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Prion Rods Contain an Inert Polysaccharide Scaffold

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A polysaccharide consisting of mainly 1,4-linked glucose units was found associated with prion rods, which are composed mainly of insoluble aggregates of the N-terminally truncated prion protein (PrP 27–30) exhibiting the ultrastructural and tinctorial properties of amyloid. The polysaccharide differs in composition from the Asn-linked oligosaccharides and the GPI-anchor of the prion protein. Prion rods were prepared from scrapie-infected hamster brains using two different purification protocols. Prolonged digestion of rods with proteinase K reduced PrP by a factor of at least 500, leaving about 10% (w/w) of the sample as an insoluble remnant. Only glucose was obtained by acid hydrolysis of the remnant and methylation analysis showed 80% 1,4-, 15% 1,6- and 5% 1,4,6-linked glucose units. The physical and chemical properties as well as the absence of terminal glucose units indicate a very high molecular mass of the polysaccharide. No evidence was found for covalent bonds between PrP and the polysaccharide. The polysaccharide certainly contributes to the unusual chemical and physical stability of prion rods, acting like a scaffold. A potential structural and/or functional relevance of the polysaccharide scaffold is discussed.

Key words: Alditol acetates / Carbohydrate analysis / Methylation analysis / Prions / PrP / Scrapie.

Introduction

Prions are the causative agents of fatal neurodegenerative diseases, among them Creutzfeld-Jakob-disease of man, bovine spongiform encephalopathy (BSE) and scrapie of sheep (cf. reviews of Prusiner, 1998; Brown and Bradlay, 1998; Wisniewski *et al*., 1998). They are defined as proteinaceous infectious particles, and so far the prion protein (PrP) is the only component correlating unequivocally with prion infectivity. PrP is encoded in the host genome by a single-copy gene; it is expressed in the non-infected organism as the cellular isoform of PrP, designated PrP^{C}. The prion protein has Asn-linked glycosylations at two sites (Endo *et al*., 1989; Stimson *et al*., 1999; Rudd *et al*., 1999) and is attached to the outer membrane of neurons and other cells via its glycosylphosphatdylinositol (GPI-) anchor (Stahl *et al*., 1993). In the infected organism, PrP is present both in the cellular as well as in an abnormal form, the scrapie isoform, designated PrP^{Sc}. Upon purification using detergents and digestion by proteinase K (PK), PrP^{Sc} is transformed into an N-terminally truncated but still infectious form of 27–30 kDa designated PrP 27–30 (McKinley *et al.,* 1991). PrP 27–30 polymerizes into prion rods, which are fairly regular structures with a diameter of 10–20 nm and a length of 75–200 nm. Both ultrastructurally and tinctorally, the prion rods were found to possess the properties of amyloid (Prusiner *et al*., 1983). Subsequently, immunohistochemistry and chemical analyses showed that amyloid plaques in prion diseases contain fragments of PrP (Tagliavini *et al*., 1991, 1994).

Prion rods are the purest infectious preparation available. Nucleic acids longer than 80 nucleotides are absent from the infectious unit (Kellings *et al*., 1992), ruling out a virus- or even viroid-like nucleic acid as essential for infectivity. Small amounts of host sphingolipids were found in prion rods (Klein *et al*., 1998), but in a molar ratio of less than one lipid molecule per PrP molecule. The finding that fractions with higher infectivity contained smaller amounts of lipids argued strongly against a functional character of these sphingolipids. A wealth of data from biochemistry, biophysics and molecular biology (cf. reviews cited above) indicates that PrP^{Sc} is essential for prion infectivity. However, at present some features of prion infectivity cannot be explained satisfactorily by the known properties of PrP^{Sc} or PrP 27–30. Most importantly, all experiments to convert PrP^{C} into PrP^{Sc} *in vitro* have failed to produce measurable quantities of infectivity (Kaneko *et al*., 1995; Hill *et al*., 1999). Furthermore, the phenomenon of prion strains has not been generally accepted as a consequence of different prion conformations (Bessen *et al*., 1995; Carlson, 1996; Carp *et al*., 1997; Safar *et al*., 1998). At present it is not clear whether those features might be explained by so-far unknown qualities of PrP^{Sc} or by an unknown second component in prion rods.

Indications for a non-protein component in prion rods had been published before. McKinley and colleagues (1986) described that the ultrastructure of prion rods remained after prolonged PK-digestion, although the amount of PrP had been reduced by a factor of more than 10^2 and that of infectivity by 10^3. Stahl and colleagues (1993) reported a non-protein compound of high molecular mass in prion rods after radiolabeling experiments with the hydrophobic crosslinker 3-(trifluoromethyl)-3-(*m*-

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iodophenyl)diazirine (TID). Safar and colleagues (1990) analyzed monosaccharides released from prion rods by CF_3COOH treatment. After all components of the Asn-linked oligosaccharides and the GPI-anchor of PrP were identified in expected proportions, one molecule glucose per molecule PrP was found in addition, but the authors did not discuss this unexpected finding.

The aim of the present work was to search systematically for a not yet identified polysaccharide in prion rods besides the known Asn-linked oligosaccharides and the GPI-anchor of PrP. Well established methods of carbohydrate analysis were applied to hamster prion rods purified in two laboratories which use different isolation protocols (Prusiner *et al.*, 1982, 1983; Diringer *et al.*, 1997).

Evidence was found for a chemically inert glucan made up of about 80% 1,4-linked, 15% 1,6-linked and 5% 1,4,6-crosslinked glucose molecules. Potential functional relevance of the inert polysaccharide is discussed, in terms of primary or secondary effects on prion infectivity and/or a phenomenon concomitant with pathogenesis.

Results

An Insoluble Remnant Is Left After Prolonged PK-Digestion of Prion Rods

Prion rods were treated according to the protocol of Figure 1 in order to extract lipids and to digest PrP. After PK-digestion and high-speed centrifugation the released amino acids, peptide fragments and soluble oligosaccharides were expected in the supernatant and any substance forming a pellet would have a molecular mass of 10^6 or more. Only non-digested aggregates of PrP 27–30 would be of this size, monomers or oligomers of PrP up to 10^6 Daltons would turn soluble.

Prion rods prepared either in the laboratory of S.B. Prusiner or H. Diringer, containing 500 μg PrP or more in the latter case, were subjected to the protocol of Figure 1. Visible, brownish pellets and colorless supernatants were observed and were saved for analysis described below. The pellet resulting after prolonged PK-digestion is referred to as 'PK-pellet' throughout this work, and the corresponding supernatant to as 'PK-supernatant' (cf. also Figure 1). In a third experiment starting with 1.1 mg prion rods (Prusiner) the PK-pellet amounted to 0.16 mg after the procedure. Although the error of the direct mass determination might be quite high (approximately 50%), the PK-pellet represents about 10% of the mass of prion rods.

