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Carbohydrate Research 338 (2003) 313–325
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Enzymatic synthesis of β-xylanase substrates: transglycosylation reactions of the β-xylosidase from *Aspergillus* sp.

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Received 31 May 2002; accepted 30 October 2002

Abstract

A β-D-xylosidase with molecular mass of 250 ± 5 kDa consisting of two identical subunits was purified to homogeneity from a cultural filtrate of *Aspergillus* sp. The enzyme manifested high transglycosylation activity in transxylosylation with *p*-nitrophenyl β-D-xylopyranoside (PNP-X) as substrate, resulting in regio- and stereoselective synthesis of *p*-nitrophenyl (PNP) β-(1→4)-D-xylooligosaccharides with dp 2–7. All transfer products were isolated from the reaction mixtures by HPLC and their structures established by electrospray mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy. The glycosides synthesised, β-Xyl-1→(4-β-Xyl-1→)$_n$4-β-Xyl-$OC_6H_4NO_2$-*p* ($n = 1–5$), were tested as chromogenic substrates for family 10 β-xylanase from *Aspergillus orizae* (XynA) and family 11 β-xylanase I from *Trichoderma reesei* (XynT) by reversed-phase HPLC and UV-spectroscopy techniques. The action pattern of XynA against the foregoing PNP β-(1→4)-D-xylooligosaccharides differed from that of XynT in that the latter released PNP mainly from short PNP xylosides (dp 2–3) while the former liberated PNP from the entire set of substrates synthesised.

Keywords: β-D-Xylosidase; *Aspergillus* sp.; transglycosylation; *p*-Nitrophenyl β-(1→4)-D-xylooligosaccharides; β-Xylanase substrates

1. Introduction

Many biologically important compounds including various oligosaccharides, glycoconjugates and neoglycoproteins can readily be synthesised using the transglycosylating potency of glycosidases.[1,2] The application of the strategy of enzyme-mediated synthesis is becoming more and more popular in view of its obvious advantages over traditional organic synthesis.[3,4] Of the retaining glycosyl hydrolases (glycosidases) employed in enzymatic synthesis, microbial β-D-xylosidases (β-(1→4)-D-xylan xylohydrolase, E.C. 3.2.1.37) have been utilized, for instance, in the synthesis of a series of alkyl xylosides using various alcohols as acceptors.[5,6] The enzyme from *Aspergillus awamori* was used for the production of 6-*O*-β-D-xylosyltrehalose and 6,6′-di-*O*-β-D-xylosyltrehalose;[7] a structural analogue of primeverose, namely, 6-*O*-β-D-xylopyranosyl-2-acetamido-2-deoxy-D-glucopyranose, was obtained by transglycosylation reaction catalysed by *A. niger* β-xylosidase with a yield of 30%.[8] Due to their high stability[9] and low commercial cost,[10] β-xylosidases from filamentous fungi are preferable to specific β-xylosyl transferases from plants[11] and micro-organisms[12] for the synthesis of xylosyl-containing oligosaccharides. Although β-xylosyl transferases are now well-characterised, the high cost of the donor substrate, namely, UDP-xylose, makes large-scale applications difficult.[13] From a practical viewpoint, the use of glycosyl donors with simpler activated leaving groups, such as, *p*-nitrophenyl β-D-xylopyranoside, for β-xylosidase-catalysed transxylosylations is more attractive.[8]

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0008-6215/03/$ - see front matter
PII: S0008-6215(02)00467-6

Enzymatic synthesis using exo-glycosidases is efficient, as a rule, for the production of di- and trisaccharides,[14–16] more rarely, tetrasaccharides;[17] syntheses of spacer-linked oligosaccharides for the preparation of neoglycoproteins[18] and glycosyl-containing drugs[19] and additives for foods[20] have been reported in single instances. This paper describes the successful application of an exo-glycosidase for the regio- and stereoselective synthesis of fairly long oligosaccharides, with a degree of polymerization up to 7. We have isolated and purified β-xylosidase from *Aspergillus* sp. capable of efficiently producing *p*-nitrophenyl β-(1→4)-xylooligosaccharides with dp 2–7 by substrate transxylosylation using *p*-nitrophenyl β-D-xylopyranoside. Compared to traditional β-xylanase substrates, such as xylans and Remazol Brilliant Blue xylan,[21] β-(1→4)-xylooligosaccharides coupled to a *p*-nitrophenyl chromophore are more convenient substrates for the enzymatic studies of β-xylanases (E.C. 3.21.8) due to the possibility of direct spectrophotometric monitoring of the reaction by following PNP liberation or using high performance liquid chromatography (HPLC) for analysis of PNP-containing products of the enzymatic reaction. Here we describe the β-xylosidase-mediated synthesis of the above-mentioned PNP β-(1→4)-D-xylooligosaccharides and the action of some xylanases of families 10 and 11 on the compounds obtained.

2. Results and discussion

2.1. Reaction of substrate transglycosylation with PNP-X and structures of the reaction products

A secreted β-D-xylosidase capable of hydrolyzing PNP-X was isolated from the cultural liquid of *Aspergillus* sp. and purified 1500-fold in four chromatographic steps. About 10% of the initial activity was recovered and the purified enzyme had a specific activity of 4.2 ± 0.1 U/mg. β-Xylosidase obtained after final purification step showed a single band at SDS-PAGE with a molecular mass of 125 ± 1 kDa while gel-permeation chromatography on Superose 12 revealed a molecular mass of 250 ± 5 kDa. Comparison of molecular masses obtained in the analytical gel filtration and SDS-PAGE showed that the enzyme consists of two idential 125 kDa subunits. Pure β-D-xylosidase contained less than 0.1% of extrinsic exo-glycosidase activities (α-/β-glucosidase, α-/β-galactosidase), less than 0.1% of activity with respect to xylan, CM-cellulose, (1→3),(1→4)-β-glucans and laminarin. The enzyme retains its activity on storage at 4 °C for a year when freeze-dried. The β-D-xylosidase manifested hydrolytic activity towards PNP-X with $K_m = 4.1 \pm 0.05$ mM and $V_{max} = (5.3 \pm 0.1)$ nmol/(min μg) at pH 4.5.

The purified β-D-xylosidase catalysed substrate transglycosylation with PNP-X, which acts both as a glycosyl donor and acceptor, over the concentration range 10–110 mM. The substrate transglycosylation products were analysed by analytical reversed-phase HPLC and thin layer chromatography (TLC), which demonstrated that a series of *p*-nitrophenyl xylooligosaccharides with different dp was formed. All of the PNP-containing transxylosylation products were purified by HPLC to greater than 95% purity and subjected to detailed structural investigations using ^1H and ^{13}C NMR techniques and electrospray mass spectrometry.

