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Comparative study of new α-galactosidases in transglycosylation reactions

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Abstract

We have studied the potential of several newly cloned α-galactosidases to catalyze the regioselective synthesis of disaccharides using 4-nitrophenylgalactoside as a donor. The kinetics of the reactions were followed by in situ NMR spectroscopy. The following thermophilic enzymes have been tested: Aga A and an isoenzyme Aga B obtained from the strain KVE39 and Aga 285 from the strain IT285 of *Bacillus stearothermophilus*; Aga T is an α-galactosidase from *Thermus brockianus* (strain IT360). Two other non-thermophilic α-galactosidases have also been evaluated: Aga 1 (*Streptococcus mutans*, strain Ingbritt) and Raf A (*Escherichia coli*, strain D1021). For all of the enzymes studied, high regioselectivity was observed leading to two (1 → 6)-disaccharides: 4-nitrophenyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside and methyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside, which were obtained in 54% (Aga B) and 20% (Aga T) yields, respectively. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Thermophilic α-galactosidases; Transglycosylation; Disaccharides

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1. Introduction

The regioselective synthesis of the glycosidic bond may be achieved by means of the standard chemical approach [1], which usually requires cumbersome and sophisticated multistep protection–deprotection sequences. The enzymatic synthesis of the saccharides has become a very powerful alternative for this purpose [2]. Thus, glycosidases cannot only catalyze the formation of the glycosidic linkage via the reverse reaction, but also via their transferase activity. Due to their stability and low cost, these enzymes are very attractive and they also induce a high stereoselectivity [3–6]. The main drawback comes from their rather low regioselectivity and the low yields usually obtained since the transferase activity remains in competition with the hydrolysis of the substrate and of the synthesized glycosides. To improve the yield of the transglycosylation reaction and the regioselectivity, suitable experimental conditions and/or new enzymatic activities must be found. Thus, high yields are usually obtained when using a donor bearing a good leaving group at the anomeric position. Nitrophenylglycosides are the most widely employed for this purpose leading, for instance, to the synthesis of blood determinant

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di- and trisaccharides [7–12]. We have also shown that vinyl β-galactoside is a good substrate for such reactions, leading in ice to very large amounts of disaccharide [13]. In a very elegant strategy, Withers et al. [14] have obtained very high yields for transglycosylation products when using fluoro α-D-galactoside as a donor and glycosidase mutants devoid of one of the carboxylates involved in the catalytic activity. The question of regioselectivity can be partially solved with the aid of some glycosidase enzymes, which are known to be highly selective. Good examples are the β-galactosidases from *Bacillus circulans* [8,15] or from *Bacillus singularis* [12], which allow the regioselective synthesis of β-(1 → 4)-disaccharides.

Many inexpensive β-galactosidases are commercially available, but this is not the case for α-galactosidases which are usually much more expensive. Up to now, the most frequently employed α-galactosidase has been extracted from green coffee beans. Recently, an α-galactosidase from *Penicillium multicolor* has been reported to catalyze efficiently highly regioselective transglycosylation reactions [16]. Thus, various products containing the α-D-Gal*p*-(1 → 3)-D-Gal*p* moiety have been synthesized in good yield by means of the transgalactosylation activity of α-galactosidases.

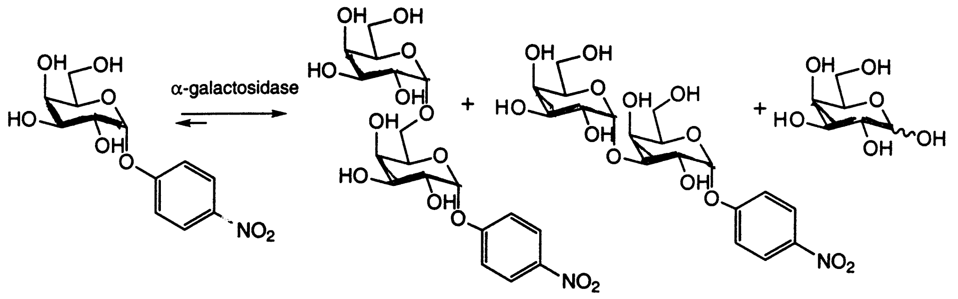
In order to provide new and low cost biocatalysts for the synthesis of glycosidic bonds, we have investigated the potential of α-galactosidases from different thermophilic species. These enzymes have been cloned and overexpressed in *Escherichia coli* [16–20,22,23]. The great advantage of such glycosidases is that the whole cells extracts of the host microorganism can be used as a catalyst. Heating the cells inactivates the Mel A α-galactosidases of *E. coli*, while the cloned thermophilic α-galactosidase remains intact thus providing the desired catalyst.