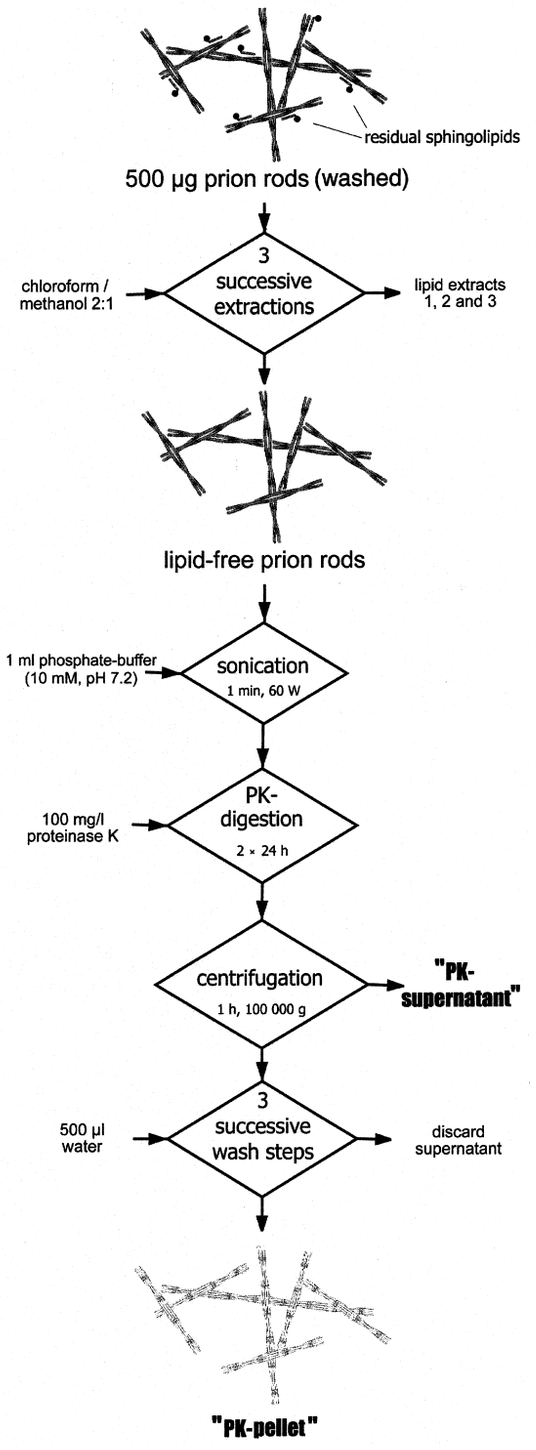

Fig. 1 Protocol for Removal of Lipids and Digestion of PrP in Prion Rods. Products in bold print were analyzed further.

PrP Is Reduced at Least 500 Times Both in the PK-Supernatant and in the PK-Pellet

Samples of the PK-supernatant of the protocol shown in Figure 1 equivalent to 5 μg PrP in the starting material were analyzed by SDS-PAGE (4/12%) as presented in Figure 2, lanes A-F. Silver staining (lane A) showed only bands of PK and PK self-cleavage fragments. 100 ng PrP 27–30 were still visible (lane D, positive control), despite the dominating bands of PK-fragments. A Western blot of the same gel visualized using the monoclonal anti-PrP-antibody 3F4 and chemiluminescence detection showed no signal for PrP in the sample of the PK-supernatant (lane A). Lanes C, D and E were positive controls containing 1000, 100 and 10 ng of PrP 27–30, indicating also the limit of sensitivity of this method (10 ng in lane E). Lane F contained 200 ng

of recombinant PrP (amino acids 90–231, 17 kDa) as an additional control.

In conclusion, no PrP could be detected in the PK-supernatant of prion rods (Prusiner) equivalent to 5 μg PrP using two methods of staining. Because of the sensitivity of Western blotting (10 ng), the reduction factor of PrP by PK-digestion was at least 500 times in this PK-supernatant. An equivalent analysis of the PK-supernatant of prion rods from the laboratory of Diringer showed similar results (not shown).

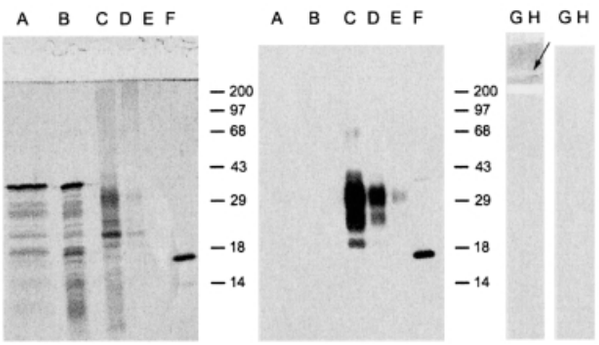

Fig. 2 SDS-PAGE Analysis of the PK-Supernatant and the PK-Pellet for the Presence of PrP.
Left panel: Silver stain of PK-supernatant and controls. A, 50 μl of PK-supernatant corresponding to a starting amount of 5 μg PrP 27–30 and 10 μg PK; B, negative control – 50 μl PK-supernatant without PrP but with 10 μg PK; C–E, positive controls – 1000, 100 and 10 ng PrP 27–30; F, positive control – 200 ng recombinant PrP (amino acids 90–231, 17 kDa).
Middle panel: Western blot of PK-supernatant and controls. Samples are identical to left panel.
Right panels: PK-pellet, silver stain (left) and Western blot (right). G, PK-pellet from 5 μg PrP 27–30; H, PK-pellet from 50 μg PrP 27–30.

Samples of the PK-pellet of prion rods (Prusiner) equivalent to 5 μg or 50 μg PrP, respectively, were analyzed by SDS-PAGE (4/12%) as shown in Figure 2 (lanes G and H). Silver staining yielded no bands in the PrP-region but a high-molecular band at the border between stacking and resolving gel in the larger sample (lane H, arrow). A similar band that contained no amino acids by gas-phase Edman degradation was described by Stahl and colleagues (1993) after radioactive labeling of prion rods. From the absence of PrP bands in Western blots (Figure 2 lanes G and H) it could be concluded that PrP was reduced at least 1000 times in the PK-pellet. Positive controls showed a sensitivity of better than 50 ng (not shown). PK-pellets of prion rods (Diringer), however, yielded signals for PrP in Western blots (not shown) that were indicative for a reduction of about 100 times. Repeated PK-digestion slowly reduced the amount of PrP further, but faint bands were still visible after 96 hours of digestion in prion rods prepared in the laboratory of Diringer.

In summary, the reduction of PrP was at least 1000 times in the PK-pellet of prion rods (Prusiner) and about 100 times in the samples of Diringer. The failure to completely remove PrP from prion rods (Diringer) may be due to the different purification method of this material employing only 1 detergent instead of 6 different detergents employed by the method of Prusiner.

