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## Fine structural analysis of the fungal polysaccharide pullulan elaborated by *Aureobasidium pullulans*, CH-1 strain

DRAGICA JAKOVLJEVIĆ<sup>1\*</sup>, MIROSLAV M. VRVIĆ<sup>2#</sup>, MILANKA RADULOVIĆ<sup>1</sup> and  
MIRJANA HRANISAVLJEVIĆ-JAKOVLJEVIĆ<sup>2</sup>

<sup>1</sup>*Institute of Chemistry, Technology and Metallurgy, Centre for Chemistry, Njegoševa 12, YU-11000 Belgrade* and <sup>2</sup>*Faculty of Chemistry, Univeristy of Belgrade, Studentski trg 12-16, P.O. Box 158, YU-11001 Belgrade, Yugoslavia*

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The structure of pullulan, the extracellular  $\alpha$ -D-glucan elaborated by the yeast-like fungus *Aureobasidium pullulans*, may be described as a linear  $\alpha$ -D-glucan consisting of maltotriosyl repeat units connected terminally by (1 $\rightarrow$ 6)- $\alpha$ -D-glucosidic bonds. Occasionally some of maltotriosyl residues are replaced by higher oligosaccharide units, most frequently with maltotetraosyl residues. Using the susceptibility of pullulan CH-1 (obtained from strain CH-1 of *Aureobasidium pullulans*) to hydrolysis catalysed by porcine  $\alpha$ -amylase, the polysaccharide was cleaved and the fragments obtained fractionated by gel-permeation chromatography. The heterogenous size of the fragments indicates that there is no apparent regular distribution of tetrasaccharide units in the pullulan chain. Enzymatic digestion of pullulan CH-1 using pullulanase, followed by gel-permeation chromatography of the resulting digest confirmed these results as did preparative paper chromatography and CI mass spectrometry of the separated components, *i.e.*, that maltotetraosyl units (about 7 %) are building units of pullulan CH-1.

**Keywords:** *Aureobasidium pullulans*, polysaccharide, pullulan, amylolysis, pullulanolysis.

The structure of the extracellular polysaccharide pullulan from *Aureobasidium pullulans* has been investigated by a number of authors.<sup>1,2</sup> It is essentially a linear  $\alpha$ -D-glucan, composed of maltotriose repeating units linked to terminal glucosyl residues by (1 $\rightarrow$ 6)- $\alpha$ -D-glucosidic bonds. Depending on the experimental conditions, some differences have been found in the yields, molecular masses, and sometimes in the fine structural features.

Concerning the structural differences, it was found that some strains of *Aureobasidium pullulans* produce pullulan consisting predominantly of maltotriose repeating units, the basic chain being interspersed by maltotetraose residues. The content of these building units varies between 1–7 %.<sup>3,4</sup> Some investigations have revealed the pres-

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ence of larger (1-4)-oligosaccharide residues having up to ten monosaccharide residues in the chain.<sup>5,6</sup>

The presence of the minor structural modifications in polysaccharides may have a profound influence on the physical properties of the polymers in solution, perhaps on the chemical properties too.

Since our intention was to make a series of pullulan derivatives or modificates, it was necessary to investigate the fine structure of pullulan elaborated by *Aureobasidium pullulans*, CH-1 strain from our departmental collection.

#### RESULTS AND DISCUSSION

Using common chemical and physical methods (*e.g.* methylation analysis, periodate oxidation, acid hydrolysis and spectral analysis), it was found that pullulan CH-1 yielded only D-glucose on total acid hydrolysis and consumed 1.30 mol of periodate, liberating at the same time 0.32 mol of formic acid per "anhydroglucose". Borohydride reduction of periodate oxidized pullulan CH-1 and subsequent acid hydrolysis yielded erythritol and glycerol indicating the presence of (1-4)- and (1-6)-glucosidic linkages in the ratio 1.8:1. On methylation analysis 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucoses (32 % and 67 %, respectively) were obtained. Lastly, glucose, maltose, maltotriose, panose and isopanose were identified as products of partial acid hydrolysis. Hence, on the basis of the mentioned results the gross structure of pullulan CH-1 shown in Fig. 1 was proposed.

