INVESTIGATION OF AN EXTRACELLULAR β-D-XYLOSIDASE FROM *ASPERGILLUS CARBONARIUS*

Ph.D. theses

Tünde Kiss

Promoter: Dr. László Kiss

University of Debrecen, Faculty of Sciences
Institute of Biochemistry
Debrecen, 2002

1. INTRODUCTION AND AIM OF THE DISSERTATION

Xylans are the most abundant renewable hemicelluloses of plant cell walls in hardwoods and cereals. The backbone of xylan is composed of β-1,4-linked D-xylopyranosyl residues. Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain. The structures of xylans vary depending on their origin, so it is not surprising that a complete hydrolysis of these complex molecules requires the synergistic action of several microbial enzymes. The presence of such xylan-degrading enzyme systems is quite widespread among fungi and bacteria. The most important xylanolytic enzymes are the endo-β-1,4-xylanase (EC 3.2.1.8), which hydrolyses the insoluble xylan backbone into shorter, soluble xylo-oligosaccharides, and the β-xylosidase (EC 3.2.1.37), which hydrolyses the soluble xylo-oligosaccharides and xylobiose from the nonreducing end to liberate xylose.

In the last decade the interest in xylan-degrading enzymes has greatly increased due to their potential biotechnological applications, especially in the paper and pulp industry, since the enzymatic degradation of xylan considerably facilitated pulp bleaching, decreasing both cost and environmental impact. From this point of view the most important xylanolytic enzymes are endoxylanases, however, the use of β-xylosidases is also essential for the overall conversion of xylans. Moreover, β-xylosidases play an important role in relieving the end product inhibition of endoxylanases. Despite their importance – in contrast to endoxylanase – limited information exists on the β-D-xylosidases, particularly their catalytic mechanism.

At the Institute of Biochemistry of University of Debrecen (Faculty of Sciences) one of the most important topics of the enzymatic research is the investigation of different β-glycosidases (β-glucosidases, β-hexosaminidases). The above mentioned facts prompted us to investigate another group of glycosidases, the β-D-xylosidases.

The aims of the present dissertation were as follows:

→ Isolation, purification and biochemical characterization of a microbial β-D-xylosidase.

→ The investigation of the mechanism of the action of the enzyme. We planned the chemical modification of the catalytically important amino acid residues in the active site of the enzyme with selective modifying reagents and with a reactive substrate analogue inactivator (affinity label) and we planned to investigate the effects of these modifications on the activity of the enzyme. One of the aims was the synthesis of an N-bromoacetyl-β-D-xylopyranosylamine as an affinity label for these investigations.

→ We also planned to investigate of the substrate binding site of the enzyme with substrate analogue inhibitors.

→ Furthermore, our aim was to determine the stereochemistry of the enzyme catalysed reactions.

2. APPLIED METHODS

The β-D-xylosidase was isolated from *A. carbonarius* cultures grown on solid medium. Modern methods of the protein separations (hydrophobic interaction chromatography, chromatofocusing, affinity chromatography) were used during the purification of the enzyme. The purify of the enzyme was checked by SDS polyacrylamide gel electrophoresis (SDS PAGE). The same method and MALDI TOF MS technique were applied for the determination of the molecular weight of the enzyme.

In the course of our work the enzyme activities were monitored with synthetic, chromogen substrates, by spectrophotometer. In kinetic studies the classical methods of enzyme kinetics were used. The kinetic parameters (K_m, V_{max}) were determined by nonlinear fitting of the classical Michaelis-Menten rate equation to the experimental data. In inhibition experiments K_i values were determined from Dixon plots. In the pH dependence studies molecular acid dissociation constants of the enzyme molecule (pK_{E1},pK_{E2}) and of the enzyme-substrate complex (pK_{ES1}) were also determined by nonlinear least-square fit.

The investigation of the catalytic amino acid residues was carried out by selective modifying reagents and by the kinetic analysis of the modifications.

