Transformation of maltose into prebiotic isomaltooligosaccharides
by a novel $\alpha$-glucosidase from Xanthophyllomyces dendrorhous

Lucía FERNÁNDEZ-ARROJO$^a$, Dolores MARÍN$^b$, Aranzazu GÓMEZ DE SEGURA$^a$, Dolores LINDE$^b$, Miguel ALCALDE$^a$, Patricia GUTIÉRREZ-ALONSO$^b$, Iraj GHAZI$^a$, Francisco J. PLOU$^{a,*}$, María FERNÁNDEZ-LOBATO$^b$ and Antonio BALLESTEROS$^a$

$^a$ Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Cantoblanco, 28049 Madrid, Spain.

$^b$ Centro de Biología Molecular Severo Ochoa, Departamento de Biología Molecular (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain.

$^*$ Corresponding author: Francisco J. Plou, Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, CSIC, Cantoblanco, Marie Curie 2, 28049 Madrid, Spain. Fax: +34-91-5854760. E-mail: fplou@icp.csic.es. URL: http://www.icp.csic.es/abg
ABSTRACT

The transglycosylation activity of a novel α-glucosidase from the basidiomycetous yeast Xanthophyllomyces dendrorhous (formerly Phaffia rhodozyma) was studied using maltose as glucosyl donor. The enzyme synthesized oligosaccharides with α-(1→2), α-(1→4) and α-(1→6) bonds. Using 200 g/l maltose, the yield of oligosaccharides was 53.8 g/l, with prebiotic oligosaccharides containing at least one α-(1→6) linkage (panose, 6-O-α-glucosyl-maltotriose and 6-O-α-isomaltosyl-maltose) being the major products (47.1 g/l). The transglycosylating yield was 3.6 times higher than the observed with the α-glucosidase from Saccharomyces cerevisiae (53.8 vs. 14.7 g/l). Moreover, when increasing the maltose concentration up to 525 g/l, the maximum production of tri- and tetrasaccharides reached 167.1 g/l, without altering the percentage of oligosaccharides in the mixture. Compared with other microbial α-glucosidases in which the main transglycosylation product is a disaccharide, the enzyme from X. dendrorhous yields a final product enriched in trisaccharides and tetrasaccharides.

Keywords: Glycosidase, Isomaltooligosaccharides Prebiotics, Transglycosylation, Alpha-glucosidase, Oligosaccharides.
1. INTRODUCTION

Several non-digestible carbohydrates are the only prebiotic molecules known to date [1]. The two requirements of a prebiotic are that it is not hydrolyzed in the small intestine and that it is selectively fermented in the colon by certain beneficial members of the colonic microbiota (Bifidobacterium and Lactobacillus genera). There are several prebiotics in use around the world, with Japan being the leader in development and consumption. Besides the fructooligosaccharides [2] and galactooligosaccharides [3], which are commercialized in USA and Europe, Japan produces other “emerging” prebiotics (isomaltooligosaccharides, soybean oligosaccharides, lactosucrose, gentiooligosaccharides, xylooligosaccharides, to cite some) that will be likely coming soon to the market [4].

Although the synthesis of oligosaccharides in vivo is performed by glycosyltransferases (EC 2.4.) [5], the widely available glycosidases (glycoside hydrolases, EC 3.2.) can be also used for in vitro synthesis of glycosidic bonds. In order to revert their hydrolytic function towards synthesis, thermodynamic control (using high substrate concentrations) and/or kinetic control (using activated glycosyl donors) have been successfully employed [6]. Despite the broad availability of glycosidases, their synthetic applications are often limited by low yield and poor regioselectivity [7]. In this context, a group of novel, site-specifically mutated glycosidases called glycosynthases were developed [8]. A glycosynthase is a specifically-mutated retaining glycosidase in which substitution of the catalytic carboxyl nucleophile by a non-nucleophilic residue (Ala, Gly or Ser) results in an enzyme that is hydrolytically inactive but yet able to catalyse the transglycosylation reaction. Although oligosaccharide yields can reach 95-98% in some cases [6], the need of activated glycosyl fluoride donors limits their practical application.

