Carbohydrate Research, 206 (1990) 251–256
Elsevier Science Publishers B.V., Amsterdam

Hydrolysis of (1→3)- and (1→2)-β-D-xylosidic linkages by an endo-(1→4)-β-D-xylanase of *Cryptococcus albidus*

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(Received December 18th, 1989; accepted for publication, February 12th, 1990)

ABSTRACT

The substrate specificity of an endo-(1→4)-β-D-xylanase of the yeast *Cryptococcus albidus* was investigated using a series of methyl β-D-xylotriosides. In addition to (1→4) linkages, the enzyme could cleave (1→3) and (1→2) linkages adjacent to a (1→4) linkage and further from the non-reducing end of the substrate. The enzyme could hydrolyse a (1→3) linkage that attached a terminal xylopyranosyl group to a (1→4)-linked xylobiosyl moiety. The enzyme did not attack α-D-xylosidic linkages. The rate of cleavage of (1→4) linkages was much higher than those of other linkages at 0.5mM substrate, but the rates were comparable at 20mM substrate when transglycosylation reactions also occurred that facilitated degradation of the substrates.

INTRODUCTION

Endoglycanases are generally considered to be more specific enzymes than exoglycanases or glycosidases. Their high substrate specificity is due to the fact that their binding sites consist of several subsites that are located on both sides of the catalytic group. Each subsite requires the same configuration and type of linkage between the sugar units[1–5]. Any change in the regularity of the structure of the substrate may result in resistance to enzymic hydrolysis.

The above considerations are not fully valid for an endo-(1→4)-β-D-xylanase (EC 3.2.1.8) of the yeast *Cryptococcus albidus*, which exhibits significant transfer activity. The enzyme can attack β-(1→3) linkages in the xylan rhodymenan that are in a special arrangement with β-(1→4) linkages[6]. At high concentrations of substrate, the enzyme catalyzes not only the expected (1→4)-β-xylosyl transfer but also (1→3)-β-xylosyl transfer[6] and 6-*O*-β-xylosyl transfer to cellobiose[7]. Although previous investigations have not provided any evidence for a (1→2)-β-xylosyl transfer, the transformations of positional isomers of xylobiose in the cells of *C. albidus* indicated that (1→2)-β-xylosidic linkages might be generated or hydrolyzed by the enzyme[8].

In order to establish its properties, the action of the *C. albidus* endo-β-D-xylanase was investigated using various synthetic methyl β-D-xylotriosides.

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0008-6215/90/$03.50

EXPERIMENTAL

Enzyme. — The endo-(1→4)-β-D-xylanase of the yeast *C. albidus* was purified as described[9] and 1 unit of activity is defined as the amount of enzyme that liberates reducing sugars from beechwood xylan equivalent to 1 μmol of D-xylose in 1 min under given conditions[9].

Substrates. — The following methyl β-D-glycosides were synthesized as described: β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-OMe[10] (**1**, Xyl-4Xyl-4Xyl-Me), β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-OMe[11] (**2**, Xyl-4Xyl-3Xyl-Me), β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→2)-β-D-Xyl*p*-OMe[11] (**3**, Xyl-4Xyl-2Xyl-Me), β-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-OMe[12] (**4**, Xyl-3Xyl-4Xyl-Me), β-D-Xyl*p*-(1→2)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-OMe[13] (**5**, Xyl-2Xyl-4Xyl-Me), α-D-Xyl*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-OMe[12] (**6**), β-D-Xyl*p*(1→4)-[β-D-Xyl*p*-(1→3)]-β-D-Xyl*p*-OMe[14] (**7**), and β-D-Xyl*p*-(1→4)-[β-D-Xyl*p*-(1→2)]-β-D-Xyl*p*-OMe[13] (**8**).

Enzyme–substrate mixtures. — The effect of the endo-β-D-xylanase on **1–8** was studied at 0.5 and 20mM in 0.05M pyridine–acetic acid buffer (pH 5.2) at 30°. The products of hydrolysis in the 20mM incubate were analysed on aliquots (1–2 μL) by t.l.c. on microcrystalline cellulose (Lucefol, Kavalier, Czechoslovakia), using ethyl acetate–acetic acid–water (17:8:10). Larger aliquots (50 μL) of the 0.5mM incubate were first heated for 2 min at 100° in order to denature the enzyme, then dried in a vacuum over P_2O_5, and a solution of the residue in water (5–10 μL) was chromatographed as above. Reducing sugars were detected with aniline hydrogenphthalate[15].

