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Artificial antigens. Synthetic carbohydrate haptens immobilized on crystalline bacterial surface layer glycoproteins

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ABSTRACT

The crystalline surface-layer glycoproteins of *Clostridium thermohydrosulfuricum* L111-69, *Bacillus stearothermophilus* NRS 2004/3a and *Bacillus alvei* CCM 2051 were used for immobilization of spacer-linked blood group A-trisaccharide {αGalNAc(1 → 3)[αFuc(1 → 2)]βGal} and of the spacer-linked, tumor-associated T-disaccharide [βGal(1 → 3)αGalNAc]. The immobilization involved the glycan portions of surface-layer glycoproteins. Different activation methods were used, namely, periodate oxidation, or treatment with epichlorohydrin or divinyl sulfone, followed by coupling of the hapten under appropriate conditions. The resulting conjugates are useful for assessing the application potential of haptenated surface layer preparations as carrier/adjuvants for the induction of immunity to poorly immunogenic molecules.

INTRODUCTION

Crystalline surface-layers (S-layers) composed of identical (glyco)protein subunits represent the outermost cell envelope component in many eubacteria and archaebacteria[1–6]. Due to the crystalline arrangement of the subunits, functional groups are present on each protomer in identical, well-defined positions. S-layers have been used as matrix for the immobilization of a broad spectrum of molecules[7,8].

Immunization of experimental animals against *O*-polysaccharide antigens of Gram-negative bacteria protects the animals against infectious challenge with

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organisms of the analogous *O*-serotype[9]. However, effective immunization with other carbohydrate antigens has proved to be more difficult, as in the cases of small tumor-associated oligosaccharides[10] and certain capsular polysaccharides[11]. Herein we describe methods for binding of oligosaccharide haptens onto the glycan chains of the S-layer glycoproteins of *Clostridium thermohydrosulfuricum* L111-69[12], *Bacillus stearothermophilus* NRS 2004/3a[13,14], and *Bacillus alvei* CCM 2051[15]. Following immunization of mice with haptenated S-layer preparations, strong anti-hapten cellular and antibody responses were observed[16].

EXPERIMENTAL

General.—Spacer-linked A-trisaccharide and T-disaccharide (both as their 8-carboxyhydrazidooctyl or 8-[*N*-2-aminoethyl]carboxamidooctyl glycosides) are commercial products of CHEMBIOMED, Ltd. Glutaraldehyde was either from Fluka Chemical Co. (Buchs, Switzerland) or Sigma Chemical Co. (St. Louis, MO, USA); lysozyme was purchased from Boehringer–Mannheim (Mannheim, Germany), and epichlorohydrin and divinyl sulfone were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals were of the highest purity available. Total carbohydrate[17], rhamnose[18], hexosamine[19], and protein[20] contents of underivatized S-layers and T-disaccharide- or A-trisaccharide-S-layer conjugates were determined according to literature procedures. The colorimetric assays were calibrated using appropriate carbohydrate mixtures as standards. Alternatively, the *N*-acetylgalactosamine content of S-layer conjugates and underivatized S-layers was estimated on a Biotronic LC 3000 amino acid analyzer (Biotronik, Maintal, Germany) after hydrolysis of the preparations with 4 N HCl for 16 h at 100°.

Bacterial strains and growth conditions.—*Clostridium thermohydrosulfuricum* L111-69 was grown anaerobically in continuous culture[21], *Bacillus stearothermophilus* NRS 2004/3a[22] was grown aerobically in batch culture, and *Bacillus alvei* CCM 2051[23] was grown aerobically in continuous culture, all as previously described.

Cell-wall preparations and S-layer self-assembly products.—Cell-wall preparations of all strains and crystalline self-assembly products, obtained by reaggregation of isolated S-layer subunits of *B. stearothermophilus* and *B. alvei*, were obtained as recently published[22]. The purification of the S-layer glycoproteins was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and by electron microscopy as previously described[24].