2. Results and discussion

The course of the reactions catalyzed by the α-galactosidases was monitored by means of in situ proton NMR spectroscopy [24]. This technique has the advantage of giving signals where intensities are directly proportional to the concentrations of the substances. The resonance region between 4.0 and 6.0 ppm, despite being rather narrow, allows a clear separation (at 500 MHz) of the anomeric protons of the saccharides present in the reaction medium and the spectrum resolution is quite good although the enzyme is present in the solutions. However, when carried out in the NMR tube, the course of the enzymatic reaction has to be studied at a very high D_2O/H_2O ratio since the residual water protons absorb at 4.6–4.8 ppm. We have already shown that no significant isotopic effect affected the enzymatic activities [24].

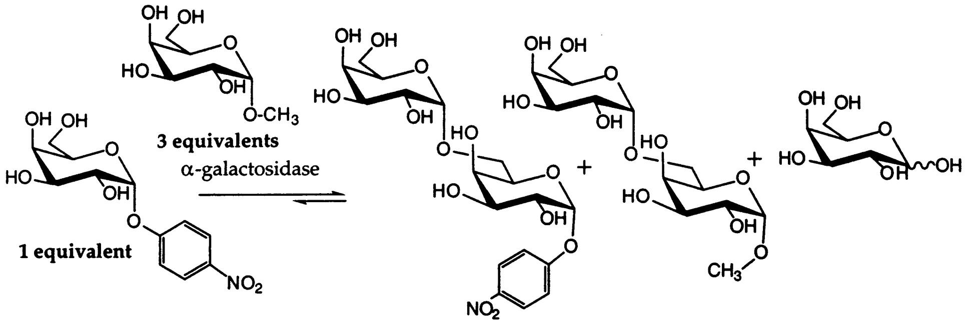
The behavior of six α-galactosidases was studied. Four of them named Aga A, Aga B (an isoenzyme of the former), Aga 285 and Aga T are thermophilic enzymes. The first three are obtained from the strains KVE39 [17,18] and IT285, respectively, of *Bacillus stearothermophilus*, while the Aga T is produced from *Thermus brockianus* (strain ITI360) [19]. All of these enzymes have been cloned and characterized [16–20,22,23]. Two other non thermophilic enzymes were also tested: Aga 1 and Raf A. The first one was produced from the strain Ingbritt of *Streptococcus mutans* [22] and the second one from the strain D1021 of *E. coli* [23].

The transglycosylation reaction induced by a glycosidase is often described as a reaction between an activated monosaccharide the donor (*p*-nitrophenylglycoside) and an acceptor. The acceptor is a molecule bearing at least one hydroxy group and may be an aliphatic alcohol, an hydroxyaminoacid (serine, threonine and peptide derivatives) or a saccharide. Since this reaction remains in competition with the hydrolysis of the donor and of the transglycosylated products, an excess of the acceptor relative to the donor is commonly used. Sometimes, when the acceptor is a rare and/or an expensive molecule, the donor is employed in excess over the acceptor. For instance, the synthesis of monogalactosylated cyclodextrins needs an eightfold excess of melibiose compared with cyclodextrin [25]. Obviously the yield of the reaction is calculated by using the less abundant reactant, the acceptor in this case. Such a yield does not



1 : major disaccharide **2** : minor disaccharide

Scheme 1. Transglycosylation reaction catalyzed by glycosylhydrolases: the autocondensation reaction.



1: major autocondensation disaccharide **3** : major condensation disaccharide

Scheme 2. Transglycosylation reaction catalyzed by glycosylhydrolases: the condensation reaction.

give any correct idea of the capacity of the enzyme to catalyze an efficient transglycosylation reaction because an important reaction is not considered: the autocondensation reaction. In fact, the donor can also act as an acceptor and the result is a competition between two transglycosylation reactions: the autocondensation and the condensation with the acceptor (Schemes 1 and 2). As a consequence, the first part of this work is devoted to the study of the transglycosylation autocondensation reaction. The second part deals with the condensation reactions in the presence of two acceptors: the methyl α- and β-galactopyranosides. All the enzymes studied are able to induce the transglycosylation reaction leading to the synthesis of α-(1 → 6)- and α-(1 → 3)-disaccharides, the former being largely predominant. Small amounts of other disaccharides were also present.