In order to maximize positive ion sensitivity by favouring the formation of a single adduct,[22] PNP-xylooligosaccharides were infused into the mass spectrometer from methanol/water solutions containing sodium chloride. Consequently, the ESI-TOF MS spectrum of each purified oligosaccharide showed only peaks corresponding to the $[M + Na]^+$ ion and associated isotopic peaks. Peaks arising from hydrogen or potassium adducts were not observed, and the formation of disodiated adducts, i.e., $[M + 2Na]^{2+}$, and monosodiated dimers, that is, $[2M + Na]^+$, was also

Table 1
Calculated and observed sodium ion adduct masses for PNP xylooligosaccharides obtained by TOF MS and lock mass calibrated MS/MS

Substrate	$[M+Na]^+$ calcd (u)	TOF MS [a]		MS/MS [b]	
		$[M+Na]^+$ observed (u)	Error (ppm)	$[M+Na]^+$ observed (u)	Error (ppm)
Xyl_2-PNP	426.1012	426.1405	92.2	426.1042	7.0
Xyl_3-PNP	558.1435	558.1883	80.3	558.1476	7.3
Xyl_4-PNP	690.1858	690.2634	112.4	690.1908	7.2
Xyl_5-PNP	822.2280	822.3053	94.0	822.2307	3.3
Xyl_6-PNP	954.2703	954.3560	89.8	954.2643	6.3
Xyl_7-PNP	1086.3125	1086.3774	59.7	1086.3101	2.2

[a] External mass calibration using CsI and NaI ion clusters over m/z 130–1980.
[b] Lock mass calibrated using the sodiated B_2 daughter ion (287.0743 u) as the internal reference mass.

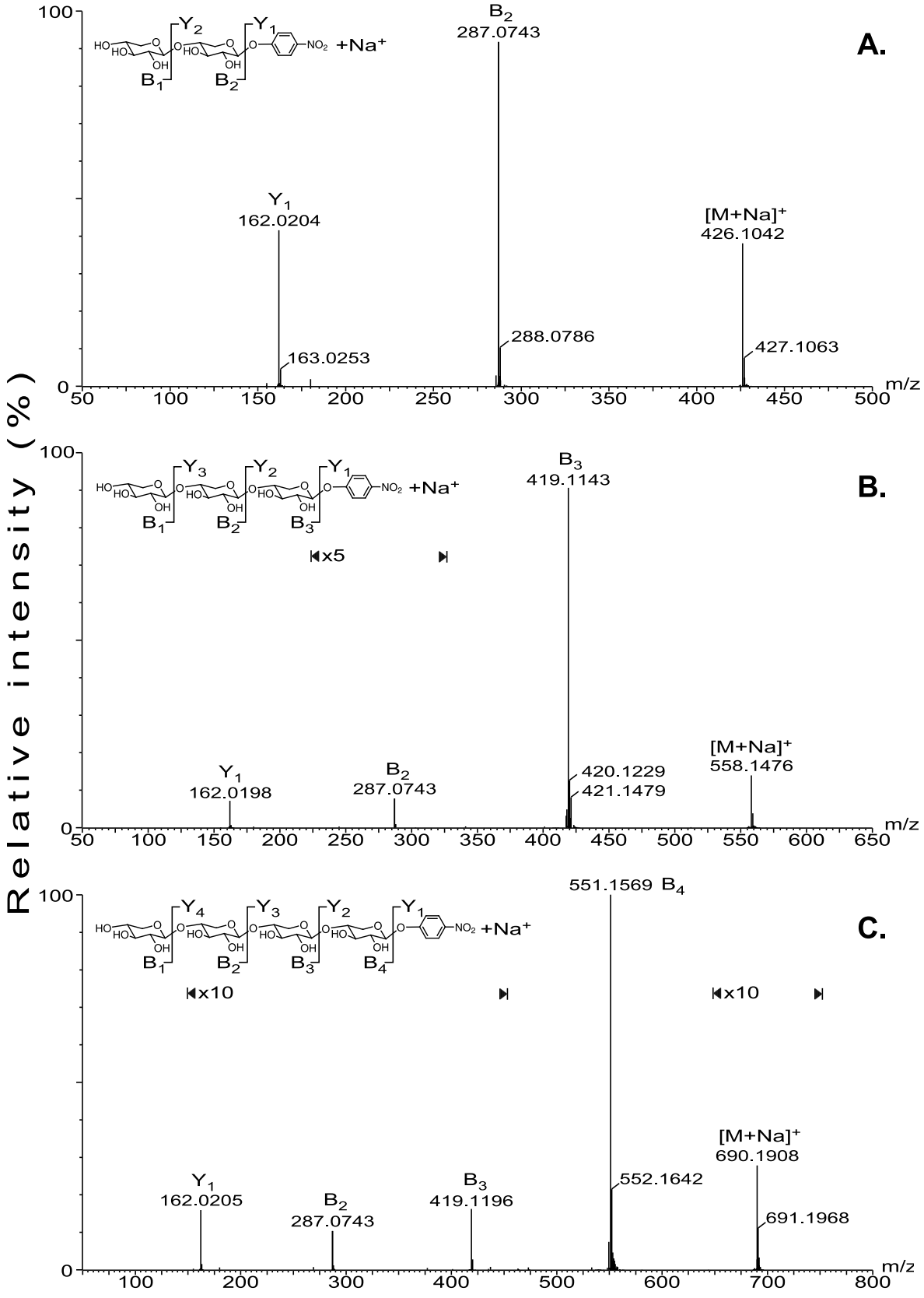

Fig. 1. MS/MS spectra of PNP-X_2 (**A**), PNP-X_3 (**B**), and PNP-X_4 (**C**).

