GAG-specific Endoglycosidase Assay using ^{35}S-Labeled Proteoglycans

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ABSTRACT

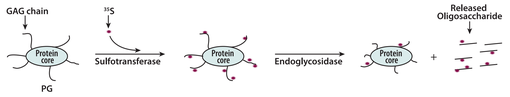
Glycosaminoglycans (GAGs) are linear amino-polysaccharides found in the extracellular matrix and on the cell membrane. They include heparan sulfate (HS), heparin, chondroitin sulfate (CS), dermatan sulfate, keratan sulfate and hyaluronan (HA). The majority of GAGs exist as components of functional proteoglycans (PGs). GAGs play roles in numerous cellular events, including cell growth, migration, and signaling through interaction with various growth factors, cytokines and other extracellular matrix proteins. Regulation of GAG synthesis and degradation is essential for these related cellular events. In mammals, GAG degradation is accomplished by GAG-specific endoglycosidases. For example, HS is degraded by Heparanase (HSPE), HA is degraded by Sperm Adhesion Molecule 1 (SPAM1) and Hyaluronidase 1 (HYAL1), and CS is degraded by Hyaluronidase 4 (HYAL4). These enzymes are key to furthering our understanding of GAG degradation and subsequent cellular events. However, unlike bacterial GAG-specific lyases, the products of these enzymes do not have UV absorption, posing a great challenge to the study of these enzymes.

Here, we describe a novel enzymatic assay for mammalian GAG endoglycosidases using ^{35}S-labeled recombinant PGs. PGs that contain GAG substrates are first radiolabeled with ^{35}S using recombinant sulfotransferases, and the labeled substrates are subsequently treated with GAG endoglycosidases. The reactions are separated using SDS-PAGE. The gels are dried and radioimages are obtained. This method allows us to visually monitor the progress of an enzymatic reaction. Given the high sensitivity of radioisotope labeling, nanogram levels of GAG degradation can be detected. As examples, HSPE is assayed using recombinant Syndecan-4 as the substrate, human HYAL4 is assayed using Serglycin as the substrate, and bovine SPAM1 is assayed using recombinant Serglycin and Neurocan as the substrates.

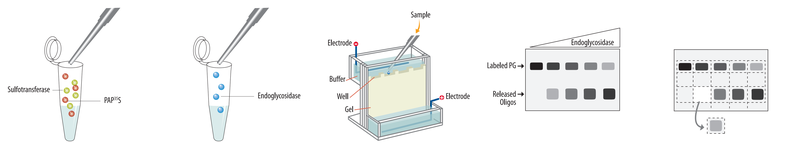
INTRODUCTION

- GAGs are linear amino polysaccharides that are generally covalently attached to the protein unit of PGs present in the extracellular matrix and on cell membranes. GAGs include CS, HS, heparin, dermatan sulfate, keratan sulfate, and HA.
- GAGs interact with growth factors, cytokines, and extracellular matrix proteins to regulate cell growth, migration, and signal transduction, and are known to be involved in numerous diseases including mucopolysaccharidoses, macular corneal dystrophy, osteoarthritis, hereditary multiple exostoses, and herpes simplex infection.
- GAG synthesis and degradation are crucial for proper cellular functioning. GAGs are degraded by GAG-specific endoglycosidases such as HSPE, SPAM1, and HYAL4.
- HSPE is the major endoglycosidase that cleaves HS *in vivo*, allowing the extracellular matrix to be remodeled for cell movement. In numerous cancer cell lines, increased metastatic potential corresponds with increased HPSE activity, thus HPSE is a potential target for cancer therapy.
- SPAM1, also called PH-20, is a hyaluronidase that degrades HA, a major structural glycosaminoglycan found in extracellular matrices and basement membranes, during fertilization. SPAM1 also digests CS proteoglycans.
- HYAL4 digests CS; however, unlike other members of the hyaluronidase family, it has negligible activity on HA.

ASSAY REACTION



ASSAY PROCEDURE



STEP 1 Add PAP^{35}S and a sulfotransferase to the sample in sulfotransferase labeling buffer at 37 ºC for 20 min. to label the proteoglycan.

STEP 2 Add the endoglycosidase of interest to the sample in digestion buffer at 37 ºC for 20 min. to digest the proteoglycan.

STEP 3 Separate samples using SDS-PAGE for 30 min.

STEP 4 Dry the gel and expose to X-ray film.

STEP 5 Excise the hot spots on the gel and count with a scintillation counter.

