Carbohydrate Research, 140 (1985) 342–346
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Hydrolysis of β-D-xylo-oligosaccharides by β-D-xylosidase from *Bacillus pumilus*

ELISA VAN DOORSLAER, HILDA KERSTERS-HILDERSON, AND CLEMENT K. DE BRUYNE
Laboratorium voor Biochemie, Rijksuniversiteit Gent, Ledeganckstraat 35, B-9000 Gent (Belgium)
(Received December 16th, 1984; accepted for publication, January 29th, 1985)

β-D-Xylosidase, induced in *Bacillus pumilus* by D-xylose, is glycon-specific (D-xylopyranose) and hydrolyses only β-D-xylo-oligosaccharides and aryl β-D-xylopyranosides[1–8]. The enzyme does not transfer the xylose residue to nucleophiles other than water and operates with inversion of configuration[3], the product being α-D-xylose. These findings suggest a single-displacement mechanism without the formation of an intermediate enzyme–xylosyl complex[5].

It has been concluded[5–7] that the active site is composed of an extended region such that at least two xylose residues of a xylo-oligosaccharide can bind at each subsite. Although the second or "aglycon" subsite accepts[5–7] alkyl and aryl aglycon groups, it is not a simple hydrophobic pocket, but has a specific three-dimensional structure so that the atoms of the groups binding to this site are forced to take up well-defined positions. To determine whether the binding of the enzyme to its substrate extends beyond this second subsite, we have investigated the effect of chain length on the enzymic hydrolysis of xylo-oligosaccharides (and certain derivatives).

The action patterns of the enzyme during hydrolysis are characteristic of an exo-hydrolase that hydrolyses the (1→4)-β-D linkage of xylo-oligosaccharides, starting at the non-reducing end. T.l.c. showed that (1→4)-β-D-xylotetraose (R_F 0.23) was hydrolysed to the triose (R_F 0.32) and thence to xylobiose (R_F 0.44); xylobiose was not released directly from xylotetraose. Phenyl β-D-xylotrioside (R_F 0.39) was attacked at the non-reducing end, and xylose units were removed in a stepwise manner since phenyl xylobioside (R_F 0.54) was formed first and then phenyl xyloside (R_F 0.78) which accumulated; no xylobiose (R_F 0.44) or xylotriose (R_F 0.32) was formed.

The hydrolysis of (1→4)-β-D-xylo-oligosaccharides (up to heptaose), phenyl β-D-xylobioside, and benzyl β-D-xylobioside (results not shown) followed the above pattern. Criteria[9,10,19] for the characterisation as a xyloside xylohydrolase or a xylan xylohydrolase are (*1*) the anomeric form of the released D-xylose and (*2*) the effect of d.p. on the relative rate of hydrolysis of the xylo-oligosaccharides.

0008-6215/85/$ 03.30

TABLE I

KINETIC PARAMETERS FOR THE HYDROLYSIS OF β-D-XYLO-OLIGOSACCHARIDES AND RELATED COMPOUNDS[a] (pH 7.15, 25°)

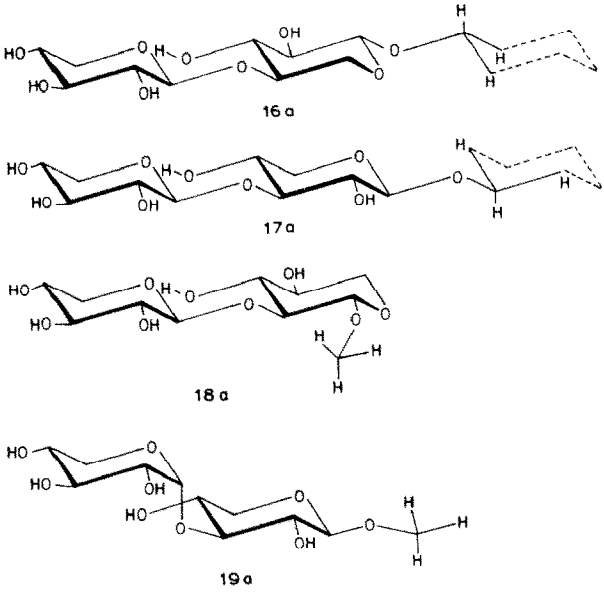
No.	*Substrate*	K_m[b] (mM)	k_{cat}[b] (s^{-1})
1	(1→4)-(Xyl*p*)$_2$	2.9	18.1
2	(1→4)-(Xyl*p*)$_3$	1.1	3.0
3	(1→4)-(Xyl*p*)$_4$	1.4	3.0
4	(1→4)-(Xyl*p*)$_5$	1.4	3.8
5	(1→4)-(Xyl*p*)$_6$	1.5	3.0
6	(1→4)-(Xyl*p*)$_7$	2.2	3.0
7	Commercial xylans	not hydrolysed	
8	Alkyl Xyl*p*	not hydrolysed	
9	Phenyl Xyl*p*	1.9	0.7
10	4-Nitrophenyl Xyl*p*	1.5	7.6
11	2-Chloro-4-nitrophenyl Xyl*p*	1.0	27.6
12	2,4-Dinitrophenyl Xyl*p*	2.1	53
13	Phenyl (1→4)-(Xyl*p*)$_2$	1.4	3.5
14	Phenyl (1→4)-(Xyl*p*)$_3$	2.2	3.0
15	Benzyl (1→4)-(Xyl*p*)$_2$	2.9	3.0
16	Methyl (1→4)-(Xyl*p*)$_2$	6.2	10.1
17	Methyl (1→3)-(Xyl*p*)$_2$	2.6	14.2
18	Methyl (1→2)-(Xyl*p*)$_2$	100	6
19	Methyl *O*-β-D-xylopyranosyl-(1→3)-α-D-xylopyranoside	not hydrolysed	