The PK-Supernatant Contains the Asn-Linked Oligosaccharides and the GPI-Anchor of PrP

The PK-supernatant of 500 μg prion rods (Prusiner) was concentrated to about 300 μl using a rotary evaporator. Soluble sugar components were analyzed by the 'alditol acetate method' (Biermann and McGinnis, 1989). The resulting chromatogram is shown in Figure 3. The compari-

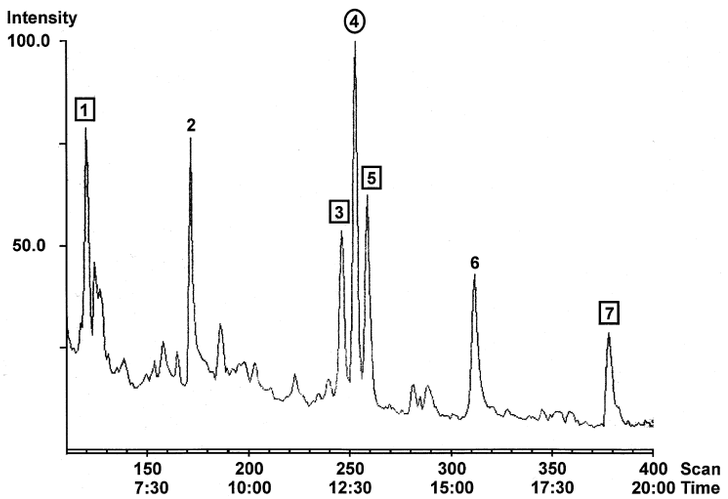

Fig. 3 Monosaccharide Component Analysis of the PK-Supernatant of 500 μg Prion Rods (Prusiner).
Gas chromatogram of alditol acetates. The numbered peaks were identified by comparison of retention time, EI and CI mass spectra with standards. (1) 1-deuterio-6-deoxy-galactitol pentaacetate, (2) palmitic acid, (3) 1-deuterio-mannitol hexaacetate, (4) 1-deuterio-glucitol hexaacetate, (5) 1-deuterio-galactitol hexaacetate, (6) stearic acid, (7) 1-deuterio-2-amio-2-deoxy-glucitol hexaacetate.

son of retention times as well as electron impact (EI) and chemical ionization (CI) mass spectra with that of standard compounds confirmed all peak assignments.

With the exception of sialic acid, all major components of the Asn-linked oligosaccharides and the GPI-anchor of PrP were found in the supernatant fraction of the PK-digestion. They are marked by squares in Figure 3. Sialic acid is decomposed by the CF_3COOH hydrolysis step of the 'alditol acetate method' (Hermentin, 1998) and therefore not detectable. In addition to the components of the Asn-linked oligosaccharides and the GPI-anchor of PrP, larger amounts of glucose (Figure 3, peak 4) and fatty acids (Figure 3, peaks 2 and 6; from the GPI-anchor and/or contamination) were observed.

The presence of glucose in the PK-supernatant can be explained by a small fraction released from the PK-pellet during PK-digestion (cf. results of next chapter). One could however argue that the prion rods employed here were purified by a sucrose gradient centrifugation step. But since they were washed four times by suspension, sonication and reprecipitation in H_2O, followed by lipid extraction, all soluble constituents should have been removed from the rods. However, even in the very unlikely case that the glucose peak of Figure 3 would be due to remaining sucrose in the PK-supernatant, this does not affect the results on the PK-pellet presented in the following paragraph.

The PK-Pellet Is a Polysaccharide and Contains Only Glucose

Attempts were made with a series of solvents to solubilize the PK-pellet. The yield of the solubilization was controlled by direct chemical ionization (DCI) mass spectrometry of the liquid after centrifugation (1 h, 100 000 *g*). The PK-pellet proved to be insoluble in water and all organic solvents tested with the exception of $(CH_3)_2SO$. In the latter signals in the mass range above 100 u were detected that were not present in $(CH_3)_2SO$ alone. However, the PK-pellet did not dissolve fully or even visibly. Total dissolution, accompanied by partial decomposition and color deepening, was achieved only by a mixture of 96% $(CH_3CO)_2O$ and 4% H_2SO_4 known for its ability to dissolve even cellulose.

The observation of ions in the higher mass range from the solution of the PK-pellet in $(CH_3)_2SO$ was investigated further. About 10 μg of the pellet were suspended in 50 μl $(CH_3)_2SO$. A DCI mass spectrum was recorded from the suspension and is shown in Figure 4. It does not exhibit a distinct molecular ion peak, but allowed us to interpret the most abundant fragments. The presence of signals at m/z = 144, 162, 180, 198 indicates a high molecular mass polysaccharide composed of hexose units. Furthermore, the most abundant ion in the higher mass range at m/z = 1194 can be explained as a fragment containing seven hexose units with one acetyl group ($162 \times 7 + 42$) as $[MNH_4]^+$. Upon loss of one hexose and water, it yields the ion at m/z = 996, as well as the ions at m/z = 792 and 630