The kinetics of hydrolysis of **1–4** by endo-β-D-xylanase in 0.05M acetate buffer (pH 5.4) were followed by determination of the reducing sugars[16] as μmol equivalents of xylobiose. The initial rates were obtained from plots of the amount of reducing sugar liberated versus time and were related to 1 enzyme unit.

RESULTS AND DISCUSSION

Of the methyl β-D-xylotriosides **1–8**, the endo-β-D-xylanase of *C. albidus* attacked only Xyl-4Xyl-4Xyl-Me (**1**), Xyl-4Xyl-3Xyl-Me (**2**), Xyl-4Xyl-2Xyl-Me (**3**), and Xyl-3Xyl-4Xyl-Me (**4**). The resistant compounds were Xyl-2Xyl-4Xyl-Me (**5**), **6** which contains an α-(1→3) linkage, and **7** and **8** which have branched structures.

In agreement with the fact that the pathways of the degradation of linear (1→4)-β-D-xylo-oligosaccharides depend on their concentration[17], the products of hydrolysis of **1–4** differed at 0.5 and 20mM initial substrate concentration (Fig. 1). At 0.5mM, **1–3** were cleaved mainly at the second linkage from the non-reducing end, *i.e.*, regardless of whether the linkage was (1→4), (1→3), or (1→2), whereas **4** was cleaved exclusively to give the trisaccharide and methanol. Reducing sugars liberated from **3** and **4** are shown on chromatograms in Fig. 2.

At 20mM substrate, linkages in **1–4** other than those noted above were hydrolyzed, indicating the formation of more than one type of productive enzyme–substrate

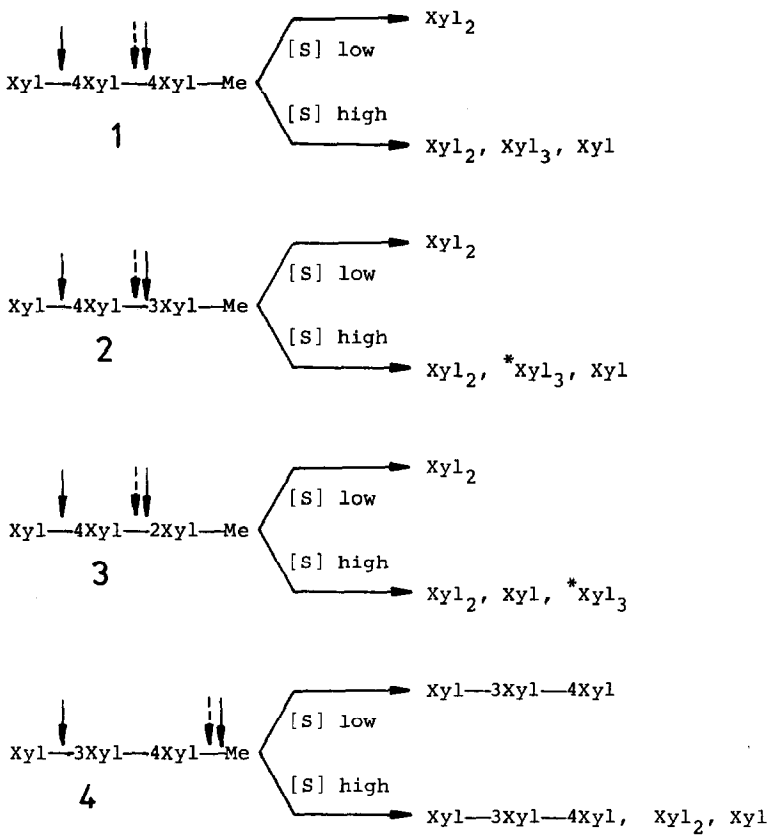

Fig. 1. Reducing sugars liberated by endo-β-D-xylanase from **1–4** at 0.5 and 20mM substrate concentration ([S]):--➨ linkages cleaved at low [S], → linkages cleaved at high [S], *denotes the products formed exclusively by transglycosylations. The scheme does not show the cleavage of primary products of hydrolysis such as Xyl-4Xyl-Me.

