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Transglycosylation activity of α-D-galactosidase from *Trichoderma reesei* An investigation of the active site

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Abstract

The transglycosylation reaction catalyzed by α-D-galactosidase from the mycelial fungus *Trichoderma reesei* was studied using *p*-nitrophenyl α-D-galactopyranoside (PNPG). An aliphatic alcohol or the substrate itself can be an acceptor of the galactose residue in this reaction. The transglycosylation products were identified as alkyl galactosides in the case of alcohols or as galactobioside and galactotrioside in the case of PNPG. The transglycosylation rates follow a first-order equation with respect to the alcohol concentrations except for methanol. Affinities of some substrates were estimated from their K_i values in the reaction of the enzyme with PNPG. Transglycosylation of the substrate suggests a model for the enzyme active center. It is proposed that the active center includes two galactose-binding sites and a hydrophobic site. © 1998 Elsevier Science Ltd.

Keywords: α-D-Galactosidase; *Trichoderma reesei*; Transglycosylation products; Alkyl galactosides; *p*-Nitrophenyl α-D-galactopyranoside

1. Introduction

Many glycosidases have been shown to possess transglycosylation activities in addition to hydrolytic activity. In transglycosylation, the glycosyl part of the substrate is transferred to hydroxyl-containing compounds other than water. Transglycosylation activity has been studied for a number of exoglycosidases (β-D-galactosidase [1], α-L-fucosidase [2], α-D-glucosidase [3], and β-D-xylosidase [4]) and endoglycosidases (endo-*N*-acetyl-α-D-galactosaminidase [5], chitinase [6], and lysozyme [7]).

It is possible to describe the glycosidase action in terms of a unified mechanism which consists of the following steps: Formation of a Michaelis complex, cleavage of the glycosidic bond in the donor followed by release of the aglycon and formation of a glycosyl–enzyme, transfer of the glycosyl residue to an acceptor within this complex, and regeneration of the

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enzyme molecule. The role of acceptor can be played by water (hydrolysis) or other hydroxylic compounds, for example, simple alcohols [8], hydrolysis products [9], saccharides [10–12], or a second substrate molecule (substrate transglycosylation) [13,14]. In the last case one might assume the formation of a triple complex, ES′S, which incorporates the enzyme (E), a glycoside residue as donor (S′), and an acceptor (S, a second substrate molecule or an other compound). The formation of such a complex and, consequently, the efficiency of transglycosylation depends on the affinity of the active center for the acceptor, or on the presence of acceptor-binding sites in the enzyme active center.

In this work, we attempt to implement this approach for the α-D-galactosidase (melibiase, E.C. 3.2.1.22) from the fungus *Trichoderma reesei* [15]. Previously, we have characterized its enzymatic properties and the main catalytically important amino acids in the active center of the enzyme [16,17]. The presence of a single galactose-binding site has been postulated for α-D-galactosidases isolated from bacteria [18], yeast [19], fungi [20,21], and plants [22]. The active center of the α-D-galactosidase from *T. reesei* was shown to have additional binding sites for a number of compounds.

2. Results

Transglycosylation activity of α-D-galactosidase toward alcohols.—Methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-butanol were studied as glycosyl acceptors. In this case, methyl, ethyl, 1-propyl, 2-propyl, 1-butyl, 2-butyl α-D-galactopyranosides were the transglycosylation products. The structures of the transglycosylation products were identified from the mass spectra of their acetates after GLC and by NMR analysis of the reaction mixture. In the case of methanol, the NMR spectrum showed groups of lines typical of methyl α-D-galactopyranoside in the region 3–4 ppm. The spectrum of the tested sample taken at an elevated temperature, showed an H1 doublet at 4.85 ppm (J 2.5 Hz), typical of methyl α-D-galactopyranoside. The initial rates of alkyl galactopyranoside formation were measured by analysis of the reaction mixture by GLC–MS as described above. It was found that in all cases except methanol the dependence of the transglycosylation rate is first-order with respect to the alcohol concentration. The dependence diverges from linear in the case of methanol (Fig. 1). The rates of transglycosylation catalyzed by α-galactosidase for alcohols are shown in Table 1.

