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β-D-Galactosidase from *Paenibacillus thiaminolyticus* catalyzing transfucosylation reactions

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A genomic library of bacterial strain *Paenibacillus thiaminolyticus* was constructed and the plasmid DNA of the clone, containing the gene encoding β-D-galactosidase with β-D-fucosidase activity, detected by 5-bromo-4-chloro-3-indoxyl β-D-galactopyranoside, was sequenced. Cells of *Escherichia coli* BL21 (DE3) were used for production of the enzyme in the form of a histidine-tagged protein. This recombinant fusion protein was purified using Ni-NTA agarose affinity chromatography and characterized by using *p*-nitrophenyl β-D-fucopyranoside (K_m value of 1.18 ± 0.06 mmol/L), *p*-nitrophenyl β-D-galactopyranoside (K_m value of 250 ± 40 mmol/L), *p*-nitrophenyl β-D-glucopyranoside (K_m value of 77 ± 6 mmol/L), and lactose (K_m value of 206 ± 5 mmol/L) as substrates. Optimal pH and temperature were estimated as 5.5 and 65°C, respectively. According to the amino acid sequence, the molecular weight of the fusion protein was calculated to be 68.6 kDa and gel filtration chromatography confirmed the presence of the enzyme in a monomeric form. In the following step, its ability to catalyze transfucosylation reactions was tested. The enzyme was able to catalyze the transfer of fucosyl moiety to different *p*-nitrophenyl glycopyranosides (producing *p*-nitrophenyl β-D-fucopyranosyl-(1,3)-β-D-fucopyranoside, *p*-nitrophenyl β-D-fucopyranosyl-(1,3)-α-D-glucopyranoside, *p*-nitrophenyl β-D-fucopyranosyl-(1,3)-α-D-mannopyranoside, and *p*-nitrophenyl β-D-fucopyranosyl-(1,6)-α-D-galactopyranoside) and alcohols (producing methyl β-D-fucopyranoside, ethyl β-D-fucopyranoside, 1-propyl β-D-fucopyranoside, 2-propyl β-D-fucopyranoside, 1-octyl β-D-fucopyranoside, and 2-octyl β-D-fucopyranoside). These results indicate the possibility of utilizing this enzyme as a promising tool for enzymatic synthesis of β-D-fucosylated molecules.

Keywords: β-D-fucosidase/β-D-galactosidase/*Paenibacillus thiaminolyticus*/transglycosylation

Introduction

In living organisms oligosaccharidic structures are involved in many biologically important processes in which, among others, they act as specific recognition markers or protein function modulators. The attachment of an oligosaccharidic chain may also affect the stability, solubility, conformation states, or protease resistance of biologically active molecules. Abnormal types of glycosylations were observed during different pathological processes, cancer being one of the most serious ones (Varki 1993; Dwek 1996). The diversity of the oligosaccharides functions and also different types of glycoconjugates is a challenge to modern biochemistry in an attempt to find new ways of synthesizing a broad spectrum of new glycosides and to test their biological activities as well as their pharmaceutical or biotechnological potential.

The approach of using enzymatic synthesis of oligosaccharides and glycoconjugates is, at present, often preferred to the time-consuming and complicated multistep chemical synthesis. Two types of enzymes are usually used for the catalysis of glycosidic linkage formation in oligosaccharidic chains of glycosylated molecules—glycosyltransferases and glycosidases. Although in the case of the former group of enzymes the transfer of the glycosyl moiety is a result of their natural function, very often glycosidases, which under normal conditions catalyze the hydrolysis of the glycosidic bond, are preferred. The advantage of glycosidases application issues from the good availability of these enzymes, their stereospecificity and ability to glycosylate a wide variety of a hydroxyl group containing molecules and the usage of relatively simple and cheap donor molecules (Crout and Vic 1998; Křen and Thiem 1997; Watt et al. 1997). In some cases lower yields and regioselectivity may complicate the work; however, these problems may be overcome by a careful choice of used glycosidase and reaction conditions (concentration of reactants, use of organic solvents, etc.) or by genetic manipulation enabling the production of glycosynthases from corresponding glycosidases (Křen and Thiem 1997; Palcic 1999).

β-D-Galactosidases (β-D-galactoside galactohydrolases, EC 3.2.1.23) are enzymes which have been for many years applied in biotechnological processes for different purposes. Their hydrolytic activity is useful in the processing of different lactose containing products of food industry, e.g. in the production of lactose-free milk for people suffering from lactose intolerance. Their ability to catalyze transgalactosylation reactions enables the production of galactooligosaccharides, beneficially affecting intestinal microflora or galactosylated molecules with the potential for pharmaceutical industry. Some of these widespread enzymes, present in animals, plants, and a wide variety of microorganisms (Panesar et al. 2006), are known to often display not only β-D-galactosidase activity, but also the β-D-fucosidase, β-D-glucosidase, or α-L-arabinosidase activity (Chang et al. 2009). This broad substrate specificity of some β-D-galactosidases together with the ability to transfer diverse monosaccharidic residues to different saccharidic or

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nonsaccharidic acceptors enables these enzymes to become promising synthetic tools. The possibility of using recombinant β-D-galactosidase from bacterial strain *Paenibacillus thiaminolyticus* (*P. thiaminolyticus*) for the synthesis of β-D-fucosylated molecules is reported in this paper. In previous publications, these compounds have been reported to display different biotechnologically interesting features. Alkyl fucosides were confirmed to be surface-active molecules and thus applicable as non-ionic surfactants (Kobayashi et al. 1999), some β-D-fucosyl group containing disaccharides (fucosylglucose and fucosylxylose) were reported as possible *Bifidus* factors (Sakai et al. 1989; Nunoura et al. 1996). Another field of investigation of β-D-fucosylated compounds is the synthesis of molecules with a reputed pharmacological potential, such as asterosaponins with antitumor and antiviral activities (Li et al. 1998).

An expression and purification of a recombinant β-D-galactosidase originating from *P. thiaminolyticus* and the characterization of its β-D-fucosidase, β-D-galactosidase, and β-D-glucosidase activity is described in this paper. Moreover, its ability to catalyze the transfucosylation reaction using different *p*-nitrophenyl glycopyranosides and alcohols as acceptors is reported. According to the obtained results β-D-galactosidase with β-D-fucosidase activity from *P. thiaminolyticus* seems to be a promising tool for the synthesis of different types of β-D-fucosylated molecules with a potential importance in biotechnology and pharmaceutical industry.

Results

Hypothesis of β-D-*fucosidase activity of the* β-D-*galactosidase from* P. thiaminolyticus *confirmation*

β-D-Fucosidase activity of the whole cells of *P. thiaminolyticus* was tested using the chromogenic substrate *p*-nitrophenyl β-D-fucopyranoside (*p*NPβ-D-Fuc). It was supposed to be possibly the second activity of enzyme β-D-galactosidase. This theory was supported by the results obtained from the native polyacrylamide gel electrophoresis (PAGE) of the cell lysate stained simultaneously with *p*NPβ-D-Fuc or *o*-nitrophenyl β-D-galactopyranoside (*o*NPβ-D-Gal) or 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). The colored (yellow or blue) bands, indicating the position of the enzyme with the ability to cleave the appropriate chromogenic substrate, appeared in the same positions in the individual lines (data not shown). On the basis of these results, the use of X-gal for screening of genomic library of *P. thiaminolyticus* and thus for the detection of the positive colony, containing plasmid deoxyribonucleic acid (DNA) with the gene of an enzyme possessing β-D-fucosidase activity, was possible.

