Biochemical and Biophysical Research Communications 379 (2009) 243–248

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

First in-gel detection and purification of human xylosyltransferase II

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ARTICLE INFO

Article history:
Received 3 December 2008
Available online 25 December 2008

Keywords:
Xylosyltransferase
Proteoglycan
Glycosaminoglycan
Turnover number
Catalytic efficiency
Pichia pastoris
Heparin

ABSTRACT

Human xylosyltransferases I and II (XylT-I, XylT-II) are key enzymes in glycosaminoglycan biosynthesis. Knowledge about the *in vivo* molecular weight, oligomeric state or turnover number are essential characteristics which have been addressed in this study. XylT-II was purified from *Pichia pastoris* by fractionated ammonium sulfate precipitation, heparin affinity and ion exchange chromatography. XylT-II was purified over 7000-fold with a final yield of 2.6%. By utilizing mass spectra analysis we can prove its first in-gel detection showing a migration pattern behavior that confirms its *in silico* molecular weight of 95.8 kDa. We could determine a turnover number of 2.18 min^{-1} or one transferred xylose molecule per one XylT-II molecule each 27.5 s. The k_{cat}/K_M ratio was 0.357 min^{-1} μM^{-1} for XylT-II using the bikunin-homologous acceptor Bio-QEEEGSGGGQKK-F. The comparison to XylT-I derived from the same organism revealed a 2.4-fold higher catalytic efficiency (0.870 min^{-1} μM^{-1}) for XylT-I.

Proteoglycans are complex macromolecules which are composed of a core protein to which glucosaminoglycan chains (GAG), such as chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate [1,2], are attached. The key enzymes for the biosynthesis of GAGs are the type II transmembrane proteins xylosyltransferase I and its isoform xylosyltransferase II (EC 2.4.2.26, XylT-I and XylT-II). Both enzymes transfer xylose from UDP-xylose to specific serine residues of the core protein. While XylT-I activity could already be shown in 2000 by Götting et al. [3], the physiological role of XylT-II was proved recently [4]. Both xylosyltransferases are different to other membrane-bound glycosyltransferases since they are proteolytically cleaved from the Golgi surface and released into the extracellular space together with proteoglycans. Therefore, XylT activity is used as a diagnostic marker for enhanced proteoglycan biosynthesis and tissue remodeling [5].

Two isoforms of xylosyltransferase are known in vertebrates, but only one isoenzyme exists in *Caenorhabditis elegans* and *Drosophila melanogaster* [6,7]. To date it has not yet been elucidated why higher organisms share two isoenzymes. Usually, isoenzymes display different kinetic parameters, regulatory properties, developmental stage expression profiles or tissue distribution. Mutation analyses of GAG attachment sites have revealed for human xylosyltransferase I and II different kinetic acceptance for these sites (manuscript under preparation). Moreover, a tissue-specific expression for XylT-II mRNA was predominantly found in the kidney, liver and lung [3,8]. It has also been shown that XylT-I is exclusively regulated by cytokines like TGF-β_1, while XylT-II expression is unaffected by this cytokine [9], indicating different cell signaling pathways.

Xylosyltransferases are present in animal tissues only in very small amounts, resulting in difficulties during isolation of these enzymes, whereas the detection of their enzymatic activity is simple and sensitive. Even the recombinant expression and purification in what are usually high-level protein producers, like yeasts or insect cells, led to difficulties in the SDS–PAGE visualization of xylosyltransferases; up to now, only the detection of XylT-I from insect cells has been possible, yielding 50 μg of purified protein from one liter culture medium [10]. An SDS–PAGE confirmation and analysis of xylosyltransferase II has, to our knowledge, not been reported. Through the lack of protein information by gel analysis, it is impossible to give some biochemical properties like protein mass determination by its migration pattern [11], oligomeric state analysis by blue native PAGE [12] or turnover number calculations based on a known amount of the protein of interest in a sample.

Here, we report the first in-gel detection through purification of catalytically active XylT-II from *Pichia pastoris* by employing a three-step purification protocol consisting of fractionated ammonium sulfate precipitation, heparin affinity and ion exchange chromatography, and with this purification we can present the first *in vivo* determined k_{cat} for XylT-II. This value will help future studies by indicating the amount of XylT-II in a sample through the correlation to its measured XylT-II activity. Additionally, we provide a kinetic analysis of XylT-II and its comparison to the isoform XylT-I derived from the same organism.

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doi:10.1016/j.bbrc.2008.12.033

Materials and methods

Construction of expression plasmid. Primers that included at their 5′-end the restriction sites *Not*I and *Eco*RI were designed based on the XylT-II cDNA sequence of pCMV-XL6-XYLT2 (Genbank accession number NM_022167, Origene, Rockville, MD). The oligonucleotides (upper primer 5′-TGAATTACGAATTCGGCCTGGAGGAGGAC GAG-3′ and lower primer 5′-TTTAAATGCGGCCGCCCTGAGTCGCCC GTCTGC-3′) were used to amplify a 2516 bp fragment which comprised the catalytic domain of XylT-II. The digested PCR product was cloned into the multiple cloning site of the pPICZ αA expression vector (Invitrogen, Karlsruhe, Germany), in frame with the alpha-factor signal sequence and with a C-terminal located myc-epitope and hexa-histidine tag.

Expression in P. pastoris. The *P. pastoris* strain X-33 (Mut^+), which harbored the XylT-II expressing plasmid controlled by the methanol-inducible AOX promoter. For that, a single colony was grown overnight in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) until an OD_{600nm} of approximately 25 was reached. Cells were harvested, washed twice in sterile water and resuspended in BMMY medium (0.5% methanol instead of glycerol) to a final OD_{600nm} of 1.0. Cells were grown on a rotary shaker for 3 days at room temperature. Expression was induced once a day by addition of methanol to a final concentration of 0.5%.

