Glycoconjugate J (1990) 7:111-120

Preparation of Oligosaccharide-Polyacrylamide Conjugates and Their Use as Antigens in Enzyme Immunoassay (EIA)

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Received January 11, 1990.

Key words: Salmonella, oligosaccharide, conjugate, lipopolysaccharide, EIA

Oligosaccharides derived from *Salmonella* lipopolysaccharides or from human milk were converted to their *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol derivatives. These derivatives were copolymerized with acrylamide to give linear, water-soluble polymers, which were used as coating antigens in EIA assays.

The immunological diagnosis of bacterial infections is mostly based on determination of specific antibodies against the cell wall components of the infectious agent. In Gram-negative bacteria like Salmonella the lipopolysaccharide (LPS) located in the outer cell wall is the primary target for the humoral immune system. The LPS molecule of Salmonella species is built up of three structurally and serologically distinct parts: (i), the lipid A moiety, known as the toxic principle of the LPS molecule and which is the most conserved portion; (ii), the core oligosaccharide, which appears to be common to all or almost all Salmonella species and (iii), the O-polysaccharide which determines the serological specificity of the bacteria and which is composed of polymerized repeating units.

The LPS from Salmonella species has been widely used in EIA for quantification of antibodies against O-antigens in human sera [1]. The use of whole LPS has, however, some drawbacks: (i), the LPS is normally extracted from submerged culture and as a consequence the preparation is a mixture of LPS molecules with variable amounts of the O-polysaccharide, also containing LPS molecules with the core polysaccharide only [2]; (ii), depending on the extraction and purification methods, the LPS preparation may be more or less contaminated with other bacterial macromolecules like outer membrane proteins and nucleic acid [3]; and (iii), LPS in aqueous solution forms aggregates of different sizes which are difficult to control.

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An alternative to LPS in EIA assays is glycoconjugates prepared from pure Salmonella O-antigenic oligosaccharides. The usual way to prepare such glycoconjugates is by coupling of synthetic or isolated oligosaccharides to natural macromolecules (e.g., proteins). This approach, however, has the drawback of introducing a new serologically active moiety, the protein [4]. Another approach is to transform oligosaccharides into high molecular weight material by copolymerization of suitable oligosaccharide derivatives with unnatural non-carbohydrate monomers. This leads to macromolecules where the only serologically active moiety is the carbohydrate part. This approach was earlier used to convert synthetic oligosaccharide fragments of bacterial O-antigens (in the form of glycosides with double-bond containing aglycons) *via* copolymerization with acrylamide into serologically active macromolecules [5-13].

In the present study we describe synthesis of polyacrylamide conjugates of oligosaccharides isolated from *Salmonella typhimurium* (serogroup B) and from *Salmonella enteritidis* (serogroup D) and their usefulness as coating antigens in EIA. The scheme of transformation involves introduction of a double-bond containing group at the reducing end of the oligosaccharides and subsequent co-polymerization of the obtained derivatives with acrylamide.

Materials and Methods

Bacterial Strains

The *Salmonella typhimurium* SH 4809 and *S. enteritidis* SH 1262 were obtained from P.H. Mäkelä, Central Public Health Laboratory, Helsinki, Finland.

Preparation of the Lipopolysaccharides

The LPS from *Salmonella typhimurium* SH 4809 (serogroup B) and from *S. enteritidis* SH 1262 (serogroup D) were prepared from submerged culture using hot phenol-water extraction [14]. The LPS's were then treated with alkali (0.25 M NaOH, 37°C, 16 h) in order to hydrolyze off the ester linked fatty acids present in the lipid A. This preparation is referred as LPS-OH.

