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Analysis of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose

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Abstract The exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in defined medium were investigated. At equal cell densities, the strain produced 95 mg l^{-1} exopolysaccharides with glucose and 30 mg l^{-1} with fructose as the carbohydrate source. High-performance size-exclusion chromatography of the exopolysaccharides produced on glucose showed the presence of two fractions with relative molecular masses (M_r) of 1.7×10^6 and 4×10^4 in almost equal amounts. The exopolysaccharides produced on fructose contained mainly a fraction of low M_r of 4×10^4. The high-M_r fraction of the purified exopolysaccharides produced on glucose appeared to have a sugar composition of galactose, glucose and rhamnose in the molar ratio of 5:1:1, whereas the low-M_r weight fraction contained galactose, glucose and rhamnose in the molar ratio of approximately 11:1:0.4. The purified exopolysaccharide fractions produced on fructose showed comparable ratios. The high-molecular-mass fractions contained terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose. The low-molecular-mass fractions contained mainly 1,3-linked galactose and 1,6-linked galactose and lower amounts of other sugar linkages. The production of the high-M_r fractions appeared to be dependent on the carbohydrate source, whereas the low-M_r fractions were produced more continuously.

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Introduction

Certain lactic acid bacteria are capable of forming exopolysaccharides (EPS) when grown in skim milk, whey ultrafiltrate, MRS broth or chemically defined media (Bouzar et al. 1996; Cerning 1990; Cerning et al. 1986, 1988, 1990, 1994; Kojic et al. 1992; Manca de Nadra et al. 1985; van den Berg et al. 1995). EPS-producing lactic acid bacteria are of major importance for the food industry, since they are assumed to play an important role in the rheological behaviour and stabilisation of fermented milk products like yoghurt and also the prevention of syneresis (Schellhaass 1983; Wacher-Rodarte et al. 1993). The utilisation of slime-producing strains in the manufacture of yoghurt is particularly of interest in France and The Netherlands, since the addition of stabilisers to unfruited yoghurt is prohibited in these countries (Cerning et al. 1986). *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are used in the manufacture of yoghurt and the utilisation of EPS-producing strains is presumed to enhance its viscosity and texture. Various EPS-producing *L. delbrueckii* subsp. *bulgaricus* strains have previously been studied in detail (Cerning et al. 1986; Garcia-Garibay and Marshall 1991; Manca de Nadra et al. 1985; Mozzi et al. 1995; Wacher-Rodarte et al. 1993).

It has also been shown that not only the amount of EPS produced by lactic acid bacteria is important for the improvement of the viscosity and texture of fermented milk products, but that the molecular mass, sugar

composition and the primary structure of the EPS also play an important role (Cerning 1990). A large number of EPS produced by various *L. delbrueckii* subsp. *bulgaricus* strains have been investigated and several different sugar compositions have been found, mostly containing glucose and galactose in varying ratios and sometimes containing one or more other neutral sugars (Cerning 1990). Bouzar et al. (1996) and Groux (1973) found that *L. delbrueckii* subsp. *bulgaricus* strains produced EPS mainly composed of galactose and glucose, with lower amounts of mannose and arabinose. In contrast, the EPS produced by *L. delbrueckii* subsp. *bulgaricus* strain CRL 420 contained glucose and fructose in a ratio of 1:2 (Manca de Nadra et al. 1985). Structural studies on the purified EPS produced by *L. delbrueckii* subsp. *bulgaricus* strain rr showed the presence of branched repeating units of galactose, glucose and rhamnose in the ratio 5:1:1 respectively (Gruter et al. 1993).

In previous work we found that *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 produced EPS when grown in batch or continuous cultures on a chemically defined medium with glucose as the carbohydrate source. The EPS had a sugar composition of galactose, glucose and rhamnose in the ratio 6.8:1:0.7. It was found that, when the strain was grown on lactose, the amount and sugar composition of the EPS produced was comparable with values for glucose-grown cultures. Lactose was cleaved to form glucose and galactose and only glucose was metabolised, whereas galactose accumulated in the growth medium and was not used for EPS synthesis (Grobben et al. 1995, 1996). When grown with fructose as the carbohydrate source, the amount of EPS produced was substantially lower and the EPS produced were composed of galactose and glucose in the ratio 2.5:1. No rhamnose residues were detected in these EPS. Analyses of the enzymes involved in the synthesis of sugar nucleotides indicated that, in glucose-grown cultures, the activity of UDP-glucose pyrophosphorylase, leading to the synthesis of UDP-glucose and UDP-galactose, was higher than that in fructose-grown cultures, whereas in fructose-grown cultures no enzyme activities were found that led to the synthesis of dTDP-rhamnose (Grobben et al. 1996). This indicated that the sugar composition and the structure of the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 are dependent on the carbon source used. Therefore, further structural characterisation of the EPS produced under different growth conditions is required.

