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Transglycosylation by barley α-amylase 1

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ABSTRACT

The transglycosylation activity of barley α-amylase 1 (AMY1) and active site AMY1 subsite mutant enzymes was investigated. We report here the transferase ability of the V47A, V47F, V47D and S48Y single mutants and V47K/S48G and V47G/S48D double mutant AMY1 enzymes in which the replaced amino acids play important role in substrate binding at subsites at −3 through −5. Although mutation increases the transglycosylation activity of enzymes, in the presence of acceptors the difference between wild type and mutants is not so significant. Oligomer transfer reactions of AMY1 wild type and its mutants were studied using maltoheptaose and maltopentaose donors and different chromophore containing acceptors. The conditions for the chemoenzymatic synthesis of 4-methylumbelliferyl-α-D-maltooligosaccharides (MU-α-D-MOSs) were optimized using 4-methylumbelliferyl-β-D-glucoside as acceptor and maltoheptaose as donor. 4-Methylumbelliferyl-α-D-maltoside, -maltotrioside, -maltotetraoside and -maltopentaoside have been synthesized. Products were identified by MALDI-TOF MS. ^{1}H and ^{13}C NMR analyses showed that AMY1 V47F preserved the stereo- and regioselectivity. The produced MU-α-D-MOSs of degree of polymerization DP 2, DP 3 and DP 5 were successfully applied to detect activity of *Bacillus stearothermophilus* maltogenic α-amylase, human salivary α-amylase and *Bacillus licheniformis* α-amylase, respectively in a fast and simple fluorometric assay.

1. Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) belong to a family of glycoside hydrolases catalysing the cleavage of α(1–4) glycosidic bonds in starch and related carbohydrates with retention of the α-anomeric configuration in the products. Glycoside hydrolases and transglycosylases are classified based on their structure and sequence similarities and grouped into 125 families according to the CAZy database [1]. α-Amylases are found in glycoside hydrolase family 13 (GH 13), which together with GH 70 and 77 constitute clan H of glycoside hydrolases (GH-H). In addition, the family GH31, GH57 and GH119 also contain a few α-amylases and enzymes showing remote sequence homologies shared with the α-amylase family GH13 [2] Retaining glycosidases and transglycosylases act by acid/base catalysis *via* a double displacement mechanism. The active site contains a pair of catalytic carboxylic acids, one acting as an acid catalyst in the glycosylation step and a base catalyst for the deglycosylation step. During the first displacement the glycosidic oxygen is protonated by this catalytic acid leading to the scission of the glycosidic bond and the formation of an oxocarbenium ion-like transition state. A covalent glycosyl-enzyme intermediate is formed by attack of the catalytic nucleophile on the sugar anomeric centre and the aglycone of the substrate leaves the active site. The second displacement proceeds by an incoming water molecule, the catalytic base promotes the attack of the water molecule leading to hydrolysis [3]. Retaining glycoside hydrolases have the capacity to catalyse transglycosylation [4,5]. In this case the free hydroxyl of a sugar or other aglycone molecule in the second displacement step attacks to the glycosyl-enzyme intermediate rather than water resulting in deglycosylation of the enzyme and release of transglycosylation product [3].

The transferase ability of amylases was observed earlier during X-ray crystallographic analysis using protein co-crystallised

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with acarbose [6–8] however, it does not mean that these enzymes are equally suitable for transglycosylation in solution. Some excellent reviews were published in the last decade on preparative enzymatic synthesis of carbohydrates including monosaccharide derivatives and oligosaccharides. Different enzymes from aldolases to coupled multi-enzyme biotransformations have been used for synthesis [9,10]. Glycosidase-catalysed syntheses resulting in rare glycostructures were summarised by Bojarová and Kren [11].

Many different oligosaccharides have been obtained by chemoenzymatic transglycosylation, which is a very attractive synthetic method because it allows the formation of well-defined oligosaccharides without use of chemical protection of hydroxyl groups [12,13]. Previously substantial transglycosylation was detected for the Y151M mutant of human salivary α-amylase (HSA) in addition to hydrolytic activity. ^{1}H and ^{13}C NMR analyses of products revealed that Y151M HSA preserved stereo- and regioselectivity [14,15]. *Bacillus stearothermophilus* maltogenic α-amylase (BSMA) was capable of transferring the acarviosine-glucose pseudotrisaccharide residue from an acarbose donor onto glucopyranosylidene-spiro-thiohydantoin with glycosylation at C-6, C-4 and C-3 positions, with retention of stereoselectivity [16]. BSMA furthermore catalysed maltosyl transfer to produce various oligosaccharides [17,18].

Glycosynthase enzymes were prepared by Withers and co-workers from an active site mutant of retaining β-glycosidases, which had only transferase activity but lost totally the hydrolytic activity [3]. Based on the same approach a mutant AMY1 enzyme was prepared in which the Asp180 catalytic nucleophile was substituted. D180G and D180A AMY1 mutants showed 10^5 to 10^6 times reduced hydrolytic activity compared to the wild type, but did not act as glycosynthase. It was found that replacement of D180 as well as E205 and D291 catalytic groups led to complete inactivation of AMY1, confirming that these residues play essential role in bond cleavage [19].

Barley α-amylase isoenzymes, AMY1 and AMY2 previously showed transglycosylation on MOSs and 4-nitrophenyl (PNP) glycoside substrates as seen by appearance of small amounts of transfer products using G4, G5, G6 and PNP-G4 substrates [20]. However, this ability for the synthesis of oligosaccharides was not further explored.

