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ORIGINAL PAPER

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Enzymatic synthesis and hydrolysis of xylogluco-oligosaccharides using the first archaeal α-xylosidase from *Sulfolobus solfataricus*

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Abstract The first, recently identified, archaeal α-xylosidase from *Sulfolobus solfataricus* (XylS) shows high specificity for hydrolysis of isoprimeverose [α-D-xylopyranosyl-(1,6)-D-glucopyranose, (X)], the *p*-nitrophenyl-β derivative of isoprimeverose, and xyloglucan oligosaccharides and has transxylosidic activity, forming, in a retaining mode, interesting α-xylosides. This article describes the synthesis of isoprimeverose, the disaccharidic repeating unit of xyloglucan, of the *p*-nitrophenyl-β derivative of isoprimeverose, and of a trisaccharide based on isoprimeverose that is one of the trisaccharidic building blocks of xyloglucan. A substrate structure–activity relationship is recognized for both the hydrolysis and the synthesis reactions of XylS, it being a biocatalyst (i) active hydrolytically only on X-ending substrates liberating a xylose molecule and (ii) capable of transferring xylose only on the nonreducing end glucose of *p*-nitrophenyl-(PNP)-β-D-cellobioside. The compounds synthesized by this enzyme are a starting point for enzymological studies of other new enzymes (i.e., xyloglucanases) for which suitable substrates are difficult to synthesize. This study also allows us to define the chemical characteristics of the xylose-transferring activity of this new archaeal enzyme, contributing to building up a library of different glycosidases with high specific selectivity for oligosaccharide synthesis.

Key words Glycosidase · Xyloglucan · Oligosaccharides · *Sulfolobus* · Archaea · Xylosidase · Thermophilic enzymes

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Introduction

Glycosyl hydrolases characterized from the hyperthermophilic archaeon *Sulfolobus solfataricus* are a secreted α-amylase, an intracellular α-glucosidase from strain 98/2 (Rolfsmeier and Blum 1995; Haseltine et al. 1996), and a β-glycosidase from strains MT4 and P2 (Pisani et al. 1990; Grogan 1991). In an effort to determine the full set of glycosyl hydrolases produced by this archaeon, we have identified a novel α-xylosidase (XylS) that was classified in family 31 of glycosyl hydrolases and showed high specificity for isoprimeverose [α-D-xylopyranosyl-(1,6)-D-glucopyranose, (X)], for the *p*-nitrophenyl-β derivative of isoprimeverose, and for xyloglucan oligosaccharides (Moracci et al. 2000).

Xyloglucan is widely distributed in plants, as it is the principal hemicellulose component in the primary cell wall (20%–30% of the total cell wall) and one of the most abundant storage polysaccharides in seeds (>40% in weight in some species). This polymer is composed of a β-(1,4)-glucan backbone, with α-(1,6)-D-xylose groups linked to about 75% of the glucosyl residues. A degree of complexity is added in some species by the presence of other differently linked monosaccharides such as α-L-Fuc and β-D-Gal, among others. Xyloglucans not only have structural functions but can also be involved in more complex biological functions. In particular, some xyloglucan oligosaccharides can promote the elongation of stem segments (Lorences and Fry 1994), playing a role in the regulation of plant growth.

Recently, a significant need for purified xyloglucan oligosaccharides and isoprimeverose for enzymological and metabolic studies has emerged (Lorences and Fry 1994; Chaillou et al. 1998). These compounds were prepared by several steps of hydrolysis of the natural polymer xyloglucan catalyzed enzymatically by different glycosyl

hydrolases such as *endo*-β-1,4-glucanases, β-galactosidases, α-fucosidases, isoprimeverose-producing oligoxyloglucan hydrolases, α-xylosidases, and β-glucosidase (Kato and Matsuda 1980). The problems arising from this approach are the huge number of different enzymatic activities required as well as the production of mixtures of oligosaccharides that are difficult to purify and always contaminated by monosaccharides which are usually removed microbiologically.

Enzymatic synthesis of carbohydrate and derivatives is an important area of carbohydrate chemistry. This technique has been greatly improved by the newfound roles for carbohydrates in a variety of biological functions (Palcic 1999; Kuberan and Linhardt 2000) and in the application of these compounds as therapeutic agents, as substrates for the characterization of new enzymes, and as model compounds for biological studies (Crout and Vic 1998; Mayer et al. 2000; Whymer and Toone 2000). Enzymatic strategies for synthesis are based on the use of at least two enzymes, glycosyl transferases and glycosyl hydrolases used in transglycosylating mode. The alternative glycosidase approach for the synthesis of oligosaccharides overcomes some important drawbacks intrinsic in the use of glycosyl transferases and chemical routes (Crout and Vic 1998).

