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Enzymatic properties of α-D-galactosidase from *Trichoderma reesei*[1]

Andrew N. Savel'ev[a], Farid M. Ibatyllin[b], Elena V. Eneyskaya[b], Anatoly M. Kachurin[b], Kirill N. Neustroev[b,*]

[a] *St. Petersburg Technical University, Department of Biophysics, 29 Polytechnicheskaya str., St. Petersburg, 195251, Russia*

[b] *Petersburg Nuclear Physics Institute, Molecular and Radiation Biophysics Division, Gatchina, St. Petersburg, 188350, Russia*

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Abstract

The kinetics of hydrolysis of a number of natural and synthetic substrates [melibiose, raffinose, stachyose, methyl α-D-galactopyranoside, and *p*-nitrophenyl α-D-galactopyranoside (PNPG)], catalyzed by α-D-galactosidase from the fungus *Trichoderma reesei*, has been studied. A number of *N*-acyl-α-D-galactopyranosylamines, which are competitive inhibitors of α-D-galactosidase, have been synthesized, and the K_I values for these compounds have been obtained. The inhibiting properties of the competitive inhibitors of D-galactose, 1,5-anhydro-D-galactitol, and 2-deoxygalactose have been compared, and reasons for differences in K_I values between these compounds have been discussed. It has been shown that α-D-galactosidase exhibits transglycosylating activity; the main product of transglycosylation in the reaction with PNPG is *p*-nitrophenyl 6-*O*-α-D-galactopyranosyl-α-D-galactopyranoside. The hydrolysis inhibition in the presence of a substrate has been shown to correlate with the substrate transglycosylation. Data of steady-state kinetics together with data of presteady-state kinetics obtained by the stop–flow method suggest that an intermediate galactosyl–enzyme complex is formed in the reaction and is of particular importance in the processes under study. A minimal kinetic scheme describing the experimental data obtained is proposed. © 1996 Elsevier Science Ltd.

Keywords: α-D-Galactosidase; *Trichoderma reesei*; Galactopyranosylamines; Kinetic scheme; Transglycosylation

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[*] Corresponding author. Tel: + 7-(812-71)-32014; fax: + 7-(812-71)-32303; e-mail: neustk@hep486.pnpi.spb.ru.

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1. Introduction.

α-D-Galactosidase (melibiase, EC 3.2.1.22), belonging to the group of exoglycosidases, catalyzes the hydrolysis of terminal α-D-galactosyl groups in galactooligosaccharides and galactomannans. Although a considerable number of α-D-galactosidases of different origin (bacteria [1], yeast [2], fungi [3,4], plants [5,6]) have now been isolated, data on the structure of the active sites of these enzymes are lacking. Little is known about the action of these glycosidases.

α-D-Galactosidase used in the present work was isolated from the mesophyllic fungus *Trichoderma reesei*. The enzyme was isolated in high purity, which enabled us to crystallize the protein for preliminary X-ray investigations [7], which became the first stage in the structure study. In the earlier investigations we have found the presence of a catalytically important sulfhydryl group and methionine in the active site of α-D-galactosidase [8,9]. The present study deals with specific features of the kinetic mechanism of this enzyme.

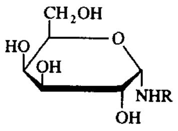
2. Results and discussion

Synthesis of N-acyl-α-D-galactopyranosylamines.—*N*-Acyl-α-D-galactopyranosylamines (Table 1) were synthesized by the following procedure. An ammonium complex of α-D-galactopyranosylamine, prepared as reported [10], was acylated with the corresponding anhydride in aqueous solution at 0 °C in the presence of pyridine, added to neutralize the acid formed, instead of sodium hydrogencarbonate, which was used, for

Table 1
K_I values for various inhibitors in reaction with α-D-galactosidase

Inhibitor	K_I (mM)
D-Galactose	0.09
1,5-Anhydro-D-galactitol	1.4
2-Deoxygalactose	13.0
N-Acyl-α-D-galactopyranosylamines[a]	
1	0.72
2	0.16
3	0.53
4	0.25
5	0.36

[a]



1 R = Bz
2 R = Ac
3 R = p-IC_6H_4CO
4 R = $ClCH_2CO$
5 R = NH_2CH_2CO

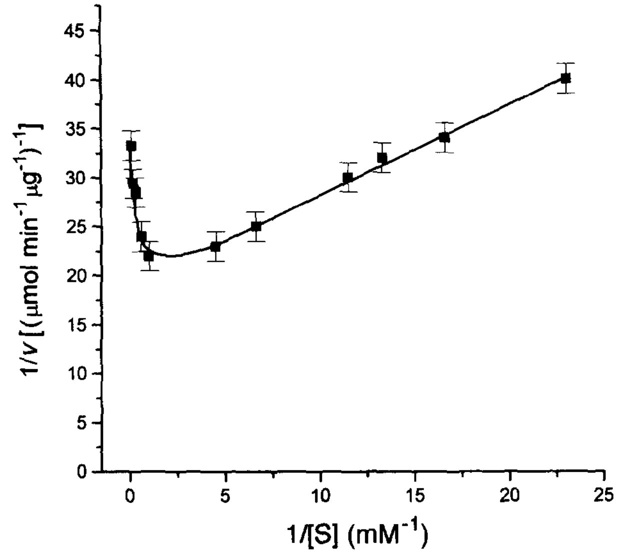


Fig. 1. Lineweaver–Burk plot for hydrolysis of PNPG catalyzed by α-D-galactosidase.