Amylolytic enzymes are extensively used in biotechnology, as they have important applications in both the food and the pharmaceutical industries. Among them, α-glucosidases (α-D-glucoside glucohydrolases, EC 3.2.1.20) are exo-glycosidases that catalyze the release of glucose from the non-reducing end of short oligosaccharides [9-11]. On the basis of
amino acid sequence homology, $\alpha$-glucosidases are distributed in two groups of glycoside hydrolases, i.e. GH-family 13 and 31 [12]. The former enzyme group strongly recognizes the $\alpha$-glucosyl-moiety in heterogeneous substrates, such as synthetic $\alpha$-glucosides and sucrose. The members of GH-family-31 are specific for the maltosyl-structure of maltooligosaccharides rather than for the $\alpha$-glucosyl-structure of heterogeneous substrates [13].

Interestingly enough, some $\alpha$-glucosidases exhibit transglycosylation activity, e.g. those from *Aspergillus niger* [14], *Bacillus stearothermophilus* [15], *Saccharomyces cerevisiae* [16] or brewer’s yeast [15]. This transglycosylation activity is being applied to produce prebiotic isomaltooligosaccharides [17;18] or to glucosylate compounds such as L-menthol [15], pyridoxine [19] and hydroquinone [20]. The basidiomycetous yeast *Xanthophyllumyces dendrorhous* (formerly *Phaffia rhodozyma*) was isolated from deciduous trees [21], and it produces astaxanthin (3-3’-dihydroxi-β,β-carotene-4,4-dione) as the main carotenoid [22]. Its amylolytic activity has been also reported, and we recently purified and characterized a novel $\alpha$-glucosidase from *X. dendrorhous*, which releases glucose from maltooligosaccharides and soluble starch [23].

In this work, we have studied the transglycosylation activity of *X. dendrorhous* $\alpha$-glucosidase [24]. Its synthetic activity was compared with that of the $\alpha$-glucosidase from another yeast, namely *Saccharomyces cerevisiae*. 
2. EXPERIMENTAL PROCEDURES

Materials
The α-glucosidase from Saccharomyces cerevisiae was from Roche. Glucose (≥ 99.5%), maltose monohydrate (99%), p-nitrophenyl-α-D-maltoside (PNPαMalt) (≥ 98%), kojibiose (≥ 95%), panose (≥ 98%), maltotriose (≥ 95%), maltotetraose (≥ 96%) and 6-O-α-glucosyl-maltotriose (≥ 96%) were from Sigma. Sucrose was from Merck. All other reagents and solvents were of the highest available purity and used as purchased.

Enzyme purification and quantification
The X. dendrorhous α-glucosidase was obtained from 1 litre of culture filtrates prepared as previously described [23]. The filtrate was concentrated using a VivaFlow 50 tangential flow filtration module (Vivascience, Sartorius Group) with 30,000 MWCO polyethersulfone (PES) membrane, and dialyzed against 20 mM sodium phosphate buffer pH 7.0 (buffer A). It was then applied to a DEAE-Sephacel column (10 ml) equilibrated with buffer A. The protein was eluted with a 0 to 0.5 M NaCl gradient at a flow rate of 1 ml/min. Active fractions (2.5 ml) eluting at approx. 0.05 M NaCl were pooled, dialyzed against 20 mM sodium acetate pH 4.5 (buffer B), and applied to the DEAE-Sephacel column equilibrated with buffer B. The proteins were eluted with a 0 to 0.2 M NaCl gradient, active fractions (2.5 ml) eluting at 0.2 M NaCl. These fractions were pooled, dialyzed against water and stored at -70 ºC until use. Silver- and Coomassie Blue-stained SDS/PAGE of the samples confirmed the enzyme purification. Protein concentration was determined with the Bio-Rad microprotein determination assay, following the manufacturer's specifications, with bovine serum albumin as standard.