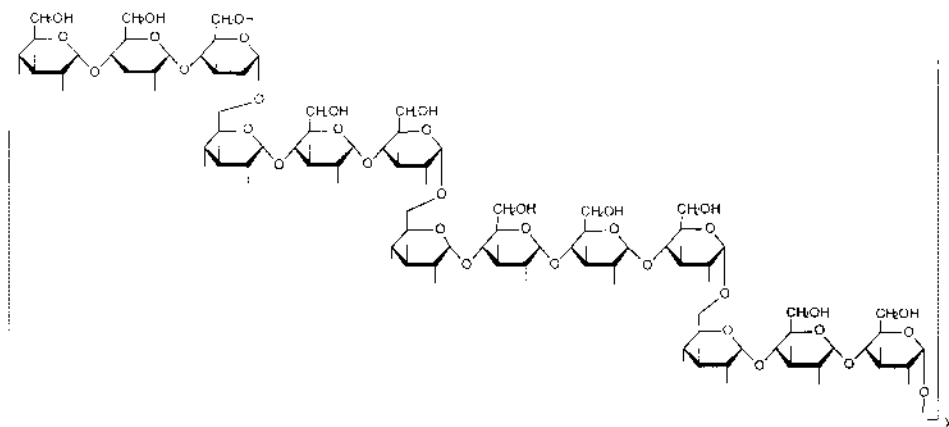


Fig. 1. Proposed structure of pullulan from *Aureobasidium pullulans*, strain CH-1.

These results and proposed structure are in conformity with results of earlier investigations.<sup>4,7</sup>

For further investigation aimed at elucidating the fine structural features of this polymer, the enzymic method was chosen as being the most reliable. It was decided to use porcine *alpha*-amylase and pullulanase. From earlier investigations<sup>8</sup> it is known

that enzymic hydrolysis of pullulan with fungal amylase (EC 3.2.1.57) yields isopanose (6<sup>1</sup>-D-glucosylmaltose), with bacterial amylase<sup>8</sup> (EC 3.2.1.57), panose (6<sup>2</sup>-maltosyl-D-glucose) was obtained and with pullulanase<sup>9</sup> (EC 3.2.1.41), maltotriose was the main product. *Alpha*-amylase (EC 3.2.1.37) from human saliva or porcine pancreas are generally assumed to be without the action on pullulan. This inactivity is explained by the fact that the minimum structure required for catalysed hydrolysis to occur with these enzymes is the 6<sup>1</sup>-maltotriosyl-D-glucose unit. Since porcine *alpha*-amylase hydrolysed some pullulans, it can be concluded that pullulan is not a simple sequence of maltotriosyl residues linked by (1-6)-D-linkages.<sup>10</sup>

The hydrolysis of pullulan CH-1 by *alpha*-amylase (EC 3.2.1.1) was monitored by viscosimetric measurements and paper chromatography. The components formed during hydrolysis after inactivation of the enzyme were subjected to gel-filtration on a Sepharose CL-4B column.

The viscosimetric measurements showed the hydrolysis to be fast. The paper chromatography indicated that the fragments formed by hydrolysis must have relatively high molecular masses since they did not move far from the starting line with the solvent used.

Based on the gel-chromatography profile (Fig. 2) of *alpha*-amylase treated pullulan, it is evident that hydrolysis catalysed by this enzyme resulted in heterogenous fragments of differing molecular masses. At least four fragments were noticeable, corresponding to elution volumes of 22–30 mL, 35–45 mL, 47–55 mL and 57–65 mL.