example, in the synthesis of some 1-*N*-glycyl β-oligosaccharide derivatives. All compounds, except *N*-acetyl-α-D-galactopyranosylamine, were prepared as a mixture of α and β anomers. The high quantitative yield of the α anomer when acetic anhydride was used may result from the high reaction rate as compared with that of other acylating compounds. No attempts were made to separate the mixture of α and β anomers by crystallization. We used reversed-phase HPLC, which allows isolation of pure *N*-acyl-α-D-galactopyranosylamines. The inhibitors prepared, in contrast to α- and β-D-galactosylamines and their *N*-alkyl derivatives, are stable in aqueous solutions at pH 3.5–7.0, which allows their application in enzymatic experiments.

Analysis of steady-state kinetics of the hydrolysis reaction catalyzed by α-D-galactosidase.—α-D-Galactosidase from *Trichoderma reesei* catalyzes the hydrolysis of a number of natural and synthetic substrates. The dependence of the initial hydrolysis rate on the substrate concentration is generally of a complex character (Fig. 1). Inhibition by an excess of a substrate is typical of hydrolysis of *o*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl α-D-galactopyranoside (PNPG), 4-methylumbelliferyl α-D-galactopyranoside (MUG), and methyl α-D-galactopyranoside. In the hydrolysis of the natural compounds melibiose, raffinose, and stachyose this effect was not observed at the corresponding concentrations of the compounds (Fig. 2). The kinetic parameters K_m and k_{cat} of the reactions studied (Table 2) were evaluated by measuring the initial rates under conditions for which no noticeable inhibition of the reaction by substrate was observed. In the case of PNPG, this range of substrate concentrations is less than 0.3 mM (Fig. 1). PNPG is the substrate of α-D-galactosidase with the highest hydrolysis rate, its experimental k_{cat} value in hydrolysis being approximately an order of magnitude higher than the corresponding values of the other substrates. The K_m values in hydrolysis of

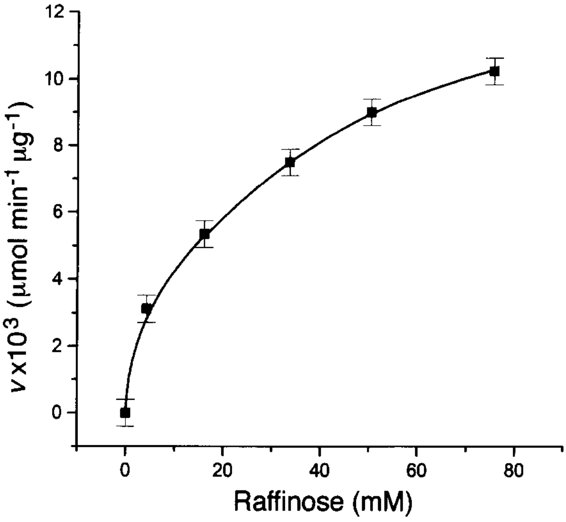


Fig. 2. Dependence of initial rate for hydrolysis of raffinose catalyzed by α-D-galactosidase upon substrate concentration; 0.05 M NaOAc buffer, pH 5.6, 37 °C.

PNPG at pH 5.6 also differ substantially from those in reactions with other substrates. D-Galactose competitively inhibits enzymatic hydrolysis of PNPG (Table 1), exhibiting in this case a high affinity to the active site of the enzyme. The binding site for the galactose residue is obviously the main place of binding for all substrates of α-D-galactosidase.

The inhibition of the reaction by D-galactose on the one hand, and, for a number of substrates, by the excess of the substrate on the other hand, allowed us to estimate only approximately the kinetic K_m and k_{cat} constants under conditions of low concentration of the substrate and its low conversion into the product. These estimates indicate that all substrates used in the present study can be divided into "fast" (PNPG) and "slow" (the others). The Michaelis constant for PNPG determined in the kinetic experiments is substantially lower than those for the other substrates. These peculiarities of the steady-state kinetics of the reaction studied can be explained with a model describing the reaction as the formation of an intermediate galactosyl–enzyme complex. The kinetic

Table 2
Kinetic parameters of reactions catalyzed by α-D-galactosidase from *Trichoderma reesei*

Substrate	K_m (mM)	k_{cat} (µmol min^{-1}µg^{-1})
PNPG	0.05	5.1×10^{-2}
Methyl α-D-galactopyranoside	4.0	1.8×10^{-3}
Melibiose	3.0	3.5×10^{-3}
Raffinose	11.0	1.2×10^{-2}
Stachyose	5.0	2.0×10^{-3}

scheme of the reaction at low concentrations of substrate, without considering inhibition by D-galactose, may be presented as follows:

$$\begin{array}{ccccccc} & & & P_1 & & & \\ & k_1 & & \uparrow & & k_5 & \\ E+S & \leftrightarrow & ES & \rightarrow & ES' & \rightarrow & E+P_2 \\ & k_{-1} & & k_2 & & & \end{array}$$

where E is the enzyme; S is the substrate; ES is the enzyme–substrate complex (Michaelis complex); ES′ is the intermediate galactosyl–enzyme complex; P_1 is the reaction product formed from the aglycon part of the substrate (*p*-nitrophenol for PNPG, methanol for methyl α-D-galactopyranoside, etc.); P_2 is D-galactose; k_1, k_{-1}, k_2, and k_5 are the rate constants in the corresponding stages ($k_5 = k_5' \cdot [H_2O]$).

