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Characterisation of a recombinant β -xylosidase (xylA) from *Aspergillus oryzae* expressed in *Pichia pastoris*

Narin Kirikyali, Jonathan Wood and Ian F Connerton*

Abstract

β -xylosidases catalyse the hydrolysis of short chain xylooligosaccharides from their non-reducing ends into xylose. In this study we report the heterologous expression of *Aspergillus oryzae* β -xylosidase (XylA) in *Pichia pastoris* under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. The recombinant enzyme was optimally active at 55°C and pH 4.5 with K_m and V_{max} values of 1.0 mM and 250 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ respectively against 4-nitrophenyl β -xylopyranoside. Xylose was a competitive inhibitor with a K_i of 2.72 mM, whereas fructose was an uncompetitive inhibitor reducing substrate binding affinity (K_m) and conversion efficiency (V_{max}). The enzyme was characterised to be an exo-cutting enzyme releasing xylose from the non-reducing ends of β -1,4 linked xylooligosaccharides (X_2 , X_3 and X_4). Catalytic conversion of X_2 , X_3 and X_4 decreased (V_{max} and k_{cat}) with increasing chain length.

Keywords: *Aspergillus oryzae*; Xylose; β -xylosidase; Enzyme kinetics; Protein expression

Introduction

Xylanolytic and cellulolytic enzymes encoded by filamentous fungi have been employed in several industrial applications for improving digestibility in animal feed, production of sweeteners, pharmaceuticals, additive chemicals for bio-fuel production and for the replacement of hazardous chemicals in textile and paper manufacture (Michelin et al. 2012). As a consequence it has been a necessity for enzymes involved in cellulose and hemicellulose hydrolysis to be individually identified and characterised in order to utilise them in the process of converting waste agricultural materials into valuable products with greater efficiency.

Hemicellulose is comprised of a linear main chain β -1,4 linked D-xylose backbone with short lateral side chains of different lower molecular weight sugar residues (Dyk and Pletschke 2012). Enzymatic hydrolysis of hemicellulose commences with the removal of side chains that block the sites where xylanases cleave the xylan backbone. Endo-1,4- β -xylanase enzymes cleave the glycosidic bonds in a selective manner depending on the chain length, degree of branching of substrate molecules and the presence of

alternative carbohydrate moieties (Polizeli et al. 2005). Cleavage of the xylan backbone yields xylooligosaccharides and the final trimming is carried out by β -xylosidase, whereby short chain oligosaccharides and xylobiose are hydrolysed from the non-reducing termini to release xylose monomers (Polizeli et al. 2005; Teng et al. 2011).

Among xylanolytic enzymes, endo-xylanases and β -xylosidases have attracted attention as they commence and complete the breakdown of hemicellulose fraction respectively (Kulkarni et al. 1999). For comprehensive hydrolysis β -xylosidases play an important role in the removal of xylooligosaccharides from the catalytic environment, which assists by the elimination of the end-product inhibitors of endo-xylanases.

The gene encoding for XylA was previously identified by Kitamoto et al. (1999) and was reported to be responsible for the rapid browning of soy sauce. In addition Kitamoto et al. (1999) were interested in the antisense inhibition of XylA expression in order to hinder the translation in *Aspergillus oryzae* KBN616 to produce a mutant strain that could be used in Japanese food industry. However, XylA is a potentially efficient candidate for the facilitation of hydrolysis of hemicellulose applications in industrial processes. The work presented here reports the expression

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of a β -xylosidase from *Aspergillus oryzae* in *Pichia pastoris* and the kinetic characterisation of the recombinant enzyme.

Materials and methods

Construction of expression vector

The β -xylosidase encoding gene (*xylA*) was kindly provided by Noriyuki Kitamoto (Aichi Industrial Technology Institute, Japan). The gene sequence appears in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB013851. The gene was sub-cloned into pCR⁺2.1 and subsequently into the *Pichia pastoris* expression vector pPpHis4_GAP_BglII (TU Graz) under control of the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter.