The fact that pullulan CH-1 is susceptible to enzymic hydrolysis catalysed by *alpha*-amylase means that tetrasaccharide units (as mentioned above) must exist in its struc-

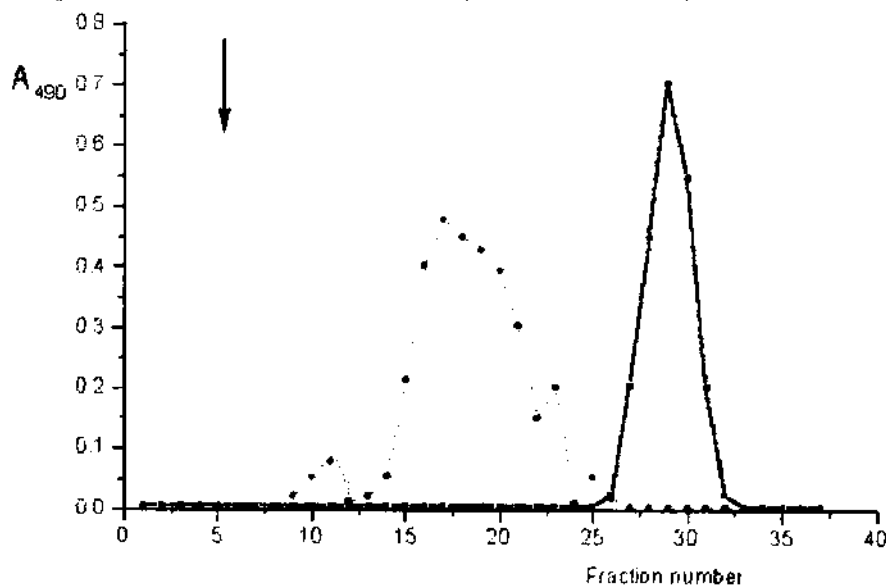


Fig. 2. Gel-permeation chromatography on a Sepharose CL-4B column (1 80 cm) at a flow rate of 6 mLh<sup>-1</sup>: pullulan digested with *alpha*-amylase (.....) and pullulan digested with pullulanase (—). The elution volume of Blue Dextran (BD) is indicated by the arrow.

ture, which enables enzymic hydrolysis to occur. Further, a regular distribution of these units would give oligomers of a particular size. The length of the polymer is not of significance. On the contrary, an irregular distribution of tetrasaccharide units in the polymer chain would yield oligomeric products of differing molecular masses.<sup>11</sup> It can be concluded from the hydrolysis of pullulan CH-1 by *alpha*-amylase that the tetrasaccharide units are randomly distributed in the polymer chain.

The next step was the investigation of the effect of the enzyme pullulanase (EC 3.2.1.41) on pullulan CH-1. This enzyme catalyses the hydrolysis of (1 → 6)-D-glucosidic linkages. Descending paper chromatography proved the presence of two components in the hydrolysate. The most abundant was shown to be maltotriose, and the second was tentatively identified as maltotetraose. After inactivation of pullulanase, the hydrolysate was subjected to gel-filtration on a Sepharose CL-4B column. The elution profile of this gel filtration is presented in Fig. 2. As is evident, only one fraction of low molecular mass was present in the eluate, corresponding to an elution volume between 65 mL to 75 mL. However, as two components were unequivocally found by paper chromatography, it was decided to separate them by preparative paper chromatography with the aim of identifying their structures.

Each of the separated oligosaccharides was reduced with sodium borohydride and then permethylated with sodium methylsulphonylmethanide and methyl iodide in dimethyl sulfoxide.<sup>12</sup> The oligosaccharide alditols obtained were further analysed by mass spectrometry.<sup>13</sup>

Interpretation of the mass spectra made possible to establish the monosaccharide sequence in permethylated alditols using the characteristic A-series fragments gener-

