
WHITE PAPER

Simultaneous site-specific characterization of N- and O-glycosylation of Bovine Fetuin-A using LC/MS glycopeptide analysis

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1. Introduction

Glycosylation is a post-translational modification critical to protein folding, cell signaling, and immune function. N-linked glycans attach predictably to asparagine within Asn-X-Ser/Thr sequons, while O-linked glycans attach to serine or threonine with no consensus sequence, making sites difficult to predict. Traditional glycan-release methods strip glycans from the protein, pooling signal across all sites and destroying site-of-attachment information. O-glycoprotease IMPa cleaves the peptide backbone N-terminal to O-glycosylated residues, preserving both glycan structure and site identity.

Bovine fetuin-A carries three N-glycosylation sites and multiple O-glycosylation sites concentrated in a proline/alanine-rich C-terminal domain unique to the bovine orthologue, making it an ideal reference standard for demonstrating the combined N, O-glycopeptide workflow.

2. Why Use N, O-Glycopeptide Analysis?

Glycopeptide LC-MS/MS with IMPa retains glycans on their peptides, enabling simultaneous site-specific identification and quantitation of N- and O-glycoforms in a single experiment without extra sample prep or LC-MS runs. Key advantages include: (1) precise site-specific glycoform analysis; (2) preserved glycan structure; (3) NeuAc vs. NeuGc differentiation; and (4) compatibility with complex samples, including serum-derived standards.

3. Proof of Concept

The combined IMPa and trypsin workflow resolved both N- and O-glycosylation on bovine fetuin-A in a single LC-MS/MS experiment. Three N-glycosylation sites and six O-glycosylation sites were detected and characterized. Detailed results for N-glycan and O-glycan types are presented in sections 3.1 and 3.2, respectively.

3.1 N-Glycan Results

Three N-glycosylation sites were detected and fully characterized: Asn-99, Asn-156, and Asn-176. All sites carried fully sialylated N-glycans comprising NeuAc and bovine-specific NeuGc. Triantennary forms (A3G3S3 and A3G3S4) were predominant, with minor biantennary (A2G2S2) and tetraantennary (A4G4S4, site 1 only) forms. Core fucosylation was not detected, and high-mannose or hybrid glycans were absent. NeuGc-containing glycoforms were observed at Asn-99, Asn-156, but absent at Asn-176, while a minor alpha-galactose xenoantigen glycoform appeared at Asn-156 only. The figure below illustrates the N-glycoform

distribution at a single representative site; complete glycoform profiles for all remaining sites are available upon request.