Increased transglycosylation activity of M53A, M53W and M53Y AMY1 mutants was found together with decreased hydrolytic activity and maltooligosaccharides (MOSs) of degree of polymerization (DP) 7–11 released using PNP-G5 and PNP-G6 substrates, amounting to a total of 15% for the M53W mutant [21]. Transglycosylation was observed also for T212W AMY1, which formed 3% PNP-G6 transfer products at 35% PNP-G5 substrate conversion. However, the double mutant Y105A/T212W did not catalyse synthesis [22]. In the present work we examined barley AMY1 and mutants, in which V47 and S48 guided by the three-dimensional structure [23] were engineered by a random mutagenesis and selection approach to produce enzymatically active single and binding site double mutants for the investigation of the enzyme–substrate interactions at the −3 through −5 subsites [24]. The Y105A and Y105A/T212Y active site mutants prepared earlier at the outer subsites −6 and +4 were also examined [25].

The goal of the present study was to explore application of AMY1 and the new −3 through −5 subsite mutants in enzymatic synthesis of oligomeric glycosides using maltooligosaccharide donors and different chromophore containing acceptors. Furthermore, considerable effort was made to explore the transglycosylation ability of the V47F AMY1 mutant for the preparation of 4-methylumbelliferyl-maltooligosaccharides (MU-α-D-MOS) of DP 2–5. MU-MOSs should be good fluorogenic substrates for assay of α-amylases, but access to these substrates is limited as they are not commercially available except for MU-α-D-glucoside. The V47F catalysed enzymatic synthesis thus represents an effective way to overcome the problem with availability of MU-MOSs.

2. Materials and methods

2.1. Substrates

Maltoheptaoside (G7) [26] was an in-house stock, 4-methylumbelliferone and maltopentaoside (G5) were purchased from SIGMA. Amylose was obtained from GENAY and was used in a concentration of 0.25% in sodium acetate buffer pH 5.5. 2-Chloro-4-nitrophenyl-β-maltooligosaccharide (CNP-MOS DP 3–11) substrates were synthesized based on the method reported previously [27,28].

Acceptors from SIGMA: PNP-α-D-glucoside, PNP-β-D-glucoside, PNP-β-D-galactoside, PNP-β-D-fucoside, PNP-β-D-xyloside, PNP-α-D-mannoside, PNP-α-L-arabinoside, PNP-β-D-N-acetyl-glucosaminide, PNP-β-D-N-acetyl-galactosaminide, PNP-β-D-galacturonide, PNP-β-D-glucuronide, salicin, amygdalin, chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), benzylalcohol, 4-methylumbelliferyl-α-D-neuraminic acid, 4-methylumbelliferyl-α-D-glucoside, 4-methylumbelliferyl-β-D-glucoside.

Acceptors from in-house stock: 4-methoxyphenyl-β-D-N-acetyl-glucosaminyl-β-(1–6)-N-acetyl-glucosaminide, glucopyranosylidene-spirohydantoin, 4-fluorophenyl-(β-D-gluco-pyranosylidene)-spiro-oxathiazole (synthetic 1 in Table 2), N-(β-D-glucopyranosyl) benzylurea (synthetic 2 in Table 2) and N-(β-D-glucopyranosyl) phenylacetamide (synthetic 3 in Table 2) were generous gifts from Prof. L. Somsák.

2.2. Enzymes

Wild type barley α-amylase 1 (AMY1) was prepared as reported previously [21]. V47F, S48Y, V47K/S48G, V47A, V47D and V47G/S48D active site mutants of AMY1 were designed and selected to alter the region at subsites −3 through −5 as identified on the basis of the three-dimensional structure of the complex of maltoheptaose bound to the inactive catalytic nucleophile mutant AMY1 D180A (PDB codes: 1RP8 and 1RP9) [23]. The mutants have been screened for activity both on a plate assay as well as in minicultures and subsequently selected mutants were prepared in *Pichia pastoris* and purified essentially as previously described by Svensson and co-workers [21]. α-Amylases (EC 3.2.1.1) from human saliva (Type IXA) (HSA) and from *Bacillus licheniformis* (Type XII-A) (BLA) were purchased from SIGMA. *B. stearothermophilus* maltogenic amylase (BSMA) was a generous gift of K. H. Park [29]. Y105A, Y105F, Y105W, T212Y and Y105A/T212Y AMY1 mutants were produced in *P. pastoris* (Invitrogen, Carlsbad, CA) [30] as reported previously [22].

2.3. Subsite mapping

2-Chloro-4-nitrophenyl-β-maltooligosaccharide (CNP-MOS DP 3–10) substrates (~1 mM) were incubated at 37 °C in 20 mM sodium acetate pH 5.5, 5 mM $CaCl_2$. Reactions were initiated by addition of appropriate diluted enzyme and aliquots (10 μL of 100 μL total volume of reaction mixtures) were removed at different time intervals and injected onto a reversed phase HPLC column to determine cleavage frequencies. A Hewlett-Packard 1090 Series II liquid chromatograph equipped with diode array detector, automatic sampler, and ChemStation was used for HPLC. Samples were separated on ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm, 150 mm) with isocratic elution of MeCN:water = 1:9 flowing at a rate of 1 mL/min at 40 °C. Effluent was monitored for the CNP-

glycosides at 302 nm and the products of the hydrolysis were identified and quantified by using relevant standards. The acetonitrile was gradient grade and the water was obtained from a laboratory purification system. Subsite binding energies, for subsite map evaluation, were calculated by the SUMA program [31] on the basis of Arrhenius equation using bond cleavage frequencies derived from relative cleavage rates of different productive complexes.

2.4. Study of transferase activity

For acceptor screening test reactions were started by addition of 5 μL wild type AMY1 (100 nM) to 100 μL buffer (20 mM sodium acetate, 5 mM $CaCl_2$) pH 5.5, containing 5 mM acceptor and 10 mM maltopentaose donor. Reaction mixtures were incubated at 25 °C. Release of products was followed at 302 nm, 317 nm, 254 nm or 210 nm for PNP-, MU-, phenyl- and other aglycon respectively, using ZORBAX Eclipse XDB-C18 column (5 μm, 150 mm × 4.6 mm) and/or Supelcosil C18 (3 μm, 150 mm × 4.6 mm) with isocratic elution of MeCN:water = 1:9 flowing at a rate of 1 mL/min.

