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Carbohydrate Research 332 (2001) 373–379

CARBOHYDRATE
RESEARCH

The hydrolytic and transferase action of alternanase on oligosaccharides☆

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Received 12 February 2001; accepted 9 April 2001

Abstract

Alternanase is an enzyme which endo-hydrolytically cleaves the α-(1 → 3), α-(1 → 6)-linked D-glucan, alternan. The main products are isomaltose, α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-D-Glc and the cyclic tetrasaccharide *cyclo* {→ 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 →}. It is also capable of acting on oligosaccharide substrates. The cyclic tetrasaccharide is slowly hydrolyzed to isomaltose. Panose and the trisaccharide α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc both undergo transglycosylation reactions to give rise to the cyclic tetrasaccharide plus D-glucose, with panose being converted at a much faster rate. The tetrasaccharide α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc is hydrolyzed to D-glucose plus the trisaccharide α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-D-Glc. Alternanase does not act on isomaltotriose, theanderose (6^{Glc}-*O*-α-D-Glc*p* sucrose), or α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-α-D-Glc. The enzyme releases 4-nitrophenol from 4-nitrophenyl α-isomaltoside, but not from 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl α-isomaltotrioside, or 4-nitrophenyl α-isomaltotetraoside.

Keywords: Alternan; Alternanase; Cyclic oligosaccharide; Panose; Isomaltose; Transglycosylation

1. Introduction

Alternanase is an endoglucanase that cleaves the α-(1 → 3), α-(1 → 6)-linked bacterial D-glucan known as alternan.[1] Its main products include isomaltose, the trisaccharide α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-D-Glc and the cyclic tetrasaccharide *cyclo* {→ 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 →}.[2] Other higher products include branched cyclic saccharides. The formation of the unusual cyclic tetrasaccharide and the absence of nigerose and α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc from the products of alternan hydrolysis suggested that the action of alternanase on various oligosaccharides may yield useful information regarding the enzyme's specificity and mode of action.

2. Experimental

Enzymes.—*Leuconostoc mesenteroides* NRRL B-512 dextransucrase was a powdered preparation which had been stored at rt in a

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PII: S0008-6215(01)00106-9

sealed bottle since 1952.[3] The activity was within 10% of that stated on the original label, despite storage for nearly 50 years. Endodextranase from *Penicillium* sp. was a gift from Novo Nordisk (Danbury, CT).

Alternanase from *Bacillus sp.* NRRL B-21195 was purified to electrophoretic homogeneity by affinity chromatography and isoelectrophoresis.[4] The preparation used in reactions described herein contained 0.018 mg/mL of purified protein (0.47 U/mg) dissolved in 50 mM sodium 3-(*N*-morpholino)-2-hydroxypropane sulfonate buffer, pH 7.0, containing 50 mM KCl, 1 mM $CaCl_2$, and 1.5 mM NaN_3.

Carbohydrates.—Maltose, nigerose, panose and 4-nitrophenyl α-D-glucopyranoside were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Pullulan was from Pfanstieh Chemical Co. (Waukegan, IL). All other compounds were reagent grade. Alternan was prepared as previously described.[5] Theanderose (6^{Glc}-*O*-α-D-Glc*p* sucrose) was purchased from Wako Fine Chemicals (Japan) and purified by gel-filtration chromatography over Bio-Gel P-2 (Bio-Rad, Richmond, CA). 6-*O*-α-D-Glucopyranosyl α,α-trehalose and 6,6′-di-*O*-α-D-glucopyranosyl α,α-trehalose[6] were generously provided by Dr M. Kurimoto of Hayashibara Biochemical Laboratories (Okayama, Japan).

