Chemical modification of pullulan: 1. Periodate oxidation

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The present paper describes the periodate oxidation of pullulan. Special attention is paid to the structures and characteristics of the activated products. Pullulan contains three different anhydroglucoside moieties in the repeating unit. Therefore, periodate oxidation of pullulan results in different types of dialdehyde structures. From titrimetric analysis of the formic acid generated during the oxidation of pullulan, the numbers of singly and doubly oxidized anhydroglucoside units can be calculated. The aldehyde content in completely periodate-oxidized pullulan as determined by reaction with hydroxylamine hydrochloride is only 67%. A plausible explanation is the presence of a stable, six-membered hemiacetal. The presence of aldehyde groups with different reactivities is also evidenced by the data collected from reduction experiments and by ^{13}C n.m.r. Finally, the hydrolytic stability of the polysaccharide backbone was studied.

(Keywords: periodate oxidation; pullulan; hemiacetal formation; hydroxylamine hydrochloride; hydrolytic stability)

INTRODUCTION

In recent years, there has been an increasing interest in the preparation of macromolecular prodrugs[1]. Several polysaccharides are being used as carrier molecules, including dextran, starch and cellulose derivatives[2]. We recently were interested in exploring the possibilities of using pullulan in the preparation of macromolecular drug derivatives.

Pullulan is a linear polysaccharide which is produced by the yeast-like fungus *Aureobasidium pullulans* (*Pullularia pullulans*). It consists of α-1,6-linked maltotriose units. The selection of pullulan in the preparation of drug derivatives is justified on the basis of the blood compatibility and biodegradation properties of the polysaccharide[3,4] (*Figure 1*).

Direct coupling of polysaccharides with drugs is only feasible if the latter have the appropriate functionality. In most cases the carrier molecule or the drug must be transformed into a suitable reactive derivative enabling covalent coupling. A large number of methods are available to activate polysaccharides[5]. Among them is the periodate oxidation which leads to polyaldehyde structures. The present paper describes the periodate oxidation of pullulan. Special attention is paid to the structures and characteristics of the activated products.

MATERIALS AND METHODS

Materials and instruments

Pullulan was obtained from the Sigma Chemical Company (St Louis, MO, USA). Sodium periodate, hydroxylamine hydrochloride, sodium borohydride and sodium cyanoborohydride were obtained from Janssen Chimica (Beerse, Belgium).

Methods

Periodate oxidation of pullulan. The preparation of a pullulan derivative having 50% of the anhydroglucoside units oxidized is given as an example.

To a solution of pullulan (1 g, 6.17 meq anhydroglucoside units) in 50 ml double-distilled water was added sodium periodate (0.877 g, 4.1 mmol). The reaction mixture was stirred in the dark for a few hours at room temperature and then subjected to dialysis (molar mass cut-off = 1000 g mol^{-1}) against double-distilled water for 48 h. The resulting polymer was freeze dried.

Determination of the aldehyde content. The aldehyde content was determined by the hydroxylamine hydrochloride method[6]. The periodate-oxidized pullulan reacts almost instantaneously with hydroxylamine hydrochloride to form the corresponding oxime. The hydrogen chloride liberated can be titrated with alkali solution.

Ten millilitres of the aldehyde solution in H_2O containing ca. 150 mg product were added to 25 ml of 0.4 M hydroxylamine hydrochloride. The solution was

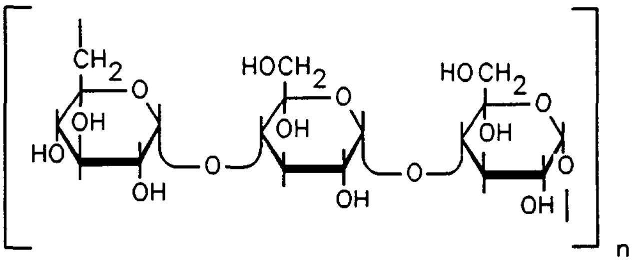

Figure 1 Structure of pullulan

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stirred overnight at room temperature and titrated with 0.1 N NaOH. The titration was followed potentiometrically.