Detection, sequencing, and homology determination of the gene encoding β-D-*galactosidase from* P. thiaminolyticus

The genomic library of *P. thiaminolyticus* was constructed in cells *Escherichia coli* (*E. coli*) DH5α using plasmid pUC19ΔlacZ. The advantage of detection of positive colonies with β-D-galactosidase activity directly on ampicillin containing Luria–Bertani medium (LBA) plates with X-gal was taken, because the absence of lacZ does not allow the expression of active β-D-galactosidase of *E. coli*. Plasmid DNA from the positive colony containing active β-D-galactosidase (indicated by the blue color of the colony) was isolated by Gen Elute™ HP Plasmid Midiprep kit and used as a template for sequencing carried out by the Geneart company, Germany. After the comparison of the determined sequence with NCBI database using program WU-BLAST2, an open reading frame was found with a 60% identity with the gene of β-D-galactosidase from *Paenibacillus* JDR-2. The summary of β-D-galactosidases exhibiting more than 50% identity with the amino acid sequence of β-D-galactosidase from *P. thiaminolyticus* is presented in Table I. The complete nucleotide sequence of the gene encoding β-D-galactosidase from *P. thiaminolyticus* is available in EMBL Nucleotide Sequence Database with accession no. FN397629.

Table I. β-D-Galactosidases exhibiting more than 50% identity with the amino acid sequence of β-D-galactosidase from *P. thiaminolyticus*

Source of β-D-galactosidase (EMBL Database accesion number)	Identity (%) with β-D-galactosidase from *P. thiaminolyticus*
Paenibacillus sp. JDR-2 (EDS51475)	60
Geobacillus sp. Y412MC10 (EDV73651)	59
Geobacillus sp. Y412MC10 (EDV79034)	58
Paenibacillus sp. JDR-2 (EDS54839)	57
Bacillus circulans (BAA21669)	53
Catenulispora acidiphila DSM 44928 (EEN34964)	52

The results were obtained using program WU-BLAST2.

For production of the recombinant protein, plasmid pET16b enabling the production of histidine-tagged recombinant proteins was chosen. The vector was ligated to the polymerase chain reaction (PCR) product containing β-D-galactosidase encoding sequence and the correctness of the prepared construct (named pET16b-βgal-2M) was confirmed by sequencing.

Expression and purification of recombinant β-D-*galactosidase*

The recombinant enzyme was expressed in cells *E. coli* BL21 (DE3) and purified after the disintegration process using affinity chromatography on the Ni-NTA agarose column. The processes of expression, disintegration, and purification were analyzed by sodium dodecyl sulfate (SDS)–PAGE electrophoresis under denaturating conditions (Figure 1). Only one single band was observed after the purification process (using Coomassie Brilliant Blue for visualization), corresponding to the calculated molecular mass of 68.6 kDa. The supposed molecular weight was calculated from the amino acid sequence of the histidine-tagged fusion protein using a program ProtParam tool on the ExPASy Proteomics server. (Gel filtration chromatography was used to determine the native form of the enzyme and the results indicated the presence of the enzyme only in the monomeric form (data not shown).)

After purification the enzyme preparates were used for biochemical characterization of the recombinant β-D-galactosidase and subsequently for transfucosylation reactions.

Characterization of β-D-*fucosidase,* β-D-*galactosidase, and* β-D-*glucosidase activity of recombinant* β-D-*galactosidase*

As mentioned above, β-D-galactosidase from *P. thiaminolyticus* was supposed to possess not only the β-D-galactosidase but also β-D-fucosidase activity, which was, alongside the possible ability to catalyze transfucosylation reactions, the central point of this study. In addition to these two activities the ability of

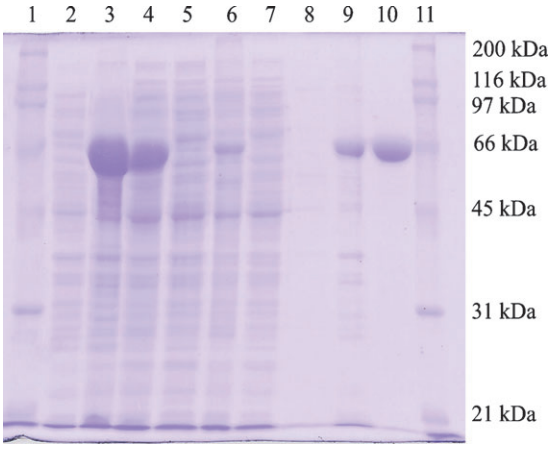

Fig. 1. SDS–PAGE analysis of the expression and purification of the recombinant β-D-galactosidase from *P. thiaminolyticus* produced in *E. coli* BL21 (DE3). Electrophoresis was carried out in 10% polyacrylamide gel and proteins were visualized with Coomassie Brilliant Blue R-250. Lines 1 and 11 – SDS–PAGE Molecular Weight Standards, Broad Range, line 2 – cells *E. coli* BL21 (DE3) before induction of expression, line 3 – cells *E. coli* BL21 (DE3) 4 h after induction of expression by IPTG, line 4 – supernatant after disintegration of cells after expression, line 5 – fraction of supernatant proteins, which did not bind to the Ni-NTA agarose column after the application of the supernatant sample, line 6 – Ni-NTA agarose after application of the supernatant sample, line 7 – fraction after wash of the Ni-NTA agarose column with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 10 mM imidazole, line 8 – fraction after wash of the Ni-NTA agarose column with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 40 mM imidazole, line 9 – Ni-NTA agarose after wash with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 40 mM imidazole, line 10 – recombinant β-D-galactosidase from *P. thiaminolyticus* eluted by the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 250 mM imidazole.

the enzyme to cleave the chromogenic substrate, containing the β-D-glucopyranoside moiety, was also tested.

Four different substrates (*p*NPβ-D-Fuc, *p*-nitrophenyl β-D-galactopyranoside (*p*NPβ-D-Gal), *p*-nitrophenyl β-D-glucopyranoside (*p*NPβ-D-Glc), and lactose) were used for the basic characterization. The K_m values and average specific activities of the purified samples were measured as (1.18 ± 0.06) mmol/L and 1.7 μmol/min/mg for *p*NPβ-D-Fuc, (250 ± 40) mmol/L and 270 μmol/min/mg for *p*NPβ-D-Gal, (77 ± 6) mmol/L and 0.1 μmol/min/mg for *p*NPβ-D-Glc, and (206 ± 5) mmol/L and 0.9 μmol/min/mg for the lactose (37°C, 50 mM phosphate buffer (pH 6)).

At this stage it is necessary to point out complications arising when using in experiments the *p*NPβ-D-Gal as a substrate. Considering the high K_m value and relatively low solubility of *p*NPβ-D-Gal in the buffer system, it was not possible to measure the whole scale of substrate concentrations necessary to approach the maximal reaction rate; therefore, the accuracy of the result decreased. Because of a strong influence of the solvents on the enzyme activity, it was not possible to measure the dependence of the enzyme activity on the *p*NPβ-D-Gal concentration in organic solvents (e.g. dimethylformamide (DMF)), which would increase the solubility of the substrate.

The temperature profile exhibited its optimum at 65°C and the pH of 5.5 was determined as optimal for the enzyme. However also in the pH range of 5–7, the enzyme possessed more than 90% of maximal activity. During the entire research, the enzyme was stored at – 20°C and its activity did not decrease below 90% of its original activity.

Transfucosylation reactions using p-nitrophenyl glycopyranosides and alcohols as acceptors

The ability of recombinant β-D-galactosidase to catalyze transfucosylation reactions was tested for two different types of acceptor molecules, *p*-nitrophenyl glycopyranosides and alcohols. The possibility of transferring the fucosyl moiety from *p*NPβ-D-Fuc to different saccharidic molecule parts and hydroxyl groups of alcohols, the regiospecificity of the enzyme and influence of length of alcohol alkyl chains as well as hydroxyl groups positions were monitored.

