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# Structural analysis of the exopolysaccharides produced by *Lactobacillus* spp. G-77

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**Abstract**

The exopolysaccharide produced by a ropy strain of *Lactobacillus* spp. G-77 in a semi-defined medium, was found to be a mixture of two homopolymers composed of D-Glc. The two polysaccharides were separated and, on the basis of monosaccharide and methylation analyses, $^{1}H$, $^{13}C$, 1D and 2D NMR experiments, one of the polysaccharides was shown to be a 2-substituted-(1–3)-$\beta$-D-glucan, identical to that described for the EPS from *Pediococcus damnosus* 2.6 (M.T. Dueñas-Chasco, M.A. Rodríguez-Carvajal, P. Tejero-Mateo, G. Franco-Rodríguez, J.L. Espartero, A. Irastorza-Iribar, and A.M. Gil-Serrano, *Carbohydr. Res.*, 303 (1997) 453–458), and the other polysaccharide was shown to consist of repeating units with the following structure

$$\begin{array}{l}\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\quad \alpha\text{-D-Glc}p\end{array}$$



*Keywords:* *Lactobacillus* spp. G-77; Exopolysaccharide; Repeating unit

## 1. Introduction

Many bacteria are known to produce exopolysaccharides (EPSs) that are either excreted in the growth medium or remain attached to the bacterial cell wall [1]. Recently a growing interest has been observed in EPS produced by lactic acid bacteria (LAB), which may have suitable rheological properties for the dairy industry [2]. However, in ciders and wines, the polysaccharides produced by LAB can cause the alteration known

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as oiliness or ropiness. The polysaccharide increases the consistency of the beverage and causes economic losses in the Basque Country cider industry. Some strains of *Pediococcus cerevisiae* have been described as polysaccharide-producing bacteria in both ciders and wines [3,4] and their structures have been determined [5,6]. In ciders, ropy *Lactobacillus* spp. strains have been isolated by Dueñas et al. [7] but their structures have not been studied. The structures of EPS produced by several strains of *Lactobacillus* species including *L. kefiranofaciens* [8], *L. hilgardii* [9], *L. delbrueckii* subsp. *bulgaricus* [10], *L. helveticus* [11–13], and *L. paracasei* [14] in the dairy industry have been elucidated. In addition, an EPS-producing *Lactobacillus sake* strain has been isolated from fermented meat and their polysaccharides have been characterized [15,16]. This study was designed with the aim of determining the polysaccharide structure produced by a ropy strain of *Lactobacillus* spp. in a semidefined medium. This knowledge is important for the selection of specific enzymes for the elimination of cider viscosity.

## 2. Experimental

*Bacterial and culture conditions.*—*Lactobacillus* spp. G-77 was isolated from a ropy Basque Country cider [7]. This strain was identified on the basis of the morphological, physiological and biochemical characteristics and it resembles quite closely the species called *L. collinoides* and *L. brevis*.

The medium used both for the preparation of inocula and for the maintenance was the MRS broth [17] containing 6% ethanol (pH 4.8). For exopolysaccharide production, *Lactobacillus* spp. G-77 was grown in a semidefined medium (SMD). The semidefined medium that was used for culturing EPS production had the following composition: glucose 20 g/L, casaminoacids 5 g/L, bacto yeast nitrogen base (Difco) 6.7 g/L, $MnSO_4 \cdot H_2O$ 0.05 g/L, $K_2HPO_4$ 2 g/L, NaAcO 5 g/L, adenine 0.005 g/L, guanine 0.005 g/L, xanthine 0.005 g/L, uracil 0.005 g/L, and L-malic acid 4 g/L. The pH of the SMD medium was 4.8. The glucose and bacto yeast nitrogen base were sterilized by passing through a 0.2 mm sterile filter and added after autoclaving. For inocula, cells harvested from 48 h cultures in MRS medium with ethanol were washed and resuspended in phosphate-buffered saline with peptone. *Lactobacillus* spp. G-77 was cultured in semidefined medium and incubated for 7 days (nonshaken, nonaerated) at 28 °C. The flasks were inoculated to give an initial viable count of approximately $10^7$ CFU/mL ($OD_{600} = 0.2$).

*Isolation and purification of the exopolysaccharide.*—Cells were removed by centrifuging for 30 min at 20000 *g*. The clear supernatant was collected, and the EPS was precipitated by adding 3 volumes of cold ethanol, followed by storage overnight at 4 °C. The precipitate was recovered by centrifugation at 4500 *g* for 20 min at 4 °C and dissolved in distilled water. The polysaccharide was purified by precipitation with ethanol three times and the final precipitate was dissolved in and dialyzed against distilled water for 2 to 3 days (changed twice each day) and lyophilized.

