

Estimation of Exo-β-1 → 4-glucanase in Crude Cellulase Solutions

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A method has been developed for the estimation of exo-β-1 → 4 glucanase in cellulolytic solutions based on the relative susceptibility of cellotetraose and cellobiose to hydrolysis. By its use we have found that most cellulolytic organisms produce some exo-β-1 → 4 glucanase. Trichoderma viride QM6a is most productive of those tested. (Oddly enough it is also the best producer of C₁, but not of the endo-β-1 → 4 glucanase.) The exo-β-1 → 4 glucanase, like the endo-, appears to be an induced enzyme.

The exo-polysaccharases, as a group, are relatively new (except for β-amylase). Their investigation goes back less than thirty years to the discovery of the exo-α-1,4-glucanase known also as glucoamylase or amyloglucosidase. Most of these enzymes remove a single sugar unit from the non-reducing end of the polymer. Action is most rapid on the longer chains, such small molecules as the dimer being highly resistant to hydrolysis. Within the past 10 years, additional exo-glucanases have been discovered, the best known of which is the exo-β-1,3-glucanase. The exo-β-1,4-glucanase which is part of the cellulolytic system has received little attention (1, 2). These enzymes are usually found in association with endo-enzymes and glucosidases, and, as yet, little effort has been expended in detecting the presence of the exo-enzyme.

Further work on exo-β-1 → 4-glucanase demands a method for its detection and estimation in crude solutions—i.e., in the presence of both endo-β-1 → 4-glucanase and cellobiase. For simplification and other reasons (see below), let us ignore the contribution of the endo-β-1 → 4-glucanases. If our generalizations on exo-glucanases (1, 2) are correct, we may approach the problem in several ways. The one which seems most promising depends upon the differences in hydrolysis rates of dimer

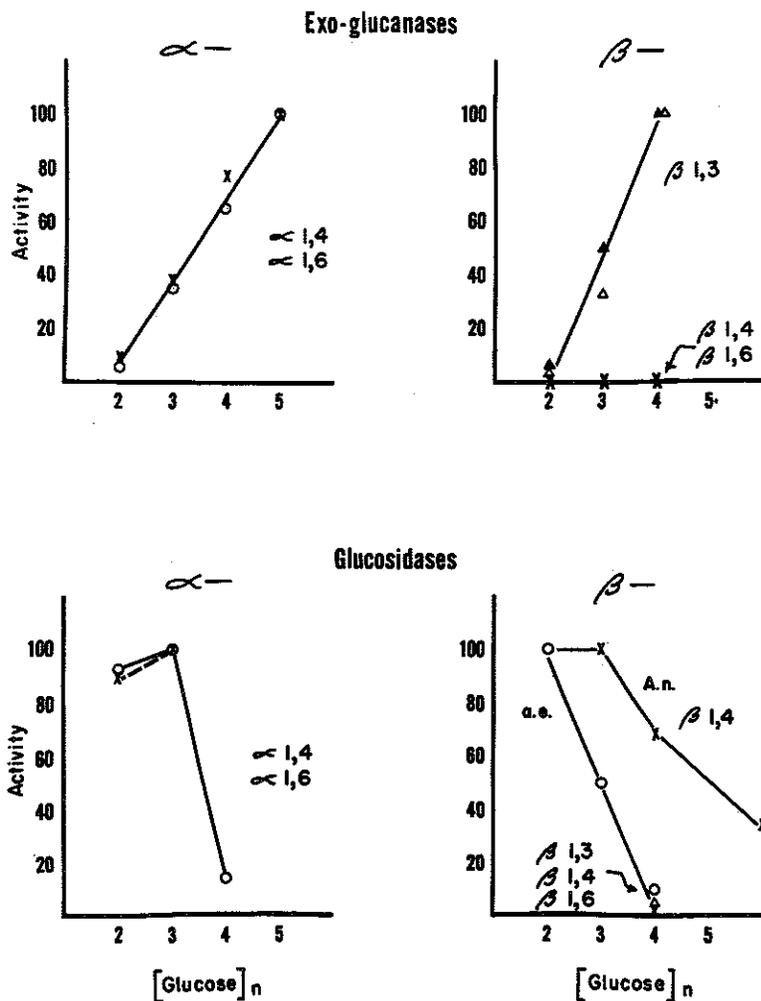


Figure 1. Activity of glucosidases and of exo-gluconases as a function of degree of polymerization (DP) of substrate (Reference 2). The exo- β -glucanases shown here act only on β -1 \rightarrow 3 linkages

and tetramer by the two enzyme types (Figure 1). Cellotetraose (G_4) is much more rapidly hydrolyzed than cellobiose (G_2) by exo- β -1 \rightarrow 4-gluconase (1), and much less rapidly hydrolyzed than cellobiose by cellobiase. Therefore, a high activity ratio (cellotetraose/cellobiose) indicates a predominance of exo-gluconase; a low ratio indicates cellobiase.

