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COUPLING OF POLYSACCHARIDES ACTIVATED BY MEANS OF CHLOROACETALDEHYDE DIMETHYL ACETAL TO AMINES OR PROTEINS BY REDUCTIVE AMINATION

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ABSTRACT

A novel method has been developed for the coupling of modified polysaccharides to proteins or other amines. Chloroacetaldehyde dimethyl acetal has been used for the introduction of *O*-(2,2-dimethoxyethyl) groups into amylose, dextran, and a linear (1→3)-β-D-glucan. In amylose and the (1→3)-β-D-glucan, these groups were attached preponderantly at O-6 and in dextran at O-2. Mild treatment with acid then gave polysaccharide derivatives substituted with aldehyde groups which were coupled in good yields to proteins and other amines by reductive amination with sodium cyanoborohydride in aqueous solution at pH 7. An aminated (1→3)-β-D-glucan derivative that induced antitumor activity in mouse macrophages *in vitro* is reported.

INTRODUCTION

Many methods are now available for attaching carbohydrates to proteins[1]. The attachment often involves two stages: (*a*) activation of the carbohydrate and (*b*) coupling of the intermediate to the protein[2]. The coupling reaction should be fast and proceed under mild conditions so that biologically active compounds are not inactivated.

Reductive amination with sodium cyanoborohydride is a mild method for coupling carbonyl compounds to amino compounds, and several reducing disaccharides have been coupled to proteins by this method[3,4]. Aldehyde groups in polysaccharides having suitable vicinal hydroxyl groups are generated by periodate oxidation and these polysaccharide derivatives have been coupled to proteins[5]. 1-Thioglucosides with ω-aldehydo aglycons have been prepared and coupled to proteins by reductive amination[6]. In agarose, aldehyde groups have been introduced

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by treatment with glycidol followed by periodate oxidation[7]. Carbonyl groups have also been introduced into polysaccharides by bromine oxidation[8]. Ideally, carbohydrates should be activated under conditions to which they are stable and the procedure should be sufficiently versatile to be applicable to different sugars.

We now report on the activation of amylose, dextran, and a linear (1→3)-β-D-glucan with chloroacetaldehyde dimethyl acetal followed by mild hydrolysis with acid, and the coupling to some proteins (HSA, IgG) and amines (L-lysine, ammonium acetate) by reductive amination.

Water insoluble (1→3)-β-D-glucans have strong stimulatory effects on peritoneal mouse macrophages *in vitro*[9]. In earlier publications, we reported that macrophages were stimulated when cultivated on plastic dishes[10] covered with immobilised (1→3)-β-D-glucan and that (1→3)-β-D-glucan attached to plastic beads will activate macrophages *in vivo*[11].

A water-soluble (1→3)-β-D-glucan derivative prepared by bromine oxidation of the glucan followed by reductive amination with sodium cyanoborohydride and ammonium acetate had a stimulating effect similar to that of the insoluble (1→3)-β-D-glucan[12]. We also report another soluble, aminated (1→3)-β-D-glucan derivative which stimulates macrophages *in vitro*.

EXPERIMENTAL

Materials. — Curdlan (Wako), human serum albumin (Sigma, HSA), and amylose (Sigma), gamma globulin (Kabi, IgG), a lyophilised powder of normal human immunoglobulin, were commercial products. Dextran T70, DEAE-Sepharose CL-6B, and CM-Sepharose CL-6B were obtained from Pharmacia, and mice from Gl. Bomholt Gård Ltd. (Ry, Denmark).

General methods. — Solutions were concentrated under reduced pressure at <40°. Optical rotations were measured on aqueous solutions with a Perkin–Elmer 141 polarimeter. G.l.c. was performed with a Packard 427 instrument, fitted with a flame-ionisation detector and an OV-225, WCOT fused-silica capillary column (12 m × 0.23 mm i.d.); helium was the carrier gas. Temperature programme: 140→180° at 2°/min. For quantification, an Autolab minigrator was used. G.l.c.–m.s. was performed with a Finnigan 4021 instrument including a Finnigan 9610 gas chromatograph and Incos data system. E.i. mass spectra were recorded at 70 eV. N.m.r. spectra were recorded at 85°, using a Jeol FX 90 Q instrument and solutions in D_2O (internal TPS).