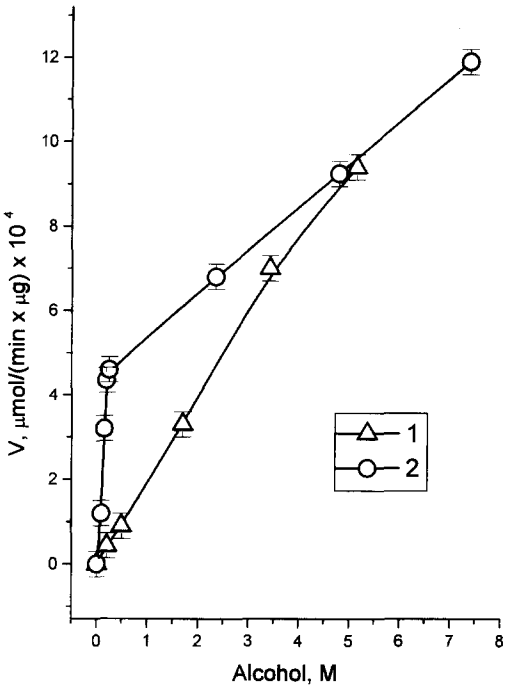


Fig. 1. Dependencies of the alkyl galactoside formation initial rates on concentration of EtOH (1) and MeOH (2). The measurements were carried out at 37 °C in 0.02 M sodium phosphate buffer, pH 6.2, initial PNPG concentration 25 mM.

Structures of the transglycosylation products.—Products of transglycosylation in reaction with *p*-nitrophenyl α-D-galactopyranoside (PNPG) were shown to be *p*-nitrophenyl α-D-galactobiosides (PNP α-galactobiosides) and *p*-nitrophenyl α-D-galactotriosides (PNP α-galactotriosides). We have shown previously that *p*-nitrophenyl 6-*O*-α-D-galactopyranosyl-α-D-galactopyranoside (PNP α-(1 → 6)-galactobioside) is formed preferentially [17]. Transglycosylation products were separated by HPLC on Lichrosorb–NH_2 into two fractions, identified as

Table 1
Rate constants of the alkyl galactoside formation for some alcohols

Alcohol	k / μmol/(min μg)×10^{-4}
Methanol	5.85
Ethanol	2.20
1-Propanol	2.40
2-Propanol	0.20
1-Butanol	6.65
2-Butanol	3.75

The incubation was carried out at 37 °C in 0.05 M NaOAc buffer, pH 5.6.

PNP α-D-galactobiosides and PNP α-D-galactotriosides. Each of the two fractions collected was subfractionated on Lichrosorb Si100. The first fraction was separated into PNP α-(1 → 3)-galactobioside and PNP α-(1 → 6)-galactobioside. The second fraction was separated into four PNP α-D-galactotriosides. ^{1}H NMR data for PNP α-(1 → 6)-galactobioside (D_2O) are δ 8.42 (d, 2 H, *J* 9 Hz, Ar group), 7.28 (d, 2 H, *J* 9 Hz, Ar group), 5.90 (d, 1 H, $J_{1,2}$ 3.2 Hz, H–1), 5.23 (d, 1 H, $J_{1',2'}$ 3.7 Hz, H–1'), and 4.32–3.71 (m, 12 H), in agreement with the literature [14]. According to HPLC, the yield of PNP α-(1 → 6)-galactobioside is 60% of the total amount of transglycosylation products. Similar estimates were provided by ^{1}H NMR analysis of the transglycosylation products directly in the reaction mixture without chromatographic separation. α-(1 → 3)-Galactobioside and α-(1 → 6)-galactobioside, prepared from corresponding PNP α-galactobiosides, were used as standards for GLC–MS to analyze galactobiosides resulting from the transglycosylation reaction with galactose as an acceptor. It was found that two galactobiosides being formed were the same as the standards. No galactotriosides were detected in this case. The ratio of α-(1 → 6)-galactobioside and α-(1 → 3)-galactobioside was 3:1.

Analysis of the substrate transglycosylation.—The initial rate of transglycosylation in PNPG hydrolysis was studied as a function of the substrate concentration. The results obtained by two independent methods, HPLC and NMR, show good agreement. Estimation of the maximal transglycosylation rate under the conditions chosen gives a value of 2×10^3 μmol/(min μg), with a saturation concentration of PNPG of about 10 mM (Fig. 2). The dependence of the PNP α-galactobioside formation on the PNPG concentration correlates with the inhibition of PNPG hydrolysis at high substrate concentration (Fig. 3).