In the synthesis of the reactive substrate analogue (N-bromoacetyl-β-D-xylopyranosylamine) micro, half-micro and macro methods of the preparative organic chemistry were used. The reactions were monitored by thin-layer chromatography. The isolation and purification of the products were carried out by column chromatography. The prepared products were identified by NMR technique. In the investigation of the transfer reactions besides these methods HPLC was applied.

3. NEW RESULTS OF THE DISSERTATION

3. 1. Isolation of a microbial β-D-xylosidase

We compared the extracellular β-D-xylosidase productions of different *Aspergillus* strains (*A. carbonarius*, *A. nidulans*, *A. niger* and *A. oryzae*) grown on wheat bran medium. Among these strains *A. carbonarius*, which has not been investigated so far, proved to be the best enzyme producer (its β-D-xylosidase production was 2-5 times higher than that of the other strains), therefore this strain was selected as an enzyme source for further investigations. We determined the optimal fermentation time for the β-D-xylosidase production of *A. carbonarius*, and on the basis of these results the enzyme purification was carried out from four-day-old *A. carbonarius* cultures.

3. 2. Purification and biochemical properties of β-D-xylosidase from *A. carbonarius*

We developed a rapid and efficient purification procedure of the β-D-xylosidase from *A. carbonarius* and we in detailed investigated its biochemical properties. The enzyme was purified to homogeneity by using hydrophobic interaction chromatography, chromatofocusing and affinity chromatography. The procedure resulted in an overall yield of 48,5 %, and the specific activity increased 61-fold (Table 1). The purified enzyme appeared as a single protein band on SDS PAGE.

We established, that the purified enzyme was a monomer protein, its molecular weight proved to be 100 kDa estimated by SDS-PAGE and 108 kDa estimated by MALDI TOF MS. The isoelectric point of the β-D-xylosidase estimated by chromatofocusing was at pH 4.4, indicating that the enzyme was an acidic protein. The pH and temperature optimum values were 3.0–4.0 and 60 °C, respectively. The enzyme remained stable over a pH range of 3.5–6.5 and up to 50 °C for 30 min.

3. 3. Investigation of the mechanism of action of the β-D-xylosidase from *A. carbonarius*

3.3.1. Enzyme kinetic investigations

The purified enzyme possessed β-D-xylosidase and low α-L-arabinosidase activities, as well. In order to determine whether these activities could be attributed to a common catalytic site, mixed substrate experiments were carried out. The results revealed that a single catalytic centre was responsible for the splitting of the both synthetic substrates (*p*-nitrophenyl

Table 1

Summary of purification of β-D-xylosidase from *A. carbonarius*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield* (%)
Culture filtrate	568	30.5	0.054	1.0	100
HIC (Phenyl-Sepharose CL-4B)	16.7	26.4	1.58	29.3	86,6
Chromatofocusing (PBE 94)	5.6	17.0	3.04	56.3	55,7
Affinity chromatography	4.5	14.8	3.29	60.9	48,5

* Yield was calculated based on total activity.

β-D-xylopyranoside (*p*NP-Xyl) and *p*-nitrophenyl α-L-arabinopyranoside (*p*NP-Ara)). This observation was also proved by inhibition experiments. The inhibition experiments at the same time indicated that not only the splitting site, but also the binding site was the same for the corresponding substrates and inhibitors.

We determined the kinetic parameters of the β-D-xylosidase (Table 2). These results demonstrated that *p*NP-Xyl bound to the β-D-xylosidase more tightly (according to K_m) and it was hydrolysed also more efficiently (according to V_{max}/K_m) than *p*NP-Ara. However, the maximum velocity value was higher with the arabinoside substrate than with the xyloside. These facts indicated that the *equatorial* C-4 hydroxyl group on the pyranoside ring played an important role in substrate binding, while the *axial* orientation at position C-4 could be more advantageous in the splitting.

