iTRAQ strategy to quantify and identify phosphorylation level of biologically active human factor IX used for treatment of hemophilia B

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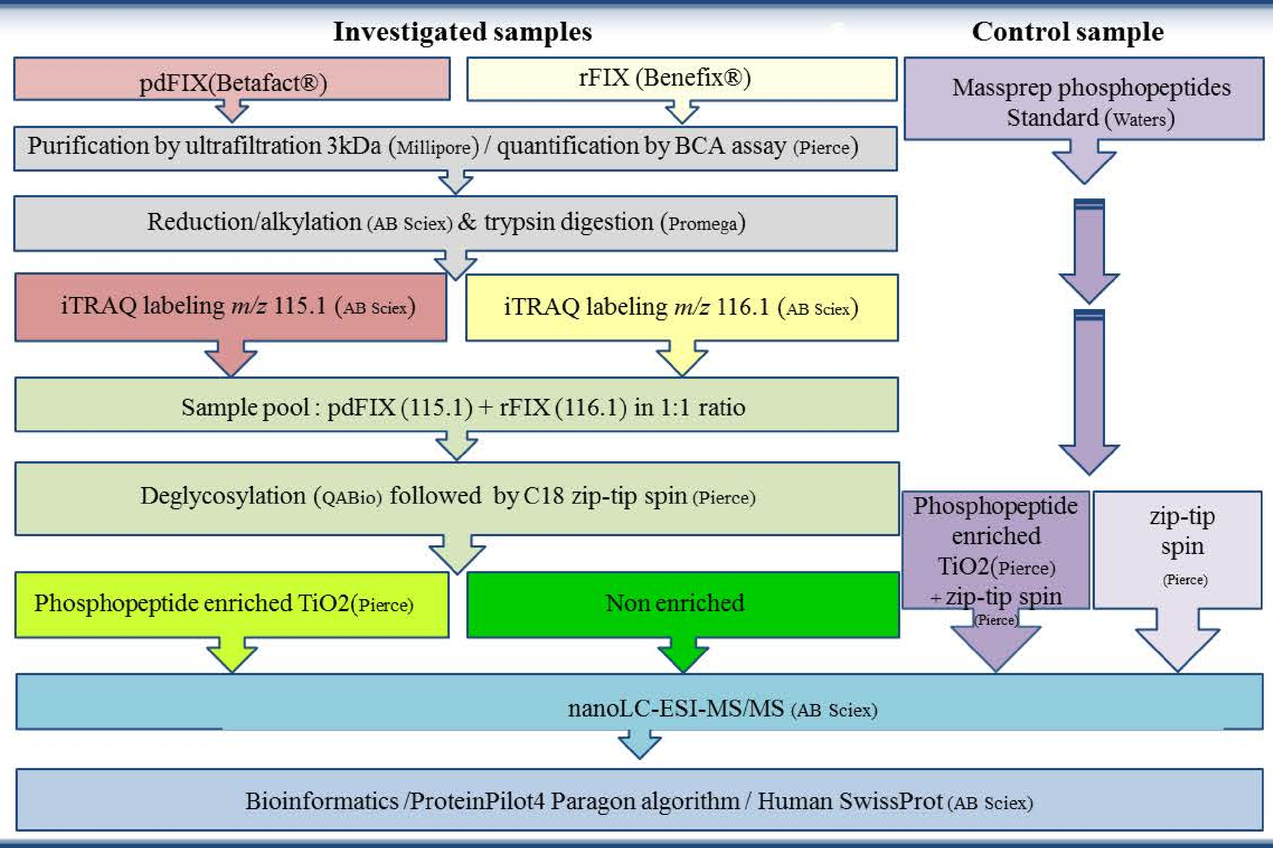
Introduction

Hemophilia B is an inherited coagulation defect characterized by a deficiency in human factor IX (FIX). Patients are commonly treated with plasma-derived FIX (pdFIX) prepared from pooled human plasma or with recombinant FIX (rFIX) produced by transfected Chinese Hamster Ovary cells (CHO) [1]. Although the treatment with rFIX is more suitable to limit potential infectious contaminants of plasma-derived clotting factors, *in vivo* recovery is 30%-lower with rFIX compared to that obtained with pdFIX [2-3] . This difference observed between rFIX and pdFIX is known to depend on post-translational modifications (PTMs) such as phosphorylation. Although several phosphorylations on FIX were previously explored, the low *in vivo* recovery for rFIX is not totally elucidated [4].

The aim of this study was to better understand the reasons of the low recovery of rFIX by identifying all differences between pdFIX and rFIX molecules in terms of phosphorylation sites as well as the level of phosphorylation. These information are crucial to produce novel rFIX molecules exhibiting PTMs similar to pdFIX, and therefore, to increase recovery rates in hemophilia patients.

Experimental setup



Phosphorylation Identification & Quantitation of Human Coagulation Factor IX

Standard : mixture of 4 phosphorylated peptides

Peptide	Sequence	$[M+H]^{2+}$
T18 p	NVPLpYK	407.19
T43 p	VNQIGpTLSESIK	684.84
T43 pp	VNQIGpTLSEpSIK	724.82

Table 1. Identified synthetic enolase phosphopeptides from phosphopeptides enriched TiO2 standard sample. These peptides identifications allowed to validate our strategy.

