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Recombinant, truncated *B. circulans* keratanase-II: Description and characterisation of a novel enzyme for use in measuring urinary keratan sulphate levels via LC–MS/MS in Morquio A syndrome

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ABSTRACT

Objective: Morquio A syndrome (mucopolysaccharidosis IVA; MPS IVA) is an autosomal recessive lysosomal storage disorder caused by deficient *N*-acetylgalactosamine-6-sulphatase (GALNS) activity. Early and accurate diagnosis of this condition is critical for improved patient outcomes, particularly as enzyme replacement therapy has recently become available. An LC–MS/MS assay utilising keratan sulphate (KS) disaccharides derived from keratanase-II digestion provides a sensitive and specific means for quantitation of urinary KS, a screening biomarker for Morquio A (Oguma et al., 2007; Martell et al., 2011). To ensure a reliable supply of keratanase-II, we sought to produce a *Bacillus circulans*-derived enzyme via a recombinant approach in *Escherichia coli*.

Design and methods: Bioinformatics analysis of the *B. circulans* keratanase-II enzyme identified likely dispensable C-terminal domains amenable to enhancement via protein engineering. A truncated form of the enzyme was designed to remove the domains predicted to be unnecessary for catalytic activity and detrimental to recombinant expression in *E. coli*.

Results: C-terminally truncated, recombinant *B. circulans* keratanase-II was purified to >98% homogeneity and extensively characterised, demonstrating desired activity, specificity and utility in LC–MS-based quantitation of urinary KS from Morquio A and control samples, and is functionally indistinguishable from full-length, native *B. circulans*-derived keratanase-II.

Conclusions: This novel, recombinant keratanase-II meets all performance requirements and can be produced in a rapid and reproducible manner. We speculate that other related bacterial enzymes of biomedical or industrial interest may be amenable to similar engineered enhancements.

Introduction

The glycosaminoglycan keratan sulphate (KS) consists of β-(1 → 3) repeats of a galactosyl β-(1 → 4)-*N*-acetylglucosamine disaccharide unit [1]. KS has been identified as a key mediator in a multitude of biological processes, ranging from embryonic development [2], axonal guidance, metastatic tumour invasion [3] and modulation of immune recognition via both the innate [4] and adaptive systems [5]. In normal metabolism, KS is catabolised in the lysosome into its component sugars and sulphate which are then recycled or excreted. Deficient activity of *N*-acetylgalactosamine-6-sulphatase (GALNS; EC 3.1.6.4), an enzyme that catabolises KS and chondroitin-6-sulphate, causes Morquio A syndrome (mucopolysaccharidosis IVA; MPS IVA), a disorder in which accumulated KS leads to progressive tissue and organ dysfunction [6,7]. Management of the disorder until recently has been limited to supportive and symptom-based care; however, enzyme replacement therapy (ERT) with elosulfase alfa is now a treatment option [8]. Urinary KS has been proposed as a screening biomarker for Morquio A syndrome, and a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for KS quantitation in urine has been described [9]. A key component of this assay is the use of a keratanase enzyme to digest urinary KS into disaccharides for subsequent quantitation.

KS disaccharides are generated by use of *Bacillus circulans* keratanase-II (endo-β-*N*-acetylglucosaminidase), which cleaves *N*-acetylglucosamine linkages within the KS chain to yield

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disaccharides (Gal(1–4)GlcNAc, either mono- or di-sulphated) or tetrasaccharides consisting of two repeats of this unit linked via a 1–3 bond (Gal(1–4)GlcNAc(1–3)Gal(1–4)GlcNAc). Unlike other keratanases, the enzyme from *Bacillus* sp. is not inhibited by the presence of adjacent fucose on *N*-acetylglucosamines [10], making it attractive for the analysis of this substrate. The keratanase-II gene from *B. circulans* is 5808 bp long and encodes a 1936 amino acid protein devoid of cysteine residues [11]. The N-terminal 34 amino acids of the protein likely represent a signal peptide (SignalP 4.0; [12]) downstream from which is a large domain with little overall homology to other known proteins, assumed to be the catalytic domain (Fig. 1). Homology with other proteins is highest at the C-terminus which contains three repeats of a bacterial surface layer homology (SLH) domain. SLH domains [13] are found in many bacterial enzymes that play a role in the degradation of complex polysaccharides. Each individual SLH domain is approximately 60 residues long and mediates binding to non-classical, pyruvylated secondary cell wall polymer (SCWP) components of both Gram positive and Gram negative bacterial cell walls [14].

To facilitate the expression and characterisation of the *B. circulans* keratanase-II in *Escherichia coli*, we conducted a detailed bioinformatics analysis of the protein resulting in the expression of a truncated version of the enzyme. Unlike a full-length recombinant version, the truncated form was fully soluble and maintained both the activity and specificity of the enzyme purified from its native source. Furthermore, we demonstrate that it facilitates digestion and subsequent quantitation of KS in urine from Morquio A-affected and unaffected individuals in a manner indistinguishable from the full-length, native *B. circulans*-derived enzyme.

