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(54) Title: PURIFICATION OF VWF FOR INCREASED REMOVAL OF NON-LIPID ENVELOPED VIRUSES

(57) Abstract: The present invention provides methods for purifying Von Willebrand factor (VWF) for increased removal of non-lipid enveloped viruses.

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PURIFICATION OF VWF FOR INCREASED REMOVAL OF NON-LIPID ENVELOPED VIRUSES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/235,570, filed August 20, 2009, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] Generally, the invention relates to methods of purifying VWF for increased removal of non-lipid enveloped viruses.

BACKGROUND OF THE INVENTION

[0003] Von Willebrand factor (VWF) is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of VWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds. VWF mediates initial platelet adhesion to the sub-endothelium of the damaged vessel wall. Only the larger multimers exhibit hemostatic activity. It is assumed that endothelial cells secrete large polymeric forms of VWF and those forms of VWF which have a low molecular weight (low molecular weight VWF) arise from proteolytic cleavage. The multimers having large molecular masses are stored in the Weibel-Pallade bodies of endothelial cells and liberated upon stimulation.

[0004] VWF is synthesized by endothelial cells and megakaryocytes as prepro-VWF that consists to a large extent of repeated domains. Upon cleavage of the signal peptide, pro-VWF dimerizes through disulfide linkages at its C-terminal region. The dimers serve as protomers for multimerization, which is governed by disulfide linkages between the free end termini. The assembly to multimers is followed by the proteolytic removal of the propeptide sequence (Leyte et al., Biochem. J. 274 (1991), 257-261).

[0005] The primary translation product predicted from the cloned cDNA of VWF is a 2813-residue precursor polypeptide (prepro-VWF). The prepro-VWF consists of a 22 amino acid signal peptide and a 741 amino acid propeptide, with the mature VWF comprising 2050 amino acids (Ruggeri Z.A., and Ware, J., FASEB J., 308-316 (1993)).

[0006] Defects in VWF are causal to Von Willebrand disease (VWD), which is characterized by a more or less pronounced bleeding phenotype. VWD type 3 is the most severe form in which VWF is completely missing, and VWD type 1 relates to a quantitative loss of VWF and its phenotype can be very mild. VWD type 2 relates to qualitative defects of VWF and can be as severe as VWD type 3. VWD type 2 has many sub forms, some being associated with the loss or the decrease of high molecular weight multimers. Von Willebrand syndrome type 2a (VWS-2A) is characterized by a loss of both intermediate and large multimers. VWS-2B is characterized by a loss of highest-molecular-weight multimers. Other diseases and disorders related to VWF are known in the art.

[0007] The removal or inactivation of non-lipid enveloped viruses from therapeutic protein solutions has traditionally been accomplished by treatment with physical methods like high temperature (*e.g.*, dry heat, vapor heat, pasteurization), irradiation with high energy rays (e.g., ultraviolet (UV) rays or beta radiation), low pH, nanofiltration or by chromatographic procedures, in particular affinity chromatography. However, these procedures are often ineffective when purifying a high molecular weight protein such as VWF which does not pass through a nanofilter and/or loses its potency or molecular integrity upon treatment with heat or radiation.

[0008] Current regulatory guidelines ask manufacturers to address the issue of reduction and/or inactivation of both lipid enveloped and non-lipid enveloped viruses for recombinant pharmaceutical products. The ICH "Guideline on Viral Safety Evaluations of Biotechnology Products" (Federal Register, 1998, 63(185): 51074- 51084) gives manufacturers flexibility how to address viral issues taking into account the type of product, the production process and the risk of potentially contaminating viruses. These guidelines point out that the risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production.

[0009] Whereas the inactivation of lipid-enveloped viruses can be performed very effectively by a solvent/detergent (S/D) treatment approach, the inactivation or removal of non-lipid-enveloped model viruses (NLEV's) can be challenging due to their small size and physical stability.

[0010] Thus there exists a need in the art to develop methods to efficiently inactivate or remove non-lipid enveloped viruses during the purification of VWF.

SUMMARY OF THE INVENTION

[0011] The present invention provides an efficient method for purifying VWF for increased removal of non-lipid enveloped viruses. The present invention provides a novel method of purifying VWF for increased removal of NLEV's by performing the product loading step and the wash step of the purification process at a high pH.

[0012] One method known in the art for purifying polypeptides from NLEV's involves the use of nanofiltration. The principle behind efficient separation of protein and virus using nanofiltration exploits the size difference between the polypeptide and the virus; efficient separation requires the polypeptide to have an effective size smaller that the virus, which allows the polypeptide to pass through the pores of the nanofilter while the virus is retained. If the polypeptide and virus are of a comparable size relative to each other, however, separation is problematic because either the polypeptide and virus both pass through the nanofilter pores or neither do. The methods disclosed herein overcome this problem by using a cation exchange resin rather than nanofiltration and loading and/or washing the resin at a sufficiently high pH to separate the polypeptide from the virus.

[0013] Without being bound by theory, the methods disclosed herein are useful for improved removal of NLEV from polypeptide solutions wherein the polypeptide is of a certain size and/or conformation. A polypeptide of a sufficiently large size is likely to have localized charge characteristics at or above the isoelectric point of the polypeptide, *i.e.*, regions of the polypeptide can maintain localized positive or negative charges, thereby allowing the polypeptide to adsorb to the column resin while the virus flows through. This uneven charge distribution over the length of a polypeptide allows the polypeptide to remain attached to the resin despite loading and/or washing of the resin at a high pH.

[0014] The invention provides a method for removing a non-lipid enveloped virus from a protein-containing solution comprising loading a protein in the solution onto a cation

exchange resin, and washing the resin with a buffer at a pH higher than the isoelectric point of the protein to elute the virus. In one aspect, the protein is loaded onto the resin in a buffer at a pH higher than that of the isoelectric point of the protein to elute the virus. In another aspect, the protein is loaded onto the resin in a buffer that is not the buffer used in the wash step, and the resin is subsequently washed with the buffer that is at a pH higher than an isoelectric point of the protein.

[0015] In one embodiment, a method for removing a non-lipid enveloped virus from a protein-containing solution is provided comprising applying the solution to a cation exchange resin at a pH higher than the isoelectric point of the protein, and washing the cation exchange resin with a first wash buffer to form an eluate, said first wash buffer having a pH that is equal to or lower than the solution applied to the cation exchange resin.

[0016] In one aspect, the pH of the solution is about 1 pH unit above the isoelectric point of the protein. In other aspects, the pH of the solution is about 1.1, or about 1.2, or about 1.3, or about 1.4, or about 1.5, or about 1.6, or about 1.7, or about 1.8, or about 1.9, or about 2.0, or about 2.1, or about 2.2, or about 2.3, or about 2.4, or about 2.5, or about 2.6, or about 2.7, or about 2.8, or about 2.9, or about 3.0, or about 3.1, or about 3.2, or about 3.3, or about 3.4, or about 3.5, or about 3.6, or about 3.7, or about 3.8, or about 3.9, or about 4.0, or about 4.1, or about 4.2, or about 4.3, or about 4.4, or about 4.5, or about 4.6, or about 4.7, or about 4.8, or about 4.9, or about 5.0, or about 5.1, or about 5.2, or about 5.3, or about 5.4, or about 5.5, or about 5.6, or about 5.7, or about 5.8, or about 5.9, or about 6.0or more pH units or more above the isoelectric point of the protein. In these embodiments, the pH is greater than about 7. In a related aspect, the pH of the protein-containing solution is about 7.0. In other aspects, the pH of the protein-containing solution is about 7.1, or about 7.2, or about 7.3, or about 7.4, or about 7.5, or about 7.6, or about 7.7, or about 7.8, or about 7.9, or about 8.0, or about 8.1, or about 8.2, or about 8.3, or about 8.4, or about 8.5, or about 8.6, or about 8.7, or about 8.8, or about 8.9, or about 9.0, or about 9.1, or about 9.2, or about 9.3, or about 9.4, or about 9.5, or about 9.6, or about 9.7, or about 9.8, or about 9.9, or about 10.0, or about 10.1, or about 10.2, or about 10.3, or about 10.4, or about 10.5, or about 10.6, or about 10.7, or about 10.8, or about 10.9, or about 11.0, or about 11.1, or about 11.2, or about 11.3, or about 11.4, or about 11.5, or about 11.6, or about 11.7, or about 11.8, or about 11.9, or about 12.0, or about 12.1, or about 12.2, or about 12.3, or about 12.4, or about 12.5, or about 12.6, or about 12.7, or about 12.8, or about 12.9, or about 13.0 or higher.

[0017] In another embodiment, a method is provided for removing a non-lipid enveloped virus from a protein-containing solution comprising applying the solution to a cation exchange resin, washing the cation exchange resin with a first wash buffer at a pH higher than the pH of the solution applied to the cation exchange resin, and washing the cation exchange resin with a second wash buffer to form an eluate, said first eluant having a pH that is equal to or lower than the first wash buffer. In one aspect, the pH of the first wash buffer is about 1 pH unit above the pH of the solution applied to the cation exchange resin. In other aspects, the pH of the first wash buffer is about 0.1, or about 0.2, or about 0.3, or about 0.4, or about 0.5, or about 0.6, or about 0.7, or about 0.8, or about 0.9, or about 1.1, or about 1.2, or about 1.3, or about 1.4, or about 1.5, or about 1.6, or about 1.7, or about 1.8, or about 1.9, or about 2.0, or about 2.1, or about 2.2, or about 2.3, or about 2.4, or about 2.5, or about 2.6, or about 2.7, or about 2.8, or about 2.9, or about 3.0, or about 3.1, or about 3.2, or about 3.3, or about 3.4, or about 3.5, or about 3.6, or about 3.7, or about 3.8, or about 3.9, or about 4.0, or about 4.1, or about 4.2, or about 4.3, or about 4.4, or about 4.5, or about 4.6, or about 4.7, or about 4.8, or about 4.9, or about 5.0, or about 5.1, or about 5.2, or about 5.3, or about 5.4, or about 5.5, or about 5.6, or about 5.7, or about 5.8, or about 5.9, or about 6.0, or about 6.1, or about 6.2, or about 6.3, or about 6.4, or about 6.5, or about 6.6, or about 6.7, or about 6.8. or about 6.9, or about 7.0, or about 7.1, or about 7.2, or about 7.3, or about 7.4, or about 7.5, or about 7.6, or about 7.7, or about 7.8, or about 7.9, or about 8 or about 8.1, or about 8.2, or about 8.3, or about 8.4, or about 8.5, or about 8.6, or about 8.7, or about 8.8, or about 8.9, or about 9, or about 9.1, or about 9.2, or about 9.3, or about 9.4, or about 9.5, or about 9.6, or about 9.7, or about 9.8, or about 9.9, or about 10 or more pH units or more above the isoelectric point of the protein. In these embodiments, the pH of the first wash buffer is greater than about 7. In other aspects, the pH of the first wash buffer is about 7.1, or about 7.2, or about 7.3, or about 7.4, or about 7.5, or about 7.6, or about 7.7, or about 7.8, or about 7.9, or about 8.0, or about 8.1, or about 8.2, or about 8.3, or about 8.4, or about 8.5, or about 8.6, or about 8.7, or about 8.8, or about 8.9, or about 9.0, or about 9.1, or about 9.2, or about 9.3, or about 9.4, or about 9.5, or about 9.6, or about 9.7, or about 9.8, or about 9.9, or about 10.0, or about 10.1, or about 10.2, or about 10.3, or about 10.4, or about 10.5, or about 10.6, or about 10.7, or about 10.8, or about 10.9, or about 11.0 or higher.

