Supporting Information for Publication

Similar albeit not the same; in-depth analysis of the proteoforms of human serum, bovine serum and recombinant human fetuin

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Supplementary data – Annotated HCD/EThcD spectra of all glycopeptides observed for hFet

(Supplementary data.pdf)

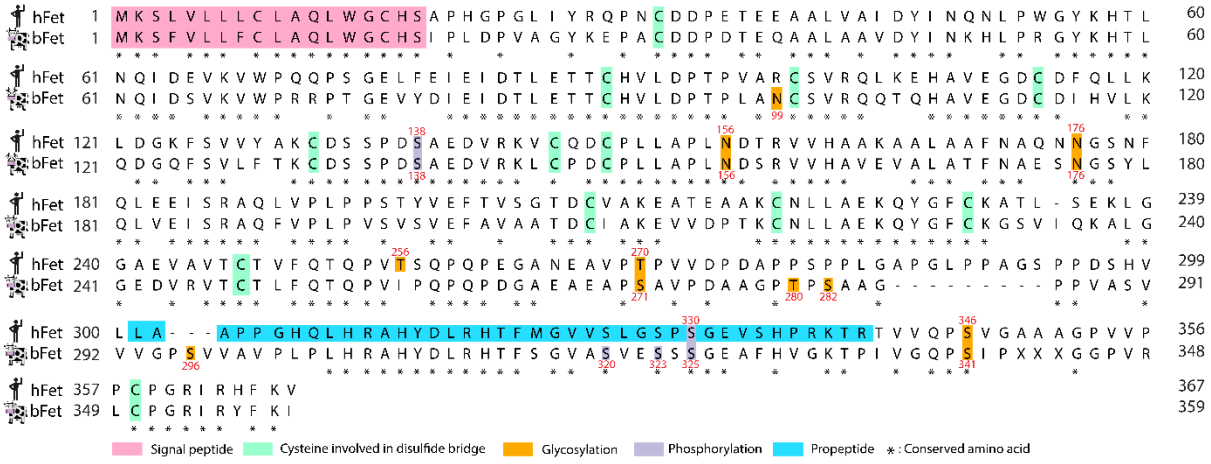

Figure S1. Amino acid sequence alignment between hFet and bFet reveals around 70% sequence similarity. Identical amino acids are marked by '*'. Glycosylation and phosphorylation sites are boxed in orange and purple, respectively; the signal peptide is boxed in pink; the propeptide in hFet is boxed in blue. The observed modified amino acids are indicated in red. Both hFet and bFet contain 12 conserved cysteine residues (boxed in light green) involved in 6 disulfide bridges.

