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Transglycosylation Activities of Exo- and Endo-Type Cellulases from *Irpex lacteus* (*Polyporus tulipiferae*)

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Two highly purified cellulases, Ex-1 [exo-type, exo-cellobiohydrolase, EC 3.2.1.91] and En-1 [endo-type, EC 3.2.1.4] obtained from Driselase, a commercial enzyme preparation from *Irpex lacteus* (*Polyporus tulipiferae*), were used in this work. Both cellulases produced ^{14}C-cellooligosaccharides such as ^{14}C-G_2 and ^{14}C-G_3 by transglycosylation when G_3, G_5, or β-PNPC was used as a donor and ^{14}C-G_1 as an acceptor. However, the transglycosylation activity of Ex-1 was far higher than that of En-1.

When Ex-1 or En-1 was incubated with β-PNPG only, no *p*-nitrophenol was released, but it was readily released when G_3 was added to the reaction mixture. In this reaction, the optimal donor (G_3) concentration for Ex-1 was 1.0 mM, and the optimal pH values of Ex-1 were at 2.7 and 3.7 for β-PNPG and β-PG as acceptors, respectively, these values being far lower than the ordinary optimal pH values of the cellulase (4.0–5.0).

It is well known that carbohydrases have transglycosylation activity in general. However, few studies have been done on such activity of cellulases [1,4-β-D-glucan 4-glucanohydrolases EC 3.2.1.4], except for the work of Nisizawa and Hashimoto on a cellulase preparation from *Irpex lacteus* (*1*), and those of Toda *et al.* (*2*) and Okada and Nisizawa (*3*) on several cellulase preparations from *Trichoderma viride*. However, the cellulase preparations used in those studies were of an endo-type and no such work seems to have been carried out so far on exo-type cellulase.

In previous papers (*4–6*), we reported the enzymatic properties of Ex-1 (an exo-cellulase), which has a different mode of action from that of En-1 (an endo-cellulase) in the hydrolysis of cellulosic substrates. In addition, Ex-1 hydrolyzed G_4 and G_6 as readily as the long-chain substrates

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Abbreviations: G_1, glucose; G_2, cellobiose; G_3, cellotriose; G_4, cellotetraose; G_5, cellopentaose; G_6, cellohexaose; ^{14}C-G_1, ^{14}C-glucose; ^{14}C-G_2, ^{14}C-cellobiose; ^{14}C-G_3, ^{14}C-cellotriose; β-PNPG, *p*-nitrophenyl β-D-glucoside; β-PNPC, *p*-nitrophenyl β-D-cellobioside; β-PG, phenyl β-D-glucoside; SDS, sodium dodecyl sulfate.

to produce exclusively G_2 at the early stage of hydrolysis. However, it produced a small amount of G_1 and a trace of G_3 on prolonged incubation with G_4 and G_6, whereas En-1 has produced large amounts of these sugars at the early stage of hydrolysis. Moreover, G_1 was also detectable, though in a very small amount, besides G_2 on prolonged incubation of Ex-1 with long-chain substrates, such as viscose-rayon, absorbent cotton and *Valonia*-cellulose. These observations could not be explained at that time.[2]

We then considered whether the production by Ex-1 of G_1 and G_3 besides G_2 might be related to its possible transglycosylation activity. Thus, we examined this possibility using G_3, G_5, and β-PNPC as substrates for this exo-cellulase.

Furthermore, we also examined the features of the possible two-step reaction "transglycosylation and subsequent hydrolysis" by Ex-1, using a reaction mixture containing G_3 as a glycosyl donor and β-PNPG or β-PG as an acceptor which is itself resistant to the action of Ex-1. We also used En-1 (an endo-cellulase) for comparison.

