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ORIGINAL PAPER

Heterologous expression of β-xylosidase gene from *Paecilomyces thermophila* in *Pichia pastoris*

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Abstract β-xylosidase from thermophilic fungi *Paecilomyces thermophila* was functionally expressed in *Pichia pastoris* with a his tag in the C-terminal under the alcohol oxidase 1 (AOX1) promoter and secreted into the medium at 0.22 mg l^{-1}. Its molecular mass was estimated to be 52.3 kDa based on the SDS-PAGE analysis, which is 1.3 times higher than the predicted 39.31 kDa from its amino acid compositions, although no potential N- or O- glycosylation sites were predicted from its amino acid sequence. This is presumed to be caused by some unpredictable post-translational modifications based on mass spectrum analysis of the recombinant protein. The enzyme was most active at 60 °C and pH 7. It showed not only a β-xylosidase activity with a K_m of 8 mM and a V_{max} of 54 μmol min^{-1} mg^{-1} for hydrolysis of *p*-nitrophenyl β-D-xylopyranoside but also an arabinofuranosidase activity (6.2 U mg^{-1}) on *p*-nitrophenyl arabinofuranoside.

Keywords *Paecilomyces thermophila* · β-Xylosidase · *Pichia pastoris* · Expression · Enzyme activity

Introduction

Hemicellulose, interwoven between cellulose and lignin, is the second major carbohydrate polymer present in plant biomass (Pérez et al. 2002). It is a branched and heterogeneous polymer composed of pentose (D-xylose and D-arabinose) and hexose (D-mannose, D-glucose and D-galactose) sugars with xylose being the major component (Kumar et al. 2008). Complete depolymerization of hemicellulose needs the synergistic action of a group of enzymes including exo-xylanase, endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and acetylxylan esterase. Among them, exo-xylanase, endo-xylanase and β-xylosidase (collectively xylanases) are the key enzymes responsible for the complete hydrolysis of xylan backbone. Exo and endo-xylanases act on the homopolymeric backbone of 1, 4-linked β-D-xylopyranose producing xylooligomers (Ahmed et al. 2009) while β-xylosidase cleaves from the non-reducing end of these xylooligomers releasing xylose (Knob et al. 2010).

Xylanases are produced by a wide spectrum of microorganisms in nature (Ahmed et al. 2009; Knob et al. 2010). They have great potential for applications in pretreatment of lignocellulose for biorefinery industries. Production of xylanases from various origins by recombinant DNA technology is a more effective way to obtain these enzymes in large amount and cheap cost compared to producing them from native strains. In addition, β-xylosidase is often insufficient in cultures of native strains as considerable amount of β-xylosidase is still kept inside the cells (Biely et al. 1980; Knob et al. 2010). So its overexpression in a secretion mode is essential for preparing hemicellulase cocktails with well balanced xylanase compositions. The functional expression of both exo- and endo-xylanases (EC 3.2.1.8) has been successful in many hosts including bacteria, yeasts, fungi and plants with yeasts being the most promising expression systems (Buckholz and Gleeson 1991). However, functional expression of β-xylosidases (E.C.3.2.1.37) is more challenging possibly due to their complicated structures. The β-xylosidase genes from *Aspergillus japonicus* (Wakiyama et al. 2008) and *Aureobasidium pullulans* (Ohta et al. 2010) have been functionally expressed in the methylotrophic yeast *Pichia pastoris,* a

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host with many excellent properties such as the ability to perform eukaryotic post-translational modifications, high level of protein expression and secretion of high molecular mass proteins (Daly and Hearn 2005; Macauley-Patrick et al. 2005; Cereghino and Cregg 2000; Cregg et al. 1985). Here we report the functional expression and characterization of a β-xylosidase gene from the thermophilic sac fungus *Paecilomyces thermophila* J18 in *P. pastoris*.