[0018] In an embodiment, the protein in the solution is a polypeptide having a molecular mass of at least about 150 kilodaltons. In various aspects, the protein in the solution is a polypeptide having a molecular mass of at least about 175 kilodaltons, or about 180

kilodaltons, or about 190 kilodaltons, or about 200 kilodaltons, or about 210 kilodaltons, or about 220 kilodaltons, or about 230 kilodaltons, or about 240 kilodaltons, or about 250 kilodaltons, or about 260 kilodaltons, or about 270 kilodaltons, or about 280 kilodaltons, or about 290 kilodaltons, or about 300 kilodaltons, or about 310 kilodaltons, or about 320 kilodaltons, or about 330 kilodaltons, or about 340 kilodaltons, or about 350 kilodaltons, or about 360 kilodaltons, or about 370 kilodaltons, or about 380 kilodaltons, or about 390 kilodaltons, or about 400 kilodaltons, or about 410 kilodaltons, or about 420 kilodaltons, or about 430 kilodaltons, or about 440 kilodaltons, or about 450 kilodaltons, or about 460 kilodaltons, or about 470 kilodaltons, or about 480 kilodaltons, or about 490 kilodaltons, or about 500 kilodaltons or more. As described herein, polypeptides also comprise multimeric structures and such multimeric structures, in various aspects, have a molecular mass of at least about 500 kilodaltons. In related aspects, the multimeric structures have a molecular mass of at least about 510, or about 520, or about 530, or about 540, or about 550, or about 560, or about 570, or about 580, or about 590, or about 600, or about 610, or about 620, or about 630, or about 640, or about 650, or about 660, or about 670, or about 680, or about 690, or about 700, or about 710, or about 720, or about 730, or about 740, or about 750, or about 760, or about 770, or about 780, or about 790, or about 800, or about 810, or about 820, or about 830, or about 840, or about 850, or about 860, or about 870, or about 880, or about 890, or about 900, or about 910, or about 920, or about 930, or about 940, or about 950, or about 960, or about 970, or about 980, or about 990 kilodaltons, or about 1 megadalton, or about 1.1 megadaltons, or about 1.2 megadaltons, or about 1.3 megadaltons, or about 1.4 megadaltons, or about 1.5 megadaltons, or about 1.6 megadaltons, or about 1.7 megadaltons, or about 1.8 megadaltons, or about 1.9 megadaltons, or about 2.0 megadaltons, or about 2.1 megadaltons, or about 2.2 megadaltons, or about 2.3 megadaltons, or about 2.4 megadaltons, or about 2.5 megadaltons, or about 2.6 megadaltons, or about 2.7 megadaltons, or about 2.8 megadaltons, or about 2.9 megadaltons, or about 3.0 megadaltons, or about 3.1 megadaltons, or about 3.2 megadaltons, or about 3.3 megadaltons, or about 3.4 megadaltons, or about 3.5 megadaltons, or about 3.6 megadaltons, or about 3.7 megadaltons, or about 3.8 megadaltons, or about 3.9 megadaltons, or about 4.0 megadaltons, or about 4.1 megadaltons, or about 4.2 megadaltons, or about 4.3 megadaltons, or about 4.4 megadaltons, or about 4.5 megadaltons, or about 4.6 megadaltons, or about 4.7 megadaltons, or about 4.8 megadaltons, or about 4.9 megadaltons, or about 5.0 megadaltons or more.

[0019] In some embodiments, the cation exchange resin has a negatively charged group selected from the group consisting of carboxymethyl (CM), sulphoalkyl (SP, SE), sulphate and methylsulfonate (S) as well as any other negatively charged ligand.

[0020] In a further embodiment, the protein is a blood coagulation protein. In various aspects, the blood coagulation protein is selected from the group consisting of Factor VIII, von Willebrand factor, FI (Fibrinogen), FV (Proaccelerin), FXI (plasma- thromboplastin antecedent), and FXIII (fibrin stabilizing factor).

[0021] In an embodiment, a method for removing a non-lipid enveloped virus from a von Willebrand (VWF)-containing solution is provided comprising applying the solution to a cation exchange resin at a pH higher than the isoelectric point of the protein and washing the cation exchange resin with a first wash buffer to form an eluate, said first wash buffer having a pH that is equal to or lower than the solution applied to the cation exchange resin.

[0022] In another embodiment, a method for removing a non-lipid enveloped virus from a VWF-containing solution is provided comprising applying the solution to a cation exchange resin, washing the cation exchange resin with a first wash buffer at a pH higher than the pH of the solution applied to the cation exchange resin and washing the cation exchange resin with a second wash buffer to form an eluate, said first eluant having a pH that is equal to or lower than the first wash buffer.

[0023] In a further embodiment, a method for removing a non-lipid enveloped virus from a VWF-containing solution comprising applying the solution to a cation exchange resin at a pH higher than the isoelectric point of the protein and washing the cation exchange resin with a first wash buffer at a pH higher than the isoelectric point of the protein applied to the cation exchange resin; and washing the cation exchange resin with a second wash buffer to form an eluate, said first eluant having a pH that is equal to or lower than the first wash buffer.

[0023a] Definitions of the specific embodiments of the invention as claimed herein follow.

[0023b] According to a first embodiment of the invention, there is provided a method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin at a pH of at least 1.6 units above the isoelectric point of a protein in the protein-containing solution; and

washing the cation exchange resin with a wash buffer to form an eluate, said wash buffer having a pH that is lower than the pH of the protein-containing solution applied to the cation exchange resin but having a pH higher than the isoelectric point of the protein, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

[0023c] According to a second embodiment of the invention, there is provided a method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin;

washing the cation exchange resin with a first wash buffer having a pH higher than the pH of the protein-containing solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein; and

washing the cation exchange resin with a second wash buffer to form an eluate, said eluate having a pH that is equal to or lower than the first wash buffer, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

[0023d] According to a third embodiment of the invention, there is provided a method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the solution to a cation exchange resin at a pH higher than the isoelectric point of the protein in the protein-containing solution; and

washing the cation exchange resin with a first wash buffer at a pH higher than the isoelectric point of the protein in the protein-containing solution applied to the cation exchange resin; and

washing the cation exchange resin with a second wash buffer to form an eluate, said eluate having a pH that is equal to or lower than the pH of the first wash buffer, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

[0023e] According to a fourth embodiment of the invention, there is provided a method for removing a non-lipid enveloped virus from a von Willebrand Factor (VWF)-containing solution comprising:

applying the VWF-containing solution to a cation exchange resin at a pH of at least 1.6 units above the isoelectric point of VWF; and

washing the cation exchange resin with a first wash buffer to form an eluate, said wash buffer having a pH that is equal to or lower than the pH of the VWF-containing solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein, and whereby the non-lipid enveloped virus is removed from the VWF-containing solution.

[0023f] According to a fifth embodiment of the invention, there is provided a method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin, wherein the pH of the protein-containing solution is at least 2.0 pH units or more above the isoelectric point of a protein in the protein-containing solution; and

washing the cation exchange resin with a wash buffer to form an eluate, said wash buffer having a pH that is equal to or lower than the pH of the solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein, wherein the protein has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

BRIEF DESCRIPTION OF THE FIGURES

[0024] **Figure 1** shows the result of an SDS-PAGE separation followed by silver staining (**A**) and Western Blot (**B**) analysis for residual rFVIII.

[0025] **Figure 2** shows the stained gel of the UNO S runs with MMV and REO virus spiked samples.

[TEXT CONTINUES ON PAGE 8]

[0026] **Figure 3** shows the stained gel of the UNO S runs with MMV and REO virus spiked samples.

[0027] **Figure 4** shows the results of subjecting the purified preparations of rVWF obtained by the process variants to proteolytic digestion by V8 protease in the native state and separating the resulting peptides by RP-HPLC.

[0028] **Figure 5** shows the results of subjecting the purified preparations of rVWF obtained by the process variants to trypsin in the denatured state and separating the resulting peptides by RP-HPLC.

DETAILED DESCRIPTION OF THE INVENTION

[0029] The present invention relates to a method for purifying VWF with increased removal of non-lipid enveloped viruses. The methods of the invention are applicable in column (*i.e.*, chromatography) as well as batch (*i.e.*, without column hardware) mode.

[0030] The method of the present invention utilizes a purification method on a cation exchange resin for the increased removal of non-lipid enveloped viruses. Previous methods of purification of VWF using cation exchange chromatography were performed at a neutral pH. These methods allowed for the manufacture of purified VWF of good yield and purity, but surprisingly the process had no capacity to remove non-lipid enveloped viruses.

Definition of terms

[0031] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY (2d ed. 1994); THE CAMBRIDGE DICTIONARY OF SCIENCE AND TECHNOLOGY (Walker ed., 1988); THE GLOSSARY OF GENETICS, 5TH ED., R. Rieger, et al. (eds.), Springer Verlag (1991); and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY (1991).

[0032] Each publication, patent application, patent, and other reference cited herein is incorporated by reference in its entirety to the extent that it is not inconsistent with the present disclosure.

[0033] It is noted here that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise.

[0034] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0035] As used herein the terms "express," "expressing" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g. the resulting protein, may also be said to be "expressed." An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means inside a cell. The term "extracellular" means outside a cell, *e.g.*, certain types of transmembrane proteins. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

[0036] As used herein a "polypeptide" refers to a polymer composed of amino acid residues, structural variants, related naturally-occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds. Synthetic polypeptides can be prepared, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides. The term "polypeptide" also includes polymeric structures. Therefore, a "polypeptide" may be a monomer, dimer, trimer, or larger multimeric structure. These multimeric structures can be up to 5 megadaltons or larger.

[0037] As used herein, the "isoelectric point" is the pH value at which the net electric charge of a polypeptide in an aqueous solution is zero.

[0038] As used herein a "fragment" of a polypeptide is meant to refer to any portion of a polypeptide or protein smaller than the full-length polypeptide or protein expression product.

[0039] As used herein an "analog" refers to any of two or more polypeptides substantially similar in structure and having the same biological activity, but can have varying degrees of activity, to either the entire molecule, or to a fragment thereof. Analogs differ in the composition of their amino acid sequences based on one or more mutations involving substitution of one or more amino acids for other amino acids. Substitutions can be

conservative or non-conservative based on the physico-chemical or functional relatedness of the amino acid that is being replaced and the amino acid replacing it.

[0040] As used herein a "variant" refers to a polypeptide, protein or analog thereof that is modified to comprise additional chemical moieties not normally a part of the molecule. Such moieties may modulate the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule and eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences (1980). Procedure for coupling such moieties to a molecule are well known in the art. For example, the variant may be a blood clotting factor having a chemical modification which confers a longer half-life in vivo to the protein. In various aspects, polypeptides are modified by glycosylation, pegylation, and/or polysialylation.

Recombinant VWF

[0041] The polynucleotide and amino acid sequences of prepro-VWF are set out in SEQ ID NO:1 and SEQ ID NO:2, respectively, and are available at GenBank Accession Nos. NM_000552 and NP_000543, respectively. The amino acid sequence corresponding to the mature VWF protein is set out in SEQ ID NO: 3 (corresponding to amino acids 764-2813 of the full length prepro-VWF amino acid sequence).

[0042] One form of useful rVWF has at least the property of *in vivo*-stabilizing, e.g. binding, of at least one Factor VIII (FVIII) molecule and having optionally a glycosylation pattern which is pharmacologically acceptable. Specific examples thereof include VWF without A2 domain thus resistant to proteolysis (Lankhof et al., Thromb. Haemost. 77: 1008-1013, 1997), and the VWF fragment from Val 449 to Asn 730 including the glycoprotein lb-binding domain and binding sites for collagen and heparin (Pietu et al., Biochem. Biophys. Res. Commun. 164: 1339-1347, 1989). The determination of the ability of a VWF to stabilize at least one FVIII molecule can be carried out in VWF-deficient mammals according to methods known in the state in the art.

[0043] The rVWF of the present invention may be produced by any method known in the art. One specific example is disclosed in WO86/06096 published on Oct. 23, 1986 and U.S. Patent Application No. 07/559,509, filed on Jul. 23, 1990, which is incorporated herein by reference with respect to the methods of producing recombinant VWF. Thus, methods are known in the art for (i) the production of recombinant DNA by genetic engineering, e.g. via

reverse transcription of RNA and/or amplification of DNA, (ii) introducing recombinant DNA into procaryotic or eucaryotic cells by transfection, e.g. via electroporation or microinjection, (iii) cultivating said transformed cells, e.g. in a continuous or batchwise manner, (iv) expressing VWF, e.g. constitutively or upon induction, and (v) isolating said VWF, e.g. from the culture medium or by harvesting the transformed cells, in order to (vi) obtain purified rVWF, e.g. via anion exchange chromatography or affinity chromatography. A recombinant VWF may be made in transformed host cells using recombinant DNA techniques well known in the art. For instance, sequences coding for the polypeptide could be excised from DNA using suitable restriction enzymes.