MATERIALS AND METHODS

Enzyme Source—The exo-cellulase (Ex-1) used in the present work was obtained by the reported procedure (*4*), from Driselase, a commercial product of *Irpex lacteus* (*Polyporus tulipiferae*) manufactured by Kyowa Hakko Co. The endo-cellulase (En-1) was the same enzyme preparation as had been used in a previous work (*6*). The molecular weights of these two cellulases were 65,000 and 15,500, respectively.

Substrates—β-PNPG and β-PG were products of Nakarai Chemical Co. β-PNPC was synthesized by the modified method of Nisizawa and Wakabayashi (*7*), mp = 244–245°C $[\alpha]_D^{16.5} = -83.3°$ ($c = 1.5$, 40% CH_3OH). G_3 and G_5 were prepared from fine crystalline cellulose powder (Toyo Roshi Co.) by the method of Miller *et al.* (*8*), and were purified by paper chromatography. ^{14}C-G_1 was a product of The Radiochemical Centre Ltd., and was purified by paper chromatography. Before use, it was diluted to a specific activity of 1.73 μCi/mg glucose with highly purified glucose.

Enzyme Reactions—*i*) The reaction mixture for the possible two-step reaction consisted of an appropriate concentration of enzyme (Ex-1 or En-1), G_3 and β-PNPG in 0.05 M sodium acetate buffer, pH 4.0. After incubation of the mixture at 30°C for an appropriate period, 1 ml of 1% Na_2CO_3 was added to 0.2 ml of reaction mixture and *p*-nitrophenol liberated was estimated colorimetrically at 420 nm. When β-PG was used as an acceptor, phenol liberated was measured by the Folin method (*9*).

ii) The reaction mixture for hydrolysis consisted of 0.5 μmol of substrate, 0.05 M sodium acetate buffer, pH 4.0 and a cellulase (2.4×10^{-3} μmol or 2.5×10^{-3} μmol of Ex-1 or En-1) in a total volume of 0.5 ml. After various periods of incubation at 30°C, a 0.2 ml aliquot was removed from the reaction mixture and the reducing power was measured by the method of Somogyi (*10*) and Nelson (*11*).

Paper Chromatography—After an appropriate period of incubation at 30°C, a 0.1 ml aliquot of reaction mixture was inactivated by heat and then spotted on Whatman No. 1 paper together with authentic sugars as standards. Chromatograms were developed twice by the ascending technique with 1-butanol : pyridine : water (6 : 4 : 3, v/v) at room temperature. Spots of transglycosylation or hydrolysis products were detected by a dipping procedure using the silver nitrate reagent (*12*).

Autoradiography of Transglycosylation Products—The reaction mixture consisted of 0.87 μCi/mg glucose, 0.05 M sodium acetate buffer, pH 4.0, 3.4×10^{-3} μmol of Ex-1 or 3.2×10^{-3} μmol of En-1 and a desired amount of substrate (6.3 μmol for G_3, 3.9 μmol for G_5, and 6.9 μmol for β-PNPC) in a total volume of 0.8 ml. After incubation at 30°C for various periods, a 0.2 ml aliquot of the mixture was analyzed by paper chromatography to determine the products formed. The autoradiograms of transglycosylation products containing ^{14}C-G_1 were developed on Fuji X-ray film by exposure at room temperature for 7 days.

Polyacrylamide Gel Electrophoresis of Ex-1—SDS polyacrylamide gel electrophoresis of Ex-1 was performed in 10% acrylamide gel containing 1% SDS by the method of Weber and Osborn (*13*), as described in a previous paper (*6*). As a test sample, 5 μg of Ex-1 was used. The enzyme

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mixture was analyzed for *p*-nitrophenol released. As shown in Fig. 5, the release of *p*-nitrophenol from β-PNPC by Ex-1 was far greater than that by En-1.

Effect of Enzyme Concentration on the Release of p-Nitrophenol—The reaction was carried out in a buffered solution containing 3.3 μmol G_3 as a donor and 2.0 μmol β-PNPG as an acceptor in a total volume of 0.2 ml, at various enzyme concentrations; 0.2×10^{-3} μmol to 1.3×10^{-3} μmol for Ex-1 and 0.8×10^{-3} μmol to 8.1×10^{-3} μmol for En-1. After incubation for 3 h, *p*-nitrophenol released in each reaction mixture was measured.