Crosslinking with glutaraldehyde.—Cell-wall fragments of *C. thermohydrosulfuricum* or *B. stearothermophilus* were treated with glutaraldehyde (0.5% final concentration in 0.1 M sodium cacodylate buffer, pH 7.2) for 20 min at room temperature. The reaction was then terminated by the addition of an excess of tris(hydroxymethyl)aminomethane (Tris). Subsequently, the peptidoglycan was digested by incubation with lysozyme[25]. After thorough washing of the pellet with 0.2 M NaCl and water, the sample was lyophilized. These preparations are referred to

as glutaraldehyde-fixed S-layer fragments. Some immobilization experiments with *B. stearothermophilus* were performed with wet pellets of glutaraldehyde-fixed S-layer fragments. *B. alvei* S-layer self-assembly products were prepared according to ref. 22, fixed with glutaraldehyde as described before, and lyophilized.

Immobilization experiments.—(A) Periodate oxidation[26]. Typically, about 2–3 mg of lyophilized, glutaraldehyde-fixed S-layer fragments or the equivalent amount of wet pellets (to give 2–3 mg dry weight) of *B. stearothermophilus* or *C. thermohydrosulfuricum* were used. With *B. alvei*, lyophilized, glutaraldehyde-fixed S-layer self-assembly products had to be used. The fragment approach is not feasible with *B. alvei* because the peptidoglycan of that strain is not degraded by the action of lysozyme[23]. To determine differences in the immobilization of haptens between glutaraldehyde-fixed S-layer fragments and unfixed S-layer self-assembly products, both types of preparations from *B. stearothermophilus* S-layer were compared. All preparations were suspended in a solution of 0.1 M sodium periodate in 50 mM sodium acetate buffer (1 mL) in 1.5 mL Eppendorf vials. Depending on the strain, the pH values of the buffers were chosen between 4.5 and 5.5. Oxidation times at 4° in the dark were generally between 3 and 5 h for the glutaraldehyde-fixed preparations (preferentially 4 h), and usually 30 min for unfixed S-layers. The optimal oxidation time for each preparation was determined by oxidation of the S-layer material for various times, and estimation of the free aldehyde groups formed from the extinction values of their 2,4-dinitrophenylhydrazone derivatives[27]. Following oxidation, the samples were centrifuged in an Eppendorf bench centrifuge and the pellets washed with 1.5 mL of 0.2 M NaCl and 2×1.5 mL of water. Subsequently, the pellets were suspended in a solution containing the spacer-linked haptens (1 mL, approximately 1 μmol/mL of A-trisaccharide or T-disaccharide as their 8-[*N*-2-aminoethyl]carboxamidooctyl or 8-carboxyhydrazidooctyl glycosides in 0.2 M freshly prepared $NaHCO_3$ solution) and 0.2 mL $NaBH_4$ (1 mg/mL of 0.2 M $NaHCO_3$). In parallel experiments, identical S-layer preparations were oxidised, but buffer was added instead of hapten solution. The reaction mixtures were incubated for 1 h at room temperature on a hematology mixer. Haptenized materials and blanks were washed thoroughly (2×1.5 mL of 50 mM freshly prepared $NaHCO_3$ solution and 3×1.5 mL of water) to remove all unbound hapten. With unfixed S-layer self-assembly products, the centrifugation steps were replaced by exhaustive dialysis against water. The supernatants of the final centrifugations and all washes were combined to determine the amount of unbound hapten. After lyophilization of both the combined supernatants and the remaining wet pellets in a Savant Speedvac concentration centrifuge, the amount of bound hapten was determined by the phenol–H_2SO_4 assay[17]. The difference between haptenized sample and blank, after normalization to dry weight, was taken as the amount of coupled hapten. A different approach was the direct determination of bound hapten (blood group A-trisaccharide or T-disaccharide) by estimation of the amount of *N*-acetylgalactosamine in the lyophilized S-layer-hapten-conjugate either colorimetrically[19] after hydrolysis with 4 N HCl for 3–5 h