Reduction of partially oxidized pullulan. To a solution of 1 g of the polyaldehyde in 20 ml water was added 0.5 g of sodium borohydride. The same experiment was done with sodium cyanoborohydride. The reaction mixture was stirred overnight at room temperature and subsequenttly acidified to pH 6 by adding acetic acid. The polysaccharide derivative was isolated by preparative size exclusion chromatography on Sephadex G-25, using water as eluent. The appropriate fraction was collected and freeze dried.

Acidic hydrolysis of reduced pullulan dialdehyde. The reduced polymer (0.5 g) was dissolved in 25 ml of 0.1 N HCl. The solution was placed in a bath, thermostatted at 37°C and 10 ml of this solution were transferred into an Ostwald viscosimeter. The viscosity of the solution was measured at regular time intervals.

RESULTS AND DISCUSSION

Introduction

Polyaldehyde derivatives of polysaccharides containing vicinal diol structures are easily obtained by reaction with periodate salts in aqueous solution. The polyaldehydes thus prepared can be isolated by precipitation, dialysis or preparative gel permeation chromatography. The aldehyde content can be varied over a broad range by changing the periodate/polysaccharide ratio.

The resulting polyaldehydes can be coupled with amino compounds to give Schiff base structures or alkylamines if coupling occurs in the presence of a reducing agent, e.g. sodium cyanoborohydride (*Figure 2*).

Periodate oxidation of polysaccharides is a complicated process. The kinetics of the oxidation reaction as well as the accessibility of the aldehyde functions are generally hampered by hemiacetal formation[7–13].

Structural analysis of partially oxidized pullulan

Pullulan contains three different anhydroglucoside moieties in the repeating unit. Consequently, it contains different types of vicinal diol groups. Therefore, periodate oxidation of pullulan results in different types of dialdehyde structure (*Figure 3*). In polysaccharides with repeating units having three adjacent hydroxy groups (e.g. dextran, pullulan) the periodate oxidation is a two-step process[7–13].

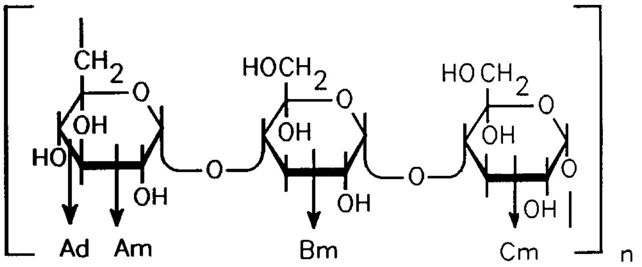

Figure 3 Different places of oxidation in a periodate-oxidized pullulan. A_m, B_m and C_m are singly oxidized units, A_d is a doubly oxidized unit

The oxidation of an α-1,4-linked anhydroglucoside unit results in the consumption of 1 mol of periodate and the formation of two aldehyde groups. Total oxidation of an α-1,6-linked anhydroglucoside unit is a two-step process, first generating an α-hydroxy aldehyde group which can be further oxidized to give formic acid and a dialdehyde group (*Figure 4*).

The aldehyde content in a partially oxidized polysaccharide can be determined titrimetrically. A conventional method for aldehyde determination is the hydroxylamine hydrochloride method[6]. In addition, the formic acid generated during total oxidation of pullulan can be determined titrimetrically. From these data the numbers of singly and doubly oxidized anhydroglucoside units can be calculated. This is illustrated in *Table 1*.

Discussion

The purpose of the present periodate oxidation study of pullulan is to analyse the different types of aldehydes in the end-products rather than analyse the kinetics of the oxidation reaction.

By titrimetric analysis of the aldehyde content and the formic acid formed, the numbers of different dialdehydes can be estimated. It must be indicated that the experimental error in the determination of the formic acid content results in less accurate values. Furthermore, it is anticipated that the aldehydes are either present in their hydrated or hemiacetal form, which further complicates the structure of the partially oxidized polymer.