Autocondensation reactions.—The kinetics of the formation of the 4-nitrophenyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside (**1**), the main product resulting from the transglycosylation reaction with 4-nitrophenyl α-D-galactoside is shown in Fig. 1. With all the enzymes used, the minor disaccharide was 4-nitrophenyl α-D-galactopyranosyl-(1 → 3)-α-D-galactopyranoside (**2**) (see Table 1). The

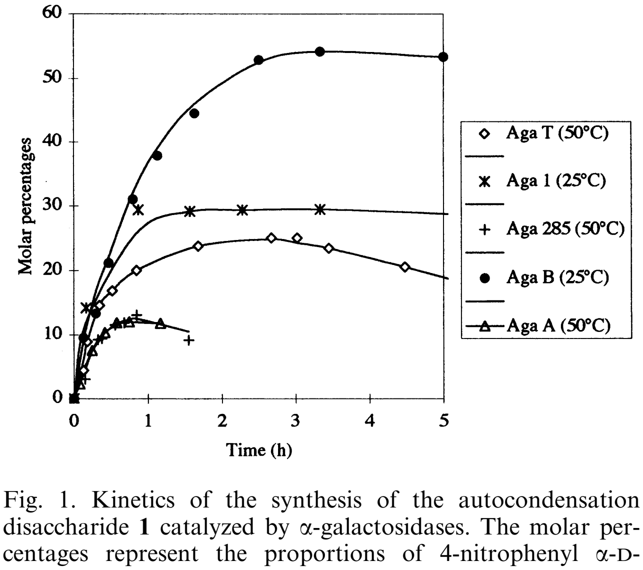


Fig. 1. Kinetics of the synthesis of the autocondensation disaccharide **1** catalyzed by α-galactosidases. The molar percentages represent the proportions of 4-nitrophenyl α-D-galactopyranoside which have reacted to form **1** and thus are also the yields for the formation of this compound. For each enzyme the percentages of conversion were 97% (Aga B), 90% (Aga A), 51% (Aga 1), 99% (Aga 285) and 88% (Aga T).

Table 1
Molar percentages (yields) obtained for each compound in the autocondensation of 4-nitrophenyl α-D-galactopyranoside (pNPGal) catalyzed by α-galactosidases

Glycosidase	Time (h)	**1** (%)	**2** (%)	Other (%)	pNPGal (%)
Aga B (25 °C)	3.2 [a]	53	4		12
	5 [b]	53	4		3
Aga B (50 °C)	2 [a]	49	3		34
	5 [b]	49	3		29
Aga A (50 °C)	0.6 [a]	12	7	3	22
	1.2 [b]	12	9	4	10
Aga 1 (25 °C)	2.5 [a]	29	2		48
	6.5 [b]	28	3		48
Aga 285 (50 °C)	0.7 [a]	12	8	<1	26
	1.6 [b]	9	9	<1	1
Aga T (25 °C)	12 [b]	17	2	2	48
Aga T (50 °C)	2.7 [a]	25	4	9	22
	6.45 [b]	15	8	12	7

[a] Time at which the maximum yield of the major disaccharide **1** was obtained.

[b] Time at which the highest concentration of 4-nitrophenyl α-D-galactopyranoside has been consumed.

molar percentages used in this work, calculated from the integrations of the NMR signals of the various anomeric protons, represent the proportions of the 4-nitrophenyl α-D-galactoside which have reacted to produce a given species. Since the synthesis of one equivalent of **1** or **2** needs two equivalents of the donor, the concentrations of such disaccharides are half of the molar percentage values (or yields). The transglycosylation ability of each enzyme can easily be compared by means of Fig. 1.