It can be readily shown that the solution to this scheme under the conditions of steady-state ES and ES′ complexes will be the Michaelis–Menten equation:

$$v = \frac{d[P_1]}{dt} = \frac{d[P_2]}{dt} = \frac{k_{cat} \cdot [E]_o \cdot [S]}{K_m + [S]}$$

where v is the initial reaction rate;

$$k_{cat} = \frac{k_2 \cdot k_5}{k_2 + k_5}; \; K_m = \frac{k_5 \cdot (k_{-1} + k_2)}{(k_2 + k_5) \cdot k_1}.$$

The stage of deglycosylation of the ES′ complex (the stage characterized by the k_5 constant) is common for all substrates, either "fast" or "slow". Therefore, the differences in experimental kinetic constants may result from the different rates of formation of the galactosyl–enzyme ES′ complex, that is, from differences in k_2. The low value k_2 ($k_2 \ll k_5$) gives $k_{cat} \approx k_2$ and $K_m \approx (k_{-1} + k_2)/k_{-1}$; the high k_2 ($k_2 \gg k_5$) value gives $k_{cat} \approx k_5$ and

$$K_m \approx \frac{k_5 \cdot (k_{-1} + k_2)}{k_2 \cdot k_1}.$$

In such a situation, the K_m value for the "slow" rate substrate determined in the kinetic experiments should agree with the real constant of dissociation for the enzyme–substrate complex. As Table 2 shows, these values (except in the case of raffinose) are in the range 3–5 mM, being substantially higher than the corresponding values for D-galactose and its derivatives. It is expected that the 1-OH group is of significant importance in binding galactose in the active site of the enzyme. Changing the oxygen to nitrogen in *N*-acyl-α-D-galactopyranosylamines slightly impairs the binding, and the introduction of a glycoside bond into substrates results in the affinity decreasing by more than an order of magnitude. In the case of 1,5-anhydro-D-galactitol K_I is substantially higher than that of D-galactose (Table 1), which proves this assumption. The decreased affinity with 2-deoxygalactose ($K_I = 13$ mM) is quite unexpected. The presence of the OH-group at position 2 is the necessary condition for binding. In the case of the "fast" substrate, the experimental K_m value is equal to the true value of the dissociation constant of the PNPG–enzyme complex multiplied by the parameter k_5/k_2.

Kinetic parameters in the enzymatic hydrolysis of PNPG as a function of pH.—The K_m and k_{cat} values in hydrolysis of PNPG were determined in the pH range from 2.2 to 8.0 (Fig. 3). Measurements in solutions with pH below 2.2 are hampered, owing to the fast enzyme inactivation under these conditions. At low pH we observed a sharp increase in both kinetic parameters. A plot of $\log(k_{cat}/K_m)$ against pH indicates the dissociation of two groups of the active site of the enzyme with $pK_a \approx 3.2$ and $pK_a \approx 7.0$, which are catalytically important. Interpreting these data, we come to the following conclusion. The true dissociation constant of PNPG does not differ significantly from the K_m values for the other substrates. With pH decreasing to 2.2, the K_m value in hydrolysis of α-PNPG increases to 3.0 mM. For this substrate the behaviour of k_{cat} with pH can be explained by the increasing k_5 value in the "acid" region. A similar influence of pH on the rate of enzymatic reaction was observed in the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside catalyzed with β-D-glucosidase from *Trichoderma viride* [11]. The plot of (k_{cat}/K_m) against pH allows the estimation of the ionization constants of the catalytic groups of the native enzyme. These groups may be presented by the carboxyl ($pK_{a1} \approx 3.2$) and also sulfhydryl ($pK_{a2} \approx 7.0$) groups. Note that the presence of the catalytically valuable sulfhydryl group in α-D-galactosidase from *T. reesei* was previously reported [8].

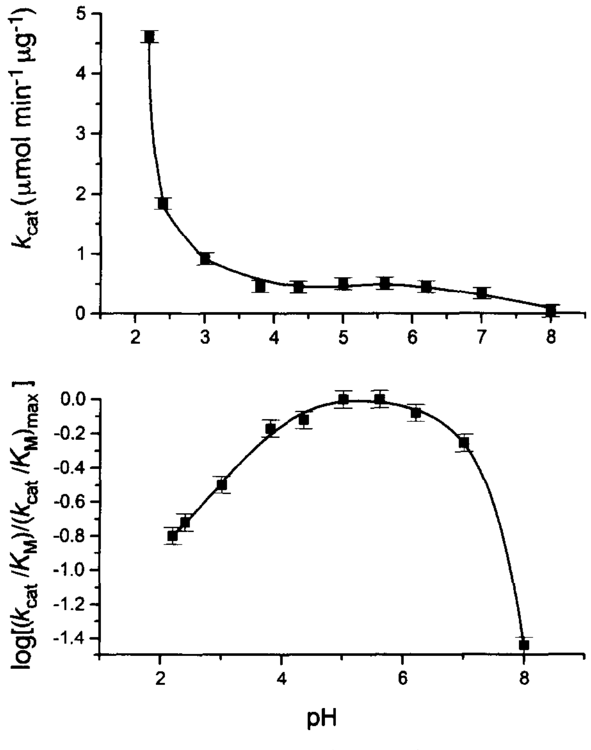


Fig. 3. Dependencies on pH of k_{cat} and $\log(k_{cat} / K_m)$ for hydrolysis of PNPG.