Partial degradation of curdlan. Curdlan, a linear (1→3)-β-D-glucan, was degraded[13] with aqueous 90% formic acid at 90° for 20 min in order to obtain a water-soluble product after final derivatisation. The slightly degraded glucan was separated into water-soluble and water-insoluble fractions, and to a solution of the latter (1 g) in 0.25M sodium hydroxide (200 mL) was added sodium borohydride (0.5 g). After 8 h at room temperature, the solution was dialysed and freeze-dried.

2,2-Dimethoxyethylation. — Solutions of amylose, Dextran T70, or degraded

curdlan (100 mg) in dry methyl sulfoxide (2 mL; for curdlan, 4 mL) in a serum vial were flushed with nitrogen, and 2M sodium methylsulfinylmethanide (dimsyl sodium) in methyl sulfoxide (0.8 mL) was added[14]. After agitation in an ultrasonic bath for 30 min, the mixture was left at room temperature overnight, freshly distilled chloroacetaldehyde dimethyl acetal (0.2–1.0 mL) was then added, and, after agitation for 1 h at 50° and 30 min in an ultrasonic bath, the mixture was dialysed against distilled water. The polysaccharide derivatives were recovered quantitatively.

Ethylation analysis[15]. — The substituted glucans (5 mg) were hydrolysed[6] with 50mM hydrochloric acid (2 mL) at 100° for 20 min, and the solutions were neutralised with 0.5M NaOH. Sodium borohydride (1 mg) was added and the solutions were dialysed against distilled water and freeze-dried to give the products (4 mg).

Each resulting 2-hydroxyethyl derivative (4 mg) was dissolved in dry methyl sulfoxide (2 mL) and treated with 2M dimsyl sodium in methyl sulfoxide (1 mL) for 12 h. Iodoethane (1 mL) was added, and the mixture was dialysed and concentrated to dryness. The residues were treated with aqueous 90% formic acid (2 mL) at 100° for 2 h, the solutions were concentrated to dryness, and the residues were hydrolysed with 0.5M trifluoroacetic acid (3 mL) at 100° for 16 h. The resulting ethyl-2-hydroxyethyl sugar derivatives in these hydrolysates were converted into alditols by reduction with sodium borodeuteride[16], and then ethylated as described above. The products were recovered and purified by reversed-phase chromatography on Waters Sep-Pak C_{18} cartridges[17] and analysed by g.l.c. and g.l.c.–m.s.

Coupling of the modified polysaccharides to proteins. — The 2,2-dimethoxyethylated (1→3)-β-D-glucan (CII, 10 mg; Table I) was treated with 50mM hydrochloric acid (2.5 mL) for 20 min at 100°. To the neutralised (pH 7) mixture was added 0.2M phosphate buffer (pH 7.0, 2.5 mL), HSA (8 mg), and sodium cyanoborohydride (1 mg). The mixture was gently stirred for 18 h at 20°, then dialysed against 50mM Tris buffer (pH 8.6), transferred to a column (10 × 1 cm) of DEAE-Sepharose CL-6B, and eluted (12 mL/h) with 50mM Tris buffer (pH 8.6) until the polysaccharide emerged. The column was then eluted with a linear gradient of 0→0.5M sodium chloride in the same buffer. IgG (8 mg) was coupled in the same way and dialysed against 50mM sodium acetate buffer (pH 4.0), and the product was fractionated on a column of CM Sepharose Cl-6B by elution first with acetate buffer and then with a linear gradient of 0→0.5M sodium chloride. Fractions (~4 mL) were analysed for protein (absorbance at 279 nm; A_{279} 1% = 5.3) and carbohydrate (phenol–sulfuric acid[18]). Fractions containing the protein–carbohydrate conjugates were combined, dialysed against distilled water, and freeze-dried. The amount of coupled carbohydrate was determined by hydrolysis of the conjugates with 0.5M trifluoroacetic acid at 100° for 12 h and analysis of the neutral sugars as alditol acetates by g.l.c. with *myo*-inositol as the internal standard.

