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THE STUDY OF THE ACTION PATTERN OF AN EXO-β-(1 → 3)-D-GLUCANASE

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SUMMARY

1. The action pattern of an exo-β-(1 → 3)-D-glucanase (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) from Basidiomycete sp. QM 806 was investigated from the point of view of whether it acts by single-chain or multi-chain mechanism.

2. Labeled substrate was prepared by reduction of insoluble laminarin with NaB^3H_4. The kinetics of its hydrolysis with the enzyme were monitored by following loss of polysaccharide, production of oligosaccharide and appearance of glucose. The specific determination of glucose and paper chromatographic separation followed by radioactivity measurements were used as the main analytical methods.

3. The rate of loss of polysaccharide was found to be approximately the same as the rate of glucose appearance. The principal products (excluding glucose) were tetra- and trisaccharide (depending on enzyme concentration). Only a small amount of products with molecular weight in the range between polysaccharide and tetrasaccharide was formed during the whole period of hydrolysis.

4. A similar picture was observed when the enzymic hydrolysis of unlabeled soluble laminarin was monitored by chromatography of the products on Biogel P-2.

5. The data obtained prove that the enzymic hydrolysis is a single-chain process and that the normal end-products of this process are tetrasaccharide or trisaccharide. Some speculations are made as to the mechanism of the process.

INTRODUCTION

Exo-glycanases can hydrolyze linear homopolysaccharides in one of two different ways, the so-called single-chain and multi-chain attack mechanisms. In the single-chain mechanism, the result of an attack of the enzyme on a substrate molecule is the complete cleavage of a given chain of substrate and only after this process is complete can the enzyme attack the next molecule of substrate. In the multi-chain process,

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enzyme attack results in the cleavage of only one glycosidic linkage after which the enzyme and substrate molecules lose contact and the next enzyme attack can be directed either at the same substrate molecule, or a different one, in a purely random fashion. Theoretical treatments of these two mechanisms have been developed recently[1,2]. The most extensive investigations of single- and multi-chain types of hydrolysis have been carried out in studies on β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) (see reviews[1,3]). We report here investigations on the action pattern of the exo-β-(1 → 3)-D-glucanase (β-1,3-glucan glucanohydrolase, EC 3.2.1.39) from Basidiomycete sp. QM 806 (ref. 4,5) using kinetic experiments that were monitored by simultaneous determination, in the reaction mixtures, of the appearance of monomer (D-glucose) and oligomers, and disappearance of polymer. Such an analysis allows one to distinguish between single and multi-chain mechanisms.

MATERIALS AND METHODS

Determination of D-*glucose*

This was done either by the method of HUGGET AND NIXON[6], using the glucose oxidase reagent of C. J. Boehringer and Soehne GmbH, or by a determination of reducing power[7].

Determination of the concentration of polysaccharide solutions

An aliquot (0.10 ml) containing about 100–200 μg of polysaccharide was mixed with 0.10 ml of 3 M H_2SO_4 and heated in a sealed tube at 100° for 4 h. The solution was then neutralized with 0.40 ml of 1.5 M NaOH and glucose determined in aliquots of this hydrolyzate. That complete hydrolysis occurred under the above conditions was established by the fact that the amounts of glucose found by the two different methods (see above) were the same. Control experiments established that the decomposition of glucose under the above hydrolysis conditions was negligible.

Measurements of radioactivity

Determinations of count rate were done in toluene liquid scintillator with Unilux II, Mark I and Mark II counters (Nuclear Chicago Corporation). Samples were prepared in the following fashions: aqueous solutions (0.10 ml) were mixed with Tween X-100 (1.0 ml) and liquid scintillator (5.0 ml). Efficiency of counting was determined using an external standard and the channel ratios procedure (see ref. 8 and the instructions for the instrument used); paper chromatogram sections were immersed in 5.0 ml of liquid scintillator and counted. The efficiency was close to 10% in this case. The time of counting was 1 min in a typical measurement and the background was 30 counts/min.

Enzymes

The exo-β-(1 → 3)-D-glucanase was prepared by the method of HUOTARI *et al.*[4], and gave only one band upon acrylamide gel electrophoresis.

