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## Effect of substitution at C-6 on the susceptibility of pullulan to pullulanases. Enzymatic degradation of modified pullulans

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Pullulan, with all of the primary hydroxyl groups modified, is an excellent substrate for defining the effect of degree of substitution on biodegradability because of the uniform distribution of substituents on the polysaccharide. 6-Chloro-6-deoxypullulan and 3,6-anhydropullulan are highly resistant to hydrolysis by the four different types of pullulanase. 6-Azido-6-deoxypullulan is resistant to three types but susceptible to hydrolysis by the fourth, isopullulanase. Neopullulanase is strongly inhibited by 6-chloro-6-deoxypullulan and 6-azido-6-deoxypullulan, the other pullulanases much less so.

*Key words:* 3,6-anhydropullulan, 6-azido-6-deoxypullulan, biodegradability, 6-chloro-6-deoxypullulan, pullulan, pullulanases.

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Le pullulane, avec tous les groupes hydroxyles primaires modifiés, est un excellent substrat pour définir l'effet de degré de substitution sur la biodégradabilité à cause de la distribution uniforme des substitués sur le polysaccharide. Le 6-chloro-6-déoxypullulane et le 3,6-anhydropullulane sont très résistants à l'hydrolyse par l'un ou l'autre de quatre types de pullulanase. Le 6-azido-6-déoxypullulane est résistant à trois types mais est hydrolysable par la quatrième isopullulanase. La néopullulanase est fortement inhibée par 6-chloro-6-déoxypullulane et 6-azido-6-déoxypullulane alors que les autres pullulanases le sont beaucoup moins.

*Mots clés :* 3,6-anhydropullulane, biodégradabilité, 6-azido-6-déoxypullulane, 6-chloro-6-déoxypullulane, pullulane, pullulanases.

[Traduit par la rédaction]

### Introduction

The prevention of rotting of natural polymers was an acute problem for the military 50 years ago. In tropical environments, the useful life of untreated materials is extremely short. The solution to the problem was (i) application of biocides or (ii) modification of the polymer in such a way that the organism could no longer attack it. Today, emphasis has shifted from prevention to promotion of deterioration to minimize accumulation of unwanted wastes. A search is continuing for readily degradable biopolymers to replace nondegradable films and other plastics. When these new polymers lack some desired property, attempts will be made to confer that property by chemical modification. The effect of the chemical change on subsequent biodegradability needs to be recognized.

Microorganisms degrade polysaccharides by producing enzymes specific for each substrate. The degree of susceptibility to degradation can be determined by incubation of the substrate with the required enzymes. Susceptibility of the chemically modified polymer can be similarly assessed. Cellulose, for example, is readily degraded by many microorganisms; cellulose triacetate is not.

An important question is how much of a change is required to prevent deterioration or how much of a change can be made without losing biodegradability? The degree of substitution (DS) is one measure of the extent of chemical change, e.g., DS = 3 indicates that all three hydroxyl groups of a hexose unit have been modified, as in cellulose triacetate. Because many of the polymers are insoluble,

uniformity of substitution is often unattainable, and DS values are average values for the entire sample. Modification of fibers or fabrics tends to give a high DS at the surfaces in contact with the reagent(s) and a low DS for material inside the fiber (Bhattacharjee and Perlin 1971). Early studies (Reese *et al.* 1968) indicate that a single substituent on each glucose unit of cellulose affords complete resistance to enzymatic degradation; but because of the uneven distribution of substituents, the overall substitution must be somewhat greater (DS > 1.25).

Whether resistance to degradation can be achieved at less than one substituent per sugar unit (DS < 1.0) is a more difficult problem, the solution of which is impossible until chemical methods are developed that can place substituents uniformly at precise positions in every sugar unit of the chain. A convenient method for the selective substitution of chlorine for the primary hydroxyl group of glycosides was developed by Evans *et al.* (1968). More recently, stereospecific substitution at all positions in disaccharides has been accomplished (Bock and Sigurdkjold 1990). For polysaccharides, the method for chlorine substitution at primary hydroxyl groups has been successfully extended to cellulose (Horton *et al.* 1973a) and amylose (Horton *et al.* 1973b). When applied to pullulan, two of the three glucoses in the repeating unit have chloro groups at C-6 (the third is linked through C-6; i.e., a DS of 0.67). Subsequent treatment with alkali introduces 3,6-anhydro groups; and reaction with azide ion affords 6-azido derivatives. The uniformly substituted products should be suitable substrates for observing the effects of these modifications on enzymatic hydrolysis.

