Title of the invention

Process for the isolation of biologically active granulocyte colony stimulating factor

Field of the invention

The invention relates to a new process for the isolation of biologically active granulocyte colony stimulating factor (G-CSF) by using the immobilised metal affinity chromatography (IMAC).

G-CSF belongs to the group of colony stimulating factors, which regulate the differentiation and proliferation of hematopoietic precursor cells and activation of mature neutrophils. G-CSF is used in medicine in the field of haematology and oncology. Two types of G-CSF are clinically available: a glycosylated form (lenograstim), which is produced by using the expression in mammalian cells and nonglycosylated form (filgrastim), which is produced by using the expression in bacteria *Escherichia coli* (*E. coli*).

Summary of the invention

According to the present invention correctly folded and biologically active molecules of G-CSF can be separated from incorrectly folded or biologically inactive molecules of G-CSF under native conditions by using IMAC. The process for isolation of biologically active G-CSF of the present invention comprises the separation of correctly folded and biologically active molecules of G-CSF from incorrectly folded or biologically inactive molecules of G-CSF and also from the majority of host proteins by using IMAC under native conditions. By this process the correctly folded and biologically active molecules of G-CSF specifically bind to the IMAC support whereas the incorrectly folded, biologically inactive forms of G-CSF and the majority of the impurities remain in the eluate. The process for isolation of the present invention also comprises the separation of the biologically active monomeric forms of G-CSF from oligomeric, polymeric and biologically inactive monomeric forms

of G-CSF. The biologically active monomeric forms of G-CSF are specifically bound to the IMAC support whereas oligomeric, polymeric and biologically inactive monomeric forms of G-CSF remain in the eluate.

The process for isolation of the present invention presents an effective step of purification and concentration of correctly folded biologically active monomeric forms of G-CSF and can be used as the key step in the entire isolation process of G-CSF.

The process for isolation of the present invention can be used in cases where G-CSF after the expression is secreted directly via the secretion pathway to the medium or where G-CSF is secreted in the form of inclusion bodies to the cytoplasm, periplasm or to any other cell organel. It can also be used for the isolation of biologically active G-CSF directly from the solubilised inclusion bodies and in all cases where G-CSF had been previously denatured and then renatured. The process of the isolation of biologically active G-CSF of the present invention is maintained under the native conditions all along the process.

The process for isolation of biologically active G-CSF of the present invention results in the production of biologically active G-CSF with a purity of greater than 95%. Only two additional chromatographic steps, the cationic exchange chromatography and the gel filtration, which also relate to the present invention, can further be used for the removal of traces of the remaining impurities (polishing).

The entire process for isolation of the present invention therefore leads to the production of biologically active G-CSF with a purity of greater than 99%. The process is suitable for the production of large quantities of biologically active G-CSF and is suitable for the industrial production of biologically active G-CSF.

Background of the invention

The nonglycosilated form of G-CSF (filgrastim) and its production are described in EP 237545 whereas the glycosilated form of G-CSF and its production are described in EP 169566.

The processes for isolation of G-CSF, which are known from the patent and scientific literature comprise different combinations of ion chromatography,

chromatofocusing, hydrophobic interaction chromatography, gel filtration and some other methods.

Generally, the first step of the process for isolation of G-CSF depends on the host organism of the heterologous expression of G-CSF. In case of the expression in *E. coli*, G-CSF is found in the insoluble inclusion bodies. The processes for the isolation of G-CSF therefore comprise additional steps for the isolation of G-CSF from the inclusion bodies leading to correctly folded biologically active form. These additional steps usually comprise washing with detergents or chaotropic substances, solubilisation with strong denaturing agents (e.g. guanidine hydrochloride (GndHCl), urea) or with high concentrations of detergents (e.g. sarcosyl or sodium dodecil sulfate), partial purification of solubilised denatured protein by using gel filtration, reverse phase HPLC (RP-HPLC) and renaturation by using dilution of the denaturanting agent or by using dialysis. In case of the expression of G-CSF in yeast or mammalian cells the inclusion bodies or similar structures are found rarely and the process for the isolation in these cases starts directly after the secretion of G-CSF. The processes for the isolation of G-CSF are described in the following patent applications and patents: EP 169566, EP 237545, EP 215126, EP 243153, US 5055555 and WO 0104154. The processes for the isolation of G-CSF are also described in the scientific literature: Lu,H.S. *et al.* in *Protein Expr Purif* 4, 465-472 (1993), Kang, S.H. *et al.* in *Biotechnol. Lett.* 17, 687-692 (1995), Wingfield, P et al in *Biochem J* 256, 213-218 (1988), (Kang, S.H. et al. v *Biotechnol. Lett.* 17, 687-692 (1995), (Yamasaki,M. et al in *Biosci Biotechnol Biochem* 62, 1528-1534 (1998), Wingfield, P. *et al.* in *Biochem J* 256, 213-218 (1988), Bae, C.S. *et al.* in *Biotechnol. Bioeng.* 57, 600-609 (1998).