The biotechnological potential of thermophilic glycosidases could be considered in the synthesis of different oligosaccharides and recently such glycosidases have been proposed for possible exploitation in commercial bioprocessing of transgenic plant carbohydrates (starch, cellulose) (Montalvo-Rodriguez et al. 2000). In particular, we recently converted the thermophilic β-glycosidase into a glycosynthase able to synthesize complex oligosaccharides, opening new avenues in the challenging production of biologically important carbohydrates (Moracci et al. 1998). Our previous results for the synthesis of branched oligosaccharides (Trincone et al. 2000) clearly showed the synthetic potential of this enzyme, confirming interest in thermophilic enzymes with new biocatalytic characteristics.

In the course of our studies on archaeal XylS (Moracci et al. 2000), the need for isoprimeverose-based substrates emerged, and we planned the enzymatic synthesis of isoprimeverose, the *p*-nitrophenyl-(PNP)-β derivative of isoprimeverose, and the trisaccharidic unit of xyloglucan by transxylosidic activity of XylS. We present here the results of this novel enzymatic approach for the synthesis, an alternative to enzymatic hydrolysis starting from natural xyloglucan. The pure compounds obtained can be used as suitable substrates for the characterization of new enzymes (xyloglucanases). The results described here also allow detailed recognition of the regioselectivity of transxylosidic and hydrolytic activities of this thermophilic enzyme (XylS), confirming interest in these biocatalysts with new properties.

Materials and methods

Enzyme preparation

The enzyme XylS was expressed in *Escherichia coli* and purified as previously reported (Moracci et al. 2000). The specific activity of the purified enzyme on PNP-α-xylopyranoside was 2.33 U mg^{-1}. Prepurified biocatalyst obtained after the three simple thermoprecipitation steps (0.15 U mg^{-1}) was also used in the preparative experiments to avoid time-consuming and expensive total protein purification.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) studies were conducted on a Bruker AMX-500 instrument (Bruker, Rheinstetten, Germany) (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C). Monodimensional (^{1}H, ^{13}C, and distortionless enhanced phase transfer [DEPT]) experiments were conducted using CD_3OD, D_2O, or, for peracetylated compounds, $CDCl_3$ as solvents and using the solvent signal as an internal reference. Two-dimensional NMR spectroscopy studies were conducted on the peracetylated derivatives.

Substrates and commercial enzymes

α-Xylosyl fluoride was obtained from xylose after acetylation with Ac_2O (perchloric acid catalyst) and reaction of the α-anomer so obtained with HF/pyridine as previously reported (Malet and Planas 1998). Free α-xylosyl fluoride was obtained by treatment with MeOH/sodium carbonate: 1,2,3,4-tetra-*O*-acetyl α-D-xylopyranose, 6.19 (H-1, J = 3.5 Hz), 4.92–4.97 (H2 and H4), 5.40 (H3), 3.89–3.65 (H5); 1-fluoro-1-deoxy 2,3,4-tri-*O*-acetyl α-D-xylopyranose, 5.61–5.73 (dd, H-1, J=53 Hz, J=2.7 Hz), 4.92 (H2), 5.48 (H-3), 5.05 (H-4), 3.79–3.97 (H5). Xylogluco-oligosaccharides (XXXG and GXXG [G, glucose]) were obtained from tamarind seed xyloglucan (commercial name, Glyroide) obtained from Dainippon Pharmaceuticals (Osaka, Japan) (Kato and Matsuda 1980; Matsushita et al. 1985). At first, Glyroide was treated by partially purified β-galactosidase from *Aspergillus oryzae* (Sigma, St. Louis, MO, USA), followed by treatment of *Trichoderma endo*-β-glucanase (Sigma) at pH 5.5, 45°C, for 20 h; the polysaccharide was enzymatically transformed to the mixture of Glc, Gal, and XXXG. XXXG was then purified by gel filtration chromatography using Bio-gel P2 (Bio-Rad; Nippon Bio-Rad, Yokohama, Japan). To obtain GXXG, XXXG was next treated by α-xylosidase purified from cellulase preparation of *Aspergillus niger* (Sigma). After complete hydrolysis of the terminal xylose of XXXG by alpha-xylosidase, GXXG was isolated by Bio-gel P2 column chromatography.