Radioactive substrate

Insoluble laminarin from *Laminaria hyperborea* (30.5 mg sample No. 1, see ref. 9) was dissolved with heating in 0.20 ml of water. The solution was then cooled

to 30° and 0.1 ml of an aqueous solution of NaB^3H_4 (6 mg) was added immediately. After 24 h at room temperature, 1 drop of glacial acetic acid was added (gas formation), followed by 3 ml of ethanol and the resulting precipitate was centrifuged off, washed with ethanol (2 × 3 ml) and with ether (5 ml) and evaporated* 3 times with absolute methanol. This residue was dissolved in water (1 ml), ethanol (4 ml) was added and the precipitate which formed after 24 h was centrifuged off, washed with ethanol (2 × 5 ml) and ether (5 ml) and dried. The yield was 20.1 mg (67%).

The working solution of substrate was prepared by dissolving about 10 mg of this substance in 5 ml of water. The polysaccharide concentration determined as described above was 1.57 mg/ml and its specific activity was 2700 disint./min per μg glucose.

Periodate oxidations

The position of label in the polysaccharide was determined as follows: The polysaccharide (0.7 mg) was dissolved in water (0.11 ml) and an aliquot of this solution (0.03 ml) was mixed with 0.05 ml of 20 mM sodium metaperiodate and the solution kept in the dark for 1.5 h. Then 1 ml of 3 mM ethyleneglycol in ethanol was added, the mixture was left for 1 h and then deionized with cation exchange resin QU-2 (H^+) and anion exchange resin Amberlite IRA-410 (HCO_3^-) and then evaporated to dryness. A freshly prepared aqueous solution of $NaBH_4$ (0.2 ml of 0.1 M) was added to the residue and the solution left overnight. Na^+ was removed as above, the solution was evaporated and the residue re-evaporated 3 times with absolute methanol and the resulting residue dissolved in 0.50 ml water (Solution A). A second 0.03-ml aliquot of the initial polysaccharide solution was treated as above, the residue obtained was dissolved in 0.03 ml of water and then the whole procedure repeated. The residue from this was then dissolved in 0.50 ml of water (Solution B). Finally a mixture of 0.05 ml of 20 mM sodium metaperiodate and 1 ml of 3 mM ethyleneglycol in ethanol was kept for 1 h in the dark and a third aliquot (0.03 ml) of the initial polysaccharide solution then added. The mixture was then deionized and reduced as described above and this Solution C was used as a control. The glucose content and radioactivity of the three solutions was determined as described above. The specific activities found (disint./min per μg glucose) were: C, 2590; A, 144 (5.6% of C); B, 177 (6.7% of C). It is evident that at least 94% of the total radioactivity in the substrate is located at C-1 of the D-glucitol moiety; unspecific incorporation of label is not more than 5–6%.

It has been shown previously[10] that periodate oxidation of sorbitol substituted at C-3 under similar conditions gives rise to not more than 1.3–1.5 mole of formaldehyde, *i.e.* one of the primary–secondary pairs of vicinal glycol groups cannot be oxidized quantitatively. It was assumed[11] that the unoxidizable bond in the C-5–C-6 bond and that the C-1–C-2 bond is quantitatively split. The data presented here show that indeed the first oxidation results in total cleavage of the C-1–C-2 bond since the second treatment does not decrease the specific activity of the polysaccharide. The cause of the small increase in specific activity (per μmole of glucose) after the second oxidation is probably the decrease in the amount of glucose in the polymer as a result of the oxidation.

* All evaporations were done, *in vacuo*, at 40°.

Enzymic hydrolysis of the labeled substrate

The working solution of polysaccharide (0.60 ml) was mixed with acetate buffer (pH 4.8) and with enzyme solution such that the total volume was 2.40 ml, the buffer concentration 0.1 mM and the enzyme concentration 0.03 I.U.*/ml (low concentration) or 0.16 I.U./ml (high concentration). Aliquots were taken at the stated times for determination of glucose and for chromatographic analysis. The reaction was stopped by heating the aliquot to 100° for 5 min.

Paper chromatography

This was carried out on Leningradskaya S paper in the solvent system 1-butanol–pyridine–water (6:4:3, by vol.) using the descending technique for a period of 40–45 h. The chromatograms were marked off in squares (either 15 mm × 15 mm or 20 mm × 15 mm) which were numbered beginning from the origin and proceeding to the front. The positions of the components of the reaction mixtures were determined through the use of unlabeled reference substances.