Materials and methods

Construction of vectors for expression in *P. pastoris*

Paecilomyces thermophila β-xylosidase gene sequence was obtained from NCBI with the accession number GU937001 (Teng et al. 2011). The coding sequence is 1,017 bp long coding a protein of 338 amino acids. It was synthesized, ligated onto plasmid pUC57 (Genescript, NJ, USA) between the *Eco*RI and *Xba*I sites, amplified in *Escherichia coli* TOP10 and further sub-cloned onto plasmid pPICZα A between the *Xho*I and *Xba*I sites after the α-factor signal sequence. During the sub-cloning PCR, the Kex2 and Ste13 signal cleavage sites were introduced following the *Xho*I site in the forward primer and a polyhistidine (6) tag sequence plus a stop codon was introduced before the *Xba*I site in the reverse primer.

Gene transformation, mutant selection and analysis of *P. pastoris*

The plasmid DNA (ca 8 μg) for yeast transformation was isolated from the recombinant *E. coli* top 10 cells using a miniprep kit (QIAGEN, Hilden, Germany). Prior to transformation, the plasmid DNA was linearized in the 5′ AOX1 promoter using the Fast Digest *Sac*I restriction enzyme (Fermentas, EU), purified on a PCR column (QIAGEN, Hilden, Germany) and eluted into a final volume of 10 μl as instructed by the supplier.

Electrocompetent cells of *P. pastoris* KM71 (Invitrogen, Carlsbad, USA) were prepared by harvesting the cells at $OD_{600} = 1$ followed by washing with 1 M sorbitol. The linearized plasmid (4.0 μg) was transformed into 50 μl yeast competent cells in a Micro Pulser (Bio-Rad, Hercules, CA) using the pre-set program PIC specific for *P. pastoris* (1.99 kV, 6.10 ms). The transformants were regenerated in 2 ml ice-cold 1 M YPS (1 % yeast extract, 2 % peptone and 1 M sorbitol) at 30 °C for 1 h without shaking. Afterwards, 200 μl portions of the regeneration culture were plated onto YPDS (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol and 2 % agar) plates containing 100 μg ml^{-1} zeocin. To isolate multi-copy strains, the regeneration culture was also plated onto YPDS plates containing zeocin with increased concentrations (200–400 μg ml^{-1}).

Expression of β-xylosidase by fermentation in shaking flasks

The selected single- and multi-copy yeast transformants were inoculated into 250 ml baffled conical flasks containing 50 ml of YPD medium with 100 μg ml^{-1} zeocin. The flasks were incubated at 28 °C and 250 rpm for 48 h. The primary induction of the stationary phase cell cultures (50 ml) was done by mixing with 50 ml of minimal medium with methanol (1 %, v/v) for 8 h, followed by addition of 1 % (v/v) of absolute methanol and incubation for another 16 h. Then 0.5 % (v/v) of absolute methanol was supplemented and the culture was continued for 8 h followed by addition of 1 % (v/v) of absolute methanol for further cultivation for 16 h. The above induction operation was repeated for another 3 days. During the induction period, 10 ml of aliquots were collected every 24 h and centrifuged at 4 °C and 4,000 rpm for 10 min. At the same time, 10 ml of minimal medium without methanol was supplemented into the culture to reimburse the depleted volume. The supernatant was collected by centrifugation and kept on ice for various analyses.

Purification of β-xylosidase and gel electrophoresis

Culture supernatant (15 ml) containing the recombinant β-xylosidase was purified using the HiTrap Chelating HP column (GE Healthcare, Uppsala, Sweden) following the supplier's protocol. The column was loaded with 0.1 M $NiSO_4$ (5 ml) followed by washing with distilled water (15 ml) to remove the excess $NiSO_4$ and equilibrating with the binding buffer (5 ml) without imidazole (0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.4). After being loaded with the supernatant, the column was washed with binding buffer (5 ml) to remove impurities. The bound protein was eluted by washing with 5 ml of elution buffer (0.02 M Na_2HPO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). The purity of the eluted protein was verified over NuPAGE Novex Tris–Acetate Mini Gels (Invitrogen, Carlsbad, USA).