Inhibition of α-D-galactosidase activity by N*-acyl-α-D-galactopyranosylamines.*— The results of the inhibition studies are summarized in Table 1. The inhibition has a competitive character in all cases. The difference in K_I values of *N*-acetyl-α-D-galactopyranosylamines may be explained by the inductive effect of the acyl group on the nitrogen atom, which directly reacts with the carboxyl group in the active site. Thus, *N*-acetyl-α-D-galactopyranosylamine, with the highest charge density on the amide nitrogen atom (K_I = 0.16 mM) among the inhibitors considered, should have the highest affinity; the next is *N*-chloracetyl-α-D-galactopyranosylamine (K_I = 0.25 mM) with a lower charge density on the same nitrogen atom.

Note also that changing pH weakly affects the inductive effect of the acyl group. The increase in the affinity with pH increase (Fig. 4) results exclusively from the changing charge of the carboxyl group at the active site in the case of *N*-benzoyl-α-D-galactopyranosylamine. We can also expect that a fairly weak affinity in the case of *N*-*p*-iodobenzoyl-α-D-galactopyranosylamine and *N*-benzoyl-α-D-galactopyranosylamine results from steric hindrance caused by the presence of the benzene ring. The active site of α-D-galactosidase apparently does not contain any hydrophobic binding site in the immediate vicinity of the catalytically valuable carboxyl group. This is why *N*-acyl-α-D-galactopyranosylamines containing an aromatic group show low affinity for the active site of α-D-galactosidase. Note that *N*-alkyl-β-D-galactopyranosylamines containing an aromatic or a bulky alkyl group — inhibitors of β-D-galactosidase from *E. coli* — have the lowest K_I value, which points to the presence of a hydrophobic site in the enzyme [12].

Analysis of transglycosylating activity.—Transglycosylation is typical of a number of carbohydrases [13], including the α-D-galactosidase from coffee bean [5]. α-D-Galactosidase from *T. reesei* shows a valuable transglycosylating activity in a number of cases. One of the possible pathways for this reaction is substrate glycosylation, when a galactose residue (donor) is transferred to the substrate molecule (acceptor). The transglycosylation products were obtained using PNPG, *o*-nitrophenyl α-D-galactopyranoside, MUG, and even methyl α-D-galactopyranoside, which inhibits the enzyme

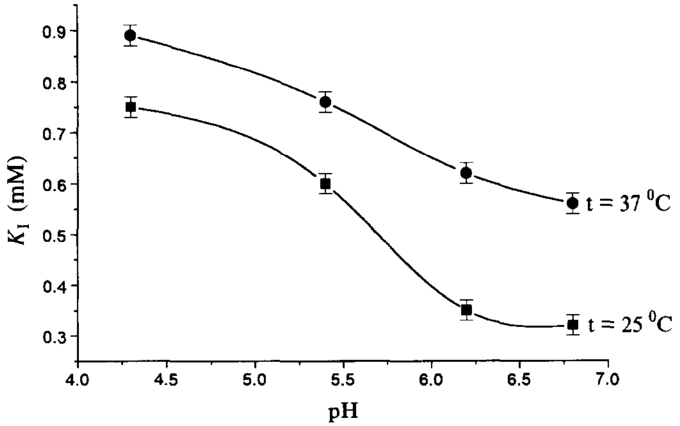


Fig. 4. Dependence on pH of K_I for *N*-benzoyl-α-D-galactopyranosylamine.

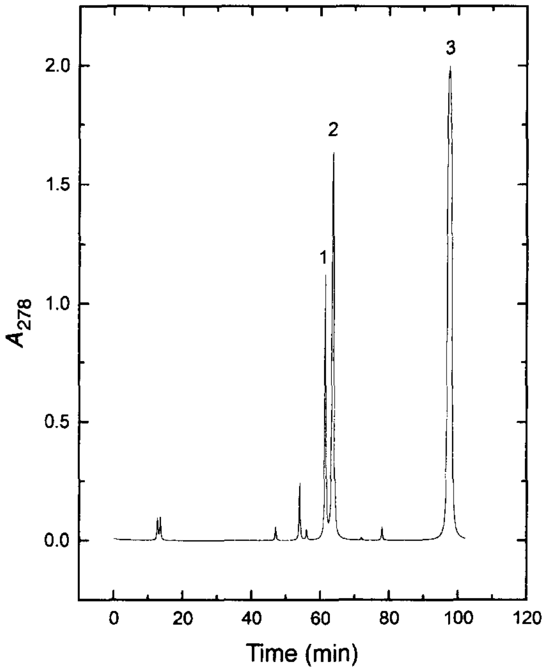


Fig. 5. Separation of the α-D-galactosidase transglycosylation products on the PREP-ODS column (250 × 20 mm) with a gradient (0–90%) of MeCN in water: 1, PNP-Gal-Gal; 2, PNPG; 3, *p*-nitrophenol.

at high concentrations. The presence of transglycosylation products in the case of synthetic substrates was determined analytically using TLC as well as HPLC on a column of Lichrosorb–NH_2. The main transglycosylation product of the reaction with PNPG was isolated and purified by reversed-phase HPLC (Fig. 5). It is, according to NMR data, *p*-nitrophenyl 6-*O*-α-D-galactopyranosyl-α-D-galactopyranoside (PNP-Gal-Gal), which agrees with data obtained for the products of transglycosylation by α-D-galactosidase from coffee bean [14].

