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STUDIES ON DEXTRANASES

PART IV*. MODE OF ACTION OF DEXTRANASE D_1 ON OLIGOSACCHARIDES

GEOFFREY N. RICHARDS† AND MARGARET STREAMER

Department of Chemistry and Biochemistry, James Cook University of North Queensland, Townsville, Queensland (Australia)

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ABSTRACT

The products of action of a purified, extracellular *endo*-dextranase D_1, isolated from a new species of *Pseudomonas*, on pure isomaltose oligosaccharides have been investigated. Reduced and tritiated oligosaccharides have also been studied, and a model is postulated for the enzyme active-site, based on substrate specificity.

INTRODUCTION

In an earlier paper[1], the isolation of a dextran-degrading bacterium from soil was described, together with the isolation of two different extracellular dextranases (D_1 and D_2). The bacterium has now been identified by Professor V. B. D. Skerman (University of Queensland) as a new species of *Pseudomonas*[2]. The dextranase D_1 appears to be a pure enzyme, as judged primarily by gel electrophoresis at two different pH values. This paper further describes the properties of dextranase D_1 with a possible model for its mode of action on dextran and on isomaltose oligosaccharides.

Throughout this paper, the abbreviation IM_x refers to an oligosaccharide of the isomaltose series containing x D-glucose residues, and "the dextranase" refers to our enzyme D_1.

EXPERIMENTAL

Paper chromatography (p.c.). — Prior to chromatography, solutions were deionised by addition of equal volumes of Amberlite IR-45(HO^-) and IR-120(H^+) resins, shaking at room temperature for 15 min, and then filtering. Chromatography was performed on Whatman No. 1 or 3MM paper, using ethyl acetate–pyridine–water (10:4:3, v/v), or on Whatman No. 17 paper, using propan-1-ol–ethyl acetate–water (14:2:7, v/v), with detection by silver nitrate–sodium hydroxide[3]. The No. 1 paper was used for qualitative analysis of oligosaccharides of low d.p. (up to IM_3). The

*Part III: N. W. H. Cheetham and G. N. Richards, *Carbohyd. Res.*, 30 (1973) 99.
†To whom communications should be addressed.

3MM paper was used for qualitative and quantitative analysis of oligosaccharides of medium d.p. (up to IM_7). The quantitative analyses were carried out by elution of appropriate sections of paper (having sprayed guide strips) and analysis[4] of the extracts with phenol–sulphuric acid. Whatman No. 17 paper was used for oligosaccharides of high d.p. and for preparative chromatography.

Preparation of isomaltose oligosaccharides (IM_x). — The linear series of oligosaccharides was produced in collaboration with Dr. G. J. Walker, Institute of Dental Research, Sydney, by partial, acid hydrolysis of Sigma dextran type 200C, followed by separation on a charcoal–Celite column. The higher oligosaccharides from the column were contaminated by the neighbouring homologues and were further purified by preparative p.c. The products were in each case obtained as freeze-dried powders.

Tritiated, reduced oligosaccharides ($TRIM_x$). — An aqueous solution (2 ml) containing sodium borohydride (5 mg) and tritiated sodium borohydride (0.94 mg, 6.6 mCi, Radiochemical Centre, Amersham, England) was added to an aqueous solution (1 ml) of the oligosaccharide (5 mg). After 3 h at room temperature, Amberlite IR-120(H^+) resin (0.5 ml) was added and, after filtration, the solution was concentrated to dryness and boric acid was removed by distillation of methanol from the residue. The products were finally freeze-dried from aqueous solution and were not purified further. They will be referred to as $TRIM_x$, to represent a tritiated, reduced oligosaccharide of the isomaltose series, originally containing x D-glucose residues.

Enzymic hydrolysis of isomaltose oligosaccharides. — A solution of the oligosaccharide (3.5mM, d.p. 2–10) and dextranase (7.8×10^{-2} units.ml^{-1}, *cf.* Ref. 1) in 54mM sodium citrate buffer (pH 5.5) was kept at 33°, and samples were removed at intervals for determination of reducing power by the Nelson–Somogyi method[5] and for identification of products by p.c.

For IM_6, the effect of substrate concentration was determined by similar experiments using 1.5 and 15.2mM oligosaccharide and 9×10^{-2} units.ml^{-1} of enzyme.

