PURIFICATION METHOD FOR DIVALENT CATION BINDING PROTEINS ON ANION EXCHANGE RESIN

FIELD OF THE INVENTION

[001] The present invention relates to a method for the purification of divalent cation binding proteins with high purity on an anion exchange resin material, to divalent cation binding proteins obtainable by said method, and to a kit comprising means for carrying out said method.

BACKGROUND OF THE INVENTION

[002] So far, many mammalian proteins are produced in host cells by e.g. transfecting cells with DNA encoding said proteins and growing the recombinant cells under conditions favorable for the expression of said proteins. The proteins secreted by the cells into the cell culture medium, or residing inside the cells, can be separated from the culture medium and other components using chromatographic techniques, e.g. ion exchange chromatography, affinity chromatography, and the like. For further pharmaceutical applications, purity is of particular importance. However, at the same time the biological activity of the protein must be preserved after thorough purification of the proteins of interest.

[003] The concept of eluting calcium binding proteins from anion exchange resins by divalent cations was firstly reported almost thirty years ago. Although bovine Factor VII was successfully isolated from bovine plasma, the purification of human Factor VII was still problematic, *i.e.* the material produced was only partially pure or was obtained in such small quantities that it was characterized as activity without detectable protein. Workers in the field succeeded in the isolation of human Factor VII from human plasma in sufficient quantities (with a yield of approx. 30%) by means of adsorbing proteins to a divalent cation, *i.e.* barium citrate, and then separating the protein by anion exchange chromatography. Further, methods were available for recovering and purifying vitamin K-dependent proteins from the medium of a cell culture producing vitamin K-dependent proteins with different specific activities by means of conventional ion-exchange resins, e.g. anion exchange resins, and using an eluant containing divalent cations, e.g. calcium ion (Ca^{2+}), barium ion (Ba^{2+}), and strontium ion (Sr^{2+}).

[004] Furthermore, methods were available for the purification of Factor IX (FIX) in a solution, comprising the steps of applying the solution containing FIX to an anion exchange resin, washing the anion exchange resin with a solution having a conductivity that is less than required to elute FIX from the resin, and eluting FIX from the anion exchange resin with a first eluant including divalent cations to form a first eluate. The first eluate is then applied to a heparin or heparin-like resin to form a second eluate, and the second eluate is applied to hydroxyapatite to form a third eluate, utilizing a high conductivity washing agent in the washing step.

[005] Factor IX (FIX) is a vitamin K-dependent serine protease of the coagulation system, belonging to the peptidase family S1. FIX is inactive unless activated by Factor XIa or Factor VIIa. For its activation, calcium, membrane phospholipids, and Factor VIII are required. Deficiency of FIX causes the

hereditary recessive bleeding disorder hemophilia B, which can be successfully treated by administration of posttranslational modified, *i.e.* phosphorylated and sulfated FIX.

[006] Further, Factor VII (FVII) is a vitamin K-dependent serine protease which plays a significant role in the coagulation cascade, where it initiates the process of coagulation with tissue factor (TF). Upon vessel injury, TF is exposed to the blood and circulating FVII. Once bound to TF, FVII is activated to FVIIa by thrombin, Factor Xa, IXa, XIIa, and the FVIIa-TF complex whose substrates are FX and FIX. Furthermore, Annexin V is a cellular protein in the annexin group, having the ability to bind in a calcium-dependent manner to phosphatidylserine and to form a membrane-bound two dimensional crystal lattice. It may play a role in blood coagulation, apoptosis, phagocytosis and formation of plasma membrane-derived microparticles.

[007] Thus, the problem underlying the present invention is to provide an improved method for the purification of divalent cation binding proteins with a high purity. The solution to the above technical problem is achieved by the embodiments characterized in the claims.

