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Analysis of Glycosylation of Bovine Folate Binding Protein by MALDI and LC Mass Spectrometry

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OVERVIEW

- A systematic approach is described to determine the number of *N*-glycosylation sites as well as the extent of glycosylation at each site in folate binding protein (FBP) from cow's milk.
- The results showed that the folate binding protein is partially glycosylated at Asn-49 (95%) and Asn-141(75%).

INTRODUCTION

Low folate nutrition, even of a very mild degree, is associated with three major disease states: the severe birth defects spina bifida and anencephaly; elevated blood homocysteine levels which can lead to arteriosclerosis of the coronary, cerebral and peripheral arteries; and cancer at a number of sites.

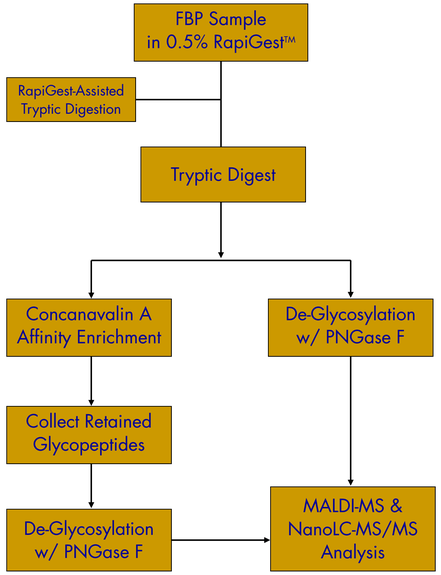
Bovine folate binding protein (FBP) is used as a critical component for human folate deficiency assays. Despite the wide usage of FBP, the glycosylation nature of FBP is still not well characterized. In this poster, a systematic study of the nature of FBP glycosylation sites by mass spectrometric analysis using a combination of enzymatic digestion, glycopeptide affinity capture and endoglycosidase cleavage is reported. Previously unconfirmed glycosylation site of FBP is successfully elucidated.

EXPERIMENTAL

Sample Processing:

- Bovine folate binding protein (from Sigma) was dissolved in a solution of 50 mM NH_4HCO_3 with 0.5% RapiGest™ SF at a concentration of 2 mg/mL. After reduction (DTT) and alkylation (Iodoacetamide), the protein was digested using trypsin. A portion of the digest was treated with peptide N-Glycosidase F (Sigma) to generate a sample for quantifications of glycosylation.
- A portion of the tryptic digest was loaded onto a home-made spin column packed with lectin affinity sorbents (Con A) to isolate glycopeptides. The enriched peptides were eluted off the spin column using 0.5M Methyl a-D-Mannopyranoside/0.5% TFA. The eluents were combined, lyophilized, reconstituted in 50 mM NH_4HCO_3, and finally treated with PGNase F to generate peptides for nano-LC-MS/MS sequencing.

EXPERIMENTAL WORK-FLOW



Mass Spectrometry Conditions

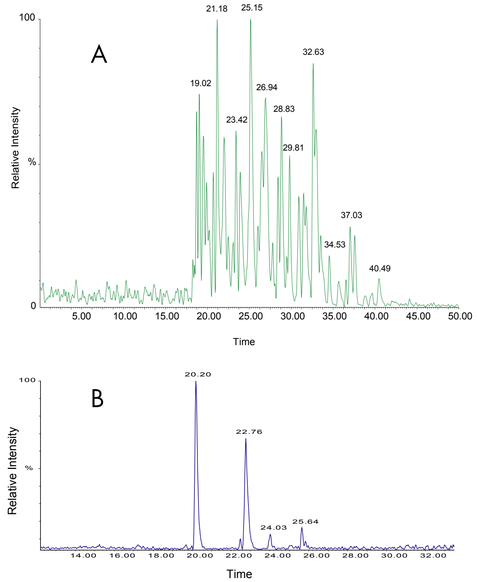
- Mass spectrometers used during the course of the study were a LCT, Q-Tof API/US and MALDI-LR (Waters, Milford, MA).
- MALDI-LR was operated in the reflectron mode.
- Q-Tof API/US was operated with a NanoLockSpray source to provide the exact mass of all eluting peptide species.
- With Q-Tof, data were acquired using an alternating low collision energy (10eV) and elevated collision energy function. During the course of the elevated energy, the collision energy was stepped from 23 to 33 eV. Data was acquired with Tof analyzer operating in the W-mode of operation with a mass resolution of >17500 FWHM.
- An integration time of 1.0 seconds was used for each scan. The reference, lock mass, channel was sampled every 30 seconds.