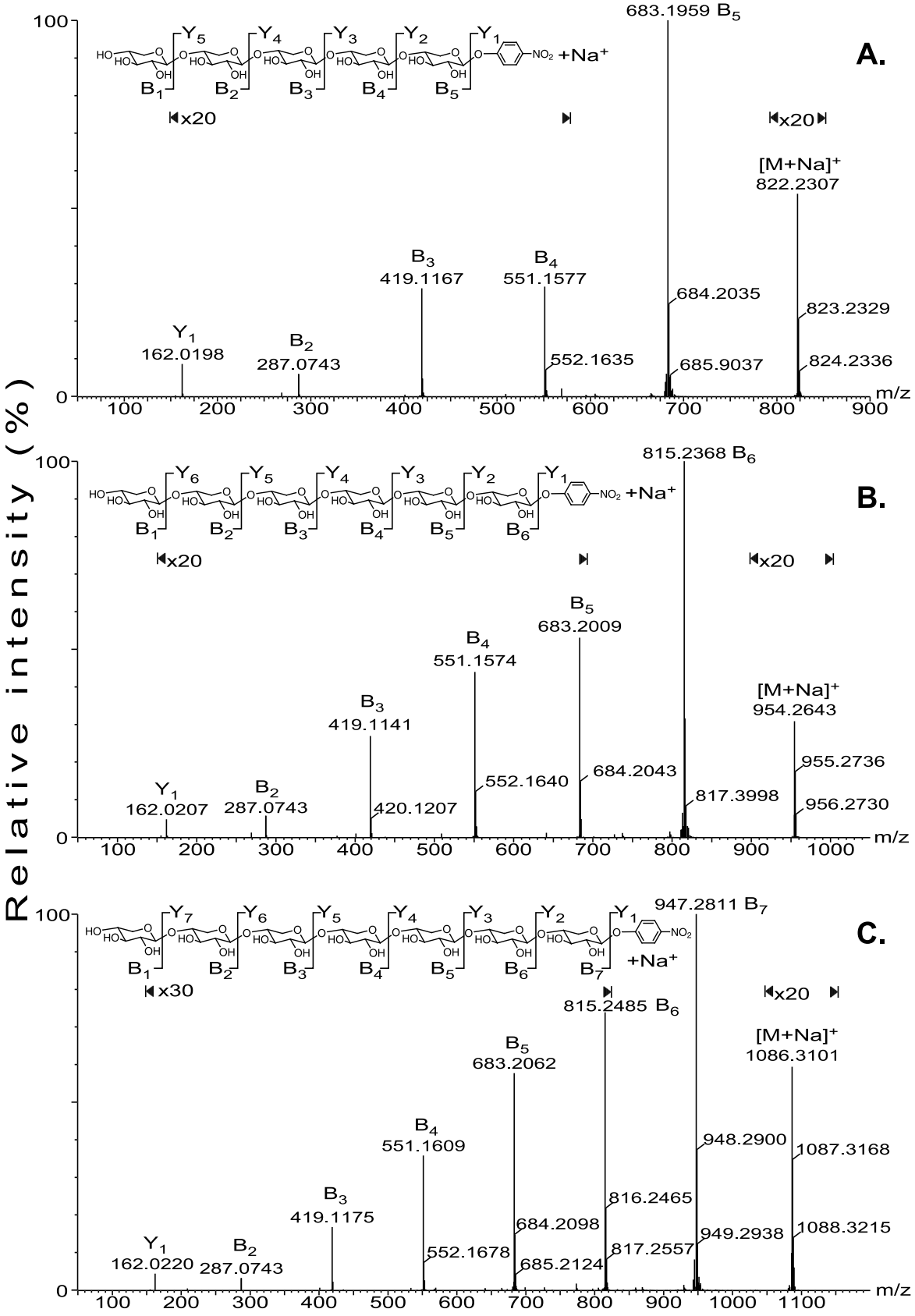

Fig. 2. MS/MS spectra of PNP-X_5 (**A**), PNP-X_6 (**B**), and PNP-X_7 (**C**).

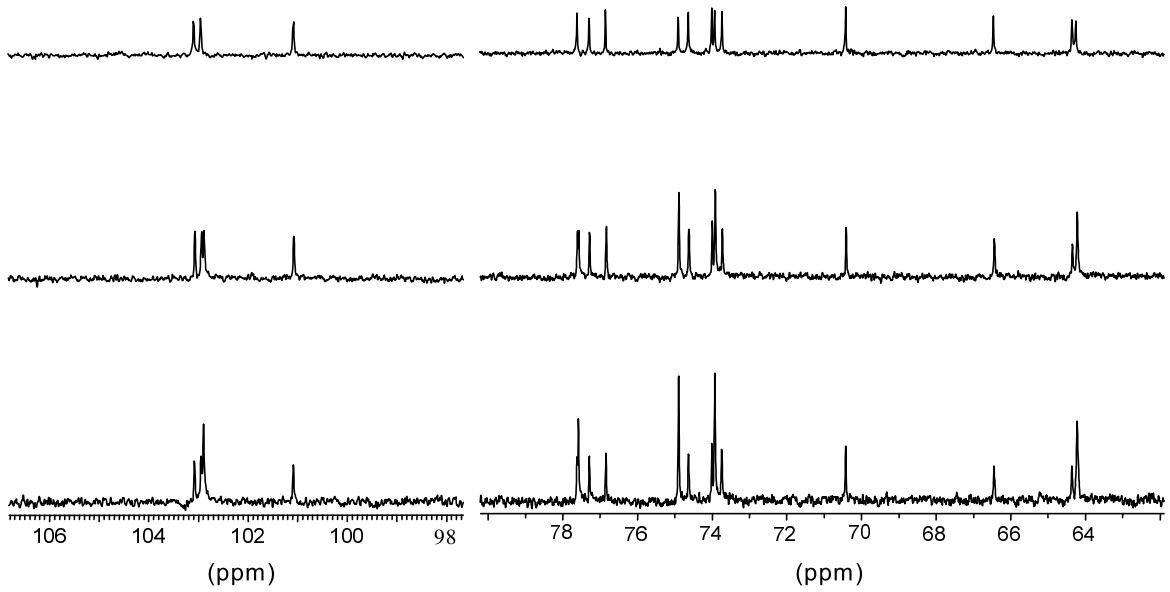

Fig. 3. ^{13}C NMR spectra of PNP-X_2 (top), PNP-X_3 (middle), and PNP-X_4 (bottom).

suppressed under the conditions used. Table 1 lists the theoretical masses of the $[M + Na]^+$ ions of PNP-X_2 to PNP-X_7 and those obtained by ESI-TOF MS.

To provide further evidence for the proposed structures of the PNP-xylooligosaccharides, MS/MS experiments were carried out on each of the monosodiated molecular ion peaks. The quadrupole analyzer of the mass spectrometer was set to allow transmission of the $[M + Na]^+$ ion and related isotopic peaks and the collision energy was adjusted to produce daughter ion whilst allowing some parent ions to pass unfragmented. Figs. 1 and 2 show the resulting centroid mass spectra obtained by centering combined continuum spectra of each compound. The predominant fragmentation observed for each compound was the loss of neutral *p*-nitrophenol upon glycosidic bond cleavage to yield a sodiated B-series ion (nomenclature according to Ref. 23). In each spectrum, a sodiated B-ion series is readily identified from B_n–B_2, where n corresponds to the number of xylose units in the oligosaccharide. Also present in each spectrum is a peak of m/z corresponding to the sodium adduct of *p*-nitrophenol (Y_1 fragment). The fragmentation pattern thus obtained for each compound in the series clearly indicates the stoichiometry of xylose residues with increasing molecular mass.