Parameters were optimized for transglycosylation. Reactions were started by addition of 5 μL wild type or mutant AMY1 (0.2–1000 nM) to 50 μL 20 mM sodium acetate, 5 mM $CaCl_2$, pH 4.5–6.5, containing 1–15 mM acceptor and 1–40 mM donor. Reaction mixtures were incubated at 25 °C. Release of products was followed at 317 for MU-glycosides, using HPLC separation.

For preparative synthesis of MU-α-MOSs 10 mg MU-α-D-Glc (0.730 mL, 40 mM, solved in DMSO) was added to sodium acetate buffer (6.25 mL, 20 mM; 5 mM $CaCl_2$; 25 mM G7; pH 5.5) and the reaction mixture was incubated at 25 °C for 12 h with V47F AMY1 (0.5 nM). To inactivate the enzyme the reaction mixture was boiled three times for 1 min at 100 °C followed by centrifugation and the supernatant was passed through a 0.2 μm syringe filter (Whatman, Maidstone, UK). After lyophilisation the remaining material was dissolved in a mixture of 500 μL DMSO and 1.6 mL acetonitrile/water (70/30) and products were separated and purified using HPLC acetonitrile/water gradient elution (0–15 min: 70–30% → 60–40%) (YMC-Pack Polyamine II. column; 5 μm, 250 mm × 10 mm, 3 mL/min). The obtained lyophilised products are white, amorphous powders.

2.5. Activity measurements

MU-α-D-MOSs (0.25–10 mM) were used to investigate the activity of HSA, BLA and BSMA. Activity of HSA was detected on MU-α-D-G3 as substrate in MES 50 mM, 5 mM $Ca(OAc)_2$, 51.6 mM NaCl, 152 mM NaN_3, pH 6.0. Activity measurement of BLA was performed using MU-α-D-G5 while MU-α-D-G2 was the substrate in case of BSMA in MES 50 mM, 5 mM $Ca(OAc)_2$, 51.6 mM NaCl, pH 6.0. Fluorescence was measured in microtiter plate at room temperature using Wallac VICTOR^2 1420 fluorimeter-luminometer (Wallac Oy, Turku, Finland) at 355 nm excitation (λ_{ex}) and 460 nm emission (λ_{em}) wavelengths. Calibration was performed using 4-methylumbelliferone in the 0.1–250 μM range.

2.6. ^1H and ^{13}C NMR

Structural parameters of MU-MOSs were determined by ^1H and ^{13}C NMR spectroscopy. The ^1H (500.13 MHz) and ^{13}C NMR (125.76 MHz) spectra were recorded with Bruker DRX-500 spectrometer in D_2O.

2.7. Mass spectrometry

The MALDI spectra of oligosaccharide products were obtained in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionisation of the sample molecules was effected with a 337 nm nitrogen laser. Spectra from multiple (at least 100) laser shots were summarised using 19 kV accelerating and 20 kV reflectron voltage. External calibration was applied using the $[M+Na]^+$ peaks of cyclodextrins DP 6–8, *m/z*: 995.306, 1157.359, 1319.412 Da, respectively. The spectrum was obtained in 2,5-dihydroxy benzoic acid (DHB) matrix using the dry-droplet method.

Table 1
Percentage of transglycosylation products compared to the total amount of product (%) as obtained using CNP-MOS substrates of DP 3–8. Peak areas of products were determined based on HPLC-chromatograms and used to evaluate the ratio of products released by hydrolysis and obtained by transglycosylation.

Enzyme	Substrate					
	CNP-G3	CNP-G4	CNP-G5	CNP-G6	CNP-G7	CNP-G8
AMY1	0	8.0	0	0	0	0
Y105A	4.0	9.7	3.0	0	0	0
Y105A/T212Y	10.1	25. 0	7.4	0	0	0
V47A	16.6	32.0	3.5	1.8	0	2.2
V47F	20.3	35.0	23.2	6.7	0	3.7
V47D	16.4	30.7	17.9	6.0	0	2.3
S48Y	14.8	31.7	25.9	16.0	25.0	15.1
V47K/S48G	10.2	32.8	25.9	16.2	17.8	15.6
V47G/S48D	20.8	43.2	35.0	23.7	19.9	19.4

3. Results and discussion

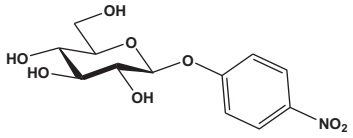
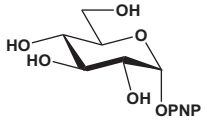
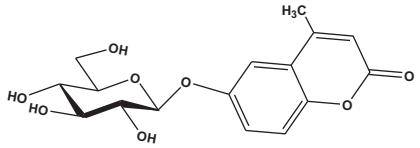
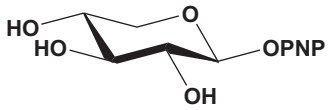
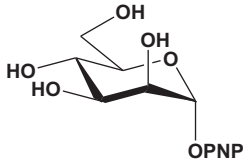
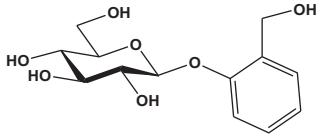
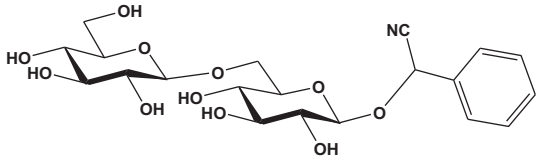
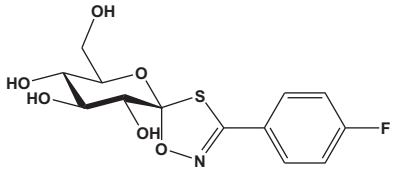
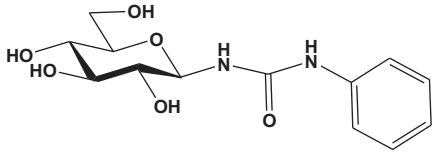
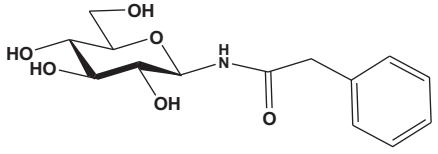
3.1. Effect of AMY1 subsite mutation on transferase activity

