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Chromatographic Analysis of the *Escherichia coli* Polysialic Acid Capsule

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Summary

Polysialic acid capsules are the major virulence factors in *Escherichia coli* K1, K92, and groups B and C meningococci. The sialic acid monomers (2-keto-3-deoxy-5-acetamido-7,8,9-D-glycero-D-galacto-nonulosonic acids) comprising these homopolymeric polysaccharide chains can be selectively modified with 1,2-diamino-4,5-methylenedioxy-benzene to produce highly fluorescent quinoxalinone derivatives distinguished by their elution times during reverse phase chromatography. Here, we describe methods to release the constituent capsular polysialic acid monomers, detect, and quantify them by sensitive fluorometry. There are relatively few 2-keto acids in bacteria, making it possible to rapidly analyze samples even without prior purification of capsular polysaccharides.

Keywords

Escherichia coli; polysialic acid capsules; virulence factors; release of sialic acid monomers; reverse phase chromatography; fluorometry

1. Introduction

The physiochemical, immunological, and host-interactive properties of bacterial cell surfaces are dominated by polysaccharides composed of one or more types of carbohydrate monomers. Capsular polysaccharides represent one class of cell surface structure that receives intensive research scrutiny due to its value as target for vaccine and new drug development (1, 2). Capsules are usually linked to the bacterial cell surface and are most often composed of one or two different carbohydrates. The *Escherichia coli* capsular polysialic acid chains are homopolymers composed of about 200 sialic acid (Neu5Ac) residues connected by alpha-2,8-glycosidic linkages. The chains are attached to the surface by an acid labile phosphodiester bond such that, depending on growth conditions, the capsule is continually sloughed into the medium. Growth in highly buffered defined chemical medium maximizes the amount of capsule retained at the cell surface (3). We have adapted a variety of preparative and release procedures that allow chemical analyses of virtually any sample regardless of its purity (4). These methods are described in this article.

Most clinical *E. coli* K1 isolates are variably modified with O-acetyl groups (CH₃-COO-) attached to carbon-7 or -9 of the Neu5Ac monomer (Fig. 1). These groups, but not the acetamido at carbon-5, are base labile and can be lost during analytical or preparative procedures. Therefore, attention must be given to how the bacteria are grown and handled during preparation for subsequent analysis by labeling with 1,2-diamino-4,5-methylenedioxy-benzene (DMB) (Fig. 1). The variable O-acetylation at carbons-7 or -9 is controlled by a random translational switch and expression of a specific polysialic acid O-acetyltransferase encoded by *neuO* (4-9). The enzyme uses acetyl-coenzyme A as donor to transfer acetyl groups to Neu5Ac residues during polymerization of the nascent polysialic acid chains inside the cell. Modified capsule chains are ultimately transported to the outer surface of the outer bacterial membrane where the constituent sialic acids, either with or without prior purification, can be analyzed after enzymatic or chemical hydrolysis of the polysaccharides. The degree of O-acetylation is quantified by reacting the resulting alpha-keto acids with DMB, giving rise to fluorescent quinoxalinones that are separated by reverse phase chromatography. Hara and associates first described the DMB labeling method to measure the degree of O-acetylated sialic acids in serum glycoproteins (10). Our modified methods describe the use of the DMB labeling procedure for analyzing bacterial polysialic acids. To designate acetylation of Neu5Ac, the abbreviations Neu5,7Ac₂, Neu5,8Ac₂, Neu5,9Ac₂, or Neu4,5Ac₂ identify O-acetyl groups at the indicated carbon positions, respectively. The other major Neu5Ac derivative has an *N*-glycolyl group instead of acetamido at carbon-5 and is abbreviated Neu5Gc; this derivative elutes earlier than Neu5Ac during reverse phase chromatography but is not known to be a component of any bacterial surface polysaccharide.

2. Materials

Clinical *E. coli* K1 isolates are class 2 pathogens that must be grown and harvested using biosafety level 2 procedures. Organic effluent and wastes should be collected and disposed of according to local chemical hazard requirements. Neither spent growth media nor live bacteria should be disposed of in sinks without prior sterilization.