Preparation of Oligosaccharides

O-Specific oligosaccharides were prepared by specific degradation of the LPS-OH by using the endo-rhamnosidase activity associated with bacteriophage P22 tail protein according to [15]. Shortly, to a dialysis bag containing phage P22 previously dialysed against 5 mM ammonium carbonate buffer, pH 7.1, LPS-OH was added at a ratio of 1g LPS-OH/10^{14} p.f.u. of phage P22. The dialysis bag was then immersed in approximately 600 to 800 ml of the same buffer and dialysed at 37°C. After approximately 50 h, the dialysis buffer was renewed and dialysis was continued for an additional 50 h. The outer dialysates were pooled and concentrated under reduced pressure to dryness. The oligosaccharides, octasaccharide representing two repeating units and dodecasaccharide representing three repeating units of the O-polysaccharide, were separated by gel chromatography using Bio-Gel P-2 and P-

4 columns (5.0 x 90 cm, Bio-Rad, Richmond, CA, USA) eluted with distilled water and monitored using a differential refractometer (Waters, Milford, MA, USA). As preservative, trichlorobutanol (TCB) was used at a concentration of 0.05%.

The oligosaccharides used in the present study have the following structures:

$$[\alpha\text{-D-Gal}p\text{-}(1\text{-}2)\text{-}\alpha\text{-D-Man}p\text{-}(1\text{-}4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\text{-}3)]_n \text{ with R linked at } 3 \text{ of Man}p$$

R
|
3
[α–D-Gal*p*-(1–2)-α-D-Man*p*-(1–4)-α-L-Rha*p*-(1-3)]$_n$.

In octasaccharide n is two and in dodecasaccharide n is 3. R is abequosyl in *S. typhimurium* and tyvelosyl in *S. enteritidis.*

Lacto-*N*-tetraose, β-D-Gal*p*-(1-3)-β-D-GlcNAc*p*-(1-3)-β-D-Gal*p*-(1-4)-D-Glc, was isolated from human milk [16].

Preparation of Oligosaccharide-Acrylamide Conjugates

Method A: A solution of *p*-trifluoroacetamidoaniline [17] (75 mg, 0.365 mmol) and sodium cyanoborohydride (25 mg, 0.40 mmol) in ethanol-H_2O (2:1, 1.5 ml) was adjusted to pH 6 with acetic acid. A solution of oligosaccharide (0.05 mmol) in H_2O (0.75 ml) was added. The mixture was incubated at 25°C for 24 h under constant stirring. After incubation the excess of reagent was removed by repeated washings with diethyl ether. After concentration at reduced pressure the product was desalted on a Bio-Gel P-2 column (2.6 x 90 cm) eluted with acetic acid-pyridine buffer, pH 5.0. The fractions containing the desired product were pooled and lyophilized. This material (35 mg) was dissolved in 2 ml H_2O and 1.5 ml MeOH was added. The mixture was stirred at 0°C while Amberlyst A26 HCO_3^- ion exchange resin was added, followed by 30 µl of acetic acid anhydride. After incubation for 2 h at 0°C the ion exchange resin was removed by filtration, washed with aqueous methanol (10 ml) and the combined filtrate and washings were concentrated to 0.5 ml. To this mixture 3 ml 25% aqueous NH_3 was added, and the mixture was kept under N_2 at 25°C. After 4 h the product was concentrated to dryness under reduced pressure, then the residue was dissolved in 2 ml H_2O and 1.5 ml MeOH was added, followed by Amberlyst A26 HCO_3^- resin. While stirring at 0°C, 30 µl acryloyl chloride was added. The mixture was kept at 0°C for 2 h. After filtration and concentration under reduced pressure the product was desalted on a Bio-Gel P-2 column (2.6 x 90 cm) eluted with acetic acid-pyridine buffer, pH 5.0. The obtained oligosaccharide *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol was analyzed by ^1H NMR spectroscopy.