Isomaltose and isomaltotriose were isolated from an endodextranase hydrolyzate of commercial dextran.[7] The tetrasaccharide α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc was prepared by the dextransucrase-catalyzed acceptor reaction with maltose.[8,9] The trisaccharide α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc was prepared in similar manner, using the B-512 dextransucrase acceptor reaction with nigerose. Likewise, methyl α-isomaltoside was synthesized by acceptor reaction with methyl α-D-glucopyranoside.[10] The tetrasaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc was prepared using the acceptor reaction of alternansucrase with maltose.[11] It was purified by gel-filtration chromatography, followed by preparative HPLC.[2] 4-Nitrophenyl α-isomaltooligosaccharides were synthesized enzymatically from 4-nitrophenyl α-D-glucopyranoside using *L. mesenteroides* NRRL B-512 dextransucrase.[12] 4-Nitrophenyl α-isomaltoside was recrystallized from water to eliminate traces of 4-nitrophenol and 4-nitrophenyl α-nigeroside. For preparative purposes, oligosaccharides were separated by size-exclusion chromatography over Bio-Gel P-2.

Chromatography.—Oligosaccharides were isolated from reaction mixtures by size-exclusion chromatography over Bio-Gel P-2, using either a 2.5 × 90 a 5 × 150-cm column eluted with water at rt. Fractions were analyzed for saccharide content by thin-layer chromatography (TLC). Oligosaccharides were separated on Whatman K5 silica gel plates that were developed with two ascents in 1:4:3:2 nitroethane–MeCN–EtOH–water.[2] Compounds were detected with *N*-(1-naphthyl)-ethylenediamine dihydrochloride.[13] 4-Nitrophenyl α-isomaltooligosaccharides were separated on silica gel plates with a single ascent in 17:3 MeCN–water. Detection was by quenching of the fluorescent indicator under UV light or by the method of Bounias.[13]

3. Results

*Action on α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc and panose.*—Alternanase acted on the trisaccharide α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc to yield D-glucose and the cyclic tetrasaccharide *cyclo*{ → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → }. The identity of the cyclic product was confirmed by ^{13}C NMR spectroscopy and by methylation analysis.[2] Alternanase acted on panose in a similar manner, giving rise to D-glucose and *cyclo*{ → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc*p*-(1 →} as the two major products. In Fig. 1, the relative rates of alternanase action on these two oligosaccharides are compared by thin-layer chromatography. The conversion of panose to cyclic tetramer plus glucose is clearly faster than the conversion of α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc to the same products. Although glucose and cyclic tetramer are the major products of both reactions, other products are also formed. Early in the reaction, two slower-migrating products appear. The faster-migrating of these eventually disappears, whereas the slower-migrating remains at a relatively constant level. At later stages of the reaction, isomaltose appears.

*Action on α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc.*—The tetrasaccharide α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 4)-D-Glc was rapidly hydrolyzed by alternanase (Fig. 2). Initial products revealed by TLC included D-glucose, the trisaccharide α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-D-Glc, and unknown oligosaccharides with R_{glc} values in the range corresponding approximately with pentamers to heptamers. After digestion had proceeded to completion, these higher oligomers had vanished, leaving D-glucose and the trimer α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-D-Glc as the major end-products. Traces of other oligosaccharides in the range of tetramers were also visible with TLC, but their identities could not be established.

Action on cyclic tetrasaccharide.—As previously reported,[2] prolonged incubation of alternanase with *cyclo*{ → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → } eventually results in the formation of isomaltose as well as traces of a slower-migrating product (Fig. 3). Neither nigerose nor D-glucose is formed. This reaction is extremely slow compared to the formation of cyclic tetramer from alternan or panose.

Action on 4-nitrophenyl α-isomaltooligosaccharides.—Although little or no conversion of 4-nitrophenyl α-isomaltoside to other oligosaccharide products could be detected by TLC, measurable amounts of 4-nitrophenol were released by the action of alternanase. Fig. 4 shows the increase in absorbance at 415 nm over time as a result of alternanase action on 4-nitrophenyl α-isomaltoside. This reaction appears to be specific for the isomaltoside, as no 4-nitrophenol was released from 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl α-isomaltotrioside, or 4-nitrophenyl α-isomaltotetraoside.

