

Mini-review on “A novel one-step purification of mouse factor IX”

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Factor IX (FIX) is a 70-kDa, single-chain, vitamin K-dependent glycoprotein present in trace amounts (~95 nM) in blood plasma^{1,2}. FIX is synthesized as a zymogen that is converted to a serine protease, FIXa, by the activated form of factor XI^{3,4}. FIXa has a central function in the intrinsic pathway of blood coagulation, in that it acts as an activator of factor X (FX), directly upstream of the common pathway^{1,5}. Upon activation, FXa concomitantly converts prothrombin to thrombin to initiate the formation of the fibrin lattice^{6,7}.

Isolation of Factor IX and Factor IXa

Purified FIX is essential for the treatment of Hemophilia B, and many animal model investigations, regarding possible augmentations in Hemophilia B therapy, also require abundant sources of FIX (see below). A number of FIX purification approaches have been reported, but all are either multi-step and time-consuming or of low yield^{1,2}. Thus, the report by Pilli et al. represents a welcome advancement in meeting patient and research requirements for purified FIX. The authors describe a rapid, single-step human FIX purification process using a *IXSelect* affinity column (GE Healthcare Life Sciences)⁸. The human *IXSelect* affinity medium consists of a single-chain antibody fragment directed against FIX (Data file 29-1084-88 AA; https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1402917750761/litdoc29108488_20140616222347.pdf). From 100 mL of human plasma, the authors purified 200 µg of FIX, a 30% yield similar to that of other purification methods⁹. Significantly, the affinity medium was also capable of isolating a substantial amount of murine FIX (80 µg from 100 mL of mouse plasma). Using a modified activated partial thromboplastin time clotting assay¹⁰, the authors showed that the purified proteins were fully functional. As detailed below, the *IXSelect* affinity medium should find many applications in the clinic and in hemostasis research.

Hemophilia B

Hemophilia B is an X-linked bleeding disorder resulting from FIX deficiencies¹¹. More than 1000 different genomic mutations are known to cause Hemophilia B, affecting an estimated 20,000 people in the US, alone¹²⁻¹⁴. Current treatment consists of intravenous infusion of recombinant or plasma-derived FIX¹⁵, both of which are subject to rapid turnover following injection; prophylactic dosing is required, approximately every 3-7 days, making abundant sources of inexpensive FIX essential to the economic treatment of patients.

The human *IXSelect* affinity medium was designed specifically for purification of FIX in industrial processes at large scale. Human *IXSelect* has a capacity of 6 mg FIX/mL medium. The agarose-based matrix is highly cross-linked and rigid, which allows for high flow rates and rapid processing of large sample volumes. The resin can be used indefinitely, provided proper use, regeneration, and storage protocols are employed. Thus, *IXSelect* would appear to be an ideal medium for supplying the FIX needed for Hemophilia B individuals, and the report by Pilli et al. substantiates that notion. Furthermore, the resin is devoid of any other mammalian component (the antibody fragment ligand is produced in yeast), thereby satisfying FDA regulatory conditions.

As indicated above, Hemophilia B is treated with either plasma-derived or recombinant FIX. Plasma FIX, synthesized in the liver as the zymogen, requires a number of critical post-translational modifications, in addition to proteolytic processing, before it is secreted as a functional protease. The most notable post-translational modification is the gamma (γ)-carboxylation of 12 N-terminal glutamic acid residues to form γ -glutamic acid (7, 9); this N-terminal region, referred to as the Gla domain, is involved in membrane binding and is required for activation.

Although plasma FIX is fully functional, purification of the protein from plasma may entail a risk for the transmission of infectious agents upon administration to Hemophilia B individuals. Thus, recombinant FIX is now the most often prescribed form, as a safer and more economic alternative therapy for Hemophilia B (7). However, production of completely γ -carboxylated recombinant human FIX protein, in high yield, has been challenging¹⁶(8). To obtain fully modified recombinant FIX, Jianming et al.¹⁷ engineered a CHO cell line that co-expresses FIX with the necessary modification enzymes (γ -glutamyl carboxylase, vitamin K epoxide reductase, and furin/PACE cleaving enzyme). The product, BeneFix (Wyeth), contains an average of 11.5 γ -carboxyglutamic acid residues; full FIX activity requires γ -carboxylation of only the first 10 Glu residues. BeneFix is purified with four chromatographic steps and subjected to nanofiltration to remove potential viral contaminants¹⁸. The GE data bulletin referred to previously describes *IXSelect* purification of FIX from a CHO cell lysate, although it is not reported whether the cell line is the same as that engineered by Jianming et al.