In addition to the above common chromatographic procedures, IMAC is also described to be used for partial purification and renaturation of some proteins. IMAC was firstly described in Porath *et al.* in *Nature* 258, 598-599 (1975) and is based on the binding of proteins to immobilised metal ions, which are chelated to different IMAC supports. The electron donor groups in the amino acid sequence of the protein are responsible for the co-ordinate binding to the support, especially the imidazole ring in the histidine residues. The isolation of recombinant proteins with engineered histidine surface clusters and histidine affinity tags on either N- or C- termini of the

protein by using the IMAC was described by Hochuli, E. *et al.* in *Bio/Technology* 1321-1325 (1988), Chaga, G. *et al.* in *Biotechnol Appl Biochem* 29 (part 1), 19-24 (1999) and Jeong, J.K. and Lee S.Y. Protein Expr. Purif, 23: 311-318 (2001). The use of IMAC for the investigation of small topographical differences among the protein molecules was described by Sulkowski in *Trends Biotechnol* 3, 1-7 (1985) and by Hemdan *et al.* in *Proc Natl Acad Sci U S A* 86, 1811-1815 (1989).

The process for the purification by using IMAC of some proteins which comprise histidine residues was described in US 5932102.

The process for the purification of proteins with surface amino acids capable of binding metal ions is described in WO 9012803. In this process IMAC is used as an additional step after partial protein purification by using several other chromatographic methods. Neither isolation nor separation of biologically active molecules of G-CSF from the biologically inactive molecules of G-CSF under native conditions by using IMAC are not described.

The comparative studies of G-CSF, its Ser-17 and $(His)_6$-tagged forms interaction with metal ions by means of affinity partitioning were described (Zaveckas, M. *et al.* in J Chromatogr A 904, 145-169 (2000); Gelunaite, L. *et al.* in J Chromatogr A 904, 131-143 (2000)). IMAC (Sepharose IDA (iminodiacetate) with immobilised Zn (II) or Ni (II) ions) was also used for renaturation of GndHCl denaturated interleukin-3, G-CSF and granulocyte macrophage colony factor (GM-CSF). The denatured proteins were bound to the support under denaturating conditions (Rozenaite, V. *et al.* in Poster abstract , P-104., Cordoba, Spain, 19-22. April (1998)).

IMAC was also used as a method for renaturation of proteins after expression in *E. coli* and denaturation in strong denaturanting agent and the method was described by Zaveckas, M. *et al.* in J Chromatogr A 904, 145-169 (2000); Rozenaite,V. et al in Poster abstract , P-104., Cordoba, Spain, 19-22. April (1998)). Again, the denaturated proteins were bound to the IMAC support.

The description of the separation of biologically active monomeric correctly folded molecules of G-CSF from oligomeric, polymeric or biologically inactive monomeric forms or incorrectly folded molecules of G-CSF by using IMAC under native conditions is not found neither in the scientific nor in the patent literature.

There are also no descriptions of isolation of biologically active G-CSF by binding to the IMAC support under native conditions of the correctly folded biologically active monomeric forms of G-CSF, which are already found in the solution. Additionally, the separation of molecules of G-CSF according to their conformational state by using IMAC has not been described.

All methods for isolation of G-CSF found in prior art comprise several steps. The use of only one chromatographic step as for separation of G-CSF according to its biological activity and correct folding and as well for the separation from other impurities present in the solution and also as an effective method for concentration of G-CSF and production of biologically active G-CSF with a purity of greater than 95%, so that only additional polishing is necessary, has not been described in prior art.

The detailed description of the invention

The present invention relates to the use of IMAC as an effective chromatographic method in the process for isolation of biologically active G-CSF. The correctly folded biologically active monomeric forms of G-CSF are selectively bound to different IMAC supports under native conditions whereas the incorrectly folded or aggregated molecules of G-CSF and most of the solubilized proteins from inclusion bodies after *E. coli* expression are not bound to these supports and are eluted without retaining. This feature is supposed to occur due to specific distribution of natural histidine residues.

The term 'native conditions' used herein refers to the conditions by which the molecule (protein) preserves the native confirmation and the biological activity.

The term 'denaturing conditions' refers to the conditions by which the native confirmation of proteins is not preserved, the biological activity is changed and is not preserved.

The term 'aggregated molecules' used herein refers to the molecules which form clusters of molecules bound together by hydrophobic or also some other bonds (e.g. disulphide bonds). These molecules are not biologically active.

The term 'elution' used herein refers to washing or extraction of the adsorbed material from the chromatographic column.

The term 'eluate' used herein refers to the solution which is obtained by washing and extraction from the chromatographic column.

