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Preparation of Polysaccharide-Enzyme Conjugates for Competitive Binding Assays*

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A variety of bacterial O-polysaccharides were covalently linked to enzymes and it was demonstrated with three discrete monoclonal antibodies that enzyme-glycoconjugates function as convenient labelled antigens in direct enzyme immunoassays, particularly competitive assays that quantify bacterial O-antigens. Two strategies, each involving reductive amination, were used to couple O-polysaccharides to enzymes, while retaining high enzymic activity. Reduction of the Schiff base formed between 1,3-diaminopropane and the terminal reducing ketodeoxyoctanoic acid (KDO) residue present in the majority of the lipopolysaccharide (LPS) core domains, following mild acid removal of Lipid A, offered the most direct route to mono-aminated polysaccharide. Alternatively, mild periodate oxidation of KDO and heptose residues generated multiple aldehyde targets for Schiff base formation, without affecting the O-antigenic determinant. Hetero- and homobifunctional coupling reagents, sulphosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate and disuccinimidyl suberate, activated polysaccharide for coupling to enzymes at amino and sulphydryl sites and produced conjugates that retained at least 95% of the original enzymic activity. The most suitable enzyme conjugates, especially for competitive inhibition EIA were those bearing one polysaccharide chain, and these were easily prepared from horse-radish peroxidase. Although the extent of conjugation of activated polysaccharide to β-galactosidase and alkaline phosphatase could be controlled by reaction stoichiometry, the use of these enzymes was a less effective utilization of valuable antigen and enzyme.

Direct competitive enzyme immunoassays [1, 2] employing antigen-coupled enzyme have considerable appeal and we recently reported an assay based upon the use of oligosaccharide or polysaccharide enzyme conjugates [3, 4]. Although oligosaccharides synthesized with a linking arm [5] could be efficiently coupled to enzymes by existing techniques [5, 6] without adversely affecting enzyme activity, polysaccharide-enzyme conjugates that retain high specific activity were more difficult to prepare [4].

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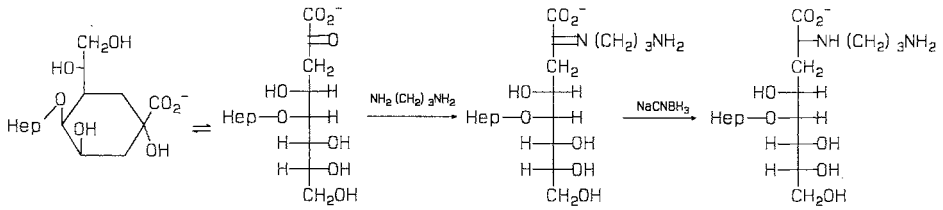


Figure 1. Proposed chemistry for attachment of 1,3-diaminopropane by reductive amination of the terminal reducing KDO residue exposed after mild acid treatment of LPS. The inner core heptose substituent at O-5 of the KDO is the point of attachment for the outer core and O-polysaccharide [26].

In studies of covalent attachment of polysaccharide antigens to immunogenic proteins for application in vaccine development difficulties most commonly centre around the coupling chemistry [7,8]. The objective in these instances is to enhance the immunogenicity of the polysaccharide by coupling to a highly immunogenic protein through selective bond formation, while avoiding the introduction of obtrusive features, that could subvert the desired anti-polysaccharide response. However, in coupling of polysaccharide to enzymes this point is unimportant and it is desirable to retain high specific activity, while controlling the number of polysaccharide chains that will be coupled to each enzyme molecule. The ability to control the latter detail is crucial if the specific antibody is to possess an affinity for enzyme glycoconjugate that is within a usable range, such that low concentrations of conjugate may be employed for the assay, while at the same time limiting the avidity so that the reporter signal responds to low concentrations of inhibitor.

