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ISOLATION AND STRUCTURE DETERMINATION OF A DIACETAMIDODIDEOXYURONIC ACID-CONTAINING GLYCAN CHAIN FROM THE S-LAYER GLYCOPROTEIN OF *Bacillus stearothermophilus* NRS 2004/3a*

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ABSTRACT

A glycan isolated from the surface-layer glycoprotein of *Bacillus stearothermophilus* strain NRS 2004/3a was shown by ^{1}H- and ^{13}C-n.m.r. spectroscopy to have the tetrasaccharide repeating-unit →4)-β-Man*p*A2,3(NAc)$_2$-(1→3)-α-Glc*p*NAc-(1→4)-β-Man*p*A2,3(NAc)$_2$-(1→6)-α-Glc*p*(1→

INTRODUCTION

During the characterization of crystalline surface layers (S-layers[1]) of thermophilic *Bacillus stearothermophilus* strains[2–4], the S-layer protein of *B. stearothermophilus* NRS 2004/3a was observed to contain covalently linked carbohydrate[5]. Two glycopeptide fractions (GP-I and GP-II) have been obtained upon digestion of this S-layer glycoprotein with pronase. The glycan portion of GP-I has been identified as an unbranched rhamnan[6] composed of the repeating unit →2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→. We now report on the glycan of GP-II.

RESULTS AND DISCUSSION

GP-II was separated from GP-I by chromatography over DEAE Bio-Gel A (Fig. 1), and was further purified by chromatography over Bio-Gel P-10. Its elution volume from the latter column indicated a molecular weight of ~12,000.

During amino acid analysis of GP-II, the presence was noted of an unknown

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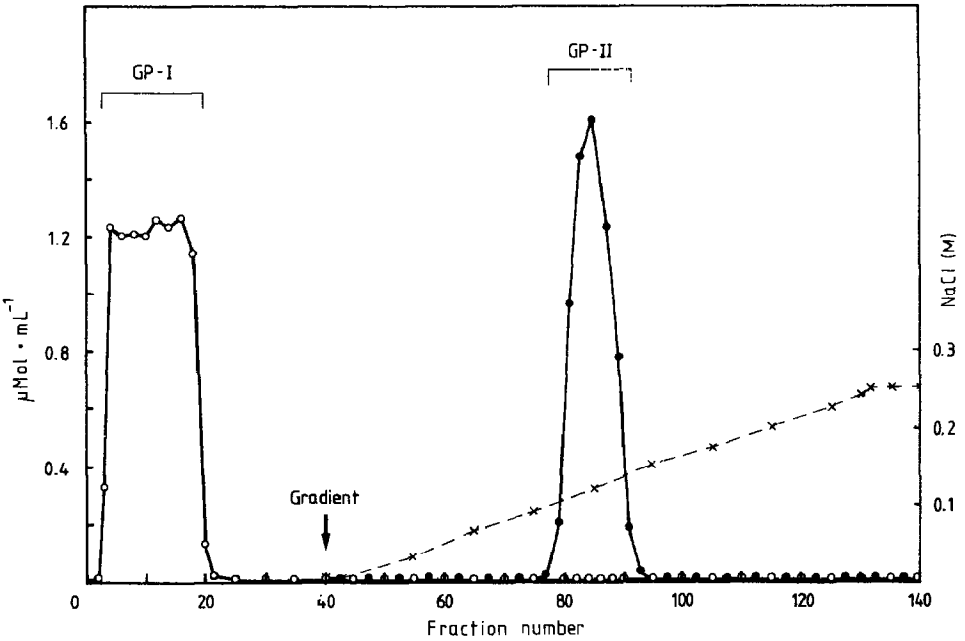

Fig. 1. Chromatography of the glycopeptides GP-I and GP-II on DEAE Bio-Gel A (see Experimental): —●—, hexose[19]; —○—, rhamnose[20].

material displaying the elution behavior of histidine. Also detected was a total of 188 nmol/mg of amino acids (Asp, Glu, Ala, Leu, Ser, Thr, and Gly in decreasing amounts). Carbohydrate analysis revealed glucose and 2-amino-2-deoxyglucose.