In order to obtain a higher mass accuracy than can be achieved through calibration of the TOF analyzer with an external standard alone, a lock mass calibration was applied during the generation of the centroid MS/MS spectra from continuum data. The sodiated B_2 ion (287.0743 u) present in all spectra was chosen as the reference peak. Application of this calibration resulted in the reduction of mass errors on the parent and fragment ions to below 10 ppm, which further corroborates structural assignments. Accurate mass and corresponding mass error values for the parent ions are shown in Table 1.

Data from NMR spectroscopy of the oligosaccharides with dp 2–5 corroborate completely their structures (Tables 2 and 3; Fig. 3)

All peaks in the ^1H and ^{13}C NMR spectra could be assigned so that it was possible (i) to delineate the spin system for each individual β-D-xylopyranose residue and (ii) to establish intersaccharide connectivities. The spin–spin coupling constants of vicinal protons available from 1D ^1H NMR spectra had values indicative of β-D-xylopyranose residues ($J_{1,2}$ 7.2–8.0; $J_{2,3}$ 9.2–9.5; $J_{3,4}$ 8.8–9.3; $J_{4,5a}$ 10.0–10.9; $J_{4,5b}$ 5.0–5.5 Hz). The β anomeric configurations of all the monosaccharide residues followed also from the chemical shift values for the anomeric carbon atoms.

Correlation spectroscopy (COSY) was used for identifying groups of protons belonging to separate xylopyranosidic residues. This was facilitated by the fact that the anomeric proton of the glycoside ('reducing') unit is the most downfield shifted as compared with other anomeric protons.

The relationships between sets of signals belonging to glycosidically-linked residues were established using the 1D NOE technique in a difference mode with pre-irradiation of anomeric protons. For instance, the following connectivities were established for the trioside: δ 4.47 (H-1″)/3.80 (H-4′) and 4.51 (H-1′)/3.89 (H-4). The marked signal enhancement for the H-3 and H-5a atoms of the respective residues is additional evidence of the β configuration of the glycosidic bonds. The type of the glycosidic linkages in the oligosaccharides ob-

Table 2
Chemical shifts obtained from ^{1}H NMR spectra of PNP xylooligosaccharides

	PNP-X_2		PNP-X_3			PNP-X_4				PNP-X_5				
	X_1	X_2	X_1	X_2	X_3	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	X_5
H-1	5.2080	4.4863	5.2388	4.5108	4.4695	5.2448	4.5159	4.4958	4.4680	5.2494	4.5134	4.4920	4.4859	4.4621
H-2	3.6458	3.2940	3.6567	3.3239	3.2624	3.6661	3.3327	3.3069	3.2683	3.6650	3.3275	3.3017	3.2993	3.2616
H-3	3.7138	3.4502	3.7182	3.5702	3.4340	3.7252	3.5790	3.5683	3.4390	3.7224	3.5737	3.5649	3.5607	3.4325
H-4	3.8775	3.6469	3.8925	3.8025	3.6292	3.8988	3.8307	3.7961	3.6351	3.8988	3.8070	3.7951	3.7898	3.6293
H-5a	4.1759	3.9939	4.1856	4.1259	3.9759	4.1917	4.1319	4.1180	3.9800	4.1904	4.1277	4.1139	4.1098	3.9746
H-5b	3.6067	3.3271	3.6186	3.3935	3.3140	3.6219	3.4005	3.3916	3.3158	3.6214	3.3961	3.3871	3.3830	3.3105
$J_{1,2}$	7.5	7.9	7.5	7.7	7.9	7.3	7.7	7.8	7.9	7.3	7.74	7.75	7.79	7.80
$J_{2,3}$	9.3	9.4	9.3	9.4	9.3	9.3	9.3	9.3	9.3	9.3	9.3	9.3	9.30	9.40
$J_{3,4}$	8.7	9.0	8.8	9.0	8.8	8.9	9.1	8.9	9.0	8.7	8.8	8.7	8.5	9.1
$J_{4,5a}$	5.3	5.4	5.2	5.1	5.5	5.3	5.3	5.4	5.5	5.27	5.5	5.6	5.3	5.5
$J_{4,5b}$	10.2	10.4	10.0	10.3	10.5	10.3	10.3	10.3	10.6	10.05	10.25	10.40	10.25	10.6
$J_{5a,5b}$	11.8	11.7	11.8	12.0	11.6	11.8	12.0	11.9	11.6	11.7	11.8	11.85	11.8	11.60

Table 3
Chemical shifts obtained from ^{13}C NMR spectra of PNP xylooligosaccharides

	PNP-X_2		PNP-X_3			PNP-X_4				PNP-X_5				
	X_1	X_2	X_1	X_2	X_3	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	X_5
C-1	101.040	103.130	101.041	102.929	103.067	101.072	102.932	102.885	103.067	101.085	102.946	102.892	102.892	103.081
C-2	73.697	733.994	73.711	73.903	73.984	73.718	73.913	73.913	73.987	73.738	73.927	73.927	73.927	74.001
C-3	74.614	76.825	74.608	74.877	76.829	74.884	74.614	74.614	76.832	77.629	74.891	74.891	74.891	76.839
C-4	70.408	77.311	77.257	77.584	70.398	77.277	77.601	77.567	70.401	77.284	77.614	77.574	77.574	70.415
C-5	64.327	66.471	64.325	64.213	66.431	64.341	64.213	64.213	66.439	64.355	64.213	64.213	64.213	66.445

tained followed unequivocally also from the positions of the signals for C-5 in the ^{13}C NMR spectra. Indeed, on going from the lower to higher oligosaccharides, the number of the higher-field signals due to the β-effect of glycosylation increases progressively. This suggests the presence of only (1→4)-linkages and therefore the linear structures for the oligosaccharides.

Assignments of the signals for carbon atoms were made using DEPT and HMQC technidues from the assigned signals for the protons taking into account (i) the characteristic downfield shifts—the α-glycosylation effects—for the C-4 atoms bearing carbohydrate substituents as compared with those of the corresponding non-glycosylated (terminal) units and (ii) characteristic upfield shifts—the β-glycosylation effects—for C-3 and C-5 of the glycosylated xylopyranose units.