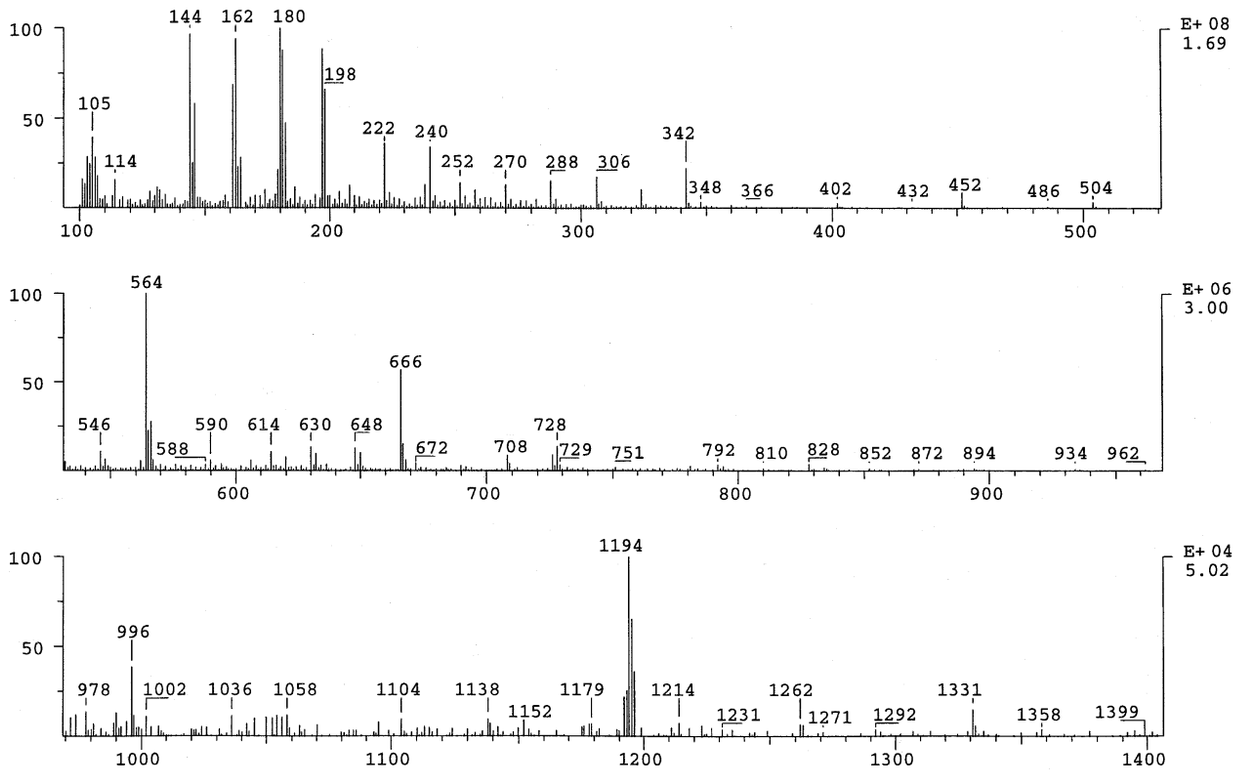

Fig. 4 Mass Spectrum of a Suspension of the PK-Pellet in $(CH_3)_2SO$.
Direct chemical ionization, reactand gas: NH_3 at ca. 0.5 Pa. – X-axis: m/z-values from 100–1400 u. Y-axis: left side: intensity in percent of highest peak, right side: detector current in arbitrary units.

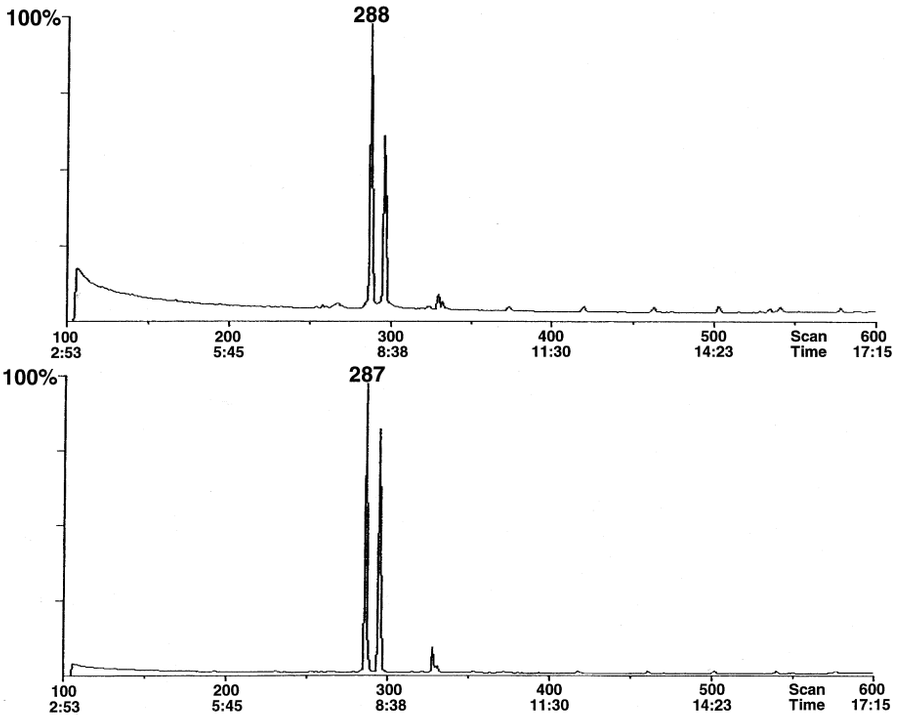

Fig. 5 Comparative GC Analysis of the PK-Pellet After Hydrolysis and of Glucose.
Samples were derivatized by a mixture of BSTFA and C_5H_5N 1:1(v/v).

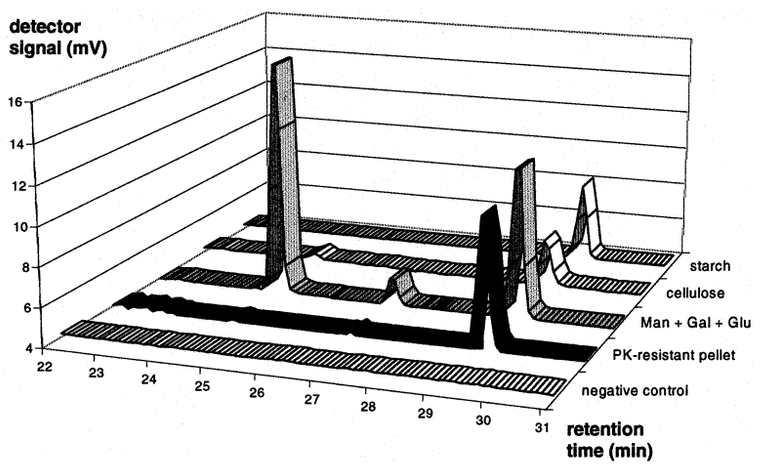

Fig. 6 Results of the 'Alditol Acetate Method'.
Shown are from bottom to top: negative control; products obtained by hydrolysis and derivatization of the PK-pellet; products obtained by derivatization of a mixture of mannose, galactose and glucose (in the order of retention time); products from cellulose; products from starch.

by further loss of ketene (from an acetyl group) and one or two hexoses. Another ion at m/z = 666 could be interpreted as a fragment consisting of four hexose units (162×4) as $[MNH_4]^+$. Loss of hexoses than leads to the ions at m/z = 504, 342 and 180. A third series of fragments with an interval of 162 u (m/z = 564, 402, 240) may be explained by DMSO-clusters of three or less hexose units.

The slight solubility of the PK-pellet in $(CH_3)_2SO$ opened a first possibility to chemically cleave the suspected polysaccharide. Even very small concentrations of the pellet would be sufficient if hydrolysis led to further solubilization, i.e. equilibrium turnover. About 30 μg of the PK-pellet were mixed with 70 μl $(CH_3)_2SO$ and 30 μl HCl-saturated CH_3OH. After 3 days at room temperature no solid material was left. The volatile constituents were removed under vacuum, the remainders taken up and silylated by a mixture of BSTFA and pyridine 1:1 (v/v). Figure 5 shows two GC peaks from the hydrolyzed PK-pellet being identical in retention time and relative height to the peaks obtained from glucose. Mass spectra of the respective peaks were identical, too (not shown). Those two peaks are indeed expected for glucose because of the separation of the four-fold silylated α- and β-methyl glucosides.

