Short-chain oligosaccharide synthesis by endo-β-1,3 glucanase from *Oerskovia xanthineolytica**

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1. Introduction

Complex carbohydrates or their conjugates are important compounds of different cellular and sub-cellular structures of living organisms. The latest progress in glycobiology has proved that carbohydrates can also play a role as informational molecules. Their structural diversity makes them suitable for events requiring molecular specificity. On cell surfaces, many carbohydrate moieties of glyco-conjugates are involved in various types of biochemical recognition processes, including growth, development, immune responses, infection, cell adhesion, metastasis, and numerous signal-transduction phenomena (1-4).

Full understanding of functional and physiological properties of oligosaccharides and their derivatives in biological systems still requires extensive interdisciplinary research. The progress in these scientific areas strongly depends on availability of considerable amounts of structurally well defined oligosaccharides. The most reasonable approach to fulfill these demands is synthesis *de novo* or modification of the existing structures using enzymatic or chemoenzymatic methods (5-7).

Enzymatic synthesis of oligosaccharides *in vitro* with glycosyl hydrolases is widely accepted as the most appropriate biocatalytic approach, because

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they are easily obtainable, relatively stable in reaction media and their natural substrates are not expensive (8,9). Much attention has, however, been focused on the exo-glycosidases (10-15), whereas the endo-glycosidases have been largely neglected (16-18).

In this paper, we describe the use of pure endo-β-1,3-glucanase obtained from *Oerskovia xanthineolytica* for short-chain oligosaccharides synthesis, using laminarin as the glucosyl donor and different nitrophenyl glycosides as acceptors.

2. Material and methods

2.1. Source and purification of enzyme

Crude enzymatic preparation containing mixtures of exo- and endo-glucanases was obtained from the culture filtrate of *Oerskovia xanthineolytica* by $(NH_4)_2SO_4$ precipitation. Proteins having endo-β-1,3-glucanase activity were then separated from other enzymes by gel filtration on Sephacryl S-200 HR at pH 5.5. Further purification of the enzyme was done by chromatofocusing, using PBE 94 and a pH range of 8.3-5.0, followed by re-chromatography on Sephacryl S-200 HR. The isolated endo-β-1,3-glucanase, which showed a single protein band after SDS-PAGE, was freeze-dried and stored at -20°C until needed.

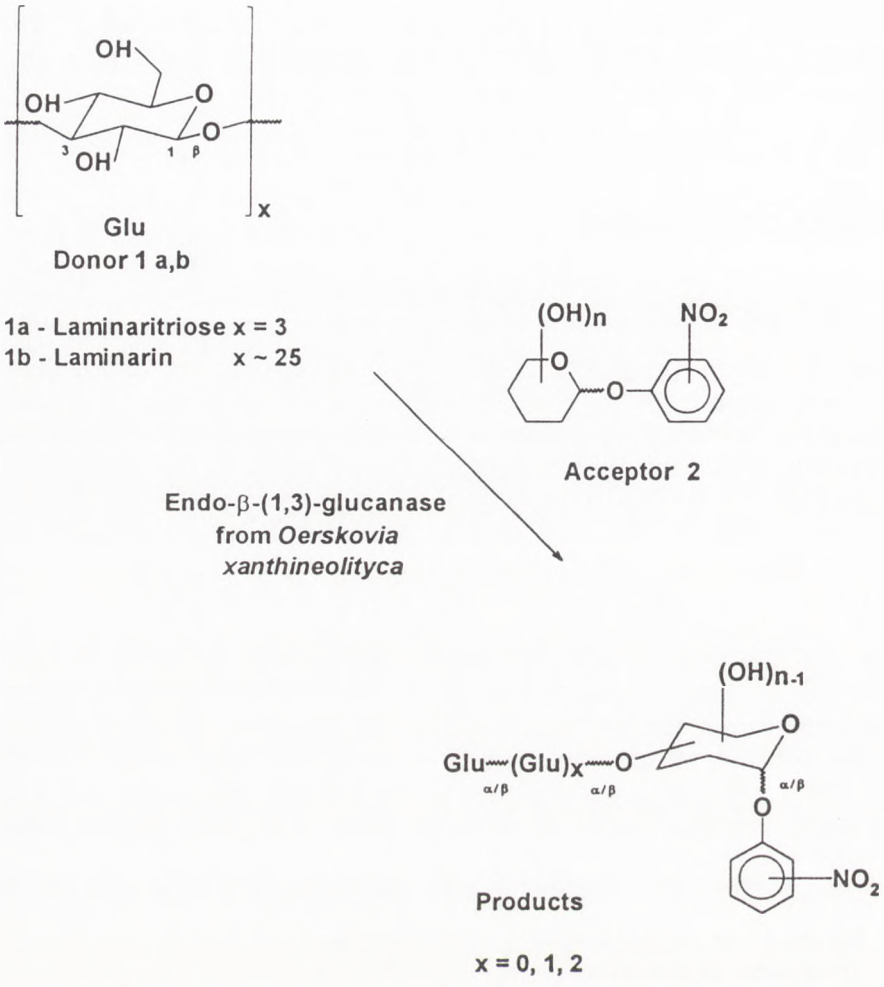
Endo-β-1,3-glucanase activity was determined by measuring the amount of reducing sugars (glucose equivalent) released after incubation of the enzyme preparation for 5 min. at 50°C in citric acid-K_2HPO_4 buffer (pH 5.5) containing 0.5 mg/ml of laminaran. This buffer was used in all experiments except stated otherwise. One unit of glucanase activity is defined as the activity that liberates 1μmol of reducing sugars per min. under the above assay conditions.