The ^{13}C NMR spectra of the di-, tri- and tetra-saccharides prepared by transxylosylation virtually coincided with those prepared by chemical synthesis.[24]

As shown, reaction of substrate transglycosylation catalysed by *Aspergillus* β-D-xylosidase occurs with high regio- and stereoselectivity, resulting in only β-(1→4)-linked PNP xylooligosaccharides. All data taken together allow us to conclude that PNP-X transxylosylation reactions catalysed by the β-D-xylosidase under consideration yielded at least six products with the general formula β-Xyl-1→(4-β-Xyl-1→)$_n$4-β-Xyl-$OC_6H_4NO_2$-*p* ($n = 0–5$).

PNP glycosides are widely used in transglycosylation reactions catalysed by various glycosidases[25] and are effective donors for the enzymatic synthesis of different di- and trisaccharides.[26] *p*-Nitrophenyl and *o*-nitrophenyl glycosides are generally useful both because they are 'fast' substrates for exo-glycosidases which hydrolyse the aryl glycosidic bonds with retention of the anomeric configurations,[27] and because the hydrophobic nature of the aglycon allows for efficient isolation of individual products from enzymatic syntheses.[27,28] However, the β-D-xylosidase from *Aspergillus* sp. described here differs in some respect from other glycosidases reported earlier. In the case of α-galactosidases from *Bacillus stearothermophilus* and *Thermus brockianus*,[25] only *p*-nitrophenyl α-(1→6)- and α-(1→3)-digalactosides were formed as a result of transgalactosylation with PNP α-galactoside as a substrate. β-Galactosidases from *E. coli*[26] and porcine liver produce PNP β-(1→6)-galactobiosides.[29] PNP α-galactotrioside was obtained in addition to the corresponding PNP galactobioside using an α-galactosidase from *Trichoderma reesei*, while PNP galactooligosaccharides with higher dp were not detected among the transglycosylation products.[30] The only successful attempt to use enzymic transglycosylation to produce oligosaccharides with dp greater than 3 was reported using β-galactosidase from *Bacillus circulans*.[29] With the aid of this enzyme, PNP galactooligosides with dp up to 6 were obtained with a high stereospecificity. Therefore, we suggest that in our case the success in high-yield production of higher PNP xylooligosaccharides is associated with the specific structure of the active centre of the β-D-xylosidase. Investigations into structure–function aspects of transglycosylation activity of the enzyme are in progress now.

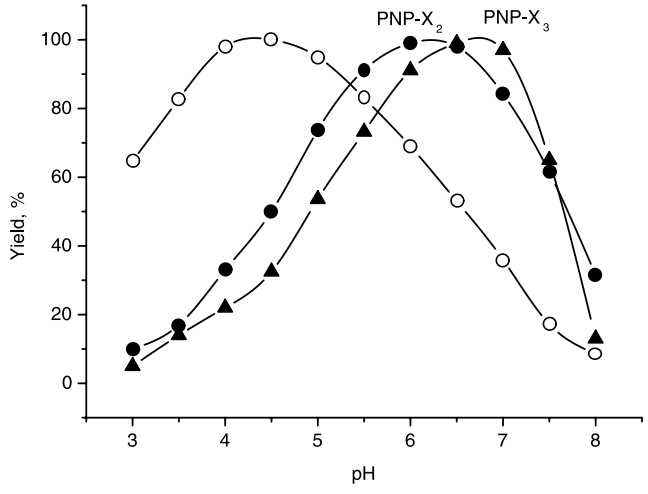

Fig. 4. pH-Dependences of the hydrolytic activity of the β-xylosidase from *Aspergillus* sp. (○) and yeilds of transglycosylation product formation: PNP-X_2 (●) and PNP-X_3 (▲).

2.2. Enzymatic synthesis PNP β-(1→4)-D-xylooligosaccharides

We investigated conditions of the enzymatic synthesis that allowed us to obtain maximum yields of PNP β-xylooligosaccharides with dp 2–7. The pH-dependences of the formation of all PNP β-xylooligosaccharides were investigated in the range of pH from 3 to 8.0. Quantitative reversed-phase HPLC analysis of all PNP β-xylooligosaccharides formed (dp 2–7) showed that the ratio of the transfer products was independent of pH in this range; pH-optimum of transglycosylation was about 6.5. Fig. 4 shows pH-dependences of formation of PNP-X_2 and PNP-X_3 by the reaction of substrate transglycosylation. It is noteworthy that the pH-optimum of the transglycosylation activity differs from that of the hydrolytic activity displayed by the enzyme under investigation (Fig. 4).

PNP xylobioside and PNP xylotrioside were efficiently produced by substrate transglycosylation using PNP-X, while the maximum yields of higher PNP β-xylooligosaccharides (dp 4–7) were achieved with a two-step synthesis using PNP xylotrioside as the substrate. The maximum yields of PNP xylobioside and PNP xylotrioside were observed at the optimum concentration of PNP-X of 110 mM and at 70–80% con-

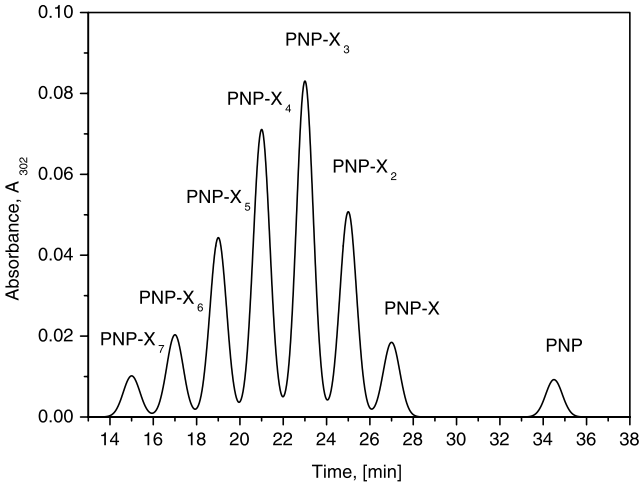

Fig. 5. Separation of the β-D-xylosidase transglycosylation products on a Waters Spherisorb C8 column.

Table 4
The yields of transfer products produced by the β-D-xylosidase in the reactions of substrate transglycosylation

Product	Yield (%)
β-Xyl-(1→4)-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_2)	29.0
β-Xyl-(1→4)-β-Xyl-(1→4)-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_3)	7.0
β-Xyl-1→(4-β-Xyl-1→)$_2$-4-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_4)	29.15
β-Xyl-1→(4-β-Xyl-1→)$_3$-4-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_5)	12.63
β-Xyl-1→(4-β-Xyl-1→)$_4$-4-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_6)	3.91
β-Xyl-1→(4-β-Xyl-1→)$_5$-4-β-Xyl-$OC_6H_4NO_2$-*p* (PNP-X_7)	0.88

version of the substrate. The yields of these products according to[31] were equal on average to 30%. PNP xylooligosaccharides with dp 4–7 were produced using PNP-X_3 as a substrate at a concentration 330 mM and at a degree of conversion of 70%. In this case, the yields of PNP-X_{4-7} were about 50%. The HPLC profile of the products of enzymatic synthesis obtained in the second stage is represented in Fig. 5. The yields of PNP xylosides synthesised enzymatically were calculated according to[29] and are presented in Table 4.