To investigate the action of the enzyme at a very high concentration of substrate, 300-mg samples of IM_2, IM_3, and IM_4 in 54mM sodium citrate buffer (0.5 ml, pH 5.5) were incubated with the enzyme (3.5×10^{-2} units) at 33° and the products determined by p.c.

For the quantitative determination of products, a solution (2.6 ml) containing 54mM citrate buffer (pH 5.5), dextranase (9.0×10^{-2} units), and IM_6 (3.7mM) was kept at 33°, and 600-μl samples were removed after 1, 4, and 8 h. After 24 h, more enzyme (4.8×10^{-2} units) was added, and after a further 8 h, the final sample (600 μl) was removed. The relative amounts of different oligosaccharides in each sample were determined by quantitative p.c. The experiment was subsequently repeated, using IM_7 (3.9mM) in place of IM_6.

Enzymic hydrolysis of tritiated, reduced oligosaccharides. — A solution (0.5 ml) containing 54mM citrate buffer (pH 5.5), 4.0mM $TRIM_7$ (17,000 counts.min^{-1}.μmole^{-1}) and dextranase (1.1×10^{-2} units) was kept at 33°, and 100-μl samples were

removed after 1 and 24 h. In a separate experiment, 6.0mM $TRIM_{10}$ (9,000 counts. $min^{-1}.\mu mole^{-1}$) and 0.7×10^{-2} unit of enzyme were used.

After p.c., the position of products on the paper was determined by spraying guide strips, and appropriate areas of the chromatogram were cut into pieces (1.5-cm square) and immersed in a 0.5% solution of 2,5-diphenyloxazole in toluene for counting in a liquid scintillation spectrometer (Packard Tri-carb).

Activity of the dextranase against other polysaccharides. — The enzyme (8×10^{-2} units.ml^{-1}) was incubated with 1% solutions of the polysaccharides in 54mM citrate buffer (pH 5.5) at 40° for 24 h. The Nelson–Somogyi reducing power of the solution was then measured and, if it was significant, the products were examined by p.c.

RESULTS AND DISCUSSION

Qualitative analysis of products of degradation of isomaltose oligosaccharides. — The time course of degradation of the oligosaccharides of d.p. 5–10 is shown in Fig. 1. Under the conditions described, no increase in reducing power was detected

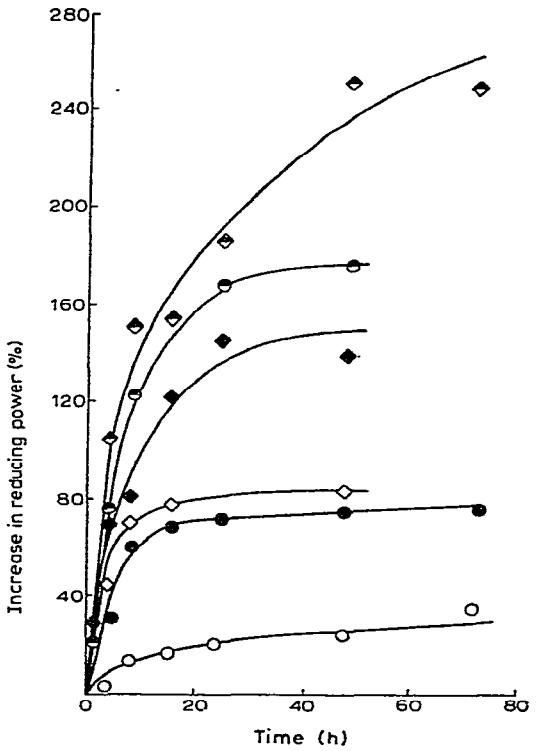


Fig. 1. Degradation of oligosaccharides by dextranase: ○, IM_5; ●, IM_6; ◊, IM_7; ◆, IM_8; ⬭, IM_9; ⬬, IM_{10}. Dextranase, 7.8×10^{-2} units.ml^{-1}. Oligosaccharide concentration, 3.5 ±0.1mM. Temperature of incubation, 33°.

for oligosaccharides of d.p. less than 5. The initial rate of degradation increased with d.p. up to IM_8 (Table I) and decreased slightly at higher d.p. The products of the degradation are shown in Table II, where the values shown are approximate and represent visual estimation on a ten-point scale of the relative amounts of the different products seen on paper chromatograms.