Standard activity microassay
The enzymatic activity towards p-nitrophenyl-α-D-maltoside (PNPαMalt) was measured following p-nitrophenol release at 410 nm from 72 mM substrate in 0.2 M sodium acetate
buffer (pH 5.6) in a final reaction volume of 100 μl and using a microplate reader (Versamax, Molecular Devices). The molar absorption coefficient (ε) of ρ-nitrophenol at 410 nm (pH 5.6) was determined to be 8620 M⁻¹ cm⁻¹. One unit (U) of activity was defined as that corresponding to the hydrolysis of 1 μmol of PNPαMalt per min.

Production of isomaltooligosaccharides

The reaction mixture (1 ml) consisted of 900 μl of maltose solution in 0.2 M sodium acetate buffer pH 5.6 (to reach a final maltose concentration of 200 g/l or 525 g/l) and 100 μl enzyme (to reach a final activity of 0.02 U/ml and 2.5 U/ml for X. dendrorhous and S. cerevisiae α-glucosidases, respectively, measured in the hydrolysis of PNPαMalt). The mixtures were incubated at 45 ºC in an orbital shaker (Vortemp) at 200 rpm. At different times, 40 μl aliquots were removed from the reaction mixture, diluted with 160 μl water and incubated 5 min at 95 ºC to inactivate the enzyme. Samples were centrifuged 5 min at 6000 rpm using an eppendorf with a 0.45 μm Durapore® membrane (Millipore), and analyzed by HPLC.

HPLC analysis

The concentration of the different products was analyzed by HPLC with a quaternary pump (Delta 600, Waters) coupled to a Lichrosorb-NH2 column (4.6 x 250 mm) (Merck). The column temperature was kept constant at 25 ºC. Detection was performed using an evaporative light-scattering detector DDL-31 (Eurosep) equilibrated at 85 ºC. Acetonitrile:water 85:15 (v/v), conditioned with helium, was used as mobile phase (flow rate 0.9 ml/min) for 8 min. Then, a gradient from this mobile phase to acetonitrile:water 75:25 (v/v) was performed in 2 min, and this eluent was maintained during 7 min. Finally, a gradient from this composition to acetonitrile:water 70:30 (v/v) was performed in 5 min, and maintained for 20 min. The data obtained were analyzed using the Millennium Software.
3. RESULTS AND DISCUSSION

Transglycosylation activity of X. dendrorhous α-glucosidase

In a previous study, the α-glucosidase from the yeast X. dendrorhous exhibited a notable hydrolytic activity towards maltooligosaccharides and soluble starch, with maltose as the best substrate [23]. No hydrolytic activity was detected towards α-(1→6)-glycosidic bonds (e.g. isomaltose and isomaltotriose) or α-glucosyl groups present in heterogeneous substrates (e.g. sucrose). Some α-glucosidases only exhibit hydrolytic activity [25], whereas others also show transglycosylating activity towards sugar acceptors [26] or non-glycosylated molecules [27]. For that reason, the transglycosylating activity of X. dendrorhous α-glucosidase was tested using maltose, the common glucosyl donor.

The transglycosylation activity was investigated with 200 g/l maltose at the optimal pH (5.5) and temperature (45 °C). Fig. 1 shows the progress of the reaction based on HPLC chromatograms. To identify the components of the mixture, the retention time of each peak was compared with those of glucosaccharide standards. Two trisaccharides (maltotriose, peak 5; panose, peak 6) were initially formed; however, the latter was resistant to hydrolysis as it contained an α-(1→6) bond. Two tetrasaccharides (6-O-α-glucosyl-maltotriose, peak 7; 6-O-α-isomaltosyl-maltose, peak 8), which contained mixed α-(1→4) and α-(1→6) bonds, were obtained by α-(1→6) glucosylation of maltotriose and panose, respectively. The amount of maltotetraose formed was negligible throughout the process. In addition, two disaccharides, kojibiose and isomaltose (peaks 3 and 4) –formed when the glucose acts as acceptor– were detected at the final stages of the reaction. Kojibiose contains an α-(1→2) glucosidic linkage and it is characterized by its low cariogenicity and digestibility [28].