Pool (pdFIX + rFIX) iTRAQ

Confidence	Peptide sequences	Modifications/cleavages	M_W
99%	Y_{441}GIYTK$_{446}$	iTRAQ4plex@N-term; iTRAQ4plex(K)@6	1031.65
99%	I_{275}TVVAGEHNIEETEHTEQK$_{293}$	iTRAQ4plex@N-term; Deamidated(Q)@18; iTRAQ4plex(K)@19	2451.07
87%	A_{192}ETVFPDVDYVNST$_{205}$	iTRAQ4plex@N-term; **Deamidated(N)@12**; **1 phospho(S/T)@13/14**	1781.45
85%	A_{192}ETVFPDVDYVNSTEAETILDNITQSTQ$_{219}$	iTRAQ4plex@N-term; **2 Phospho(S/T/T)@13/14/18** **Deamidated(N)@22**	3405.75

Table 2. Identified peptides for Human Coagulation factor IX from pool sample, iTRAQ labeled, deglycosylated and phosphopeptide enriched.

Peptide sequence	Phosphorylated sites	Relative quantitation of FIX (%)	
		rFIX (m/z=116)	pdFIX (m/z=115)
A_{192}ETVFPDVDYVNSTEAETILDNITQSTQ$_{219}$	S_{204} or T_{205}, T_{209}	0 % ±17%	100%

Table 4. The relative phosphorylation between the two forms of FIX determinated by using of iTRAQ reagent.

Identification

Post-translational modifications	Sites	Identified peptide (confidence index)
Phosphorylation/ sulfonation	S_{204} or T_{205}	A_{192}-Q_{219} (85%), A_{192}-T_{205} (87%)
	T_{209}	A_{192}-Q_{219} (85%)
N-glycosylation	N_{203}	A_{192}-T_{205} (87%)
	N_{213}	A_{192}-Q_{219} (85%)
O-glycosylation	/	/

Table 3. Identified Post-Translational Modifications (PTMs) sites in Human Coagulation factor IX by LC-MS/MS.

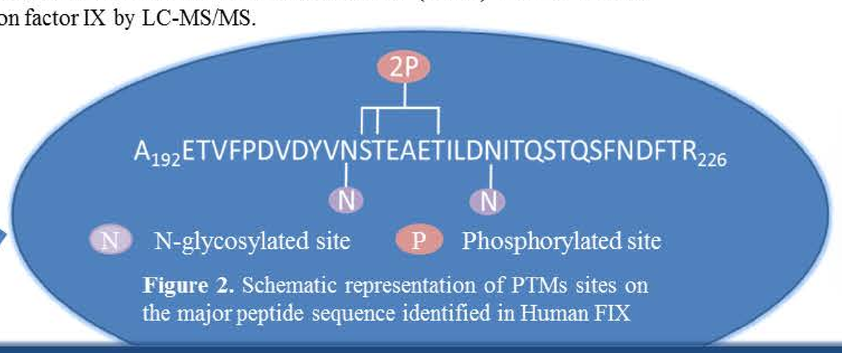


Figure 2. Schematic representation of PTMs sites on the major peptide sequence identified in Human FIX

Quantitation

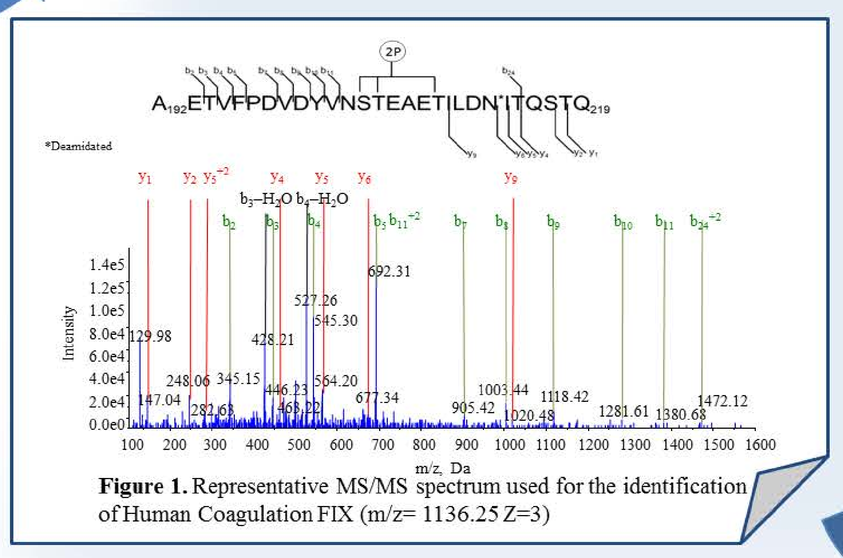


Figure 1. Representative MS/MS spectrum used for the identification of Human Coagulation FIX (m/z= 1136.25 Z=3)

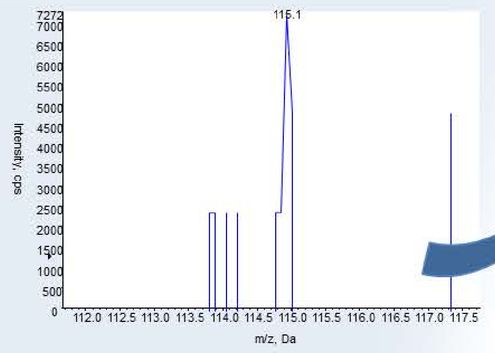


Figure 3. This phosphorylated peptide is observed to be abundant in the pdFIX form *versus* the rFIX form (intensity of 115 *versus* 116) as shown in the expanded mass region 110-117.5 Da

Conclusions and Perspectives

In addition to identification of well-known N-glycosylation sites, we reported new phosphorylation sites in pdFIX form and showed that the pdFIX form was 100% phosphorylated while no phosphorylation was detected on rFIX form. Thus, our approach represents an original way to identify new phosphorylation sites and to quantify phosphorylation level of rFIX molecules exhibiting higher degrees of phosphorylation for a better *in vivo* recovery.

References

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