This paper reports successful attempts to develop and apply this strategy to a variety of O-polysaccharides derived from the lipopolysaccharides (LPS) of *Salmonella essen, Shigella flexneri, Brucella abortus, Brucella melitensis* and *Yersinia enterocolitica.*

Methods

Preparation of O-Polysaccharides

Lipopolysaccharides were isolated by aqueous phenol extraction of phenol killed bacteria and the crude preparation was subsequently purified by ultracentrifugation [9]. A solution of LPS (10 mg/ml), was hydrolysed in 4% (by vol) aqueous acetic acid for 2 h at 100°C and lipid A was removed by centrifugation. The liberated O-polysaccharide was purified on a Sephadex G-50 column (2.6 x 95 cm; Pharmacia, Uppsala, Sweden) using pyridine/acetic acid water (5/3/992 by vol) buffer pH 5.0 and an elution rate of 40 ml/h. Lyophilization gave the pure polysaccharide as a white powder.

N-Acetylation of O-Polysaccharide

A freshly prepared 5% (by vol) solution of aqueous acetic anhydride was added to an equal volume of saturated sodium hydrogen carbonate solution in which O-polysaccharide had been dissolved (10-50 mg/ml), and after 10 min at 20°C the solution was heated at 100°C for 3 min. The solution of *N*-acetylated polysaccharide was desalted on a Sephadex G-25 column (1.0 x 50 cm) and recovered as a solid following lyophilization.

Mild Periodate Oxidation of O-Polysaccharide

N-Acetylated O-polysaccharide was dissolved in 0.01 M sodium metaperiodate solution (10 mg/ml) and incubated at 20°C for 8 min. Excess periodate was destroyed by reaction with ethylene glycol (31 µl/ml) for 1 h at 20°C. Oxidized O-polysaccharide was desalted on a Sephadex G-25 column as described above and the material collected at the column void volume was lyophilized.

Introduction of Amino Groups to the O-Polysaccharide

A solution of *N*-acetylated O-polysaccharide (20 mg/ml) and 0.5 M diaminopropane dihydrochloride in 0.25 M potassium dihydrogen phosphate/disodium phosphate buffer pH 6.0 containing sodium cyanoborohydride (2.48 mg/ml) was stirred for 24 h at 37°C [8]. The desalted (Sephadex G-25 column chromatography) polymer was recovered by freeze drying.

Introduction of Amino Groups to the Oxidized O-Polysaccharide

Sodium cyanoborohydride (20 mg/ml) was added to a solution of the oxidized O-polysaccharide (20 mg/ml) containing 0.1 M diaminopropane dihydrochloride in 0.2 M disodium hydrogen phosphate buffer, brought to pH 8.0 by addition of 2.0 M sodium hydroxide and the solution was stirred at 20°C for 1 h. The derivatized polysaccharide was recovered after desalting and freeze drying.

Maleimide Activation of Aminated O-Polysaccharides

O-Polysaccharide (20 mg) containing a single amino group and sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulpho SMCC, 4.4 mg, 10 molar excess) were dissolved in dry dimethylsulfoxide (200 µl) and reacted for 1 h at 20°C. The reaction mixture was worked-up by addition of water (800 µl) and desalted by column chromatography on Sephadex G-25. Activated, lyophilized polysaccharide was stored at -20°C in the presence of dessicant.

N-Hydroxysuccinimide Ester Activation of O-Polysaccharides

O-Polysaccharide (20 mg) bearing one or several amino groups and an equal weight of disuccinimidyl suberate (DSS, 40 molar excess) were dissolved in DMSO (200 µl) and allowed to react for 1 h at 20°C. The reaction was stopped by addition of cold (1°C) water (800 µl) and precipitated DSS was removed by filtration. The sample was quickly desalted on a column of Sephadex G-25 pre-cooled to 1°C and eluted with cold water at 40 ml/h. The O-polysaccharide activated by succinimide (NHS) esters was lyophilized and stored at -20°C with desiccant.