Transglycosylation was observed by AMY1 during CNP-G4 hydrolysis of which 8% of CNP-G5 and CNP-G6 transfer products were released (Table 1). The transglycosidase activity of AMY1 was studied systematically, using several donors and more than 20 chromophore-group containing acceptors to demonstrate the effect of the structure of acceptor for transglycosylation. The results obtained after 1 day reaction using G5 donor and suitable acceptors catalysed by AMY1 were collected in Table 2. No products were detected in case of PNP-β-D-galactoside, PNP-β-D-fucoside, PNP-α-L-arabinoside, PNP-β-D-N-acetyl-glucosaminide, PNP-β-D-N-acetyl-galactosaminide, PNP-β-D-galacturonide, PNP-β-D-glucuronide and chlorogenic acid. The results confirm that the equatorial position of 4-OH is critical since monosaccharides with axial OH at position 4 (for example galactose, fucose, L-arabinose) were not acceptors for AMY1. In addition, the monosaccharide derivatives N-acetyl-glucosamine and glucuronic acid are unfavourable for transglycosylation. The anomeric configuration is not so important; β- and α-glycosides are similarly efficient acceptors. The aglycon part of acceptors, however, affects transferase efficiency as demonstrated in Table 2. Conversions for PNP-β-Glc, salicin and synthetic 2 were 37%, 15% and 3%, respectively. MOSs of different degree of polymerization (DP 2–7) were used as donors. Noticeably reverse hydrolysis did not occur in the presence of glucose and maltose. Maltotetraose, maltopentaose and maltoheptaose were good donors while amylose did not result in products. The present findings are valid for mutants in which mutations were carried out not at the aglycone binding site.

AMY1 mutants were found to show enhanced transglycosylation activity compared to the wild type enzyme. Each active site AMY1 mutants formed more than one transfer product at the same reaction conditions furthermore, transglycosylation by V47 and S48 single and double mutants was observed not only on CNP-G4 but also on CNP-G3 and CNP-G5–8 substrates (Table 1).

Action patterns and subsite maps of wild type AMY1 and Y105A, Y105F, Y105W, T212Y and Y105A/T212Y AMY1 mutants

Table 2
Screening test of acceptors for AMY 1 catalysed transglycosylation reactions.

Name	Conversion[a] (%)	Formula
PNP-β-Glc	37	
PNP-α-Glc	35	
Me-umbellyferyl-β-Glc	21	
PNP-β-Xyl	2	
PNP-α-Man	18	
Salicin	15	
Amygdalin	9	
Synthetic 1	7	
Synthetic 2	3	
Synthetic 3	3	

[a] Reaction conditions: acceptor concentration 5 mM; enzyme 100 nM AMY1; donor G5 (10 mM) in 20 mM acetate buffer pH 5.5 containing 5 mM Ca^{2+}, after 1 day at RT.

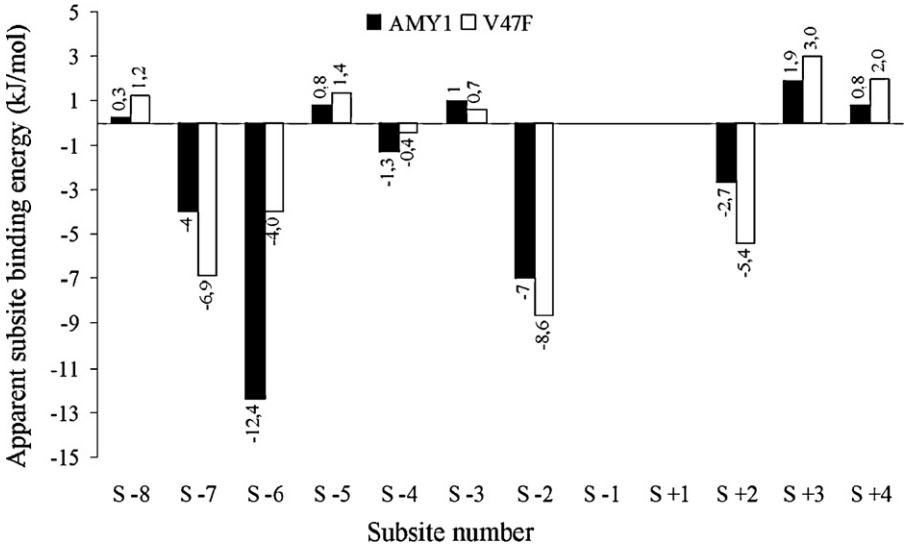


Fig. 1. Subsite map of AMY1 (■) (energy values derived from Ref. [31].) and V47F (□) mutant. Hydrolysis of the glycosidic bond occurs between subsites −1 and +1. The reducing end of maltooligodextrins is situated towards the right side of the subsite map. Negative energy values indicate binding between the enzyme and aligned glucopyranosyl residues, while positive values indicate repulsion.

have been reported previously [25]. Recently we determined action patterns and calculated the subsite maps of AMY1 V47A, V47F, V47D, S48Y, V47G/S48D and V47K/S48G mutants at subsites −3 through −5 to explore the importance of V47 and S48 amino acids in substrate binding. The mutants used for the transglycosylation analysis in the present study have all been selected because the showed enzymatic activity. The effect of V47F mutation on the subsite map is shown in Fig. 1. AMY1 has nine subsites: seven glycone and two aglycone binding sites, flanked by barrier subsites. Subsite −6 has a big favourable energy in AMY1 wild type (−12.4 kJ/mol) and substrates shorter than maltoheptaose are therefore cleaved slowly due to the non-productive complexes formed. The change of valine to phenylalanine decreased dramatically the subsite binding affinity of subsite −6 (−4.0 kJ/mol) where the mutation has occurred. Substrate specificity has also changed, thus longer substrates were hydrolysed relatively slower by the mutants.