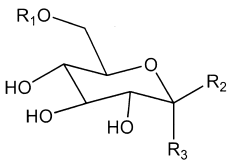
Enzymatic syntheses

Enzymatic syntheses of PNP-β-isoprimeveroside and isoprimeverose

PNP-α-D-xylopyranoside (34 μmol, 9.2 mg) was dissolved in 1 ml sodium acetate buffer (50 mM, pH 5.0) and added to 67 μmol PNP-β-D-glucopyranoside. The reaction was started at 65°C by addition of 0.2 mg pure XylS. The reaction was complete after 2 h, as indicated by the complete

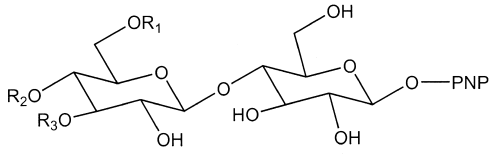
disappearance of the donor as monitored by thin-layer chromatography (TLC) (EtOAc/MeOH/H_2O, 70:20:10, by volume). The reaction mixture was rotary evaporated and purified by preparative TLC, obtaining 2.6 mg (6 μmol) of the disaccharide PNP-β-isoprimeveroside.

The same reaction was conducted on a millimolar scale using XylS purified by thermoprecipitation. At the end of the reaction, prepurification of the reaction mixture was obtained on RP-8 reverse-phase column chromatography eluting the free xylose and the residual acceptor with water and with MeOH/H_2O, 1:1 v/v; the fractions containing compound 1 (Fig. 1) were collected, and complete purification was secured by acetylation and silica gel column chromatography (*n*-hexane/EtOAc 7:3, v/v). The yield of acetylated derivative of compound 1 (Fig. 1) in this semi-preparative scale reaction was 11% with respect to the donor.



1. R_1 = α-Xyl; R_3 = H; R_2 = *p*-nitrophenol

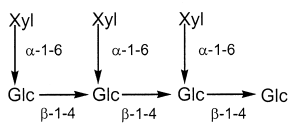
2. R_1 = α-Xyl; R_3 = R_2 = H or OH



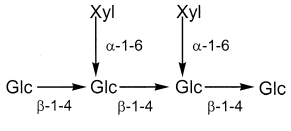
3. R_1 = α-Xyl; R_2 = R_3 = H

4. R_1 = H; R_2 = H; R_3 = α-Xyl

5. R_1 = H; R_2 = α-Xyl; R_3 = H



6. XXXG



7. GXXG

Fig. 1. Compounds synthesized and subjected to hydrolysis by α-xylosidase from *Sulfolobus solfataricus*. Synthesized: *1*, PNP-β-isoprimeveroside; *2*, isoprimeverose, α- and β-form; *3*, trisaccharidic building unit of xyloglucan; *4* and *5*, regioisomers of *3* formed in minor amounts. Subject to hydrolysis: *6*, X-ending heptasaccharide, XXXG; *7*, G-ending hexasaccharide, GXXG. Compound *6* is substrate for α-xylosidase from *Sulfolobus solfataricus*, liberating xylose and GXXG. (Abbreviations are in accord with the nomenclature proposed by Fry et al. 1993)

^{1}H-NMR (anomeric section) and ^{13}C-NMR (sugar moiety) spectra in CD_3OD/D_2O of compound 1 (see Fig. 1) showed the following signals: δ 5.18 (J=7.0 Hz, H-1 β-Glc), 4.84 (J, 3.5 Hz, H-1 α-Xyl); δ 101.2, 99.7, 77.4, 76.3, 74.9, 74.3, 73.2, 71.0, 70.9, 67.2, 62.6. After acetylation (Pyr/Ac_2O overnight at room temperature), the spectra of acetylated derivative in $CDCl_3$ showed the following signals: ^{1}H-NMR spectra, δ 5.20 (H-1 β-Glc), 5.28 (H-2 β-Glc), 5.34 (H-3 β-Glc), 5.02 (H-4 β-Glc), 3.99 (H-5 β-Glc), 3–81–3.47 (H-6 β-Glc), 5.00 (H-1 α-Xyl), 4.80 (H-2 α-Xyl), 5.50 (H-3 α-Xyl), 4.91 (H-4 α-Xyl), 3.67–3.47 (H-5 α-Xyl); ^{13}C-NMR spectra, disaccharide moiety signals, δ 98.4, 71.3, 72.7, 69.1, 73.7, 66.5 (C1–C6 β-Glc), 95.9, 71.3, 69.0, 69.3, 58.7 (C1–C5 α-Xyl), $[\alpha]_D^{20}$, 57.5 (c=0.26, chloroform). The COSY spectrum and ^{1}H and ^{13}C correlation allowed assignments, as indicated here, for all carbon and proton signals in the acetylated derivative and secured the interglycosidic linkage of the carbohydrate moiety.