In order to clarify the differences between the EPS from fructose-grown and glucose-grown cultures we purified the EPS produced by *L. delbrueckii* subsp. *bulgaricus* grown in a continuous culture on defined medium with either glucose or fructose as the carbohydrate source. The purified EPS were analysed to determine the molecular mass, the sugar composition and the presence of different types of sugar linkages.

Materials and methods

Bacterial strain, growth conditions and isolation of the EPS

L. delbrueckii subsp. *bulgaricus* NCFB 2772 was obtained from the National Collection of Food Bacteria (Reading, UK). The strain was grown in a N_2-flushed continuous culture at 40 °C, pH 6.0 ± 0.1 and at a dilution rate of 0.075 h^{-1}, using a chemically defined medium as described before (Grobben et al. 1995). Either glucose or fructose was used as the sole carbohydrate source with a final concentration of 139 mM, which resulted in a carbohydrate excess. After steady-state conditions had been re-established (after at least five volume changes), cultures were treated with 132 g trichloroacetic acid/liter and centrifuged (20 min, 27 000 *g*, 4 °C). The pH of the supernatant was corrected to 4.0 with NaOH and three volumes of cold absolute ethanol were added to precipitate the EPS. After 3 h at 4 °C, the precipitated EPS were collected by centrifugation (30 min., 27 000 *g*, 4 °C), dissolved in distilled water, dialysed at 4 °C against running tap water (24 h) and distilled water (24 h, 3 × 10 l) and lyophilised.

High-performance size-exclusion chromatography

Freeze-dried EPS were dissolved in 0.4 M sodium acetate pH 3.0 to a concentration of 4.0 g l^{-1}. Insoluble fragments were removed by centrifugation at 13 000 rpm in an Eppendorf centrifuge. High-performance size-exclusion chromatography analysis was performed using a SP 8700 HPLC equipped with 3 Bio-Gel TSK columns in series: 60XL, 40XL and 30XL (each 300 × 7.5 mm, Bio-Rad), together with a TSK XL guard column (40 × 6 mm). The system was eluted with 0.4 M sodium acetate pH 3.0 at a flow rate of 0.8 ml min^{-1} at 30 °C (Verbruggen et al. 1995). Peaks were detected with a combined refractive-index detector and viscometer (Viscotek, model 250; Viscotek, Houston, Tex., USA) and a right-angle laser light-scattering detector (Viscotek, LD 600). Molecular masses and intrinsic viscosities were calculated using the light-scattering module of the Trisec software (Viscotek).

Purification of the EPS

Approximately 300 mg isolated EPS were dissolved in 50 mM sodium acetate buffer, pH 5.0, to a concentration of 2.0 g l^{-1} and centrifuged (10 000 *g*, 10 min, 4 °C) to remove insoluble residues. The EPS fractions were purified by size-exclusion chromatography (SEC; Biopilot System, Pharmacia) using a Sephacryl S-500 column (length 1020 mm × internal diameter 100 mm, Pharmacia). The column was eluted with 50 mM sodium acetate buffer pH 5.0 at a flow rate of 37 ml min^{-1} and peaks were detected with a refractive-index detector (Shodex RI-72; Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands) and with a UV detector (Pharmacia) at 280 nm. Fractions of 210 ml were collected using a Super-Frac fraction collector (Pharmacia). All isolated fractions were analysed for their neutral sugar content using the orcinol/sulphuric acid method (Tollier and Robin 1979) on a Skalar autoanalyzer (Skalar Analytical, Breda, The Netherlands), with galactose as a standard. Fractions containing a sugar peak were pooled, concentrated at 37 °C using a vacuum evaporator, dialysed at 4 °C against running tap water (24 h) and distilled water (48 h, 5 × 10 l) and lyophilised.