*Fractional precipitation with ethanol* [18].—To a 0.4% aqueous solution (4 mL) of exopolysaccharide (PSI) in a centrifuge bottle, ethanol was added dropwise with stirring to incipient turbidity. After 5 min the dispersion was centrifuged and the precipitate material washed and dried. To the supernatant from the centrifugation, ethanol was added as before until incipient turbidity appeared and this insoluble fraction was isolated as before. The precipitation procedure was repeated until a concentration of 70% ethanol was reached. Five fractions (40, 55, 60, 65, and 70% v/v ethanol) were isolated.

*Sugar analysis.*—Monosaccharides were determined as their trimethylsilylated methyl glycosides [19]. The polysaccharide was treated with 0.625 M methanolic HCl at 80 °C for 16 h and then silylated with 1:1 pyridine-BSTFA for 16 h at 80 °C. Isobutanol was added to the mixture and then dried under a stream of nitrogen. The TMS derivatives were analyzed by GLC-MS performed with a Kratos MS80RFA instrument fitted with a CP-Sil5-CB WCOT column (25 m×0.32 mm i.d.). The temperature programme was isothermal at 140 °C for 2 min followed by an 8 °C/min gradient up to 250 °C.

*Determination of* D *and* L*-configuration.*—The polysaccharide was methanolized with 0.625 M methanolic HCl, treated with 0.625 M (+)-2-butanolic-HCl under the same conditions and then trimethylsilylated. The trimethylsilylated 2-butyl glycosides were analyzed by GLC–MS [20] as above. The temperature programme was isothermal at 130 °C followed by a 2 °C/min gradient up to 250 °C.

*Methylation analysis*.—The polysaccharide was methylated twice by the method of Ciucanu and Kerek [21]. The product was further purified by reversed-phase chromatography on a Sep-Pak $C_{18}$ cartridge [22] and hydrolysed with 2 M trifluoroacetic acid. The products were then reduced and acetylated by using the method described by Blakeney [23]. The partially methylated alditol acetates were analyzed by GLC-MS and the temperature programme was isothermal at 100 °C for 1 min followed by a 5 °C/min gradient up to 250 °C.

*NMR Spectroscopy*.—The samples were deuterium exchanged several times by freeze drying from $D_2O$ and then examined as solutions (3 mg/mL) in 99.98% $D_2O$. Spectra were recorded at 303 K or 333 K on a Bruker AMX500 spectrometer operating at 500.13 MHz ($^1H$) and 125.75 MHz ($^{13}C$). Chemical shifts are given in ppm, using the HDO signal (4.75 ppm, 303 K or 4.33 ppm, 333 K) ($^1H$) and external dimethyl sulfoxide (39.5 ppm) ($^{13}C$) as references. The 2D homonuclear proton double-quantum filtered correlation experiment (DQF-COSY) [24] was performed in the phase-sensitive mode using the Bruker standard pulse sequence. A data matrix of 512×1 K points was used to digitize a spectral width of 4500 Hz; 64 scans were used per increment with a delay between scans of 1 s. Prior to Fourier transformation, zero-filling was used in $F_1$ to expand the data to 1 K×1 K. The 2D ROESY experiment [25] was carried out in the phase-sensitive mode, with a mixing time of 300 ms. The r.f. carrier was set at 6.0 ppm in order to minimize spurious Hartmann–Hahn effects. A data matrix of 256×1 K points was used to digitize a spectral width of 6000 Hz; 64 scans were acquired per increment. Squared sine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 1 K×1 K. The 2D heteronuclear one-bond proton–carbon correlation experiment [26] was registered in the $^1H$-detection mode via single-quantum coherence (HSQC). A data matrix of 256×1 K points was used to digitize a spectral width of 4500 and 26,000 Hz in $F_2$ and $F_1$; 128 scans were used per increment with a delay between scans of 1 s and a delay corresponding to a $J$ value of 150 Hz. $^{13}C$ decoupling was achieved by the GARP scheme. Squared cosine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 1 K×1 K. Selective excitation 1D experiments were carried out by application of the DANTE-Z pulse train ($n = 300$, $\tau = 100\ \mu s$, $\theta = 0.3°$) [27]. This train was concatenated to a TOCSY sequence (isotropic mixing times of 35, 100, and 170 ms, $\pi/2$ pulse width of 49 $\mu$s) [28] to yield the 1D-TOCSY subspectra. The number of accumulated scans was 512 for each subspectrum. Besides, 1D-NOESY subspectra were obtained by allowing the free evolution of magnetization during a mixing time of 250 ms after the selection. In these experiments the HDO signal was presaturated by a low-power pulse (transmitter attenuation, 65 dB) of 1 s.