[a]Except for **19**, all glycosidic bonds are β. [b]Estimated standard error: on k_{cat}, 3%; on K_m, ≤10%.

The anomeric configuration of the D-xylose was determined by using an α-specific D-xylose isomerase coupled to D-glucitol dehydrogenase[3,11]. From the amount of α-D-xylose produced after one min (negligible mutarotation) of hydrolysis, it was concluded that the D-xylose released from the non-reducing end of (1→4)-β-D-xylobiose was α. This inversion (β→α) of configuration on hydrolysis is indicative of exo-type degradation by xylan xylohydrolases and accords with that observed for aryl β-D-xylopyranosides[3,5].

The k_{cat} and K_m values (pH 7.15, 25°) for several xylo-oligosaccharides and some derivatives were calculated from the initial rates of release of xylose (Table I). For the series of six (1→4)-β-D-xylo-oligosaccharides (Table I, derivatives **1–6**), xylobiose was the best substrate. When the reducing xylose residue (the "aglycon") in xylobiose was replaced by an alkyl group, the resulting alkyl β-D-xylopyranoside, although it bound to the enzyme[6,7], was not hydrolysed. Phenyl β-D-xylopyranoside was hydrolysed, but at a rate considerably lower than that for xylobiose. Introduction of electron-withdrawing substituents (Table I, derivatives **10–12**) increased k_{cat} but had virtually no effect on K_m. Thus, when the xylose ring of alkyl and aryl xylosides is bound correctly to the glycon site, the characteristics of the group in the aglycon site determine the value of k_{cat}.

The fact that the alkyl derivatives **15** and **16** are hydrolysed proves that the non-reducing xylosyl group binds to the glycon site and the second xylose residue



to the "aglycon" site so that the holosidic bond is cleaved. The close similarity between the kinetic parameters for the (1→4)-β-D-xylobiose derivatives is evidence that all those derivatives bound and reacted in essentially the same way.

Derivatives **2–6** and **13–16** (Table I) can be considered as (1→4)-β-D-xylobiose molecules in which HO-1 has been replaced by MeO (**16**), BzlO (**15**), PhO (**13**), xylosyloxy (**2**), or a xylo-oligosaccharide moiety (**14**, **3–6**). This "linear" elongation of the xylobiose molecule (*e.g.*, **16a** for the methyl derivative) does not prevent the correct positioning of the second xylose residue into the aglycon site, but an effect on kinetic parameters is observed. Furthermore, it is clear that, for these derivatives (**1–6** in Table I), binding of the enzyme to the substrate does not extend beyond the aglycon site.

The enzyme hydrolyses (1→3)- and (1→2)-linked β-D-xylobiosides (Table I, **17** and **18**). Each derivative can assume a structure (**17a** and **18a**) closely resembling that of the (1→4)-β-D derivatives and thus allowing the correct positioning of the "glycon"-xylose and of the holosidic bond. In each compound, the aglycon site on the enzyme is occupied by the second xylose residue.

For derivative **17**, the methyl group can be regarded as a "linear" elongation of the xylobiose unit and thus it should have only a limited influence on the kinetic parameters [*cf.* the (1→4)-β-D-series] as reflected by values in Table I.

For derivative **18**, the methyl group is oriented in a different way (**18a**). Although, once the substrate is bound to the enzyme, the holosidic bond in **18** is hydrolysed at about the same maximal rate (see k_{cat} in Table I), the orientation of the methyl group has a significant effect on K_m, and thus probably on the binding of this derivative.

Derivative **19**, having a (1→3)-α-D-holosidic bond, does not bind to the

enzyme, probably because the derivative (**19a**) cannot mimic the (1→4)-β-D-xylobiose structure.

Because of the observed inversion of configuration (β→α), the enzyme should be classified as a xylan xylohydrolase[9,10,19] and thus act more rapidly on long polymers than on dimers[19]. However, the values in Table I for **1–6** show that the maximal rate of hydrolysis decreases and then becomes virtually independent of the d.p. According to this criteron, the enzyme should be characterised as a xyloside xylohydrolase and even as a xylobiase. These findings indicate that the classification according to the aforementioned criteria is not absolute.

