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Investigations on the regioselective hydrolysis of a branched β-1,3-glucan

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Schizophyllan, an extracellular polysaccharide secreted by the fungus *Schizophyllum commune* ATCC 38548, shows interesting technical and pharmaceutical applications. Especially in the degraded form, this homoglucan is applicable as an antitumor, antihepatitis, anti-HIV and antiviral agent. Due to the fact that the side chains of the biopolymer are essential for its activities, a regioselective degradation of the β-1,3 basic chain by maintaining the β-1,6 side chains is necessary. This paper covers investigations which deal with the regioselective cleavage of the basic chain.

It was found that the hydrolysis of aqueous polysaccharide solutions by incubation in DURAN glass bottles at 121°C and 1 bar was the most successful method. A slight decrease of pH, a rapid loss of viscosity, a constant increase in reducing end groups and a continual release of glucose over an incubation time of 100 h indicated a degradation especially at the basic chain. Stepwise ultrafiltration of degraded solutions yielded fractions with varying molar masses and the ratio of the fractions depended on the total incubation time. The maintenance of the side chains was verified by ^{13}C-NMR spectra. The regioselectivity of this degradation method can be explained by a pore theory. One attempt to justify this theory is the suppression of hydrolysis after hydrophobization of the glass surface by using dichloromethylsilane.

INTRODUCTION

For several years, studies have been conducted on the production (Rau *et al.*, 1990, 1992*b*; Steiner *et al.*, 1987) and primary physicochemical characterization (Norisuye *et al.*, 1980; Kashiwagi *et al.*, 1981) of Schizophyllan, an extracellular polysaccharide produced by the basidiomycete *Schizophyllum commune* ATCC 38548. The polysaccharide consists of a β-1,3-linked main chain of glucose monomers with β-1,6-linked glucose residues at approximately every third basic chain molecule (Fig. 1).

During the characterization of this biopolymer the monomers, in this case glucose, were identified by total acid hydrolyses at high temperature (12 N H_2SO_4, 121°C, 1 bar) (Münzer, 1989). However, no studies were carried out dealing with the regioselective cleavage of the β-1,3-chain while maintaining the β-1,6-linked side chains. Subsequent fractionation of the hydrolysed Schizophyllan by stepwise ultrafiltration could then be carried out.

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MATERIALS AND METHODS

Materials

The wood rotting basidiomycete *Schizophyllum commune* ATCC 38548 was used for the production of the extracellular polysaccharide Schizophyllan. The conditions of the 50 dm^3 bioreactor cultivation have been previously described (Rau *et al.*, 1990). After the cells had been separated by continuous centrifugation and subsequent filtration through glass fiber membranes (Sartorius, Göttingen, Germany) to remove small hyphal fragments, the cell-free glucan solution was purified and concentrated by a lab-scale ultrafiltration system (DC 10 LA, Amicon, Witten, Germany), equipped with a gear pump and two hollow-fiber cartridges H5P100-43 (molecular cut-off 100,000 g per mole, inner diameter 1.1 mm, surface area 0.45 m^2). Depending on conditions of both cultivation and downstream processing, the molecular weight of Schizophyllan ranges between 6 and 15×10^6 g per mole (Rau *et al.*, 1992*a*). After the above-mentioned separation and purification processes, the molecular weight of Schizophyllan is approximately

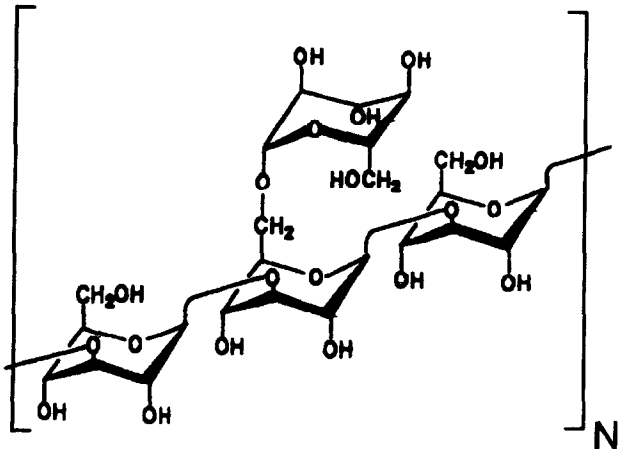

Fig. 1. Primary molecular structure of the branched glucan (repeating unit). N = number of repeating units; N of Schizophyllan (starting material) $\simeq$ 11,600.

