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Oligosaccharide synthesis by reversed catalysis using α-amylase from *Bacillus licheniformis*

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Abstract

Evidence is provided for the synthesis of hetero-oligosaccharides by Termamyl, the thermotolerant α-amylase from *Bacillus licheniformis*. In the presence of soluble starch and added non-starch sugars (isomaltose, cellobiose, mannose, melibiose, stachyose, xylose and 1-*O*-methyl-β-D-glucopyranoside), oligosaccharides were produced by the reversed catalytic reaction. New peaks of homo- and hetero-oligosaccharides (DP1-10) were detected by high performance anion exchange chromatography (HPAEC) in equilibrium reaction mixtures. Attempts to increase the extent of synthesis of oligosaccharides using organic solvents (ethanol, methanol, *N*-propanol, propanediol, *N*-butanol, dioxane and dimethyl sulfoxide (DMSO)) to lower the water activity of the reaction mixture were unsuccessful. Raising the temperature of the reaction mixture from 37°C to 55°C was ineffective.

Keywords: Enzymatic synthesis; Oligosaccharide synthesis; Enzyme reversal; α-Amylase; Organic solvents

1. Introduction

Carbohydrates are widely used in various industries such as food, beverage, paper and textile production and are increasing used for medical purposes. Consequently, there is a commercial desire for research into the synthesis of novel oligosaccharides and polysaccharides. The best methods for the synthesis of oligosaccharides in vitro use enzymes, especially those isolated from microorganisms [7]. The enzymatic synthesis of oligosaccharides may be conducted through transferring reactions using activated sugars or by the reversal of polysaccharide hydrolysis reactions. These methods are described as ''kinetically controlled'' and ''equilibrium-controlled'' reactions, respectively [4].

The extent of equilibrium-controlled synthesis may be increased by lowering the water activity of enzymatic reaction mixtures to change the reaction equilibrium and effectively drive the reaction *in reverse*. Several successful examples of the equilibrium-controlled enzymatic synthesis of oligosaccharides have been reported, such as the synthesis of *O*-α-mannobiose from D-mannopyranose in organic sol-

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vents by α-mannosidase [1], the synthesis of five disaccharides and two trisaccharides by glucoamylase in aqueous ether mixtures [6] and the synthesis of *N*-octyl-β-glucoside by β-glucosidase in acetate buffer mixed to 50% saturation with octanol. [19]. Other microbial enzymes have been used successfully in equilibrium-controlled syntheses of oligosaccharides. For example, cyclomaltodextrin glucosyltransferase (CGTase) from *Bacillus macerans* synthesizes cyclodextrins from maltose in a water-organic solvent system [14] and the 1,2-α-mannosidase of *Aspergillus phoenicis* catalyses the synthesis of mannobiose and mannotriose from mannose [22].

Kinetically controlled oligosaccharide and glycoside syntheses using microbial enzymes include the synthesis of methyl fructoside from sucrose by the β-fructofuranosidase from *Saccharomyces cerevisiae* in 20%(v/v) methanol [20]; synthesis of ethyl- and 2-fluoroethyl-β-galactopyranosides from lactose in ethanol by β-glycosidases from *Streptococcus thermophilus, Aspergillus oryzae, Candida saccharolyticum* and *Caldocellum saccharolyticum* [24,25]; and production of *N*-alkyl-β-glucosides by β-glucosidases from *Fusarium oxysporum* in *N*-alkanols [28].

In kinetically controlled synthesis systems, glycosidases show broad acceptor selectivity and can catalyse the synthesis of many novel oligosaccharides. Cheap oligosaccharides such as lactose and raffinose have been used successfully as acceptors [15–17,26]. An example of the synthesis of a potentially valuable product is the production of Gal(β-1,3)GlcNAc from lactose and 2-acetamido-2 deoxy-D-glucopyranose (GlcNAc) by the β-galactosidase from *Lactobacillus bifidus* [16, 18]. The β-*N*-acetylhexosaminidase from *A. oryzae* catalyses the synthesis of di-*N*-acetyl chitobiose, chitotriose and chitotetraose: the disaccharide acts as acceptor to give the trisaccharide and as the trisaccharide accumulates it, in turn acts as acceptor to give the tetrasaccharide [23]. Santin et al. [21] used the β-glycosidases from the thermophilic archaebacterium *Sulfobolus sulfotaricus* to synthesize 2-β-D-galactopyranosyloxyethyl-methacrylate (GalEMA) from hydroxyethyl methacrylate (HEMA) as acceptor and ρ-nitrophenyl-β-D-galactopyranoside (ρ-NTG) as donor. Lee et al. [11] produced glucooligosaccharides (GOS) using two types of glucansucrases (GTF-S and GTF-I) from *Streptococcus sobrinus* with maltose as acceptor for glucose residues from sucrose.