The effect of mutation on transglycosylation using CNP-maltotetraoside as donor and acceptor is shown in Fig. 2. CNP-G5–6 was formed by transglycosylation while shorter products (CNP-MOS of DP 1–3) released by hydrolysis. To minimize influence of secondary hydrolysis on transfer products, transglycosylation reaction was observed at <10% substrate conversion. The ratio of transglycosylation catalysed by V47F (Fig. 2B) increased significantly compared to the wild type (Fig. 2A). Product formation by transglycosylation was also observed with the use of longer CNP-MOSs. Mutations resulted in diverse product conversion with substrates of DP 3–8 used both as donors and acceptors (Table 1). The conversion was lowest on the pentamer substrate using Y105A (3.5%), while V47G/S48D produced the highest conversion for the tetramer (43.2%). V47 mutants resulted in remarkable transglycosylation only on the shorter substrates (CNP-G3–5). S48Y mutant showed significant transglycosylation on every substrate, the highest value (31.7%) was observed in case of tetramer. Interestingly, double mutants showed high transglycosylation activity on shorter and longer substrates, as well. The present increased transglycosylation activity encouraged us to use these mutants for enzymatic synthesis.

PNP-β-glucoside and maltoheptaose were selected as model acceptor and donor, respectively, to study the transfer reaction. Fig. 3 shows a comparison between the reactions catalysed by the wild type AMY1 (Fig. 3A) and the active site mutant Y105A (Fig. 3B). The mutation alters the profile of transglycosylation products, Y105A released longer products compared to the AMY1, but these products were present only in trace amounts. After

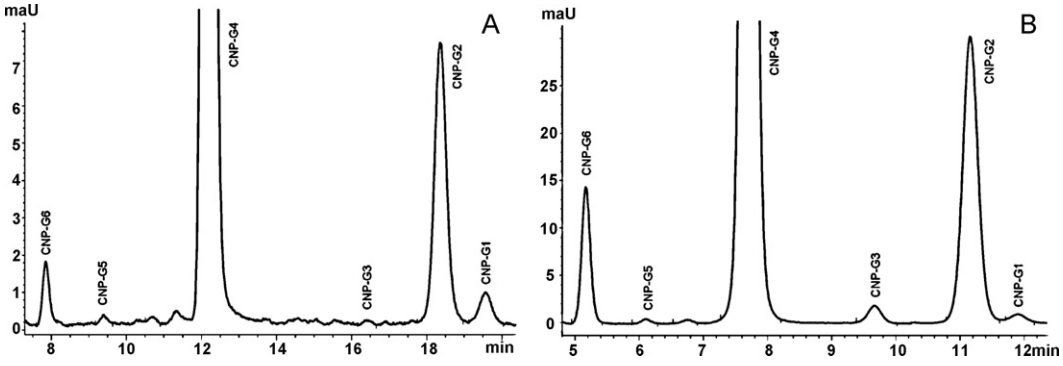


Fig. 2. Transglycosylation of CNP-G4 catalysed by barley AMY1 and its V47F mutant. AMY1 released 8% (A) and V47F 35% (B) of products by transglycosylation.

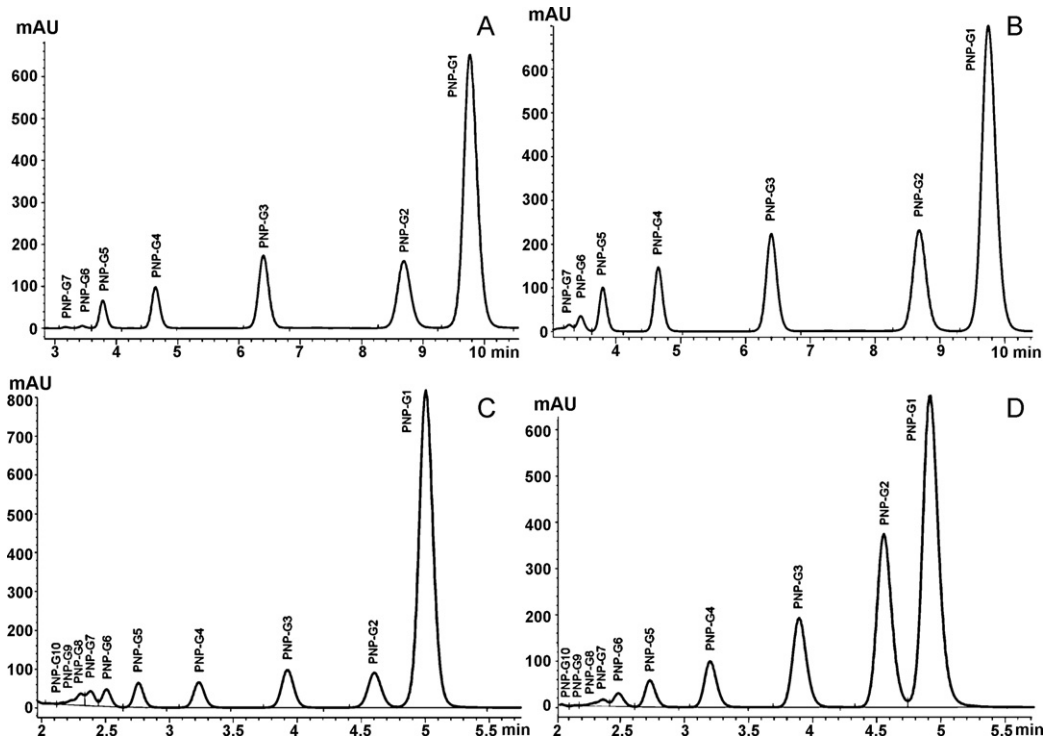


Fig. 3. Transglycosylation reaction of PNP-Glc acceptor and G7 donor catalysed by barley. AMY1 (A) and Y105A (B) after 4 h incubation, as well as V47G/S48D after 24 h (C) and 48 h (D) incubation.