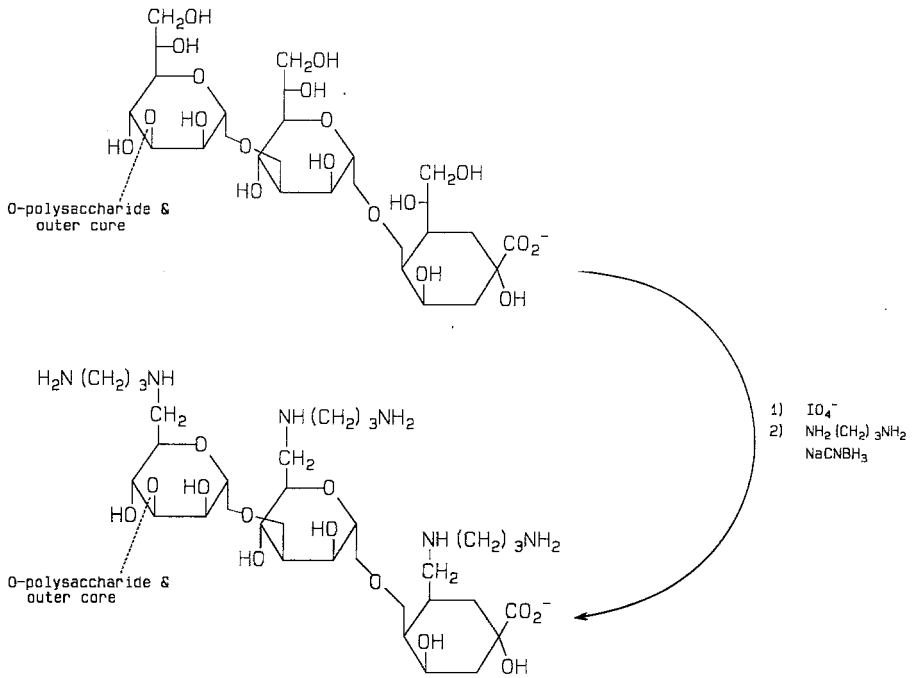


Figure 2. Vicinal diol groups that occur in acyclic chains are oxidized by mild periodate whereas diol groups in pyranose rings are resistant to cleavage. Heptose units yield a 6-aldehydo function and these sites are the probable sites for reductive amination by 1,3-diaminopropane.

Determination of Amino Groups

Amino groups introduced into the O-polysaccharide were assayed with 2,4,6-trinitrobenzenesulphonic acid (TNBS) [10]. Polysaccharide samples (0.25-1.0 mg) in 250 µl were mixed with 250 µl each of 0.2 M borate buffer pH 9.0 and 0.2% (w/v) TNBS and the mixture was heated for 30 min at 55°C. The sample was cooled, water (250 µl) was added and the absorbance was measured at 420 nm. Quantification was based on $\varepsilon_{420} = 13{,}000\ M^{-1}cm^{-1}$.

Quantification of N-Hydroxysuccinimide Esters

The quantification of NHS esters in O-polysaccharide preparations was done spectrophotometrically [11]. Samples (0.25-1.0 mg) in water (500 µl) were reacted with 0.1 M ammonium hydroxide (500 µl) and after 2 min the absorbance at 260 nm was recorded. Estimation of NHS groups present was based upon $\varepsilon_{260} = 9{,}700\ M^{-1}cm^{-1}$.

Quantification of Maleimide Groups

Maleimide groups were estimated by a difference method. After reaction with an excess of 2-mercaptoethanolamine (MEA), the unreacted thiol groups were quantified with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [12]. Samples (0.4 mg) were dissolved in 5 mM sodium acetate buffer pH 5.0 containing 0.2 mM MEA (400 µl). After 30 min at 20°C the sample was diluted with 50 mM sodium phosphate buffer pH 7.0 (500 µl) followed by addition of 10 mM DTNB (100 µl). Absorbance was read at 412 nm after 5 min and thiol groups quantified as previously described.

O-Polysaccharide β-Galactosidase Conjugate via Maleimide Coupling

Polysaccharide (19-190 µg, 1-10 mole equivalents), activated by introduction of maleimide groups, dissolved in 0.1 M K_2HPO_4/Na_2HPO_4 containing 5 mM EDTA pH 6.0 (100 µl) was added to a solution of β-galactosidase (500 µg; Boehringer Mannheim, Dorval, Quebec, enzyme label grade) in the same buffer (100 µl). The solution was kept at 4°C for 24 h and unreacted polysaccharide was reacted with excess 20 mM MEA (5 µl) for 1 h at 20°C.

The pH of the reaction mixture was adjusted to 8.0 by addition of 0.1 M sodium hydroxide (160 µl) and free sulphydryl groups were alkylated over 1 h at 20°C by addition of 10 mM iodoacetamide (40 µl). The conjugated enzymes were then separated from free polysaccharide on a column of Sepharose CL-6B (1.6 x 64 cm) eluting with 50 mM KH_2PO_4/Na_2HPO_4, 0.5 M NaCl, pH 7.0.