Briefly, the procedure is as follows: Substrate (G_4 8 mg./ml., or G_2 4 mg./ml.) in $M/20$ citrate, pH 4.5 (0.5 ml.), is added to an enzyme solution properly diluted (0.5 ml.) and incubated at 40° for 30 minutes.

Three ml. of glucose oxidase solution (Worthington; buffered to pH 7.0) are added, and the incubation continued for 15 minutes. One ml. of 5M HCl is added, and the color measured at 425 m μ . The rate of production of glucose is thereby determined for each substrate.

The values for the ratio G_4/G_2 for pure cellobiase, and for pure exo-glucanase vary depending upon the source of enzyme (2). For our example (Figure 2), we assign for cellobiase a ratio $G_4/G_2 = 0.2$, and for exo- β -1 \rightarrow 4 glucanase $G_4/G_2 = 100$. These values are based on data with purified enzymes, and approach the low and the high limits which the ratios may reach. The curve (Figure 2) is plotted from the formula:

$$\text{Exo-glucanase, \%} = 100 \times \frac{(Y_T/Y_D) - 0.2}{0.99(Y_T/Y_D) + 0.8}$$

Where Y_T = rate of glucose formation from tetramer, and
 Y_D = rate of glucose formation from dimer

(This formula was worked out by H. J. Hoge for this specific case, and for the generalized condition.)

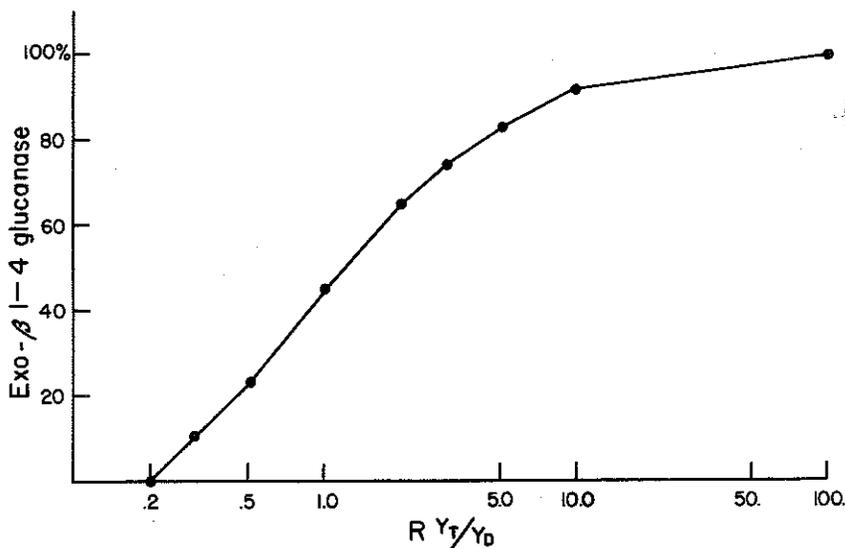


Figure 2. Estimation of exo- β -1 \rightarrow 4-glucanase in solutions containing cellobiase

Plotted from equation shown in text

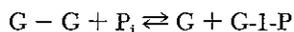
The procedure was applied to a variety of available cellulase solutions (Table I). There are two points to consider in estimating exo- β -1 \rightarrow 4-glucanase. First is the rate at which cellotetraose is hydrolyzed (here indicated in units of enzyme, where one unit yields 0.5 mg. glucose).

Second, is the proportion of this hydrolysis which can be assigned to the exo-enzyme (here represented by the ratio Y_T/Y_D). The product of these two is a measure of exo-glucanase.

The interpretation is not as simple as outlined. There is the complication which we have ignored above—*e.g.*, the contribution of endo- β -1 \rightarrow 4-glucanase to the glucose produced from tetramer. Endo-glucanases act on glucose polymers in a fashion which approaches randomness. End linkages are, however, much less susceptible to attack than internal linkages. This was first observed for the α -amylases (3) and later for the cellulase of *Myrothecium* (4). In some cases, the end linkages are completely resistant, and hydrolysis of the glucan yields only dimer and trimer (salivary α -amylase; endo- α -1 \rightarrow 6-glucanase = dextranase; endo- β -1 \rightarrow 4-glucanase of *Streptomyces*). In other cases, the final hydrolysis products are dimer and monomer, the relative amounts being 5.5:1 for *B. subtilis* α -amylase (3) acting on amylose, and about 7:1 for *Myrothecium* cellulase acting on tetramer (4). There is always the possibility that the latter enzymes may have been contaminated with either exo-glucanase or glucosidase, accounting for the appearance of glucose. This is particularly likely for exo-glucanase since its very existence was unsuspected until quite recently. But until this is proven, we must accept the possibility that some of the glucose produced by the enzyme mixtures acting on tetramer may arise from endo- β -1 \rightarrow 4-glucanase activity. On the other hand, none of the glucose from the dimer hydrolysis is due to endo-enzyme.