SUMMARY OF THE INVENTION

[008] The present invention relates to a method for the purification of a divalent cation binding protein comprising the steps of:

(a) loading an anion exchange resin material with the divalent cation binding protein in a loading buffer in the absence of divalent cations, and optionally washing the loaded anion exchange resin material with a washing buffer in the absence of divalent cations; and

(b) eluting the divalent cation binding protein with an eluant comprising at least one divalent cation to form an eluate containing the divalent cation binding protein;

wherein the eluant in step (b) has a pH higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a).

[009] Further, the present invention relates to purified divalent cation binding proteins obtainable by the above method, and to a kit comprising means for carrying out the above method.

DETAILED DESCRIPTION OF THE INVENTION

[010] In one aspect, the present invention relates to a method for the purification of a divalent cation binding protein comprising the steps of:

(a) loading an anion exchange resin material with the divalent cation binding protein in a loading buffer in the absence of divalent cations, and optionally washing the loaded anion exchange resin material with a washing buffer in the absence of divalent cations; and

(b) eluting the divalent cation binding protein with an eluant comprising at least one divalent cation to form an eluate containing the divalent cation binding protein;

wherein the eluant in step (b) has a pH higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a).

[011] The term "in the absence of divalent cations" as used herein refers to the absence of free divalent cations in the buffer, as well as of any protein-bound divalent cations, wherein divalent cations that are complexed with or by a chelator, e.g. EDTA, may be present.

[012] Loading of the anion exchange resin material with the divalent cation binding protein in a loading buffer in the absence of divalent cations can be carried out by any method known in the art. In particular, conditions suitable for loading the divalent cation binding protein to the anion exchange resin material are well known to a person skilled in the art. The specific conditions for the conductivity of the loading buffer that allows binding of the product depend on the particular properties of the protein and the anion exchange resin material used (e.g. ligand density, ligand presentation, etc.). Divalent cations bind to proteins in regions that are usually highly acidic (*i.e.* negatively charged). The negative charges are masked when the divalent cation is bound. However, by loading the anion exchange material with the divalent cation binding protein in the absence of divalent cations, e.g. by stripping off the bound divalent cation by a chelator, e.g. EDTA, the protein carries highly negatively charged patches on the surface that allow strong binding to an anion exchange ligand. The conditions for loading a protein onto an anion exchange resin material further always require a balance between pH and the concentration of the counterions, e.g. Cl^-. The chemistry of the counterion also influences the elution behavior, e.g. Cl^- carries one negative charge, and phosphate at neutral pH carries two negative charges. The latter can have a higher eluting power compared to Cl^-, even when the conductivity is lower.

[013] The salt concentrations of the solutions and buffers used in the present invention are typically in the range between 20 and 200 mM, preferably between 100 and 150 mM.

[014] Further, suitable loading buffers for loading a divalent cation binding protein to an anion exchange material in step (a) of the method of the present invention, providing conditions under which the divalent cation binding protein is bound to the anion exchange material are well known in the art. For example, the loading buffer can have a pH $\leq$ pH 7.4, preferably $\leq$ pH 7.0, more preferably $\leq$ pH 6.5, and most preferably $\leq$ pH 6.0. It may contain any salt concentrations suitable for binding the divalent cation binding protein to the anion exchange resin material which may be easily determined by a person skilled in the art. In a preferred embodiment, the loading buffer may contain a chelating agent, e.g. EDTA, preferably 2 mM EDTA. A loading buffer containing the divalent cation binding protein which may be applied to the anion exchange resin material in the method of the present invention may contain for example 20 mM Tris, 150 mM NaCl, and 2 mM EDTA.

[015] The method of the present invention optionally comprises the step of washing the loaded anion exchange resin material with a washing buffer in the absence of divalent cations. This washing step can be carried out by any method known in the art. Suitable washing buffers for washing impurities off the anion exchange material essentially without eluting the divalent cation binding protein are well known in the art. For example, the washing buffer can have a pH $\leq$ pH 7.4, preferably $\leq$ pH 7.0, more preferably $\leq$