The 'alditol acetate method' (Biermann and McGinnis, 1989) yields in most cases only one peak per monosaccharide and produces very stable products. The result of the analysis of about 30 μg PK-pellet and of standards is shown in Figure 6. It can be clearly seen that the single peak from the PK-pellet is identical to glucose. Mannose and galactose give peaks at different retention times. The last two lines of Figure 6 indicate only glucose in cellulose and starch used as positive controls.

The results presented above identify the PK-pellet from prion rods as a polysaccharide consisting of glucose as the only building block. The solubility data, the band at the interface between stacking and resolving gel of Figure 2 and the mass spectrum of Figure 4 indicate a high molecular mass of the polysaccharide.

The Glucose Units Are Mainly 1,4-Linked

As next step in the structure determination of the insoluble carbohydrate component of prion rods, called PK-pellet, the linkage of the glucose units was analyzed. The standard method for this purpose is 'methylation analysis' (Jay, 1996; Biermann and McGinnis, 1989). Table 1 shows the results of methylation analysis of PK-pellets from prion rods and from controls. All samples contain mainly 1,4-linked glucose besides 10–15% 1,6-linked and about 5% 1,4,6-branched glucose. The presence of small amounts of 1,3,4-branched glucose in one PK-pellet (Diringer) may be due to partial undermethylation in the first step of the methylation analysis. This sample had been known to contain small amounts of residual PrP not digestible by PK (cf. chapter on reduction of PrP in the PK-pellet). The partial undermethylation might therefore be due to inaccessibility of some parts masked by residual PrP.

A carbohydrate with identical features was found in a control preparation (Diringer) from the brain of non-infected hamsters, amounting to about 18% of the value seen in infected brains (cf. Table 1). We therefore conclude that a polysaccharide similar to the one found in prion rods is already present in non-infected hamster brain in smaller quantities.

Discussion

Highly purified, infectious prion rods contain approximately 10% (w/w) of an insoluble polysaccharide. By two independent methods of carbohydrate component analysis only glucose was detected in this polysaccharide of high molecular mass. Methylation analysis identified 80% 1,4-linked, 15% 1,6-linked and 5% 1,4,6-branched glucose units. Very similar results were obtained from prion rods prepared by two laboratories using different purification protocols. Figure 7 shows a schematic structural element of the polysaccharide drawn according to the results of the methylation analysis and the fragmentation pattern of the DCI mass spectrum.

Components of prion rods other than the prion protein have been analyzed before. Small amounts of nucleic acids (Kellings *et al*., 1992) and lipids (Klein *et al*., 1998) had been found, but it could be shown that these are not necessary for infectivity. Amyloid deposits of PrP in infected brain tissue are associated with small amounts of sulfated glycosaminoglycans (GAGs) already early in the pathological process (Snow *et al*., 1989, 1990; McBride *et al*., 1998). However, no GAGs were described in purified infectious prion rods. Taking the solubility of sulfated GAGs into account, it is not astonishing that they were removed in the purification process of rods without loss of infectivity.

Whether the insoluble polysaccharide described here is a functional component of the infectious unit or a secondary effect of pathogenesis is the main point of interest arising from the results of this work. Indeed, the high proportion of the polysaccharide in prion rods, its tight association with PrP and the preservation of structural characteristics even after prolonged PK-digestion of the rods (McKinley *et al*., 1986) point to a phenomenon of outstanding interest.

Before recognizing PrP^{Sc} as the indispensable part of infectivity, a polysaccharide had been the subject of speculation on the nature of the infectious agent of scrapie. Field (1966, 1967) expressed his 'polysaccharide hypo-

Table 1 Carbohydrate Linkage Analysis of the PK-Pellet from Prion Rods.

Sample	Mass of detected glucose building blocks in μg[a]					
	sum	1,4-linked	1,6-linked	1,4,6-linked	1-end	(1,3,4-linked)[b]
Prion rods (253 μg PrP 27–30; Prusiner)	50	41	8	1	–	–
Prion rods from 20 scrapie-infected hamster brains (Diringer)	101	82	7	6	–	(6)[b]
Control: 20 non-infected hamster brains (Diringer)	18	13	4	1	–	–
660 μg cellulose	655	655	–	–	–	–

[a] Mass values given were derived from comparison to an internal standard (inositol), which had been validated using cellulose as positive control (last row).

[b] Possibly due to undermethylation - since the OH-group in position 3 is least reactive, a similar signal for 1,3,4-linked glucose was neither detected in the samples of row 1 nor row 3, and this sample was the most PK-resistant, possibly interfering with complete methylation.

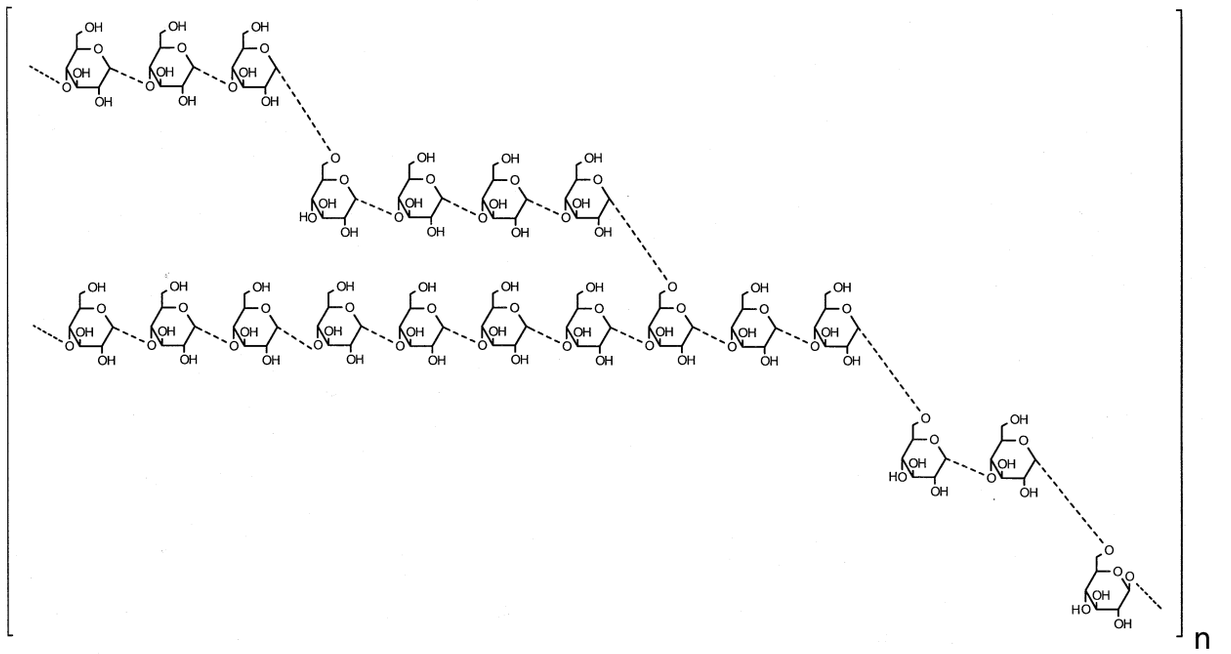

Fig. 7 Hypothetical Structure Element of the Polysaccharide Forming the PK-Pellet of Prion Rods.
Experimental basis of this scheme are the data reported in Table 1 for the PK-pellet from 253 µg PrP 27–30 rods (Prusiner) and the DCI mass spectra of Figure 4. The type of glycosidic linkage (dashed) was not determined.