Sugar composition

Purified EPS fractions were pre-hydrolysed with 72% (w/w) H_2SO_4 (1 h, 30 °C) and hydrolysed with 1 M H_2SO_4 (3 h, 100 °C) and the sugars released were converted into alditol acetates (Englyst and Cummings 1984; Verbruggen et al. 1995). The carboxyl groups were reduced using $NaBH_4$ prior to acetylation. Alditol acetates were separated by gas chromatography on a 15 m × 0.53 mm DB

225 column (J & W Scientific, Folsom, Calif., USA) using a Carlo Erba 4200 GC (Carlo Erba Strumentazione, Rodano, Italy). The oven temperature was set at 230 °C and the flame ionisation detector at 260 °C. Hydrogen was used as carrier gas. Inositol was used as an internal standard. Uronic acid was determined using the *m*-hydroxydiphenyl assay as described by Thibault (1979).

Sugar linkage analysis

The relative amount and type of glycosidic linkages in the EPS were detected by methylation analysis. Approximately 5 mg purified EPS fractions were dried for 24 h in vacuo in the presence of P_2O_5, dissolved in 1 ml dimethylsulphoxide and methylated using 1 ml dimethylsulphinyl carbanion and 1 ml iodomethane according to a modified Hakomori method (Hakomori 1964; Sandford and Conrad 1966). The methylated EPS fractions were dissolved in methanol/chloroform 1:1 (vol/vol), dialysed against running tap water (24 h) and distilled water (48 h, 5 × 10 l) and hydrolysed in 2 M trifluoroacetic acid (1 h, 121 °C). The partially methylated monomers were neutralised using 0.1 ml 1.5 M NH_3, reduced with 0.1 ml 0.5 M NH_3 containing 150 g l^{-1} $NaBH_4$ and acetylated with 0.45 ml 1-methylimidazole and 3 ml acetic acid anhydride. The partially methylated alditol acetates were extracted with dichloromethane, washed with distilled water, dried in a stream of air at below 10 °C, washed with acetone and dried again. Finally they were dissolved in ethyl acetate and analysed by gas/liquid chromatography. Peaks were identified on the basis of their relative retention times using inositol as an internal standard. The identity of the components was confirmed by gas chromatography mass spectrometry (GC-MS) (Sandford and Conrad 1966; Verbruggen et al. 1995).

Results

Growth and EPS production in continuous culture

To determine the effect of fructose and glucose on the amount and composition of the EPS produced by *L. delbrueckii* subsp. *bulgaricus*, the strain was grown in a continuous culture with defined medium supplemented with either 139 mM fructose or 139 mM glucose. EPS were isolated from the supernatant of cultures growing under steady-state conditions. *L. delbrueckii* subsp. *bulgaricus* produced 30 mg l^{-1} EPS when grown on fructose and 95 mg l^{-1} EPS when grown on glucose at an absorbance at 600 nm of 2.8 and 3.0 respectively.

Molecular mass analysis of the EPS

The measurements on the relative molecular mass (M_r) and intrinsic viscosity of the EPS produced on glucose showed that the EPS were composed of two populations, which appeared to have M_r values of 1.7×10^6 and 4.2×10^4 and intrinsic viscosities of 4.3 and 0.3 dl g^{-1} respectively. The EPS produced on fructose showed the presence of two peaks with $M_r = 1.3 \times 10^6$ and 3.8×10^4. The intrinsic viscosities of both EPS fractions were slightly lower than those of the EPS fractions of glucose-grown cultures. In the EPS produced on glucose, the high-M_r and the low-M_r fractions were present in almost equal amounts, whereas the EPS produced on fructose were mainly composed of the low-M_r fraction (Table 1).

Table 1 Relative molecular mass (M_r) and intrinsic viscosity of the exopolysaccharides (EPS), produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on either glucose or fructose, obtained by high-performance size-exclusion chromatography measurement. Each value represents the results of duplicate measurements and varied from the mean by not more than 5%

EPS fraction	Relative concentration (%)	M_r	Intrinsic viscosity (dl g^{-1})
Glucose, high-M_r	51.5	1.7×10^6	4.3
Glucose, low-M_r	48.5	4.2×10^4	0.3
Fructose, high-M_r	13.6	1.3×10^6	3.3
Fructose, low-M_r	86.4	3.8×10^4	0.2

Purification of the different EPS peaks

The EPS produced on glucose and on fructose were dissolved in sodium acetate buffer and insoluble parts were removed by centrifugation prior to applying the supernatants on the SEC column. All fractions containing sugar peaks were pooled and purified. The high-M_r fraction of the EPS produced on glucose showed a low absorption at 280 nm, which points to a low amount of protein. The high-M_r fraction of the EPS produced on fructose showed a slightly higher absorption at 280 nm. Both low-M_r fractions appeared to have no absorption at 280 nm.