pH 6.5, and most preferably ≤ pH 6.0. It may contain any salt concentrations suitable for washing the anion exchange resin material without eluting the divalent cation binding protein in a significant amount which may be easily determined by a person skilled in the art. For example, the washing buffer may contain a suitable buffer agent like for example Tris or MES, preferably 20 mM Tris or 20 mM MES. Additionally, it may contain a chelating agent like for example EDTA, preferably 2 mM EDTA. Further, it may contain a suitable salt for regulating the conductivity of the washing buffer, like for example NaCl, which may be present in a concentration of ≤ 200 mM, preferably from 100 mM to 200 mM, more preferably from 150 mM to 200 mM, more preferably from 170 mM to 190 mM, and most preferably from 175 mM to 185 mM. In another preferred embodiment of the present application, the washing buffer contains 100 to 200 mM NaCl. The absolute value for the salt concentration depends on the divalent cation binding protein to be purified, wherein it is within the knowledge of the person skilled in the art to determine which divalent cation binding proteins require lower or higher salt concentrations to get the optimal purity.

[016] Additionally, the method of the present invention may further comprise at least one additional washing step after step (a) and before step (b), wherein the conductivity of the additional washing buffer is equal or lower than the conductivity of the eluant. This additional washing step is carried out in the absence of divalent cations. In one embodiment, the additional washing buffer has a pH of 7.4. The additional washing buffer may contain any suitable salt concentration known to a person skilled in the art. Further, the additional washing buffer may contain a buffering agent like for example Tris, preferably 20 mM Tris, HEPES, Tris/acetate, Histidine, Gly-Gly, MOPS, or Tricine at typical concentrations of 5 to 50 mM and may contain a counterion, like e.g. Cl^-, at a concentration that provides a lower conductivity than the elution buffer. In a preferred embodiment of the present invention, the additional washing buffer has a lower salt concentration than the eluant. In another preferred embodiment, the additional washing buffer contains a chelating agent like for example EDTA, preferably 2 mM EDTA.

[017] Eluting the divalent cation binding protein with an eluant comprising at least one divalent cation to form an eluate containing the divalent cation binding protein can be carried out by any method known in the art. However, according to the present invention, the eluant in step (b) has a pH higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a). Preferably, the eluant has a pH ≥ 7.4, more preferably ≥ 8.0, and the washing buffer in step (a), or, in case no washing step is carried out, the loading buffer in step (a) has a pH of ≤ 7.4, more preferably ≤ 7.0, more preferably ≤ 6.5, and most preferably ≤ 6.0, provided the pH of the eluant in step (b) is higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a). In a preferred embodiment, the pH of the eluant in step (b) is at least 0.5 pH units, preferably 1.0 pH units, more preferably at least 1.5 pH units, most preferably at least 2.0 pH units higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of

the loading buffer in step (a).

[018] As used herein, the eluant may contain any salt concentrations suitable for eluting the divalent cation binding protein from the anion exchange resin material without eluting impurities in a significant amount which may be easily determined by a person skilled in the art. For example, it may contain a suitable buffer agent like for example Tris, preferably 20 mM Tris, HEPES, Tris/acetate, histidine, Gly-Gly, MOPS, or tricine, at concentrations ranging typically from 5 to 50 mM. It may also contain a suitable salt for regulating the conductivity of the washing buffer, like for example NaCl, which may be present in a concentration of $\geq$ 150 mM, more preferably $\geq$ 180 mM, and most preferably $\geq$ 195 mM. In a preferred embodiment of the present invention, the conductivity of all buffers used in the method of the present invention is about the same. In another preferred embodiment, the washing buffer in step (a), or, in case no washing step is carried out, the loading buffer in step (a) has a conductivity that is equal or lower than the conductivity of the eluant in step (b).