For experiments with *p*-nitrophenyl glycopyranosides, serving as acceptors, three molecules that the enzyme was not able to cleave were chosen: *p*-nitrophenyl α-D-galactopyranoside (*p*NPα-D-Gal), *p*-nitrophenyl α-D-glucopyranoside (*p*NPα-D-Glc), and *p*-nitrophenyl α-D-mannopyranoside (*p*NPα-D-Man). In addition to these molecules also the possibility of transferring fucosyl moiety to *p*NPβ-D-Fuc was determined. During transglycosylation experiments, *p*-nitrophenyl β-D-glucuronide (*p*NPα-D-GlcA) was also tested as a potential acceptor molecule, but in the presence of this substrate in the reaction mixture, the β-D-fucosidase activity of the enzyme was completely inhibited. Other tested molecules (*p*NPβ-D-Gal, *o*NPβ-D-Gal, and *p*NPβ-D-Glc) were cleaved by the enzyme and thus are unsuitable for intended purposes.

In transglycosylation reactions containing alcohols as acceptors, methanol and ethanol were chosen as the simplest alcohol molecules, 1-propanol and 2-propanol as an example of the shortest alcohols differing in the position of the hydroxyl group, and 1- and 2-octanol represented alcohols with a longer alkyl chain.

All transglycosylation reactions were carried out at 50°C. This temperature was chosen as a compromise between the high activity and good stability of the enzyme (activity of the enzyme did not decrease below 70% of the original activity after 4 h incubation at 50°C) and relatively good solubility of chromogenic substrates in a 50 mM phosphate buffer (pH 6).

Thin-layer chromatography (TLC) in the mobile phase ethylacetate:acetic acid:water (7:2:2, v/v/v) was used for the first determination of transglycosylation products. The results for *p*-nitrophenyl glycopyranosides used as acceptors are presented in Figure 2, and for alcohols in Figure 3. These results confirmed the ability of the enzyme to transfer the fucosyl residue to all chosen acceptor molecules.

The main transglycosylation products of reactions with *p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-Man, and *p*NPβ-D-Fuc were isolated by extraction from the TLC plates to methanol and prepared for mass spectrometry and nuclear magnetic resonance (NMR) analysis, so as to solve the question of the regiospecificity of the enzyme. Both methods confirmed the presence of the supposed transglycosylation products. The standard NMR experiments (^{1}H, ^{13}C, COSY, HMQC, HMBC, NOE, and TOCSY) enabled the assignment of all proton and carbon resonances. The type of glycosidic linkage was based on the ^{1}H-^{13}C HMBC experiment, possessing two and three bond ^{1}H-^{13}C correlations. Thus, the key HMBC contacts establishing the (1′→ 3) glycosidic linkage for products obtained by fucosylation of *p*NPβ-D-Fuc, *p*NPα-D-Glc, and *p*NPα-D-Man are cross

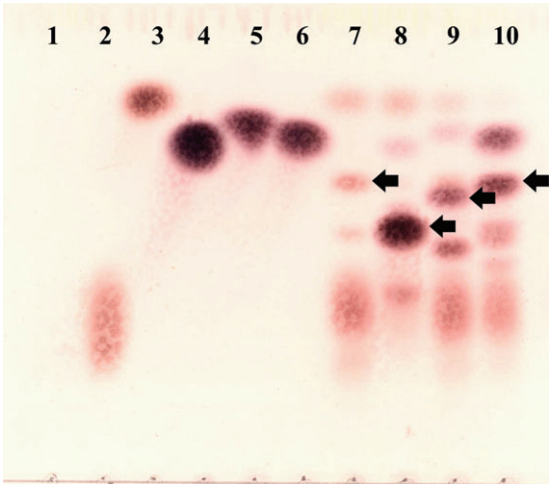

Fig. 2. The results of the transfucosylation reactions catalyzed by recombinant β-D-galactosidase from *P. thiaminolyticus* using 50 mM (or 80 mM as indicated below) *p*NPβ-D-Fuc as a donor and different *p*-nitrophenyl glycopyranosides as acceptors of fucosyl residue. All reactions were performed at 50°C for a period necessary for the maximal production of the desired transfucosylation product. Standard molecules (lines 1–6) and reaction mixtures (lines 7–10) were spotted on the Silica gel TLC plate. The separation was carried out in the mobile phase ethylacetate:acetic acid:water (7:2:2, v/v/v) and the compounds containing saccharidic moiety were visualized by 2-methylresorcinol. Line 1 – *p*-nitrophenol, line 2 – D-fucose, line 3 – *p*NPβ-D-Fuc, line 4 – *p*NPα-D-Gal, line 5 – *p*NPα-D-Man, line 6 – *p*NPα-D-Glc, line 7 – reaction mixture containing 80 mM *p*NPβ-D-Fuc serving as a donor as well as an acceptor, line 8 – reaction mixture containing 33 mM *p*NPα-D-Gal as an acceptor, line 9 – reaction mixture containing 33 mM *p*NPα-D-Man as an acceptor and line 10 – reaction mixture containing 33 mM *p*NPα-D-Glc as an acceptor. Desired transglycosylation products isolated for NMR analysis are indicated by arrows.

Table II. Results of transfucosylation reactions catalyzed by recombinant β-D-galactosidase from *P. thiaminolyticus* using *p*-nitrophenyl glycopyranosides as acceptors

Donor	Acceptor	Product linkage	Yield (%)
*p*NPβ-D-Fuc	*p*NPβ-D-Fuc	(1→3)	16
	*p*NPα-D-Gal	(1→6)	42
	*p*NPα-D-Glc	(1→3)	13
	*p*NPα-D-Man	(1→3)	69

Yields are calculated according to the amount of added acceptor.

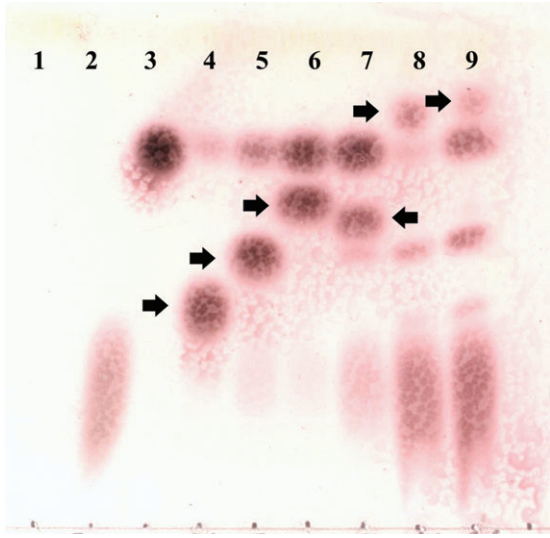

Fig. 3. The results of the transfucosylation reactions catalyzed by recombinant β-D-galactosidase from *P. thiaminolyticus* using 60 mM *p*NPβ-D-Fuc as a donor and alcohols as acceptors of the fucosyl residue. All reactions were performed at 50°C for a period necessary for the maximal production of the desired transfucosylation product. Standard molecules (lines 1–3) and reaction mixtures (lines 4–9) were spotted on the Silica gel TLC plate. Separation was carried out in the mobile phase ethylacetate:acetic acid:water (7:2:2, v/v/v) and the compounds containing saccharidic moiety were visualized by 2-methylresorcinol. Line 1 – *p*-nitrophenol, line 2 – D-fucose, line 3 – *p*NPβ-D-Fuc, line 4 – reaction mixture containing 20% methanol as an acceptor, line 5 – reaction mixture containing 20% ethanol as an acceptor, line 6 – reaction mixture containing 10% 1-propanol as an acceptor, line 7 – reaction mixture containing 10% 2-propanol as an acceptor, line 8 – reaction mixture containing 10% 1-octanol as an acceptor and line 9 – reaction mixture containing 10% 2-octanol as an acceptor. Desired transglycosylation products are indicated by arrows.