In cases of low degrees of oxidation, the number of singly oxidized units (A_m) is smaller than the number of doubly oxidized units (A_d). The conversion of singly oxidized to doubly oxidized units is not significantly faster than the conversion of the intact unit to the singly oxidized unit. At higher degrees of oxidation, both singly

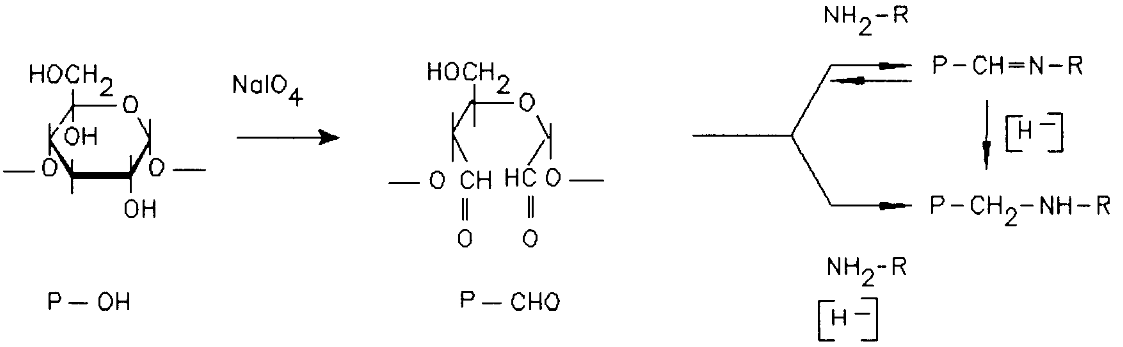

Figure 2 Reaction of a polysaccharide aldehyde with an amine gives a Schiff base structure or an alkylamine if coupling occurs in the presence of a reducing agent, e.g. sodium cyanoborohydride

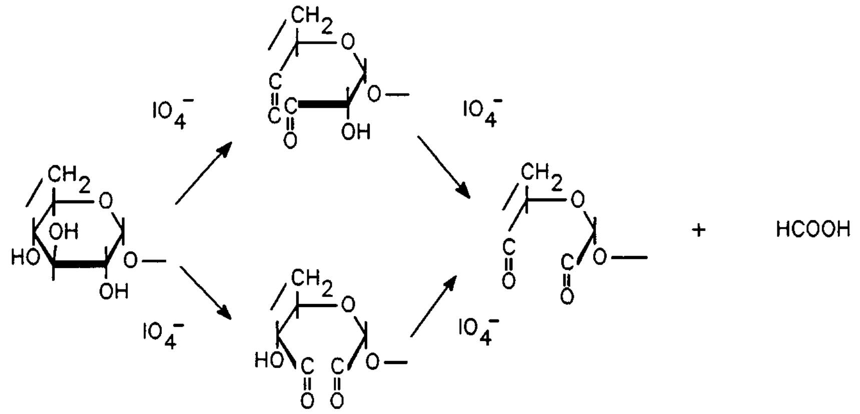

Figure 4 Oxidation of an α-1,6-linked anhydroglucoside unit

Table 1 Structural analysis of pullulan

IO_4^- (mol%)[a]	Aldehydes (%)[b]	Oxidation (%)[c]	A_m	A_d	$B_m = C_m$
33	20	28	9	5	14
53	36	46	24	7	15
67	40	58	18	9	31
79	45	70	17	9	44
93	53	79	21	14	44
106	58	90	13	17	60
119	66	101	16	18	67
332	67	107	0	28	79

[a] Mol% defined as $[IO_4^-]/[\text{glucoside}]_0 \times 100$

[b] Aldehyde content determined by hydroxylamine hydrochloride titration

[c] Degree of oxidation is defined as the number of oxidized units per 100 anhydroglucoside units, i.e.

$$\text{degree of oxidation} = A_m + B_m + C_m + A_d$$

where A_m, B_m and C_m are singly oxidized units and A_d is a doubly oxidized unit. A_d equals the number of equivalents of formic acid titrated per 100 structural units. The results are corrected for H_2O content in pullulan and oxidized pullulan

and doubly oxidized units are present. In the case of total oxidation there are doubly oxidized units A_d and singly oxidized units B_m and C_m.