Method B. A solution of oligosaccharide (0.1 mmol) in water (1.5 ml) was mixed with 4-acrylamidoaniline (100 mg) [18], and sodium cyanoborohydride (50 mg) in ethanol-water (2:1, 3 ml). After stirring overnight, acetic anhydride (300 µl) was added, the mixture was stirred for additional 3 h at 25°C, and was then partitioned between water and ethyl acetate. The aqueous phase was concentrated, then dissolved in water (10 ml) and applied on a Bond-Elut C-18 cartridge (2 g, Analytical International, Harbor City, USA). After an aqueous wash (10 ml) the product was eluted from the cartridge with 25% aqueous methanol, and

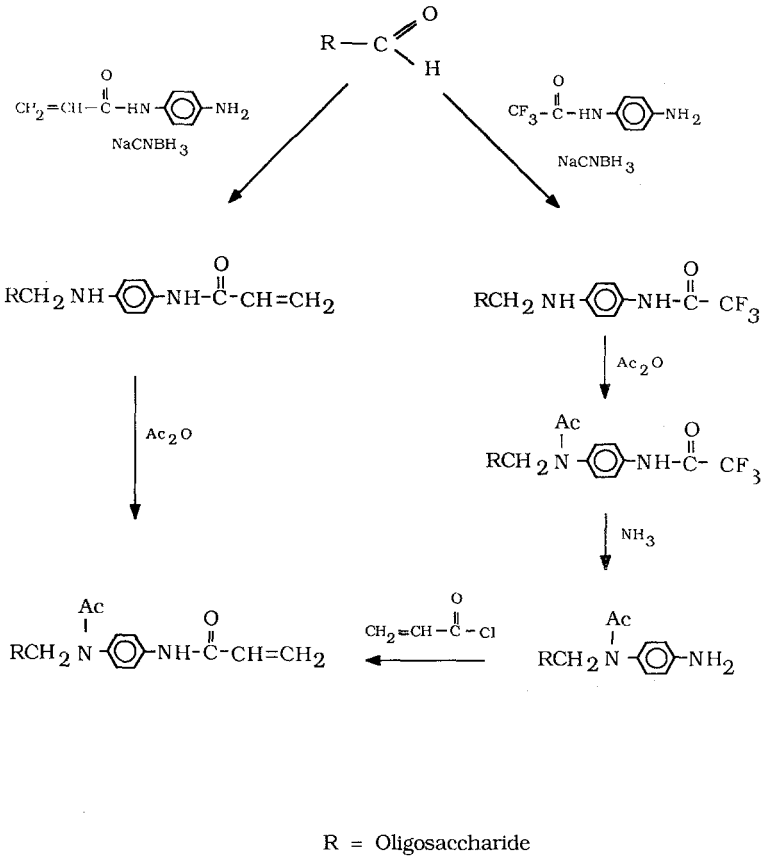


Figure 1. Conversions of reducing oligosaccharides into the corresponding *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditols.

the eluate lyophilized. After purification by gel filtration on a Bio-Gel P-2 column, the product was indistinguishable from that obtained by method A (as determined by ^{1}H NMR spectroscopy).

Polymerization

In order to obtain one oligosaccharide chain / 10 acrylamide units, 14.6 mg of the oligosaccharide *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol was mixed with 6.3 mg acrylamide. The mixture was dissolved in 0.5 ml de-aerated H_2O, and kept under argon while N,N,N',N'-tetramethylethylenediamine (2 µl) and ammonium persulfate (1 mg) was added. After stirring overnight at 25°C, the product was purified by gel chromatography on a Bio-Gel P-4 column (2.6 x 90 cm) eluted with distilled water.

Analytical Methods

The gel chromatography fractions were assayed with the phenol-sulphuric acid method [19]. ^{1}H NMR Spectra were recorded at 27°C for 1-2% solutions in deuterium oxide with a Bruker AM 500 instrument. Acetone (δ = 2.225 ppm) was used as internal reference.

Enzyme Immunoassay

Microtiter plates were coated with different concentrations of either oligosaccharide-polyacrylamide conjugate or native LPS. After incubation at 25°C for 18 h the plates were washed four times with 0.15 M sodium chloride containing 0.05% Tween 20. Salmonella O-antigen 4 or 9 specific rabbit polyclonal antibodies were then added and incubated for 2 h at 25°C. The plates were washed as above and incubated with swine anti-rabbit alkaline phosphatase-conjugated antibodies at 25°C for 18 h. After subsequent washing, *p*-nitrophenylphosphate 1 mg/ml in 1 M diethanolamine-HCl buffer, pH 9.8, with 0.5 M $MgCl_2$ was added. The enzymatic reaction was measured spectrophotometrically at 405 nm.

