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PERIODATE OXIDATION OF LEVAN FROM *Zymomonas mobilis*

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Fructose polymer levan from Zymomonas mobilis *113 S possesses various biological activities. Its oxidation with potassium periodate was studied, for further investigation of its application in pharmacology. Levan aldehyde groups were reduced by sodium borohydride to the corresponding alcohols to prevent its high reactivity. Various analysis methods for measurement of oxidation degree were compared. The best accuracy and reliability were achieved by the method of anion-exchange chromatography for direct determination of aldehyde groups in oxidised levan purified from iodate ions. The oxidation degree of levan was found to depend on its molmass (75 and 2,000 kDa) and the conditions of levan biosynthesis. The highest possible oxidation degree (60–70 %) was achieved for both above-mentioned molmasses of the levans that had been obtained by enzymatic biosynthesis in glucose medium. The lowest oxidation degree (17–23 %) of high molmass levans was observed in different fermentation procedures in sucrose medium, likely due to its high branched structures which prevent the approach of the oxidant KIO_4 to the interior of the molecule, retarding more complete oxidation of levan. The present investigation is a part of studies on the immunomodulating mechanism of levan, using the method of chemical modification.*

Key words: *levan, periodate oxidation, borohydride reduction, analysis methods.*

INTRODUCTION

Fructose homopolysaccharide levan, synthesised by *Zymomonas mobilis* 113 S, has immunostimulating, antitumour, radioprotective, and other activities (Liepa et al., 1993; Vina et al., 1997).

It is known that the biological and pharmacological properties of polysaccharides can be controlled or induced by chemical modification (Casu, 1987). Oxidation with periodate ions is a successful method for structural investigations of a polysaccharide. It has been used to determine the possible glycosidic linkages (Scamparini et al., 1997), to identify sugar residues and molar ratio in the polysaccharide molecule (Matsuda and Okutani, 1997; Pramanik and Islam, 1998) and to characterise the conformation of a polysaccharide in aqueous solution (Coviello et al., 1998). The utility of the periodate oxidation method for structural analysis was extended by application to other bacterial levans (*Leuconostoc mesenteroides*, etc.) (Rankin and Jeanes, 1954) and short-chain fructosans (levans) from rye-grass *Lolium perenne* (Harwood et al., 1954). It was established that periodate oxidation of some immunomodulating polysaccharides influences the anti-complementary and mitogenic activity of lymphocytes, phagocytic activity of macrophages (Zhang et al., 1997), and antigenicity of polysac- charides (Casal et al., 1998). Moreover, oxidised polysaccharides have been used for enzyme immobilisation by their covalent attachment to an activated carrier (Wileman et al., 1986; Lenders and Crishton, 1987).

The literature on other natural polysaccharides suggests that one of the essential elements for immunochemical binding can be the C3-C4 region of the fructose unit in the levan molecule (Glaudemans, 1975). In the present study, we investigated modification of hydroxy groups of levan at C3 and C4 positions by periodate oxidation, as well as the influence of levan molmass and conditions of its biosynthesis on the oxidation degree. The extent of reduction of aldehyde groups of oxidised levan was also studied.

MATERIALS AND METHODS

Materials. Levan from the *Zymomonas mobilis* 113 S strain was obtained from the Laboratory of Technical Microbiology and Food Biotechnology, Institute of Microbiology and Biotechnology of the University of Latvia (Bekers et al., 1993). Levans synthesised under different conditions of biotechnological process and having different molmass were used: LI (75 kDa) and LII (2,000 kDa) were enzymatically synthesised in glucose-based medium; and LIII and LIV (2,000 kDa), in sucrose-based medium with or without, respectively, presynthesis of extracellular levansucrose by fermentation in sucrose-based medium.

Modification procedure. Different molmass levan (2 g) was dissolved in 100 ml of water and mixed in light with 2.86 g (in 440 ml) of potassium periodate (100 % of the theoretical requirement). Reaction was performed at 25 °C for different times (1–120 h) using a magnetic stirrer. After oxidation, the levan solution was purified from periodate and iodate ions by passage through a column (3 × 28 cm) containing anion-exchange resin ARA ("Reakhim", Russia) in acetate form.