The behavior of Aga B is of particular interest since more than 50% of the 4-nitrophenyl α-D-galactoside is transformed to produce **1**. This result is in contradiction with that obtained in the presence of the isoenzyme Aga A. In this case, only small amounts of **1** and **2** were synthesized, while large quantities of galactose were formed. This fact is not surprising since Aga B displays a very high K_M value (~ 130 mM) for melibiose, while the corresponding K_M value for Aga A is 3.3 mM [17,18]. Therefore, it is likely that disaccharide **1** is hydrolyzed much more faster by Aga A than by Aga B. In order to verify this hypothesis, we have studied the hydrolysis of **1** with both enzymes. In such reactions only tiny amounts of transglycosylated products were synthesized while the hydrolysis took place. For instance, the presence of 4-nitrophenyl α-D-galactoside was undetectable on the NMR spectra showing that the hydrolysis of the latter was faster than the hydrolysis of disaccharide **1**. Thus, after 1 h of incubation at 50 °C, 60 and 15% of **1** were, respectively, hydrolyzed in the presence of Aga A and Aga B. Fig. 2 compares the rate of consumption of 4-nitrophenyl α-D-galactoside with these two enzymes: Aga A is known to exhibit the maximum activity at 65 °C and this enzyme still remains stable at this temperature [17]. Thus, Aga A is very active and stable at 50 °C. Conversely, Aga B has a limited stability at

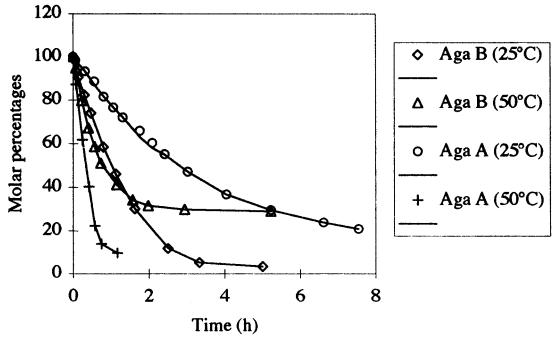


Fig. 2. Evolution of the concentration of 4-nitrophenyl α-D-galactoside at 25 and 50 °C in the reactions catalyzed by Aga A and Aga B enzymes.

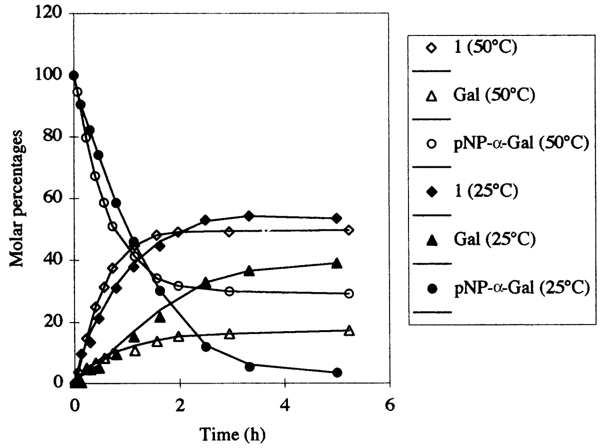


Fig. 3. Evolution of the molar percentages of the main components in the autocondensation reaction of 4-nitrophenyl α-D-galactopyranoside (pNPGal) catalyzed by the enzyme Aga B at 25 and 50 °C. Gal = α- + β-galactose, **1** = 4-nitrophenyl (α-D-galactopyranosyl)-(1 → 6)-α-D-galactopyranoside.

this temperature since the reaction stops after 2 h at about 70% conversion. Furthermore, the kinetics curves obtained at 25 and 50 °C for Aga B look rather similar at the beginning of the reaction (see Figs. 2 and 3) thus showing that the effect of the higher temperature is balanced by a loss in enzyme stability. Since the formation of **1** is faster than its hydrolysis and of the hydrolysis of 4-nitrophenyl α-D-galactoside, the time during which Aga B remains stable at 50 °C is sufficient to synthesize large amounts of **1**, but the enzymatic activity which decreases with time produces lower percentages of hydrolysis of the disaccharide **1** at 50 °C than at 25 °C. This property can be used for the synthesis of **1**: at 25 °C, a yield of about 50% was obtained while 15% of 4-nitrophenyl α-D-galactoside was remained, at 50 °C the same yield was reached but 34% of the reactant was still present. Considering the possibility of recycling the unreacted 4-nitrophenyl α-D-galactoside, the real yields for **1** are 60% at 25 °C and 75% at 50 °C.

