Structural characterisation and enzymic modification of exopolysaccharides from *Lactococcus lactis*

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Stellingen

1. Hoewel de suikersamenstellingsanalyse anders doet vermoeden, is de chemische structuur van de repeterende eenheid van EPS B40 identiek aan die gerapporteerd voor SBT 0495.
   - H. Nakajima, T. Hirota, T., Toba, T., Itoh, and S. Adachi; Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. *Carbohydr. Res.*, 224 (1992) 245-253.
   - Dit proefschrift.

2. Bij de weergave van repeterende eenheden van exopolysacharide structuren ligt het blijkbaar niet altijd voor de hand om voor de positie van eventuele substituenten de geaccepteerde weergave van suikerresiduen (waarbij de koolstofnummering met de klok meegaat) in gedachte te houden.
   - H. Nakajima, T. Hirota, T., Toba, T., Itoh, and S. Adachi; Structure of the extracellular polysaccharide from slime-forming *Lactococcus lactis* subsp. *cremoris* SBT 0495. *Carbohydr. Res.*, 224 (1992) 245-253.
   - G.W. Robijn, J.R. Thomas, D.J.C. van den Berg, H. Haas, J.P. Kamerling, and J.F.G. Vliegenthart; The structure of the exopolysaccharide produced by *Lactobacillus helveticus* 766. *Carbohydr. Res.*, 276 (1995) 137-154.

3. Bij het weglaten van de legenda (E=enzym; S=substraat; P=product 1; P^*=product 2) kan het in formulevorm weergeven van transferase reacties ten onrechte doen vermoeden dat exopolysachariden (EPS) daarbij een rol spelen:

$$\begin{array}{ccccccccc} & & & & P^* & & S & & \\ & & & & \uparrow & & \downarrow & & \\ E & + & S & \rightarrow & ES & \rightarrow & EP & \rightarrow & EPS \rightarrow E + PS \end{array}$$

4. Het opnieuw laten invullen van een medische vragenlijst bij het verplicht overgaan tot een andere verzekeringsvorm bij dezelfde ziektekostenverzekeraar is een vorm van machtsmisbruik.

5. De afname van het aantal werkzoekenden op de arbeidsmarkt komt het beleefd afhandelen van sollicitatieprocedures door werkgevers ten goede.

6. Indien bewezen is dat er in Rotterdam privé-uitgaven gedeclareerd zijn, dienen de gePEPERde rekeningen aan het gemeentebestuur te worden terugbetaald.

7. Het is een voorrecht om veel belasting te moeten betalen.

8. De energie die wordt gestoken in het opwaarderen van afvalstromen staat in schril contrast met het gemak waarmee voedsel maar al te vaak wordt weggegooid.

Stellingen behorende bij het proefschrift:
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Abstract

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Key words *Lactococcus lactis*, (exo-)polysaccharides, EPS, structural analysis, NMR, modification, enzymes, endoglucanase, phosphatase, β-galactosidase, *Trichoderma viride*, *Aspergillus aculeatus*.

The chemical structures of three exopolysaccharides (EPSs) from different strains of *Lactococcus lactis* subsp. *cremoris* (*i.e.* B40, B39 and B891) were elucidated, using enzymes in addition to chemical methods. Enzymically and chemically modified exopolysaccharides were used to characterise enzymes with activity towards these polysaccharides. Modified EPS structures were also produced to study their structure-function relationship.

EPS B40 consists of a repeating unit of →4)-[α-L-Rha*p*-(1→2)][α-D-Gal*p*1P→3]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→. The structural elucidation of (chemically modified) EPS B40 was facilitated by characterising the oligosaccharides released after incubation with an endoglucanase from *Trichoderma viride*. EPS B39 is composed of a heptasaccharide repeating unit: →2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)]-α-L-Rha*p*-(1→. EPS B891 consists of a repeating unit of →4)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)]-α-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→, in which the glucosyl residues in the branches are partially acetylated (ca. 50%) at the oxygen attached to C-6. Structural information on the side chains of both EPS B39 and EPS B891 was obtained from the action of a β-galactosidase from *Aspergillus aculeatus*. The chemical structures of these two novel exopolysaccharides in relation to EPSs from other lactic acid bacteria is discussed.

The known chemical structure of modified EPS B40 was used to obtain information about the mode of action of the purified endoglucanase from *T. viride*. Furthermore, a phosphatase from *T. viride* was recognised to act on de-galactosylated and de-rhamnosylated EPS B40. Finally, the β-galactosidase from *A. aculeatus* was purified and characterised and its activity towards EPS B39 and (*O*-deacetylated) EPS B891 is described.

Aan mijn ouders

Contents

Chapter 1

General introduction

Introduction

Bacterial exopolysaccharides (EPSs) are extracellular polysaccharides which are either associated with the cell surface in the form of capsules or secreted into the environment in the form of slime [1,2]. In their natural environment, EPSs are thought to play a role in the protection of the cell against desiccation, osmotic stress, toxic compounds, phage attack, and predation by protozoans. Furthermore, they may be involved in the adhesion of cells to solid surfaces, biofilm formation, cellular recognition, and immune response [2]. EPSs do not appear to function as energy reserve, since the bacteria are usually not capable of metabolising the EPS they produce [3,4].

Bacterial exopolysaccharides in food

In the food industry, polysaccharides are used as thickeners, gelling agents, stabilisers, emulsifiers, bodying agents, foam enhancers, or fat replacers. By volume, polysaccharides from higher plants (e.g. cellulose, starch, galactomannans, and pectins) and seaweeds (e.g. alginate and carrageenan) are the most important polysaccharides [5]. Regarding microbial polysaccharides, the two most important functional additives for food products (at least in the USA) are xanthan and gellan [6]. Xanthan is produced by *Xanthomonas campestris* [7] and has been approved for food use in the U.S.A. since 1969 and in Europe since 1974 [8]. It is used for a wide range of industrial applications due to its unusual physical properties: high specific viscosity and pseudoplasticity [9]. Illustrations of its utility in food are dressings, dry mixes, syrups, sauces, toppings, and baked goods [10]. Gellan gum is commercially produced by *Aureomonas elodea* [6] and has been accepted for food use in Japan since 1988 [8] and approved by the US Food and Drug Administration for food use in 1990 [6]. It is marketed as a gelling agent [8] and applications are found in, for example, confectionery, jams, pie fillings, puddings, and dairy products [11]. Since not all functionalities within food products can be obtained from one single polysaccharide and in some instances synergistic interactions between polysaccharides can occur, many combinations of polysaccharides are used in food industry [8]. For example, solutions of xanthan do not in themselves form gels while also solutions of plant gluco- or galactomannans normally fail to form gels. However, when solutions of xanthan and gluco- or galactomannans are mixed, heated and cooled, firm gels may be formed at relatively low polysaccharide concentrations [1]. Blends of xanthan with other polysaccharides are used in, for example, dairy products and fruit beverages [10].

Normally, if a new bacterial exopolysaccharide is addressed to uses in food products a long time is required before it may be officially approved [6]. This is not the case if food grade micro-organisms are used. For this reason, EPSs from lactic acid bacteria have received considerable attention in view of their potential application as replacers of presently applied thickeners [17]. However, since the EPS production by lactic acid bacteria (0.1-1.5 g/L) can hardly compete with for example the production level of xanthan (30-50 g/L), a ten-fold increase in EPS production by lactic acid bacteria is required from an economic point of view to use these EPSs as food additives [2].

An alternative to the use of bacterial polysaccharides as additives is the development of starter cultures producing polysaccharides that might be considered natural ingredients or natural food products [8]. The last decade, industrial interest has arisen in EPSs from lactic

acid bacteria. Lactic acid bacteria are Gram-positive bacteria comprising the genera *Lactococcus, Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus*. Since these bacteria are food grade they can be used for *in situ* production of EPSs. These polysaccharides contribute to the peculiar rheology and texture of milk and milk-derived products [6]. For example, mesophilic strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are used in Scandinavian countries for the manufacture of fermented milk products possessing a ropy or mucoid texture (e.g. viili, långmjölk, långfil and taette). The rheology of these products relies upon the souring capacity of these strains and the concomitant production of EPS [2]. Furthermore, thermophilic, slime-producing lactic acid bacteria can be used to improve yoghurt viscosity and to decrease the susceptibility to syneresis [3]. In general, the apparent viscosity of stirred yoghurt increases with the ropiness of the culture. However, no direct correlation was found between the concentration of the EPS secreted and the apparent viscosity of stirred yoghurt produced by different cultures [12]. One explanation might be that the polysaccharide structure is more important for its functionality than the polysaccharide concentration [13]. Furthermore, the production of EPS by different bacteria influences the spatial structure of the protein network, which seems to be correlated with the apparent viscosity [12]. Besides the interesting physical properties, EPSs from lactic acid bacteria have gained attention because they might be beneficial for health; animal studies have suggested antitumoral effects [14], cholesterol lowering effects [15] and immune response stimulating effects [16]. These health aspects need further investigation, since most studies were incomplete with regard to control experiments and the evidence provided is far from conclusive [17].

Exopolysaccharide biosynthesis by lactic acid bacteria

Since ancient times, lactic acid bacteria have been used for the preservation of many food products, because of their ability to convert lactose into lactate. As described above, the rheology of many fermented dairy products relies upon the souring capacity of these bacteria and the concomitant production of EPS. There has been much speculation about the biological need for lactic acid bacteria to produce EPS. On the one hand, a protecting role against lytic bacteriophage attack has been ascribed to EPS from *Lactococcus lactis* subsp. *cremoris* [18]. On the other hand, the trait of producing EPS is very unstable in lactic acid bacteria and is frequently lost upon cultivation and the advantage to produce EPS is not evident. Therefore, it has been suggested that EPS synthesis might be a trait which was kept in the gene pool of lactic acid bacteria by selection for ropy strains in the dairy industry [19].

Some EPSs are synthesised extracellularly by lactic acid bacteria via a transglycosylation process [2]. These polysaccharides include α-D-glucans (e.g. dextrans and mutans), β-D-glucans and fructans (e.g. levan) and will not be considered in this thesis. The biosynthesis of other EPSs is an intracellular process and will be described here for lactococci, which have received considerable attention as organisms targeted for improvement due to their importance in milk fermentations, the ease of handling in the laboratory, and the fact that many metabolic properties are plasmid mediated [20]. Lactococci are able to convert the glucose moiety of lactose into several sugar nucleotides, necessary for the synthesis of polysaccharides. The galactose moiety of lactose is metabolised, supplying energy for the production of biomass and EPS [reviewed in 2]. For EPS synthesis, the sugar nucleotides,

together with the sugar activating and modifying enzymes, play a crucial role in the formation of oligosaccharide building blocks (repeating units) and thus the final EPS composition [2]. Lactococcal EPS synthesis occurs at lipid carriers located on the cytoplasmic membrane of the bacterium. At the cytoplasmic side of this membrane, the production of repeating units takes place through the sequential addition of sugar residues from sugar nucleotides by several glycosyl transferases. Repeating units are expected to be translocated across the membrane, where they are polymerised resulting in an extension of the polymer at the reducing end [21].

Chemical structures of exopolysaccharides from lactic acid bacteria

Generally, intracellularly produced exopolysaccharides from lactic acid bacteria [17,22] consist of repeating units, which contain between two and eight sugar residues possibly decorated with (in)organic substituents [1]. Since different sugar residues (and other groups) can be present and each hexose residue can be in the pyranose or furanose form, in the α- or β-configuration and linked at various positions, the possible range of structures is enormous. However, comparison of the sugar compositions of many (recently) investigated EPSs from lactic acid bacteria [23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39] reveals that there are structural similarities. Galactosyl, glucosyl and rhamnosyl residues are the most abundant (and often only) sugar residues present in varying amounts. Furthermore, *N*-acetyl-glucosamine residues [27,32], *N*-acetyl-galactosamine residues [23,31], glucuronosyl residues [32], and fucosyl residues [39] have been reported. Another almost common feature is the repeating units being branched. In addition, the repeating units can be decorated with *sn*-glycerol-3-phosphate [29,31], acetyl groups [29], and phosphate groups [24]. The simultaneous production of two types of EPSs by one strain of *L. lactis* subsp. *cremoris* has been mentioned in literature [17,40]. Recently, overviews of primary EPS structures produced by lactic acid bacteria have been given [2,17,21]. Here, only the two known primary structures of EPSs produced by lactococci (*i.e. L. lactis* subsp. *cremoris* H414 [25] and SBT 0495 [24]) are depicted for illustration (Fig. 1.1).

a. →4-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→
3 2
↗ ↖
α-D-Gal*p*1P α-L-Rha*p*-1

b. →4-β-D-Gal*p*(1→3)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→
3
↑
β-D-Gal*p*-(1→3)-β-D-Gal*p*-1

Fig. 1.1. Primary structures of EPSs produced by *L. lactis* subsp. *cremoris* SBT 0495 (a) [24] and H414 (b) [25].

Knowledge of the primary structures of polysaccharides (including EPSs) is a prerequisite to obtain insight into their functionality, because the chemical structures determine the three-dimensional configuration of these polymers. Structural differences can greatly affect the physical properties of polysaccharides. This can be illustrated by a β-(1→4)-linked-D-glucan (cellulose) being water insoluble and crystalline, whereas an α-(1→4)-linked-D-glucan (amylose) is sparingly soluble in water, crystallises less well than cellulose and can form rigid gels [41]. The consequences of some structural subtleties in microbial polysaccharides have been reviewed by Sutherland [42]. Gellan, for example, only forms weak or rubbery gels unless the acyl groups (*i.e. O*-acetyl and L-glyceryl groups) are removed; deacylated gellan yields hard and brittle gels, which find applications for food and other uses.

Modification of exopolysaccharides

To enable tailoring of polysaccharides with specific functionality, insight into the relationship between the chemical structure and the physical properties of polysaccharides is required. This insight can be obtained by examination of microbial polysaccharides with closely related structures, which can be obtained in various ways [42].

(I) Different microbial species or strains may produce a range of polysaccharides with close structural similarities. For example, the EPSs produced by *Streptococcus thermophilus* OR 901 [36], Rs, Sts [37] have structural similarities with EPS from *S. thermophilus* SFi12 [35].

(II) Through the careful control of culture conditions, variations in EPS compositions have been found [43,44,45].

(III) Modified EPS structures can also be obtained by mild chemical degradation procedures, like *O*-deacetylation using an ammonium hydroxide solution [29,43] or dephosphorylation using a hydrofluoric acid solution [24].

(IV) Enzymes having a specific action towards EPS structures can be used to tailor the chemical structures and hence the physical properties [2].

(V) Finally, genetic modification of micro-organisms is promising for future EPS engineering. Mutants of *Xanthomonas campestris* [9,46] and *Lactobacillus sakei* 0-1 [47] were found to produce EPSs with a different structure than the polysaccharide from the wild-type micro-organism. Furthermore, the gene cluster responsible for EPS biosynthesis in *S. thermophilus* Sfi6 has recently been transferred into a non-EPS producing host, *L. lactis* MG1363, yielding a different structure than the EPS from the native host [48].

Starting from EPS, produced by a given micro-organism under given circumstances, modified structures can be obtained by chemical or enzymic treatment. A problem in comparing the physical properties of the different polysaccharide preparations, is to ensure that the only *real* difference lies in structure and that they do not differ in molecular mass [49], since this parameter is also very relevant to these properties. For instance, Faber *et al.* [37] showed that two EPSs produced by *S. thermophilus* Rs and Sts have the same repeating unit, but differ in the viscosity of their milk cultures, probably because of differences in molecular mass. Since chemical modification is often accompanied by unintended degradation and enzymic modification is much more specific, enzymic modification is preferable.

The use of enzymes in exopolysaccharide research

Enzymes are useful in (exo)polysaccharide research not only to obtain modified polymers, but also as valuable tools, supplementary to chemical methods, for structural characterisation. Recently, a review on polysaccharide hydrolases (polysaccharases) for microbial EPSs has been written by Sutherland [4] and some aspects, relevant for the present work, will be mentioned here. Polysaccharases can be divided into endo-acting and exo-acting enzymes. Endo-enzymes randomly act on the backbone of the polymer, resulting in a rapid decrease in the degree of polymerisation (DP) [4]. Cleavage by a single, highly specific endo-acting polysaccharase yields a series of oligosaccharides representing either the repeating unit structure or multiples or fragments of it. These oligosaccharides are amenable to nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) analyses and proved to be extremely useful in providing information about EPS structures [1,4]. In addition, these enzymes may permit the production of fragments still having acyl groups, whereas these labile groups are usually lost when chemical methods are involved. Furthermore, they provide the basis for determining the uniformity of polysaccharide structure [1]. Exo-enzymes cleave the polymer in a terminal fashion, releasing mono- or oligosaccharides. These enzymes cause only a slow reduction in the DP of the polysaccharides. In addition to polysaccharases, glycosidases might as well act on polymeric substrates to release some terminal sugar residues [4]. Both, exo-acting polysaccharases and glycosidases, might be able to debranch the substrate, leading to possible changes in physical properties. Other enzymes which may affect the physical properties of EPSs are enzymes able to remove acyl substituents (e.g. ester-linked acetyl groups) [4].

Background and aims of the research project

This thesis is part of a multidisciplinary research project on the relationship between the chemical structure and the physical properties of food grade polysaccharides, funded by the Netherlands Association of Biotechnology Centres in the Netherlands (ABON). Insight in this relationship can lead to tailor-made exopolysaccharides, which meet particular requirements in terms of structure and function. The coherence of the five research areas involved is illustrated in Fig. 1.2.

The production of EPSs by different strains is studied and optimised by changing the fermentation conditions and the medium composition, in order to achieve the highest yield of EPS. The obtained polysaccharides are characterised for their rheological and physical properties and their chemical structures. To be able to use enzymes as a tool for the structural characterisation of EPSs, screening procedures to find enzyme activities are set up. Moreover, enzymes are also useful for the production of structurally different but related polysaccharides in order to study the structure-function relationship. Furthermore, the equilibrium and enzyme kinetics are studied in order to be able to design a reactor for the enzymic synthesis of oligosaccharides and for the enzymic modification of polysaccharides.

The polysaccharides of interest are produced by different strains of the lactic acid bacterium *L. lactis* subsp. *cremoris*. This bacterium is physiologically, biochemically and genetically well-characterised and interesting in view of genetic modification to produce structurally different exopolysaccharides.

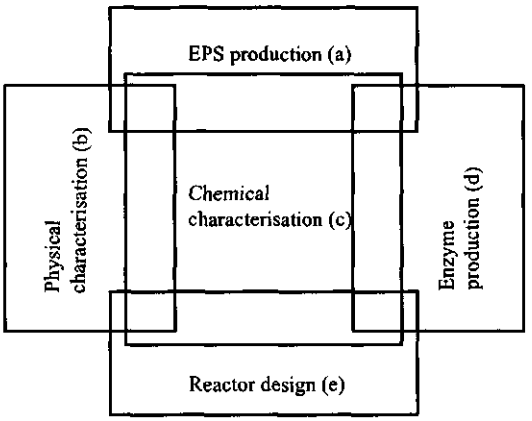

Fig. 1.2. Scheme of the strategic interactions between the different research areas within the project concerning food grade polysaccharides. The participating groups were: NIZO food research (a, b), Laboratory for Physical Chemistry and Colloid Science, Wageningen University (WU), (b), Laboratory of Food Chemistry, WU (c), Laboratory of Industrial Microbiology, WU (d), and Laboratory of Food and Bioprocess Engineering, WU (e).

Aim and outline of this thesis

The aim of this thesis is (I) to characterise the chemical structures of different EPSs produced by *L. lactis* subsp. *cremoris*, using enzymes as a tool if possible, (II) to (enzymically) modify the elucidated EPS structures in order to obtain structurally related polysaccharides for physical characterisation, (III) to characterise enzymes having activity towards EPS, using the obtained structural information of these EPSs, and (IV) to unravel structure-function relationships.

First, the chemical structure of EPS from *L. lactis* subsp. *cremoris* B40 was characterised and chemically modified, since no enzymes were found to be active on native EPS (chapter 2). After chemical modification, EPS B40 appeared to be susceptible for endoglucanase and phosphatase activities in an enzyme preparation from *Trichoderma viride*. The enzymic degradation of EPS B40 was not only helpful for the structural characterisation of the polysaccharide, but was also useful to unravel the mode of action of the endoglucanase used (chapter 3). The novel primary structures of EPSs produced by *L. lactis* subsp. *cremoris* B39 (chapter 4) and B891 (chapter 5) were elucidated. These EPSs could both be modified by a β-galactosidase from *Aspergillus aculeatus*, which was useful for the structural characterisation of the side chains of these polysaccharides. Furthermore, the β-galactosidase can also be used in structure-function studies. The purification and characterisation of this enzyme is described (chapter 6). Finally, the results of chapters 2 to 6, combined with additional data on the structure-function relationship of EPSs, are discussed (chapter 7).

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Chapter 2

Characterisation and modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40

Abstract

The chemical structure of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B40 is studied, explaining earlier reported analytical discrepancies. The EPS was found to have a molecular mass of 6.8×10^5 g/mol and a molar ratio of rhamnose:galactose: glucose:phosphorus of 1:1.3:2:1.1. ^{31}P NMR indicated that a single phosphate group is present as a phospho*di*ester. EPS B40 was chemically modified using 0.3 M H_2SO_4, 28 M HF or 2 M NaOH. From these modifications it could be concluded that galactose 1-phosphate was linked at the 3-position of 2,3,4-trisubstituted galactose in the backbone of the EPS. Furthermore, it appeared that during the hydrolysis step of the sugar composition analysis the galactose 3-phosphate linkages were only partially split and that as a result, the amount of galactose was underestimated in presence of phosphate. Finally, it was demonstrated that a crude cellulase preparation was able to degrade dephosphorylated and partially de-rhamnosylated EPS.

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Introduction

Lactococci are generally recognised as safe (GRAS) in food production and are widely used as components of starter cultures for fermented dairy products [1]. Many lactic acid bacteria, among which lactococci, produce exopolysaccharides (EPSs) which play an important role in the rheological behaviour and texture of fermented milk products [2]. In order to gain insight in the physical properties of EPS from lactic acid bacteria, detailed structural studies have been performed on EPS from lactobacilli [3,4,5,6,7,8,9,10], streptococci [11] and lactococci [12,13].

Van Kranenburg *et al.* [14] reported that EPS from *Lactococcus lactis* subsp. *cremoris* B40 consists of rhamnose, galactose and glucose in the ratio of 0.9:1.2:2.0 and that the molar ratio of carbohydrate and phosphorus is 4.7:1. From these data and from the ^{1}H and ^{13}C NMR spectra they suggest that EPS B40 is probably identical to EPS SBT 0495 [13] and consists of the repeating unit as presented in Fig. 2.1.

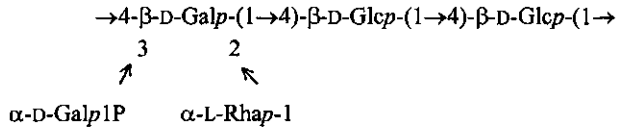

Fig. 2.1. Structure of the repeating unit from EPS SBT 0495 [13].

This chapter describes a more detailed study of the chemical structure of EPS B40 to clarify the discrepancy between the ratio of galactose reported by van Kranenburg *et al.* [14] and Nakajima *et al.* [13] and to gain a better understanding of the relationship between the chemical structure and the physical properties. For this purpose, effort is directed towards the modification of this chemical structure in order to be able to vary the physical properties. Since enzymes act very specifically, they can be used to bring about modifications in the EPS changing its functional properties. Therefore, in this study commercial enzyme preparations were screened for activity towards (chemically modified) EPS.

Experimental

Production and isolation of the exopolysaccharide.–EPS was produced by *L. lactis* subsp. *cremoris* B40 in a medium based on whey permeate. The fermentation broth was centrifuged, ultrafiltrated and freeze dried as described by Tuinier *et al.* [15] and kindly supplied as crude EPS by NIZO food research (Ede, The Netherlands).

To remove proteins, crude EPS was stirred in 4% (w/v) CCl_3CO_2H for 2 h at 4 °C (1.7 g/L) and centrifuged (28100 *g*, 30 min, 4 °C). The supernatant was collected, neutralised with 2 M NaOH and two volumes of 96% EtOH (4 °C) were added in order to precipitate EPS (2 h, 4 °C). After centrifugation (28100*g*, 30 min, 4 °C), the pellet was dissolved in distilled water, dialysed and freeze dried.

Further purification was performed using ion-exchange chromatography on a column (52×5 cm) of DEAE Sepharose fast flow (Amersham Pharmacia Biotech, Uppsala, Sweden), using a Biopilot System (Amersham Pharmacia Biotech, Uppsala, Sweden). Therefore, CCl_3CO_2H/EtOH purified EPS (0.76 g) was dissolved in 5 mM NaOAc pH 6 (375 mL) and applied to the column equilibrated with the same solution. The flow rate varied from 10 mL/min during application to max. 50 mL/min during the gradient of 5 mM NaOAc pH 6 → 2 M NaOAc pH 6. The absorption of the eluent at 280 nm was measured on-line and the protein concentration was estimated by using the extinction coefficient of β-casein (0.46 cm^2/mg; [16]). Material still bound to the column was eluted with 0.5 M NaOH (20 mL/min). The fractions (ca. 100 mL) were analysed for their total sugar content using the orcinol method [17] with glucose as standard. The sugar composition and phosphorus content of the pooled fractions were analysed as described below.

H_2SO_4-treated EPS B40.–To a solution of 25 mg EPS in 6.25 mL distilled water, 6.25 mL 0.6 M H_2SO_4 was added. The solution was kept at 37 °C for 2 h, cooled on ice and neutralised (2 M NaOH).

HF-treated EPS B40.–50 mg EPS was stirred (48 h, 0 °C) in 2.5 mL 28 M (= 48%) HF and neutralised on ice with 6 M NaOH.

NaOH-treated EPS B40.–12.5 mL 4 M NaOH containing 1 mg/mL $NaBH_4$ was added to a solution of 25 mg EPS in 12.5 mL distilled water. After 3 min at room temperature, the solution was heated for 4 h at 80 °C, cooled on ice and neutralised with 2 M HOAc.

After the various chemical modifications, samples were taken for analysis by high-performance anion-exchange chromatography (HPAEC). The remaining samples were dialysed and split; one part was concentrated and analysed by high performance size-exclusion chromatography (HPSEC) and light scattering analysis and the other part was freeze dried for sugar composition analysis and phosphorus analysis.

Enzymic degradation of EPS.–(Chemically modified) EPS B40 was incubated with over 15 commercial polysaccharide degrading enzyme preparations (e.g. pectinases, cellulases and hemicellulases). The incubation conditions depended on the optimal conditions for the enzymes and since the cellulase Maxazyme Cl from *Trichoderma viride* (Gist brocades, Delft, The Netherlands) was the only preparation which showed activity towards (modified) EPS B40, only this incubation method is described below.

Purified EPS, H_2SO_4-treated EPS, NaOH-treated EPS and HF-treated EPS were dissolved (2 mg/mL) in 50 mM NaOAc buffer pH 5.0 + 0.01% (w/v) NaN_3. Maxazyme Cl was dialysed against the same buffer and the final protein concentration was determined [18] (Bradford, 1976) using bovine serum albumin (BSA, Sigma A 4503) as a standard. The substrates were incubated with dialysed Maxazyme Cl (protein content: 0.02%) for 24 h at 30 °C. After incubation, the samples were heated (15 min, 100 °C) to inactivate the enzymes, centrifuged and analysed by HPSEC and HPAEC.

Sugar composition.–EPS was pre-treated in 12 M H_2SO_4 (1 h, 30 °C) and hydrolysed in 1 M H_2SO_4 (3 h, 100 °C), using inositol as internal standard. The released sugars were converted into their alditol acetates [19] and analysed on a J&W DB-225 column (15 m×0.53 mm) in a Carlo Erba 4200 GC. The temperature program was: 1 min isothermal at 180 °C, 180→220 °C at 2.5 °C/min and 3 min isothermal at 220 °C. The FID detector temperature was 275 °C and He was used as carrier gas.

Methylation analysis.–Purified EPS and HF-treated EPS were methylated according to Hakomori [20] and subsequently dialysed against water and freeze dried. The methylated

polysaccharide was hydrolysed in 2 M CF_3CO_2H (1 h, 121 °C). After evaporation (air stream, <20 °C), the partially methylated sugars were converted into alditol acetates [19] and analysed by GC-FID in a Carlo-Erba HRGC 5160 gas chromatograph as described by Vincken *et al.* [21]. Partially methylated alditol acetates were quantified according to their effective carbon response [22]. Identification of the compounds was confirmed by GC-MS using a CP Sil 19 CB capillary column (25 m×0.25 mm, 0.2 μm film thickness, Chrompack) in a HP 6890 gas chromatograph coupled to a HP 5973 mass-selective detector and using a HP Chem Station (Hewlett-Packard). The temperature program was 160→185 °C at 0.5 °C/min, 185→230 °C at 10 °C/min and 230 °C for 5.5 min. Besides the samples mentioned above, methylated EPS was also treated with HF prior to derivatisation into alditol acetates to obtain information on the position of phosphate within the EPS.

Phosphorus determination.–The amount of phosphorus in the samples was analysed with and without destruction in 72% $HClO_4$ (20 min, 180 °C) to correct for free phosphate. After dilution, the samples were analysed as described before [23]. To find out whether all sugar phosphate linkages were cleaved during the hydrolysis in the sugar composition analysis, hydrolysed samples were analysed for their free phosphate content as well.

^{31}P NMR analyses.–121.500 MHz ^{31}P NMR spectra were recorded in H_2O with 5-10% D_2O on a Bruker AMX-300 spectrometer, equipped with a 10 mm probe, at a probe temperature of 27 °C. Chemical shifts were referenced to added L-α-glycero-phosphorylcholine (GPC; Sigma) (δ 0.49). Chemical shifts caused by pH changes were measured to establish the presence of phosphomonoesters and/or phosphodiesters. The pH of the sample was adjusted by adding 25 mM HCl or 25 mM NaOH.

^1H and ^{13}C NMR.–1D 400.13 MHz ^1H NMR spectra and proton-decoupled 100.63 MHz ^{13}C NMR spectra of HF-treated EPS B40 were recorded in D_2O (8 mg/mL) at 60 °C on a Bruker DPX-400 spectrometer, equipped with a 5 mm probe.

Monosaccharide release.–HPAEC was performed using a Dionex system, which included a gradient pump, a eluent degas (He) module, a CarboPac PA1 column (4×250 mm) with CarboPac PA100 guard column and a pulsed electrochemical detector (PED-2) in the pulsed amperometric detection (PAD) mode. A Spectra Physics AS3000 autosampler was used and chromatograms were recorded using PC1000 software. The effluent was monitored using the PED-2 detector (reference electrode Ag/AgCl) containing a gold electrode. Potentials of E1 0.1, E2 0.7 and E3 -0.1 were applied for duration times of t_1 0.4 s, t_2 0.2 s and t_3 0.4 s. The gradient (1 mL/min) was obtained by mixing distilled water, 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH. After equilibration with 16 mM NaOH, 20 μL of the sample was injected and the elution program was 0→20 min, 16 mM NaOH isocratic, followed by regeneration: 20→25 min, 0→1 M NaOAc; 25→30 min, 1 M NaOAc; 30→35 min, 0.1 M NaOH; 35→40 min, 0.1 M →16 mM NaOH; 40→55 min, re-equilibration using 16 mM NaOH isocratic.

Galactose 1-phosphate release.–Galactose 1-phosphate was analysed by HPAEC using the system described above. The gradient (1 mL/min) was obtained by mixing 0.1 M NaOH and 1 M NaOAc in 0.1 M NaOH. The elution program was 0→5 min, 0.1 M NaOH isocratic; 5→72 min, linear gradient of 0→0.6 M NaOAc; 72→77 min, 0.6 M NaOAc isocratic, followed by regeneration: 77→82 min, 0.6→1 M NaOAc; 82→87 min, 1 M NaOAc; 87→102 min, re-equilibration using 0.1 M NaOH. α-D-Galactose 1-phosphate (Sigma) was used as a standard.

Molecular mass determination.–The molecular mass and radius of gyration of (modified) EPS B40 were determined by HPSEC with on-line refractive index and static light scattering (SLS) analysis as described before [15].

HPLC analyses after enzyme treatment.–Enzyme-treated samples were analysed for degradation by HPAEC (*vide supra*) and HPSEC. HPSEC was performed on a SP8700 HPLC (Spectra-Physics) equipped with three Bio-Gel TSK columns in series (60XL, 40XL and 30XL; each 300×7.8 mm) preceded by a TSK XL guard column (40×6 mm). The system was eluted (30 °C) with 0.4 M NaOAc pH 3.0 at 0.8 mL/min and the eluate was monitored on line using a refractometer (Viscotek). Dextrans (Mw 4,000-500,000 g/mol) were used for calibration.

Results and discussion

Purification and characterisation of EPS.–EPS produced by *L. lactis* subsp. *cremoris* B40 was extracted from the crude EPS with CCl_3CO_2H, followed by precipitation with EtOH. The yield, sugar content and the sugar composition of the crude EPS and the obtained fractions are shown in Table 2.1. The total sugar yield of the fractions was 94%. Some lactose (ca. 5%), which was still present in the crude EPS, was not recovered since the fractions were analysed after dialysis. The EtOH-precipitated material contained the bulk of the sugars, as was expected. The total sugar content increased from 51% in the crude EPS to 63% in the EtOH-precipitated material due to removal of protein-like material. Although the sugar composition of the EtOH-precipitated material was similar to the sugar composition of crude EPS small amounts of rhamnose, xylose and mannose were removed during purification with CCl_3CO_2H/EtOH.