The ^{1}H-n.m.r. spectrum of GP-II contains signals for 4 anomeric protons at δ 4.92 (s, 1 H), 4.95 (s, 1 H), and 5.1 (2 H, J <4 Hz), and 5 signals for acetyl groups (δ 1.89, 2.00, 2.01, 2.05, and 2.08).

The proton-decoupled, ^{13}C-n.m.r. spectrum of GP-II contained 29 signals corresponding to 34 carbon atoms (Table I). Considering the ^{1}H-n.m.r. spectrum, ten of these signals correspond, presumably, to *N*-acetyl groups, so that GP-II is a regular polysaccharide with a tetrasaccharide repeating-unit. There were 3 signals (5 carbons) in the range 22.5–22.9 p.p.m. (acetyl CH_3), 5 between 52.3 and 54.3 p.p.m., where the resonances of nitrogen-linked, skeletal carbon atoms are expected, 1 at 60.8 p.p.m. (CH_2OH of pyranoses), 7 (of 9 carbons) in the range 68.8–73.3 p.p.m., where ring carbon atoms of pyranoses are expected, 3 between 78.1 and 81.8 p.p.m. (secondary ether carbons), 3 (4 carbons) in the anomeric region (97.6–100.5 p.p.m.), and 7 between 174.5 and 176.0 p.p.m. (carbonyl carbon atoms of acetyl and carboxyl groups).

In a spin-echo experiment, the number of carbon atoms carrying two hydrogen atoms was determined as two. In accordance with the results of carbohydrate analysis, these would correspond to CH_2OR of Glc*p* and Glc*p*NAc with R = H (60.62 p.p.m.) and glycosyl (68.75 p.p.m.).

TABLE I

^{13}C-N.M.R. DATA FOR GP-II (FREE ACID AND AMMONIUM SALT) AND RELATED COMPOUNDS[a]

Glycosyl residue	*Substance*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
A[b]	GP-II-NH_4^+	100.52[e]	52.39	54.06	72.22	78.35	174.12[i]
	GP-II-H^+	100.71	52.58	54.16	72.17	79.55	175.63
	Pseudomonas aeruginosa O:3a lipopolysaccharide[7]	100.7	52.2	54.1	71.7	77.2	—
	GP-II-Tf	100.67	52.11	54.71	67.26	78.94	—
	Propionibacterium acne strain C-7, degradation product[8,c]	100.4	51.9	54.6	67.5	79.4	—
B	GP-II-NH_4^+	97.64[f]	52.78	81.75	68.75	72.61	60.62[j]
	GP-II-H^+	97.64	52.95	81.92	69.20	75.50	60.84
	GP-II-Tf	97.70	52.98	82.00	69.30	72.54	60.85
	3-OMe-α-Glc*p*NAc-OMe[9]	98.7	52.6	81.1	69.6	72.2	61.2
C[b]	GP-II-NH_4^+	100.52[g]	52.00	54.06	71.96	78.11	174.95[i]
	GP-II-H^+	100.53	52.29	54.30	71.68	79.36	175.94
	Pseudomonas aeruginosa O:3a lipopolysaccharide[7]	100.7	52.2	54.1	71.7	77.2	—
D	GP-II-NH_4^+	99.56[h]	71.40	73.28	69.44	71.74	68.75[j]
	GP-II-H^+	99.26	72.17	73.47	69.47	71.68	68.68
	Micrococcus luteus cell-wall teichuronic acid[10,d]	99.1	72.4	73.5	69.7	71.6	68.7

[a]Shifts are in p.p.m. downfield from the signal for Me_4Si (67.40 p.p.m. up-field from the signal of 1,4-dioxane in D_2O at 25°). A and C, →4)-β-Man*p*A(NAc)$_2$ unit; B, →3)-α-Glc*p*NAc unit; D, →6)-α-Glc*p* unit. [b]The signals of the carbonyl carbons appear in the range 174.5–176 p.p.m., and those of the methyl carbons at 22.6–22.9 p.p.m. [c]Shifted +0.9 p.p.m. [d]Shifted −1.9 p.p.m. [e]$^1J_{C,H}$ 165 Hz. [f]$^1J_{C,H}$ 171 Hz. [g]$^1J_{C,H}$ 165 Hz. [h]$^1J_{C,H}$ 174 Hz. [i]Singlet in the proton-coupled spectrum. [j]Determined by means of a spin-echo experiment.