As shown in Fig. 6, the plots of released *p*-nitrophenol against the concentration of each enzyme were linear for both Ex-1 and En-1. This indicates that the liberation of *p*-nitrophenol is due to the enzymatic reaction.

Effect of Substrate Concentration on the Release of p-Nitrophenol—The reaction was carried out for 3 h in a buffered solution containing various concentrations of G_3 (0.02 μmol to 4.0 μmol) as a donor, 3.3 μmol β-PNPG as an acceptor and Ex-1 (1.2×10^{-3} μmol) or En-1 (2.2×10^{-3} μmol) in a total volume of 0.2 ml. *p*-Nitrophenol released is plotted in Fig. 7. In the reaction mixture of Ex-1, *p*-nitrophenol increased in proportion to the concentration of G_3, and reached a maximum at 1.0 mM G_3. This suggests that the liberation of *p*-nitrophenol under these experimental conditions is due to the transglycosylation reaction, and the cellulase action may be inhibited by higher concentration of G_3. In particular, the transglycosylation activity of En-1 was strongly inhibited even by such a low concentration of G_3 as 0.2 mM. In this respect, En-1 is very different from Ex-1.

In order to investigate the inhibition of Ex-1 action by G_3 in detail, the following experiment was carried out using β-PNPC in place of β-PNPG. The reaction was carried out in a buffered solution containing 2.2 μmol β-PNPC, various concentrations of G_3 (0.04 μmol to 8 μmol) and 1.9×10^{-3} μmol Ex-1 in a total volume of 0.4 ml. Incubation was carried out for 20 min or 60 min, and *p*-nitrophenol liberated is shown in Fig. 8. The

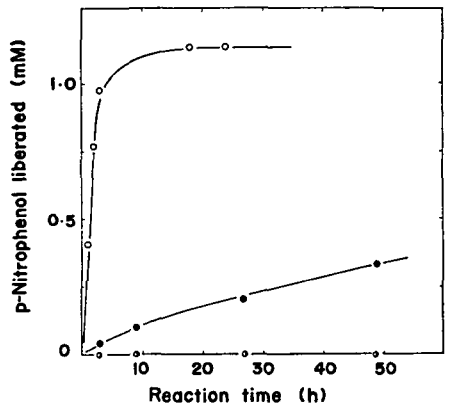

Fig. 5. Release of *p*-nitrophenol from β-PNPC by Ex-1 or En-1. ○, Ex-1; ●, En-1; ◑, hydrolysis of β-PNPC without enzyme.

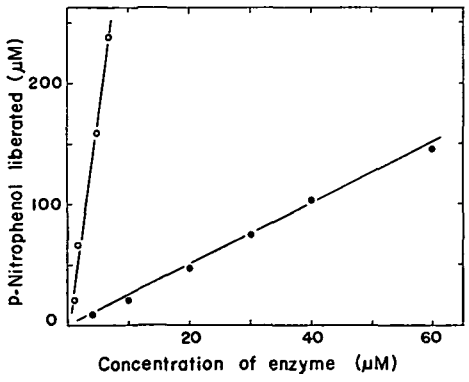

Fig. 6. Effect of enzyme concentration on the release of *p*-nitrophenol from a mixture of G_3 and β-PNPG catalyzed by Ex-1 or En-1. ○, Ex-1; ●, En-1.