Evidence for the existence of additional binding sites was found by analyzing the inhibition of the transglycosylation. Galactose is an inhibitor of transglycosylation, while glucose is not. This confirms the existence of an additional galactose-binding site at the active center of the enzyme, which is responsible for the acceptor binding (Fig. 4). Independent experiments have shown (Fig. 5) that the inhibition of PNP α-galactobioside formation by galactose correlates with the formation of α-(1 → 3)-galactobioside and α-(1 → 6)-galactobioside as products of a transglycosylation reaction. It should be emphasized that the inhibition of transglycosylation by *p*-nitrophenol is fairly strong, which suggests that this compound acts

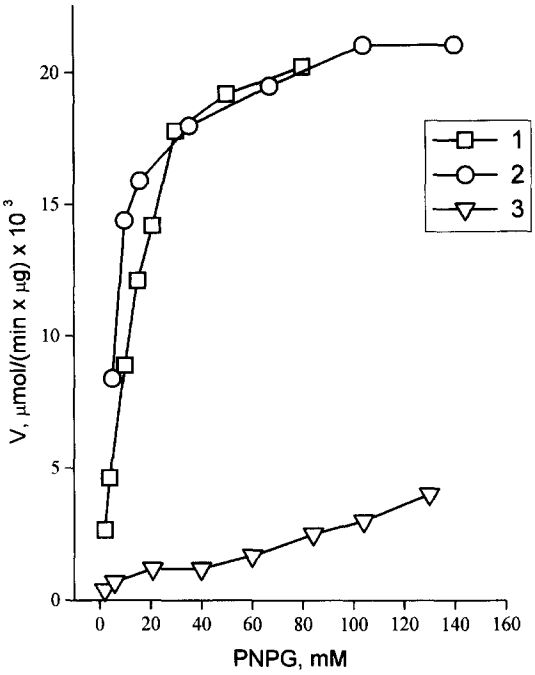


Fig. 2. Dependencies of the PNP α-(1 → 6)-galactobioside (1) and PNP α-galactobiosides (2) formation rate on the PNPG concentration in reaction with native α-D-galactosidase, and PNP α-galactobiosides formation rate on PNPG concentration in reaction with modified α-D-galactosidase (3). The measurements were carried out at 37 °C in 0.02 M sodium phosphate buffer, pH 6.2.

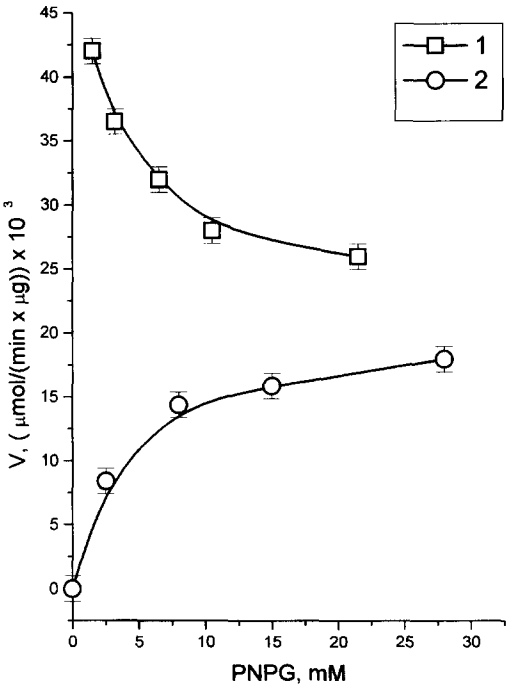


Fig. 3. Dependencies of the PNPG hydrolysis rate (1) and the PNP α-galactotriosides production rate (2) on PNPG concentration. Reactions were carried out at 37 °C in 0.05 mM NaOAc buffer, pH 5.6.

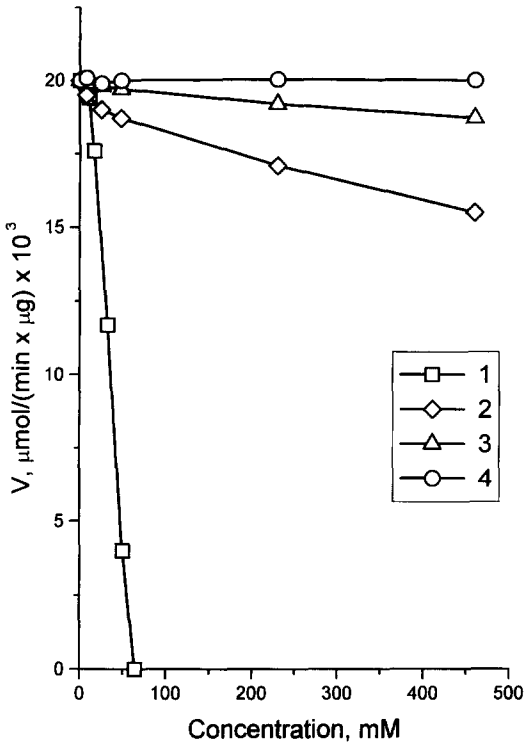


Fig. 4. Formation of PNP α-galactobiosides from PNPG in reaction of transglycosylation. Dependence of initial rate on concentrations of PNP (1), galactose (2), melibiose (3), raffinose (4). Reactions were carried out at 37 °C in 0.05 mM NaOAc buffer, pH 5.6, 15 mM PNPG.