7.5×10^6 g per mole. The aqueous solution of Schizophyllan was analyzed for:

(i) formic acid (enzymatically with formiate dehydrogenase), which was added to the culture broth after cultivation to prevent microbial contamination;
(ii) free glucose (enzymatically with glucose oxidase); and
(iii) protein content (Lowry method).

No formic acid and free glucose was detected and the protein content was less than 1.3% (wt/wt).

Hydrolysis of Schizophyllan

All reactions were carried out in DURAN glass bottles prewashed in chloric acid (Jena Glass, Schott, Mainz, Germany) at 121°C and 1 bar using an autoclave (Tecnomara, Fernwald, Germany).

Analysis

The concentration of Schizophyllan was determined by adding 15 ml of 70% (wt/vol) propan-2-ol to 5 ml of cell-free glucan solution, storing it at 4°C for 2 h to complete precipitation, followed by centrifugation at 15,000 *g* and drying of the residue to constant weight.

The progress of hydrolysis was observed by measuring the number of reducing end groups (method of Somogyi and Nelson (Somogyi, 1952)), the released glucose monomers were detected by a glucose analyzer (Yellow Springs, YSI 27, Ohio, USA).

Shear viscosity

The viscosities of native and degraded Schizophyllan solutions were determined at 20°C using a rotational viscometer Rotovisko RV 100 (Haake, Karlsruhe, Germany).

Fractionation

After hydrolysis of the glucan solution, various molecular weight fractions were obtained by a stepwise ultrafiltration using different crossflow filtration systems:

1. One hollow-fiber cartridge H1P100-43 (molecular cut-off 100,000 g per mole, inner diameter 1.1 mm, surface area 0.03 m^2, Amicon, Witten, Germany) equipped with a gear pump.
2. Three flat-channel cartridges type Ultrasette Omega Screen Channel with tangential feed overflow (molecular cut-offs 10 000 g per mole, 5000 g per mole and 1000 g per mole, surface area 0.08 m^2, Filtron, Karlstein, Germany) equipped with a piston pump.

The first step was to purify the degraded solution in deionized water by diafiltration, thus separating compounds smaller than 100,000 g per mole. This was continued for as long as a decrease in the number of reducing end groups in the retentate was noticed. After concentrating the permeate with a rotary evaporator at 40 C and 50 mbar the second step was to diafiltrate it again using a membrane with a smaller molecular cut-off of 10 000 g per mole. The third (5000 g per mole) and fourth (1000 g per mole) steps were carried out in the same way as mentioned above, and finally the permeate that included compounds smaller than 1000 g per mole was rejected. Finally, each fraction was lyophilised (Delta 1-20 K, Heraeus-Christ, Osterode im Harz, Germany).

^{13}C-NMR spectroscopy

Proton decoupled ^{13}C-NMR spectra were recorded on a Bruker WM-400 spectrometer operating at 100 MHz. All spectra were recorded in dimethyl sulfoxide-d_6 at 80°C.

Determination of molecular weights

Two methods were used to determine the molecular weights of Schizophyllan and the molar mass fractions. Initially, native Schizophyllan was analyzed by Low Angle Laser Light Scattering to obtain the constants a and b of the Mark–Houwink equation: $[\eta] = a \times M_W{}^b$; $a = 3.39 \times 10^{-5}$, $b = 1.26$ valid for $[\eta] > 445$ ml/g (Cordes *et al.*, 1989). After determination of the intrinsic viscosity using a low shear rotational viscometer of the Zimm/Crothers-type (Krannich KG, Germany) the molecular weights could be calculated. Afterwards, the fractions with lower masses were analyzed by high performance size exclusion chromatography (HPSEC)

using a TSK 6000 PW column (Toyo Soda, Japan) and a refractive index detector. The column was calibrated by the use of PEG standards.