The great majority of the syntheses listed above were catalysed by exo-acting glycosidases. The ability of endo-acting enzymes to synthesize novel oligosaccharides has rarely, if ever, been tested. This paper reports the synthesis of novel oligosaccharides from starch and added non-starch sugars catalysed by a bacterial α-amylase from *Bacillus licheniformis*.

2. Materials and methods

2.1. Enzyme and analytical methods

2.1.1. Enzyme

Termamyl, the commercially used α-amylase from *B. licheniformis*, was a gift from Novo Nordisk, Bagsvaerd, Denmark. The enzyme was purified by gel filtration and shown to be a single protein with RMM of 67 kD. In the presence of starch and 20–70 ppm calcium at pH 5.5–6.5, the enzyme is stable to elevated temperatures.

2.1.2. Enzyme assay

The enzyme activity was assayed by the determination of reducing sugars liberated by the hydrolytic reaction with 1% (w/v) soluble starch in 0.1 M acetate buffer pH 5.6, containing 0.0043 M $CaCl_2$. The reaction mixture was incubated for 10 min at the stated temperature. The reducing sugars produced were measured using the 3,5-dinitrosalicylate method using glucose as standard. One unit of enzyme activity is defined as producing 1 μmol of reducing sugar equivalent to glucose per minute.

2.1.3. Sugars analysis

Analysis of hydrolysis products and novel oligosaccharides was done qualitatively using a Dionex high performance anion exchange chromatography (HPAEC) system isocratically with 0.15 N NaOH containing 0.1 M sodium acetate as mobile phase run at 1 ml/min at room temperature. Temperature compensation was 1.7. Samples were adjusted to pH 6.5 before application of 50 μl to the column. Products were detected and analysed using a Pulsed Amperometric Detector. The system was calibrated using glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose standards (G1–G7) (Sigma). The HPAEC system separates oligosaccharides up to DP 10.

2.1.4. Protein determination

Protein was determined either by Lowry's method [12] or by the Coomassie Blue binding method [2]. Protein in column eluates was measured by the absorbency at 280 nm.

2.1.5. Purification and molecular weight checking

Purified enzyme was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Phast system and protein was stained with coomassie blue. Molecular weight of the enzyme was compared with standard protein, phosphorylase, Bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin [5].

2.2. Equilibrium-controlled synthesis of oligosaccharides

Termamyl (1 unit) was incubated with 0.5 ml 1% (w/v) soluble starch in 0.1 M Na acetate buffer pH 5.6 containing 0.0043 M calcium chloride with 0–80% (v/v) organic solvent. The solvents used were methanol, ethanol, *N*-propanol, *N*-butanol, propanediol, dimethyl sulfoxide (DMSO) and 1,4-dioxane. Reaction mixtures were incubated at the stated temperature for 10 min.

2.3. Kinetically controlled synthesis of oligosaccharides

The enzyme reaction was allowed to reach equilibrium at 55°C in the presence of starch and an acceptor sugar. The enzyme was incubated with 1% (w/v) soluble starch buffered as previously containing 200 μg/ml of each of the following acceptor sugars, isomaltose, cellobiose, mannose, melibiose, stachyose, xylose and methylglucoside. Reaction products were determined by HPAEC and the peaks compared with those obtained using the standard hydrolytic reaction without acceptor.

3. Results and discussion

3.1. Effects of organic solvents on oligosaccharide synthesis

Study on the effects of increasing concentrations, up to 80% (v/v) of various solvents on the hydrolytic activity of the enzyme, at 37°C and 55°C, the results showed that at solvent concentrations above 20–30% at either temperature the enzyme activity decreased simultaneously when the concentration of organic solvent increased. At 37°C, the enzyme was fully inactivated in 60% methanol and ethanol, 80% propanol, 40% propanediol and 50% DMSO. In contrast, the enzyme retained approx. 50% of its initial activity in 70% butanol and 70% dioxane (data not shown). At 55°C, the enzyme was fully inactivated in 50% ethanol, 60% methanol, 50% propanediol and 60% DMSO, but not in propanol, butanol, or dioxane, in which the activity remained at 50–60% of the control level. The results are in accord with those of Asakura et al. [1], who noted that there were slight differences in the denaturation effects of organic solvents on different proteins.