O-Polysaccharide Alkaline Phosphatase Conjugates via Succinimide Ester Coupling

Alkaline phosphatase (Type VII-N, Sigma Chemical Co., St. Louis, MO, USA.) was extensively dialysed against 50 mM KH_2PO_4/Na_2HPO_4 pH 7.0 and diluted to a final concentration of 2.5 mg/ml with the same buffer. Samples of O-polysaccharide (0.1-4.0 mg, 1-40 molar equivalents) activated by coupling to the bifunctional spacer, DSS, were dissolved in 200 µl of the enzyme solution and allowed to react for 16 h at 4°C.

The product was dialyzed against 20 mM Tris(hydroxymethyl)aminomethane (TRIS) pH 8.0 and applied to a Mono Q (Pharmacia) anion exchange column (5 x 50 mm) at a flow rate of 1 ml/min. After 12 min a linear sodium chloride gradient (0-0.35 M in 10 ml of the same buffer) was used to elute the bound material. Uncoupled polysaccharide passed straight through the column and free enzyme together with polysaccharide-enzyme conjugate was eluted 6 min after application of the gradient.

O-Polysaccharide-Horse-radish Peroxidase Conjugates via Succimide Ester Coupling

Horse-radish peroxidase (HRP, 500 µg; Type VI, Sigma) and DSS activated O-polysaccharide (5 mg) containing from 1 to 3 succimide ester groups per polysaccharide chain were dissolved in 0.1 M KH_2PO_4/Na_2HPO_4 pH 7.0 (200 µl) and allowed to react for 16 h at 4°C. The conjugated enzyme was separated from free enzyme and polysaccharide on a column of Sephacryl S-200 (1.6 x 95 cm) eluting with 50 mM KH_2PO_4/Na_2HPO_4 pH 7.0 at a flow rate of 15 ml/h.

Glycoconjugates were evaluated with their respective monoclonal antibodies. O-Polysaccharide-enzyme conjugates prepared from the polysaccharide antigens of *B. abortus* [13], *B. melitensis* [14] and *Yersinia enterocolitica* 0:9 [15] were tested against the antibody YsT9-2 [16], while conjugates prepared from the O-polysaccharides of *Salmonella essen* [17] and *S. flexneri* variant Y [18] were tested against the antibodies Se 155-4 (Bundle, unpublished results) and GC-4 [19]. The concentrations of antibody and enzyme conjugates to be employed in the direct competitive ELISA were determined for each conjugate/antibody pair by a preliminary checker board assay format in which conjugate and antibody concentrations were simultaneously varied. EIA Plates were coated with purified monoclonal antibody (100 µl) dissolved in 10 mM sodium phosphate buffered saline pH 7.0 (PBS) (10-30 µg/ml) for 3 h at 20°C. The plates were washed three times with PBS, enzyme-polysaccharide conjugate solution (100 µl) with or without inhibitor was added to each well, and specific binding was allowed to reach equilibrium over an 18 h incubation at 20°C. Polysaccharide-enzyme conjugate solutions were prepared in PBS containing 1% bovine serum albumin (BSA) (alkaline phosphatase and β-galactosidase) or in the case of horse-radish peroxidase conjugates, 0.01% BSA. The plates were again washed three times with PBS and substrate solution (100 µl) was added to each assay well. Specific absorbance was read after 1 h incubation at 20°C. The substrates employed were as follows: β-galactosidase, 2-nitrophenyl β-D-galactopyranoside (1 mg/ml in 50 mM KH_2PO_4/Na_2HPO_4 buffer pH 8.0, containing 4 mM $MgCl_2$, 0.1% mercaptoethanol); alkaline phosphatase, 4-nitrophenyl phosphate (1 mg/ml in 50 mM sodium carbonate buffer, pH 9.8 containing 1 mM $MgCl_2$); and HRP, 2,2'-azido-di-(3-ethyl benzthiazoline sulphonic acid) (ABTS) (0.55 mg/ml in 50 mM sodium citrate-sodium hydroxide buffer pH 4.0, containing 0.003% H_2O_2). The absorbance for each system was read at the respective wavelengths 405 nm and 414 nm.

