation. By completing this step rapidly, however, virtually quantitative yields have been obtained (9).
the incubation times were increased to detect levels of activity in Fig. 4, the \( \text{P-galactosidase} \) flows through this column. The contaminating glycosidase activities were estimated by incubation of the purified enzyme with appropriate substrates. The aliquot of enzyme and time of incubation were increased in the standard assay to detect activities of neuraminidase (10 \( \mu \text{l} \) for 180 min), endo-\( \beta \)-galactosidase (10 \( \mu \text{l} \) for 20 h), \( \beta \)-galactosidase (10 \( \mu \text{l} \) for 180 min), endo-\( \alpha \)-N-acetylglactosaminidase (10 \( \mu \text{l} \) for 210 min), \( \beta \)-N-acetylgalactosaminidase (10 \( \mu \text{l} \) for 180 min), and endo-\( \beta \)-N-acetylgalactosaminidase (10 \( \mu \text{l} \) for 185 min). The results are shown as units of contaminating activity present in 1000 units of the major activity. Endo-\( \beta \)-N-acetylgalactosaminidase activity was not detected in any purified glycosidase.

Contaminating enzyme activities were estimated by incubation of the purified enzyme with appropriate substrates. The aliquot of enzyme and time of incubation were increased in the standard assay to detect activities of neuraminidase (10 \( \mu \text{l} \) for 180 min), endo-\( \beta \)-galactosidase (10 \( \mu \text{l} \) for 20 h), \( \beta \)-galactosidase (10 \( \mu \text{l} \) for 180 min), endo-\( \alpha \)-N-acetylglactosaminidase (10 \( \mu \text{l} \) for 210 min), \( \beta \)-N-acetylgalactosaminidase (10 \( \mu \text{l} \) for 180 min), and endo-\( \beta \)-N-acetylgalactosaminidase (10 \( \mu \text{l} \) for 185 min). The results are shown as units of contaminating activity present in 1000 units of the major activity. Endo-\( \beta \)-N-acetylgalactosaminidase activity was not detected in any purified glycosidase.

### Table II

<table>
<thead>
<tr>
<th>Activity</th>
<th>Neuraminidase</th>
<th>( \beta )-Galactosidase</th>
<th>Endo-( \alpha )-N-acetylglactosaminidase</th>
<th>Endo-( \beta )-N-acetylgalactosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>1,000</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>( \beta )-Galactosidase</td>
<td>0.3</td>
<td>1,000</td>
<td>26</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Endo-( \alpha )-N-acetylglactosaminidase</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>1,000</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Endo-( \beta )-N-acetylgalactosaminidase</td>
<td>1.2</td>
<td>0.78</td>
<td>0.16</td>
<td>0.27</td>
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</tbody>
</table>

**DISCUSSION**

Following the procedure reported in this paper, five of the six glycosidases from *S. pneumoniae* have been purified free of other glycolytic, hemolytic, and proteolytic activities. The enzymes were separated on DEAE-Sephadex (Fig. 1) as reported by others (6, 7, 10) but all had significant activities of each of the other enzymes at this stage. Affinity chromatographic techniques were used to purify the \( \beta \)-galactosidase, the endo-\( \alpha \)-N-acetylglactosaminidase, and the \( \beta \)-N-acetylgalactosaminidase to homogeneity and to purify all five of the enzymes free of contaminating activities.

The neuraminidase and the endo-\( \alpha \)-N-acetylglactosaminidase were purified using degradable affinity adsorbents prepared from their glycoprotein substrates, ovine submaxillary mucin, and antifreeze glycoprotein, respectively. Particular attention to the amount of enzyme applied to a column and the total time of chromatography were important factors in obtaining optimal purification. Under the conditions employed here, the ovine submaxillary mucin-agarose was extensively degraded during chromatography and no attempt was made to use the column a second time. This is not a practical problem since gram quantities of ovine mucin are easily prepared. The antifreeze glycoprotein-agarose was much more stable; however, since the endo-\( \alpha \)-N-acetylglactosaminidase has very little activity at 4°C and is severely inhibited by the 2 M sodium chloride present during elution at 37°C. When 1 ml of the adsorbent containing 10 units of the endo-\( \alpha \)-N-acetylglactosaminidase was kept overnight at 4°C, the adsorption and elution properties of the adsorbent remained unchanged.

