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 sub-cellular 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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July, 2009

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ΔUA	unsaturated uronic acid
amu	atomic mass units
AUX	auxiliary gas
BME	basal modified eagle's medium
BSA	bovine serum albumin
BTH	bovine testicular hyaluronidase
CAD	collision gas
CE	collision energy
CHO	Chinese hamster ovary
CNS	central nervous system
CS	chondroitin sulphate
CUR	curtain gas
CXP	collision cell exit potential
Da	Dalton
DMG	3,3-dimethylglutaric acid
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DP	declustering potential
DS	dermatan sulphate
DSPG	dermatan sulphate proteoglycan
ECM	extra-cellular matrix
EP	entrance potential
ER	endoplasmic reticulum
ESI	electrospray ionisation
FCS	foetal calf serum
FGF	fibroblast growth factor
FP	focussing potential
GAG	glycosaminoglycan
Gal	galactose
GalNAc	*N*-acetylgalactosamine
GlcA	glucuronic acid
GlcN	glucosamine
GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol

HC II	heparin cofactor II
HNAc	*N*-acetylhexosamine
HPLC	high performance liquid chromatography
IdoA	iduronic acid
IS	ion spray voltage
ISTD	internal standard
MPS	mucopolysaccharidosis
MPSs	mucopolysaccharidoses
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
NEB	nebuliser gas
PBS	phosphate-buffered saline
PG	proteoglycan
PMP	1-phenyl-3-methyl-5-pyrazolone
PMSF	phenylmethanesulphonylfluoride
RHhyal-1	recombinant human Hyal-1
S	sulphate
SPAM-1	sperm adhesion molecule-1
TEM	temperature
UA	uronic acid
UDP	uridine diphosphate
UV	ultraviolet
V_0	void volume
V_t	total volume
Xyl	xylose

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PUBLICATIONS

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

Peer-reviewed journals

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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)

.....for Isabella

"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)*