When the distance from the origin to the front was 30 cm, the distribution of the compounds among the squares was: polysaccharides, 1st and 2nd squares; higher oligosaccharides (hepta- through pentasaccharide), 3rd to 6th squares; tetrasaccharide (laminaritetraitol), 7th and 8th; trisaccharide (laminaritriitol), 10th and 11th; disaccharide (laminaribitol), 12th and 13th; and glucitol, 15th through 17th squares. The chromatograms were dried, the squares carefully cut out, and their radioactivity determined as described above. The percentage of the components in the mixture was calculated as the ratio (as percent) between the activity in the given square to the sum of the activities of all squares from the chromatogram (1st through the 20th).

Corrections for non-specific label were made by subtracting, from each square, the activity in the corresponding square from a chromatogram of unhydrolyzed substrate (squares 3 through 20) or from a chromatogram of the products of total enzymic hydrolysis (squares 1 and 2). These corrections were in the region of 1–2% and the total activity on a given chromatogram was from $8 \cdot 10^3$–$12 \cdot 10^3$ counts/min.

Enzymic hydrolysis of the unlabeled substrate

Soluble laminarin isolated from *Laminaria cycharioides* by the method of BLACK[12] was hydrolyzed at 37° in 5 ml of solution that contained 10 mg of substrate, 1.0 I.U. of the enzyme and was 0.05 M in acetate buffer (pH 5.6). Aliquots (1.0 ml) of this solution were taken with time and passed over a Biogel P-2 column (1.5 cm × 40 cm, rate of elution 0.2 ml/min) which was eluted with water. 1-ml fractions were collected and the sugar content estimated by the phenol–H_2SO_4 method[13].

At the same time aliquots of the reaction mixture were taken for the determination of glucose content.

RESULTS AND DISCUSSION

The kinetics of enzymic hydrolysis of labeled substrate were studied at both high and low enzyme concentration. The results are shown in Tables I and II and in Figs. 1 and 2. The enzyme used in these studies is an exoglucanase with no trace of

* International unit, see ref. 4 for definition.

TABLE I

THE KINETICS OF HYDROLYSIS OF INSOLUBLE LAMINARIN, LABELED AT THE REDUCING TERMINUS, WITH LOW CONCENTRATIONS OF EXO-β-(1→3)-D-GLUCANASE

Time (min)	*Components of the reaction mixture (moles × 100/moles of initial polysaccharide)*					*Free glucose (% of total)*
	Polysaccharide	*Higher oligosaccharides**	*Tetrasaccharide*	*Trisaccharide*	*Disaccharide*	
3	97.8	0.55	1.2	0.3	0.0	3
6	96.2	0.6	1.8	0.6	0.5	5.9
15	92.2	1.5	4.8	1.5	0.0	12.1
30	87.0	1.6	9.3	1.9	0.2	15.9
60	74.3	3.1	16.3	5.5	0.3	32.4
300	14.0	8.3	66.0	9.8	1.0	78.5

* Penta-, hexa- and heptasaccharides.

either endo-glucanase or transglycosylating activity[4,5,14]. It is evident, both from the method of preparation and the periodate oxidation data, that the label in the substrate is confined essentially to the glucitol units that occupy the position of the original reducing termini. It follows from this that loss of labeled polysaccharide and appearance of labeled oligosaccharides during enzymic hydrolysis are a real measure of the total cleavage of polysaccharide chains to low molecular weight oligosaccharides. The fact that loss of polysaccharide and appearance of oligosaccharide take place in the initial part of the kinetic plots, and without a lag phase (see Figs. 1 and 2), shows that this is a single-chain hydrolytic process. For a multi-chain process one would expect, in the initial part of the kinetic curve, the formation of large amounts of glucose without a significant decrease in the amount of polymer, since only a decrease in the number-average degree of polymerization is taking place. Further, at these times there would not be measurable amounts of oligosaccharide produced.