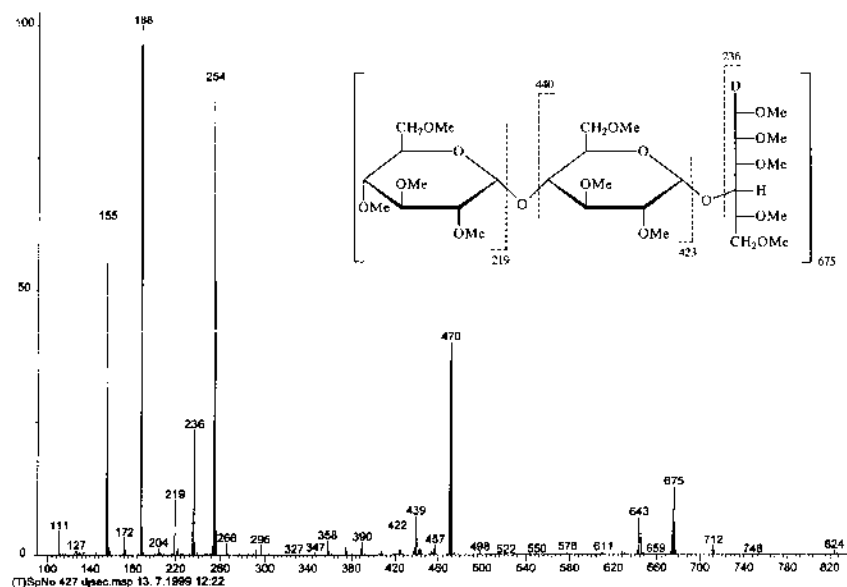


Fig. 3. CI mass spectrum and diagnostic fragment ions of the per-*O*-methylated maltotriosyl-alditol produced by pullulanolysis of pullulan CH-1.

ated by the homolytic breakage of the glycosidic linkages. The alditol part of the oligomer is assumed to be produced by the D-sequence of fragmentations.

So, in the mass spectrum of the most abundant fraction in the hydrolysate (Fig. 3), the primary fragment  $aA_1$   $m/z$  219 shows nonreducing hexose. The next fragment,  $baA_1$   $m/z$  423 with  $aA_1$  fragment gives the sequence hex-hex. The fragment generated from hexitolisald  $m/z$  236, fragment  $cald$   $m/z$  440 is characteristic for the sequence hex-hex-ol, while the fragment  $baald$   $m/z$  644 comes from hex-hex-hex-ol sequence.

The secondary fragments  $aA_2$   $m/z$  187,  $aA_3$   $m/z$  155,  $baA_2$   $m/z$  391, and  $baA_3$   $m/z$  187 obtained by the process of elimination from the fragment  $A_1$  confirmed the proposed sequence.

In this way the most abundant component in the pullulanase catalysed hydrolysis of pullulan CH-1 was identified as maltotriose.

In the similar way, the second component in the hydrolysate of pullulan CH-1 was found to contain in its mass spectrum the following primary fragments:  $aA_1$   $m/z$  219,  $baA_1$   $m/z$  423, and  $cbaA_1$   $m/z$  622, characteristic for the sequence hex-hex-hex. The fragments generated from the alditol part of the molecule:  $ald$   $m/z$  236,  $bald$   $m/z$  440, and  $bcald$   $m/z$  644 confirm the sequence to be hex-hex-hex-hex-ol, *i.e.*, this component must be maltotetraose (Fig. 4).

