Glycobiology Advance Access published May 30, 2013

α-L-Fucosidase from *Paenibacillus thiaminolyticus*: its hydrolytic and transglycosylation abilities

Eva Benešová Department of Biochemistry and Microbiology, ICT Prague, Prague, 166 28, Czech Republic, Fax: +420 220 445 167, Tel: +420 220 443 028, e-mail: Eva.Benesova@vscht.cz

- corresponding author (proofs etc.)

Petra Lipovová Department of Biochemistry and Microbiology, ICT Prague, Prague, 166 28, Czech Republic

Hana Dvořáková Laboratory of NMR spectroscopy, ICT Prague, Prague, 166 28, Czech Republic

Blanka Králová Department of Biochemistry and Microbiology, ICT Prague, Prague, 166 28, Czech Republic

Key words: α-L-fucosidase/ L-fucose/ *Paenibacillus thiaminolyticus*/ transglycosylation/

Abstract

In this work, focused on possible application of α-L-fucosidases from bacterial sources in synthesis of α-L-fucosylated glycoconjugates, several nonpatogennic aerobic bacterial strains were screened for α-L-fucosidase activity. Among them *Paenibacillus thiaminolyticus* was confirmed as a potent producer of enzyme with the ability to cleave the chromogenic substrate *p*-nitrophenyl α-L-fucopyranoside. The gene encoding α-L-fucosidase was found using genomic library of *Paenibacillus thiaminolyticus* constructed in the cells of *Escherichia coli* DH5α and sequenced (EMBL database: FN869117, CAZy database: glycosidase family 29). The enzyme was expressed in form of polyhistidine tagged protein (51,2 kDa) in *Escherichia coli* BL21 (DE3) cells, purified using Ni-NTA agarose affinity chromatography and characterized using the chromogenic substrate *p*-nitrophenyl α-L-fucopyranoside (K_m = (0.44 ± 0.02) mmol/L, K_S = (83 ± 8) mmol/L (substrate inhibition), $pH_{optimum}$ = 8.2, $t_{optimum}$ = 48 °C).

By testing the ability of the enzyme to catalyze the transfer of α-L-fucosyl moiety to different types of acceptor molecules it was confirmed that the enzyme is able to catalyze the formation of α-L-fucosylated *p*-nitrophenyl glycopyranosides containing α-D-galactopyranosidic, α-D-glucopyranosidic, α-D-mannopyranosidic or α-L-fucopyranosidic moiety. This enzyme is also able to catalyze α-L-fucosylation of aliphatic alcohols of different lenght of alkyl chain and hydroxyl group position (methanol, ethanol, 1-propanol, 2-propanol and 1-octanol) and hydroxyl group containing amino acid derivatives (*N*-(*tert*-butoxycarbonyl)-L-serine methyl ester and *N*-(*tert*-butoxycarbonyl)-L-threonine methyl ester).

Obtained results indicate the possibility to exploit studied enzyme in synthesis of different types of α-L-fucosylated molecules representing compounds with potential application in biotechnology and pharmaceutical industry.

Introduction

L-Fucose (6-deoxy-L-galactose) is a monosaccharide that is a constituent of oligosaccharidic chains of many glycoconjugates (glycoproteins, glycolipids) involved in many life important biochemical events. This saccharide moiety is usually situated on the nonreducing end of saccharidic chains, connected by α(1→2) glycosidic linkage to D-galactosyl residue or by α(1→3), α(1→4) or α(1→6) glycosidic bond to *N*-acetyl-D-glucosamine. Unlike most other common monosaccharides, which occur as D-isomers, fucose is in nature usually present in form of L-isomer (Ajisaka et al 1998; Becker and Lowe 2003; Flowers 1981; Moriwaki and Miyoshi 2010; Nelson and Cox 2005; Tarling et al 2003; Vanhooren and Vandamme 1999). The presence of D-fucose was confirmed e.g. in some compounds with interesting biological effects, as antibiotics (chartreusin, curamycin, labilomycin, avilamycin A), cardiac glycosides (e.g. ledienoside, cheirotoxin), spider venoms (*Tegenaria agrestis*, *Hololena curta*) or asterosaponins (D´Auria et al 1993; Cesar et al 2005; Flowers 1981; Kon et al 1990; Taggi et al 2004; Zayni et al 2007).

L-Fucose containing compounds were confirmed to participate in processes as growth regulation, ontogenesis, fertilization, apoptosis, inflammation etc. L-Fucosyl residues form also a part of erythrocyte surface antigens, which are responsible for determining of ABO blood groups, and are also present in oligosaccharides of human milk, which represent a natural protection of infants against some pathogens as e.g. *Campylobacter jejuni*. Changes in L-fucosylation patterns were observed also during pregnancy or in some pathological processes (Becker and Lowe 2003; Flowers 1981; Ma et al 2006; Osanjo et al 2007; Ruiz-Palacios et al 2003; Staudacher et al 1999; Vanhooren and Vandamme 1999; Wada et al 2008; Zeng et al 2003).

Two pathological events, probably most frequently mentioned in context of L-fucose, are fucosidosis and cancer diseases. Fucosidosis, caused by deficiency of α-L-fucosidase,

belongs to lysosomal storage diseases and is characterized by accumulation of α-L-fucose containing glycolipids, glycoproteins and oligosaccharides in lysosomes of different tissues. Consequences of this incorrect metabolism of α-L-fucose containing molecules are serious mental and neurological retardation and motoric degeneration of patients (Michalski and Klein 1999; Önenli-Mungan et al 2004; Osanjo et al 2007; Provenzale et al 1995; Sulzenbacher et al 2004; Tarling et al 2003). Changes in L-fucosylation of different glycoproteins and glycolipids were confirmed during some types of cancer diseases (e.g. lung carcinoma, colon cancer, hepatocarcinoma, ovarian and breast cancer) and are caused by an altered activity of fucosyltransferases. In some cases also changes in activity of α-L-fucosidase were observed in tumor tissues. It is supposed that α-L-fucosyl residues containing oligosaccharides on the malignant tumor cells are involved in process of metastasis propagation (Becker and Lowe 2003; Cordero et al 2001; Flowers 1981; Ma et al 2006; Miyoshi et al 2008; Moriwaki and Miyoshi 2010; Staudacher et al 1999; Vanhooren and Vandamme 1999).

α-L-Fucose containing glycans may also serve as ligands for some pathogens, e.g. *Campylobacter jejuni*, *Helicobacter pylori, Pseudomonas aeruginosa* or *Ehrlichia phagocytophila* (Becker and Lowe 2003; Ma et al 2006; Moriwaki and Miyoshi 2010; Osanjo et al 2007; Ruiz-Palacios et al 2003; Wada et al 2008). As other pathological events connected directly or indirectly with α-L-fucose residues it is possible to name leukocyte adhesion deficiency type II, cystic fibrosis, rheumatoid arthritis or diabetes mellitus type 1 (Becker and Lowe 2003, Cordero et al 2001; Eneyskaya et al 2001; Ma et al 2006; Staudacher et al 1999; Vanhooren and Vandamme 1999).

The ability to specifically synthetize α-L-fucose containing compounds may enable further investigation and deeper understanding of all named biologically important events and thus also effective designing of treatment of some severe diseases. As example the treatment

of cancer diseases (anti-cancer vaccines, targeting of cytotoxic agents to the carcinoma cells), enhancement of fruitfullness of transplantations or utilization of antiadhezive therapy can serve (Becker and Lowe 2003; Ma et al 2006; Osanjo et al 2007; Ruiz-Palacios et al 2003; Staudacher et al 1999; Vanhooren and Vandamme 1999; Wada et al 2008).

There are several different approaches enabling the synthesis of oligosaccharides or glycoconjugates: chemical synthesis, enzymatic synthesis or combination of these two technics. The advantage of enzymatic synthesis lies in the simplicity of the process, stereospecificity and sometimes also regiospecificity of the enzymatic reactions, in mild reaction conditions and in avoidance of toxic compounds sometimes necessary in chemical synthesis (Bojarová and Křen 2009; Crout and Vic 1998; Křen and Thiem 1997; Mackenzie et al 1998; Perugino et al 2004; Seeberger and Haase 2000; Watt et al 1997).

Two groups of enzymes may be used for the catalysis of glycosidic bond formation - glycosyltransferases and glycosidases, both of them having their advantages and limitations. Glycosyltransferases are enzymes responsible for the synthesis of oligosaccharidic structures in living organisms. They are strictly stereo- and regiospecific, but their limitations consist primarily in high costs of suitable substrates and in specificity to the acceptor molecule. Glycosidases belong to the class of hydrolases and under normal conditions they cleave glycosidic bonds in oligosaccharides and glycoconjugates. After suitable change of reaction conditions these enzymes are also able to catalyze the transfer of monosacharidic (or oligosaccharidic) residue to the acceptor molecule. The usage of glycosidases enables glycosylation of wide variety of acceptor molecules with utilization of relatively cheap donor molecules. The application of glycosidases is restricted by low regiospecificity of some of these enzymes and by the ability to cleave glycosylation product and thus decrease the final reaction yields. The solution for overcoming of all previously named complications that accompany glycosidases application consists among other in isolation and characterization of

new enzymes, in gene manipulation producing enzymes with more suitable features and in new technics as e.g. enzymatic solid fase synthesis or utilization of whole cells synthetic apparatus (Bojarová and Křen 2009; Crout and Vic 1998; Koeller and Wong 2000; Křen and Thiem 1997; Mackenzie et al 1998; Osanjo et al 2007; Perugino et al 2004; Trincone and Giordano 2006; Watt et al 1997; Wymer and Toone 2000).

α-L-Fucosidases (EC 3.2.1.51) are widespread hydrolytic enzymes, which catalyze the cleavage of α-L-fucosyl residue from nonreducing ends of oligosaccharidic chains. The ability of some of them to catalyze synthesis of glycosidic bonds via transfucosylation reaction using different types of acceptor molecules was described in different publications. α-L-Fucosidases from *Aspergillus niger*, *Corynebacterium* sp., ampullaria, *Penicillium multicolor*, *Pecten maximus*, *Alcaligenes* sp., *Thermus* sp. Y5 or canine α-L-fucosidase can be mentioned as examples. In some cases interesting results were obtained as e.g. production of higher oligosaccharides or even of branched structures (Ajisaka and Shirakabe 1992; Ajisaka et al 1998; Berteau et al 2004; Eneyskaya et al 2001; Osanjo et al 2007; Vetere et al 1997; Zeng et al 2003). However, strictly regioselective and high yielded synthesis of α-L-fucosylated molecules is still a challenging task and searching for new enzymes with transfucosylation abilities applicable to biosynthesis of particular products is of highest interest.