The kinetics of single-chain and multi-chain hydrolysis of linear polymers by

TABLE II

THE KINETICS OF HYDROLYSIS OF INSOLUBLE LAMINARIN, LABELED AT THE REDUCING TERMINUS, WITH EXO-β-(1→3)-D-GLUCANASE AT HIGH ENZYME CONCENTRATION

Time (min)	*Components of the reaction mixture (moles × 100/moles of initial polysaccharide)*					*Free glucose (% of total)*
	Polysaccharide	*Higher oligosaccharides**	*Tetrasaccharide*	*Trisaccharide*	*Disaccharide*	
5	77.0	2.1	13.5	7.3	0.0	27
10	56.6	3.3	22.1	16.5	1.4	44
15	39.4	4.5	30.5	23.6	1.0	62
30	2.0	1.9	30.9	61.8	2.1	71
60	1.7	2.4	3.6	86.5	5.0	86
150	1.4	0.6	1.0	81.3	12.0	86
300	0.0	2.0	0.3	77.6	19.6	—
24 h	0.0	1.9	0.7	67.3	29.6	87

* Penta, hexa-, hepta- and probable octasaccharides.

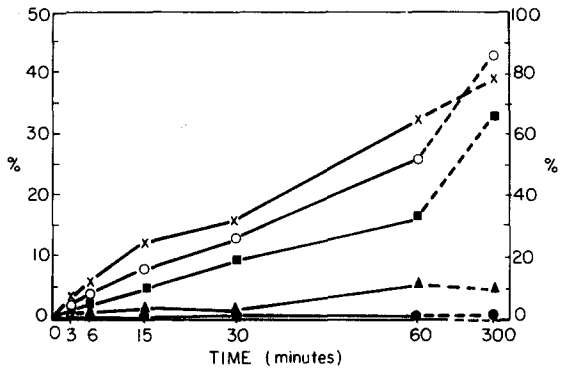

Fig. 1. The kinetics of hydrolysis of insoluble laimnarin with exo-β-(1→3)-D-glucanase at low enzyme concentration. The left ordinate for points corresponding to 3, 6, 15, 30 and 60 min, the right ordinate for points corresponding to 300 min only. ○—○, loss of polysaccharide (moles × 100/moles of initial polysaccharide); ■—■, tetrasaccharide content (moles × 100/moles of initial polysaccharide); ▲—▲, trisaccharide content (moles × 100/moles of initial polysaccharide); ●—●, disaccharide content (moles × 100/moles of initial polysaccharide); ×—×, free glucose (% of total).

exo-enzyme has recently been analyzed using mathematical models[2]. It was shown that, making all possible assumptions, the loss of polymeric material must be considerably smaller than the appearance of monomer (up to 40–50% of hydrolysis), if a multi-chain process is taking place. It was also suggested that a very useful plot for this kinetic analysis was one of loss of polymer (as a per cent of initial) against the appearance of monomer (as a percent of hydrolysis). The results obtained by us are plotted in this fashion in Fig. 3. The theoretical dependence[2] for single and multi-chain mechanisms are also plotted on the graph. It is evident that for both enzyme concentrations the plot indicates good agreement with the theoretical for a pure single chain process, up to a high percentage of total hydrolysis.

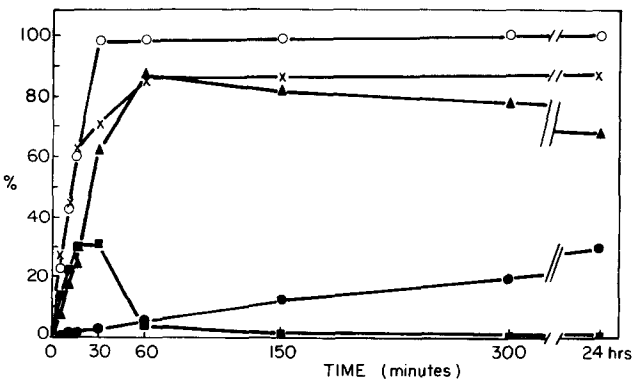

Fig. 2. The kinetics of hydrolysis of insoluble laminarin with exo-β-(1→3)-D-glucanase at high enzyme concentration. ○—○, loss of polysaccharide (moles × 100/moles of initial polysaccharide); ■—■, tetrasaccharide content (moles × 100/moles of initial polysaccharide); ▲—▲, trisaccharide content (moles × 100/moles of initial polysaccharide); ●—●, disaccharide content (moles × 100/moles of initial polysaccharide); ×—×, free glucose (% of total).