The aldehyde content in completely periodate-oxidized pullulan as determined by hydroxylamine hydrochloride titration is only 67% of that calculated from the periodate consumed and formic acid formed. Hence, 33% of the aldehydes cannot be detected via the hydroxylamine hydrochloride titration method. That method involves a nucleophilic attack of the reagent at the carbonyl carbon atom. Obviously, one of the aldhehydes generated in the periodate-oxidized glucopyranoside unit is not easily accessible.

A plausible explanation for these observations is the presence of a stable, six-membered, intra-residual hemiacetal which protects the C2 aldehyde from further reaction under the given reaction conditions. In a totally oxidized maltotriose unit, two of the six aldehydes are protected by intra-residual hemiacetal formation (*Figure 5*).

Hydroxylamine hydrochloride titration of totally oxidized pullulan results in a determination of 66.6% of the formed aldehydes. The experimental value (67%) estimates the oxidation limit (*Figure 5*). The formation of stable, intra-residual hemiacetal structures has been reported by Vermeersch for inulin[14] and by Painter and Larsen for starch[11].

Evidence for the presence of aldehydes with different reactivities

Analytical data. For the determination of the aldehyde content in a partially oxidized pullulan the hydroxylamine hydrochloride titration method was used. Hydroxylamine hydrochloride reacts with aldehydes to form oximes, and the hydrogen chloride liberated can be titrated with alkali solution (*Figure 6*).

In the titration of oxidized pullulan via the hydroxylamine hydrochloride method[6] it was observed that the titrated aldehyde content depended on the time the polyaldehyde was allowed to react with hydroxylamine before being titrated. This result is illustrated in *Figure 7*.

From *Figure 7* it is clear that about 80% of the aldehydes present in that particular periodate-oxidized pullulan reacted almost instantaneously with hydroxylamine to form the corresponding oxime. The remaining aldehydes eventually react, but much more slowly. This difference in reactivity can be attributed to the presence of the intra-residual hemiacetal structure in singly oxidized pullulan units.

Spectroscopic evidence. The presence of aldehyde groups with different reactivities in partially oxidized pullulans was further evidenced by the data collected from reduction experiments.

In *Table 2* the signals in the ^{13}C n.m.r. spectrum of pullulan are given.

In the ^{13}C n.m.r. spectrum of completely oxidized pullulan that was subsequently reduced with sodium borohydride the three signals of C1 are shifted to lower field. The signals in the region 70–74 ppm, present in intact pullulan, are moved to the region 61–63 ppm. There are no signals for secondary alcohol groups (*Table 3*).

In a further investigation, the completely oxidized pullulan was reduced with sodium cyanoborohydride at pH 5–5.5 and the ^{13}C n.m.r. spectrum of the resulting compound was taken. Sodium cyanoborohydride is a milder reductant than sodium borohydride. It was shown by Vermeersch[14] that inuline aldehyde functions in the intra-residual hemiacetal form could be reduced with sodium borohydride but not with sodium cyanoborohydride. The results for pullulan are given in *Table 3*.