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TABLE 1. Sources of pullulanases

Enzyme	pH optimum	Dominant product	Enzyme source	Reference
Glucamylase EC 3.2.1.3	5.0	Glucose (G)	<i>Aspergillus niger</i> (Diazyme 455)	Miles Laboratory
Pullulanase EC 3.2.1.41	6.0 (7.0)	Maltotriose G1,4 $\alpha$ G1,4 $\alpha$ G	<i>Aerobacter aerogenes</i>	Bender and Wallenfels 1961
Neopullulanase EC 3.2.1.-	6.0	Panose G1,6 $\alpha$ G1,4 $\alpha$ G	<i>Bacillus subtilis</i>	Kuriki <i>et al.</i> 1988
Isopullulanase EC 3.2.1.57	3.5	Isopanose G1,4 $\alpha$ G1,6 $\alpha$ G	<i>Aspergillus niger</i>	This article

NOTE: The activity (mg reducing sugar·h<sup>-1</sup>·mg enzyme<sup>-1</sup>) of these preparations varied from 0.8 (glucamylase) to 26 (neopullulanase) when determined under our conditions of assay. The  $K_M$  values for the endopullulanases on pullulan are about 1 mg/mL; the value for exopullulanase (glucamylase) is about 150 mg/mL (literature values).

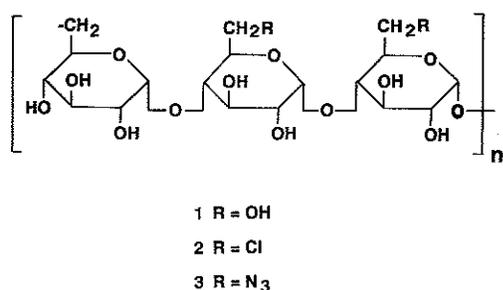


FIG. 1. Pullulan and derivatives modified at C-6.

In this paper, we examine the action of four enzymes on three different modified pullulans (Fig. 1).

### Materials and methods

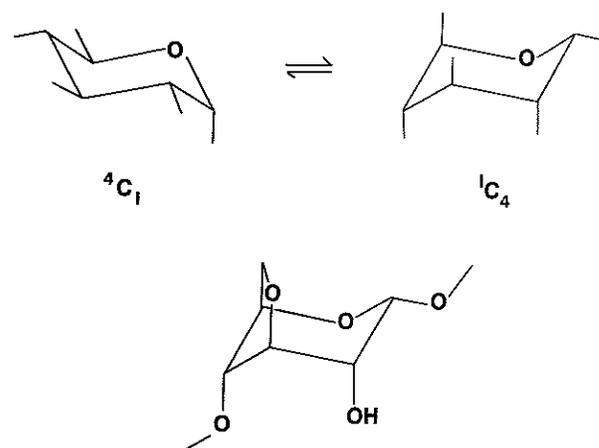
Pullulan was generously supplied by Hayashibara Laboratories, Okayama, Japan. 6-Chloro-6-deoxypullulan (2) was prepared by treatment of pullulan in dimethylformamide with methanesulfonyl chloride (Evans *et al.* 1968). Treatment of this derivative with alkali resulted in intramolecular cyclization and the formation of 3,6-anhydropullulan. The reaction of 2 with sodium azide in boiling water gave 6-azido-6-deoxypullulan (3). All structures were confirmed by <sup>13</sup>C nmr spectroscopy and full details will be published elsewhere.

The enzymes were generously donated by the investigators who first prepared them (Table 1), except for isopullulanase. This was prepared from the same *Aspergillus niger* strain (QM 386) used by Sakano *et al.* (1972). The crude enzyme was purified by adsorption on bentonite, and the eluate was sufficiently free of glucamylase that, after treatment of pullulan, the characteristic trimer (isopanose) could be detected by paper chromatography.