The free disaccharide isoprimeverose was prepared by the action of Driselase on PNP-β-isoprimeveroside (Moracci et al. 2000); the product was purified and characterized by NMR spectroscopy after acetylation. Diagnostic signals in the ^{13}C-NMR spectra of acetylated disaccharide at 58.43 ppm (C-5 α-Xyl) and 66.24 ppm (C-6 of glucose unit) indicated no change in the carbohydrate sequence in the product. Free sugar was reobtained after methanolysis (sodium carbonate in anhydrous methanol).

Direct enzymatic syntheses of isoprimeverose

Two set of experiments were conducted at different glucose/xylosyl fluoride ratios to assess the best conditions for this reaction. To a constant amount of the donor α-fluoroxylose (1.5 mg) dissolved in a total volume of 100 μl sodium acetate buffer (500 mM, pH 5.6), different amounts of glucose were added (1.8, 3.6, and 9 mg for the first set; 0.9, 0.45, 0.18 for the second set); the reaction was started at 65°C by adding 10 μl pure enzyme and was continued for 2 h. Preliminary investigation (by TLC analysis) confirmed that even at maximum glucose concentration all of the donor was consumed in 2 h. Accurate blank experiments were conducted in parallel in the absence of enzyme or donor. Glucose oxidase peroxidase (GOD-POD) analysis permitted evaluation of the remaining free glucose in the reaction mixture and calculation, as yield, of total xylosylated glucose. Yields are relative to the minor component in the reaction mixture.

The preparative experiment was conducted as follows: in 50 ml sodium acetate buffer (500 mM, pH 5.6), 8.526 g (47.3 mmol) glucose was solubilized, and to this solution 1,800 mg (11.8 mmol) α-fluoroxylose was added. The reaction was started at 65°C under stirring after the addition of 13 ml prepurified XylS. The reaction was strictly monitored by TLC (EtOAc/MeOH/H_2O, 47:40:13 by volume), and at total donor consumption the whole reaction mixture was chromatographed on a RP-18 reverse-phase glass column.

Final purification of isoprimeverose was obtained by peracetylation of crude disaccharide and silica gel column chromatography (*n*-hexane/EtOAc, 8:2 and 7:3 v/v). The

yields after acetylation in this semipreparative scale synthesis averaged 10%–12%. Small amounts (<5%) of regioisomers were also formed and easily purified by the same column chromatography. After acetylation, the product is obtained as a mixture, at the reducing glucose end, of β- and α-forms in a ratio of about 3:1. Diagnostic ^{13}C- and ^{1}H-NMR correlation signals observed were δ 96.1–5.01 (C-1/H-1, α-Xyl, β-form at reducing glucose end); 91.5–5.66 (C-1/H-1, β-Glu, β-form); 95.7–5.01 (C-1/H-1, α-Xyl, α-form); 89.6–6.03 (C-1/H-1, α-Glu, α-form); 58.45–3.63/3.72 (C-5/H-5, α-Xyl, β- and α-forms); and 66.24–3.60/3.74 (C-6/H6 Glc, β- and α-forms). The remaining signals in the proton and carbon spectra of acetylated isoprimeverose are in agreement with the structure proposed but were not selectively assigned.

Enzymatic hydrolysis of xylogluco-oligosaccharides

For this step, 3 mg XXXG or GXXG was dissolved in 300 µl 50 mM acetate buffer, pH 5.6, and the reaction was started at 60°C after adding 50 µl prepurified XylS. Blank experiments were conducted without enzyme on isoprimeverose and XXXG and GXXG. The reaction was monitored by TLC (EtOAc/MeOH/H_2O, 47:40:13 by volume) to total consumption of substrate as judged by TLC (5 h). Identity of products was secured by fingerprint analysis of the ^{13}C-NMR spectra of the total reaction mixture and authentic XXXG and GXXG.