at 100°, or in the amino acid analyzer after hydrolysis with 4 N HCl for 16 h at 100°. Since no significant differences in the amount of bound hapten were observed with either method, we have used the less time-consuming colorimetric assays as a routine procedure in all experiments. Coupling of the haptens was also performed under acidic conditions. After oxidation and washing of the pellets, they were suspended in a solution of hapten in 0.2 M sodium acetate buffer, pH 4.0. Sodium cyanoborohydride (0.2 mL; 1 mg/mL of 0.2 M sodium acetate buffer, pH 4.0) was used as a reducing agent[28]. Incubation and washing was performed as described above.

(B) Epichlorohydrin activation[29,30]. Typically, 2–3 mg of glutaraldehyde-fixed S-layer fragments were suspended in 2.5 mL of either 0.2 M Na_2CO_3/Na_2CO_3 buffer, pH 9.1–10.0, or 0.2 M Na_2CO_3 solution pH 11.4, or 0.4 M NaOH solution containing 25 mg sodium borohydride. Upon addition of epichlorohydrin (0.2 mL), the reaction mixtures were incubated for 30 min to 16 h at room temperature or at 40°, with rotation on a rotary mixer (150 rpm). Controls were prepared similarly, but either epichlorohydrin or hapten was omitted. After centrifugation and washing with water (5 × 1.5 mL) to remove all of the activating agent, the combined supernatants of the first centrifugation and all washes were analyzed for carbohydrate material shed into the supernatant from the S-layer under the strongly alkaline reaction conditions. The remaining pellets were suspended in hapten solution (1.0–1.4 mL; 1–2 μmoles/mL of 0.2 M $NaHCO_3$ solution) and incubated for 2–6 h with shaking on the hematology mixer at room temperature. Excess of hapten was removed by washing with water (5 × 1.5 mL) prior to the blocking of unreacted epoxy groups by treatment with 0.02 M ethanolamine in 50 mM $NaHCO_3$ solution for 4–18 h at room temperature. Subsequently, the samples were washed with water (5 × 1.5 mL), lyophilized and assayed for carbohydrate, as described for the conjugates prepared via periodate oxidation.

(C) Divinyl sulfone activation[30,31]. Typically, about 2–3 mg of glutaraldehyde-fixed S-layer fragments were suspended in 2 mL of appropriate buffer (pH 9.1–11.4; see epoxy activation), and then divinyl sulfone (0.2 mL) was added to the suspensions. Extensive mixing was necessary to disperse the reagent. Treatment of controls, estimation of shedded S-layer glycan, incubation times with hapten solutions, and blocking of unreacted vinyl groups were performed exactly as described for the epoxy activation.

RESULTS

Structure of the glutaraldehyde-fixed S-layer fragments.—Electron microscopic studies have revealed that, following glutaraldehyde fixation of cell-wall fragments of *Clostridium thermohydrosulfuricum* L111-69 or *Bacillus stearothermophilus* NRS 2004/3a, both faces of the peptidoglycan sacculus were covered by an S-layer lattice (Fig. 1b). After removal of the peptidoglycan by digestion with lysozyme, a double layer of S-layer glycoprotein fragments remained which was held together

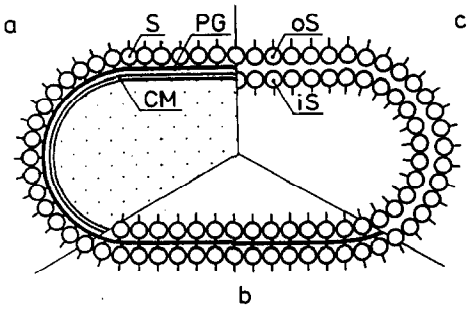

Fig. 1. Preparation scheme of glutaraldehyde-fixed S-layer fragments; (a) intact bacterial cell; (b) empty peptidoglycan sacculus after glutaraldehyde fixation. A second S-layer has formed on the inner surface; (c) glutaraldehyde-fixed S-layer fragments consisting of two S-layers after lysozyme treatment. PG, Peptidoglycan; CM, cytoplasmic membrane; S, S-layer—iS and oS, inner and outer S-layer (adapted from Sára and Sleytr[25]).