TABLE I

INITIAL RATE OF DEGRADATION OF OLIGOSACCHARIDES BY DEXTRANASE[a]

Oligosaccharide (d.p.)	5	6	7	8	9	10
Increase in reducing power after 1 h (%)	1.8	8.0	9.1	27.8	22.5	20.3

[a]Oligosaccharide concentration, 3.5 ± 0.1mM; dextranase D_1, 7.8×10^{-2} units.ml^{-1}.

TABLE II

PRODUCTS OF DEGRADATION OF ISOMALTOSE OLIGOSACCHARIDES[a] BY DEXTRANASE D_1

Products	*Substrate*																	
	IM_2		IM_3		IM_4		IM_5		IM_6		IM_7		IM_8		IM_9		IM_{10}	
D-Glucose	—	—	—	1[b]	—	1[b]	—	1[b]	—trace[b]		—	—	—	—	—	—	—	—
IM_2	10	10	—	1	—	1	1	3	2	6	1	2	1	5	1	3	1	2
IM_3			10	10	1	2	1	3	2	4	2	3	2	5	2	3	2	3
IM_4					10	10	2	3	4	6	3	4	4	6	6	8	6	9
IM_5							10	6	2	2	3	4	4	5	6	8	6	8
IM_6							1	1	9	1	1	—	2	—	3	1	6	—
IM_7							1	1	1	1	8	2	—	—	3	1	4	trace
IM_8											1	—	8	1	—	—	3	—
IM_9											1	—			8	1	—	—
IM_{10}																	8	1

[a]Substrate concentration, 3.5 ± 0.1mM; dextranase, 7.8×10^{-2} units.ml^{-1}. The first figure in each column represents samples taken after 4h incubation, and the second figure, samples taken after 24 h. [b]Samples taken after incubation for 4 days.

Although IM_3 and IM_4 showed no measurable increase in reducing power, there was paper-chromatographic evidence of slight attack by the enzyme after incubation for 4 days, with IM_3 yielding a trace of D-glucose and IM_2, while IM_4 yielded traces of D-glucose, IM_2, and IM_3. The low yields of these products suggested that the enzyme would not normally attack oligosaccharides smaller than IM_5 in the presence of more readily degradable substrates. These results are therefore consistent with the earlier observation[1] that IM_{2-4} are the products of action of D_1 on dextran.

Of the oligosaccharides which were readily degraded to give an increase in reducing power, only IM_5 showed a single, predominant mode of degradation. This resulted in production of IM_2 and IM_3. The degradation of oligosaccharides larger than IM_5 yielded a range of products which indicated that more than one of the glycosidic bonds was susceptible to attack. Thus, IM_6 was degraded to give mainly

IM_2, IM_3, and IM_4, which indicated hydrolysis of the central glycosidic bond and also one or both of the bonds on either side of the central bond. Similarly, IM_{10} gave IM_6, IM_5, and IM_4 as the major products, again indicating hydrolysis of the central bond and one or both bonds on either side. For IM_{10}, however, a slower hydrolysis of other bonds also occurred to yield smaller amounts of IM_8, IM_7, IM_3, and IM_2.

D-Glucose was produced only from oligosaccharides smaller than IM_5 and then very slowly, and yet IM_5 and IM_6 both yielded significant, but small, amounts of products containing one less D-glucose residue (*i.e.*, IM_4 and IM_5, respectively). At the same time, it was observed that IM_5, IM_6, and IM_7 yielded products containing more D-glucose residues than the original. Both of the last two observations suggested the possibility of some synthetic activity by the enzyme, and this was further investigated.

Synthetic activity of the enzyme. — In Table III, the figures represent visual estimations of relative yields of products on paper chromatograms. These show that the synthetic activity can be detected with 15.2mM IM_6, but not with 1.5mM solutions. Table IV reports experiments at very high concentration of substrate for oligosaccharides which are relatively resistant to enzymic hydrolysis. These results give a clear indication that the dextranase will catalyse the combination of two oligo-

TABLE III

EFFECT OF CONCENTRATION OF IM_6 ON ACTION PATTERN[a]

Products	*1.5mM*		*15.2mM*	
	4 h	*80 h*	*4 h*	*80 h*
IM_2	4	6	4	6
IM_3	2	4	2	4
IM_4	6	8	6	8
IM_5	—	—	1	1
IM_6	10	4	10	4
$>IM_6$	—	—	1	—

[a]Dextranase, 9.0×10^{-2} units.ml^{-1}.

