Biotechnology Letters **23:** 1575–1581, 2001.

Transglycosylation reactions by exoglycosidases from the termite *Macrotermes subhyalinus*

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Received 1 June 2001; Revisions requested 2 July 2001; Revisions received 23 July 2001; Accepted 24 July 2001

Key words: β-galactosidase, α-glucosidase, β-glucosidase, invertase, *Macrotermes subhyalinus*, termites, transglycosylation

Abstract

The ability of four exoglycosidases (β-galactosidase, β-glucosidase, α-glucosidase and invertase) from the termite *Macrotermes subhyalinus* to catalyse tranglycosylation reactions was tested using lactose, cellobiose, maltose and sucrose as glycosyl donors and 2-phenylethanol as glycosyl acceptor. The experimental conditions were optimized in relation to the time course of the reaction, pH and concentrations of glycosyl donor and acceptor. Whereas the hydrolytic activity was largely predominant over the transferase activity with β-galactosidase and β-glucosidase, the transglycosylation activity represented 68% with α-glucosidase. In addition, as demonstrated by the transglycosylation product formed, the hydrolysis of sucrose was catalysed by α-glucosidase and not by invertase. On the basis of this work, α-glucosidase from *M. subhyalinus* appears to be a valuable tool for the preparation of neoglycoconjugates.

Introduction

The study of the biological systems mediated by glycoconjugates needs efficient methods for the synthesis of carbohydrates. Because the chemical synthetic procedures require tedious protection-deprotection multisteps, the enzymatic approach has become more and more valued (Toone *et al.* 1989, Cote & Tao 1990, Ichikawa *et al.* 1992). Among the enzymes used, glycosidases are often chosen because, in addition to reversible hydrolysis, they catalyse reactions of glycosyl transfer to acceptors other than water. These enzymes are easy to purify, generally possess a good thermostability and need only cheap substrates.

Although the potential of glycosidases is now largely exploited for the *in vitro* synthesis of various glycoconjugates (Leparoux *et al.* 1996, Singh *et al.* 1996, Vetere *et al.* 1997, Zeng *et al.* 2000), moderate yields are always obtained in aqueous medium since the glycosidase-catalysed reactions are always shifted in the direction of hydrolysis. To improve the transglycosylation activity of glycosidases, experimental devices can be envisaged like, for example, aqueous-organic reaction systems (Finch & Yoon 1997, Becker & Kuhl 1999) associated with high concentrations of glycosyl acceptor other than water (Vulfson *et al.* 1990). Such conditions cannot be considered in the context of our project since the final objective is the *in vitro* glycosylation of peptides which have necessarily a limited number of glycosyl acceptor (hydroxyl) groups. Under conditions of kinetically controlled transglycosylation, the yields are also largely dependent on the enzyme nature and origin (Leparoux *et al.* 1994, Yoon & Ajisaka 1996). This is why the search for new sources of glycosidases with high transglycosylation activities appears to be of great interest.

Termites are known to be very rich in digestive glycosidases but most of the work on these enzymes was undertaken in order to specify the termite nutrition mode and to elucidate the respective roles played by

termites and symbiotic microorganisms in the degradation of plant carbohydrates (Rouland 1986, Veivers *et al.* 1991, Matoub 1993). In the present report, glycosidases from the higher termite *Macrotermes subhyalinus* were studied in order to investigate their potential as a source of transglycosylation enzymes.

Materials and methods

Enzymatic source and preparation of crude extracts

The termites, *Macrotermes subhyalinus*, were collected to Lamto (Ivory Coast) directly from the nest, then stored at −20 °C.

After thawing, the termites (20 g) were washed with distilled water, then harvested by centrifugation and resuspended in 100 ml 0.9% (w/v) NaCl solution. After disruption of termites in an Ultra-Turrax type T25, the solution was centrifuged at 15 000 *g* for 15 min at 4 °C. The obtained supernatant constituted the crude extract.