Results

The reducing residue of O-polysaccharide liberated from LPS by mild acid hydrolysis [20] was exploited in the strategy to couple polysaccharide to enzyme. In general a substituted 2-deoxyoctulosonic acid (KDO) terminates O-polysaccharides released from LPS [21, 22] and this provides the point of attachment for coupling chemistry (Fig. 1). Reductive amination of KDO with 40 mM sodium cyanoborohydride at pH 6.0 had previously been reported [8]. Using similar conditions with an excess of 1,3-diaminopropane, 1.0-1.2 amino groups per polysaccharide chain were introduced, assuming an average molecular weight of 15,000 Da for a polysaccharide with one reducing centre per chain.

An alternative activating scheme was investigated to generate aldehyde groups that would be more reactive than isolated KDO residues at the terminal reducing end of the O-polysaccharide. The O-polysaccharide was subjected to mild periodate oxidation such that only the alditol residue or exocyclic diols functionalities were cleaved to yield aldehyde groups [23, 24] (Fig. 2). Reductive amination [25] then provided a polysaccharide bearing several amino groups, since typical LPS core structures [26] contain two heptose and at least a terminal KDO residue, there were in general multiple sites susceptible to mild periodate oxidation. The O-polysaccharides of *Yersinia enterocolitica* 0:9, *Brucella abortus* and

Table 1. Formation of *Yersinia* O-polysaccharide-enzyme conjugates: effect of activated polysaccharide to enzyme ratio on coupling efficiency with three enzymes.

Enzyme	Coupling ratio, Active Polysaccharide: Enzyme	Yield of conjugate (%)	Conjugate (ng/ml) required for EIA with YsT9-2 antibody[a]	
β-Gal	1:1	24	600	
β-Gal	3:1	64	200	
β-Gal	10:1	84	100	
AP	1:1	23	100	
AP	5:1	69	40	
AP	13:1	96	20	
AP	50:1	100	20	
HRP	27:1	71	10	
AP (*S. essen*)	2:1	20	200	(Se155.4)[b]
AP (*S. flex*)	1:1	10	300	(GC-4)[b]
HRP (*S. essen*)	10:1	48	20	(Se155.4)[b]

[a] Concentration of polysaccharide-enzyme conjugate required to give an absorbance of 1.00 following binding to solid phase antibody and subsequent 1 h incubation with enzyme substrate.
[b] Antibody used instead of YsT9-2.

Brucella melitensis, all of which are homopolymers of 4,6-dideoxy-4-formamido-D-mannose [13-15], experienced partial *N*-deformylation during removal of Lipid A. Consequently, prior to periodate oxidation of these polysaccharides, free amino groups were *N*-acetylated in order to eliminate the possibility of periodate cleavage of the O-polysaccharide at potentially reactive sites.

Activation of polysaccharides that possess 1-2 or 3-4 amino groups per chain by disuccinimidyl suberate proceeded efficiently in DMSO and after removal of the organic solvent by a short gel permeation column, the derivatized polysaccharide was coupled to enzyme. When monoaminated polysaccharide was activated by a 40:1 ratio of DSS, polysaccharides with 0.7 to 0.9 active esters per chain were obtained. O-Polysaccharides with 2-3 NHS esters were obtained with the polysaccharides bearing 3-4 amino groups. Preparations of activated polysaccharides were quite stable when stored in the lyophilized state at -20°C. The choice of coupling reagent was determined by the enzyme to be conjugated. Thus the heterobifunctional maleimide reagent sulpho-SMCC was used to prepare β-galactosidase conjugates by alkylation of the multiple sulphydryl groups present in this enzyme.