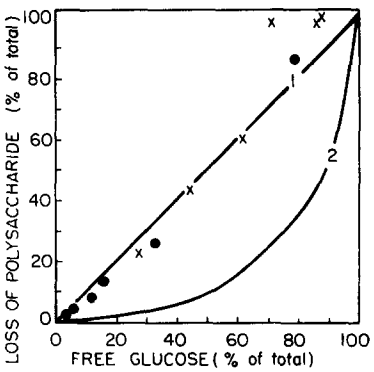

Fig. 3. The relation between the loss of polysaccharide and appearance of free glucose during hydrolysis of insoluble laminarin with exo-β-(1→3)-D-glucanase. ●, hydrolysis at low enzyme concentration; ×, hydrolysis at high enzyme concentration; Curve 1, theoretical dependence for single-chain mechanism (see ref. 2); Curve 2, theoretical dependence for multi-chain mechanism (see ref. 2).

The formation of higher oligosaccharides (degree of polymerization > 4) is negligible during the whole course of hydrolysis (see Tables I and II). This, of course, is additional evidence for a single chain mechanism at the low enzyme concentration (see Table I and Fig. 1). The principle reaction product (ignoring glucose) is the tetrasaccharide which is then very slowly hydrolyzed to trisaccharide. At the high enzyme concentration, tetrasaccharide and trisaccharide appear at similar rates during the initial stages of hydrolysis. Hydrolysis of tetrasaccharide to trisaccharide and of the latter to disaccharide are observed in the later stages of hydrolysis. Glucitol is not released at any point in the hydrolytic process. It is important to note that disaccharide formation proceeds with a lag-phase and the rate of its appearance is nearly the same as the sums of the rates of loss of tri- and tetrasaccharides. It would thus appear that the disaccharide is a secondary product which is formed as a result of

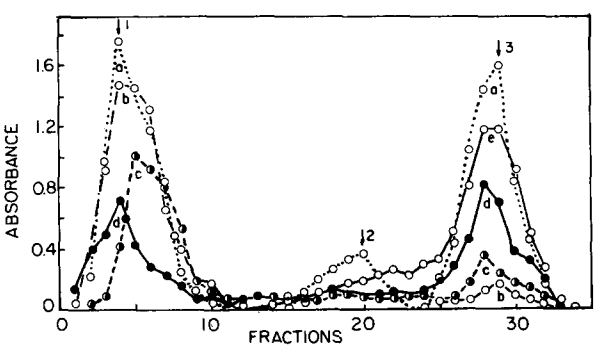

Fig. 4. The chromatography on Biogel P-2 of the products of hydrolysis of soluble laminarin with exo-β-(1→3)-D-glucanase. (a) ○...○, standards (1-soluble laminarin, 2-laminaritetraitol, 3-glucose); (b) ○—○, reaction mixture after 5 min (free glucose content is 10% of total); (c) ◑---◑, reaction mixture after 15 min (free glucose content is 30% of total); (d) ●—●, reaction mixture after 30 min (free glucose content is 50% of total); (e) ○—○, reaction mixture after 17 h (free glucose content is 93% of total). Sugar content is expressed as absorbance, using the phenol–H_2SO_4 method[13].

the hydrolysis of tetra- and trisaccharide. Hence the hydrolytic process proceeds by a pure single-chain mechanism up to the formation of either tri- or tetrasaccharide, depending on the enzyme concentration, and these fragments can be regarded as the normal end-products of the hydrolysis of linear β-(1 → 3)-linked glucan chains.

Further evidence for a single-chain mechanism of hydrolysis was produced by monitoring the hydrolysis of *L. cycharioides* laminarin by means of gel filtration (Fig. 4). A decrease in the size of the polysaccharide peak (without notable change in its position (*i.e.* molecular weight)) and a simultaneous increase in the peak of low molecular weight products was observed. It should be noted that there is no formation of significant amounts of products with a molecular weight between polysaccharide and tetrasaccharide during the whole course of hydrolysis. This evidence, again, supports a single-chain in contrast to a multi-chain mechanism. If a multi-chain mechanism were operating one would expect a progressive shift in the polysaccharide peak in the direction of lower molecular weight, together with a broadening of the peak and the formation of glucose.