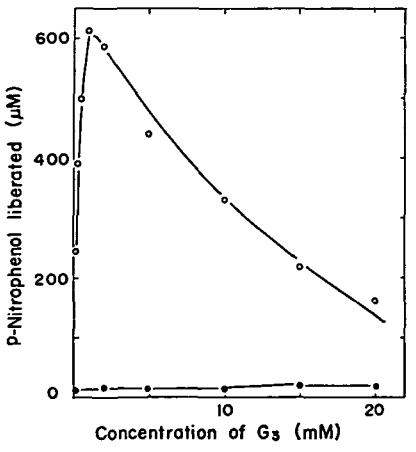

Fig. 7. Effect of the concentration of G_3 as a donor on the release of *p*-nitrophenol from reaction mixture containing β-PNPG as an acceptor, catalyzed Ex-1 or En-1. ○, Ex-1; ●, En-1.

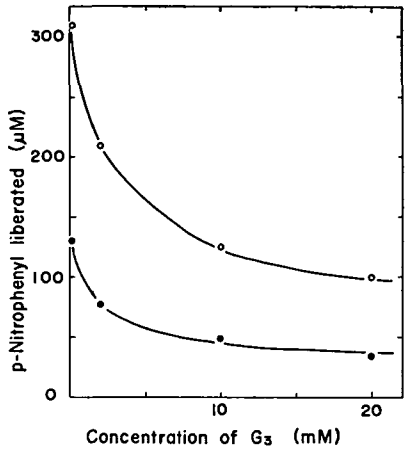

Fig. 8. Inhibition of G_3 during the hydrolysis of β-PNPC by Ex-1. ●, 20 min incubation; ○, 60 min incubation.

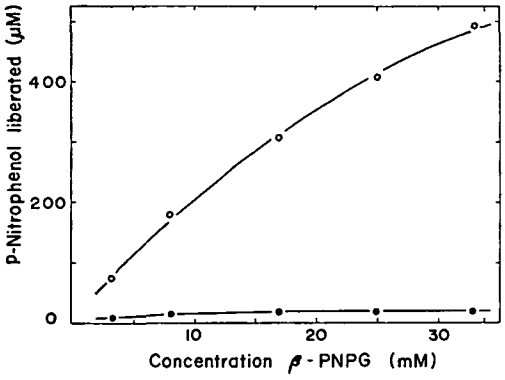

Fig. 9. Effect of the concentration of β-PNPG as an acceptor on the release of *p*-nitrophenol from reaction mixture containing G_3 as a donor, catalyzed by Ex-1 or En-1. ○, Ex-1; ●, En-1.

results showed that the hydrolysis of the aglycone linkage of β-PNPC by Ex-1 was greatly inhibited by G_3 at high concentrations.

Effect of Acceptor Concentration on the Release of p-Nitrophenol—The reaction was carried out in a buffered solution containing 2.0 μmol G_3 as a donor, 1.9×10^{-3} μmol Ex-1 or 2.2×10^{-3} μmol En-1 and various concentrations of β-PNPG (0.6 μmol to 6.6 μmol) as an acceptor in a total volume of 0.2 ml. After incubation for 3 h, *p*-nitrophenol liberated was measured. Its amount increased remarkably with increasing concentration of β-PNPG, while the amount of *p*-nitrophenol released from β-PNPG by En-1 was only small and did not increase with increasing concentration of β-PNPG (Fig. 9). The result for En-1 showed that the inhibition of En-1 by G_3 (at 10 mM) was not reversed by the increasing concentrations of β-PNPG used as an acceptor.

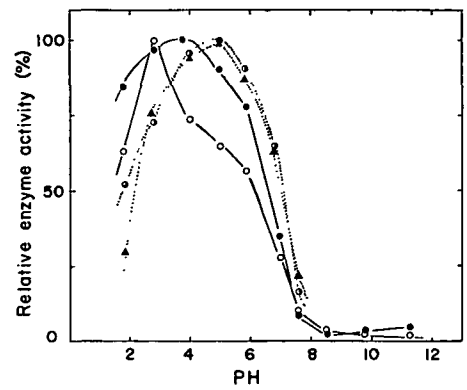

Fig. 10. Effect of pH on the release of *p*-nitrophenol catalyzed by Ex-1, using β-PNPG or β-PG as an acceptor and G_3 as a donor substrate, and effects of pH on the hydrolysis of β-PNPC and cotton. ○, β-PNPG; ●, β-PG; ◑, β-PNPC; ▲, cotton. Similar patterns of pH-activity curve were obtained from the hydrolysis of Avicel, viscose-rayon and alkali-cellulose catalyzed by Ex-1.