It is noteworthy that the enzyme activity showed stimulation in 5–10% ethanol and in 5–20% propanol at 55°C (Fig. 1a and b). The maximum stimulation was 35% over the control, by propanol at 55°C. In general, the activ-

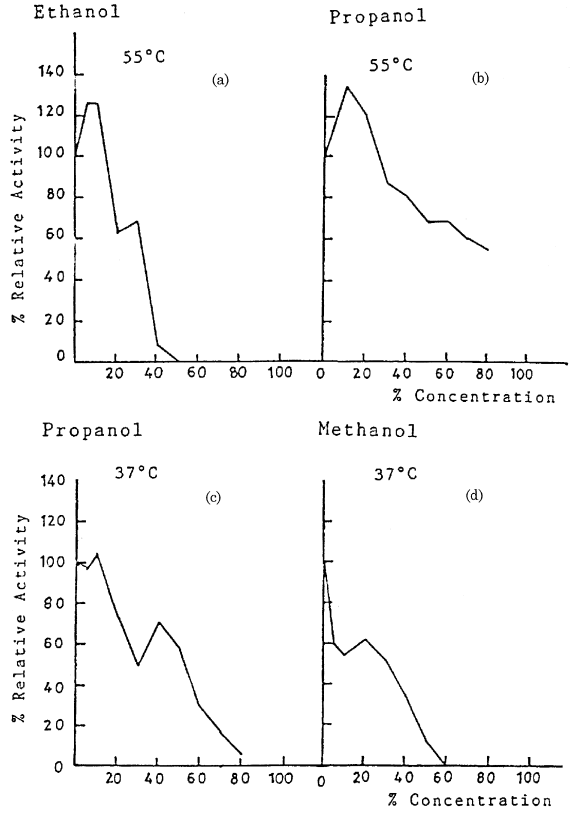


Fig. 1. Effects of increasing concentrations of alkanols on the hydrolytic activity of Termamyl, at stated temperatures; (a) ethanol and (b) propanol at 55°C; (c) propanol and (d) methanol at 37°C.