[0044] Alternatively, the DNA molecule could be synthesized using chemical synthesis techniques, such as the phosphoramidate method. Also, a combination of these techniques could be used.

[0045] The invention also provides vectors encoding polypeptides of the invention in an appropriate host. The vector comprises the polynucleotide that encodes the polypeptide operatively linked to appropriate expression control sequences. Methods of effecting this operative linking, either before or after the polynucleotide is inserted into the vector, are well known. Expression control sequences include promoters, activators, enhancers, operators, ribosomal binding sites, start signals, stop signals, cap signals, polyadenylation signals, and other signals involved with the control of transcription or translation. The resulting vector having the polynucleotide therein is used to transform an appropriate host. This transformation may be performed using methods well known in the art.

[0046] Any of a large number of available and well-known host cells may be used in the practice of this invention. The selection of a particular host is dependent upon a number of factors recognized by the art, including, for example, compatibility with the chosen expression vector, toxicity of the peptides encoded by the DNA molecule, rate of transformation, ease of recovery of the peptides, expression characteristics, bio-safety and costs. A balance of these factors must be struck with the understanding that not all host cells are equally effective for the expression of a particular DNA sequence. Within these general guidelines, useful microbial host cells include bacteria, yeast and other fungi, insects, plants, mammalian (including human) cells in culture, or other hosts known in the art.

[0047] Next, the transformed host is cultured and purified. Host cells may be cultured under conventional fermentation conditions so that the desired compounds are expressed.

Such fermentation conditions are well known in the art. Finally, the polypeptides are purified from culture by methods well known in the art.

[0048] Depending on the host cell utilized to express a compound of the invention, carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids not counting proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (e.g., in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

[0049] Alternatively, the compounds may be made by synthetic methods. For example, solid phase synthesis techniques may be used. Suitable techniques are well known in the art, and include those described in Merrifield (1973), Chem. Polypeptides, pp. 335-61 (Katsoyannis and Panayotis eds.); Merrifield (1963), J. Am. Chem. Soc. 85: 2149; Davis et al. (1985), Biochem. Intl. 10: 394-414; Stewart and Young (1969), Solid Phase Peptide Synthesis; U.S. Pat. No. 3,941,763; Finn et al. (1976), The Proteins (3rd ed.) 2: 105-253; and Erickson et al. (1976), The Proteins (3rd ed.) 2: 257-527. Solid phase synthesis is the preferred technique of making individual peptides since it is the most cost-effective method of making small peptides.

Fragments, variants and analogs of VWF

[0050] Methods for preparing polypeptide fragments, variants or analogs are well-known in the art.

[0051] Fragments of a polypeptide are prepared using, without limitation, enzymatic cleavage (e.g., trypsin, chymotrypsin) and also using recombinant means to generate a polypeptide fragments having a specific amino acid sequence. Polypeptide fragments may be

generated comprising a region of the protein having a particular activity, such as a multimerization domain or any other identifiable VWF domain known in the art.

[0052] Variants of a polypeptide are contemplated to include human and non-human forms of VWF (*e.g.*, murine VWF). Also contemplated by the methods herein are chimeric polypeptides comprising, *e.g.*, a mouse/human fusion polypeptide.

[0053] Methods of making polypeptide analogs are also well-known. Amino acid sequence analogs of a polypeptide can be substitutional, insertional, addition or deletion analogs. Deletion analogs, including fragments of a polypeptide, lack one or more residues of the native protein which are not essential for function or immunogenic activity. Insertional analogs involve the addition of, e.g., amino acid(s) at a non-terminal point in the polypeptide. This analog may include insertion of an immunoreactive epitope or simply a single residue. Addition analogs, including fragments of a polypeptide, include the addition of one or more amino acids at either of both termini of a protein and include, for example, fusion proteins.

[0054] Substitutional analogs typically exchange one amino acid of the wild-type for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide without the loss of other functions or properties. In one aspect, substitutions are conservative substitutions. By "conservative amino acid substitution" is meant substitution of an amino acid with an amino acid having a side chain of a similar chemical character. Similar amino acids for making conservative substitutions include those having an acidic side chain (glutamic acid, aspartic acid); a basic side chain (arginine, lysine, histidine); a polar amide side chain (glutamine, asparagine); a hydrophobic, aliphatic side chain (leucine, isoleucine, valine, alanine, glycine); an aromatic side chain (phenylalanine, tryptophan, tyrosine); a small side chain (glycine, alanine, serine, threonine, methionine); or an aliphatic hydroxyl side chain (serine, threonine).

[0055] Analogs may be substantially homologous or substantially identical to the recombinant VWF from which they are derived. Preferred analogs are those which retain at least some of the biological activity of the wild-type polypeptide, *e.g.* blood clotting activity.

[0056] Polypeptide variants contemplated include polypeptides chemically modified by such techniques as ubiquitination, glycosylation, including polysialation, conjugation to therapeutic or diagnostic agents, labeling, covalent polymer attachment such as pegylation (derivatization with polyethylene glycol), introduction of non-hydrolyzable bonds, and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not

normally occur in human proteins. Variants retain the same or essentially the same binding properties of non-modified molecules of the invention. Such chemical modification may include direct or indirect (e.g., via a linker) attachment of an agent to the VWF polypeptide. In the case of indirect attachment, it is contemplated that the linker may be hydrolyzable or non-hydrolyzable.

[0057] Preparing pegylated polypeptide analogs will generally comprise the steps of (a) reacting the polypeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the binding construct polypeptide becomes attached to one or more PEG groups, and (b) obtaining the reaction product(s). In general, the optimal reaction conditions for the acylation reactions will be determined based on known parameters and the desired result. For example, the larger the ratio of PEG: protein, the greater the percentage of poly-pegylated product. In some embodiments, the binding construct will have a single PEG moiety at the N-terminus. Polyethylene glycol (PEG) may be attached to the blood clotting factor to provide a longer half-life *in vivo*. The PEG group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG ranges from about 2 kiloDalton ("kD") to about 100 kDa, from about 5 kDa to about 50 kDa, or from about 5 kDa to about 10 kDa. The PEG groups are attached to the blood clotting factor via acylation or reductive alkylation through a natural or engineered reactive group on the PEG moiety (e.g., an aldehyde, amino, thiol, or ester group) to a reactive group on the blood clotting factor (e.g., an aldehyde, amino, or ester group) or by any other technique known in the art.

[0058] Methods for preparing polysialylated polypeptide are described in United States Patent Publication 20060160948, Fernandes et Gregoriadis; Biochim. Biophys. Acta 1341: 26-34, 1997, and Saenko et al., Haemophilia 12:42-51, 2006. Briefly, a solution of colominic acid containing 0.1 M $NaIO_4$ is stirred in the dark at room temperature to oxidize the CA. The activated CA solution is dialyzed against, e.g., 0.05 M sodium phosphate buffer, pH 7.2 in the dark and this solution was added to a rVWF solution and incubated for 18 h at room temperature in the dark under gentle shaking. Free reagents can then be separated from the rVWF-polysialic acid conjugate by ultrafiltration/diafiltration. Conjugation of rVWF with polysialic acid may also be achieved using glutaraldehyde as cross-linking reagent (Migneault et al., Biotechniques 37: 790-796, 2004).

[0059] It is further contemplated that a polypeptide of the invention may be a fusion protein with a second agent which is a polypeptide. In one embodiment, the second agent

which is a polypeptide, without limitation, is an enzyme, a growth factor, an antibody, a cytokine, a chemokine, a cell-surface receptor, the extracellular domain of a cell surface receptor, a cell adhesion molecule, or fragment or active domain of a protein described above. In a related embodiment, the second agent is a blood clotting factor such as Factor VIII, Factor VII, Factor IX. The fusion protein contemplated is made by chemical or recombinant techniques well-known in the art.

[0060] It is also contemplated that prepro-VWF and pro-VWF polypeptides may provide a therapeutic benefit in the formulations of the present invention. For example, US Patent No. 7,005,502 describes a pharmaceutical preparation comprising substantial amounts of pro-VWF that induces thrombin generation in the presence of platelets *in vitro*. In addition to recombinant, biologically active fragments, variants, or analogs of the naturally-occuring mature VWF, the present invention contemplates the use of recombinant biologically active fragments, variants, or analogs of the prepro-VWF (set out in SEQ ID NO:2) or pro-VWF polypeptides (amino acid residues 23 to 764 of SEQ ID NO: 2) in the formulations described herein.

[0061] Polynucleotides encoding fragments, variants and analogs may be readily generated by a worker of skill to encode biologically active fragments, variants, or analogs of the naturally-occurring molecule that possess the same or similar biological activity to the naturally-occurring molecule. These polynucleotides can be prepared using PCR techniques, digestion/ligation of DNA encoding molecule, and the like. Thus, one of skill in the art will be able to generate single base changes in the DNA strand to result in an altered codon and a missense mutation, using any method known in the art, including, but not limited to site-specific mutagenesis. As used herein, the phrase "moderately stringent hybridization conditions" means, for example, hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook et al., 9.47-9.51 in Molecular Cloning, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Methods of Producing VWF

[0062] Industrially, VWF, in particular human recombinant VWF (rVWF), is synthesized and expressed together with rFVIII in a genetically engineered CHO cell line. The function

of the co-expressed rVWF is to stabilize rFVIII in the cell culture process. rVWF is synthesized in the cell as the pro-form, containing a large pro-peptide attached to the N-terminus. Upon maturation in the endoplasmatic reticulum and Golgi apparatus, the pro-peptide is cleaved off by the action of the cellular protease furin and is secreted as a homopolymer of identical subunits, consisting of dimers of the expressed protein.

Purification of VWF

[0063] Provided herein is a method for removing a non-lipid enveloped virus from a protein-containing solution is provided comprising applying the solution to a cation exchange resin at a pH higher that the isoelectric point of the protein, and washing the cation exchange resin with a first wash buffer to form an eluate, said first wash buffer having a pH that is equal to or lower than the solution applied to the cation exchange resin.

[0064] In one aspect, the pH of the solution is about 1 pH unit above the isoelectric point of the protein. In other aspects, the pH of the solution is about 1.2, or about 1.4, or about 1.6, or about 1.8, or about 2.0, or about 2.2, or about 2.4, or about 2.6, or about 2.8, or about 3.0, or about 3.2, or about 3.4, or about 3.6, or about 3.8, or about 4.0, or about 4.2, or about 4.4, or about 4.6, or about 4.8, or about 5.0, or about 5.5, or about 6.0 pH units or more above the isoelectric point of the protein.

[0065] In these embodiments, the pH is greater than about 7.. In other aspects, the pH is about 7.1, or about 7.2, or about 7.3, or about 7.4, or about 7.5, or about 7.6, or about 7.7, or about 7.8, or about 7.9, or about 8.0, or about 8.1, or about 8.2, or about 8.3, or about 8.4, or about 8.5, or about 8.6, or about 8.7, or about 8.8, or about 8.9, or about 9.0, or about 9.1, or about 9.2, or about 9.3, or about 9.4, or about 9.5, or about 9.6, or about 9.7, or about 9.8, or about 9.9, or about 10.0, or about 10.1, or about 10.2, or about 10.3, or about 10.4, or about 10.5, or about 10.6, or about 10.7, or about 10.8, or about 10.9, or about 11.0, or about 11.1, or about 11.2, or about 11.3, or about 11.4, or about 11.5, or about 11.6, or about 11.7, or about 11.8, or about 11.9, or about 12.0, or about 12.1, or about 12.2, or about 12.3, or about 12.4, or about 12.5, or about 12.6, or about 12.7, or about 12.8, or about 12.9, or about 13.0 or higher.

[0066] In another embodiment, a method is provided for removing a non-lipid enveloped virus from a protein-containing solution comprising applying the solution to a cation exchange resin, washing the cation exchange resin with a first wash buffer at a pH higher than the pH of the solution applied to the cation exchange resin, and washing the cation

exchange resin with a second wash buffer to form an eluate, said second wash buffer having a pH that is equal to or lower than the first wash buffer. In one aspect, the pH of the first wash buffer is about 1 pH unit above the pH of the solution applied to the cation exchange resin. For this stage it is contemplated that the ion exchange media is UNOsphere™ S (BioRad Laboratories, Inc., Hercules, CA), but other cation exchange systems may be used in the practice of the methods. These cation exchange systems are known to those of ordinary skill in the art.