2.2. Synthesis of nitrophenyl-oligosaccharides by transglycosylation (general procedure)

The general scheme for the synthesis of nitrophenyl oligosaccharides by transglycosilation is shown in Scheme 1. Reaction mixtures consisted of 1.0 mg of donor (laminarin) and 0.9 mg of acceptor (different nitrophenyl glycopyranosides) dissolved in 10 μl of 20 mM buffer (pH 5.5) containing 30% acetonitrile (v/v). The mixture was incubated overnight with 2 U of the enzyme at 50°C. The reaction was stopped by immersion in a boiling water bath for 10 min., and analysed by TLC.

SCHEME 1

GENERAL SCHEME FOR NITROPHENYL OLIGOSACCHARIDES SYNTHESIS BY TRANSGLYCOSYLATION USING ENDO-β-1,3-GLUCANASE



Transglycosylation reaction was carried out with laminaritriose (1a) or laminarin (1b) as donors and different nitrophenyl glycosides as acceptors (c.f. Tab. 1).

2.3. Analytical procedures

Analytical TLC: reacted samples were subjected to HPTLC for qualitative analysis using Kieselgel 60 F_{254} plates (Merck). Aliquots of appropriately

diluted samples were spotted on plates and developed with a solvent system consisting of ethyl acetate/acetic acid/water (2:1:0.5 by vol.). Products were detected by UV light or visualized by spraying with EtOH solution containing 0.5% α-naphthol and 5% H_2SO_4 and heating at 120°C for 10 min.

Semi-preparative TLC was performed with similar TLC plates and the areas corresponding to the new products on unsprayed plates were scrapped-off, and eluted from the silica gel with a solvent mixture consisting of acetonitrile and methanol (3:2). The pure isolates were freeze-dried and subjected to FAB MS analysis. Meta-nitrobenzyl alcohol was used as the matrix and LiCl as the source of lithium.

3. Results and discussion

Enzymatic synthesis of novel oligosaccharides mediated by microbial endo-β-1,3-glucanase occurs via the general process presented in Scheme 1. The reaction mixture, in addition to the biocatalyst, contained essentially linear β-1,3-glucan (laminarin) as the donor, a nitrophenyl-glycoside as the acceptor, and the buffer (pH=5.5) with 30% (v/v) of acetononitrile as the co-solvent.

Preliminary experiments proved that synthesis occurs in a wide range of temperatures (20-50°C), pH (8-5.5) and in the presence of different aprotic solvents, namely DMF, DMSO and acetononitrile (data not shown). The solvents chosen for our experiments play at least two roles in the reaction milieu i.e. they increase the solubility of the nitrophenyl glycosides (acceptors) and reduce the content of water molecules which act as competitive acceptors of glycosyl residues transfered by the enzyme. The best results of synthesis were obtained at pH 5.5 in the presence of 30% CH_3CN (v/v). Higher concentrations of this co-solvent lowers the activity of the enzyme.