[019] The eluant according to the present invention further contains at least one divalent cation. In a preferred embodiment, the eluant comprises a divalent cation, selected from the group consisting of Ca^{2+}, Be^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, or combinations thereof. The cation is preferably present in the eluant in a concentration from 1 mM to 20 mM, more preferably in a concentration of 2 mM. The cation is present in the eluant in the form of a suitable salt with an anion. Suitable anions are known to a person skilled in the art and comprise for example sulfate-, phosphate-, carbonate-, and borate-based anions, or combinations thereof. The salt comprising the divalent cation may be present in the eluant in a concentration from 1 mM to 20 mM, preferably 2 mM. In a preferred embodiment, the salt is $CaCl_2$. In a particularly preferred embodiment, the eluant contains 2 mM $CaCl_2$. In further preferred embodiments, the eluant contains 2 mM Ca^{2+} and has a pH that is at least 0.5 pH units, preferably at least 1.0 pH units, more preferably at least 1.5 pH units, most preferably at least 2.0 pH units higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a).

[020] As used herein, the term "anion exchange resin material" does not underlie a specific restriction. According to the present invention, the resin includes any material suitable for anion exchange chromatography known in the art, like for example an agarose based chromatography material, e.g. sepharoses like Fast Flow or Capto, polymeric synthetic material, e.g. polymethacrylate like Toyopearls, polystyrene/divinylbenzene, e.g. Poros, Source, or cellulose, e.g. Cellufine. In a specific example of the present invention, the anion exchange resin material is sepharose, which is based on modified agarose, the polysaccharide chains of which are crosslinked to form a three-dimensional network. In a preferred embodiment, the anion exchange resin material includes, but is not limited to a resin that carries a primary amine as ligand, e.g. aminohexyl sepharose, benzamidine sepharose, lysine sepharose, or arginine sepharose. In another preferred embodiment, the anion exchange resin material includes, but

is not limited to a resin having a positively charged moiety at neutral pH, such as alkylaminoethane, like diethylaminoethane (DEAE), dimethylaminoethane (DMAE), or trimethylaminoethyl (TMAE), polyethyleneimine (PEI), quaternary aminoalkyl, quaternary aminoethane (QAE), quaternary ammonium (Q), and the like. In a particularly preferred embodiment the anion exchange resin material is Q-Sepharose Fast Flow (Q-Sepharose FF).

[021] The divalent cation binding protein according to the present invention may be any divalent cation binding protein, like for example a calcium binding protein and/or a vitamin K-dependent protein. In a preferred embodiment, the divalent cation binding protein is selected from the group, consisting of factor IX (FIX), factor VII (FVII), and Annexin V.

[022] The divalent cation binding protein may be obtained using methods known to a person skilled in the art like, e.g. plasma derived proteins, transgenically produced proteins, or recombinantly produced proteins, for example using CHO cells. Secretory and non-secretory methods for extracting proteins from cell culture are well known to a person skilled in the art. This may include any method 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) the introduction of recombinant DNA into prokaryotic or eukaryotic cells by transfection, e.g. via electroporation or microinjection, (iii) the cultivation of said transformed cells, e.g. in a continuous or batchwise manner, (iv) the expression of a divalent cation binding protein, e.g. constitutive or upon induction, and (v) the isolation of the protein, e.g. from the culture medium or by harvesting the transformed cells, in order to obtain a crude divalent cation binding protein. Additionally, the recombinant DNA encoding a divalent cation binding protein, e.g. a plasmid, may also contain a DNA sequence encoding a selectable marker for selecting the cells which have been successfully transfected with the recombinant DNA.

[023] The proteins may be pre-purified to reduce impurities, for example by gel electrophoresis, chromatography, gel filtration, centrifugation, filtration, precipitation, crystallization or any other method known in the art. The term "impurity" as used herein includes any impurity originating from the production of the divalent cation binding protein and may include e.g. host cell protein impurities, nucleic acid impurities, polypeptide impurities, buffer and salt impurities, impurities originating from the cell culture medium, product related impurities, such as dimers or fragments, and combinations thereof.

[024] In a preferred embodiment, the divalent cation binding protein which has been purified according to the method of the present invention has a purity with respect to host cell protein impurities of at least 95% w/w, more preferably at least 98% w/w, more preferably at least 99% w/w, and most preferably at least 99.5% w/w divalent cation binding protein in total protein. Accordingly, in a preferred embodiment, the content of impurities in the purified divalent cation binding protein is less than 5% w/w, more preferably less than 2% w/w, more preferably less than 1% w/w, and most preferably less than 0.5% w/w. The percentage values of the impurities refer to w/w of product, *i.e.* the purified divalent

cation binding protein, and can be measured, for example, by HPLC or ELISA.

