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Note

Hydrolysis of (1→3)-α-D-glucosidic linkages in oligosaccharides and polysaccharides by *Cladosporium resinae* exo-(1→3)-α-D-glucanase

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Many micro-organisms[1–3] are induced to secrete a variety of glucanases and glucosidases capable of hydrolysing glucan when they are grown in media containing the glucan as sole carbohydrate source. Examples are provided by *Trichoderma viride*[2] and *Penicillium funiculosum*[3], both of which release endo- and exo-(1→3)-α-D-glucanases when grown in the presence of (1→3)-α-D-glucans, and by *Cladosporium resinae*, which has the unique property of producing several enzymes[4] capable of hydrolysing substrates containing (1→3)-α-D-glucosidic linkages, even when grown in the absence of (1→3)-α-D-glucan. A previous report[5] described the separation from *C. resinae* cell-free filtrates of an endo-(1→3)-α-D-glucanase having little or no action on nigerose, and another enzyme which displayed high activity towards nigerose but which was without any detectable action on water-insoluble mutan. We now report on the properties and specificity of the latter enzyme.

The culture conditions for the growth of *C. resinae* QM7998 were described previously[5]. The mycelia were harvested 14 days after inoculation, and the cell-free filtrate (150 ml) was fractionated with acetone. The precipitate in the 50–66% acetone fraction was dissolved in 10 ml of 20mM sodium phosphate buffer (pH 6.0). This solution contained, per ml, protein (6.2 mg), endo-(1→3)-α-D-glucanase (16 i.u.), mycodextranase (8.3 i.u.), amyloglucosidase (113 i.u.), (1→3)-β-D-glucanase (105 i.u.), maltase (48 i.u.), and exo-(1→3)-α-D-glucanase (7.7 i.u.). Amyloglucosidase, (1→3)-β-D-glucanase, and maltase activities were determined by measuring the release of D-glucose from glycogen, laminarin, and maltose, respectively. The activities of the other enzymes were determined as described previously[5]. Endo-(1→3)-α-D-glucanase, mycodextranase, and the bulk of the amyloglucosidase were removed by chromatography on DEAE-Sephadex, and exo-(1→3)-α-D-glucanase was eluted together with (1→3)-β-D-glucanase by 0.26M sodium chloride[5]. The fractions containing the highest activity towards nigerose were combined, and the exo-(1→3)-α-D-glucanase activity was completely separated from (1→3)-β-D-glucanase by isoelectric focusing on a glycerol density-gradient and a pH gradient of 3–6. The recovery of exo-(1→3)-α-D-glucanase was 59%, and the isoelectric point was at pH 4.7. The

enzyme preparation was contaminated with amyloglucosidase, the rate of hydrolysis of maltose being 43 relative to that of nigerose (100). Isomaltose was not a substrate.

The effect of various conditions on the activity of the enzyme was tested in digests (0.25 ml) containing nigerose (50 μg), sodium citrate buffer (25mM), and enzyme. The reaction was terminated by the addition of 0.25 ml of 0.5M Tris buffer (pH 7), and the release of D-glucose was determined with D-glucose oxidase reagent in 0.5M Tris buffer. The optimum temperature for the activity of the enzyme was 50°, and maximum activity and stability were shown at pH 5.

Action of exo-(1→3)-α-D-glucanase on oligosaccharides. — The relative activity towards oligosaccharides of the nigerose series was tested in digests containing substrate (0.75 μmol), sodium citrate buffer (pH 5, 25mM), and enzyme (0.004 i.u.). Nigerose, nigerotriose, and nigerotetraose were hydrolysed at equivalent rates, the release of D glucose after 30 min being 21.4, 11.2, and 10.2 μg, respectively. The value of K_m for nigerose was 1.4×10^{-3}M.