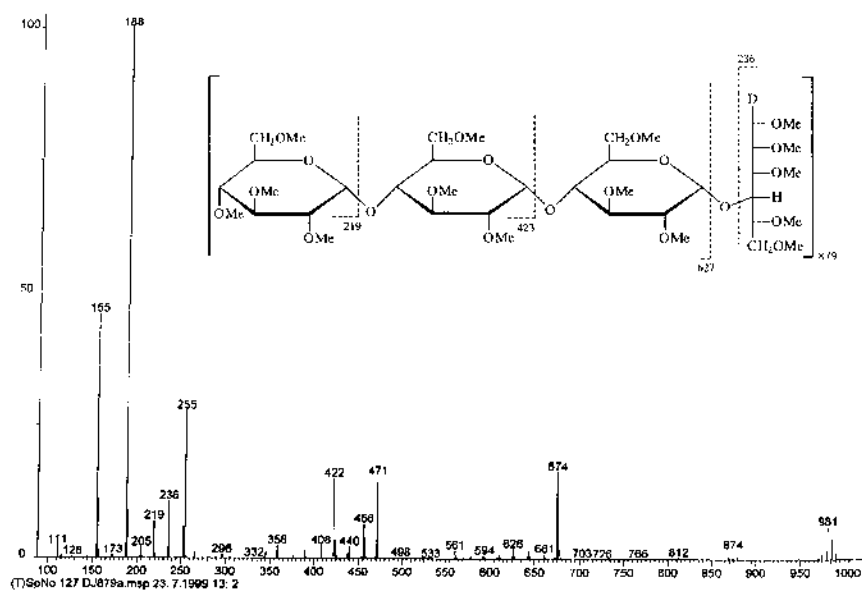


Fig. 4. CI mass spectrum and diagnostic fragment ions of the per-*O*-methylated maltotetraosyl-alditol produced by pullulanolysis of pullulan CH-1.

On the basis of the results obtained in the course of this investigation, the structure of pullulan CH-1 was found to be a linear  $\alpha$ -D-glucan consisting of maltotriose repeating units mutually connected by (1-6)- $\alpha$ -D-linkages, maltotetraose units being randomly inserted in the polymer chain (see Fig. 1).

## EXPERIMENTAL

*Micro-organisms and culture conditions*

*Aureobasidium pullulans* (CH-1, Departmental collection) was grown in a liquid culture as described previously.<sup>14</sup>

*Isolation and purification of pullulan CH-1*

The cells were harvested after 5 days of growth and centrifuged at 4000 rpm at 20 °C. The biomass was discarded. From the clear solution, the crude polysaccharide was precipitated by the addition of two volumes of ethanol. After storage for 18 h, the precipitate was collected by centrifugation at 13000 rpm at 4 °C for 20 min, and washed thrice with aqueous 66 % ethanol followed by cold ethanol (twice). The ethanol was decanted and the residual white polysaccharide was dried *in vacuo* at room temperature over anhydrous calcium chloride, to give pullulan CH-1.

*Enzymic digestions*

A solution of pullulan (150 mg) in phosphate buffer (pH 6.9; 200 μmol) was mixed with porcine *α*-amylase (4 kat) to a final volume of 10 mL and incubated at 37 °C. The viscosity, measured using an Ostwald viscometer, rapidly dropped to a steady value after 20 min, but the digestion was continued for further 45 min before inactivation by heating at 100 °C for 5 min. Paper chromatography of the obtained fragments was performed using descending elution with a mobile phase of ethyl acetate–pyridine–water<sup>15</sup> (10:4:3, v/v/v), and detection with alkaline silver nitrate.<sup>16</sup>

The enzyme-catalyzed hydrolysis of (1 → 6)-D-glucosidic bonds in pullulan was effected by dissolving the polysaccharide (5 mg) in 0.03 M citrate-phosphate buffer (pH 5.0, 0.5 mL) containing 20 nkat of pullulanase and incubated for 24 h at 37 °C. The products were chromatographed by descending paper chromatography (Whatman No. 1) with ethyl acetate–pyridine–water (10:4:3, v/v/v) as the eluent<sup>15</sup> and visualised with a silver nitrate-sodium hydroxide dip.<sup>16</sup> Both *α*-amylase and pullulanase were purchased from the Merck Corporation (Darmstadt).

*Gel permeation of pullulan and fragments of pullulan*

Carbohydrate samples (5 mg, 1 mL) were chromatographed at room temperature on a column (1 × 80 cm) of Sepharose CL-4B with water at a flow-rate of 6 mL h<sup>-1</sup>. The void volume was determined using Blue Dextran 2,000. The fractions (2.5 mL) were monitored for carbohydrate by the phenol-sulphuric acid procedure,<sup>17</sup> and for Blue Dextran by the absorbance at 620 nm. Sepharose CL-4B was obtained from Sigma Laboratories.