Table 2
The kinetic constants for hydrolysis of some substrates by α-galactosidase

Substrate	K_i /mM	K_m /mM
Melibiose	6.2	3.0
Raffinose	5.8	11
Stachyose	4.0	5.0
PNP α-(1 → 6)-galactobioside	2.6×10^{-2}	2.7×10^{-2}
PNP α-galactotriosides	1.6×10^{-1}	nd

Incubations were carried out at 37 °C in 0.05 M sodium acetate buffer, pH 5.6.

competitively in the PNPG binding in the acceptor region (Fig. 4). PNP glycosides which do not contain a galactose residue in the α configuration (PNP α-D-mannopyranoside, PNP α-D-glucopyranoside, and PNP β-D-galactopyranoside) fail to inhibit transglycosylation or to accept a galactose residue at the concentrations studied. Likewise, an α-galactosidase inhibitor, *N*-benzoyl-α-D-galactopyranosylamine (K_i = 0.53 mM) shows no acceptor properties, at least at concentrations lower than 100 mM.

The main product of the substrate transglycosylation reaction, PNP α-(1 → 6)-galactobioside, was isolated in preparative quantities to be used in further experiments. It was found that PNP α-(1 → 6)-

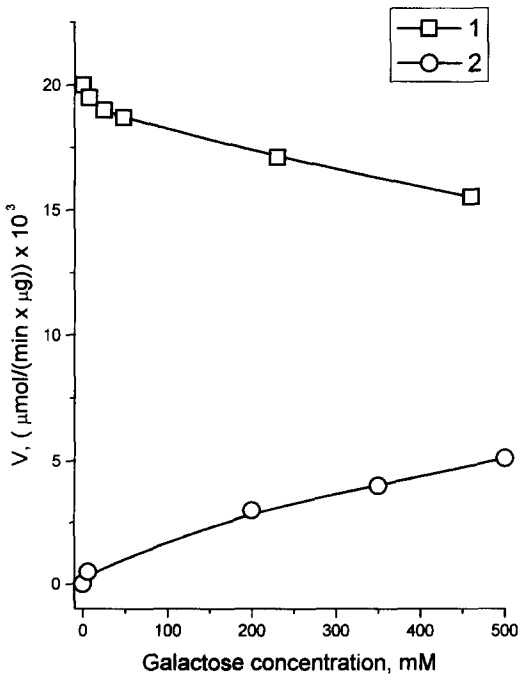


Fig. 5. Dependence of the PNP α-galactobiosides (1) and α-galactobiosides (2) formation initial rates on galactose concentration. Concentration of PNPG was 15 mM; 0.05 M NaOAc buffer, pH 5.6; 37 °C.

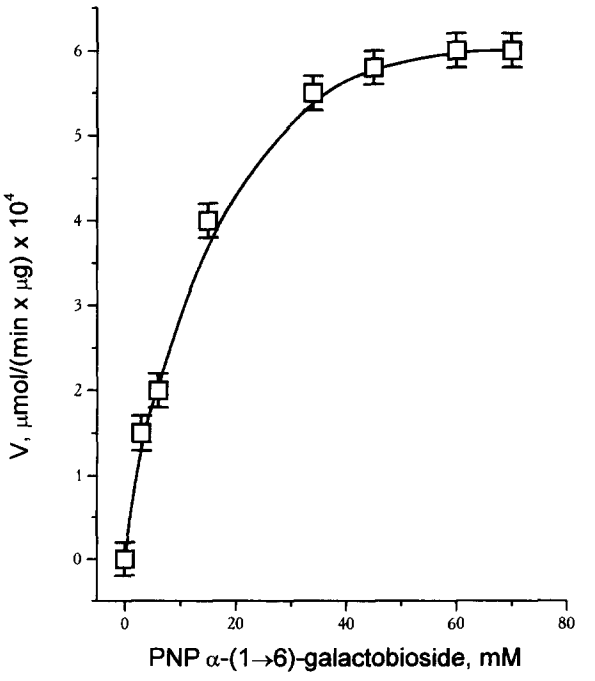


Fig. 6. Dependence of the PNP α-galactotriosides formation initial rate on PNP α-galactobioside concentration. The reaction mixture was incubated in 0.05 M NaOAc buffer, pH 5.6 at 37 °C.