Table III. Results of transfucosylation reactions catalyzed by recombinant β-D-galactosidase from *P. thiaminolyticus* using alcohols as acceptors

Donor	Acceptor	Yield (%)
*p*NPβ-D-Fuc	Methanol	94
	Ethanol	72
	1-propanol	56
	2-propanol	24
	1-octanol	15
	2-octanol	5

Yields are calculated according to the amount of added donor.

peaks between H-1′–C-3 and H-3–C-1′. On the other hand, cross peaks between H-1′–C-6 and H-6–C-1′ characterized the (1′→6) glycosidic bond for the compound formed by fucosylation of *p*NPα-D-Gal. The results of the NMR analysis are summarized in Table II. Presented yields are calculated according to the amount of acceptor molecule added to reaction mixture and the amount of transfucosylation products isolated by the extraction to methanol during the preparation of samples for NMR analysis. It is necessary to emphasize that the yield of the transglycosylation reaction using *p*NPβ-D-Fuc as a donor as well as an acceptor is not easily comparable with yields from other transglycosylations, for the final yield is relative to the entire amount of *p*NPβ-D-Fuc added to the reaction mixture.

The results indicate the ability of the enzyme to create different glycosidic bonds, depending on the type of used saccharidic acceptor. The hydroxyl group at C3 of the fucose, mannose, and glucose is preferred while the C6 position is used for the transfer to galactose. The bonds involving the hydroxyl groups at C2 and C4 were not detected.

For the transglycosylation products of reactions containing alcohol molecules as acceptors, only mass spectrometry analysis was carried out, for only one possible bond between the alcohol and fucosyl residue exists, the glycosidic bond between the C1 of the fucosyl moiety and hydroxyl group of the alcohol, resulting from the reaction mechanism. The transfucosylation products were not isolated from the reaction mixtures in these experiments; only the enzyme was removed before analysis from the mixture by filtration on Cellulose Triacetate filters Vecta Spin Micro. Mass spectrometry confirmed the presence of all supposed glycosylated alcohols. The results of transglycosylation reactions are summarized in Table III. The yields

of transfucosylation reactions were, in these cases, calculated using the data of the TLC spot densities (obtained by using program TotalLab) of substrates and hydrolysis and transfucosylation products. Presented yields were calculated according to the amount of the *p*NPβ-D-Fuc used as the donor in the reaction mixture. The results indicate that the amount of transglycosylation products decreases with the length of the alkyl chain and that the primary hydroxyl group is more easily accessible for the enzyme than the secondary group of acceptor alcohols.

The yields of transglycosylation reactions using *p*-nitrophenyl glycopyranosides as acceptors, presented in Table II, are (in comparison with the results of reactions with alcohols, analyzed by program TotalLab) decreased by the extraction process. Program TotalLab was not correctly applicable in the case of *p*-nitrophenyl glycopyranosidic acceptors owing to the presence of different types and combinations of monosaccharidic moieties that may influence the results of staining by 2-methylresorcinol (Brückner 1955). (This problem did not appear during the analysis of TLC results of transglycosylation reactions when using alcohols as acceptors, where only one type of saccharidic moiety was present.) This is the reason why the yields were calculated for samples obtained by extraction during the process of sample preparation for the NMR analysis. Not identical contaminant compounds of the molecular weights of 304 Da (measured in the positive mode) and 894 Da (measured in the negative mode) were detected by mass spectrometry analysis in the samples of *p*-nitrophenyl β-D-fucopyranosyl-(1,6)-α-D-galactopyranoside and *p*-nitrophenyl β-D-fucopyranosyl-(1,3)-α-D-glucopyranoside after the extraction, respectively. Their presence was considered in the final yield calculation.

Discussion

The gene of β-D-galactosidase from *P. thiaminolyticus* was found and sequenced. According to the glycosyl hydrolases classification introduced by Henrissat (1991), based on the primary sequences similarities, β-D-galactosidases generally belong to the glycoside hydrolase families 1, 2, 35, and 42, all of which are ordered to the clan GH-A (Henrissat and Davies 1997). According to the amino acid sequence it was determined that β-D-galactosidase from the *P. thiaminolyticus* exhibits the highest similarity to the enzymes from the glycoside hydrolase family 35. This family encloses the enzymes with β-D-galactosidase (EC 3.2.1.23) or exo-β-glucosaminidase (EC 3.2.1.165) activity from all three domains, i.e., Archaea, Bacteria, and Eukaryota. β-D-Galactosidases from *Bacillus circulans* ATCC 31382, *Arabidopsis thaliana,* or human β-D-galactosidase could be mentioned as examples of the most interesting members of the glycoside hydrolase family 35 (Cantarel et al. 2009).

According to our results β-D-galactosidase from *P. thiaminolyticus* is a monomeric enzyme with the molecular weight of 66.0 kDa (68.6 kDa in the form of a histidine-tagged fusion protein). As noted earlier, β-D-galactosidases are enzymes occurring in microorganisms as well as in plants and animals (Panesar et al. 2006). Their molecular weight ranges from 19 kDa to 750 kDa and both monomeric and multimeric proteins are known. The presence of β-D-galactosidase in the monomeric form was referred to in different organisms including bacteria, fungi, plants, and mammals (molecular weights between 41 kDa and 145 kDa) (data published in *The Comprehensive Enzyme Information System BRENDA* (Chang et al. 2009)). As an example, the data published by Fujimoto and co-workers can be mentioned as being in good agreement with our results. They described the β1–3 linkage specific β-galactosidase from *Bacillus circulans*, occurring in the monomeric form with the deducted molecular weight of 66.9 kDa (Fujimoto et al. 1998).

As presumed in the very beginning of this work, the enzyme from *P. thiaminolyticus* exhibited two different activities, β-D-fucosidase and β-D-galactosidase. In addition to these two activities an ability to cleave β-D-glucosides was also observed.

The ability to cleave the chromogenic substrate *p*NPβ-D-Fuc or *o*-nitrophenyl β-D-fucopyranoside was observed by many different β-D-galactosidases (Chang et al. 2009). They included salt-tolerant β-galactosidase from psychrophilic Antarctic *Planococcus* isolate (Sheridan and Brenchley 2000) or thermostable β-galactosidases from *Thermotoga maritima* (Li, Zhang, et al. 2009) and *Sterigmatomyces elviae* CBS8119 (Onishi and Tanaka 1995). Even two successful strategies for enhancing the β-fucosidase activity of β-galactosidase from *E. coli* were described in works of Zhang et al. and Parikh and Matsumura. In the first mentioned paper, the authors used the method of DNA shuffling (Zhang et al. 1997), while the second work applied the strategy of site-saturation mutagenesis (Parikh and Matsumura 2005).

Regarding the interest of finding new synthetic tools for the synthesis of β-D-fucosylated compounds, two types of molecules (*p*-nitrophenyl glycopyranosides and alcohols) were tested for the possibility of serving as acceptors in transglycosylation reactions catalyzed by the recombinant β-D-galactosidase from *P. thiaminolyticus*. As mentioned earlier, all transglycosylation reactions were followed by TLC analysis and the products containing saccharidic moieties were visualized by 2-methylresorcinol. In the case of *p*-nitrophenyl glycopyranosidic acceptors, ultraviolet light (UV) detection, visualizing donor, acceptor, and transglycosylation products containing the *p*-nitrophenyl part, was also useful.

According to TLC results it was possible to predict the glycosylation of all chosen acceptors, for not only the spots corresponding to substrates of the reactions (donor and acceptor molecules) or to hydrolysis products, but also one or more additional spots were observed, representing the transglycosylation products. The presence of all expected transglycosylation products was confirmed by mass spectrometry analysis. In the case of *p*-nitrophenyl glycopyranosidic acceptor molecules, a further structural analysis, enabling the identification of glycosylated hydroxyl groups positions, was necessary. NMR spectroscopy was used for these purposes and the main transfucosylation products were isolated by extraction to methanol. Other molecules, present in small amounts and detectable by UV, are assumed to be the products of transfucosylation of the donor molecule (in all cases the *p*NPβ-D-Fuc may serve as a donor as well as an acceptor) or products containing more than one attached fucosyl residue. Other minor products, visible only after staining by 2-methylresorcinol, are supposed to be the products of transglycosylation reactions using D-fucose, liberated during hydrolysis, as an acceptor.