complex. In addition to the cleavage of the linkages observed at 0.5mM substrate, the terminal non-reducing xylopyranosyl group was also released. This mode of cleavage was accompanied by (1→4)-β-xylosyl transfer to another substrate molecule, to yield tetrasaccharide glycosides. From such products formed from **2** and **3**, xylotriose was released as a secondary product (Figs. 2 and 3). An alternative pathway to xylotriose may be xylosyl transfer to xylobiose. Xyl-3Xyl and Xyl-2Xyl, which can be distinguished easily by chromatography[8], were not detected. The presence of Xyl-4Xyl in the hydrolysate of 20mM **4** (Fig. 2) points to the cleavage of its (1→3) linkage.

The above results clearly demonstrate the ability of the yeast endo-β-D-xylanase to hydrolyze β-(1→3) and β-(1→2) linkages, but they do not indicate the rate of cleavage in comparison with that of the (1→4) linkage. Therefore, the rates of liberation of reducing sugars (as xylobiose) from **1–4** were determined (Table I). Since **1–3** each undergo a single cleavage at 0.5mM, the initial rates of hydrolysis correspond to the initial rates of hydrolysis of the (1→4), (1→3), and (1→2) linkages, respectively.

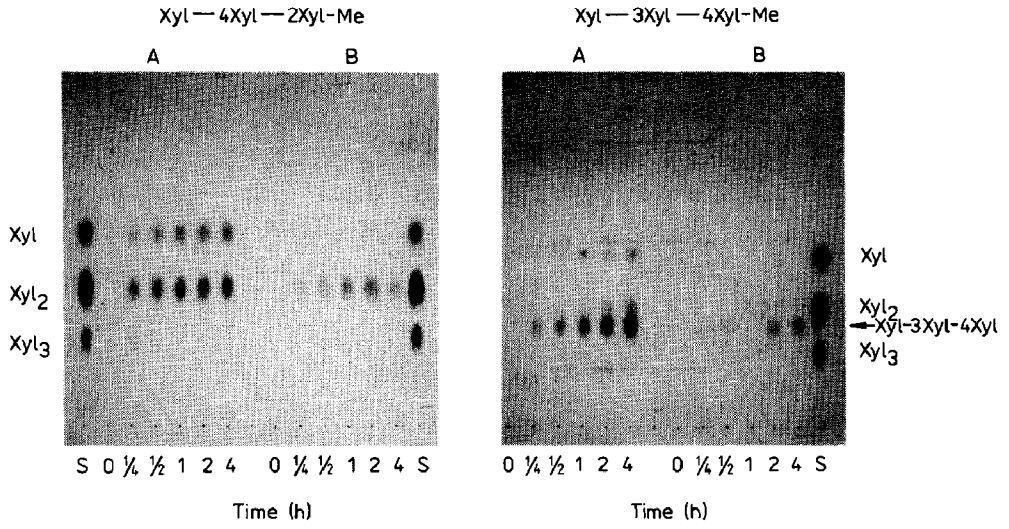

Fig. 2. T.l.c. of the reducing saccharides formed by the action of endo-β-D-xylanase on Xyl-4Xyl-2Xyl-Me (**3**) and Xyl-3Xyl-4Xyl-Me (**4**) at A, 20mM; and B, 0.5mM substrate concentration: S, standards; Xyl, D-xylose; Xyl_2, xylobiose; Xyl_3, xylotriose [both β-(1→4)].

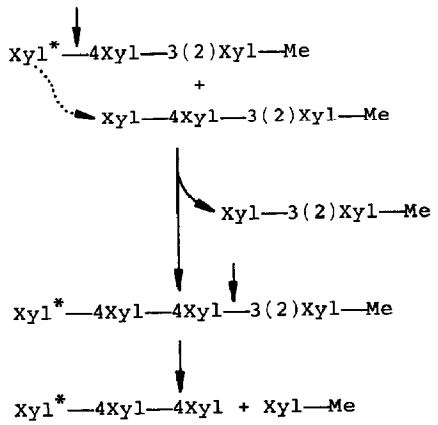

Fig. 3. Schematic representation of the formation of xylotriose from **2** and **3** in the reaction with β-D-xylanase of *C. albidus*. The short arrows indicate the positions of cleavage, and * marks the xylosyl residue transferred.