Another complication, which we ignore because so few exo-glucanases have been studied, is the possible variability between enzymes from different sources. The basic reaction is common to all, but even now minor differences are recognized, such as the effects of branching of the substrate molecule, and of linkage specificity. There may, also, be "relative" differences in the action on tetramer as compared to action on dimer. These factors will influence the absolute values obtained by the procedure here outlined. (It is quite likely that changes in the method will be required as more information becomes available.)

The only other enzymes which could yield glucose from dimer and tetramer are the phosphorylases (cellobiose phosphorylase, etc.).



Fortunately, these enzymes are not found among the extra-cellular cellulases with which we are involved.

"The proof of the pudding is in the eating." If the above procedure can effectively locate good sources of exo- β -1 \rightarrow 4-glucanase, it will have served its purpose.

Table I. Estimation of Exo- β -1 \rightarrow

Organism	Growth* Substrate	Activity in μ /ml.	
		4 mg./ml. vs. G_2	8 mg./ml. vs. G_3
<i>Trichoderma viride</i> 6a	CB + Lactose	0.24	2.4 [†]
<i>Trichoderma viride</i> 6a	Cellulose	0.6	18.0
<i>Basidiomycete</i> sp. 806	CB + Lactose	0.3	0.6
<i>Basidiomycete</i> sp. 806	Cellulose	4.0	8.0
<i>Chrysosporium pruinatum</i> 826	CB + Lactose	0.9	1.0
<i>Chrysosporium pruinatum</i> 826	Cellulose	8.0	13.0
<i>Penicillium pusillum</i> 137g	CB + Lactose	2.6	2.6
<i>Penicillium pusillum</i> 137g	Cellulose	2.4	9.2
<i>Aspergillus foetidus</i> 328	Bran	60.	18.
<i>Aspergillus luchuensis</i> 873	CB + Lactose	15.0	8.
<i>Aspergillus terreus</i> 442	CB + Lactose	5.4	5.4
<i>Aspergillus terreus</i> 72f	CB + Lactose	1.9	2.0
<i>Penicillium brasilianum</i> 6947	CB + Lactose	1.5	1.2
<i>Penicillium funiculosum</i> 474	CB + Lactose	14.0	7.0
<i>Penicillium parvum</i> 1878	CB + Lactose	9.8	5.0
<i>Fusarium moniliforme</i> 1224	Cellulose	2.5	1.5
<i>Lenzites trabea</i> 1009	Cellulose	0.1	2.0
<i>Myrothecium verrucaria</i> 460	Cellulose	0.18	1.0
<i>Pestalotiopsis westerdijkii</i> 381	Cellulose	0.4	0.4
<i>Polyporus cinnabarinus</i> 8846	Cellulose	0.12	0.5
<i>Polyporus versicolor</i> 1013	Cellulose	0.1	0.3
<i>Stachybotrys atra</i> 94d	CB Acetate	0.3	0.5

* Growth: CB + Lactose = Cellobiose 0.3% + Lactose 0.3%.

[†] Col. 4 \times Col. 6. Activity 40°C., 30 min.; μ = 0.5 mg. glucose.

According to our data (Table I) and our interpretation of them, most cellulolytic preparations contain exo- β -1 \rightarrow 4-glucanase. *Trichoderma viride* QM6a cultures produce the most exo-enzyme and the best proportion of exo-enzyme to cellobiase. While *Aspergillus foetidus* is just as active as *Trichoderma* on the tetramer, most of this activity is attributable to cellobiase. All good cellulolytic fungi produce much more of the exo-enzyme when grown on cellulose than when grown on cellobiose plus lactose.

The data further suggest that *Chrysosporium pruinatum*, and *Penicillium pusillum* are good sources of exo- β -1 \rightarrow 4 enzyme, if one does not object to the cellobiase present. *Polyporus cinnabarinus* produces less exo-enzyme, but as in *Trichoderma viride*, it is nearly free of cellobiase. *Streptomyces* sp. B814 is the only organism which appears to lack exo-glucanase in its cellulase complex.