Melibiose, raffinose, and stachyose neither inhibited hydrolysis at high concentrations nor yielded transglycosylation products. The absence of transglycosylation products was confirmed by the GLC–MS method after transformation of products of enzymatic reaction into trimethylsilyl derivatives. The formation of an intermediate galactosyl–enzyme complex is, presumably, the crucial point explaining two other specific features of the α-D-galactosidase reaction under study — inhibition by an excess of the substrate and substrate transglycosylation. Interaction of the galactosyl–enzyme complex with a second molecule of the substrate, resulting in the formation of the triple complex,

$$ES' + S \quad \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} \quad ES'S,$$

is formally expressed in the observed effect of substrate inhibition. In our opinion, it is reasonable to propose that substrate transglycolysation occurs by the formation of the same triple complex. The general minimal kinetic scheme of the reaction catalyzed with α-D-galactosidase from *T. reesei*, taking into account the above-presented considerations, may be written as follows:

$$\begin{tikzcd}
 & & P_1 & & \\
E+S \arrow[r, leftrightarrow, "k_1", "k_{-1}"'] & ES \arrow[r, "k_2"'] \arrow[ur] & ES'+S \arrow[d, "k_5"] \arrow[r, leftrightarrow, "k_3", "k_{-3}"'] & ES'S \arrow[r, "k_4"] & E+S'S \\
 & & E+P_2 & &
\end{tikzcd}$$

with the above-mentioned designations, and where S′S is a product of substrate transglycosylation, and k_4 is the rate constant for this stage. The solution of the scheme may be obtained in analytical form under the conditions of the steady-state ES, ES′, and ES′S complexes. Expressions for the initial rates have the form

$$v_1 = \frac{d[P_1]}{dt} = \frac{k_1 \cdot k_2 \cdot (k_4 \cdot k_5 + k_{-3} \cdot k_5 + k_3 \cdot k_4 \cdot [S]) \cdot [E]_o \cdot [S]}{Z}$$

$$v_2 = \frac{d[P_2]}{dt} = \frac{k_1 \cdot k_2 \cdot k_5 \cdot (k_{-3} + k_4) \cdot [E]_o \cdot [S]}{Z}$$

$$v_3 = \frac{d[SS']}{dt} = \frac{k_1 \cdot k_2 \cdot k_3 \cdot k_4 \cdot [E]_o \cdot [S]_2}{Z}$$

where $Z = k_5 \cdot (k_{-1} + k_2) \cdot (k_{-3} + k_4) + \{k_1 \cdot k_5 \cdot (k_{-3} + k_4) + k_3 \cdot k_4 \cdot (k_{-1} + k_2) + k_1 \cdot k_2 \cdot (k_{-3} + k_4)\} \cdot [S] + k_1 \cdot k_3 \cdot (k_2 + k_4) \cdot [S]^2$.

It can be easily verified that at low concentrations of substrate and in the supposed absence of transglycosylation ($k_4 \approx 0$), the expressions for v_1 and v_2 are transformed into the above-mentioned form of the Michaelis–Menten equation.

The formation of the ES′S complex most probably results from the presence of an additional site (sites) for substrate binding in the active site. This site should have a lower affinity for the substrate than the main site since its saturation is achieved only at relatively high concentrations of the substrate. We may assume that for all substrates of α-D-galactosidase the common reaction mechanism is the same; however, the low affinities for the second site of raffinose, stachyose, and melibiose do not allow us to observe the effects connected with the formation of the ES′S complex at the concentrations used.

With increasing concentrations of the substrate, the v_1 and v_3 rates approach a limiting value, which is equal to

$$\frac{k_2 \cdot k_4 \cdot [E]_o}{k_2 + k_4}$$

and the v_2 rate tends to zero.

Analysis of the presteady-state kinetics.—The characteristic curve for PNPG hydrolysis (at very short incubation times, initial burst products are formed) is shown in Fig. 6. The character of the initial burst kinetics indicates the biphasic nature of the product-formation curve. Thus, *p*-nitrophenol is produced rapidly in the presteady-state phase and

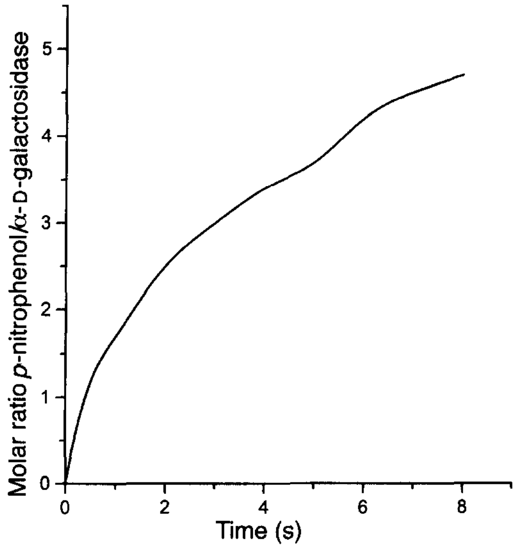


Fig. 6. Initial burst kinetics of PNPG hydrolysis with α-D-galactosidase; 0.05 M sodium phosphate (pH 6.5), 4 °C, $\lambda = 420$ nm.

more slowly after the steady state has been achieved. This proves that the reaction proceeds through the formation of a galactosyl–enzyme complex. Extrapolation of the absorbance (Fig. 6) to zero time for the reaction allows determination of the ratio of *p*-nitrophenol and α-D-galactosidase that are present in the incubation mixture. This fact enabled us to confirm the presence of one active site in this enzyme.