TABLE IV

DEXTRANASE ACTIVITY AGAINST HIGH CONCENTRATIONS OF GLUCOSE AND ISOMALTOSE OLIGOSACCHARIDES[a]

Substrate	*Products of higher d.p.*
D-Glucose	—
IM_2	—
IM_3	IM_6 present after 24 h, increased during 3 days.
IM_4	IM_8 present after 4 h, increased during 3 days. IM_6 present after 7 h, slight increase during 3 days.

[a]Substrate concentration, 300 mg/1.3 ml; dextranase, 7.0×10^{-2} units.ml^{-1}.

saccharides to form a new oligosaccharide (*e.g.*, $2IM_3 \rightarrow IM_6$; $2IM_4 \rightarrow IM_8$). It is possible that the formation of IM_6 from IM_4 may occur *via* the intermediate formation and subsequent hydrolysis of IM_8.

We still have to explain the production of relatively large amounts of IM_5 from IM_6 in the absence of any D-glucose product. The synthetic activity demonstrated in the preceding experiments seems inadequate to account for this effect, because, although it may be postulated that IM_5 is derived from subsequent hydrolysis of a primary, synthetic product (*e.g.* IM_{12}), such subsequent hydrolysis would be expected to be very slow at the low concentration of substrate. A more likely explanation is the reaction of an enzyme–product complex with unreacted IM_6 to produce a higher oligomer which may hydrolyse rapidly despite its low concentration, because it is still complexed with an enzyme molecule. A likely mode of reaction is summarised as follows, where "E" represents the enzyme:

$$IM_6\text{–}E \rightarrow IM_2\text{–}E + IM_4$$
$$\downarrow IM_6$$
$$IM_8\text{–}E \rightarrow IM_2 + IM_3 + IM_4 + IM_5 + IM_6 + E$$

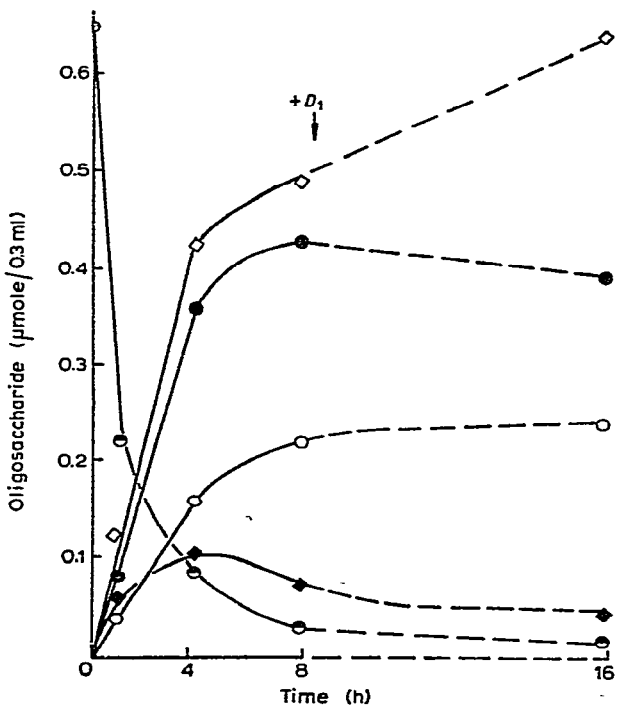


Fig. 2. Degradation of IM_6 by dextranase: ◇, IM_2; ○, IM_3; ●, IM_4; ◆, IM_5; ⊖, IM_6. IM_6 concentration, 3.7mM. D_1, 3.46×10^{-2} units.ml^{-1} initially; 5.3×10^{-2} units.ml^{-1} added later.