It is possible to explain a single chain hydrolytic process in one of three ways: (i) An increase in the rate of hydrolysis of an n-mer with decrease in the value of n. This explanation must be rejected in the present case because the reverse dependence has been shown[4,5]. (ii) An explanation of the type already given for β-amylase[1,15]. The enzyme and substrate form a complex and glucose is cleaved off. The complex then does not dissociate but the next glucose moiety cleaves off, *etc.* Such a scheme fits the present case and we can add the following considerations. The dissociation of the complex does not take place and the hydrolysis goes by a pure single-chain process until the substrate molecule reaches a critical value of degree of polymerization in the region 3–4. The stability of the complex is then abruptly decreased and dissociation takes place. Subsequent hydrolysis of the oligosaccharides proceeds much more slowly by an ordinary glycosidase type of action.

One can propose the following model for such a mechanism. The binding site of the enzyme probably includes a sequence extending from the non-reducing terminus into the third glucose residue. A similar conclusion has been reached on the basis of studies on the substrate specificity of the enzyme[5]. After cleavage of the first glucose residue the complex rearranges (without dissociation) such that the polysaccharide chain shifts the length of one glucose residue along the enzyme molecule (see the scheme already proposed for β-amylase[1,15]). The complex then becomes active again, because of a juxtaposition of a glycosidic bond and the catalytic site, and another glucose residue is cleaved off. This sequence of events can be represented by:

$$E + S_n \rightleftharpoons ES_n \xrightarrow[-\mathrm{Glc}]{} EP_{n-1} \rightarrow ES_{n-1} \xrightarrow[-\mathrm{Glc}]{} EP_{n-2} \rightarrow ES_{n-2} \xrightarrow[-\mathrm{Glc}]{} EP_{n-3} \xrightarrow{etc.}$$
$$EP_4 \rightleftharpoons E + P_4$$

where E is the enzyme; S_n, P_n are substrate and product, respectively, of enzymic action, n is the degree of polymerization of each substance, Glc is glucose. Obviously this reaction sequence is in good agreement with the kinetics observed.

(iii) A third possible explanation of the observed kinetics can be based on an assumed conformational inhomogeneity of the substrate. One can assume that the substrate exists in two conformational forms, one attackable by the enzyme, the other not, and that these two conformational forms are in equilibrium. If the rate of attain-

ment of this equilibrium is considerably slower than the rate of enzyme attack, then one would expect "single-chain" kinetics. After cleavage of the first bond and dissociation of the enzyme–product complex, the enzyme will show a preferential attack on the desorbed molecules since they will conserve the active conformation. Such a process would be "multi-chain" in its molecular mechanism but single-chain in its kinetics and would proceed until the size of the substrate molecule becomes too small to maintain the active conformation (*e.g.* as a result of cooperative stabilization). In order that such a scheme operate it is necessary that there be no excess of substrate molecules in the active conformation. The concentration of such molecules will increase in direct proportion to the overall substrate concentration and this should result, if the above mechanism is operating, in a tendency for the kinetics to depart from single-chain as the substrate concentration is raised. No such tendency is observed with the present enzyme, since the substrate concentrations used were approx. 4 times the value of K_m (ref. 4). We think it highly unlikely that such a mechanism is operative in the present case, but it cannot be eliminated on the basis of the data at hand.

The loss of polysaccharide at the final stages of the hydrolysis is greater than the appearance of glucose (see Figs. 2 and 3). Obviously this is not possible for an homogenous substrate. The labeled substrate used here was prepared from insoluble laminarin which is an approximately 1:1 mixture of two polysaccharides with closely related structures[9,10]. The so-called "M-chains" have D-mannitol at their reducing termini; the so-called "G-chains" have D-glucose in the same position. The method of synthesis used here would incorporate label only into the G-chains, and as a result, the loss of polysaccharide and appearance of oligosaccharide is measured for G-chains only. However, the appearance of glucose is measured for both G- and M-chains. It is reasonable to assume that there is a fine difference between the structure of these two polysaccharides that results in a slightly higher rate of enzymic hydrolysis of the G-chains in comparison to the M-chains. If this were so the formation of glucose will not be as rapid as the loss of labeled polysaccharide.

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