thesis' in 1966 with the argument of high chemical resistance of the scrapie agent. The hypothesis described a cellular polysaccharide aggregating in the cytoplasm of astrocytes as the replicating agent of scrapie, acting as a 'seeding focus' when transmitted to another animal. Adams and Caspary (1967) elaborated the hypothesis postulating a small nucleic acid enclosed in a polysaccharide coat. This theoretical phase culminated in the proposition that a polysaccharide without nucleic acid may act as 'linking substance' of an incomplete cellular factor (Adams and Field, 1968). Later some experiments were carried out to test the hypothesis. Hunter and colleagues (1969) published that 0.01 M periodate, a carbohydrate-specific reagent, inactivated scrapie infectivity in brain homogenates almost completely. However, Adams and colleagues (1972) could not reproduce this result. Therefore and because no other arguments for a replicating polysaccharide could be found the 'polysaccharide hypothesis' of the scrapie agent was abandoned even before the prion model was developed.

Later observations pointing to a carbohydrate were not discussed as an argument against the prion model. Prusiner's laboratory (McKinley *et al.*, 1986) published that after digestion of prion rods with high concentrations of PK for 30 hours the amount of PrP was reduced by a factor of 10^2 and infectivity by 10^3. Nevertheless, the rod-like ultrastructure visible in the electron microscope by negative staining was unaltered compared to the starting material. However, the chemical nature of the remaining material has not been determined. In the present work we have shown that after prolonged PK-digestion of prion rods under conditions similar to those employed by McKinley and colleagues approximately 10% of the starting mass remained as an insoluble pellet and that the amount of PrP in this pellet was reduced by a factor of 10^3. Thus, the earlier observation (McKinley *et al.*, 1986) could be reproduced. Another hint to the presence of an inert material in prion rods published again by Prusiners laboratory (Stahl *et al.*, 1993) was also reproduced. The arrow in Figure 2 highlights a band of high molecular mass at the interface between the stacking and resolving gel of a 4/12% SDS-PAGE, similar to the earlier work. The third study pointing to the presence of carbohydrates in prion rods (Safar *et al.*, 1990) reported small amounts of glucose in hydrolysates of undigested rods in 2 M CF_3COOH, which was however not discussed in terms of an additional component. This finding can now be explained by the presence of a glucose-polysaccharide partially hydrolyzed by CF_3COOH. In summary, the findings of the present study are in agreement with earlier results whenever comparable. However, for the first time a chemical analysis was carried out to the point to prove the presence of substantial amounts of an inert polysaccharide in prion rods.

The Structure of the Polysaccharide Scaffold

The polysaccharide found in prion rods contains glucose as the only building block, which is mainly 1,4-linked. Its polymeric nature can be deduced convincingly from quite different features all pointing into the same direction. The insolubility of the polysaccharide in water and all solvents tested except $(CH_3)_2SO$ or a mixture of 96% $(CH_3CO)_2O$ and 4% H_2SO_4 indicates a very high molecular mass. This conclusion is further substantiated by the existence of a

band not migrating into the resolving gel in SDS-PAGE (Figure 2), the fragmentation pattern of the DCI mass spectrum in Figure 4 and the absence of terminal glucose units in methylation analysis (Table 1). Also the finding that the ultrastructure of the rods after almost complete digestion of PrP is retained (McKinley *et al*., 1986) points to the polymeric nature of this component. The polysaccharide shows all functional features of a scaffold closely related to prion rod structure.

However, several questions on the chemical characterization of the polysaccharide scaffold are still open.

First, the stereochemistry of the glycosidic bonds, i. e. α- or β-, could not be determined. NMR spectroscopy or digestion experiments using specific enzymes as usual methods for determining the stereochemistry of glycosidic bonds in oligosaccharides were not suitable for the analysis of tiny amounts of an insoluble polysaccharide.

Second, a quantitative value for the average molecular mass of the polysaccharide could not be given, only a lower limit can be presented. The absence of terminal glucose units in methylation analysis sets this limit to 15 kDa taking the sensitivity of the method into account. The fact that the polysaccharide does not enter the resolving gel of a 4/12% SDS-PAGE and its almost complete insolubility however point to a very high molecular mass. Matrix-assisted laser desorption/ionization (MALDI) mass spectra of polysaccharides up to a molar mass of 75000 (Hao *et al*., 1998) were published, but experiments with the prion polysaccharide gave only fragments of low molecular mass (data not shown). Even after complete methylation no peaks of high molecular mass were observed by MALDI or electrospray ionization (ESI) mass spectrometry.

Third, potential covalent links between PrP and the polysaccharide scaffold could not be ruled out. In prion rods the polysaccharide is tightly associated with the prion protein. Nevertheless, more than half of the PrP contained in the rods could be solubilized by ultrasonication in buffer containing 0.2% SDS (Riesner *et al*., 1996). Another fact arguing against covalent bonds between PrP and the polysaccharide is the absence of signals with increased molecular mass for PrP in the SDS-PAGE analysis and in MALDI mass spectra of prion rods (Prusiner *et al*., 1983; Stahl *et al*., 1992). Therefore, covalent bonds between PrP and the polysaccharide should not exist or only with a minority of the PrP molecules. Such bonds might be formed between free amino groups of the protein, i. e. arginine or lysine residues, and the reducing end of the polysaccharide. However, the high molecular mass of the polysaccharide would limit the number of such bonds considerably.

The linkage of the glucose units in the polysaccharide scaffold of prion rods has similarities with glycogen, but the physical properties of the polysaccharide are more like cellulose. Glycogen is a mainly α-1,4-linked glucan containing 1,4,6-branches every 6–12 glucose units. Brain tissue contains about 0.1% (wet weight) of glycogen, which is in fast metabolic equilibrium with soluble glucose and is completely soluble in water. In contrast, the polysaccharide scaffold of prion rods is nearly insoluble, contains about 15% 1,6-linked glucose units and 1,4,6-branches only every 20 glucose unit. Cellulose contains exclusively β-1,4-linked glucose but is known as a plant structural polysaccharide. It is totally insoluble in water and organic solvents. Quasi-crystalline regions of stacked β-sheets formed between the long glucose chains are responsible for the very high stability of cellulose. The physical properties of the polysaccharide scaffold of prion rods correspond in some respect to the so-called 'mammalian cellulose' discussed in early studies that were not followed up later (Hall *et al*., 1958, 1959; Cruise and Jeffrey, 1959; Hall and Saxl, 1960, 1961; Toriumi *et al*., 1972).