The courses of the reactions catalyzed by Aga 285 were quite similar to those provided by Aga A (see Fig. 1). Considering the transglycosidase activity, the enzymes Aga 1 and Aga T occupy an intermediate position between Aga A and Aga B since they were able to produce 20–25% of the disaccharide **1**. The activity of Aga 1 seems to be inhibited by either galactose or disaccharide **1** since the reaction at 25 °C stopped at 52% conversion. The galactosidase Aga T (as well as Aga A) certainly exhibits a high transglycosylation activity, but the relatively low yield obtained in this case is explained by the high rate of hydrolysis of the disaccharide **1** (data not shown). Table 1 summarizes the results obtained with the different glycosidases and shows that the enzyme Aga B exhibits a high potential to perform the synthesis of disaccharide **1** with a yield of up to 50% (calculated from the initial 4-nitrophenyl α-D-galactoside).

Condensation reactions with methyl α-D-galactopyranoside and methyl β-D-galactopyranoside.—The condensation reactions in the presence of either methyl α-galactopyranoside or methyl β-D-galactopyranoside produced mainly two disaccharides: 4-nitrophenyl (α-D-galactopyranosyl)-(1 → 6)-α-D-galactopyranoside (**1**) and methyl (α-D-galactopyranosyl)-(1 → 6)-α-D-galactopyranoside (**3**) [or methyl (α-D-galactopyranosyl)-(1 → 6)-β-D-galactopyranoside (**4**)). In that case also, minor quantities of the corresponding (1 → 3)-disaccharides and traces of other compounds were also present (Scheme 2).

Considering the results obtained for the autocondensation reactions, we have only studied the behavior of α-galactosidases able to synthesize disaccharide **1** with good yields. Thus, Fig. 4 shows the kinetics of formation of compounds **1** and **3** for the enzymes Aga B, Aga T, Aga 1 and Raf A. The particular behavior of Aga B is once more clearly exemplified: the large preference for the 4-nitrophenyl α-D-galactopyranoside as an acceptor induced the formation of large quantities of **1** at the expense of the condensation disaccharide **3**, even when a threefold amount of methyl α-D-galactopyranoside was used. A similar trend is also observed with the α-galactosidase Aga 1. In this case, we have stopped the reaction at 55% conversion of the 4-nitrophenyl α-D-galactopyranoside because the hydrolysis of the disaccharide **3** took place very soon.

More interesting is the high transglycosylation potential of the enzyme Aga T, which produced more condensation product **3** than the autocondensation did **1**. This was also true when the acceptor methyl α-D-galactopyranoside was replaced by the β anomer. In that case, good yields of the methyl (α-D-galactopyranosyl-(1 → 6)-β-D-galactopyranoside (**4**) have also been obtained (see Fig. 5). The enzyme Raf A produced similar amounts of the two disaccharides, but the enzymatic activity seemed to be inhibited because the reaction stopped after 1.5 h for a conversion close to 75%.

In conclusion, we have shown that new cloned α-galactosidases are powerful and inexpensive tools for the synthesis of disaccharides which are difficult to obtain via classical organic synthesis methodologies. Thus, the enzyme Aga B was shown to produce very efficiently 4-nitrophenyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside **1** with high yield (see Section 3). This enzyme, which recognizes strongly the 4-nitrophenyl α-D-galactoside as an acceptor, was not able to perform the transglycosylation reaction in good yields with other acceptors. This goal was reached when using the thermophilic α-galactosidase Aga T. Despite the competition with the autocondensation reaction, this enzyme provided yields of 20% for the synthesis of methyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside (**3**) or methyl α-D-galactopyranosyl-(1 → 6)-β-D-galactopyranoside (**4**) (see Section 3).