[025] Further, in another aspect of the present invention, a purified divalent cation binding protein is provided which is obtainable by the method of the present invention, as well as a kit comprising means for carrying out the method of the present invention. In particular, the present invention relates to a kit comprising an eluant comprising at least one divalent cation, suitable for eluting a divalent cation binding protein from an anion exchange resin material in a buffer, wherein the pH of the eluant is higher than the pH of the buffer. For example, the kit may contain a loading buffer and/or an eluant and/or a washing buffer and/or an additional washing buffer which are suitable for the purification of a divalent cation binding protein using an anion exchange resin material according to the present invention. In a preferred embodiment, the loading buffer, the washing buffers and/or the eluant are as defined above. Further, the kit of the present invention may contain a suitable anion exchange resin material.

[026] The present invention further relates to the use of the method of the present invention as defined above and/or of the kit of the present invention as defined above for the purification of a divalent cation binding protein.

[027] The present invention provides an efficient method for the purification of a divalent cation binding protein using an anion exchange resin material allowing a high reduction of process related impurities of the protein with concomitantly high product yields.

[028] In particular, the method of the present invention is based on the following principles. Generally, binding of proteins to anion exchange resin materials is increased at lower conductivities and higher pH values. Vice versa, binding of proteins to anion exchange resin materials is decreased at higher conductivities and lower pH values. In the method of the present invention, the divalent cation binding protein is preferably loaded and/or washed at a low pH which still allows binding of the divalent cation binding protein to the anion exchange material and does not harm the structural integrity or the activity of the divalent cation binding protein. At such conditions, many protein impurities, in particular those having a higher isoelectric point (pI) than the divalent cation binding proteins, do not bind to the anion exchange resin material, and, therefore, binding of impurities to the anion exchange resin material is greatly reduced. Protein impurities that do bind to the anion exchange resin material under these conditions, i.e. protein impurities having a low pI, will not coeluate with the divalent cation binding protein since the increase of the pH in the elution buffer causes all proteins, including the divalent cation binding protein, to bind even stronger to the anion exchange resin material. According to the method of the present invention, only the divalent cation binding protein is specifically eluted under such elution conditions due to the divalent cations present in the elution buffer. In this context, it should be noted that elution from an anion exchange resin material at increased pH values is very atypical, since, as has been stated above, proteins generally bind stronger to anion exchange resin materials at higher pH values. By specifically eluting the divalent cation binding proteins with an eluant comprising at least one

divalent cation under the above conditions, the method of the present invention surprisingly and advantageously achieves superior purities of the divalent cation binding protein product with a single purification step by anion exchange chromatography.

[029] In particular, the present invention advantageously modifies the surface charge of the proteins to be purified by increasing the pH between the loading step and/or the washing step of step (a) of the method of the present invention, and the step of eluting the divalent cation binding protein. An increased pH in the elution step forces impurities to remain adsorbed to the anion exchange resin material while specific elution of the protein to be purified with divalent cations at a high pH is carried out. In particular, a high pH is normally an unfavorable condition for driving elution from an anion exchange resin material, since proteins bind stronger to an anion exchange resin material at higher pH values. However, according to the present invention, the above conditions result in a high purity as well as high yields of divalent cation binding proteins. The method of the present invention may provide a significant reduction of process related polypeptide impurities e.g. by loading the protein solution at neutral pH onto an anion exchange resin material, applying a wash step at reduced pH, and eluting the product at an increased pH in the presence of a divalent cation. Instead of the low pH wash the sample can already be loaded at a low pH followed by elution at increased pH in the presence of a divalent cation.