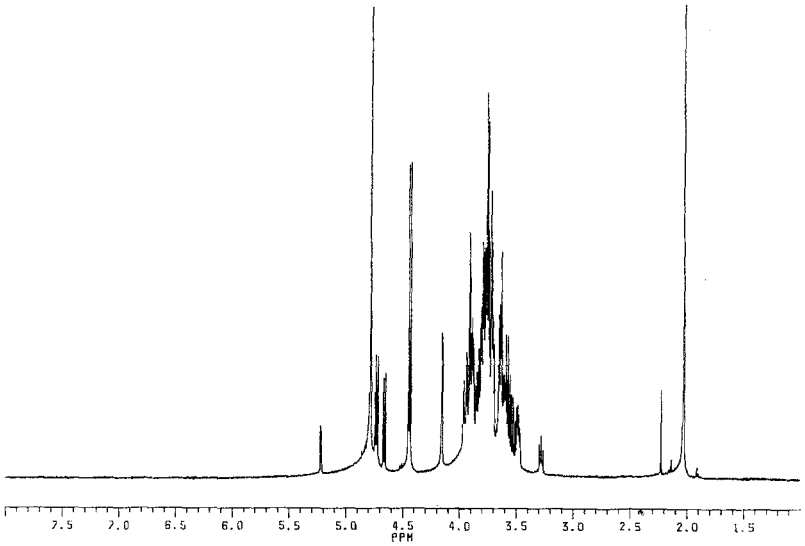
Results and Discussion

Two methods were used for obtaining the same type of acrylamide-oligosaccharide monomer derivatives. In method A, derivatization of reducing oligosaccharides with 4-trifluoroacetamidoaniline and sodium cyanoborohydride as described [17] produced oligosaccharide 1-amino-1-deoxy-*N*-(4-trifluoroacetamidophenyl)-alditols, which could readily be *N*-acetylated [20] with acetic anhydride. De-*N*-trifluoroacetylation with aqueous ammonia produced the corresponding amino derivatives (Fig. 1). *N*-Acryloylation of these with acryloyl chloride gave the oligosaccharide *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol derivatives, a typical yield (for the lacto-*N*-tetraose derivative) was 54%, calculated from the oligosaccharide. In method B, identical derivatives, in similar yields, were obtained if the oligosaccharides were reductively aminated with 4-acrylamidoaniline [18], and the product then was acetylated with acetic anhydride (Fig. 1).

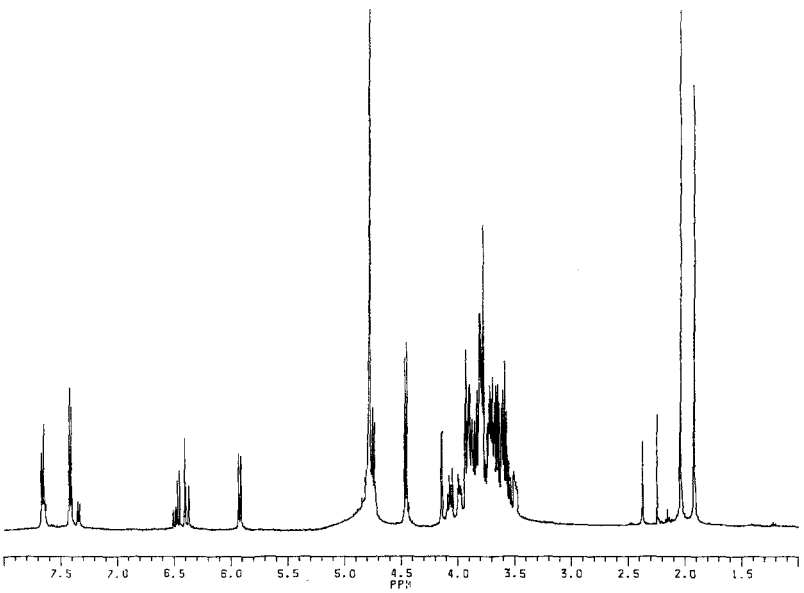
The ^{1}H NMR spectra of lacto-*N*-tetraose and the corresponding *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol derivative are shown in Fig. 2. It should be noted that in the latter spectrum there are two sets of signals with unequal intensities in the aromatic (7.30-7.65 ppm) as well as the acetyl group region (1.90 and 2.35 ppm). This is due to the presence of unequal amounts of two rotational isomers around the tertiary amide bond, a phenomenon known [21] to occur also with other amides.