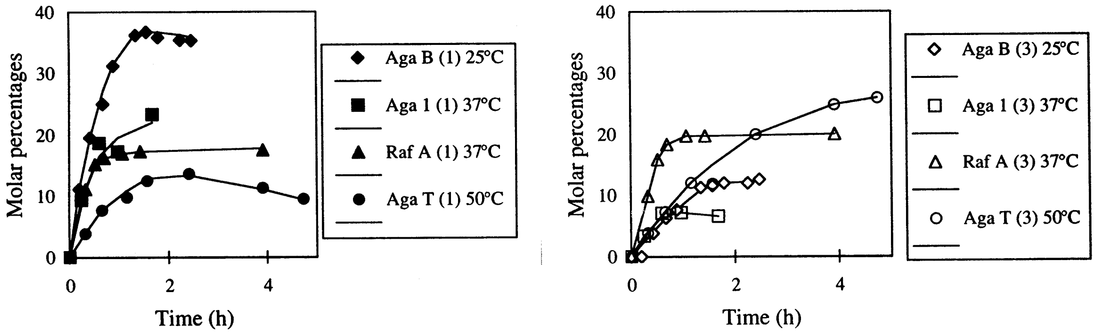


Fig. 4. Kinetics of formation of the major (1 → 6)-disaccharides **1** (resulting from the autocondensation reaction, left curves) and **3** (resulting from the condensation with methyl α-D-galactopyranoside, right curves) in the presence of α-galactosidases. Comment for the legend: Aga B(3) 25 °C for instance denotes the evolution of the molar percentage of the disaccharide **3** at 25 °C in the presence of the enzyme Aga B.

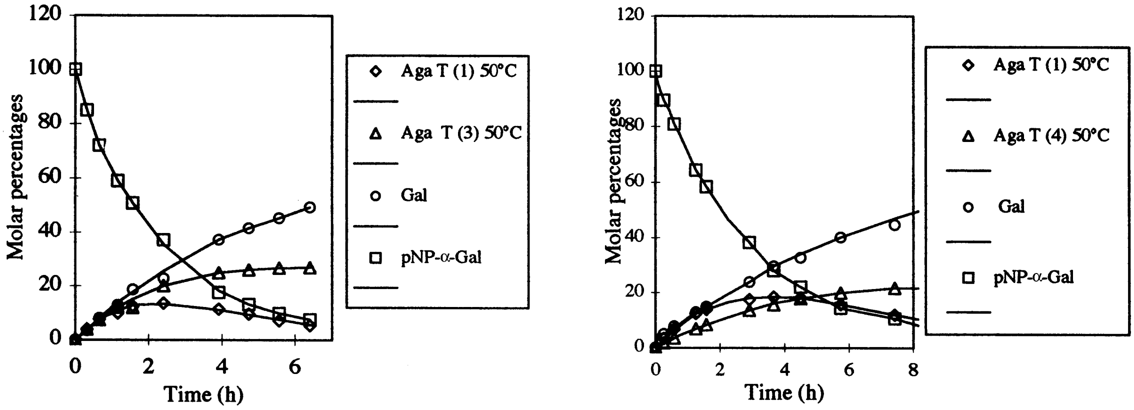


Fig. 5. Left: kinetics of formation of the major (1 → 6)-disaccharides **1** (resulting from the autocondensation reaction) and **3** resulting from the condensation with methyl α-galactopyranoside in the presence of α-galactosidase Aga T at 50 °C. Right: same study in the presence of methyl β-D-galactopyranoside (**4** = methyl (α-D-galactopyranosyl)-(1 → 6)-β-D-galactopyranoside). Comment for the legend: Aga T(3) 50 °C for instance denotes the evolution of the molar percentages of the disaccharide **3** at 50 °C in the presence of the enzyme Aga T.

3. Experimental

General procedures.—The α-galactosidase from green coffee beans was purchased from Sigma. The chemicals supplied by Sigma were used without further purification. The enzymatic preparation of α-galactosidases Aga A (from *B. stearothermophilus*), Aga B (from *B. stearothermophilus*), Aga 285 (from *B. stearothermophilus*), Aga T (from *Thermus* ssp.), Aga 1 (from *S. mutans*) and Raf A (from *E. coli*) were supplied by Professor R. Mattes (Institut for Industrial Genetics, University of Stuttgart, Germany). These enzymatic activities have been prepared according to Refs. [17–20]. The deuterium oxide was purchased from Eurisotop (isotopic purity 99.9%). The course of the reactions was followed by means of thin-layer chromatography (TLC) (precoated Silica Gel 60 sheets E. Merck F254) and by ^{1}H NMR spectroscopy at 500 MHz (Bruker AX500 spectrometer). The components of the reaction mixtures of autocondensation reactions were separated on silica gel columns (Seymour eluent: 60:30:3:5 MeOH–$CHCl_3$–AcOH–water) and the components of condensation reactions on 1:1 charcoal (Darco G-60, 100 mesh, Aldrich)–Celite (Fluka 535) columns (eluent: 5–25, EtOH–water). The complete analysis of the NMR ^{1}H and ^{13}C resonances and the subsequent structure assignment were made using standard 2D sequences (COSY HH and HCOOR correlations) and by comparison with previously published data [9,17,21,26]. The spectra were recorded with a Bruker AX500 spectrometer operating at 500 MHz for ^{1}H (solvent D_2O, chemical shifts in ppm quoted from the resonance of (trimethylsilyl)-3-propansulfonic acid at 0.0 ppm) and 126 MHz for ^{13}C (solvent D_2O, chemical shifts in ppm quoted from the methyl acetone resonance at 29.8 ppm).