Recently, Takeo et al. reported a successful chemical synthesis of PNP β-(1→4)-xylooligosaccharides with dp = 2–4.[24] The use of PNP-X_3 and, possibly PNP-X_4, synthesised in good yield using organic chemistry methods can allow an effective production of PNP β-xylooligosaccharides with dp > 5 by the chemo-enzymatic approach employing β-D-xylosidase described here.

2.3. Mode of action of β-xylanases against PNP xylooligosaccharides

The action patterns of β-xylanases from *Aspergillus orizae*[28] and *T. reesei*[30] were studied using PNP xylooligosaccharides with dp 2–7 as substrates. Hydrolysis of PNP-X_2 by both XynA and XynT led to the liberation of *p*-nitrophenol and D-xylobiose, which was identified by TLC using an authentic sample of xylobiose as a standard. This suggests that the above substrate can be used for β-xylanase assays using the detection of liberated chromophore by spectrophotometric methods. Similar β-xylanase action was observed in the hydrolysis of PNP-X_3 in the initial stage of the reaction yielding xylotriose and *p*-nitrophenol. The concentrations of *p*-nitrophenol produced in reactions of β-xylanases with PNP xylosides (with dp 2 and 3) were linear with respect to reaction time in the substrate concentration range from 0.4 to 1 mM and substrate conversion of 20–25%. Typical elution profiles obtained by reversed-phase HPLC separation of reaction products of the hydrolysis of PNP-X_4 catalysed by both β-xylanases are shown in Fig. 6(A and B). Reproducible values for the yields of PNP xylooligosaccharides and free PNP for all the specimens tested are given in Tables 5 and 6.

In these studies of modes of action, only chromogenic reaction products, namely, free *p*-nitrophenol or PNP β-(1→4)-xylooligosaccharides, were detected. The data obtained suggest that the action patterns for both β-xylanases towards PNP xylobioside and PNP xylotrioside are similar but differ significantly towards longer oligosaccharides (Tables 5 and 6). The K_m values in the xylanase-induced hydrolysis of PNP-X_2 and PNP-X_3 were calculated from Lineweaver–Burk equations. A specific chromogenic substrate that is cleaved by β-xylanase exclusively at the aglycon chromogenic site appears to be quite useful for detailed studies of catalytic function of these enzymes. Traditional methods of monitoring xylan hydrolysis by measuring the amount of reducing ends released[21] are more complicated than the detection of the liberated chromophore. Due to high extinction of *p*-nitrophenol, the sensitivity of xylanase measurements increases significantly. Moreover, evaluation of xylanase subsite structure can be made with the aid of PNP xylooligosaccharides since one can easily separate the products of the hydrolysis by HPLC techniques (Fig. 6(A,B)). Therefore, the synthesised PNP β-D-xylooligosaccharides (dp 2–7) are promising for various enzymatic experiments and search for new xylanase-producing strains.

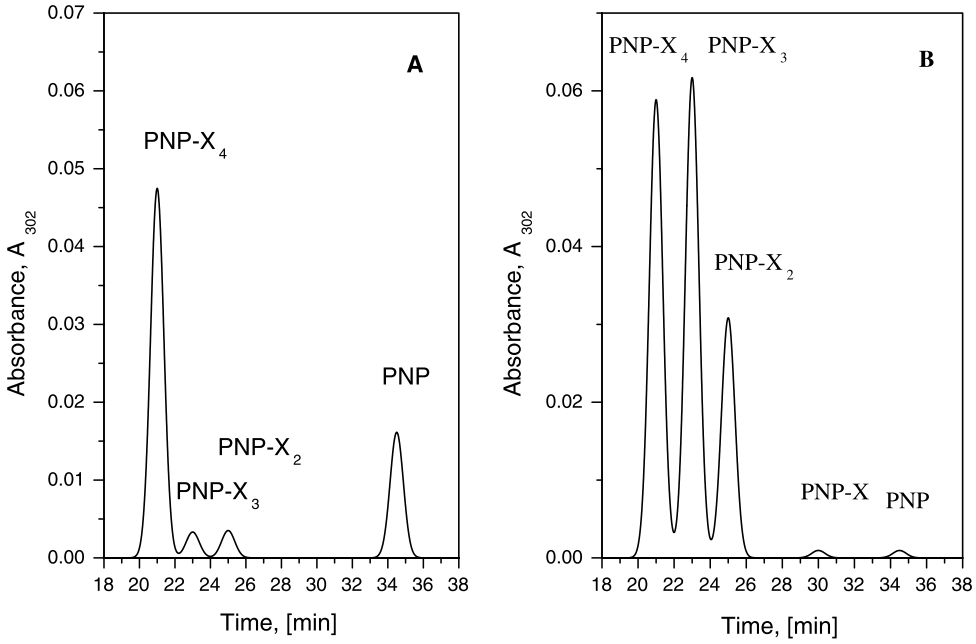

Fig. 6. A: HPLC separation of the products of PNP-X_4 hydrolysis catalysed by XynA. B: HPLC separation of the products of PNP-X_4 hydrolysis catalysed by XynT.

Table 5
Yields [a] of products from hydrolysis of *p*-nitrophenyl xylooligosaccharides by XynA

Substrate	Products (mol/mol of *p*-nitrophenyl products) [b]						
	PNP	PNP-X	PNP-X_2	PNP-X_3	PNP-X_4	PNP-X_5	PNP-X_6
PNP-X_2	0.886	0.114					
PNP-X_3	0.615	0.188	0.197				
PNP-X_4	0.702		0.153	0.145			
PNP-X_5	0.248		0.103	0.286	0.363		
PNP-X_6	<0.05			0.073	0.244	0.683	
PNP-X_7	<0.04				0.066	0.190	0.702

[a] The yields given are an average of at least three determinations.
[b] Experimental error is $\pm 5\%$ of values given.

Table 6
Yields [a] of products from hydrolysis of *p*-nitrophenyl xylooligosaccharides by XynT

Substrate	Products (mol/mol of *p*-nitrophenyl products) [b]						
	PNP	PNP-X	PNP-X_2	PNP-X_3	PNP-X_4	PNP-X_5	PNP-X_6
PNP-X_2	0.8	0.2					
PNP-X_3	0.09	0.58	0.33				
PNP-X_4	0.03		0.32	0.65			
PNP-X_5	0.08			0.21	0.71		
PNP-X_6	0.06			0.08	0.43	0.43	
PNP-X_7	0.09			0.09	0.36	0.35	0.11

[a] The yields given are an average of at least three determinations.
[b] Experimental error is $\pm 5\%$ of values given.