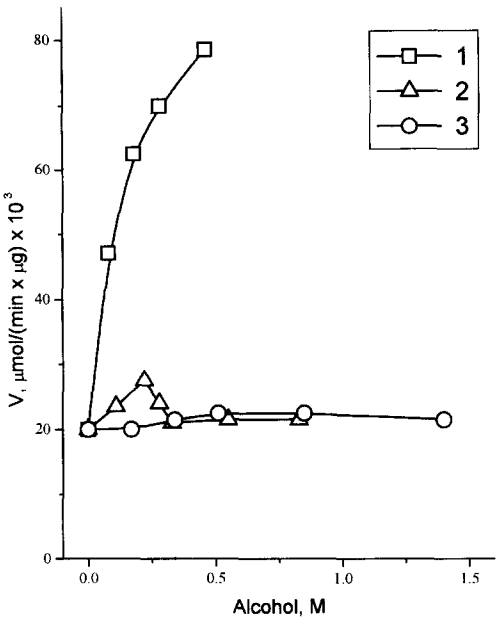


Fig. 7. Influence of 1-pentanol (1), 1-butanol (2) and ethanol (3) on the PNP α-galactobiosides formation initial rate in reaction of transflycosylation. The measurements were carried out at 37 °C in 0.02 M sodium phosphate buffer, pH 6.2, 15 mM PNPG.

galactobioside is hydrolyzed by α-galactosidase from *T. reesei* as well as that it participates in the transglycosylation reaction at high concentrations. The kinetic constants for the reaction of the PNP α-(1 → 6)-galactobioside hydrolysis were estimated by measuring the galactose concentration at the initial stages of the hydrolysis using GLC–MS. The K_m value for the PNP α-(1 → 6)-galactobioside hydrolysis is listed in Table 2. The reaction of the PNP α-(1 → 6)-galactobioside transglycosylation (Fig. 6) differed in having a lower rate $V = 6 \times 10^{-4}$ μmol/(min μg) and a higher saturation concentration than PNPG.

The influence of alcohols on the PNPG transglycosylation is ambiguous. Methanol, 1-propanol and ethanol do not affect the reaction rate up to 15% (v/v) concentration. 1-Butanol at 0.2 M concentration causes a small rate increase. 1-Pentanol increases the PNPG transglycosylation rate (Fig. 7).

Inhibition of α-D-galactosidase by natural and synthetic compounds.—It is obvious that interactions of natural substrates and products of substrate transglycosylation with the active center of α-D-galactosidase involves sorption at the active site and is responsible for acceptor binding in the transglycosylation reaction. To evaluate quantitatively these interactions, the following approach was applied: The inhibition constant K_i was measured for the various compounds acting as inhibitors of PNPG hydrolysis. The resulting value, as a good approximation, should be equal to the true dissociation constant for the complex of the enzyme with these substrates (Table 2). Note that in all cases the inhibition of PNPG hydrolysis was competitive. It should be emphasized that the K_i value for PNP α-galactotriosides differs by an order of magnitude, and for PNP α-(1 → 6)-galactobioside by 2 orders of magnitude, from the corresponding values for oligosaccharide substrates.

Transglycosylation properties of modified α-D-galactosidase.—It has been shown previously that treatment of α-D-galactosidase with hydrogen peroxide converts methionine in the active site of a protein into methionine sulfoxide. This modification increases the PNPG hydrolysis rate by a factor of 10–12 [16]. It was found that the affinity of PNPG to the acceptor-binding region in the active center of modified enzyme decreases and the saturation concentration could not be reached even at 120 mM of PNPG (Fig. 4).

3. Discussion

The approach used in the present work may lead to certain conclusions about the structure of the enzyme active site. Methanol is the only alcohol which exhibits concentration-dependent behavior in the transglycosylation reaction at low concentrations. The methanol-binding site is saturated at a concentration of the ligand of 1 mM. Methanol, similarly to other alcohols, has little or no effect on the hydrolysis of PNPG and melibiose and, consequently, the methanol-binding site should be distinctive from the substrate-binding site. Other alcohols form no equilibrium complexes with α-galactosidase (possibly for steric reasons), and the rate of the transglycosylation reaction in their presence is explicated by a first-order equation with respect to the acceptor concentration.

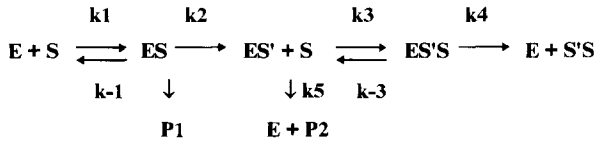
An initial rate of transfer of the alkyl group on the galactose is lower for a branched alcohol molecule than for a linear one. The pairs 1-butanol-2-butanol and 1-propanol-2-propanol were compared to estimate this difference. The lower rate in the case of the branched alcohol presumably is a result of steric hindrance. No correlation exists between the atomicity of an alcohol and the transfer rate.