Sugar composition

The sugar compositions of the purified EPS fractions were determined after hydrolysis and conversion into alditol acetates. Table 2 shows that the high-M_r fraction of the EPS produced on glucose contained galactose, glucose and rhamnose monomers in a molar ratio of approximately. 5:1:1 respectively. The low-M_r fraction of the EPS produced on glucose contained mainly galactose and glucose, whereas rhamnose was only present in a very low amount, resulting in a molar ratio of approximately. 11:1:0.4. The sugar compositions of the high-M_r fraction and the low-M_r fraction produced on fructose were comparable to those found in the EPS fractions produced on glucose, although the galactose to glucose ratio varied slightly. Other neutral sugars and uronic acids could not be detected.

Table 2 Sugar compositions of the purified EPS fractions from *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture with glucose or fructose as the carbohydrate source. The maximum deviation between duplicate measurements was 2%

EPS fraction	Composition of EPS (mol %)		
	Galactose	Glucose	Rhamnose
Glucose, high-M_r	70	15	15
Glucose, low-M_r	89	8	3
Fructose, high-M_r	65	20	15
Fructose, low-M_r	84	13	3

Table 3 Sugar linkage composition of the purified EPS fractions from *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture on glucose and fructose. Each value represents the average of duplicate measurements. *Gal* galactose, *Glc* glucose, *Rha* rhamnose. The ratio is calculated by dividing the percentage terminally linked sugars by the percentage branched sugars

Linkage type	Composition (mol %)			
	Glucose, high-M_r fraction	Glucose, low-M_r fraction	Fructose, high-M_r fraction	Fructose, low-M_r fraction
(Gal)1→	27.9	8.7	33.7	11.1
→2(Gal)1→	4.5	–	–	–
→3(Gal)1→	6.7	32.3	1.3	27.1
→4(Gal)1→	–	–	1.1	8.7
→6(Gal)1→	1.6	37.8	0.5	26.5
↑3 → 2(Gal)1 →	9.3	4.6	9.9	5.5
↑3 → 4(Gal)1 →	22.6	7.4	27.0	8.8
→3(Glc)1→	9.6	4.5	10.8	6.5
(Rha)1→	17.9	4.7	15.7	5.7
Terminally linked (%)	45.8	13.4	49.4	16.8
Branched (%)	31.9	12.0	36.9	14.3
Ratio	1.44	1.12	1.34	1.17

Methylation analysis

The sugar linkage compositions of the purified EPS fractions were determined by methylation analysis. As shown in Table 3, the high-M_r EPS fractions produced on either glucose or fructose contained mainly terminally (1-)linked galactose and 1,3,4-linked galactose with lower amounts of 1,2,3-linked galactose, 1,3-linked glucose and terminally linked rhamnose. Trace amounts of linearly linked galactose residues were also detected. The low-M_r EPS fractions contained mainly 1,3-linked galactose and 1,6-linked galactose residues, with a lower amount of terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose. In the low-M_r EPS fraction produced in fructose-grown cultures, 1,4-linked galactose residues were also found.

Discussion

In previous papers we reported that *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on fructose produced a lower amount of EPS than cultures grown on glucose or lactose and that there were differences in the monomeric sugar composition of the EPS (Grobben et al. 1995, 1996). It appeared that these differences were independent of the growth rates on these substrates (data not shown). A possible explanation of this observation might be that this strain produces more than one type of EPS. Cerning et al. (1994) suggested that *L. casei* produces different types of EPS depending on the carbon source. Two extracellular polysaccharides with different sugar compositions were isolated from *Serratia marcescens*: one had a relatively high content of rhamnose monomer, whereas the other contained no rhamnose (Adams and Martin 1964).