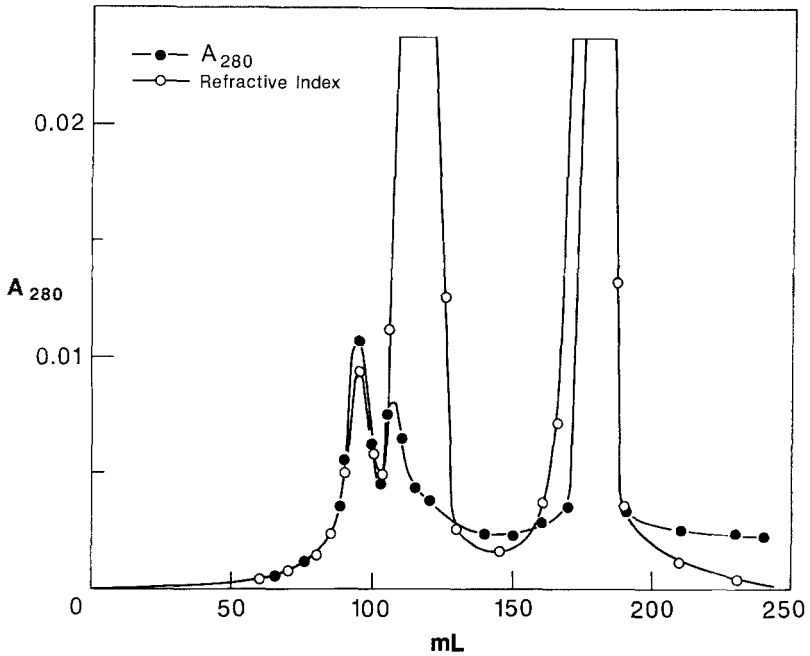


Figure 3. Elution profile of *Y. enterocolitica* 0:9 polysaccharide-HRP conjugate from a Sephacryl S-200 column (1.6 x 95 cm; flow rate 15 ml/h). Unconjugated enzyme overlapped by a large excess of free polysaccharide elutes as the second peak. Low molecular weight side products such as succinimide are contained in the last peak.

Enzyme was coupled to activated polysaccharide by incubating appropriately buffered solutions of the two macromolecules. Multiply-substituted enzymes exhibited undesirable binding characteristics and were not efficiently inhibited by hapten or competing polysaccharide. The ideal conjugate of one polysaccharide chain per enzyme could be obtained with horse-radish peroxidase, which has only one coupling site per molecule for NHS esters. Conjugates that meet this condition were prepared from polysaccharides activated at single or several sites (Table 1). Both alkaline phosphatase and β-galactosidase possessed more than one potential conjugation site and a compromise had to be reached between the number of polysaccharide chains introduced per enzyme and the number of moles of enzyme conjugated. This was controlled by using only polysaccharide activated at one site and then varying the ratio of activated polysaccharide to enzyme (Table 1). It is seen that a large variation in conjugate yield and avidity accompanies the increasing polysaccharide to enzyme ratio.

In all cases it is essential to remove uncoupled polysaccharide from conjugate. This was straight-forward for horse-radish peroxidase conjugates using a Sephacryl S-200 column (Fig. 3), a step which also removed unconjugated enzyme. However, unreacted alkaline phosphatase and β-galactosidase could not be readily separated from conjugated enzyme, although anion exchange chromatography was successful for resolving mixtures of unreacted polysaccharide from conjugated alkaline phosphatase. Either anion exchange or gel permeation chromatography accomplished this task for β-galactosidase.

The yield of conjugated enzyme in both cases, alkaline phosphatase and β-galactosidase, was established by affinity quantification. Conjugated enzyme bound to antibody coated EIA plates, while unconjugated enzyme was removed in the washing step. Complete measurement of conjugate was ensured in three sequential binding steps. Data collected in this way indicated that at a 1:1 ratio of polysaccharide to enzyme there was a 25% yield of conjugate. The data presented in Table 1 for the *Yersinia* polysaccharide are representative of those obtained with other polysaccharides, e.g. *Salmonella essen* (Table 1).

The enzyme conjugates prepared from the *Yersinia enterocolitica* 0:9 antigen with alkaline phosphatase, β-galactosidase and horse-radish peroxidase were evaluated in competitive EIA. Assays were set up with and without polysaccharide inhibitor using solid phase antibody and the working conjugate concentration (Table 1). The inhibition curves (Fig. 4A and 4B) depend upon the extent of enzyme substitution by polysaccharide and it is seen that conjugates with increasing numbers of these antigen chains require higher concentrations of inhibitor to attain 50% inhibition. The HRP conjugate which may not in principle exceed the optimal target of one antigen chain per enzyme is observed to be readily inhibited by 15 ng/ml of the homologous polysaccharide (Fig. 4A and 4B).