For subsequent reduction of oxidised levan (800 mg in 80 ml of water), sodium borohydride (200 mg) was added and stirred for 1 h at 25 °C. The solution was then extensively dialysed against distilled water at 4 °C, until pH in the dialysate was constant (6–6.5). Solution of oxidised and subsequently reduced levan was lyophilised and stirred at 3–5 °C.

The methods of oxidation and the subsequent reduction of levan mentioned above were used in repeated oxidation–reduction (re-oxidation) experiments under the same conditions.

Methods of analysis. The oxidation degree, equivalent to the quantity of oxidised fructose units, was determined by two methods.

According to the method of Fleury and Lange (Rankin and Jeanes, 1954), a sample of oxidised levan (1 ml) was diluted with water to about 5 ml and added to 10 ml of saturated sodium bicarbonate solution; then, 5 ml of 0.1 N sodium arsenite solution and 1 ml of 20 % potassium iodide were added immediately. After standing at least 15 minutes, the solution was rapidly back-titrated with 0.1 N iodine solution to a starch end-point using a microburet. The quantity of oxidised fructose units was calculated from the periodate uptake (moles) and expressed as a percentage (X).

According to the method described in (Линденбаум и др., 1977), samples of a known concentration of oxidised levan (1 ml) purified from oxidant ions were added to 9 ml of water, 20 ml of 1 M NaOH and 20 ml of 0.01 N iodine solution. After standing at least 30 minutes, the solution was added to 35 ml 1 M HCl. The excess of unreacted iodines was back-titrated with 0.01 N $Na_2S_2O_3$ to a starch end-point. Control analysis was performed under the same conditions in water (10 ml) without levan. The oxidation degree as a percentage (γ) was calculated using the following formula:

$$\gamma = \frac{V_c - V_s}{2 \times 12.4 \times c} \times N_T \times 100\%,$$

where V_c and V_s stand for $Na_2S_2O_3$ volume to control and sample titration, respectively (ml); c, levan quantity in sample (g); N_T, normality of $Na_2S_2O_3$ solution; 12.4, coefficient, equivalent to aldehyde group mmol at full oxidation of 1 g levan.

The content of levan was determined by spectrophotometry, using a resorcinol reagent according to Roe et al. (1949). The optical density was determined with a Shimadzu UV-260 (Japan) spectrophotometer at 540 nm.

The kinematic viscosity (St) was measured using an OSTWALD capillary viscometer with a 2.37 mm capillary diameter. A 10 ml sample was used, and all measurements were conducted at 25 °C.

RESULTS

Measurement of levan oxidation degree. In the present study, two principially different methods of oxidation degree analysis were used: the method of Fleury and Lange (Jackson, 1944; Rankin and Jeanes, 1954) and the method described by Lindenbaum (Линденбаум и др., 1977). In the first method, the number of oxidised fructose units (oxidation degree) (X) was calculated from the final periodate consumption. By the second method, oxidation degree (γ) was calculated directly from aldehyde analysis after removal of iodate and periodate by anion-exchange chromatography. The comparative results of the analytical methods (Table 1) showed that levan oxidation degree values and the precision of measurements obtained by the first method were lower. Therefore, we show further only values for the levan oxidation degree (γ) obtained by aldehyde analysis.

Table 1

COMPARATIVE ANALYSIS OF DIFFERENT METHODS TO DETERMINE THE LEVAN IV OXIDATION DEGREE

Time of oxidation, h	Method of Rankin and Jeanes (1954)	Method of Линденбаум и др. (1977)
	Average value of X, %	Average value of γ, %
12	3.4±1.5	3.9±0.8
48	10.1±5.1	10.5±1.4
120	17.7±1.5	19.8±1.8