Assays of protein concentration and enzyme activities

Protein concentration was measured by Bradford method using bovine serum albumin as the standard. *p*-Nitrophenyl β-D-xylopyranoside (*p*-NPX) and *p*-nitrophenyl α-L-arabinofuranoside were used as the substrates for assays of β-xylosidase and arabinofuranosidase activities, respectively. The experiments were conducted by incubating 0.65 ml of 5 mM substrates in 50 mM sodium phosphate buffer (pH

7) with 100 µl of enzyme elute for 10 min at 60 °C followed by addition of 0.75 ml of 1 M Na_2CO_3 to terminate the reactions. The reaction mixtures were diluted 30-fold and the released *p*-NP was detected at 405 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 µmol of *p*-NP per minute.

Determination of optimal conditions for β-xylosidase activity

The optimal pH for β-xylosidase activity was investigated at pH 3–8 using 50 mM citrate buffer (pH 3–6) and 50 mM sodium phosphate buffer (pH 7–8) to get the desired pHs.

The optimal temperature was investigated at 30–80 °C in 50 mM sodium phosphate buffer at pH 7. The Michaelis–Menten constants were obtained at pH 7 and 60 °C for *p*-NPX concentrations up to 25 mM. The K_m and V_{max} were calculated from the Lineweaver–Burk plots using the Graphpad Prism software (GraphPad Software, Inc, California, USA).

Xylose production from oat hay by purified β-xylosidase

Dried oat hay was purchased from a local shop and used as a source for xylan extraction. Grinded oat hay (1 %, w/v) in 500 ml water was autoclaved at 120 °C for 20 min. The solid was separated from the supernatant by centrifugation at 4,000 rpm and 4 °C for 10 min. To the supernatant were added 10 % volume of 3 M sodium acetate and 2 volumes of absolute ethanol. The mixture was incubated overnight at room temperature to precipitate xylan. The precipitated xylan was harvested by centrifugation at 4,000 rpm and 4 °C for 10 min, washed with 80 % ethanol and freeze-dried for use for β-xylosidase activity assay.

The oat hay xylan (10 %, w/v) in 6 ml sodium phosphate buffer (50 mM, pH 7) was incubated with 0.64 mg of the enzyme at 60 °C and 200 rpm for 24 h followed by placing the mixture in liquid nitrogen to terminate the reaction. The reaction mixture was thawed and centrifuged at 6,000 rpm for 10 min. The supernatant was collected after filtering through a membrane with a pore size of 0.22 µm. The filtrate was analyzed by HPLC with an Aminex HPX-42A carbohydrate column (300 mm × 7.8 mm) at 70 °C to detect the released xylose. Samples (50 µl) were eluted by de-ionized water at 0.6 ml min^{-1} and detected by a refractive index detector.

Mass spectrum analysis of β-xylosidase

The protein sample was diluted 1:1 with 200 mM triethylammonium bicarbonate (pH 8.5), reduced with 10 mM TCEP at 55 °C for 60 min and alkylated with 20 mM iodoacetamide at room temperature for 60 min. Three aliquots of the sample were digested with 1 µg of trypsin, chymotrypsin and endoproteinase Glu-C at 37 °C for 16 h, respectively. After being acidified with 1 % formic acid, the samples were lyophilized and re-suspended in 0.2 % formic acid. The samples were analyzed on an AGILENT QTOF 6520 with online nano-flow HPLC and chip-cube. The chromatographic column was a 150 mm × 75 µm REPROSIL C18 with a 40 µl trap-column in the AGILENT chip format. Samples (5 µl) were loaded onto the trap-column at 4 µl min^{-1}, the bound peptides were eluted from the trap-column and separated with a gradient formed from 0.2 % formic acid in water (A) and 0.2 % formic acid in acetonitrile (B). A linear gradient from 8 % B to 35 % B over 40 min was used for the elution. Instrument parameters were optimized prior to analysis with a 100 fmole tryptic BSA digest. For protein identification, 3 precursor scans per second were taken and 1 fragment was scanned per second.