Kinetic study of the autocondensation reaction of 4-nitrophenyl α-D-galactopyranoside catalyzed by α-galactosidases.—On one hand, the amount of salts necessary for the preparation of 0.78 mL of a mixture of phosphate buffer (0.1 M, pH 7.0) and the silylated reference, [3-(trimethylsilyl)-propanesulfonic acid sodium salt, 10 mmol/L] were lyophilized, dissolved in D_2O and lyophilized once more. On the other hand, 21 mg (70 μmol) of 4-nitrophenyl α-D-galactoside was prepared using the same procedure. Then, the α-galactosidase preparation was dried in a dessicator containing P_2O_5. The lyophilized buffer reference mixture was redissolved in 0.78 mL of D_2O and adjusted to pD 7.0. A part of this solution (0.6 mL) was used to dissolve the substrate. The resulting solution was immediately filtered in a NMR tube and warmed up to the desired temperature reaction. The dried α-galactosidase was redissolved in the remaining buffer reference mixture (0.18 mL) prior the addition to reactants in the NMR tube. The reaction was allowed to proceed in the magnet of the spectrometer pre-adjusted to the temperature of the reaction. Using this procedure, it was possible to perform the first measurements about 4 min after the introduction of the enzyme in the NMR tube.

Kinetic study of the condensation reaction of 4-nitrophenyl α-D-galactoside with methyl α-D-galactopyranoside or methyl β-D-galactopyranoside catalyzed by the α-galactosidases.—The procedure and the amounts of all the components were identical to those used in the above reactions apart from the presence of the methylgalactoside (3 equiv for 1 of 4-nitrophenyl α-D-galactopyranoside).

Kinetic study of the hydrolysis of disaccharide **1** *catalyzed by α-galactosidases*.—A solution of phosphate buffer (0.1 M, pH 7.0) containing 10 mM (trimethylsilyl)-3-propanesulfonic acid was prepared and adjusted to pD 7. Disaccharide **1** (14 μmol) was dissolved in 500 μL of the phosphate buffer–reference mixture and immediately filtered in a NMR tube. The solution was warmed up to the desired reaction temperature before plotting a NMR spectrum (without the enzyme preparation). The α-galactosidases preparation (1.75 U) were dried in a dessicator containing P_2O_5 and redissolved in 56 μL of the phosphate buffer–reference mixture and then added to the reactants solution. The reaction was then allowed to proceed in the magnet of the thermostated spectrometer.

4-Nitrophenyl α-D-galactopyranosyl-(1→6)-α-D-galactopyranoside (**1**).—To 4-nitrophenyl α-D-galactopyranoside (900 mg, 3 mmol) in phosphate buffer (0.1 M, pH 7.0, 33.33 mL) was added at 25 °C α-galactosidase Aga B from *B. stearothermophilus* (105 U calculated from the hydrolysis of 4-nitrophenyl α-D-galactopyranoside at 25 °C) and the solution was incubated at 25 °C. After 150 min of incubation, the reaction was quenched by adding 500 μL of 1 M NaOH. Silica gel 60 (E. Merck F254, 2 g) was added under stirring to the resulting solution and the water was removed under reduced pressure. The powder obtained was deposited at the head of the silica gel column (3.5 cm diameter, 80 g of silica gel) and the products were allowed to elute (Seymour eluent: 60:30:3:5 $CHCl_3$–MeOH–water–AcOH). The course of the purification was followed by TLC (pre-coated Silica Gel 60 plates, E. Merck F254). 4-Nitrophenol and 4-nitrophenyl α-D-galactopyranoside were eluted first, then the disaccharide **2** (23 mg), the disaccharide **1** (375 mg; $R_f = 0.63$ with the eluent above, overall yield: 54%) and then the galactose were obtained (118 mg).