Chemicals

Glucose oxidase, peroxidase, 2-phenylethanol, synthetic substrates: phenylethyl-β-D-galactopyranoside (PEGal), *p*-nitrophenyl-β-D-galactopyranoside (PNPβGal), *p*-nitrophenyl-β-D-glucopyranoside (PNPβGlc), *p*-nitrophenyl-β-D-fucopyranoside (PNPβFuc), *p*-nitrophenyl-β-D-mannopyranoside (PNPβMan), *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (PNPβGalNAc), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (PNPβGlcNAc), *p*-nitrophenyl-β-D-xylopyranoside (PNPβXyl)), *p*-nitrophenyl-α-D-galactopyranoside (PNPαGal), *p*-nitrophenyl-α-D-glucopyranoside (PNPαGlc), *p*-nitrophenyl-α-D-mannopyranoside (PNPαMan), *p*-nitrophenyl-α-L-arabinopyranoside (PNPαAra), *p*-nitrophenyl-α-D-fucopyranoside (PNPαFuc) 6-bromo-2-naphtyl-β-D-glucopyranoside (BNβGlc), 6-bromo-2-naphtyl-α-D-glucopyranoside (BNαGlc) and natural substrates: lactose, cellobiose, gentiobiose, maltose, melibiose and sucrose were purchased from Sigma-Aldrich. All other chemicals and reagents were of analytical grade.

Enzyme assays

Glycosidase activities towards *p*-nitrophenylglycosides were determined at 37 °C for 10 min in reaction mixtures (1 ml) containing 0.1 M sodium acetate buffer (pH 5.4), 5 mM synthetic substrate and 50 μl enzymatic preparation. The reactions were stopped by adding 2 ml of 1 M Na_2CO_3. The liberation of *p*-nitrophenoxide anion (PNP) was quantified at 420 nm. The calibration curves (PNP) were carried out under the same conditions of pH and temperature (Fourage *et al.* 1999).

In the case of the determination of activities towards PNPβGalNAc, PNPβGlcNAc, BNβGlc and BNαGlc, a substrate concentration of 2.5 mM was used.

Experimental conditions for the determination of activities towards natural substrates were as described above but with a concentration of di- and tri-saccharide of 10 mM. The reactions were stopped by heating at 100 °C for 5 min. The released glucose was determined by the glucose oxidase-peroxidase method (Kunst *et al.* 1984).

Protein concentration was determined by the method of Lowry using bovine serum albumin as the standard. The unit of activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 nmol of substrate per min at 37 °C under the conditions described above.

Transglycosylation reactions

In a typical experiment, the transglycosylation reaction was carried out at 37 °C in 1 ml 0.1 M sodium acetate buffer or 0.1 M sodium phosphate buffer containing an appropriate amount of the crude extract corresponding to 15 units of the considered enzymatic activity and various concentrations (10–800 mM) of glycosyl donor (sucrose, cellobiose, maltose, lactose) and of glycosyl acceptor (phenylethanol). The progress of the reaction was monitored at different times (between 1 and 130 h) by withdrawing aliquots (50 μl) which were heated at 100 °C for 5 min. After filtration through a 0.45 μm hydrophilic Durapore membrane (Millipore), the reaction mixture (20 μl) was analysed quantitatively by HPLC at room temperature. Chromotographic separations of sugars (disaccharides and products generated by their hydrolysis) were performed on a Supelcosyl LC-NH2 (5 μm) column (0.46 × 25 cm) from Supelco using acetonitrile/water (75:25, v/v) as the eluent, and monitored by refractometric detection. The flow rate was maintained at 0.75 ml min^{-1}. Phenylethanol and phenylethylglycosides were analysed on a Hypersil (5 μm) column (0.46 × 25 cm) from Shandon using methanol/water (35:65, v/v) as the eluent. Chromatographic separa-

tions were monitored at 257 nm using a constant flow rate of 0.45 ml min^{-1}.