The 4 peptides with the highest signal intensity and charges of 2^+ and 3^+ were taken for fragmentation. The mass calibration was maintained with a lock mass. The raw data were processed with AGILENT Qualitative Analysis software mzData.xml and submitted to database searches using PEAKS and SPIDER tools in PEAKS software (Bioinformatics Solutions, Canada). A database with the sequences of the species was used to search against the uniprot-swissprot database to confirm the absence of sequences from other species. The allowed modifications include carboxymethylation as fixed for cysteine, as variable modifications oxidation of methionine, deamidation of glutamate and aspartate, and phosphorylation of serine, threonine and tyrosine. A maximum of three missed cleavage were allowed. Intact protein molecular mass was determined on AGILENT 6224 ESI-TOF with a capillary-flow HPLC (AGILENT 1200) with a Poroshell C8 300 1 × 75 mm (AGILENT) column at 20 µl min^{-1} with a gradient from 5 % B to 50 % B within 5 min.

All the experimental data were the average of at least triplicate experiments with standard deviations of ±5 %.

Results and discussion

Gene transformation, expression and protein purification

Pichia pastoris KM71 transformants carrying the β-xylosidase gene showed clear colonies on YPDS plates with different zeocin concentrations (100–500 µg ml^{-1}) after incubation at 28 °C for 2 days. The single- and multi-copy transformants were randomly picked up and cultivated in 250 ml shaking flasks. The multi-copy strain that resisted

200 μg ml^{-1} zeocin gave the highest total protein expression at 0.22 mg l^{-1}. The multi-copy strains that resisted higher concentrations (>200 μg ml^{-1}) of zeocin showed protein expression levels equal to that of the single-copy strain or even lower (data not shown), possibly due to the formation of inclusion bodies at higher copy numbers as a result of the incorrect protein folding (Cereghino and Cregg 2000). Figure 1 shows the enzyme expression and β-xylosidase activity of the best transformant. Both total protein and enzyme activity in the supernatant reached their maximums at 48 h and became rapidly declined afterwards. The prolonged methanol induction enhances cellular stress responses leading to the movement of excess vacuolar proteases to cellular cytoplasm and eventually releasing to the fermentation broth. The formaldehyde produced during the methanol metabolism makes the cell wall become fragile and leaky. These factors collectively fasten the cell lysis, reducing the enzyme expression and fastening enzyme cleavage thus decreasing enzyme activity (Sinha et al. 2005). Therefore, in subsequent studies, the cell cultures were harvested at 48 h after induction with methanol for protein purification.

Characterization of recombinant β-xylosidase

The expressed β-xylosidase is expected to contain a His tag at its C terminal with a predicted molecular mass of 39.31 kDa before post-translational modifications. The purified enzyme showed a single band on SDS PAGE with an estimated molecular mass of 52.3 kDa (Fig. 2), which is 1.3 times higher than the predicted 39.31 kDa prior to post-translational modifications but is same with that (52.3 kDa) of the native protein from *P. thermophila* J18 (Teng et al. 2011). Analysis of the amino acid sequence did not predict