[030] Various modifications and variations of the described method and products of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should not be unduly limited to such embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[031] Figure 1: SDS-PAGE of fractions obtained after purification of Annexin V on Q-Sepharose Fast Flow. The SDS-PAGE analysis was carried out on a 12% gel under reducing conditions. The load, column, flow through, wash and elution fractions A2 to A8 were analyzed by a gelelectrophoretic method and the separated polypeptides were visualized by silver staining (total protein stain) or Western blotting using anti-Annexin specific antibodies. Annexin V is a single chain polypeptide with about 36 kDa and can bind up to 10 divalent cation molecules at three different types of binding sites. The position of the Annexin V band is indicated with an arrow.

EXAMPLES

[032] The following examples are provided as a guide for a person skilled in the art. The examples should not be construed as limiting the invention, the examples merely provide specific methodology useful in understanding and practicing an embodiment of the invention.

Example 1: Purification of rFIX on Q-Sepharose FF

[033] A Q-Sepharose Fast Flow resin was activated with 2 column volumes (CV) of 2 M NaCl and equilibrated with 4 CV of an equilibration buffer (20 mM Tris, 2 mM EDTA, pH 7.4, conductivity

approximately 2 mS/cm). Thereafter, a FIX containing filtered cell culture supernatant obtained from a genetically engineered CHO cell line supplemented with 3 mM EDTA was loaded onto the column at a linear flow rate of about 150 cm/h having a conductivity of about 13 to 15 mS/cm. The column was then washed with 2 CV of equilibration buffer followed by a second wash with 5 CV washing buffer (20 mM MES, 2 mM EDTA, 175 mM NaCl, pH 6.0) to remove the majority of protein impurities that do not bind onto the anion exchange resin material at pH 6.0. Before the elution, the conductivity in the column was again reduced by applying a 2 CV wash with equilibration buffer. Bound FIX was eluted with 5 CV of the eluant (20 mM Tris, 2 mM $CaCl_2$, 180 mM NaCl, pH 8.0). The results in Table 1 show that by applying this procedure, the divalent cation binding protein product with a purity of 98% could be obtained with a single purification step by anion exchange chromatography out of the cell culture supernatant matrix.

Table 1: Purification of rFIX on Q-Sepharose FF

	FIX Antigen	CHO protein	FIX purity	CHO impurity level
	µg/ml	µg/ml	%	ng CHO/ µg FIX Antigen
Load	6.2	13.4	32	2161
Eluate pool	105.3	2.43	98	23.1

Example 2: Purification of FVII on Q-Sepharose FF

[034] A Q-Sepharose Fast Flow resin was activated with 2 CV of 2 M NaCl and equilibrated with 5 CV of an equilibration buffer (20 mM Tris, 2 mM EDTA, pH 7.5, conductivity approximately 2 mS/cm). Thereafter, a FVII containing filtered cell culture supernatant obtained from a genetically engineered CHO cell line supplemented with 9 mM EDTA was loaded onto the column at a linear flow rate of about 46 cm/h having a conductivity of about 16 mS/cm. The column was then washed with 4 CV of equilibration buffer followed by a second wash with 5 CV washing buffer (20 mM MES, 2 mM EDTA, 170 mM NaCl, pH 6.0) to remove the majority of protein impurities that do not bind onto the anion exchange resin material at pH 6.0. Before the elution, the conductivity in the column was again reduced by applying a 2 CV wash with equilibration buffer. Bound FIX was eluted with 5 column volumes of the eluant (20 mM Tris, 2 mM $CaCl_2$, 180 mM NaCl, pH 8.0). The results in Table 2 show that by applying this procedure the divalent cation binding product with a purity of 97% could be obtained with a single purification step by anion exchange chromatography out of the cell culture supernatant matrix.