Transglycosylation activities of many β-D-galactosidases originating from numerous sources have been investigated in the past few years. Enzymes with the ability to catalyze the formation of different glycosidic bonds were found and

characterized. Thus, the ability to transfer the galactosyl residue to a number of different acceptors was described. Although the β-D-fucosidase activity of some β-D-galactosidases has been known for a long time, the possibility of utilizing these enzymes for the synthesis of β-D-fucosylated molecules has not been so far sufficiently described.

The recombinant β-D-galactosidase from *P. thiaminolyticus* seems to be an enzyme preferring the formation of β(1→6) and β(1→3) glycosidic bonds, depending on the glycosylated molecule. The β(1→6) glycosidic linkage was found in the main product in the transfucosylation reaction, using *p*NPα-D-Gal as an acceptor. In all other analyzed products the fucosyl moiety was attached to the acceptor by the β(1→3) linkage.

The formation preference of the (1→6) glycosidic linkage during the transglycosylation reaction catalyzed by β-D-galactosidase from porcine liver, using *p*NPα-D-Gal and *p*NPβ-D-Gal as acceptors and *o*NPβ-D-Gal as a donor, was published by Zeng and co-workers. They also reported a significant influence of acceptor *p*-nitrophenyl group orientation, because yields obtained by using the α-glycosidic acceptors were higher than for the β-linkage containing derivatives. In the same work, the formation of (1→3) and (1→6) glycosidic bonds was reported to be catalyzed by the β-D-galactosidase from *Bacillus circulans* ATCC 31382 in the reaction with *p*NPα-D-Gal, serving as an acceptor, but only linkage (1→6) was formed when using *p*NPβ-D-Gal (Zeng et al. 2000).

The regioselectivity toward D-mannose used as an acceptor was tested for a series of β-D-galactosidases of different origins. Only β-1,3-galactosidase from *Bacillus circulans* ATCC 31382 was able to catalyze the formation of products containing (1→3) and (1→6) glycosidic bonds, in the ratio favorable for the (1→3) linkage-containing product (final ratio 2:1). β-Galactosidases from *E. coli*, *Aspergillus oryzae*, and *Penicillium multicolor* preferred the formation of the (1→6) glycosidic bond, and in the case of β-1,4-galactosidase from *Bacillus circulans* dominated the formation of the (1→4) linkage (62%), while the yields of (1→6) and (1→3) linkage-containing products were almost similar (18% and 20%, respectively). β-Galactosidase from *Bifidobacterium bifidum* catalyzed the formation of (1→3), (1→4), and (1→6) linkage-containing products, approximately in the ratio of 1:2:2. The enzyme from *Streptococcus* 6646K catalyzed exclusively the formation of the (1→4) glycosidic linkage (Miyasato and Ajisaka 2004).

Recombinant β-glycosidase from *Thermus thermophilus*, favoring the formation of the (1→3) glycosidic linkage during transglycosylation reactions, was used in the work of Fourage and Colas for the transfucosylation reaction using *p*NPβ-D-Fuc as a donor and glucose as an acceptor. During the research the preparative synthesis of forming disaccharide was tested. The final yield (expressed as a percentage of initial concentration in *p*NPβ-D-Fuc) after purification of the product was 25% (Fourage and Colas 2001).

The ability to catalyze the transfucosylation reaction, using *p*NPβ-D-Fuc as a donor and glucose as an acceptor molecule, was also confirmed for the β-D-glucosidase I from *Bifidobacterium breve* clb. A mixture of transfucosylation products, containing (1→2), (1→3), (1→4), and (1→6) glycosidic linkages was obtained and its successful assimilation by bifidobacteria was determined (Sakai et al. 1989).

Glycosidase Gly-001-09 from CLONEZYME™ thermophilic glycosidase library was chosen by Li and co-workers for the production of β-D-fucopyranosyl-β-D-xylopyranosides containing (1→2) and (1→3) glycosidic bonds. According to the used xylopyranoside derivative (an acceptor), the final yield could reach 88%. The ratio of arising products, containing the (1→2) or (1→3) glycosidic linkage, could be influenced by the character of the aglyconic part of the xylopyranoside derivative (Li et al. 1998).

Growing interest in possible applications of alkylglycosides in different biotechnological areas was the reason for testing the ability of the recombinant β-D-galactosidase from *P. thiaminolyticus* to utilize also primary or secondary alcohols of a different alkyl chain length as acceptors in transfucosylation reactions. A significant influence of both mentioned features was observed. The enzyme was able to use all of the tested alcohols (methanol, ethanol, 1- and 2-propanol, and 1- and 2-octanol) as acceptors (products confirmed in the reaction mixtures by mass spectrometry analysis); however, primary hydroxyl groups were preferred to secondary ones and the transglycosylation yields decreased markedly from methanol to alcohols with a longer alkyl chain.

Similar trends were obtained using the lipid-coated β-D-galactosidase from *E. coli* and *Aspergillus orzyae* in the two-phase aqueous-organic system. Okahata and Mori (1998) reported the ability of both enzymes to catalyze transgalactosylation reactions (lactose used as a galactosyl donor) and the decrease in the reaction yield from the primary to secondary hydroxyl group of 1-butanol and 2-butanol used as acceptors. Only β-D-galactosidase from *E. coli* was able to galactosylate the tertiary hydroxyl group of tert-butanol, the yield being approximately one half as much as for 2-butanol. Different results were observed for the lipid-coated β-D-galactosidase from *Bacillus circulans*, where the secondary hydroxyl group was preferred to the primary one, but where the tertiary hydroxyl group was not glycosylated at all. In the same work the influence of the alkyl chain length over the transgalactosylation activity of lipid-coated β-D-galactosidase from *E. coli* was also tested. High yields were obtained for 1-butanol or 1-octanol used as acceptors (67% and 82% of conversion, respectively), but a rapid decrease was observed when using 1-decanol or 1-dodecanol (Okahata and Mori 1998). Similar results were obtained during the study of galactosylation of 1-butanol, 1-hexanol, and 1-octanol using β-D-galactosidase from *Bacillus megaterium* 2-37-4-1 for all three acceptors, yields ranging from 15–20% (Li, Wang, et al. 2009).

High transfucosylation activity was confirmed for β-glucosidase from Thai rosewood and for almond β-glucosidase in reactions containing methanol, ethanol, 1-propanol, and 2-propanol as acceptors and *p*NPβ-D-Fuc as a donor molecule (Lirdprapamongkol and Svasti 2000). Almond β-glucosidase was also confirmed to be able to catalyze the condensation reactions producing alkyl fucosides. The trend of decreasing conversion was observed from 1-hexanol to 1-octanol used as acceptors (Kobayashi et al. 1999).

In conclusion, during research the gene of β-D-galactosidase with β-D-fucosidase activity from *P. thiaminolyticus* was found and sequenced and the enzyme was expressed in the form of the recombinant histidine-tagged fusion protein. The ability of the enzyme to catalyze the transfucosylation reactions was confirmed and, regarding broad acceptor specificity and in some cases relatively high yields of transglycosylation reactions, the enzyme seems to be a promising biocatalyst for synthesis of

β-D-fucosylated molecules. Based on these results, the study deepening the knowledge on the possibility of using this enzyme in transglycosylation reactions will continue. The main emphasis will be directed at testing the enzyme's ability to use further acceptor types in transfucosylation reactions.

Material and methods

Cultivation of P. thiaminolyticus

Bacterial strain *P. thiaminolyticus* was cultivated in the liquid Luria–Bertani medium (LB) for 16 h at 30°C in a platform shaker at 250 RPM or on LB plates under same temperature conditions. After cultivation, cells were harvested by centrifugation (4000 g, 20 min, 4°C).