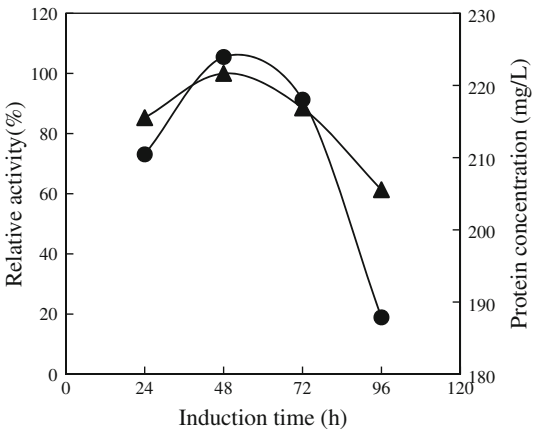

Fig. 1 Effects of induction time on enzyme concentration (*circle*) and β-xylosidase activity (*triangle*). Enzyme activity was assayed using 5 mM *p*-nitrophenyl β-D-xylopyranoside (*p*-NPX) at pH 7 and 60 °C. The enzyme activity was set as 100 % when it was the highest

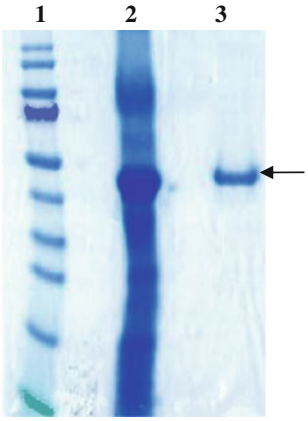

Fig. 2 SDS-PAGE of β-xylosidase. *Lane 1* page ruler pre-stained protein ladder (Fermentas, EU), *Lane 2* broth supernatant, *Lane 3* purified his-tagged protein

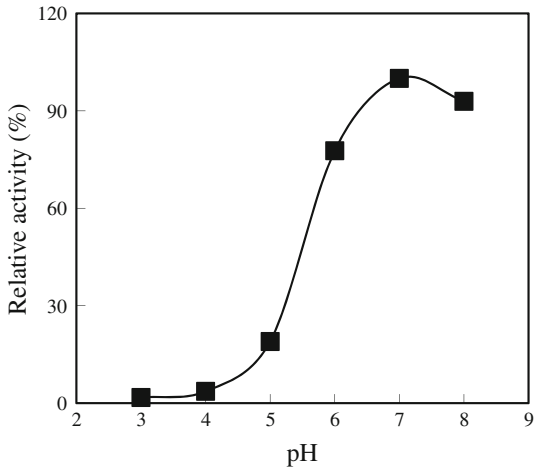

Fig. 3 Effect of pH on β-xylosidase activity of purified protein. Enzyme activity was assayed in 50 mM citrate (pH 3–6) and sodium phosphate (pH 7–8) buffers. The enzyme activity was set as 100 % when it was the highest

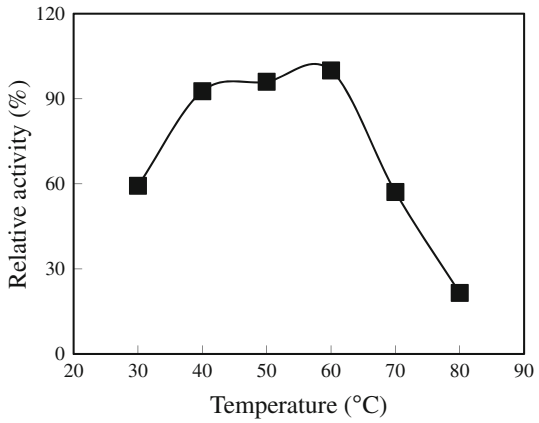

Fig. 4 Effect of temperature on β-xylosidase activity. Enzyme activity was assayed in 50 mM sodium phosphate buffer of pH 7. The enzyme activity was set as 100 % when it was the highest