in a fixed position by the penta-1,5-diylidene bridges resulting from the glutaraldehyde treatment[25] (Fig. 1c). In the case of *Bacillus alvei* CCM 2051, glutaraldehyde-fixed S-layer self-assembly products were used instead of glutaraldehyde-fixed cell-wall fragments. Depending on the lattice type, each morphological unit of the S-layer lattice consists of six identical subunits in *C. thermohydrosulfuricum* and two identical subunits in both *B. stearothermophilus* and *B. alvei*. The glycan chains used for the immobilization experiments protrude from these subunit matrices[32].

Covalent attachment of haptens.—The results of the immobilization experiments are summarized in Table I.

(A) Periodate oxidation. This method was used to generate aldehyde functions which could then be used for coupling of spacer-linked carbohydrate haptens by reductive amination[33] (see Fig. 2). The glycan chains of *C. thermohydrosulfuricum* consist of up to 60 disaccharide repeats of the structure 4αDMan$p(1 \rightarrow 3)\alpha$LRha$p1$. The carbohydrate content of this S-layer glycoprotein was estimated at ~ 10%[12].

TABLE I

Maximum immobilization of haptens to S-layer subunits

Immobilization method	pH	Hapten/S-layer (mol/mol)	S-layer producing bacterial strain
Periodate oxidation	4.5–5.5	23	*C. thermohydrosulfuricum*
		17	*B. stearothermophilus*
		12	*B. alvei*
Epichlorohydrin activation	9.1–11.4	8	*C. thermohydrosulfuricum*
		8	*B. stearothermophilus*
Divinyl sulfone activation	9.1–10.5	60	*C. thermohydrosulfuricum*
		11	*B. stearothermophilus*

Upon periodate oxidation for 4 h at pH 5.5, only half of the initial amount of sugars was detected by the phenol–sulfuric acid assay[17]. This decrease indicates a complete oxidation of the mannose residues in the glycan chains (Fig. 2A). Concomitantly, formation of aldehyde groups was demonstrated by reaction with 2,4-dinitrophenylhydrazine[27]. By decreasing the pH value of the sodium acetate buffer to 4.5, oxidation time was only slightly shortened. Thus, the oxidation is preferentially performed in the dark at 4° for 4 h. Immobilization of up to 23 molecules of spacer-linked hapten (8-[*N*-2-aminoethyl]carboxamidooctyl or 8-carboxyhydrazidooctyl glycosides) per S-layer subunit of *C. thermohydrosulfuricum* was achieved. Successful immobilization was possible only when the newly formed Schiff bases were immediately reduced (Fig. 2B). This figure shows that only ~ 20% of the aldehyde functions actually undergo Schiff base formation in this S-layer glycoprotein. Reduction at pH 4.0, by the action of sodium cyanoborohydride, gave inferior results and only 25% of the incorporation value obtained with sodium borohydride were observed. *B. stearothermophilus* S-layer protomers have two different glycan chains; the total carbohydrate content was estimated at