3. Experimental

Chemicals.—*p*-Nitrophenyl α-D-galactopyranoside (PNPG), 4-methylumbelliferyl α-D-galactopyranoside (MUG), D-galactose, melibiose, raffinose, stachyose, methyl α-D-galactopyranoside, bovine serum albumin (BSA), D-glucose oxidase, and peroxidase were purchased from Sigma (USA), and 2-deoxy-D-*lyxo*-hexose (2-deoxygalactose) from Reanal (Hungary). Reagents used for syntheses of inhibitors and solvents were purchased from Fluka and E. Merck (Germany).

General methods.—α-D-Galactosidase quantities were measured by the Lowry procedure with BSA as standard. In addition, the concentration of α-D-galactosidase was determined by spectrometry using a value 1.86 $\text{L g}^{-1}\text{cm}^{-1}$ as specific absorption coefficient at 278 nm.

The ^{1}H NMR spectra were recorded at 300 MHz in the Fourier-transform mode with quadrature detection using a Bruker Spectrospin CPX-300 spectrometer. One-dimensional (1D) spectra were collected by using a 90° pulse of 5.7 μs. Two-dimensional (2D) phase-sensitive DQF-COSY spectra were recorded using the time proportional phase increment (TPPI). A total of 512 FIDs, each consisting of 16 scans, was acquired with an F2 time domain size of 1K. Prior to Fourier transformation, the data were zero-filled and apodized using a shifted sine-bell function ($\pi/4$ in F1 and F2). Resolution was 1.4 Hz/point.

TLC was performed on Silica Gel F_{254} (E. Merck). Fluorescence was measured on a Hitachi 4000 fluorescence spectrometer. The stop–flow experiments were carried out using an AMINCO stop–flow spectrometer at wavelength $\lambda = 420$ nm.

Enzyme and enzymatic assays.—*α*-D-Galactosidase was purified by a procedure reported [7]. The activity of α-D-galactosidase toward PNPG as substrate was measured as previously described [15]. The unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 μmol of PNPG per min at 37 °C, pH 6.0. The activities of α-D-galactosidase in hydrolyses of raffinose and stachyose were determined by measuring the concentration of reducing saccharide during the reaction course [16], and, in the case of hydrolysis of melibiose, by the glucose oxidase method [17]. The activity of α-D-galactosidase in hydrolysis of MUG was monitored by the liberation of 4-methylumbelliferol, by measuring the fluorescence with the excitation wavelength of 350 nm and the emission wavelength of 446 nm.

K_m, k_{cat}, and K_I values.—The K_m and k_{cat} values were determined by the method of initial rates in the hydrolysis of natural and synthetic substrates. Linearization was carried out by the Lineweaver–Burk method. The inhibition constants were measured by the Dixon method, by varying the concentration of the substrate (PNPG) in the reaction mixture. The dependencies of the kinetic constants of hydrolysis of PNPG on pH were determined using 50 mM phosphate–citrate (pH 2.2–4.0), acetate (pH 4.0–5.6), and phosphate (pH 6.0–8.0) buffers containing 0.1 M NaCl.

Transglycosylating activity of α-D-galactosidase.—Experiments on the transglycosylating activity of α-D-galactosidase were carried out under the following conditions: pH 6.2, 0.02 M sodium phosphate buffer, 37 °C. The reaction was terminated by adding 2 M HCl to the mixture in the ratio 10% v/v. TLC was used for qualitative analysis of the transglycosylation products. The reactions with methyl α-D-galactopyranoside and oligosaccharides were analyzed using a 2:2:1 EtOH–1-butanol–H_2O system of solvents; for the reaction with synthetic substrates, 4:2:1 1-butanol–CH_3COOH–H_2O was used. The analysis for the presence of transglycosylation products, or their absence, in the case of methyl α-D-galactopyranoside and other galactose-containing oligosaccharides (melibiose and raffinose), in the form of trimethylsilyl derivatives, was performed by GLC–MS on a D-3 column with a temperature gradient from 120 to 360 °C, using a Finigan Mat-90 instrument. Sucrose was used as internal standard in the case of methyl α-D-galactopyranoside, and maltotetraose in other cases.

A quantitative analysis of the transglycosylation products in the case of hydrolysis of PNPG was carried out by the following procedure. After the reaction was terminated, the mixture was lyophilized and analyzed by HPLC on a column packed with Lichrosorb–NH_2; a quantitative interpretation was accomplished by integrating peaks of each *p*-nitrophenyl derivative of oligosaccharides. The isocratic elution was carried out using 4:1 MeCN–5 mM NaOAc, pH 4.2.

For structural studies of the main transglycosylation product in the reaction with PNPG, the mixture of α-D-galactosidase (2 mg) and PNPG (50 mg) in 0.02 M sodium phosphate buffer (2 mL, pH 6.2, 37 °C, 20 min) was lyophilized and, after chromatographic separation, analyzed by NMR spectroscopy.