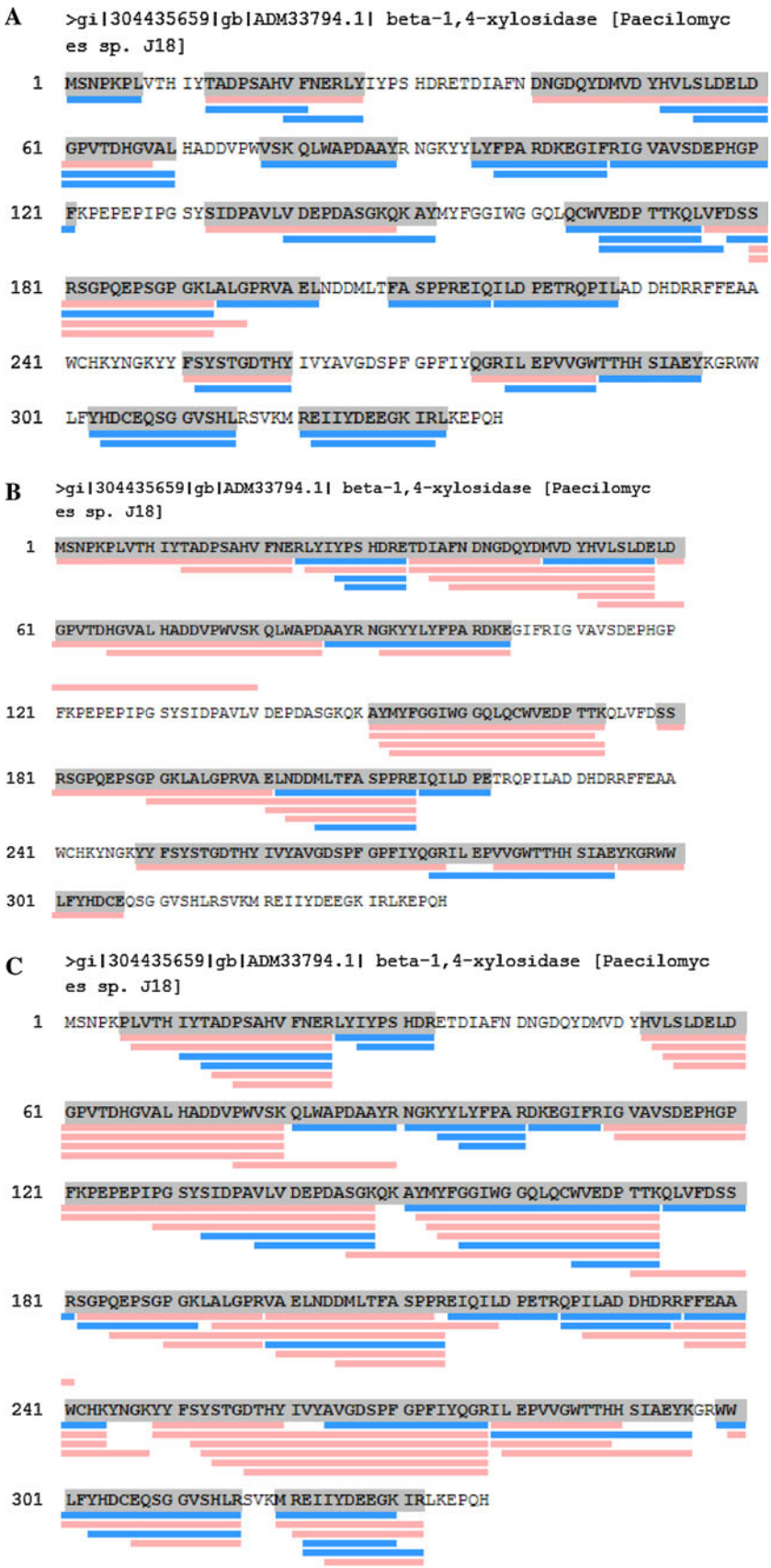

Fig. 5 Amino acid sequences of the recombinant protein digested by 3 different proteases, chymotrypsin **a**, endoproteinase glu-C **b** and trypsin **c**, obtained by analysis against the uniprot database. *Blue line* exact match of peptide sequence with database sequence, *pink line* modified peptide fragments during sample preparation, *grey color* homologous sequences between mass spectrum fragments and database sequences. (Color figure online)