The enzyme was incubated with 3-α-isomaltosyl-D-glucose and with 3^3-α-D-glucosylisomaltotriose under the conditions described above for nigerose oligosaccharides. There was no release of D-glucose from 3-α-isomaltosyl-D-glucose during 20 h, indicating that the enzyme could only hydrolyse (1→3)-α-D-linkages that occur at the non-reducing end of oligosaccharides. Glucose (1 mol. prop.) was released from 3^3-α-D-glucosylisomaltotriose (B_4), showing that adjacent (1→6)-linkages were not inhibitory. The products of the reaction, after separation by p.c., were shown to be D-glucose and isomaltotriose. Hydrolysis of B_4 with exo-(1→3)-α-D-glucanase followed by an incubation with *Streptococcus mitis* (1→6)-α-D-glucan glucohydrolase[6] gave a complete conversion into D-glucose. The branched oligosaccharides B_5 and B_6 (mainly 3^3-α-D-glucosylisomaltosaccharides), which are among the products of *P. funiculosum* dextranase action on *Leuconostoc mesenteroides* NRRL B-512(F) dextran[7], were not substrates for the enzyme. Apparently, *C. resinae* exo-(1→3)-α-D-glucanase could not hydrolyse (1→3)-α-D-glucosidic branch-linkages, and the absence of glucose release from B_5 and B_6 confirmed that these oligosaccharides did not contain isomers having the (1→3) linkage located at the non-reducing terminal.

It was recently proposed[7] that the pentasaccharide fraction (B_5) obtained from various dextrans after hydrolysis with *P. funiculosum* dextranase did not consist entirely of the branched component 3^3-α-D-glucosylisomaltotetraose (rB_5), but also contained a proportion of 3^3-α-isomaltosylisomaltotriose (sB_5). Only the latter isomer was susceptible to hydrolysis by *S. mitis* (1→6)-α-D-glucan glucohydrolase, and the products were D-glucose and a tetrasaccharide. The subsequent action of

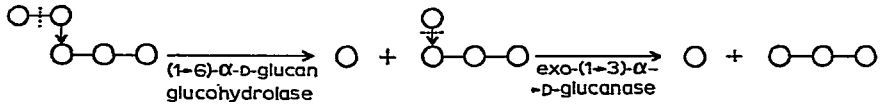

Fig. 1. Hydrolysis of the mixed-linkage pentasaccharide sB_5 by *S. mitis* (1→6)-α-D-glucan glucohydrolase, followed by *C. resinae* exo-(1→3)-α-D-glucanase. ○, D-Glucose residue; ——, (1→6)-α-D-glucosidic linkage; ↓, (1→3)-α-D-glucosidic linkage. Dotted lines drawn at right angles to a linkage indicate the point of hydrolysis.

C. resinae exo-(1→3)-α-D-glucanase on the tetrasaccharide yielded D-glucose and isomaltotriose, thus proving its structure to be 3^3-α-D-glucosylisomaltotriose, and confirming the structure proposed for sB_5 (Fig. 1).

Action of exo-(1→3)-α-D-glucanase on polysaccharides. — The enzyme was unable to hydrolyse (1→3)-α-D-glucosidic branch-linkages in dextrans. The soluble dextran synthesized by *Streptococcus mutans* glucosyltransferase-S, which contains 32% of (1→3)-α-D-glucosidic branch-linkages[8], was virtually resistant to hydrolysis. After incubation of the glucan (1 mg) with exo-(1→3)-α-D-glucanase (0.1 unit) for 48 h, the release of D-glucose was less than 1%. Following this treatment, the glucan, which was previously resistant to hydrolysis by exo-dextranases, became susceptible to hydrolysis by *Arthrobacter globiformis* (1→6)-α-D-glucan isomaltohydrolase (G_2-dextranase)[9]; the limit of hydrolysis was 16%. This result indicated that *C. resinae* exo-(1→3)-α-D-glucanase removed one or more (1→3)-linked D-glucose residues from the non-reducing terminals, thus allowing the exo-dextranase to recognize the (1→6)-linked main-chain.