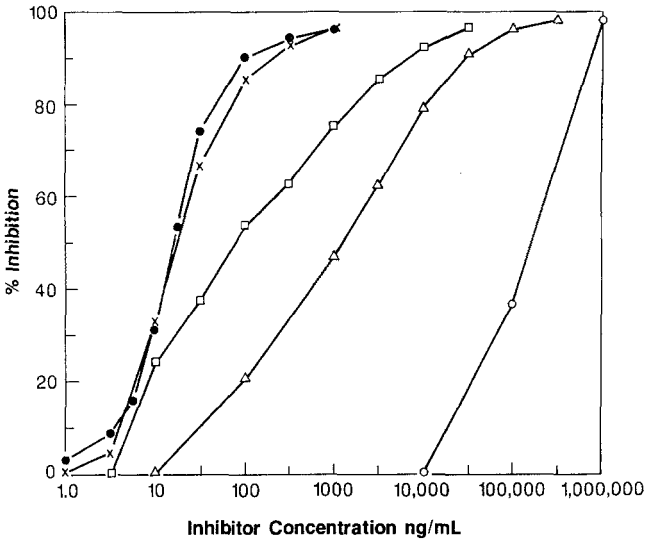
The most effective reduction of background colour in EIA employing carbohydrate enzyme conjugates was achieved by using 1% BSA as an internal blocking agent to the enzyme conjugate [4]. Conjugate was diluted to working strength in buffer containing the BSA, and washing steps excluded BSA or any of the other detergents used to alleviate non-specific binding effects. These general conditions reported in the earlier studies [4] were found to hold for all the conjugates reported here with the exception of the HRP-polysaccharide conjugates. Although these conjugates were bound in the presence of 1% BSA, the presence of 0.001% (w/v) sodium azide in commercial BSA solutions was sufficient to effectively poison horse-radish peroxidase. Consequently the EIA was set up by first coating the assay plate with antibody followed, after washing, by a 1% BSA blocking step. When this was done in combination with dilution of the conjugate solution in buffer containing 0.01% BSA, low background readings comparable to those seen in the earlier study were observed.

Discussion

Optimal strategies for the preparation of O-polysaccharide-enzyme conjugates were established so that minimal modification of the polysaccharide and little if any loss of enzyme activity occurred. Since direct reductive amination [25] of aldose or ketose sugars by amino groups in proteins is inefficient [8] and results in substantial loss of enzymic activity [4], conjugates were prepared by first modifying the polysaccharide at sites within the LPS core region by introduction of 1,3-diaminopropane, followed by activation of the aminated polysaccharide with hetero- or homo-bifunctional coupling reagents and covalent attachment to enzyme.

Two approaches were investigated for amination of O-polysaccharides. Direct, reductive amination of polysaccharide at the terminal reducing KDO, exposed by acid removal of Lipid A from the inner LPS core domain, with a large excess of 1,3-diaminopropane introduced one amino group per polysaccharide chain (Fig. 1). Roy *et al.* [8] showed that

A



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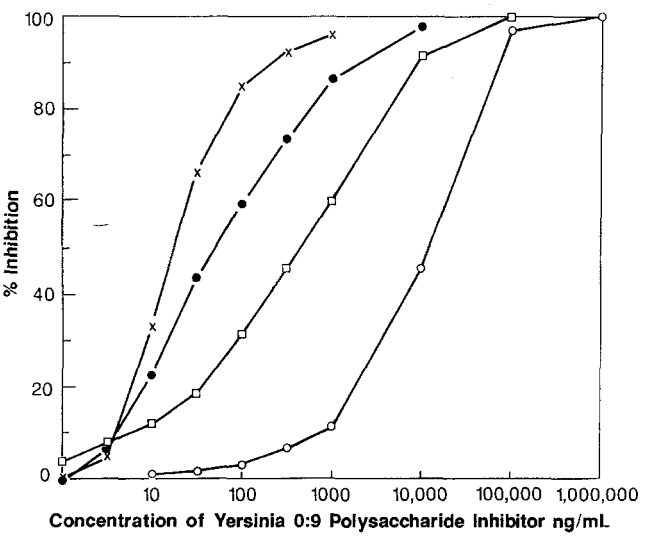