Table 2.1
Sugar yield, sugar content and sugar composition of crude EPS and of the different fractions obtained after purification of EPS B40 with CCl_3CO_2H and EtOH.

	Yield[a] (w/w%)	Sugar content (w/w%)	Rha	Xyl	Man	Gal	Glc
			(mol%)				
Crude EPS	100	51	23	1	5	29	42
CCl_3CO_2H precipitate	8	17	22	2	10	24	42
EtOH precipitate	84	63	23	1	4	29	44
EtOH supernatant	2	9	41	8	9	17	26

[a] expressed as mg sugar per 100 mg sugar in crude EPS.

The EtOH-precipitated material was purified further on an anion-exchange chromatography column. The elution patterns of sugar and protein are shown in Fig. 2.2. The retention of EPS B40 on the DEAE column illustrates the negative charge of the polysaccharide. After elution with 2 M NaOAc a sugar recovery of only 72% was obtained, while another 25% was recovered after elution with 0.5 M NaOH (NaOH concentration is not shown in Fig. 2.2). From preliminary results, the first part of the sugar peak was known to contain a relatively high amount of mannose and was therefore pooled separately.

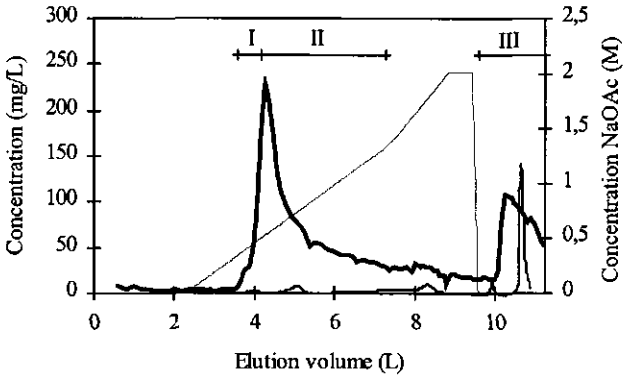

Fig. 2.2. Fractionation of EPS B40 on DEAE Sepharose Fast Flow, eluted with a gradient of NaOAc (—), followed by 0.5 M NaOH. The total sugar content (▬) is expressed as glucose equivalents. The protein concentration (—) was estimated by dividing the measured A280 by the extinction coefficient of β-casein.

In Table 2.2, the sugar yield, the total sugar content, the sugar composition and the phosphorus content of the different pools are given. Pool I indeed contained a relatively high amount of mannose in addition to rhamnose, galactose, glucose and phosphorus. It appears that in this pool mannan-rich material, for which we have strong indications that it originates from the yeast extract in the medium (unpublished results), co-eluted with EPS. Pool II appeared to be similar to pool III in sugar composition, but the analysed total sugar content in pool II was higher than in pool III, which agrees with the higher protein concentration in pool III (Fig. 2.2). The molar ratio of Rha:Gal:Glc in both pools was 1:1.3:2 and the phosphorus content was 0.05 g/g sugars, which corresponds to 1.1 mol phosphorus per 4.3 mol sugar. However, since not all galactose was detected during the sugar analysis (*vide infra*), it is more accurate to express the phosphorus content relative to the amount of rhamnose or glucose instead of relative to the analysed total amount of sugar.

Table 2.2
Sugar yield, sugar content, sugar composition and phosphorus content of the different pools obtained after fractionation of EPS B40 on a DEAE Sepharose column.

	Yield	Total sugar content	Rha	Xyl	Man	Gal	Glc	Phosphorus
Pool	(w/w%)	(w/w%)			(mol%)			(g/g sugars)
I	9	57	18	1	35	19	28	0.03
II	50	57	23	1	2	30	45	0.05
III	25	45	23	1	1	29	46	0.05

Purified EPS SBT 0495 contained Rha:Gal:Glc:P in a ratio of 1.5:2.4:2:1.1 [24]. The amount of phosphorus, relative to the amount of glucose, in EPS B40 was the same as in SBT

0495. Although the relative amount of rhamnose analysed in EPS B40 was lower than in EPS SBT 0495, it approaches the proposed structure for EPS SBT 0495 [13] better. Most striking is the relative amount of galactose, which is much lower in EPS B40 than reported for EPS SBT 0495. This was analysed further in pool II and will be discussed below.

Chemical modifications of EPS.–EPS B40 was chemically modified for three reasons. Firstly, because it might be useful in the structure elucidation of EPS B40. Secondly, because screening on chemically modified EPS besides native EPS enlarges the chance of detecting EPS-degrading enzymes. Finally, chemically modified EPSs are of interest for physical studies. Therefore, EPS B40 was treated with H_2SO_4, HF and NaOH and the sugar composition and phosphorus content of the resulting polymers were analysed (Table 2.3).

Table 2.3
Total sugar content, sugar composition and phosphorus content of purified EPS B40 before and after chemical modification. (Traces of mannose and xylose were still present.)

	Total sugar content (w/w%)	Rha	Gal	Glc	Phosphorus
		(molar ratio)			
Purified native EPS	57	1.0	1.3	2.0	1.1
0.3 M H_2SO_4-treated EPS	42	1.0	0.6	2.0	1.1
28 M HF-treated EPS	85	0.8	1.0	2.0	0.0
2 M NaOH-treated EPS	60	1.0	1.0	2.0	0.1

It appears (Table 2.3) that after H_2SO_4 treatment the relative amount of galactose had decreased by a factor 2, whereas the relative amount of rhamnose, glucose and phosphorus did not change. Directly after H_2SO_4 treatment, the sample was analysed for the presence of sugar monomers using HPAEC. Only galactose monomer was detected and the amount was consistent with the loss of galactose found for the polymer. Since it is known that sugar 1-phosphate linkages are sensitive towards dilute acid [25], galactose is probably terminally linked to phosphate, which agrees well with the proposed structure [13,14]. The liberated amount of galactose was, however, lower than was expected from this proposed structure. Possible explanations are that not each terminal galactose is released by H_2SO_4 treatment, or that not each repeating unit contains a terminally linked galactose. This was further analysed with ^{31}P NMR and will be discussed later.

HF treatment removed all phosphate from the EPS and resulted in a polysaccharide with lower amounts of galactose and rhamnose relative to glucose (Table 2.3). Under these conditions, HF selectively cleaves sugar-phosphate linkages without hydrolysis of sugar-sugar bonds [13,25]. However, some cleavage of rhamnose linkages during treatment with HF has been reported before [5,26,27]. As discussed below (Fig. 2.3), the removal of phosphate and rhamnose did not cause a large decline in hydrodynamic volume and, consequently, they must have been present as side groups of the EPS and not in the backbone. Moreover, the removal of galactose indicates that galactose is linked to the backbone of the polysaccharide via phosphate. The amount of rhamnose monomer released during HF treatment and detected by HPAEC was consistent with the rhamnose removal in the polymer. However, the amount of galactose detected by HPAEC was larger than the loss of galactose in the polymer (Table 2.3).

Converted into a relative amount, 0.7 mol galactose per 2 mol glucose in the starting EPS was recovered by HPAEC. It can therefore be concluded that the removal of galactose from the polymer during HF treatment was much larger than determined as alditol acetates.

During HF treatment all phosphate and therefore all terminally linked galactose was removed, while in H_2SO_4-treated EPS at least most of the terminally linked galactose but almost no phosphate was removed. Remarkably, the relative amount of galactose in the polymer found after H_2SO_4 treatment was lower than the relative amount of galactose found after HF treatment (Table 2.3). These results strongly suggest that galactose in the backbone (see also the results of the methylation analyses) of the EPS is only partially analysed in presence of phosphate. In order to investigate whether the low amount of galactose found as alditol acetates is caused by incomplete hydrolysis of galactose-phosphate linkages, hydrolysates were analysed (prior to further derivatisation) for the amount of phosphorus as well. The results showed that almost no free phosphate was present in native EPS. After hydrolysis, 30-40% of the total amount of phosphorus in the EPS was detected as free phosphate. This means that only part of the phosphate linkages to the galactose in the backbone of the EPS were hydrolysed. As a result, the galactose in the backbone was determined for only 30-40% whereas the terminally linked galactose was determined completely after hydrolysis. This explains why a Gal:Glc ratio of 1.3:2 (Table 2.3) was found in the native EPS while a ratio of 2:2 was expected according to the proposed structure. It also explains the relatively small decrease in the galactose content of the polymer after HF treatment; the real removal of (terminally linked) galactose was diminished by the fact that all galactose in the backbone was analysed in our assay due to the removal of phosphate.

NaOH treatment of the EPS revealed a polysaccharide with a decreased content of phosphorus and galactose (Table 2.3). Unlike HF treatment, NaOH treatment did not remove all phosphate from the EPS and all rhamnose linkages were kept intact. Despite the fact that the galactose content had decreased after NaOH treatment, no galactose was found by HPAEC. There was, however, a peak that eluted at the retention time of galactose 1-phosphate followed by a second peak of similar size, which has not further been characterised.

From the chemical modifications it can be concluded that the galactose in the backbone of purified EPS B40 was underestimated because of incomplete hydrolysis of the phosphate ester linked to the galactose in the backbone. This probably explains the relatively low amount of galactose found after hydrolysis in 4 N HCl, as reported by van Kranenburg *et al.* [14] as well. Nakajima *et al.* [24] treated their EPS with 48% HF followed by hydrolysis with 2 N CF_3CO_2H. Since their EPS was not dialysed after HF treatment, no galactose was lost and a ratio of Rha:Gal:Glc of 1.5:2.4:2 was found. In conclusion, the discrepancy between the relative amount of galactose in EPS B40 [14] en EPS SBT 0495 [24] was caused by differences in methods rather than differences in EPS structures.

Methylation analysis of EPS.–Methylation analyses were performed on native EPS and HF-treated EPS, while methylated samples of native EPS were also HF-treated prior to derivatisation into alditol acetates. The data (Table 2.4) show that in native EPS terminally linked rhamnose, 4-substituted glucose and traces of 2,4-disubstituted and 2,3,4-trisubstituted galactose were found. A poor stoichiometry in the methylation analyses of phosphorylated polysaccharides, due to partial dephosphorylation during methylation and incomplete hydrolysis of phosphate esters, has been reported previously [5,7,13,27]. HF treatment after methylation caused a higher recovery of 2,3,4-trisubstituted galactose compared to native EPS. This can probably be explained by the fact that since all sugar-phosphate linkages are

split during HF treatment, phosphate can no longer interfere with the hydrolysis and, accordingly, all galactose in the backbone of the EPS is analysed. HF treatment prior to methylation analysis instead of HF treatment after methylation caused a change of 2,3,4-trisubstituted galactose into 2,4-disubstituted galactose. This demonstrates that 2,3,4-trisubstituted galactose in native EPS is substituted with phosphate at O-3, which again agrees with the proposal of van Kranenburg *et al.* [14] that the structure of EPS B40 resembles the structure of EPS SBT 0495 [13].

Table 2.4
Methylation analysis data of native EPS (**1**), methylated and HF-treated EPS (**2**) and HF-treated and methylated EPS (**3**)

Derivative	Molar ratio[a]		
	1[b]	**2**[b]	**3**
2,3,4-Rha[c]	1	1	1
2,3,6-Glc	2	2	2
3,6-Gal	trace	trace	1
6-Gal	trace	1	trace

[a] 2,3,6-Glc was taken as 2; [b] Unmethylated material was left out of consideration (see text); [c] 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-rhamnitol, etc.

It should be remarked that the methylation analyses of native EPS B40 resulted in undermethylation, probably due to the low solubility of EPS in Me_2SO. However, the sugar composition of the unmethylated material (not shown) resembled that of the EPS. Moreover, the results (Table 2.4) were quite reproducible and no di-*O*-methyl sugars and mono-*O*-methyl sugars were observed for terminally linked rhamnose and 4-substituted glucose. Therefore, it was assumed that the methylated part of the EPS was representative for the total EPS.

The results of the methylation analysis of EPS B40 (Table 2.4) correspond to the results of EPS SBT 0495 reported by Nakajima *et al.* [13], except for the fact that we could not detect any terminally linked galactose. For HF-treated EPS the latter was expected since galactose was split off during HF treatment and was lost during dialysis. For native EPS, however, the loss of terminally linked galactose was not expected. Since the methylation analysis takes place under strong alkaline conditions, it seems possible that the galactose 3-phosphate linkage is split during methylation and that galactose 1-phosphate is lost during dialysis. However, Table 2.4 shows that HF treatment after methylation results in 2,3,4-trisubstituted galactose, which means that during methylation the phosphate group was still present. Nakajima *et al.* [13] found some terminally linked galactose after purifying the partially methylated, HF-treated and again methylated polysaccharide by extraction, but less than they had expected. Since they removed the excess of HF under vacuum over KOH in a desiccator, they retained the terminally linked galactose that was released during HF treatment. If, however, terminally linked galactose was split off during methylation, they would have retained the galactose as well. Therefore, the difference in recovery of terminally linked galactose reported here and reported EPS SBT 0495 [13], might be explained by differences in the purification procedure.

NMR analyses of EPS.–The ^{31}P NMR spectrum of purified EPS showed one resonance (δ -0.8) at pH 7.0, indicating the presence of a single phosphate group in a repeating unit [28]. The chemical shift of this resonance did not virtually change during titration experiments (pH 6.1-10.0), corroborating the presence of a phosphodiester [29]. The fact that after purification no phosphate monoester was found shows that during purification with CCl_3CO_2H the acid labile galactose 1-phosphate was kept intact and that all phosphate groups are present in diester form. However, it should be noticed that prolonged exposure of EPS to CCl_3CO_2H could result in degradation of the phosphodiester. In that case we observed a phosphomonoester besides a phosphodiester resonance in the ^{31}P NMR spectrum.

H_2SO_4-treated EPS gave a resonance (δ 0.1 at pH 4.0) which shifted during titration experiments (δ 3.1 at pH 8.4), which is indicative for a titratable phosphomonoester. Since only a very small phosphodiester signal was found in this sample, almost all terminally linked galactose must have been removed during H_2SO_4 treatment.

HF-treated EPS was measured as well and no resonances could be detected in ^{31}P NMR experiments, which corresponds with complete dephosphorylation of EPS.

^1H and ^{13}C NMR spectra (not shown) of dephosphorylated EPS B40 were similar to the spectra of dephosphorylated EPS SBT 0495 [13]. These results agree with the suggestion of van Kranenburg *et al.* [14], that the structure of the repeating unit of EPS B40 is identical to the structure published for EPS SBT 0495 [13].

Molecular mass and radius of gyration of (modified) EPS.–The change in hydrodynamic volume caused by the different chemical treatments of EPS B40 are illustrated in Fig. 2.3 (lines I).

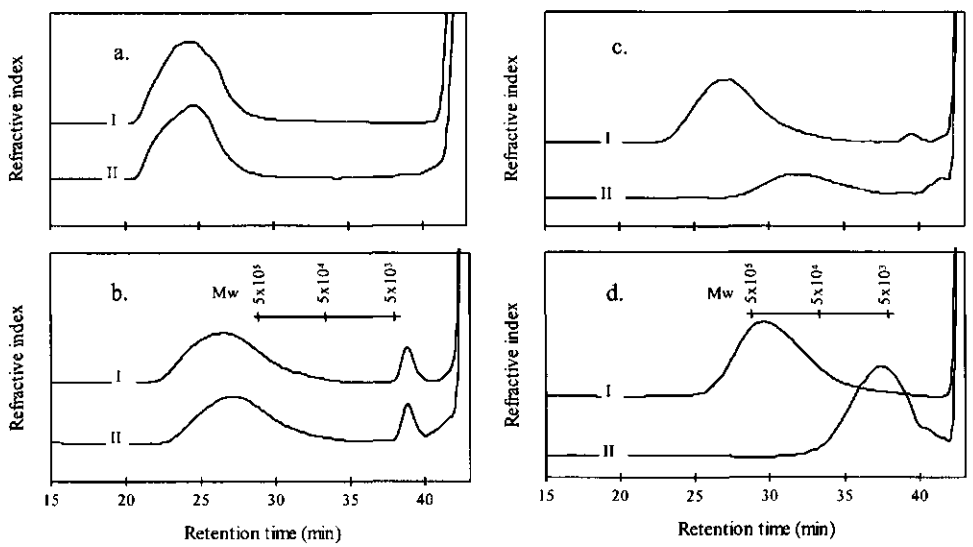

Fig. 2.3. HPSEC patterns of (modified) EPS B40 after incubation with a crude cellulase: (a.) purified EPS (b.) H_2SO_4-treated EPS (c.) NaOH-treated EPS (d.) HF-treated EPS; (I) substrate (II) substrate with enzyme. Calibration was performed by using dextrans and the molecular mass is given in g/mol.

All treatments caused a small decrease in hydrodynamic volume. To find out whether this decrease can be totally explained by the removal of substituents, native and chemically modified EPS were analysed by HPSEC/SLS to determine the molecular mass (M_n), the polydispersity (M_w/M_n) and radius of gyration (R_g). The results for purified EPS B40 were: $M_n = 6.8\times10^5$ g/mol, $M_w/M_n = 1.7$ and $R_g = 76.7$ nm and the results after H_2SO_4 treatment were: $M_n = 5.2\times10^5$ g/mol, $M_w/M_n = 1.4$ and $R_g = 67.2$ nm. The reduction in molecular mass during H_2SO_4 treatment approached the loss of all terminally linked galactose. It can therefore be concluded that this chemical modification was rather specific and that the resulting polymer might be interesting for further physical characterisation. The results of NaOH-treated EPS and HF-treated were not reliable due to problems with the solubility of modified EPS and a bad fit of the Debye plot, respectively.

Enzymic degradation of EPS.–Since enzymes can be used to modify polysaccharides very specifically, (chemically modified) EPS was incubated with various commercial enzyme preparations and analysed for degradation. In case of endo-activity, a shift in hydrodynamic volume can be observed (HPSEC), while exo-activity causes a release of sugar monomers or galactose 1-phosphate (HPAEC). Most of the tested commercial enzyme preparations showed no activity towards EPS B40 at all. There was however a crude cellulase preparation from *T. viride* (Maxazyme Cl) which showed endo-activity towards HF-treated EPS and, to a much lesser extent, towards NaOH-treated EPS (Fig. 2.3). Since (H_2SO_4-treated) EPS B40 was not degraded by this crude cellulase, it can be concluded that the removal of phosphate and/or rhamnose is essential for the enzyme to be active on this EPS. Preliminary research on the degradation products on HPAEC showed a regular series of oligomers and it seems that although a crude enzyme preparation was used only one enzyme acted very specifically. In the future, more effort will be put in the further identification of the oligosaccharides and in revealing the mode of action of this enzyme since a well-characterised enzyme can be used as a tool for characterising other polysaccharides [30].

Conclusions

As a first step in the study of the relationship between the chemical structure and the physical characteristics of EPS B40, the structure was characterised. The results indicate that EPS B40 is probably identical to the structure that was reported for EPS SBT 0495 by Nakajima *et al.*, [13]. This has been proposed before by van Kranenburg *et al.* [14] based mainly on similarities in sugar composition, phosphorus content and ^1H and ^{13}C NMR spectra.

Chemical modification of the EPS B40 with H_2SO_4, HF and NaOH appeared to be helpful not only to get information about the structure, but also to find an enzyme able to degrade modified EPS B40. This enzyme is subject of further investigation.

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Chapter 3

Endoglucanase V and a phosphatase from *Trichoderma viride* are able to act on modified exopolysaccharide from *Lactococcus lactis* subsp. *cremoris* B40

Abstract

EPS B40 from *Lactococcus lactis* subsp. *cremoris* consists of a repeating unit of →4) - [α-L-Rha*p*-(1→2)] [α-D-Gal*p*1P→3] - β-D-Gal*p*- (1→4) - β-D-Glc*p*- (1→4)-β-D-Glc*p*- (1→. A phosphatase from *Trichoderma viride* was able to release phosphate, but only after removal of rhamnosyl and galactosyl residues by mild CF_3CO_2H treatment. Purified endoV from *T. viride* was able to act on the backbone of the polymer, but only if rhamnosyl substituents and phosphate had been removed. After complete removal of phosphate and partial removal of rhamnosyl residues by HF treatment, incubation with endoV resulted in a homologous series of oligomers. Purification of these oligomers and subsequent characterisation by NMR demonstrated that endoV was able to cleave the β-(1→4) linkage between two glucopyranosyl residues when the galactopyranosyl residue towards the nonreducing end is unsubstituted. The mode of action of endoV on HF-treated EPS B40 is discussed on the basis of the subsite model described for endoV [J.-P. Vincken, G. Beldman, and A.G.J. Voragen, *Carbohydr. Res.*, 298 (1997) 299-310].

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Introduction

Some types of endocellulases (EC 3.2.1.4) are able to cleave β-(1→4)-glycosidic linkages in a variety of substrates such as cellulose, carboxymethylcellulose, (1→3), (1→4)- glucan [1,2], xylan [3,4], xyloglucan [5], mannan [6], and a deacetylated heteroglycan produced by *Pseudomonas flavescens* [7]. The cellulase complex of *Trichoderma viride* contains six endoglucanases [8]. Based on their ability to degrade xylans, some of these endoglucanases (endo IV, V and VI) have a broader substrate specificity than others (endo I, II and III) [3]. The differences in specificity of glycosyl hydrolases can be defined precisely by the use of substrates with known, and preferably regular, structures [1]. Since microbial heteropolysaccharides are almost all composed of repeating units [9] their regular structures could be very useful for the characterisation of glycosyl hydrolases.

To study the relationship between the chemical structure and the physical properties of exopolysaccharide (EPS) B40, crude enzyme preparations from the major enzyme manufacturers were screened for activity towards this EPS. It was shown that a cellulase preparation from *T. viride* was able to degrade HF-modified EPS B40 [10]. HF-treated EPS B40 consists of repeating units: probably →4)[α-L-Rha*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→, in which part of the rhamnosyl substituents (ca. 20%) have been removed by the HF treatment [10]. Although the endo-activity found was not helpful for studying the structure-function relationship of EPS, the regular structure of this EPS could be very useful for the characterisation of the enzyme. Therefore, the present study investigates the endoglucanase in the cellulase preparation that is responsible for the degradation of HF-treated EPS B40. The influence of side groups in the substrate on the hydrolysing capability of the enzyme was analysed by incubating the enzyme with chemically modified EPS B40. In addition, the homologous series of oligomeric end products derived from HF-treated EPS B40 was purified and characterised to examine the mode of action of the endoglucanase.

Experimental

EPS B40.–Crude EPS produced by *Lactococcus lactis* subsp. *cremoris* B40 [11] was kindly supplied by NIZO food research (Ede, The Netherlands). Further purification of EPS B40 was performed using CCl_3CO_2H and EtOH as described by van Casteren *et al.* [10].

HF-treated EPS B40.–EPS B40 was chemically modified by treatment with 28 M hydrofluoric acid (48 h, 0 °C). During this treatment, terminally linked galactose and phosphate are removed and as a side effect ca. 20% of the rhamnosyl substituents are also removed [10].

CF_3CO_2H-treated (and HF-treated) EPS B40.–(HF-treated) EPS B40 (3 mg) was partially hydrolysed in 2.5 mL 0.1 M CF_3CO_2H (1 h, 100 °C) to remove rhamnosyl substituents. CF_3CO_2H was then removed by repeated evaporation followed by freeze drying.

H_2SO_4-treated EPS B40.–purified EPS B40 was treated with 0.3 M H_2SO_4 (2 h, 37 °C) as described by van Casteren *et al.* [10] to remove terminally linked galactose.

Enzymes.–Maxazyme Cl from *T. viride* (Gist-brocades, Delft, The Netherlands) and endoglucanase V (endoV, EC 3.2.1.4) purified from this preparation by Beldman *et al.* [8] were used. Since the nomenclature of our endoglucanases differs from the one generally adopted in literature, endoV has been tentatively identified as being EGI [4,12].

Degradation of modified EPS B40 by Maxazyme Cl.–HF-treated EPS B40, HF- *and* CF_3CO_2H-treated EPS B40 and CF_3CO_2H-treated EPS B40 were incubated with Maxazyme Cl as described before [10]. The resulting degradation products were analysed by high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). To obtain sufficient oligomeric fragments for purification and subsequent characterisation by NMR, HF-modified EPS B40 (0.3 g) was dissolved in 150 mL 50 mM NaOAc buffer (pH 5). Maxazyme Cl (0.33 g) was dissolved and dialysed against the same buffer (final volume: 23 mL). The substrate (140 mL) was incubated (24 h, 30 °C) with dialysed enzyme solution (20 mL). The protein content was approximately 0.01% according to the procedure of Bradford [13] using bovine serum albumin as a standard. After incubation, the enzymes were denatured by heating (15 min, 100 °C). Finally, the sample was centrifuged and the supernatant was freeze dried and used for isolation of the oligosaccharides.

Incubation of HF-treated EPS B40 with endoV.–HF-treated EPS B40 in 50 mM NaOAc, pH 5.0 (0.25 mL, 2 mg/mL) was incubated (24 h, 30 °C) with purified endoV (ca. 0.5 μg protein). The sample was then heated (15 min, 100 °C), centrifuged and analysed by HPAEC and MALDI-TOF MS.

Incubation of CF_3CO_2H-treated (and HF-treated) EPS B40 with endoV.–CF_3CO_2H-treated samples (3 mg) were dissolved in 1 mL 50 mM NaOAc pH 5.0. Part of this solution (0.1 mL) was incubated (24 h, 30 °C) with purified endoV (ca. 0.2 μg protein). Then, the samples were heated (15 min, 100 °C), centrifuged and analysed by HPAEC and MALDI-TOF MS.

Release of oligosaccharides from HF-treated EPS B40 in time.–Purified endoV (ca. 0.2 μg) was added to HF-treated EPS B40 in 50 mM NaOAc pH 5.0 (1.7 mL, 2.2 mg/mL). During incubation at 20 °C, a sample was taken each hour for analysis by HPAEC. The amount of an oligomer released in a certain time interval, was expressed as a percentage of the PAD response of this oligomer peak after incubation for 20.5 h.

Size-exclusion chromatography.–The oligomers obtained after preparative degradation of HF-treated EPS B40 with Maxazyme Cl were purified using columns (100×2.6 cm, i.d.) of BioGel P-6 and BioGel P-2 (BioRad). After the sample (at most 120 mg per run) had been applied onto the column, elution with distilled water (0.5 mL/min) was performed at 60 °C. The refractive index was monitored on-line (Shodex RI-72 detector) and appropriate fractions were pooled. The purity of the pools was checked using HPAEC and if necessary pools were rechromatographed.

HPAEC analysis of the digests.–Degradation products were analysed on a CarboPac PA-1 column using HPAEC equipment as described earlier [10]. After equilibration with 16 mM NaOH, 20 μL sample was injected and the elution program was started: 0→20 min, 16 mM NaOH isocratic; 20→25 min, 0.1 M NaOH isocratic; 25→65 min, linear gradient of 0→0.4 M NaOAc in 0.1 M NaOH; 65→70 min, 1 M NaOAc in 0.1 M NaOH isocratic; followed by a conversion step to NaOH (70→75 min, 0.1 M NaOH isocratic) and re-equilibration (75→90 min, 16 mM NaOH isocratic).

HPSEC analysis of CF_3CO_2H-treated polymers.–High-performance size-exclusion chromatography (HPSEC) was performed as has been described by van Casteren *et al.* [10], although this time a Spectra System P1000 pump and AS 3000 autosampler were used.

MALDI-TOF MS analysis of the digest.–The digest of modified EPS B40 (10 μL) was desalted with dowex (AG 50W-X8 Resin, BioRad 143-5441) and then mixed on plate (1 μL) with 1 μL matrix. The matrix was made by mixing isocarbostyril (3 mg), 2,5-

dihydroxybenzoic acid (9 mg), acetonitril (0.3 mL) and distilled water (0.7 mL). MALDI-TOF MS was performed using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, USA) in the positive mode. The negative mode was used as well to examine whether phosphate-containing oligosaccharides were released. The laser intensity was set at 2,000 which equals 7.5 μJ per pulse. The pulse delay time was 200 ns, the accelerating voltage was 12,000 V, the grid voltage was 7,200 V and the guide wire voltage was 9.6 V. The instrument was used in the reflector mode. The mass spectrometer was calibrated using a mixture of galacturonic acid oligomers. Each run, the calibration was checked by analysing a sample of glucosyl oligomers.

Phosphate analysis.–The relative amount of bound and free phosphate in the samples was determined by comparing the samples with and without destruction in 72% $HClO_4$ (20 min, 180 °C). After dilution, the samples were analysed as described by Chen *et al.* [14].

Absolute configurations of monosaccharides.–The absolute configurations of the monosaccharides were determined as described by Gerwig *et al.* [15]. The GC-FID analysis of the trimethylsilated (–)-2-butyl glycosides was performed in a Carlo Erba HRGC 5160 gas chromatograph, equipped with a CP-Sil 5 CB column (25 m×0.32 mm, Chrompack). The temperature program was: 135 °C→160 °C at 0.5 °C/ min; 160 °C→200 °C at 10 °C/min. The injection-port and detector temperatures were 200 °C and 250 °C, respectively. The He flow-rate was 3 mL/min and the samples (ca. 0.05 μL) were injected directly on the column without stream splitter.

NMR Spectroscopy.–Prior to NMR analysis, the samples were exchanged three times in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, USA) with intermediate freeze drying. Finally, samples were dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at probe temperatures of 27 °C (oligosaccharides) or 70 °C (polysaccharide) on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts are expressed in ppm relative to internal acetone: δ 2.225 for ^1H and δ 31.077 for ^{13}C. For small sample amounts Shigemi tubes (Campro Scientific, Veenendaal, The Netherlands) with a sample volume of 190 μl were used.

In 1D ^1H NMR spectra, suppression of the HOD signal was achieved by using presaturation during relaxation delay for 1 s. Proton-decoupled ^{13}C spectra were recorded at 125.770 MHz. For 1D ^1H and ^{13}C NMR spectra, data sets of 16,384 and 32,768 data points were recorded, respectively.

2D COSY spectra were acquired in the magnitude mode. 2D TOCSY and ROESY spectra were basically acquired as described by Fransen *et al.* [16] using the time-proportional phase increment (TPPI) method [17]. For 2D HMBC spectra [18] a standard gradient-enhanced 2D-heteronuclear proton detected multiple-quantum coherence pulse-sequence (HMQC) delivered by Bruker was changed into a HMBC sequence by setting the delay between the first proton and first carbon pulse to 60 ms. For all homonuclear 2D experiments, 512 experiments of 2048 data points were acquired with 32-320 scans per increment; the 2D HMBC spectra were acquired in 1024 experiments of 2048 data points.

Time domain data were multiplied by phase-shifted (squared-)sine-bell functions or with Lorentzian-to-Gaussian multiplication. After zerofilling and Fourier transformation data sets of 2048×1024 or 2048×2048 points were obtained, which were baseline corrected when necessary.

Results

Degradation of modified EPS B40 by Maxazyme Cl. HF-treated EPS B40 was incubated with Maxazyme Cl and the digest was analysed by HPAEC. Fig. 3.1 shows that enzymic digestion resulted in a series of oligomers eluting between 35 and 60 min. The peaks at 12, 14 and 27 min originated from the enzyme preparation. Using on-line HPAEC/MS [19] masses could be assigned to the HPAEC peaks. However, since the HPAEC/MS setup was not always available, masses of the released oligomers throughout the research were monitored using MALDI-TOF MS.

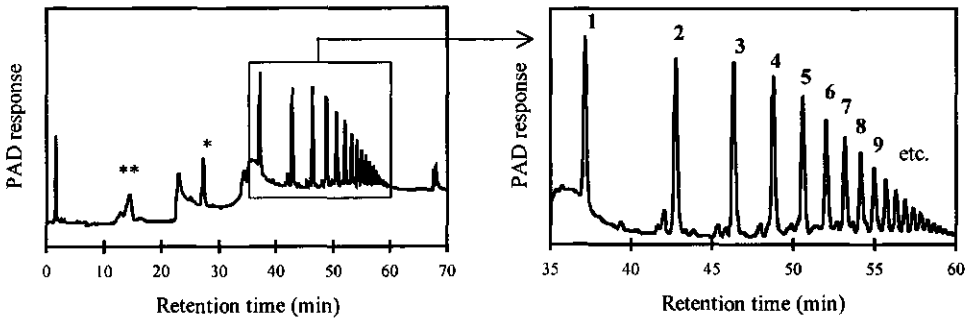


Fig. 3.1. HPAEC elution profile of a digest of HF-treated EPS B40 after incubation with Maxazyme Cl. *, peaks that originate from the enzyme preparation. The numbering of the oligomer peaks in the enlarged part of the chromatogram indicates the amount of 'repeating units' within the backbone of the oligomer concerned and this numbering is used throughout this chapter.

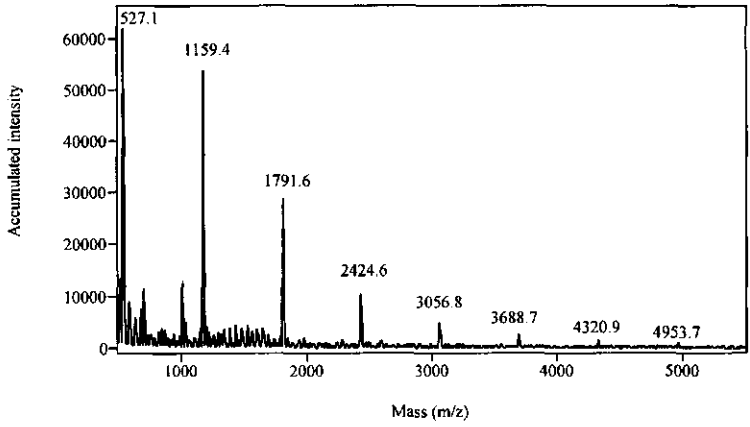


Fig. 3.2. MALDI-TOF mass spectrum of the digest of HF-treated EPS B40 after incubation with Maxazyme Cl. The masses could be assigned to the HPAEC peaks in Fig. 3.1: 527.1 = trimer = **1**, 1159.4 = heptamer = **2**, 1791.6 = undecamer = **3**, etc.