Transformation and expression of recombinant β -xylosidase in *Pichia pastoris*

Plasmid DNA (10 μ g) containing the *xylA* gene sequence was linearized with *Bgl*II for integration at the GAP locus and was transformed into *P. pastoris* GS115 using Invitrogen *Pichia* Easycomp kit (as per manufacturers' guidelines). Positive transformants, displaying the His⁺ phenotype on Regeneration Dextrose medium (RD) agar plates (1 M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 0.005% amino acids (L-glutamic acid, L-methionine, L-lysine, L-leucine, L-isoleucine) and 1.5% agar), before transfer onto RD plates containing 50 μ g ml⁻¹ bromo-4-chloro-3-indolyl β -D-xylopyranoside (X-xyl) (Sigma-Aldrich, UK) and incubated at 30°C for 2 days. Functional expression of the β -xylosidase under the control of GAP promoter was tested by cleavage of xylopyranoside from the synthetic indicator X-xyl.

A single colony displaying the highest level of blue precipitate was sub-cultured from the RD plate onto a YPD plate (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and incubated at 30°C for 3 days. A seed culture was raised using a single colony inoculated into 50 ml YPD liquid medium, incubated at 28°C overnight. One hundred millilitres of YPD broth was then inoculated to 1 OD_{600nm} and incubated for 72 h at 28°C. One ml of culture was removed every 24 h to test expression levels using the synthetic 4-nitrophenyl β -xylopyranoside (PNPX) as described in section 2.4. Following incubation the cells were harvested by centrifugation at 4000 \times g for 10 min.

Purification of recombinant β -xylosidase

Following centrifugation the culture supernatant was concentrated using a Sartorius Sartoclon Slice then diafiltered with 10 volumes of Tris-salt buffer (10 mM Tris, 50 mM NaCl, pH 7.5). The concentrate was then stabilised using 30% (w/v) sucrose based on protein concentration and frozen for long term storage at -20°C. Prior

to enzymatic assays the 30% (w/v) sucrose was removed from the recombinant enzyme concentrates using a Vivaspin concentrator (GE Healthcare, UK) with a 10 kDa molecular weight cut off membrane filter and the filtrate was washed with Tris-salt buffer (10 mM Tris, 50 mM NaCl, pH 7.5).

Enzyme assays using synthetic substrates

Assays for β -xylosidase activity were performed by measuring the pNP released from p-nitrophenyl glycoside synthetic substrates 4-nitrophenyl- β -D-xylopyranoside (PNPX), 4-nitrophenyl- β -D-glucopyranoside (PNPG) and 4-nitrophenyl- α -L-arabinofuranoside (PNPAf) in a final volume of 4 ml for 20 min in 50 mM sodium phosphate buffer pH 6.0 at 50°C. Reactions were terminated by the addition of 1 M Na₂CO₃ and the amount of released pNP was measured at OD_{410nm}. One unit (U) of β -xylosidase activity is defined as the amount of enzyme required to release 1 μ mol of pNP per min under assay conditions. Kinetic parameters (K_m and V_{max}) were determined by the measurement of activity against pNPX using different substrate concentrations (0.5 - 12 mM) using the standard assay procedure. Enzyme assays were performed in triplicate and are presented as mean values with standard error.

Enzyme assays using xylooligosaccharides

Activities against xylobiose, xylotriose and xylotetraose were determined at varying substrate concentrations (0.25 - 4 mg ml⁻¹) in a final volume of 1 ml for 10 min in 50 mM sodium phosphate buffer pH 6.0 at 50°C. All assays were carried out in triplicate and were terminated by the addition of 1 M Na₂CO₃. Reaction products were separated according to molecular size by HPLC (Dionex ICS-3000 SP) with CarboPacTM PA20 column (3 \times 150 mm) and a gradient of 10 - 50 mM sodium hydroxide was applied for 20 min at a flow rate of 1 ml min⁻¹. The products were quantified on the basis of standard peak areas from various concentrations of control xylose, xylobiose, xylotriose and xylotetraose solutions. Enzyme assays were performed in triplicate and are presented as mean values with standard deviations.