2.1 Bacterial Culture Media

1. General purpose LB medium: Weigh 10 g tryptone, 5 g NaCl, and 5 g yeast extract. Dissolve the ingredients in 600 mL water in a 2 L Erlenmeyer flask then bring to 1 L in a graduated cylinder, transfer back to the flask or aliquot into smaller containers and sterilize (*see* Note 1). Store at room temperature.
2. Defined minimal medium: Prepare medium according to the recipe given by Finne and colleagues (*see* Ref. 3). Luxurious growth is obtained by including glucose and casamino acids as carbon sources in a highly phosphate-buffered salts solution (*see* Note 2). The buffering prevents any significant drop in pH such that the labile

¹The ingredients can be either purchased separately or as a preweighed mix from Fisher. Sterilize in autoclave for 20 min.

²Dissolve medium components in order in water with stir bar spinning in a 1-2 L beaker. Bring to the desired volume and filter sterilize. Store at 4° C. Casamino acid is a casein digest; we use NZ Amine from Sigma. Glycerol can be substituted for glucose in the event that catabolite repression is not desired.

phosphodiester linkage between the terminal non-reducing sialic acid residue and phospholipid membrane anchor is stabilized during growth.

2.2 DMB Reagent and Chromatography Components

1. DMB labeling reagent: Dissolve 0.63 mg DMB and 1.2 mg sodium hydrosulfite in 200 μ L of 1.4 M acetic acid; add 22 μ L beta-mercaptoethanol and vortex the mixture for a few seconds to dissolve the components. Store protected from light for up to one week at 4° C (*see* Note 3).
2. Solvent: Mix 45 mL HPLC-grade acetonitrile, 35 mL methanol, and 420 mL ultrapure water, mix with stir bar and add another 500 mL of water. Filter solvent through 47 mm diameter, 0.2- μ m porosity nylon (Alltech #2034, Deerfield, IL, USA) into a side-arm Erlenmeyer flask with magnetic stir bar; mix under vacuum to degas the solvent (*see* Note 4).
3. Reverse phase column: TOSOH Bioscience TSK gel Super-ODS column (#R0129-82PM), Dionex AS50 Autosampler, GP50 Gradient Pump, RF2000 Fluorescence Detector, and Chromeleon software for data management (*see* Note 5).

2.3 Standards

1. 2-Keto acids: Dissolve 1 mg Neu5Ac, or any of its commercially available derivatives (*see* Note 6), KDO, or pyruvate in 0.1-1 mL water and store at -20° C.
2. Bovine submaxillary gland mucin (SGM): Dissolve 1 mg Type I-S SGM (Sigma) in 0.1-1 mL water (*see* Note 7). Store at -20° C.

3. Methods

Carry out all procedures except bacterial growth at room temperature unless indicated otherwise. Bacteria should be grown at 37° C with vigorous (200-250 rpm) shaking.

³The DMB labeling reagent is prepared as a 2x solution that is sufficient for up to 10 reactions. Although it can be stored for up to 1 week in the refrigerator, we prefer to use it shortly after preparation. If a precipitate forms, heat the solution at 50° C until the precipitate dissolves. Since the DMB component is relatively expensive, we use a balance with 5 decimals to weigh sub-mg amounts of reagents. Store sodium hydrosulfite (dithionite) under argon gas, and beta-mercaptoethanol in a fume hood. Store DMB powder at -20° C.

⁴The relative proportions of chromatography solvent ingredients may need adjustment depending on the separation desired. The original procedure (*see* Ref. 9) uses 9:7:84 (vol/vol/vol) acetonitrile/methanol/water. We modified the ratio to achieve good separation of Neu5Gc, Neu5Ac, and Neu5,9Ac₂ standards with our column and HPLC equipment.

⁵As long as a fluorescence detector and data management system are available, any HPLC system or reverse phase column should be adequate. We use the Dionex system because of its versatility for different kinds of carbohydrate analyses.

⁶Neu5Ac, Neu5Gc, KDO and pyruvate are purchased from Sigma. The only commercial supplier of Neu5,9Ac₂ is Applied Biotechnology, Austria. This company also markets Neu4,5Ac₂, which is not a component of polysialic acids but is found as a constituent of some mammalian glycoconjugates. Any sialic acid with a potential reducing end should be amenable to DMB labeling, but with the exception of those found on SGM, we have tested Neu4,5Ac₂ (*see* Ref. 9), and the 2-deoxy-anhydro derivative of sialic acid from Sigma. As expected, no labeling is detected because this compound lacks a free reducing end.