Copolymerization of the oligosaccharide *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditols with acrylamide in aqueous solution using N,N,N',N'-tetramethylethylenediamine-ammonium persulfate as initiators gave water-soluble polymers with molecular weight of 10^5 - 10^6, as indicated by gel filtration data. The ratio of oligosaccharide chains to acrylamide units in the polymer was generally around 1/10, as determined by the phenol-sulfuric acid colorimetric method. A typical yield of polymer, calculated from the used amount of oligosaccharide *N*-acetyl-*N*-(4-acryloylamidophenyl)-alditol, was 70% (lacto-*N*-tetraose polymer).

a


7.5
7.0
6.5
6.0
5.5
5.0
4.5
PPM
4.0
3.5
3.0
2.5
2.0
1.5

b


7.5
7.0
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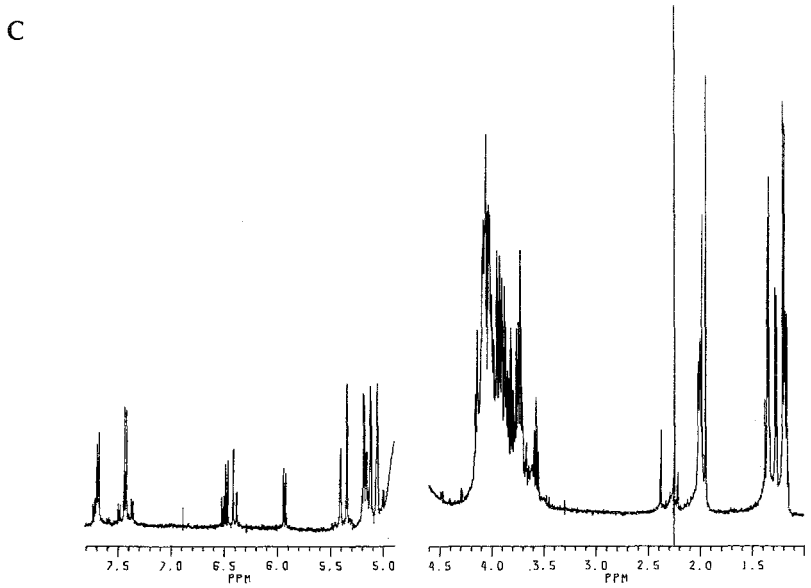

Figure 2. ^{1}H NMR spectra in $^{2}H_2O$ (27°C) of lacto-*N*-tetraose (a), the *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol of lacto-*N*-tetraose (b), and the *N*-acetyl-*N*-(4-acrylamidophenyl)-1-amino-1-deoxyalditol of *S. typhimurium* octasaccharide (c). The reference signal (acetone) was set to 2.225 ppm.

The bacterial oligosaccharide-polyacrylamide conjugates i.e. the conjugated octa- (two repeating units) and dodecasaccharides (three repeating units) from strains *Salmonella typhimurium* SH 4809 (serogroup B) and from *S. enteritidis* (serogroup D) were used as antigens in an EIA system. Rabbit antibodies against the whole bacteria were used. The coating efficiency of the conjugates was compared to that of the corresponding lipopolysaccharides. The coating curves for the *S.typhimurium* SH 4809 antigens are shown in Fig. 3. The octa- and dodecasaccharide polyacrylamide conjugates showed an optimal coating dose of 0.1 µg/ml. The optimal coating dose for the corresponding LPS was 10 µg/ml. Similar results were obtained with the *S. enteritidis* SH 1262 antigens (Fig. 4). These results show that the polyacrylamide conjugates are extremely efficient as coating antigens with an optimal coating dose that is 100 times lower than the corresponding LPS. The use of octa- or dodecasaccharides isolated from Salmonella for detection of specific antibodies in an EIA system is of advantage since only the antibodies reacting with the O-antigen are detected. The Salmonella LPS is build of a lipid A unit, a core oligosaccharide and the repeating O-chain. The lipid A and core oligosaccharide are the same in all or almost all Salmonella species. The O-antigen differ between the different species both in chemical composition