Activity of the enzymes was determined as described previously (Reese *et al.* 1968). A typical assay contained pullulan (3 mg) and enzyme in 0.05 M acetate buffer of desired pH (Table 1). Incubation was for 30 min at 40°C. For resistant substrates, the amount of enzyme was increased and the incubation period prolonged. Reducing sugars were determined by the dinitrosalicylic acid method. Scarcity of enzyme, and of some substrates, limited the extent of the investigation. In the inhibition tests, enzyme and inhibitor were incubated for 5 min prior to the addition of substrate. The maximum concentration of inhibitor tested was equal to that of the pullulan (e.g., 3 mg/mL).

### Results and discussion

In 6-chloro-6-deoxy- and 6-azido-6-deoxy-pullulan, the individual glucose and substituted glucose units presumably retain the <sup>4</sup>C<sub>1</sub> conformation common to all the units in pullulan. The secondary structures of these derivatives should therefore be similar to that of native pullulan. Formation of the anhydro ring in 3,6-anhydropullulan forces these



### 1,4-Linked 3,6-anhydro- $\alpha$ -D-glucopyranose unit

FIG. 2. Chair conformations and the effect of 3,6-anhydro ring formation.

glucose units into the <sup>1</sup>C<sub>4</sub> conformation (Fig. 2) and leads to a radically different secondary structure.

At pH 2.6, the chloro derivative gave a clear solution. Addition of buffers (above pH 3.0) resulted in decreased solubility, evidenced by turbidity and some precipitation. Azidopullulan solutions were slightly coloured, and little precipitation occurred.

Substitution at C-6 and introduction of 3,6-anhydro rings effectively reduced the hydrolysis of pullulan by all four types of pullulanase (Table 2): the reduction was about three orders of magnitude. In these cases, protection against hydrolysis was obtained at a DS of 0.67, and the nature of the substituent was of minor importance.

The minimum requirement for activity of endoenzymes appears to be the need for two adjacent unsubstituted sugar units, as shown for xylan hydrolysis (Perlin and Reese 1963) and for dextran hydrolysis (Hutson and Weigl 1963). In these examples, both terminal units of the oligosaccharide products were unsubstituted. If this principle extends to pullulanases, our derivatives had essentially no pairs of adjacent unsubstituted units.

There was, however, one important exception: the extent of hydrolysis of 6-azido-6-deoxypullulan by isopullulanase (Table 2). Not only was the relative rate of hydrolysis appreciable, but the extent of hydrolysis on long incubation was of similar magnitude (i.e., ca. 20% that of pullulan). It would perhaps be predicted that isopullulanase is the most likely of the endoenzymes to act on the C-6 substituted pullulans, since it is the only system where the

TABLE 2. Susceptibility of C-6 modified pullulans to enzymatic hydrolysis

Enzyme	Hydrolysis rate (relative)*			
	Pullulan	3,6-Anhydropullulan	6-Chloropullulan	6-Azidopullulan
Glucamylase	100	0	0.03	0.05
Pullulanase	100	0	0.01	0.03
Neopullulanase	100	0	0.02	0.01
Isopullulanase	100	0	0	23.0

\*Hydrolysis at rates less than 0.0001 that of pullulan are assigned a 0 value.

TABLE 3. Inhibition by C-6 modified pullulans of enzymatic hydrolysis of pullulan

Enzyme	Inhibition $R_{1/5}50^*$		
	3,6-Anhydropullulan	6-Chloropullulan	6-Azidopullulan
Glucamylase	10	10	10
Pullulanase	10	5+	1.5
Neopullulanase	10	0.06	0.02
Isopullulanase	10	8+	2+

\* $R_{1/5}50$  is the ratio of inhibitor to substrate to get 50% inhibition. Where no inhibition was detectable at an inhibitor concentration equal to that of the substrate, an arbitrary value of 10 was assigned.