Table 2: Purification of rFVII on Q-Sepharose FF

	FVII Antigen	CHO protein	FVII purity	CHO impurity level
	µg/ml	µg/ml	%	ng CHO/ µg FVII Antigen
Load	7.4	26.3	22	3554
Eluate pool	13.0	0.43	97	30

Example 3: Purification of human Annexin V on Q-Sepharose FF

[035] A Q-Sepharose Fast Flow resin was activated with 4 CV of 2 M NaCl and equilibrated with 15 CV of an equilibration buffer (20 mM Tris, 2 mM EDTA, pH 7.5, conductivity approximately 2 mS/cm). Thereafter, an Annexin V protein preparation purified from human placenta was spiked with a protein stock solution obtained from a CHO cell line to generate a protein load with an Annexin purity of below 20%. The Annexin containing protein mixture was supplemented with 1 mM EDTA and loaded onto the column at a linear flow rate of about 23 cm/h having a conductivity of about 4 mS/cm. The column was then washed with 10 CV of equilibration buffer followed by a second wash with 10 CV washing buffer (20 mM MES, 2 mM EDTA, 155 mM NaCl, pH 6.0) to remove the majority of protein impurities that do not bind onto the anion exchange resin material at pH 6.0. Before the elution, the conductivity in the column was again reduced by applying a 10 CV wash with equilibration buffer. Bound Annexin was eluted with 30 CV of the eluant (20 mM Tris, 2 mM $CaCl_2$, 150 mM NaCl, pH 8.0). The elution buffer had the same conductivity as the wash buffer. The resulting fractions were analyzed by SDS polyacrylamide gelelectrophoresis on a 12% gel under reducing conditions. The gels with the separated polypeptides stained for total protein (silver stain) and Annexin V (anti-Annexin Western blot) are depicted in Figure 1. The results show that in the elution fractions A2 to A4 Annexin V is contained in a high purity compared to the protein mix that was loaded onto the column (fraction load). The data indicate that after a single purification step by anion exchange chromatography the divalent cation binding protein could be obtained in a high purity in the elution fractions.

Example 4:

[036] Various conditions for purifying FIX from host cell proteins (HCP) using Q-Sepharose Fast Flow were tested. In particular, the procedure included the following conditions:

[037] Run 1: Load neutral pH, wash low pH 6.5, elution high pH 7.9, conductivity equal in wash and elution buffer.

Run 2: Load neutral pH, wash low pH 6.0, elution high pH 8.0, conductivity in wash buffer lower than in elution buffer.

Run 2.1: same as run 2, salt concentration in wash increased.

[038] In all experiments a second wash was performed immediately before the elution with a buffer of low conductivity. In all experiments the conductivity of the elution buffer is higher than the loading buffer. The results are shown in Table 3.

Table 3

	Load	Wash 1	Wash 2	Elution	Comment	purity of eluate pool	CHO reduction factor	
						ng HCP/ µg FIX	reduction of spec. impurity	µg load/ µg eluate pool
run 1	20 mM Tris, approx. 150 mM NaCl, 2 mM EDTA, pH=7.4	20 mM MES, 160 mM NaCl, 2 mM EDTA, pH=6.5	20 mM Tris, pH=7.4	20 mM Tris, 180 mM NaCl, 2 mM $CaCl_2$, pH=7.9	Wash low pH, elution buffer high pH conductivity equal in wash and elution buffer	21	56	175
run 2	20 mM Tris, approx. 150 mM NaCl, 2 mM EDTA, pH=7.4	20 mM MES, 165 mM NaCl, 2 mM EDTA, pH=6.0	20 mM Tris, pH=7.4	20 mM Tris, 180 mM NaCl, 2 mM $CaCl_2$, pH=8.0	Wash low pH, elution buffer high pH conductivity in wash buffer lower than in elution buffer	28	81	195
run 2.1	20 mM Tris, approx. 150 mM NaCl, 2 mM EDTA, pH=7.4	20 mM MES, 175 mM NaCl, 2 mM EDTA, pH=6.0	20 mM Tris, pH=7.4	20 mM Tris, 180 mM NaCl, 2 mM $CaCl_2$, pH=8.0	Wash low pH, elution buffer high pH conductivity in wash buffer lower than in elution buffer	23	94	143

Example 5: Comparative purification of rFIX on Q-Sepharose FF

[039] In order to demonstrate the beneficial effects of the method of the present invention, two comparative chromatographic runs were performed. In particular, rFIX from a clarified CHO cell culture supernatant was purified on Q-Sepharose FF, wherein Run 1 was performed according to the method of the present invention, *i.e.* the pH of the eluant in step (b) had a pH higher than the pH of the washing buffer in step (a), and Run 2 was performed in the same manner but without any pH difference between loading/washing in step (a) and elution in step (b). The respective buffer condition are summarized in Table 4.