Incubation of exo-(1→3)-α-D-glucanase with *L. mesenteroides* NRRL B-1355 soluble dextran also resulted in the release of a small amount of D-glucose (~1%). The "limit" dextran was then hydrolysed by G_2-dextranase to give mainly isomaltose. The amount of the minor product 3^2-α-D-glucosylisomaltose seen on paper chromatograms was far less than that obtained from the native dextran. It is concluded that exo-(1→3)-α-D-glucanase released D-glucose by hydrolysis of (1→3)-linkages at non-reducing terminals of the dextran (Fig. 2, *A*), and that the trisaccharide released by G_2-dextranase from B-1355 soluble-dextran (Fig. 2, *B*) is mainly derived from the ends of the chains. The extent of hydrolysis of the dextran by the two enzymes acting together did not exceed that obtained by G_2-dextranase alone (61%), showing that newly exposed, inner (1→3)-α-D-glucosidic linkages that were not by-passed or hydrolysed by G_2-dextranase were also resistant to exo-(1→3)-α-D-glucanase.

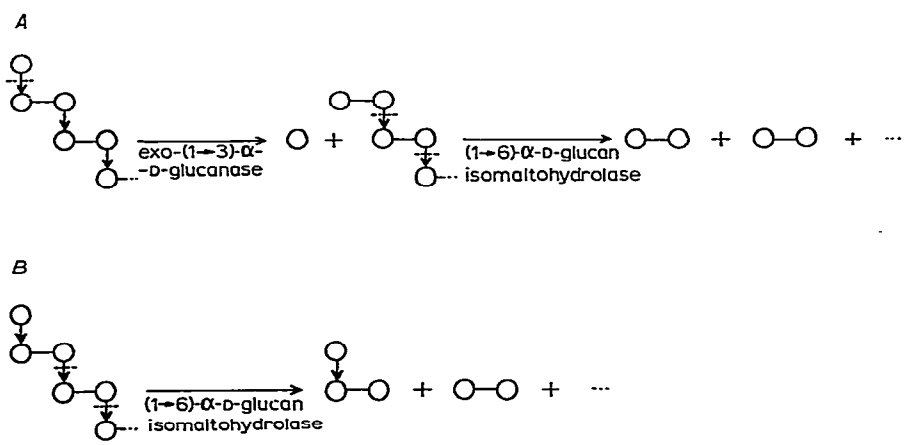

Fig. 2. Hydrolysis of *L. mesenteroides* B-1355 soluble dextran by G_2-dextranase: with (*A*) and without (*B*) prior treatment with exo-(1→3)-α-D-glucanase. A fragment of the structure proposed for a non-reducing terminal of the dextran is shown. The symbols are the same as in Fig. 1.

The ability of the enzyme to release D-glucose from some of the non-reducing terminals of certain polysaccharides, as well as oligosaccharides, supports its description as an exo-α-D-glucanase. Furthermore, its specificity towards nigerose is superior to that shown by plant[10] and animal[11] α-D-glucosidases, most of which will hydrolyse isomaltose. Chiba and Shimomura[12] recently compared the relative rates of hydrolysis of nigerose and maltose reported for a variety of highly purified α-D-glucosidases. Not one enzyme was significantly more active towards nigerose than towards maltose, and nigerose activities were in the range 26–105% of those towards maltose. Activities towards nigerotriose were not reported.

(1→3)-α-D-Glucanases are generally produced by fungi that grow on other fungi[13], and their ability to release a variety of enzymes capable of completing the hydrolysis of fungal cell-wall (1→3)-α-D-glucans is an obvious benefit. The two enzymes produced by *C. resinae* can be utilized with advantage as experimental tools for exploring the structure of the glucans synthesized from sucrose by *L. mesenteroides* strains and by oral strains of *Streptococcus spp.*[5,8].

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