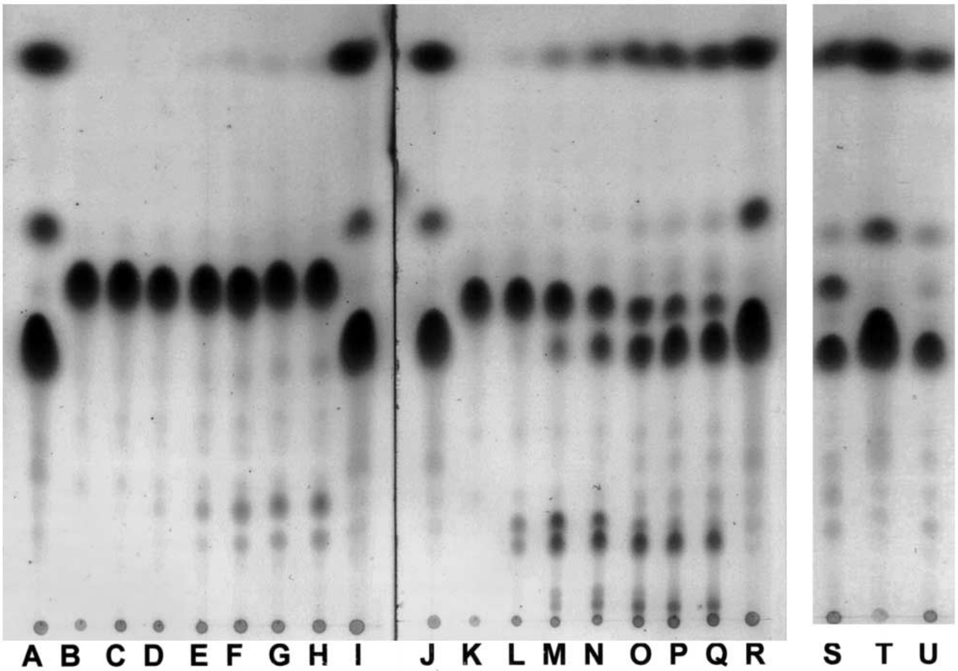

Fig. 1. Thin-layer chromatogram showing the action of alternanase on α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-D-Glc (lanes B–H and S) and panose (lanes K–Q and U) as a function of time. Reaction mixtures consisted of 0.1 mL of buffered alternanase solution (0.018 mg protein per mL) and 0.1 mL of trisaccharide solution (75 mM) in the same buffer. At timed intervals, 1-μL samples were applied and dried. The plate was developed and compounds were detected as described under Section 2. Lanes A, I, J, R, and T contain a standard mixture of D-glucose, isomaltose, and *cyclo*{ → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1 → } (from top to bottom). Lanes B and K represent oligosaccharide samples without the enzyme. Samples of reaction mixtures were spotted at 5 mm (C, L), 1 h (D, M), 2 h (E, N), 4 h (F, O), 6 h (G, P), 8 h (H, Q) and 5 days (S, U).

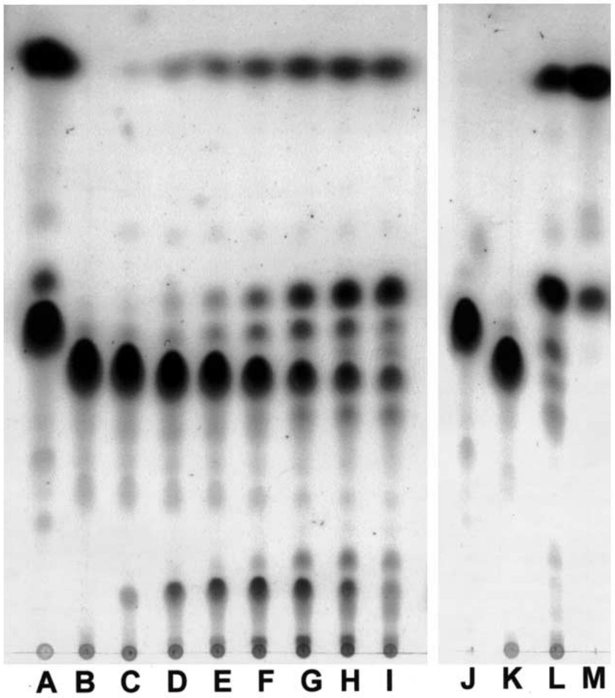

Fig. 2. Thin-layer chromatogram showing the action of alternanase on α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc as a function of time. Reaction mixture consisted of 0.1 mL of buffered alternanase solution (0.018 mg protein per mL) and 0.1 mL of tetrasaccharide solution (36 mg/mL) in the same buffer. At timed intervals, 1-μL samples were applied and dried. Chromatographic conditions are described in Section 2. Lane A: D-glucose, α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-D-Glc and cyclic tetrasaccharide standards. Lane J: cyclic tetrasaccharide; Lane M: D-glucose and α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-D-Glc standards. Lanes B and K: control (buffer and substrate only, no enzyme). Remaining lanes represent reaction aliquots taken at 5 min (C), 30 min (D), 1 h (E), 2 h (F), 4 h (G), 6 h (H), 8 h (I), and 24 h (L).