Animal studies

Hemophilia B has an extensive history of study using animal models, and new therapeutics^{3,15} for Hemophilia B, which are being explored with such animal models, often require large amounts of purified experimental versions of FIX. Thus, the most important outcome of the study by Pilli et al. is the finding that the *IXSelect* affinity medium can

efficiently purify non-human FIX. This result is impressive, given a 82% sequence similarity between mouse and human factor IX. Importantly, human and murine FIX interact with the same proteins in each system, resulting in similar behaviors in a range of coagulation assays common in the clinical setting¹⁵. Hence, the mouse is a useful FIX model for human coagulation therapeutics, particularly for the ability to modify its genetics¹⁹ and for when large groups of genetically defined strains are to be studied. Murine models, currently in use, include mice deficient in FIX and mice engineered to express FIX variants²⁰.

Canine and primate models of Hemophilia B are also in use for testing the efficiency of scaling up therapeutics for long-term follow up studies, and for studies requiring larger blood volumes^{15,21,22}. Primate models are especially useful, because the animals are most similar to humans in size and anatomy^{23,24}. Thus, it will be of interest to determine the efficacy of the *IXSelect* medium in isolation of FIX from other species. One would expect the medium to perform well with chimpanzee and macaque FIX, which share with human FIX 99.8% and 96.4% amino acid identity, respectively. Considering the result with mouse plasma, other sources of FIX that will likely be amenable to *IXSelect* purification are dog (86% identity), cow (84% identity), pig (86% identity), rat (82% identity), and rabbit (83% identity). To compare efficiencies of purification, it will be necessary to determine the circulating concentrations of FIX in the plasmas of non-human species. Also, as noted above for human FIX, Pilli et al. initially reported a 30% yield from the *IXSelect* medium; this yield may be limited by the nature of the antibody fragment ligand used as the affinity medium, but perhaps some optimization testing to boost yield is warranted. Efficient FIX isolation from multiple sources is essential for assays in different organisms and, ultimately, for the progression to human clinical trials.

Future applications

The highly specific *IXSelect* column and media, tagged with a humanized FIX antibody, previously held promise for the production of large yields of pure human FIX from industrial-sized sample volumes²⁵. The results of Pilli et al.⁸ should extend the applicability of the column to the high yield purification of FIX from the plasmas of a wide range of mammals that are frequently used to test Hemophilia therapeutics. Furthermore, the authors have readily isolated multiple highly pure, mutant forms of FIX, which have been characterized by in vitro activity assays (unpublished results). These mutant proteins are vital for identifying the means by which FIX interacts with other pro- and anti-coagulant factors⁸. Similarly, the >1000 mutations already known to affect human FIX activity and abundance represent a rich pool of targets for identification of small molecule therapeutics; screening for such compounds will likely require isolation of numerous mutants in

recombinant form, which will be greatly facilitated by the simple, economical, and rapid one-step *IXSelect* column.

Finally, BeneFix has been in use since 1997, and it is the only FDA-approved recombinant FIX. Notably, the amount of FIX secreted by engineered CHO cells is relatively low, about 30-40 mg/L²⁶. Thus, transgenic animals that secrete FIX from mammary glands are being explored as sources of more abundant recombinant FIX. One of the most successful systems is pig, which has been reported to secrete 375 mg FIX/L of milk ("reported FIX secretion values, corrected to actual concentration of active form"²⁶), with a native degree of specific activity and complete carboxylation²⁷. It will be interesting to test the efficacy of the *IXSelect* medium in purification of FIX from milk.

In conclusion, the *IXSelect* medium should continue to show its range of applicability in purification of wild-type and mutant variants of FIX from numerous cellular environments. Less expensive and more efficient isolation of these proteins is advantageous towards bringing Hemophilia B studies to the human level. The ease of FIX isolation, described by Pilli et al., should provide a significant enhancement in the use of multiple animal models in the future development of new therapeutic agents for Hemophilia B.

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