Inhibition studies

To investigate the effect of end product xylose on catalytic activity, reactions were carried out in the presence of various xylose concentrations from 1 mM to 80 mM using synthetic substrate concentrations of either 1 mM or 4 mM pNPX. To further confirm the type of inhibition, kinetic constants were determined from experiments carried out using fixed inhibitor concentrations of 5 mM xylose at varying substrate concentrations from 0.25 mM to 8 mM under standard assay conditions.

The effects of monosaccharide sugars (20 mM glucose, mannose, galactose, arabinose, fructose and xylose), metal ions and chemicals (10 – 20 mM LiCl, KCl, ZnCl₂, SDS, EDTA and DTT) on enzyme activity were tested using 50 mM sodium phosphate buffer pH 6, 1 mM pNPX and 2 µg of enzyme at 50°C for 10 min in a final volume of 4 ml and measured optical density of released p-nitrophenyl at OD_{410nm}.

Determination of protein concentration

Protein concentrations were determined by the standard assay procedure using Pierce Coomassie® Plus Protein Assay Reagent. Sample diluents were used as the blank and the absorbance measured at OD_{595nm}. All assays were performed in triplicate and the OD_{595nm} readings of unknown sample were compared against BSA protein standard series which covered the range of concentrations between 50 and 1500 µg ml⁻¹.

Determination of molecular mass by SDS-PAGE

SDS-PAGE was performed using 8% polyacrylamide gels according to the method described by Laemmli (1970). Protein bands were stained with colloidal Coomassie Blue. Bands from SDS-PAGE were excised and were subjected to trypsin digestion prior to mass spectrometry analysis.

Mass spectrometry

Analyses of samples were carried out by LC-ESI-tandem MS on a Q-TOFII mass spectrometer fitted with a nano-flow ESI (electrospray ionization) source (Waters Ltd, UK). Peptides were separated on a PepMap C18 reverse phase, 75 µm i.d., 15-cm column (LC Packings) and delivered on-line to the MS via a CapLC HPLC system. Sequence interpretation for individual peptides was performed using the PepSeq MASCOT tool of the MassLynx™ 4.0 software package (Waters).

Results

Characterisation of recombinant β-xylosidase

The β-xylosidase gene is contained within an open reading frame of 2397 nucleotides with no introns, which encodes a protein of 798 amino acids. A putative signal peptide was identified by SignalP software, thus the mature protein was predicted to be 778 amino acids with a molecular mass of 84.7 kDa. The recombinant enzyme was recovered from *Pichia pastoris* culture supernatant at approximately 100 mg L⁻¹. NetNGly 1.0 predicted 12 potential N-glycosylation sites for β-xylosidase, and consistent with this, the recombinant enzyme was heterogeneous with a molecular mass estimated between 153 and 165 kDa on SDS-PAGE (Figure 1). The recombinant protein was excised from SDS-PAGE and the masses and protein sequences of tryptic peptides were determined using mass spectrometry to confirm the protein

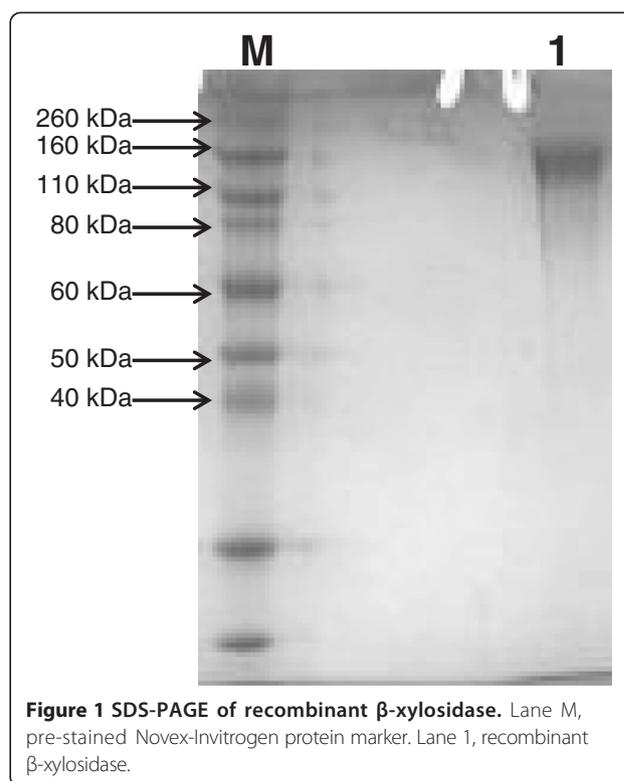


Figure 1 SDS-PAGE of recombinant β-xylosidase. Lane M, pre-stained Novex-Invitrogen protein marker. Lane 1, recombinant β-xylosidase.

product was exo-1,4 β-xylosidase originating from *Aspergillus oryzae*.