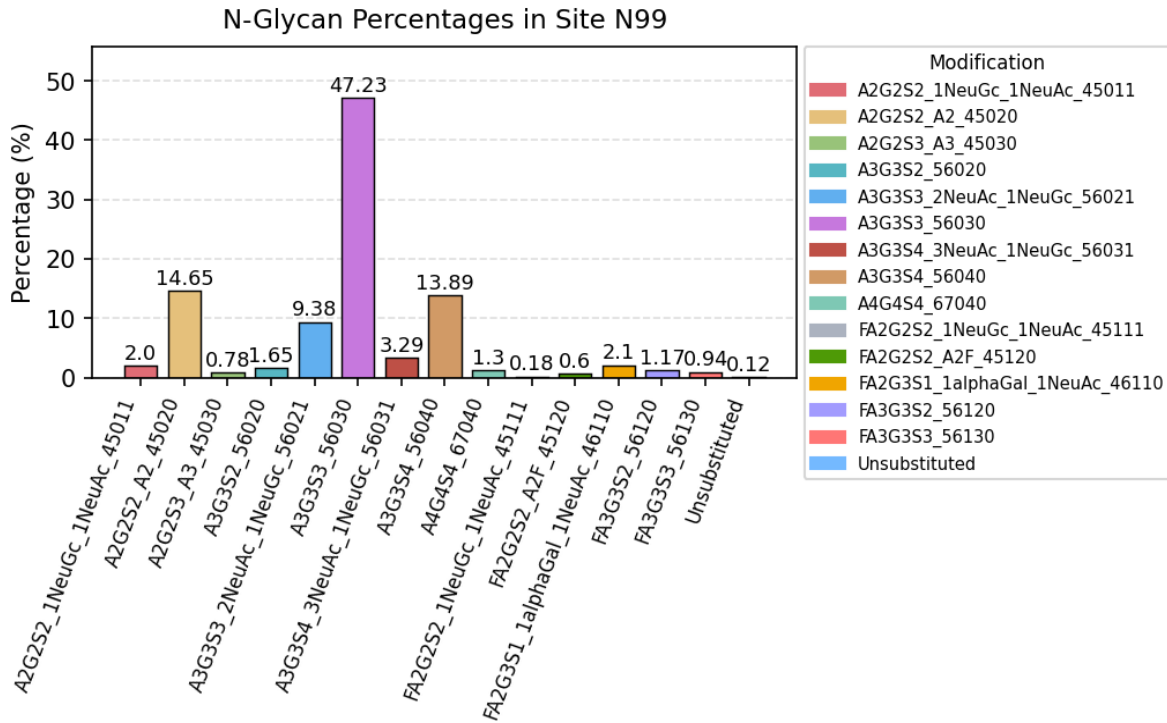


Figure 1. N-glycoform percentage distribution at Asn-99 (KLCPDCPLLPLNDSR). Triantennary-dominant profile, with NeuGc variants present. Core fucosylation absent.

3.2 O-Glycan Results

Six O-glycosylation sites were detected in the proline-rich C-terminal domain of bovine fetuin-A. Four sites showed appreciable occupancy, while two sites (TPIVGQP and HTFSGVA regions) were below 1%. All O-glycans were mucin-type core 1 structures built on a GalNAc- α -O-Ser/Thr core, exclusively sialylated with NeuAc; NeuGc and core 2 branched forms were not detected. The predominant form at all sites was monosialylated core 1 (NeuAcHexHexNAc), with disialylated core 1 (NeuAcHexNeuAcHexNAc) as a secondary component, most prominent at S-271. Minor unsialylated precursors (HexNAc, HexHexNAc) were also observed. The figure below depicts the O-glycoform distribution at a single representative site; complete profiles for all remaining sites are available upon request.