Enzymatic syntheses of the trisaccharidic unit of xyloglucan and isomers

For this step, 0.71 mmol α-fluoroxylose dissolved in 3 ml sodium acetate buffer (500 mM, pH 5.3) was mixed with 0.11 mmol PNP-β-cellobioside, and 0.5 ml prepurified XylS was added. The reaction was started under stirring at 65°C and stopped when the fluorinated xylose donor (TLC: EtOAc/MeOH/H_2O, 70:20:10 by volume) was consumed. A prepurification step on a RP-18 column as described for the synthesis of compounds 1 and 2 (see Fig. 1) was conducted on the reaction mixture, obtaining approximately 10 mg (15% yield based on the acceptor) of a mixture of PNP trisaccharides in which a xylose molecule was added on PNP-β-cellobioside as indicated by preliminary ^{13}C-NMR spectra of the mixture.

Acetylation (Ac_2O/pyridine, 24 h at room temperature) of this mixture and analysis of the peracetylated compounds by TLC (*n*-hexane/EtOAc, 6:4 v/v) showed the presence of three single spots well separated at R_f 0.1 (acetylated derivative of compound 4, 3 mg; see Fig. 1), 0.18 (acetylated derivative of compound 3, 8 mg, Fig. 1), 0.27 (acetylated derivative of compound 5, 2.4 mg, Fig. 1), purified by preparative TLC, and characterized by one- and two-dimensional NMR spectroscopy. For compound 3 (acetylated derivative), ^{13}C-NMR signals of the sugar moiety were 99.9, 97.78, 96.20, 75.2, 73.1 (×2), 72.7, 72.3, 71.6, 71.1, 70.9, 69.2, 69.0, 68.9, 67.2, 61.7, and 58.9; for compound 4 (acetylated derivative), ^{13}C-NMR signals of the sugar moiety were 100.8, 97.7, 96.4, 77.9, 75.6, 73.2, 72.1 (×2), 72.0, 71.2, 71.01, 68.9, 68.8, 68.7, 61.8, 61.5, and 58.9; and for compound 5 (acetylated derivative), ^{13}C-NMR signals of the sugar moiety were 100.3, 97.8, 95.9, 75.9, 75.4, 73.1, 72.4, 72.3, 72.1, 72.0, 71.1, 70.1, 68.8, 68.7, 62.8, 61.6, and 59.4. Assignment of diagnostic signals from ^{1}H- and ^{13}C-NMR spectroscopy correlation studies is described next in the Results and discussion section.

Results and discussion

The PNP derivative of isoprimeverose (compound 1; see Fig. 1) was previously synthesized by chemical means (Sone et al. 1989). The free disaccharide isoprimeverose (compound 2, see Fig. 1), to the best of our knowledge, is not commercially available and has been obtained only by the enzymatic hydrolytic action of *endo*-β-1,4-glucanase, β-galactosidase, and isoprimeverose-producing oligoxyloglucan hydrolase activities on xyloglucan with time-consuming purification and biological elimination of contaminant monosaccharides (Chaillou et al. 1998). The enzymatic synthesis of the PNP-β derivative of the trisaccharide unit of xyloglucan (compound 3, Fig. 1) by transxylosylation has not been described elsewhere.

Family 31 of the α-glycosyl hydrolases, to which XylS belongs (Moracci et al. 2000), follows a retaining reaction mechanism in two steps: in the first step, the enzyme catalyzes the departure of the aglycon group from the substrate (donor) and the consequent formation of a glycosyl ester intermediate. In the second step, the enzyme is deglycosylated by a nucleophile (acceptor) that attacks the anomeric carbon of the donor and cleaves the covalent intermediate, leading to the overall retention of the anomeric configuration of the substrate. When a nucleophile different from water intercepts the glycosyl enzyme intermediate, transglycosylation occurs, producing glycosylated products (Crout and Vic 1998; Mayer et al. 2000).