## 3. Results and discussion

The EPS (PSI) of *Lactobacillus* spp. G-77 purified as described in Experimental was shown to be composed of D-Glc. Methylation analysis of the PSI revealed the presence of terminal D-Glc, 3-linked D-Glc, 6-linked D-Glc, 2,3-linked D-Glc, and 2,6-linked D-Glc (Table 1) in a ca. 1.8:0.7:1.0:0.8:1.1 molar ratio. In the 1D $^1H$ NMR spectrum (Fig. 1(a)) six anomeric protons were observed, labelled A–F from low to high field. From the $\delta$ and $^3J$ values, the protons A–C were identified as belonging to *a*-glucopyranose residues in 1:1:1 ratios and the protons D–F as belonging to $\beta$-glucopyranose residues in 0.7:1.4 ratio. These results suggested that PSI was a mixture of two polysaccharides: one all-$\alpha$ polysaccharide with terminal, 6-linked and 2,6-linked D-Glc (1:1:1.1) and one all-$\beta$ polysaccharide with terminal, 3-linked and 2,3-linked D-Glc (0.8:0.7:0.8). In order to confirm this assumption, a fractionated precipitation with ethanol was performed. Five fractions (40, 55, 60, 65, and 70% v/v) were isolated. The $^1H$ NMR anomeric region and the polysaccharide

Table 1
Methylation analysis data for the EPSs from *Lactobacillus* spp. G-77

| Methylated sugars (as alditol acetates) | Molar ratio [a] | | |
|---|---|---|---|
| | PSI | PS$\alpha$ | PS$\beta$ |
| 2,3,4,6-$Me_4$-Glc[b] | 1.8 | 1.1 | 1.1 |
| 2,4,6-$Me_3$-Glc | 0.7 | | 1.0 |
| 2,3,4-$Me_3$-Glc | 1.0 | 1.0 | |
| 4,6-$Me_2$-Glc | 0.8 | | 0.9 |
| 3,4-$Me_2$-Glc | 1.1 | 1.2 | |

[a] Key: PSI, EPS mixture; PS$\alpha$, EPS precipitated from 40% ethanol; PS$\beta$, EPS precipitated from 70% ethanol.
[b] 2,3,4,6-$Me_4$-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol.