Modification of levan. Oxidised forms of levan were obtained by potassium periodate oxidation according to the scheme showed in Figure 1, II. Highly branched levan containing free 1,2-glycol groups (Figure 1, I) is composed of a large number of potentially oxidisable fructose units. A sufficient degree of oxidation was obtained at a molar ratio of potassium periodate per fructose unit of at least 1.0 (comparative data not shown). In the present studies, we used a 100 % molar amount of KIO_4 of the theoretical requirement. The oxidation of levan by potassium periodate was studied as a function of the oxidation time. The results of oxidation of levans which were obtained under different biotechnological conditions, are presented in Table 2. The best results of oxidation were achieved with low molmass (75 kDa) levan I and high molmass (2,000 kDa) levan II obtained by enzymatic synthesis in glucose medium. In these experiments, a maximum 60–70 % oxidation degree was reached. In contrast, only 23 % oxidation was obtained for levan IV over a 120 h period of reaction. Oxidation of

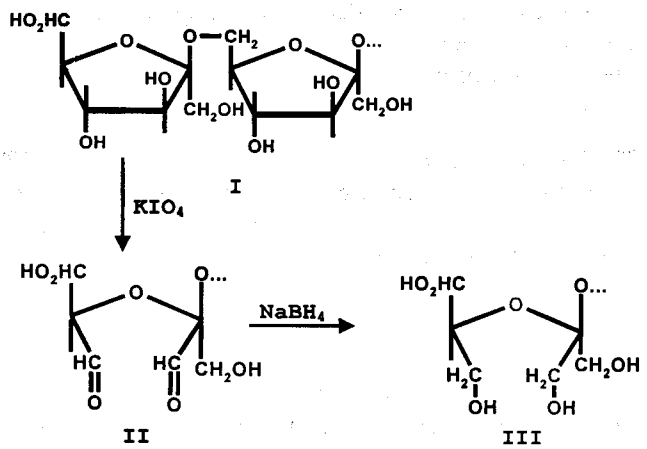

Fig. 1. Periodate oxidation of levan and subsequent reduction with sodium borohydride:
I, levan, fragment of structure; II, oxidised form; III, reduced form.

Table 2

OXIDATION DEGREE OF DIFFERENT LEVANS

Levans	Molmass of levan, kDa	Oxidation time, h	γ, %
LI	75	1	24.2
		2	31.8
		3	42.6
		5	69.4
LII	2,000	5	60.5
		17	62.7
LIII	2,000	12	16.8
LIV	2,000	48	9.1
		120	23

levans began at pH 4.0, and this pH was maintained (pH 3.8–4.0) during the entire period of oxidation. In these conditions, the kinematic viscosity (St) of modified high molmass levans in the reaction mixture was also practically constant (0.024 to 0.026 St).

The obtained results after reduction of aldehyde groups (Figure 1, III) in oxidised levan IV are presented in Table 3. Measurements of γ after reduction showed that practically all aldehyde groups were reduced already after 1 h of reaction.

Table 3

COMPLETENESS OF ALDEHYDE REDUCTION IN OXIDISED LEVAN IV

Time of reduction, h	γ, %	
	before reduction	after reduction
1	18	0.20
3	18	0.18
24	18	0.32

After re-oxidation of levan IV aldehyde groups (Table 4), the maximal oxidation degree of levan, obtained by repeated oxidation after corresponding reduction, was unchanged (~23 %), showing an absence of formation of hemialdals and hemiacetals which can retard complete oxidation of levan.

Table 4

RE-OXIDATION OF LEVAN IV AFTER REPEATED REDUCTION

Succession of the modification stages	Total time of oxidation, h	Total γ, %
Oxidation	120	23.0
Reduction		
Re-oxidation	240	26.4
Reduction		
Re-oxidation	360	24.0

DISCUSSION

We used levan specific chemical modification for the study of its immunomodulating mechanism. Such an approach may be particularly useful for possible variation of levan binding with active components of the immune system and to identify its immunological determinants.

Each (2–6)-linked D-fructose unit in a levan molecule contains only a 3,4-diol system, and therefore the periodate oxidation takes place in one step without liberation of formic acid (in contrast to the glucose units in glucose polymer). This oxidation consumes one molecule of periodate with cleavage of the bonds only between C3 and C4 (Figure 1).