Preparation of recombinant α-L-fucosidase from *Paenibacillus thiaminolyticus*, characterization of its hydrolytic activity and testing the possibility to utilize this enzyme in synthesis of α-L-fucosyl containing molecules are the main topics of this paper.

Results

Determination of the α-L-fucosidase producing bacterium

Twenty-four aerobic and nonpathogenic bacterial strains (for concrete information see Methods) were screened for α-L-fucosidase activity using the chromogenic substrate *p*-nitrophenyl α-L-fucopyranoside (*p*NPα-L-Fuc). Different mesophilic, psychrotrophic and psychrophilic bacterial strains were chosen for the screening. α-L-Fucosidase activity was measured in cell extracts obtained after disintegration. *Paenibacillus thiaminolyticus* (*P. thiaminolyticus*) was confirmed as only one producer of an enzyme with desired activity.

Determination of the nucleotide sequence of the gene encoding α-L-fucosidase from P. thiaminolyticus and construction of expression plasmid pET16b-αLF1

The gene encoding the enzyme with α-L-fucosidase activity was searched using the genomic library of *P. thiaminolyticus* constructed in cells *Escherichia coli* (*E. coli*) DH5α. Because of absence of enzymes with α-L-fucosidase activity in cells of *E. coli* DH5α, positive colonies, producing enzyme with the ability to cleave chromogenic substrate 5-bromo-4-chloro-3-indolyl α-L-fucopyranoside (X-Fuc), were detected directly on ampicillin containing Luria-Bertani medium (LBA) plates with aforesaid substrate. The process of preparation of genomic library of *P. thiaminolyticus* and following detection of colonies producing an enzyme with desired activity is illustrated in Figure 1.

One positive colony was found and its plasmid DNA was isolated by Gen Elute^{TM} HP Plasmid Midiprep kit. The size of isolated plasmid was estimated to be approximately 6 500 bp. This plasmid DNA was further used as a template for sequencing by the method of primer walking (Geneart company, Germany). Determined sequence was compared with known sequences included in the NCBI database using program WU-BLAST2. Based on sequence similarities (an open reading frame with a 67% identity with a gene of α-L-

fucosidase from *Paenibacillus* sp. JDR-2 (ACT04757) was found) the gene of α-L-fucosidase from *P. thiaminolyticus* (1278 bp) was identified. Determination of presumable Shine-Dalgarno sequence 5´-AAGGAGAGAA-3´ enabled the identification of the start of the gene. Complete nucleotide sequence of this gene is available in EMBL Nucleotide Sequence Database with Accession No FN869117.

With consideration of advantages coming from production of enzymes in form of fusion proteins with histidine-tag, plasmid pET16b was chosen as a vector for the gene of α-L-fucosidase from *P. thiaminolyticus*. Prepared expression plasmid (for more details see the Methods) was named pET16b-αLF1 and its correctness was confirmed by sequencing.

Expression and purification of the recombinant α-L-fucosidase

Recombinant α-L-fucosidase was produced in competent cells *E. coli* BL21 (DE3) using prepared expression plasmid pET16b-αLF1. After cultivation the cells were disintegrated and cell extract containing produced recombinant enzyme was applied at the Ni-NTA agarose column, which is able to specifically bind histidine-tagged proteins. After the washing out of nonspecifically bind proteins α-L-fucosidase in form of fusion protein was eluted and samples of the purified enzyme were immediately transferred to the 25 mM EPPS buffer (pH 8) by gel filtration using PD10 desalting column. Processes of cultivation, disintegration and purification were followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and are summarized in the Figure 2.

It is possible to see that after induction of expression a protein of anticipated molecular weight (51.2 kDa) started to be produced. The weight of fusion protein was calculated from the amino acid sequence using program ProtParam tool on ExPASy Proteomics server. Also the method of affinity chromatography was confirmed to be very successful, because only one

single band is present after the elution of specifically bind proteins and their analysis by SDS-PAGE. Contaminants visible in the line 9 were either not eluted from the column or their concentration in the final solution was too low to be detected by Coomassie Brilliant Blue used for visualization.

Purified enzyme was further used for the characterization of hydrolytic and transglycosylation activities. In enzyme preparations after the purification process, which were used for the characterization, concentration of the proteins was on the average 0.1 mg/mL and the specific activity reached 80 μmol/min/mg.

Characterization of the hydrolytic activity of the recombinant α-L-fucosidase

Characterization of hydrolytic activity of recombinant α-L-fucosidase was carried out with the substrate *p*NPα-L-Fuc. Simultaneously it was determined that this enzyme is not able to cleave chromogenic substrates as *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), which were later tested to be possible acceptor molecules for transglycosylation reactions.

Effects of pH and temperature on the a-L-fucosidase activity: The influence of reaction temperature and pH on the activity of the enzyme was measured. Also the stability of the enzyme at different temperatures was determined (for more details see the Methods).

By measuring the temperature profile it was found that the highest activity was exhibited (under chosen conditions and reaction time of 10 min) at 48 °C. The optimal pH for the enzyme, determined using a set of Britton-Robinson buffers of different pH, was 8.2 and the zone of maximal activity was very narrow. Buffers suitable for the work with the enzyme

were 25 mM EPPS (pH 8) or 100 mM phosphate buffer (pH 8), but 50 mM Tris buffer inhibited notably the activity of the enzyme.

During the storage of the enzyme at - 20 °C its activity was continuously controlled for a period of 15 weeks. It was determined that the consequence of the freezing was a lost of 30% of original activity of the enzyme, which was measured immediately after the purification and desalting process. The time of storage did not exhibit further influence on the enzyme activity.

Apparent kinetic parameters for the recombinant a-L-fucosidase: The dependence of initial velocity of the reaction catalyzed by α-L-fucosidase on the concentration of pNPα-L-Fuc in the reaction mixture was measured in concentration range from 0 to 19 mmol/L. During the experiment it was discovered that the measured dependence corresponds to the behavior of substrate inhibition caused probably by contemporary binding of several substrate molecules into the active site of the enzyme. The K_m value was measured to be (0.44 ± 0.02) mmol/L and K_s as (83 ± 8) mmol/L.

Transfucosylation activity of the recombinant α-L-fucosidase

Three different types of acceptor molecules were used for transfucosylation reactions catalyzed by purified preparations of recombinant α-L-fucosidase. *p*-Nitrophenyl glycopyranosides (*p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-Man and *p*NPα-L-Fuc) constituted acceptors with saccharidic part. Aliphatic alcohols (methanol, ethanol, 1-propanol, 2-propanol, 1-octanol and 2-octanol) and hydroxyl group containing amino acid derivatives (*N*-(*tert*-butoxycarbonyl)-L-serine methyl ester (Boc-L-Ser-OMe) and *N*-(*tert*-butoxycarbonyl)-L-threonine methyl ester (Boc-L-Thr-OMe)) were tested as possible acceptors of nonsaccharidic character.

During designing of suitable reaction conditions for tranglycosylations it was necessary to consider relatively low solubility of *p*-nitrophenyl glycopyranosidic substrates serving as donor or acceptor molecules in buffer systems at 45 °C. This temperature was chosen as a suitable compromise among high enzyme activity, good stability and sufficient substrate solubility. The possibility to use dimethylformamide (DMF) for increase of *p*-nitrophenyl glycopyranosides solubility was also tested and α-L-fucosidase activity of the recombinant enzyme was measured in set of reaction mixtures containing 5%, 10%, 25% and 50% DMF. It was determined that in the presence of 10% of this organic solvent in reaction mixture the activity of α-L-fucosidase did not decrease under 90%. Stability of the enzyme at 45 °C in 10% DMF and 25 mM EPPS buffer (pH 8) for 6 hours was tested, too. More than 85% of original activity was preserved. Based on this results 10% DMF was used for the increase of the solubility of *p*-nitrophenyl glycopyranosidic donor and acceptor molecules in transglycosylation reactions utilizing *p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-Man, *p*NPα-L-Fuc, Boc-L-Ser-OMe and Boc-L-Thr-OMe as acceptors. In case of alcohol acceptors the solubility of the donor molecule was ensured by presence of acceptor molecules.

During experiments with *p*-nitrophenyl glycopyranosidic acceptors it was confirmed that the enzyme is able to catalyze the transfer of α-L-fucosyl moiety to *p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-Man and *p*NPα-L-Fuc. Results of analysis by thin layer chromatography (TLC) and visualization by 2-methylresorcinol (saccharidic parts of molecules) are demonstrated in the Figure 3. Visualisation by ultraviolet (UV) light (aromatic parts of molecules) is presented in supplementary data. Spots corresponding not only to donor and acceptor molecules or to products of hydrolysis, but also to transglycosylation products are visible. Formation of all transglycosylation products, predicted according to TLC analysis, was confirmed by mass spectrometry and NMR analysis as described in following paragraphs.

The determination of regiospecificity of the enzyme was a next task of this work. Samples of main transglycosylation products for the analysis by mass spectrometry and nuclear magnetic resonance (NMR) were prepared.

In case of *p*NPα-D-Man the amount of transglycosylation products was too low for an extraction and NMR analysis, but the presence of a compound of anticipated molecular weight was confirmed in the whole reaction mixture (after removing the enzyme by filtration - Cellulose Triacetate filters Vecta Spin Micro(12kDa)) by mass spectrometry.