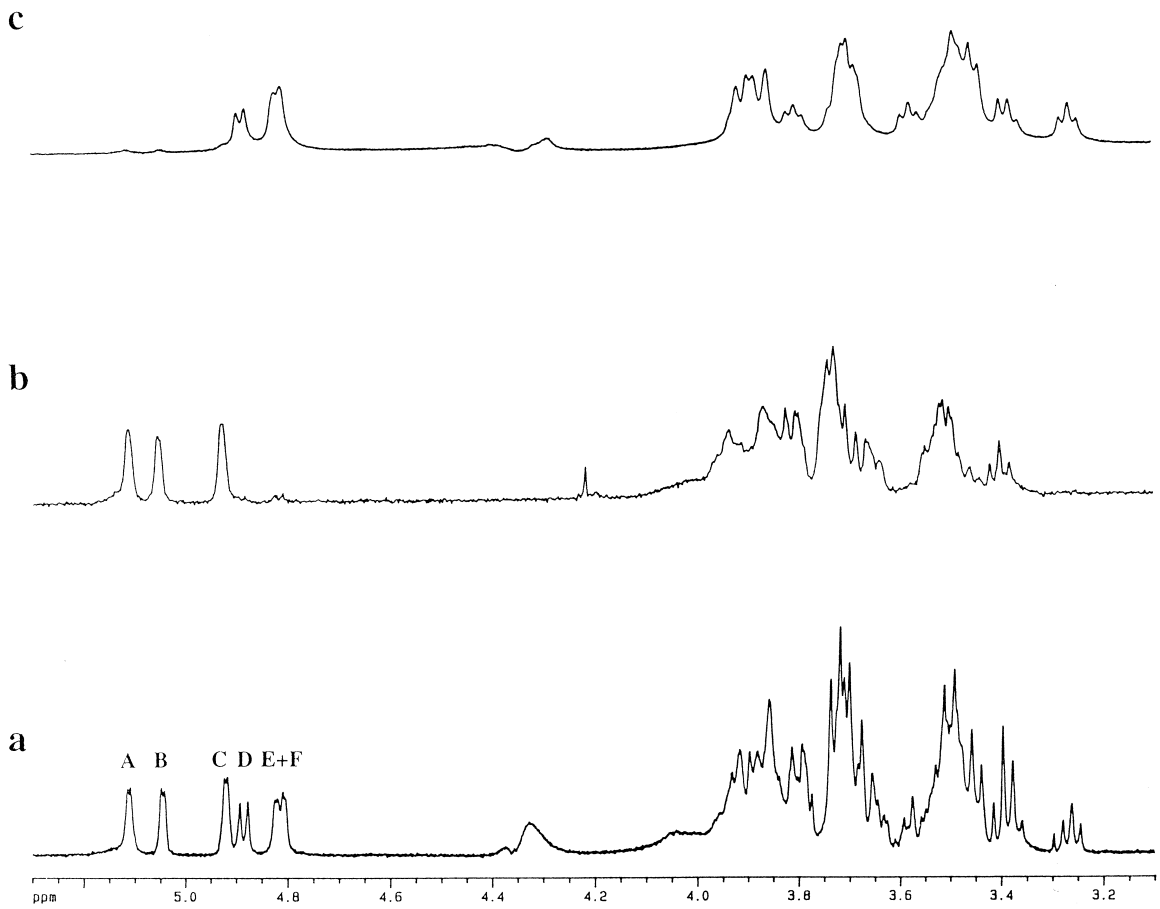



Fig. 1. 500 MHz $^1$H NMR spectra at 333 K of the EPSs from *Lactobacillus* spp. G-77. (a) PSI, (b) PS$\alpha$, and (c) Ps$\beta$. The integrals for the anomeric protons are: (a) ca. 1:1:1:0.7:1.4; (b) ca. 1:1:1; and (c) 1:2.

$\alpha$: polysaccharide $\beta$ ratio for each, are shown in Fig. 2. These results showed clearly that PSI was a mixture of two polysaccharides: PS$\alpha$ (40% ethanol fraction) and PS$\beta$ (70% ethanol fraction). The methylation analyses of both polysaccharides are shown in Table 1, and the 1D $^1$H NMR in Fig. 1(b) and (c), respectively. The exopolysaccharide PS$\beta$ was found to be identical to the EPS isolated from *Pediococcus damnosus* 2.6 [5]. The exopolysaccharide PS$\alpha$ was subjected to a 1D and 2D $^1$H and $^{13}$C NMR analysis.

*NMR spectroscopic analysis of PS*$\alpha$.—Chemical shifts for the $^1$H and $^{13}$C NMR signals of the fraction PS$\alpha$ were assigned from COSY, TOCSY, and HSQC experiments (Tables 2 and 3). In the 1D $^1$H NMR spectrum of PS$\alpha$ (Fig. 1(b)), three different signals at 5.11, 5.04, and 4.92 ppm were observed for anomeric protons and labelled A–C from low to high field as mentioned above. In order to completely assign the spectrum, selective excitation 1D experiments were carried out (Fig. 3). Thus, Fig. 3(b)–(d) shows the TOCSY subspectra from selective inversion of the anomeric proton signal in unit A at 5.11 ppm via a DANTE-Z pulse train for 35 ms of spin-lock time (Fig. 3(b)). The magnetization is transferred from H-1 mainly to H-2 proton at 3.64 ppm, and to some extent to H-3 proton at 3.79 ppm. Increasing the spin-locking time (Fig. 3(c) and (d)) allows the observation of all protons belonging to unit A, which were easily assigned. The same procedure was followed to completely assign the resonance signals from units B and C (spectra not shown). These assignments were confirmed by a homonuclear DQF-COSY experiment (not shown). Chemical shifts for the $^{13}$C resonances were assigned from an HSQC experiment (Table 3).

Correlation of the three different spin systems identified in the NMR experiments with the three different $\alpha$-D-glucopyranose residues identified by methylation analysis was made as follows: signals for C-2 at 77.9 ppm and the attached H-2 at 3.64 ppm appeared shifted downfield for unit A, with respect to the same nucleus in units B and C.