It is very important to quantify the oxidation degree by employing an analytical method with high accuracy and reliability of measurements. In the present study, a higher reliability of results was obtained by direct determination of aldehyde groups in oxidised levan. The advantage of this method is the termination of the oxidation reaction by immediate isolation of oxidised levans from periodate and iodate ions before measurement.

Using the alternative Fleury-Lange method of periodate analysis in reaction mixture, we achieved lower accuracies in measurement of the reduced periodate during periodate oxidation of levan, and lower estimations of the reduced periodate. Similar results were observed by Rankin and Jeanes in the study of dextran oxidation (Rankin and Jeanes, 1954). The authors suggested that this inaccuracy resulted in part from reaction of iodine with periodate oxidised polysaccharide present in the bicarbonate-buffered solution during analysis of periodate. The other disadvantage of this method is that, to obtain higher precision and greater accuracy of the results, the iodometric titration must be carried out at 4 °C. We can recommend the use of the Fleury-Lange method only for intermediate control of the oxidation degree during the prolonged process of levan oxidation, when purification of the sample before analysis is difficult.

It has been shown that some high molmass bacterial levans can be completely oxidised almost within 70 to 100 h (Rankin and Jeanes, 1954). We established that the oxidation degree of levans from *Zymomonas mobilis* 113 S depends on its biosynthesis conditions and molmass, due to

the structural difference of high and low molmass levans. Low molmass levans have linear or low branched structure (Tanaka and Yamamoto, 1980), while high molmass levans are highly branched. The average length of linear parts of a chain of high molmass bacterial levans, which appeared to contain > 1000 branches, was estimated to be only 9 to 12 fructosyl residues (Feingold and Gehatia, 1957; Tanaka and Yamamoto, 1980). Differences in the oxidation degree of high molmass levans produced under different biotechnological conditions may be partly explained by spatial branching. We suggest that the low oxidation degrees of levans III and IV (17–23%) are due to their highly branched structures which retard or prevent the approach of oxidant KIO_4 to the interior of the levan molecule. Bacterial high molmass levans with branched morphology exist as compact globular-shaped molecules in aqueous solutions (Sivala and Bahary, 1978; Simms et al., 1990). Such structures may be stabilised by hydrogen bonds between nearly arranged carbohydrate chains, preventing levan oxidation. Therefore, it is conceivable that fructosyl residues on the surface of levan molecule were preferentially modified during the oxidation reaction, and that hydrolysis of the glucosidic bonds during oxidation did not occur. We found that the viscosity of slightly acidic levan solutions did not decrease within 120 h. Also, the viscosity of high molmass *Zymomonas mobilis* levan solutions at low concentrations (≥1 %) is quite stable and reversible at room temperature over a wide range of pH from 3 to 11 (Vina et al., 1998). Levan solution viscosity, which is mainly determined by pH, temperature and other conditions of medium, is one of the important parameters for the characterisation of the spatial structure of the high molmass polysaccharide (Kasapis et al., 1994; Kitamura et al., 1994). According to gel chromatography on a calibrated Sepharose 6B column, levan IV contains predominantly the immunochemically most active high molmass (up to 2,000 kDa) fractions (Linde et al., 1996). In the present study, we used only this levan for further detailed investigation of chemical modifications.

In various biological test-systems, to prevent the high reactivity of aldehyde groups, they were reduced to the corresponding alcohols by addition of $NaBH_4$ (Figure 1, II). However, the observed reactions in the presence of a low concentration of sodium borohydride may have resulted in the degradation of the oxidised polysaccharides by β-alkoxycarbonyl elimination (Guthrie, 1961; Lindberg et al., 1975). In order to prevent further elimination, the reaction may be performed in the presence of a high concentration of $NaBH_4$ for a minimal reducing time, such that the carbohydrate is reduced before it react further. Therefore, the reduction of aldehyde groups was performed by addition of a twofold molar excess of $NaBH_4$ per aldehyde, during a period of no more than for 1 h.