Table 1 summarizes the carbohydrates present in the reaction mixture throughout the process. It is worth mentioning that the synthetic specificity of many glycosidases may differ
substantially from the specificity of hydrolysis [29]. Thus, various \(\alpha\)-glucosidases –whose function in nature is the hydrolysis of \(\alpha-\(1\rightarrow4\) \) bonds– transfer glucosyl groups to the less-hindered, primary 6-OH of the acceptor, yielding products such as isomaltose and panose [30]. In addition, transfer to secondary hydroxyl groups (2-OH, 3-OH, 4-OH) has been reported [31]. As a result, a mixture of oligosaccharides consisting of \(\alpha-\(1\rightarrow2\)\), \(\alpha-\(1\rightarrow3\)\), \(\alpha-\(1\rightarrow4\)\) and/or \(\alpha-\(1\rightarrow6\)\) bonds is usually obtained [9;15]. Our results indicate that \(X.\ dendrorhous\ \alpha\)-glucosidase is able to transfer glucosyl moieties to the 2-OH, 4-OH and 6-OH of a glucose residue.

----- Insert Table 1 -----

Fig. 2 illustrates the kinetics of oligosaccharide formation. Maltotriose concentration reached a maximum of 16.2 g/l at 100 h, after which it was progressively hydrolyzed. The maximum yield of tri- and tetrasaccharides (the kinetic maximum) with the \(X.\ dendrorhous\ \alpha\)-glucosidase was 53.8 g/l, which corresponded to 26.9% (w/w) of the total amount of carbohydrates in the mixture. Most of them (47.1 g/l) contained \(\alpha-\(1\rightarrow6\)\) bonds, which are responsible of the prebiotic properties of the products. The enrichment of the mixture in oligosaccharides with \(\alpha-\(1\rightarrow6\)\) bonds is a consequence of their higher resistance to hydrolysis, as the enzyme is highly \(\alpha-\(1\rightarrow4\)\) specific for cleavage of glycosidic bonds. In addition, the formation of new disaccharides accounted for 30.6 g/l (15.3% referred to the total weight of carbohydrates in the sample).

-----Insert Fig. 2-----

Regarding the degree of polymerization (d.p.), it has been reported that an enzyme having a poor ability to hydrolyze a tetrasaccharide is unlikely to synthesize such molecules, as the binding conditions for the enzyme-substrate complex will be the same in both reactions [15]. For example, the \(\alpha\)-glucosidase from the acidophilic archaeon \(Ferroplasma acidiphilum\) is not able to synthesize oligosaccharides with a d.p. higher than three as its hydrolytic
substrate specificity is restricted to disaccharides [32]. Accordingly, the X. dendrorhous α-glucosidase is able to hydrolyze maltodextrins and also to synthesize tetrasaccharides; however, pentasaccharides were not detected under our reaction conditions.

**X. dendrorhous and S. cerevisiae comparative analysis**

The transglycosylation activity of X. dendrorhous α-glucosidase was compared with that of commercial α-glucosidase from the yeast S. cerevisiae, as they are good representatives of yeasts α-glucosidases with different hydrolytic activity. X. dendrorhous α-glucosidase has a narrow hydrolysis range towards maltooligosaccharides whereas the aglycon specificity of S. cerevisiae α-glucosidase is rather broad [14]. HPLC chromatograms (data not shown) clearly indicated that the main reaction catalyzed by S. cerevisiae α-glucosidase was the hydrolysis of maltose. In the initial stages of the reaction (0.5-2 h), a peak of maltotriose appeared, but this trisaccharide was quickly hydrolyzed as it only contained α-(1→4) bonds. At the end-time of the reaction, only two transglycosylation products were present, isomaltose and panose, formed, respectively, by α-(1→6) glucosylation of glucose and maltose.

Fig. 3 illustrates the kinetics of the process with S. cerevisiae α-glucosidase. The reaction rate towards maltose was much faster than with X. dendrorhous enzyme. This was a consequence of the higher volumetric activity of the S. cerevisiae working solution (24.9 vs. 0.20 U/ml towards synthetic p-nitrophenyl-α-D-maltoside). However, the specific activity of X. dendrorhous α-glucosidase was higher (33.0 vs. 1.1 U/mg protein), but the low protein concentration in the extract (0.007 mg/ml) resulted in only a total of 0.02 enzyme units for the transglycosylation experiments compared with 2.5 enzyme units with the S. cerevisiae enzyme. It is worth mentioning that the maximum yield of oligosaccharides shows no dependency with the enzyme concentration in the mixture, but it is obtained for a longer or shorter time of reaction [33]. The amount of enzyme added must be a compromise to reach the maximum transglycosylation rate in a relatively short time, without fast hydrolysis of the
transglycosylation products to facilitate stopping the reaction at the kinetic maximum.