3. Experimental

3.1. Materials

p-Nitrophenyl β-D-xylopyranoside (PNP-X), other *p*-nitrophenyl α-/β-glycosides, xylobiose, xylan from birchwood, CM-cellulose, β-glucan from barley, laminarin from *Laminaria digitata* were obtained from Sigma (St. Louis, MO, USA). All reagents were of analytical or research grade.

3.2. Analytical methods

Protein concentrations were measured following the Lowry procedure with BSA as a standard.[32] The molecular mass of the protein was estimated by SDS-PAGE on a 7.5% polyacrylamide gel according to the Laemmli method[33] using molecular weight calibration kit MW SDS 200 (29,000–205,000) from Sigma. Determination of the apparent molecular mass of the protein in non-denaturing conditions was performed by analytical chromatography on a Superose 12 (Pharmacia) column (10 × 300 mm) equilibrated with 20 mM NaOAc, pH 4.2, 100 mM NaCl. A kit containing series of calibration standards (MW-GF-1000, Sigma) for molecular masses from 12 to 200 kDa was used.

All ^{1}H and ^{13}C NMR spectra were recorded with an AMX-500 Bruker spectrometer (^{1}H at 500.13 MHz, ^{13}C at 125 MHz) in D_2O at ambient temperature with acetone as an internal standard (δ_H 2.225; δ_C 31.45). All samples for NMR analysis were dissolved in D_2O. Chemical shifts are given relative to internal acetone, 2.225 ppm for ^{1}H and 31.5 ppm for ^{13}C. Spectra were recorded on a Bruker AMX500 operating at 500.13 MHz for ^{1}H and 125.13 MHz for ^{13}C.

One-dimensional ^{1}H, NOE and ^{13}C spectra and phase-sensitive two-dimensional spectra (COSY-DQF, NOESY and ^{1}H–^{13}C heteronuclear corelations) were recorded using standard pulse programs at 20 °C and 50 °C. Data were analysed using software package XWINNMR (Bruker). The assigment of individual sugar residues was based on two-dimensional phase-sensitive spectroscopy COSY. The sequential assigment was based on NOE cross-peaks in NOESY experiment using a mixing time of 180 ms and was obsd as an NOE between the anomeric proton and proton at the substitution position. Assigment of ^{13}C signal was based on the proton–carbon correlations obsd in the ^{1}H–^{13}C correlations spectra. Determination of the anomeric configuration was based on chemical shifts obsd, and the $J_{1,2}$ coupling constants measured from one-dimensional ^{1}H spectra.

Positive-ion mass spectra were recorded on a Micromass Q-TOF2 orthogonal acceleration quadrupole/time-of-flight mass spectrometer fitted with a nanoflow ion source (Micromass, Manchester, UK). With the TOF analyzer operating in single-reflectron 'V' mode, typical peak resolution on singly-charged sodiated carbohydrate adducts was 9500 FWHM. TOF MS mass calibration was obtained over the m/z range 130–1980 using a soln of NaI (2 g/L) and CsI (0.05 g/L) in 1:1 Pr^{i}OH–water.

Solutions of PNP xylooligosaccharides (typical concn 10–50 μM, in 1:1 MeOH–water containing 0.5 mM NaCl) were infused into the mass spectrometer by a syringe pump at 500 nL/min. The electrospray capillary voltage was maintained at 3.0 kV and the cone voltage was varied (40–80 V) to optimise the intensity of the $[M + Na]^+$ ion signal. Argon was present in the collision cell at a pressure of $3.6–3.7 \times 10^{-5}$ mbar (ANALYZER Penning gauge) during both MS and MS/MS experiments. For MS/MS experiments, the collision energy was varied (25–56 V), depending on the resilience of the $[M + Na]^+$ ion, to achieve an optimal balance between parent and fragment ion intensities. A scan time of 2.5 s with an interscan delay of 0.1 s was used in all MS modes. Data were collected until an acceptable signal-to-noise ratio was achieved after the combination of individual spectra. For the production of MS/MS spectra, the monoisotopic mass of the B-2 fragment ion (287.0743 u) was used to apply a lock mass calibration during generation of centroided spectra from typically 60–80 individual MS/MS spectra.

Oligosaccharides, products of enzymatic hydrolysis and transglycosylation products were analysed qualitatively by TLC on Kieselgel 60 plates (Merck) with a 1-butan-ol–acetic acid–water (3:1:1, v/v) solvent system.

3.3. Purification of enzymes

Aspergillus sp. used in this study was a wild-type strain from the culture collection Biokemis-Charlock Ent, UK. The growth medium contained (g/L) $NH_4H_2PO_4$, 24.6; KH_2PO_4, 10; KCl, 0.5; $FeSO_4$, 0.01; $MgSO_4$, 0.5; peptone, 5; wheat bran, 10; glucose, 5; yeast extract, 2.5. The microorganism was grown in 20-liter fermenter for 96 h at 30 °C with aeration. All purification procedures were carried out at 4 °C. Mycelium was removed by centrifugation (3000*g*, 40 min), and the supernatant was concentrated 30-fold and transferred to 20 mM Tris–HCl buffer, pH 7.5 (buffer A), using hollow fibres ('Kirishi', Russia). Crude β-xylosidase soln was applied onto a DEAE Toypearl column (20 × 500 mm) equilibrated with buffer A and eluted with 1 M NaCl in the same buffer. The combined fractions were concentrated on an Amicon PM-30 membrane to 20 mL, dialysed against buffer A and applied onto a TSK DEAE 5PW column (21.5 × 150 mm) (Pharmacia-LKB) equilibrated with the same buffer. The β-xylosidase was eluted with linear gradient (0–0.5 M) of NaCl in buffer A. Fractions containing β-xylosidase activity were pooled, con-

centrated on an Amicon PM-30 membrane to 5 mL, and dialysed against 20 mM sodium acetate buffer, pH 4.0 (buffer B). The obtained protein soln was chromatographed on a TSK SP 5PW column (21.5 × 150 mm, Pharmacia) with a linear gradient (0–0.5 M) of NaCl in buffer B. Then, β-xylosidase was concentrated using an Amicon PM-30 membrane to 4 mL, transferred to 1.7 M $(NH_4)_2SO_4$ in buffer B, and applied onto a Phenyl Superose HR 5/5 (Pharmacia) column (5 × 50 mm) equilibrated with the same buffer. β-Xylosidase-containing fractions were eluted with a linear gradient (0–20 mM) sodium acetate buffer, pH 4.0. Finally, β-xylosidase was purified by gel-exclusion chromatography on a Sephacryl S-200 column (35 × 700 mm, Pharmacia) equilibrated in buffer B containing 0.1 M NaCl, dialysed against deionised water, and freeze-dried. The activity of the enzyme during purification was monitored using PNP-X as a substrate as described below.