Information exists on the various forms of oxidation product of polysaccharides (Guthrie, 1961). The possibility of hemialdal and hemiacetal formation in some oxidised polysaccharides is very high, because these links may be intermolecular (Figure 2, a) and/or intramolecular (Figure 2, a,

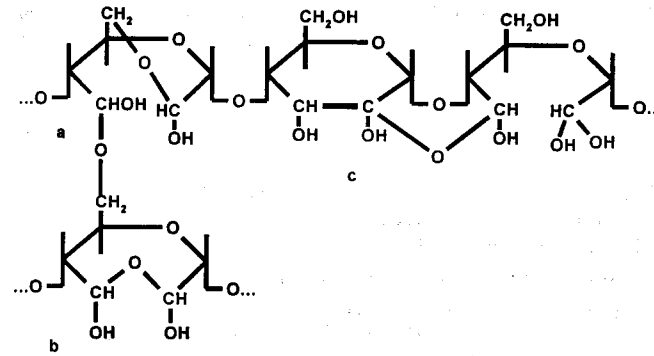

Fig. 2. Possible oxidation products of polysaccharides: hemiacetals (a, b, c) and hemialdals (b) (partly from Guthrie, 1961).

b). Hemialdals (Figure 2, b) can be formed from one aldehyde group and the hydrated form of another. It has been shown that the aldehyde groups of an oxidised unit may form hemiacetals with the hydroxyl groups of unoxidised residues in the same chain (Figure 2, c) (Painter and Larsen, 1970). These links are so stable that the theoretical consumption of periodate in many cases is impossible under normal conditions (Lindberg et al., 1975). However, all oxidation products can be easily reduced with $NaBH_4$. Oxidised polysaccharides after reduction may be re-oxidised, analysed for their oxidation degree, again reduced with $NaBH_4$, re-oxidised, etc., to achieve the maximal oxidation and reduction degree. Thus, the existence of various forms of oxidation products is associated with re-oxidation cycles. Since we established (Table 4) that these forms practically were not formed or formed insignificantly, the low maximal oxidation degree of levans III and IV may be explained, as mentioned above, only by its very high branched structures. This observed effect has an important role in our research, as only the reducing ends of the chain, which are arranged on the surface of the levan molecule, have predominant significance for the immunological activity of bacterial levans (Cisar et al., 1974; Glaudemans, 1975).

The immunomodulatory properties of the modified levans are presently being studied in various test-systems.

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Zymomonas mobilis LEVĀNA PERJODĀTA OKSIDĀCIJA

Pētīti levāna imūnmodulējošie mehānismi, izmantojot ķīmiskās modifikācijas metodi. *Zymomonas mobilis* 113 S fruktozes polimērs levāns, kam piemīt dažāda veida bioloģiskā aktivitāte, oksidēts ar kālija perjodātu. Bioloģiskās testsistēmās oksidētā levāna aldehīdgrupas reducēja ar nātrija borhidrīdu līdz atbilstošiem spirtiem, lai ierobežotu modificētā levāna augsto reakcijspēju. Levāna oksidācijas pakāpes mērīšanai salīdzinoši izpētītas dažādas analīzes metodes. Rezultātu lielāku precizitāti un ticamību nodrošināja metode tiešai oksidētā levāna aldehīdgrupu noteikšanai pēc neizreaģējušo jodāta jonu atdalīšanas ar jonu apmaiņas hromatogrāfijas paņēmienu. Noskaidrots, ka levāna oksidācijas pakāpe ir atkarīga no tā molmasas (75 un 2,000 kDa) un levāna biosintēzes apstākļiem. Maksimālā iespējamā oksidācijas pakāpe (60–70 %) sasniegta abu molmasu levāniem, kas fermentatīvi sintezēti glikozi saturošā vidē. Zemu maksimālās oksidācijas pakāpi (17–23 %) lielmolekulāram levānam, kas iegūts fermentatīvi saharozi saturošā vidē, iespējams, nosaka tā stipri zarotā struktūra; tā kavē oksidanta KIO_4 iekļūšanu molekulas iekšienē un ierobežo levāna oksidāciju. Pieļaujam, ka modificējas fruktozes atlikumi uz lielmolekulārā levāna virsmas.