Fig. 3.2 shows the MALDI-TOF mass spectrum of HF-treated EPS B40 after incubation with Maxazyme Cl. A homologous series of sodiated oligomers was detected, corresponding to the results of HPAEC/MS. Therefore, the masses in Fig. 3.2 could be assigned to the oligomer peaks in Fig. 3.1: **1** = 527.1 g/mol; **2** = 1159.4 g/mol; **3** = 1791.6 g/mol, etc. Since MS shows masses of sodiated oligomers, the mass of oligomer **1** corresponds to a trimer of hexoses. Oligomer **2** represents a heptamer of 6 hexoses and 1 deoxyhexose. Oligomer **3** is an undecamer of 9 hexoses and 2 deoxyhexoses, etc. Thus, it could be concluded that the enzyme preparation was able to release a series of oligomers starting with a trimer with an increment of 632 g/mol: three hexoses + [three hexoses + one deoxyhexose]$_n$. Considering the sugar composition of HF-treated EPS B40 [10], the hexoses must be glucose and galactose and the deoxyhexose rhamnose.

HF-treated EPS B40 consists of repeating units: →4)[α-L-Rha*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→ (*vide infra*), in which part of the rhamnosyl substituents (ca. 20%) have been removed during the HF treatment [10]. The absolute configuration of the sugar residues was confirmed. Since all oligomers released during enzyme incubation contained one 'repeating unit' without rhamnosyl substitution, apparently the enzyme is only able to cleave the polymer if rhamnosyl residues have been removed. To confirm this, HF-treated EPS B40 was partially hydrolysed using CF_3CO_2H to remove more rhamnosyl residues. It was calculated from the amount of rhamnose released (HPAEC, not shown) that after this treatment ca. 60% of the repeating units were free of rhamnosyl substitution. Although HPSEC analysis after treatment with CF_3CO_2H showed partial depolymerisation of the EPS, no significant amounts of oligomers were released during this acidic treatment (HPAEC). Incubation of HF- *and* CF_3CO_2H-treated EPS B40 with Maxazyme Cl and analysis by HPAEC showed that there was an increase of oligomer **1** (×6) and oligomer **2** (×2), a decrease of oligomers **3** and **4** and disappearance of oligomer **5** and larger oligomers in comparison with the products of enzyme incubation with HF-treated EPS B40. These results show that indeed the enzyme is able to release smaller oligomers when more rhamnosyl substituents have been removed.

Maxazyme Cl appeared not to be active on EPS B40 [10], which consists of a repeating unit (*vide infra*): →4)[α-L-Rha*p*-(1→2)][α-D-Gal*p*1P→3]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→. One of the reasons for this is the presence of rhamnosyl substituents. However, it is possible that the galactose 1-phosphate side groups also obstruct enzyme action. To examine whether this is the case, EPS B40 was partially hydrolysed using CF_3CO_2H. During this treatment, not only ca. 60% of rhamnosyl residues are removed but also the galactosyl residues terminally linked to phosphate, since galactose 1-phosphate is sensitive towards dilute acid [10,20]. Incubation of CF_3CO_2H-treated EPS B40 with Maxazyme Cl did not result in a significant release of phosphate-containing oligomers as was determined by using HPAEC and MALDI-TOF MS. Surprisingly, oligomer **1** (trimer) was released, whereas oligomers **2**, **3**, **4**, etc. were not detected (data not shown). The release of oligomers free of phosphate was unexpected and indicates that either the galactose 3-phosphate linkage was hydrolysed upon CF_3CO_2H treatment or the crude enzyme preparation contains a phosphatase that is able to cleave this linkage. This was investigated further using the purified enzyme and is discussed below.

Degradation of modified EPS B40 by endoV.–Incubation of HF-treated EPS B40 with endoV purified from Maxazyme Cl released the same oligomers, identified by HPAEC and MALDI-TOF MS, as Maxazyme Cl, indicating that only one enzyme was involved in this

degradation. Incubation of HF- *and* CF_3CO_2H-treated EPS B40 with endoV also showed the same degradation pattern, identified by HPAEC and MALDI-TOF MS, as Maxazyme Cl. Since all oligomers released during enzyme incubation contained one 'repeating unit' without rhamnosyl substitution, it was confirmed that endoV is blocked by rhamnosyl residues in EPS B40.

To examine whether endoV is additionally blocked by the phosphate groups on EPS B40, CF_3CO_2H-treated EPS B40 was incubated with endoV. The resulting HPAEC pattern showed no oligomer peaks and the MALDI-TOF mass spectrum did not show a series of 'neutral' oligomers or phosphate-containing peaks. These results indicate that indeed endoV is blocked by phosphate on EPS B40. The fact that endoV did not release oligomer **1** from CF_3CO_2H-treated EPS B40 while Maxazyme Cl did, suggests that the galactose 3-phosphate linkage was not hydrolysed during the CF_3CO_2H treatment. It is likely then, that Maxazyme Cl contains a phosphatase able to cleave this linkage. Since rhamnosyl residues had already been removed during the CF_3CO_2H treatment, the release of phosphate by such an enzyme would make the substrate accessible to endoV, explaining the release of oligomer **1**.

Degradation of modified EPS B40 by a phosphatase.–The indication for the presence of a phosphatase in Maxazyme Cl was supported by the amount of free phosphate in the digests, expressed as percentage of the total phosphate content: during incubation of CF_3CO_2H-treated EPS B40 with Maxazyme Cl the amount of free phosphate increased from <10% to ca. 60%. Maxazyme Cl itself did not contribute to the total amount of phosphate. The fact that there was almost no free phosphate in native EPS B40 after treatment with Maxazyme Cl suggests that the phosphatase only acts if terminally linked galactose and/or rhamnose is absent from the EPS. Treatment of EPS B40 with dilute H_2SO_4 (37 °C) results in a release of galactose, since the galactose 1-phosphate linkage is sensitive towards dilute acid [10]. During this treatment, the rhamnosyl linkages were not hydrolysed. Incubation of H_2SO_4-treated EPS B40 with Maxazyme Cl did not result in a release of phosphate indicating that phosphatase in Maxazyme Cl is hindered by rhamnosyl residues in EPS B40. Since we were not able to modify EPS B40 to generate a substrate without rhamnosyl residues but still carrying terminally linked galactose moieties, we were not able to check whether the phosphatase is also hindered by the presence of terminally linked galactose.

Release of oligosaccharides from HF-treated EPS B40 in time.–Fig. 3.3 shows the time course of the release of oligomers **1**, **7** and **11** from HF-treated EPS B40 during incubation with endoV. The oligomers shown are representative of the trend for other oligomers released: the larger the oligomers the faster they accumulate. For oligomers larger than **11**, the amount released after 12-18 h was higher than after 20 h. This indicates that large oligomers were not only released but also further degraded during incubation. Due to a decreasing resolution of the HPAEC analysis with increasing size of oligomers there is no discrimination between end products in which one 'repeating unit' is free of rhamnosyl residues and intermediate products with, for example, two 'repeating units' free of rhamnosyl residues. Thus, the results in Fig. 3.3 can be explained by the fact that before reaching the end point of the incubation, the HPAEC peaks of large oligomers contain both intermediate products and end products.

As stated above, incubation of HF-treated EPS B40 with endoV resulted in a series of oligomers starting with a trimer: three hexoses + [three hexoses + one deoxyhexose]$_n$. Since the exact structure of these oligomers is still unknown, no information is provided on the precise mode of action of endoV towards this substrate. To enable identification of the oligomers, the substrate was treated with the enzyme on a larger scale and the oligomers

obtained purified and analysed by NMR. Since the availability of pure endoV was limited and since incubation with Maxazyme Cl resulted in the same series of oligomers, Maxazyme Cl was used for the preparative incubation. The oligomers were purified using size-exclusion chromatography and the effectiveness confirmed by HPAEC.

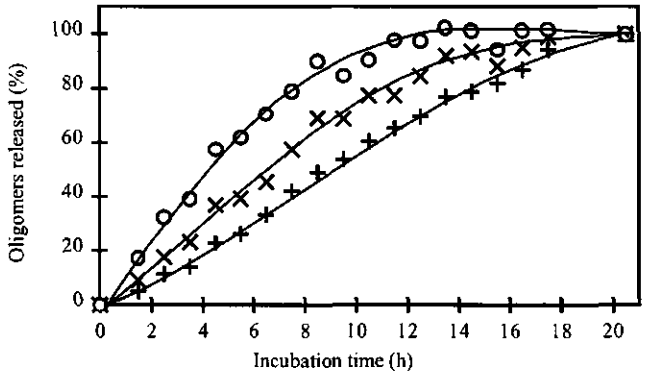


Fig. 3.3. Release of oligomers **1**, **7** and **11** (DP 3, 27, 43) from HF-treated EPS B40 in time during incubation with endoV. The amounts of released oligomers are expressed as a percentage of the PAD response of this oligomer peak after incubation for 20.5 h. +, oligomer 1 (Fig. 3.1); ×, oligomer **7** (Fig. 3.1); O, oligomer **11** (Fig. 3.1). It should be noted that the decreasing resolution of the HPAEC system with increasing size of oligomers influenced the results (see text).

NMR spectroscopy of trimer (1).–The 1D ^{1}H-NMR spectrum of the trimer (**1**) showed 4 resonances in the anomeric region, which were designated **A** H-1 - $\mathbf{C}^{\alpha/\beta}$ H-1 (Fig. 3.4a). The coupling constant of the narrow doublet at δ 5.22 ($\mathbf{C}^{\alpha}$ H-1, $^3J_{1,2}$ 3.8 Hz) suggests the presence of an α-hexopyranosyl residue. The coupling constants of the broader doublets at δ 4.67 (**A** H-1, $^3J_{1,2}$ 8.1 Hz), 4.66 ($\mathbf{C}^{\beta}$ H-1, $^3J_{1,2}$ 8.1 Hz) and 4.48 (**B** H-1, $^3J_{1,2}$ 7.8 Hz) indicate the presence of β-hexopyranosyl residues. The intensity ratio of **A** H-1:**B** H-1:$\mathbf{C}^{\alpha}$ H-1:$\mathbf{C}^{\beta}$ H-1 was 1:1:0.3:0.7. Therefore, the resonances at δ 5.22 and 4.66 were assigned to the sugar residue at the reducing end (residue **C**) since both anomers exist because of mutarotation.

The 2D COSY, TOCSY and ROESY measurements (not shown) allowed the assignment of the protons as is given in Table 3.1. The resonances of **A** H-1 and $\mathbf{C}^{\beta}$ H-1 had correlations to H-2, 3, 4, 5, 6a, 6b in the TOCSY spectrum, identifying glucose residues. The resonance of **B** H-1 only showed TOCSY cross-peaks to H-2, 3, 4; due to inefficient magnetisation transfer through the small $^3J_{3,4}$ and $^3J_{4,5}$ couplings no correlations to H-5 could be seen [16,21]. This, combined with the characteristic resonance position of H-4, allowed the assignment of residue **B** as a galactosyl residue.

Methylation analysis of HF-treated EPS B40 demonstrated the presence of 4-substituted glucosyl residues, 2,4-disubstituted galactosyl residues and terminally linked rhamnosyl residues [10]. For the trimer, the differences in the chemical shifts (Table 3.1) of residue **A** H-4 (Δδ -0.005), **B** H-4 (Δδ 0.235), $\mathbf{C}^{\alpha}$ H-4 (Δδ 0.255) and $\mathbf{C}^{\beta}$ H-4 (Δδ 0.255) compared to the chemical shifts for the corresponding aldohexoses reported by Bock and Thøgersen [22]

indicate that **B** and $\mathbf{C}^{\alpha/\beta}$ are 4-substituted but residue **A** is not. The 2D ROESY spectrum showed two intense inter-residual cross-peaks (**A** H-1, **B** H-4 and **B** H-1, $\mathbf{C}^{\beta}$ H-4) resulting in structure **1** shown in Fig. 3.4a. Unfortunately, the results could not be confirmed with a HMBC experiment due to the low signal/noise ratio as result of the low sample concentration.

Table 3.1
^{1}H NMR chemical shifts[a] of the trimer (**1**) isolated from HF-modified EPS B40 after treatment with Maxazyme Cl as determined from COSY, TOCSY and ROESY.

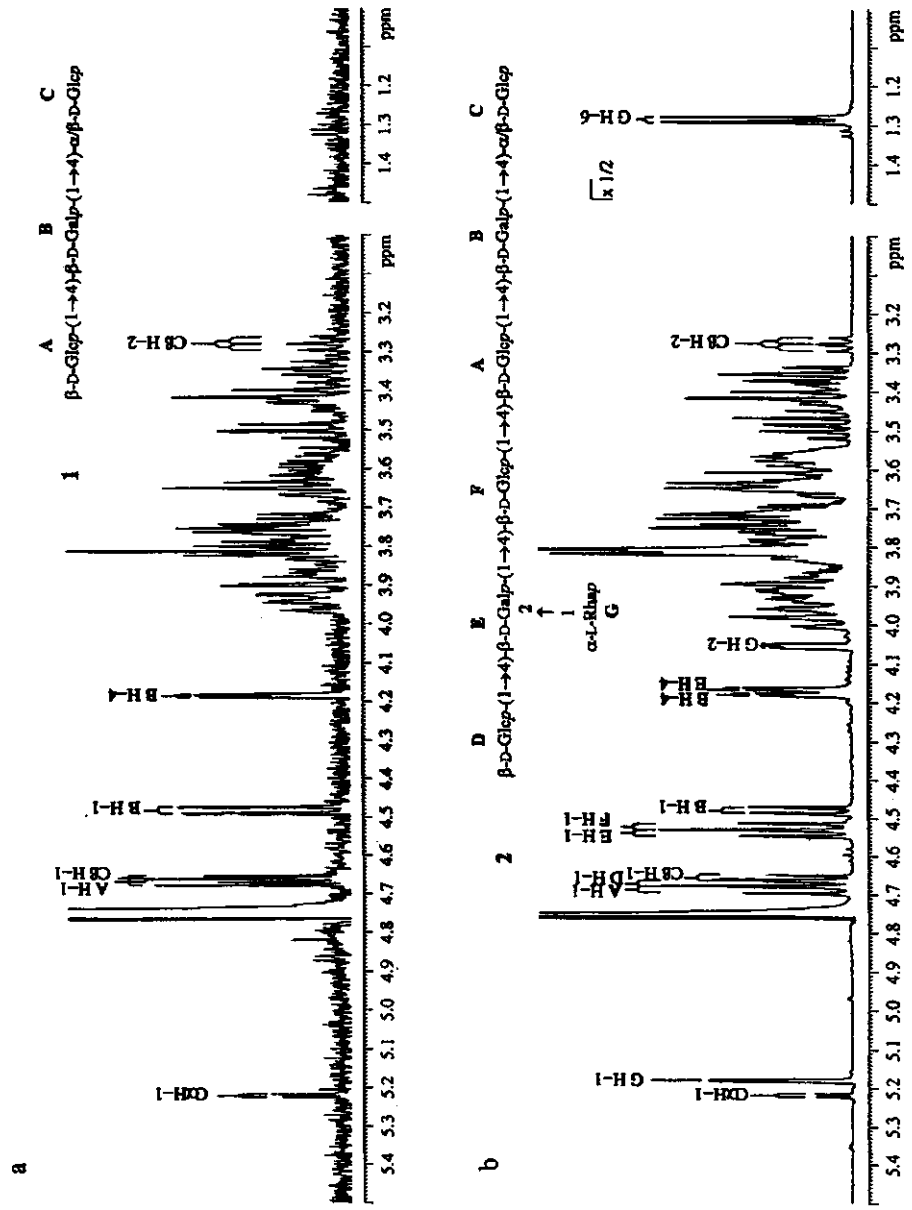
Residue (1)	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	4.67	3.34	3.50	3.40	3.43	3.91	3.73
B	4.48	3.61	3.77	4.18	3.80	[b]	[b]
$\mathbf{C}^{\alpha}$	5.22	3.57	3.83	3.65	3.95	[c]	[c]
$\mathbf{C}^{\beta}$	4.66	3.28	3.63	3.66	3.59	3.95	3.80

[a] in ppm relative to the signal of acetone at δ 2.225; [b] between 3.85 and 3.70; [c] between 3.96 and 3.82.

Since the incubation of HF-treated EPS B40 with Maxazyme Cl resulted in the release of oligomer **1** (amongst others), endoV must have cleaved the polymer between two glucosyl residues. As shown, endoV is hindered by the presence of rhamnosyl residues in the polymer and all oligomers released contained exactly one repeating unit free of terminally linked rhamnose. These results suggest that endoV releases oligomers containing an unsubstituted 'repeating unit' at the reducing side *or* the nonreducing side of the glycosidic linkage cleaved. This was investigated further by NMR analysis of the heptamer.

NMR spectroscopy of heptamer (2).–The 1D ^{1}H-NMR spectrum of the heptamer (**2**) showed the presence of eight resonances in the anomeric region, which were designated **A-G** (Fig. 3.4b). The coupling constants of the resonance at δ 5.23 ($\mathbf{C}^{\alpha}$ H-1, $^{3}J_{1,2}$ 3.8 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 169 Hz) suggested the presence of an α-hexopyranosyl residue. The coupling constants of the resonances at δ 4.69 (**A** H-1, $^{3}J_{1,2}$ 8.7 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 166 Hz), 4.67 (**D** H-1, $^{3}J_{1,2}$ 8.2 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 163 Hz), 4.66 ($\mathbf{C}^{\beta}$ H-1, $^{3}J_{1,2}$ 7.9 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 163 Hz), 4.54 (**E** H-1, $^{3}J_{1,2}$ 8.2 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 166 Hz), 4.52 (**F** H-1, $^{3}J_{1,2}$ 8.4 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 166 Hz), and 4.48 (**B** H-1, $^{3}J_{1,2}$ 7.8 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 163 Hz) indicate the presence of β-hexopyranosyl residues. The resonances at δ 5.23 and 4.66 showed an intensity ratio of 0.3:0.7 (the intensities of the other anomeric resonances being 1) and were assigned to the sugar at the reducing end (residue $\mathbf{C}^{\alpha/\beta}$). The rhamnosyl residue **G** (**G** H-1 δ 5.19, $^{3}J_{1,2}$ 1.4 Hz, $^{1}J_{C\text{-}1,H\text{-}1}$ 175 Hz) showed a high-field doublet at δ 1.28 (**G** H-6, $^{3}J_{5,6}$ 6.3 Hz) arising from the methyl group.

Using the ^{1}H NMR data, the 2D COSY (not shown) and TOCSY (not shown) measurements a complete assignment of the rhamnosyl (residue **G**) protons, starting from the methyl doublet (Table 3.2) was possible. The chemical shift of **G** H-5 at δ 3.90 strongly suggests an α-configuration for residue **G** [23]. From the 2D COSY, TOCSY and ROESY measurements (not shown), most protons of the other residues (**A-F**) could also be assigned (Table 3.2). The resonances of **B** H-1 and **E** H-1 only showed cross-peaks to H-2, 3, 4 in the TOCSY spectra, whereas the other anomeric protons had correlations to H-2, 3, 4, 5, 6a, 6b. Together with the characteristic resonance positions of **B** H-4 and **E** H-4, residue **B** and **E** were assigned to galactosyl residues. Residues **A**, $\mathbf{C}^{\alpha/\beta}$, **D** and **F** were identified as glucosyl


a
b
A
B
C
D
E
F
G
1
2
β-D-Glcp-(1→4)-β-D-Galp-(1→4)-α/β-D-Glcp
β-D-Glcp-(1→4)-β-D-Galp-(1→4)-β-D-Glcp-(1→4)-β-D-Galp-(1→4)-α/β-D-Glcp
α-L-Rhap
x 1/2
ppm

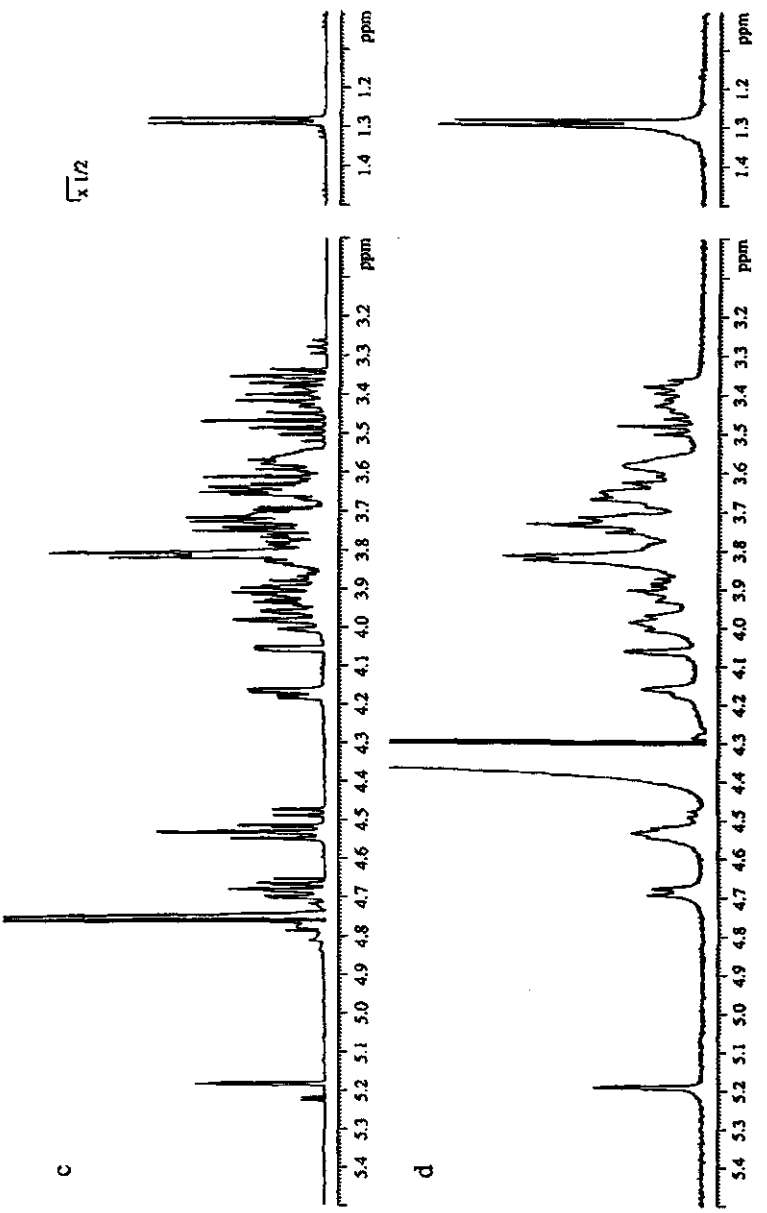


Fig. 3.4. 500-MHz ^{1}H NMR spectrum of trimer (1) (a), heptamer (2) (b), undecamer (3) (c) and HF-treated EPS B40 (d), recorded in D_2O at 27 °C (oligosaccharides) or 70 °C (polysaccharide).

residues. The differences in the chemical shifts (Table 3.2) of residue **A** H-4 (Δδ 0.245), **B** H-4 (Δδ 0.235), $\mathbf{C}^{\alpha}$ H-4 (Δδ 0.245), $\mathbf{C}^{\beta}$ H-4 (Δδ 0.255), **D** H-4 (Δδ -0.005), **E** H-4 (Δδ 0.225) and **F** H-4 (Δδ 0.345) compared to the chemical shifts of the corresponding aldohexoses reported by Bock and Thøgersen [22], suggest that all of these residues except for residue **D** are substituted at H-4. As a consequence, **D** was assigned to the sugar residue at the nonreducing end of the heptamer. The relative large chemical shift of **E** H-2 (δ 3.72) compared to **B** H-2 (δ 3.62) suggests that galactosyl residue **E**, unlike residue **B**, is substituted at O-2.

Table 3.2
^{1}H NMR chemical shifts[a] of the heptamer (**2**) isolated from HF-modified EPS B40 after treatment with Maxazyme Cl as determined from COSY, TOCSY and ROESY.

Residue (**2**)	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	4.69	3.38	3.65	3.65	3.56	3.97	3.82
B	4.48	3.62	3.78	4.18	3.81	n.d.[b]	n.d.
$\mathbf{C}^{\alpha}$	5.23	3.57	3.83	3.65	3.94	n.d.	n.d.
$\mathbf{C}^{\beta}$	4.66	3.28	3.63	3.66	3.60	3.95	3.80
D	4.67	3.35	3.51	3.40	3.43	3.91	3.74
E	4.54	3.72	3.92	4.17	n.d.	n.d.	n.d.
F	4.52	3.35	3.61	3.75	3.57	4.00	3.79
G	5.19	4.06	3.70	3.47	3.90	1.28	1.28

[a] in ppm relative to the signal of acetone at δ 2.225; [b] not determined.

Comparison of the proton chemical shifts of **1** (Table 3.1) and **2** (Table 3.2) shows close similarities for residues designated with the same letter, except for residue **A**. This exception was caused by the fact that **A** is a 4-substituted glucosyl residue in **2** while it is a terminally linked glucosyl residue in **1**. The designations used are **A** for a glucosyl residue linked to an unsubstituted galactosyl residue and **D** for a glucosyl residue linked to a substituted galactosyl residue (*vide infra*). The consequence of this designation is that residues with similar chemical shifts (**A** in **1** and **D** in **2**; both glucosyl residues at the nonreducing end) are designated differently.

The 1D ^{13}C-NMR spectrum (not shown) of **2** showed eight anomeric signals. This is in agreement with the heptasaccharide since two resonances were expected for residue **C**. Based on their chemical shifts, the C-1 signals at δ 96.6 and 92.7 were assigned to the glucosyl residue at the reducing end ($\mathbf{C}^{\beta}$ and $\mathbf{C}^{\alpha}$, respectively). The CH_3 signal of the rhamnosyl residue (**G**) was observed at δ 17.3 and the C-6 signals of the other residues were found between δ 62.0 and 60.0. These positions of the C-6 signals are in favour of the absence of 6-substituted hexosyl residues and the occurrence of pyranose rings only (comparison of chemical shifts: [22]).

The 2D ROESY spectrum showed intense inter-residual cross-peaks between: **G** H-1, **E** H-2; **A** H-1, **B** H-4; **D** H-1, **E** H-4; **B** H-1, $\mathbf{C}^{\alpha/\beta}$ H-4 or **A** H-4; **F** H-1, $\mathbf{C}^{\alpha/\beta}$ H-4 or **A** H-4. The correlation between **E** H-1 and **F** H-4 could only be assigned tentatively, due to overlap in the spectra. The 2D ^{13}C-^{1}H HMBC spectrum of **2** (Fig. 3.5) allowed the complete assignment of the anomeric ^{13}C signals: δ 104.6: **D** C-1; 104.3: **A** C-1; 103.7: **B** C-1; 103.3: **F** C-1; 101.8: **G**

C-1; 101.2: **E** C-1; 96.6: $\mathbf{C}^{\beta}$ C-1 and 92.7: $\mathbf{C}^{\alpha}$ C-1. Most other ^{13}C resonances could be assigned as well. Using the assignments of the ^{13}C and ^{1}H signals in the HMBC spectrum, the long range correlations observed across the glycosidic linkages could be interpreted: **D** C-1, **E** H-4; **E** C-1, **F** H-4; **F** C-1, **A** H-4; **A** C-1, **B** H-4; **B** C-1, $\mathbf{C}^{\alpha/\beta}$ H-4; and **G** C-1, **E** H-2. The possibility of having confused the assignments of **A** H-4 and $\mathbf{C}^{\alpha/\beta}$ H-4 was ruled out since the strong interaction between **A** C-1 and **B** H-4 excludes interaction between **B** C-1 and **A** H-4. The correlations in the HMBC and ROESY spectra determined the complete monosaccharide sequence of the heptamer (**2**) resulting in the structure shown in Fig. 3.4b.

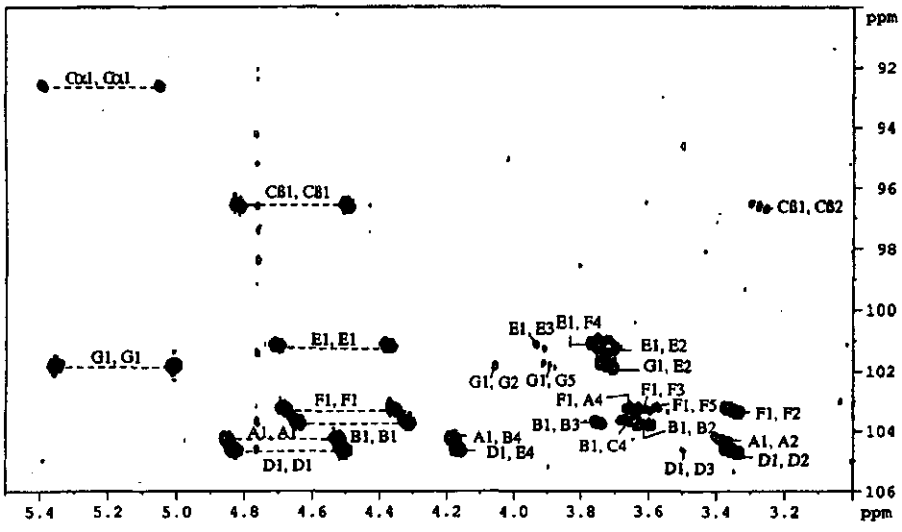


Fig. 3.5. Partial 500-MHz 2D ^{13}C-^{1}H HMBC NMR spectrum of the heptamer (**2**) recorded in D_2O at 27 °C. The code E1,F4 corresponds to the coupling between **E** C-1 and **F** H-4, etc.

NMR spectroscopy of undecamer (3) and HF-treated EPS B40. In Fig. 3.4c and d, the 1D ^{1}H-NMR spectra of the undecamer (c) and HF-treated EPS B40 (d) are shown. The fact that a homologous series of oligomers was released makes it reasonable to extrapolate the results from **1** and **2** to the undecamer and the polymer. Comparison of the spectra from the heptamer (Fig. 3.4b), the undecamer (Fig. 3.4c) and the polymer (Fig. 3.4d) shows a gradual decrease in intensity of the resonances from the anomeric protons of sugar residues **A, B,** $\mathbf{C}^{\alpha/\beta}$ and **D** with increasing degree of polymerisation. In the undecamer, a new anomeric resonance was present (δ 4.69; having partial overlap with **A** H-1) arising from the 4-substituted residue **D**. If we extrapolate the results from the oligomers to the polymer, the latter consists of sugar residues **D**, **E**, **F**, and **G**, confirming the proposed structure. In the spectrum of the polymer, low intensities of unsubstituted galactosyl residues (**B**) can be seen, which agrees with the partial removal of rhamnosyl substituents upon HF modification.

The NMR results of (the oligomers of) HF-treated EPS B40 together with the analysis of the absolute configuration prove the strong suggestion [10,24] that EPS B40 has the same chemical structure as EPS SBT 0495 [25]. However, in HF-modified EPS our assignment of the proton signals of the β-D-glucopyranosyl residues was different from the assignment reported by Nakajima *et al.* [25], whereas the anomeric ^{13}C assignments were similar. Based on our assignment of the protons of the trimer and the heptamer, we cannot conclude other than that the proton chemical shifts of residue **F** (H-1 δ 4.52, etc.) belong to the glucosyl residue designated as Glc *B* by Nakajima *et al.* [25] (H-1 δ 4.653, etc.). Furthermore, the proton chemical shifts of 4-substituted glucosyl residue **D** (H-1 δ 4.69 in the undecamer) belong to the glucosyl residue designated as Glc *A* by Nakajima *et al.* [25] (H-1 δ 4.501, etc.).

Discussion

The results of the NMR analyses of the trimer (**1**) and the heptamer (**2**) demonstrated that endoV was able to cleave the β-(1→4) linkage between two glucosyl residues, resulting in oligomers containing an unsubstituted galactosyl residue adjacent to the glucose at the reducing side of the linkage cleaved. Based on the specific series of oligomers released by endoV and on the NMR results of **1** and **2**, the mode of action of endoV towards HF-treated EPS B40 can be summarised as is shown in Fig. 3.6. It should be realised that the relative amount of unsubstituted galactosyl residues drawn in this figure is too high; it was measured to be ca. 20% [10]. Therefore, the possibility for the liberation of larger oligomers (Fig. 3.1 and 3.2) is not depicted in Fig. 3.6. Nevertheless, Fig. 3.6 clearly shows that endoV is *not* able to release oligomers with a rhamnosyl-containing 'repeating unit' at the reducing end, which is important information about the mode of action of endoV towards this substrate.

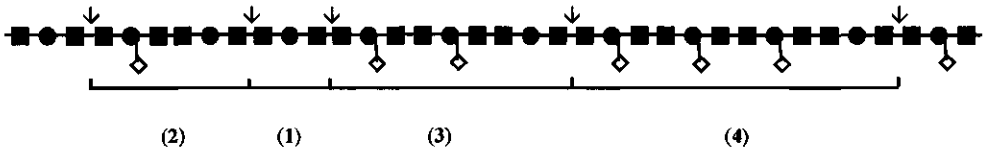


Fig. 3.6. Mode of action of endoV towards HF-treated EPS B40. In order to show the diversity of oligomers, the relative amount of rhamnosyl substitution drawn in the figure is lower than has been measured in the HF-treated polymer. ■, β-^{D}-Glc*p*-(1→4); ●, β-^{D}-Gal*p*-(1→4); ◊, α-^{L}-Rha*p*-(1→2). Sites of endoV attack are indicated by arrows. The resulting oligomers in this example are: trimer (**1**), heptamer (**2**), undecamer (**3**) and pentadecamer (**4**).