Results

Glycosidase activities towards natural and synthetic substrates

Macrotermes subhyalinus is a termite of the subfamily Macrotermitinae. The biodegradation of plant polysaccharides by the termites is, to a great extent, due to their double symbiosis: endosymbiosis with gut microflora and exosymbiosis with a fungus from the genus *Termitomyces* (Rouland *et al.* 1990). Starch, cellulose and xylan are the most abundant polysaccharides produced by plants. Their degradation involves the synergistic activities of various endo- and exo-glycosidases. Today, most glycosidases used for synthetic purposes are exo-glycosidases (Crout & Vic 1998). Glycosyl transfer takes place only to the nonreducing terminal monosaccharide unit of substrates. This is why, although the termites contain various endoglycosidases (Rouland 1986, Veivers *et al.* 1991, Matoub 1993), the results reported in Table 1 are focused on the exoglycosidase activities using a variety of disaccharides and synthetic substrates. Considering the neuter castes, all activities were higher in termite workers than in major and minor termite soldiers. For this reason, only the crude extracts prepared from workers were used as an enzymatic source for the study of transglycosylation reactions. As seen in Table 1, the best enzymatic activities using both natural and synthetic substrates were obtained with β-galactosidase, β-glucosidase, β-fucosidase, α-glucosidase and β-fructosidase (invertase).

Transglycosylation reactions

The ability of exoglycosidases from extracts of *Macrotermes subhyalinus* to catalyse transglycosylation reactions was tested with sucrose, maltose, lactose and cellobiose as glycosyl donors and 2-phenylethanol as the glycosyl acceptor. The latter has already been used to study the transglycosylation activities of β-galactosidases from *Aspergillus oryzae* (Fortun & Colas 1991) and *Achatina achatina* (Leparoux *et al.* 1997) and β-glycosidase from *Thermus thermophilus* (Dion *et al.* 1999) owing to the easy and quantitative measurement by UV-absorbance of the transglycosylation products (phenylethylglycosides).

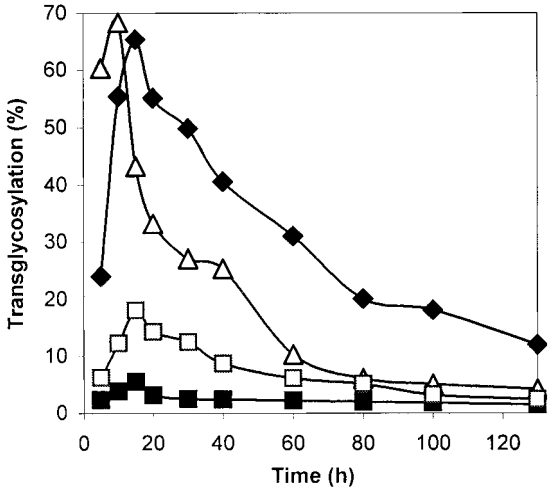

Fig. 1. Time course of 2-phenylethylglycoside synthesis by glycosidases of *M. subhyalinus* extracts. The transglycosylation activities were tested in a 1 ml reaction volume at 37 °C using 400 mM of glycosyl donor, 100 mM of phenylethanol as the glycosyl acceptor and 15 units of enzyme activity. The nature and the pH of the buffer depended on the substrate tested: 100 mM sodium acetate buffer at pH 5 for cellobiose (■) and at pH 5.5 for maltose (△); 100 mM sodium phosphate buffer at pH 6 for lactose (□) and at pH 6.5 for sucrose (♦). The transglycosylation products were identified and quantified as described in Materials and methods.

The experimental conditions were optimized in relation to those factors able to have an influence on the rate of transglycosylation. Thus, the time course of the reaction is an important parameter since the products formed during the transglycosylation reactions can be used as substrates by the enzyme and can be hydrolysed. Therefore strict control of glycosylation kinetics is necessary. The maximum yield of the phenylethylglycoside production at 37 °C depended on the substrate tested and could, in each case, be obtained whatever the enzyme concentration but for a longer or shorter time of reaction. For the glycosidases studied, an enzymatic activity corresponding to 15 units appeared to be a good compromise to reach the maximum transglycosylation rate in a relatively short time (about 10–15 h), without forward hydrolysis of the transglycosylation product (Figure 1). A smaller enzyme amount required a much longer incubation time whereas a higher concentration caused a rapid hydrolysis of the desired product.