nano-LC Conditions

nano-LC Instrument:	Waters® nanoACQUITY UPLC™ System
nano-LC Columns:	
	On-line Trapping Column: Waters nanoACQUITY UPLC™ Trap Column, 180µm x 20 mm, Symmetry® C_{18}, 5 µm
	Analytical Column: Waters nanoACQUITY UPLC™ Column, 75µm x 150mm, Atlantis® dC_{18}, 3 µm
Mobile Phase A:	Aqueous 0.1% (v/v) Formic acid (FA)
Mobile Phase B:	Acetonitrile containing 0.1% (v/v) FA
Gradient:	5-50% mobile phase B over 45 min
Flow Rate:	250 nL/min

RESULTS

Figure 1. (A) Total ion chromatograms of a LC-ESI-MS analysis of tryptic digest of FBP sample. (B) Total ion chromatogram (BPI) of a LC-MS analysis of the enriched glycopeptides from the FBP tryptic digest via a lectin affinity spin column.



RESULTS

Figure 2. Analyses of lectin-enriched peptides after the treatment with PNGase F enzyme. (A) MALDI-Tof spectrum of the lectin-enriched peptides after the sample was treated with PNGase F. (B) LC-MS elution profile of the lectin-enriched peptides after the sample was treated with PNGase F. (C) Low energy CID spectrum of the peptide eluted at 21.35 min in Figure 2B.

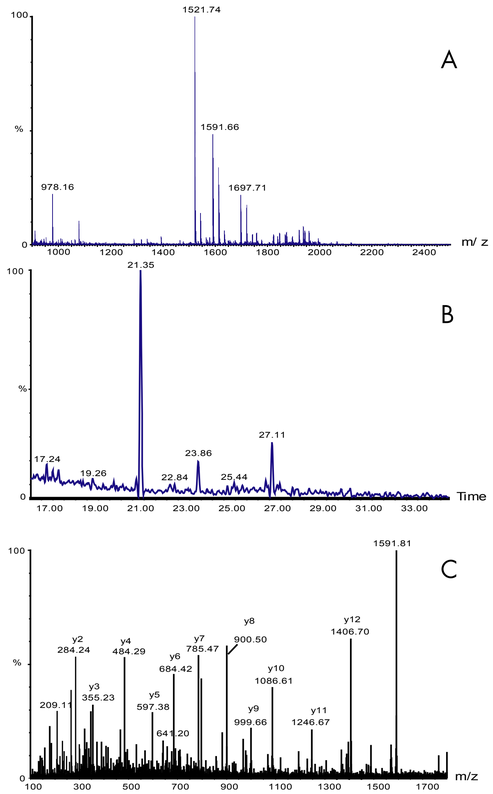


Figure 3. Amino acid sequence of bovine folate binding protein (P02702). The sequences of the two tryptic peptides that each contains a N-glycosylation site are underlined. The modification residue is labeled as red in the text.

AQAPRTPRARTDLLNVCMDAKHHKAEPGPEDSLHEQCSPWRKNACCSV[49]NTSIE
AHKDISYLYRFNWDHCGKMEPACKRHFIQDTCLYECSPNLGPWIREVNQRWRKE
RVLGVPLCKEDCQSWWEDCRTSYTCKSNWHKGW[141]NWTSGYNQCPVKAAH
CRFDFYFPTPAALCNEIWSHSYKVSNYSRGSGRCIQMWFDPFQGNPNEEVARFY
AENPTSGSTPQGI

Table I

Stoichiometry* of Glycosylation Sites on Folate Binding Protein

	Ion Intensity (Area)			
	Peptide: NACCSVNTSIEAHK		Peptide: GWNWTSGYNQCPVK	
Trials	Non-modified Form	Deamidated Form	Non-modified Form	Deamidated Form
1	162	4722	34	101
2	147	3492	25	76
3	124	3152	28	71
Glycosylated Percentage	95%		74%	

*Stoichiometry of glycosylation was determined following quantification of the deamidated peptide intensities, normalized to the sum of the intensities for the deamidated and the non-modified forms of the same peptide.

CONCLUSIONS

- The presented approach provided concrete data, for the first time, in elucidating the glycosylation sites as well as the extent of glycosylation at each site for bovine folate binding protein (FBP).
- The results showed that the folate binding protein is partially glycosylated at Asn-49 (95%) and Asn-141(75%).

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