Suppression of hydrolysis

The glass surface was prewashed with 1 N HCl, rinsed with distilled water and then silanized for 1 h using dichlorodimethylsilane in toluene with a ratio of 1 ml silanization reagent per cm^2 glass surface. After this pretreatment, the hydrolysis was carried out in the same way as without hydrophobization.

RESULTS AND DISCUSSION

Catalytic hydrolysis of aqueous Schizophyllan solutions at glass surfaces

For these tests, purified solutions of 1 g/l (wt/vol) Schizophyllan were used. Fifty ml were incubated for a few hours in 100 ml DURAN glass bottles at 121°C and 1 bar. Due to the fact that the reactions were carried out in a closed system (autoclave) at a high temperature, the oxygen concentration remained at a low level (less than 1%) during the entire period of reaction, and, therefore, the influence of oxygen was ignored. During the degradation procedure, samples were taken and analyzed for the number of reducing end groups, glucose concentration, pH and shear viscosity. The results are presented in Fig. 2. During the incubation time of 100 h, the number of reducing end groups increased up to nearly 9% glucose equivalents. As a result of diminishing kinetic inhibition (diffusion and adsorption processes) caused by a reduction in viscosity, the rate of degradation or release of reducing end groups smoothly increased with reaction time. During the first phase of the reaction, the rate of hydrolysis was approximately 0.05% glucose equivalents per hour and increased up to approximately 0.1% glucose equivalents per hour at the end of hydrolysis. The rate of depolymerization over the total incubation time was 0.08% glucose equivalents per hour.

In spite of the extensive hydrolysis, the glucose concentration remained at a low level during the entire period of incubation. This indicates a regioselective degradation of the β-1,3-basic chain while maintaining the β-1,6- side chains. During hydrolysis of Schizophyllan under strong acid conditions (e.g. by adding sulphuric acid to the biopolymer solution) there will be a rapid decrease in pH and a scission of side chains and the main chain (25% of the measured glucose equivalents were found as free glucose) will be obtained. As determined by these investigations, the decrease of pH from 5.3 to 3.8 indicates mild acid hydrolysis. This is caused by protons which are released from the glass surface by the action of water at higher temperatures.

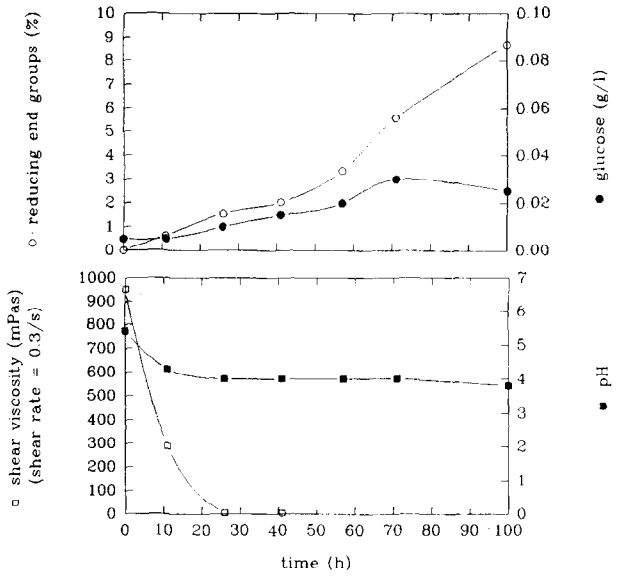

Fig. 2. Hydrolysis of a 1 g/l aqueous Schizophyllan solution in DURAN glass bottles. The solutions were autoclaved at 121°C at a pressure of 1 bar. Analysis: reducing end groups as glucose equivalents by the method of Somogyi–Nelson; glucose as free glucose enzymatically by glucose oxidase; shear viscosity at 20°C and a shear rate of 0.3 s^{-1} using a rotational viscometer.

Due to these facts, it can be stated that strong acid conditions will cause a total scission of the biopolymer, while milder conditions result in a main chain scission.

Two reasons can be stated to explain the extremely rapid decrease in viscosity over the first 26 h.

1. A scission of the biopolymer mainly at the side chain or a total scission of the molecule will cause a loss of viscosity because of the fact that the side chains influence the viscosity of the solution.

2. A scission of the biopolymer primary at the main chain only causes a reduction of the molecular weight and does not influence the impact of the side chains. As the viscosity is related to the molecular weight (Mark–Houwink equation), a reduction in the molecular weight will cause a decrease in viscosity.