The previously reported general characterization of the enzyme XylS indicated that this new α-xylosidase from *S. solfataricus* efficiently hydrolyzes PNP-α-xyloside (Moracci et al. 2000). We took advantage of this preference for the aryl substrate as a donor by using the enzyme in transxylosylation mode for the synthesis of interesting compounds. Syntheses also occurred using α-fluoroxylose as donor. The acceptors used were PNP-β-D-glucoside, PNP-β-cellobioside, and free glucose.

Enzymatic synthesis of *p*-nitrophenyl β-D-isoprimeveroside

Synthesis of the disaccharide PNP-β-D-isoprimeveroside was performed using PNP-β-D-glucoside and PNP-α-D-xyloside as acceptor and donor, respectively. The xylosyl enzyme intermediate formed by the reaction of donor and enzyme was cleaved by the acceptor to form compound 1 (Fig. 1), characterized by two-dimensional NMR spectroscopy. Different regioisomers in which xylose was transferred to different glucose positions (probably the OH in compounds 3 and 4) are formed in trace amounts and are

visible in the total reaction mixture before purification by TLC but were not isolated and characterized.

Inspection of the XylS kinetic constants data (Moracci et al. 2000) could in principle discourage ascribing any synthetic potential to the biocatalyst because the product of the transxylosidic reaction (PNP-β-D-isoprimeveroside) seems to be a better substrate than the donor used (PNP-α-D-xyloside), with values of 9.30 versus 0.28 K_{cat}/K_M, respectively. However, this preference could explain the rather low yield (11%) observed in the semipreparative experiments, likely a result of balanced activity of the enzyme for hydrolysis of the substrate and product in the synthetic reaction conditions (i.e., in the presence of 0.67 M of acceptor), conditions that are very different from those in which the kinetic parameters were calculated.

Enzymatic synthesis of isoprimeverose

The production of free isoprimeverose, compound 2 (see Fig. 1), starting from compound 1 (Fig. 1), was previously obtained by selective enzymatic cleavage of the β-bond (Moracci et al. 2000). For the direct enzymatic synthesis of isoprimeverose, free glucose and α-xylosyl fluoride were used as acceptor and donor, respectively. Glycosyl fluorides, similar to aryl glycosides, are very reactive and are commonly used as substrates for glycosidases. Glucose is inexpensive, and its determination at the end of the reaction permits estimation of the yields of the synthetic reactions by calculating the amount of enzymatically xylosylated glucose.

Preliminary experiments were conducted on an analytical scale to assess the best glucose/xylosyl fluoride ratio. The set of experiments in which molar ratios were 1, 2, and 5 indicated that the yield of the reaction was not greatly affected by increasing glucose concentration from 0.1 to 0.5 M, ranging from 10% to 16% even at the highest glucose concentration used. Similar yields were obtained in experiments in which the ratio of acceptor to donor is less than 1, namely, 0.5, 0.25, and 0.1: the product was synthesized even in the reaction conducted at 0.01 M glucose, as judged by TLC analysis of the reaction mixture. Moreover, no dixylosyl fluoride (coupling of the donor) or extensive chemical degradation of the donor itself occurred in the reaction mixture (500 mM sodium acetate buffer, 65°C, 2 h).

The body of these results leads to the conclusion that the similarity of the yields in the two sets of experiments represents an intrinsic characteristic for the transferring action of this enzyme as a result of balanced activity on substrate and product in the adopted reaction conditions. The yield for isoprimeverose in the reaction conducted on a semipreparative scale after acetylation was 10%–12% with respect to the α-fluoroxylose used. Less than 5% of regioisomers were removed by chromatographic purification, and the free isoprimeverose was used for enzymological and microbiological studies (Moracci et al. 2000). Although the activity of XylS was not depressed at the high salt concentration (500 mM acetate buffer; see Material and methods) adopted with fluorine-possessing donors, inhibition of enzymatic activity was reported in the presence of organic solvents (Moracci et al. 2000), preventing further investigation following this approach.

Selectivity of XylS in the hydrolysis of xylogluco-oligosaccharides

We have recently reported that XylS, in the presence of xyloglucan oligosaccharides, promotes the release of trace amounts of xylose (Moracci et al. 2000). However, the heterogeneity of the xyloglucan oligosaccharide mixture, obtained after endoglucanase action on the natural polymer, hampered any precise estimation of the efficiency and molecular specificity of the hydrolytic action of our thermophilic α-xylosidase. The mode of action of XylS was studied here with two simple model compounds, 6 and 7 (Fig. 1), termed XXXG and GXXG, respectively, according to the adopted nomenclature (Fry et al. 1993).