Disintegration of cells of P. thiaminolyticus

After cultivation the harvested cells were resuspended in the 25 mM EPPS buffer (pH 8) (Sigma-Aldrich, USA) and disrupted using lysozyme (final concentration of 5 mg/mL) (Fluka, USA), natrium deoxycholate (final concentration 0.1%) (Sigma-Aldrich), DNase (30 U to 1 mL) (Sigma-Aldrich) and sonication (20 W, 6 × 30 s) at Sonicator® 3000 ultrasonic liquid processor (Misonix Inc., USA). Cell debris was discarded by centrifugation (20,000 g, 20 min, 4°C).

Native electrophoresis in polyacrylamide gel

Native electrophoresis in polyacrylamide gel (Laemli 1971) was performed in 6.8% running gel under a constant voltage of 100 V at 4°C. After separation the gels were incubated in the (a) 6.6 mM *p*NPβ-D-Fuc (MP Biomedicals Inc., France) in 0.2 M HEPES buffer (pH 8) (Sigma-Aldrich) or (b) 6.6 mM *o*NPβ-D-Gal (Sigma-Aldrich) in 0.2 M HEPES buffer (pH 8) or (c) 2.2 mM X-gal (purchased from Duchefa Biochemie, Netherlands) dissolved in a 2% solution of DMF (Sigma-Aldrich) in 25 mM EPPS buffer (pH 8) to visualize the proteins with β-D-fucosidase and β-D-galactosidase activity.

Preparation of genomic library

Genomic DNA of *P. thiaminolyticus* was isolated from the overnight culture with a Genomic tip 500/G kit (Qiagen, USA) and partially digested by the restriction enzyme *Sau3*AI. Resulting fragments were ligated into *Bam*HI linearized and Calf Intestinal Alkaline Phosphatase (CIAP) dephosphorylated vector pUC19ΔlacZ (derived from commercial pUC19 (GibcoBRL, USA) by Karasová-Lipovová et al. (2003)) using T4 DNA ligase (CIAP was purchased from Invitrogen (USA); all other enzymes were purchased from New England Biolabs (USA)). Competent cells of *E. coli* DH5α (GibcoBRL, USA) were transformed with the ligation reaction mixture and spread on LBA plates (final concentration of ampicillin was 0.1 mg/mL) (AppliChem GmbH, Germany), X-gal (40 μg/mL), and isopropyl β-D-thiogalactopyranoside (IPTG, 40 μg/mL) (purchased from Duchefa biochemie, Netherlands). Plasmid DNA was isolated by alkaline lysis from positive colonies (production of β-D-galactosidase indicated by the blue color). The procedures of ligation, transformation, and alkaline lysis were performed according to standard protocols (Sambrook and Russel 2001).

Sequence analysis and expression plasmid construction

The inserted part of isolated plasmid DNA from the positive colony was sequenced (Geneart, Germany) using the method of primer walking; thus, the sequence of both strands was determined. The obtained sequence was compared to the NCBI database using program WU-BLAST2, and the open reading frame of the gene encoding β-D-galactosidase was determined. According to the obtained results, two primers were designed for PCR producing DNA fragment for insertion into the expression vector. The restriction site for endonuclease *Nde*I (emphasized by underlining) was inserted into primer designed for the 5′-terminal part (ATCGGCGAAGGAGAGAAGCATATGACAACG) and the restriction site for *Bam*HI was contained in the primer for the 3′-terminal part (CACAGCGCTGGGGATCCGAACTAAAG) of the amplified sequence. Genomic DNA of *P. thiaminolyticus* was used as a template in the PCR reaction catalyzed by Kod Hot Start DNA polymerase (Novagen, USA). Reaction conditions were adjusted according to the instructions of the manufacturer. The PCR product was specifically cleaved by restriction endonucleases *Nde*I and *Bam*HI to be applicable for ligation into the expression plasmid pET16b (Novagen, USA) digested with the same enzymes and dephosphorylated by CIAP. The competent cells of *E. coli* BL21 Gold (DE3) (Stratagene, USA) were transformed by the ligation mixture and cultivated on LBA plates with X-gal and IPTG, as described previously.

The plasmid DNA of colonies producing active β-D-galactosidase (indicated by the blue color of colony) was isolated by alkaline lysis. The accuracy of the construct was confirmed by sequencing and the construct (named pET16b-βgal-2M) was used for the expression of the histidine-tagged protein.

Expression, disintegration, and purification of recombinant β-D-*galactosidase*

The competent cells of *E. coli* BL21 (DE3) (Novagen, USA) were transformed with plasmid pET16b-βgal-2M and grown on LBA plates. One colony was used for the preparation of overnight inoculum (10 mL of LBA, 37°C, 250 RPM), subsequently used the following day for 1% inoculation. After inoculation the cells were grown in a shaking incubator (LBA, 37°C, 250 RPM) and the increase of optical density (OD) at 600 nm was followed. At an OD of 0.5 IPTG was added to the final concentration of 0.3 mmol/L to induce expression. Four hours after induction of expression, the cells were harvested by centrifugation (4000 × *g*, 20 min, 4°C), suspended in the 25 mM EPPS buffer (pH 8), and disrupted, as described previously, for disintegration of cells of *P. thiaminolyticus*. The supernatant, obtained by centrifugation (20,000 × *g*, 20 min, 4°C) after disintegration, was applied on the Ni-NTA agarose affinity chromatography column (Qiagen GmbH, Germany) equilibrated with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl (Lach-Ner, s.r.o, CZ) and 10 mM imidazole (Sigma-Aldrich, USA). The contaminants were washed out of the column by the same buffer with 40 mM imidazole. The recombinant histidine-tagged β-D-galactosidase was eluted with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl and 250 mM imidazole. Fractions containing the recombinant protein (determined by the activity assay) were put together and desalted by gel chromatography using column PD10 (GE Healthcare, UK). Processes of expression, disintegration, and purification were followed by

SDS–PAGE electrophoresis (Laemli 1971) in 10% running gel under reduction conditions of dithiotreitol (Amresco, USA). Separated proteins were visualized by Coomassie Brilliant Blue R-250 (Amresco).

Protein concentration estimation

Protein concentration was estimated parallelly by measuring the absorbance at 280 nm and by Bradford protein assay (Bradford reagent was purchased from Amresco, USA) using bovine serum albumin (SERVA Electrophoresis GmbH, Germany) as standard (Bradford 1976).

Enzyme assay

β-D-Fucosidase activity was measured in the 50 mM phosphate buffer (pH 6) at 37°C for 10 min using as a substrate 15 mM *p*NPβ-D-Fuc in the reaction mixture. The reaction was terminated by the addition of an equal volume of 10% Na_2CO_3. The absorbance of the reaction mixture was detected at 405 nm and the amount of released *p*-nitrophenol was calculated using the calibration curve carried out under same conditions. The amount of the enzyme, able to release 1 μmol of *p*-nitrophenol per minute at 37°C, was defined as one unit.

The pH dependence of the enzyme activity was determined in a set of Britton–Robinson buffers of different pH covering the range from 2 to 10. The influence of the temperature on activity was tested for temperatures ranging from 10°C to 85°C. Kinetic parameters were calculated by the nonlinear regression method.

The dependence of the enzyme activity on the substrate concentration was also measured for substrates *p*NPβ-D-Gal, *p*NPβ-D-Glu, and lactose (37°C, 50 mM phosphate buffer (pH 6)). The experimental procedure for chromogenic substrates differed from the measurement with *p*NPβ-D-Fuc only in the reaction time (1 h) in the experiment using *p*NPβ-D-Glu as a substrate. In the case of lactose used as a natural substrate of β-D-galactosidases, the reaction was terminated after 15 min by 5 min incubation at 90°C. The concentration of released glucose was determined using Glu God BIO-LA-TEST (Pliva-Lachema Diagnostika s.r.o., CZ). Kinetic parameters were again calculated using the method of nonlinear regression.

