IDENTIFICATION AND CHARACTERISATION OF ENDOGLYCOSIDASE ACTIVITIES TOWARDS DERMATAN SULPHATE BY TANDEM MASS SPECTROMETRY

A thesis presented for the degree of

DOCTOR OF PHILOSOPHY

by

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SUMMARY

Dermatan sulphate (DS) is a sulphated glycosaminoglycan (GAG) that is widely distributed as proteoglycan throughout the extracellular matrix and at cell surfaces where it plays an important role in many key biological processes. The intra-cellular catabolism of DS commences with endohydrolysis of the polysaccharide chains to oligosaccharides, which are then sequentially degraded from the non-reducing terminus by lysosomal exoenzymes to monosaccharides and inorganic sulphate for transport out of the lysosome and re-utilisation by the cell. Both endo-β-N-acetylhexosaminidase (Hyal-1 hyaluronidase) and endo-β-glucuronidase activities towards DS have been proposed. The present study was undertaken to: 1) determine the substrate specificities and sub-cellular locations of these endoglycosidase activities; and 2) compare endoglycosidase activities and substrate specificities in the mucopolysaccharidoses, where a defect in one of the lysosomal exoenzymes required to degrade DS results in the lysosomal accumulation of partially degraded DS oligosaccharide fragments. To this end, a series of oligosaccharide substrates designed to represent aspects of the physiological substrate was prepared, and an assay was developed to measure endoglycosidase activities and determine their substrate specificities by quantifying specific oligosaccharide products.

Assay substrates rich in glucuronic acid (GlcA) or iduronic acid (IdoA) were prepared by limited chondroitinase ABC digestion of chondroitin sulphate A and DS, respectively. The resulting tetra- to hexadecasaccharides were separated by size-exclusion chromatography and characterised by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS). These substrates, which were not susceptible to degradation by lysosomal exoenzymes, were then incubated with Chinese hamster ovary (CHO)-K1 cell homogenate (source of endoglycosidase activity), and the oligosaccharide products generated from the non-reducing end of the substrate were measured by ESI-MS/MS. Endo-β-N-acetylhexosaminidase and endohexuronidase activities were detected towards the oligosaccharide substrates, with both activities preferentially degrading the GlcA-rich substrates and only minor activity observed towards IdoA-rich substrate. The endo-β-N-acetylhexosaminidase activity had a minimum-sized substrate requirement of a hexasaccharide and was observed to sequentially remove tetrasaccharides from the non-reducing end of oligosaccharides, whereas the
endohexuronidase activity had a minimum substrate of an octasaccharide, acted randomly and was comparatively low. The activities displayed the same acidic pH optimum and responded in the same manner to changes in buffer composition and substrate concentration, and to the presence of divalent cations, NaCl, detergent and protease inhibitors. Both activities were modestly affected by the hyaluronidase inhibitor, apigenin. Percoll density gradient subcellular fractionation confirmed that the activities were primarily in the lysosomes and late endosomes. The endo-β-N-acetylhexosaminidase and endohexuronidase activities detected here in CHO-K1 cells are consistent with the Hyal-1 and endo-β-glucuronidase enzymes described previously. These data suggest that Hyal-1 and endo-β-glucuronidase are predominantly lysosomal enzymes that act in concert to degrade the low-sulphate, GlcA-rich domains of DS, but are less active towards the highly sulphated regions containing IdoA.