[0067] In other aspects, the pH of the first wash buffer is about 1.1, or about 1.2, or about 1.3, or about 1.4, or about 1.5, or about 1.6, or about 1.7, or about 1.8, or about 1.9, or about 2.0, or about 2.1, or about 2.2, or about 2.3, or about 2.4, or about 2.5, or about 2.6, or about 2.7, or about 2.8, or about 2.9, or about 3.0, or about 3.1, or about 3.2, or about 3.3, or about 3.4, or about 3.5, or about 3.6, or about 3.7, or about 3.8, or about 3.9, or about 4.0, or about 4.1, or about 4.2, or about 4.3, or about 4.4, or about 4.5, or about 4.6, or about 4.7, or about 4.8, or about 4.9, or about 5.0, or about 5.1, or about 5.2, or about 5.3, or about 5.4, or about 5.5, or about 5.6, or about 5.7, or about 5.8, or about 5.9, or about 6.0 pH units or more above the isoelectric point of the protein. In these embodiments, the pH of the first wash buffer is greater than about 7. In other aspects, the pH of the first wash buffer is about 7.1, or about 7.2, or about 7.3, or about 7.4, or about 7.5, or about 7.6, or about 7.7, or about 7.8, or about 7.9, or about 8.0, or about 8.1, or about 8.2, or about 8.3, or about 8.4, or about 8.5, or about 8.6, or about 8.7, or about 8.8, or about 8.9, or about 9.0, or about 9.1, or about 9.2, or about 9.3, or about 9.4, or about 9.5, or about 9.6, or about 9.7, or about 9.8, or about 9.9, or about 10.0, or about 10.1, or about 10.2, or about 10.3, or about 10.4, or about 10.5, or about 10.6, or about 10.7, or about 10.8, or about 10.9, or about 11.0, or about 11.1, or about 11.2, or about 11.3, or about 11.4, or about 11.5, or about 11.6, or about 11.7, or about 11.8, or about 11.9, or about 12.0, or about 12.1, or about 12.2, or about 12.3, or about 12.4, or about 12.5, or about 12.6, or about 12.7, or about 12.8, or about 12.9, or about 13.0 or higher.

[0068] The following examples are not intended to be limiting but only exemplary of specific embodiments of the invention.

EXAMPLES

Example 1

[0069] Viruses and cells used in the assays described below are as follows.

[0070] REO-3 (Family Reoviridae ; non-enveloped dsRNA virus), Strain Dearing (ATCC VR-824) was obtained from the ATCC. The virus was propagated and titrated on Vero cells obtained from ECACC (84113001). MMV (Family Parvoviridae ; non-enveloped ssDNA virus), prototype strain (ATCC VR-1346), was obtained from the American Type Culture Collection, Rockville, Maryland. The virus was propagated and titrated on A9 cells (ATCC CCL-1 .4). PPV (Family Parvoviridae ; non-enveloped ssDNA virus), strain Tennessee (BRFF #PP951024), was obtained from Biological Research Faculty & Facility, Ijamsville, Maryland. The virus was propagated and titrated on PK-13 cells (ATCC CRL-6489). EMCV (Family Picornaviridae ; non-enveloped ssRNA) (ATCC #VR-129B) was obtained from the American Type Culture Collection. The virus was propagated and titrated on Vero cells (European Collection of Cell Cultures, ECACC, #84113001). HadV (Family Adenoviridae ; non-enveloped dsDNA), strain Adenoid 75 (ATCC VR-5), was obtained from the American Type Culture Collection. The virus was propagated and titrated on HeLa cells (ATCC CCL-2).

[0071] The steps involved in an exemplary VWF purification process comprise:

- Immune affinity chromatography of cell culture supernatant
    i. Flow-through fraction
- Anion Exchange (*e.g.*, trimethylaminoethyl anion exchange column)
- Filtration (0.45/0.2 μm)
- Anion Exchange (*e.g.*, Mustang Q (Pall Corporation))
- Virus Inactivation (*e.g.*, using solvent/detergent treatment)
- Filtration (0.8/0.65 μm)
- Cation Exchange (*e.g.*, UNO S column)
- Ultrafiltration/Concentration
- Filtration (0.45/0.2 μm)
- Gel Filtration (Superose 6 prep grade (GE Life Sciences))

[0072] **Optimization of UNO S Step**. During the UNO S step rVWF is bound to a strong cation exchange resin while some of the impurities pass through. After washing the column with increased conductivity buffers the bound rVWF is released from the column with a salt

step. During the initial virus removal studies, this step showed at least a significant removal rate for the model REO virus. The conditions of the applied parameters and the corresponding results are listed in Table 1, below.

Table 1

Parameter	Standard value	Changed value	MMV reduction factor (log10)
Conductivity load/wash	15 mS/cm	25 mS/cm	n.a.
pH load/wash/elution	6.5	8.0	0.9
Wash buffer 2	TQA buffer*	TQA buffer with 200mM betaine	0.7
Wash buffer 3	TQA buffer	TQA buffer with 20% ethylen glycol	
Wash buffer 4	TQA buffer	TQA buffer with 10 mM $CaCl_2$	0.8
Wash buffer 5	TQA buffer	TQA buffer with 10 mM EDTA	
pH load/wash	6.5	9.0	2.0, 2.11, 2.12 and 2.12
pH load/wash	6.5	9.0	2.12 for REO virus

*TQA Buffer: Tris, NaAc, mM NaCl in WFI
pH 6.3 - 6.7 at 20 - 25°C

[0073] As can be seen in Table 1 the moderate changes in the process parameters (modification of conductivity, pH 8.0 and additives to wash buffers) did not result in a significant improvement of the MMV removal rates . Increasing the pH further to 9.0 reproducibly resulted in a significant removal rate of more than 2 logs for MMV as well as REO virus. This process change is technically easy to implement and the exposure of rVWF to the high pH environment can be kept relatively short (max. 6 hours). Elution of the bound rVWF is performed under neutral conditions.

[0074] The analysis of the virus inactivation capacity of the processes was carried out according to the recommendations of the CPMP guideline 268/95, using the following formula: $R = \log\left(\frac{V_1 \times T_1}{V_2 \times T_2}\right)$

where

R = virus reduction factor

V1 = volume of starting material [ml]

T1 = concentration of virus in starting material [TCID50/ml]

V2 = volume of material after the step [ml]

T2 = concentration of virus after the step [TCID50/ml]

[0075] The volumes and the titers of each spiked sample before and after treatment were used to calculate R. Whenever virus was undetectable, the detection limit was taken as the virus titer for calculation. Calculations were carried out with virus titers (log_{10}[TCID50/ml] given to two decimal places, and only final results, i.e. reduction factors (R), were rounded to the first decimal place.

Example 2

[0076] The UNO S eluate was concentrated to approximately 800 µg rVWF antigen/ml by ultrafiltration using 30 kDa cut-off modified cellulose membranes to facilitate the trace analysis of impurities and product variants.

Testing of rVWF

[0077] **Ristocetin Activity**. The Ristocetin Cofactor Activity is determined by a turbidimetric analyzer using a von Willebrand reagent containing stabilized thrombocytes and the antibiotic "ristocetin". The von Willebrand Factor contained in the sample (= Ristocetin Cofactor) causes agglutination of stabilized thrombocytes in the presence of ristocetin. The agglutination reduces the turbidity of the reagent preparation, and the change in optical density is measured by the turbidimetric analyzer. Calibration is performed by the WHO concentrate reference standard #00/514.

[0078] **VWF Antigen**. VWF- samples are tested for their content of vWF-Antigen in an ELISA assay - double sandwich system with two polyclonal antibodies. Measurement of the color reactions on the microtitre plates is performed with a photometer at 490 nm. The concentration of each sample is calculated towards the standard curve with a computer supported ELISAAnalysis Program (curve algorithm : Cubic regression). All readings are corrected against the blank.

[0079] **FVIII Binding Activity**. FVIII binding of rVWF under static conditions was determined by an ELISA chromogenic assay (ECA) by incubating a constant amount of rFVIII with a diluted VWF-containing sample. The VWF- FVIII- complex formed was then transferred to a microtiter plate coated with a commercially available polyclonal rabbit anti-human VWF antibody. After incubation, unbound FVIII was removed by a subsequent washing step. Bound FVIII was quantified by a commercially available FVIII chromogenic assay (Technochrom FVIII : C reagent kit, Technoclone, Austria). The blank corrected optical densities (in mOD/min at 405 nm) were plotted against the VWF:Ag concentrations in logarithmic scale.

[0080] **SDS-PAGE Analysis.** Conventional 8% SDS-PAGE analysis under reducing conditions and staining of the gels with Coomassie Blue and Silver Stain can provide insight in the protein composition of rVWF. After transfer of the separated protein bands to a nitrocellulose membrane and immunological staining of the protein with appropriate antibodies against VWF, FVIII and Furin respectively, a comparison of VWF related proteins to total proteins can be made.

[0081] **Multimer Analysis.** The multimeric structure of VWF is analyzed by high-density horizontal SDS agarose gel electrophoresis. In brief, samples are diluted to the same concentration in the range of 0.3-1.0 IU/ml VWF:Ag, incubated with Tris-EDTA-SDS buffer and the multimers separated under non-reducing conditions on an agarose gel. VWF multimers were visualized by in-gel immunostaining with a polyclonal rabbit anti human VWF antibody, followed by alkaline phosphatase (ALP) conjugated goat anti-rabbit IgG using the ALP color development kit. Alternatively, agarose gels were blotted onto a blotting membrane and staining was performed by a polyclonal rabbit anti-human VWF antibody followed by horse radish peroxidase conjugated anti-rabbit IgG. For visualization, electro-chemi-luminescence was used which increases the sensitivity of detection for VWF by at least two magnitudes. Low (1 % agarose) and high resolution (2.5 % agarose) conditions were used to analyze the size distribution of VWF multimers and the multimeric structure, respectively.

[0082] **HPLC Analysis.** Recombinant VWF can be cleaved by GluC (V8 protease) under native conditions to give two main fragments (N-terminal and C-terminal homodimer fragment), which are separated on a reverse phase HPLC C4-column. The fragments are detected by by monitoring the UV absorbance at 280 nm.

[0083] **Peptide Mapping.** The primary structure of rVWF was investigated using a peptide mapping approach. Samples of purified rVWF were reduced with dithiothreitol (DTT) and the free, sulfhydril groups were blocked with 4-vinylpyridin. Sequencing grade trypsin was added to the rVWF and allowed to react for 18 hours. The resulting peptide mixture was separated by reverse phase chromatography. Eluting peptides were detected by on-line UV detection at 214 nm and on-line electrospray ionization mass spectrometry.

[0084] **Test for deamidated rVWF.** The analytical method for the detection of iso-aspartate (one reaction product originating from the de-amidation of asparagine) employs tryptic digestion, followed by the Protein Isoaspartyl Methyltransferase (PIMT) enzymatic

reaction using the ISOQUANT IsoAspartate Detection Kit supplied by Promega. PIMT catalyzes the transfer of a methyl group from the substrate S-adenosyl-L methionine (SAM) to IsoAsp at the carboxyl position, generating S-adenosyl homocysteine (SAH). The stoichiometrically released SAH is detected at a wavelength of 260 nm by a RP HPLC method.

[0085] The analytical data for the product obtained by the different processes are summarized in Table 2.

Table 2: Analytical Data for pH 9 and Control Runs

Sample	UNO S #1 (pH 9 run)	UNO S #2 (Control run)	UNO S #3 (Control run pilot scale)
VWF RcoF Activity (U/ml)	36.7	64.8	84.7
VWF Antigen (µg/ml)	715	1090	2010
Specific activity (U/mg)	51.3	59.4	42.1
Collagen binding (U/ml)	63.2	106.7	141.8
Specific collagen binding activity (U/mg)	88.4	97.9	70.5
FVIII binding (%)	51.7	64.6	63.1
CHO protein (µg/ml)	0.09	0.17	n.d.
CHO DNA (pg/ml)	Non detectable	10	n.d.
Furin activity (mU/mi)	< 6.25	< 6.25	<6.25
FVIII Ag (mUlml)	< 125	152	1650
FVIII Activity (mUIml)	71	1452	6222
Deamidation (mol%)	5.1	3.7	5.0
SDS-PAGE	See Figure 1		
Multimer pattern (low resolution)	See Figure 2		
Multimer pattern (high resolution)	See Figure 3		
RP-HPLC	See Figure 4		
Peptide Mapping	See Figure 5		

[0086] As can be seen from Table 2 the biochemical properties of rVWF purified by the different process variants are comparable.