*Analysis of the fragments derived from the pullulanolysis*

A solution of the fragments derived from the pullulanolysis (15.84 mg) was separated by preparative paper chromatography (Whatman 3 MM, solvent ethyl acetate–pyridine–water (10:4:3, v/v/v), using double development. The resolved components (14.69 mg and 1.10 mg) were analyzed as permethylated oligosaccharide alditols by MS after reduction, methylation, and acetylation.

*Oligosaccharide alditols*

Oligosaccharides (0.5–2 mg) were reduced in 2 mL aqueous NaBD<sub>4</sub> (1 mg/mL) at room temperature for 24 h. The excess NaBD<sub>4</sub> was destroyed by the addition of Dowex 50 (H<sup>+</sup>) resin. The mixture was filtered through glass wool, and the boric acid was removed by co-distillation from methanol (3–2 mL). The resulting oligosaccharide alditols were dissolved in dimethyl sulfoxide and methylated.<sup>12</sup> The permethylated oligosaccharide alditols were purified by chromatography on a column of Sephadex LH-20 (1 × 20 cm) eluted with acetone–chloroform, 2:1. Fractions containing anthrone-positive<sup>18</sup> material were pooled, and solvents were removed by rotary evaporation to dryness.

*Mass-Spectrometry*

Mass spectra were recorded on a Finnigan-Mat spectrometer Model 8230. The spectra were recorded at 150 eV with an ionic source temperature of 200 °C and a pressure of 1.33 × 10<sup>-1</sup> Pa. Isobutane was used as the ionisation reagent.

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## ИЗВОД

ФИНА СТРУКТУРНА АНАЛИЗА ФУНГАЛНОГ ПОЛИСАХАРИДА ПУЛУЛАНА  
ДОБИЈЕНОГ ПОМОЋУ *Aureobasidium pullulans*, СОЈ СН-1

ДРАГИЦА ЈАКОВЉЕВИЋ,<sup>1\*</sup> МИРОСЛАВ М. ВРВИЋ,<sup>2</sup> МИЛАНКА РАДУЛОВИЋ<sup>1</sup> и МИРЈАНА  
ХРАНИСАВЉЕВИЋ-ЈАКОВЉЕВИЋ<sup>2</sup>

<sup>1</sup>Институт за хемију, технологију и металургију, Центар за хемију, Њевошева 12, 11000 Београд и <sup>2</sup>Хемијски факултет, Универзитет у Београду, Студентски брџ 12-16, б. бр. 158, 11001 Београд

Пулулан, екстрацелуларни полисахарид који производи квасцу слична гљива *Aureobasidium pullulans*, јесте линеарни глукан чију структуру претежно чине поли-малтотриозил остаци повезани међусобно  $\alpha$ -(1-6)-гликозидним везама. Варијације у структури овог полисахарида могу настати услед замене малтотриозних остатака на појединим местима полимерног ланца олигосахаридним фрагментима дужег низа, најчешће малтететраозним остацима. У овом раду испитан је пулулан који производи *A. pullulans*, сој СН-1, ензимском хидролизом са панкреасном  $\alpha$ -амилазом и пулулазаом, са циљем да се утврди да ли и у ком односу овај полисахарид садржи олигосахаридне фрагменте, као и да се одреди дужина олигосахаридног низа, односно број моносахаридних остатака који улазе у његов састав. Применом уобичајених аналитичких метода (гел-филтрациона хроматографија, метилациона анализа, препаративна хроматографија комбинована са масеном спектрометријом метилованих олигосахаридних алдитола), утврђено је да су малтететраозне јединице (7%) интегрални део полисахаридног ланца пулулана СН-1. Распоред ових јединица је насумичан.

(Примљено 29. децембра 2000)

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