Clear results were obtained after analysis of the hydrolysis products by TLC and ^{13}C-NMR spectroscopy of reaction products: xylose was obtained by enzymatic action only from X-ending compound 6, which was completely transformed at the end of the reaction in compound 7. No xylose or glucose was obtained from G-ending compound 7, which is not a substrate for the enzyme. This result indicates a sharp selectivity of the enzyme for the position of xylose at the nonreducing end of the molecule, leading to the possible exploitation of XylS in the synthesis of XG and derivatives.

Synthesis of trisaccharides

PNP-β-cellobioside is the model acceptor chosen for constructing the trisaccharidic unit of xyloglucan and for testing the selectivity of xylose transfer with respect to the internal or external glucose molecule of the donor. Because of the seven free OH groups of PNP-β-cellobioside, this synthesis is challenging because of the possible formation of multiple products that are difficult to purify. In our case, only three of seven compounds are formed in 15% yields, compounds 3, 4, and 5 (Fig. 1); their acetylated derivatives are easily purified and characterized by NMR spectroscopy. Compound 3 is the most abundant in the reaction mixture (60%).

In the ^{13}C-NMR spectra (see Material and methods section) of the total mixture, signals from the acetylated α-linked xylose molecule are present; two of these signals that best characterize the xylose moiety are the C-5, at approximately 58 ppm, and the anomeric carbon signal at about 96 ppm. The latter signal is related , in the ^{1}H/^{13}C correlation spectra, to the anomeric proton signal, with J ~3.6 Hz establishing the α-configuration of the xylose enzymatically attached. The interglycosidic linkages in the three trisaccharides were established by complete assignment of signals of the acetylated PNP-β-cellobioside and comparison with spectra of pure acetylated compounds 3, 4, and 5 (Fig. 1).

Confirmation and securing of the assignment of signals in the spectra of trisaccharides were obtained by COSY and ^{1}H/^{13}C correlation spectra analysis as follows. In the spectra of acetylated derivatives of the most abundant product (60%), compound 3 (Fig. 1), three methylene signals are

present: one at 61.79/4.54–4.13 ppm for acetylated C-6 of glucose directly attached to PNP, one for xylose at 58.9 ppm, and the last for a nonacetylated (i.e., xylosylated) methylene signal at 67.2/3.65–3.72 ppm that in the COSY spectrum is assigned, through pyranose ring coupling, to the C-6 of the external glucose of aryl cellobioside (anomeric signals: 4.58/99.9 ppm). This kind of analysis established that xylose in compound 3 (Fig. 1) is α-linked to O-6 of the more external glucose molecule. Similar spectroscopic studies were conducted on compounds 4 and 5 (Fig. 1): in compound 4, the xylose is attached on O-3 (77.9/3.82 ppm), and in compound 5, xylose is attached on O-4 (72.4/4.03), always on the external glucose molecule. This finding demonstrates that the exo-acting characteristic of this enzyme is also operative in the synthetic mode as is found in the hydrolytic reaction.

Conclusion

This article shows evidence that the new enzyme XylS from the archaeon *Sulfolobus solfataricus* has transxylosidic activity and is able to synthesize α-xylosides. The attachment of xylose is greatly favored on the oxygen of the C-6 of (i) a free glucose molecule, (ii) the PNP-β derivative of glucose, and (iii) the PNP-β derivative of cellobiose. In the latter case, exclusive functionalization of the nonreducing end glucose unit results from the absolute exo-acting characteristic of this enzyme, as is also observed in the hydrolytic mode of action on XXXG.

The transferring capability has been exploited for the synthesis of isoprimeverose and its β-aryl derivative, which have been of great utility for enzymatic characterization of XylS and in the study of its expression in vivo when the thermophilic archaeon is grown on isoprimeverose as the sole carbon source. A degree of complexity is added when PNP-β-cellobioside is used as acceptor: only three trisaccharides were formed, the most abundant (60%) being a trisaccharide that is the building unit of the xyloglucan.

Interest in these molecules is high because they can be useful substrates for the study of xyloglucanases, for NMR spectroscopy and chromatographic studies (A. Faik, personal communication) in the characterization of xyloglucan fragments (Watt et al. 2000), and for conformational and interaction studies of the polymer with cellulose in natural sources (Lorences and Fry 1994; Vincken et al. 1995).

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