Høj *et al.* [2] suggested that the substrate specificity of glycosyl hydrolases is determined by their ability to bind different polysaccharides in their substrate-binding sites and by the position of the glycosidic linkage in relation to the catalytic amino acids. Thus, the first step in enzymic hydrolysis of polysaccharides can be envisaged as binding of several glycosyl residues with an array of subsites. The subsite structure of endoV from *T. viride* (Fig. 3.7a) has been elucidated according to the method of Suganuma *et al.* [26] using homologous

cellodextrins [12]. Subsite affinity is defined as the decrease in free energy upon interaction of an enzyme subsite with a glycosyl residue. Each subsite interacts with one sugar residue. By fitting HF-treated EPS B40 along the subsites in a way that endoV is able to cleave between the adjacent (1→4)-linked glucosyl residues (Fig. 3.7a and b) it is remarkable that the subsites with the highest affinity (-II and II), at least for glucosyl residues, interact with galactosyl residues. Since D-glucose and D-galactose are C-4 epimers, it can be concluded that the subsites -II and II are tolerant of the different configurations of the hydroxyl group at C-4. The ability of other glucanases to accept epimeric monosaccharides in their binding sites has previously been recognised for *Rhizopus arrhizus* (1→3)-β-glucan endohydrolase (EC 3.2.1.6) which also recognises galactose [27] and mannose [28]. The binding of HF-treated EPS B40 with the subsites of endoV is not hindered by the presence of a rhamnosyl residue linked to the galactosyl residue at subsites II and V, since endoV is still able to cleave (Fig. 3.7a and b). As described above, endoV is not able to release oligomers with a rhamnosyl substitution in the 'repeating unit' at the reducing end. In Fig. 3.7c, HF-treated EPS B40 is fitted along the subsites in a way that endoV is not able to cleave between the glucosyl residues. It is clear that endoV is hindered by the presence of a rhamnosyl residue linked to the galactosyl residue at subsite -II.

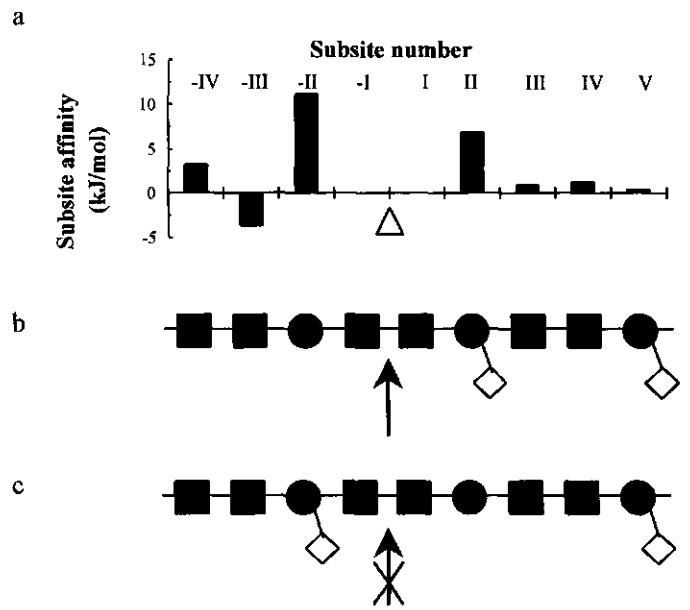


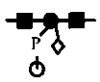
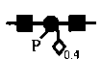
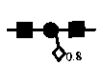
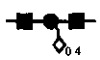
Fig. 3.7. Subsite interaction of endoV with HF-treated EPS B40. (a) Simplified histogram of subsite interaction energy with Glc residues of cellodextrins for endoV of *T. viride* as described by Vincken *et al.*, [12]. (b) HF-treated EPS B40 fitted in the subsite of endoV in a way that cleavage will occur. (c) HF-treated EPS B40 fitted in the subsite of endoV in a way that cleavage will *not* occur. Δ, catalytic group; ■, β-D-Glc*p*-(1→4); ●, β-D-Gal*p*-(1→4); ◊, α-L-Rha*p*-(1→2). Sites of endoV attack are indicated by arrows.

The mode of action of endoV towards potato xyloglucan as described by Vincken *et al.* [5] indicates that endoV is not hindered by the presence of a xylosyl residue linked to a glucosyl residue at subsite -II. This suggests that the activity of endoV is influenced by the type of sugar of the substituent and/or the type of linkage involved in the substitution of the residue at subsite -II, rather than substitution itself.

The release of oligomer **1** (trimer) (Fig. 3.3) is very interesting for the mode of action of endoV, especially since the pattern of release is similar to that of oligomer **2** (not shown). EndoV is able to release **1** if at least two adjacent repeating units do not contain rhamnosyl substituents (Fig. 3.6). This means that subsite II is filled with a galactosyl residue without rhamnosyl substitution, for at least one of the two sites of attack necessary to release oligomer **1**. For the release of oligomers **2**, **3**, **4**, etc. subsite II is always filled with a galactosyl residue substituted with a rhamnosyl residue. Since the release of oligomer **1** at the beginning of the incubation is similar to the release of **2**, it can be concluded that endoV does not prefer unsubstituted galactosyl residues at subsite II above galactosyl residues substituted with a rhamnosyl residue.

The specificity of EndoV from *T. viride* has been shown to be broader than that of the true cellulases since it is able to degrade xylan [3], xyloglucan and possibly mannan [5]. Present study shows that endoV is also able to degrade HF-modified EPS B40. Table 3.3 summarises

Table 3.3
Summary of the different modifications of EPS B40 and the release of phosphate and oligosaccharides from the resulting polymers after incubation with Maxazyme Cl or purified endoV. P, phosphate; ■, β-D-Glc*p*-(1→4); ●, β-D-Gal*p*-(1→4); ○, α-D-Gal*p*-(1→P); ◊, α-L-Rha*p*-(1→2); the approximate relative amount of the remaining rhamnosyl residues (100% = 1.0) is given in case rhamnose had been partially removed. -, no release; +, release; ++, high release of small oligomers.

EPS B40 modification	Structure of 'repeating unit'	Maxazyme Cl		Purified endoV
		P-release by phosphatase	Oligosaccharide release	Oligosaccharide release
Native EPS		–	–	n.d.[a]
H_2SO_4-treated		–	–	n.d.
CF_3CO_2H-treated		+	+[b]	–
HF-treated		n.r.[c]	+	+
HF *and* CF_3CO_2H-treated		n.r.	++	++

[a] not determined since the crude enzyme preparation was negative. [b] only the trimer was released. [c] not relevant since phosphate was not present in the substrate.

the different modifications of EPS B40 and the enzyme activity of Maxazyme Cl and purified endoV on the resulting polymers. Chemical modification with HF was performed because native EPS B40 appeared to be resistant to degradation by all enzyme preparations examined and we thought that this was caused by the phosphate substituent. The removal of phosphate with HF simultaneously released ca. 20% of the rhamnosyl substitutions and this appeared to be critical for the activity of endoV. Later we found that removal of rhamnosyl and galactosyl substituents by mild CF_3CO_2H treatment made the substrate accessible to a phosphatase. In retrospect, mild CF_3CO_2H hydrolysis can be used as an alternative to HF-treatment to modify EPS B40 since it liberates terminally linked galactose and (at least part of) rhamnose. The resulting polymer can be dephosphorylated by a phosphatase present in Maxazyme CL and subsequently degraded by endoV in Maxazyme CL.

The present study shows that (enzymic) modifications of EPS are not only helpful for the structural characterisation of EPS, but that they can also be useful to unravel the mode of action of the enzymes used.

Acknowledgements

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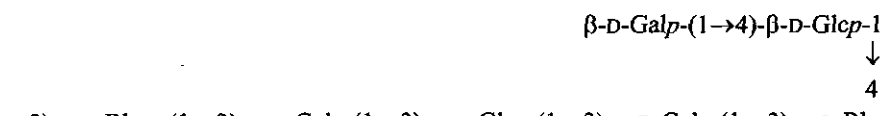
Chapter 4

Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39

Abstract

Lactococcus lactis subsp. *cremoris* B39 grown on whey permeate produced an exopolysaccharide containing L-Rha, D-Gal and D-Glc in a molar ratio of 2:3:2. The polysaccharide was modified using an enzyme preparation from *Aspergillus aculeatus*, resulting in a release of Gal and a polymer with approximately the same hydrodynamic volume as the native polysaccharide. Methylation analysis and ^{1}H NMR studies of both native and modified exopolysaccharides elucidated that terminally linked Gal was released during modification and that the chemical structure of the branches within the repeating units is: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→. 2D NMR experiments (both ^{1}H-^{1}H and ^{1}H-^{13}C) revealed that exopolysaccharide B39 consists of a branched heptasaccharide repeating unit with the following structure:



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Introduction

Polysaccharides are often incorporated into food products essentially to alter the balance between free and bound water and to change the rheological properties, the 'mouth feel' and the texture of the product [1]. Besides polysaccharides from higher plants and seaweeds, bacterial exopolysaccharides (EPSs) are used in food products [2].

Since lactic acid bacteria have the GRAS-status (generally recognised as safe), an increasing interest has been developed for the polysaccharides produced by these bacteria [3]. To obtain insight in the structure-function relationship of EPSs, the chemical structures of several EPSs produced by lactic acid bacteria like lactobacilli [4-12], lactococci [13-14] and streptococci [15-18] have been reported.

The effect of structural changes on the physical properties of EPSs can be determined by examination of related polysaccharide structures [19]. One way to prepare microbial polysaccharides with altered structures is through use of enzymes [19]. Moreover, enzymes are not only useful for studying the structure-function relationship of EPSs, but they have also proven to be very helpful in characterising the chemical structures of these polysaccharides [5-6,20-23].

Here, we report the structural determination of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B39 in whey permeate. An enzyme preparation from *Aspergillus aculeatus* was used for the unravelling of the chemical structure and will be used in future research to study the physical properties of related EPS structures.

Experimental

Production, isolation and purification of EPS.–The production and isolation of EPS from *L. lactis* subsp. *cremoris* B39 was performed as described for EPS B40 [24] and the crude EPS was kindly supplied by NIZO food research (Ede, The Netherlands). The crude material was purified with CCl_3CO_2H and EtOH [25], followed by separation on a size-exclusion column (100×10 cm i.d.) of Sephacryl S500 (Amersham Pharmacia Biotech, Uppsala, Sweden), irrigated with 50 mM NaOAc, pH 5.0. The refractive index and A_{280} were measured on-line (Biopilot System; Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions were assayed for their total neutral sugar content [26], pooled accordingly, dialysed and freeze dried.

Enzyme preparation.–The commercial enzyme preparation Pectinex Ultra SP-L produced by *A. aculeatus* (Novo Nordisk Ferment Ltd., Dittingen, Switzerland) was used for modifying EPS B39.

Enzymic modification of the EPS.–In the search for enzymes which are able to modify EPS B39, partially purified (using only CCl_3CO_2H and EtOH) EPS B39 (0.5 mL, 2 mg/mL in 50 mM NaOAc pH 5.0 + 0.01% (w/v) NaN_3) was incubated (24 h, 30 °C) with 50 µl Ultra SP, which was previously dialysed against the same buffer. After incubation, the enzymes were inactivated (15 min, 100 °C) and the precipitate formed was removed by centrifugation. The supernatant was analysed by high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC). To obtain sufficient amounts of modified EPS B39 for sugar composition analysis, methylation analysis and NMR analyses, purified EPS B39 (30 mg) was dissolved in 50 mM NaOAc buffer pH 5.0 (15 mL) containing

0.01% (w/v) NaN_3. Ultra SP (1 mL) was dialysed against the same buffer and diluted to 10 mL. The EPS solution was incubated (24 h, 30 °C) with enzyme solution (1.5 mL) and subsequently heated (30 min, 100 °C) to inactivate the enzymes. After centrifugation, part of the supernatant was analysed by HPAEC. The remaining part was dialysed, centrifuged again and freeze dried.

Sugar composition.–Samples were pre-treated in 12 M H_2SO_4 (1 h, 30 °C) and hydrolysed in 1 M H_2SO_4 (3 h, 100 °C), using inositol as internal standard. The released sugars were converted into their alditol acetates [27] and analysed by GC-FID [25].

Methylation analysis.–Samples were methylated according to Hakomori [28] and subsequently dialysed against water and freeze dried. The methylated samples were hydrolysed in 2 M CF_3CO_2H (1 h, 121 °C). After evaporation (stream of air, <20 °C), the partially methylated sugars were converted into alditol acetates [27] and analysed by GC-FID as described by Vincken *et al.*, [29]. Partially methylated alditol acetates were quantified according to their effective carbon response [30]. Identification of the compounds was confirmed by GC-MS [25].

Absolute configurations of monosaccharides.–The absolute configurations of the monosaccharides were determined as described by Gerwig *et al.* [31]. The trimethylsilated (–)-2-butyl glycosides were analysed using GC-FID [20].

HPAEC analysis of the digest.–The release of monomers and/or oligomers caused by enzymic hydrolysis of EPS B39 was verified by HPAEC, which was performed as described earlier [20].

Hydrodynamic volume.–HPSEC was performed as described earlier [20,25].

NMR spectroscopy.–Prior to NMR analysis, the samples were exchanged three times in D_2O (99.9 atom% D, Cambridge Isotope Laboratories, USA) with intermediate freeze drying. Finally, samples were dissolved in 99.96% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at probe temperatures of 80 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Incidentally, 1D ^1H and 2D COSY and TOCSY spectra of EPS B39 were recorded at 70 °C in order to shift the HOD signal. Chemical shifts are expressed in ppm relative to internal acetone: δ 2.225 for ^1H and δ 31.55 for ^{13}C.

In 1D ^1H NMR spectra, suppression of the HOD signal was achieved by using presaturation during relaxation delay for 1 s. Proton-decoupled ^{13}C spectra were recorded at 125.77 MHz. For 1D ^1H and ^{13}C NMR spectra, data sets of 16,384 and 32,768 data points were recorded, respectively.

The 2D COSY spectra (70 °C and 80 °C) were acquired in the magnitude mode. In addition, a 2D DQF-COSY spectrum at 80 °C was recorded according to Derome and Williamson [32]. 2D TOCSY and ROESY spectra were basically acquired as described by Fransen *et al.* [33] using the time-proportional phase increment (TPPI) method [34]. The 2D NOESY experiment was performed with a mixing time of 250 ms. For ^1H-^{13}C 2D-heteronuclear proton-detected multiple bond correlation spectroscopy (HMBC) spectra [35] a standard gradient-enhanced ^1H-^{13}C 2D-heteronuclear proton detected multiple-quantum coherence (HMQC) pulse-sequence delivered by Bruker was changed into a HMBC sequence by setting the delay between the first proton and first carbon pulse to 40, 60, 80 and 140 ms, respectively. For 2D HMQC spectra, this delay was 3.0 ms. For all homonuclear 2D experiments, 512 experiments of 2048 data points were acquired with 80-120 scans per increment; all heteronuclear 2D spectra were acquired in 1024 experiments of 2048 data

points, except for the decoupled HMQC which was acquired in 1024 experiments of 1024 data points.

Time domain data were multiplied by phase-shifted (squared-)sine-bell functions or with Lorentzian-to-Gaussian multiplication. After zerofilling and Fourier transformation data sets of 2048×1024 (homonuclear experiments) or 2048×2048 points (heteronuclear experiments) were obtained, which were baseline corrected when necessary.

Results

Isolation, purification and chemical characterisation of EPS B39.–Crude EPS produced by *L. lactis* subsp. *cremoris* B39 was purified by CCl_3CO_2H extraction and EtOH precipitation, followed by size-exclusion chromatography on Sephacryl S-500. The purification step using CCl_3CO_2H and EtOH removed most of the proteins present in the crude EPS, while size-exclusion chromatography successfully removed a mannan population (probably originating from the medium) and remnants of protein. No A_{280} absorbing material co-eluted with EPS B39, indicating that the EPS was (almost) free of protein.

Sugar composition analysis of native EPS B39 (**1**) and determination of absolute configurations revealed the presence of L-Rha, D-Gal and D-Glc in a molar ratio of 2.0:3.0:2.0. Methylation analysis of **1** showed the presence of 2-substituted rhamnose, 3,4-disubstituted rhamnose, terminally linked galactose, 2-substituted galactose, 3-substituted galactose, 3-substituted glucose and 4-substituted glucose (Table 4.1). According to these results, the results of enzymic modification of EPS B39 (*vide infra*) and NMR experiments (*vide infra*) all hexose residues are in the pyranose ring form.

Table 4.1
Methylation analysis data of native EPS B39 (**1**) and enzymically modified EPS B39 (**2**)

	Molar ratio[a]	
Derivative	**1**	**2**
3,4-Rha[b]	1.0	1.0
2-Rha	0.7	0.7
2,3,4,6-Gal	1.0	0.4
3,4,6-Gal	1.0[c]	1.0[c]
2,4,6-Gal	0.8	0.8
2,3,4,6-Glc	-	0.9
2,4,6-Glc	0.6	0.6
2,3,6-Glc	1.0[c]	0.4[c]

[a] 3,4-Rha was taken as 1.0; [b] 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-rhamnitol, etc.; [c] 2-substituted Gal and 4-substituted Glc were not separated on GC and a mixed mass spectrum was found on GC-MS. Their total molar ratio was 2.0 for **1** and 1.4 for **2**. Based on the sugar composition data and the methylation analysis data of **1**, it was supposed that in this sample both components were present in equal amounts. For **2**, the decrease in the signal was totally ascribed to 4-substituted Glc because of the presence of t-Glc.

Modification of EPS B39.–After incubation of EPS B39 with Ultra SP, analysis by HPAEC showed that monomeric galactose had been released while no oligomers were found. The hydrodynamic volume of the modified EPS had only been decreased very slightly compared to the native EPS (HPSEC). Sugar composition analysis of modified EPS B39 (**2**) resulted in a molar ratio of Rha, Gal and Glc of 2.0:2.4:2.1. The lower amount of galactose in **2** compared to **1** is in accordance with the release of galactose as analysed by HPAEC. Methylation analysis (Table 4.1) showed a decrease in terminally linked galactose and 4-substituted glucose while a new sugar derivative, originating from terminally linked glucose, appeared. Consequently, terminally linked galactose is attached to the 4-substituted glucose residue and the branches attached to the backbone of native EPS B39 contain at least two sugar residues.

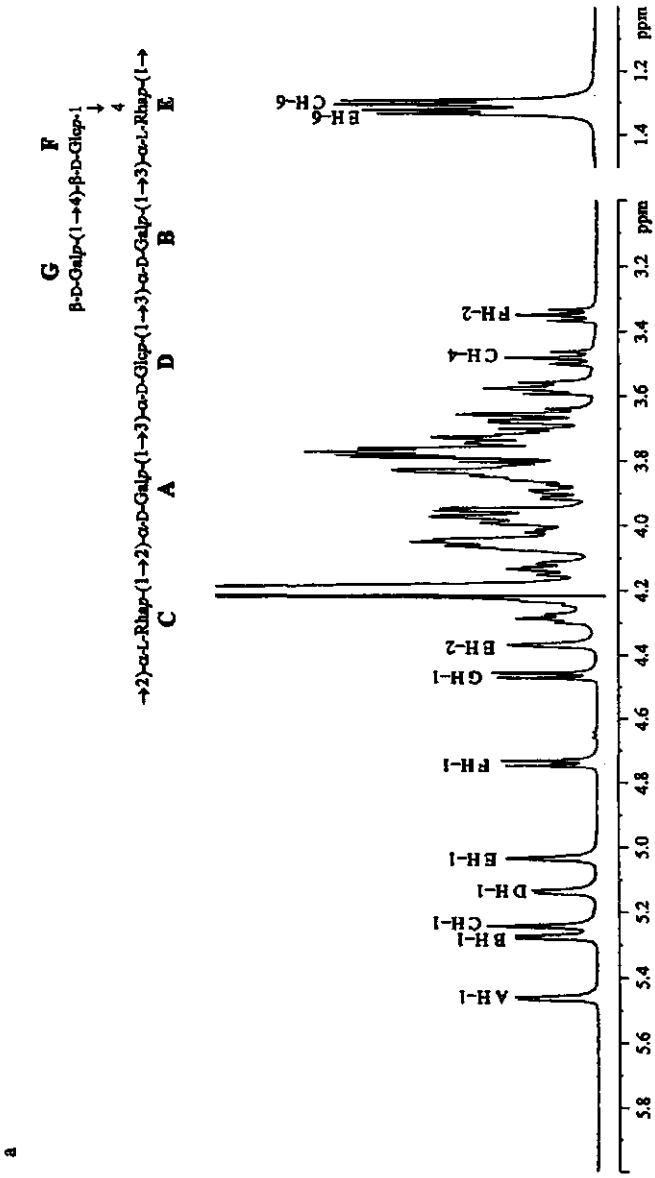
During incubation of EPS B39 with Ultra SP not all terminally linked galactosyl residues were removed (Table 4.1). Nevertheless, the enzymic modification provided important structural information about EPS B39.

1D NMR spectroscopy.–The ^{1}H NMR spectrum of **1** (Fig. 4.1a) showed seven signals with similar intensity in the anomeric region (δ 5.5-4.4), suggesting a heptasaccharide repeating unit. The seven monosaccharide residues were designated **A**-**G** as indicated in the spectrum. The chemical shifts and coupling constants of the signals at δ 5.462 (**A** H-1, $^3J_{1,2}$ 3.51 Hz), δ 5.276 (**B** H-1, $^3J_{1,2}$ 3.81 Hz) and δ 5.136 (**D** H-1, $^3J_{1,2}$ 3.66 Hz) indicated the presence of three α-hexopyranosyl residues, whereas the chemical shifts and coupling constants of the signals at δ 4.739 (**F** H-1, $^3J_{1,2}$ 7.93 Hz) and δ 4.464 (**G** H-1, $^3J_{1,2}$ 7.78 Hz) suggested two β-hexopyranosyl residues. The coupling constants $^3J_{1,2}$ of the signals at δ 5.243 (**C** H-1) and δ 5.033 (**E** H-1) were too small to be determined. Together with two high-field doublets (δ 1.331, $^3J_{5,6}$ 5.95 Hz and δ 1.302, $^3J_{5,6}$ 6.10 Hz), arising from methyl groups, these signals are indicative for two rhamnosyl residues.

Comparison of the ^{1}H NMR spectrum of **2** (Fig. 4.1b) with the ^{1}H NMR spectrum of **1** (Fig. 4.1a) showed that the intensity of the signals at δ 4.739 (residue **F**) and δ 4.464 (residue **G**) decreased, while a new anomeric signal appeared at δ 4.713. According to the methylation analyses, the new anomeric resonance could be assigned to H-1 of a terminally linked glucopyranosyl residue (residue **F***). Furthermore, it was concluded that both sugar residues in the branches of EPS B39 have a β-configuration. The combined results, from methylation analyses and NMR studies, suggested that (at least) a disaccharide fragment is involved in the branches of EPS B39: β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→, corresponding to residues **G** and **F**. The specific residue assignments follow from 2D NMR experiments (*vide infra*).

The ^{13}C NMR spectrum of **1** (not shown) confirmed the proposed heptasaccharide repeating unit since it showed seven anomeric signals: δ 104.5, 103.6, 103.3, 101.5, 99.3, 97.1, and 94.7. Furthermore, the signals from the methyl carbons of the two rhamnosyl residues were present at 18.5 and 18.1 ppm.

2D NMR spectroscopy.–Assignments of ^{1}H chemical shifts of **1** (Table 4.2) were performed by means of 2D (DQF)-COSY, TOCSY, ROESY and HMQC measurements. As starting points, the anomeric protons of residues **A**-**G** and the methyl protons of the rhamnosyl residues **C** and **E** were used. From the (DQF)-COSY spectra (not shown), all protons of the rhamnosyl residues **C** and **E** could be assigned. TOCSY spectra confirmed these assignments and the spectrum with a mixing time of 140 ms is shown in Fig. 4.2 as a typical example. The chemical shifts of **C** H-5 at δ 3.83 and **E** H-5 at δ 3.86 (Table 4.2)


a
G F
β-D-Galp-(1→4)-β-D-Glcp-1
↓ 4
→2)-α-L-Rhap-(1→2)-α-D-Galp-(1→3)-α-D-Glcp-(1→3)-α-D-Galp-(1→3)-α-L-Rhap-(1→
C A D B E
AH-1
BH-1
CH-1
DH-1
EH-1
FH-1
GH-1
EH-2
FH-2
CH-4
EH-6
CH-6
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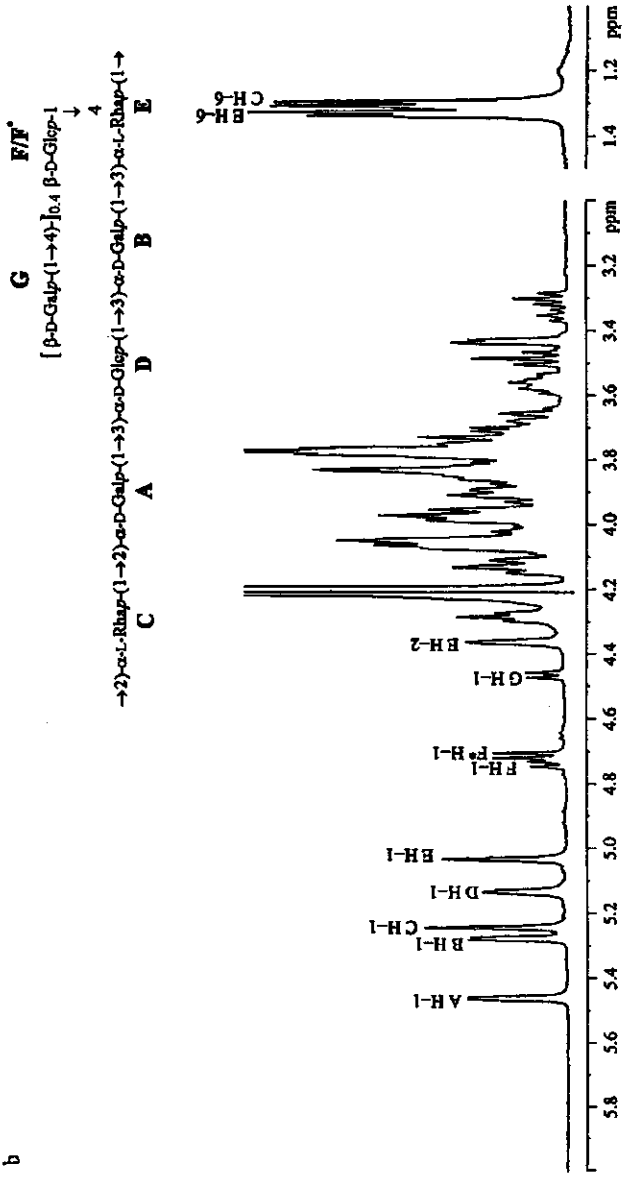
1.4 1.2 ppm



b

Fig. 4.1. 500-MHz ^{1}H NMR spectra of EPS B39 (1) (a) and modified EPS B39 (2) (b), recorded in D_2O at 80 °C. In modified EPS B39, the code **F** refers to 4-substituted β-D-Glc*p*, whereas **F*** refers to terminally linked β-D-Glc*p*. The approximate relative amount of remaining terminally linked β-D-Gal*p* residues in **2** is indicated by $[\ ]_{0.4}$ (100%= 1.0).

Table 4.2
^{1}H NMR chemical shifts[a] of EPS B39 (**1**), recorded in D_2O at 80 °C.

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3
A (1,2-α-D-Gal*p*)	5.462	3.96	4.07	4.05	4.286	3.76	3.76	–
B (1,3-α-D-Gal*p*)	5.276	4.05	4.12	4.19	4.23	3.77	3.77	–
C (1,2-α-L-Rha*p*)	5.243	4.07	3.90	3.482	3.83	–	–	1.302
D (1,3-α-D-Glc*p*)	5.136	3.73	3.99	3.73	4.03	3.83	3.83	–
E (1,3,4-α-L-Rha*p*)	5.033	4.369	4.13	3.96	3.86	–	–	1.331
F (1,4-β-D-Glc*p*)	4.739	3.351	3.69	3.65	3.56	3.98	3.84	–
G (t-β-D-Gal*p*)	4.464	3.57	3.66	3.95	3.73	3.79	3.79	–

[a] in ppm relative to the signal of acetone at δ 2.225.

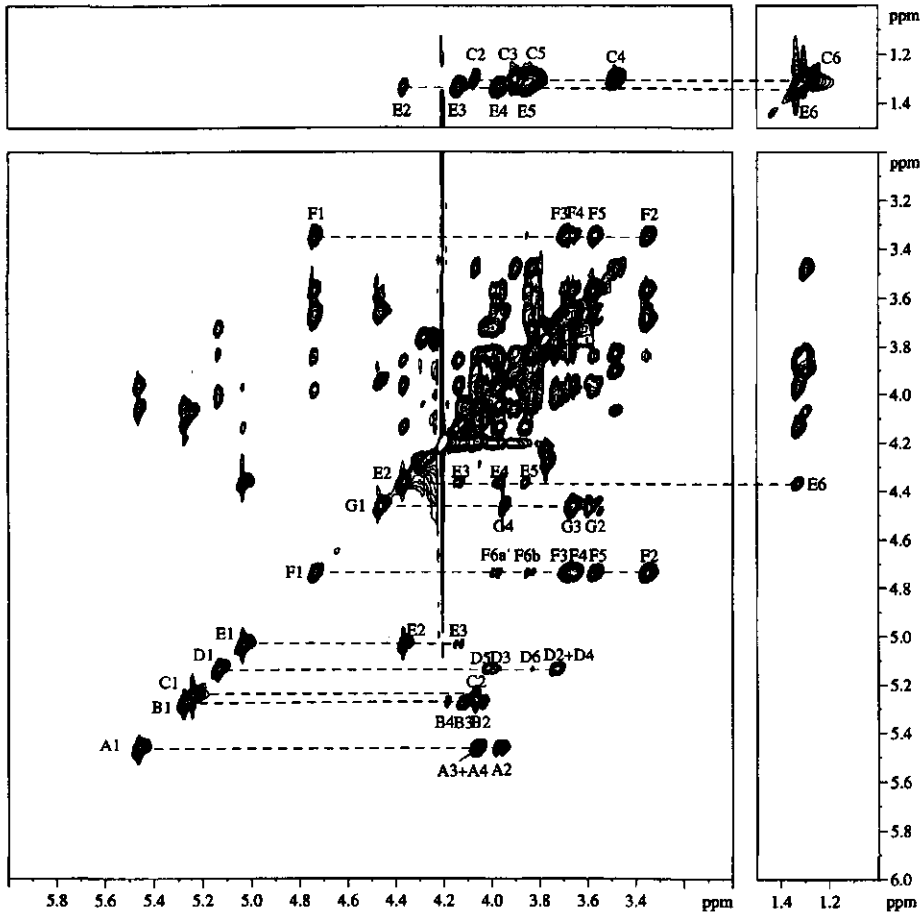


Fig. 4.2. 500-MHz 2D TOCSY spectrum (mixing time = 140 ms) of EPS B39 (**1**) recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons, of H-2 of residues **E** and **F**, and H-6 of residues **C** and **E** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

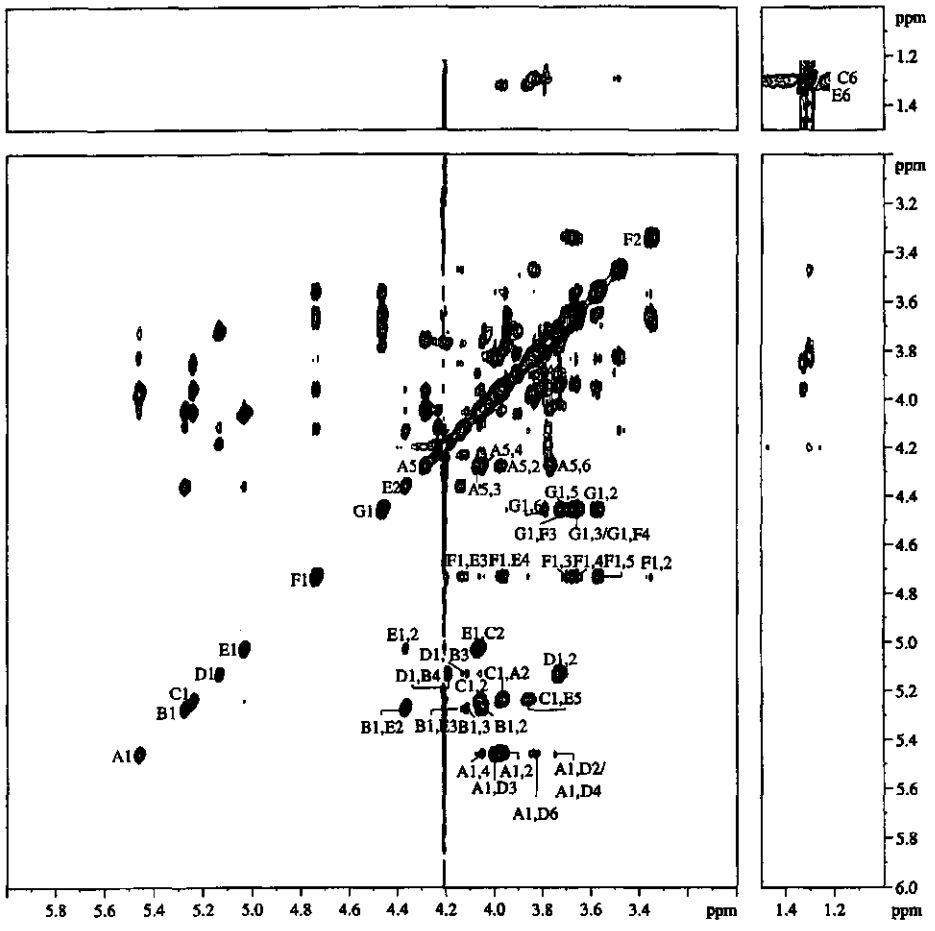


Fig. 4.3. 500-MHz 2D ROESY spectrum (mixing time = 250 ms) of EPS B39 (1) recorded in D_2O at 80 °C. The code A1 corresponds to the diagonal peak belonging to **A** H-1; A1,2 refers to an intra-residual cross-peak between **A** H-1 and **A** H-2; A1,D3 refers to an inter-residual connectivity between **A** H-1 and **D** H-3, etc.