Ten combinations of donor/acceptor (Tab. 1) were subjected to the synthesis process. Synthetic nitrophenyl glycosides of different monosaccharides and the naturally occurring glycoside, amygdalin, served as acceptors in the enzymatic transglycosylation reaction. End products were detected by qualitative TLC-analysis, as shown in Fig.1. Only the reaction mixtures containing *p*-nitrophenyl-α-D-mannopyranoside or *o*-nitrophenyl-β-D-galactopyranoside had traces, or no detectable products of synthesis, respectively. The other 8 combinations of the reaction mixtures resulted in a total of 38 new product spots (Tab. 1). This indicates that the endo-β-1,3-glucanase has low selectivity towards the examined acceptors. However, the specificity of this enzyme varies and depends on the structure of the acceptor molecule. This phenomenon is indicated by different amounts of products that were obtained with different acceptors. The number and intensity of the spots correspond to the yield of the synthesis. The TLC analysis of all reaction mixtures showed that *p*-nitrophenyl-β-D-xylopyranoside was the best acceptor among the ones examined (see products b_1-b_6; Fig.1).

TABLE 1

RESULTS OF TRANSGLYCOSYLATION REACTION CATALYZED BY ENDO-β-1,3-GLUCANASE WITH DIFFERENT NITROPHENYL GLYCOSIDES AS ACCEPTORS AND LAMINARIN AS DONOR

Symbol of acceptor	Acceptor	Number of products
2a	p-Np-α-D-xylopyranoside	6 (a_1-a_6)
2b	p-Np-β-D-xylopyranoside	7 (b_1-b_7)
2c	o-Np-β-D-xylopyranoside	6 (c_1-c_6)
2d	p-Np-α-D-glucopyranoside	5 (d_1-d_5)
2e	p-Np-β-D-glucopyranoside	6 (e_1-e_6)
2f	p-Np-α-D-mannopyranoside	traces
2g	p-Np-α-D-galactopyranoside	4 (g_1-g_4)
2h	o-Np-β-D-galactopyranoside	none
2i	p-Np-α-L-fucopyranoside	2 (i_1-i_2)
amygdalin	D-mandelonitril-β-gentobioside	2

In the subsequent experiment we, therefore, scaled-up (x10) the synthesis with *p*-nitrophenyl-β-D-xylopyranoside and isolated 6 transglycosylation products using semi-preparative TLC, and determined the molecular weights of

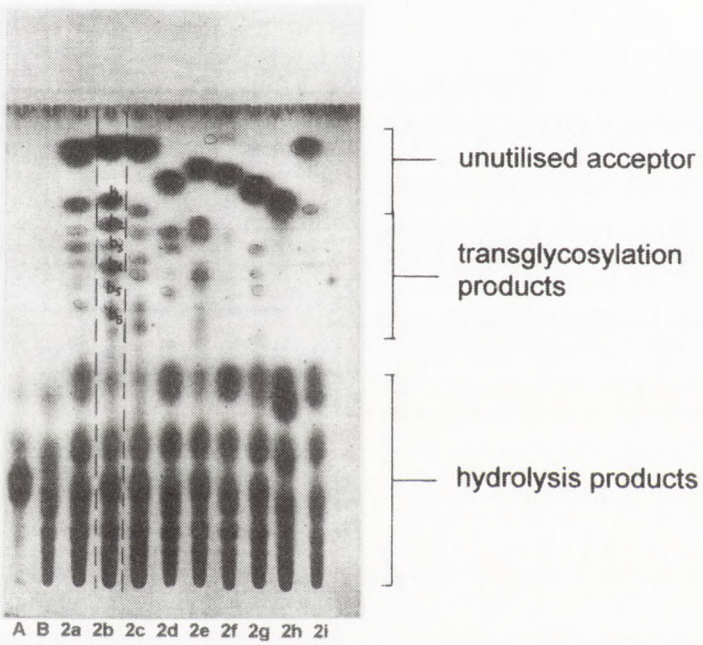


Fig. 1. Thin layer chromatogram of transglycosylation reaction mixtures containing laminarin (1b) as a donor and acceptors (2a-2i). TLC plates were developed in ethyl acetate/acetic acid/water (2:1:0,5). A, enzymatic hydrolysate of laminaritriose; B, enzymatic hydrolysate of laminarin; 2a-2i transglycosylation reaction mixtures.