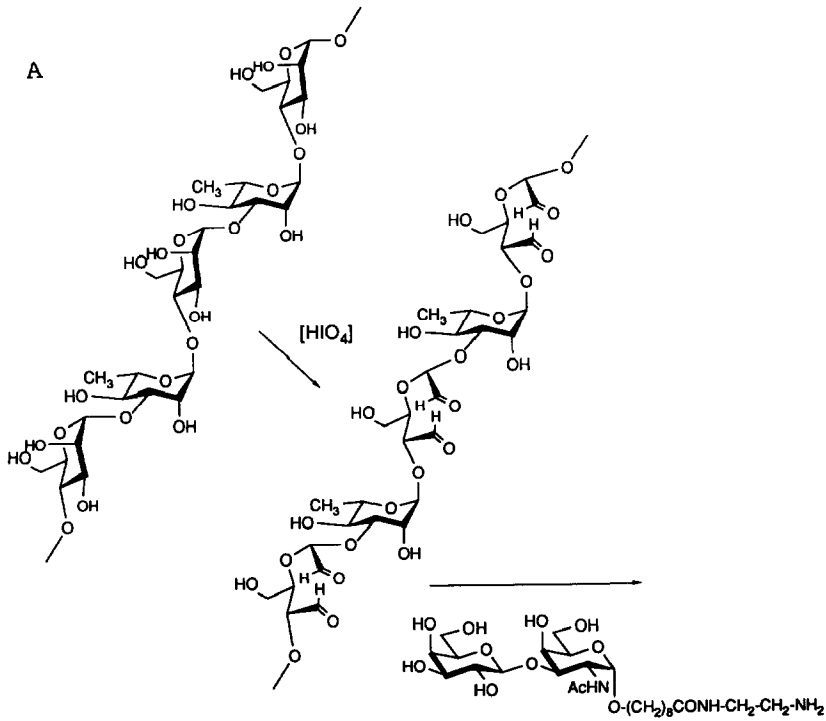

Fig. 2. Schematic representation of the immobilization reaction of a spacer-linked hapten to the S-layer glycoprotein of *Clostridium thermohydrosulfuricum* L111-69. (A) Periodate oxidation of the glycan, oxidative ring cleavage, and formation of aldehyde functions. (B) Covalent linkage of spacer-linked T-disaccharide (8-[*N*-2-aminoethyl]carboxamidooctyl glycoside) and reduction of the Schiff bases.

~ 7.5%, with approximately equal amounts of both glycans present[13,14]. The structure of glycan I is 2αLRha$p(1 \rightarrow 2)\alpha$LRha$p(1 \rightarrow 3)\beta$LRha$p1$ with ~ 50 repeats[13] and that of glycan II is 4βMan$p$UA2,3(NAc)$_2(1 \rightarrow 3)\alpha$Glc$pNAc(1 \rightarrow 4)\beta$Man$p$UA2,3(NAc)$_2(1 \rightarrow 6)\alpha$Glc$p1$ with ~ 15 repeats[14]. Thus, three different sites susceptible to oxidative ring cleavage are present in the repeats of both glycans (two rhamnose residues in glycan I and the glucose residue in glycan II). Up to 17 molecules of hapten per S-layer subunit were immobilized which indicates that ~ 15% of the aldehyde groups have reacted with ligands. No difference in the amount of immobilized hapten was detected when wet pellets of unfixed S-layer self-assembly products of *B. stearothermophilus* were used. With the latter, however, severely lower overall yields of material were obtained, possibly due to partial solubilization of the protein. The reaction conditions like pH value and oxidation time were similar for the two strains. Apparently the S-layer from *B. stearothermophilus* is less sensitive to harsher oxidation conditions. In *B. alvei*, the glycan chains of the S-layer consist of branched trisaccharide repeats of the structure 3βDGal$p(1 \rightarrow 4)[\alpha$DGlc$p(1 \rightarrow 6)]\beta$DMan$pNAc1$[15]. By the