-----Insert Fig. 3 ------

As shown in Fig. 3, the maltotriose concentration reached a maximum of 13.7 g/l, after which the trisaccharide was completely hydrolyzed. The \textit{S. cerevisiae} $\alpha$-glucosidase yielded a maximum of 14.7 g/l of tri- and tetrasaccharides, corresponding to a 7.4\% (w/w) of the total carbohydrates in the mixture. In contrast, isomaltose and panose were not significantly hydrolyzed throughout the process, as they contained $\alpha$-(1$\rightarrow$6) bonds. At the end of the process, the isomaltose concentration attained 21.4 g/l.

\textbf{Effect of maltose concentration on oligosaccharide production}

The synthesis of isomalto-oligosaccharides from maltose is a kinetically controlled reaction that involves a glucosyl-enzyme intermediate \cite{34}. The nucleophiles H$_2$O and maltose compete for the glucosyl-enzyme intermediate \cite{35}. When H$_2$O is the nucleophile, the enzyme acts as a hydrolase (releasing glucose). When maltose is the nucleophile, the enzyme acts as a glucosyltransferase. The condensation products can be also hydrolyzed by the enzyme. In fact, the reaction must be monitored and stopped at the point of maximum yield of condensation products \cite{36}. The maximum yield of oligosaccharides depends on two parameters: the concentration of maltose and the intrinsic transferase/hydrolase ratio of the enzyme \cite{37}.

In order to improve the yield of transglycosylation products, a high maltose concentration (525 g/l) was assayed. Fig. 4 shows the progress of tri- and tetrasaccharide formation. As expected, the increase in maltose concentration caused an improvement in the yield of tri- and tetrasaccharides. The maltotriose concentration reached again a maximum, after which its rate of hydrolysis was higher than its rate of synthesis. On the contrary, the oligosaccharides that contained at least one $\alpha$-(1$\rightarrow$6) linkage were very resistant to hydrolysis. The maximum production of tri- and tetrasaccharides was 167.1 g/l, which
corresponded to 31.4% of the total weight of carbohydrates.

These values indicated that the transglycosylation activity of \textit{X. dendrorhous} $\alpha$-glucosidase was not significantly maltose-dependent (26.9% and 31.4% w/w tri- and tetrasaccharides production at 200 and 525 g/l, respectively). Yields in kinetically controlled synthesis usually range from 20 to 40\% [38]. In this context, it has been reported that the ratio of transglucosylating to hydrolysing activity for the $\alpha$-glucosidase from \textit{Aspergillus niger} notably decreases when lowering maltose concentration [14].

Table 2 summarizes the carbohydrate composition (in weight) of the mixture at different times. The maximum percentage of trisaccharides was 20\% at 200 g/l and 28.1\% at 525 g/l maltose. In contrast, the tetrasaccharide production reached maxima of 7.4\% and 3.6\% at 200 and 525 g/l, respectively. However, the values presented in Table 2 suggest that the tetrasaccharide production could be further increased, in detriment of trisaccharide yield, by just allowing to progress the reaction for an extended period. The higher tetrasaccharide production found at 200 g/l maltose may be explained considering that, at lower maltose concentration, there is more competition between trisaccharides and maltose to accept a glucosyl moiety, thus favouring the formation of high polymerization degree products [39;40].