To test the hypothesis that endoglycosidase activities are altered in the mucopolysaccharidoses, an attempt was made to compare Hyal-1- and endo-β-glucuronidase-like activities and their substrate specificities in mucopolysaccharidosis (MPS)-affected and unaffected control skin fibroblasts. However, no activity was detected towards octa- to hexadecasaccharide substrates in control fibroblast homogenates, and in homogenates of MPS fibroblasts deficient in the lysosomal exoenzymes α-L-iduronidase and N-acetylgalactosamine-4-sulphatase, despite the fact that: 1) what appear to be the products of Hyal-1 and endo-β-glucuronidase activities towards endogenous DS could be detected in the lysosomes of the MPS cells by sub-cellular fractionation; and 2) the ESI-MS/MS assay was demonstrated sensitive enough to detect endoglycosidase activities in homogenates of a number of different mouse tissues (including whole skin). We hypothesise that this absence of detectable endoglycosidase activity in skin fibroblasts results from enzyme non-recognition of the exogenous assay substrates tested, and hence that these cells contain heretofore undescribed Hyal-1 and endo-β-glucuronidase isoforms with unique substrate specificities.

In conclusion, the development of an ESI-MS/MS assay to measure the products of endoglycosidase activities has enabled the characterisation of these activities towards DS. This strategy may be useful for the future study of endoglycosidase activities towards a variety of other GAGs such as heparan sulphate, where particular oligosaccharide structures have been shown to possess unique biological activities.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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TC NIELSEN

July, 2009
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>ΔUA</td>
<td>unsaturated uronic acid</td>
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<tr>
<td>amu</td>
<td>atomic mass units</td>
</tr>
<tr>
<td>AUX</td>
<td>auxiliary gas</td>
</tr>
<tr>
<td>BME</td>
<td>basal modified eagle’s medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BTH</td>
<td>bovine testicular hyaluronidase</td>
</tr>
<tr>
<td>CAD</td>
<td>collision gas</td>
</tr>
<tr>
<td>CE</td>
<td>collision energy</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulphate</td>
</tr>
<tr>
<td>CUR</td>
<td>curtain gas</td>
</tr>
<tr>
<td>CXP</td>
<td>collision cell exit potential</td>
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<tr>
<td>Da</td>
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<td>DMG</td>
<td>3,3-dimethylglutaric acid</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<td>DNA</td>
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<tr>
<td>DP</td>
<td>declustering potential</td>
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<td>DSPG</td>
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<td>ECM</td>
<td>extra-cellular matrix</td>
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<td>ER</td>
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<td>fibroblast growth factor</td>
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<td>GalNAc</td>
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<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<tr>
<td>HC II</td>
<td>heparin cofactor II</td>
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<td>HNAc</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>IS</td>
<td>ion spray voltage</td>
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<td>ISTD</td>
<td>internal standard</td>
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<td>mucopolysaccharidosis</td>
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<td>MPSs</td>
<td>mucopolysaccharidoses</td>
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<td>MRM</td>
<td>multiple reaction monitoring</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<td>MS/MS</td>
<td>tandem mass spectrometry</td>
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<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>NEB</td>
<td>nebuliser gas</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PG</td>
<td>proteoglycan</td>
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<tr>
<td>PMP</td>
<td>1-phenyl-3-methyl-5-pyrazolone</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulphonylfluoride</td>
</tr>
<tr>
<td>RHhyal-1</td>
<td>recombinant human Hyal-1</td>
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<td>S</td>
<td>sulphate</td>
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<td>SPAM-1</td>
<td>sperm adhesion molecule-1</td>
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<td>TEM</td>
<td>temperature</td>
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<tr>
<td>UA</td>
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</tr>
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<td>uridine diphosphate</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>V₀</td>
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<tr>
<td>Vₜ</td>
<td>total volume</td>
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<tr>
<td>Xyl</td>
<td>xylose</td>
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PUBLICATIONS

The following publications resulted from the work described in this thesis:

Peer-reviewed journals


Conference abstracts

Nielsen, T.C., Meikle, P.J., Hopwood, J.J. and Fuller, M. A method to measure endohydrolase products by mass spectrometry *Proceedings of the Australian Health and Medical Research Congress 2006* (abstract #1525)
“I cannot express strongly enough my unbounded admiration for the greatness of mind of these men who conceived [the heliocentric system] and held it to be true….in violent opposition to the evidence of their own senses…..”

- Galileo, Dialogue concerning Two Principal Systems of the World (Third Day)