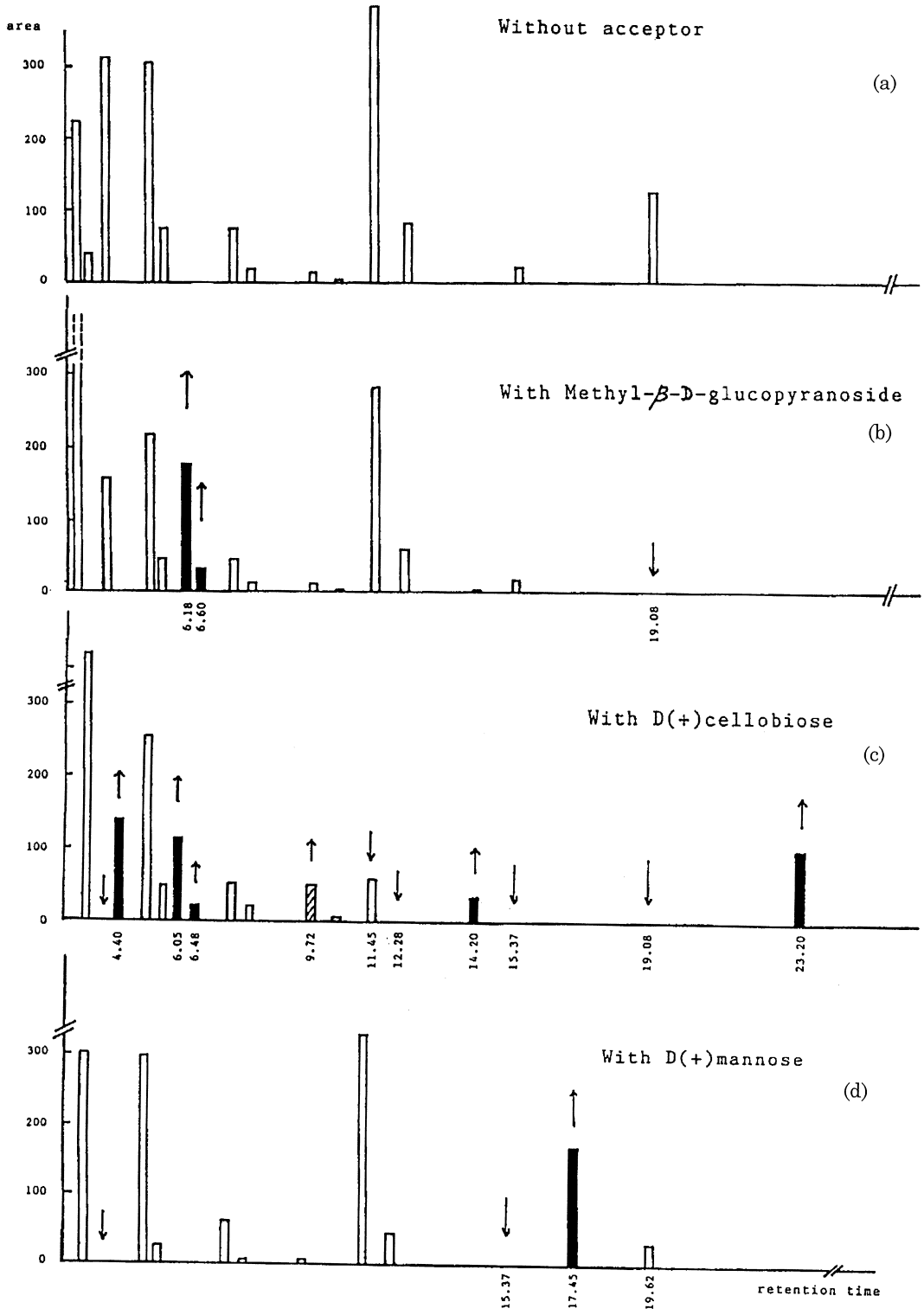
ity decreased with increasing solvent concentration, but, again in general, the decline in activity did not follow a smooth curve and in some cases, for instance propanol and methanol at 37°C (Fig. 1c and d) and with most of the solvents at 55°C (data not shown), there was an increase in activity, or at least a plateau of activity, after an initial fall in activity with increasing solvent concentration. Clearly, the overall fall in enzyme activity results from several factors, not merely the denaturation of the enzyme protein. These may include partial unfolding of the enzyme protein, thus altering the accessibility of the active centre and binding sites to the substrate, alteration of the dielectric constant of the medium, hence changing the extent of dissociation of amino acid residues in the active centre and changes in the conformation of the substrate molecules. Larout and Willemot [10] reported the effects of organic solvents on the stability and synthetic ability of glucosidases and glucoamylase, demonstrating significant differences in the response of the enzymes to different solvents. When alkanols are used as co-solvents it is more than likely that they will act as acceptors; for instance, Rodriguez et al. [20] showed that the β-fructofuranosidase from *S. cerevisiae* could catalyse the synthesis of methyl-fructoside when provided with 20%(v/v) methanol. Similar observations have been made by Panintrarux et al. [19], Stevenson and Furneaux [25] and Tsitsimpikon et al. [28], using β-glucosidase, β-galactosidase and β-glucosidase, respectively. To the best of our knowledge, there are no reports of alkyl-oligosaccharides being synthesized by endo-enzymes.

The results of the HPAEC profiles of sugars liberated on the incubation of Termamyl with starch at 55°C in the absence of those alkanol used and with 5%, 20% and 30% (v/v) alkanols confirm that the hydrolytic activity is enhanced by those alkanols at the concentrations but there is no evidence for the synthesis of alkyl-oligosaccharides, as evidenced by no appearance of new peaks in the HPAEC profiles. We conclude that, in the circumstances we used, Termamyl does not use alkanols as acceptors for alkyl-oligosaccharide synthesis.