Effect of pH on Transglycosylation by Ex-1—Transglycosylation activity of Ex-1 was examined in a pH range from 1.8 to 11.2, using 0.02 M Britton-Robinson wide range buffer. The reaction was carried out in a buffered solution containing 0.2 μmol G_3 as a donor, 3.3 μmol β-PNPG or β-PG as an acceptor and 1.9×10^{-3} μmol Ex-1 in a total volume of 0.2 ml. After incubation for 2 h, *p*-nitrophenol or phenol released was estimated. The results are shown in Fig. 10. The profile of the pH-dependence of transglycosylation by Ex-1 showed maxima at about 2.7 and 3.7 for β-PNPG and β-PG, respectively.

On the other hand, the effects of pH on the hydrolysis of β-PNPC, Avicel, cotton, viscose-rayon and alkali-cellulose by Ex-1 were examined in the presence of 2.4×10^{-3} μmol Ex-1 and 2.7 μmol β-PNPC (total volume of 0.2 ml) or 2% cellulosic substrate (total volume of 0.5 ml) in the same buffered solution for comparison (Fig. 10).

DISCUSSION

It was found in this work that the transglycosylation by Ex-1 (an exo-cellulase) is similar to that by En-1 (an endo-cellulase) in terms of product patterns, but different in terms of capacity. This may be due to the mutarotation property of Ex-1 (in an upward direction) being similar to that of En-1 (*4*, *6*); the anomeric carbon atom of the products retains the same configuration upon the bond cleavage (*3*, *14*, *15*).

When ^{14}C-G_1 was used as an acceptor and G_3, G_5, or β-PNPC as a donor in the reaction mixture of Ex-1, ^{14}C-G_3 was predominantly produced. A similar trend was observed with En-1, except for much lower transglycosylation activity, possibly due to very slow attack of this cellulase on G_3 and β-PNPC as compared with that by Ex-1 (Table I). However, the production of ^{14}C-G_2, even though ^{14}C-$G_3 > {}^{14}C$-G_2, is difficult to explain without the assumption that Ex-1 can transfer the G_1-residue of the donor (G_3 or G_5) *in addition to the G_2-residue-transferring activity.*

This assumption may be supported by the fact that Ex-1, which usually cuts off a G_2 residue from lower and higher cellulosic substrates, attacked β-PNPC (corresponding to G_3 in having two β-glucosic bonds) readily at both the holoside and aglycone linkages to release G_1 or *p*-nitrophenol just as β-glucosidase does.[3] Therefore, Ex-1 may be expected to split the G_1 residue at the non-reducing end of G_3 and transfer it to ^{14}C-G_1 to form ^{14}C-G_2. When G_5 was used as donor, the G_1 residue-transfer would occur after splitting of the donor into G_3 and G_2. Thus, it seems reasonable to assume that Ex-1, which splits off G_2-residues from longer chain substrates, becomes able to split off G_1-residues too from the non-reducing end in the case of shorter chain substrates such as G_3 and β-PNPC. If this is the case, Ex-1 acts as a real cellobiohydrolase upon longer chain substrates, but it acts as a glucohydrolase too (like β-glucosidase or even like an endo-cellulase) upon shorter chain substrates. In fact, it has been reported that various purified cellulases, which have often been named 1,4-β-D-glucan cellobiohydrolase by some workers, produced G_1 besides G_2 from even-numbered cellooligosaccharides such as G_4 and G_6 (*16–19*). Consequently, the exo-type cellulases should not be called 1,4-β-D-glucan "cellobiohydrolase" in a strict sense.