The sample (5 mg) was dissolved in 0.05 M D_2O–acetate buffer, pD = 5.0, $T = 300$ K. The water resonance was suppressed by continuous low-power (0.2 W × 20 dB) irradiation during 4 s. The signal from aromatic protons was suppressed using a low-frequency filter. 2D-COSY was used to assign the proton signals. ^{1}H NMR data for the *p*-nitrophenyl galactoside fragment of PNP-Gal-Gal: δ 5.850 (d, 1 H, $J_{1,2}$ 4.0 Hz, αH-1), 4.150 (dd, 1 H, H-2), 4.190 (dd, 1 H, $J_{2,3}$ 11 Hz, H-3), 4.255 (d, 1 H, $J_{3,4}$ 2.0, $J_{4,5} < 0.5$ Hz, H-4), 4.185 (t, 1 H, $J_{5,6}$ 7.2 Hz, H-5), 3.72 (d, 2 H, H-6ab); for terminal galactose: δ 5.160 (d, 1 H, αH-1), 3.84 (dd, 1 H, $J_{1,2}$ 4.2 Hz, H-2), 3.91 (dd, 1 H, $J_{2,3}$ 10.4 Hz, H-3), 3.98 (d, 1 H, $J_{3,4}$ 2.4 Hz, H-4), 3.91 (t, 1 H, H-5), 3.66 (m, 2 H, $J_{5,6}$ 7.8 Hz, H-6ab).

Syntheses of α-D-galactosidase inhibitors.—*N*-Benzoyl-α-D-galactopyranosylamine (**1**), *N*-acetyl-α-D-galactopyranosylamine (**2**), *N*-*p*-iodobenzoyl-α-D-galactopyranosylamine (**3**), *N*-chloracetyl-α-D-galactopyranosylamine (**4**), and *N*-aminoacetyl-α-D-galactopyranosylamine (**5**) were synthesized by the following methods. The corresponding anhydride (22 mmol) (benzoic anhydride for **1**, acetic anhydride for **2**, *p*-benzoic anhydride for **3**, chloroacetic anhydride for **4**) was added to a solution of α-D-galactopyranosylamine (1.95 g, 10 mmol; which was synthesized as reported [10]) in H_2O (10 mL) and pyridine (2.4 mL), and the mixture was kept with stirring for 20 min at 0 °C. Then the mixture was evaporated at reduced pressure and, without pretreatment, the product was purified by reversed-phase HPLC on a column packed with Octadecyl Si 100, 5 μm (20 × 500 mm, Serva) with a gradient (0–100%) of MeCN in water.

Compound **1** had $[\alpha]_D^{25}$ +25.0° (*c* 0.02, H_2O); ^{1}H NMR (D_2O): δ 5.825 (d, 1 H, $J_{1,2}$ 5.2 Hz, H-1), 4.168 (dd, 1 H, $J_{2,3}$ 10.5 Hz, H-2), 4.020 (dd, 1 H, $J_{3,4}$ 2.4 Hz, H-3), 4.039 (d, 1 H, H-4), 3.880 (m, 1 H, $J_{5,6}$ 6.8 Hz, H-5); ^{13}C NMR (D_2O): δ 133.57 (para C), 129.59 (2 meta C), 128.32 (2 ortho C), 69.51, 70.09, 74.44, 77.66, 81.18 (C-2–C-5, C-1), 61.74 (C-6).

In the case of **2**, chromatography under the same conditions was used for additional purification; $[\alpha]_D^{25}$ +127.3° (*c* 0.015, H_2O); ^{1}H NMR (D_2O): δ 5.599 (d, 1 H, $J_{1,2}$ 5.1 Hz, H-1), 4.050 (dd, 1 H, $J_{2,3}$ 10.1 Hz, H-2), 3.842 (dd, 1 H, $J_{3,4}$ 3.2 Hz, H-3), 3.983 (d, 1 H, H-4), 3.765 (dd, 1 H, $J_{5,6}$ 6.3 Hz, H-5), 2.093 (s, 3 H, Me); ^{13}C NMR (D_2O): δ 182.16 (C=O), 24.05 (Me), 67.02, 69.62, 70.17, 72.65, 77.51 (C-4, C-2, C-3, C-5, C-1), 61.91 (C-6).

To prepare compounds **3** and **4**, the α and β anomers were separated by reversed-phase chromatography, as reported above. Compound **3** had $[\alpha]_D^{25}$ +60.1° (*c* 0.015, H_2O); ^{1}H NMR (D_2O): δ 5.608 (d, 1 H, $J_{1,2}$ 6.0 Hz, H-1), 4.156 (dd, 1 H, $J_{2,3}$ 11.2 Hz, H-2), 3.858 (d, 1 H, $J_{3,4}$ 5.5 Hz, H-3), 4.017 (t, 1 H, H-4), 3.868 (m, 1 H, $J_{5,6}$ 6 Hz, H-5), 3.726 (m, 2 H, H-6); ^{13}C NMR (D_2O): δ 61.07 (CH_2), 68.87, 69.45, 76.84, 80.44, 84.18 (C-1–C-5).