The effect of alcohols on the glycosidase activity has been reported, e.g., for β-galactosidase from guinea pig liver [1]. It has been found that alcohols act as nonessential activators for this enzyme which has a site of interaction with the alcohol molecule. In

our case, alcohols do not influence directly the rate of enzymatic hydrolysis, but some of them enhance the initial transglycosylation rate. Such an effect of methanol on the rate of *p*-nitrophenyl α-maltopentaoside formation was observed in a transglycosylation reaction catalyzed by α-amylase from *Pseudomonas stutzeri* [23]. Two possible reasons for the increase in the transglycosylation rate are proposed: (1) increase of the acceptor solubility in the presence of an alcohol; (2) a specific interaction of an alcohol molecule with the protein followed by conformational changes. In the case under study, the complex character of the dependence for 1-butanol and the selective nature of the activation of transglycosylation indicate that the transglycosylation activity increases as a result of the interaction between the activator, the 1-pentanol or 1-butanol, and α-D-galactosidase.

A structural analysis of transglycosylation products has shown that solely α-(1 → 6) or α-(1 → 3) linkages are formed for PNP α-galactobiosides and for galactobiosides. The preferential formation of the α-(1 → 6) bond was observed in transglycosylation products of α-mannosidase from jack bean and α-galactosidase from coffee bean [14]. In the course of the present study, no consideration was given to cases where the acceptor concentrations much exceeded the saturation concentrations of the additional binding sites. PNP α-galactotriosides were not found previously among transglycosylation products [24–26].

A general kinetic scheme of the α-galactosidase action described previously in detail [17] is presented below:



where E is the enzyme; S is the substrate; ES is the enzyme-substrate complex (Michaelis complex); ES′ is the intermediate galactosyl–enzyme complex, P1 is the reaction product formed from the aglycone part of the substrate (*p*-nitrophenol for PNPG, methanol for methyl α-D-galactopyranoside, etc.), P2 is galactose, S′S is a transglycosylation product.

The shapes of the curves in Figs. 2 and 3, with a characteristic saturation at high substrate concentrations, correlate well with the above-presented kinetic scheme and confirm the assumption that the triple complex ES′S is formed in the course of the reaction. Formation of such a complex is possible in the presence in the active center of a substrate-binding site (sites) that is (are) distinctive from the primary binding site. Note, that inhibition by excess of substrate (PNPG) described previously for α-D-galactosidase from coffee bean [25] occurs at high substrate concentration. There exists an obvious correlation between the formation of PNP α-galactobiosides (formation S′S) and a decreasing of hydrolysis rate (P2 formation).

p-Nitrophenol inhibits the reaction of substrate transglycosylation with PNPG, having at the same time no noticeable effect on the melibiose hydrolysis. Considering this fact, an assumption may be made that *p*-nitrophenol competes with a second PNPG molecule in the formation of the ES′S complex. The same assumption is confirmed indirectly by the results of the inhibitory analysis. PNP α-(1 → 6)-galactobioside has the highest affinity with the active site of the enzyme among all the compounds studied here (Table 2). Presumably, in the formation of the complex of this substrate with α-D-galactosidase, contributions are made by all three fragments of the PNP α-galactobioside molecule, namely, the galactose residue at the first, primary, binding site; the galactose residue at the second, additional, site; and the *p*-nitrophenol residue at the PNP-binding (hydrophobic) site. Addition of one more galactose residue (PNP α-galactotrioside) to the substrate only impairs the binding ($K_i = 1.6 \times 10^{-4}$ M). We should conclude that the only additional galactose-binding site exists inside the enzyme active center.

The relatively large K_i value for raffinose and melibiose, and the very weak inhibition of the PNPG transglycosylation reaction by these two oligosaccharide substrates (Fig. 4) confirms the presence of a hydrophobic region that is responsible for PNP binding. Since both the PNP α-galactobiosides and PNP α-galactotriosides show, nevertheless, lower K_i values than the oligomeric substrates, it is conceivable that some extended hydrophobic region exists in the acceptor-binding region inside the active center of the enzyme. The model of the active site of the enzyme, consisting of the galactose-binding sites 1 and 2, a PNP-binding (hydrophobic) site, and a methanol-binding region, is presented in Fig. 8. The fact that PNP α-D-glucopyranoside, PNP α-D-mannopyranoside, and PNP β-D-galactopyranoside exhibit no acceptor properties indicates that site 2 has a significant affinity only for galactose and only for the α configuration of the glycosidic bond.