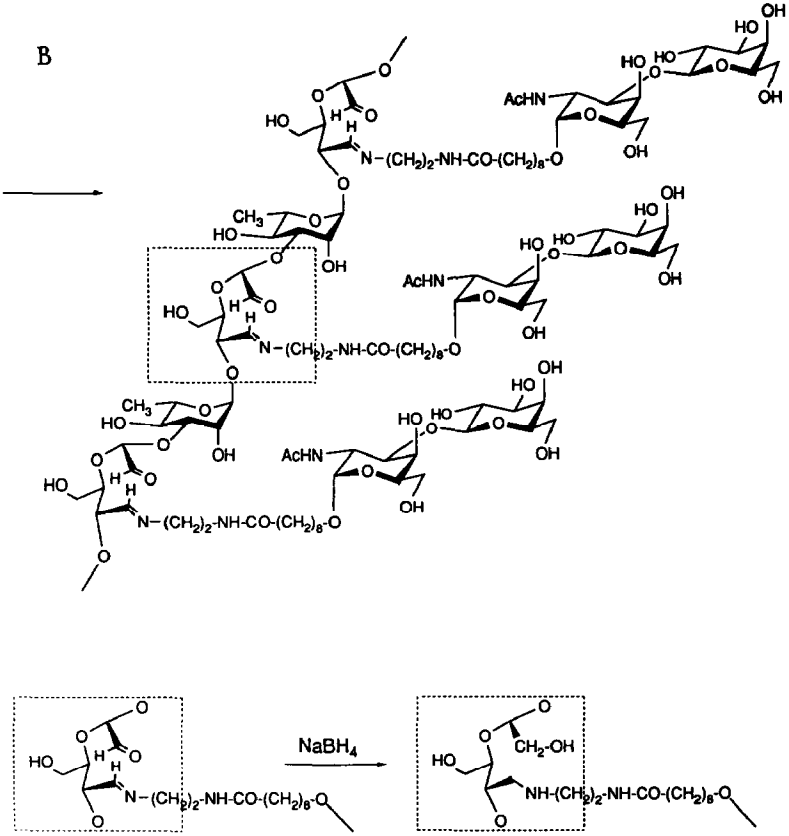

Fig. 2 (continued).

phenol–sulfuric acid assay[17] a sugar content of ~7% was found. After periodate oxidation, this value was decreased to approximately half. Up to 12 moles of hapten per mole of S-layer subunit were immobilized. This incorporation rate represents a yield of ~23% and compares with those obtained via periodate oxidation of the S-layers of the other strains.

(B) Epichlorohydrin activation. This method was used for the immobilization of spacer-linked carbohydrate haptens onto the glycan chains. The optimal pH value for epoxyalkylation by this method is ~pH 13 (ref. 29). Investigations of the supernatants prior to the addition of the hapten solutions demonstrated that, at this pH value, 60% of the S-layer glycan was lost within 30 min at room temperature, and almost 90% was lost within 2 h at 40°. For this reason, milder reaction conditions were chosen. The best results with *C. thermohydrosulfuricum* were obtained at room temperature for epoxyalkylation either at pH 10.0 for 1 h or at pH 9.1 overnight. Under these conditions, ~8 moles of hapten were immobilized. As toward the conditions of the periodate oxidation, the *B. stearothermophilus* glycoproteins were more resistant to the more strongly alkaline conditions. With this strain, ~8 moles of hapten per mole of S-layer subunit could be bound at pH 11.4 after epoxyalkylation for 1 h at room temperature. Epoxyalkylation at this pH for several hours, or at lower pH overnight, apparently led to crosslinking of glycan so that the ability for immobilization of hapten was lost. The "shedding" of S-layer carbohydrates under milder alkaline conditions (pH 9–10) amounted to 10–15% for both strains. The relatively low degree of hapten immobilization achieved by this method is due to the compromise in reaction conditions necessary to prevent a more extensive degradation of the S-layer glycans.