Table 2

Kinetic parameters of β-D-xylosidase from *A. carbonarius*

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m ($U \times mg^{-1} \times mM^{-1}$)
*p*NP-Xyl	0.198 ± 0.002	3.64 ± 0.01	18.4 ± 0.19
*p*NP-Ara	5.5 ± 0.64	6.3 ± 0.26	1.2 ± 0.14

3.3.2. Investigation of the substrate binding site of the β-D-xylosidase

In order to obtain further information on the binding site in the active centre of the enzyme inhibition studies with substrate analogues were carried out. The results revealed that the carbohydrate moiety of the substrate (or the inhibitor) was a significant factor in the formation of the enzyme-substrate (-inhibitor) complex, in contrast to other, previously investigated β-D-glycosidases. Investigations with hexopyranosides demonstrated that the C-5 hydroxylmethyl group on the sugar ring stericly hindered the substrate binding, therefore its lack was essential for the formation of the enzyme-substrate (-inhibitor) complex. We found out that the C-2 hydroxyl group on the sugar ring was also relevant for the binding. Interactions (probably important hydrogen bonds) with the hydroxyl group at C-2 were required for the stabilization of the transition state and for the optimal orientation of the catalytic groups with respect to the bond to be cleaved. This observation was proved by the experiments with 2-deoxy-D-xylose. The introduction of a 1,2-unsaturation into the molecule (e.g. D-xylal and D-xylal derivatives) drastically decreased the inhibitor activity. A possible

explanation of this observation could be – besides the lack of the C-2 hydroxyl group – the rigidity of structure around C-1 of the pyranose ring, thus the inhibitor cannot fit well or at all into the active centre of the enzyme. The fact that 5-thio-D-xylose was a better inhibitor of the enzyme than D-xylose itself suggested that the heteroatom in the sugar ring could play a remarkable role in the binding of the substrates or the inhibitors. The investigations with inhibitors with different aglycon demonstrated that the hydrophobicity and electronic properties of the aglycon also proved to be important for the inhibition, although steric factors cannot be neglected either.

3.3.3. Investigation of catalytic amino acid residues of the enzyme

We determined the pH dependence of kinetic parameters of the hydrolysis of *p*NP-Xyl. The pH-dependence curves gave apparent pK values of 2.7 and 6.4 for the free enzyme, while value of 4.0 was obtained for the enzyme-substrate complex using *p*NP-Xyl as a substrate. These values permit us to conclude that two ionizable amino acid residues, a carboxylate group and a protonated group – presumably another carboxyl residue or a histidine– took part in the binding of *p*NP-Xyl, while a glutamic acid or an aspartic acid residue was essential in the cleavage of the substrate.

In order to investigate the exposed carboxyl groups in the β-D-xylosidase, we modified the enzyme with selective modifying agents Woodward's reagent K (WRK) and with water-soluble carbodiimide (EDAC) in the presence of glycine methyl ester. Kinetic analysis of the modifications indicated that the reagents inactivated the β-D-xylosidase according to pseudo-first order kinetics, proving that the enzyme included carboxyl residues, which were relevant for the catalysis and that at least one of these carboxyl groups was essential in the action of the enzyme. The enzyme was protected against EDAC- and WRK-mediated modification by the presence of a competitive inhibitor indicating that the modified, catalytically active carboxyl group was located in the active centre of the enzyme. We also investigated the pH dependence of the inactivations. The results demonstrated that the modified carboxyl group, on the basis of its pK_A value, was an Asp or Glu residue. A similar pK value was determined with the pH dependence of the V_{max} suggesting that the carboxyl residue of catalytically active Asp or Glu was modified. This suggestion was proved by the determination of the changes in the kinetic parameters during inactivation. During the modification of the enzyme V_{max} decreased considerably and than the enzyme inactivated, while K_m did not change significantly. These results clearly indicated that the modified carboxyl group was involved in substrate cleavage and not in binding. On the basis of the results of the modifications of the

enzyme we established, that the β-D-xylosidase from *A. carbonarius* included carboxyl residues, which were relevant for the catalysis, among these carboxyl groups at least one – probably an Asp or Glu residue – was present in the active centre of the enzyme and presumably as a protondonor it played an essential role in the splitting of the glycosid bond of *p*NP-Xyl.