^{1}H NMR (D_2O) δ 3.14 (dd, $J_{1',2'}$ 3.4, $J_{2',3'}$ 10.2 Hz, H-2′), 3.46 (m, H-5′), 3.49 (dd, $J_{2'-3'}$ 10.2, $J_{3',4'}$ 4.4 Hz, H-3′), 3.53 (dd, $J_{4',5'}$ 3.0, $J_{3',4'}$ 4.4 Hz, H-4′), 3.64 (m, 2 H, H-6′), 3.65 (m, H-5), 3.69 (dd, $J_{3,4}$ 3.4, $J_{2,3}$ 10.2 Hz, H-3), 3.89 (dd, $J_{2,3}$ 10.2, $J_{1,2}$ 3.9 Hz, H-2), 3.94 (m, H-6b), 3.96 (m, H-6a), 3.98 (d, $J_{3,4}$ 3.4 Hz, H-4), 4.65 (d, $J_{1',2'}$ 3.4 Hz, H-1′), 5.75 (d, $J_{1,2}$ 3.9 Hz, H-1). ^{13}C NMR (D_2O) $\delta = 60.5$ (C-6′), 68.0 (C-4), 67.7 (C-4′), 68.0 (C-4), 69.0 (C-6), 69.2 (C-2), 69.3 (C-2′), 70.7 (C-3), 71.8 (C-3′), 72.7 (C-5′), 75.1 (C-5), 96.3 (C-1), 97.6 (C-1′).

Anal. Calcd for $C_{18}H_{25}NO_{13}$ (463): C, 46.65; H, 5.40; N, 3.02. Found: C, 46.61; H, 5.35; N, 3.11.

Methyl α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranoside (**3**).—To a solution of 4-nitrophenyl α-D-galactopyranoside (602 mg, 2 mmol) and methyl α-D-galactopyranoside (1165 mg, 6 mmol) in phosphate buffer (0.1 M, pH 7.0, 22 mL) was added 70 U of the enzymatic preparation of α-galactosidase Aga T (calculated from the hydrolysis of 4-nitrophenyl α-D-galactoside at 25 °C). The solution was incubated at 25 °C for 7 h and the reaction was quenched by adding 330 μL of 1 M NaOH. The water was removed under reduced pressure and the compounds were separated on a charcoal–celite column. The eluent was composed of a mixture of EtOH and water (an EtOH gradient was used). In such conditions all the 4-nitrophenylsaccharides were retained. The first fractions, eluted with a 5% EtOH/water mixture, contained the galactose (135 mg), while the second ones were composed of methyl-α-D-galactopyranoside (1040 mg).The disaccharides were allowed to elute with a 10% EtOH–water solution. Small amounts of α-Gal-(1 → 3)-α-Gal-O-Me came first ($R_f = 0.39$, Seymour eluent). Further elution provided 143 mg of the disaccharide **3** ($R_f = 0.33$, Seymour eluent, yield 20%).

^{1}H NMR (D_2O) δ 3.28 (s, 3 H, OCH_3), 3.61 (m, 1 H, H-6b), 3.72 (m, 2 H, H-6′a and H-6b′), 3.76 (m, 4 H, H-2, H-2′, H-3, H-3′), 3.79 (m, 1 H, H-6a), 3.84 (dd, H-4′), 3.86 (ddd, H-5′), 3.89 (dd, H-4), 3.95 (H-5), 4.70 (d, H-1), 4.84 (d, H-1′). ^{13}C NMR (D_2O): δ 54.7 (OCH_3), 58.4 (C 6′), 58.5 (C-6), 63.7 (C-3), 65.5 (C-4), 65.8 (C-2), 66.5 (C-2′), 66.7 (C-3′ and C-4′), 68.0 (C-5), 68.3 (C-5′), 96.7 (C-1), 95.5 (C-1′). Anal. Calcd. for $C_{13}H_{24}O_{11}$ (356): C, 43.82; H, 6.74. Found: C, 43.73; H, 6.82.

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