Transglycosylation products of reactions with *p*NPα-D-Glc and *p*NPα-L-Fuc as acceptors were isolated by extraction from TLC plates to methanol and following evaporation, the main products of transfucosylation of *p*NPα-D-Gal had to be isolated by extraction to deionized water and by lyophilization because of presence of unidentified compounds soluble in methanol and insoluble in water. The presence of all products of molecular weights corresponding to used acceptor molecules with bind α-L-fucosyl moiety was confirmed by mass spectrometry. During the NMR analysis (using standard experiments as ^{1}H, ^{13}C, COSY, HMQC, HMBC, NOE and TOCSY) the formation of 2 different regioisomers was determined for *p*NPα-D-Glc and *p*NPα-L-Fuc serving as acceptors and even 5 regioisomers in case of *p*NPα-D-Gal. Based on results of ^{1}H-^{13}C HMBC experiments the types of formed glycosidic linkages of transfucosylation products of *p*NPα-D-Glc and *p*NPα-L-Fuc were determined. In both cases the cross peaks between H-1' – C-2 and H-2 – C-1' confirmed the presence of (1'→ 2) glycosidic bond. The second position of *p*NPα-L-Fuc used for the transfer of α-L-fucosyl moiety was the hydroxyl group at the carbon C4, in case of *p*NPα-D-Glc the utilized hydroxyl group was positioned at the carbon C6. Source materials for this results constitute again cross peaks between H-1' – C-4 and H-4 – C-1' or H-1' – C-6 and H-6 – C-1', respectively. In both cases the amounts of formed regioisomers were approximately 1:1.

In transfucosylation reactions utilizing *p*NPα-D-Gal as acceptor three of five formed regioisomers were produced in major amount in ratio 1:2.5:4. Particular types of glycosidic bonds were not possible to determine as the full NMR characterization could not be performed due to heavy overlaps of ^{1}H and ^{13}C resonances of different saccharide moieties. Formation of five regioisomers suggests the impossibility of the use of *p*NPα-D-Gal as an acceptor for application in biotechnological practice and thus further experiments were not carried out.

The results of NMR analysis and yields of reactions are summarized in Table I, assignments of detected NMR signals are mentioned in Methods. Final reaction yields were calculated according to the amount of acceptor added to reaction mixture and amount of transglycosylation product isolated by the extraction process. Yield presented for *p*NPα-L-Fuc serving as acceptor is not easily comparable with other yields because of the fact, that the acceptor molecule is also a donor of the α-L-fucosyl residue.

For transglycosylation reactions testing the ability of recombinant α-L-fucosidase to catalyze the transfer of α-L-fucosyl moiety to aliphatic alcohols methanol, ethanol, 1-propanol, 2-propanol, 1-octanol and 2-octanol were chosen as suitable acceptor molecules. Methanol and ethanol represent simplest alcohol structures, 1-propanol and 2-propanol are the shortest alcohols differing in position of hydroxyl group and 1-octanol and 2-octanol serve for monitoring of influence of the length of aliphatic chain on transglycosylation activity. Suitable amount of acceptor molecules was determined according to results of a set of experiments differing only in quantity of acceptor molecules in reaction mixtures.

Results of analysis of reaction mixtures by TLC and both visualization methods are demonstrated in the Figure 4 and in supplementary data. Products of transglycosylation of methanol, ethanol, 1-propanol and 2-propanol are of good visibility. All reaction mixtures were after filtration again analyzed by mass spectrometry and this method confirmed also the

presence of 1-octanol transglycosylation product. Further structural analysis of transglycosylation products by NMR was not necessary, for only one hydroxyl group is possessed by donor molecules.

For the determination of concrete reaction yields programs Total Lab and Image J were used in this case. TLC spot densities of remaining substrate and of hydrolysis and transglycosylation products, obtained as outputs of used analyzing programs, were used for the final yield calculation. It was not possible to use this method for the determination of reaction yields of α-L-fucosylation of *p*-nitrophenyl glycopyranosidic acceptors because of the presence of different types and combinations of saccharidic residues that influence the staining by 2-methylresorcinol (Brückner 1955). Transglycosylation products of alcohol α-L-fucosylation contain only one type of monosaccharidic moiety (α-L-fucosyl residue) and thus the results of Total Lab and Image J analysis are not affected. All results are summarized in Table II, this time the yields are calculated according to the amount of the donor molecule in the reaction mixture (the sum of spot densities of all molecules containing α-L-fucosyl residue after the end of reaction is calculated to be 100%).

Last acceptor molecules tested in transfucosylation reactions were amino acid derivatives Boc-L-Ser-OMe and Boc-L-Thr-OMe. Simultaneously with the monitoring of the ability of the enzyme to catalyze the transfer of the α-L-fucosyl moiety to hydroxyl group in side chains of amino acids the influence of the position of this hydroxyl group was tested again. Plates illustrating the results of TLC analysis after visualization are present in the Figure 5 and in supplementary data. The advantage of contemporary visualization by 2-methylresorcinol and UV light is well demonstrated here, for the same mobility of *p*NPα-L-Fuc and transglycosylation products. Spots indicated in Figure 5 by arrows are visible only after 2-methylresorcinol visualization and it is therefore possible to conclude that these spots have to belong to molecules with saccharidic part but without an aromatic ring. As only one

hydroxyl group was present in acceptor molecules only mass spectrometry was used for the analysis of whole reaction mixtures after removing enzyme by filtration. Compounds of anticipated molecular weights were confirmed in both cases.

Final reaction yields were calculated using programs Total Lab and Image J as described previously for alcohol acceptors. Results (calculated again according to the amount of donor molecule added to the reaction mixture) are presented in Table II.

Discussion

This paper represents our effort to find new enzymes for fucosylated molecules production. In our previous publication we demonstrated the possibility to use recombinant β-D-galactosidase from *P. thiaminolyticus* for β-D-fucosylation of different types of acceptor molecules (Benešová et al 2010). In this phase of our research we have successfully confirmed the possibility to use recombinant α-L-fucosidase originating from the same bacterial strain as used in our first work for the synthesis of α-L-fucosylated molecules.

The gene encoding the enzyme with α-L-fucosidase activity was found and sequenced. According to glycosidase classification introduced by Henrissat this enzyme belongs to glycoside hydrolase family 29 (Henrissat 1991) together with more than 600 other α-L-fucosidases (EC 3.2.1.51 and EC 3.2.1.111) from different origines (Archaea, Bacteria and Eukaryota). All these enzymes share among others the same reaction mechanism, it means the double-displacement mechanism with retaining of configuration at the anomeric carbon of arising product. This finding was important for following research due to the fact that α-L-fucosidases are at present members of only two glycoside hydrolase families, i.e. the families 29 and 95. Enzymes from glycoside hydrolase family 95 (EC 3.2.1.51 and 3.2.1.63) act by a mechanism resulting in an inversion of anomeric configuration and they are generally not able to catalyze transglycosylation reactions (Cantarel et al 2009; Trincone and Giordano 2006).

Expression plasmid pET16b-αLF1 containing the found nucleotide sequence was prepared for its ability to produce recombinant α-L-fucosidase in form of histidine-tagged enzyme. Fusion tag attached to the N-terminus of protein of interest didn´t mean any restriction for the testing of the enzyme ability to catalyze transglycosylation reactions and for the appraisal of possible application in α-L-fucosylated molecules biosynthesis. It simplified

the purification process and enabled the work with highly purified enzyme preparations without any significant influence of contaminating proteins.

Characterization of hydrolytic activity of purified recombinant enzyme (pH and temperature profil, temperature stability, the dependance of initial velocity on *p*NPα-L-Fuc concentration) was carried out in order to find the best conditions for transglycosylation reactions.

According to known stereospecificity of glycosidases it was possible to assume that α-L-fucosidase would not be able to cleave molecules containing D-isomer of particular sugar moiety bound by α-glycosidic linkage, as e.g. *p*NPα-D-Gal, *p*NPα-D-Glc and *p*NPα-D-Man, which were chosen as possible acceptors for testing of transglycosylation abilities of studied enzyme. The ability of the enzyme to catalyze the transfer of α-L-fucosyl moiety to *p*NPα-L-Fuc (donor molecule in all transglycosylation reactions) was tested, too. Two next groups of tested acceptor molecules were aliphatic alcohols of different length of alkyl chain and position of hydroxyl group and amino acids containing hydroxyl group in side chain, which serves in glycoproteins for attachment of oligosaccharides to a protein by *O*-glycosidic linkage.

All above mentioned acceptor molecules were chosen according to their importance for further possible applications. Enzymatic synthesis of α-L-fucosyl moiety containing oligosaccharidic chains of defined structure enables a detailed basic research of impact of particular motives in natural biological processes. Results of this research can consequently help in designing of new treatment of several serious diseases. For the same reason also synthesis of different compounds of new structure, not usually occuring in the nature, is of high importance (Ajisaka et al 1998; Berteau et al 2004; Wada et al 2008; Zeng et al 2003). Different alkyl glycosides represent as easily biodegradable surfactants an important improve for pharmaceutical, food, chemical and cosmetic industry (Das-Bradoo et al 2004; Hu et al

2008; Sarney and Vulfson 1995). Fucosylated amino acids with hydroxyl group in their side chain can be used as building blocks in synthesis of glycopeptides of defined structure and thus enable the deepening of findings in basic research and their application in pharmaceutical practice (Liu et al 2006; Pratt and Bertozzi 2005).

Our research confirmed recombinant α-L-fucosidase from *P. thiaminolyticus* as an enzyme with the ability to catalyze a transfer of L-fucosyl moiety to all tested saccharidic acceptors. However, this transfer is not regioselective, as in all samples analyzed by NMR a mixture of transfucosylation products with different glycosidic bonds was detected. Although not regioselective transfer was determined, the fact, that α(1→2) and α(1→6) linkages were formed when *p*NPα-D-Glc was used as acceptor molecule, should be stressed as important, because in many papers of other authors formation of α(1→3) glycosidic bond was reported. Exactly this property makes this enzyme interesting for subsequent research. Nevertheless, the relatively low yields represent in this moment the most significant complication for the practical application of studied enzyme and should be solved in the first place. Genetic manipulations, for example, can offer solution for some shortcomings, which we recognized during the work with "wild-type" recombinant α-L-fucosidase from *P. thiaminolyticus*.