(C) Divinyl sulfone activation. The optimal pH values for the addition of OH-groups of the glycan chains to one of the double bonds of divinyl sulfone were slightly lower than those required for the epoxyalkylation. Excellent immobilization results could be obtained with S-layers from *C. thermohydrosulfuricum* and *B. stearothermophilus*. On *C. thermohydrosulfuricum* S-layers, 50–60 molecules of spacer-linked hapten per S-layer subunit were immobilized at pH 10.0 following activation for 30–45 min at room temperature. About 11 moles of hapten per mole of S-layer were bound to *B. stearothermophilus* S-layers using similar reaction conditions. Obviously, these yields represent the highest amounts of hapten which can be attached to the glycan chains without steric hindrance. About 10% of the glycan material was "shed" into the supernatant under these conditions prior to the addition of the hapten solutions.

DISCUSSION

The glycans of S-layer glycoproteins consist of regular polysaccharide chains[34], made up of oligosaccharide repeat units similar to the *O*-antigens of Gram-negative bacteria. Functional groups suitable for effective binding of spacer-linked

haptens can be introduced into these polysaccharide chains either by oxidation with periodate or by the action of bifunctional reagents such as epichlorohydrin or divinyl sulfone. Following either "activation" step, the desired antigens or haptens are attached to the modified glycan chains through Schiff base formation, nucleophilic opening of oxirane rings, or addition of polar groups to activated vinyl groups. Depending on the chosen activation reaction, the immobilization rates (Table I) are quite different but, in general, they are in accordance with published data[30] on the efficacy of the respective coupling procedure. Haptenation of S-layer glycoprotein glycans has been performed on peptidoglycan-containing cell-wall fragments after digestion of the peptidoglycan layer. Prior to the digestion with lysozyme, the cell-wall fragments were crosslinked by the action of glutaraldehyde to retain the original shape of the bacterium. Alternatively, purified and recrystallized S-layer protomers (S-layer self-assembly products) were used. These preparations were used both in native and crosslinked form. Glutaraldehyde-fixed cell-wall fragments, under electron microscopic examination, were seen to contain two S-layers, one oriented toward the outside, the other toward the inside of the sacculus (Fig. 1). In previous cases, the glycan chains have been shown to be oriented towards the aqueous medium[32]. Therefore, the haptens attached to the glycan chains would protrude away from the surface of the sacculi and would present themselves to the environment much in the way the *O*-antigens of Gram-negative bacteria are displayed.

Alternatively to the reactions on the carbohydrate chains, binding of haptens was also performed on 1-ethyl-3,3′-dimethyl-(aminopropyl)-carbodiimide (EDC) activated carboxyl groups of the protein portion of the S-layer glycoprotein (not shown). These results have clearly demonstrated the application potential of S-layer glycoproteins as a novel carrier/adjuvants system.

The immune response of mice to several of the haptenated S-layer preparations has been studied. Even with poorly immunogenic antigens, such as the T-disaccharide βGal(1 → 3)αGalNAc, the anti-hapten cellular and humoral immune responses observed were superior to those seen with conventional antigen carrier proteins such as bovine serum albumin[16].