strongly suggest the α-configuration for both rhamnosyl residues [22]. The TOCSY H-1 track of residue **A** showed cross-peaks with H-2,3,4, indicating a galactosyl residue. The partial overlap of the signals of **A** H-3 and **A** H-4 was inferred from **A** H-5, **A** H-3 and **A** H-5, **A** H-4 cross-peaks in the ROESY spectrum (Fig. 4.3) and a weak connectivity of **A** H-5 with **A** H-4 in the DQF-COSY spectrum. The (un)decoupled HMQC spectra confirmed the partial overlap of **A** H-3 and **A** H-4. The resonances for **A** H-6a,6b were found via connectivities with **A** H-5 in both the (DQF)-COSY and the TOCSY spectra and since the undecoupled HMQC spectrum (Fig. 4.4) showed only one set of cross-peaks between **A** C-6 and **A** H-6, the resonances of **A** H-6a and **A** H-6b had to overlap. The TOCSY H-1 track of residue **B** showed cross-peaks with H-2,3,4, indicating a galactosyl residue. The resonance for **B** H-5 was found

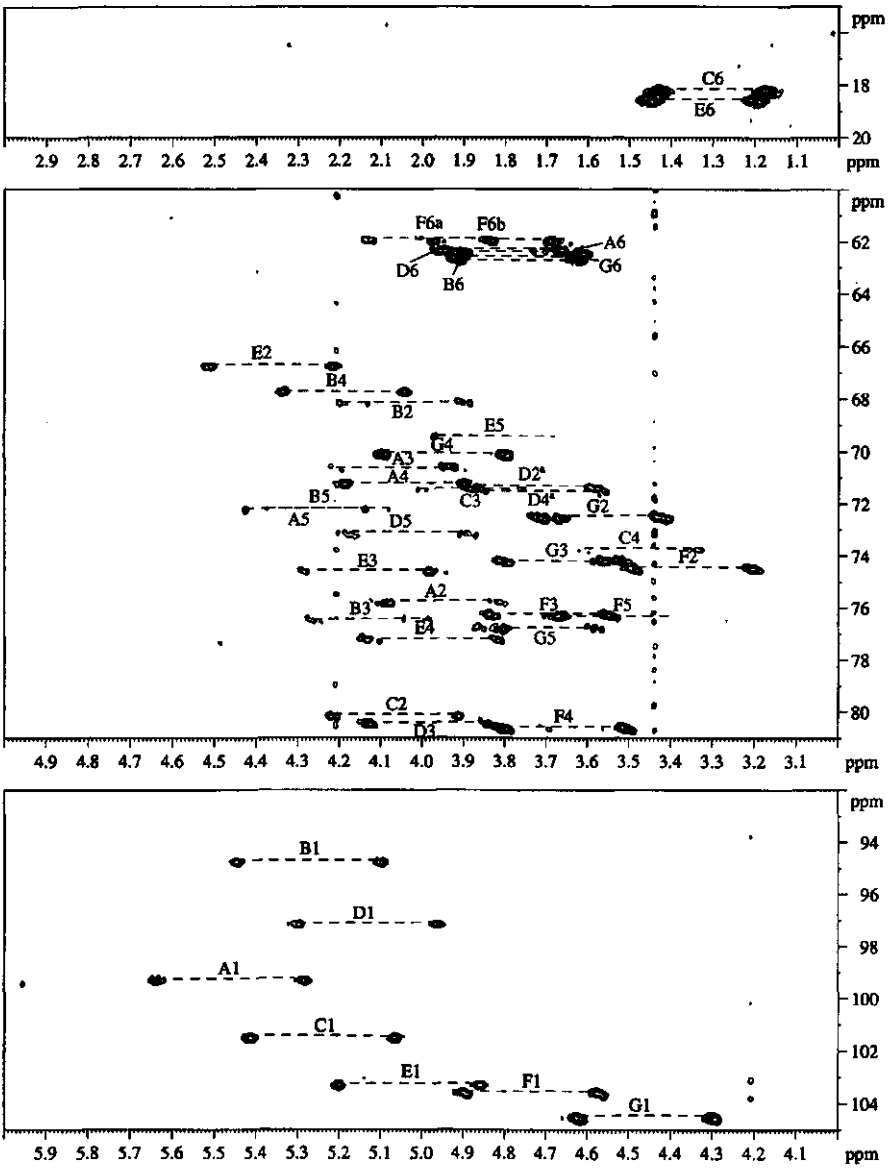


Fig. 4.4. 500-MHz 2D ^{1}H-^{13}C undecoupled HMQC spectrum of EPS B39 (**1**) recorded in D_2O at 80 °C. A1 refers to the set of cross-peaks between C-1 and H-1 of residue **A**, etc. [a] Assignments may have to be interchanged.

via an intra-residual connectivity with **B** H-3 in the ROESY spectrum. This assignment was confirmed in COSY and TOCSY spectra recorded at 70 °C (not shown); weak cross-peaks between **B** H-4 and **B** H-5 were found. The resonances for **B** H-6a,6b were found via connectivities with **B** H-5 in both the (DQF)-COSY and the TOCSY spectra and the

(un)decoupled HMQC spectra indicated that the resonances of **B** H-6a and **B** H-6b overlap. The TOCSY track of **D** H-1 showed cross-peaks with **D** H-2,3,4,5,6a,6b, which is indicative for a glucosyl residue. The (DQF)-COSY and TOCSY spectra already suggested overlap between **D** H-2 and **D** H-4, and this was confirmed by the (un)decoupled HMQC spectra. The latter spectra also indicated that the resonances of **D** H-6a and **D** H-6b overlap. The TOCSY track of **F** H-1 showed the complete series of cross-peaks with **F** H-2,3,4,5,6a,6b, indicating that **F** corresponds to a glucosyl residue. Using these connectivities and the cross-peaks in the (DQF)-COSY spectrum, all protons of residue **F** could be assigned. The **G** H-1 track in the TOCSY spectrum showed cross-peaks with **G** H-2,3,4, suggesting a galactosyl residue. **G** H-5 and **G** H-6 were found via the **G** H-1 and **G** H-4 track in the ROESY spectrum. Again, the (un)decoupled HMQC spectra indicated that the resonances of **G** H-6a and **G** H-6b overlap.

The undecoupled 2D HMQC spectrum (Fig. 4.4) together with the decoupled 2D HMQC spectrum allowed the assignment of the ^{13}C resonances (Table 4.3). The one-bond coupling constants of the anomeric carbon atoms of residues **B**, **D** and **E** ($^1J_{C\text{-}1,H\text{-}1}$ 170-173 Hz) and residues **A** and **C** ($^1J_{C\text{-}1,H\text{-}1}$ 175-177 Hz) are indicative for an α-configuration of these residues, whereas residues **F** and **G** ($^1J_{C\text{-}1,H\text{-}1}$ 162-163 Hz) are in the β-configuration [36]. The relatively high coupling constants of residues **A** and **C** are most likely caused by the 2-substitution [18]. Comparison of the ^{13}C data in Table 4.3 with the corresponding methyl aldosides [37] shows low-field chemical shifts for **A** C-2 and **C** C-2, suggesting that residues **A** and **C** are 2-substituted. Likewise, the downfield chemical shifts of **B** C-3 and **D** C-3 indicate that these residues are 3-substituted, whereas the downfield chemical shifts of **E** C-3 and **E** C-4 shows that residue **E** is 3,4 disubstituted. Residue **F** was assigned as the 4-substituted residue since **F** C-4 was downfield shifted and residue **G** was identified as the terminally linked sugar residue. The assignments of the type of sugar residues and the type of linkages based on NMR experiments are in agreement with the results from the methylation analyses (Table 4.1).

Table 4.3
^{13}C NMR chemical shifts[a] of EPS B39 (**1**), recorded in D_2O at 80 °C.

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A (1,2-α-D-Gal*p*)	99.3	75.8	70.6	71.2	72.2	62.5
B (1,3-α-D-Gal*p*)	94.7	68.2	76.5	67.8	72.2	62.8
C (1,2-α-L-Rha*p*)	101.5	80.2	71.5	73.8	n.d.[b]	18.1
D (1,3-α-D-Glc*p*)	97.1	71.4[c]	80.5	71.6[c]	73.2	62.4
E (1,3,4-α-L-Rha*p*)	103.3	66.8	74.6	77.3	69.4	18.5
F (1,4-β-D-Glc*p*)	103.6	74.5	76.3	80.6	76.3	62.0
G (t-β-D-Gal*p*)	104.5	72.5	74.2	70.1	76.8	62.5

[a] in ppm relative to the signal of acetone at δ 31.55; [b] not determined; [c] Assignments may have to be interchanged.

The complete monosaccharide sequence of EPS B39 was determined via a 2D HMBC spectrum with a delay time of 80 ms (Fig. 4.5) and 2D ROESY (Fig. 4.3) and NOESY analysis. In other HMBC spectra (delay times 40, 60, 140 ms; not shown) no new inter-residual connectivities could be detected. Since the inter-residual connectivities in the

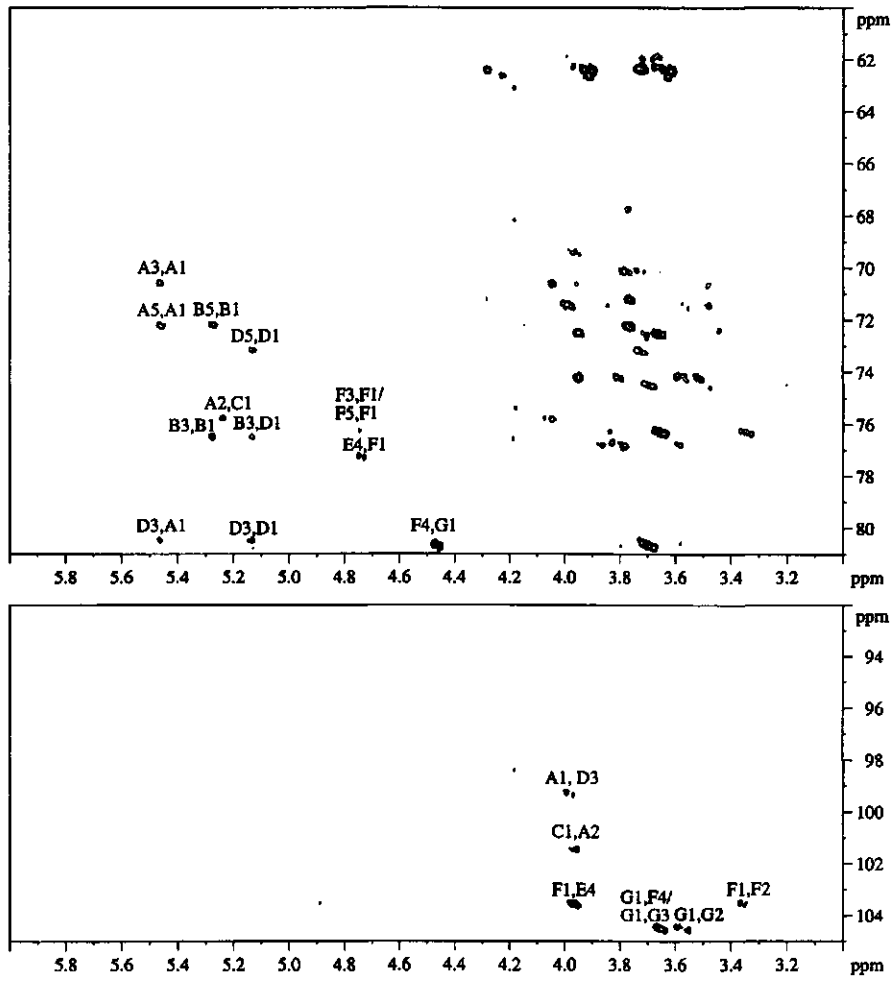


Fig. 4.5. Partial 500-MHz 2D ^{1}H-^{13}C undecoupled HMBC spectrum (delay time between the first proton pulse and the first carbon pulse = 80 ms) of EPS B39 (**1**) recorded in D_2O at 80 °C. The code G1,F4 corresponds to a long-range coupling between **G** C-1 and **F** H-4, etc.

NOESY and ROESY experiments were essentially identical, only the results in the ROESY spectrum are described. In the latter spectrum inter-residual connectivities **A** H-1,**D** H-3, **A** H-1,**D** H-2 and/or **A** H-1,**D** H-4 and **A** H-1,**D** H-6 demonstrated the **A**→**D** sequence, which was also proven by **A** C-1, **D** H-3 and **D** C-3, **A** H-1 cross-peaks in the HMBC spectrum. The connectivities in the ROESY spectrum between **D** H-1,**B** H-3 and **D** H-1,**B** H-4 indicated the **D**→**B** sequence. Moreover, a weak inter-residual connectivity in the HMBC spectrum was found between **B** C-3 and **D** H-1, confirming the **D**(1→3)**B** linkage. The **B**(1→3)**E** linkage could not be revealed by HMBC analyses, but methylation analysis in combination with carbon chemical shifts demonstrated that residue **E** is 3,4-disubstituted. Therefore, the strong

connectivity of **B** H-1 and **E** H-2 and the weaker connectivity of **B** H-1 and **E** H-3 in the ROESY spectrum showed the **B→E** sequence, but the strongest connectivity did not reflect the glycosidic linkage. The observation of a strong inter-residual non-glycosidic NOE cross-peak was reported before for this α-(1→3) linkage [18]. The strong inter-residual cross-peak of **E** H-1,**C** H-2 in the ROESY spectrum indicated the **E**(1→2)**C** linkage, but this could not be confirmed by HMBC analyses. The strong cross-peak in the ROESY spectrum between **C** H-1,**A** H-2 revealed the structural element **C**(1→2)**A** and this sequence was proven by **C** C-1, **A** H-2 and **A** C-2, **C** H-1 connectivities in the HMBC spectrum. The **F→E** linkage was suggested by connectivities in the ROESY spectrum, **F** H-1,**E** H-3 and **F** H-1,**E** H-4, and inter-residual cross-peaks between **F** C-1, **E** H-4 and **E** C-4, **F** H-1 in the HMBC spectrum confirmed the **F**(1→4)**E** sequence. The **G**(1→4)**F** linkage could only be assigned tentatively by ROESY experiments since **G** H-1,**G** H-3 and **G** H-1,**F** H-4 overlap. However, the **G**(1→4)**F** linkage was proven by inter-residual connectivities between **G** C-1, **F** H-4 and **F** C-4, **G** H-1 in the HMBC spectrum.

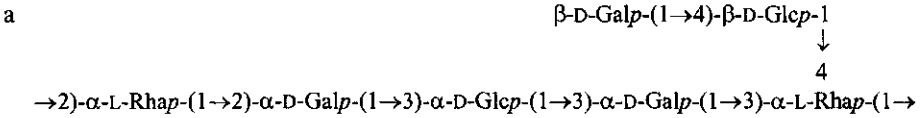
Both, in 2D NOESY and ROESY spectra, long-range NOE contacts between **C** H-1 and **E** H-5 were found, indicating a conformation for the polysaccharide in which residue **C** is close to residue **E**.

The combined results from enzymic modification, chemical studies and NMR experiments demonstrated that the EPS produced by *L. lactis* subsp. *cremoris* B39 is composed of the heptasaccharide repeating unit with the structure as is shown in Fig. 4.1a.

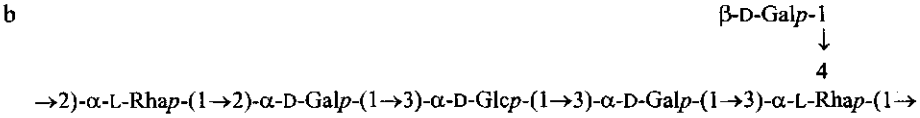
Discussion

In order to be able to study the effect of structural changes on the physical properties of exopolysaccharides, the chemical structure of the EPS from *L. lactis* subsp. *cremoris* B39 was

a



b



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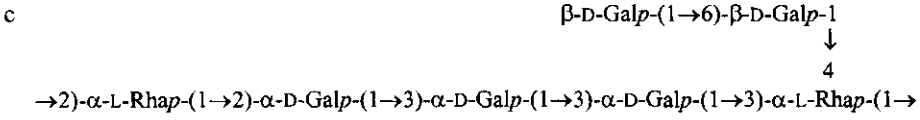

Fig. 4.6. Chemical structures of EPSs produced by *S. thermophilus* with close structural similarities to the EPS produced by *L. lactis* subsp. *cremoris* B39 (a): SFi12 [16] (b), OR 901 [17] and Rs and Sts [18] (c).

elucidated. To our knowledge, this chemical structure (Fig. 4.6a) was not reported before for EPS produced by lactic acid bacteria. However, related EPS structures (Fig. 4.6b and c) have been described for different strains of *Streptococcus thermophilus*. The backbone of EPS B39 is identical to the backbone reported for EPS SFi12 [16] (Fig. 4.6b) and this was reflected in the similar ^{1}H NMR chemical shifts of the backbone sugar residues (within 0.06 ppm). The only difference between these two EPSs is the 4-substituted glucosyl residue in the branches of EPS B39, which is absent in EPS SFi12. Another structure which is related to EPS B39 was reported for EPS from *S. thermophilus* OR 901 [17], Rs and Sts [18], three presumably different strains with an identical repeating unit [18] (Fig. 4.6c). In comparison with EPS B39, all glucosyl residues were replaced by galactosyl residues in this EPS and the terminally linked galactosyl residue is linked to the 6-position instead of the 4-position of the next sugar residue.

It has been noted before [19] that different microbial species or strains may produce a range of polysaccharides with close structural similarities. Examination and comparison of the physical properties of all available, related EPS structures, including enzymically modified EPS, enables us to determine the effect of structural changes on the physical properties of these macromolecules. Work on the comparison of physical properties of related EPS structures is in progress.

Acknowledgements

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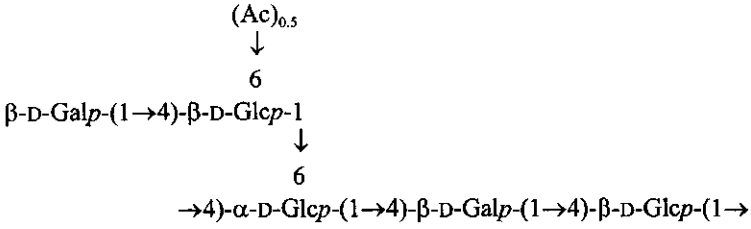
Chapter 5

Structural characterisation and enzymic modification of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* B891

Abstract

Lactococcus lactis subsp. *cremoris* B891 grown on whey permeate produced an exopolysaccharide containing D-Gal and D-Glc in a molar ratio of 2:3. The polysaccharide was partially *O*-acetylated. By means of HF solvolysis, *O*-deacetylation, enzymic modification, methylation analysis and 1D/2D NMR studies the exopolysaccharide was shown to be composed of repeating units with the following structure:



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Introduction

Over the past 20 years, the food industry has developed an enormous range of convenience food products, but it is believed that the scientific understanding of the roles of hydrocolloids used has not kept pace adequately with innovations and changes in technology [1]. To be able to produce tailor-made polysaccharides for food applications, it is a prerequisite to know the influence of the chemical structure on the physical properties. Extracellular polysaccharides (EPSs) frequently consist of repeating units [2] and these regular structures make them very suitable for structure-function studies.

The last decade, the polysaccharides of lactic acid bacteria have received considerable attention in view of their potential application as replacers of presently applied thickeners [3]. EPSs may also represent safe additives for novel food formulations, because of their contribution to the peculiar rheology and texture of milk-derived products [2]. As a first step in structure-function studies, the chemical structures of several EPSs produced by lactic acid bacteria have been elucidated. For *Lactococcus lactis* subsp. *cremoris*, the investigated strains include H414 [4], SBT 0495 [5], LC330 [6], B40 [7,8], and B39 [9].

Here, we report the structural analysis of the exopolysaccharide produced by *L. lactis* subsp. *cremoris* B891.

Experimental

Production, isolation and purification of EPS.–The production and isolation of EPS from *L. lactis* subsp. *cremoris* B891 was performed as described for EPS B40 [10] and the crude EPS was kindly supplied by NIZO food research (Ede, The Netherlands). The crude material was purified as described before [9].

O-deacetylation of the native polysaccharide.–The native EPS (1 mg/mL) was *O*-deacetylated by treatment with 5% NH_4OH at room temperature for 8 h [11]. The *O*-deacetylated EPS was recovered by freeze drying.

Enzyme preparation.–The commercial enzyme preparation Pectinex Ultra SP-L produced by *Aspergillus aculeatus* (Novo Nordisk Ferment AG, Dittingen, Switzerland) was used to modify *O*-deacetylated EPS B891.

Enzymic modification of the O-deacetylated EPS.–In the search for enzymes that are able to modify *O*-deacetylated EPS B891, partially purified EPS B891 was *O*-deacetylated. The resulting polymer (0.5 mL, 2 mg/mL in 50 mM NaOAc pH 5.0) was incubated (24 h, 30 °C) with 50 µl Ultra SP, which was dialysed against the same buffer. After incubation, the enzymes were inactivated (15 min, 100 °C) and the precipitate formed was removed by centrifugation. The supernatant was used to analyse the released monosaccharides and to determine the hydrodynamic volume of the remaining polymer. To obtain sufficient amounts of enzymically modified EPS B891 for methylation analysis and NMR analysis, purified and *O*-deacetylated EPS B891 (10 mg) was dissolved in 50 mM NaOAc buffer pH 5.0 (5 mL). Ultra SP (1 mL) was dialysed against the same buffer and diluted to ca. 4 mL. The EPS solution was incubated (24 h, 30 °C) with 0.5 mL enzyme solution and subsequently heated (15 min, 100 °C). After centrifugation, the supernatant was dialysed against distilled water and freeze dried for further analysis.

Partial HF solvolysis.–The solvolysis of EPS B891 with anhydrous liquid HF was performed according to Mort [12] at the Department of Plant Biochemistry and Biotechnology, Wesfälische Wilhelms-Universität Münster, Germany. The polysaccharide was partially solvolysed with HF in two batches. Therefore, freeze dried polysaccharide (ca. 40 mg per batch) was treated with anhydrous, liquid HF (ca. 0.5 mL) for 30 min at –40 °C. To stop the reaction, 25 mL of ether, pre-cooled with dry ice, was added. After the ether-HF mixture had warmed to room temperature, it was filtered through a Teflon filter using N_2 pressure. The precipitate was washed with more ether to remove any residual HF and then dried under vacuum. Finally, the precipitate was dissolved in distilled water and freeze dried. The effect of the solvolysis was evaluated by high-performance anion-exchange chromatography (HPAEC) and matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) analysis.

Partial purification of oligosaccharides obtained by HF solvolysis.–Freeze dried oligosaccharides of EPS B891 (47 mg) were dissolved in distilled water (0.5 mL) and centrifuged. The supernatant was applied onto a Bio-Gel P-2 column (84×2.6 cm i.d., Bio-Rad Laboratories, Richmond, CA) and elution with distilled water (0.5 mL/min) was performed at 60 °C. The refractive index was monitored on-line (Shodex RI-72 detector) and relevant fractions (RI-signal) were analysed for their oligomeric content by HPAEC and MALDI-TOF MS.

Sugar composition and methylation analysis.–The sugar composition, the methylation analysis and the absolute configurations of the monosaccharides were determined as described before for EPS B39 [9].

Monosaccharide release.–The release of monomers caused by enzymic hydrolysis of EPS B891 was verified by HPAEC, which was performed as described earlier [7].

Hydrodynamic volume of the polysaccharides.–High-performance size-exclusion chromatography (HPSEC) was performed as has been described before [7,8].

Oligosaccharide release.–The release of oligomers caused by solvolysis of EPS B891 using HF was examined by two methods: (I) The reaction product was analysed using the HPAEC equipment as described earlier [7]. After equilibration with 20 mM NaOAc in 100 mM NaOH, 20 µL sample was injected and the elution program was started: 0→35 min, linear gradient of 20→50 mM NaOAc in 100 mM NaOH; 35→50 min, linear gradient of 50 mM→1 M NaOAc in 0.1 M NaOH; 50→55 min, 1 M NaOAc in 0.1 M NaOH isocratic; followed by re-equilibration (55→70 min, 20 mM NaOAc in 100 mM NaOH isocratic). (II) The reaction product was analysed by MALDI-TOF MS as described earlier [8].

NMR spectroscopy.–Samples were analysed by NMR spectroscopy at probe temperatures of 80 °C (polysaccharides) or 27 °C (oligosaccharides) as was described for EPS B39 [9].

Results

Isolation, purification and chemical characterisation of EPS B891.–Crude EPS produced by *L. lactis* subsp. *cremoris* B891 was purified by CCl_3CO_2H extraction and EtOH precipitation followed by size-exclusion chromatography on Sephacryl S-500. The purification step using CCl_3CO_2H and EtOH removed most of the proteins present in the crude EPS, while size-exclusion chromatography successfully removed a mannan population and remnants of protein. The mannan population is believed to originate from the yeast

extract in the growth medium, as has been reported before [13]. The population was absent when EPS B891 was produced on a chemically defined medium [14] without yeast extract.

Sugar composition analysis of purified, native EPS B891 (**1**) and determination of absolute configurations revealed the presence of D-Gal and D-Glc in a molar ratio of 2.0:3.0. Methylation analysis of **1** showed the presence of terminally linked galactose, 4-substituted galactose, 4-substituted glucose, and 4,6-disubstituted glucose (Table 5.1). According to the results of NMR experiments (*vide infra*) and the methylation analyses before and after enzymic modification of EPS B891 (*vide infra*) all hexose residues are in the pyranose ring form.

Table 5.1
Methylation analysis data of native EPS B891 (**1**), *O*-deacetylated EPS B891 (**2**) and enzymically modified *O*-deacetylated EPS B891 (**3**). It should be kept in mind that the acetyl groups in **1** were removed during the procedure due to alkaline conditions.

	Molar ratio[a]		
Derivative	**1**	**2**	**3**
2,3,4,6-Gal[b]	1.0	0.9	-
2,3,6-Gal	0.5	0.5	0.5
2,3,4,6-Glc	-	-	1.2
2,3,6-Glc	2.1	2.1	1.2
2,3-Glc	1.0	1.0	1.0

[a] 2,3-Glc was taken as 1.0; [b] 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactitol-1-*d*, etc.

O-deacetylation and enzymic modification of EPS B891.–Treatment of EPS B891 with 5% NH_4OH at room temperature resulted in complete *O*-deacetylation as determined by NMR spectroscopy (*vide infra*). After incubation of *O*-deacetylated EPS B891 with Ultra SP, analysis by HPAEC showed that monomeric galactose had been released. The hydrodynamic volume of the enzymically modified EPS (**3**) had only been decreased slightly compared to *O*-deacetylated EPS (**2**) (HPSEC). After enzymic modification, methylation analysis (Table 5.1) showed the disappearance of terminally linked galactose and a decrease of 4-substituted glucose while a new sugar derivative, originating from terminally linked glucose, appeared. Consequently, terminally linked galactose is attached to a 4-substituted glucose residue and the branches, attached to the backbone of EPS B891, contain at least two sugar residues.

1D NMR spectroscopy.–The ^{1}H NMR spectra of native EPS (**1**), *O*-deacetylated EPS (**2**) and enzymically modified *O*-deacetylated EPS (**3**) are shown in Fig. 5.1. In native EPS, the signal at δ 2.162 was assigned to the protons of an acetyl group. Based on the intensities of the relevant signals in the spectrum, the extent of *O*-acetylation of native EPS was determined to be 50%. Because of *O*-acetylation, the spectrum of native EPS (**1**) was more complex and showed less resolution than the spectra of (enzymically modified) *O*-deacetylated EPS (**2** and **3**). Therefore, the latter polysaccharides were used to solve the chemical structure of the repeating units, leaving the acetyl groups out of consideration for the moment. The ^{1}H NMR spectrum of **2** (Fig. 5.1) showed five signals in the anomeric region (δ 5.0-4.4), corresponding to a pentasaccharide repeating unit. The five monosaccharide units were labelled **A**-**E**. The

coupling constant of the anomeric signal of residue **A** ($^3J_{1,2}$ 3.51 Hz) indicates the presence of an α-linked residue, whereas the coupling constants of the anomeric signals of residues **B-E** ($^3J_{1,2}$ 7.3-7.8 Hz) suggest four β-linked residues. In the ^{1}H NMR spectrum of enzymically modified *O*-deacetylated EPS (**3**) (Fig. 5.1), the anomeric signal of residue **E** (δ 4.463) disappeared almost completely and based on the results of the methylation analysis this residue was assigned to the terminally linked galactosyl residue. Since the chemical shift of **C*** H-1 in **3** shifted upfield relative to **C** H-1 in **2**, residue **C** was assigned to the 4-substituted glucosyl residue that changed into a terminally linked residue (residue **C***) upon enzymic modification.

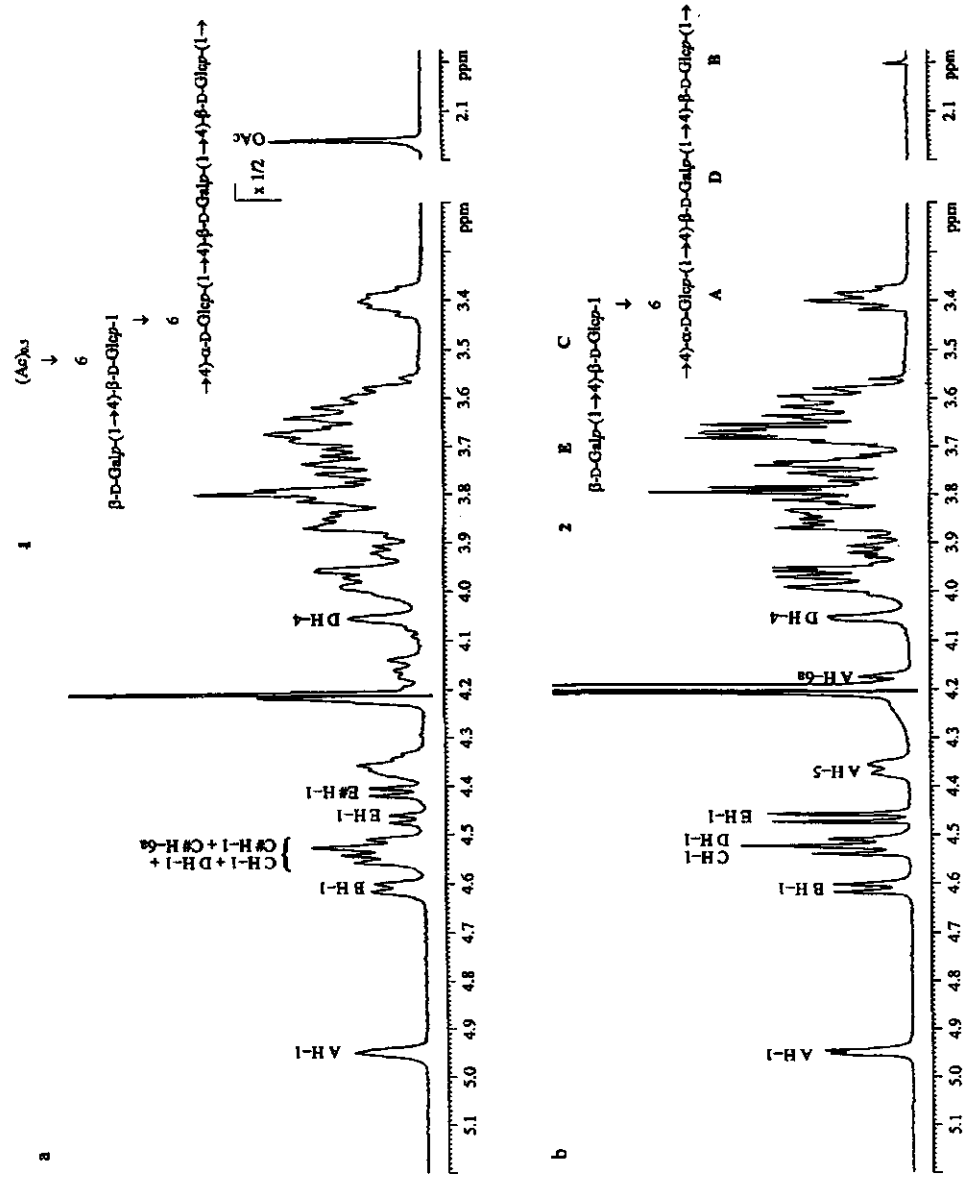
The ^{13}C NMR spectrum (not shown) of native EPS B891 (**1**) did not contain signals typical for the carbon atoms of an acetyl group, probably because of the instability of acetyl groups at 80 °C (*vide infra*). The ^{13}C NMR spectrum (not shown) of *O*-deacetylated EPS B891 (**2**) is in agreement with the suggested pentasaccharide repeating unit, since five signals were observed in the anomeric region (δ 110-95). Based on their chemical shifts, the C-1 signal at δ 101.2 was assigned to the α-hexopyranosyl residue (**A**), while the other anomeric signals (δ104.6, 104.4, 103.8, 103.6) were assigned to four β-hexopyranosyl residues. The resonances at δ 62.4, 61.9 and 61.7 were assigned to hydroxymethyl carbons (unsubstituted at position 6) of hexopyranosyl residues, and one of these signals represents two carbons (*vide infra*). The signal at δ 68.9 was assigned to C-6 of the 4,6-disubstituted hexopyranosyl residue.