Activity on other saccharides.—When alternanase was incubated with the following isomaltosyl-containing saccharides at the same levels used for the conversion of panose and alternan, no products were detected by TLC: isomaltotriose, methyl α-isomaltoside, 6-*O*-α-D-glucopyranosyl α,α-trehalose, 6,6′-di-*O*-α-D-glucopyranosyl α,α-trehalose, theanderose (α-D-Glcp-(1→6)-α-D-Glcp-(1↔2)-β-D-Fru*f*), and α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc. After prolonged treatment of pullulan with alternanase (approximately 2–3 weeks), traces of isomaltose and cyclic tetramer were discernible.

4. Discussion

Upon hydrolysis of alternan by alternanase, the main products are isomaltose, the trisaccharide α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-D-Glc, and the cyclic tetrasaccharide *cyclo*-{→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→}. The absence of D-glucose, nigerose and the trimer α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-D-Glc in the product mixture suggested that the enzyme cleaves the α-(1→3) linkages between isomaltosyl units of alternan. This was also supported by our observation that the cyclic tetrasaccharide product was slowly cleaved to isomaltose.[2]

It was previously noted that the conversion of alternan to oligosaccharides did not pro-

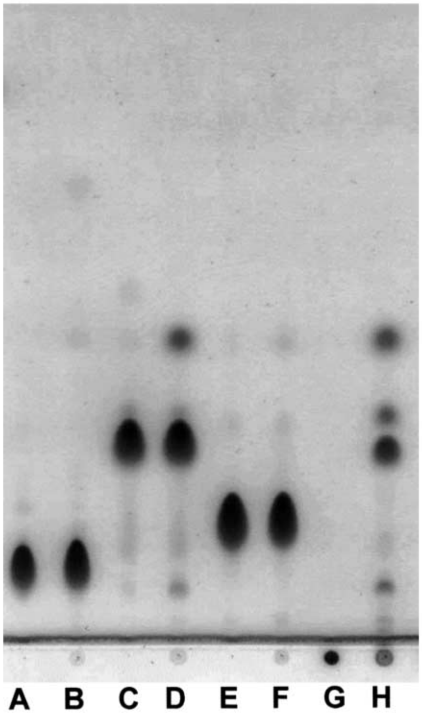

Fig. 3. Thin-layer chromatogram showing action of alternanase on α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc, *cyclo* {→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→}, isomaltotriose, and alternan after 18 days. Lane A, α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc in buffer only; lane B, α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc plus alternanase; lane C, cyclic tetrasaccharide only; lane D, cyclic tetramer plus enzyme; lane E, isomaltotriose only; lane F, isomaltotriose plus enzyme; lane 6, alternan only; lane H, alternan plus enzyme. Reaction mixtures consisted of 0.1 mL of alternanase solution (0.018 mg protein per mL) plus 0.1 mL of substrate solution (38 mg/mL). The plate was developed as in Fig. 1.