Figure 4. The degree of enzyme substitution by polysaccharide affects the sensitivity of response to inhibitors. A) Inhibition by *Y. enterocolitica* 0:9 polysaccharide of enzyme conjugate binding to immobilized antibody (Yst9-2). Conjugates tested were a 1:1 HRP-polysaccharide conjugate (x), alkaline phosphatase conjugates prepared from reactions in which the ratios enzyme:activated polysaccharide were 1:1 (●), 1:5 (□), 1:13 (△) and 1:50 (○). B) Inhibition by β-galactosidase conjugates prepared from reactions in which the ratio of enzyme:activated polysaccharide was 1:1 (●), 1:3 (□), 1:10 (○). The inhibition curve obtained using 1:1 polysaccharide-HRP conjugate is included for comparison.

KDO and other ketoses react almost quantitatively with low molecular weight amines such as glycine, when these are present in excess and a reaction pH of 6.0 is maintained. Under similar conditions the KDO residue reacted quantitatively with 1,3-diaminopropane as judged by quantification of amino groups. A more heavily aminated polysaccharide derivative (Fig. 2) was obtained when mild periodate oxidation was used to generate aldehyde groups under conditions that are known to oxidize only vicinal diol groups of acyclic chains [23], such as the L-*glycero*-D-*manno*-heptose residue [24, 27]. As this heptose appears to be common to a very large number of LPS inner core structures [26] its use for coupling strategies constitutes a general method by which O-antigens may be conjugated to proteins or solid supports without altering epitopes that reside in the O-polysaccharide domain [3, 28, 29].

Activation of aminated polysaccharides with disuccinimidyl suberate and sulphosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate resulted in a stable amide bond to the modified polysaccharide. The half life of succinimide esters attached to polysaccharide activated by the homo-bifunctional succinimide ester reagent was extended by carrying out activation with disuccinimidyl suberate in dimethylsulphoxide solution. After removal of this solvent, amide bond formation to protein was performed in aqueous buffer. Polysaccharide activated in this way gave the most effective conjugates when coupled to horse-radish peroxidase, an enzyme with only one coupling site per molecule. Monogamous horseradish peroxidase-polysaccharide conjugates could be prepared by reaction with polysaccharides activated at either single or multiple sites. These conjugates showed a large incremental increase in molecular weight (from 40 to 50 KDa) that provides the added advantage that they were readily separated from unreacted polysaccharide and enzyme (Fig. 3). Although the presence of the latter was transparent to the assay, it was essential to remove unreacted polysaccharide.

Enzyme conjugates prepared by amide bond formation with alkaline phosphatase or maleimide alkylation of thiol groups of β-galactosidase were less suitable for EIA because it was difficult to control the degree of polysaccharide incorporation. Conjugates bearing several polysaccharide chains exhibited enhanced affinity for solid phase antibody and required high concentrations of inhibitors for competitive EIA. This property was attributed to high functional affinity resulting from polyvalency [30] of multiply substituted enzymes. The shallow slope of the inhibition curves obtained with conjugates that possess several polysaccharide chains per enzyme yields a less sensitive measurement of inhibitory activity as well as requiring more inhibitor.

Although limited polyvalency is desirable so that high functional affinity may augment the relatively low intrinsic affinity of carbohydrate antibodies (K_A 10^4-10^6 M^{-1}) [30], the affinities that accompany the incorporation of two or more polysaccharide chains per enzyme molecule detract from assay sensitivity. Similar results were obtained when high ratios of monoactivated polysaccharide to enzyme were employed. Although the problem of multiple substitution of alkaline phosphatase or β-galactosidase could be controlled by reducing the ratio of polysaccharide to enzyme in conjugation reactions, low yields of product represented an inefficient use of enzyme and polysaccharide. Due to their higher molecular weight it was impossible to separate conjugated from unconjugated enzyme, although it was still feasible to remove the critical impurity, unreacted polysaccharide.

Horse-radish peroxidase-conjugated polysaccharides are thus well defined and sensitive reagents for direct competitive EIA. Applications not only permit the convenient measurement of relative affinity for large numbers of structurally-related monovalent ligands, but they also provide for the sensitive estimation of bacterial O-antigens in the low nanogram range [31].

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