3.2. Synthesis of hetero-oligosaccharides

Inclusion of non-starch sugars in reaction mixtures as potential acceptors and incubation at 55°C for 1 and 18 h appeared to speed the hydrolysis of starch. This implies that none of the acceptors used had inhibitory effects on

Fig. 2. Peak areas obtained by HPAEC of oligosaccharides liberated in the reaction mixtures incubation at 55°C for 18 h (a) without acceptors and with (b) 1-*o*-methyl-β-D-glucopyranoside, (c) D(+) cellobiose and (d) D(+) mannose as acceptors. ↑ = indicates the new peaks of oligosaccharides synthesized. ↓ = indicates the peaks of oligosaccharides eliminated.


area
300
200
100
0
Without acceptor
(a)
With Methyl-β-D-glucopyranoside
(b)
6.18
6.60
19.08
With D(+)cellobiose
(c)
4.40
6.05
6.48
9.72
11.45
12.28
14.20
15.37
19.08
23.20
With D(+)mannose
(d)
15.37
17.45
19.62
retention time

α-amylase activity in the conditions used. The reaction mixtures remaining after 18 h were analysed by HPAEC. This showed that new products were synthesized in the presence of β-methylglucoside, cellobiose and mannose (Fig. 2a–d), but not in the presence of xylose, isomaltose, melibiose and stachyose (data not shown). Fig. 2b provides the peak areas obtained by HPAEC of the reaction mixture containing β-methylglucoside and compares them with the control reaction (Fig. 2a). This indicates that two new oligosaccharides are formed with retention times 6.18 and 6.60 min (between maltotriose and maltotetraose), apparently at the expense of maltoheptaose (retention time 19.08 min). Fig. 2c makes a similar comparison of the peak areas obtained in the presence of cellobiose and that of the control reaction and show that several new products are synthesized with peaks at 4.40, 6.05, 6.48, 14.70 and 23.20 min, also a peak at 9.72 min is enhanced. As before, these appear to be synthesized at the expense of maltoheptaose but now there appears to be no maltohexaose (retention time: 15.37 min) nor of an unidentified material giving a peak at 12.28 min in the reaction mixture. The amount of maltopentaose (11.45 min) is significantly reduced. In the presence of mannose (Fig. 2d), one new oligosaccharide is synthesized (retention time: 17.45 min), plausibly a hexa or heptasaccharide, at the expense of maltoheptaose, maltohexaose and maltose. The nature of these new oligosaccharides is unknown, at present.

4. Conclusion

In contrast to synthetic reactions involving exo-acting glycosidases, the synthesis of oligosaccharides catalysed by endo-acting glycosidases cannot be described satisfactorily as either kinetically controlled or equilibrium-controlled. However, synthesis of oligosaccharides by transglycosylation is a normal component of the complex of reactions in which starch is hydrolysed by bacterial α-amylases [13]. It is reasonable to assume that transglycosylation occurs by the transfer of oligosaccharide units (rather than single glucose residues) from donor oligosaccharides to acceptor oligosaccharides. It is also plausible that maltoheptaose is a product of transglycosylation. The basis of this suggestion comes from the work of, among others, Torgerson et al. [27] who demonstrated that the α-amylase of *Bacillus amyloliquefaciens* has 10 binding sites for anhydroglucose residues in α-1,4-glucan chains and that the point of hydrolysis occurs between sites 6 and 7. Maltoheptaose (G7) could be hydrolysed to G5 + G2, G4 + G3, G3 + G4, G2 + G5, but probably not to G6 + G1 or G1 + G6, and similarly, maltoheptaose might be synthesised by transglycosylation from G5 + G2 or G4 + G3. The absence of maltoheptaose from reaction mixtures containing β-methylglucoside, cellobiose or mannose suggests that these sugars act as alternative acceptors in that transglycosylation and prevent synthesis of maltoheptaose. The retention time of the novel products formed in the presence of β-methylglucoside suggests that those products are tetra or pentasaccharides (standard materials are not available), which implies that the donor was G3 or G4. It is less easy to explain the consequences of including cellobiose in the reaction mixture: again no maltoheptaose is synthesised but a new oligosaccharide, probably an octasaccharide, a small amount of a hexasaccharide, a tetrasaccharide and a trisaccharide are produced. These are plausibly derivatives of cellobiose. The synthesis of new oligosaccharides seems to prevent the synthesis of maltoheptaose, which implies that hetero-oligosaccharides inhibit the condensation reaction.

Bucke [3,4] reviewed the in vitro synthesis of oligosaccharides using exo-enzymes, glycosidases such as α-glucosidases from yeast, buckwheat, *Tetrahymena pyriformis* and jackbean α-mannosidases [9] which wide range of acceptors could be used and β-glucosidase from *Penicillium emersonii*, which a few sugars could be used as acceptors to synthesize hetero-oligo-

saccharides, too. Moreover, an in vitro synthesis of oligosaccharides by β-galactosidase from *Escherichia coli* was also reported using *N*-acetyl galactosamine as the acceptor [8].

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