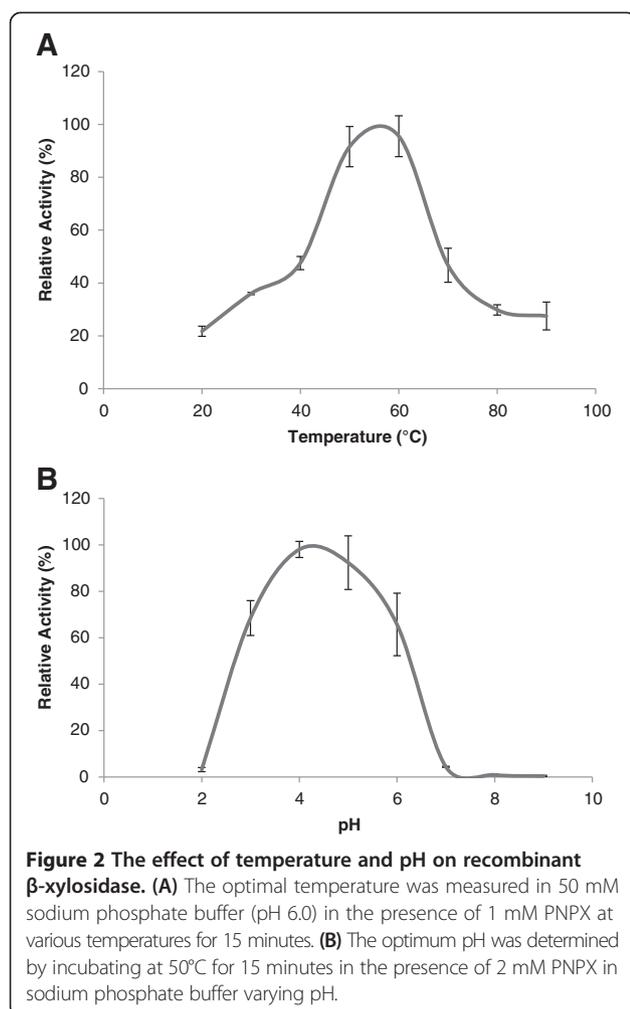
Determination of optimal conditions

The optimum temperature was determined by incubation of the recombinant enzyme in the presence of 50 mM sodium phosphate buffer pH 6.0 and 2 mM 4-nitrophenyl β-xylopyranoside (PNPX) for 15 min at varying temperatures between 20°C and 90°C. The enzyme displayed optimum activity at 55°C and retained more than 90% catalytic activity between the ranges of 50 – 60°C (Figure 2A).

The optimum pH of enzymatic activity was assayed in phosphate buffer system of varying pH values from 2–9 in the presence of 2 mM PNPX. The enzyme displayed activity within a narrow pH range, with an optimum of pH 4.5 and at least 65% activity from pH 3 – 6; less than 5% activity was observed at pH 7 – 9 (Figure 2B).

Substrate specificity and kinetic analysis

The substrate specificity of the recombinant enzyme was determined using various 4-nitrophenyl glycoside synthetic substrates and xylooligosaccharides. Recombinant XylA hydrolysed 4-nitrophenyl β-xylopyranoside efficiently but had trace hydrolytic activities against 4-nitrophenyl-β-D-glucopyranoside or 4-nitrophenyl-α-L-arabinofuranoside. Specific activities were determined as 150, 2 and 0.9 U mg⁻¹ for PNPX, PNPAf, PNPG respectively.