The effect of pH on the rates of transglycosylation was studied between pH 3.5 and 10.5 (Figure 2). Maximum tranglycosylations were obtained in the range pH 5–6.5 depending on the substrate considered.

The efficiency of glycosidases from the termite *Macrotermes subhyalinus* in catalysing of transglyco-

Table 1. Glycosidase activities of *M. subhyalinus* extracts.

Enzyme	Substrate[a] (mM)	Specific activities of crude extract (U mg^{-1})		
		Worker	Minor soldier	Major soldier
β-Galactosidase	PNPβGal (5)	82	47	23
	Lactose (10)	28	20	15
β-Glucosidase	PNPβGlc (5)	640	328	302
	BNβGlc (2.5)	40	38	24
	Cellobiose (10)	334	31	19
	Gentiobiose (10)	19	18	17
β-Fucosidase	PNPβFuc (5)	733	380	297
β-Xylosidase	PNPβXyl (5)	29	25	12
β-Mannosidase	PNPβMan (5)	44	32	18
β-Fructosidase	Sucrose (5)	53	32	23
β-*N*-Acetylgalactosaminidase	PNPβGalNAc (2.5)	34	33	27
β-*N*-Acetylglucosaminidase	PNPβGlcNAc (2.5)	28	26	23
α-Galactosidase	PNPαGal (5)	65	35	11
	Melibiose (10)	30	28	23
α-Glucosidase	PNPαGlc (5)	336	174	165
	Maltose (10)	47	31	25
	BNαGlc (2.5)	40	38	24
α-Mannosidase	PNPαMan (5)	23	22	11
α-Fucosidase	PNPαFuc (5)	37	18	11
α-Arabinosidase	PNPαAra (5)	49	26	15
α-Galactosidase + α-glucosidase	Raffinose (10)	28	21	14

[a]For abbreviations, see Materials and methods.

sylation reactions was also largely dependent on the respective concentrations of glycosyl donor and glycosyl acceptor. The best yields were obtained with a concentration of around 400 mM of glycosyl donor (disaccharides) (Figure 3) and 100 mM of glycosyl acceptor (2-phenylethanol) (Figure 4).

When sucrose was used as glycosyl donor, the product formed was the phenylethyl-α-D-glucoside and not the phenylethyl-β-D-fructoside (as demonstrated by its hydrolysis by a specific α-glucosidase) indicating that the enzyme responsible for the transglycosylation reaction is an α-glucosidase and not a β-fructosidase (invertase). Table 2 shows that the α-glucosidase activity offers the best potential to catalyse transglycosylation reactions compared with the β-galactosidase and β-glucosidase activities.

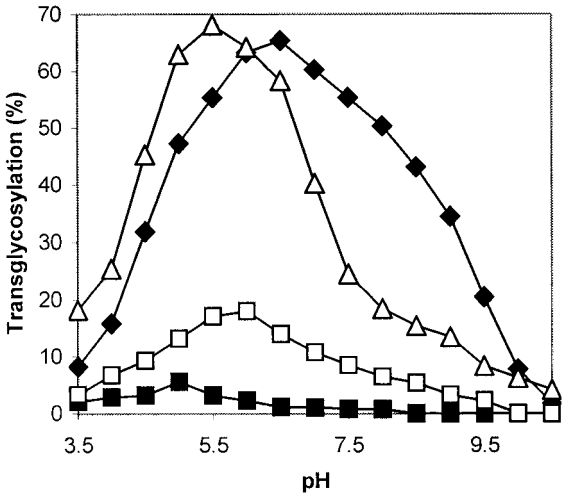

Fig. 2. Effect of pH on transglycosylation reactions catalysed by glycosidases of *M. subhyalinus* extracts. The assays were carried out in the following buffer systems: 100 mM sodium acetate buffer from pH 3.5 to 5.5, 100 mM sodium phosphate buffer from pH 6 to 8 and 100 mM glycine/NaOH buffer from pH 8.5 to 10.5. The reaction time was 15 h when sucrose (♦), cellobiose (■) or lactose (□) was used as glycosyl donor and 10 h in the case of maltose (△). The other experimental conditions were identical to those given in Figure 1.