When a partially oxidized pullulan was treated with sodium borohydride, it was found that all aldehydes were reduced. However, for reduction with sodium cyanoborohydride some remaining aldehydes are observed. From this data it can be concluded that

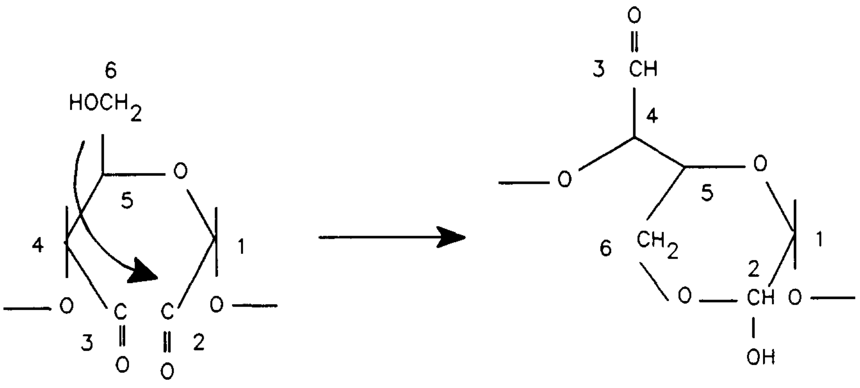

Figure 5 Formation of a stable, six-membered, intra-residual hemiacetal

$$R-CH=O \; + \; H_2N-OH\,.\,HCl \longrightarrow RCH=N-OH \; + \; HCl$$

Figure 6 Hydroxylamine hydrochloride titration method

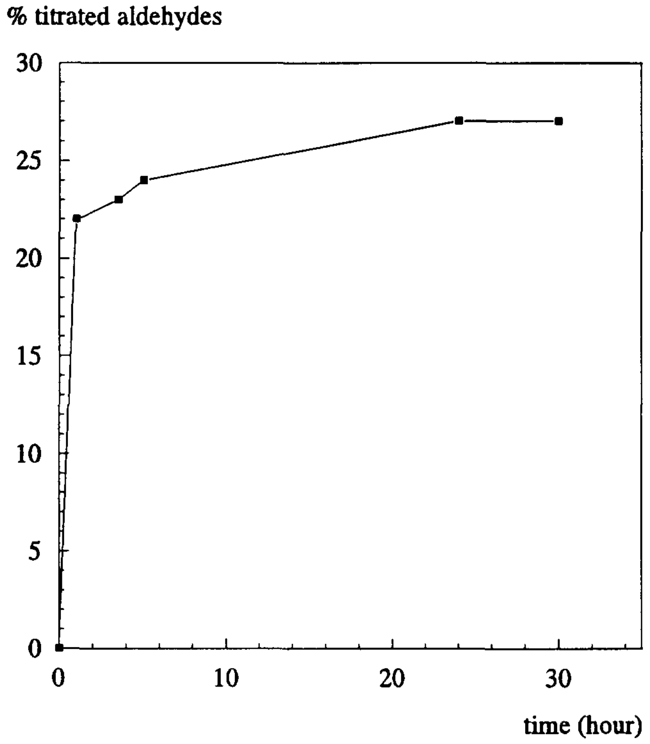

Figure 7 Dependence of the titrated aldehyde content in a periodate-oxidized pullulan on the reaction time of the polyaldehyde with an excess of hydroxylamine hydrochloride

Table 2 ^{13}C n.m.r. data of pullulan in D_2O

	C1 (α-1,4)	C1 (α-1,6)	C6	C2,3,4,5
δ (ppm)	100.1–100.6	98.8	61.3–61.6	70–74

aldehydes with different reactivities are present in partially oxidized pullulan. This can be explained by the existence of the intra-residual hemiacetal (*Figure 5*).

Effect of periodate oxidation on the hydrolytic stability of the polysaccharide backbone

Dextran and pullulan are hydrolytically stable in mildly alkaline and acidic media. However, since periodate oxidation involves the cleavage of anhydroglucoside residues the subsequent hydrolytic stability may be seriously impaired.