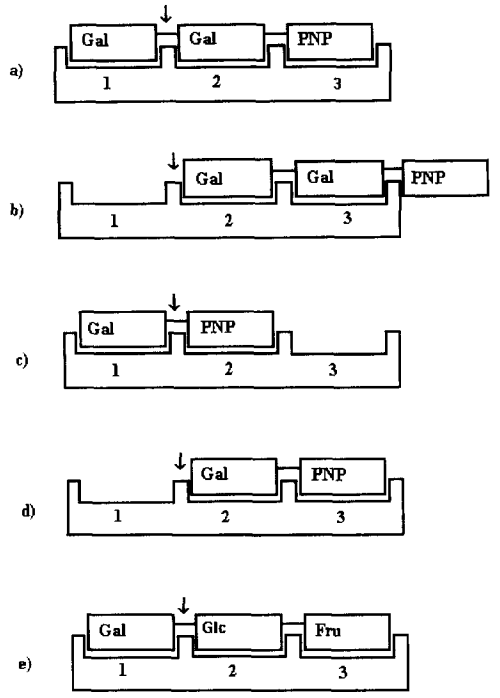


Fig. 8. Schematic representation of the α-galactosidase active center and its binding sites. Site 1, site having a maximal affinity for a galactosyl residue; site 2, site having affinity for a galactosyl residue and having no affinity for residues like PNP; site 3, PNP-binding site; MeOH-binding site is not shown. Arrow represents protein catalytic groups. (a) PNP α-galactobioside has maximal affinity in the productive complex due to interaction with sites 1-3 in the active center; (b) The nonproductive complex of PNP α-galactobioside is also possible but its formation should be less than (a). The relation between **k2** and **k5** for this substrate should have an intermediate value in comparison with 'fast' and 'slow' substrates; (c) The PNPG affinity is determined by binding of a galactosyl residue in site 1. The PNP residue is a good leaving group because its affinity to site 2 is close to zero or even negative; **k2** ≫ **k5** in this case; (d) PNPG is the most probable substrate for formation of a nonproductive complex due to a strong binding of a galactosyl residue in site 2 and a PNP residue in site 3; (e) Only a productive complex was observed for 'slow' substrates: melibiose, raffinose (shown) and stachyose. The main contribution to the binding appears to be made by the interaction only at site 1. The leaving group is not as good as in the case of PNPG, and **k2** ≪ **k5**.

Particular attention should be given to the fact that *N*-benzoyl galactopyranosylamine does not act as an acceptor in transglycosylation. It can be explained due to selective binding of this compound by galactose exclusively at the first site. The presence of an NH group prevents the binding of this compound in the acceptor-binding region at the active center.

Modification of methionine at the active site of α-galactosidase reduces the affinity of PNPG for acceptor-binding sites at the active center. Presumably, methionine plays an important role in the binding of the acceptor. Such an approach to the study of the active site, by modifying certain amino acid residues and monitoring changes in the transglycosylation reaction has been applied to α-amylase from *Saccharomyces fibuligera* [27].

In the formation of a complex between the enzyme and substrates (PNPG, methyl α-D-galactopyranoside, melibiose, stachyose) the main contribution to the variation of the standard free energy appears to be made by the interaction at site 1. *p*-Nitrophenyl, the aglycon of the PNPG substrate, is a good-leaving group. probably, due to the existence of site 3. In this case, the ES′ complex rapidly becomes accessible to an attack by a water molecule to give a galactose molecule. This second stage is the rate-limiting stage of PNPG hydrolysis, the actually measured k_{cat} and K_m values are determined by the rate constant in this stage.

The first stage of hydrolysis of other substrates results in the release of a poor leaving group. In the case of methyl α-D-galactopyranoside, the interaction of methanol with the methanol-binding site may prevent the access of a water molecule to the ES′ complex. For melibiose, raffinose, and stachyose the decisive role is presumably played by the affinity of these substrates to site 2. Therefore, the first stage of the reaction is the rate-limiting stage for slow substrates.

For PNP α-galactobioside the leaving group is PNPG, that leads to a low hydrolysis rate for this substrate. The reason for a low hydrolysis rate is the high affinity of PNPG. Substrate transglycosylation becomes possible in cases when the triple ES′S complex is formed. Evidently, PNPG is the most beneficial substrate for formation of this complex.

The proposed kinetic scheme should be, in principle, modified, since the formation is possible of a nonproductive complex between the enzyme and a substrate, where the substrate occupies sites 2 and 3. However, such a modification introduces no fundamental changes into the above speculation.

4. Experimental

Chemicals.—PNP glycosides, saccharides, salts, and BSA were from Sigma, the Me_3Si reagent was from Serva. Xylitol and arabinitol were synthesized

by $NaBH_4$-treatment of xylose and arabinose, respectively, as described [28].

Enzyme purification.—α-D-Galactosidase from *T. reesei* was purified as outlined in Ref. [15]. Oxidation-induced activation of α-galactosidase was performed following the procedure reported in Ref. [16].