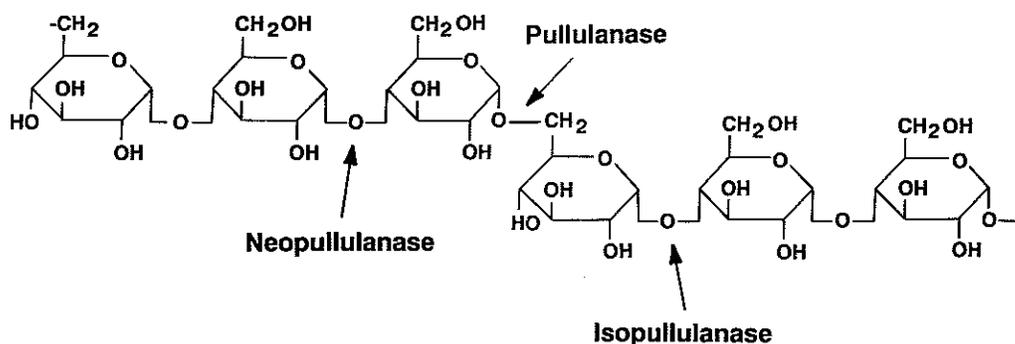


FIG. 3. Sites of action of various enzymes on pullulan.

potential reducing sugar at the cleavage site is unchanged from that in the parent polysaccharide (see Fig. 3). This does not explain, however, why the azidopullulan is hydrolyzed and the chloropullulan is not.

Similar results were obtained in our previous study (Reese *et al.* 1968) with glucosides modified at C-6. The 6-deoxy-, 6-chloro-6-deoxy-, and other derivatives of methyl  $\alpha$ -D-glucopyranoside were completely resistant to hydrolysis by five different  $\alpha$ -glucosidases. The 6-azido-6-deoxyglucoside was not available. The modified  $\beta$ -D-glucopyranosides were similarly resistant to hydrolysis by  $\beta$ -glucosidases. Here again, one exception was noted: the  $\beta$ -glucosidase of *Penicillium melinii* (QM 1931) was able to hydrolyze the 6-O-acetyl- $\beta$ -D-glucoside at a rate 25% that of unmodified methyl  $\beta$ -D-glucopyranoside. Work with other glycosidases supports these results. Thus the 6-deoxygalactose group of methyl 6'-deoxy- $\beta$ -lactoside was hydrolyzed at a rate only 4% that of the parent lactoside by *Escherichia coli*  $\beta$ -galactosidase (Bock 1990).

The lack of action of the exoenzyme glucamylase on C-6 substituted pullulans is consistent with the inability of exoenzymes to act on the nonreducing terminal unit when that unit has been modified. Such inhibition has been reported for exoglucanases acting on modified starch (Marshall 1978),

on  $\beta$ -1,3-glucans (Nelson *et al.* 1963), and on carboxymethyl pullulan (McCleary and Anderson 1980).

The C-6 modified pullulans have been tested as inhibitors of the enzymatic hydrolysis of pullulan (Table 3). In most cases, there was little or no inhibition when the inhibitor concentration was equal to that of the pullulan (substrate).

However, here again, there was one major exception. The neopullulanase of *Bacillus subtilis* was strongly inhibited by both 6-chloro- and 6-azido-pullulans. Other inhibitors have been evaluated for this effect but only on the pullulanase of *Aerobacter aerogenes*.  $\beta$ -Cyclodextrin, for example, was found to inhibit this enzyme at the  $R_{1/5}50$  (ratio of inhibitor to substrate to get 50% inhibition) of 0.001 (Marshall 1973).

Thus, the modification of substrate appears usually to result in a marked decrease in the affinity of the enzyme for the substrate. The increased affinity of neopullulanase for the modified pullulan (over the natural), however, is of a "nonproductive" nature, since there is no subsequent hydrolysis. With isopullulanase (*A. niger*) the binding affinity for the 6-azidopullulan is less, but still of the "productive" type, as subsequent hydrolysis does take place. The complete lack of inhibition shown by the 3,6-anhydropullulan in all enzyme systems is not surprising since its

radically different secondary structure would almost certainly preclude approach to the active sites of the enzymes.

Assuming uniformity of substitution, the critical DS for degradation of a modified polysaccharide by microbial enzymes appears to be between 0.5 and 0.7. Hydrolysis will occur below this range but not above it. This interpretation further assumes that the organism produces no enzyme capable of removing the substituent. An organism has been discovered that does remove acetate groups from cellobiose octaacetate. It cannot, however, remove acetate groups from the polymeric cellulose triacetate (Reese 1957). Until an organism can be shown to remove substituent groups from polysaccharides, this assumption appears reasonable.

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