However, the (1→4) linkages were cleaved more rapidly by one or two orders of magnitude. The (1→2) linkage was hydrolyzed more rapidly than the (1→3) linkage in spite of the fact that the enzyme did not catalyze (1→2)-β-glycosyl transfer. The rates of hydrolysis of **1**–**3** were fairly comparable at 20mM substrate when reactions other than simple hydrolysis occurred. The transglycosylations facilitated the degradation of the substrates through the formation of intermediates that were more susceptible to hydrolysis than the original xylotriosides.

TABLE I

Initial rates[a] (μmol.min^{-1}) of formation of reducing sugars as xylobiose from the methyl β-D-xylotriosides **1–4** by the endo-β-D-xylanase of *C. albidus*

Compound	*Substrate concentration*			
	0.5mM		*20mM*	
	Expt. 1	*Expt. 2*	*Expt. 1*	*Expt. 2*
Xyl-4Xyl-4Xyl-Me (**1**)	0.725	0.70	0.183	0.325
Xyl-4Xyl-3Xyl-Me (**2**)	0.005	0.008	0.10	0.13
Xyl-4Xyl-2Xyl-Me (**3**)	0.038	0.045	0.264	0.35
Xyl-3Xyl-4Xyl-Me (**4**)	0.006	0.004	0.0625	-

[a] The rates are referred to one unit of enzyme activity.

Both compounds containing a β-(1→3) linkage, *i.e.*, Xyl-4Xyl-3Xyl-Me (**2**) and Xyl-3Xyl-4Xyl-Me (**4**), were hydrolyzed at the (1→3) linkage. In contrast, of the two analogous compounds that contained a (1→2) linkage, only Xyl-4Xyl-2Xyl-Me (**3**) was hydrolyzed at the (1→2) linkage. The resistance of Xyl-2Xyl-4Xyl-Me (**5**) can be ascribed to a steric effect, namely the inability of subsite -II (Fig. 4) of the enzyme binding site to accommodate a 2-*O*-substituted β-xylosyl residue.

In view of the four subsite binding sites of *C. albidus* endo-β-D-xylanase[18], the productive complexes of the enzyme with **1–4** (Fig. 4) point to the specificity of the subsite pairs. The subsites -I and -II have a strict requirement for a (1→4)-β-D-xylobiosyl moiety. The non-reducing xylopyranosyl unit of the xylobiosyl moiety is

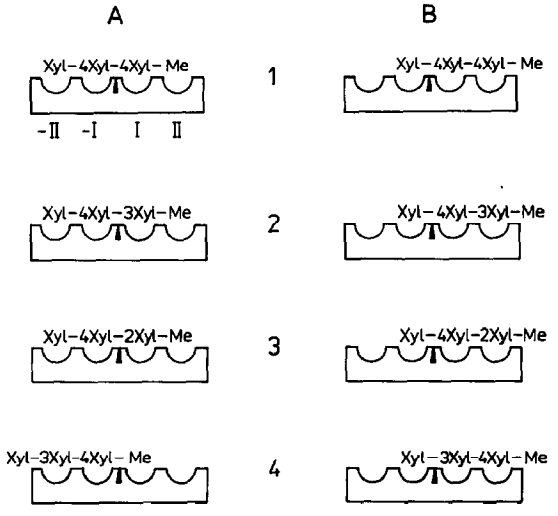

Fig. 4. Productive complexes of *C. albidus* endo-β-D-xylanase with **1–4** formed at A, 0.5 and 20mM substrate concentration; and B, additional complexes formed at the latter concentration. Decomposition of the B complexes is accompanied by transglycosylation.

accommodated at subsite -II when unsubstituted or 3- or 4-substituted with a β-xylopyranosyl residue, but not when 2-substituted. Thus, the enzyme will not hydrolyze any linkage adjacent to a (1→2) or (1→3) linkage and further from the non-reducing end. The subsites to the right of the catalytic groups (Fig. 4) do not have such a strict requirement for a (1→4)-linked β-xylopyranosyl residue. It may be noted that an acidic endo-β-D-xylanase of *Aspergillus niger*[19], which has a substrate binding site larger than that of the *C. albidus* endo-β-D-xylanase, behaves as a more specific endoglycanase when tested on methyl β-D-xylotriosides used in the present work.

ACKNOWLEDGMENT

We thank Mrs. M. Cziszárová for technical assistance.

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