As it is known that during transglycosylation reactions catalyzed by glycosidases two processes are competing i.e. synthesis of the product (glycosylation) and hydrolysis of the formed product (caused by natural activity of glycosidases), it is obvious that suppression of hydrolytic activity could strongly influence final reaction yields. Therefore, genetic manipulations producing so called glycosynthases, i.e. mutants of glycosidases with preserved transglycosylation abilities (when suitable substrate molecules are used) and suppressed hydrolytic activity, represent an interesting tool for final transglycosylation yields increasing (Cobucci-Ponzano et al 2009; Mackenzie et al 1998; Perugino et al 2004; Wada et al 2008; Wang 2009).

In 2009 results of Cobucci-Ponzano and co-workers were published reporting a successfull preparation of two α-L-fucosynthases. These enzymes, originating from *Sulfolobus solfataricus* and *Thermotoga maritima*, were able to use β-L-fucopyranosyl azide as donor molecule for transglycosylation reactions using several different acceptor molecules. In some cases interestingly high final yields reaching e.g. 91% (fucosynthase from *Thermotoga maritima*, *p*-nitrophenyl β-D-xylopyranoside used as acceptor) or 86% (fucosynthase from *Sulfolobus solfataricus*, *p*-nitrophenyl β-*N*-acetyl-D-glucosamine used as acceptor) were found (Cobucci-Ponzano et al 2009; Wang 2009). The increase of transfucosylation activity of α-L-fucosidase from *Thermotoga maritima* was also the goal of the work of Osanjo and co-workers. In this case the method of directed evolution was used and a significant improvement of tranglycosylation yield (from original 7% to more than 60%) was achieved (Osanjo et al 2007).

According to above-mentioned results of authors interested in altering of enzyme abilities by genetic manipulations, it is obvious that methods of molecular biology can offer a new direction for the work with studied recombinant α-L-fucosidase originating from *P. thiaminolyticus*. However, it is also necessary to emphasise that genetic manipulations and glycosynthase preparation is not in any way a routine task. Nevertheless, obtained results can contribute to better understanding of this process and its mechanism.

Also other aspects of presented work should be taken into account. The main goal of this work was to characterize molecules arising during transglycosylation reactions and thus verify the ability of the enzyme to catalyze synthetic reactions. Therefore, attention was not paid to the optimization of isolation and purification process of formed compounds. These steps should be solved during following experiments. Substantial increase of achieved yields can by very likely assumed as a result of extraction optimization. Also the fact that suitable conditions for individual reactions were designed according to results obtained from

experiments with small reaction mixtures (ten to thirty-five times smaller than mixtures used for samples isolation for NMR analysis) could influenced the time course of the reaction and thus also final yields and should be considered in future tests.

As mentioned earlier, primary and secondary aliphatic alcohols of a different alkyl chain length (methanol, ethanol, 1- and 2-propanol and 1- and 2-octanol) represent the second group of molecules tested to be able to serve as acceptors in transfucosylation reactions catalyzed by α-L-fucosidase from *P. thiaminolyticus*. The enzyme was able to catalyze the transfer of L-fucosyl moiety to short alcohols with relatively high yields (nearly 70% in experiments with methanol as acceptor), which decreased with the length of the alkyl chain. Also an influence of position of hydroxyl group was observed and it was evident that primary alcohols were preferred as acceptor molecules to secondary alcohols with the same length of alkyl chain. Because of combination of these two facts transfucosylation products of 2-octanol - if arising - were under the limit of used detection methods (TLC and mass spectrometry).

Many papers interested in chemical or enzymatic synthesis of alkylglycosides with different types of saccharidic moieties (glucose, galactose, mannose) and in their properties and possible applications were published (e.g. Das-Bradoo et al 2004; Kouptsova et al 2001; Lirdprapamongkol and Svasti 2000; Matsumura et al 1990; Okahata and Mori 1998; von Rybinski and Hill 1998).

In comprehensive work Vermeer and co-workers tested the possibility to fucosylate ethanol, 1-propanol, allyl alcohol, 1-butanol, 3-bromopropanol, 3-azidopropanol, 5-azidopentanol and 1-decanol by different chemical methods. Although in some cases interestingly high yields overcoming 90% were achieved, a mixture of α and β anomers in different molar ratios was obtained in all cases (Vermeer et al 2001). Strictly stereospecific

glycosidases could thus represent an advantageous tool for synthesis of products with particular anomeric configuration.

From results obtained by us in experiments with alcohols as acceptor molecules it is evident that a lot of research remains to be done before the studied α-L-fucosidase from *P. thiaminolyticus* can be declared as suitable for practical application or definitely rejected. Besides the above-mentioned possibility to prepare genetically modified enzyme also other possibilities will be tested. One of them would be the test of suitability of enzyme protection by encapsulation, as in some experiments negative influence of high concentration of acceptor molecules on enzyme activity was observed. Also further molecules of different structure will be tested as possible acceptors.

Last group of tested acceptor molecules was presented by amino acid derivatives Boc-L-Ser-OMe and Boc-L-Thr-OMe, where both (α-carboxy and α-amino) functional groups are protected, as recommended in literature (Nilsson and Sciegelova 1994; Cantacuzene and Attal 1991).

According to achieved yields (3.5% for Boc-L-Ser-OMe α-L-fucosylation and 3.2% for α-L-fucosylated Boc-L-Thr-OMe synthesis) it is obvious that α-L-fucosidase from *P. thiaminolyticus* is not suitable for application in biotechnological practice under tested conditions, as our results cannot compete e.g. with yields of chemical synthesis published by Mereyala and co-workers. They employed iodomethane activation with achieved final yields of 79% of *N*-Boc-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-L-serine methyl ester and 73% of *N*-Boc-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-L-threonine methyl ester (Mereyala et al 1997). However, many advantages of enzymatic synthesis were mentioned above and for that reason it would be interesting to determine possible effect of different reaction conditions changes on final results. The fact, that the enzyme studied in our research is able to use both tested molecules as suitable acceptors is crucial. Interestingly, in this case (it means in

transglycosylation reactions using amino acid derivatives as acceptor molecules) the enzyme doesn´t exhibit any significant preference for acceptor containing primary hydroxyl group over a molecule with secondary hydroxyl group. In the future, the influence of amino acid derivatives protecting group will be tested, as their character could cause substantial differences in final yields of transglycosylation reactions (Cantacuzene and Attal 1991; Leparoux et al 1994). Naturally, also here the final yields result from two parallel phenomena, namely hydrolytic and transglycosylation reactions and thus can be influenced by application of genetically modified enzyme.

In conclusion, in this part of our research work we found that the bacterial strain *P. thiaminolyticus* produces the enzyme, which is able to catalyze transfucosylation reactions. Recombinant form of this α-L-fucosidase expressed with histidine tag in *E. coli* BL21(DE3) was able to catalyze the transfer of α-L-fucosyl moiety to structurally different acceptor molecules including *p*-nitrophenyl glycopyranosides, aliphatic alcohols and amino acid derivatives. This fact is the most important finding of this research. Although the results achieved during the work are not directly applicable in the biotechnological practice, they are opening the ways for further research.

Materials and methods

Microorganisms and their cultivation

Used bacterial strains originated from Czech Collection of Microorganisms in Brno (an acronym CCM), from the Collection of Microorganisms at the Institute of Chemical Technology in Prague, Department of Biochemistry and Microbiology (an acronym DBM), from National Collection of Industrial and Marine bacteria (an acronym NCIMB) or were kindly provided by Professor N. J. Russell, MA, PhD (Camb) (Imperial College London).

All bacterial strains were cultivated in platform shaker under following conditions:

a) *Agrobacterium rhizogenes* A4 (DBM 3131), *Arthrobacter nicotianae* (CCM 1648), *Arthrobacter ureafaciens* (CCM 1644), *Bacillus* sp. (DBM 3069), *Bacillus amyloliquefaciens* (DBM 3129), *Bacillus megaterium* (CCM 2007), *Bacillus pumilus* (DBM 3128), *Brevibacterium ammoniagenes* (DBM 1075), *Brevibacterium linens* (DBM 3111), *Brevibacterium stationis* (CCM 317), *Micrococcus luteus* (CCM 169), *Micrococcus roseus* (CCM 679) and ***P. thiaminolyticus* (CCM 3599):** liquid Luria-Bertani (LB) medium, 16 hours, 30 °C, 250 RPM. Bacterial strain *P. thiaminolyticus* was also cultivated on LB plates under same temperature conditions.

b) *Bacillus licheniformis* (DBM 3100) and *Enterococcus faecalis* (DBM 3075): liquid LB medium, 16 hours, 37 °C, 250 RPM

c) *Arthrobacter* sp. C1-1 (Prof. Russell), *Arthrobacter* sp. C1-2a (Prof. Russell), A*rthrobacter* sp. C2-2 (Prof. Russell), *Arthrobacter* sp. CH07 (Prof. Russell), *Arthrobacter globiformis* (NCIMB 8907), CO85 (Prof. Russell) and GY26 (Prof. Russell): liquid Brain Heart Infusion medium, 40 hours, 15 °C, 150 RPM

d) *Leuconostoc mesenteroides* subsp. *dextranicum* (CCM 2086) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (CCM 1807): liquid Man-Rogosa-Sharpe medium, 16 hours, 30 °C, 250 RPM

All bacterial cultures were after cultivation harvested by centrifugation (4 000 g, 20 min, 4 °C) and stored at -20 °C.

Bacterial strains *E. coli* DH5α (GibcoBRL, USA) used for genomic library contruction and *E. coli* BL21 (DE3) (Novagen, USA) exploited for protein expression process were cultivated on liquid LBA medium (final concentration of ampicillin was 0.1 mg/mL (AppliChem GmbH, Germany)) for 16 hours at 37 °C and 250 RPM or LBA plates under same temperature conditions.

Disintegration of bacterial cells

Harvested bacterial cells were resuspended in 25 mM EPPS buffer (pH 8) (Sigma-Aldrich, USA) and disintegrated using lysozyme (final concentration of 5 mg/mL, 30 min incubation at laboratory temperature) (Fluka, USA) and natrium deoxycholate (final concetration 0.1%, 30 min incubation at 4 °C) (Sigma-Aldrich, USA). Described steps were followed by DNase addition (20 U to 1mL, 15 min incubation at laboratory temperature) (Sigma-Aldrich, USA) and sonication (20 W, 6 x 30 s) at Sonicator® 3000 ultrasonic liquid processor (Misonix Inc., USA). Cell debris was discarded by centrifugation (20 000 g, 20 min, 4 °C) and cell lyzates were stored at -20 °C.