Functional Implications of the Polysaccharide Scaffold

Prion rods show high stability against chemical, enzymatic or physical treatments that inactivate almost all other pathogens. The altered conformation and aggregation of PrP certainly contributes to this high stability, but whether it fully explains the observed effects remains open. Experimental evidence exists that reaggregated insoluble PrP 27–30 with a high content of β-sheet is much less stable against PK compared to native prion rods (Caughey *et al*., 1995; Kaneko *et al*., 1995; Post *et al*., 1998; Hill *et al*., 1999). This leaves room for the polysaccharide scaffold being partly responsible for the extreme stability of prion rods.

In the context of discussing a structural polysaccharide component of prions, several publications describing an analogous component in Alzheimer neurofibrils are worth mentioning. Structures quite similar to prion rods have been isolated from brain tissue of Alzheimer patients. These paired helical filaments (PHF) are insoluble in many solvents (Selkoe *et al*., 1982). They are composed mainly of an aggregated, hyperphosphorylated isoform of the Tau-protein. When inspecting PHF in the electron microscope before and after prolonged protease digestion, rod-like structures are seen in both cases (Wischik *et al*., 1988). Therefore, a structured, protease-resistant component may also be contained in PHF. This protease-resistant core of PHF has been described as being non-proteinaceous (Sparkman, 1993) and later as 'glycolipid' containing mainly 1,6-linked glucose (Goux *et al*., 1995, 1996). However, the experimental details mentioned in the publications above can as well be interpreted as a polysaccharide scaffold with a minor extent of fatty acid modification or impurities.

The elucidation of the *in vivo* formation of the polysaccharide scaffold of prion rods would possibly also provide the answer to its functional role. At present, however, only inferences from the data obtained so far can be stated and discussed.

The polysaccharide scaffold could be a second component of the infectious unit, i. e. essential for infectivity. However, this assumption would only hold true if future

reconstitution experiments of prion infectivity from monomeric, non-infectious PrP 27–30 solubilized from prion rods and the non-infectious polysaccharide scaffold will be successful.

Another potential role of the polysaccharide scaffold could be a secondary step involved in the pathogenic process of the prion disease. In this case the scaffold would not be a necessary component of the infectious unit but its formation an inevitable step in the progression of the disease.

The tight but supposedly non-covalent association of the polysaccharide with prion rods makes it plausible to postulate an exaggerated, but finally not successful host response to the accumulation of the pathological conformation of PrP. A similar hypothesis has been advanced as the cause of the formation of so-called corpora amylacea (Shingrao *et al*., 1995), a topic we will investigate further. In this sense, the aggregated prion protein may provide a trigger and matrix for the formation or deposition of the polysaccharide. Whether a similar host response triggered by the aggregation of an insoluble protein occurs in the mammalian brain during Alzheimer and prion disease now becomes an interesting question. The answer may shed light on a common pathological process of neurodegeneration.

Materials and Methods

All chemicals used were of the highest grade commercially available. Conical glass vials (1.0 or 2.5 ml) with Teflon-lined screw-caps for carbohydrate hydrolysis and derivatization were purchased from Macherey and Nagel GmbH, Düren, Germany.

Prion Rods and Control Samples

PrP 27–30 rods purified from scrapie-infected Syrian hamster brain according to published protocols (Prusiner *et al*., 1982, 1983; Diringer *et al*., 1997) were kindly provided by Dr. Stanley B. Prusiner and Hana Serban (Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, California, USA) as well as Dr. Heino Diringer (Robert-Koch-Institut, Bundesinstitut für Infektionskrankheiten und nicht übertragbare Krankheiten, Berlin, Germany). Dr. Heino Diringer also provided control samples prepared from non-infected Syrian hamster brain by the same protocol (Diringer *et al*., 1997).

SDS/PAGE, Staining and Western Blotting

SDS/PAGE (4/12%) was performed according to Laemmli (1970). Prior to gel electrophoreses all samples were boiled for five minutes in loading buffer. A pre-stained protein molecular mass standard 14–200 kDa (HEW 407, Gibco) was used. The separated protein bands were silver-stained (Blum *et al*., 1987) or blotted onto PVDF membranes (Millipore). The membranes were saturated with 5% nonfat dry milk in TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0,05% Tween 20) for 2 hours. After rinsing twice with TBST the primary antibody anti-PrP 3F4 (1:10 000 in TBST) (Kascsak *et al*., 1987) was incubated overnight at 4 °C. The membrane was washed twice in TBST and developed by a horseradish peroxidase coupled secondary antibody followed by the enhanced chemiluminescence detection reagent (Amersham).

Preparation of the PK-Pellet from Prion Rods

500 μg prion rods were washed four times by suspension in 500 μl H_2O, sonication (1 min) and reprecipitation (1 h, 100 000 *g*). They were transferred into micro glass centrifuge tubes with tightly fitting Teflon stoppers using 60 μl CH_3OH. 120 μl $CHCl_3$ was added and the mixture left for 5 minutes at room temperature while mixing occasionally. Then the rods were pelleted for 5 minutes at 13 000 *g* (Hettich EBA-12 centrifuge, fixed angle rotor) and the extract collected. The extraction was repeated two times using 180 μl $CHCl_3/CH_3OH$ 2:1 (v/v) in each step. The prion rods remaining in the centrifuge tube were dried for 10 minutes by vacuum, taken up in 1 ml phosphate buffer (10 mM, pH 7.2) and sonicated for 1 minute at 50–70 W (beaker sonicator Labsonic U, B. Braun GmbH, Melsungen, Germany). 100 μg protease K (PK; Boehringer, Mannheim) was added to achieve a concentration of 0.1 g/l. After intensive mixing the suspension was left at room temperature for 24 hours. Thereafter the same amount of protease K (100 μg) was added, mixed intensively and left for another 24 hours at room temperature. Finally the clear liquid was centrifuged for 1 hour at 100 000 *g*. The PK-supernatant was collected and stored at –78 °C. The pellet was resuspended in 500 μl H_2O and centrifuged as above. The supernatant was discarded and the wash step repeated twice again. The final PK-pellet was stored at –78 °C.