[0087] The major band of the rVWF protein is very similar in all products whereas the extent of impurities is lower in sample #1 (denominated as VWF#07 in figure 1) by both silver staining and western blot analysis for residual rFVIII. The banding pattern for rFVIII

is comparable between all batches which suggests that no degradation due to the pH 9.0 conditions occurred.

[0088] Low and high resolution agarose gel electrophoresis revealed the high similarity of the rVWF preparations. No differences in multimer composition by low resolution multimer analysis could be seen. Figure 2 shows the stained gel of the UNO S runs with MMV and REO virus spiked samples. Also the high resolution multimer analysis revealed the intact multimer pattern suggesting no damage to the rVWF multimers occurred due to the pH 9.0 conditions. Figure 3 shows the stained gel of the UNO S runs with MMV and REO virus spiked samples.

[0089] By the Isoquant assay no enhanced de-amidation could be detected due to the dwell time of rVWF at pH 9.0. Generally the molar percentage of deamidated rVWF is very low. Subjecting the purified preparations of rVWF obtained by the process variants to proteolytic digestion by either V8 protease in the native state (see Figure 4) or trypsin (see Figure 5) in the denatured state and separating the resulting peptides by RP-HPLC resulted in similar chromatograms for all samples.

[0090] Minor differences in the peak patterns are due to the presence of different amounts of impurities (mainly residual rVWF propeptide as can be seen in Figure 1) in the preparations which was confirmed by mass spectrometry or N-terminal sequence analysis.

Example 3

[0091] Purification of rVWF by Cation Exchange chromatography at high pH with MMV spike.

[0092] A UNOsphere S resin packed into a column was activated with 1 CV of 2 M NaCl and equilibrated with 25 CV of an equilibration buffer (pH=9.0). Thereafter, a rVWF containing solution adjusted to a conductivity of 15 mS/cm and a pH of 9.0 and spiked with mouse minute virus (MMV) was loaded onto the column at a linear flow rate of about 10.0 cm/h. The column was then washed with 10 CV of equilibration buffer (pH=9.0) and the product was eluted with 3.5 CV of elution buffer (pH=7.5) at a linear flow rate of 65cm/h. The increased pH during the loading and wash phase significantly reduced the binding of the virus particles to the resin but retained full binding of the product VWF. As a result, most of the loaded virus particles were found in the non-binding (flow through) and wash fraction separated from the product that was recovered in the eluate pool at high yields. The results in

Table 3 show that by applying this procedure a virus removal capacity of 2 logs could be obtained with the non-enveloped model virus mouse minute virus (MMV).

[0093] The TCID50 assay was performed as follows. Briefly, serial 1/2 log dilutions of the samples were prepared in the appropriate tissue culture medium and 100 µl of each dilution were added to each of 8 wells of a microtiter plate seeded with the indicator cell tine. The cells were then incubated for 7 days at 36°C ± 2°C before the cytopathic effect was evaluated by visual inspection of the cells under a microscope. Median tissue culture infectious doses (TCID50) were calculated according to the Poisson distribution and expressed as $\log_{10}$[TCID50/ml].

Table 3: Purification of rVWF on UNOsphere S

	Volume	Virus titer (TCID50)	Virus content (TCID50)	Reduction
	ml	Log_{10}/ml	Log_{10}	Log_{10}
Load	400	5.26	7.86	-
Eluate pool	89.4	3.8	5.75	2.11

[0094] The purification was performed using a column with 15 mm diameter and a bed height of 14 cm. Data shown are virus titers of active mouse minute virus.

Example 4

[0095] Purification of rVWF by Cation Exchange chromatography at high pH with Reo Type 3 virus spike.

[0096] A UNOsphere S resin packed into a column was activated with 1 CV of 2 M NaCl and equilibrated with 25 CV of an equilibration buffer (pH=9.0). Thereafter, a rVWF containing solution adjusted to a conductivity of 15 mS/cm and a pH of 9.0 and spiked with various non-enveloped viruses was loaded onto the column at a linear flow rate of about 100 cm/h. The column was then washed with 10 CV of equilibration buffer (pH=9.0) and the product was eluted with 3.5 CV of elution buffer (pH=7.5) at a linear flow rate of 65 cm/h. The increased pH during the loading and wash phase significantely reduced the binding of the virus particles to the resin but retained full binding of the product VWF. As a result, most of the loaded virus particles were found in the non-binding (flow through) and wash fraction separated from the product that was recovered in the eluate pool at high yields. The results in Table 4 show that by applying this procedure a virus removal capacity of 2 logs could be obtained with the non-enveloped model virus mouse Reo Virus Type 3 (REO-III).

Table 4: Purification of rVWF on UNOsphere S

	Volume	Virus titer (TCID50)	Virus content (TCID50)	Reduction
	ml	Log_{10}/ml	Log_{10}	Log_{10}
Load	897	3.73	6.68	-
Eluate pool	89.3	2.61	4.56	2.12

[0097] The purification was performed using a column with 15 mm diameter and a bed height of 14 cm. Data shown are virus titers of active mouse Reovirus Type III (REO-III).

Example 5

[0098] Purification of rVWF on UNOsphere S according the standard procedure (neutral pH).

[0099] A UNOsphere S resin packed into a column was activated with 1 CV of 2 M NaCl and equilibrated with 25 CV of an equilibration buffer (pH=6.5). Thereafter, a rVWF containing solution adjusted to a conductivity of 15 mS/cm and a pH of 6.5 and spiked with various non-enveloped viruses was loaded onto the column at a linear flow rate of about 100 cm/h. The column was then washed with 10 CV of equilibration buffer (pH=6.5) and the product was eluted with 3.5 CV of elution buffer (pH=7.5) at a linear flow rate of 65 cm/h. The virus titer of the various viruses tested were evaluated in the different chromatographic fractions (load, column flow through, wash, eluate , post eluate) and the reduction factors were calculated. The results in Table 5 show that by applying the standard purification procedure for VWF on UNOsphere S the removal capacity for non-enveloped viruses was insufficient for the different model viruses tested to claim a robust chromatographic step for removal of non-lipid enveloped viruses.

Table 5: VWF purification according to the standard procedure and the corresponding reduction capacities for non-enveloped viruses.

	Reduction	Comment
	Log_{10}	Virus Characteristics
PPV (porcine Parvovirus)	<1	small, DNA virus
hAdV (human Adenovirus)	1.8	large , DNAvirus
EMCV (Enzephalo Myocarditis Virus)	<1	small, RNA
Reo Virus Type III	1.8	large, RNA
MMV (mouse Minute Virus)	<1	small, DNA

[0100] The reduction rate is calculated as the total virus load in the load fraction divided by the total virus load in the eluate fraction expressed in logarithmic values.

[0101] The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

[0102] Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

CLAIMS:

1. A method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin at a pH of at least 1.6 units above the isoelectric point of a protein in the protein-containing solution; and

washing the cation exchange resin with a wash buffer to form an eluate, said wash buffer having a pH that is lower than the pH of the protein-containing solution applied to the cation exchange resin but having a pH higher than the isoelectric point of the protein, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

2. The method of claim 1 wherein the protein-containing solution applied to the cation exchange resin is at least 1.8 pH units above the isoelectric point of the protein in the protein-containing solution.

3. A method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin;

washing the cation exchange resin with a first wash buffer having a pH higher than the pH of the protein-containing solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein; and

washing the cation exchange resin with a second wash buffer to form an eluate, said eluate having a pH that is equal to or lower than the first wash buffer, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

4. The method of claim 3 wherein the pH of the first wash buffer is at least 1 pH unit above the isoelectric point of the protein in the protein-containing solution applied to the cation exchange resin.

5. A method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the solution to a cation exchange resin at a pH higher than the isoelectric point of the protein in the protein-containing solution; and

washing the cation exchange resin with a first wash buffer at a pH higher than the isoelectric point of the protein in the protein-containing solution applied to the cation exchange resin; and

washing the cation exchange resin with a second wash buffer to form an eluate, said eluate having a pH that is equal to or lower than the pH of the first wash buffer, wherein the protein in the protein-containing solution has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

6. The method of claim 5 wherein the protein-containing solution applied to the cation exchange resin is at least 1 pH unit above the isoelectric point of the protein in the protein-containing solution.

7. The method of claim 5 wherein the pH of the first wash buffer is at least 1 pH unit above the pH of the protein-containing solution applied to the cation exchange resin.

8. The method of any one of claims 2, 4, 6 and 7 wherein the pH of the protein-containing solution applied to the cation exchange resin is greater than pH 7.0.

9. The method of any one of claims 1-8, wherein the cation exchange resin has a negatively charged group selected from the group consisting of carboxymethyl (CM), sulphoalkyl (SP, SE), sulfated esters of cellulose, heparin and methylsulfonate (S).

10. The method of any one of claims 1-9, wherein the protein in the protein-containing solution is a blood coagulation protein.

11. The method of claim 10, wherein the blood coagulation protein is selected from the group consisting of Factor VIII and von Willebrand factor.

12. A method for removing a non-lipid enveloped virus from a von Willebrand Factor (VWF)-containing solution comprising:

applying the VWF-containing solution to a cation exchange resin at a pH of at least 1.6 units above the isoelectric point of VWF; and

washing the cation exchange resin with a first wash buffer to form an eluate, said wash buffer having a pH that is equal to or lower than the pH of the VWF-containing solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein, and whereby the non-lipid enveloped virus is removed from the VWF-containing solution.

13. The method of claim 3, wherein the protein is von Willebrand Factor (VWF).

14. The method of claim 5, wherein the protein is von Willebrand Factor (VWF).

15. The method of claim 1, wherein the protein-containing solution applied to the cation exchange resin is at least 2 pH units above the isoelectric point of the protein in the protein-containing solution.

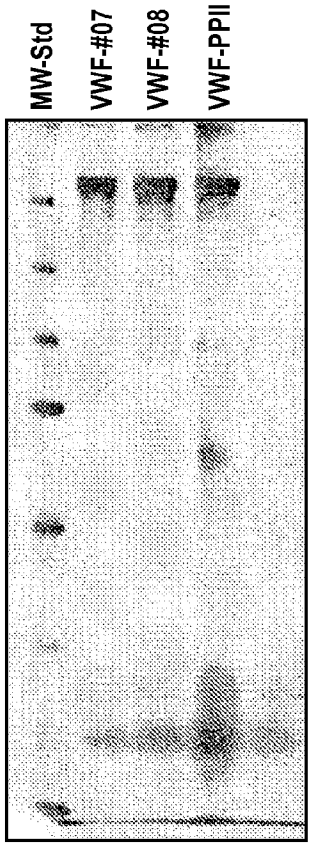
16. A method for removing a non-lipid enveloped virus from a protein-containing solution comprising:

applying the protein-containing solution to a cation exchange resin, wherein the pH of the protein-containing solution is at least 2.0 pH units or more above the isoelectric point of a protein in the protein-containing solution; and

washing the cation exchange resin with a wash buffer to form an eluate, said wash buffer having a pH that is equal to or lower than the pH of the solution applied to the cation exchange resin and having a pH higher than the isoelectric point of the protein, wherein the protein has a molecular mass of at least about 150 kilodaltons, and whereby the non-lipid enveloped virus is removed from the protein-containing solution.

17. The method of claim 16, wherein the pH of the protein containing-containing solution and the pH of the wash buffer is about 9.0.