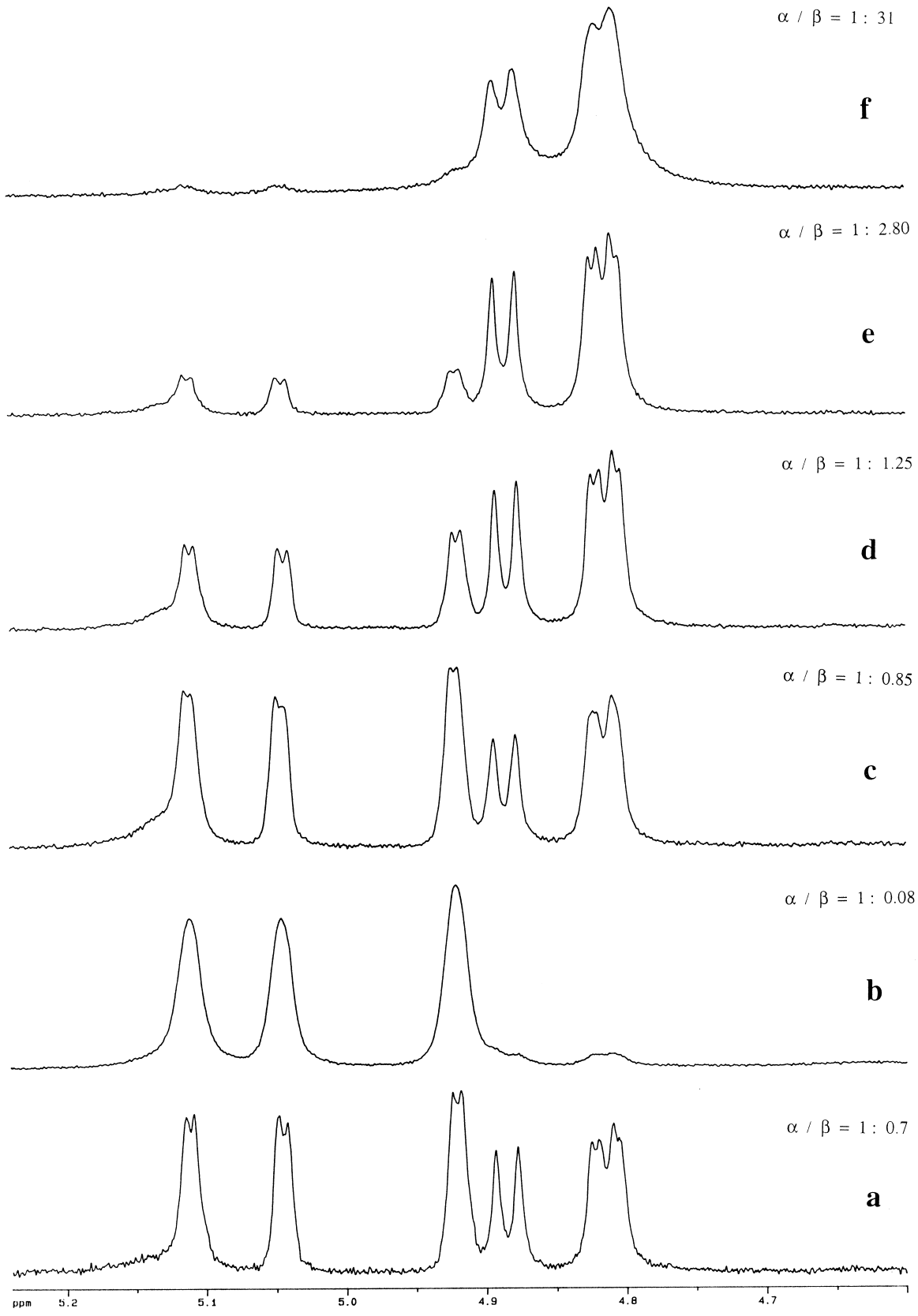



Fig. 2. Fractional precipitation with ethanol. $^1$H NMR anomeric region of the different precipitates and polysaccharide $\alpha$/polysaccharide $\beta$ ratio of: (a) whole EPS (PSI), (b–f) 40, 55, 60, 65, and 70% ethanol (recording parameter as in Fig. 1).

Table 2
$^1$H NMR chemical shifts ($\delta$, ppm) and coupling constants ($J$, Hz) for PS$\alpha$ from *Lactobacillus* spp. G-77

| Unit | H-1 | H-2 | H-3 | H-4 | H-5 | H-6 | H-6′ |
|---|---|---|---|---|---|---|---|
| → 2,6)-α-D-Glc*p*-(1→(**A**) | 5.11 | 3.64 | 3.79 | 3.50 | 3.85 | 3.72 | 3.90 |
| α-D-Glc*p*-(1→(**B**) | 5.04 | 3.49 | 3.71 | 3.40 | 3.85 | 3.71 | 3.80 |
| →6)-α-D-Glc*p*-(1→(**C**) | 4.92 | 3.53 | 3.67 | 3.48 | 3.82 | 3.71 | 3,90 |
| | $J_{1,2}$ | $J_{2,3}$ | $J_{3,4}$ | $J_{4,5}$ | $J_{5,6}$ | $J_{5,6'}$ | $J_{6.6'}$ |
| →2,6)-α-D-Glc*p*-(1→(**A**) | 2.8 | 8.6 | 9.9 | 10.0 | — | — | — |
| α-D-Glc*p*-(1→(**B**) | 2.8 | 9.0 | 9.2 | 9.9 | — | — | — |
| →6)-α-D-Glc*p*-(1→(**C**) | 3.0 | ca. 8.5 | ca. 8.5 | ca. 9.5 | — | — | — |

Table 3
$^{13}$C NMR chemical shifts ($\delta$, ppm) for PS$\alpha$ from *Lactobacillus* spp. G-77

| Unit | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|
| →2,6)-α-D-Glc*p*-(1→(**A**) | 97.8 | 77.9 | 73.5 | 71.5 | 72.0 | 67.5 |
| α-D-Glc*p*-(1→(**B**) | 98.5 | 73.2 | 74.8 | 71.5 | 73.8 | 62.5 |
| →6)-α-D-Glc*p*-(1→(**C**) | 99.5 | 73.2 | 75.0 | 71.5 | 72.3 | 67.5 |