Genomic library construction and sequence analysis

Commercial plasmid pUC19 (GibcoBRL, USA) was used for the construction of genomic library of *P. thiaminolyticus* in cells *E. coli* DH5α. This vector was cleaved by restriction enzyme *Bam*HI and subsequently dephosphorylated by CIAP. Wizard® DNA Clean-Up System (Promega Corporation, USA) was used for the purification of prepared plasmid DNA.

Chromosomal DNA of *P. thiaminolyticus* was isolated from cells after cultivation process by Genomic tip 500/G (Qiagen, Germany) and partially digested by restriction endonuclease *Sau*3AI. Arised fragments were ligated into prepared vector pUC19 using T4 DNA ligase.

CIAP was purchased from Invitrogen (USA), all other enzymes were purchased from New England Biolabs (USA). Reaction conditions stated by the producer were followed.

Competent cells of *E. coli* DH5α were transformed (Sambrook and Russel 2001) with prepared ligation mixture and spread on LBA plates with X-Fuc (40 μg/mL) (Biosynth AG®, Switzerland) and isopropyl β-D-thiogalactopyranoside (IPTG, 40 μg/mL) (Duchefa biochemie, Netherlands). Positive colony producing enzyme with α-L-fucosidase activity was identified according to its blue color caused by the ability to cleave chromogenic substrate X-Fuc. Plasmid DNA of this colony was isolated by alkaline lysis (Sambrook and Russel 2001) and by GenElute™ HP Plasmid Midiprep Kit (Sigma-Aldrich, USA) and used as a template for sequencing analysis (Geneart, Germany) by "primer walking" method. Commercial primers „M13-21 (forward)“ and „M13-R“ were used for the first sequencing analysis. An open reading frame of α-L-fucosidase encoding gene was determined using program WU-BLAST2.

Construction of an expression plasmid

Plasmid pET16b (Novagen, USA) was chosen as a suitable vector for expression plasmid containing a gene of α-L-fucosidase construction. Chosen plasmid was cleaved by restriction endonucleases *Nde*I and *Bam*HI, whose restriction sites are situated in pET16b cloning region, dephosphorylated by CIAP and purified by Wizard® DNA Clean-Up System. Primers **GGCAAAGGAGAGAACA<u>CATATG</u>ACGTTAACCGC** containing a restriction site for *Nde*I (emphasized by underlining) and

CAGAAAATACAGAAAACAGGATCCCAAGTAG with a restriction site for *Bam*HI were used for production of DNA fragment (1382 bp) containing desired gene sequence in the PCR reaction catalyzed by Kod Hot Start DNA polymerase (Novagen, USA). Chromosomal DNA of *P. thiaminolyticus* served as template in this reaction. After specific cleavage by *Nde*I and *Bam*HI and purification by commercial kit Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA) the PCR product was ligated into the prepared plasmid pET16b. Molar concentration ratio of plasmid DNA and PCR product was 1:3. Ligation catalyzed by T4 DNA ligase (3 h at 16 °C and 10 min at 22 °C) was followed by transformation of competent cells *E. coli* BL21 (DE3) (Novagen, USA) by obtained ligation mixture and by their cultivation on LBA plates with X-Fuc and IPTG, as described previously. The plasmid DNA of positive colony was isolated by alkaline lysis and GenElute™ HP Plasmid Midiprep Kit and the accuracy of the prepared expression vector was confirmed by sequencing. The name pET16b-αLF1 will be used for this expression plasmid in the text.

Expression and purification of recombinant histidine-tagged α-L-fucosidase

Prepared expression plasmid pET16b-αLF1 was used for transformation of the competent cells of *E. coli* BL21 (DE3) and the cells were cultivated on LBA plates for 16 h at 37 °C. One colony was used for inoculation of 10 mL of LBA, which was subsequently cultivated overnight at 37 °C and 250 RPM and afterwards used for 1% inoculation of 100 mL of LBA. Optical density at 600 nm (OD_{600}) of the growing culture (37 °C, 250 RPM) was followed and at the value OD_{600} of 0.5 expression of the recombinant protein was induced by addition of IPTG to the final concentration of 0.3 mmol/L. The cultivation was finished four hours after induction of recombinant protein expression and the cells were harvested by centrifugation (4 000 g, 20 min, 4 °C).

Harvested cells were resuspended in 25 mM EPPS buffer (pH 8) and disintegrated by the same protocol as described for *P. thiaminolyticus* cells disruption.

Ni-NTA agarose column (Qiagen GmbH, Germany) for affinity chromatography was equilibrated with 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 sample after *E. coli* BL21 (DE3) cells disintegration, containing recombinant histidine-tagged α-L-fucosidase, was applied on the Ni-NTA agarose column. The contaminants were washed out of the column by 50 mM phosphate buffer (pH 7.5) with 150 mM KCl with 40 mM imidazole. For the elution of the recombinant α-L-fucosidase 50 mM phosphate buffer (pH 7.5) with 150 mM KCl and 250 mM imidazole was used. All fractions were assayed for α-L-fucosidase activity. Samples displaying this activity were put together and desalted by gel chromatography (column PD10 (GE Healthcare, UK)). This chromatographic step served also for the transfer of the protein of interest into 25 mM EPPS buffer (pH 8).

Electrophoretic analysis

Different samples of chromosomal or plasmid DNA were analysed by agarose electrophoresis in 1% gel. Lambda DNA/Hind III Marker (Invitrogen, USA) or 100 bp DNA Ladder (Invitrogen, USA) were used as molecular weight standards. Ethidium bromide (Amresco, USA) was used for the visualization.

Expression and purification of recombinant α-L-fucosidase were followed by SDS-PAGE in 5% stacking gel and 10% running gel (Laemmli 1970). Reduction conditions in the presence of dithiotreitol (Amresco, USA) were used. SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad Laboratories, USA) served as molecular weight standard. Coomassie Brilliant Blue R-250 (Amresco, USA) was used for proteins visualization.

Protein concentration assay

Protein concentration was in analyzed samples determined by Bradford protein method (Bradford 1976). Bradford reagent was purchased from Amresco, USA. Bovine serum albumin (SERVA Electrophoresis GmbH, Germany) was used as standard protein in the concentration range 0-0.1 mg/mL.

α-L-Fucosidase assay

Enzymatic activity of α-L-fucosidase was measured in 25 mM EPPS buffer (pH 8) at 37 °C for 10 min using *p*NPα-L-Fuc (Biosynth AG®, Switzerland) as a substrate (7 mM in the reaction mixture). An equal volume of 10% Na_2CO_3 was used for reaction termination and the absorbance of reaction mixture was measured at 405 nm. Calibration curve, constructed for *p*-nitrophenol in the concentration range 0-100 μmol/L, was constructed under same reaction conditions and served for the calculation of realeased amount of *p*-nitrophenol. Enzyme amount, which was able to release 1 μmol of *p*-nitrophenol per minute at 37 °C, was defined as one unit.

α-L-Fucosidase characterization

Samples of recombinant α-L-fucosidase after purification process were used for enzyme characterization. Temperature profil (i.e. the dependence of α-L-fucosidase activity on reaction temperature) was measured for different temperatures ranging from 20 °C to 70 °C. Stability of the enzyme was tested by incubation of enzyme sample at different temperatures (37 °C, 45 °C, 50 °C and 55 °C) for defined time intervals and subsequent standard activity assay. Stability of the enzyme at -20 °C (storage conditions) was controled weekly during 15 weeks by standard activity assay. For the determination of the influence of reaction mixture pH on the enzyme activity a set of Britton-Robinson buffers was used. Final

reaction mixture pH covered the range from 2 to 12. Kinetic parameters were determined by measuring the dependance of initial velocity on the concentration of *p*NPα-L-Fuc in reaction mixtures and subsequent calculation by the nonlinear regression method. According to obtained data it was necessary to use an equation (1) taking into account inhibition caused by high substrate concentrations.

$$v_0 = \frac{V_{\lim} \cdot [S]}{K_M + \left(1 + \frac{[S]}{K_S}\right) \cdot [S]} \qquad (1),$$

v_0 represents initial velocity, [S] represents substrate concentration, K_M is used for Michaelis constant, V_{lim} for maximum velocity and K_S for inhibition constant (Copeland, 2000). Confidence intervals are expressed in standard deviations.

Transglycosylation reactions

The ability of recombinant α-L-fucosidase to catalyze transfer of α-L-fucosyl moiety to different types of acceptor molecules (*p*-nitrophenyl glycopyranosides, alcohols and amino acid derivatives) was tested. All transglycosylation reactions were carried out in 25 mM EPPS buffer (pH 8) at 45 °C. In all cases *p*NPα-L-Fuc served as a donor molecule. Due to low solubility of *p*-nitrophenyl glycopyranosides (especially *p*NPα-L-Fuc) in 25 mM EPPS buffer (pH 8) the possibility to use 5%, 10%, 25% and 50% DMF in reaction mixtures containing *p*-nitrophenyl glycopyranosidic and amino acid acceptors was tested. Stability of the recombinant enzyme at 45 °C in the presence of 10% DMF was measured for 6 hours. Preserved activity was determined using standard activity assay. According to obtained results final reaction mixtures contained 10% DMF. In all cases the composition of reaction mixtures and reaction time were designed to achieve maximal transglycosylation product yields. The volumes of reaction mixtures depended on the method used for product analysis and on the expected amount of arising product.

a) p-nitrophenyl glycopyranosides as acceptors

Chromogenic substrates *p*NPα-D-Gal, *p*NPα-D-Glc, *p*NPα-D-Man (purchased from Sigma-Aldrich (USA)) and also the compound serving as donor molecule (*p*NPα-L-Fuc) were tested to be suitable acceptor molecules for transglycosylation reactions. Reaction mixtures composition is summarized in Table III. Composition of final reaction mixtures after transglycosylation reaction was analyzed by TLC. Main transglycosylation products were isolated from TLC plates by extraction into methanol (*p*NPα-D-Glc and *p*NPα-L-Fuc used as acceptors) or into deionized water (*p*NPα-D-Gal used as acceptor). Different isolation procedures were caused by the presence of methanol soluble contaminants in the sample with *p*NPα-D-Gal as acceptor. After decreasing of sample volume by methanol evaporation (rotary evaporator Büchi, Rotavapor R114 with water bath B480 (Büchi Labortechnik AG, Switzerland)) or water lyophilisation (FreeZone 1 Liter Benchtop Freeze Dry System (Labconco, USA)) samples were filtered using Puradisc 4 Syringe Filter (0.45 μm) (Whatmann, UK). Remaining methanol/deionized water was evaporated/lyophilised, the samples were dissolved in D_2O, and analyzed by mass spectrometry and NMR spectroscopy.