A change in the pH value from 2.8 to 7.3 affected the shifts of the signals of 2 carbonyl carbon atoms, which were identified therefore as carboxyl carbon atoms (174.12 and 174.95 p.p.m.). Also shifted were the signals at 78.1 and 78.4 p.p.m. and, hence, the corresponding carbon atoms were linked to the carboxyl groups. Together with the other data, this evidence suggested that the two remaining, unknown glycosyl units of the repeat unit would be diacetamidodideoxyuronic acids.

Further assignments were made with the aid of the proton-coupled, ^{13}C-n.m.r. spectrum. The 2 signals (174.12 and 174.95 p.p.m.) for carbonyl carbon atoms appeared as singlets (no two-bond coupling) and were confirmed as carboxyl carbon atoms of uronic acid groups. The remaining 5 signals for carbonyl carbon atoms showed $^{2}J_{C,H}$ values of 6 Hz, identifying them as acetyl carbonyl carbon atoms. The signals for anomeric carbon atoms had $^{1}J_{C,H}$ values of 165, 165, 171, and 174 Hz. Considering that one Glc*p* and one Glc*p*NAc residue were present, and that all four $^{3}J_{H\text{-}1,H\text{-}2}$ values were small, the two signals having $^{1}J_{C,H}$ 171 and 174 Hz were attributed to α-Glc*p*NAc and α-Glc*p* residues, respectively. The other two signals for anomeric carbon atoms were assigned to diacetamidodideoxy-β-pyranosyluronic acid residues having an axial 2-substituent. By comparison to spectra of well-documented reference compounds[7-12], the structure of the unknown constituent is suggested to be 2,3-diacetamido-2,3-dideoxy-β-mannopyranosyluronic acid (Table I).

Smith degradation of GP-II gave a product containing Glc*p*NAc, but no Glc*p*, which was eluted from a column of Bio-Gel P-4 in the molecular-weight-

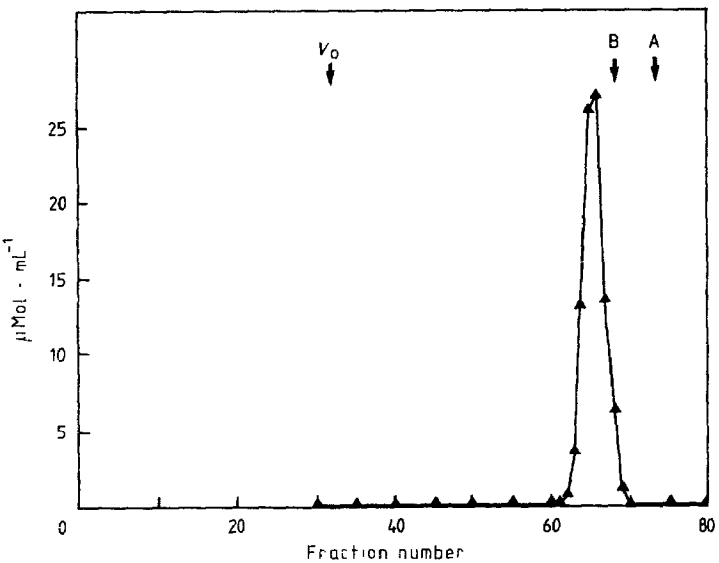

Fig. 2. Elution of the product of the Smith degradation of GP-II from a column (1 × 113 cm) of Bio-Gel P-4 with 50mM ammonium hydrogencarbonate; ——▲——, hexosamine[21]. V_0, A, and B refer to the void volume (polysaccharide), a disaccharide (A), and a trisaccharide (B).

range of a trisaccharide (Fig. 2). Thus, the Glc*p*NAc unit was substituted at C-3 or C-4. Given the resistance to oxidation of the two Man*p*A(NAc)$_2$ residues, the Glc*p* residue was concluded to be located within the polysaccharide chain of GP-II.