Our concern (above) about interference by endo- β -1 \rightarrow 4-glucanase was unwarranted. The Y_T/Y_D values are relatively constant for a par-

4-glucanase in Cellulolytic Filtrates

R G_4/G_2	<i>Exo-glucanase</i>		C_x $\mu/ml.$	<i>Lactone</i> <i>Inhib.</i>
	% ^b	$\mu/ml.$ ^c		
10.*	92.	2.2*	2.	
30.	96.	17.3	38.	9. %
2.	65.	0.4	1.	
2.	65.	5.3	32.	68.
1.1	47.	0.5	1.	
1.6	58.	7.5	42.	83.
1.0	45.	1.2	10.	
3.8	80.	7.4	88.	40.
0.3	11.	2.0	22.	81.
0.54	26.	2.1	0.8	95.
1.0	45.	2.4	5.0	
1.1	47.	1.9	3.	
0.75	36.	0.4	2.	
0.5	23.	1.6	0.3	94.
0.5	23.	1.2	3.	
0.6	29.	0.4	2.8	61.
20.0	94.	1.9	5.4	11.
5.	83.	0.8	46.	NT
1.	45.	0.2	50.	72.
4.	80.	0.4	45.	NT
3.	74.	0.2	2.9	19.
1.7	60.	0.3	5.9	NT

^b From Figure 2.

ticular organism over wide ranges of C_x concentration (Table I). Thus, the R value for *Basidiomycete* 806 is 2, both for a solution containing 1 C_x unit and for a solution containing 32 C_x units per ml. C_x activity is determined with carboxymethyl cellulose as substrate. It is chiefly a measure of endo- β -1 \rightarrow 4-glucanase activity, since adding substituents—*e.g.*, carboxymethyl—to a substrate inhibits action of exo-glucanase much more than it inhibits action of endo-glucanase. As a result, it should be possible to find much exo-glucanase in a culture filtrate low in endo-glucanase.

An alternate means of estimating exo-glucanase is by means of glucono-lactone. This agent inhibits cellobiase, but is without effect on exo- β -glucanases (2). A ratio of lactone to substrate of 0.1 (by weight) is suitable for making the determination. The results (Figure 3) show that the procedure is useful only for solutions of relatively high exo-glucanase. Cellotriose or cellotetraose may be used as substrates, but cellobiose cannot be used because of its resistance to hydrolysis by the exo-enzyme.

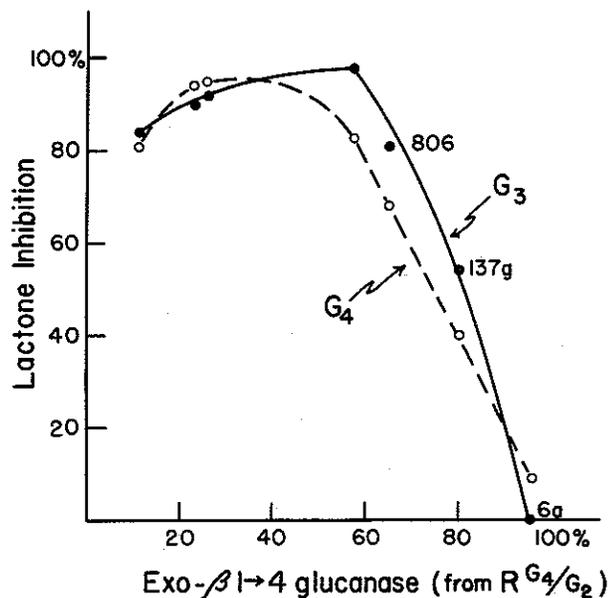


Figure 3. Estimation of $\text{exo-}\beta\text{-1} \rightarrow 4\text{-glucanase}$ from inhibition by gluconolactone. $R \text{ Lactone/substrate} = 0.1 \text{ (wt.)}$

●—● Cellotriose (G_3) as substrate; ○—○ cellotetraose (G_4) as substrate; 6a = *Trichoderma viride*; 137g = *Penicillium pusillum*; 806 = *Basidiomycete sp.*

Literature Cited

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Discussion

Dr. King: "The method appears to be suitable for the detection of $\text{exo-}\beta\text{-1} \rightarrow 4\text{-glucanase}$. I believe, however, that one needs more data on

exo-glucanases from different organisms—*i.e.*, how similar or how different may these be in their relative activities on the tetramer and the dimer ($R G_4/G_2$)."

Dr. Reese: "This is quite right. The proposed method is to help us find new exo-glucanases. Then we can determine their $R G_4/G_2$ values, and refine the procedure if need be."