Final yields were calculated according to isolated molar amounts of transglycosylation product and added acceptor molecule. Due to low transglycosylation yield in case of *p*NPα-D-Man the presence of anticipated reaction product was confirmed only by mass spectrometry in the whole reaction mixture after filtration on VectaSpin Centrifuge Tube Filter (Whatmann, UK) with cut-off of 12 kDa.

b) aliphatic alcohols as acceptors

Aliphatic alcohols with 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, USA)) were tested to be possible acceptor molecules for transfucosylation reactions catalyzed by recombinant α-L-fucosidase. During the designing of suitable reaction mixture composition a set of experiments differing in quantity of used acceptor (ranging from 5% to 30% of particular alcohol in reaction mixture) was carried out. Final reaction mixtures compositions were chosen according to highest achieved yields of transfucosylation products. In some cases also decrease in donor molecule concentration influenced the final yield and was therefore used in final experiments. Composition of used reaction mixtures is summarized in Table IV. Reaction mixtures after transglycosylation reactions were analyzed by TLC and samples of complete reaction mixtures (transfucosylation products were not isolated from reaction mixtures) were prepared for mass spectrometry characterization by filtration on VectaSpin Centrifuge Tube Filter (cut-off of 12 kDa).

Programs TotalLab (Nonlinear Dynamics, UK) and Image J (National Institutes of Health) determining TLC spots densities were used for transglycosylation reactions evaluation. Transglycosylation products yields were calculated using data for the amount of donor molecule, obtained transglycosylation products and released L-fucose in reaction mixture after transfucosylation reaction. The sum of spot densities of all above mentioned compounds represent 100% of L-fucosyl moieties in reaction mixtures.

c) amino acid derivatives as acceptors

Amino acid derivatives Boc-L-Ser-OMe and Boc-L-Thr-OMe (Sigma-Aldrich, USA) were used as possible candidates for accepting L-fucosyl moiety during transfucosylation reactions. Reaction mixtures composition is summarized in Table V. Samples after transfucosylation reactions were analyzed by TLC and prepared for mass spectrometry

characterization as described previously. Programs TotalLab and Image J were used for the final transfucosylation product yields calculation as in case of alcoholic acceptors.

Thin layer chromatography

The samples of the reaction mixtures after transfucosylation reactions were without terminating the reaction spotted on Silica gel TLC plates with fluorescent indicator (254 nm) (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). Molecules containing *p*-nitrophenyl group were after separation visualized by UV light. Saccharidic parts containing molecules were detected by 0.1 M 2-methylresorcinol (Alfa Aesar GmbH & Co. KG, Germany) dissolved in 5% (v/v) solution of sulfuric acid (Lach-Ner, s.r.o, CZ) in ethanol (after heating of TLC plate).

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

All transglycosylation products were confirmed by mass spectrometry analysis in mode ESI+ (Q-Tof micro mass spectrometer, Waters Micromass, USA) with direct inlet.

Products of *p*-nitrophenyl glycopyranosides transfucosylation were analyzed also by NMR spectroscopy. ^{1}H, ^{13}C, COSY, HMQC and HMBC, NOE and TOCSY spectra were measured on a Bruker Avance^{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. Tetramethylsilane was used as internal standard. Chemical shifts are given in δ-units (ppm).

p-nitrophenyl α-L-fucopyranosyl-(1,2)-α-L-fucopyranoside:

^{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 5.94 (d, 1H, J = 3.7 Hz, H-1), δ 4.99 (d, 1H, J = 4.2 Hz, H-1'), δ 4.24 (q, 1H, J = 6.6 Hz, H-5'),

δ 4.17 (dd, 1H, J = 3.4 Hz, J = 10.3 Hz, H-3), δ 4.10 (overlapped, 1H, H-5), δ 4. 00 (dd, 1H, J = 3.5 Hz, J = 10.4 Hz, H-2), δ 3.88 (overlapped, 1H, H-3'), δ 3.86 (overlapped, 1H, H-4), δ 3.76 (overlapped, 1H, H-4'), δ 3.68 (dd, 1H, J = 4.0 Hz, J = 10.5 Hz, H-2'), δ 1.19 (d, 3H, J = 6.6 Hz, 6'-CH_3), δ 1.10 (d, 3H, J = 6.6 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''), 96.2 (C-1'), 94.2 (C-1), 71.8 (C-2), 71.8 (C-4'), 71.6 (C-4), 69.2 (C-3'), 68.0 (C-5), 67.8 (C-3), 67.7 (C-2'), 67.1 (C-5'), 15.3 (6-CH_3), 15.3 (6'-CH_3).

p-nitrophenyl α-L-fucopyranosyl-(1,4)-α-L-fucopyranoside:

^{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 = 3.2 Hz, H-1), δ 5.07 (d, 1H, J = 4.3 Hz, H-1'), δ 4.30 (q, 1H, J = 6.6 Hz, H-5'), δ 4.10 (overlapped, 1H, H-4), δ 4.09 (overlapped, 1H, H-2), δ 4.07 (overlapped, 1H, H-5), δ 4.05 – 4.02 (m, 1H, H-3), δ 3.92 (dd, 1H, J = 3.5 Hz, J = 10.4 Hz, H-3'), δ 3.78 (overlapped, 1H, H-2'), δ 3.79 (overlapped, 1H, H-4'), δ 1.19 (d, 3H, J = 6.6 Hz, 6'-CH_3), δ 1.10 (d, 3H, J = 6.6 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''), 96.7 (C-1), 95.4 (C-1'), 74.4 (C-4), 71.8 (C-4'), 69.3 (C-3'), 68.0 (C-3), 68.0 (C-5), 68.0 (C-2'), 67.0 (C-5'), 65.9 (C-2), 15.3 (6-CH_3), 15.3 (6'-CH_3).

p-nitrophenyl α-L-fucopyranosyl-(1,2)-α-D-glucopyranoside:

^{1}H NMR: δ 8.24 (d, 2H, J = 9.3 Hz, *p*-NO_2Ph), δ 7.27 (d, 2H, J = 9.3 Hz, *p*-NO_2Ph), δ 5.90 (d, 1H, J = 3.5 Hz, H-1), δ 5.06 (d, 1H, J = 4.2 Hz, H-1'), δ 4.05 (dd, 1H, J = 9.4 Hz, H-3), δ 3.86 (overlapped, 1H, H-5'), δ 3.83 (overlapped, 1H, H-3'), δ 3.73 (overlapped, 1H, H-2'), δ 3.70 (overlapped, 1H, H-2), δ 3.69 (overlapped, 2H, CH_2-6), δ 3.62 overlapped, 1H, H-4'),

δ 3.62 (overlapped, 1H, H-5), δ 3.55 (overlapped, 1H, H-4), δ 0.61 (d, 3H, J = 6.7 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''), 101.6 (C-1'), 95.9 (C-1), 80.8 (C-2), 72.7 (C-5), 71.5 (C-3), 71.5 (C-4'), 69.3 (C-3'), 68.8 (C-4), 68.2 (C-2'), 67.5 (C-5'), 60.1 (6-CH_2), 15.0 (6-CH_3, 6'-CH_3).

p-nitrophenyl α-L-fucopyranosyl-(1,6)-α-D-glucopyranoside:

^{1}H NMR: δ 8.24 (d, 2H, J = 9.3 Hz, *p*-NO_2Ph), δ 7.27 (d, 2H, J = 9.3 Hz, *p*-NO_2Ph), δ 5.82 (d, 1H, J = 3.8 Hz, H-1), δ 4.78 (d, 1H, J = 3.9 Hz, H-1'), δ 3.89 (overlapped, 1H, H-3), δ 3.76 (overlapped, 1H, H-2), δ 3.73 – 3.84 (overlapped, 2H, CH_2-6'), δ 3.69 (overlapped, 1H, H-5), δ 3.61 (overlapped, 1H, H-2'), δ 3.47 (overlapped, 1H, H-3'), δ 3.44 (overlapped, 1H, H-4), δ 3.43 (overlapped, 1H, H-5'), δ 3.29 (overlapped, 1H, H-4'), δ 0.91 (d, 3H, J = 6.6 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''), 99.8 (C-1'), 96.1 (C-1), 72.8 (C-3), 72.5 (C-5), 71.6 (C-4), 71.5 (C-4'), 70.8 (C-2), 69.3 (C-3'), 68.3 (6'-CH_2), 67.9 (C-2'), 66.6 (C-5'), 15.2 (6-CH_3, 6'-CH_3).

Acknowledgements

This work was supported by the Ministry of Education, Youth and Sports [MSM 6046137305] and by ESF COST Chemistry Action CM1002 "MultiGlycoNano" (MŠMT LD13024).