Molecular mass analysis of the EPS showed the presence of a high-M_r fraction (approx. 1.7×10^6) and a low-M_r fraction (approx. 4×10^4). The production of the high-M_r fractions of the EPS appeared to be dependent on the carbohydrate source, whereas the low-M_r fractions were produced more independently of the growth substrate. Cerning et al. (1986) found that *L. delbrueckii* subsp. *bulgaricus* strain 416a produced a single polysaccharide with a M_r of approximately 5×10^5. The M_r of the EPS produced by strain CRL 420 appeared to be 2×10^5 (Manca de Nadra et al. 1985). Cerning (1990) found that *Streptococcus thermophilus* strains produced two different polymers at the same time with $M_r = 2 \times 10^6$ and 3.5×10^4, the EPS fractions appeared to have an identical sugar composition and the latter was possibly a degradation product. Marshall et al. (1995) found that *Lactococcus lactis* subsp. *cremoris* LC330 was able to produce two polysaccharides concurrently, with $M_r > 1 \times 10^6$ and 1×10^4, and with different sugar compositions. In contrast to the large polysaccharide, the production of the smaller polysaccharide by this strain was not affected by changes in growth conditions and it appeared to be associated with the cell wall. The EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 were isolated by the usual procedure for the isolation of extracellular (unattached) polysaccharides. Therefore, it is unlikely that capsular polysaccharides are isolated using this procedure, since capsular polysaccharides have often to be detached from cell walls, although the association of the capsular polysaccharides with cell walls is dependent on the bacterial strain (Cerning 1990; Sutherland 1972). In *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, the intrinsic viscosities of the high-M_r EPS fractions were comparable with the intrinsic viscosity of purified EPS of *L. delbrueckii* subsp. *bulgaricus*, obtained by Cerning (1995). The low-M_r fractions showed almost no thickening properties. Therefore, it is expected that EPS produced on glucose are probably more viscous than an equal amount of the isolated EPS produced in fructose-grown cultures.

The results of our methylation analyses indicate that the high-M_r fractions of the EPS produced on fructose and glucose have a repeating unit that is composed of terminally linked galactose, 1,2,3-linked galactose, 1,3,4-linked galactose, 1,3-linked glucose and terminally linked rhamnose residues. The repeating unit of the EPS produced by *L. delbrueckii* subsp. *bulgaricus* rr (Gruter et al. 1993) showed a similar sugar linkage composition and it is very possible that the high-M_r fractions of the EPS of our strain and the EPS produced by *L. delbrueckii* subsp. *bulgaricus* strain rr have repeating units with identical structures, although we did not perform any measurements to determine the order of the glycosidic residues of the EPS repeating unit. The low-M_r fractions of the EPS produced on glucose and fructose contained high amounts of 1,3-linked galactose and 1,6-linked galactose residues. These were not, or only in trace amounts, found in the high-M_r fractions. The relative amounts of branched and terminally linked sugar residues were significantly lower in the low-M_r fractions, so that they appeared to be more linear than the high-M_r EPS fractions. It is concluded that the production of both EPS fractions is regulated in different ways. In all EPS fractions investigated by methylation analysis, the amount of glucose residues was lower than might be expected in comparison to the neutral sugar compositions (Table 2) and the monomeric sugar compositions found in earlier work (Grobben et al. 1995, 1996). On the other hand, the relative amount of rhamnose was higher than expected. This may be explained by the fact that the results obtained by methylation analysis give more qualitative rather than quantitative information (Schols and Voragen 1994). In contrast to our previous results, it was observed that the amount of galactose residues in the EPS produced on fructose was higher than in the EPS produced on glucose. It is still unclear why this discrepancy occurred. Measurements of the sugar composition of the different EPS fractions purified in this work, using the HPLC method described before (Grobben et al. 1995), gave similar results (Table 2) to those obtained by the alditol acetate method (data not shown). Since the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown on fructose were mainly composed of the low-M_r fraction, it is concluded that this is apparently responsible for the low concentration of rhamnose in the total EPS produced on fructose (Grobben et al. 1996).

Analysis of the activities of key enzymes leading to the production of sugar nucleotides indicated that, following growth on fructose, no activity could be detected of enzymes involved in the synthesis of dTDP-rhamnose. Therefore, we concluded that the absence of these activities resulted in the absence of rhamnose in the EPS produced on fructose (Grobben et al. 1996). In this work, however, we found that *L. delbrueckii* subsp. *bulgaricus* grown on fructose produced EPS containing rhamnose monomers. Therefore, the enzymes involved in the production of dTDP-rhamnose must be active when grown on fructose, although their activities may be below the detection level of the enzyme assays described in our previous work (Grobben et al. 1996).

In conclusion, *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 is able to produce two types of EPS concurrently, which differ in molecular size, intrinsic viscosity and sugar composition. The production of the large polysaccharide is dependent on the carbohydrate source, whereas the small polysaccharide is probably produced more continuously. It is unclear in what way the production of the two types of EPS is regulated in *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, so further investigations will be needed to clarify the biosynthesis of both EPS fractions and how this biosynthesis is regulated.

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