PHOTO-AFFINITY LABELING OF β-D-XYLOSIDASE

Eric SAMAN, Marc CLAEYSSENS, H. KERSTERS-HILDERSON and Clemens De BRUYNE
Laboratorium voor Algemene en Biologische Scheikunde, K. L. Ledeganckstraat, 35, B-9000 Gent, Belgium

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1. Introduction

As part of a comparative study of the structure and function of a group of glycosidases, we are particularly interested in the properties of a β-D-xylosidase (β-D-xyloside xylohydrolase, EC 3.2.1.37) induced in *Bacillus pumilus* [1]. In an attempt to obtain further information on the catalytic site of this enzyme, several potential affinity-labels have been prepared [2–4] and tested on this enzyme.

This communication reports on the interaction of a photoaffinity label with the β-D-xylosidase. The choice of a photolytic activatable ligand is founded on practical considerations, the most important being the easy handling of these products, since the reactive species is formed in situ.

The advantage of the aromatic azides, used in this work, over other photolabile molecules (e.g. diazoketones), is their thermal and pH stability. On illumination they produce a highly reactive aryl nitrene, which is thought to be able to react with all amino acid residues.

It is concluded that *p*-azidophenyl xylopyranoside acts on the β-D-xylosidase as a pseudo photo-affinity label, rather than a true photo-affinity label.

2. Materials and methods

The β-D-xylosidase, induced in a *Bacillus pumilus* strain was isolated and purified as described earlier [1,5]. It was stored in 80% ammonium sulphate at −18°C and, immediately before use, dissolved and dialysed in the appropriate buffer.

The synthesis of the azido compounds is described elsewhere [3]. The preparation of other glycosides, was performed as described in [6].

The activity of the enzyme preparations was assayed with *p*-nitrophenyl β-D-xylopyranoside as routine substrate. The standard conditions, defined for these measurements were: 1.5 ml of 1.33 mM substrate solution in 10 mM Na–K phosphate buffer pH 7.2, containing 1 mM EDTA, and incubated at 25°C. All other solutions were also prepared in this buffer. The enzymic activity was measured by following the extinction of the reaction-mixture at 400 nm as a function of time, with a Beckman DBGT spectrophotometer, equipped with a 10 inch recorder. The definition of the unit of enzyme activity is as reported elsewhere [1]. Photo-inactivation experiments were performed as follows: to an enzyme solution (0.20 mg/ml) containing 1–2 U per ml was added a suitable concentration of the photo-inactivator and in some cases a competitive inhibitor, or another substance to be tested, was included. The mixture was then placed in a quartz cuvette and irradiated in the dark in a thermostated room (20°C) with a 30 cm u.v. lamp (15 W) with emission maximum at 325 nm. In the scavenging experiments with oxygen, air was bubbled through the reaction mixture. At appropriate times, aliquots (5 μl) were taken from the inactivation mixture and diluted 200 times (in 1 ml of 10 mM phosphate buffer, 1 mM EDTA) to stop inactivation. Subsequently, substrate solution (4 mM in the same buffer) was added to a total volume of 1.5 ml and the enzymic activity was measured. A blank experiment, in which irradiation was omitted, was run simultaneously and revealed no significant decrease in enzymic activity (see Results). It was therefore more convenient to illuminate an enzyme solution

without azido-compounds as a blank. The enzymic activity of the inactivation mixture was then expressed as a percentage of the activity found in this blank experiment. The double-cell experiments were performed in 2 × 1 cm quartz cells (HELLMA) that were illuminated through the front cell. Thin-layer chromatography of reaction mixtures was performed on Silica Gel G (Merck) in ethyl acetate–acetic acid–water (3 : 1 : 1, v/v/v). Detection was effected with 5% sulphuric acid in ethanol followed by heating for 10 min at 110°C.

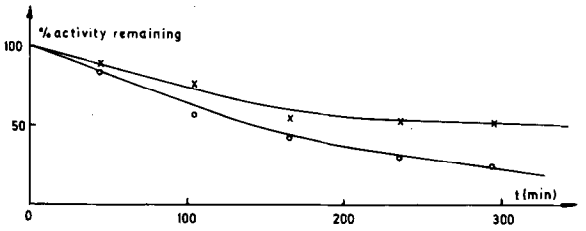

Fig.2. β-D-Xylosidase treated with 0.075 mM *p*-azidophenyl β-D-xylopyranoside. Illumination was interrupted after 15 min and solution 1 (○) was kept in the dark, whereas solution 2 (×) also kept in the dark was treated with oxygen. Illumination was started at zero time.

3. Results and discussion

As shown in fig.1, the activity of the enzyme, illuminated in the presence of *p*-azidophenyl xyloside, decreases linearly with time, whereas in a control solution, without the azido compound, the activity remained constant for the time of the experiment. To test the nature of the inactivation, the experiment, depicted in fig.2, was performed. This demonstrates that the inactivation is due to a radical chain reaction, since the inactivation proceeds even when illumination is ceased, but slows down and even stops, when oxygen, as a scavenger [7], is bubbled through the solution. The results obtained when a *p*-nitrophenyl glycoside is added to the reaction mixture, as shown in table 1a, also are in favour of a radical reaction. The excellent protection observed here must be attributed to the scavenging properties of the nitrophenyl moiety of the glycoside [8]. Table 1b shows that the protection by the nitro derivatives cannot be attributed to absorption of the photolyzing radiation by the nitrophenyl moiety. Table 2 gives some more data on the inactivation by various azido compounds, and the protection by several inhibitors of the β-D-xylosidase. One may notice that the *o*-azidophenyl β-D-xyloside is not an inactivator at all (experiment 4). From experiment 7 it is obvious that illumination of the reaction mixture is necessary for the inactivation to occur. In fact, when the mixture of the enzyme and compound A is kept in the dark, this xyloside behaves as a normal substrate, as does the ortho-derivative (C). When the para-substituted xyloside (A) was added as cosubstrate, the apparent constant for competitive inhibition with respect to the routine substrate, was 1.44 mM, as determined by double reciprocal plotting.