Protein determination.—Protein quantities were measured by the Lowry procedure with BSA as a standard. Concentrations of pure α-galactosidase were also determined spectrophotometrically (extinction coefficient, 1.86 mL/(mg cm) at 278 nm).

Enzyme assay.—The α-D-galactosidase activity was measured using PNPG as a substrate [29]. One unit of the activity was defined as the amount of the enzyme that hydrolyzed 1 μmol of PNPG per 1 min at 37 °C. The activity was measured in 0.02 M sodium phosphate buffer, pH 6.2. The α-galactosidase activity in the hydrolysis of PNP α-(1 → 6)-galactobioside was measured using the following procedure. The reaction was terminated by adding 2 M HCl to 10% (v/v), the mixture was lyophilized, and then analyzed as sugar acetates by GLC–MS. A DB5 column with a temperature gradient from 120 to 260 °C and an Incos 50 mass spectrometer (Finnigan MAT) were used. Xylitol was used as internal standard. The inhibition constants were determined using the Dixon method by varying the concentration of the substrate in the reaction mixture. The K_m and V_{max} values in the hydrolysis of PNP α-(1 → 6)-galactiobioside were found by the method of initial velocities. Linearization was done by the Lineweaver–Burk method. All measurements were carried out at 37 °C in 0.05 M NaOAc buffer, pH 5.6.

Measurement of the transglycosylation activity.—All measurements were carried out at 37 °C in 0.02 M sodium phosphate buffer, pH 6.2 [17]. The reaction was terminated by adding 2 M HCl to 10% (v/v), the mixture was lyophilized and then analyzed.

The transglycosylation activity towards alcohols was studied by the following procedure: α-D-galactosidase (2 mg/mL) was incubated with 25 mM PNPG in the presence of varying amounts of alcohol in 0.05 M NaOAc buffer, pH 5.6 at 37 °C. Products were analyzed as sugar acetates by GLC–MS on a DB3 column (Finnigan MAT) with a temperature gradient from 120 to 260 °C. Arabinitol or xylitol were used as internal standard.

The transglycosylation activity towards PNP galactosides was measured analyzing the reaction products by reversed-phase HPLC on an Octadecyl Si-100 column with a gradient (0–90%) of MeCN in water with detection at 278 nm. Quantities of products formed were measured by integrating the corresponding chromatographic peaks. The transglycosylation products were also analyzed qualitatively by TLC on Kieselgel 60 (Merck) plates using 4:2:1 1-butanol–HOAc–water.

The transglycosylation activity towards methyl α-D-galactopyranoside was determined by analyzing the products of the reaction in the form of trimethylsilyl derivatives by GLC–MS on a DB3 column using a temperature gradient from 120 to 360 °C, with sucrose as internal standard.

Measurements of transglycosylation activity towards galactose followed a similar procedure. To analyze qualitatively the products of transglycosylation of methyl α-D-galactopyranoside and galactose, TLC was performed on Kieselgel 60 (Merck) plates using 2:2:1 EtOH–1-butanol–water.

NMR measurements.—^{1}H NMR spectra were recorded at 270 MHz on a Bruker HX-270 Spectrospin spectrometer. The measurements were made in 20 mM sodium phosphate buffer (pD 6.4) and 50 mM 4,4-dimethyl-4-silapentane sodium sulphonate (DSS) was used as internal standard in 0.5 mL 99.8% D_2O. The solvent resonance was presaturated for 0.5 s with a decoupler operating at 24 dB in the CW mode. Data were acquired after a 75° pulse into 16K points, with a spectral width of 10 kHz and 2053 scans, including first 5 dummy scans. The spectra were Lorentz-broadened by 1 Hz. The DSS signal was used for adjustment of phase and amplitude parameters in order to obtain correct differential spectra. The transglycosylation activity of α-galactosidase toward PNPG was studied as a function of the substrate concentration by ^{1}H NMR by recording the H–1α doublets at 5.910 and 5.238 ppm.

Structures of transglycosylation products.—To obtain the PNP-containing transglycosylation products, the mixture of 25 mM PNPG and α-D-galactosidase (2 mg/mL) in 0.2 M potassium phosphate buffer, pH 6.2, was incubated for 30 min at 37 °C. The reaction products were separated by HPLC as described above. The elimination of PNP from PNP α-galactobioside was carried out in 0.5 M NaOH at 60°C during 2.5 h, and the product was purified by HPLC on a Lichrosorb C-18 column in 20% MeCN in H_2O (v/v). The yield of α-galactobiosides was 15%. The conversion of PNP α-galactotriosides to PNP α-galactobiosides was made by treatment with α-galactosidase in 0.02 M potassium phosphate buffer, pH 6.2 at 37 °C. Structures of resulting products were determined as described in Section 2.

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