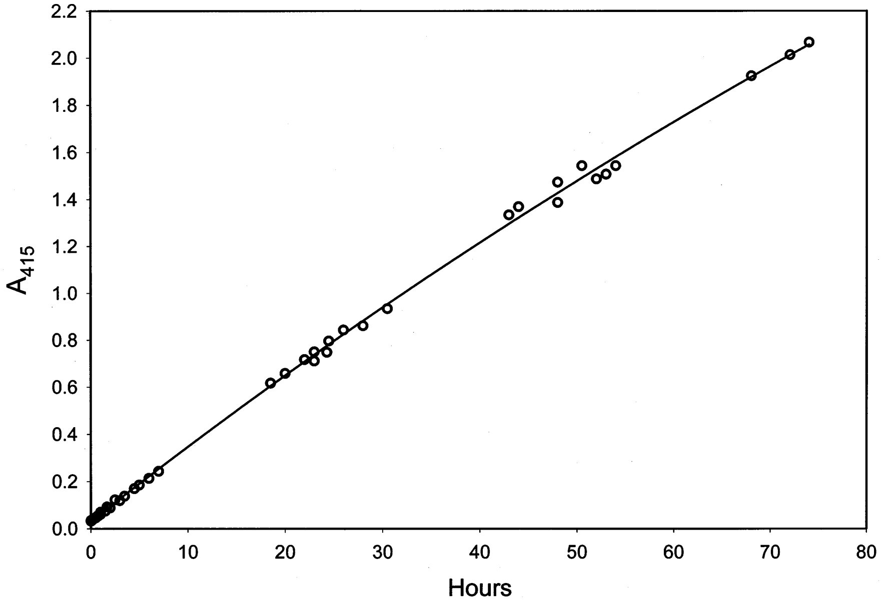

Fig. 4. Hydrolysis of 4-nitrophenyl α-isomaltoside by alternanase as measured by spectrophotometric determination of 4-nitrophenol at 415 nm. The reaction mixture consisted of 1.0 mL of 6.6 mM 4-nitrophenyl α-isomaltoside in pH 7 buffer plus 0.2 mL of alternanase (0.018 mg protein per mL). Reaction was monitored at room temperature in a 1-cm cuvette, against a blank containing substrate in buffer without enzyme. Under identical conditions, 4-nitrophenyl α-D-glucopyranoside, 4-nitrophenyl α-isomaltotrioside and 4-nitrophenyl α-isomaltotetraoside gave no significant increase in A_{415}.

ceed to completion.[1] Subsequent work has shown that the products, in particular isomaltose and the cyclic tetrasaccharide, exhibit an inhibitory effect on the hydrolysis of alternan by alternanase,[14] indicating that the enzyme can bind to relatively small substrates. Alternanase also binds to isomaltosyl agarose,[14] lending support to the hypothesis that the enzyme recognizes the isomaltosyl structure of alternan. On the other hand, its inability to hydrolyze dextran[2] indicates that the enzyme cannot act on substrates containing three glucosyl units that are linked through two consecutive α-(1→6) linkages. Based on these data, we decided it would be appropriate to study the action of alternanase on a variety of saccharides that contain the isomaltosyl functional group.

Since the trisaccharide α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc is an analogue of the repeating unit of alternan, yet does not appear in the product mixture, we suspected that it would be cleaved by alternanase. However, we expected the products to be D-glucose and isomaltose. It was therefore surprising to discover that this trisaccharide was converted to D-glucose and *cyclo*{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}. Since the trisaccharide α-D-Glcp-(1→6)-α-D-Glc*p*-(1→3)-D-Glc is relatively scarce, we also looked at its more readily available analogue α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc (panose). As Fig. 1 clearly illustrates, panose yields the same major products, but at a much faster rate. Equally interesting is the appearance of two minor products in both trisaccharide reaction mixtures, migrating more slowly than the cyclic tetramer. The faster-migrating of these two appears only transiently, then disappears. The slower-migrating product remains at a relatively constant low level throughout the course of the reaction. Neither of these two compounds has been isolated.

The discovery that panose is hydrolyzed by alternanase suggested that other isomaltosides might also serve as substrates. In view of the fact that dextran is not a substrate,[2] it was not surprising to find that isomaltotriose was not hydrolyzed (Fig. 3). The tetrasaccharide α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc is also inert to alternanase action (Fig. 3), supporting the idea that consecutive α-(1→6)-linked sequences of D-glucose cannot bind productively to the enzyme's active site. However, the tetrasaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc was rapidly hydrolyzed by alternanase (Fig. 2). Unlike panose, it did not give rise to any significant amount of cyclic product. Instead, it was hydrolyzed at the (1→4)-α-D-glucosidic bond, to yield D-glucose and the trisaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc. This trisaccharide is the same as that which arises from hydrolysis of alternan,[2] and was identified based on its chromatographic mobility and resistance to further alternanase hydrolysis. Other, higher DP oligosaccharides also appeared transiently in this reaction mixture, but were not isolated for structural identification. These are believed to represent intermediate transglycosylation products.