Table 5.2
^{1}H NMR chemical shifts[a] of *O*-deacetylated EPS B891 (**2**), recorded in D_2O at 80 °C.

Residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A (1,4,6-α-D-Glc*p*)	4.948	3.63	3.87	3.75	4.364	4.18	3.98
B (1,4-β-D-Glc*p*)	4.608	3.39	3.69	3.67	3.63	3.99	3.84
C (1,4-β-D-Glc*p*)	4.531	3.40	3.68	3.65	3.60	3.98	3.82
D (1,4-β-D-Gal*p*)	4.515	3.60	3.75	4.053	3.79	3.92	3.85
E (*t*-β-D-Gal*p*)	4.463	3.57	3.67	3.95	3.73	3.81	3.77

[a] in ppm relative to the signal of acetone at δ 2.225.

2D NMR spectroscopy of O*-deacetylated EPS B891 (2).*–By means of 2D COSY, TOCSY, and NOESY experiments, the ^{1}H chemical shifts of **2** (Table 5.2) were assigned. Taking the sugar composition and the methylation analysis data into account, the complete series of cross peaks on the H-1 track of residues **A**, **B**, and **C** in the TOCSY spectrum (Fig. 5.2) together with the chemical shifts of **A** H-4, **B** H-4 and **C** H-4 indicate that these residues are glucosyl residues. For residues **D** and **E**, the H-1 tracks in the TOCSY spectrum showed cross-peaks to H-2,3,4 of the corresponding residue. Together with the typical chemical shifts of **D** H-4 and **E** H-4, this indicates that these residues are galactosyl residues. The H-5 chemical shifts of residues **D** and **E** were found via connectivities with H-4 in the COSY spectrum (not shown) and the H-5 assignment of residue **E** could be confirmed via the H-4 track in the TOCSY spectrum (not indicated in Fig. 5.2). The assignments of H-6a,6b from residues **D** and **E** were based on correlations with the corresponding H-5 in both COSY and TOCSY spectra,


a
b
A H-1
B H-1
C H-1 + D H-1 + C# H-1 + C# H-6a
E H-1
B# H-1
D H-4
x 1/2
OAc
ppm

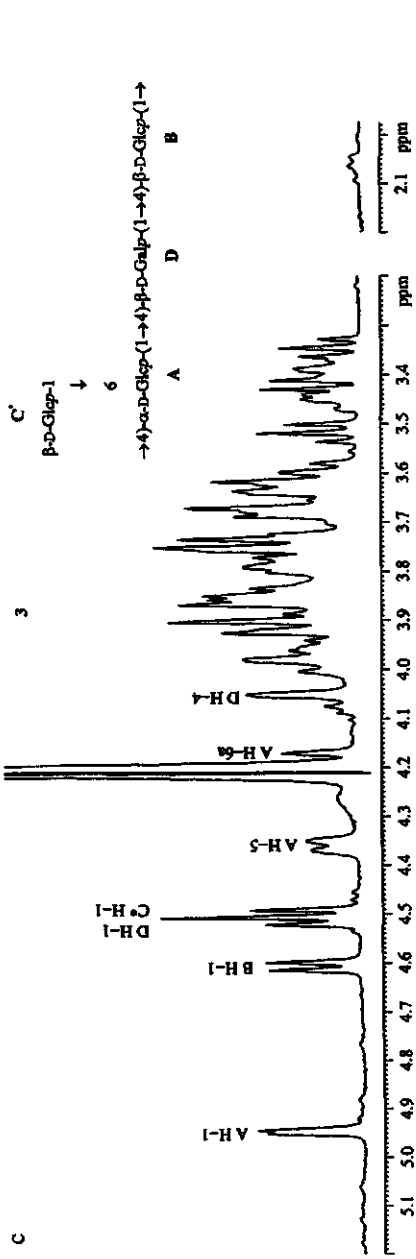


Fig. 5.1. 500-MHz ^{1}H NMR spectra of native EPS B891 (1) (a), *O*-deacetylated EPS B891 (2) (b), and enzymically modified *O*-deacetylated EPS B891 (3) (c), recorded in D_2O at 80 °C. The labelling of sugar residues in (a) is the same as in (b), and the signals of sugar residues which were shifted because of the presence of an acetyl group were labelled (*). For native EPS B891, the approximate relative amount of acetyl groups present (100%= 1) is given between brackets.

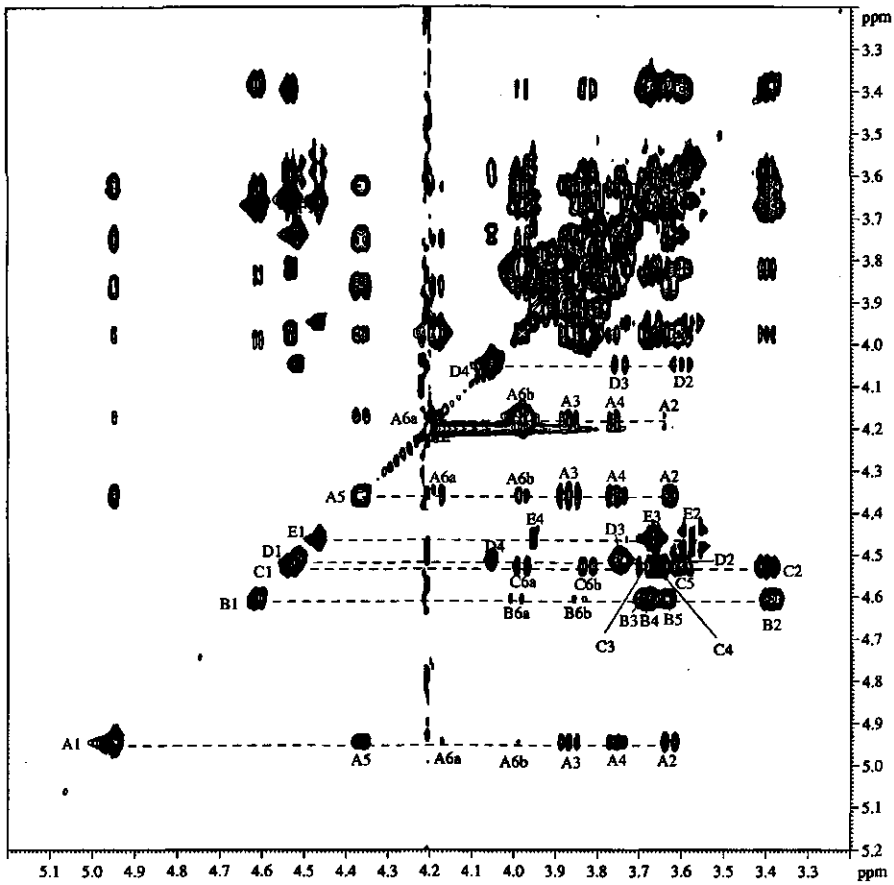


Fig. 5.2. 500-MHz 2D TOCSY spectrum (mixing time = 140 ms) of *O*-deacetylated EPS B891 (2) recorded in D_2O at 80 °C. Diagonal peaks of the anomeric protons, of H-5 and H-6a of residue **A**, and of H-4 of residue **D** are indicated. Cross-peaks belonging to the same scalar-coupling network are indicated near a dotted line starting from the corresponding diagonal peak.

although for residue **E** no precise values could be given due to overlap. For this residue, the H-6a,6b chemical shifts were derived from HMQC measurements. The anomeric configuration was confirmed for all residues by intra-residual interactions in the NOESY spectrum (not shown).

Using the assignment of the ^1H signals, almost all ^{13}C resonances could be assigned as well by means of HMQC and HMBC experiments (Table 5.3). In conjunction with the methylation analysis data, the relative downfield chemical shifts of **A** C-4 ($\Delta\delta$ 9.4), **A** C-6 ($\Delta\delta$ 7.3), **B** C-4 ($\Delta\delta$ 9.7), **C** C-4 ($\Delta\delta$ 9.8), and **D** C-4 ($\Delta\delta$ 9.3) compared to the ^{13}C chemical shifts of the corresponding methyl aldosides [15] demonstrate that residue **A** is 4,6-disubstituted and residues **B**, **C** and **D** are 4-substituted. The $^1J_{C\text{-}1,\ H\text{-}1}$ values of 171 Hz for residue **A** and 162-164 Hz for residues **B-E** agree with the α-configuration for residue **A** and the β-configuration for all other residues.

Table 5.3
^{13}C NMR chemical shifts[a] of *O*-deacetylated EPS B891 (**2**), recorded in D_2O at 80 °C as determined from 1D ^{13}C and 2D ^{1}H ^{13}C- HMQC and HMBC experiments.

Residue	C-1	C-2	C-3	C-4	C-5	C-6
A (1,4,6-α-D-Glc*p*)	101.2	73.1	72.8	80.0	71.2	68.9
B (1,4-β-D-Glc*p*)	103.6	74.5	n.d.[b]	80.3	76.2	61.9
C (1,4-β-D-Glc*p*)	103.8	74.4	75.9	80.4	76.2	61.9
D (1,4-β-D-Gal*p*)	104.6	72.5	73.8	79.0	76.8	61.7
E (*t*-β-D-Gal*p*)	104.4	72.6	74.2	70.1	76.7	62.4

[a] in ppm relative to the signal of acetone at δ 31.55; [b] not determined.

The complete monosaccharide sequence of EPS B891 was determined via methylation analysis after enzymic modification (*vide supra*), 2D HMBC spectra and 2D NOESY analysis. The results of the enzymic modification of EPS B891 already showed that residue **E** was linked to position 4 of residue **C**. This information appeared to be important for the correct sequence of the sugar residues within the repeating unit of EPS B891, since the chemical shifts of residues **B** and **C** are very alike. In the NOESY spectrum (not shown), no inter-residual connectivities were found with **E** H-1, but the HMBC spectrum (Fig. 5.3) showed cross-peaks between **E** C-1 and **C** H-4 and between **C** C-4 and **E** H-1. The **C**(1→6)**A** sequence was suggested by correlations between **C** H-1 and **A** H-6a,6b in the NOESY spectrum and by the cross-peak between **A** C-6 and **C** H-1 in the HMBC spectrum. Inter-residual connectivities in the NOESY spectrum between **A** H-1 and **D** H-4,5,6a,6b indicate the **A**→**D** sequence. This was confirmed by the **D** C-4, **A** H-1 cross-peak in the HMBC spectrum. Due to overlap in the NOESY spectrum, the **D** H-1, **B** H-4 cross-peak could only be assigned tentatively. However, the HMBC spectrum showed a cross-peak between **B** C-4 and **D** H-1, suggesting the **D**(1→4)**B** linkage. The **B**(1→4)**A** sequence was suggested by a strong inter-residual cross-peak between **B** H-1 and **A** H-4 in the NOESY spectrum and this sequence was proven by **B** C-1, **A** H-4 and **A** C-4, **B** H-1 correlations in the HMBC spectrum.

The combined results from chemical, enzymic and NMR studies have demonstrated that *O*-deacetylated EPS B891 is composed of pentasaccharide repeating units with structure **2** as is shown in Fig. 5.1b.

NMR spectroscopy of EPS B891 (1).–1D and 2D NMR experiments were performed on native EPS B891 (**1**) in order to locate the acetyl group within the repeating unit. Since **1** was only partially *O*-acetylated, the spectra were expected to contain signals of both *O*-acetylated and non-*O*-acetylated repeating units. Thus, the assignments of chemical shifts of **2** could be used to interpret the spectra of 1.1D ^{1}H NMR spectra of **1** and **2** (Fig. 5.1) showed changes in signal intensities in the anomeric region (δ 5.0 - 4.4): **2**: **A** H-1 : **B** H-1 : [**C** H-1+**D** H-1] : **E** H-1 = 1:1:2:1, whereas **1**: **A** H-1 : **B** H-1 : [**C**$^{\#}$ H-6+**C** H-1+**C**$^{\#}$ H-1+**D** H-1] : **E** H-1 : **E**$^{\#}$ H-1 = 1:1:2.5:0.4:0.7. Signals of sugar residues, which were shifted because of the presence of an acetyl group, were labelled ($^{\#}$). In the ^{1}H spectrum of **1**, the signal at δ 4.410 was assigned to the anomeric proton of sugar residue **E**$^{\#}$. This assignment was based on the observation that the COSY and TOCSY (both not shown) correlation patterns of residue **E**$^{\#}$ in **1** were similar (but shifted) to the corresponding patterns of residue **E**. Furthermore, addition of the 1D signal intensities of **E** H-1 and **E**$^{\#}$ H-1 resulted in a ratio of 1 compared to the other anomeric

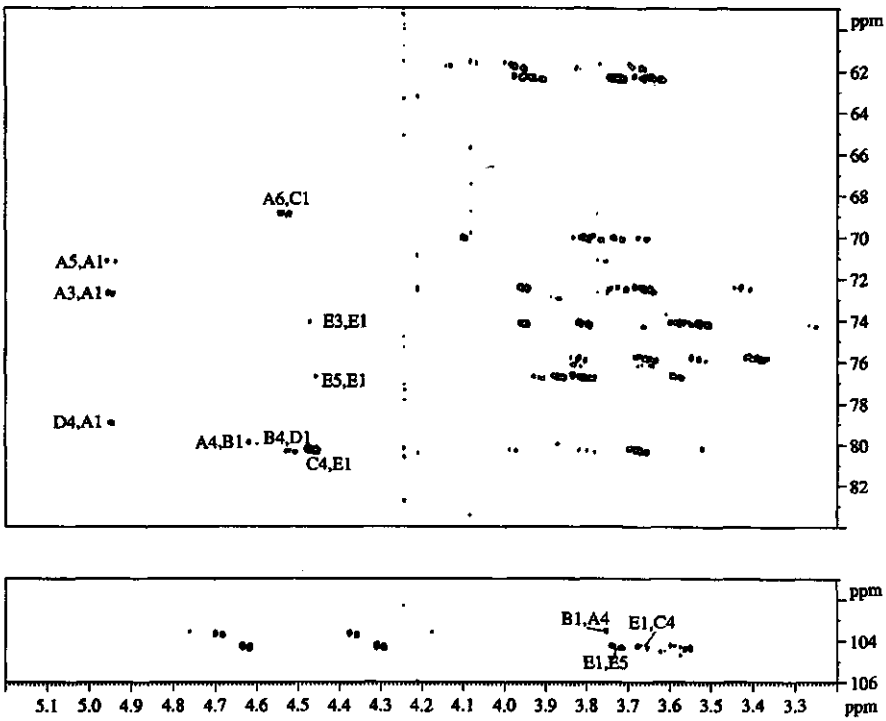


Fig. 5.3. 500-MHz 2D ^{1}H-^{13}C undecoupled HMBC spectrum (delay time between the first proton pulse and the first carbon pulse = 60 ms) of *O*-deacetylated EPS B891 (**2**) recorded in D_2O at 80 °C. The code B1,A4 corresponds to a long-range coupling between **B** C-1 and **A** H-4, etc.

proton signals. The series of signals in the anomeric region of **1** with a total intensity of 2.5 was more difficult to unravel. However, strong indications were found in the COSY and TOCSY spectra of **1** (both not shown) that the chemical shifts of the protons of residue **C** shifted upon *O*-acetylation. Since the largest downfield shift was found for **C**$^{\#}$ H-6a,6b (signal **C**$^{\#}$ H-6a appearing in the anomeric region), EPS B891 is probably partially acetylated at O-6 of the glucosyl residue in the branches of the repeating unit (residue **C**$^{\#}$). In the NOESY spectrum of **1** (not shown), the CH_3 protons of the acetyl group had no cross-peaks with other protons. To prove the position of the acetyl group, a HMBC spectrum of **1** was recorded. However, in this spectrum (not shown) the expected multiple bond correlation between the C=O carbon of the acetyl group and the proton attached to the *O*-acetylated carbon could not be found. This was probably because **1** was only partially *O*-acetylated and because the acetyl groups appeared to be unstable at 80 °C; during the NMR measurements the degree of *O*-acetylation decreased from ca. 50% to ca. 20%. Performing measurements at higher concentrations or at lower temperature was no option because the sample viscosity would lead to considerable broadening of the signals. To overcome this problem, EPS B891 was partially solvolysed and the largest oligomers obtained were analysed by NMR.

HF solvolysis of EPS B891.–From work with other polysaccharides [16-18], the solvolysis of EPS B891 with liquid HF was expected to produce *O*-acetylated oligosaccharides useful to

locate the acyl substituents. Especially the presence of only one α-linked sugar residue and its position in the backbone of the repeating unit of EPS B891 was expected to be in favour of the release of the repeating unit, since preferential cleavage of α- over β-linkages in HF has been noted previously [16,17]. Therefore, EPS B891 was treated with liquid HF and the obtained reaction product was analysed for the presence of oligomers. The HPAEC chromatogram (not shown) demonstrated the release of several oligosaccharides but since the retention time of oligosaccharides depends not only on the type and amount of sugar residues but also on the position of glycosidic linkages and on the overall conformational structure [19], no information was obtained about the identity of the oligomeric structures. Further analysis of the reaction product by MALDI-TOF MS confirmed the presence of oligomers. In fact, the series of signals in the spectrum (Fig. 5.4) indicated the presence of non-, mono- and di-*O*-acetylated oligosaccharides. The mono-*O*-acetylated pentahexosyl fluoride (sodiated,

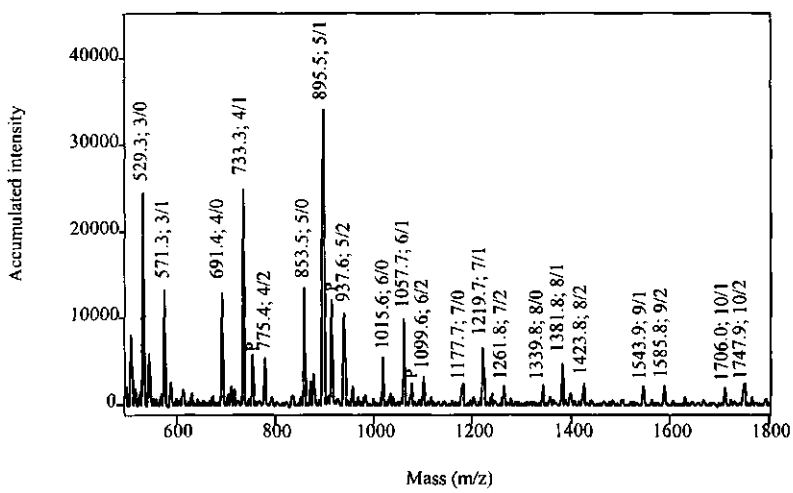


Fig. 5.4. MALDI-TOF mass spectrum of the reaction products obtained after treatment of EPS B891 in liquid HF (30 min at –40 °C). The masses of sodiated oligomers are indicated, followed by the corresponding amount of hexoses and acetyl groups. P indicates the presence of potassium adducts. It should be kept in mind that the use of liquid HF gives glycosyl fluorides as products.

mass = 895.5) had the highest accumulated intensity and probably corresponds to the repeating unit of EPS B891. Attempted purification of the repeating unit from the HF reaction products on Bio-Gel P-2 resulted in the elution pattern shown in Fig. 5.5. Analysis of the fractions by HPAEC and MALDI-TOF MS (not shown) indicated that all oligomers were purified only partially. Since the presence of (non)reducing ends in a mixture of oligomers complicate NMR spectra, the fractions containing the largest oligomers (degree of polymerisation ≥ 6) were pooled for NMR analysis. To prove the multiple-bond correlation(s) between the C=O carbon of the acetyl group with the proton(s) attached to the *O*-acetylated

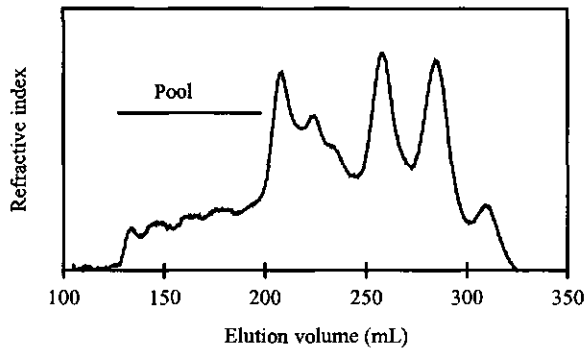


Fig. 5.5. Elution pattern on Bio-Gel P-2 of the oligosaccharides produced by treatment of EPS B891 in liquid HF for 30 min at –40 °C. The oligomers pooled for NMR analyses are indicated.

carbon, a 2D HMBC spectrum of this pool was recorded. As expected, cross-peaks were found (not shown) at ^{1}H δ 4.53; ^{13}C δ 175.3 and ^{1}H δ 4.35; ^{13}C δ175.3, proving that the acetyl group is indeed located at O-6 of sugar residue **C**#. Thus, the combined results from the analyses on *O*-(de)acetylated EPS B891 prove the chemical structure of the repeating unit of native EPS B891 (**1**) as is shown in Fig. 5.1a.

Discussion

The chemical structure of EPS from *L. lactis* subsp. *cremoris* B891 was elucidated (Fig. 5.1a). As many other microbial polysaccharides, the EPS appeared to be *O*-acetylated. In native EPS B891, the acetyl groups were found in a non-stoichiometric ratio relative to the monosaccharides present. It has been mentioned before [11] that during the isolation and purification procedure of native EPS partial *O*-deacetylation may have occurred. Furthermore, it is possible that EPS contains acetyl substituents on alternate repeating units or more randomly distributed [20]. Since esterase activities capable of removing these substituents from exopolysaccharides have not been demonstrated [21] until very recently [22], EPS B891 was *O*-deacetylated using alkali and the chemical structure of the resulting polysaccharide was characterised.

Methylation analysis of polysaccharides **1**, **2** and **3** did not provide a stoichiometric ratio of 4-substituted galactose to 4,6-disubstituted glucose (50% of theoretical, Table 5.1). These results were reproducible and there were no signals pointing towards undermethylation. Since base-catalysed *O*-acetylation has been shown to be not quantitative for all partially methylated alditols because of borate complexing [23], the partially methylated hydrolysate of EPS B891 was also *O*-acetylated under acid-catalysed conditions based on the method described by Harris *et al.* [23]. After acid-catalysed *O*-acetylation, the ratio of 4-substituted galactose to 4,6-disubstituted glucose appeared to be stoichiometric, although this time the amount of terminally linked galactose was underestimated.

In order to prove the location of the acetyl group within the repeating unit, it was necessary to produce oligomers that could be analysed by NMR spectroscopy at low temperature and high concentration. Since the screening of enzymes on *O*-acetylated EPS B891 did not result in any endo-activity (unpublished results), oligomers were produced by solvolysis using HF. From the basic structure of EPS B891 and the behaviour of various polysaccharides in HF, a single cleavage was expected to occur at the α-linkage within the repeating unit. According to the analysis of the HF reaction product by MALDI-TOF MS, the solvolysis was not that selective, since not only single repeating units (and multimers of the repeating unit) were released. In the ^{1}H NMR spectrum of the pooled oligomers obtained after HF solvolysis (not shown), the signal of the anomeric proton of the α-linked sugar residue decreased drastically and a new doublet, probably α-glucosyl fluoride [18] based on $J_{1,F}$ 52.6 Hz, was centred at δ 5.69. Furthermore, the RI signal of the separation of oligomers on Bio-Gel P-2 (Fig. 5.5) combined with the MALDI-TOF MS spectra of the obtained fractions (not shown) proved that the reaction product of HF solvolysis contained a lot of oligosaccharides with degree of polymerisation ≤ 4. These results indicate that the HF-solvolysis was rather selective, but that 30 min in liquid HF probably was too long and led to increased undesired cleavage of β-linkages. Nevertheless, the location of the acetyl group was proven by recording a 2D HMBC spectrum of the pooled oligomers. The approach for locating acetyl groups by measuring HMBC spectra has been described before for polysaccharides from fibre flax [24]. In EPS B891, the acetyl groups were present at the O-6 position of the 4-substituted glucosyl residues in the branches of the repeating units. The chemical shifts of the protons attached to the *O*-acetylated carbon were measured to be 4.53 and 4.35 ppm. These values are similar to the chemical shifts reported for the protons at the acetylated O-6 of a 4-substituted glucosyl residue in xyloglucan oligomers: 4.60 and 4.30 ppm [25]. Moreover, the effect of *O*-acetylation on the protons attached to the carbon which bears the *O*-acetyl group in EPS B891 correspond to the observed shifts for the protons at the *O*-acetylated primary carbon (C-9) of sialic acid (0.5-0.6 ppm), which are lower than the observed shifts for the protons at the *O*-acetylated secondary carbons of sialic acid (1-1.5 ppm) [26].

Knowing the chemical structure of native EPS B891, most masses in the MALDI-TOF MS spectrum of the HF reaction product (Fig. 5.4) can be explained. However, the presence of masses corresponding to [four hexoses + two acetyl groups] and [five hexoses + two acetyl groups] indicates that the *O*-acetylation pattern has minor variants. Similar results have been reported before for gellan gum oligomers obtained by treatment in liquid HF [27]. Since the NMR spectra of native EPS B891 showed the presence of only one acetyl group, no further attention was paid to the unexpected masses in the MALDI-TOF MS spectrum of the HF reaction product.

The exact chemical structure of the repeating unit of EPS B891 can be represented in different ways. For the representation, data from the biosynthesis of the repeating unit by *L. lactis* subsp. *cremoris* B891 are taken into account. According to the results of van Kranenburg *et al.* [28], who demonstrated a glucosyltransferase linking glucose to the lipid carrier and a galactosyltransferase linking galactose to the lipid-linked glucose, the first two sugar residues synthesised for the repeating unit of B891 form lactose. These results led to the representation of the repeating unit of EPS B891 as is shown in Fig. 5.1a.

As a first step in studying the structure-function relationship of exopolysaccharides by using EPS B891, the chemical structure was elucidated. Since the presence or absence of an

acetyl group on each repeating unit can greatly alter the properties of a number of exopolysaccharides [29], comparison of the physical properties of *O*-acetylated and *O*-deacetylated EPS B891 would be very interesting. Likewise, additional physical analysis of enzymically modified *O*-deacetylated EPS B891 could give information about the influence of the presence or absence of the terminally linked galactosyl residue in the branches of the repeating units.

To our knowledge, the chemical structure of EPS B891 has not been reported before for EPS produced by *L. lactis* subsp. *cremoris* or other lactic acid bacteria. Yet, the chemical structure of the branches of *O*-deacetylated EPS B891 are identical to the branches reported for EPS B39 [9]. This is reflected by the fact that the enzyme preparation Ultra SP is able to release galactose from both EPS B39 [9] and *O*-deacetylated EPS B891 (this research). Work on the purification and characterisation of the β-galactosidase responsible for the applied modification of both EPSs is in progress.

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Chapter 6

Purification and characterisation of a ß-galactosidase from *Aspergillus aculeatus* with activity towards (modified) exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* B39 and B891

Abstract

β-Galactosidase from *Aspergillus aculeatus* was purified from a commercial source for its hydrolytic activity towards (modified) exopolysaccharides (EPSs) produced by *Lactococcus lactis* subsp. *cremoris* B39 and B891. The enzyme had a molecular mass of approximately 120 kDa, a pI between 5.3-5.7 and was optimally active at pH 5.4 and 55-60 °C. Based on the N-terminal amino acid sequence, the enzyme probably belongs to family 35 of the glycosyl hydrolases. The catalytic mechanism was shown to be retaining and transglycosylation products were demonstrated using lactose as a substrate. The β-galactosidase was also characterised using its activity towards two EPSs having lactosyl side chains attached to different backbone structures. The enzyme degraded *O*-deacetylated EPS B891 faster than EPS B39. Furthermore, the presence of acetyl groups in EPS B891 slowed down the hydrolysing rate, but the enzyme was still able to release all terminally linked galactosyl residues.

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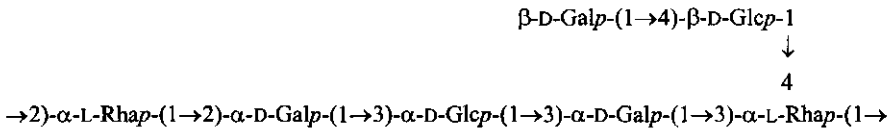
Introduction

Enzymes can be very useful in polysaccharide research for at least two purposes. Firstly, they can be helpful during structural elucidation. Secondly, they can be used to modify the chemical structure of polysaccharides in order to alter their physical properties. Relatively few polysaccharases with activity towards microbial exopolysaccharides (EPSs) have been isolated and characterised [1]. Most enzymes active on these polysaccharides are highly specific and seldom act on more than one substrate, unless the structures are very similar [2]. Since there are very few commercially available enzymes acting on EPSs, the laboratory must normally isolate its own enzymes [1].

For these reasons, many crude enzyme preparations were screened for endo- and exo-activity towards EPSs produced by *Lactococcus lactis* subsp. *cremoris* B40 [3,4], B39 [5] and B891 [6]. It appeared to be extremely difficult to find enzyme preparations able to degrade these EPSs and only a few preparations showed some activity towards these (chemically modified) polysaccharides. Interestingly, a crude enzyme preparation from *Aspergillus aculeatus* was shown to contain one or more β-galactosidase(s) able to release galactose from EPS B39 [5] and from EPS B891 (after *O*-deacetylation) [6]. For both polysaccharides, the enzyme preparation was very useful in determining the structure of the side chains. Knowing the chemical structures of EPS B39 and EPS B891 (Fig. 6.1) and noticing the similarities between the side chains, the activity towards both substrates can probably be attributed to *one* β-galactosidase in this enzyme preparation.

In the present study, we report the purification and characterisation of this β-galactosidase (E.C. 3.2.1.23) from *A. aculeatus* and its action towards several (chemically modified) EPSs.

a.



b.

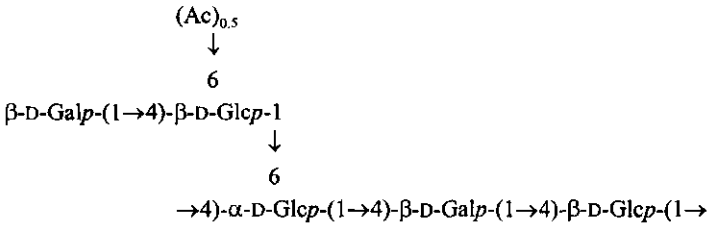


Fig. 6.1. Chemical structures of the repeating units of the exopolysaccharides produced by *L. lactis* subsp. *cremoris* B39 (a) [5] and B891 (b) [6]. The approximate relative amount of acetyl groups present (100% = 1) is given between brackets.

Experimental

Substrates.–The origin and chemical structures of EPSs produced by *L. lactis* subsp. *cremoris* EPS B39 and EPS B891 have been described before [5,6]. For both EPSs, two grades of purity were used: (I) Partially purified EPS was obtained by selective extraction/precipitation using CCl_3CO_2H and EtOH. (II) Purified EPS was obtained from the partially purified EPS by size-exclusion chromatography. (Partially) purified EPS B891 was *O*-deacetylated as described [6]. *p*-Nitrophenyl β-D-galactopyranoside (*p*np-β-D-Gal*p*) was obtained from Koch and Light Ltd. (Haverhill, England) and lactose from Merck (Darmstadt, Germany).

Enzyme purification.–β-Galactosidase was purified from the commercial enzyme preparation Pectinex Ultra SP produced by *A. aculeatus* (Novo Nordisk Ferment AG, Dittingen, Switzerland) starting from 60 mL. Purification involved desalting on Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, CA), followed by separation on DEAE Sepharose Fast Flow, MonoS HR 5/5 and Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden). Experimental details are given in Fig. 6.2. Separation on the Bio-Gel P-10 column was carried out at 4 °C, whereas the other purification steps were performed at 20 °C. All buffers contained 0.01% (w/v) NaN_3 to prevent microbial growth, except for the buffers used for the DEAE Sepharose column. Therefore, NaN_3 was directly added to the fractions obtained after this separation step. In each purification step, the protein content (A_{280}) was monitored and the fractions were screened for β-galactosidase activity towards *p*np-β-D-Gal*p*. Fractions containing activity were also screened towards partially purified, *O*-deacetylated EPS B891 and the fractions having the highest activity were pooled. The resulting pool after separation on the DEAE column was dialysed against 20 mM NaOAc pH 4 (4 °C) before further purification on the MonoS column. After the last two separation steps, the active fractions were pooled according to their purity as judged from native polyacrylamide gel electrophoresis (PAGE). The resulting pool after separation on Superdex 200 was dialysed against 50 mM NaOAc pH 5 (4 °C) and characterisation of the β-galactosidase was performed using this pool.

Enzyme assays.–Column fractions containing activity towards *p*np-β-D-Gal*p* were screened for activity towards partially purified *O*-deacetylated EPS B891. The release of galactose was determined from the formation of NADH from NAD^+ after addition of β-galactose dehydrogenase S (Roche Molecular Biochemicals, F. Hoffmann-La Roche Ltd., Basel, Switzerland). This fast method, based on the method described by Sturgeon [7], was used to pool the collected fractions properly and no enzyme activities were calculated from these data.