Discussion

Glycosidases from termites have been largely studied with regard to their hydrolytic activities in order to understand the symbiotic relationship with bacte-

Table 2. Optimization of experimental conditions for transglycosylation reactions catalysed by glycosidases of *M. subhyalinus* extracts. All assays were performed at 37 °C with phenylethanol (100 mM) as glycosyl acceptor and 15 units of enzymatic activity.

Enzyme	Substrate (400 mM)	Incubation time (h)	Optimum pH	Transglycosylation (%)
β-Galactosidase	Lactose	15	6	18
β-Glucosidase	Cellobiose	15	5	6
α-Glucosidase	Maltose	10	5.5	68
	Sucrose	15	6.5	65

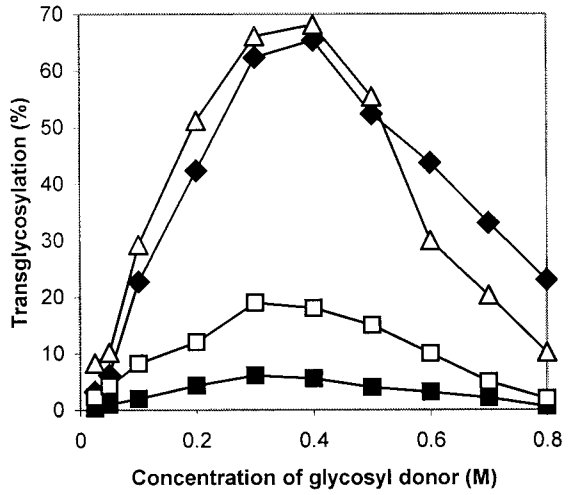

Fig. 3. Effect of the glycosyl donor concentration on transglycosylation reactions catalysed by glycosidases of *M. subhyalinus* extracts. Experimental conditions specific to each type of glycosyl donor were as follows: sucrose (pH 6.5, incubation time 15 h) (♦), cellobiose (pH 5, 15 h) (■), maltose (pH 5.5, 10 h) (△) and lactose (pH 6, 15 h) (□). For all assays: temperature: 37 °C; phenylethanol: 100 mM; enzyme: 15 units.

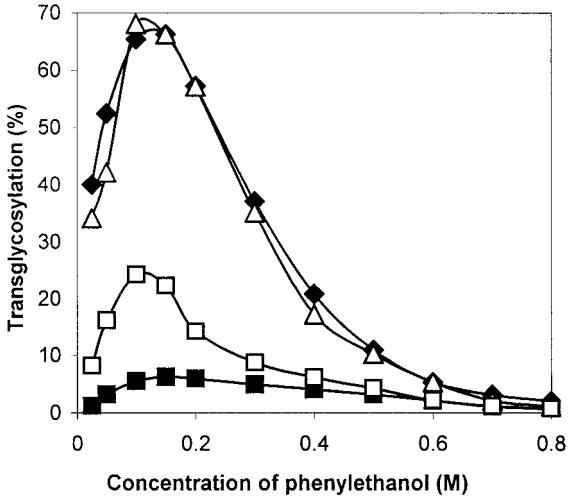

Fig. 4. Effect of the glycosyl acceptor (2-phenylethanol) concentration on transglycosylation reactions catalysed by glycosidases of *M. subhyalinus* extracts. All the glycosyl donors were at the concentration of 400 mM: sucrose (♦), cellobiose (■), maltose (△) and lactose (□). The conditions of pH, temperature, enzymatic concentration and incubation time were identical to those given in Figure 3.

ria and the fungus *Termitomyces* sp. which grows on structures (fungus comb) built by termite workers (Rouland *et al.* 1990, Matoub 1993). However, no work concerning the transglycosylation activities of these enzymes has been so far reported.