The results of the Smith-degradation experiment, as well as reported data on model compounds[9], supported the assignment of the signal at 81.75 p.p.m. to the glycosylated C-3 of the Glc*p*NAc residue, and that at 68.75 p.p.m. to the C-6 of a Glc*p* glycosylated at C-6. The alternative (2- or 4-substituted Glc*p*) was ruled out because of the absence of additional signals to the low-field side of 73.3 p.p.m. [the two signals at 78 p.p.m. were pH-dependent and are assigned to C-5 of the Man*p*-A(NAc)$_2$ residues]. It also followed that the remaining CH_2 signal (60.62 p.p.m.) belongs to GlcNAc. The product of Smith degradation did not lend itself to ^{13}C-n.m.r. analysis, owing to its poor solubility.

The remaining signals belonging to the fragments →3)-α-Glc*p*NAc-(1→ and →6)-α-Glc*p*-(1→ were next assigned by using appropriate data from the literature[9–12]. The two, nearly identical signal clusters belonging to the Man*p*A(NAc)$_2$ units[7] were assigned in the same manner.

Because of the poor solubility of the product of Smith degradation, an attempt was made to obtain a defined oligosaccharide by degradation of GP-II with trifluoromethanesulfonic acid[13]. However, only a mixture of oligosaccharides (GP-II-Tf) was obtained, the ^{13}C-n.m.r. spectrum of which (Table I) contained one portion that was assigned to a nonreducing-terminal Man*p*A(NAc)$_2$ residue. The signal corresponding to C-4 of this unit appeared at 67.26 p.p.m. (*cf.* 67.5 p.p.m.[8]). A concomitant shift difference was observed for the signal of one of the two nitrogen-bearing carbon atoms.

Finally, the sequence of the four monosaccharide units in the repeating unit of GP-II (Fig. 3) was derived from the following argument. In the lipopolysaccharide[7] from *Pseudomonas aeruginosa* O:3a,b and O:3a,d, the signal for C-4 of Man*p*A(NAc)$_2$ was observed at 71.7 p.p.m. if substituted with α-D-Fuc*p*NAc and 75.5 p.p.m. if substituted with β-D-Fuc*p*NAc or with β-D-Man*p*A(NAc)$_2$. By comparison with these data, it was concluded that the values of 72.22 and 71.96 p.p.m. indicate that C-4 of each Man*p*A(NAc)$_2$ unit was glycosylated with an α-pyranose residue. Hence, the structure of GP-II shown in Fig. 3 appears the most likely.

The absolute configurations of the monosaccharide constituents were determined by using the following arguments. Comparison of the observed α- and β-effects of glycosidation on the ^{13}C-n.m.r. shifts of the monosaccharides involved with those of synthetic models[14] showed that the constituent monosaccharides belong to the same stereochemical series. For comparison of optical rotations, the methyl glycoside of α-D-Man*p*(NAc)$_2$[11] was used as a model for that of β-D-Man*p*A(NAc)$_2$. By application of the rules[15] and assuming that the effects of the change CH_2OH to COOH and CH_2OH to CH_3 were equal, the $[M]_D$ of −240° of the methyl glycoside of β-D-Man*p*A(NAc)$_2$ was calculated from the $[\alpha]_D^{20}$ +8° of the methyl glycoside of α-D-Man*p*(NAc)$_2$[11]. Together with the $[\alpha]_D^{20}$ values of +158° and +105° for the methyl glycosides of α-D-Glc*p*[16] and α-D-Glc*p*NAc[17], the $[\alpha]_D$

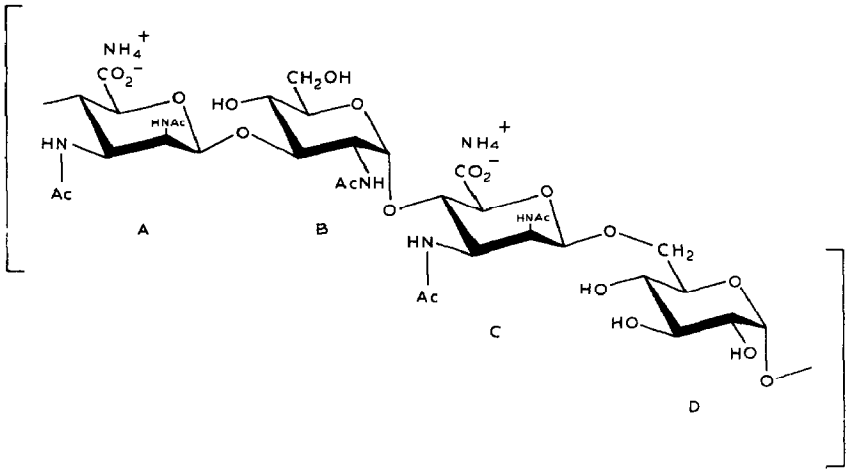

Fig. 3. Proposed structure of the polysaccharide GP-II elaborated by *Bacillus stearothermophilus* NRS 2004/3a.