β-Galactosidase activity towards EPS was calculated from the release of galactose as determined by high-performance anion-exchange chromatography (HPAEC) [3], whereas the release of *p*-nitrophenol from *p*np-β-D-Gal*p* was measured spectrophotometrically at 405 nm and activity was calculated using the molar extinction coefficient of 13,700 M^{-1} cm^{-1}. The substrate concentration was 0.02% (w/v) for *p*np-β-D-Gal*p* and 0.16% (w/v) for partially purified (*O*-deacetylated) EPS. One unit of enzyme activity (U) is defined as the amount of enzyme that liberates 1 μmol galactose per min (in 50 mM NaOAc pH 5.0 at 30 °C).

Determination of protein content.–The protein content was determined according to the procedure of Bradford [8] using bovine serum albumin as a standard.

Determination of temperature- and pH optimum.–The substrate concentration was 0.02% (w/v) of *p*np-β-D-Gal*p* and incubations took place for 1 h at 30 °C in 50 mM NaOAc pH 5

unless mentioned otherwise. The optimum temperature for the β-galactosidase was determined by incubating at temperatures in the range 0 to 70 °C. The optimum pH was determined using McIlvaine buffers (mixtures of 0.08 M citric acid and 0.16 M sodium hydrogenphosphate) in the pH range 3 to 7.5. The protein concentration was 0.065 μg/mL for the incubations in NaOAc buffer and 0.26 μg/mL for the incubations in McIlvaine buffer, because of the lower enzyme activity in the latter buffer.

Determination of molecular mass and pI.–The molecular mass was determined using three methods: (I) By PAGE, using a PhastSystem (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the instructions of the supplier. The molecular mass was estimated by SDS-PAGE on a PhastGel gradient 10-15 gel, using a low molecular mass kit from 14.4 kDa to 94.0 kDa for calibration. The proteins were silver stained. (II) By determination from the Superdex 200 gel filtration chromatography column (Fig. 6.2). Calibration was performed using ribonuclease A (13.7 kDa), chymotrypsin A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), and ferritin (440 kDa). (III) By matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS), using a Voyager-DE RP Biospectrometry Workstation (PerSeptive Biosystems, Framingham, USA) in the positive mode. The enzyme was concentrated and desalted in a Vivaspin 500 μl concentrator (5000 MWCO, Vivascience, Inc., Binbrook Hill, UK) and then mixed (1 μL) with 1 μL matrix (10 mg 2,5-dihydroxybenzoic acid in 1 mL distilled water). The mass spectrometer was used in the linear mode and calibrated with bovine serum albumin ($[M+H]^+ = 66{,}431$).

The pI was deduced from a pH 4-6.5 isoelectric focusing gel using a PhastSystem and the low pI calibration kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The proteins were silver stained or the activity was visualised under UV light by soaking the gel in 10 mM NaOAc pH 5 + 0.01% NaN_3 containing 1 mM 4-methylumbelliferyl-β-D-galactoside.

Deglycosylation of β-galactosidase.–Deglycosylation of the enzyme with N-glycosidase F (Roche Molecular Biochemicals, F. Hoffmann-La Roche Ltd., Basel, Switzerland) was performed according to the instructions of the Glycopro deglycosylation kit (ProZyme, Inc., San Leandro, USA). Analysis was performed by SDS-PAGE (*vide supra*).

Determination of N-terminal amino acid sequence.–The N-terminal amino acid sequence was determined after SDS-PAGE followed by electroblotting onto polyvinylidene difluoride (PVDF) membrane by the Sequence Centre of Utrecht University (The Netherlands). Analysis was performed, using automated Edman degradation, on a Perkin-Elmer Applied Biosystems Protein Sequencer 476A.

Kinetics of β-galactosidase towards pnp-β-D-Galp.–*p*np-β-D-Gal*p* was incubated with β-galactosidase (in 50 mM NaOAc pH 5.0, 30 °C, 1 h) at substrate concentrations in the range 0.008 mM (0.0002% w/v) to 8 mM (0.24% w/v). The inhibitory effect of D-galactose on β-galactosidase activity was investigated using different concentrations of *p*np-β-D-Gal*p* (0.015 to 6 mM) and D-galactose (0 to 10 mM). The protein concentration was 0.065 μg/mL.

Kinetics of β-galactosidase towards lactose.–Lactose was incubated with β-galactosidase (in 50 mM NaOAc pH 5.0, 30 °C, 1 h) with substrate concentrations in the range 0.08 mM to 80 mM. The protein concentration was 0.52 μg/mL. The β-galactosidase activity was calculated from the release of galactose as determined by HPAEC [3]. Transglycosylation products were determined by HPAEC [9] and MALDI-TOF MS [4] using a substrate concentration of 80 mM.

Stereochemical course of hydrolysis.–Substrate (0.2 mg *p*np-β-D-Gal*p* in 0.5 mL 50 mM NaOAc buffer, pH 5) and enzyme (0.16 mg β-galactosidase in H_2O) were freeze dried three times from D_2O (99.9 atom% D, Cambridge Isotope Laboratories, USA) to exchange labile ^1H atoms for D. Just prior to ^1H NMR analysis, the substrate was dissolved in 0.7 mL 99.96% D_2O (Cambridge Isotope Laboratories, USA) and the pD adjusted to approximately 5 by the addition of 1% DCl. The solution was then equilibrated at 30 °C in a 5 mm NMR tube and the initial spectrum was recorded on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. The enzyme was dissolved in 100 µl 99.96% D_2O and 20 µl of this solution was added to the NMR tube. The stereochemical course of hydrolysis was followed by recording ^1H NMR spectra at intervals during the incubation. Afterwards, a trace of acetone was added to the NMR tube and a final spectrum was recorded for calibration (δ 2.225 ppm).

Activity of β-galactosidase towards purified EPSs.–Purified EPS B39, B891 and *O*-deacetylated B891 were dissolved in 50 mM NaOAc pH 5.0 containing 0.01% (w/v) NaN_3 in a way that the resulting concentration of terminally linked galactosyl residues (ca. 160 µg/mL) was similar for all substrates. The substrates were incubated (30 °C) with β-galactosidase (protein concentration was 18 µg/mL) and the enzyme was inactivated at set time intervals by heating (10 min, 100 °C). The release of galactose was analysed by HPAEC [3].

To verify the absence of galactose in enzyme-treated EPS B39 and EPS B891 and the presence of acetyl groups in EPS B891 after incubation with β-galactosidase, incubations were repeated on a larger scale as described above. After 32 h of incubation, no heat treatment was given to inactivate the enzyme, because acetyl groups are heat-unstable [10]. The digests were dialysed against distilled water and freeze dried three times from D_2O (99.9 atom% D, Cambridge Isotope Laboratories, USA). The substrates were dissolved in 0.7 mL 99.96% D_2O (Cambridge Isotope Laboratories, USA) and analysed by ^1H NMR at 80 °C in a 5 mm NMR tube on a Bruker AMX-500 spectrometer.

Results

Purification of β-galactosidase.–The purification scheme of β-galactosidase is given in Fig. 6.2 and the results are summarised in Table 6.1. The overall purification of the enzyme on protein basis was approximately 15-fold and the overall yield of β-galactosidase activity was ca. 15%. The specific activity of the purified β-galactosidase towards *p*np-β-D-Gal*p* was 24 U/mg, which was lower than reported for purified β-galactosidase from *Aspergillus niger* (49 U/mg [11] and 33 U/mg [12]). Furthermore, it was obvious (Table 6.1) that the activity towards *p*np-β-D-Gal*p* was much higher (×333) than the activity towards *O*-deacetylated EPS B891.

Characterisation of β-galactosidase.–The molecular mass of β-galactosidase estimated by size-exclusion chromatography was approximately 130 kDa. SDS-PAGE revealed a mass of 110 kDa, indicating that the enzyme is monomeric. By using MALDI-TOF MS, three ions were found at *m/z* of approximately 120,000 $[M+H]^+$, 60,000 $[M+2H]^+$, and 40,000 $[M+3H]^+$, confirming that the molecular mass of β-galactosidase is ca. 120 kDa. Similar molecular masses have been reported for β-galactosidase from *A. niger* [11-13], *Aspergillus oryzae* [14,15], *Aspergillus fonsecaeus* [16] and *Aspergillus foetidus* [17]. Upon deglycosylation, the molecular mass found by SDS-PAGE decreased slightly. This indicates that, like most fungal glycosidases [12], the enzyme is a glycoprotein.

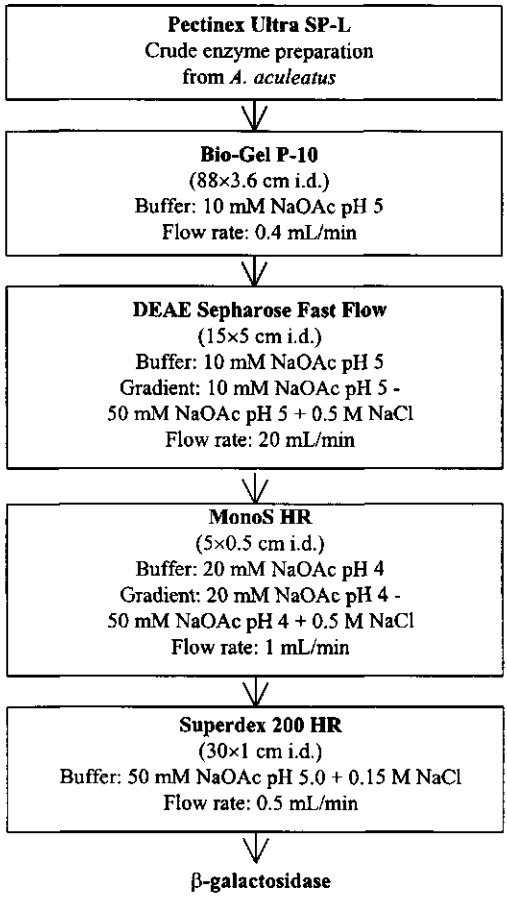


Fig. 6.2. Purification scheme of β-galactosidase from Pectinex Ultra SP produced by *A. aculeatus*.

Table 6.1
Purification of β-galactosidase from *A. aculeatus*.

Purification step	*p*np-β-D-Gal*p*		*O*-deacetylated EPS B891	
	specific activity (U/mg)	yield[a] (%)	specific activity (mU/mg)	yield[a] (%)
Bio-Gel P-10	2	100	4	100
DEAE Sepharose Fast Flow	n.d.[b]	n.d.[b]	21	64
MonoS HR	27	20	69	22
Superdex 200 HR	24	13	72	17

[a] based on activity; [b] not determined.

The isoelectric point (pI) of the β-galactosidase was determined by isoelectric focusing and 3×2 major bands were found after silver staining with pIs in the range 5.3 to 5.7. When using 4-methylumbelliferyl-β-D-galactoside for enzyme activity screening, the area with the six bands was visualised under UV light. It appeared that each group with two bands contains β-galactosidase activity and that multiple forms of the β-galactosidase are present. Differences in chromatographic behaviour and physico-chemical characteristics such as pIs are often observed for fungal extracellular enzymes [12], and no further attempts were made to investigate the presence of multiple forms. The pIs reported here are higher than the pIs reported for β-galactosidases from other Aspergilli (4.2-4.9) [11-13,15,16].

The optimum temperature of β-galactosidase in NaOAc buffer was 55-60 °C; at higher temperatures the activity decreased rapidly. This temperature optimum variates from the optima reported for β-galactosidases produced by *A. niger* (60-65 °C) [12], *A. oryzae* (45-55 °C) [14,15,18], *A. foetidus* (67 °C) [17], and *Aspergillus phoenicis* (70 °C) [19]. The optimum pH was found at pH 5.4 in McIlvaine buffers and was higher than the optima reported for the β-galactosidases of other Aspergilli (2.5-5.0) [11,12,14-21].

The N-terminal amino acid sequence was determined from the major band on SDS-PAGE. The resulting sequence was ?QKYVTWDDKSLFINGERN?. An amino acid sequence alignment was performed by using the BLAST programs [22]. The 18 N-terminal amino acid sequence returned a perfect match with the known β-galactosidase precursor from *A. niger* [23]. According to the SWISS-PROT Protein Sequence Data Bank [24], the *A. niger* β-galactosidase belongs to family 35 of the glycosyl hydrolases [25-27]. Since the N-terminal amino acid sequence of the enzyme from *A. aculeatus* matches with *A. niger* β-galactosidase and both enzymes are produced by Aspergilli, it was assumed that the β-galactosidase from *A. aculeatus* also belongs to family 35 of the glycosyl hydrolases.

Stereochemical course of hydrolysis.–There are two basic mechanisms of catalysis for glycosyl hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point [28]. The stereochemical course of hydrolysis by β-galactosidase from *A. aculeatus* was followed by recording ^{1}H NMR spectra at intervals during the incubation (Fig. 6.3). Time t = 0 shows the partial spectrum of *p*np-β-D-Gal*p* before addition of β-galactosidase. After 7 min of incubation with β-galactosidase, the substrate was completely hydrolysed and the doublet ($^3J_{1,2}$ 7.9 Hz) found in the anomeric region was assigned to the anomeric proton of β-Gal (δ 4.575 ppm) [29]. Later in the incubation, mutarotation of the initially formed β-anomers of Gal brought about the appearance of another doublet ($^3J_{1,2}$ 3.7 Hz) in the anomeric region, assigned to the anomeric proton of α-Gal (δ 5.256 ppm) [29]. After 22.5 h, the relative intensities of the α- and β-anomer resonances were 0.28:0.72, which corresponds to the mutarotational equilibrium of Gal. These results prove that β-galactosidase from *A. aculeatus* catalysed the hydrolysis of *p*np-β-D-Gal*p* with retention of anomeric configuration. Since the β-galactosidase from *A. aculeatus* probably belongs to family 35 of the glycosyl hydrolases and the mechanism of catalysis appears to be conserved within each family [30], the mechanism for family 35 of the glycosyl hydrolases is probably retaining. Inferred from sequence similarities, this has been suggested before [31].

Kinetics of β-galactosidase.–β-Galactosidase from *A. aculeatus* catalysed the hydrolysis of *p*np-β-D-Gal*p* and lactose, and the dependence of the initial hydrolysis rate on the substrate concentration was investigated for both substrates. The Lineweaver-Burk plot (Fig. 6.4) indicated that the rate of hydrolysis of *p*np-β-D-Gal*p* by β-galactosidase decreased at higher

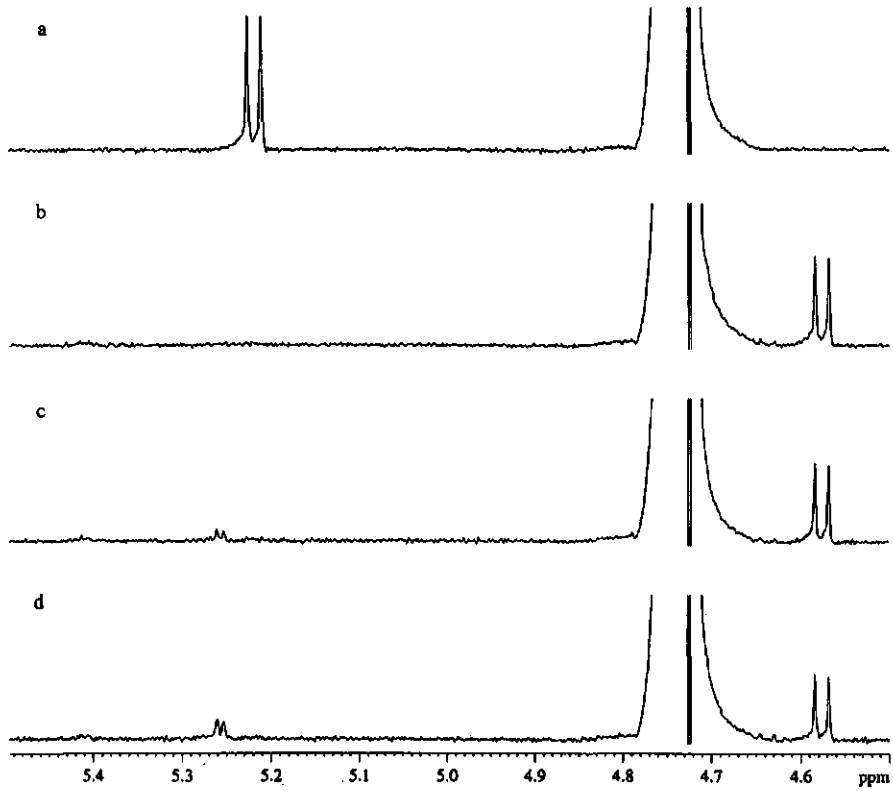


Fig. 6.3. Partial ^{1}H NMR spectra showing the stereochemical course of hydrolysis of *p*np-β-D-Gal*p* by *A. aculeatus* β-galactosidase: t = 0 min (a), t = 7 min (b), t = 49 min (c) and t = 22.5 h (d). The doublet in the spectrum at t = 0 (a) originated from *p*np-β-D-Gal*p*. The doublet at 5.256 ppm represents the anomeric proton of α-Gal*p*, and the doublet at 4.575 ppm represents the anomeric proton of β-Gal*p*.

substrate concentrations. In the hydrolysis of lactose, this effect was not observed at the corresponding substrate concentrations (Fig. 6.5a), although at the highest lactose concentrations (40 and 80 mM) this tendency was very slightly observed. A Lineweaver-Burk plot resembling Fig. 6.4b has been reported for an α-galactosidase from *Trichoderma reesei* [32] using *p*-nitrophenyl α-D-galactopyranoside as a substrate. In that study, the apparent hydrolysis inhibition in presence of a high substrate concentration was correlated with transglycosylation activity of the enzyme. In the current study, the presence of transferase reactions at lactose concentrations >10 mM was suggested by differences in the amounts of galactose and glucose released (Fig. 6.5b). Transglycosylation products are being formed when the enzyme transfers the galactose moiety of a β-galactoside to an acceptor, other than water, containing a hydroxyl group [33]. Incorporation of galactose into oligosaccharides leads to different amounts of the monomeric hydrolysis products galactose and glucose as was shown for the hydrolysis of lactose by a β-galactosidase from *Streptococcus thermophilus* [34]. To investigate the formation of transglycosylation products during lactose hydrolysis at

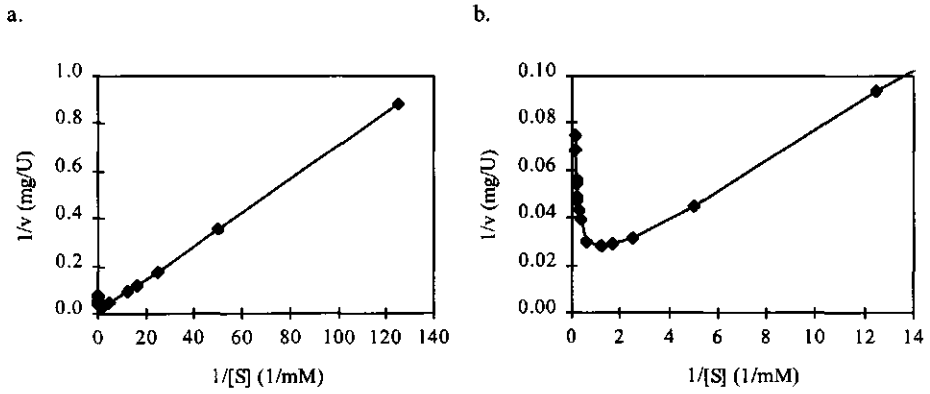


Fig. 6.4. Lineweaver-Burk plot for the hydrolysis of *p*np-β-D-Gal*p* catalysed by *A. aculeatus* β-galactosidase. The whole range of substrate concentration [S] is depicted in (a), whereas (b) zooms in on the area with high [S].

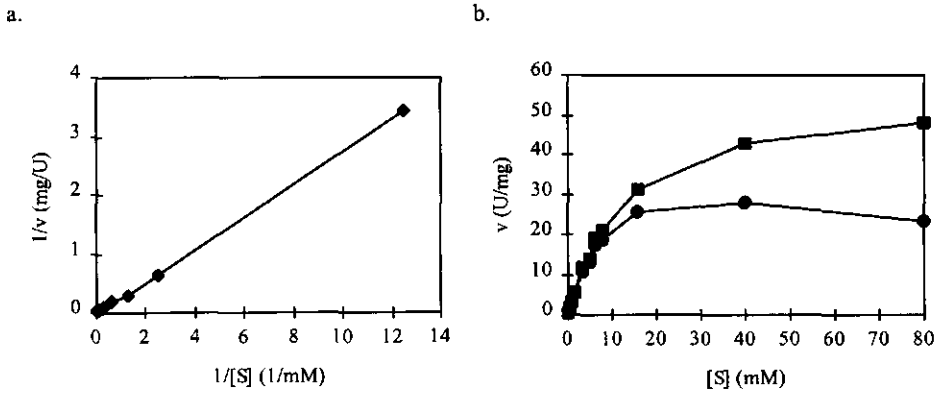


Fig. 6.5. Hydrolysis of lactose catalysed by *A. aculeatus* β-galactosidase: Lineweaver-Burk plot (a) and the rate of hydrolysis deduced from the glucose (■) and galactose (●) release upon substrate concentration (b).

a substrate concentration of 80 mM, the digest was analysed by HPAEC and MALDI-TOF MS. Both methods proved the presence of transglycosylation products, since after incubation oligomeric peaks were found by HPAEC and a mass corresponding to a trimer of hexoses appeared in the MALDI-TOF MS spectrum (not shown). No effort was made to further characterise the obtained transglycosylation products.

The kinetic parameters K_m and k_{cat} of the reactions were evaluated from the initial rates under conditions for which the Lineweaver-Burk plot was linear. Assuming that all protein represents β-galactosidase (M_w = 120 kDa), an estimation of k_{cat} was derived from the obtained V_{max}. According to Fersht [35] the ratio k_{cat}/K_m is the 'specificity constant' of a substrate. For *p*np-β-D-Gal*p*, k_{cat}/K_m was (932 s^{-1})/(3.28 mM) = 284 mM s^{-1} and for lactose k_{cat}/K_m was (254 s^{-1})/(34.8 mM) = 7 mM s^{-1}, indicating that the hydrolytic activity of β-

galactosidase towards *p*np-β-D-Gal*p* is ca. 40 times higher than towards lactose.

D-Galactose in concentrations ≤1 mM did not noticeably inhibit enzymic hydrolysis of *p*np-β-D-Gal*p*. However, 10 mM D-galactose inhibited the hydrolysis of *p*np-β-D-Gal*p* by the β-galactosidase, but only at substrate concentrations below 3 mM. These results indicate that the inhibition can be overcome by a sufficiently high substrate concentration, which means that D-galactose is a competitive inhibitor of the β-galactosidase. These results agree with the fact that galactose is generally considered to be a competitive β-galactosidase inhibitor since it competes with lactose for the substrate-binding sites in the enzyme [33].

Activity of β-galactosidase towards EPSs.–Valuable information about the substrate specificity of an enzyme can be deduced from the effects of structural modifications of a substrate on the enzymic constants k_{cat} and K_m. Ideally, these parameters should be determined for EPS B891, *O*-deacetylated EPS B891 and EPS B39 to investigate whether the β-galactosidase has different affinities and hydrolytic activities for the different polymers. However, the range of substrate concentration of the polysaccharides in which the reaction rate can be measured was limited due to the high viscosity and consequently no reliable values for the kinetic parameters could be obtained. Therefore, the differences in preference of the β-galactosidase for the different EPSs were investigated by measuring the release of galactose in time. Attempts were made to dissolve the EPSs in such amounts that the concentration of terminally linked galactosyl residues (t-Gal) was equal for all samples.

The results (Fig. 6.6) show that the amount of galactose released after 32 h from EPS B39 (155 μg/mL) and EPS B891 (152 μg/mL) was similar and that the amount for *O*-deacetylated EPS B891 (167 μg/mL) was slightly higher. The addition of new enzyme after 32 h of incubation did not release more galactose (not shown) and, consequently, the end point of the reactions had been reached at that time. The higher concentration of t-Gal in *O*-deacetylated EPS B891 might have increased the initial reaction rate. However, in another experiment (not shown), in which the initial reaction rates of β-galactosidase on *O*-deacetylated EPS B891 and EPS B39 were identical, the concentration of t-Gal using EPS B39 was much higher (30%) than the concentration of t-Gal using *O*-deacetylated EPS B891 as a substrate. Extrapolating

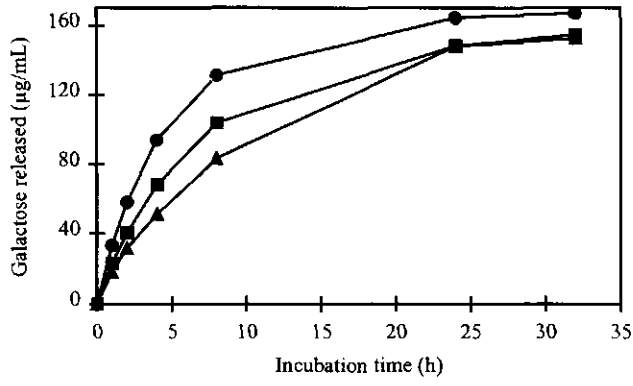


Fig. 6.6. Release of galactose from *O*-deacetylated EPS B891 (●), EPS B39 (■) and EPS B891 (▲) in time during incubation with β-galactosidase from *A. aculeatus*.

these results to Fig. 6.6, it was concluded that the higher initial reaction rate of *O*-deacetylated EPS B891 was only partially caused by the higher concentration of t-Gal. Thus, at substrate concentrations with equal amounts of t-Gal, *O*-deacetylated EPS B891 is degraded more rapidly than EPS B39, which is being degraded faster than EPS B891.

To investigate whether β-galactosidase can release all terminally linked galactosyl residues from EPS B39 and EPS B891 and to exclude the possibility that EPS B891 is slowly *O*-deacetylated during incubation at 30 °C, ^{1}H NMR experiments were performed on the resulting polymers of EPS B39 and EPS B891 after incubation with β-galactosidase. The results (not shown) proved that indeed all terminally linked galactosyl residues were released from both EPSs. Furthermore, the acetyl groups were still present in EPS B891 after incubation with β-galactosidase.

Discussion

Purification of the β-galactosidase activity from *A. aculeatus* resulted in *one* β-galactosidase, possibly consisting of multiple forms. These results conform to findings that were reported before. Different forms of β-galactosidase were found in *A. niger* [11,13], but evidence for the existence of a β-galactosidase-encoding gene family in fungi has not yet been reported [12].

The mechanism of catalysis of β-galactosidase from *A. aculeatus* was shown to result in a retention of the anomeric configuration. According to Sinnott [28], many retaining hydrolyses contain transglycosylation activity. For β-galactosidase from *A. aculeatus* transferase activity was shown using 80 mM lactose as a substrate. Based on observations with other β-galactosidases [33,34], the concentration of transglycosylation products formed by β-galactosidase from *A. aculeatus* will probably increase at higher lactose concentrations. Using EPS as a substrate, it is unlikely that there is significant transferase activity, if any at all, since the substrate concentrations are relatively low due to the high viscosity.

The β-galactosidase from *A. aculeatus* was able to act on different EPSs with similar side chains and has been used for structural elucidation of EPSs [5,6]. Yamamoto *et al.* [36,37] used a β-galactosidase from Jack bean to obtain structural information about the side chains of EPS of *Lactobacillus helveticus* TY1-2 and TN-4. Like EPS B39 and *O*-deacetylated EPS B891, these polysaccharides contain lactosyl side chains. Therefore, it is well possible that also the β-galactosidase from *A. aculeatus* is able to act on the EPSs from *Lactobacillus helveticus* TY1-2 and TN-4. Neither the position at which the lactosyl side chain is linked to the backbone of the polymer nor the type of sugar residue in the backbone which is substituted with the lactosyl fragment seems to be relevant for both enzymes (position 4 of α-L-Rha*p* for EPS B39, position 6 of α-D-Glc*p* for EPS B891, position 6 of β-D-Gal*p* for EPS TY1-2, and position 3 of β-D-Gal*f* for EPS TN-4).

Ester-linked substituents are widely found in bacterial exopolysaccharides [1]. With respect to EPSs produced by lactic acid bacteria, the polysaccharides produced by *Lactobacillus sake* 0-1 [38] and *L. lactis* subsp. *cremoris* B891 [6] are partially *O*-acetylated. According to Sutherland [2] the action of most of the polysaccharide hydrolases on EPSs is hardly effected by the presence of various acyl substituents, although the action of some polysaccharide lyases can be inhibited markedly. The present study showed that the acetyl groups did not confer total resistance to the investigated β-galactosidase and that all

terminally linked galactosyl residues could be removed in presence of these substituents. Nevertheless, it appeared that native EPS B891 was degraded slower than *O*-deacetylated EPS B891. Various parameters might be involved in the explanation of this observation. Firstly, *O*-deacetylation of EPS might change the sample viscosity, leading to a different rate at which the enzyme encounters the substrate in solution. Under the conditions used in Fig. 6.6, no obvious differences in viscosity were found by visual comparison. Furthermore, the reaction rate was linearly related to the substrate concentration for all EPSs (not shown). From this, it was concluded that it is very unlikely that the differences in reaction rates found between the EPS structures are due to differences in viscosity. Secondly, the acetyl groups present in native EPS might cause some steric hindrance for the enzyme to bind. Knowing that the acetyl groups in EPS B891 are linked to the sugar residue adjacent to the terminally linked galactosyl residues, this might very well be the case. Thirdly, the presence of acetyl groups can greatly affect the ordered structure adopted by some bacterial polysaccharides in solution [39]. Overall, the influence of the presence of acetyl substituents in EPS B891 on the hydrolysing rate of β-galactosidase seems to be directly related to the chemical structure (and ordering) of the EPS molecules. Moreover, the lower reaction rate of β-galactosidase using EPS B39 compared to *O*-deacetylated EPS B891 as a substrate also seems to be a matter of structure (and ordering).

In conclusion, the β-galactosidase investigated in the present study has been used for structural elucidation of EPSs [5,6], and can be used in future research to study the relationship between the chemical structure and the physical properties of these polysaccharides. The enzymic modification of EPSs might lead to improved physical properties, as has been found for a mutant type of xanthan after treatment with β-glucuronidase [40]. The structure-function relationship of EPSs modified using β-galactosidase is now under investigation.

Acknowledgements

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Chapter 7

General discussion

Part of this chapter will be submitted for publication by: R. Tuinier, W.H.M. van Casteren, P.J. Looijesteijn, H.A. Schols, A.G.J. Voragen, and P. Zoon.

Introduction

To be able to produce tailor-made polysaccharides with industrial potential, insight into the relationship between the chemical structure and the physical behaviour of polysaccharides is required. Since microbial exopolysaccharides (EPSs) are build up with repeating units, these polymers are very suitable for structure-function studies. For food industry, lactic acid bacteria are interesting from this point of view because they are generally recognised as safe (GRAS) and can be used for the *in situ* production of EPS. Within the framework of thesis, EPSs produced by different strains of *Lactococcus lactis* subsp. *cremoris* were investigated because of their viscosifying properties. The fact that this bacterium was physiologically and biochemically rather well-characterised and the fact that a genetic study on EPS biosynthesis in *L. lactis* [1] was already in progress were other arguments to investigate the EPSs produced by this bacterium.

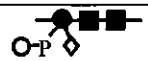
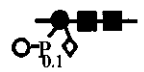
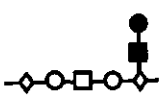
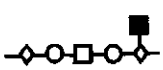
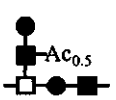
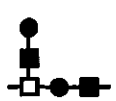
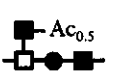
The aim of this thesis was (I) to elucidate the chemical structures of different EPSs produced by *L. lactis* subsp. *cremoris,* using enzymes as a supplement to chemical methods, (II) to prepare structurally related polysaccharides for structure-function studies by (enzymic) modification of EPSs, (III) to characterise enzymes, which are able to degrade or modify EPS, by using the obtained results on EPS structures, and (IV) to study the relationship between the chemical structure and the physical properties of (modified) EPSs. The results of these topics are successively discussed in this chapter.

Chemical structures of EPSs

The chemical structures of three EPSs from different strains of *L. lactis* subsp. *cremoris* were characterised: EPS B40, B39 and B891 (Table 7.1). The repeating unit of EPS B40 appears to be identical to the already published structure of EPS SBT 0495 [2]. In contrast, the structures of EPS B39 and B891 have not been reported before for polysaccharides produced by lactic acid bacteria. All EPS structures conform to the structural features of EPSs from lactic acid bacteria as described in chapter 1: the sugar residues within the repeating units are galactose, glucose (EPS B891) and, additionally, rhamnose (EPS B40 and B39). Furthermore, all repeating units are branched and decorations are present in EPS B40 (phosphate groups) and in EPS B891 (acetyl groups). The chemical structure of EPS B39 shows similarities with the structures of EPS SFi12 [3], EPS OR 901 [4], and EPSs Rs and Sts [5] produced by *Streptococcus thermophilus* (chapter 4). Another interesting resemblance is found between the structure of the backbone of EPS B891 and the backbone of EPS B40 (and EPS SBT 0495 [2]): they are identical, except for the fact that in EPS B891 one glucosyl residue is α-linked. However, the position(s) and chemical structures of the substituent(s) of these EPSs are totally different.

In the structural studies, enzymes were successfully used in addition to chemical methods, like sugar analysis, methylation analysis, mass spectrometry, and NMR spectroscopy. In fact, an endoglucanase and a phosphatase from *Trichoderma viride* were found to be helpful tools in the structural characterisation of EPS B40. Furthermore, a β-galactosidase from *Aspergillus aculeatus* was used to elucidate the structure of the side chains of EPS B39 and EPS B891. Unfortunately, the number of enzymes found to act on EPS was limited and, in case of EPS B40, the polysaccharide had to be modified by chemical methods first. Especially during the

Table 7.1
Chemical and enzymic modifications of EPS B40, B39 and B891, resulting in structurally related polysaccharides (■, β-D-Glc*p*; □, α-D-Glc*p*; ●, β-D-Gal*p*; ○, α-D-Gal*p*; ◊, α-L-Rha*p*; P, phosphate group; Ac, acetyl group; the approximate relative amount of remaining residues (100% =1.0) is given in case residues had been partially removed).