Abbreviations

Boc-L-Ser-OMe, *N*-(*tert*-butoxycarbonyl)-L-serine methyl ester; Boc-L-Thr-OMe, *N*-(*tert*-butoxycarbonyl)-L-threonine methyl ester; CIAP, Calf intestinal alkaline phosphatase; DMF, dimethylformamide; *E. coli*, *Escherichia coli*; IPTG, isopropyl β-D-thiogalactopyranoside; LBA, ampicillin containing Luria-Bertani medium; LB, Luria-Bertani; NMR, nuclear magnetic resonance; OD_{600}, optical density at 600 nm; *P. thiaminolyticus, Paenibacillus thiaminolyticus*; PCR, polymerase chain reaction; *p*NPα-L-Fuc, *p*-nitrophenyl α-L-fucopyranoside; *p*NPα-D-Gal, *p*-nitrophenyl α-D-galactopyranoside; *p*NPα-D-Glc, *p*-nitrophenyl α-D-glucopyranoside; *p*NPα-D-Man, *p*-nitrophenyl α-D-mannopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography; X-Fuc, 5-bromo-4-chloro-3-indolyl α-L-fucopyranoside; UV, ultraviolet

References

Ajisaka K, Shirakabe M. 1992. Regioselective synthesis of α-L-fucosyl-containing disaccharides by use of α-L-fucosidases of various origins. *Carbohydr Res.* 224:291-299.

Ajisaka K, Fujimoto H, Miyasato M. 1998. An α-L-fucosidase from *Penicillium multicolor* as a candidate enzyme for the synthesis of α(1-3)-linked fucosyl oligosaccharides by transglycosylation. *Carbohydr Res.* 309:125-129.

Becker DJ, Lowe, JB. 2003. Fucose: biosynthesis and biological function in mammals. *Glycobiology.* 13(7):41R-53R.

Benešová E, Lipovová P, Dvořáková H, Králová B. 2010. β-D-Galactosidase from *Paenibacillus thiaminolyticus* catalyzing transfucosylation reactions. *Glycobiology*. 20(4):442-451.

Berteau O, Bielicky J, Kilonda A, Machy D, Anson DS, Kenne L. 2004. α-L-Fucosidases: Exoglycosidases with unusual transglycosylation properties. *Biochemistry.* 43(24):7881-7891.

Bojarová P, Křen V. 2009. Glycosidases: a key to tailored carbohydrates. *Trends Biotechnol.* 27(4):199-209.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248-254.

Brückner J. 1955. Estimation of monosaccharides by the orcinol-sulphuric acid reaction. *Biochem J*. 60:200-205.

Cantacuzene D, Attal S. 1991. Enzymic synthesis of galactopyranosyl-L-serine derivatives using galactosidases. *Carbohydr Res.* 211:327-331.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B. 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res*. 37:D233-D238.

Cesar LMM, Mendes MA, Tormena CF, Marques MR, de Souza BM, Saidemberg DM, Bittencourt JC, Palma MS. 2005. Isolation and chemical characterization of PwTx-II: A novel alkaloid toxin from the venom of the spider *Parawixia bistriata* (Araneidae, Araneae). *Toxicon.* 46:786-796.

Crout DHG, Vic G. 1998. Glycosidases and glycosyl transferases in glycoside and oligosaccharide synthesis. *Curr Opin Chem Biol*. 2:98-111.

Cobucci-Ponzano B, Conte F, Bedini E, Corsaro MM, Parrilli M, Sulzenbacher G, Lipski A, Dal Piaz F, Lepore L, Rossi M, Moracci M. 2009. β-Glycosyl azides as substrates for α-glycosynthases: Preparation of efficient α-L-fucosynthases. *Chem Biol*. 16:1097-1108.

Copeland RA. 2000. *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*. 2nd ed. New York (NY): Wiley-VCH.

Cordero OJ, Merino A, de la Cadena MP, Bugía B, Nogueira M, Vinuela JE, Martínez-Zorzano VS, de Carlos A, Rodríguez-Berrocal FJ. 2001. Cell surface human a-L-fucosidase. *Eur J Biochem.* 268:3321-3331.

Das-Bradoo S, Svensson I, Santos J, Plieva F, Mattiasson B, Hatti-Kaul R. 2004. Synthesis of alkylgalactosides using whole cells of *Bacillus pseudofirmus* species as catalysts. *J Biotechnol.* 110:273-285.

D´Auria MV, Minale L, Riccio R. 1993. Polyoxygenated steroids of marine origin. *Chem Rev. 93*:1839-1895.

Eneyskaya EV, Kulminskaya AA, Kalkkinen N, Nifantiev NE, Arbatskii NP, Saenko AI, Chepurnaya OV, Arutyunyan AV, Shabalin KA, Neustroev KN. 2001. An α-L-fucosidase from *Thermus* sp. with unusually broad specificity. *Glycoconjugate J.* 18:827-834.

Flowers HM. 1981. Chemistry and biochemistry of D- and L-fucose. *Adv Carbohydr Chem Biochem.* 39:279-345.

Henrissat B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J.* 280:309-316.

Hu Y, Luan H, Zhou K, Ge G, Yang S, Yang L. 2008. Purification and characterization of a novel glycosidase from the chine white jade snail (*Achatina fulica*) showing transglycosylation activity. *Enzyme Microb Technol.* 43:35-42.

Koeller KM, Wong CHH. 2000. Synthesis of complex carbohydrates and glycoconjugates: enzyme-based and programmable one-pot strategies. *Chem Rev.* 100:4465-4493.

Kon K, Sugi H, Tamai K, Ueda Y, Yamada N. 1990. Synthesis and cytostatic activity of the antitumor antibiotic chartreusin derivatives. *J Antibiot.* 43(4):372-382.

Kouptsova OS, Klyachko NL, Levashov AV. 2001. Synthesis of alkyl glycosides catalyzed by β-glycosidase in a system of reverse micelles. *Russ J Bioorg Chem.* 27(6):380-384.

Křen V, Thiem J. 1997. Glycosylation employing bio-systems: from enzymes to whole cells. *Chem Soc Rev*. 26:463-473.

Laemli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227:680-685.

Leparoux S, Fortun Y, Colas B. 1994. Synthesis of β-galactosyl-(hydroxy amino acid) derivatives using β-galactosidase activity of *Achatina achatina* digestive juice. *Biotechnol Lett.* 16(7):677-682.

Lirdprapamongkol K, Svasti J. 2000. Alkyl glucoside synthesis using Thai rosewood β-glucosidase. *Biotechnol Lett*. 22:1889-1894.

Liu L, Bennett CS, Wong C-H. 2006. Advances in glycoprotein synthesis. *Chem Commun.* 21:21-33.

Ma B, Simala-Grant JL, Taylor DE. 2006. Fucosylation in prokaryotes and eukaryotes. *Glycobiology.* 16(12):158R-184R.

Mackenzie LF, Wang Q, Warren RA, Withers SG. 1998. Glycosynthases: Mutant glycosidases for oligosaccharide synthesis. *J Am Chem Soc.* 120:5583-5584.

Matsumura S, Imai K, Yoshikawa S, Kawada K, Uchibori T. 1990. Surface activities, biodegradability and antimicrobial properties of n-alkyl glucosides, mannosides and galactosides. *J Am Oil Chem Soc.* 67(12):996-1001.

Mereyala HB, Gurrala SR, Gadikota RR. 1997. Stereoselective synthesis of α-*O*-fucosylated serine, threonine, tyrosine and saccharides. *J Chem Soc, Perkin Trans*. 1:3179-3182.

Michalski JC, Klein A. 1999. Glycoprotein lysosomal storage disorders: α- and β-mannosidosis, fucosidosis and α-*N*-acetylgalactosiminidase deficiency. *Biochim Biophys Acta.* 1455:69-84.

Miyoshi E, Moriwaki K, Nakagawa T. 2008. Biological function of fucosylation in cancer biology. *J Biochem.* 143:725-729.

Moriwaki K, Miyoshi E. 2010. Fucosylation and gastrointestinal cancer. *World J Hepatol.* 2(4):151-161.

Nelson DL, Cox MM. 2005. *Principles of Biochemistry*. 4th ed. New York: W.H.Freeman&Co.

Nilsson KGI, Scigelova M. 1994. Synthesis of glycosylated serine and threonine derivatives employing glycosidases. *Biotechnol Lett.* 16(7):671-676.

Okahata Y, Mori T. 1998. Transglycosylation catalyzed by a lipid-coated β-D-galactosidase in a two-phase aqueous-organic system. *J Mol Catal B: Enzymatic.* 5:119-123.

Osanjo G, Dion M, Drone J, Solleux C, Tran V, Rabiller C, Tellier C. 2007. Directed evolution of the α-L-fucosidase from *Thermotoga maritima* into an α-L-transfucosidase. *Biochemistry*. 46:1022-1033.

Önenli-Mungan N, Özer G, Altunbasak S, Besley G, Yüksel B, Topaloglu AK, Soyupak S. 2004. Fucosidosis with hypothyroidism: a case report. *Turk J Pediatr.* 46:170-173.

Perugino G, Trincone A, Rossi M, Moracci M. 2004. Oligosaccharide synthesis by glycosynthases. *Trends Biotechnol.* 22(1):31-37.

Pratt MR, Betozzi CR. 2005. Synthetic glycopeptides and glycoproteins as tools for biology. *Chem Soc Rev.* 34:58-68.

Provenzale JM, Barboriak DP, Sims K. 1995. Neuroradiologic findings in fucosidosis, a rare lysosomal storage disease. *AJNR.* 16:809-813.

Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. 2003. *Campylobacter jejuni* binds intestinal H(O) antigen (Fucα1,2Galβ1,4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem.* 278(16):14112-14120.

Sambrook J, Russel DW. 2001. *Molecular cloning: a laboratory manual*. 3rd ed. New York: Cold Spring Harbor Laboratory Press.

Sarney DB, Vulfson EN. 1995. Application of enzymes to the synthesis of surfactants. *Trends Biotechnol.* 13:164-172.

Seeberger PH, Haase WCH. 2000. Solid-phase oligosaccharide synthesis and combinatorial carbohydrate libraries. *Chem Rev.* 100:4349-4393.

Staudacher E, Altmann F, Wilson IBH, März L. 1999. Fucose in *N*-glycans: from plant to man. *Biochim Biophys Acta.* 1473:216-236.

Sulzenbacher G, Bignon C, Nishimura T, Tarling CA, Withers SG, Henrissat B, Bourne Y. 2004. Crystal structure of *Thermotoga maritima* α-L-fucosidase. *J Biol Chem.* 279(13):13119-13128.

Taggi AE, Meinwald J, Schroeder FC. 2004. A new approach to natural products discovery exemplified by the identification of sulfated nucleosides in spider venom. *J Am Chem Soc.* 126:10364-10369.