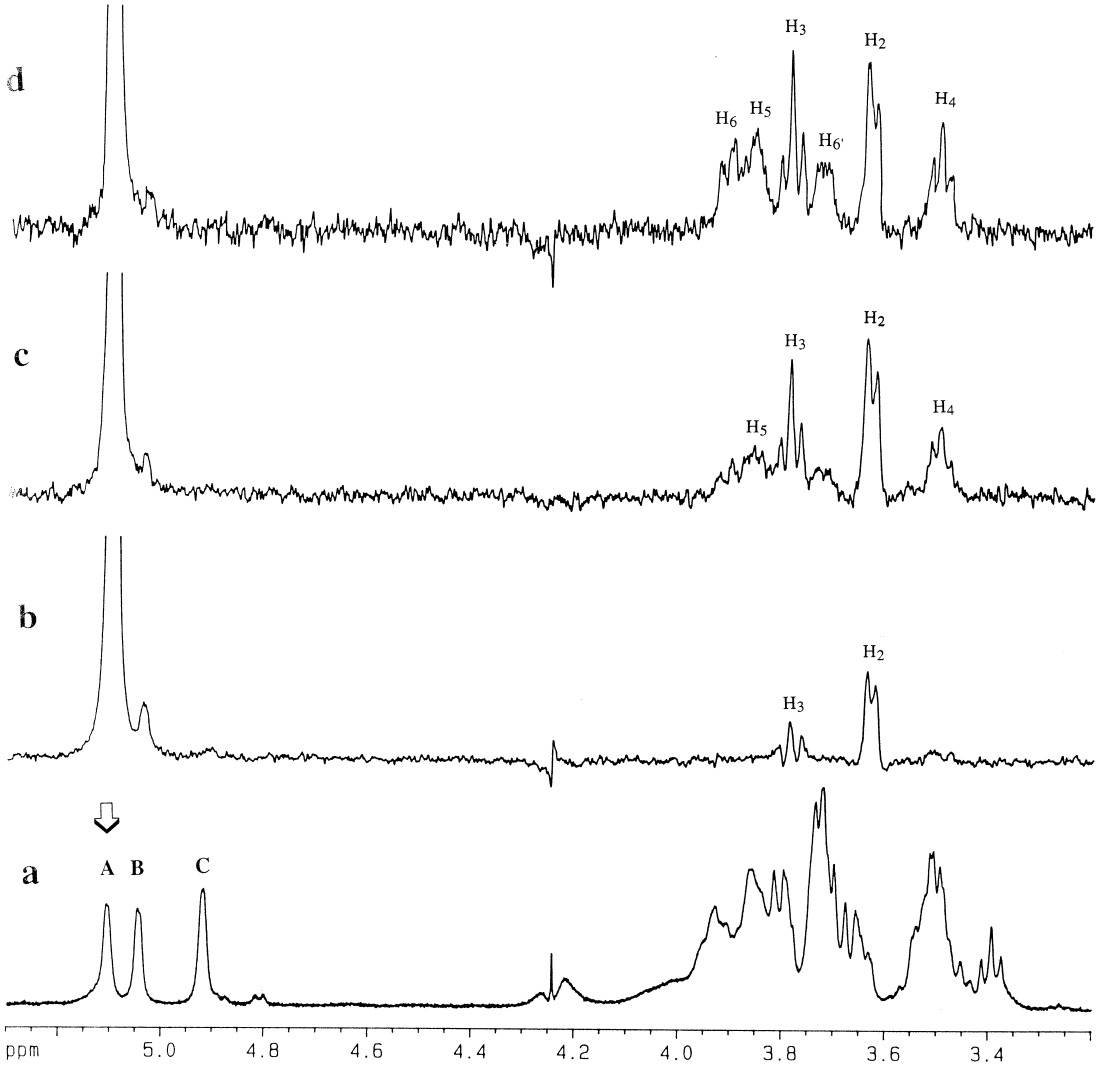



Fig. 3. (a) 500 MHz $^1$H NMR spectrum at 333 K of PS$\alpha$ and 1D TOCSY subspectra with selective inversion of the A signal at 5.11 ppm and isotropic mixing time of: (b) 35 ms, (c) 100 ms, and (d) 170 ms.