EPS	Modifying agent	Structure of repeating unit after modification
B40	–	
B40	0.3 M H_2SO_4	
B40	0.1 M CF_3CO_2H	
B40	0.2 M NaOH	
B40	28 M HF	
B40 (HF-treated)	0.1 M CF_3CO_2H	
B40 (CF_3CO_2H-treated)	phosphatase (in Maxazyme Cl)	
B39	–	
B39	β-galactosidase	
B891	–	
B891	5% NH_4OH	
B891	β-galactosidase	
B891 (NH_4OH-treated)	β-galactosidase	

structural characterisation of EPS B891, additional enzymes would have been useful: e.g. an endo-enzyme able to act on the backbone of this EPS or a glucosidase able to remove the terminally linked glucosyl residues after treatment with β-galactosidase. The former enzyme would have made the use of liquid HF, which requires specialised equipment, redundant and the degradation would have been more specific. Although these enzymes have not been found, they are most likely present in nature. Within the framework of this thesis, only commercial enzyme preparations were screened for activity towards EPSs. According to Sutherland [6], commercially available enzyme preparations are seldom capable of degrading microbial polysaccharides. This may be caused by the fact that the enzymes in these preparations have not been induced on appropriate substrates. Furthermore, commercial enzyme preparations are often produced by a pure bacterial culture, whereas in nature micro-organisms are often found as consortia capable of degrading complex substrates, which cannot be used by the individual species. A problem with bacterial mixtures, however, is to ensure that they are stable and that all necessary micro-organisms are present in sufficient numbers [6]. The exogenous production of EPS-degrading enzymes by mixed cultures of micro-organisms was investigated by another partner within this research project, but has not resulted in enzyme activities able to partly degrade or modify EPS B40, B39, and B891. Other sources of enzymes, like the endogenous production of enzymes by *L. lactis* subsp. *cremoris* and the production of polysaccharide-degrading enzymes by bacteriophages, have hardly or not at all been studied within this project.

Besides the use of enzymes, several chemical modifications of EPSs have proven to facilitate the analyses. For EPS B40, the poor stoichiometry in sugar (linkage) analyses due to incomplete hydrolysis in presence of phosphate groups could be interpreted using different chemical modification methods. Furthermore, the modification of EPS B40 (using 28 M HF) and EPS B891 (using 5% NH_4OH) resulted in better-resolved NMR spectra. Another example is the use of liquid HF for the production of oligomers of EPS B891. Since esters remain intact in liquid HF, the location of acetyl groups in the oligomers could rather easily be determined by recording NMR spectra at low temperatures. In conclusion, chemical modifications of EPSs are still very useful for structural analysis, especially in case particular enzymes are not available.

(Enzymic) modification of EPS structures

In this thesis, methods to obtain structurally related polysaccharides by chemical and enzymic modification of EPSs are described and the results are summarised in Table 7.1. These modifications were all caused by the (partial) removal of sugar residues and/or non-sugar substituents. The consequences of some of these structural modifications on the physical properties will be discussed later.

Recently, there has been a growing industrial interest in functional polymer systems. Since charged polymers (polyelectrolytes) are most appropriate for these functional purposes [7], it would be interesting to introduce charged groups into neutral polysaccharides. This can be achieved, for example, by oxidising an alcohol function into a carboxylic acid in a specific way starting with enzymic oxidation, which introduces an aldehyde group on galactose units, followed by chemical oxidation by halogen [8,9]. The neutral exopolysaccharides B39 and B891 both contain terminally linked galactosyl residues, which are suitable for oxidation by

galactose oxidase. To exclude possible steric hindrance of galactose oxidase by the acetyl groups, EPS B891 was *O*-deacetylated before oxidation. Thus, both EPSs were oxidised basically as described for guar gum [9].

> [In short, EPS in 0.1 M sodium phosphate buffer pH 7 (0.25 mg/mL) was incubated (24 h, 30 °C) with galactose oxidase (2 U/mg EPS) from *Dactylium dendroides* [10] expressed in *Aspergillus nidulans*, and catalase (Sigma; 1.7 U/U galactose oxidase). Then, KI and I_2 were added to a final weight ratio of EPS:I_2:KI of 1:3:0.6 and the pH was adjusted to pH 9 with sodium carbonate. After 24 h of mixing at room temperature (in the dark), the solution was heated, filtrated, dialysed, and freeze dried].

After oxidation, the polyelectrolytes were analysed for their hydrodynamic volume using high-performance size-exclusion chromatography. Remarkably, no polymeric material remained for oxidised EPS B39, whereas the hydrodynamic volume of *O*-deacetylated EPS B891 decreased only slightly upon oxidation. The latter is not uncommon since limited polymer degradation has also been reported during oxidation of guar gum [9], a polysaccharide consisting of a β-(1→4)-linked mannan backbone substituted with α-(1→6)-linked galactopyranosyl residues. However, the extensive degradation of EPS B39 was not expected. Although the exact mechanism of degradation is still unknown [9], some speculations can be made from the obtained results. For instance, the presence of (1→2)- and/or (1→3)-linked sugar residues might lead to increased polymer degradation compared with the presence of (1→4)- and (1→6)-linkages. Furthermore, rhamnosyl residues might be more susceptible towards degradation than galactosyl, glucosyl and mannosyl residues. So only oxidised EPS B891 was examined more carefully and analysis of the sugar composition using HPAEC after methanolysis [11] resulted in an approximate molar ratio of galactose:glucose:galacturonic acid of 1:3:0.4. Assuming that only the terminally linked galactosyl residues were oxidised, 40% of these residues were converted into uronic acids; for guar gum this was ca. 30% [9]. The consequences of the presence of these charged groups in EPS B891 on the physical properties of this EPS will be described later.

Characterisation of enzymes

In this thesis, the activity of endoglucanase V (endoV) and a phosphatase from *T. viride* towards modified EPS B40 is described [12]. Furthermore, a β-galactosidase from *A. aculeatus* was purified and characterised for its activity towards EPS B39 and (modified) EPS B891 [13]. These investigations are based on the availability of various well-characterised and structurally related polysaccharides (mostly obtained by chemical modification). The regular structures of EPSs make these polysaccharides very suitable for characterising enzyme activities. The drive behind this kind of research is the fact that purified and well-characterised enzymes have shown to be valuable tools in the structural elucidation of polysaccharides [14].

Endoglucanase V is able to cleave each β-(1→4) linkage between two glucopyranosyl residues of the unsubstituted backbone of EPS B40. The repeating unit of the backbone of EPS B891 differs from the repeating unit of the backbone of EPS B40 only in one glucosyl residue being α-linked towards a galactosyl residue (Fig. 7.1). To find out more about the mode of action of endoV, it would be interesting to know if endoV is also able to cleave the

backbone of EPS B891. All the more since the deviating linkages in the backbone of EPS B891, compared to a β-(1→4)-linked glucan, are at the same positions as the deviating linkages in the backbone of EPS B40. Unfortunately, this experiment could not be performed since no enzyme and no chemical procedure was found able to release the glucosyl substitutions from *O*-deacetylated *and* degalactosylated EPS B891. Preliminary results of an incubation of *O*-deacetylated *and* degalactosylated EPS B891 with the crude enzyme preparation indicated that endoV is not able to cleave the backbone in presence of glucosyl substitutions.

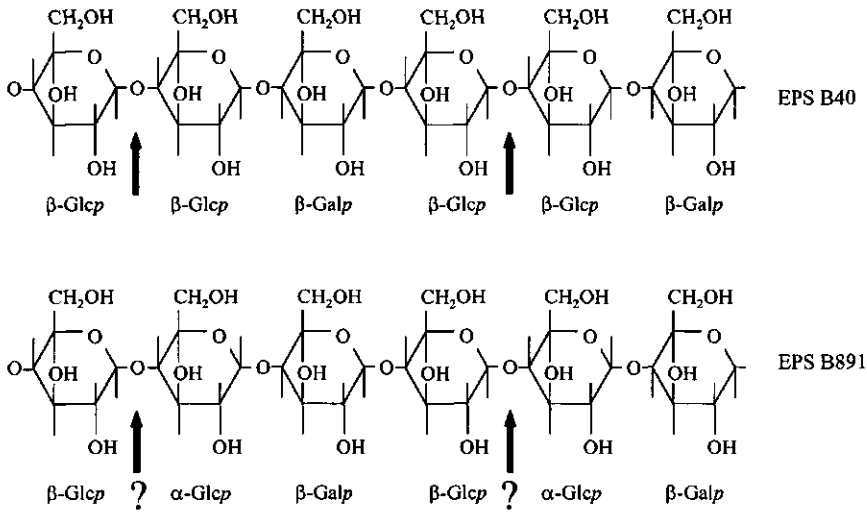


Fig. 7.1. Chemical structure of two repeating units of the backbones of EPS B40 and EPS B891. The arrows indicate the linkages that can be cleaved (EPS B40) or can possibly be cleaved (EPS B891).

The purified β-galactosidase is able to release galactose from EPS B39 and (*O*-deacetylated) EPS B891. In both polysaccharides, the released galactose residues were originally part of lactosyl side chains of the backbone. To know more about the enzyme activity, it would be interesting to find out whether the glucosyl residues in the side chains are essential for enzyme activity. This can be investigated by incubating the EPS produced by *S. thermophilus* Sfi12 [15] with the β-galactosidase, since the backbone of EPS Sfi12 is identical to the backbone of EPS B39, but the branches lack the glucosyl residues. If the enzyme is able to release galactose from this EPS, this will not only be interesting for enzyme-specificity reasons, but also for investigation of the physical consequences of the lack of side chains in EPS Sfi12 and, consequently, B39.

Structure-function relationship

The chemical structures of polysaccharides determine the shapes these polymers adopt in, for example, aqueous systems, and thus their rheological properties. In industrial applications,

polysaccharides are used to control the rheology of aqueous phases in three ways [16]. First, they are used as simple viscosifiers, where the polysaccharide molecules exist as fluctuating disordered chains (random coils), like for example dextran. Second, they are used to gel the aqueous phase. Due to permanent chain-chain interactions an ordered polymer conformation can result in a three-dimensional network (gel) if structural interruptions cause a single chain to interact with different other chains (otherwise insolubility occurs). Gelling polysaccharides include pectin and gellan. Finally, polysaccharides are used for their rheological properties intermediate between those of thickened solutions and rigid gels. Xanthan is such a polysaccharide.

Systematic physical analyses of EPSs from lactic acid bacteria have hardly been performed until very recently [17,18,19] and these studies describe the physical properties of the same primary EPS structure from *L. lactis* subsp. *cremoris* (B40, SBT 0495 or strains producing EPS with probably an identical chemical structure). These EPSs are polyelectrolytes due to the presence of phosphate groups, and they behave like random coil polymers at an ionic strength of 0.1 M [20,21].

In order to investigate the influence of some structural modifications of EPSs on the physical properties, the viscosifying features of (modified) EPSs were studied within this research project [22]. The key parameter to describe the thickening effect of flexible polymers is the intrinsic viscosity. Since the intrinsic viscosity depends only on the molecular mass and the size of the polymer in solution, the thickening effect of a polysaccharide can be deduced from the molecular mass (M) and the radius of gyration (R_g), determined from static light scattering [21]. From the relation between the radius of gyration and the molecular mass, $R_g \sim M^{\nu}$, it can be derived whether the polymer can be regarded as a flexible chain; ν should then be between 0.5 (for θ-solvent) and 0.6 (for good solvent). For (modified) EPS B39 and B891, static light scattering experiments were performed and the results are summarised in Table 7.2. Most of our polysaccharides were shown to have an exponent ν between 0.5 and 0.6, and can be regarded as flexible chains. To simplify the comparison of polymers, it was assumed that all these polymers behave as flexible chains [22].

Table 7.2
Overview of the results from static light scattering for various (modified) EPSs. EPS B39 was purified and modified [23] by incubating twice with Pectinex Ultra SP-L to release all terminally linked galactose. EPS B891 was purified and modified as described before [24], and oxidation was basically performed as described elsewhere [9].

EPS (and modification)	M_n (10^3·kg/mol)	R_g (nm)	ν
B39	1.30 ± 0.07	84 ± 3	0.68 ± 0.05
B39 (degalactosylated)	0.50 ± 0.02	48 ± 2	0.60 ± 0.03
B891	1.00 ± 0.03	70 ± 2	0.56 ± 0.03
B891 (*O*-deacetylated)	0.66 ± 0.02	54 ± 2	0.53 ± 0.02
B891 (*O*-deacetylated; degalactosylated)	0.49 ± 0.01	43 ± 1	0.41 ± 0.05
B891 (*O*-deacetylated; oxidised)	0.60 ± 0.01	54 ± 2	0.52 ± 0.03

It is remarkable that the molecular masses of all polysaccharides decreased significantly upon modification, even if modification was performed by using enzymes. These unexpected results are difficult to explain. On the one hand, there may have been other enzyme activities in the crude enzyme preparation acting towards the backbone of these EPSs. This explanation seems to be unlikely since endo-activity towards a polymer consisting of repeating units is expected to result in a dramatic decrease in degree of polymerisation (releasing repeating units or multiples of it). However, if there is a structural irregularity within the polymer, due to purification methods for example, side activities may have incidentally cleaved the EPSs. On the other hand, this does not explain the decrease in molecular mass after *O*-deacetylation.

As the reduction in molecular mass effects the radius of gyration and the intrinsic viscosity, two characteristics that are independent of the molecular mass were used to evaluate the effect of modification on the viscosifying effect of the EPSs [22]. The first characteristic was the Kuhn length, l_K, which was derived from the radius of gyration and contour length, L, of the EPS chains: $l_K = 6R_g^2/L$. The contour length was estimated by multiplying the length of one repeating unit (1.8 nm for EPS B39 and 1.2 nm for EPS B891) by the average number of repeating units in one polymer chain. The Kuhn length is a measure for the stiffness of the polymer chains. The number of Kuhn segments in a polymer chain is N_K (= L/l_K). The other characteristic was l_K^2/M_K, with M_K the molecular mass of the Kuhn segment ($M_K = M/N_K$), which is a measure for the thickening efficiency of the polymer.

Following the approach described above and assuming that the EPSs behave as ideal chains, the Kuhn parameters and the thickening efficiency were calculated (Table 7.3). Although this approach applies to solutions of uncharged polymers, oxidised EPS B891 was analysed as well, since this polymer can be regarded as quasi-neutral at an ionic strength of 0.1 M [25]. At this ionic strength, which is typical for many food products, the negatively charged groups are electrostatically screened since the distance between these groups is always larger than 1 nm. (At an ionic strength of 0.1 M the Debeye length is only 1 nm).

Table 7.3
Overview of the calculated Kuhn parameters and thickening efficiency for the various (modified) EPSs. For the purification and modification methods of the EPSs: see references in Table 7.2.

EPS (and modification)	L (μm)	l_K (nm)	N_K	l_K^2/M_K (nm^2·mol/kg)
B39	2.12 ± 0.19	20 ± 3	107 ± 27	33 ± 8
B39 (degalactosylated)	0.96 ± 0.08	14 ± 2	66 ± 16	28 ± 6
B891	1.44 ± 0.08	20 ± 2	71 ± 12	29 ± 3
B891 (*O*-deacetylated)	0.98 ± 0.07	18 ± 3	55 ± 11	27 ± 4
B891 (*O*-deacetylated; degalactosylated)	0.91 ± 0.04	12 ± 1	74 ± 10	23 ± 3
B891 (*O*-deacetylated; oxidised)	0.88 ± 0.05	20 ± 3	44 ± 8	29 ± 4

During the interpretation of the results, it should be realised that the differences in Kuhn length and thickening efficiency are small and that the errors are relatively large. Nevertheless, some trends can be seen and will be discussed here. The chemical structure of EPS B39 resulted in a similar thickening efficiency as the structure of EPS B891. For comparison, a Kuhn length of ca. 17 nm has been reported for EPS B40 [17] and a thickening

efficiency of ca. 30 $nm^2 \cdot mol/kg$ was deduced. These results suggest that, apart from the influence of the molecular mass, the thickening capacity of EPS B39, B891 and B40 is similar.

For EPS B891, removal of acetyl groups did not significantly effect the chain stiffness and thickening efficiency. However, the removal of terminally linked galactosyl residues tended to decrease the chain stiffness (l_K) for both *O*-deacetylated B891 and EPS B39. These results can be explained by the fact that the presence of galactosyl residues in the side chains of the polymer probably hinder the possible orientations of the backbone. Since the thickening efficiency decreased upon degalactosylation, it seems that the increase in chain stiffness (l_K), due to the presence of the terminally linked galactose residues in these EPSs, overcompensates the increase of the mass of the Kuhn segment (M_K).

The introduction of charges by oxidation of *O*-deacetylated EPS B891 did not seem to change the chain stiffness and thickening efficiency. (*O*-deacetylated) EPS B891 is probably quite expanded and stiff already before oxidation due to the presence of lactosyl groups. Introduction of carboxyl groups does not promote much additional chain expansion. Besides, the ionic strength of 0.1 M causes electrostatic screening of the introduced charges and, possibly, chain expansion can be observed at lower ionic strengths. Similar results have been described for (oxidised) guar gum [8,9]. The effect of the ionic strength on chains stiffness and thickening efficiency was not further investigated.

In conclusion, physical analyses of (modified) EPS B39 and B891 suggest that the thickening efficiency of both native polysaccharides is similar and that the removal of terminally linked galactosyl residues results in decreased thickening efficiency.

Concluding remarks

This thesis describes the structural elucidation of exopolysaccharides produced by *L. lactis* subsp. *cremoris* B40, B39 and B891. The approach of using enzymes as a tool during this characterisation has shown to be successful. An endoglucanase has been used to prove the chemical composition of EPS B40 and structural information has been obtained from β-galactosidase activity towards EPS B39 and EPS B891. Unfortunately, only few enzyme activities towards EPSs have been found, and chemical modification methods were used in addition. Various (modified) EPS structures have been used for characterising the activities of the enzymes mentioned above. Furthermore, a start has been made within this project to study the effect of structural changes on the physical properties of these (modified) exopolysaccharides, but we are still far from a complete understanding. In the meanwhile, the process of EPS biosynthesis by different strains of *L. lactis* subsp. *cremoris* has been studied on several aspects. In fact, the production of EPS under different growth conditions has been investigated within the project [26,27]. Furthermore, the biosynthesis of EPS has been studied on a molecular level by investigating the exopolysaccharide gene clusters [1], and metabolic engineering studies aiming at an increase in the EPS production level are in progress [28]. Present knowledge on the biosynthesis of EPS by lactococci might lead to the production of modified EPS structures by genetically engineered lactococcal strains in the near future. Then, the chemical and physical characterisation of these additional modified EPSs will contribute to a better understanding of the structure-function relationship.

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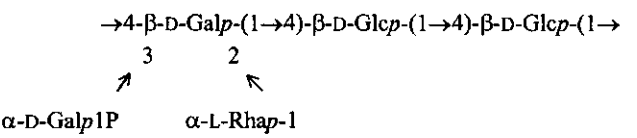
Summary

Summary

Since ancient times, lactic acid bacteria have been used for the preservation of food. Some of these bacteria are able to produce exopolysaccharides (EPSs), which may contribute to the peculiar rheology and texture of, for example, milk-derived products. Insight into the relationship between the chemical structure of EPSs and their physical properties can lead to tailor-made polysaccharides, which meet particular requirements in terms of structure and function. In this thesis, the elucidation of the chemical structures of three exopolysaccharides from *Lactococcus lactis* subsp. *cremoris* is described. Enzymes are used as a tool during the structural characterisation and modification of EPS and the action of three enzymes having activity towards (chemically modified) EPSs is investigated as well. Finally, a start has been made within this project to study the effect of structural changes of EPSs on the physical properties.

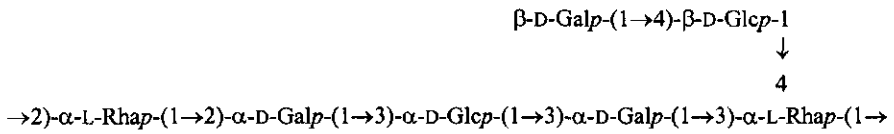
In chapter 1, a brief general introduction into the research subject is given. Besides background information about the use of bacterial EPSs in food and the biosynthesis of EPS, attention is paid to common features in EPS structures from lactic acid bacteria. Different ways to obtain structurally related EPSs are presented and the use of enzymes in polysaccharide research is outlined.

Chapter 2 describes the study of the chemical structure of EPS B40, explaining earlier reported analytical discrepancies. The EPS contains rhamnose:galactose:glucose:phosphate in a molar ratio of 1:1.3:2:1.1. ^{31}P NMR indicated that a single phosphate group is present as a phosphodiester. EPS B40 has chemically been modified using 0.3 M H_2SO_4, 28 M HF or 2 M NaOH. From these modifications it is concluded that during the hydrolysis step prior to sugar composition analysis the galactose 3-phosphate linkages are split only partially and that, consequently, the amount of galactose is underestimated. The backbone of HF-modified EPS B40 can be degraded by a crude cellulase preparation from *Trichoderma viride*. Purification and characterisation of the obtained oligomers (chapter 3), together with the characterisation of the polymer (chapter 2), has resulted in a chemical structure for EPS B40 identical to the repeating unit already described for EPS SBT 0495:

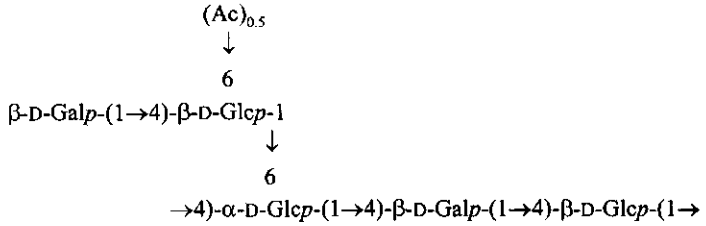


In chapter 3, the enzyme activity responsible for the degradation of HF-modified EPS B40 is identified as an endoglucanase (endoV). Thus, after complete removal of galactosyl residues and phosphate and partial removal of rhamnosyl residues, endo*glucanase* is able to cleave the backbone consisting of glucosyl and *galactosyl* residues. Characterisation of the resulting homologous series of oligomers by MS and NMR unequivocally demonstrated that endoV is able to cleave the β-(1→4) linkage between two glucopyranosyl residues when the galactopyranosyl residue towards the nonreducing end is unsubstituted. The mode of action of endoV on HF-modified EPS B40 is discussed on the basis of the subsite model for endoV, described in literature. The crude cellulase preparation from *T. viride* has also been shown to contain a phosphatase able to act on EPS B40 after removal of rhamnosyl and galactosyl residues by mild CF_3CO_2H treatment.

In chapter 4, the structural elucidation of EPS B39 is outlined. This novel exopolysaccharide structure contains L-Rha, D-Gal and D-Glc in a molar ratio of 2:3:2. Enzymic modification, methylation analysis and 1D/2D NMR experiments (both ^{1}H-^{1}H and ^{1}H-^{13}C) revealed that EPS B39 consists of a branched heptasaccharide repeating unit with the following structure:



Chapter 5 describes the chemical structure of EPS B891, which contains D-Gal and D-Glc in a molar ratio of 2:3. The polysaccharide is partially *O*-acetylated. By means of HF solvolysis, *O*-deacetylation, enzymic modification, methylation analysis and 1D/2D NMR studies the novel exopolysaccharide is shown to be composed of repeating units with the following structure:



EPS B39 and *O*-deacetylated EPS B891 both contain lactosyl side chains and it is demonstrated that the terminally linked galactosyl residues can be removed by using a crude commercial enzyme preparation from *Aspergillus aculeatus*. The purification and characterisation of the β-galactosidase responsible for this modification is described in chapter 6. The enzyme has a molecular mass of approximately 120 kDa, a pI between 5.3-5.7 and is optimally active at pH 5.4 and 55-60 °C. Based on the N-terminal amino acid sequence, the enzyme probably belongs to family 35 of the glycosyl hydrolases. The catalytic mechanism is shown to be retaining and transglycosylation products are demonstrated using lactose as a substrate. The β-galactosidase is able to release terminally linked galactosyl residues from EPS B891 in presence of acetyl groups, but the hydrolysing rate after *O*-deacetylation is higher. Furthermore, *O*-deacetylated EPS B891 is degalactosylated faster than EPS B39.

In chapter 7, the results of this thesis are discussed. Emphasis is placed on the approach of using enzymes in structure (-function) studies of exopolysaccharides. Furthermore, the use of (modified) exopolysaccharides for characterising enzyme activities is outlined. Finally, the influence of various structural modifications on the physical properties of EPSs is briefly discussed.

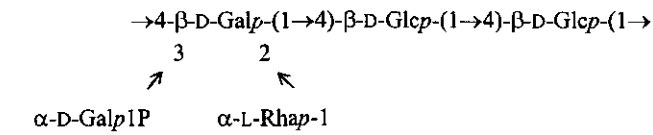
Samenvatting

Samenvatting

Van oudsher worden melkzuurbacteriën gebruikt voor de conservering van levensmiddelen. Een aantal van deze bacteriën is in staat om exopolysachariden (EPSsen) te produceren, welke bij kunnen dragen aan de typische rheologie en textuur van bijvoorbeeld melkproducten. Inzicht in de relatie tussen de chemische structuur en de fysische eigenschappen van deze polysachariden kan leiden tot op maat gemaakte polysachariden met verbeterde eigenschappen. In dit proefschrift wordt de structuuropheldering van drie exopolysachariden van *Lactococcus lactis* subsp. *cremoris* beschreven. Tijdens het onderzoek werden enzymen gebruikt als hulpmiddel bij zowel de structuuropheldering als ook bij de modificatie van de EPSsen. Tevens werd de activiteit van drie enzymen op al dan niet chemisch gemodificeerde EPSsen bestudeerd. Tot slot werd er binnen dit project een begin gemaakt met het onderzoeken van de gevolgen van structuur veranderingen op de fysische eigenschappen van EPSsen.

Hoofdstuk 1 is een inleidend hoofstuk waarin de achtergrond van het onderzoek wordt beschreven. Er wordt ingegaan op het gebruik van bacteriële EPSsen in levensmiddelen, de biosynthese van EPS en algemene structuurkenmerken van melkzuurbacterie EPSsen. Verder wordt weergegeven hoe EPSsen met kleine verschillen in chemische structuur kunnen worden verkregen en hoe enzymen gebruikt kunnen worden in polysacharide onderzoek.

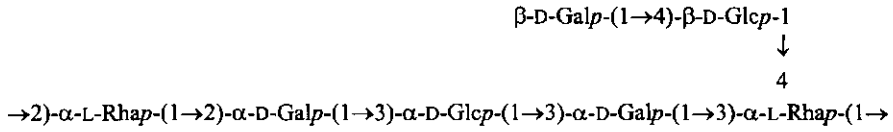
Hoofdstuk 2 beschrijft het onderzoek naar de chemische structuur van EPS B40 en verklaart tevens eerder gerapporteerde analytische discrepanties. Het EPS bestaat uit rhamnose:galactose:glucose:fosfaat in een molaire verhouding van 1:1,3:2:1,1. ^{31}P NMR liet zien dat een fosfaat groep aanwezig is in de vorm van een di-ester. EPS B40 werd chemisch gemodificeerd met 0,3 M H_2SO_4, 28 M HF of 2 M NaOH. Uit deze modificaties kon worden geconcludeerd dat de galactose-3-fosfaat bindingen niet volledig worden gehydrolyseerd tijdens de suikersamenstellingsanalyse en dat als gevolg daarvan de hoeveelheid galactose wordt onderschat. De hoofdketen van (HF-)gemodificeerd EPS B40 kan worden afgebroken met een cellulase preparaat geproduceerd door *Trichoderma viride*. De zuivering en karakterisering van de aldus verkregen oligosachariden (hoofdstuk 3) en de karakterisering van het polysacharide (hoofdstuk 2) resulteerden in de volgende chemische structuur voor de repeterende eenheid van EPS B40, welke gelijk is aan de structuur beschreven voor EPS geproduceerd door *L. lactis* subsp. *cremoris* SBT 0495:



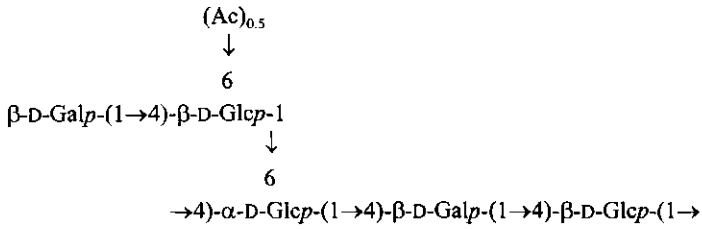
In hoofdstuk 3 wordt beschreven dat endoglucanase V (endoV) verantwoordelijk is voor de afbraak van HF-gemodificeerd EPS B40. Na het geheel verwijderen van terminaal gebonden galactose en van fosfaat en het gedeeltelijk verwijderen van rhamnose is endo*glucanase* in staat de hoofdketen bestaande uit glucosyl en *galactosyl* residuen te splitsen. Analyse van de aldus verkregen serie van oligosachariden met behulp van MS en NMR bewees dat endoV de β-(1→4)-binding tussen twee glucosyl residuen kan splitsen indien het nabije galactosyl residu in de richting van de niet-reducerende kant van het polysacharide ongesubstitueerd is. De activiteit van endoV op HF-gemodificeerd B40 wordt bediscussieerd met behulp van een

model uit de literatuur betreffende de substraatbindingsplaatsen voor endoV. Het enzym preparaat van *T. viride* bevatte ook een fosfatase die in staat is te werken op EPS B40, nadat rhamnosyl en galactosyl residuen (gedeeltelijk) verwijderd zijn met een milde CF_3CO_2H behandeling.

Hoofdstuk 4 beschrijft de structuuropheldering van EPS B39. Dit nieuwe exopolysacharide bevat rhamnose:galactose:glucose in een molaire verhouding van 2:3:2. Met behulp van enzymatische modificatie, methyleringsanalyse en 1D/2D NMR technieken werd bewezen dat de repeterende eenheid van EPS B39 bestaat uit een heptasacharide met de volgende structuur:



In hoofdstuk 5 wordt de chemische structuur van EPS B891 opgehelderd. Dit exopolysacharide bestaat uit galactose:glucose in een molaire verhouding van 2:3 en is gedeeltelijk *O*-geacetyleerd. Gebruik makend van HF-solvolyse, *O*-deacetylering, enzymatische modificatie, methyleringsanalyse en 1D/2D NMR studies werd aangetoond dat de repeterende eenheden van EPS B891 de volgende nieuwe structuur hebben:



EPS B39 en *O*-gedeacetyleerd EPS B891 bevatten beide lactosyl zijketens, waarvan de galactosyl residuen verwijderd kunnen worden met een enzym preparaat geproduceerd door *Aspergillus aculeatus*. De zuivering en karakterisering van de β-galactosidase die verantwoordelijk is voor deze modificatie is beschreven in hoofdstuk 6. Het enzym heeft een molecuulmassa van ongeveer 120 kDa, een pI tussen 5,3 en 5,7 en de activiteit is optimaal bij pH 5.4 en 55-60 °C. Op grond van de N-terminale aminozuursequentie behoort het enzym waarschijnlijk tot familie 35 van de glycosyl hydrolases. Er werd aangetoond dat het catalytische mechanisme van het enzym de anomere configuratie behoudt en dat er transglycosyleringsproducten kunnen worden gevormd met lactose als substraat. De β-galactosidase is in staat om galactose vrij te maken van *O*-geacetyleerd EPS B891, maar de hydrolyse snelheid na *O*-deacetylering is groter. Galactose residuen werden sneller vrijgemaakt van *O*-gedeacetyleerd EPS B891 dan van EPS B39.

In hoofdstuk 7 worden de resultaten van dit proefschrift bediscussieerd. De nadruk ligt hierbij op het gebruik van enzymen in structuur (-functie) studies van exopolysachariden. Verder wordt ingegaan op het gebruik van (gemodificeerde) EPSsen voor de karakterisering van enzymen. Tenslotte wordt de invloed van diverse modificaties van EPSsen op de fysische eigenschappen beschreven.

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Curriculum vitae

Wilhelmina Helena Maria (Willemiek) van Casteren werd geboren op 12 november 1970 te Ravenstein. Na het behalen van het VWO diploma aan het Liemers College te Zevenaar begon zij in 1989 aan de studie Voeding van de Mens aan de toenmalige Landbouwuniversiteit Wageningen. In de loop van deze studie begon zij tevens aan de studie Levensmiddelentechnologie aan dezelfde universiteit. In het kader van deze studies deed zij afstudeervakken bij de toenmalige vakgroepen Humane Voeding ('de stereospecifieke vetzuurconfiguratie van triglyceriden in levensmiddelen') en Levensmiddelentechnologie ('zuivering en karakterisering van een antigeen eiwit in de albuminefractie van de erwt'). In deze periode werd ook een onderzoeksstage uitgevoerd bij CSM Centraal Laboratorium te Breda ('polymeren van ruwsap tot witsuiker'). Beide studies werden *cum laude* afgesloten.

Vlak voor haar afstuderen begon zij in 1995 aan een promotie-onderzoek onder begeleiding van prof. dr. ir. A.G.J. Voragen, dr. H.A. Schols en dr. G. Beldman. Het onderzoek werd uitgevoerd bij het Laboratorium voor Levensmiddelenchemie van de Wageningen Universiteit en de resultaten zijn beschreven in dit proefschrift.

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