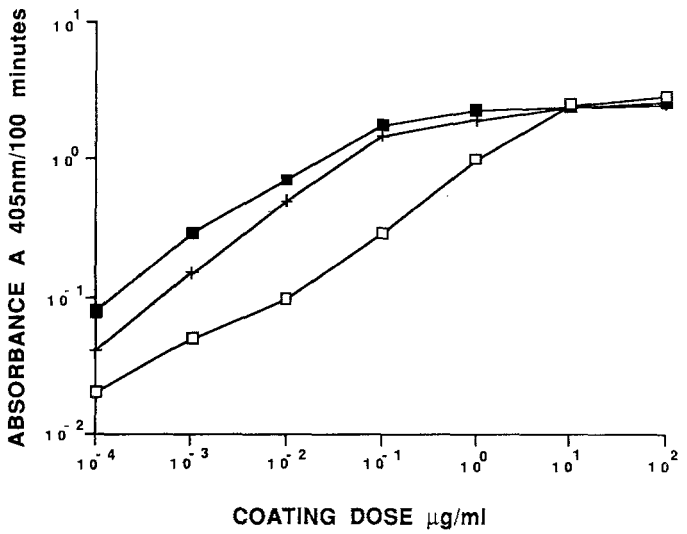

Figure 3. Coating curves in EIA of the *Salmonella typhimurium* SH 4809 oligosaccharide-polyacrylamide conjugates compared with the corresponding LPS: (+) Octasaccharide-polyacrylamide conjugate; (■) dodecasaccharide-polyacrylamide conjugate; (□) lipopolysaccharide.

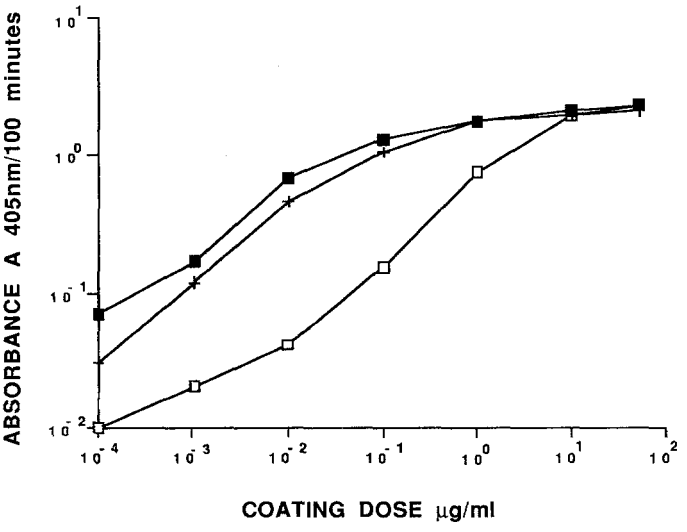

Figure 4. Coating curves in EIA of the *Salmonella enteritidis* SH1262 oligosaccharide-polyacrylamide conjugates compared with the corresponding LPS. Legends as in Fig. 3.

and in immunochemical specificity. The use of well defined oligosaccharides like the octa- or dodecasaccharides as polyacrylamide conjugates instead of the whole LPS in EIA means that only antibodies against the O-antigen will be detected (antibodies against polyacrylamide do not occur in serum). In addition the polyacrylamide derivatives are, as mentioned, much more efficient in coating the plastic surface of a microtiter plate. The method for preparation of oligosaccharide-polyacrylamide conjugates described above should be suitable for any oligosaccharide that has a free reducing end.

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