3 h reaction several products were observed. The reaction conditions were the same, but the conversion (33% and 40%), and products (DP 2–5 and DP 2–7) differed for AMY1 and Y105A. Formation of even longer products (DP 6–10) was observed for the V47G/S48D mutant catalysed transglycosylation (Fig. 3C). The hydrolytic activity of the double mutant was very low, but it had excellent transglycosylation activity, nine products (up to PNP-decaoside) in 55% conversion were produced after 48 h (Fig. 3D) . With progress of reaction the longer transglycosylation products were hydrolysed resulting in increased amounts of shorter products. Therefore the ratio of transglycosylation products changes during the course of reaction, as seen after 24 h (Fig. 3C) and 48 h (Fig. 3D) incubation.

3.2. Enzymatic synthesis by V47F AMY1 – optimization of reaction conditions

We have tested both MU-α- and β-D-Glc as acceptors for enzymatic transglycosylation to synthesize MU group-containing MOSs as new, fluorogenic substrates for α-amylase assays. Each steps of optimization procedure of the transglycosylation reaction was performed using MU-β-D-Glc as acceptor to avoid loss by hydrolysis of chromophore group, because the enzyme is not capable to cleave the β-glycosidic bond.

Various concentrations of MU-β-D-Glc (4, 8, 12 and 15 mM) were tested. Higher molar concentration of products was found with increasing acceptor concentration (Fig. 4). In subsequent experiments 15 mM acceptor was used.

Various donors were tested for enzymatic transglycosylation, i.e. the natural substrate amylose, maltopentaoside (G5) and maltoheptaoside (G7). The transglycosylation reaction reached only 0.7% conversion after 7.5 h incubation using amylose donor. Hydrolysis of amylose is slower in case of mutated enzymes compared to the wild type because of their decreased hydrolytic activity. In contrast, G7 was found to be an excellent donor for AMY1 mutants with transglycosylation reaching 40% conversion. The conversion using G5 as donor closely resembled that of G7 (Fig. 5). Enhanced conversion was observed with increased G7 concentration (10–40 mM), levelling off after 13 h at room temperature (Fig. 6).

It was found that pH 5.5 is optimal for transglycosylation resembling the optimal pH of the hydrolytic activity. The highest conversion resulted in 40.7% at pH 5.5 in the studied pH range 4.5–6.5 (Fig. 7). The lower pH decreased stability of enzyme while at higher pH reaction was slower.

MU-α-D-Glc is soluble only in presence of DMSO and therefore acceptor dissolved in DMSO was added to the sodium acetate buffer to give 10, 20, 30 and 40% DMSO in the final reaction mixture. The rate of product formation was slower and the conversion of acceptor was lower with increasing DMSO content. The highest conversion (28%) was reached at 10% DMSO (Fig. 8). There-

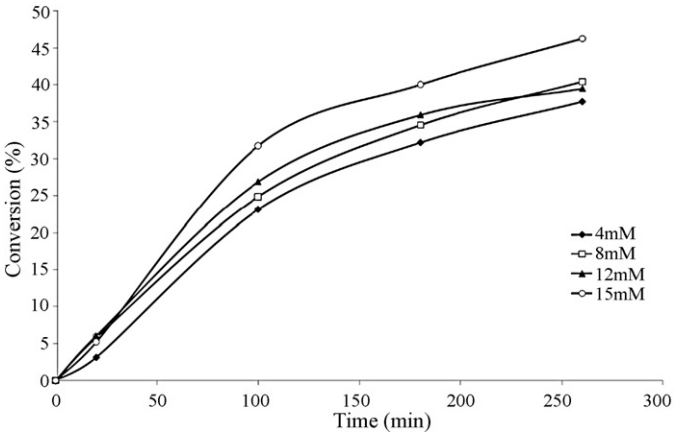


Fig. 4. Conversion of acceptor in transglycosylation reaction using different concentrations of MU-β-D-Glc acceptor (4 mM (●); 8 mM (□); 12 mM (▲); 15 mM (○)). Donor: G7 (20 mM), pH 5.5, RT.

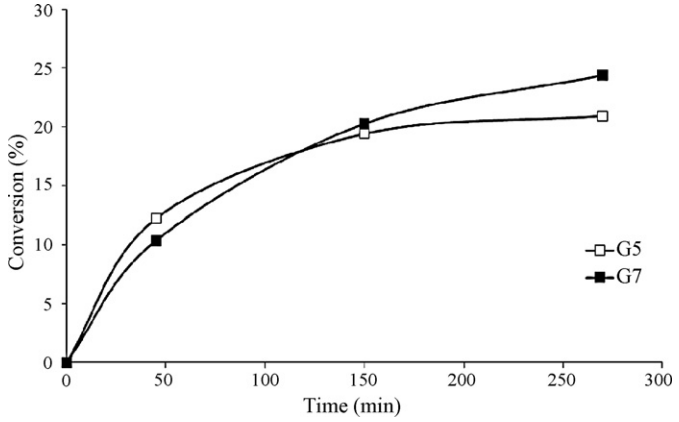


Fig. 5. Conversion of acceptor (PNP-β-D-Glc 100 mM) in transglycosylation reaction using maltopentaose (G5) (□) and maltoheptaose (G7) (■) donors (pH 5.5; G7 20 mM, G5 20 mM).

fore preparative enzymatic transglycosylation was performed in buffer containing 10% DMSO in the final reaction mixture.