Due to the fact that an increase of reducing end groups and a constancy of free released glucose was measured during hydrolysis, a scission of the β-1,3 main chain is the most probable kind of degradation, and, therefore, the decrease of viscosity can be explained by a reduction of the molecular weight.

After the initial successful catalytic hydrolysis, the procedure was repeated with a three-fold increase in Schizophyllan concentration to obtain higher yields of each fraction after the stepwise ultrafiltration.

Initially, hydrolysis was carried out for an incubation period of 44 h. In order to determine the changes in the flow behavior index n during the course of hydrolysis, shear viscosities were measured at various shear rates in the range of 0.3–300/s (Fig. 3). The results were fitted to the power law equation:

$\eta = K \times D^{(n-1)}$
K = consistency index (Pas^n)
D = shear rate (s^{-1})
n = flow behavior index (−).

The samples withdrawn at 0 and 8 h incubation time clearly show a pseudoplastic flow behavior due to the decrease of viscosity at an increasing shear rate. This is a typical characteristic of water-soluble biopolymer solutions (Rau *et al.*, 1990; Kulicke, 1986). However, the slope of the curve obtained for the samples withdrawn after 18 h is noticeably shallower, and after 25 h all samples show nearly Newtonian flow characteristics with a flow behavior index close to $n = 1$.

We wanted to know whether it was possible to obtain a defined ratio of the molar mass fractions by varying the total incubation time. For this reason, three hydrolyses were carried out with total incubation times of 23, 34.5 and 46 h. The average values of the number of reducing end groups obtained to these three hydrolyses are shown in Fig. 4.

The time course in Fig. 4 can be compared to the hydrolyses of 1 g/l Schizophyllan solution (Fig. 2). However, the number of reducing end groups per unit of time is approximately three times higher. This is a noteworthy result, because an increase in the Schizophyllan concentration is connected with an increase of the extent and rate of hydrolysis. In this case, a trebling of the concentration of Schizophyllan caused approximately a trebling of the extent of hydrolysis and a doubling of the rate of depolymerization (0.16% compared with 0.08% glucose equivalents per hour).

Fractionation of degraded Schizophyllan solutions

The fractionation of the hydrolysed solutions by stepwise ultrafiltration resulted in various ratios of the molar mass fractions (Fig. 5).

The total yield was approximately 30–40% (wt/wt) of the original weight of polysaccharide. The loss of 60–70% can be explained by adsorption at the ultrafiltration membrane surfaces during the intensive purification and fractionation steps. Depending on the incubation time, different ratios of the molar mass fractions were obtained. Generally, the yield of the fraction with molar masses > 100,000 g per mole was reduced with increasing incubation time. This contrasts with the other fractions where the longer the time of hydrolysis, the higher the yield. Finally, it is apparent that a desired ratio of the molar mass fractions can be obtained by varying the incubation time.

^{13}C-NMR spectroscopy

^{13}C-NMR spectra were recorded to find out whether the glass surface-catalyzed hydrolysis also caused structural changes (Fig. 6). All spectra showed exactly the same

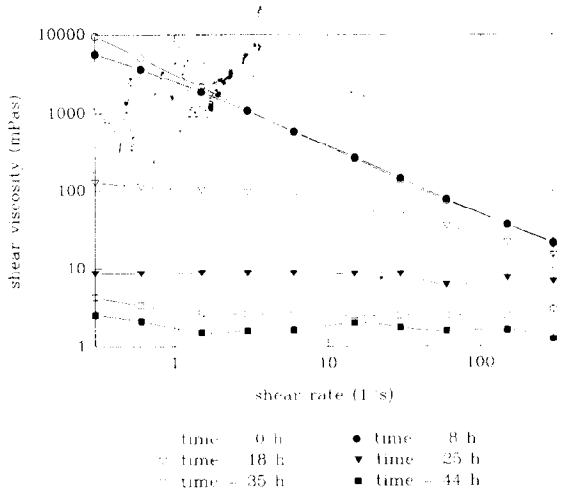

Fig. 3. Decrease of viscous behavior during the hydrolysis of a 3 g/l Schizophyllan solution in DURAN glass bottles depending on incubation time. Analysis: shear viscosity at 20°C and various shear rates (0.3, 0.6, 1.5, 3, 6, 15, 30, 60, 150, 300/s) using a rotational viscometer. Conditions as in Fig. 2.