Tarling CA, He S, Sulzenbacher G, Bignon C, Bourne Y, Henrissat B, Withers SG. 2003. Identification of the catalytic nucleophile of the family 29 α-L-fucosidase from *Thermotoga maritima* through trapping of a covalent glycosyl-enzyme intermediate and mutagenesis. *J Biol Chem*. 278(48):47394-47399.

Trincone A, Giordano A. 2006. Glycosyl hydrolases and glycosyltransferases in the synthesis of oligosaccharides. *Curr Org Chem.* 10:1163-1193.

Vanhooren PT, Vandamme EJ. 1999. L-Fucose: occurence, physiological role, chemical, enzymatic and microbial synthesis. *J Chem Technol Biotechnol.* 74:479-497.

Vermeer HJ, van Dijk, CM, Kamerling JP, Vliegenthart JFG. 2001. Fucosylation of linear alcohols: A study of parameters influencing the stereochemistry of glycosylation. *Eur J Org Chem.* 193-203.

Vetere A, Galateo C, Paoletti S. 1997. All-aqueous, regiospecific transglycosylation synthesis of 3-*O*- α-L-fucopyranosyl-2-acetamido-2-deoxy-D-glucopyranose, a building block for the synthesis of branched oligosaccharides. *Biochem Biophys Res Commun*. 234:358-361.

von Rybinski W, Hill K. 1998. Alkyl polyglycosides - properties and applications of a new class of surfactants. *Angew Chem Int Ed*. 37:1328-1345.

Wada J, Honda Y, Nagae M, Kato R, Wakatsuki S, Katayama T, Taniguchi H, Kumagai H, Kitaoka M, Yamamoto K. 2008. 1,2-α-L-Fucosynthase: A glycosynthase derived from an inverting α-glycosidase with an unusual reaction mechanism. *FEBS Lett*. 582:3739-3743.

Wang L-X. 2009. Expanding the repertoire of glycosynthases. *Chem Biol*. 16:1026-1027.

Watt GM, Lowden PAS, Flitsch SL. 1997. Enzyme-catalyzed formation of glycosidic linkages. *Curr Opin Struct Biol*. 7:652-660.

Wymer N, Toone EJ. 2000. Enzyme-catalyzed synthesis of carbohydrates. *Curr Opin Chem Biol.* 4:110-119.

Zayni S, Steiner K, Pföstl A, Hofinger A, Kosma P, Schäffer C, Messner P. 2007. The dTDP-4-dehydro-6-deoxyglucose reductase encoding *fcd* gene is part of the surface layer glycoprotein glycosylation gene cluster of *Geobacillus tepidamans* GS5-97^{T}. *Glycobiology.* 17(4):433-443.

Zeng X, Murata T, Usui T. 2003. Glycosidase-catalyzed synthesis of fucosyl di- and trisaccharide derivative using α-L-fucosidase from *Alcaligenes* sp. *J Carbohydr Chem*. 22(5):309-316.

Legends to figures

Fig. 1. Construction of genomic library of *P. thiaminolyticus* in the cells *E. coli* DH5α and the detection of colonies containing the gene of an enzyme with α-L-fucosidase activity.

Fig. 2. SDS-PAGE analysis of the expression and purification of the recombinant α-L-fucosidase 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, **line 6** - fraction after washing the Ni-NTA agarose column with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 10 mM imidazole, **line 7** - Ni-NTA agarose after washing with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 10 mM imidazole, **line 8** - fraction after washing 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 washing with the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 40 mM imidazole, **line 10** - recombinant α-L-fucosidase from *P. thiaminolyticus* eluted by the 50 mM phosphate buffer (pH 7.5) with 150 mM KCl containing 250 mM imidazole.

Fig. 3. Transfucosylation activity of the recombinant α-L-fucosidase using *p*-nitrophenyl glycopyranosides as acceptors and 50 mM (or 80 mM as indicated below) *p*NPα-L-Fuc as a donor. All reactions were performed at 45 °C for a period necessary for the maximal production of transfucosylation product and analyzed on the Silica gel TLC plate with

fluorescent indicator (254 nm). Mobile phase: ethylacetate:acetic acid:water (7:2:2, v/v/v). Visualization: a) 2-methylresorcinol and b) UV light (presented in supplementary data). **Line 1** - *p*-nitrophenol, **line 2** - L-fucose, **line 3** - *p*NPα-L-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*NPα-L-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. Transglycosylation products isolated for NMR analysis are indicated by arrows.

Fig. 4. Transfucosylation activity of the recombinant α-L-fucosidase using alcohols as acceptors and 60 mM (lines 5, 7, 8 and 9) or 20 mM (lines 4 and 6) *p*NPα-L-Fuc as donor. All reactions were performed at 45 °C for a period necessary for the maximal production of the desired transfucosylation product and analyzed on the Silica gel TLC plate with fluorescent indicator (254 nm). Mobile phase: ethylacetate:acetic acid:water (7:2:2, v/v/v). Visualization: a) 2-methylresorcinol and b) UV light (presented in supplementary data). **Line 1** - *p*-nitrophenol, **line 2** - L-fucose, **line 3** - *p*NPα-L-Fuc, **line 4** - reaction mixture containing 30% methanol as an acceptor, **line 5** - reaction mixture containing 10% ethanol as an acceptor, **line 6** - reaction mixture containing 5% 1-propanol as an acceptor, **line 7**- reaction mixture containing 10% 2-propanol as an acceptor, **line 8** - reaction mixture containig 10% 1-octanol as an acceptor and **line 9** - reaction mixture containing 10% 2-octanol as an acceptor. Transglycosylation products are indicated by arrows.

Fig. 5. Transfucosylation activity of the recombinant α-L-fucosidase from *P. thiaminolyticus* using the amino acid derivatives as acceptors and 40 mM *p*NPα-L-Fuc as donor. All reactions were performed at 45 °C for a period necessary for the maximal production of the

transfucosylation product and analyzed on the Silica gel TLC plate with fluorescent indicator (254 nm). Mobile phase: ethylacetate:acetic acid:water (7:2:2, v/v/v). Visualization: a) 2-methylresorcinol and b) UV light (presented in supplementary data). **Line 1** - *p*-nitrophenol, **line 2** - L-fucose, **line 3** - *p*NPα-L-Fuc, **line 4** - Boc-L-Ser-OMe, **line 5** - Boc-L-Thr-OMe, **line 6** - reaction mixture containing 40 mM Boc-L-Ser-OMe as an acceptor, **line 7**- reaction mixture containing 40 mM Boc-L-Thr-OMe as an acceptor. Transglycosylation products are indicated by arrows.

Tables

Table I. NMR analysis results of transfucosylation reactions catalyzed by recombinant α-L-fucosidase from *P. thiaminolyticus* using *p*-nitrophenyl glycopyranosides as acceptors.

donor	acceptor	number of products	Proportional representation of major products	types of formed linkages	yield (%)
*p*NPα-L-Fuc	*p*NPα-L-Fuc	2	1:1	(1→2) and (1→4)	14
	*p*NPα-D-Gal	5	1:2.5:4	not determined	32
	*p*NPα-D-Glc	2	1:1	(1→2) and (1→6)	21

Yields are calculated according to amount of added acceptor.

Table II. Results of transfucosylation reactions catalyzed by recombinant α-L-fucosidase from *P. thiaminolyticus* using alcohols and amino acid derivatives as acceptors.

donor	acceptor	yield (%)
*p*NPα-L-Fuc	methanol	69
	ethanol	56
	1-propanol	48
	2-propanol	34
	1-octanol	x
	2-octanol	xx
	Boc-L-Ser-OMe	3.5
	Boc-L-Thr-OMe	3.2

x - amount of arising product was to low for quantification by programs TotalLab and ImageJ
xx – product of the reaction was not detected by mass spectrometry
Yields are calculated according to amount of added donor.

Table III. Composition of reaction mixtures used for transfucosylation reactions catalyzed by recombinant α-L-fucosidase from *P. thiaminolyticus* using *p*-nitrophenyl glycopyranosides as acceptors.

Acceptor	Acceptor concentration in reaction mixture [mmol/L]	Donor concentration in reaction mixture [mmol/L]	DMF amount in reaction mixture [%]	Reaction mixture volume [µL]	Enzyme activity in reaction mixture[U]	Reaction time [h]
***p*NPα-D-Gal**	33	50	10	2100	4.7	3.5
***p*NPα-D-Glc**	33	50	10	1310	3.1	3.3
***p*NPα-D-Man**	33	50	10	180	0.2	4.0
***p*NPα-L-Fuc**	*	80	10	1400	1.8	3.5

* Donor molecule served also as an acceptor of L-fucosyl moiety.
In all cases *p*NPα-L-Fuc served as donor molecule.

Table IV. Composition of reaction mixtures used for transfucosylation reactions catalyzed by recombinant α-L-fucosidase from *P. thiaminolyticus* using aliphatic alcohols as acceptors.

Acceptor	Acceptor amount in reaction mixture [%]	Donor concentration in reaction mixture [mmol/L]	Reaction mixture volume [µL]	Enzyme activity in reaction mixture[U]	Reaction time [h]
Methanol	30	20	150	0.20	4.0
Ethanol	10	60	150	0.15	3.5
1-propanol	5	20	150	0.20	2.0
2-propanol	10	60	150	0.20	3.0
1-octanol	10	60	150	0.15	0.8
2-octanol	10	60	150	0.15	0.8

In all cases *p*NPα-L-Fuc served as donor molecule.

Table V. Composition of reaction mixtures used for transfucosylation reactions catalyzed by recombinant α-L-fucosidase from *P. thiaminolyticus* using amino acid derivatives as acceptors.

Acceptor	Acceptor concentration in reaction mixture [mmol/L]	Donor concentration in reaction mixture [mmol/L]	DMF amount in reaction mixture [%]	Reaction mixture volume [µL]	Enzyme activity in reaction mixture[U]	Reaction time [h]
Boc-L-Ser-OMe	40	40	10	175	0.2	3.5
Boc-L-Thr-OMe	40	40	10	175	0.2	3.5

In all cases *p*NPα-L-Fuc served as donor molecule.