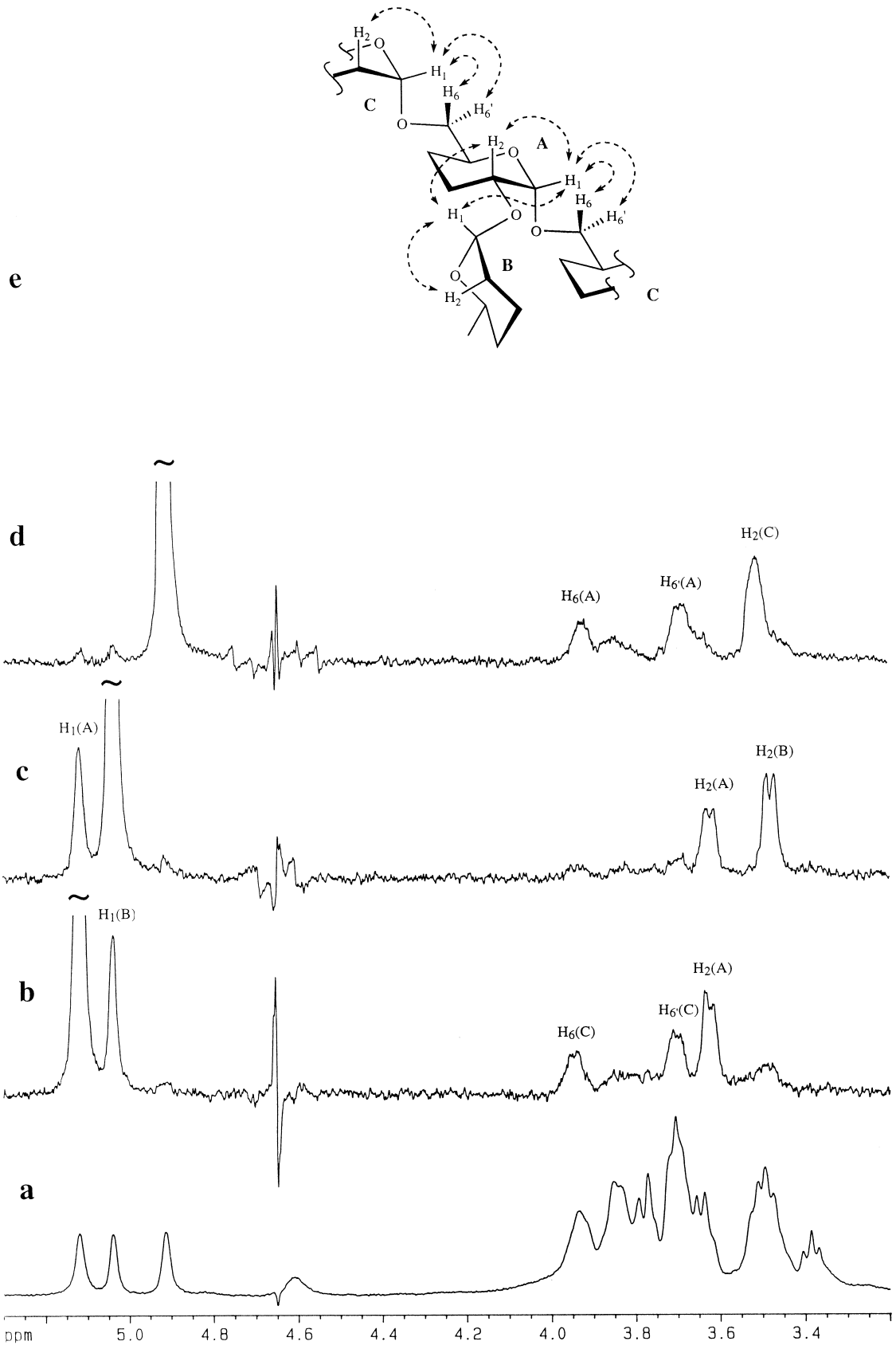
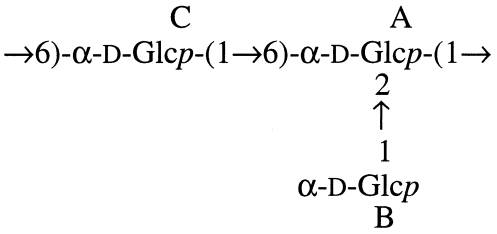



Fig. 4. (a) 500 MHz $^1$H NMR spectrum at 303 K of PS$\alpha$ and 1D NOESY subspectra obtained by selective inversion and subsequent mixing time of 250 ms of signals (b) H-1 in unit A , (c) H-1 in unit B, and (d) H-1 in unit C. (e) Schematic representation of NOE connectivities observed in PS$\alpha$.

Therefore, unit A should be substituted in position 2, and it could be identified as the 2,6-linked α-D-glucopyranose unit, the branched point of the main chain, and the only residue with such a type of substitution. On the other hand, the C-6 signal at 62.5 ppm appeared at a higher field in unit B than those for unit A and C, meaning that position 6 in unit B was unsubstituted. This fact allowed the identification of the unit B as the terminal α-D-glucopyranose unit. Finally, unit C was correlated with nonsubstituted 6-linked α-D-glucopyranose in the main chain.