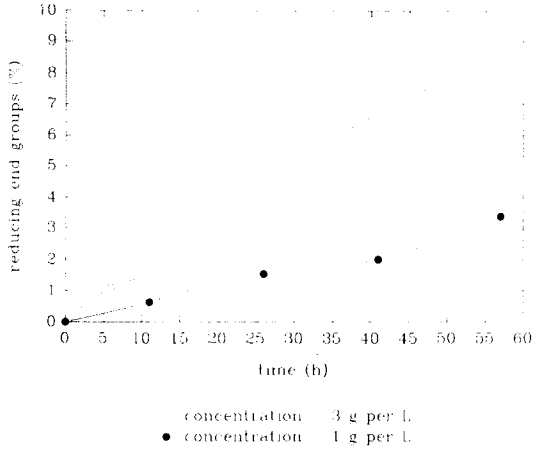

Fig. 4. Hydrolysis of a 3 g/l Schizophyllan solution at different total incubation times (average values, single data not shown) in comparison with the hydrolysis of a 1 g/l Schizophyllan solution in DURAN glass bottles (as in Fig. 2). Analysis: reducing end groups by the method of Somogyi-Nelson. Conditions as in Fig. 2.

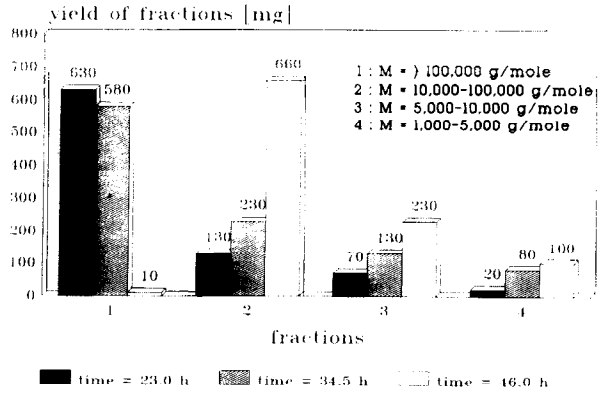

Fig. 5. Distribution of molar mass fractions yielded after the hydrolysis of the 3 g/l Schizophyllan solution shown in Fig. 4.