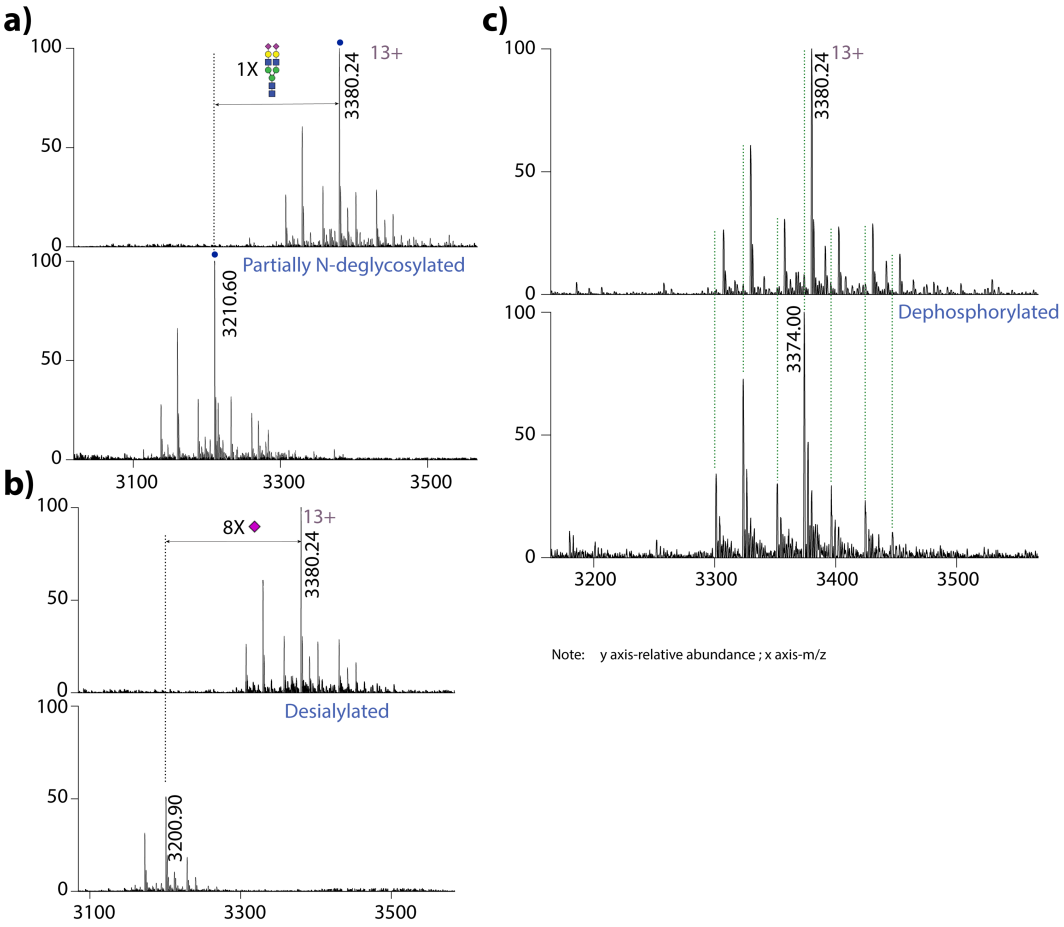

Figure S2. Zoom in on the 13^+ charge state of hFet to clearly display the changes after specific enzyme treatment. In **(a)**, hFet was treated by PNGase F and revealed partially N-deglycosylation in all major peaks; peaks marked with blue color before and after treatment represent the mass difference of one of the N-glycans (~2206 Da). In **(b)**, hFet was treated by sialidase. Comparing the most abundant peak on the charge state 13^+ with *m/z* of 3380.24 before treatment and 3200.90 after treatment, the mass difference corresponds to 8 sialic acids. In **(c)**, hFet was treated by alkaline phosphatase. The fully phosphorylated modification of hFet was confirmed by the mass shift of 80 Da of all proteoforms after the phosphatase treatment.

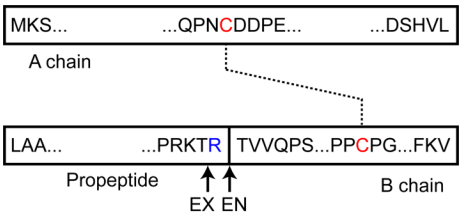

Figure S3. The two polypeptide chain structure of hFet is depicted by a schematic including the A chain at the top, and the connecting peptide and B chain at the bottom. A so far unknown endoproteinase (EN) generates the two-chain form of hFet and an exopeptidase (EX) eliminates

the Arginine at position 322, at the end of the propeptide. The A chain and B chain are in hFet connected by a disulfide bridge represented by the dashed line (…).