These correlations were confirmed by 1D-NOESY subspectra obtained by selective excitation of the respective anomeric proton signals A, B, and C. In Fig. 4(b), transfer of magnetization via NOE was observed to protons H-1 (B), H-2 (A) and H-6 and H-6′ (C), after selective excitation of the signal A (H-1 in unit A). In the same way, NOE responses for protons H-1 (A), H-2 (A) and H-2 (B) were obtained after selective excitation of signal B (H-1 in unit B) (Fig. 4(c)). Finally, by inversion of signal C (H-1 in unit C), NOE responses for protons H-2 (C) and H-6 (A) and H-6′ (A) were observed (Fig. 4(d)). All these connectivities are summarized in Fig. 4(e) and were confirmed by a 2D-ROESY experiment (not shown). The above NOE conectivities, excluded any other possible structure.

On the basis of the above chemical and spectroscopic data, *Lactobacillus* spp. G-77 secrete two exopolysaccharides. One of the polysaccharides was found to be a (1-3)-β-D-glucan, identical to that described for the EPS from *Pediococcus damnosus* 2.6 [5], and the other was shown to consist of repeating units with the following structure

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## References

[1] I.W. Sutherland, *Adv. Microbiol. Physiol.*, 8 (1972) 143–213.
[2] J. Cerning, *FEMS Microbiol. Rev.*, 87 (1990) 113–130.
[3] K. Fernandez, M. Dueñas, A. Irastorza, A. Bilbao, and G. del Campo, *J. Food Prot.*, 59 (1996) 35–40.
[4] A. Lonvaud-Funel and A. Joyeux, *Sci. Aliments.*, 8 (1988) 33–49.
[5] M.T. Dueñas-Chasco, M.A. Rodríguez-Carvajal, P. Tejero-Mateo, G. Franco-Rodríguez, J.L. Espartero, A. Irastorza-Iribar, and A.M. Gil-Serrano, *Carbohydr. Res.*, 303 (1997) 453–458.
[6] R.M. Llauberes, B. Richard, A. Lonvaud, and D. Dubordieu, *Carbohydr. Res.*, 203 (1990) 103–107.
[7] M. Dueñas, A. Irastorza, K. Fernández, and A. Bilbao, *J. Food Prot.*, 59 (1995) 76–80.
[8] T. Mukai T. Toba, T. Itoh, and S. Adachi, *Carbohydr. Res.*, 204 (1990) 227–232.
[9] M. Pidoux, G.A. De Reutier, B.E. Brooker, I.J. Colquhoun, and V.J. Morris, *Carbohydr. Polym.*, 13 (1990) 351–362.
[10] M. Gruter, B.R. Leeflang, J. Kuiper, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 239 (1993) 209–226.
[11] Y. Yamamoto, T. Numone, R. Yamauchi, K. Kato, and Y. Sone, *Carbohydr. Res.*, 275, (1995) 319–332.
[12] G.W. Robjin J.R. Thomas, H. Haas, D.J.C. van den Berg, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 276 (1995) 137–154.
[13] M. Staaf, G. Widmalm, Z. Yang, and E. Huttunen, *Carbohydr. Res.*, 291 (1996) 155–164.
[14] G.W. Robjin, H.L.J. Wienk, D.J.C. van den Berg, A.M. Ledeboer, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 285 (1996) 129–139.
[15] D.J. Van den Berg, G.W. Robijn, A.C. Janssen, M.L.F. Giuseppin, R.Vreeker, J.P. Kamerling, J.F.G. Vliegenthart, AAT M. Ledeboer, and T. Verrips, *Appl. Environ. Microbiol.*, 61 (1995) 2840–2844.
[16] G.W. Robjin D.J.C. van den Berg, H. Haas, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 276 (1995) 117–136.
[17] J.C. De Man, M. Rogosa, and M.E. Sharpe, *J. Appl. Bacteriol.*, 23 (1960) 130–135.
[18] R.L. Whistler and J.L. Sannella, *Methods Carbohydr. Chem.*, 5 (1965) 34–36.
[19] M.F. Chaplin, *Anal. Biochem.*, 123 (1982) 336–341.
[20] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[21] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.

[22] T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
[23] A.B. Blakeney, P.J. Harris, R.J. Henry, and B.A. Stone, *Carbohydr. Res.*, 113 (1983) 291–299.
[24] D.J. States, R.A. Haber Korn, and D.J. Ruben, *J. Magn. Reson.*, 48 (1982) 286–292.
[25] A.A. Bothner-By, R.L. Stephens, J.M. Lee, C.D. Warren, and R.W. Jeanloz, *J. Am. Chem. Soc.*, 106 (1984) 811–813.
[26] G. Bodenhausen and D.J. Ruben, *Chem. Phys. Lett.*, 69 (1980) 185–189.
[27] D. Boudot, D. Canet, J. Brondeau, and J.C. Bouble, *J. Magn. Reson.*, 83 (1989) 428–433.
[28] D. Boudot, C. Roumestand, F. To, and D. Canet, *J. Magn. Reson.*, 90 (1990) 221–227.