The stabilities of partially oxidized and subsequently sodium borohydride reduced dextran and pullulan in

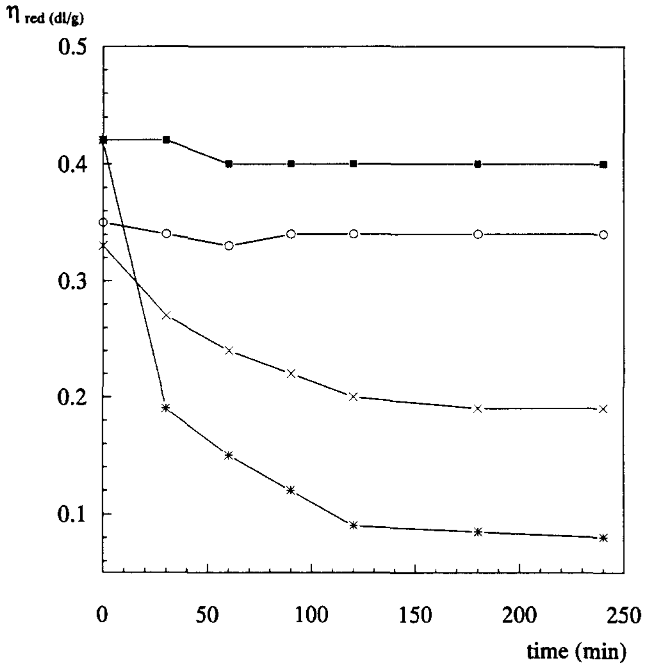

Figure 8 Plot of the reduced viscosity (η_{red}) against time for intact pullulan (■) and dextran (○) and reduced pullulan (∗) and dextran (×)

Table 3 ^{13}C n.m.r. data of pullulan reduced with sodium borohydride and sodium cyanoborohydride

	C1 (α-1,4)	C1 (α-1,6)	C2,3,4,5	C6	Hemiacetals
Pullulan	100.1	98.8	70–74	61.6	
δ (ppm)	100.6			61.3	
$NaBH_4$-reduced pullulan	104.2	103.1	61–63	61–63	
δ (ppm)	103.6				
$NaCNBH_3$-reduced pullulan	100.1	98.8	61–63	61–63	88–94
δ (ppm)	100.6				

0.1 N HCl at 37°C were compared to those of intact dextran and pullulan under the same conditions. The aldehyde functions were reduced in order to avoid intermolecular hemiacetal formation. The intrinsic viscosity was determined as a function of the incubation time and the results are plotted in *Figure 8*.

It is clear that the periodate-treated dextran and pullulan are much more sensitive to acid hydrolysis than the intact samples.

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REFERENCES

1 Bender, H., Lehmann, J. and Wallenfels, K. *Biochem. Biophys. Acta* 1959, **36**, 309
2 Molteni, L. in 'Drug Carriers in Biology and Medicine' (Ed. G. Gregoriadis), Academic Press, London, 1979, p. 107
3 Catley, B. J. and Whelan, W. J. *Arch. Biochem. Biophys.* 1971, **143**, 138
4 Kawahara, K., Ohta, K., Miyamoto, M. and Nakurama, S. *Carbohydr. Polym.* 1984, **4**, 335
5 Schacht, E. 'Dextran and Inulin as Carriers for Bioactive Materials', University of Ghent, Belgium, 1988
6 Blackader, D. A. and Hinselwood, C. *J. Chem. Soc.* 1958, 2720
7 Larsen, B. and Painter, T. J. *Carbohydr. Res.* 1969, **10**, 186
8 Painter, T. J. and Larsen, B. *Acta Chem. Scand.* 1969, **24**, 831
9 Painter, T. J. and Larsen, B. *Acta Chem. Scand.* 1970, **24**, 2366
10 Painter, T. J. and Larsen, B. *Acta Chem. Scand.* 1970, **24**, 2724
11 Painter, T. J. and Larsen, B. *Acta Chem. Scand.* 1973, **24**, 1957
12 Ishak, M. F. and Painter, T. J. *Carbohydr. Res.* 1978, **64**, 189
13 Painter, T. J. *Carbohydr. Res.* 1977, **55**, 95
14 Vermeersch, J. *PhD Thesis*, University of Ghent, Belgium, 1985