Date: 30 January 2015



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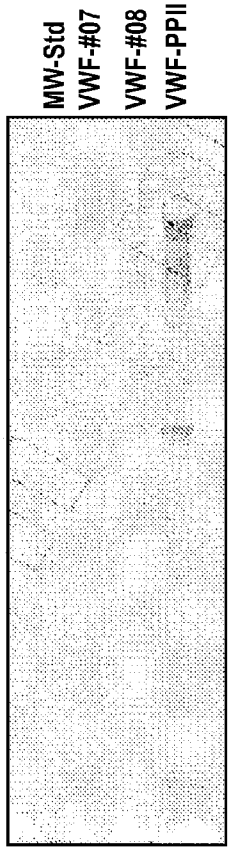

B) Western blot for residual rFVIII

FIG. 1

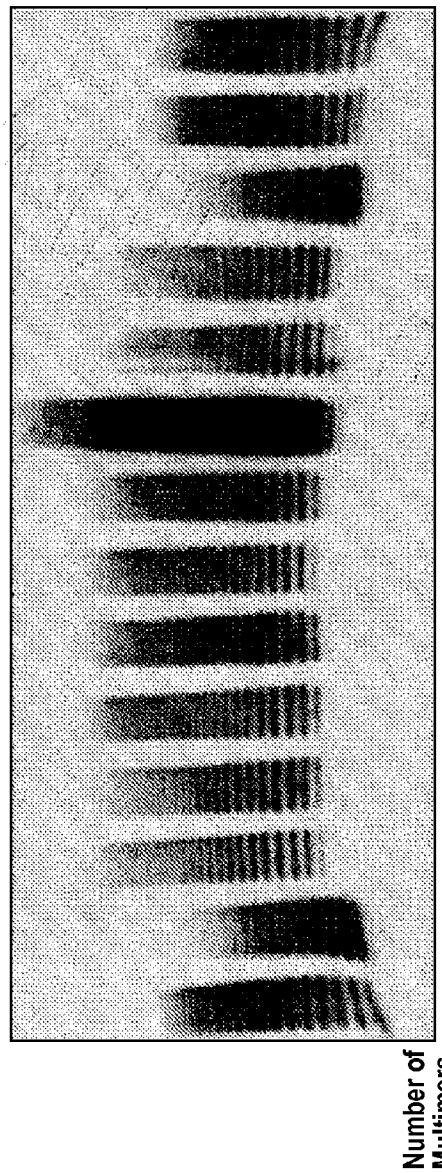
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FIG. 2

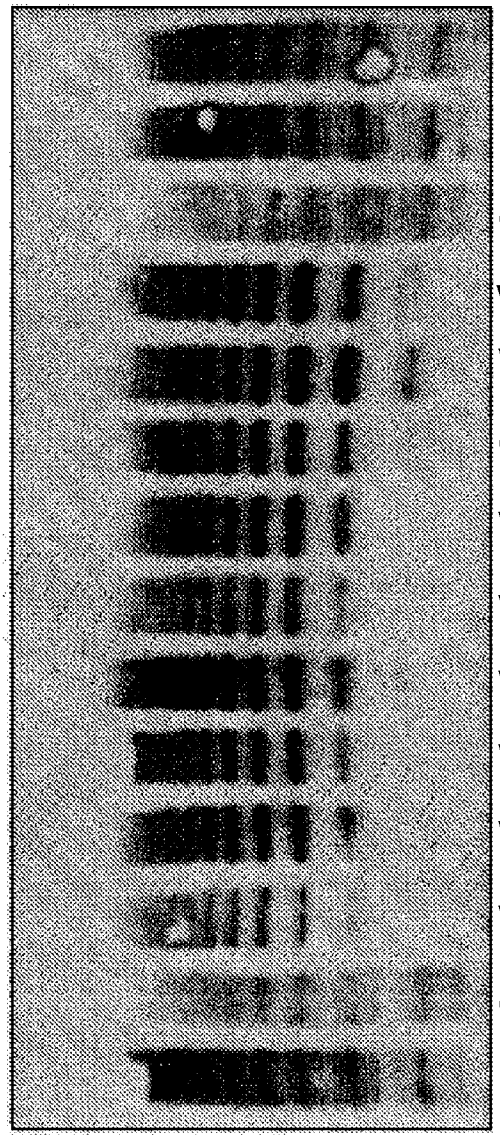
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FIG. 3

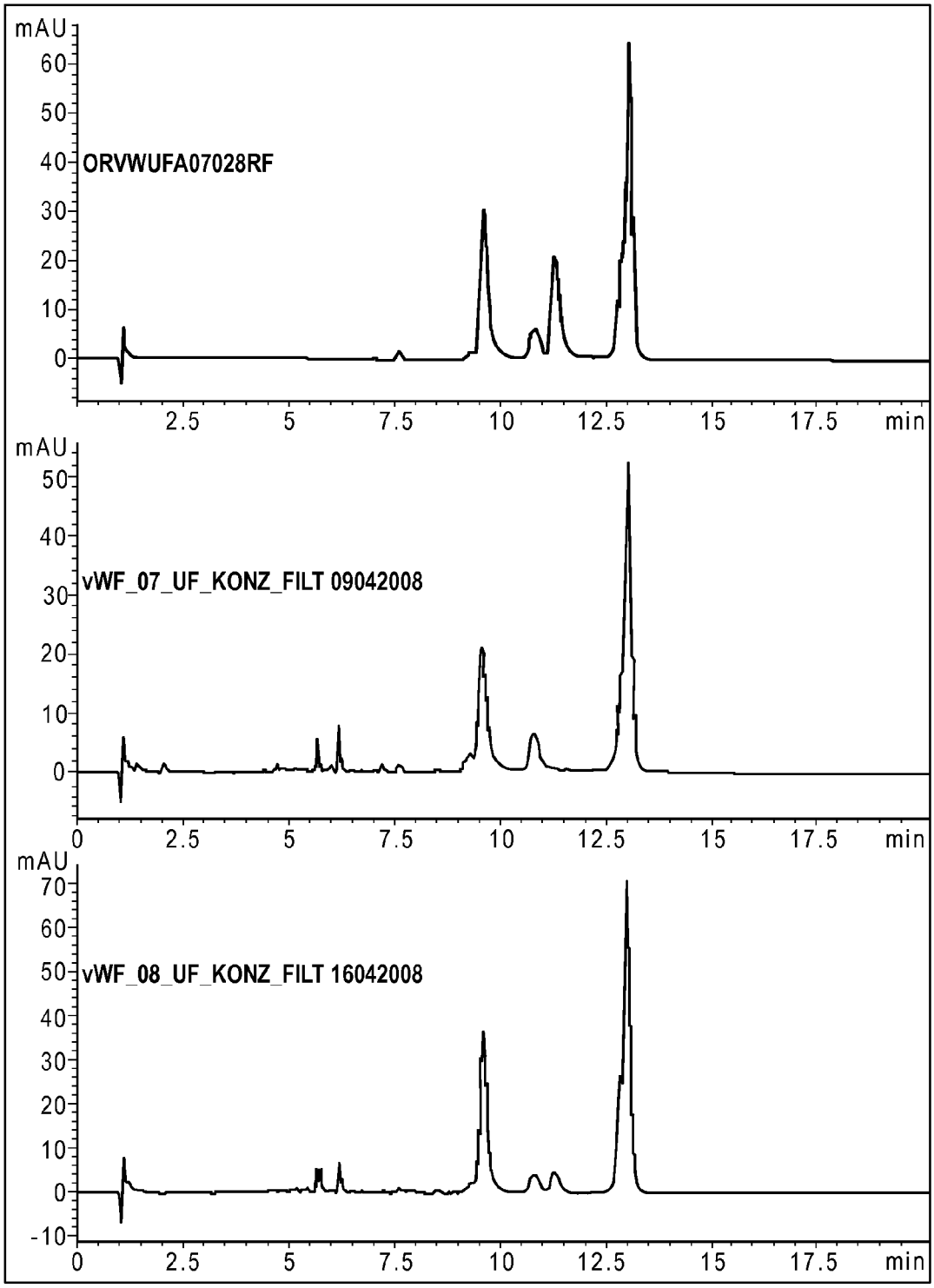
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FIG. 4

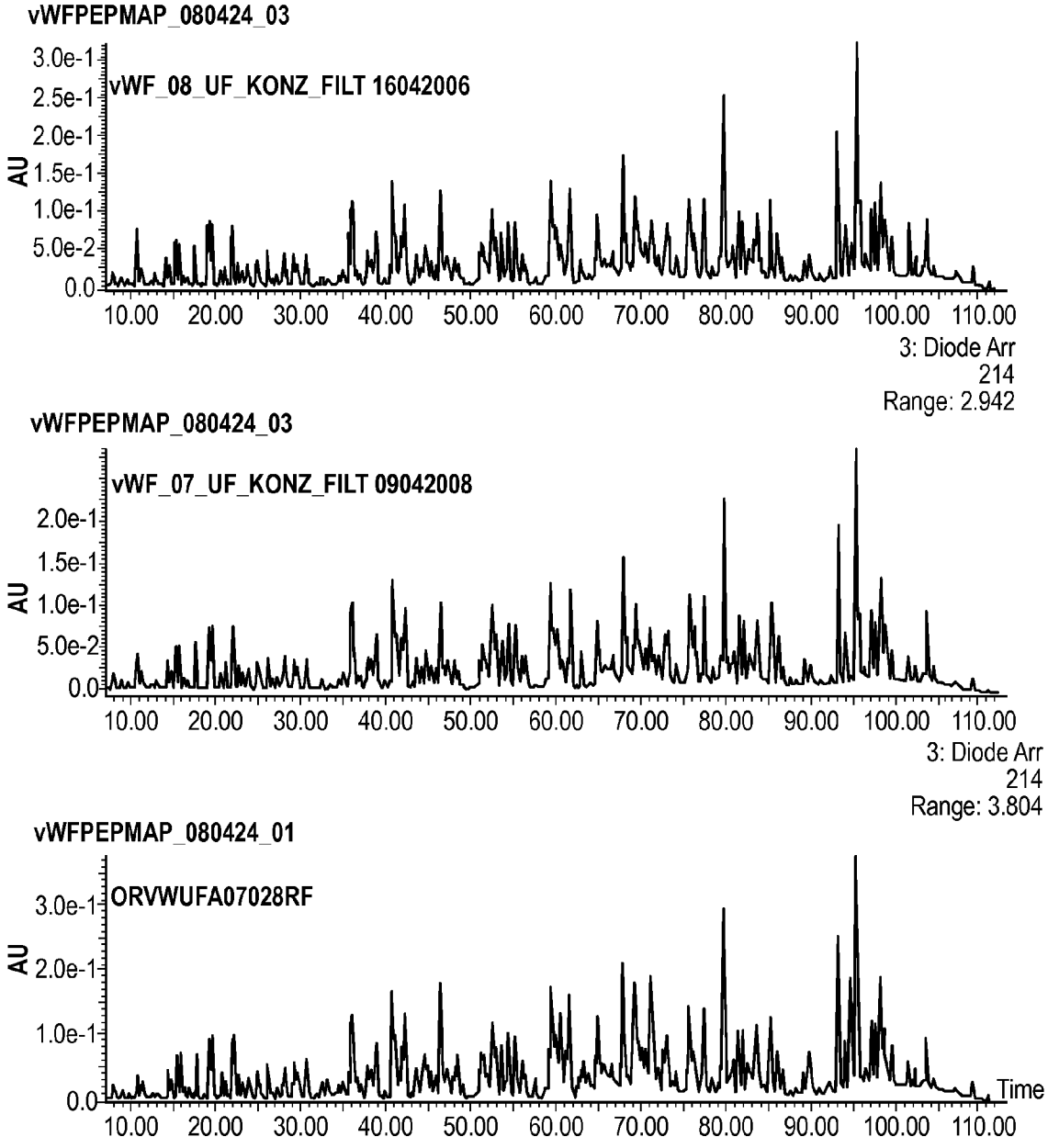
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FIG. 5

SEQUENCE LISTING

<110> Mitterer, et al.