Experiments to Solubilize the PK-Pellet

Samples of about 10 μg PK-pellet were mixed with 50 μl each of the following solvents: H_2O, CH_3OH, $CH_3(CH_2)_3OH$, $(CH_3CH_2)_2O$, $(CH_3)_2CO$, CH_3CN, 1,4-dioxane, $CHCl_3$, $(CH_3)_2SO$ and a mixture containing 4% H_2SO_4 and 96% $(CH_3CO)_2O$. After 1 hour at room temperature, part of the liquid (5 μl) was taken up with the DCI emitter and transferred into the direct inlet of an INCOS 50 quadrupol mass analyzer (Finnigan MAT, mass range 40–1150 u). Only the last two solvents gave signals in the mass range above m/z = 100 u. The sample was dissolved without visible residue only by a mixture of H_2SO_4 and $(CH_3CO)_2O$. Upon dissolution the color of this liquid changed from colorless via yellowish to dark brown. Partial oxidative destruction of the sample might be responsible for this color change.

Direct Chemical Ionization Mass Spectrometry of the PK-Pellet

10 μg PK-pellet was mixed with 50 μl $(CH_3)_2SO$. After 1 hour at room temperature, part of the suspension (5 μl) was taken up with the DCI emitter and transferred into the sample chamber of the mass analyzer. Ammonia at a pressure of about 0.5 Pa was used as reactant gas for direct chemical ionization at 80 °C source temperature.

The spectrum shown in Figure 4 was recorded using a MAT 900 S sectorfield mass analyzer (Finnigan MAT, mass range 100–2000 u).

Component Analysis I: Methanolysis and Trimethylsilyl Derivatization

The procedure was adapted from Chambers and Clamp (1971). In a conical glass vial a sample of 30 μg PK-pellet or a standard compound like cellulose was suspended in 70 μl $(CH_3)_2SO$. 30 μl of a saturated solution of gaseous HCl in CH_3OH was added and the mixture left for 3 days at room temperature. After dilution with 500 μl CH_3OH the solvent was slowly reduced to 50 μl by applying vacuum. 500 μl CH_3OH were added and the azeotropic removal of HCl using CH_3OH was repeated 5 times. Only in the last step it was evaporated to dryness, because of sample damage by concentrated HCl.

The residue was taken up in 10 µl pyridine and 10 µl BSTFA were added. After 15 minutes 1 µl of the solution now containing fully silylated methyl-glycosides was analyzed by GC/MS. A nonpolar column (Optima-1, Macherey and Nagel, *l* 25 m, ∅ 0.32 mm, film thickness 0.38 µm) was used with helium as carrier gas. The temperature of injector and transfer line was 250 °C. Temperature program: 3 min 120 °C, 10 °C per minute to 280 °C, 15 min 280 °C.

Component Analysis II: Alditol Acetate Method

A general description of methods for analysis of carbohydrates by GC/MS is given by Biermann and McGinnis (1989). All steps described below were carried out successively in the same conical glass vial with Teflon-lined screw-cap.

Acid hydrolysis (according to Albersheim *et al*., 1967): 300 µl of 2 M CF_3COOH was added to the sample, the vial flushed with Ar, closed tightly and heated to 120 °C for 2 hours. After cooling, volatile compounds were removed by a steady stream of Ar through the vial placed in a heat block at 40 °C.

Reductive ring opening (according to Harris *et al*., 1984): 500 µl of a fresh solution containing 40 g/l $NaBD_4$ in 2 M NH_4OH were added to the sample, the vial flushed with Ar and kept at 60 °C for 1 hour. Because of the formation of hydrogen the vial should not be closed tightly. The reaction was stopped by the dropwise addition of CH_3COOH until no H_2 was formed any more. 500 µl CH_3OH was added and the volatile compounds driven out by a steady stream of Ar through the vial placed in a heat block at 40 °C. The last step gently removed the H_2O and had to be repeated 3 to 6 times until less than 20 µl of H_2O were left.

Acetylation (according to Blakeney *et al*., 1983): 100 µl 1-methylimidazole (catalyst) and 1 ml $(CH_3CO)_2O$ were added to the sample and kept at room temperature for 15 minutes. The reaction was stopped by pouring the contents of the vial into 2 ml H_2O. The vial was rinsed twice with 1 ml H_2O each. The combined liquids were extracted twice using 1 ml CH_2Cl_2 each. The combined organic extracts were washed 4 times with 4 ml H_2O each and transferred back into the conical glass vial. The solvent was removed by a steady stream of Ar through the vial at room temperature. The acetylated sample was stored dry in the glass vial at – 20 °C. Directly before GC-analysis it was taken up in 40 – 100 µl CH_3OH.

GC/MS-analysis: GC column and technical parameters were identical to the component analysis I described above. Temperature program: 2 1/2 min 170 °C, 1 °C per minute to 190 °C, 10 °C per minute to 280 °C, 15 min 280 °C. Injection volume was 0.5 – 2 µl, 1 minute after injection the split valve was opened (1:10). For Figure 6 a polar BPX-70 (SGE) column was used isothermally at 240 °C.

Instruments: Varian 1700 GC coupled to a Varian MAT CH7A sectorfield mass analyzer via an open split column for electron impact ionization; Varian 3400 GC directly coupled to an INCOS 50 quadrupol mass analyzer for chemical ionization; Carlo Erba Industries HRGC 5160 coupled to FID detector.

Linkage Analysis

Complete methylation (modified from Blakeney and Stone, 1985; Parente *et al*., 1985; Hounsell *et al*., 1996): 60 µl $(CH_3)_2SO$ and 10 µl inositol-standard [5 g/l in $(CH_3)_2SO$] were added to the sample (20 – 100 µg polysaccharide, see Table 1), the vial flushed with Ar and kept at room temperature for 24 hours to better dissolve polysaccharides. Then 200 µl of a methylsulfinylmethanide-solution (prepared according to Carpita and Shea, 1989) was put in and stirred for 3 hours. Now 120 µl CH_3I were added, the Ar-atmosphere renewed and the mixture stirred for 24 hours. Finally 200 µl CH_2Cl_2, 100 µl CH_3OH and 500 µl $Na_2S_2O_3$-solution (10 mg/l) were given into the vial and shaken by hand for 2 minutes. The lower organic phase was removed and reextracted twice using 200 µl H_2O. The water phases were discarded and the washed organic phase pipetted into a fresh conical vial. 200 µl 2,2-dimethoxypropane was added to remove traces of H_2O. The volatile solvents were removed by a gentle stream of Ar at room temperature.

Further steps (according to Blakeney and Stone, 1985; Parente *et al*., 1985; Harris *et al*., 1984): Acid hydrolysis, reductive ring opening and acetylation were carried out as described above (component analysis II).

GC/MS-analysis: An nonpolar column (Optima-1, Macherey and Nagel) separated the partially methylated alditol acetates satisfactorily. Technical parameters and temperature program were equivalent to the component analysis I described above.

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