any potential *N*- or *O*-glycosylation sites for this enzyme. The same molecular mass (52.3 kDa) was also reported when the same gene was heterologously expressed in *E. coli* (Teng et al. 2011). The enzyme showed its maximal β-xylosidase activity at pH 7.0 (Fig. 3) and 60 °C (Fig. 4). Its specific activity, K_m and V_{max} were determined to be 0.26 U mg^{-1}, 8 mM and 54 U mg^{-1}, respectively. Surprisingly, the enzyme also showed a good arabinofuranosidase activity (6.2 U mg^{-1}) on 4-nitrophenyl α-L-arabinofuranoside. Other activities except the arabinofuranosidase and β-xylosidase activities were not tested but their existences could not be excluded.

The dual functions of the enzyme are expected to be beneficial for more efficient hydrolysis of hemicellulose.

For xylan preparation from oat hay, 1 g of oat hay gave ca 0.4 g of xylan. Incubation of this oat hay xylan (10 %, w/v) with 0.64 mg of β-xylosidase in 6 ml 50 mM sodium phosphate buffer of pH 7 at 60 °C for 24 h yielded 4 g l^{-1} of xylose.

Mass spectral analysis of β-xylosidase

To elucidate the reasons leading to the higher molecular mass of the recombinant β-xylosidase, mass spectrum analysis of the recombinant protein was conducted. The database search using PEAKS resulted in a nearly full coverage of the protein sequence and the missing parts were either short peptides below the minimal size for the MS to MS/MS switching or above the maximal mass for ions to select. The only parts of the protein not covered by any of the three enzymatic cleavages were the 5 C-terminal amino acids. Therefore, the combined use of the three different proteolytic cleavages gave a full coverage of the sequence with almost no gaps in the protein itself.

Besides the unmodified peptides a number of modifications were recognized by the database search (Fig. 5a–c). Some of them were generated due to the sample treatments such as deamidation, carboxymethylation, pyro-glu formation and oxidation, but others such as phosphorylation might be added to the protein during the posttranslational modifications within the cell, although they could not be predicted based on the amino acid sequence of the protein (Kameoka et al. 2003; Cantin and Yates 2004; Saito et al. 2008; Wang et al. 2012). In addition to these modifications, some unspecific cleavages were identified. As these peptides showed high identification scores and the manual inspection of the MS/MS spectra further confirmed these results, this might indicate some unspecific cleavages due to high enzyme concentrations. These unspecific cleavages cannot be explained from the database search alone. A search against the uniprot database showed no additional peptides from other species, indicating that the determined sequence could only be from the β-1, 4-xylosidase investigated. Therefore, the higher than predicted molecular mass of the recombinant β-xylosidase might be ascribed to some unpredictable posttranslational modifications, although more information of the protein structure is needed to further confirm this presumption.

In summary, β-xylosidase from thermophilic fungus *P. thermophila* was successfully expressed in *P. pastoris* in a secretory mode. The transformant resisting 200 μg ml^{-1} zeocin gave the highest protein expression level. The recombinant β-xylosidase was most active at pH 7 and 60 °C. It showed not only a β-xylosidase activity but also an arabinofuranosidase activity. SDS-PAGE and mass spectrum analyses of the recombinant protein showed a molecular mass of 52.3 kDa, which is 1.3 times higher than the predicted value from the amino acid sequence. The higher-than-predicted molecular mass is presumed to be caused by some unconventional posttranslational modifications, which needs to be further confirmed by more sophisticated experiments. The activity, stability and substrate diversity of the enzyme are expected to be improved by rational and random gene mutagenesis to meet the requirements of various industrial applications. As metagenomic sequences abound in repositories, the synthetic approach to get genes could pave a way to comparative analyses of a larger number of yet physically unexpressed sequences of various genes and their variants to screen powerful enzymes, similar to laboratory directed evolution of proteins.

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