^{1}H and ^{13}C NMR spectroscopy were used to determine the anomeric configuration and the interglycosidic bond type of MU-β-D-MOSs and the spectra revealed that the mutated enzyme preserved the stereo- and regioselectivity. The type of the interglycosidic bond and the anomeric configuration were confirmed by using 2D ^{1}H and ^{13}C NMR spectroscopy. The doublet of 1D ^{1}H NMR spectrum with 3.7 Hz $^{3}J_{1,2}$ coupling constant belongs to new α-glycosidic bond between glucose units, while that of β was 7.9 Hz. Chemical shift of C-4 (76.51 ppm) is the highest among the carbon skeleton except anomeric carbon. Moreover, interglycosidic ROE effects were clearly detected around the glycosidic bond between H1′ and H3 and H4. These results indicate the presence of α(1–4) interglycosidic bond between the two glucose units. Further interglycosidic bonds in higher oligomers originated from maltooligomer donor, therefore are assumed to be α(1–4) exclusively. In the trisaccharide both α-anomeric protons appeared as doublet, split by equal $^{3}J_{1,2}$ = 3.9 Hz couplings. C4 and C4′ carbon shifts were observed at 76.72 and 77.04 ppm due to the glycosylation shift. Also, a long-range correlation was observed between H1″ and C4′ in a HMBC experiment.

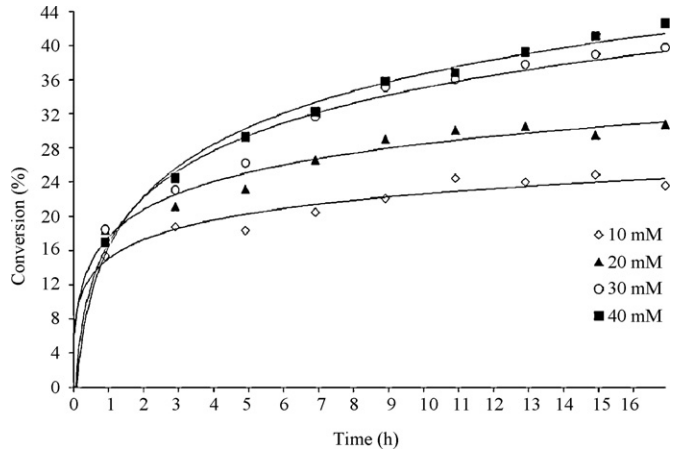


Fig. 6. Conversion of MU-β-D-Glc acceptor (25 mM) in transglycosylation reaction using different concentrations of maltoheptaose (G7) donor (10 mM (◊); 20 mM (▲); 30 mM (○); 40 mM (■)) (pH 5.5).

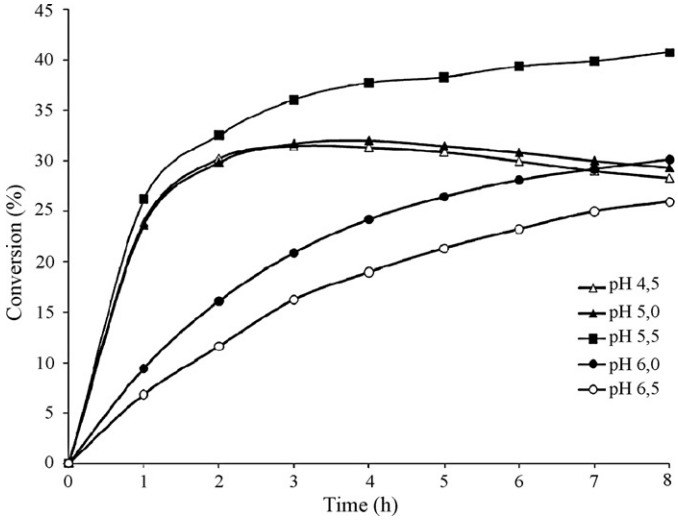


Fig. 7. Conversion of acceptor (MU-β-D-Glc 15 mM) in transglycosylation reaction using G7 donor (20 mM) in sodium-acetate buffers of different pH (pH 4.5 (△); pH 5.0 (▲); pH 5.5 (■); pH 6.0 (●); pH 6.5 (○)).

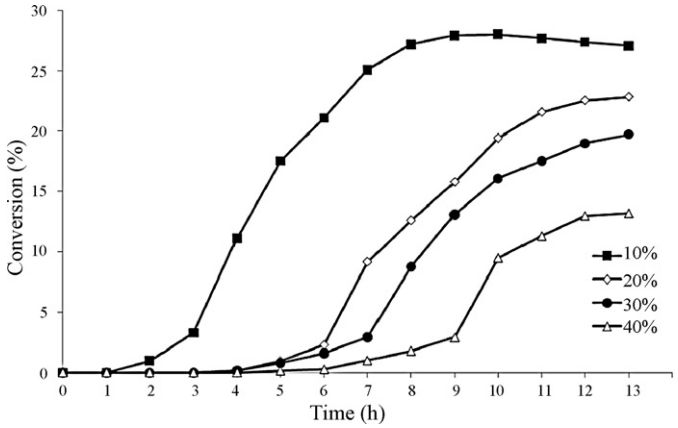


Fig. 8. Conversion of acceptor (MU-β-D-Glc 15 mM) in transglycosylation reaction using G7 donor (20 mM) in sodium-acetate buffer (pH 5.5) with different DMSO concentration (10% (■); 20% (◊); 30% (●); 40% (△)).