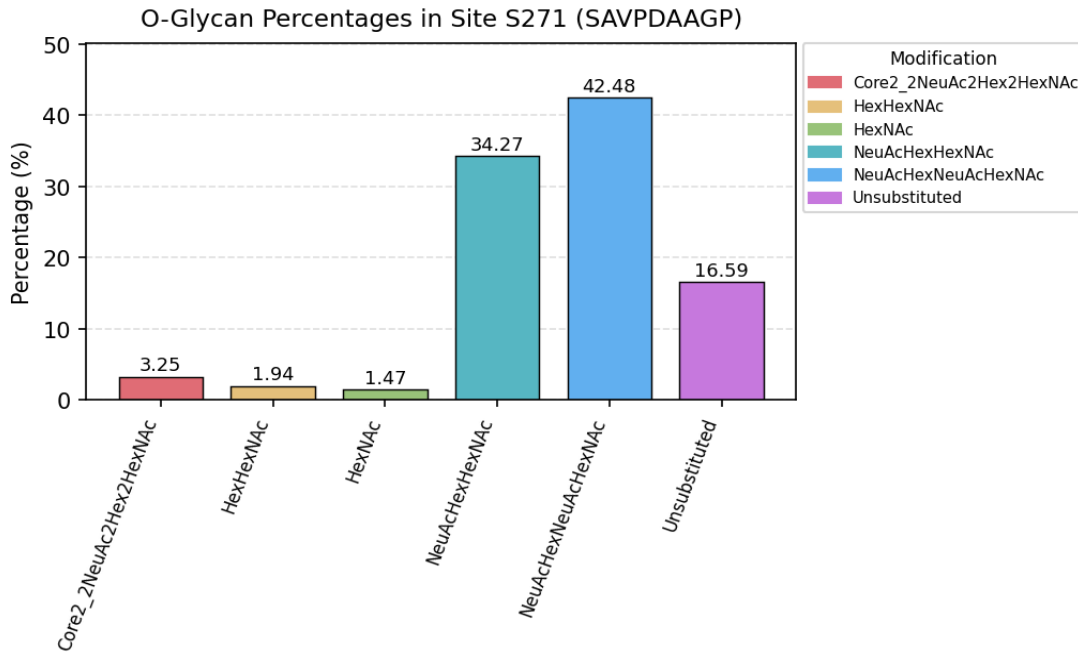


Figure 2. O-glycoform percentage distribution at Site 1 (S-271, peptide SAVPDAAGP). Disialylated core 1 is proportionally highest here compared to other sites, reflecting this site's greater capacity for dual sialylation.

3.3 Summary Comparison

The following table summarizes the key structural and compositional differences between N- and O-glycans detected on bovine fetuin-A. Each reported distinction is derived directly from the LC-MS/MS data collected in this experiment.

Feature	N-Glycans	O-Glycans	Notes
Linkage	N-GlcNAc (Asn-linked)	O-GalNAc (mucin-type)	Distinct biosynthetic pathways
Sites	3 Asn sites (cystatin domains)	9+ Ser/Thr (C-terminal domain)	O-sites in proline-rich tail
Dominant structure	Triantennary A3G3S3/S4	Sialyl-core 1 NeuAcHexHexNAc	Both fully sialylated
Sialic acid type	NeuAc dominant; NeuGc minor	NeuAc only	NeuGc unique to N-glycans
Core fucosylation	Absent (all 3 sites)	N/A	Contrast to human fetuin-A
Branching	Bi- to tetraantennary	Linear core 1 only	N-glycans structurally complex
alpha-Gal epitope	Detected (minor, site 1)	Not detected	Bovine xenoantigen
Core 2 O-glycans	N/A	Not detected	Linear core 1 predominates

Table 1. Comparative summary of N- and O-glycan features on bovine fetuin-A (P12763).

4. Why is Spectrus the Right Choice for N, O-Glycopeptide Analysis?

Spectrus combines expert glycoprotein sample preparation with high-resolution Orbitrap MS for confident, site-specific N- and O-glycosylation analysis in a single experiment. Our workflow maximizes analytical depth while minimizing sample requirements.

- Simultaneous N- and O-glycopeptide analysis in one LC/MS run
- High-resolution MS for accurate glycoform identification and mass measurement
- Quantitative site-specific occupancy and glycoform characterization with preserved structure and site localization
- NeuAc vs. NeuGc differentiation
- Suitable for biotherapeutics, biosimilar comparability, biomarker research, and more

Appendix

A.1 Methods in Brief

Bovine fetuin-A (Sigma-Aldrich F3385) was denatured with guanidine, reduced with dithiothreitol (DTT), alkylated with iodoacetamide (IAM), and digested with O-Glycoprotease (IMPa) followed by trypsin. Samples were then analyzed directly by targeted LC-MS/MS, enabling simultaneous detection of both N- and O-glycopeptides in a single run. Chromatography was performed reverse-phase using a Dionex™ UltiMate™ 3000 HPLC in tandem with a Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap™. The stationary phase was ThermoTrap C18 100 μm × 2 cm (trap); 100 μm × 15 cm, 3 μm (analytical). Mobile phase A was water + 0.1% formic acid and mobile phase B was acetonitrile + 0.1% formic acid.

A.2 Glycan Structures

All glycan structures in this white paper are drawn according to the Symbol Nomenclature for Graphical Representations of Glycans (SNFG) standard. Each monosaccharide is represented by a unique shape and color as shown in Figure 3.