In order to identify the catalytic carboxyl residue we synthesized and tested a new, reactive substrate analogue inactivator, N-bromoacetyl-β-D-xylopyranosylamine for β-D-xylosidase (Fig. 1). The *A. carbonarius* β-D-xylosidase was irreversible inactivated by the synthesised N-bromoacetyl-β-D-xylopyranosylamine and the process exhibited pseudo-first-order kinetics. A competitive inhibitor protected the enzyme from inactivation proving that the inactivation took place in the active centre. The treatment with a thiol reagent did not regenerate the enzyme indicating a stable, covalent modification and excluding the participation of a methionyl group in the inactivation process in contrast to *Escherichia coli* β-D-galactosidase.

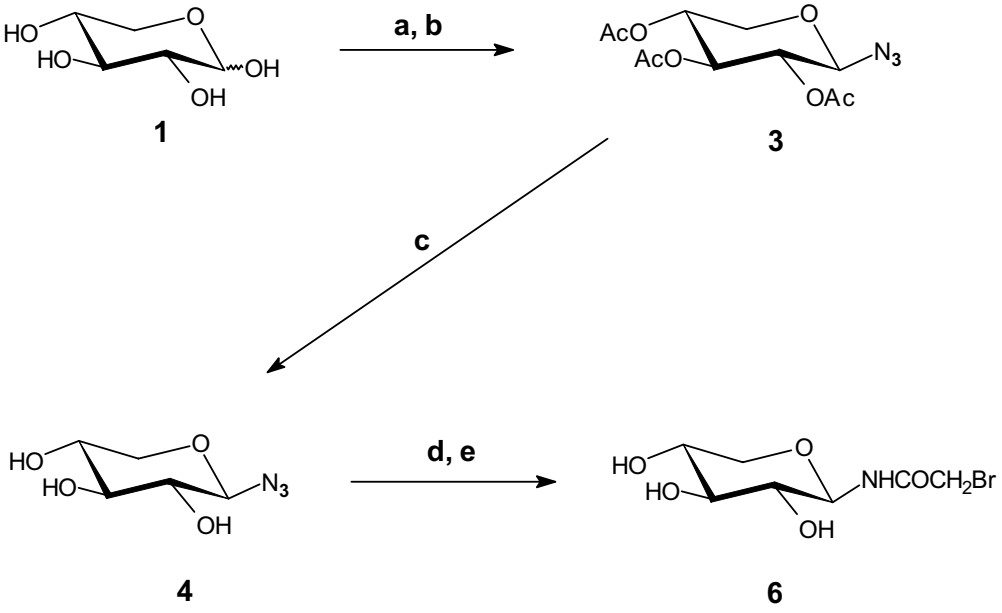

Fig. 1

Synthesis of N-bromoacetyl-β-D-xylopyranosylamine

a) pyridin/Ac_2O; **b**) $(CH_3)_3SiN_3$, $SnCl_4$, DCM; **c**) NaOMe, MeOH;

d) $H_2/Pd(OH)_2$, DMF; **e**) $(BrCH_2CO)_2O$/pyridin, CCl_4

These results clearly proved that the N-bromoacetyl-β-D-xylopyranosylamine formed a stable, covalent bond with the catalytic carboxyl residue in the active centre of the β-D-xylosidase from *A. carbonarius*, consequently it could identify after the sequencing of the enzyme.

3.3.4. Investigation of the transferase activity of the β-D-xylosidase

Our experiments revealed that under certain conditions the β-D-xylosidase from *A. carbonarius* could catalyze not only the hydrolysis, but also the formation of the glycosidic bond. We determined the stereochemistry of the β-D-xylosidase catalysed reactions. Our results indicated that the β-D-xylosidase from *A. carbonarius* operated with overall retention of the anomeric configuration, thus it belonged to the retaining ($e \rightarrow e$) glycosidases.

4. POSSIBLE UTILIZATION OF THE RESULTS

The synthesized N-bromoacetyl-β-D-xylopyranosylamine as an affinity label can be applied generally for the identification of catalytically important active-site residues in β-D-xylosidases, which is essential for understanding the catalytic mechanism of these enzymes.

As the β-D-xylosidase from *A. carbonarius* has a transferase activity, it can be a useful tool for chemoenzymatic syntheses.