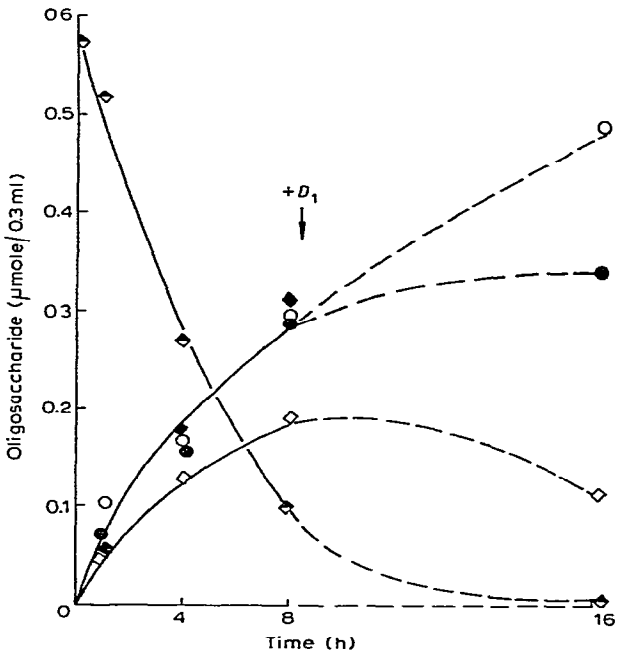


Fig. 3. Degradation of IM_7 by dextranase: ○, IM_2; ●, IM_3; ◆, IM_4; ◇, IM_5; ◈, IM_7. IM_7 concentration, 3.9mM; D_1, 3.46×10^{-2} units.ml^{-1} initially; 5.3×10^{-2} units.ml^{-1} added later.

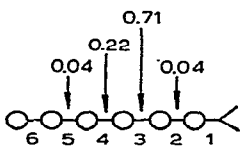
Quantitative determination of the products of degradation of IM_6 and IM_7. — Fig. 2 shows the yields of products from IM_6 against time. The values were determined by quantitative paper chromatography, and after the initial incubation of 24 h, more enzyme was added to ensure completion of the reaction. IM_4 and IM_2 are produced at approximately the same rate during the first 4 h and are the major products. IM_3 is produced to a smaller extent and, on the assumption that a molecule of IM_6 is degraded either to IM_4 and IM_2, or alternatively to $2IM_3$, then Fig. 2 shows that the IM_4,IM_2 mode of attack occurs at about five times the rate of the IM_3 mode. These results, however, do not indicate whether the IM_4,IM_2 mode of attack occurs at the glycosidic linkage penultimate to the reducing or the non-reducing end of IM_6.

The final, molar yield of IM_2 exceeded that of IM_4, even though their initial rate of production was quite similar. This effect results from a number of relatively slow, secondary hydrolyses, *e.g.*, $IM_4 \rightarrow 2IM_2$; $IM_5 \rightarrow IM_3 + IM_2$. The production and subsequent degradation of IM_5 is also shown in Fig. 2, and yet no D-glucose was detected either by paper chromatography or by analysis with the D-glucose oxidase reagent. Therefore, the IM_5 did not result from hydrolysis of a terminal, glycosidic bond in IM_6.

A similar experiment with IM_7 is reported in Fig. 3. Here, the initial rates of production of IM_4, IM_3, and IM_2 are approximately equal, with IM_5 appearing at a slightly lower rate, which is probably associated with its simultaneous, slow hydrolysis

It seems therefore that the two reactions, $IM_7 \rightarrow IM_5 + IM_2$; $IM_7 \rightarrow IM_4 + IM_3$ occur at similar rates.

Degradation of tritiated, reduced isomaltose oligosaccharides (TRIM$_x$). — Table V shows the distribution of radioactivity in the products of enzymic degradation of $TRIM_7$. Each product shown as $TRIM_{2-5}$ in Table V is likely to include some oligosaccharide of similar molecular weight and resulting from a reaction such as $TRIM_7 \rightarrow TRIM_3 + IM_4$. The relative frequency of each type of bond scission may be calculated from Table V, however, since each scission will produce only one characteristic, radioactive product. This calculation involves the assumption that all of the radioactive products are derived from primary scission of $TRIM_7$, and this assumption has been verified by the finding that reduced isomaltohexaose (RIM_6) is hydrolysed very slowly by the enzyme; therefore, presumably, $TRIM_5$ and smaller oligomers are even more stable. The results of Table V therefore indicate the following pattern of scission of $TRIM_7$:



where O, —, and < represent a D-glucose residue, an α-(1→6) linkage, and the terminal alditol group, respectively. The numbers indicate the relative frequency of cleavage of each bond. Thus, in the scission of the heptamer to trimer and tetramer, two different types of cleavage are involved, with scission at bond 3 predominating. It seems probable that degradation of IM_7 to IM_3 and IM_4 also involves both types of attack (*i.e.*, at glycosidic bonds 3 and 4 from the reducing end), perhaps in proportion similar to that shown in the above diagram, since it is unlikely that the reduction of the aldose to alditol would favour enzymic attack at bond 3 compared with bond 4.