⁷Prior to a run, wash column with 50% methanol, then chromatography solvent until a stable baseline is achieved. Wash with 50% methanol at the conclusion of a day's set of experiments. When resolution deteriorates after 200 or more runs, it is recommended that the column and guard be replaced. It is important to run at least a Neu5Ac standard prior to each set of unknowns, since retention times may change from day to day. The minimum detection limit is about 2 pmols/DMB derivative. If desired, a standard curve of 2-10 or 20 pmols Neu5Ac can be established and used to quantify unknown sample quantities. It is generally most useful to simply compare relative amounts of different sialic acids within a sample, which can be spiked with a known quantity of Neu5Ac or other standard(s) depending on sample complexity.

3.1 Analysis of Standards

1. Mix equal volumes of SGM and 4 M acetic acid to give 2 M final concentration. Incubate at 80° C for 2 h to hydrolyze sialic acid residues while retaining O-acetyl groups. After hydrolysis, centrifuge and discard any pellet.
2. Mix 20 µL of SGM supernatant, Neu5Ac, or other standards with 20 µL of 2x DMB labeling reagent. Incubate in the dark at 50° C for 2-2.5 h. Prepare SGM-derived sialic acids or other standards for chromatography by centrifuging them through 0.22 µm nylon Spin-X LC Corning-Costar microfuge tubes from Fisher (#07-200-389).
3. Inject 6-10 µL sample and perform reverse phase chromatography in an ascending manner. The column will run at about 2000 psi with a flow rate of 1 ml/min. Each run takes 30-35 min. For SGM, expect an elution profile similar to that shown in Fig. 2A (*see* Note 7).
4. To confirm the presence of O-acetyl groups in samples or standards, mix 20 µL of solution with 4 µL of 1 M NaOH. Incubate for 30 min at 37° C, and neutralize with 2 µL of 2 M acetic acid or simply proceed to the DMB labeling reaction. Fig. 2B shows an example of sialic acids derived from SGM that was treated with base prior to DMB labeling and chromatography.

3.2 Growing and Harvesting Bacterial Cells

1. Inoculate LB with a single bacterial colony and grow to saturation. Dilute culture 1:200 into the desired volume of medium and grow to an $A_{600} = 0.6$ with vigorous shaking (*see* Note 8). It is important to grow bacteria in a sterile container with at least 2x the volume of the medium to attain proper aeration and reproducible growth rates. Cultures can be grown to saturation, but capsule production is maximal during the exponential phase of growth.
2. Collect bacteria by centrifugation at $5000 \times g$ for 7 min and discard supernatant. It is generally not necessary to wash pellets derived from 5 to 100 mL cultures. Simply invert containers over paper towels for a few minutes to allow residual liquid to drain, and then wipe sides with Kimwipes folded on forceps of suitable size. Alternatively, wash pellets one or more times with water, PBS or desired buffer and resuspend to 1:10 or less the original volume of the bacterial culture. Store samples in an ice bucket or freeze at -20° C for later use.

3.3 Analysis of Polysialic Acids from Intact Bacteria

1. Mix the desired volume of bacterial sample with an equal volume of 4 M acetic acid and hydrolyze as described in section 3.1.1 above. If preferred, monomers may be obtained by enzymatic hydrolysis using commercially available sialidase(s), but due to the simplicity of acid hydrolysis only this method is described. Depending

⁸Bacterial strains are stored at -80° C in 15-20% glycerol. We have stored strains this way with no apparent phenotypic change after at least 30 years. A small amount of frozen cells is inoculated onto an LB petri dish solidified with 1.5% agar. The dish is streaked for single colony isolation and incubated at the desired temperature, usually 37° C overnight. Dishes containing grown bacteria may be wrapped with Parafilm and stored for up to 2 weeks at 4° C.

on the sample there will be some amount of precipitate that should be removed by centrifugation prior to proceeding to the next step.