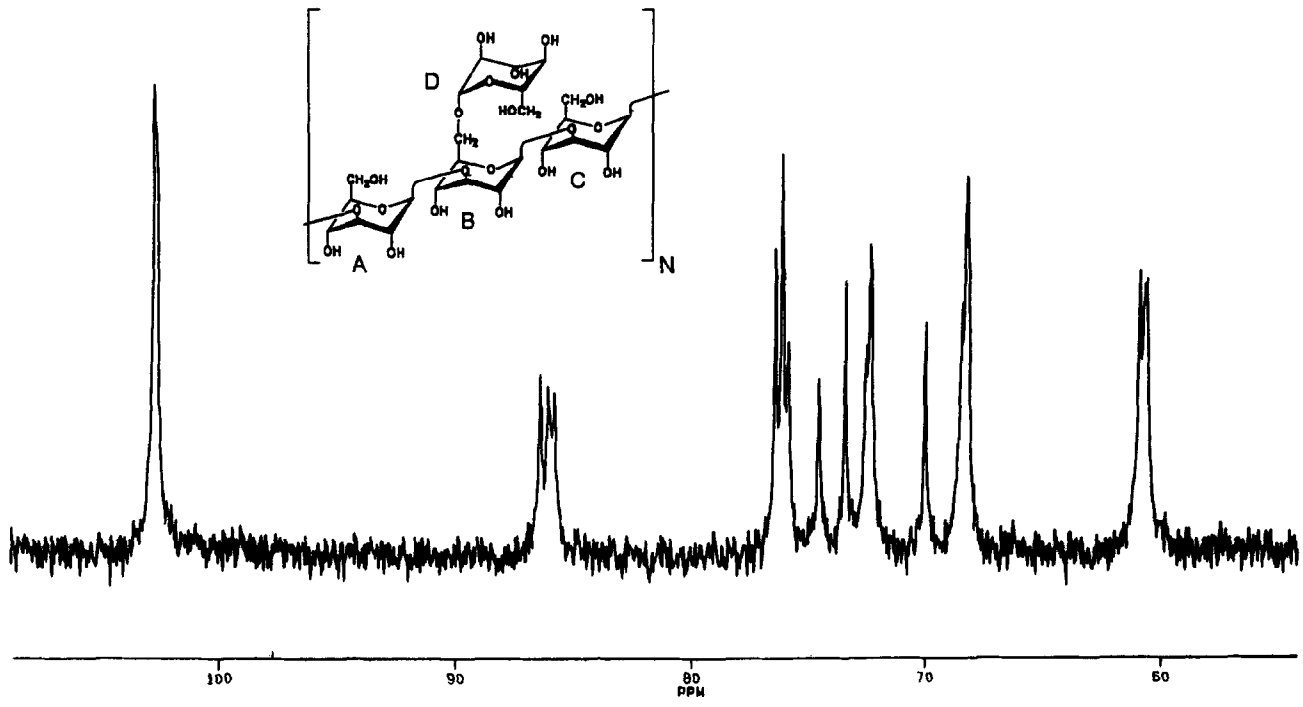

Fig. 6. Proton decoupled ^{13}C-NMR spectrum of native and degraded Schizophyllan recorded on a Bruker WM-400 spectrometer in dimethyl sulfoxide-d_6 at 80°C. Assignment of peaks: 61 ppm: C_6 (A/C/D, free); 68 ppm: C_4; 70 ppm: C_6 (B, bound); 72 ppm: C_2 (A/B/C); 73 ppm: C_2 (D); 74 ppm: C_3 (D, free); 76 ppm: C_5; 86 ppm: C_3 (A/B/C, bound); 102 ppm: C_1.

peak pattern. The signal at 70 ppm (C_6, bound) even of the molar mass fraction 1000–5000 g per mole is clear evidence for a regioselective cleavage of the β-1,3 main chain while maintaining the β-1,6 side chains.

Determination of molecular weights

The four molar mass fractions of degraded and native Schizophyllan were analyzed for their weight average molecular weights by the use of two different determination methods. The results are shown in Table 1. The results for the fractions > 100,000 g per mole and 5000-10,000 g per mole are as expected. The values for the molecular weight of the fractions 10,000–100,000 g per mole and 1000–5000 g per mole are higher than expected. Two reasons can be given for this result: first it must be borne in mind that the exclusion limits of the filtration membranes are not very sharp and that they are calibrated by the use of globular proteins and not partly linear polysaccharides. Secondly, the flow behavior of Schizophyllan solutions through the HPSEC column differs from that of polyethylene glycol and, therefore, comparisons are only possible to a limited extent.

Table 1. Weight average molecular weights of native Schizophyllan and four molar mass fractions of degraded Schizophyllan

Molar mass fraction	Weight average molecular weight
Native Schizophyllan	7500 kDa[a]
Fraction > 100 kDa	500 kDa[a]
Fraction 10–100 kDa	320 kDa[b]
Fraction 5–10 kDa	9 kDa[b]
Fraction 1–5 kDa	8 kDa[b]

[a]Molecular weights were obtained by light scattering measurements of native Schizophyllan and determination of intrinsic viscosities by a low shear rotational viscometer.
[b]Molecular weights were obtained by HPSEC experiments.

Theoretical aspects of the reaction mechanism

It is an important result that the hydrolysis of aqueous Schizophyllan solutions proceeds regioselectively by cleaving only the main chain, although normally the β-1,6-linkages would be expected to be less stable against hydrolytic influences. Although DURAN borosilicate glass 3.3 (DIN ISO 3585) is highly resistant to water, neutral and acid solutions, it is possible that at high temperatures (> 100°C) small amounts of mainly monovalent ions are eliminated from the glass surface by the action of water and acids. Released protons reduce the pH as found in these investigations. With these facts in mind, a very thin, almost two-dimensional silica gel monolayer with a pore distance of approximately 0.32 nm is formed at the glass surface. As described in the literature (Yanaki *et al.*, 1980; Yanaki *et al.*, 1983) the mean distance of the β-1,6-linked glucose residue protruding from the triple helical Schizophyllan strain is approximately 0.30 ± 0.03 nm.