<120> PURIFICATION OF VWF FOR INCREASED REMOVAL OF NON-LIPID ENVELOPED VIRUSES

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<400> 2

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35 40 45

Ser Met Tyr Ser Phe Ala Gly Tyr Cys Ser Tyr Leu Leu Ala Gly Gly
50 55 60

Cys Gln Lys Arg Ser Phe Ser Ile Ile Gly Asp Phe Gln Asn Gly Lys
65 70 75 80

Arg Val Ser Leu Ser Val Tyr Leu Gly Glu Phe Phe Asp Ile His Leu
85 90 95

Phe Val Asn Gly Thr Val Thr Gln Gly Asp Gln Arg Val Ser Met Pro
100 105 110

Tyr Ala Ser Lys Gly Leu Tyr Leu Glu Thr Glu Ala Gly Tyr Tyr Lys
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Leu Ser Gly Glu Ala Tyr Gly Phe Val Ala Arg Ile Asp Gly Ser Gly
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Asn Phe Gln Val Leu Leu Ser Asp Arg Tyr Phe Asn Lys Thr Cys Gly
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Leu Cys Gly Asn Phe Asn Ile Phe Ala Glu Asp Asp Phe Met Thr Gln
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Ser Cys Asn Ile Ser Ser Gly Glu Met Gln Lys Gly Leu Trp Glu Gln
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Cys Gln Leu Leu Lys Ser Thr Ser Val Phe Ala Arg Cys His Pro Leu
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Val Asp Pro Glu Pro Phe Val Ala Leu Cys Glu Lys Thr Leu Cys Glu
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Val Ser Pro Cys Ala Arg Thr Cys Gln Ser Leu His Ile Asn Glu Met
305 310 315 320

Cys Gln Glu Arg Cys Val Asp Gly Cys Ser Cys Pro Glu Gly Gln Leu
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Leu Asp Glu Gly Leu Cys Val Glu Ser Thr Glu Cys Pro Cys Val His
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Ser Gly Lys Arg Tyr Pro Pro Gly Thr Ser Leu Ser Arg Asp Cys Asn
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Pro Gly Glu Cys Leu Val Thr Gly Gln Ser His Phe Lys Ser Phe Asp
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Asp Cys Gln Asp His Ser Phe Ser Ile Val Ile Glu Thr Val Gln Cys
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Pro Gly Leu His Asn Ser Leu Val Lys Leu Lys His Gly Ala Gly Val
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Cys Pro Pro Gly Leu Tyr Met Asp Glu Arg Gly Asp Cys Val Pro Lys
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Ala Gln Cys Pro Cys Tyr Tyr Asp Gly Glu Ile Phe Gln Pro Glu Asp
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Ile Phe Ser Asp His His Thr Met Cys Tyr Cys Glu Asp Gly Phe Met
725 730 735

His Cys Thr Met Ser Gly Val Pro Gly Ser Leu Leu Pro Asp Ala Val
740 745 750

Leu Ser Ser Pro Leu Ser His Arg Ser Lys Arg Ser Leu Ser Cys Arg
755 760 765

Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp Asn Leu Arg Ala Glu
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Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr Asp Leu Glu Cys Met
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Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile Leu Val Gly Asn Lys
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915 920 925

Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly Glu Val Asn Val Lys
930 935 940

Arg Pro Met Lys Asp Glu Thr His Phe Glu Val Val Glu Ser Gly Arg
945 950 955 960

Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser Val Val Trp Asp Arg
965 970 975

His Leu Ser Ile Ser Val Val Leu Lys Gln Thr Tyr Gln Glu Lys Val
980 985 990

Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln Asn Asn Asp Leu Thr
995 1000 1005

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Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg Lys Val Pro
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Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
1070 1075 1080

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala
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Cys Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln
1100 1105 1110

His Gly Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln
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Ser Cys Glu Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu
1130 1135 1140

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Trp Arg Tyr Asn Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln
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His Pro Glu Pro Leu Ala Cys Pro Val Gln Cys Val Glu Gly Cys
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His Ala His Cys Pro Pro Gly Lys Ile Leu Asp Glu Leu Leu Gln
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Arg Arg Phe Ala Ser Gly Lys Lys Val Thr Leu Asn Pro Ser Asp
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Pro Glu His Cys Gln Ile Cys His Cys Asp Val Val Asn Leu Thr
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Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu Val Val Pro Pro Thr
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Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala Glu Phe
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Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr His Asp
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Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser
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Phe Ser Lys Ile Asp Arg Pro Glu Ala Ser Arg Ile Thr Leu Leu
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Leu Met Ala Ser Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val
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Gly Pro Lys Arg Asn Ser Met Val Leu Asp Val Ala Phe Val Leu
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Gln Gly Asp Arg Glu Gln Ala Pro Asn Leu Val Tyr Met Val Thr
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Pro Asp Cys Ser Gln Pro Leu Asp Val Ile Leu Leu Leu Asp Gly
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Ser Ser Ser Phe Pro Ala Ser Tyr Phe Asp Glu Met Lys Ser Phe
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Ala Lys Ala Phe Ile Ser Lys Ala Asn Ile Gly Pro Arg Leu Thr
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Gln Val Ser Val Leu Gln Tyr Gly Ser Ile Thr Thr Ile Asp Val
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Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu Ser Leu Val
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Asp Val Met Gln Arg Glu Gly Gly Pro Ser Gln Ile Gly Asp Ala
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Leu Gly Phe Ala Val Arg Tyr Leu Thr Ser Glu Met His Gly Ala
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Ser Val Asp Ser Val Asp Ala Ala Ala Asp Ala Ala Arg Ser Asn
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Arg Val Thr Val Phe Pro Ile Gly Ile Gly Asp Arg Tyr Asp Ala
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Ala Gln Leu Arg Ile Leu Ala Gly Pro Ala Gly Asp Ser Asn Val
1835                1840                1845

Val Lys Leu Gln Arg Ile Glu Asp Leu Pro Thr Met Val Thr Leu
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Gly Asn Ser Phe Leu His Lys Leu Cys Ser Gly Phe Val Arg Ile
1865                1870                1875

Cys Met Asp Glu Asp Gly Asn Glu Lys Arg Pro Gly Asp Val Trp
1880                1885                1890

Thr Leu Pro Asp Gln Cys His Thr Val Thr Cys Gln Pro Asp Gly
1895                1900                1905
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1925 1930 1935

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1940 1945 1950

Ser Thr Arg His Ile Val Thr Phe Asp Gly Gln Asn Phe Lys Leu
1955 1960 1965

Thr Gly Ser Cys Ser Tyr Val Leu Phe Gln Asn Lys Glu Gln Asp
1970 1975 1980

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1985 1990 1995

Gln Gly Cys Met Lys Ser Ile Glu Val Lys His Ser Ala Leu Ser
2000 2005 2010

Val Glu Leu His Ser Asp Met Glu Val Thr Val Asn Gly Arg Leu
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Val Ser Val Pro Tyr Val Gly Gly Asn Met Glu Val Asn Val Tyr
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Pro Lys Thr Phe Ala Ser Lys Thr Tyr Gly Leu Cys Gly Ile Cys
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Glu Cys His Lys Val Leu Ala Pro Ala Thr Phe Tyr Ala Ile Cys
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Tyr Asn His Cys Glu His Gly Cys Pro Arg His Cys Asp Gly Asn
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    2705                2710                2715

Cys Glu Glu Pro Glu Cys Asn Asp Ile Thr Ala Arg Leu Gln Tyr
    2720                2725                2730

Val Lys Val Gly Ser Cys Lys Ser Glu Val Glu Val Asp Ile His
    2735                2740                2745

Tyr Cys Gln Gly Lys Cys Ala Ser Lys Ala Met Tyr Ser Ile Asp
    2750                2755                2760

Ile Asn Asp Val Gln Asp Gln Cys Ser Cys Cys Ser Pro Thr Arg
    2765                2770                2775

Thr Glu Pro Met Gln Val Ala Leu His Cys Thr Asn Gly Ser Val
    2780                2785                2790

Val Tyr His Glu Val Leu Asn Ala Met Glu Cys Lys Cys Ser Pro
    2795                2800                2805

Arg Lys Cys Ser Lys
    2810
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<211> 2050
<212> PRT
<213> Homo sapiens

<400> 3

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Ser Leu Ser Cys Arg Pro Pro Met Val Lys Leu Val Cys Pro Ala Asp
1               5                  10                  15

Asn Leu Arg Ala Glu Gly Leu Glu Cys Thr Lys Thr Cys Gln Asn Tyr
            20                  25                  30

Asp Leu Glu Cys Met Ser Met Gly Cys Val Ser Gly Cys Leu Cys Pro
        35                  40                  45

Pro Gly Met Val Arg His Glu Asn Arg Cys Val Ala Leu Glu Arg Cys
    50                  55                  60

Pro Cys Phe His Gln Gly Lys Glu Tyr Ala Pro Gly Glu Thr Val Lys
65                  70                  75                  80
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Ile Gly Cys Asn Thr Cys Val Cys Arg Asp Arg Lys Trp Asn Cys Thr
85 90 95