Coupling of modified polysaccharide with L-lysine or ammonium acetate. — 2,2-Dimethoxyethylated (1→3)-β-D-glucan (CII, 100 mg; Table I) was hydrolysed

TABLE I

DEGREE OF SUBSTITUTION (D.S.) BY 2,2-DIMETHOXYETHYL GROUPS AS CALCULATED FROM ^{1}H-N.M.R. DATA

Polysaccharide[a]	*Cl-CH_2CH-$(OMe)_2$ (mL)*	*D.s.*	$[\alpha]_{578}^{22}$ *(degrees)*
Amylose AI	0.2	0.06	—
Amylose AII	1.0	0.42	146
Curdlan CI	0.4	0.27	—
Curdlan CII	1.0	0.57	2
Dextran D	0.5	0.07	170

[a]100 mg treated with 2M dimsyl sodium in methyl sulfoxide (0.8 mL).

with 50mM hydrochloric acid (5 mL) as described above. Ammonium acetate (500 mg in water, 2.5 mL) and sodium cyanoborohydride (12.5 mg) were added, and the mixture was left for 3 days at 20°, dialysed first against aqueous 10% acetic acid for 3 h and then against distilled water, and freeze-dried to give aminated glucan (75 mg; N, 0.8%). A solution of L-lysine (200 mg) in water (2 mL) was adjusted to pH 7.0 and used for reductive amination as described above. The yield was 80 mg; N, 1.7%.

Assay of macrophage-mediated cytostasis. — Macrophages were obtained from the peritoneal cavity of hybrid C_3D_2 mice (C3H/Tif × DBA/2). The cells were cultivated[12] in Dulbeccos modified Eagle's medium with 10% heat-inactivated (56°, 30 min) newborn-calf serum (Gibco Biocult Ltd.) in an atmosphere of 5% CO_2 in air at 37°. Target cells were the *in vitro*-transformed fibroblast line L 929[19]. After macrophage stimulation with aminated glucan for 24 h, the target cells were added so as to obtain a 15:1 macrophage–target cell ratio. After co-culture of the cells for 48 h, radioactive thymidine (0.5 μCi/mL, methyl-^{3}H) was added, and the incorporation[12] was measured for an incubation period of 24 h.

RESULTS AND DISCUSSION

When amylose, dextran, or (1→3)-β-D-glucan were severally treated with sodium methylsulfinylmethanide in methyl sulfoxide (Hakomori[14]) and then chloroacetaldehyde dimethyl acetal, a partially 2,2-dimethoxyethylated polysaccharide was obtained. In order to prevent a peeling reaction, the (1→3)-linked polysaccharide was first reduced with sodium borohydride. The d.s. (Table I) could be monitored on the basis of the relative intensity of the ^{1}H-n.m.r. signals for OMe groups (δ 3.47) and the anomeric protons [δ 5.36 and $J_{1,2}$ 3.8 Hz for amylose, δ 4.96 and $J_{1,2}$ 3.2 Hz for dextran, and δ 4.77 and $J_{1,2}$ 6.8 Hz for the (1→3)-β-D-glucan].

The substitution pattern was determined by ethylation analysis. When the 2,2-dimethoxyethylated glucans were treated with 50mM hydrochloric acid at 100° for 20 min, the dimethyl acetal groups were converted into aldehyde groups without cleavage of the glycosidic linkages (gel-filtration data). The aldehyde groups were

reduced with sodium borohydride, giving a 2-hydroxyethylated polysaccharide. The polymer was ethylated and hydrolysed, the monosaccharide derivatives were reduced with sodium borodeuteride, and the alditol derivatives were ethylated. The resulting ethyl–2-ethoxyethyl derivatives were analysed by g.l.c. and g.l.c.–m.s. The ethyl and 2-ethoxyethyl groups on each alditol were located by e.i.-mass spectrometry[15]. As shown in Table II, 2,2-dimethoxyethylation of the (1→3)-β-D-glucan occurred mainly at O-6 and to a lesser extent at O-4. In amylose, substitution occurred preferentially at O-6 with some substitution at O-2, whereas dextran was preferentially substituted at O-2.