The \textit{X. dendrorhous} $\alpha$-glucosidase shows a different transglycosylation pattern in terms of yield and regioselectivity. Thus, the \textit{Aspergillus niger} and \textit{A. nidulans} $\alpha$-glucosidases give isomaltose as the main transglycosylating product using maltose as substrate [41]. Thus, a maltose concentration of 30\% (w/v) is used for \textit{A. niger} $\alpha$-glucosidase to achieve an isomaltose content of 30\% in the final reaction product [14]. Campa et al. [42] showed that with this enzyme two trisaccharides were formed, one of them (panose) was especially fast
hydrolyzed and the second (isomaltotriose) presented a notably lower concentration than that of isomaltose throughout the process. With the A. nidulans α-glucosidase B, approximately 50% of maltose was converted to transglycosylation products, 60% was found to be isomaltose [9]. The α-glucosidase from Acremonium sp. synthetizes oligosaccharides containing α-1,2-glucosydic bonds [43], whereas the Paecilomyces lilacinus enzyme forms both α-1,2 and α-1,3 bonds [31]. Interestingly, a novel α-glucosidase from Chaetomium thermophilum var. coprophilum converts 5% (w/v) maltose into trehalose and maltooligosaccharides [26]. The pattern of transglycosylation products derived from maltose with the α-glucosidase from Geobacillus sp. appeared to be different from the oligosaccharides obtained with A. niger and A. nidulans enzymes, especially regarding the formation of tetrasaccharides [27].

In conclusion, the X. dendrorhous α-glucosidase gives a notable yield of total transglycosylating products (approx. 40% considering the di-, tri- and tetrasaccharides formed). Interestingly, the transferase to hydrolase ratio of this enzyme shows low dependency with maltose concentration. In addition, the mixtures obtained with X. dendrorhous α-glucosidase are enriched in tri- and tetrasachharides, which contrasts with other α-glucosidases that synthesize basically disaccharides.

ACKNOWLEDGEMENTS

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Reference List


Table 1. Carbohydrates present in the transglycosylation reactions catalyzed by the different α-glucosidases.

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Table 2. Carbohydrate composition (% w/w) of the reaction mixture using *X. dendrorhous* α-glucosidase and two initial maltose concentrations.

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<sup>a</sup> Experimental conditions: 0.02 U/ml, 0.2 M sodium acetate buffer, pH 5.6, 45 °C.

<sup>b</sup> Kojibiose and isomaltose.
Figure Legends

Fig. 1. HPLC progress of the transglucosylation of maltose catalyzed by *X. dendrorhous* α-glucosidase (reaction times are indicated). Conditions: 200 g/l maltose in 0.2 M sodium acetate buffer (pH 5.6), 0.02 enzyme units (33 U/mg protein), 40 °C. HPLC conditions are described in experimental section (peak assignation according to Table 1).

Fig. 2. Oligosaccharides production from maltose catalyzed by *X. dendrorhous* α-glucosidase. Experimental conditions: 200 g/l maltose, 0.02 enzyme units (33 U/mg protein), 0.2 M sodium acetate buffer, pH 5.6, 45 °C.

Fig. 3. Oligosaccharides production from maltose catalyzed by *S. cerivisiae* α-glucosidase. Experimental conditions: 200 g/l maltose, 2.5 enzyme units (1.1 U/mg protein), 0.2 M sodium acetate buffer, pH 5.6, 45 °C.

Fig. 4. Kinetics of tri- and tetrasaccharide production from maltose catalyzed by *X. dendrorhous* α-glucosidase using 525 g/l maltose. Experimental conditions: 0.2 M sodium acetate buffer, 0.02 enzyme units (33 U/mg protein), pH 5.6, 45 °C.
Fig. 1

Retention time (min)

Detector signal

0 10 20 30 40

262 h

70 h

168 h

262 h
Fig. 2

![Graph showing the reaction time (h) vs. oligosaccharide production (g/l). The graph includes various oligosaccharides such as Glucose, Maltose, Maltotriose, Panose, Isomaltose, Kojibiose, 6-O-α-glucosyl-maltotriose, and 6-O-α-isomaltosyl-maltose.](image)

- Glucose
- Maltose
- Maltotriose
- Panose
- Isomaltose
- Kojibiose
- 6-O-α-glucosyl-maltotriose
- 6-O-α-isomaltosyl-maltose
Fig. 4

Reaction time (h) vs. Oligosaccharide production (g/l)

- Maltotriose
- Panose
- 6-O-α-isomaltosyl-maltose
- 6-O-α-glucosyl-maltotriose