We further found in this work that *p*-nitrophenol was released from β-PNPG by En-1 as well as Ex-1 upon addition of G_3 to the reaction mixture, while no liberation of *p*-nitrophenol occurred in the absence of G_3. This may be explained by the assumption that the major part of G_3 was split by these cellulases into G_2 and G_1, followed by transfer of the glucosyl or cellobiosyl residue to β-PNPG to produce β-PNPC, from which *p*-nitrophenol is liberated by the action of these cellulases. Moreover, since Ex-1 attacks G_3 as well as β-PNPC much more readily than En-1, the aglycone release by Ex-1 must proceed faster than that by En-1, as can be clearly seen from the large difference of the time course curves with these cellulases (Fig. 5).

However, it should be noted here that the optimal pH values in this apparently two-step reaction by Ex-1 were shifted to such low ones as 2.7 and 3.7 for β-PNPG and β-PG used as an acceptor, respectively (Fig. 10). In contrast, the ordinary hydrolysis (H_2O acts as acceptor of glycosyl residue in a one step-reaction) by Ex-1 of not only long-chain substrates such as Avicel, cotton, viscose-rayon, and alkali-cellulose but also the aglycone linkage of β-PNPC was carried out with maximal activities at pH 4.0 to 5.0. Therefore, the pH values found in this reaction were very much lower than the ordinary one. From these results, it seems that the total reaction caused by this apparent two-step enzymatic reaction, "transglycosylation and subsequent hydrolysis" between donor (substrate; G_3 in the present work) and acceptor (non-substrate; β-PNPG in the present work), does not take place by a simple mechanism.

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REFERENCES

1. Nisizawa, K. & Hashimoto, Y. (1959) *Arch. Biochem. Biophys.* **81**, 211–222
2. Toda, S., Suzuki, H., & Nisizawa, K. (1968) *J. Ferment. Technol.* **46**, 711–718
3. Okada, G. & Nisizawa, K. (1975) *J. Biochem.* **78**, 297–306

4. Kanda, T., Nakakubo, S., Wakabayashi, K., & Nisizawa, K. (1978) *J. Biochem.* **84**, 1217–1226
5. Kanda, T., Nakakubo, S., Wakabayashi, K., & Nisizawa, K. (1979) in *Advances in Chemistry Series* (Brown, R.D., Jr. & Jurasek, L., eds.) Vol. 181, pp. 211–236, American Chemical Society, Washington, D.C.
6. Kanda, T., Wakabayashi, K., & Nisizawa, K. (1980) *J. Biochem.* **87**, 1625–1634
7. Nisizawa, K. & Wakabayashi, K. (1952) *Seikagaku* (in Japanese) **24**, 36–43
8. Miller, G.L., Dean, J., & Blum, R. (1960) *Arch. Biochem. Biophys.* **91**, 21–26
9. Folin, O. & Ciocalteu, U. (1927) *J. Biol. Chem.* **73**, 627–650
10. Somogyi, M. (1952) *J. Biol. Chem.* **195**, 19–23
11. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380
12. Trevelyan, W.E., Procter, D.P., & Harrison, J.S. (1950) *Nature* **166**, 444–445
13. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
14. Nisizawa, K., Kanda, T., Shikata, S., & Wakabayashi, K. (1978) *J. Biochem.* **83**, 1625–1630
15. Hehre, E.J., Okada, G., & Genghof, D.S. (1969) *Arch. Biochem. Biophys.* **135**, 75–89
16. Halliwell, G. & Griffin, M. (1973) *Biochem. J.* **135**, 587–594
17. Wood, T.M., McCrae, S.I., & Macfarlane, C.C. (1980) *Biochem. J.* **189**, 51–65
18. Berghem, L.E.R. & Pettersson, L.G. (1973) *Eur. J. Biochem.* **37**, 21–30
19. Streamer, M., Eriksson, K-E., & Pettersson, B. (1975) *Eur. J. Biochem.* **59**, 607–613