The screening of various glycosidase activities from the termite *M. subhyalinus* prompted us to study more particularly the properties of β-galactosidase, β-glucosidase, α-glucosidase and invertase from this source. The efficiency of the four enzymes in synthesizing of neoglycoconjugates was tested under kinetically controlled conditions. The synthesis relies on the trapping of a reactive intermediate, generated from an activated glycosyl donor, with exogenous nucleophiles to form a new glycosidic bond (Gijsen *et al.* 1996). The reactive intermediate must be trapped more rapidly by the glycosyl acceptor than by water. As the donor glycoside is consumed while the product is formed, the latter can become a substrate for the enzyme and be hydrolysed; for this reason the kinetics of the reaction must be monitored and stopped at the point of maximum yield (Nilsson 1988, Wong *et al.* 1995). Yields in kinetically controlled synthesis generally range from 20 to 40% (Wong *et al.* 1995). In the case of the transglycosylation reactions catalysed by *M. subhyalinus* glycosidases, the molar yields were 68%, with α-glucosidase, indicating that for this enzyme, the rate of transglycosylation product formation was largely favoured relative to the rate of hydrolysis.

α-Glucosidases (EC 3.2.1.20) are exoglycosidases which catalyse the splitting of the α-glucosyl residue from the nonreducing terminal of the substrate to liberate α-glucose. These enzymes have received widespread attention because *in vivo* their presence is essential for the degradation of lysosomal deposits. Many are known to catalyse the production of oligosaccharides *in vitro* and/or the formation of neoglycoconjugates via transfer reactions (Cote & Tao 1990). In most cases, the obtained transglycosylation products depend on the enzyme source. Thus, the mammalian α-glucosidase is able to form a very stable and nonreducing form of glucosylated-L-ascorbic acid through a specific transglucosylation distinct from that of microbial α-glucosidase (Yamamoto *et al.* 1990). Although most of the time the obtained yields are low, some α-glucosidases exhibit a good transglucosylation activity like for example α-glucosidase isolated from a strain of thermophilic *Bacillus* and cloned in *E. coli* (Nakao *et al.* 1994). The enzyme reacted at 60 °C with 1.8 M sucrose, used as a sole glucosyl donor and acceptor, to synthesize oligosaccharides containing theanderose, a rare trisaccharide, at a maximum yield of 35%.

In this work, the *M. subhyalinus* α-glucosidase-catalysed synthesis of phenylethylglycosides from phenylethanol was taken as a model system since it has been shown that the behavior of this glycosyl acceptor is close to that of hydroxy amino acid derivatives (Leparoux *et al.* 1994, 1997). Our results suggest that the α-glucosidase activity was solely responsible for the hydrolysis of sucrose by the crude extracts since the phenylethyl-α-D-glucoside and not the phenylethyl-β-D-fructoside was formed by transglycosylation. The use of both maltose and sucrose as substrates during the purification of the enzymes will determine whether the crude extracts of *M. subhyalinus* contain a β-fructosidase (invertase) or not. An interesting parallel can be established with the results obtained by Hoefsloot *et al.* (1988) with acid α-glucosidase from mammalian cells. Cloning of cDNA has led to the unexpected finding that lysosomal α-glucosidase has probably arisen from the same ancestral gene as sucrase. Recently, it has also been suggested that the α-glucosidase and sucrase activities from *Pyrococcus woesi* and *P. furiosus* were supported by the same enzyme (Chang *et al.* 2001). Finally, the present work demonstrates the usefulness of α-glucosidase activity of *M. subhyalinus,* as compared to other sources, for catalysing the synthesis of α-glucosylconjugates in attractive yields.

Acknowledgement

We thank the Ministère Français de la Coopération for a doctoral fellowship granted to one of us (L.P. Kouamé, permanent address: Université d'Abobo-Adjamé, BP 801, Abidjan 02, Côte d'Ivoire).

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