Kinetic constants for synthetic and natural substrates were determined using Hanes plots (Table 1). The enzyme exhibited K_m and V_{max} values of 1.0 mM and 250 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ respectively for the hydrolysis of 4-nitrophenyl β -xylopyranoside. Addition of the reaction product xylose resulted in enzyme inhibition. Kinetic constants were determined using Hanes plots from experiments carried out using a fixed inhibitor concentration of 20 mM xylose at varying PNPX concentrations (0.5 -

4 mM). The K_m was altered whilst the V_{max} remained unchanged, suggesting that xylose is a competitive inhibitor. A K_i of 2.7 mM for xylose was determined from Dixon plots. The addition of 20 mM fructose also had an inhibitory effect. Hanes plots indicated that the catalytic conversion of substrate into product was altered in terms of a reduction in the V_{max} and substrate affinity (K_m), exhibiting the characteristics of uncompetitive inhibition.

The degradation of various xylooligosaccharides (X_2 , X_3 and X_4) by recombinant XylA was analysed by HPLC. Xylose was released from all substrates and the rate of xylose released decreased with increasing chain length of the xylooligosaccharide. Table 1 shows reductions in the catalytic conversion parameters V_{max} and k_{cat} with respect to the increasing chain length of the xylooligosaccharides. The relative affinity of XylA towards the natural substrate xylo-tetraose (X_4) was significantly greater than xylobiose (X_2) or xylo-triose (X_3) with respect to the K_m values.

Effect of carbohydrates on catalytic activity

The catalytic activity of recombinant β -xylosidase was measured using 1 mM pNPX as substrate in the presence of 20 mM sugar solutions to test whether the enzyme was inhibited or enhanced (Figure 3A). In the presence of 20 mM xylose and fructose the catalytic activity was reduced to 18% and 26% respectively, with none of the others (arabinose, mannose, galactose, glucose and sucrose) showing any change in catalytic activity.

Effect of metal ions and chemical compounds on enzyme activity

The effects of various metal ions and reagents on β -xylosidase activity were assayed at 10 mM and 20 mM concentrations (Figure 3B). Most notably the addition of Zn^{2+} (10 mM) enhanced enzyme activity by 80%. The detergent SDS at 20 mM reduced the catalytic activity by 40%.

Discussion

A β -xylosidase encoding gene (*xylA*) from *Aspergillus oryzae* KBN616 was expressed in a soluble, active form under control of the constitutive GAP promoter in

Table 1 Kinetic analysis of synthetic and natural substrates determined in 50 mM sodium phosphate buffer (pH 6.0) at 50°C

| Substrate | K_m (mM) | V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) | k_{cat} (sec^{-1}) | k_{cat}/K_m ($\text{mM}^{-1} \text{s}^{-1}$) |
|-------------------------|----------------|---|---------------------------------|--|
| pNPX | 1 \pm 0.3 | 250 \pm 0.001 | 352.7 | 352.7 |
| pNPX + 20 mM Xylose | 2.9 \pm 0.5 | 250.5 \pm 23 | 353.4 | 121.9 |
| pNPX + 20 mM Fructose | 0.1 \pm 0.06 | 14.5 \pm 3 | 20.6 | 206 |
| Xylobiose (X_2) | 2.6 \pm 0.3 | 25.5 \pm 0.1 | 36.0 | 13.8 |
| Xylo-triose (X_3) | 3.07 \pm 0.3 | 21.3 \pm 0.3 | 30.1 | 9.7 |
| Xylo-tetraose (X_4) | 0.62 \pm 0.4 | 14.5 \pm 0.003 | 20.5 | 33.1 |

\pm standard deviation of the means of triplicate data.