EXPERIMENTAL

β-D-Xylosidase was isolated, purified, and standardised as described[1,8]. D-Xylose was measured by the method of Winckers[12] or by a coupled reaction of D-xylose isomerase with D-glucitol dehydrogenase[11]. Hydrolysis of non-chromogenic aryl β-D-xylopyranosides was followed discontinuously by the method of Asp[13]. All reactions were carried out at 25° in 0.01M Na–K phosphate buffer (pH 7.15) containing mM EDTA. The V and K_m values were calculated as described elsewhere[5]. T.l.c. was performed on Kieselgel GF 254 (Merck), using water–acetic acid–ethyl acetate (1:1:3) and detection by charring with sulfuric acid. *O*-β-D-Xylopyranosyl-(1→4)-D-xylopyranose [(1→4)-β-D-xylobiose] and benzyl *O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranoside (benzyl β-D-xylobioside) were synthesised by literature procedures[14,15]. Phenyl *O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranoside and phenyl *O*-β-D-xylopyranosyl-(1→4)-*O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranoside (phenyl β-D-xylo-bioside and -trioside) were obtained as products of enzymic transglycosylation using a fungal β-D-xylosidase and phenyl β-D-xylopyranoside[16,17]. Methyl *O*-β-D-xylopyranosyl-(1→4)-β-D-xylopyranoside, the corresponding (1→3)- and (1→2)-linked derivatives, and methyl *O*-β-D-xylopyranosyl-(1→3)-α-D-xylopyranoside were a gift from Professor J. Kovac (Slovak Academy of Sciences). The (1→4)-β-D-xylo-oligosaccharides with d.p. 3–5 were a gift from Professor T. Yasui (Tokyo University of Education). The (1→4)-β-D-xylo-oligosaccharides with d.p. 6 and 7 were prepared[18] by partial hydrolysis of β-D-xylan by fuming hydrochloric acid and were purified on a column (120 × 3 cm) of Biogel P-2 at 60° by elution with water.

REFERENCES

1 H. Kersters-Hilderson, M. Claeyssens, E. Van Doorslaer, E. Saman, and C. K. De Bruyne, *Methods Enzymol.*, 83 (1982) 631–639.
2 M. Claeyssens, E. Saman, H. Kersters-Hilderson, and C. K. De Bruyne, *Biochim. Biophys. Acta*, 405 (1975) 475–481.
3 H. Kersters-Hilderson, M. Claeyssens, E. Van Doorslaer, and C. K. De Bruyne, *Carbohydr. Res.*, 47 (1976) 269–273.
4 E. Saman, M. Claeyssens, and C. K. De Bruyne, *Eur. J. Biochem.*, 85 (1978) 301–307.
5 H. Kersters-Hilderson, E. Van Doorslaer, and C. K. De Bruyne, *Carbohydr. Res.*, 65 (1978) 219–227.

6 H. KERSTERS-HILDERSON, E. VAN DOORSLAER, AND C. K. DE BRUYNE, *Cárbohydr. Res.*, 78 (1980) 163–172.
7 E. VAN DOORSLAER, H. KERSTERS-HILDERSON, AND C. K. DE BRUYNE, *Carbohydr. Res.*, 78 (1980) 317–326.
8 H. KERSTERS-HILDERSON, E. VAN DOORSLAER, M. LIPPENS, AND C. K. DE BRUYNE, *Arch. Biochem. Biophys.*, 234 (1984) 61–72.
9 *Enzyme Nomenclature, Recommendations (1978) of the Nomenclature Committee of the International Union of Biochemistry*, Academic Press, New York, 1979.
10 S. CHIBA, K. HIROMI, N. MINAMIURA, M. OHNISHI, T. SHIMOMURA, K. SUGA, T. SUGANUMA, A. TANAKA, S. TOMIOKA, AND T. YAMAMOTO, *J. Biochem. (Tokyo)*, 85 (1979) 1135–1141.
11 H. KERSTERS-HILDERSON, E. VAN DOORSLAER, C. K. DE BRUYNE, AND K. YAMANAKA, *Anal. Biochem.*, 80 (1977) 41–50.
12 P. L. M. WINCKERS AND PH. JACOBS, *Clin. Chim. Acta*, 34 (1971) 401–408.
13 N. G. ASP, *Anal. Biochem.*, 40 (1971) 281–286.
14 C. E. BALLOU, *J. Am. Chem. Soc.*, 79 (1975) 165–166.
15 H. B. WOOD AND H. G. FLETCHER, JR., *J. Am. Chem. Soc.*, 80 (1958) 5242–5246.
16 M. CLAEYSSENS, E. VAN LEEMPUTTEN, F. G. LOONTIENS, AND C. K. DE BRUYNE, *Carbohydr. Res.*, 3 (1966) 32–37.
17 F. DELEYN, M. CLAEYSSENS, AND C. K. DE BRUYNE, *J. Carbohydr. Nucleosides Nucleotides*, 7 (1980) 203–217.
18 R. L. WHISTLER AND D. F. DURSO, *J. Am. Chem. Soc.*, 72 (1950) 677–679.
19 E. T. REESE, A. H. MAGUIRE, AND F. W. PARRISH, *Can. J. Biochem.*, 46 (1968) 25–34.