the synthesised nitrophenyl glycosides by mass spectroscopy (Tab. 2). From the mixture obtained after the enzymatic reaction between laminarin and *p*-nitrophenyl-β-D-xylopyranoside (acceptor 2b) isomers of di-, tri- and tetrasaccharide glycosides were isolated. Disaccharide glycosides (b_1, b_2), formed by addition of a glucosyl residue to a molecule of the acceptor, are the main products of this reaction. At the current stage of our work we can only state that carbohydrate chains of isolated glycosides consist of one residue of xylopyranose bound with one, two, or three glucosyl residue(s) in molecules of di-, tri- and tetrasaccharide glycosides, respectively. Complete stereochemistry of these products is under investigation.

TABLE 2
RESULTS OF FAB MS ANALYSIS OF THE ISOLATED PRODUCTS (b_1 - b_6)

Symbol of product	Molecular ion	Structure of product
b_1	$(M+Li)^+ = 440$	disaccharide glycoside
b_2	$M+Li)^+ = 440$	disaccharide glycoside
b_3	$(M+Li)^+ = 602$	trisaccharide glycoside
b_4	$(M+Li)^+ = 602$	trisaccharide glycoside
b_5	inconclusive results	
b_6	$(M+Na)^+ = 780$	tetrasaccharide glycoside

The pattern of spots on the TLC plate (Fig. 1) indicates, that in the reaction mixtures containing other acceptors, synthesis products show polarity similar to that of glycosides $b_1 - b_6$ mentioned above. This fact may lead to the general conclusion, that synthesis of different short-chain oligosaccharides using our endo-β-1,3-glucanase is therefore possible.

Although the mechanism of this synthesis has not yet been studied, the process of end products formation can be assumed hypothetically. It has been recognized, that during the hydrolysis process endo-glucanases randomly cleave inner glycosidic linkages in the molecules of the appropriate glucan. As a consequence, a mixture of oligosaccharides with continuously modified degree of polymerization is produced. If the reaction lasts long enough, end products of the hydrolysis will accumulate in the environment. The composition of the resulting mixture strongly depends on the substrate specificity and the kinetic characteristic of the enzyme used. This analogy is theoretically possible for the synthesis catalyzed by the endo-β-1,3-glucanase through the transglycosylation process. Nitrophenyl glycosides of different molecular weights that are probable intermediate products of the synthesis can serve as secondary donors for the enzyme. Therefore, transfer of glycosyl residues onto acceptors may proceed simultaneously with shortening of the β-1,3-linked carbohydrate chains of synthesized glycosides. As the end effect, a mixture of short-chain oligosaccharide glycosides can therefore be obtained.

The novelty of this approach for short-chain oligosaccharide glycosides' synthesis lies in the use of an enzyme belonging to the endo-glycosidases group. The low specificity of this endo-β-1,3-glucanase from *Oerskovia xanthineolytica* towards the acceptors used, offers the possibility of designing numerous oligosaccharide structures. This one step synthesis results in a mixture of easily separable products with di- and trisaccharides dominating. This type of short-chain oligosaccharides is well documented as substances of practical importance (e.g. as analytical reagents, biologically active molecules and substrates useful in organic synthesis) (19-23). Commercially available and relatively inexpensive glycans and synthetic or natural glycosides can serve as substrates for the synthesis.

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Summary

An approach for enzymatic synthesis of oligosaccharides via transglycosylation with endo-β-1,3-glucanase from *Oerskovia xanthineolytica* is described. Linear -β-1,3-glucan (laminarin) was used as the donor of glycosyl residues, whereas nitrophenyl glycosides of different monosaccharides served as acceptors. The synthesis was performed in water-organic solvent environment with several combinations of donor/acceptor. Employing p-nitrophenyl-β-D-xylopyranoside as an acceptor in the presence of 30% acetonitrile resulted in the production of six new glycosides shown by FAB MS to be di-, tri- and tetrasaccharides. This enzyme is therefore suitable for the synthesis of short-chain oligosaccharides.

Key words:
synthesis, oligosaccharides, endo-β-1,3-glucanase, *Oerskovia xanthineolytica.*

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