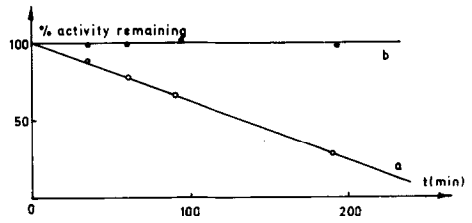

Fig.1. Activity decrease of β-D-xylosidase, illuminated (a) in the presence of 0.068 mM *p*-azidophenyl β-D-xylopyranoside and (b) in the absence of the inactivator.

The fact that both the *o*- and *p*-azidophenyl xylosides are substrates was proven by thin-layer chromatography after total hydrolysis. The *p*-azidophenol of experiment 6 was obtained by total hydrolysis of a mM solution of *p*-azidophenyl β-D-xylopyranoside.

Experiment 3 shows that the inactivator capacity of the *p*-azidophenyl xyloside (A) is drastically reduced when the product is submitted to prephotolysis. The residual inactivation may be due to the formation of long-lived intermediates, as is also proposed by Escher et al. [9].

We can thus conclude that the inactivation of the β-D-xylosidase by *p*-azidophenyl β-D-xylopyranoside, can be attributed to a radical chain reaction, probably due to the formation of a nitrene. It is furthermore obvious that inhibitors of the enzymic reaction give a considerable protection (experiment 1, table 2), that photolysis is necessary for inactivation to occur, and prephotolysis greatly reduces the inactivation rate. In addition, experiment 2 shows that compound B, which is neither a substrate nor an inhibitor of the

Table 1

(a) Protection of β-D-xylosidase against radical-inactivation by *p*-nitrophenyl β-D-glycopyranosides. Illumination was for 60 min in the presence of 0.5 mM *p*-azidophenyl β-D-xylopyranoside

Protector	Concn. (mM)	K_i[a] (mM)	Activity remaining (%)
None	–	–	37.5
p-Nitrophenyl β-D-riboside	0.98	0.033	100
p-Nitrophenyl β-D-glucoside	1	[b]	100

(b) Double-cell experiment. Illumination time was 150 min. For details see Materials and methods

Protector	Rear cell	Activity remaining (%)
p-Nitrophenyl β-D-riboside (4 mM)	enzyme + *p*-azidophenyl β-D-xyloside (0.5 mM)	18
Buffer	enzyme + *p*-azidophenyl β-D-xyloside (0.5 mM) + *p*-nitrophenylriboside (1 mM)	86

[a] Determined by double reciprocal plotting.
[b] No competitive inhibition.

Table 2

Inactivation of β-D-xylosidase with various azido compounds and protection by several ligands

Experiment No.	Inactivator (mM)	Protector (mM)	Illumination time (min)	Activity remaining (%)
1	A (0.068)	–	190	29
	A (0.068)	E (9.1)	190	70
2	B (0.091)	–	190	73
	B (0.091)	E (9.1)	190	87
3	A (0.182)	–	180	8
	A (0.182)[a]	–	180	67
4	C (0.750)	–	120	100
5	A (0.095)	–	100	17
	A (0.095)	F (2.12)	100	18.5
	A (0.095)	G (1.00)	100	49
6	D (0.083)	–	90	54.5
	D (0.083)	G (1.00)	90	66
7	A (0.091)	–	–[b]	93

[a] Prephotolysed: a 1 mM solution of inactivator in standard buffer was illuminated for 19 h before being mixed with enzyme and substrate.
[b] The solution was kept for 16 h in the dark at room temperature.

(A) *p*-azidophenyl β-D-xylopyranoside; (B) *p*-azidophenyl β-D-galactopyranoside; (C) *o*-azidophenyl β-D-xylopyranoside; (D) *p*-azidophenol; (E) pentyl β-D-xylopyranoside; (F) phenyl β-D-glucopyranoside; (G) phenyl β-D-ribopyranoside.

β-D-xylosidase, is a poor inactivator, as compared with the xyloside analog (experiment 1). The same can be concluded for *p*-azidophenol (D) as shown by experiment 6.

So far all these results are compatible with true photoaffinity labeling. Experiment 5 eliminates the possibility that the protection occurs through aspecific scavenger properties of the added ligands. Product F (not an inhibitor) gives no protection, whereas product G (strong inhibitor) gives considerable protection. On the contrary, the protection by the nitro derivatives must certainly be attributed to their aspecific scavenger properties as is shown in tables 1a and 1b. This result is in accordance with the results of fig.2, where no immediate change in reaction rate occurs when illumination is interrupted. These experiments indicate rather that a pseudophoto-affinity labeling occurs, which can be compared to ordinary affinity labeling as described by Ruoho et al. [10]. It is then easy to understand why some inactivation is also observed with the azidophenol and with aspecific azidoglycosides, since this inactivation must then be ascribed to the normal pseudo first-order reaction process of two species, one of which is present in large excess.

Further studies of the nature of the modified residue and the stoichiometry of the modification of the β-D-xylosidase are now in progress.

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