**Glycosidases of Streptococcus pneumoniae**

**Fig. 5** Purification of \( \beta \)-N-acetylgalactosaminidase on \( p \)-aminophenyl-1-thio-\( \beta \)-N-acetylglucosaminide-agarose. The concentrated \( \beta \)-N-acetylgalactosaminidase (85 ml in 10 mM sodium cacodylate, pH 6.0, 0.1 \% sodium chloride) from Step 5D was applied to a column (1.5 x 8.5 cm) of \( p \)-aminophenyl-1-thio-\( \beta \)-N-acetylglucosaminide-agarose. Elution was continued with 50 mM sodium cacodylate, pH 6.0, 0.5 \% sodium chloride (A) and the \( \beta \)-N-acetylgalactosaminidase was then eluted from the column with 50 mM Tris·HCl, pH 8.6, 1 M sodium chloride (B). Details of the procedure are given under "Experimental Procedures." Symbols denote protein concentration (\( \bullet \), \( \beta \)-N-acetylgalactosaminidase (\( O \), 1 = 1.1 units/ml), \( \beta \)-galactosidase (\( A \), 1 = 0.006 unit/ml), and endo-\( \alpha \)-N-acetylgalactosaminidase (\( A \), 1 = 0.2 unit/ml).
The S. pneumoniae β-galactosidase and β-N-acetylgalactosaminidase have been purified to homogeneity on affinity adsorbents substituted with p-aminophenyl-1-thio-β-β-N-acetylgalactoside or p-aminophenyl-1-thio-β-β-N-acetylgalactosaminide, respectively. The fact that these two enzymes can be purified free of each other on these adsorbents attests to the specificity with which the glycosidases bind the appropriate substrate analog. p-aminophenyl-1-thioglycoside substituted adsorbents have been widely used as potential affinity adsorbents for glycosidases with variable results (37-43). In most studies a spacer arm between the ligand and the gel matrix was employed and frequently, an adsorbent was found to adsorb several glycosidases in addition to the one which is expected to bind (39-41). In one case, Escherichia coli β-galactosidase has been shown to adsorb equally well to the p-aminophenyl-1-thio-β-β-N-acetylgalactoside adsorbent and to a column containing only the spacer arm (44). In the present report, the p-aminophenyl-1-thioglycosides were coupled directly to cyanogen bromide-activated Sepharose 4B. Since spacer arms provide potential sites for nonspecific adsorption of inert protein or unwanted enzyme activities, it is noteworthy that in this case they were not required. Thus spacer arms need not be considered a necessary component for the design of ligands for all glycosidase affinity adsorbents.

Specific glycosyltransferases have been utilized to synthesize radiolabeled substrates for neuraminidase and endo-β-galactosidase. For the neuraminidase, resialylation of asialo-α2,3-linked glycoprotein by incubation with pure β-galactosidase α2,6-sialyltransferase and CMP[14C]NeuAc produces oligosaccharides with the terminal sequence 14CNeuAca2,6Galβ1→4GlcNAc (24). The endo-β-galactosidase is known to act on substrates that contain the blood group A positive sequence GalNAca1→3Fucα1→2Galβ1→4GlcNAc ... (6). While the oligosaccharides of α2,3-linked glycoprotein do not normally carry this structure, it can be synthesized with the aid of two glycosyltransferases. The β-galactoside α1→2 fucosyltransferase incubated with GDP-fucose and asialo-α2,6-linked glycoprotein yields a product with the terminal sequence Fucα1→2Galβ1→4GlcNAc. This product is an acceptor for the β-fucosylase α1→2 galactoside α1→3 N-acetylactosaminyltransferase (45) which on incubation with UDP[14C]GalNAc gives the final product 14C[GalNAca1→3Fucα1→2Galβ1→4GlcNAc ... (6). In addition to the trisaccharide 14C[GalNAca1→3Fucα1→2Gal released by the endo-β-galactosidase, α2,6-N-acetylgalactosaminidase would also release a radiolabeled product as free [14C]GalNAc. This activity, however, is not found in the culture medium of S. pneumoniae when incubated with p-nitrophenyl-α2,6-N-acetylgalactosaminidase.

A third radiolabeled substrate was prepared from the ovalbumin glycopeptide with composition Asn(GlcNAc),Manβ1→3Galβ1→4GlcNAc. The procedure used to label Asn(GlcNAc)α1→3Galβ1→4GlcNAc introduces tritium by NaBH₄ reduction of periodate-oxidized mannose residues (1 mol of periodate/mol of glycopeptide (34)). At least 50% of the labeled glycopeptides were susceptible to cleavage by the S. pneumoniae endo-β-N-acetylgalactosaminidase. Thus, of the 3 terminal mannose residues susceptible to periodate oxidation at least one of these may be modified without destroying the ability of the glycopeptide to serve as a substrate.

Acknowledgments — We wish to thank J. I. Rearick and T. A. Beyer (Department of Biochemistry, Duke University Medical Center) for the preparation and characterization of the Galβ1→3GalNAc from antifreeze glycoprotein and α2,6-galactoside α1→2 fucosyltransferase, respectively.

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Glycosidases of Streptococcus pneumoniae

Systematic purification of five glycosidases from Streptococcus (Diplococcus) pneumoniae.
L R Glasgow, J C Paulson and R L Hill


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