Materials and methods

Molecular cloning

The keratanase-II gene (NCBI AY188989; UniProt Q7X0Z0) was synthesised by Life Technologies and subcloned into the BamHI and XhoI sites of pET22b (modified to remove the pelB leader), resulting in the expression construct pBM001 which placed a His6 tag at the extreme C-terminus of the enzyme. The truncated construct described (pBM002) was generated by amplifying the region of interest using CloneAmp high-fidelity polymerase (Clontech) and cloning into pET22b using Infusion cloning (Clontech). Recombinant clones were identified and plasmid DNA purified and sequenced. For expression, positive clones were transformed into the *E. coli* DE3 lysogen BL21(DE3)pLysS [15].

Recombinant protein expression and purification

BL21(DE3)pLysS *E. coli* harbouring the plasmid of interest were grown with shaking in LB medium containing 100 μg/mL ampicillin at 37 °C until an OD_{600} of 0.6 to 0.8. IPTG (1 mM final concentration) was added and growth continued for 3 h. Cells were harvested by centrifugation, re-suspended in 2–10 mL PBS/1 M NaCl supplemented with 10 μg/mL lysozyme (w/v) and frozen at −80 °C. After thawing, cells were sonicated and the soluble fraction was collected by a clarifying spin at 15,000 rpm for 60 min. Target protein was purified using pre-packed columns (GE Healthcare) on an Akta 'Pure' instrument (GE Healthcare). The truncated enzyme was purified from the soluble fraction of a 500 mL culture by application to a 5 mL HisTrap-HP column and elution with a linear gradient of 0 − 250 mL imidazole. Peak fractions were pooled and buffer exchanged into PBS/3 M NaCl, before applying to a 5 mL phenyl sepharose hydrophobic interaction (HIC) column. 2 mL fractions were collected from the gradient and characterised by SDS-PAGE. The final product was buffer exchanged into PBS/20% glycerol and stored at −80 °C until required.

SDS-PAGE and immunoblotting

Samples were prepared and analysed on SDS-PAGE using precast 'Bolt' gels and associated buffers (Life Technologies), running in MES buffer conditions, according to the manufacturer's recommendations. For immunoblotting, gels were equilibrated in 1× NuPAGE transfer buffer prior to transfer to nitrocellulose membrane for 60 min at 25 V constant voltage. Proteins were detected with a Sigma anti-His tag monoclonal antibody (Cat #H1069) at 1:2500 using buffers and detection reagents from the Life Technologies 'Western Breeze' chromogenic system.

Keratanase activity assay

Keratanase activity was assayed using a variation of the neocuproine assay described by Bureau and MacGregor [16]. Briefly, a standard curve of a representative reducing sugar (*N*-acetyl glucosamine; GlcNAc) was generated by adding 60 μL 0–100 μg/mL GlcNAc to a mixture of 100 μL neocuproine (made with 0.151 g in 100 mL water) and 100 μL 'reagent A' (made with 4.00 g Na_2CO_3, 1.63 g glycine and 47 mg $CuSO_4 \cdot 5H_2O$ in 100 mL water) and heating at 100 °C for 12 min, followed by cooling on ice. 100 μL of the above sample was diluted with 100 μL water, mixed well and the absorbance at 405 nm (A_{405}) read in a SpectraMax plate reader. The data were plotted as a scatter of A_{405} versus concentration of reducing sugar (GlcNAc) in μg/mL using Microsoft Excel. A linear fit was calculated using the software and the equation used for interpolation of the sample readings. One unit was defined as the amount of enzyme that released 1 μmol of reducing sugars per minute under the assay conditions.

To assay the enzyme, 10 μL of the purified enzyme was added to 10 μL of KS (GlycoSyn, New Zealand), from bovine cornea, 2 mg/mL in assay buffer (100 mM sodium acetate, pH 6.0), in a total volume of 60 μL assay buffer. Samples were placed at 37 °C for 30 min followed

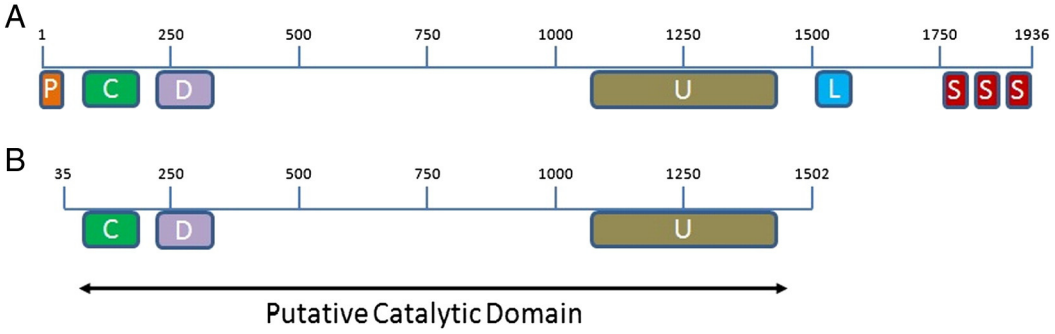

Fig. 1. Conserved domains within *B. circulans* keratanase-II (A) native, full-length enzyme and (B) C-terminally truncated enzyme. P: leader peptide (residues 1–34), C: carbohydrate binding domain (CBM_4_9), residues 81–192, D: domain of unknown function (DUF), residues 227–294; U: UgpB domain (periplasmic sugar transport), residues 1071–1421; L: low complexity 'linker' region, residues 1498–1571; S: SLH domains, residues 1750–1936. The putative catalytic domain is identified by the black arrow.

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