Our results completed with new informations the quite incomplete knowledge of the fungal β-D-xylosidases, particularly of their catalytic mechanism. In the last decade interest in the β-D-xylosidases has greatly increased due to their potential biotechnological applications, especially in biobleaching technology in the paper and pulp industry. Our results can help to study and to understand of the mechanism of action of the β-D-xylosidases, thus the results can promote their industrial application.

5. LIST OF PUBLICATIONS

1. **Tünde Kiss** and László Kiss
   Purification and characterisation of an extracellular β-D-xylosidase from *Aspergillus carbonarius*
   World Journal of Microbiology and Biotechnology (2000) **16(5),** 465-470

2. **Tünde Kiss**, Anikó Erdei and László Kiss
   Investigation of the active site of the extracellular β-D-xylosidase from *Aspergillus carbonarius*
   Archives of Biochemistry and Biophysics, (2002) **399(2),** 188-194

3. **Tünde Kiss**, Andrea Székely, Marietta Tóth, László Somsák, László Kiss
   Inhibition of β-D-xylosidase (EC 3.2.1.37) from *Aspergillus carbonarius* by β-D-xylopyranose and D-xylal derivatives
   Carbohydrate Research, közlés alatt

OTHER PUBLICATIONS

4. **Kiss Tünde**, Gyémánt Gyöngyi, Dános Béla és Nánási Pál
   A *Glycyrrhiza glabra* L. és a *Glycyrrhiza echinata* L. poliszacharidjainak vizsgálata Gyógynövény poliszacharidok II.
   Acta Pharmaceutica Hungarica (1998) **68**, 263-268

5. Marcelis van der Stelt, Minke A. Noordermeer, **Tünde Kiss**, Guus van Zadelhoff, Ben Merghart, Gerrit A. Veldink, Johannes F. G. Vliegenthart
   Formation of a new class of oxylipins from N-acyl(ethanol)amines by the lipoxygenase pathway
   European Journal of Biochemistry (2000) **267**, 2000-2007

6. Zoltán Nagy, **Tünde Kiss**, Attila Szentirmai and Sándor Biró
   β-Galactosidase of *Penicillium chrisogenum*: Production, purification and characterisation of the enzyme
   Protein Expression and Purification, (2001) **21,** 24-29

LECTURES AND POSTERS

1. **Kiss Tünde** és Kiss László
   *Aspergillus carbonarius*ból izolált β-xilozidáz enzim szubsztrátspecificitása és kinetikai vizsgálata
   MTA Szénhidrátkémiai Munkabizottsága 1997. évi előadóülése, 1997. május 21-22, Mátrafüred

2. László Kiss and **Tünde Kiss**
   Purification, substrate specificity and kinetic investigation of β-xylosidase from *Aspergillus carbonarius*
   3rd International Conference of the Hungarian Biochemical Society, 6-9 July, 1997, Pécs, Hungary

3. **Tünde Kiss**, László Kiss and Göte Johansson
   Purification of β-xylosidase by partitioning in aqueous two-phase systems
   Annual Meeting of the Committee of Carbohydrate Chemistry of the Hungarian Academy of Sciences, 26-27 May 1999, Mátrafüred, Hungary

4. **Tünde Kiss** and László Kiss
   Purification and characterisation of an extracellular β-xylosidase from *Aspergillus carbonarius*
   26th Meeting of the Federation of European Biochemical Societies, June 19-24, 1999, Nice, France

5. **Tünde Kiss**, Andrea Székely, László Somsák and László Kiss
   Synthesis of xylosidase inhibitors and their enzymatic investigations using β-D-xylosidase from *Aspergillus carbonarius*
   20th International Carbohydrate Symposium, August 27-September 1, 2000, Hamburg, Germany

6. **Kiss Tünde**, Kiss László
   Aspergillus carbonarius-ból izolált extracelluláris β-D-xilozidáz vizsgálata
   IX. Fermentációs Kollokvium, 2000. október 5-8, Debrecen
   Acta Biol. Debrecina (2000) 140-144