Of the other isomaltosyl-containing substrates tested, only 4-nitrophenyl α-isomaltoside showed evidence of hydrolysis by alternanase. The release of 4-nitrophenol proceeded in a nearly linear fashion over time (Fig. 4), but despite this clear indication of enzyme-catalyzed hydrolysis, no products were discernible by TLC. This may be explained by the much higher sensitivity of the spectrophotometric detection of 4-nitrophenol, which is in the micromolar range of concentrations, compared to detection of sugars on TLC, which is in the millimolar concentration range. Although this sensitivity suggests that 4-nitrophenyl α-isomaltoside could be useful as a sensitive substrate for the detection and assay of alternanase, our preliminary results indicate certain problems with such an assay. Firstly, the assay would not be selective, as other glycosidases may also hydrolyze 4-nitrophenyl α-isomaltoside.[15,16] Another, more troublesome problem arises from the fact that 4-nitrophenyl α-isomaltoside is poorly soluble in water (< 30 mM), and our initial experiments indicate a K_M value much higher than the K_{SP}. Thus, saturation kinetics are impossible to attain in strictly aqueous systems. Nevertheless, it may still be a useful chromogenic substrate for some purposes.

In light of the fact that 4-nitrophenyl α-isomaltoside is a poor substrate for alternanase, it comes as no surprise that methyl α-isomaltoside yielded no detectable products. The methyl glycosidic bond possesses less energy than the aryl glycosidic bond, therefore, a hydrolysis would be even less favorable. On the other hand, since the glucopyranosyl–fructofuranoside bond present in theanderose is a rather high-energy bond, some other explanation must account for the fact that theanderose is not a substrate for alternanase. Apparently, the stereochemistry of the fructofuranoside moiety prevents this compound from binding to the enzyme's active site. Similarly, the glucosyl trehaloses must also be unable to bind productively, since neither of these saccharides was hydrolyzed.

It is interesting to speculate on the much greater reactivity of panose relative to α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc, especially since this enzyme has been described as an endo-alternanase.[2] It is not too far-fetched to imagine the existence of a D-glucan composed of alternating α-(1→6) and α-(1→4) linkages that might be a better substrate than alternan for this enzyme. On the more practical side, the reaction with panose provides a means for producing the cyclic tetrasaccharide *cyclo*{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)--α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} from starch or other substrates besides alternan, since panose can be produced enzymatically from maltose[17] or pullulan,[18] as well as by alternansucrase[11] or dextransucrase[8] acceptor reactions. The stoichiometric release of glucose from panose also provides a means for the quantitative analysis of alternanase activity using a glucose oxidase-coupled assay.[19]

From these results, we conclude that alternanase is a hydrolase–transglycosylase which recognizes and binds to isomaltosyl units which are not flanked by other α-(1→6) linkages. It is capable of hydrolyzing both α-(1→3) and α-(1→4) linkages. Hydrolytic products include D-glucose, isomaltose, and the trisaccharide α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-D-Glc. Transglycosylation reactions can yield the cyclic tetrasaccharide described by Côté and Biely,[2] as well as transient oligosaccharide structures of higher DP. Studies are currently pending in which we plan to isolate these higher DP oligosaccharides, as their structures may reveal something about the mode of action of alternanase. The enzyme will also be cloned in the near future, and sequence analysis is expected to yield useful information about its relationship to other glycosyl hydrolases. MacGregor et al.[20] have recently shown how sequence analysis can be used to derive information about enzyme specificity in cyclodextrin glucanotransferases and related enzymes.

Acknowledgements

The authors thank Dr David Weisleder for performing NMR analyses of the products and substrates, James Nicholson for methylation analyses, and Drs Kevin Hicks, Peter Biely and Frank Momany for helpful discussions and suggestions. We are also grateful to Dr Masashi Kurimoto of Hayashibara Biochemical Laboratories for kindly providing us with the samples of glucosyl trehaloses.

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