Compound **4** had $[\alpha]_D^{25}$ +74.8° (*c* 0.015, H_2O); ^{1}H NMR (D_2O): δ 5.603 (d, 1 H, $J_{1,2}$ 6.57 Hz, H-1), 4.039 (dd, 1 H, $J_{2,3}$ 10.8 Hz, H-2), 3.844 (dd, 1 H, $J_{3,4}$ 3.15 Hz, H-3), 3.963 (m, 1 H, H-4), 3.753 (t, 1 H, $J_{5,6}$ 12.1 Hz, H-5), 3.688 (d, 2 H, H-6), 2.08 (m, 2 H, CH_2Cl); ^{13}C NMR (D_2O): δ 46.72 (CH_2Cl), 72.32 (CH_2), 79.33, 82.86, 83.66, 87.37 (C-2–C-5), 94.45 (C-1).

Compound **5** was prepared from **4** by ammonolysis, according to the following procedure. Compound **4** (100 mg) was dissolved in satd aq ammonium hydrogencarbonate (1 mL), and more ammonium hydrogencarbonate was added after 20 and 30 h to maintain the saturation of the solution. The total reaction time was 48 h at 30 °C. The product was separated from ammonium hydrogencarbonate in the mixture by chromatography on a column packed with Octadecyl Si 100 under the following conditions: initial buffer, H_2O; elution with 80% MeCN. NMR data for **5** (D_2O): δ 5.601 (d, 1 H, $J_{1,2}$ 5.2 Hz, H-1), 4.105 (dd, H-2), 3.854 (dd, $J_{3,4}$ 2.63 Hz H-3), 3.984 (H-4), 3.780 (H-5) 3.698 (m, H-6).

In the case of compound **1**, the α and β anomers were separated by reversed-phase HPLC on a column packed with Spherosorb ODS, 5 μm (4 × 250 mm, Pharmacia) with a gradient (0–90%) of MeCN in water, with subsequent additional purification of the α anomer by rechromatography under the same conditions.

For compound **1**, the ratio of α and β anomers was 3:5; the α anomer was prepared in quantitative yield (85%). For compound **3**, the ratio of α and β anomers was 5:1, yield 70%. For compound **4**, the ratio of α and β anomers was 3:1, yield 50%.

1,5-Anhydro-D-galactitol was synthesized by a method reported [18]. Additional purification was carried out as follows: the product was acetylated by treatment with acetic anhydride–pyridine overnight at room temperature and purified by reversed-phase chromatography on a PREP-ODS column (250 × 20 mm; Gasukuro Kogyo, Korea) with a gradient (0–100%) of MeCN in water. Fractions containing 1,5-anhydro-D-galactitol tetraacetate were lyophilized, and deacetylation was carried out with 5% Et_3N in MeOH during 12 h.

Commercial 2-deoxygalactose was used after additional purification by HPLC chromatography on a column of ODS 120T in water with refractometric detection.

References

[1] Y. Nagao, T. Nakada, M. Imoto, T. Shimamoto, S. Sakai, M. Tsuda, and T. Tsuchiya, *Biochem. Biophys. Res. Commun.*, 151 (1988) 236–241.
[2] J. Aduse-Opoku, L. Tao, J.J. Ferretti, and R. R. Russell, *J. Gen. Microbiol.*, 137 (1991) 757–764.
[3] P.S. Lazo, A.G. Ochoa, and S. Gascon, *Eur. J.Biochem.*, 77 (1977) 375–382.
[4] K.N. Neustroev, A.S. Krylov, O.N. Abroskina, L.M. Firsov, V.V. Nasonov, and A.Y. Khorlin, *Biokhimiya*, 56 (1991) 447–457.
[5] H. Carchon and C.K. De Bruyne, *Carbohydr.Res.*, 41 (1975) 175–189.
[6] J.E. Porter and M.R. Ladisch, *Biotechnol. Bioeng.*, 37 (1991) 356–363.
[7] A.M. Golubev and K.N. Neustroev, *J. Mol. Biol.*, 231 (1993) 933–934.
[8] A.M. Kachurin, K.N. Neustroev, A.M. Golubev, and F.M Ibatullin, *Biokhimiya*, 58 (1993) 550–561.
[9] A.M. Kachurin, A.M. Golubev, M.M. Geisow, O.S. Veselkina, L.S. Isaeva-Ivanova, and K.N. Neustroev, *Biochem. J.*, 308 (1995) 955–964.
[10] D.F. Bishop and R.J. Desnick, *J. Biol. Chem.*, 256 (1981) 1307–1316.
[11] I. de la Mata, P. Estrada, R. Macarron, J.M. Dominguez, M.P.Castillon, and C. Acebal, *Biochem. J.*, 283 (1992) 679–682.
[12] G. Legler and M. Herrchen, *Carbohydr. Res.*, 116 (1982) 95–103.
[13] Y. Ichikawa, G.C. Look, and C.H. Wong, *Anal. Biochem.*, 202 (1992) 215–238.
[14] K.G.I. Nilsson, *Carbohydr. Res.*, 167 (1987) 95–103.
[15] O.P. Bahl and K.M. Agrawal, *J. Biol. Chem.*, 244 (1969) 2970–2978.
[16] M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
[17] J.B. Lloyd and W.J. Whelan, *Anal. Biochem.*, 30 (1969) 467–470.
[18] R.K. Ness, H.G. Fletcher, Jr., and C.S. Hudson, *J. Am. Chem. Soc.*, 72 (1950) 4547–4549.