Therefore, fixing the Schizophyllan molecule by deposition of the side chain glucose in the silica gel layer pores is conceivable (Fig. 7). The resulting shielding of the side chains could be the reason for maintaining the β-1,6-linkage during the hydrolytic degradation of the basic chains. To find out whether it would be possible to obtain a higher rate of hydrolysis by using a larger surface of the catalyst, we carried out reactions with handmade glass fragments. Surprisingly, no increase in the rate of hydrolysis was detected.

Suppression of hydrolysis by hydrophobization of the glass surface

The first step to verify this pore theory was carried out by hydrophobization of the glass surface. In comparison with untreated glass surfaces, the hydrolysis in silanized glass bottles is suppressed, and the extent of suppression depends on the percentage of dichlorodimethylsilane in toluene (Fig. 8). While a 2% (vol/vol) solution merely resulted in a slight decrease in the extent of hydrolysis, it was possible to halve the extent of hydrolysis using a 4% (vol/vol) solution of the silanization reagent. It was not possible to achieve greater suppression of hydrolysis by the use of more highly concentrated silanization reagents. It seems that a 4% solution causes the greatest possible hydrophobization of the surface and this happens because of the larger area required by the methyl groups which cause a blocking of the pores, resulting in a decrease of the catalytic activity of the glass surface.

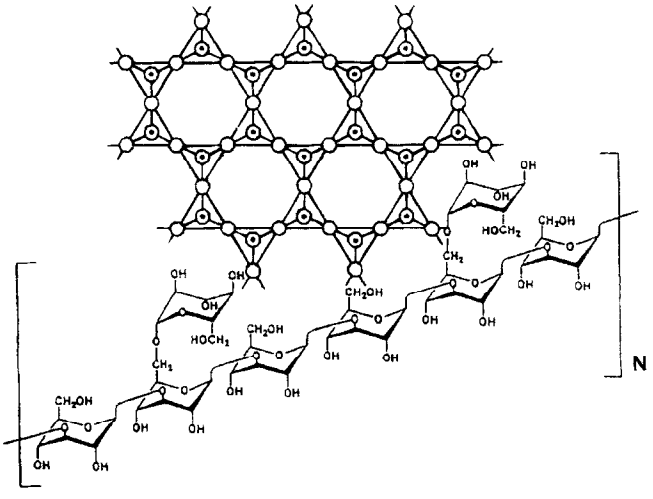

Fig. 7. Possible fixing of the side chains of Schizophyllan by deposition in the silica gel layer pores; ○ = silicon; ○ = oxygen.

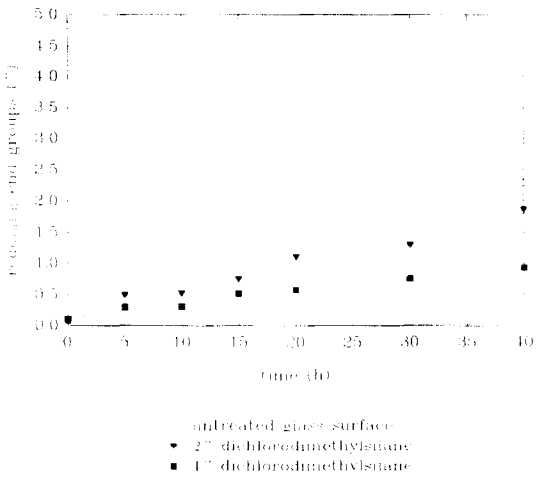

Fig. 8. Suppression of hydrolysis of 1 g/l Schizophyllan solution in DURAN glass bottles depending on the percentage of the silanization reagent. Conditions as in Fig. 2. The glass surface was prewashed with 1 N HCl, rinsed with distilled water and then silanized for 1 h with various percentages of dichloromethylsilane in toluene; the ratio was 1 ml silanization reagent per cm^2 glass surface. Analysis: reducing end groups by the method of Somogyi–Nelson.

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This paper is dedicated to Professor J. Klein on the occasion of his 60th birthday.

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