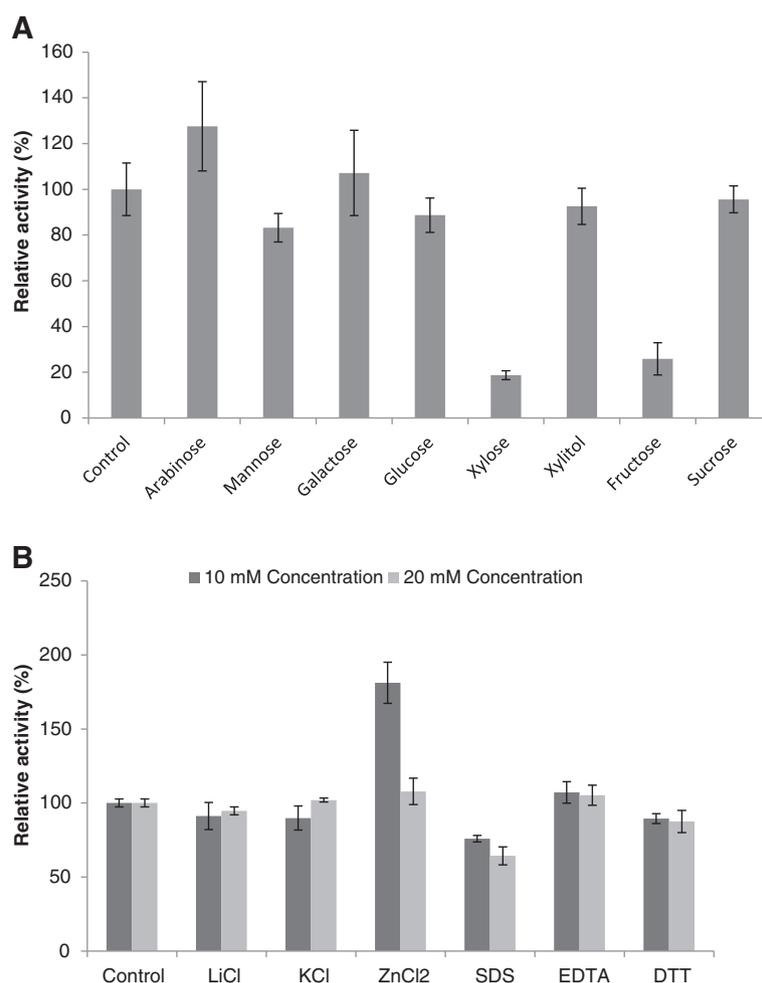


Figure 3 Enzyme activity profiles in the presence of (A) 20 mM carbohydrates and (B) metal ions and chemical compounds within reaction mixtures. All reactions were performed in 50 mM sodium phosphate buffer (pH 6.0) and 2 mM PNPX at 50°C. Control reaction performed in the absence of carbohydrates and chemicals under identical conditions.

Pichia pastoris. The predicted presence of a native signal sequence (SignalP) was confirmed through secretion of the mature protein by the expression host. XylA was predicted to have a molecular mass of 86.4 kDa and 12 potential N-linked glycosylation sites. Kitamoto *et al.* (1999) previously identified purified the native enzyme from *A. oryzae* culture supernatant, in which the enzyme produced a single protein band with an apparent molecular mass of 110 kDa on SDS-PAGE. However the molecular mass range of XylA determined by SDS-PAGE from *P. pastoris* was 153 to 165 kDa indicating differences in post-translational modification consistent with the predicted glycosylation sites. Heterologous proteins expressed in *P. pastoris* are subject to glycosylation and several plant cell wall degrading enzymes expressed in *P. pastoris* are reported to be hyper-glycosylated, including β -xylosidase from *Paecilomyces thermophila* (Juturu and Wu 2013), cellobiose dehydrogenase from *Neurospora crassa* (Zhang

et al. 2011) and endo-xylanase from *Actinomadura* sp. S14 (Sriyapai *et al.* 2011).

The biochemical properties of the recombinant β -xylosidase closely match the native enzyme with respect to the observed optimal pH range (pH 4.5 – 5) and temperature (55°C) for enzyme activity. These are comparable to other fungal xylosidases (Saha 2003; La Grange *et al.* 2001; Wakiyama *et al.* 2008; Zanoelo *et al.* 2004; Rasmussen *et al.* 2006), which exhibit optimal activities between pH 3–5 at 60°C.