Gel filtration chromatography

The molecular weight of the produced recombinant protein was determined by gel filtration chromatography using column Sephacryl S-300 HR 16/60 (AP Biotech, UK). Separation was carried out in the 0.1 M phosphate buffer (pH 6) with the flow rate of 0.5 mL/min on the BioLogic Duo-Flow System (Bio-Rad, USA). Molecular weight was calculated from the calibration curve. Blue dextran 2000 (2000 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) (all purchased from AP Biotech, UK), bovine serum albumin (67 kDa) (SERVA Electrophoresis GmbH, Germany), and lysozyme (14.5 kDa) (Fluka, USA) were used as standard proteins.

Transglycosylation reactions

For transglycosylation reactions two different types of acceptor molecules were used: *p*-nitrophenyl glycopyranosides and alcohols. In the first case chromogenic substrates, not cleavable by the enzyme (with the exception of *p*NPβ-D-Fuc), as *p*NPβ-D-Fuc, *p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-GlcA, and *p*NPα-D-Man, were used. All of them (besides *p*NPβ-D-Fuc as listed previously) were purchased from Sigma-Aldrich. In the second case alcohols containing the primary or secondary hydroxyl group (methanol, ethanol, and 1-propanol, (Penta, CZ), 2-propanol (Lach-Ner, s.r.o, CZ), 1-octanol (Fluka, USA), and 2-octanol (Sigma-Aldrich)) were used.

The reaction mixture with *p*-nitrophenyl glycopyranosides as acceptor molecules consisted of the 50 mM phosphate buffer (pH 6), 50 mM *p*NPβ-D-Fuc (as the donor molecule), and 33 mM acceptor (all acceptor molecules are listed previously). The only exception was the reaction mixture with *p*NPβ-D-Fuc serving as a donor as well as an acceptor molecule, where the concentration was 80 mmol/L. The reaction was initiated by the addition of 0.53 U of the purified β-D-galactosidase to 1050 μL of the reaction mixture containing *p*NPα-D-Glc or *p*NPα-D-Man, 0.26 U to the reaction mixture of the final volume 875 μL containing *p*NPβ-D-Fuc, and 0.12 U to 700 μL of the reaction mixture with *p*NPα-D-Gal. (Different amounts of the added enzyme result from different isolations used in particular experiments.) The basic effort was to use the highest possible amount of the enzyme and to retain the concentrations of acceptor and donor molecules. The volumes of the reaction mixtures were designed according to expected yields, so that the initial amount of *p*NPβ-D-Fuc was 10 mg (*p*NPα-D-Gal used as an acceptor), 15 mg (*p*NPα-D-Glc or *p*NPα-D-Man used as acceptors), or 20 mg (*p*NPβ-D-Fuc used as an acceptor). All the reactions were carried out at 50°C.

The reaction times (50 min for *p*NPα-D-Gal as an acceptor, 5 h for *p*NPα-D-Man, 4 h for *p*NPβ-D-Fuc and 3 h for *p*NPα-D-Glc) were determined as times of maximal production of transglycosylation products. The reactions were not terminated but immediately applied on TLC plates and separation was carried out. Desired transglycosylation products were then isolated and prepared for mass spectrometry and NMR spectroscopy analysis. The final yields of particular transglycosylation products were calculated from amounts of acceptor added to the reaction mixture and obtained compound after the isolation procedure.

Reaction mixtures containing alcohols as acceptor molecules differed in the amount of alcohol content (20% v/v of methanol and ethanol and 10% of 1-propanol, 2-propanol, 1-octanol, and 2-octanol). The content of alcohol resulted from screening experiments, where different amounts of alcohols (ranging from 10% to 30%) were used. Reaction mixtures with highest yields were used for further investigation. All reactions were performed at 50°C in the 50 mM phosphate buffer (pH 6) containing 60 mM *p*NPβ-D-Fuc. Reactions were started by the addition of 0.02 U of the purified β-D-galactosidase to 150 μL of the reaction mixtures containing methanol, ethanol, and 2-propanol and 0.05 U to the reaction mixtures with 1-propanol, 1-octanol, and 2-octanol. Different amounts of added β-D-galactosidase again resulted from different enzyme isolates used in particular experiments and from the effort to use the highest possible amount of the enzyme and to retain the concentrations of acceptor and donor molecules. After the reaction (2.5 h were determined as times of maximal production of transglycosylation products for methanol and ethanol, 4 h for 1-propanol and 2-propanol and 2 h for 1-octanol and 2-octanol), the enzyme was removed from the reaction mixture by filtration on Cellulose Triacetate filters Vecta Spin Micro (Whatmann, UK) with cut-off of 12 kDa. Prepared samples were analyzed by TLC and characterized by mass spectrometry. (Particular transglycosylation products were not isolated from reaction mixtures.) Yields of formed

transglycosylation products were calculated according to TLC spot densities determined by program TotalLab (Nonlinear Dynamics, UK) using the data for the added donor molecule, obtained transglycosylation products, and fucose released during the reaction. The sum of all these data represents 100% of fucosyl moieties in reaction mixtures.

Thin-layer chromatography

The samples of the reaction mixtures were spotted on the Silica gel TLC plate (Fluka, USA) and developed by a solvent system ethylacetate:acetic acid:water (7:2:2, v/v/v) (ethylacetate purchased from Lach-Ner, s.r.o, CZ and acetic acid from Penta, CZ). The spots of reaction products after separation were visualized by UV detection (in the case of molecules containing the *p*-nitrophenyl group) and by 0.1 M 2-methylresorcinol (Alfa Aesar GmbH & Co. KG, Germany) dissolved in the 5% (v/v) solution of sulfuric acid (Lach-Ner, s.r.o, CZ) in ethanol, detecting the saccharidic parts of molecules (after heating of the TLC plate).

Identification and characterization of transglycosylation products by mass spectrometry and NMR spectroscopy analysis

All transglycosylation products, obtained from transfucosylation reactions using *p*-nitrophenyl glycopyranosides and alcohols as acceptors, were confirmed by mass spectrometry analysis in mode ESI+ (Q-Tof micro mass spectrometer, Waters Micromass, USA) with direct inlet. In the case of alcohol acceptors the whole reaction mixture (enzyme removed by filtration on Vecta Spin Micro filters) was analyzed. Inasmuch as the reaction mechanism allows only the formation of one type of glycosidic bond (between the alcohol hydroxyl group and C1 of the fucosyl moiety), it was not necessary to analyze these products by NMR spectroscopy. Products of transglycosylation reactions with *p*-nitrophenyl glycopyranosides as acceptors were prepared by extraction into methanol directly from TLC plates after separation. Methanol was evaporated for decreasing the volume and samples were filtered using syringe filters with the polytetrafluoroethylene membrane (0.45 μm) (Whatmann International Ltd). Remaining methanol was evaporated and the samples were dissolved in D_2O, so that they were suitable not only for mass spectrometry analysis, but also for NMR spectroscopy analysis. ^{1}H, ^{13}C, COSY, HMQC and HMBC, NOE, and TOCSY spectra were measured on a Bruker Advance^{III} 600 (Bruker Corporation, Germany) spectrometer operating at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C. All spectra were acquired at 298 K in D_2O. Chemical shifts are given in δ-units (ppm) and are referenced to TMS.

p-Nitrophenyl β-D-*fucopyranosyl-(1,6)*-α-D-*galactopyranoside*: ^{1}H NMR: δ 8.21 (d, 2H, J = 9.2 Hz, p-NO_2Ph), δ 7.26 (d, 2H, J = 9.2 Hz, p-NO_2Ph), δ 5.79 (d, 1H, J = 3.7 Hz, H-1), δ 4.23 (d, 1H, J = 7.9 Hz, H-1′), δ 4.15 (m, 1H, H-5), δ 4.04–4.09 (m, 2H, H-3, H-4), δ 3.98 (dd, 1H, J = 3.7 Hz, 9.7 Hz, H-2), δ 3.92 (dd, 1H, J = 4.6 Hz, 11.3 Hz, H-6a), δ 3.74 (dd, 1H, J = 7.3 Hz, 11.3 Hz, H-6b), δ 3.61–3.65 (m, 2H, H-4′, H-5′, overlapped with impurity), δ 3.46 (dd, 1H, J = 3.4 Hz, 9.9 Hz, H-3′), δ 3.28 (dd, 1H, J = 7.9 Hz, 9.9 Hz, H-2′), δ 1.11 (d, 3H, J = 6.5 Hz, 6′-CH_3).