β-Xylanase I from *T. reesei* of family 11 was kindly donated by Dr A. Miasnikov, Danisco-Cultor Oy, Finland, and separated from admixtured β-xylosidase activity by gel-exclusion chromatography on a Sephacryl S-200 column in 20 mM sodium acetate buffer, pH 4.5, containing 0.1 M NaCl. The specific activity of the purified protein was about 300 ± 5 U/mg; the enzyme contained less than 0.01% of extrinsic β-xylosidase activity. *Aspergillus orizae* β-xylanase (family 10) was isolated from cultural liquid according to the procedures described by Kitamoto et al.[30] resulting in 250 ± 5 U/mg activity and less than 0.01% of β-xylosidase admixtures.

3.4. Enzyme assays

The β-xylosidase activity towards PNP-X was determined at 37 °C in 50 mM sodium acetate buffer, pH 4.5.[8] One unit of β-D-xylosidase was defined as the amount of the enzyme releasing 1 μmol of *p*-nitrophenol per min. Exo-glycosidase activities using different *p*-nitrophenyl α-/β-glycosides as substrates were measured at the corresponding pH values at 37 °C according to Ref. 34. β-Xylanase activity was evaluated by Somogyi–Nelson method[21] using xylan as a substrate. One unit of the β-xylanase activity was defined as an amount of the enzyme capable of releasing 1 μmol of reducing sugar from xylan with concn of 2 mg/mL at 37 °C in 20 mM sodium acetate buffer, pH 4.0, per 1 min.

The Michaelis constants were determined for PNP-X in the β-D-xylosidase-mediated hydrolysis and PNP-X_2 and PNP-X_3 in the hydrolysis by β-xylanases, respectively, from the Michaelis–Menten equation by nonlinear regression analysis.[35] Rates were determined at 6–15 different substrate concentrations ranging from approx 0.1 × the K_m value determined to 4–6 × K_m.

The effect of pH on the hydrolytic activity of the β-D-xylosidase was measured in the pH range 3–8.0 of 0.1 M sodium phosphate buffers using PNP-X as a substrate.

3.5. Kinetic studies of the hydrolysis of PNP xylooligosaccharides by XynA and XynT

Reaction mixtures containing 0.01 U of the corresponding β-xylanase and 0.4 mM of a substrate in 20 mM sodium acetate buffer, pH 4.0, were incubated at 37 °C for defined time intervals. The reaction were stopped by freezing and freeze-drying. Each reaction mixture was separated on a Waters Spherisorb C8 column with a linear gradient (0–90%) of MeCN in water.

3.6. Transxylosylation reactions of the β-D-xylosidase

In a typical experiment, the transglycosylation reaction was carried out at 37 °C in 30 mM sodium phosphate buffer, pH 6.5. The progress of the reactions was monitored by thin layer chromatography. Reactions were stopped by freeze-drying. A quantitative analysis of transfer products was carried out on a Waters Spherisorb C8 column (250 × 4.6 mm, Supelco Inc.) using a linear gradient (0–90%) of MeCN in water with detection at 302 nm. The amounts of the products formed were measured by integrating the corresponding chromatographic peaks.

The effect of pH on the transglycosylation activity of the β-D-xylosidase was evaluated at 37 °C in the pH range 5.5–8.0 in 0.1 M sodium phosphate buffers.

3.7. Synthesis of *p*-nitrophenyl β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranoside and *p*-nitrophenyl β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranoside

For the synthesis of the above PNP xylooligosaccharides, 180 mg of PNP-X and appropriate amount of the β-D-xylosidase corresponding to 8 U were mixed in 6 mL of 30 mM sodium phosphate buffer, pH 6.5, and incubated for 180 min at 37 °C. Following termination of the reaction by freezing and freeze-drying, the transglycosylation products, PNP-X_2 and PNP-X_3, were initially separated on an INERTSIL PREP-ODS column (20.0 × 250 mm) following by final purification of each individual product on the same column using a linear gradient (0–90%) of MeCN in water.

3.7.1. *p*-Nitrophenyl β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranoside. $[\alpha]_D$ −91.1° (*c* 1.0, MeOH).

3.7.2. *p*-Nitrophenyl β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-β-D-xylopyranoside. $[\alpha]_D$ −96.5° (*c* 1.0, H_2O).

3.8. Synthesis of PNP xylooligosaccharides with dp 4–7

For the synthesis of longer PNP xylooligosaccharides (dp 4–7), 48 U of the enzyme and 180 mg of PNP β-D-xylotrioside taken as a substrate were incubated in 1 mL of sodium phosphate buffer, pH 6.5, for 180 min at 37 °C. Then the products were initially separated on an INERTSIL PREP-ODS column as described above and further purified on a TSK-NH_2-60 column (4.6 × 250 mm, Pharmacia) using isocratic elution with 80% MeCN. Finally, the products were freeze-dried and used in further investigations.

3.8.1. 4-Nitrophenyl β-D-xylopyranosyl-(1→4)-bis [β-D-xylopyranosyl-(1→4)]-β-D-xylopyranoside. $[\alpha]_D$ − 102.5° (*c* 1.0, H_2O).

3.8.2. 4-Nitrophenyl β-D-xylopyranosyl-(1→4)-tris [β-D-xylopyranosyl-(1→4)]-β-D-xylopyranoside. $[\alpha]_D$ − 107.8° (*c* 1.0, H_2O).

3.8.3. 4-Nitrophenyl β-D-xylopyranosyl-(1→4)-tetrakis [β-D-xylopyranosyl-(1→4)]-β-D-xylopyranoside. $[\alpha]_D$ − 114.1° (*c* 1.0, H_2O).

3.8.4. 4-Nitrophenyl β-D-xylopyranosyl-(1→4)-pentakis [β-D-xylopyranosyl-(1→4)]-β-D-xylopyranoside. $[\alpha]_D$ − 119.6° (*c* 1.0, H_2O).

Acknowledgements

The present work was supported in part by a grant No. 00-04-48878 from the Russian Foundation for Basic Research and the grant of 6th Expertise Competition from the Presidium of the Russian Academy of Sciences. We also thank the Wallenberg Consortium North for Functional Genomics for financing the purchase of mass spectrometry equipment at the Royal Institute of Technology.

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