The recombinant enzyme was most active against p-nitrophenyl- β -D-xylopyranoside (PNPX), with minimal activities towards 4-nitrophenyl- β -D-glucopyranoside (PNPG) and 4-nitrophenyl- α -L-arabinofuranoside (PNPAf). Activity towards a broad range of synthetic substrates by other fungal β -xylosidases has been reported, although maximum activity is generally towards PNPX (Margolles-Clark *et al.* 1997; Ohta *et al.* 2010; Wakiyama *et al.* 2008;

Katapodis et al. 2006). The exception to this is *Aspergillus awamori* X-100 β -xylosidase, which is reported to exhibit a greater k_{cat} against PNPAf (Eneyskaya et al. 2007). The recombinant enzyme exhibited kinetic constants for the hydrolysis of PNPX of 1.0 mM and 353 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for K_m and V_{max} respectively. A range of kinetic constants have been reported for the hydrolysis of PNPX by fungal β -xylosidases but these values are similar to those reported for purified β -xylosidases from *Sporotrichum thermophile* (Katapodis et al. 2006) and *Fusarium proliferatum* (Saha 2003). The hydrolysis of various xylooligosaccharides (X_2 , X_3 and X_4) was monitored by HPLC. In the presence of individual xylooligosaccharides, xylose was detected as an initial product of catalysis indicating that the recombinant β -xylosidase is an exo-cutting enzyme. The k_{cat} values for the xylooligosaccharides decrease with increasing chain length in the order of X_2 (36.0 sec^{-1}) > X_3 (30.1 sec^{-1}) > X_4 (20.5 sec^{-1}). However, the reduction in the K_m value for xylotetraose (X_4) results in a greater catalytic efficiency towards this substrate. The observed changes in the kinetic constants (K_m and k_{cat}) with respect to xylooligosaccharide chain length show similar patterns to those reported for the β -xylosidases originating from *Talaromyces emersonii*, *Trichoderma reesei* and *Aspergillus nidulans* (Rasmussen et al. 2006; Dilokpimol et al. 2011). In contrast *Neurospora crassa* β -xylosidase is reported to show a reduction in catalytic efficiency towards xylotetraose compared to shorter chain xylooligosaccharides (Kirikyali and Connerton, 2014). This is largely due to a relative reduction in the affinity of the *N. crassa* enzyme towards the xylotetraose substrate (> K_m value).

Similar to the data reported for the β -xylosidases from *Penicillium sclerotiorum* (Knob and Carmona 2009), *Talaromyces thermophilus* (Guerfali et al. 2009) and *Paecilomyces thermophila* (Yan et al. 2008) the presence of 20 mM concentrations of the metal ions Li^+ , K^+ or Zn^{2+} had no effect on enzyme activity. The β -xylosidase of *Talaromyces thermophilus* has been reported to retain 44% activity in the presence of 10 mM of the detergent SDS (Guerfali et al. 2009), and similarly XylA retained catalytic activities of 75% and 60% respectively at 10 and 20 mM SDS.

Xylose has been determined to be a competitive inhibitor of recombinant *A. oryzae* β -xylosidase. In the presence of 20 mM xylose with varying substrate concentrations the K_m was altered with no corresponding effect on V_{max} . This is consistent with competitive inhibition in which the inhibitor interferes with the catalytic properties of enzyme by affecting substrate binding affinity by conferring a K_i of 2.7 mM. In this respect the recombinant enzyme displays similar characteristic to the β -xylosidases from *A. niger* (K_i 2.9 mM) (Gomez et al. 2001) and *T. Reesei* (K_i 2.4 mM) (Rasmussen et al. 2006). However, xylose tolerant β -xylosidases have been reported

to exist with K_i values up to 200 mM (Yan et al. 2008; Zanoelo et al. 2004). Fructose has the novel characteristics of an uncompetitive inhibitor in which the inhibitor interacts with the enzyme-substrate complex to prevent product formation. In this case the binding of xylooligosaccharide to the active site of β -xylosidase creates a binding site for fructose. High substrate concentrations of substrate will increase the occupancy of the active site and the binding sites for fructose, and therefore the effective inhibition. The functional and physiological consequences of this finding should be considered when the enzyme has to function in the presence of mixed substrates.

Competing interests

JW has become an employee of Biocatalysts Ltd since completion of the project. The authors declare no other competing interests.

Authors' contributions

NK carried out the biochemical studies and drafted the manuscript. JW carried out the protein expression studies and helped to draft the manuscript. IFC conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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