Asp His Val Cys Asp Ala Thr Cys Ser Thr Ile Gly Met Ala His Tyr
100 105 110

Leu Thr Phe Asp Gly Leu Lys Tyr Leu Phe Pro Gly Glu Cys Gln Tyr
115 120 125

Val Leu Val Gln Asp Tyr Cys Gly Ser Asn Pro Gly Thr Phe Arg Ile
130 135 140

Leu Val Gly Asn Lys Gly Cys Ser His Pro Ser Val Lys Cys Lys Lys
145 150 155 160

Arg Val Thr Ile Leu Val Glu Gly Gly Glu Ile Glu Leu Phe Asp Gly
165 170 175

Glu Val Asn Val Lys Arg Pro Met Lys Asp Glu Thr His Phe Glu Val
180 185 190

Val Glu Ser Gly Arg Tyr Ile Ile Leu Leu Leu Gly Lys Ala Leu Ser
195 200 205

Val Val Trp Asp Arg His Leu Ser Ile Ser Val Val Leu Lys Gln Thr
210 215 220

Tyr Gln Glu Lys Val Cys Gly Leu Cys Gly Asn Phe Asp Gly Ile Gln
225 230 235 240

Asn Asn Asp Leu Thr Ser Ser Asn Leu Gln Val Glu Glu Asp Pro Val
245 250 255

Asp Phe Gly Asn Ser Trp Lys Val Ser Ser Gln Cys Ala Asp Thr Arg
260 265 270

Lys Val Pro Leu Asp Ser Ser Pro Ala Thr Cys His Asn Asn Ile Met
275 280 285

Lys Gln Thr Met Val Asp Ser Ser Cys Arg Ile Leu Thr Ser Asp Val
290 295 300

Phe Gln Asp Cys Asn Lys Leu Val Asp Pro Glu Pro Tyr Leu Asp Val
305 310 315 320

Cys Ile Tyr Asp Thr Cys Ser Cys Glu Ser Ile Gly Asp Cys Ala Cys
325 330 335

Phe Cys Asp Thr Ile Ala Ala Tyr Ala His Val Cys Ala Gln His Gly
340 345 350

Lys Val Val Thr Trp Arg Thr Ala Thr Leu Cys Pro Gln Ser Cys Glu
355 360 365

Glu Arg Asn Leu Arg Glu Asn Gly Tyr Glu Cys Glu Trp Arg Tyr Asn
370 375 380

Ser Cys Ala Pro Ala Cys Gln Val Thr Cys Gln His Pro Glu Pro Leu
385 390 395 400

Ala Cys Pro Val Gln Cys Val Glu Gly Cys His Ala His Cys Pro Pro
405 410 415

Gly Lys Ile Leu Asp Glu Leu Leu Gln Thr Cys Val Asp Pro Glu Asp
420 425 430

Cys Pro Val Cys Glu Val Ala Gly Arg Arg Phe Ala Ser Gly Lys Lys
435 440 445

Val Thr Leu Asn Pro Ser Asp Pro Glu His Cys Gln Ile Cys His Cys
450 455 460

Asp Val Val Asn Leu Thr Cys Glu Ala Cys Gln Glu Pro Gly Gly Leu
465 470 475 480

Val Val Pro Pro Thr Asp Ala Pro Val Ser Pro Thr Thr Leu Tyr Val
485 490 495

Glu Asp Ile Ser Glu Pro Pro Leu His Asp Phe Tyr Cys Ser Arg Leu
500 505 510

Leu Asp Leu Val Phe Leu Leu Asp Gly Ser Ser Arg Leu Ser Glu Ala
515 520 525

Glu Phe Glu Val Leu Lys Ala Phe Val Val Asp Met Met Glu Arg Leu
530 535 540

Arg Ile Ser Gln Lys Trp Val Arg Val Ala Val Val Glu Tyr His Asp
545 550 555 560

Gly Ser His Ala Tyr Ile Gly Leu Lys Asp Arg Lys Arg Pro Ser Glu
565 570 575

Leu Arg Arg Ile Ala Ser Gln Val Lys Tyr Ala Gly Ser Gln Val Ala
580 585 590

Ser Thr Ser Glu Val Leu Lys Tyr Thr Leu Phe Gln Ile Phe Ser Lys
595 600 605

Ile Asp Arg Pro Glu Ala Ser Arg Ile Thr Leu Leu Leu Met Ala Ser
610 615 620

Gln Glu Pro Gln Arg Met Ser Arg Asn Phe Val Arg Tyr Val Gln Gly
625 630 635 640

Leu Lys Lys Lys Lys Val Ile Val Ile Pro Val Gly Ile Gly Pro His
645 650 655

Ala Asn Leu Lys Gln Ile Arg Leu Ile Glu Lys Gln Ala Pro Glu Asn
660 665 670

Lys Ala Phe Val Leu Ser Ser Val Asp Glu Leu Glu Gln Gln Arg Asp
675 680 685

Glu Ile Val Ser Tyr Leu Cys Asp Leu Ala Pro Glu Ala Pro Pro Pro
690 695 700

Thr Leu Pro Pro Asp Met Ala Gln Val Thr Val Gly Pro Gly Leu Leu
705 710 715 720

Gly Val Ser Thr Leu Gly Pro Lys Arg Asn Ser Met Val Leu Asp Val
725 730 735

Ala Phe Val Leu Glu Gly Ser Asp Lys Ile Gly Glu Ala Asp Phe Asn
740 745 750

Arg Ser Lys Glu Phe Met Glu Glu Val Ile Gln Arg Met Asp Val Gly
755 760 765

Gln Asp Ser Ile His Val Thr Val Leu Gln Tyr Ser Tyr Met Val Thr
770 775 780

Val Glu Tyr Pro Phe Ser Glu Ala Gln Ser Lys Gly Asp Ile Leu Gln
785 790 795 800

Arg Val Arg Glu Ile Arg Tyr Gln Gly Gly Asn Arg Thr Asn Thr Gly
805 810 815

Leu Ala Leu Arg Tyr Leu Ser Asp His Ser Phe Leu Val Ser Gln Gly
820 825 830

Asp Arg Glu Gln Ala Pro Asn Leu Val Tyr Met Val Thr Gly Asn Pro
835 840 845

Ala Ser Asp Glu Ile Lys Arg Leu Pro Gly Asp Ile Gln Val Val Pro
850 855 860

Ile Gly Val Gly Pro Asn Ala Asn Val Gln Glu Leu Glu Arg Ile Gly
865 870 875 880

Trp Pro Asn Ala Pro Ile Leu Ile Gln Asp Phe Glu Thr Leu Pro Arg
885 890 895

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Glu Ala Pro Asp Leu Val Leu Gln Arg Cys Cys Ser Gly Glu Gly Leu
            900                 905                 910

Gln Ile Pro Thr Leu Ser Pro Ala Pro Asp Cys Ser Gln Pro Leu Asp
        915                 920                 925

Val Ile Leu Leu Leu Asp Gly Ser Ser Ser Phe Pro Ala Ser Tyr Phe
    930                 935                 940

Asp Glu Met Lys Ser Phe Ala Lys Ala Phe Ile Ser Lys Ala Asn Ile
945                 950                 955                 960

Gly Pro Arg Leu Thr Gln Val Ser Val Leu Gln Tyr Gly Ser Ile Thr
                965                 970                 975

Thr Ile Asp Val Pro Trp Asn Val Val Pro Glu Lys Ala His Leu Leu
            980                 985                 990

Ser Leu Val Asp Val Met Gln Arg Glu Gly Gly Pro Ser Gln Ile Gly
        995                 1000                1005

Asp Ala Leu Gly Phe Ala Val Arg Tyr Leu Thr Ser Glu Met His
    1010                1015                1020

Gly Ala Arg Pro Gly Ala Ser Lys Ala Val Val Ile Leu Val Thr
    1025                1030                1035

Asp Val Ser Val Asp Ser Val Asp Ala Ala Ala Asp Ala Ala Arg
    1040                1045                1050

Ser Asn Arg Val Thr Val Phe Pro Ile Gly Ile Gly Asp Arg Tyr
    1055                1060                1065

Asp Ala Ala Gln Leu Arg Ile Leu Ala Gly Pro Ala Gly Asp Ser
    1070                1075                1080

Asn Val Val Lys Leu Gln Arg Ile Glu Asp Leu Pro Thr Met Val
    1085                1090                1095

Thr Leu Gly Asn Ser Phe Leu His Lys Leu Cys Ser Gly Phe Val
    1100                1105                1110

Arg Ile Cys Met Asp Glu Asp Gly Asn Glu Lys Arg Pro Gly Asp
    1115                1120                1125

Val Trp Thr Leu Pro Asp Gln Cys His Thr Val Thr Cys Gln Pro
    1130                1135                1140

Asp Gly Gln Thr Leu Leu Lys Ser His Arg Val Asn Cys Asp Arg
    1145                1150                1155
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Gly Leu Arg Pro Ser Cys Pro Asn Ser Gln Ser Pro Val Lys Val
1160                1165                1170

Glu Glu Thr Cys Gly Cys Arg Trp Thr Cys Pro Cys Val Cys Thr
1175                1180                1185

Gly Ser Ser Thr Arg His Ile Val Thr Phe Asp Gly Gln Asn Phe
1190                1195                1200

Lys Leu Thr Gly Ser Cys Ser Tyr Val Leu Phe Gln Asn Lys Glu
1205                1210                1215

Gln Asp Leu Glu Val Ile Leu His Asn Gly Ala Cys Ser Pro Gly
1220                1225                1230

Ala Arg Gln Gly Cys Met Lys Ser Ile Glu Val Lys His Ser Ala
1235                1240                1245

Leu Ser Val Glu Leu His Ser Asp Met Glu Val Thr Val Asn Gly
1250                1255                1260

Arg Leu Val Ser Val Pro Tyr Val Gly Gly Asn Met Glu Val Asn
1265                1270                1275

Val Tyr Gly Ala Ile Met His Glu Val Arg Phe Asn His Leu Gly
1280                1285                1290

His Ile Phe Thr Phe Thr Pro Gln Asn Asn Glu Phe Gln Leu Gln
1295                1300                1305

Leu Ser Pro Lys Thr Phe Ala Ser Lys Thr Tyr Gly Leu Cys Gly
1310                1315                1320

Ile Cys Asp Glu Asn Gly Ala Asn Asp Phe Met Leu Arg Asp Gly
1325                1330                1335

Thr Val Thr Thr Asp Trp Lys Thr Leu Val Gln Glu Trp Thr Val
1340                1345                1350

Gln Arg Pro Gly Gln Thr Cys Gln Pro Ile Leu Glu Glu Gln Cys
1355                1360                1365

Leu Val Pro Asp Ser Ser His Cys Gln Val Leu Leu Leu Pro Leu
1370                1375                1380

Phe Ala Glu Cys His Lys Val Leu Ala Pro Ala Thr Phe Tyr Ala
1385                1390                1395

Ile Cys Gln Gln Asp Ser Cys His Gln Glu Gln Val Cys Glu Val
1400                1405                1410
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Ile Ala Ser Tyr Ala His Leu Cys Arg Thr Asn Gly Val Cys Val
1415 1420 1425

Asp Trp Arg Thr Pro Asp Phe Cys Ala Met Ser Cys Pro Pro Ser
1430 1435 1440

Leu Val Tyr Asn His Cys Glu His Gly Cys Pro Arg His Cys Asp
1445 1450 1455

Gly Asn Val Ser Ser Cys Gly Asp His Pro Ser Glu Gly Cys Phe
1460 1465 1470

Cys Pro Pro Asp Lys Val Met Leu Glu Gly Ser Cys Val Pro Glu
1475 1480 1485

Glu Ala Cys Thr Gln Cys Ile Gly Glu Asp Gly Val Gln His Gln
1490 1495 1500

Phe Leu Glu Ala Trp Val Pro Asp His Gln Pro Cys Gln Ile Cys
1505 1510 1515

Thr Cys Leu Ser Gly Arg Lys Val Asn Cys Thr Thr Gln Pro Cys
1520 1525 1530

Pro Thr Ala Lys Ala Pro Thr Cys Gly Leu Cys Glu Val Ala Arg
1535 1540 1545

Leu Arg Gln Asn Ala Asp Gln Cys Cys Pro Glu Tyr Glu Cys Val
1550 1555 1560

Cys Asp Pro Val Ser Cys Asp Leu Pro Pro Val Pro His Cys Glu
1565 1570 1575

Arg Gly Leu Gln Pro Thr Leu Thr Asn Pro Gly Glu Cys Arg Pro
1580 1585 1590

Asn Phe Thr Cys Ala Cys Arg Lys Glu Glu Cys Lys Arg Val Ser
1595 1600 1605

Pro Pro Ser Cys Pro Pro His Arg Leu Pro Thr Leu Arg Lys Thr
1610 1615 1620

Gln Cys Cys Asp Glu Tyr Glu Cys Ala Cys Asn Cys Val Asn Ser
1625 1630 1635

Thr Val Ser Cys Pro Leu Gly Tyr Leu Ala Ser Thr Ala Thr Asn
1640 1645 1650

Asp Cys Gly Cys Thr Thr Thr Thr Cys Leu Pro Asp Lys Val Cys
1655 1660 1665

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Val His Arg Ser Thr Ile Tyr Pro Val Gly Gln Phe Trp Glu Glu
1670                1675                1680

Gly Cys Asp Val Cys Thr Cys Thr Asp Met Glu Asp Ala Val Met
1685                1690                1695

Gly Leu Arg Val Ala Gln Cys Ser Gln Lys Pro Cys Glu Asp Ser
1700                1705                1710

Cys Arg Ser Gly Phe Thr Tyr Val Leu His Glu Gly Glu Cys Cys
1715                1720                1725

Gly Arg Cys Leu Pro Ser Ala Cys Glu Val Val Thr Gly Ser Pro
1730                1735                1740

Arg Gly Asp Ser Gln Ser Ser Trp Lys Ser Val Gly Ser Gln Trp
1745                1750                1755

Ala Ser Pro Glu Asn Pro Cys Leu Ile Asn Glu Cys Val Arg Val
1760                1765                1770

Lys Glu Glu Val Phe Ile Gln Gln Arg Asn Val Ser Cys Pro Gln
1775                1780                1785

Leu Glu Val Pro Val Cys Pro Ser Gly Phe Gln Leu Ser Cys Lys
1790                1795                1800

Thr Ser Ala Cys Cys Pro Ser Cys Arg Cys Glu Arg Met Glu Ala
1805                1810                1815

Cys Met Leu Asn Gly Thr Val Ile Gly Pro Gly Lys Thr Val Met
1820                1825                1830

Ile Asp Val Cys Thr Thr Cys Arg Cys Met Val Gln Val Gly Val
1835                1840                1845

Ile Ser Gly Phe Lys Leu Glu Cys Arg Lys Thr Thr Cys Asn Pro
1850                1855                1860

Cys Pro Leu Gly Tyr Lys Glu Glu Asn Asn Thr Gly Glu Cys Cys
1865                1870                1875

Gly Arg Cys Leu Pro Thr Ala Cys Thr Ile Gln Leu Arg Gly Gly
1880                1885                1890

Gln Ile Met Thr Leu Lys Arg Asp Glu Thr Leu Gln Asp Gly Cys
1895                1900                1905

Asp Thr His Phe Cys Lys Val Asn Glu Arg Gly Glu Tyr Phe Trp
1910                1915                1920
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Glu Lys Arg Val Thr Gly Cys Pro Pro Phe Asp Glu His Lys Cys
    1925                1930                1935

Leu Ala Glu Gly Gly Lys Ile Met Lys Ile Pro Gly Thr Cys Cys
    1940                1945                1950

Asp Thr Cys Glu Glu Pro Glu Cys Asn Asp Ile Thr Ala Arg Leu
    1955                1960                1965

Gln Tyr Val Lys Val Gly Ser Cys Lys Ser Glu Val Glu Val Asp
    1970                1975                1980

Ile His Tyr Cys Gln Gly Lys Cys Ala Ser Lys Ala Met Tyr Ser
    1985                1990                1995

Ile Asp Ile Asn Asp Val Gln Asp Gln Cys Ser Cys Cys Ser Pro
    2000                2005                2010

Thr Arg Thr Glu Pro Met Gln Val Ala Leu His Cys Thr Asn Gly
    2015                2020                2025

Ser Val Val Tyr His Glu Val Leu Asn Ala Met Glu Cys Lys Cys
    2030                2035                2040

Ser Pro Arg Lys Cys Ser Lys
    2045                2050
```