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REFERENCES

1 U.B. Sleytr, *Int. Rev. Cytol.*, 53 (1978) 1–64.
2 U.B. Sleytr and P. Messner, *Annu. Rev. Microbiol.*, 37 (1983) 311–339.
3 J. Smit, in M. Inouye, (Ed.), *Bacterial Outer Membranes as Model Systems*, Wiley, New York, 1987, pp. 343–376.
4 S.F. Koval, *Can. J. Microbiol.*, 34 (1988) 407–414.
5 U.B. Sleytr and P. Messner, *J. Bacteriol.*, 170 (1988) 2891–2897.
6 P. Messner and U.B. Sleytr, in A.H. Rose and D.W. Tempest, (Eds.), *Advances in Microbial Physiology*, Vol. 33, Academic Press, London, 1992, pp. 213–275.
7 U.B. Sleytr, W. Mundt, P. Messner, R.H. Smith, and F.M. Unger, U.S. Pat. 5043158 (1991).
8 M. Sára and U.B. Sleytr, *Appl. Microbiol. Biotechnol.*, 30 (1989) 184–189.
9 A. Mizrahi (Ed.), *Bacterial Vaccines, Advances in Biotechnological Processes*, Vol. 13, Wiley, New York, 1990.
10 S. Hakomori and R. Kannagi, in D.M. Weir, L.A. Herzenberg, C. Blackwell, and L.A. Herzenberg (Eds.), *Handbook of Experimental Immunology*, 4th ed., Vol. 1, Blackwell, Oxford, pp. 9.1–9.39.
11 R. Bell and G. Torrigiani (Eds.), *Toward Better Carbohydrate Vaccines*, Wiley, Chichester, 1987.
12 R. Christian, P. Messner, C. Weiner, U.B. Sleytr, and G. Schulz, *Carbohydr. Res.*, 176 (1988) 160–163.
13 R. Christian, G. Schulz, F.M. Unger, P. Messner, Z. Küpcü, and U.B. Sleytr, *Carbohydr. Res.*, 150 (1986) 265–272.
14 P. Messner, U.B. Sleytr, R. Christian, G. Schulz, and F.M. Unger, *Carbohydr. Res.*, 168 (1987) 211–218.
15 E. Altman, J.-R. Brisson, P. Messner, and U.B. Sleytr, *Biochem. Cell Biol.*, 69 (1991) 72–78.
16 R.H. Smith, P. Messner, L.R. Lamontagne, U.B. Sleytr, and F.M. Unger, *Cell. Immunol.*, submitted.
17 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
18 G. Keleti and W.H. Lederer (Eds.), *Handbook of Micromethods for the Biological Sciences*, Van Nostrand-Reinhold, New York, 1974, p. 52.
19 G. Keleti and W.H. Lederer (Eds.), *Handbook of Micromethods for the Biological Sciences*, Van Nostrand-Reinhold, New York, 1974, pp. 71–72.
20 O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 133 (1951) 265–275.
21 M. Sára, I. Kalsner, and U.B. Sleytr, *Arch. Microbiol.*, 149 (1988) 527–533.
22 U.B. Sleytr, M. Sára, Z. Küpcü, and P. Messner, *Arch. Microbiol.*, 146 (1986) 19–24.
23 M. Sára, K. Moser-Thier, U. Kainz, and U.B. Sleytr, *Arch. Microbiol.*, 153 (1990) 209–214.
24 P. Messner, F. Hollaus, and U.B. Sleytr, *Int. J. Syst. Bacteriol.*, 34 (1984) 202–210.
25 M. Sára, and U.B. Sleytr, *J. Bacteriol.*, 169 (1987) 4092–4098.
26 J.H. Bobbitt, *Adv. Carbohydr. Chem.*, 11 (1956) 1–41.
27 J. Bartos and M. Pesez, *Pure Appl. Chem.*, 51 (1979) 1805–1814.
28 R.F. Borch, M.D. Bernstein, and H.D. Durst, *J. Am. Chem. Soc.*, 93 (1971) 2897–2904.
29 I. Matsumoto, Y. Mizuno, and N. Seno, *J. Biochem.*, 85 (1979) 1091–1098.
30 J.M. Egly (Ed.), *Ultrogel, Magnogel, and Trisacryl, Practical Guide for Use in Affinity Chromatography and Related Techniques*, Réactifs IBF—Société Chimique Pointet-Girard, Villeneuve-la-Garenne, 1983.
31 J. Porath, T. Låås, and J.C. Janson, *J. Chromatogr.*, 103 (1975) 49–62.
32 M. Sára, S. Küpcü, and U.B. Sleytr, *Arch. Microbiol.*, 151 (1989) 416–420.
33 H.J. Jennings and C. Lugowski, *J. Immunol.*, 127 (1981) 1011–1018.
34 P. Messner and U.B. Sleytr, *Glycobiology*, 1 (1991) 545–551.