The relatively small extent of scission at bonds 2 and 5 in $TRIM_7$ is in contrast with the observation that IM_7 yields major amounts of IM_5 and IM_2. This suggests

TABLE V

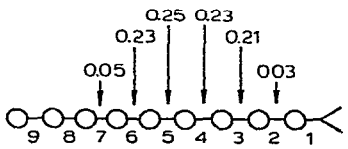
DEGRADATION OF $TRIM_7$[a]

Oligosaccharide	*Counts/min (% total)*	*Frequency of production*[b]
$TRIM_2$	3	0.04
$TRIM_3$	59	0.71
$TRIM_4$	18	0.22
$TRIM_5$	3	0.04
$TRIM_7$	17	

[a]$TRIM_7$, 4.0mM, dextranase D_1, 2.2×10^{-2} units.ml^{-1}, 24-h incubation. [b]Counts (%) in each oligosaccharide product/total counts (%) for oligosaccharide products.

that, for the latter oligomer, this mode of hydrolysis occurs mainly at the second glycosidic bond from the reducing end and that the conversion of the aldose group into alditol tends to inhibit hydrolysis at proximate glycosidic bonds. The latter hypothesis is supported by the lower rate of total hydrolysis of $TRIM_7$ compared with IM_7.

Results for degradation of $TRIM_{10}$ are shown in Table VI and, neglecting any secondary hydrolysis of $TRIM_7$ product, these results indicate the following action pattern:



No quantitative results are available for the products of degradation of the parent IM_{10}, but visual examination of a paper chromatogram (Table II) indicated that isomaltose oligosaccharides of d.p. 3–7 were the major, initial products. By analogy with the results from $TRIM_{10}$, we can now suggest that IM_{10} is attacked rapidly at bonds 4 and 6 to produce IM_4 and IM_6, and at bond 5 to produce IM_5. The production of heptamer and trimer from $TRIM_{10}$ evidently occurs mainly by scission of bond 3 rather than bond 7. It is probable, by analogy, that the same situation occurs in the parent IM_{10}, so that again (as with IM_7) an asymmetrical mode of scission occurs preferentially near the reducing rather than the non-reducing end of the oligosaccharide.

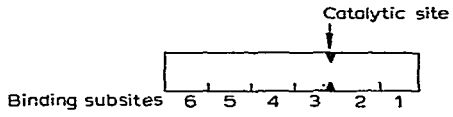
TABLE VI

DEGRADATION OF $TRIM_{10}$[a]

Oligosaccharide	*Counts/min (% total)*	*Frequency of production*
$TRIM_2$	2	0.03
$TRIM_3$	13	0.21
$TRIM_4$	14	0.23
$TRIM_5$	15	0.25
$TRIM_6$	14	0.23
$TRIM_7$	3	0.05
$TRIM_{10}$	39	

[a] $TRIM_{10}$, 6.0mM; dextranase, 1.4×10^{-2} units.ml^{-1}; 24h incubation.

On the basis of the above results, we postulate the following model for the active site of the enzyme:



Each binding subsite occupies one D-glucose residue of a dextran chain and the (1→6)-linkage direction is (*e.g.*) from subsite 2→1. Inadequate amounts of pure oligosaccharides were available for a detailed kinetic study, but from the above model, we might anticipate both substrate and product inhibition resulting from inactive binding, *e.g.* at subsites 3–6.

Activity against other polysaccharides. — Using the methods described in the experimental section, no action of dextranase D_1 was detected with potato starch, amylopectin, amylose, glycogen, or sucrose. With pullulan, however, there was an increase in reducing power corresponding to an apparent 3.6% conversion into maltotriose in 24 h. The major oligosaccharide product was maltotriose and no D-glucose, maltose, or isomaltose were detected. The enzyme, therefore, is apparently capable of slow attack of the α-(1→6) linkage in pullulan. It must be concluded that the α-(1→4)-linked sectors of pullulan are to some extent compatible with the binding site of the enzyme, but that similar regions in amylopectin fail to bind to the enzyme because of hindrance by other parts of the polysaccharide molecule.

ACKNOWLEDGMENTS

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