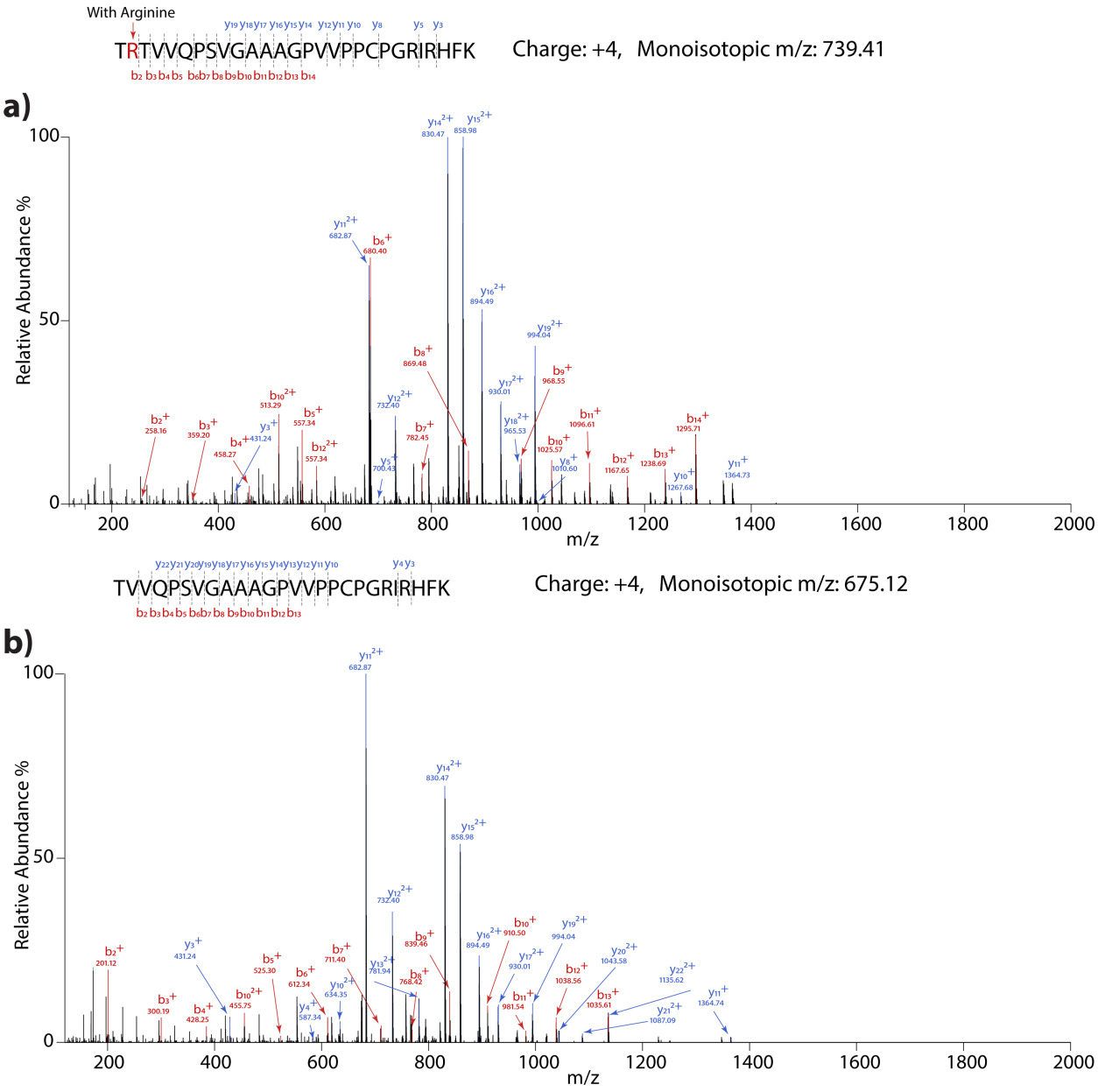

Figure S4. LC-MS/MS spectra of peptides derived from Lys-C/Glu-C digestion of rhFet providing evidence of the co-existence of rhFet proteoforms with and without an Arginine at position 322 (a) a peptide harboring the Arginine and (b) a peptide without the Arginine.

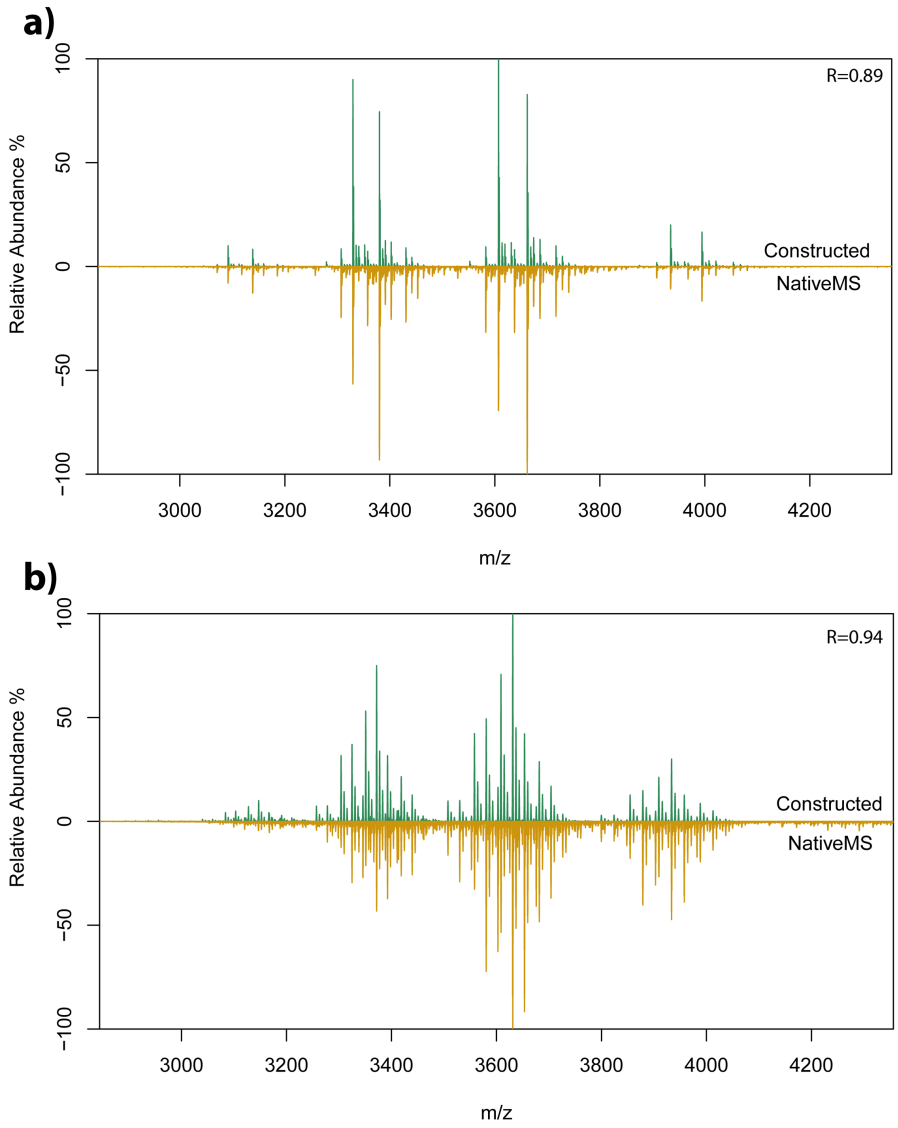

Figure S5. A comparison of the intact protein native MS spectrum with the constructed spectrum based on the peptide-centric proteomics data in **(a)** hFet and **(b)** bFet. The correlation between the spectra is very good, being 0.89 and 0.94 for hFet and bFet, respectively.