2. It is usually useful to concentrate the samples prior to DMB labeling. Concentration of small volumes (up to 2 mL) is conveniently done in a vacuum centrifuge under medium heat. After concentrating, centrifuge and discard any precipitate. Lyophilization is recommended for larger sample volumes. Dried samples should be dissolved in the desired volume of 1.4 M acetic acid; remove any precipitate by centrifugation.
3. Perform DMB labeling and chromatography. Fig. 3 shows examples of results for a strain lacking modification (EV36), and SP#4, which is a naturally occurring clinical K1 isolate that, because of the particular nature of its translational switch, expresses *neuO* constitutively. Because there is much more lipopolysaccharide than polysialic acid in the bacteria, KDO is an inevitable contaminant in the analysis, but is readily distinguished from Neu5Ac or its derivatives by the lower retention time (*see* Note 9).

3.4 Semi-purification and Analysis of Polysialic Acids

1. Grow bacteria in defined minimal medium and collect by centrifugation. Wash with PBS and suspend in 1:10 the volume of the original culture volume with water.
2. Add an equal volume of 100 mM pyridine-acetate, pH 5.5; incubate with shaking for 2 h at 37° C. Remove intact bacteria by centrifugation and lyophilize supernatant to dryness.
3. Dissolve the dried material in the desired volume of pyridine buffer and remove any precipitate by centrifugation. Dialyze against three changes of 50 mM pyridine buffer or water in a cold room (*see* Note 10).
4. Concentrate by vacuum centrifugation and carry out DMB labeling and chromatography. Expect 13-15 mg of polysialic acid isolated from 200 mL of bacteria grown to saturation in this medium. Fig. 4A shows the results with a negative control sample of strain SP#4 grown at 15° C, a growth condition that inhibits polysialic acid synthesis. By contrast, SP#4 grown at 37° C shows the expected presence of O-acetylated sialic acids (Fig. 4B). If desired, the relatively low amount of KDO in the analysis can be prevented by ultracentrifugation immediate after the pyridine extraction step to remove small membrane blebs containing lipopolysaccharide.

⁹Because K1 polysialic acid is a repeating homopolymer with connecting alpha-2,8-linkages, Neu5,8Ac₂ is likely produced after hydrolysis by a spontaneous transesterification, or branch migration of acetyl groups from carbon positions 7 to 9. Alternatively, it is conceivable that Neu5,8Ac₂, synthesized by an O-acetyltransferase other than NeuO prior to polymerization is used to terminate chain growth (*see Ref. 4*). Thus, the methods described here can be used to study sialic acid metabolism in general, and not just the analysis of capsular polysaccharide chains described here.

¹⁰Pyridine also will extract low molecular weight intracellular compounds, and if monomeric sialic acids or pyruvate are overproduced these will be detected in the analysis of polysialic acid. To distinguish between sialic acids derived from polymer or the intracellular environment, dialyze using standard 12000-MWCO membrane tubing.

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Fig. 1.

Fluorescent labeling of α -keto acids. DMB reacts with 2-keto acids under acidic conditions to produce fluorescent quinoxalinones that are detected after excitation at 373 nm and emission at 448 nm. The solution structure of Neu5Ac is shown in parentheses with carbons 1-9 numbered by the subscripts. 3-deoxy-D-manno-octulosonic acid (KDO) is an α -keto sugar that is a usual component of lipopolysaccharide; depending on the preparative conditions it can be a contaminant in the analysis of polysialic acids, as can pyruvate that accumulates in some bacterial strains (*see* Ref. 4).

Fig. 2. Chromatographic analysis of SGM-derived standards. A. SGM is a glycoprotein with about 15% sialic acids as terminal residues on the constituent sugar chains. Peaks represent the biologically most common sialic acids as indicated. B. Base hydrolysis collapses acetylated sialic acids to their Neu5Ac or Neu5Gc forms.

Fig. 3. Analysis of capsule using intact bacteria. A. Strain EV36, lacking *neuO*, produces little or no modified sialic acid. B. Strain SP#4 constitutively expresses *neuO* (see Ref. 4).

Fig. 4. Semi-purified polysialic acid from strain SP#4 grown at permissive and non-permissive temperatures. As described in the text, bacteria grown at 15° C synthesize little or no capsular polysaccharide (A) relative to the normal growth temperature of 37°C (B).