value of the polysaccharide (all constituents of the D series) was calculated to be +8° by means of Klyne's rule[18], which agrees well with the measured value of +15° (*c* 1, water).

EXPERIMENTAL

General. — Total hexose[19], rhamnose[20], hexosamine[21], uronic acids[22], and protein[23] were determined by literature procedures, neutral hexoses and amino sugars were analysed by g.l.c. of the alditol acetates[24], and the $[\alpha]_D^{20}$ value of GP-II was determined with a Perkin–Elmer Model 141 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker WM 250 instrument at 25° for solutions in D_2O (external sodium trimethylsilylpropionate in D_2O). The spectral width was 2 kHz and 16k of memory were used. ^{13}C-N.m.r. spectra were recorded at 62.9 MHz on the same instrument for solutions in D_2O (external 1,4-dioxane in D_2O; δ 67.40 relative to the signal of Me_4Si), using 32k of memory and a spectral width of 12 kHz.

Growth of bacteria and isolation of glycopeptide. — *Bacillus stearothermophilus* strain NRS 2004/3a was grown and the S-layer glycoprotein was prepared as described previously[5]. The guanidine hydrochloride extract (up to 50 mg of cell walls in 10 mL of 5M guanidine hydrochloride in water) was chromatographed on a column (2.6 × 100 cm, equilibrated with the same solution) of Sepharose CL-6B (Pharmacia). Fractions containing protein were combined and dialysed against water, and the retentate was lyophilised. The product (1.8 g) was suspended (50 mg/mL) in 0.1M Tris-HCl buffer (pH 7.5), containing 10mM calcium chloride and 0.2% of sodium dodecylsulfate. After preincubation for 1 h at 39°, pronase E (Serva, 5 mg/mL) and a few drops of toluene (to prevent bacterial contamination) were added[25]. After incubation for 12 h at 39°, more pronase E was added and

incubation was continued for 12 h at 56°. Insoluble material was removed by centrifugation (25,000*g*, 20 min, 4°) and the clear supernatant solution was passed through a column (1 × 15 cm) of Dowex 50W-X8 (H^+) resin in water. The eluate was neutralised with sodium hydrogencarbonate and concentrated, and a solution of the residue in aqueous 1% acetic acid was chromatographed on a column (1 × 113 cm) of Bio-Gel P-4 (Bio-Rad). Fractions giving positive tests for hexose[19] and rhamnose[20] were combined and applied to a column (1.6 × 10 cm) of DEAE Bio-Gel A (Bio-Rad) equilibrated with Tris-HCl buffer (pH 7.2). The column was washed with 10 bed-volumes of the starting buffer, and the glycopeptides were eluted with a linear gradient from the starting buffer to 0.25M sodium chloride in the same buffer (total volume of the gradient, 700 mL). Fractions (8 mL) giving positive tests for rhamnose or hexose (Fig. 1) were combined, and desalted on a column (1.8 × 95 cm) of Bio-Gel P-10 equilibrated with aqueous 1% acetic acid. Appropriate samples, designated GP-I (38 mg) and GP-II (43 mg), were combined and lyophilised.

Smith degradation[8]. — This was performed on GP-II (12 mg) for 40 h, the solution was neutralised with solid ammonium hydrogencarbonate and concentrated, and the residue was subjected to gel filtration on a column (1 × 113 cm) of Bio-Gel P-4 in 50mM ammonium hydrogencarbonate. Fractions giving a positive reaction for hexosamine[21] were combined and lyophilised.

Deglycosylation with trifluoromethanesulfonic acid. — A dry sample (8.5 g) of GP-II was treated as described[13] but, instead of dialysis, the product was fractionated on a column (1 × 113 cm) of Bio-Gel P-4.

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