Table 4: Buffer conditions of two chromatographic rFIX purification runs

	pH		Ca^{2+}
	Run 1	Run 2	
Loading	6.0	7.6	absent, 2 mM EDTA
Wash 1	6.0	7.6	absent, 2 mM EDTA
Wash 2	7.6	7.6	absent, 2 mM EDTA
Elution	7.6	7.6	present

The fractions obtained were analyzed for rFIX activity yield, rFIX antigen yield, reduction factor of CHO host cell proteins (CHO HCP), and rFIX specific activity. The reduction factor of CHO HCP was calculated as the ratio of total CHO HCO loaded to CHO HCP found in the eluate. The respective data are summarized in Table 5.

Table 5: Results of two chromatographic rFIX purification runs

	rFIX activiy yield [%]	rFIX antigen yield [%]	CHO HCP reduction factor	rFIX spec. activity [U/mg rFIX]
Run 1	88	33	239	266
Run 2	95	36	112	134

These results indicate that the method of the present invention, in particular the pH change between load/wash and elution, can significantly improve reduction of contaminant host cell proteins and specific activity of the purified protein.

CLAIMS

1. A method for the purification of a divalent cation binding protein comprising the steps of:
   (a) loading an anion exchange resin material with the divalent cation binding protein in a loading buffer in the absence of divalent cations, and optionally washing the loaded anion exchange resin material with a washing buffer in the absence of divalent cations; and
   (b) eluting the divalent cation binding protein with an eluant comprising at least one divalent cation to form an eluate containing the divalent cation binding protein;
   wherein the eluant in step (b) has a pH higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a).
2. The method according to claim 1, wherein the pH of the eluant in step (b) is at least 0.5 pH units higher than the pH of the washing buffer in step (a), or, in case no washing step is carried out, of the loading buffer in step (a).
3. The method according to claim 1 or 2, wherein the washing buffer in step (a), or, in case no washing step is carried out, the loading buffer in step (a) has a conductivity that is equal or lower than the conductivity of the eluant in step (b).
4. The method according to any of claims 1 to 3, wherein the at least one divalent cation in step (b) is selected from the group consisting of Ca^{2+}, Be^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+}, or combinations thereof.
5. The method according to any one of claims 1 to 4, wherein the anion exchange resin material has a positively charged group which is selected from the group, consisting of diethylaminoethane (DEAE), dimethylaminoethane (DMAE), trimethylaminoethyl (TMAE), polyethyleneimine (PEI), quaternary aminoalkyl, quaternary aminoethane (QAE), and quaternary ammonium (Q).
6. The method according to any one of claims 1 to 5, wherein the anion exchange resin material carries a primary amine as ligand which is selected from the group, consisting of aminohexyl, benzamidine, lysine, and arginine.
7. The method according to any one of claims 1 to 6, wherein the divalent cation binding protein is a calcium binding protein.
8. The method according to any one of claims 1 to 7, wherein the divalent cation binding protein is a vitamin K-dependent protein.
9. The method according to any one of claims 1 to 8, wherein the divalent cation binding protein is selected from the group, consisting of Factor IX, Factor VII, and Annexin V.
10. A purified divalent cation binding protein obtainable by a method according any one of

claims 1 to 9.

11. A kit comprising means for carrying out the method according to any one of claims 1 to 9.

12. The kit according to claim 11, comprising an eluant comprising at least one divalent cation, suitable for eluting a divalent cation binding protein from an anion exchange resin material in a buffer, wherein the eluant has a pH higher than the pH of said buffer.