3.3. Enzymatic synthesis – preparative reaction

Enzymatic transglycosylation was stopped after 12 h incubation at 37.5% conversion and MU-α-D-MOS products of DP 2–6 were separated and purified by HPLC (Fig. 9). MU-α-D-G2 was

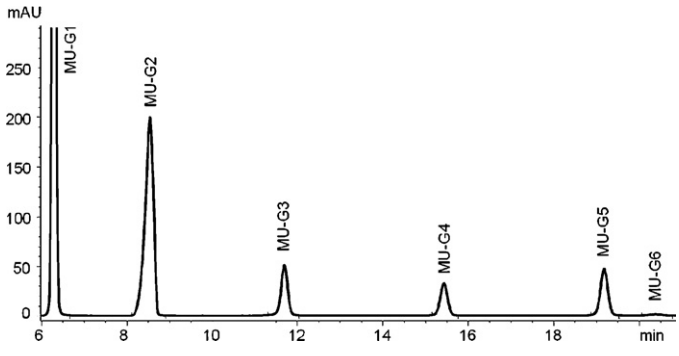


Fig. 9. HPLC profile of MU-α-D-MOSs released by enzymatic transglycosylation. Products were separated and purified using HPLC (YMC-Pack Polyamine II. column; 250 mm × 10 mm) with detection at 317 nm. The first peak corresponds to the MU-α-D-Glc acceptor, the other peaks from left to right correspond to MU-MOSs of DP 2–6.

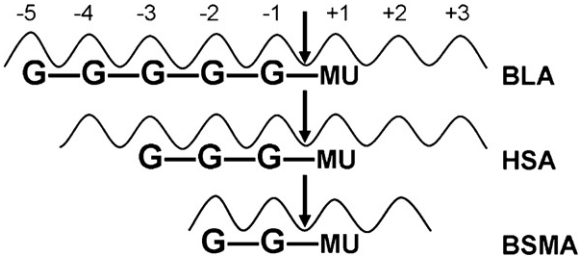


Fig. 10. Binding of MU-G5, MU-G3 and MU-G2 to the active site of α-amylases of different origin BLA, HSA and BSMA. Glycone binding sites are labelled with negative and aglycone binding sites with positive numbers, arrows indicate the cleavage point.

the main product (3.2 mg), and also MU-α-D-G3 (1.0 mg), MU-α-D-G5 (2.0 mg) were obtained as white amorphous powder. MU-α-D-G2 might be generated from the secondary hydrolysis of initial products. MU-α-MOS products were identified according to the good agreement between the calculated and the measured *m*/*z* values of $[M+Na]^+$ species from the MALDI-TOF MS spectrum of purified reaction products. MU-α-D-G5, calculated (1009.23 Da) measured (1009.30 Da); MU-α-D-G3, calculated (685.19 Da) measured (685.12 Da,); MU-α-D-G2, calculated (523.31 Da) measured (523.14 Da). Products of DP 2, 3 and 5 were used as substrates for detection of enzyme activity of selected amylases.

3.4. Activity measurements using the fluorogenic substrate

To test the utility of the synthesized fluorogenic MU-α-D-MOS substrates activities were investigated for BLA, HSA and BSMA. The substrates for activity measurement were selected on the basis of the subsite maps. The subsite maps of BLA [32] and HSA [33] have been published earlier. HSA has four glycone and three aglycone subsites, BLA has five glycone and three aglycone binding sites followed by a barrier subsite, and the subsite map of BSMA consists of two glycone and two aglycone subsites. We proved that enzymes liberate the methylumbelliferone (MU) exclusively from the substrates. Decrease in peak area of the MU substrate without the release of other products was observed by HPLC. MU-G5 substrate binds to subsites −5 through +1 in BLA, while MU-G3 binds to subsites −3 through +1 in HSA and MU-G2 binds to subsites from −2 through +1 in BSMA (Fig. 10). These are the only productive binding modes for the release of the fluorophore. Release of MU increased in time and was proportional of substrate concentration.

4. Conclusion

Enhanced transglycosylation activity of active site AMY1 mutant was observed during previous subsite mapping experiments [21,22]. We report here firstly the transglycosylation activity of Y105A, Y105A/T212Y, V47A, V47F, V47D, S48Y, V47K/S48G and V47G/S48D AMY1 active site mutants. Although the mutant enzymes produce higher amounts of transfer products than AMY1, in the presence of acceptor the difference was not so remarkable. Transglycosylation was observed using AMY1 in presence of G5 donor and chromophore containing acceptor, since AMY1 catalysed hydrolysis of G5 is very slow. The rate of hydrolysis of G7 catalysed by the studied −6 subsite mutants is also slow, namely the transfer reaction is under kinetic control. The success of transfer reaction is supported by the slow hydrolysis of donor and products.

The transglycosylation by AMY1 and mutants were studied using different MOS donors and chromophore containing acceptors. It was found that the equatorial position of 4-OH in the acceptor is critical, but the anomeric configuration did not influence the transfer reaction. Increased donor and acceptor concentration resulted in higher products conversion. Parameters of enzymatic reaction for the synthesis of MU-α-D-MOSs were optimized, with the use of MU-β-D-Glc acceptor and G7 donor molecules. 4-Methylumbelliferyl-α-D-maltoside, -maltotrioside, and -maltopentaoside have been successfully synthesized. Products were identified by molecular masses determined using MALDI-TOF MS, and ^1H and ^{13}C NMR studies revealed that the AMY1 V47F preserved stereo- and regioselectivity. MU-α-D-MOS of DP 2, DP 3 and DP 5 were successfully used for the qualitative detection of the activity of BSMA, HSA and BLA in a fast and simple fluorometric assay.

Some chromophore containing molecules proved to be useful as acceptors in an AMY1 catalysed transglycosylation and have the potential to synthesize their derivatives for inhibition or other enzymatic studies. The results show that enzymatic transglycosylation by AMY1 V47F is a useful approach for the synthesis of MU-α-D-MOSs and AMY1 mutants could be used for enzymatic synthesis of other oligosaccharide derivatives. Although MU-MOSs are good fluorogenic substrates for α-amylase assays they are not commercially available up to now and the present enzymatic synthesis published provides an effective solution for preparation of these α-amylase substrates.

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