Monitoring intracellular activity of Arylsulfatase B on its natural substrates in a functional bioassay using LIF-CZE

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Arylsulfatase – B (ASB)

Arylsulfatase B (ASB, N-acetylgalactosamine-4-sulfatase) – a lysosomal sulfatase participating in the lysosomal degradation of Dermatan Sulfate (DS) and Chondroitin Sulfate (CS)

\[ \ldots [\text{IduA} - \text{GalNAc}]_{N_1} - [\text{GlcA} - \text{GalNAc}]_{M_1} - [\text{IduA} - \text{GalNAc}]_{N_2} \ldots \]

with variable sulfation on 3 sites

hydrolyzing sulfate from N-acetylgalactosamine-4-sulfate on the non-reducing end of the oligosaccharides

Biological role

DS and CS are synthesized and secreted by cells as components of glycosaminoglycans (GAGs) in the extracellular matrix

DS and CS internalized by cells for degradation through specific receptors to the lysosome— to supply building blocks for synthetic pathways

ASB is one of the enzymes degrading DS and CS (in collaboration with \( \beta \)-hexosaminidase, iduronate-2-sulfatase, glucuronate-2-sulfatase, \( \alpha \)-iduronidase, \( \alpha \)-glucuronidase N-acetylgalactosamine-6-sulfatase, etc.)
Functional (efficacy indicating) bioassay

The regulatory expectation:

an assay “that describes the specific ability or capacity of a product to achieve a defined biological effect” (ICH Q6B)

Utility of the functional bioassay

in vitro product comparability, in lieu of demonstrating efficacy in animal or human studies
demonstrates cellular activity of rhASB (the active ingredient in Naglazyme®, Galsulfase)

enzyme needs to be taken up by the target cell
locate to the lysosome
show activity at the site on the natural substrate
Indicator cell for an efficacy indicating bioassay

First choice: human cells

- several published studies note differences in metabolic activities between human and animal disease model cells in lysosomal storage disorder

Cells with a single (ASB) deficiency in the lysosomal enzyme repertoire and the ability to take up rhASB

- if the single deficiency in the lysosome is corrected by the uptake of the enzyme of interest (partially or fully), the entire pathway will be turned on
- amplification system by sequentially degrading the natural substrate GAGs

Cells should be able to synthesize, secrete and re-import DS and/or CS to avoid the need to supplement the natural substrate into the culture medium

- cells with lysosomal degradation deficiency tend to accumulate GAGs from the medium more than normal cells
- imported GAGs will be degraded until the degradation reaches the deficient step – partially degraded GAGs will be stored → natural substrate for the missing enzyme

Selected a fibroblast line (GM00519) from an MPS VI patient for the study
Monitoring options

accumulation of degradation intermediates or end products

depletion of intracellular GAG pool

• avoids the potential problem of the transient nature of intermediates or degradation end products being re-utilized by the cell (monosaccharides, oligosaccharides and inorganic sulfate)
Monitoring accumulation and depletion of DS and CS

Digestion with Chondroitin ABC lyase (EC 4.2.2.4) breaks down larger DS and CS oligosaccharides to disaccharides

- possibly one trisaccharide or tetrasaccharide/chain

All these disaccharides are negatively charged and only 1 of 8 is not sulfated

Detection options

- LC/MS/MS
- Fluorescent labeling + capillary zone electrophoresis with laser induced fluorescence (CZE-LIF)
  - labeling disaccharides with a neutral dye can allow a highly selective detection through CZE without complicated sample treatments
Monitoring DS and CS by LIF-CZE

Very simple sample preparation from lysis to LIF-CZE

- lysate centrifuged, supernatant digested, dried, labeled, reconstituted, centrifuged
- taking advantage of the selectivity of CZE for negative targets with neutral dye

CZE conditions:
- pressure injection (0.5 p.s.i., 10 s)
- capillary: N-CHO Coated Capillary, 40 cm, 50 mm, I.D
- buffer: ProteomeLab Carbohydrate Separation Gel Buffer-N
- separation at 18 kV for 20 minutes (0.18 minute ramp, reverse polarity).
Establishing assay conditions

Spike recoveries, calibrations with the internal standard GlcNAc-6S

recovery of commercial (porcine) DS spike

<table>
<thead>
<tr>
<th>Sample</th>
<th>4S disaccharide [nmol]</th>
<th>Spike recovery [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In lysis buffer</td>
<td>In cell lysate</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>0.16</td>
</tr>
<tr>
<td>700 ng Dermatan Sulfate</td>
<td>0.44</td>
<td>0.59</td>
</tr>
<tr>
<td>350 ng Dermatan Sulfate</td>
<td>0.22</td>
<td>0.37</td>
</tr>
<tr>
<td>175 ng Dermatan Sulfate</td>
<td>0.12</td>
<td>0.26</td>
</tr>
</tbody>
</table>

In water

In lysate + Chondroitin ABC lyase
Accumulation of intracellular DS/CS in GM00519 cells

GM00519 cells on 12 well plates

1 nmol (0.6 \(10^{15}\) molecules) of 4S disaccharides in 200 µL of cell lysate

8.0 \(10^5\) cells/well - 0.8 \(10^9\) molecules/cell

Protein content in wells remains constant (consistent with confluence)

- estimated number of cells (150 pg protein/cell) approximately 800,000

Faster growing cells accumulate DS/CS faster

Same general profile (no plateau)
Localization of rhASB when taken up by GM00519 cells

GM00519 cells
  - cultured for 3 days
  - in presence of variable concentrations of rhASB
  - washed, permeabilized
  - stained with primary antibodies against rhASB, lysosomal antigen LAMP1 or with DAPI (nuclear)
Depletion of intracellular DS/CS by supplementing cell culture medium with rhASB

GM00519 cells on 12 well plates
rhASB added 4 weeks post confluence for 5 days
Depletion of intracellular DS/CS by supplementing cell culture medium with rhASB

GM00519 cells on 12 well plates
rhASB added 4 weeks post confluence

Response dependent on rhASB dose and incubation time
Residual CS/DS disaccharide can not be digested with increased dose
- likely transient in synthesis or export/import
Effect of an inactive rhASB mutant in the assay

Mutant: C53S, active site mutation

- Cys - formylglycine conversion needed for catalytic activity can not take place

Mutant is taken up same as regular molecule

No effect on the depletion of CS/DS
The functional bioassay is stability indicating.

rhASB samples were exposed to acid, base and peroxide treatment.

Linear segments of profiles can be compared with parallel line analysis or hyperboloid segments can be compared after linear transformation.
Summary

Functional assay “... describes the specific ability or capacity of a product to achieve a defined biological effect” (ICH Q6B)

Assay characteristics

- *ex vivo* setting (cells from MPS VI patient)
- natural (heterodisperse) substrates
- collaboration of taken-up rhASB with other lysosomal enzymes when degrading the natural substrates
- rate of the multi-enzyme process can be controlled by rhASB
- readily applicable to other lysosomal enzymes
- very simple sample preparation, utilizing selectivity and resolving power of LIF-CZE by labeling negatively charged targets with neutral dye