SYMBOL NOMENCLATURE FOR GLYCANS (SNFG)




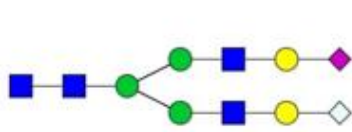
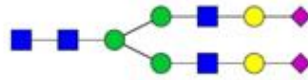
	N-acetylglucosamine		Fucose
	N-acetylgalactosamine		N-acetylneuraminic acid
	Galactose		N-glycolylneuraminic acid
	Mannose		

Figure 3. SNFG symbol key. GlcNAc = blue square; GalNAc = yellow square; Gal = yellow circle; Man = green circle; Fuc = red triangle; NeuAc = purple diamond; NeuGc = teal diamond

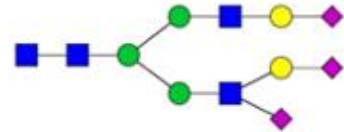
A.2.1 N-Glycan Structures



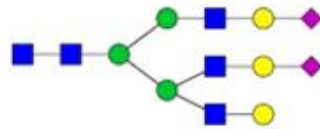
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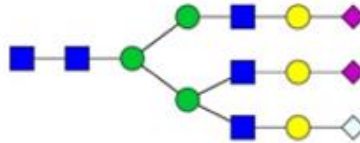
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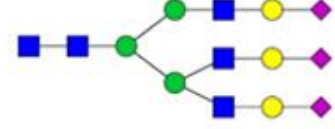
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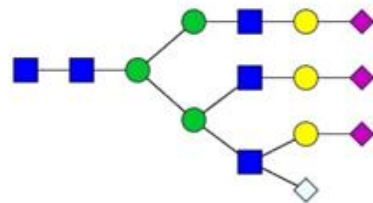
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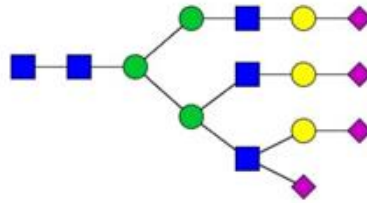
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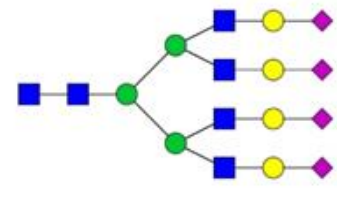
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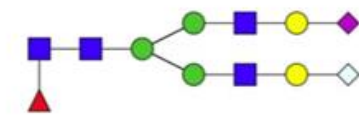
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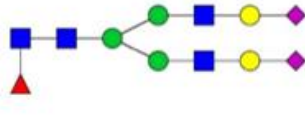
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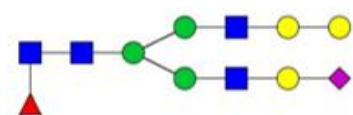
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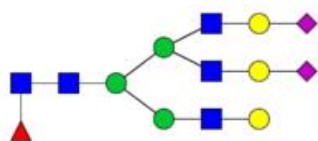
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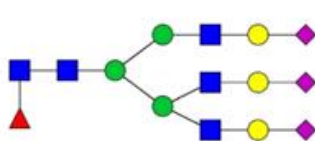
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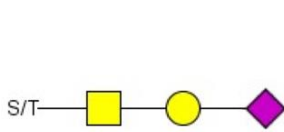


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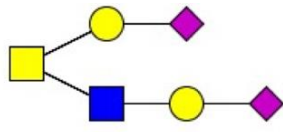


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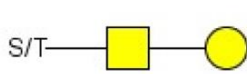
A.2.2 O-Glycan Structures



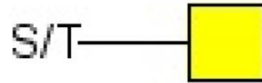
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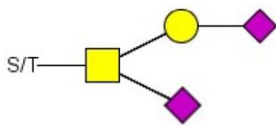
Core2_2NeuAc2Hex2HexNAc



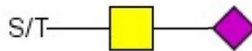
HexHexNAc



HexNAc



NeuAcHexNeuAcHexNAc



NeuAcHexNAc

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