Electrophoresis buffer: 40 mM Tris, pH 8.0, 40 mM acetic acid, 1 mM EDTA

FIGURE 1

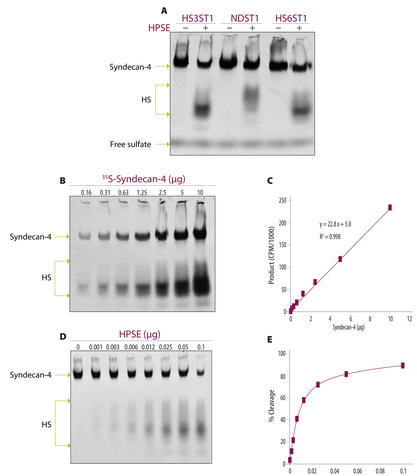


FIGURE 1 HPSE Assay. (**A**) 10 µg of Recombinant Human Syndecan-4 (Catalog # 2918-SD) was sulfated using 0.5 µg each of either Recombinant Human HS3ST1 (Catalog # 5968-ST), Recombinant Human NDST1 (Catalog # 5967-ST), or Recombinant Human HS6ST1 (Catalog # 5057-ST). Subsequently, 2 µg of ^{35}S-labeled Syndecan-4 was digested with 1 µg of HPSE. Both the undigested and digested samples were separated using SDS-PAGE. HS3ST1-labeled Syndecan-4 was selected for use in subsequent HPSE assays. (**B**) Increasing amounts of ^{35}S-labeled Syndecan-4 were incubated with 1 µg HPSE. (**C**) The radioactivities of the hot spots corresponding to the released HS in (B) were plotted against Synedan-4 input. A straight line was obtained, suggesting a first-order rate reaction. (**D**) 0.2 µg of ^{35}S-labeled Syndecan-4 was digested with increasing amounts of HPSE. (**E**) The percentage of the released radioactivity in (D) was plotted against HPSE input. Approximately 10 ng of HPSE is sufficient to release 50% of the ^{35}S-labeled HS from Syndecan-4.

Sulfotransferase labeling buffer: 12.5 mM MES, 0.25% Triton X®-100, 1.25 mM $MgCl_2$, 1.25 mM $MnCl_2$, 0.625 mM $CaCl_2$, pH 7.0
HPSE digestion buffer: 50 mM Citrate, 0.1% Triton X-100, 0.6 mM $MgCl_2$, 0.6 mM $MnCl_2$, 0.8 mM $CaCl_2$, 0.2 mg/mL BSA, pH 4.0

FIGURE 2

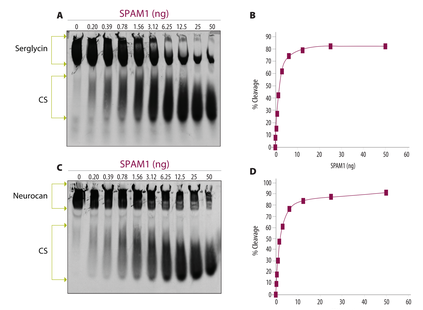


FIGURE 2 SPAM1 Assay. (**A**) 1 µg of recombinant mouse Serglycin was sulfated using 0.5 µg of Recombinant Mouse CHST3 (Catalog # 5356-ST) and then digested with increasing amounts of Recombinant Bovine SPAM1 (Catalog # 6436-GH). (**B**) The percentage of the released radioactivity in (A) was plotted against SPAM1 input. (**C**) 1 µg of Recombinant Mouse Neurocan (Catalog # 5800-NC) was sulfated using 0.5 µg of CHST3 and then digested with increasing amounts of SPAM1. (**D**) The percentage of the released radioactivity in (C) was plotted against SPAM1 input. Approximately 1.5 ng of SPAM1 is sufficient to release 50% of the ^{35}S-labeled CS from Serglycin and Neurocan.

SPAM1 digestion buffer: 100 mM NaOAc, pH 5.5

FIGURE 3

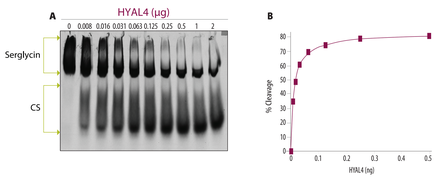


FIGURE 3 HYAL4 Assay. (**A**) 1 µg of the ^{35}S-labeled Serglycin was digested with increasing amounts of Recombinant Human HYAL4 (Catalog # 6904-GH). (**B**) The percentage of the released radioactivity in (A) was plotted against HYAL4 input. Approximately 50 ng of HYAL4 is sufficient to release 50% of ^{35}S-labeled CS from Serglycin.

HYAL4 digestion buffer: 100 mM NaOAc, pH 4.5

CONCLUSIONS

- ^{35}S-labeled proteoglycans are good substrates for GAG-specific endoglycosidases.
- All of the GAG-specific endoglycosidases studied followed typical Michaelis-Menten kinetics.
- Using this novel enzymatic assay, HYAL4 was shown to degrade chondroitin sulfate.