^{13}C NMR: δ 161.7 (p-NO_2Ph-1″), 142.4 (p-NO_2Ph-4″), 126.1 (p-NO_2Ph-3″), 117.0 (p-NO_2Ph-2″), 102.8 (C-1′), 97.0 (C-1), 72.9 (C-3′), 71.2 (C-5′), 70.9 (C-5), 70.8 (C-4′), 70.4 (C-2′), 69.1 (C-3, C-4), 68.6 (6-CH_2), 67.8 (C-2), 15.3 (6′-CH_3).

p-Nitrophenyl β-D-*fucopyranosyl-(1,3)*-α-D-*mannopyranoside*: ^{1}H NMR: δ 8.21 (d, 2H, J = 9.2 Hz, p-NO_2Ph), δ 7.25 (d, 2H, J = 9.2 Hz, p-NO_2Ph), δ 5.76 (d, 1H, J = 1.5 Hz, H-1), δ 4.52 (d, 1H, J = 7.8 Hz, H-1′), δ 4.33 (dd, 1H, J = 3.2 Hz, H-2), δ 4.15 (dd, 1H, J = 3.2 Hz, 9.6 Hz, H-3), δ 3.82 (dd, 1H, J = 9.7 Hz, H-4), δ 3.79 (m, 1H, H-5′), δ 3.75–3.66 (m, 3H, H-4′, H-6, overlapped), δ 3.65 (dd, 1H, J = 3.6 Hz, 9.9 Hz, H-3′), δ 3.63 (m, 1H, H-5), δ 3.53 (dd, 1H, J = 7.9 Hz, 9.8 Hz, H-2′), δ 1.23 (d, 3H, J = 6.6 Hz, 6′-CH_3).

^{13}C NMR: δ 160.8 (p-NO_2Ph-1″), 142.3 (p-NO_2Ph-4″), 126.1 (p-NO_2Ph-3″), 116.7 (p-NO_2Ph-2″), 101.2 (C-1′), 97.5 (C-1), 78.4 (C-3), 73.5 (C-5), 72.8 (C-3′), 71.3 (C-4′), 71.1 (C-5′), 70.6 (C-2′), 67.5 (C-2), 65.0 (C-4), 60.6 (6-CH_2), 15.4 (6′-CH_3).

p-Nitrophenyl β-D-*fucopyranosyl-(1,3)*-β-D-*fucopyranoside*: ^{1}H NMR: δ 8.21 (d, 2H, J = 9.3 Hz, p-NO_2Ph), δ 7.25 (d, 2H, J = 9.3 Hz, p-NO_2Ph), δ 5.19 (d, 1H, J = 7.7 Hz, H-1), δ 4.58 (d, 1H, J = 7.8 Hz, H-1′), δ 4.04 (d, 1H, J = 3.1 Hz, H-4), δ 4.00 (q, 1H, J = 6.6 Hz, H-5), δ 3.92 (dd, 1H, J = 7.7 Hz, 9.8 Hz, H-2), δ 3.87 (dd, 1H, J = 3.2 Hz, 9.8 Hz, H-3), δ 3.73 (q, 1H, J = 6.5, H-5′), δ 3.69 (d, 1H, J = 3.4 Hz, H-4′), δ 3.61 (dd, 1H, J = 3.4 Hz, 9.8 Hz, H-3′), δ 3.53 (dd, 1H, J = 7.8 Hz, 9.8 Hz, H-2′), δ 1.26 (d, 3H, J = 6.6 Hz, 6-CH_3), δ 1.24 (d, 3H, J = 6.5 Hz, 6′-CH_3).

^{13}C NMR: δ 161.8 (p-NO_2Ph-1″), 142.5 (p-NO_2Ph-4″), 126.1 (p-NO_2Ph-3″), 116.3 (p-NO_2Ph-2″), 104.1 (C-1′), 99.4 (C-1), 81.8 (C-3), 72.7 (C-3′), 71.3 (C-4′), 71.2 (C-2′), 70.9 (C-5), 70.8 (C-4, C-5′), 69.3 (C-2), 15.5, 15.3 (6-CH_3, 6′-CH_3).

p-Nitrophenyl β-D-*fucopyranosyl-(1,3)*-α-D-*glucopyranoside*: ^{1}H NMR: δ 8.22 (d, 2H, J = 9.3 Hz, p-NO_2Ph), δ 7.25 (d, 2H, J = 9.3 Hz, p-NO_2Ph), δ 5.80 (d, 1H, J = 3.5 Hz, H-1), δ 4.60 (d, 1H, J = 7.8 Hz, H-1′), δ 4.05 (dd, 1H, J = 9.3 Hz, H-3), δ 3.92 (dd, 1H, J = 3.5 Hz, 9.7 Hz, H-2), δ 3.80 (q, 1H, J = 6.5 Hz, H-5′), δ 3.73 (d, 1H, J = 3.0 Hz, H-4′), δ 3.62–3.70 (m, 4H, H-6a, H-5, H-6b, H-3′, overlapped with impurity), δ 3.45–3.60 (m, 2H, H-2′, H-4, overlapped with impurity), δ 1.23 (d, 3H, J = 6.5 Hz, 6′-CH_3).

^{13}C NMR: δ 161.2 (p-NO_2Ph-1″), 142.4 (p-NO_2Ph-4″), 126.0 (p-NO_2Ph-3″), 116.7 (p-NO_2Ph-2″), 103.2 (C-1′), 96.5 (C-1), 82.2 (C-3), 72.7 (C-3′), 72.6 (C-5), 71.2 (C-2′), 71.0 (C-4′), 71.0 (C-5′), 70.2 (C-2), 67.8 (C-4), 60.2 (6-CH_2), 15.4 (6-CH_3, 6′-CH_3).

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Abbreviations

CIAP, calf intestinal alkaline phosphatase; DMF, dimethylformamide; DNA, deoxyribonucleic acid; *E. coli*, *Escherichia coli*; IPTG, isopropyl β-D-thiogalactopyranoside; LBA, ampicillin containing Luria-Bertani medium; LB, Luria-Bertani medium; NMR, nuclear magnetic resonance; OD, optical density; *P. thiaminolyticus*, *Paenibacillus thiaminolyticus*; PAGE, polyacrylamide gel; PCR, polymerase chain reaction; *p*NPβ-D-Fuc, *p*-nitrophenyl β-D-fucopyranoside; *p*NPα-D-Gal, *p*-nitrophenyl α-D-galactopyranoside; *p*NPβ-D-Gal, *p*-nitrophenyl

β-D-galactopyranoside; *p*NPα-D-Glc, *p*-nitrophenyl α-D-glucopyranoside; *p*NPβ-D-Glc, *p*-nitrophenyl β-D-glucopyranoside; *p*NPα-D-GlcA, *p*-nitrophenyl β-D-glucuronide; *p*NPα-D-Man, *p*-nitrophenyl α-D-mannopyranoside; *o*NPβ-D-Gal, *o*-nitrophenyl β-D-galactopyranoside; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; UV, ultraviolet light.

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