Aldehydes react reversibly with primary amines to form aldimines which can be reduced to secondary amines with sodium cyanoborohydride[20]. The optimum pH for imine formation is 6–8; in this pH range, the rate of carbonyl reduction is low compared to that of imine reduction. Hence, despite the unfavourable equilibrium for imine formation in aqueous solutions, a good yield of amine can be obtained.

Reductive amination is one of the most successful techniques for modifying proteins whilst preserving the overall structure[21]. Synthetic glycoproteins have been prepared by treating a protein with reducing disaccharides in the presence of sodium cyanoborohydride; the protein is linked through the ε-amino groups of the lysine residues[3]. The low rate of sugar incorporation in these reactions is presumably due to the very low concentrations of acyclic sugars in aqueous solution. Free aldehydes should react much faster than cyclic sugars[6].

By reductive amination at pH 7 in the presence of sodium cyanoborohydride, the amino groups of HSA or human IgG were coupled to the aldehyde substituents on the modified (1→3)-β-D-glucan. Nearly all of the protein added was recovered as a protein–carbohydrate conjugate after ion-exchange chromatography. Glucose analyses of the conjugates revealed 10% of carbohydrate in the HSA-conjugate and 15% in the IgG conjugate. Gel filtration revealed that the molecular weights of the conjugates were higher than those of the (respective) parent proteins. The HSA–carbohydrate conjugate differed in mobility from HSA on sodium dodecyl sulfate–polyacrylamide gel electrohoresis.

An aminated (1→3)-β-D-glucan derivative was prepared by reductive amina-

TABLE II

PROPORTIONS (MOL %) OF X-*O*-(2-HYDROXYETHYL)-D-(1-^{2}H)GLUCITOLS IN THE ETHYLATION ANALYSIS OF ACTIVATED AMYLOSE AI, CURDLAN (CI AND CII), AND DEXTRAN D

X	*AI*	*CI*	*CII*	*D*
—	93.5	75.0	39.5	94.0
2	1.5	—	—	6.0
4		4.0	6.0	—
6	5.0	21.0	39.5	—
4,6	—	—	15.0	—

TABLE III

INCORPORATION OF (^{3}H)THYMIDINE INTO L-929 CELLS AND IN CO-CULTURES OF MACROPHAGES (mϕ) AND L-929 CELLS

Cells	*(1→3)-β-D-Glucan*	*C.p.m.*	*Percentage of maximum incorporation*
L-929	—	40531 ±1537	100 ±3.8
L-929	aminated	37592 ±3080	92.7 ±8.2
mϕ + L-929	—	13251 ±3370	32.7 ±25.4
mϕ + L-929	soluble[a]	12474 ±870	30.8 ±7.0
mϕ + L-929	aminated	1518 ±55	2.8 ±3.6
mϕ	—	<100 ±10	<0.25 ±10
mϕ	aminated	<100 ±12	<0.25 ±10

[a]Degraded curdlan, water-soluble fraction.

tion of the activated polysaccharide with ammonium acetate. Table III shows that the aminated glucan derivative had little effect on L-929 tumour cells alone. Unstimulated macrophages had a profound inhibitory effect on the growth of these cells which could not be potentiated by a neutral, water-soluble (1→3)-β-D-glucan. However, when the macrophages were stimulated with an aminated, soluble (1→3)-β-D-glucan for 24 h before the addition of the tumour cells, there was an almost complete arrest of their growth as measured by the incorporation of radioactive (^{3}H)thymidine. These results were in good agreement with microscopic observations. Unstimulated macrophages or macrophages stimulated with the aminated (1→3)-β-D-glucan derivative did not incorporate (^{3}H)thymidine.

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