Ultrafiltration. Forty liters of culture supernatant containing XylT-II were collected by harvesting the cells at 4000 rpm for 10 min and concentrated about 33-fold to 1215 ml using ultrafiltration cells with polyethersulfone membranes (Vivascience, Hanover, Germany).

Fractionated ammonium sulfate precipitation. Solid ammonium sulfate was added to the supernatant to 25% saturation. After 1 h at room temperature, the suspension was centrifuged at 4000 rpm for 2 h, the supernatant was decanted and the precipitate removed. Additional ammonium sulfate was added to reach the point of 60% saturation and the suspension was allowed to incubate for another 1 h. To recover the precipitate, the supernatant was decanted after the suspension was centrifuged at 4000 rpm for 2 h. The precipitate was dissolved in 150 ml 40 mM sodium acetate, pH 6.0 and subjected to heparin affinity chromatography.

Heparin affinity chromatography. A POROS 50 HE column (10 cm × 16.0 mm, 50 μm particle size) was equilibrated with 40 mM sodium acetate, pH 6.0 (60 ml) at a flow rate of 10 ml/min. After loading a 2 ml sample from the previous ammonium sulfate precipitation step, the column was washed with 40 mM sodium acetate, pH 6.0 (60 ml). XylT-II was eluted with a step gradient of sodium chloride. The NaCl concentration was increased as follows: 40 mM sodium acetate, pH 6.0, 150 mM NaCl (40 ml), 40 mM sodium acetate, pH 6.0, 200 mM NaCl (40 ml), 40 mM sodium acetate, pH 6.0, and 500 mM NaCl (40 ml) and 40 mM sodium acetate, pH 6.0, 2.88 M NaCl (40 ml). Protein elution was monitored at A_{280}. This procedure was repeated 75 times by cyclic chromatography. Fractions were assayed for XylT-II activity and selected fractions were pooled, concentrated and desalted by buffer exchange into 35 ml 20 mM sodium acetate, pH 6.0.

Ion exchange chromatography. A POROS 20 HQ column (10 cm × 4.6 mm, 20 μm particle size) was equilibrated with 20 mM sodium acetate, pH 6.0 (45 ml) at a flow rate of 10 ml/min. After loading a desalted sample from the heparin affinity chromatography step, the column was washed with 20 mM sodium acetate, pH 6.0 (33 ml). XylT-II was eluted with a step gradient of sodium chloride. The NaCl concentration was increased as follows: 20 mM sodium acetate, pH 6.0, 70 mM NaCl (42 ml), 20 mM sodium acetate, pH 6.0, 180 mM NaCl (42 ml), 20 mM sodium acetate, pH 6.0, 360 mM NaCl (42 ml), 20 mM sodium acetate, pH 6.0, 500 mM NaCl (33 ml) and 20 mM sodium acetate, pH 6.0, 2 M NaCl (25 ml). Protein elution was monitored at A_{280}. Fractions were assayed for XylT-II activity.

TCA precipitation. XylT-II-containing fractions were TCA precipitated using a modified protocol from Tornqvist and Belfrage [13]. Briefly, concentrated XylT solution was expanded to a volume of 1 ml by adding water if necessary. Twenty-five microliters of a 1% solution of sodium deoxycholate were added and incubated for 30 min at 4 °C. One milliliter of a 12% solution of cold TCA was added and centrifuged for 30 min at 13,000 rpm. The precipitated solution was washed once with water and resuspended overnight at 37 °C with 20–100 μl of a 2.5% SDS solution.

XylT-II enzyme activity assay. Measurement of xylosyltransferase II activity was performed with the radiochemical assay described in [14]. For the determination of K_M values, various concentrations of synthetic acceptor peptide were incubated with desalted XylT enzyme solution. The assay conditions are the same as described under the activity assay section. The ratio k_{cat}/K_M is a function of acceptor activity with increased k_{cat}/K_M values, reflecting higher acceptor activity because of either a high k_{cat} or a low K_M.

Protein Identification. Protein Identification was performed by mass spectrometry with a matrix-assisted laser desorption/ionization time of flight/time of flight mass spectrometry (MALDI-TOFTOF-MS) system at Proteome Factory (Berlin, Germany).

ImageJ analysis. ImageJ (NIH, Bethesda, USA) was used to create lane profile plots from SDS polyacrylamide gels. Peak area measurements were performed according to the manual [15].

Results

Expression of human xylosyltransferase II in P. pastoris

Recombinant xylosyltransferase II was heterologously produced in *P. pastoris* as a soluble active protein, which was designated for secretion into the medium since both enzymes are naturally secreted. Because this organism secretes only low levels of endogenous proteins [16], XylT-II comprises the vast majority of the total protein in the medium, thus facilitating protein purification.

Table 1
Summary of purification steps employed for the enrichment and in-gel detection of XylT-II from 40 l of *Pichia pastoris* cell culture supernatant.

	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Purification (-fold)	Recovery (%)
Concentrated culture supernatant	1215	9.81[a]	29,802	0.0003	1	100.0
Ammonium sulfate precipitation	150	8.73[a]	6398	0.0014	4	89.0
Heparin affinity chromatography	35	3.92	22.3	0.1755	533	39.9
Ion exchange chromatography	3	0.25	0.11 0.011[b]	2.3327 22.7272[b]	7087	2.6

[a] Cause of the inhibitory effect of ammonium sulfate on XylT-II activity. An aliquot was subjected to gel filtration, fractions collected, assayed for activity and summed up for the recalculation of the original XylT-II activity.
[b] Values were calculated on the basis of a 10.2% XylT-II protein portion from the total protein amount. This number reflects the situation as if the protein had been purified.