The term 'inclusion bodies' used herein refers to insoluble compact aggregates of incorrectly folded or partially correctly folded proteins.

The term 'inclusion bodies solution' used herein refers to the solution which comprises inclusion bodies.

The term 'biologically active G-CSF' used herein refers to G-CSF with biological activity equal or greater than $1x10^8$ IU/mg, measured by a method based on stimulation of cellular proliferation as described in example 5.

The process for isolation of biologically active G-CSF of the present invention comprises the following steps:

a) loading of the solution comprising biologically active G-CSF or a mixture of biologically active and inactive G-CSF on the IMAC support under native conditions.

b) selective binding of biologically active G-CSF, which is correctly folded and occurs in monomeric form, to the IMAC support under native conditions.

c) washing of IMAC column and elution of biologically active G-CSF from the column under native conditions.

The process for isolation of biologically active G-CSF of the present invention can additionally comprise further purification of biologically active G-CSF and comprises the following steps which are performed after the isolation of biologically active G-CSF by IMAC.

d) cationic exchange chromatography and

e) gel filtration.

The entire process for isolation results in the production of biologically active G-CSF suitable for clinical use in medicine.

The process for isolation of biologically active G-CSF of the present invention can also be used in case of isolation of G-CSF derivatives such as methionyl G-CSF (Met-G-CSF), glycosilated, enzymatically of chemically modified (e. g. pegylated) G-CSF, G-CSF analogues and the fusion proteins which comprise G-CSF.

The essential feature of the process for isolation of the present invention is that:

1. the molecules of G-CSF are separated according to their conformational state and therefore according to their biological activity,
2. the process enables binding of biologically active molecules of G-CSF to the IMAC support under native conditions and consecutively the separation of biologically active molecules of G-CSF from biologically inactive molecules of G-CSF under native conditions,
3. the process enables binding of correctly folded molecules of G-CSF to the IMAC support under native conditions and consecutively the separation of correctly folded molecules of G-CSF from the incorrectly folded molecules of G-CSF under native conditions,
4. the process enables binding of biologically active monomeric forms of G-CSF to the IMAC support under native conditions and consecutively the separation of biologically active monomeric forms of G-CSF from the biologically inactive monomeric forms of G-CSF,
5. the process enables binding of monomeric forms of G-CSF to the IMAC support and consecutively the separation of monomeric forms of G-CSF from the oligo- and polymeric forms of G-CSF under native conditions,
6. the process enables the separation under native conditions of correctly folded monomeric biologically active molecules of G-CSF from other proteins and impurities which are present in the solution,
7. the entire process for isolation of biologically active G-CSF, which further comprises the purification with cationic exchange chromatography and gel filtration, results in the production of larger quantities of G-CSF with the purity of greater than 99% and is therefore suitable for the industrial production of biologically active G-CSF,
8. the entire process for isolation of biologically active G-CSF, which comprises further purification by cationic exchange chromatography and gel filtration does not involve any additional steps of purification of G-CSF and is maintained under native conditions all along.

The entire process for isolation of biologically active G-CSF of the present invention is maintained under native conditions.

The process for isolation of G-CSF of the present invention is not a renaturation process of incorrectly folded molecules G-CSF, but is the process, which comprises the specific binding to the IMAC support of the correctly folded biologically active monomeric molecules of G-CSF, which are already present in the solution.

The separation of monomeric from oligo- and polymeric forms of G-CSF occurs in such a way that the biologically active monomeric forms of G-CSF are bound to the IMAC support, whereas the oligo and polymeric forms, which can also occur in the aggregate form, remain in the eluate.

Instead of IMAC, gel filtration could be used for the separation of monomeric form of G-CSF from oligo- and polymeric forms of G-CSF. The advantage of IMAC over the gel filtration is its better binding capacity and the ability of separation of correctly folded monomeric forms of G-CSF from the incorrectly folded monomeric forms of G-CSF. Correctly folded monomeric forms of G-CSF can not be separated from the incorrectly folded monomeric forms of G-CSF by gel filtration. Thus, by using IMAC better yields are obtained.

Another advantage of the process for isolation of biologically active G-CSF of the present invention over other isolation procedures known from the prior art is that (under the native conditions maintained all along the process) the isolation of correctly folded molecules of G-CSF from the mixture of different proteins and also from the mixture of molecules G-CSF occurring in different conformational states is enabled. Therefore a direct isolation of G-CSF from the inclusion bodies under native conditions is possible.

Additional advantages of the process of the present invention over other similar processes are also: reduced use of detergents, the absence of denaturating agents, which are either toxic or environmentally unfavourable (e.g. GndHCl or urea), reduced use of buffers and other solutions.

The advantage of the process for isolation of biologically active G-CSF of the present invention over the methods by which strong denaturing agents are used is that there is no need of use of active reducing agents like dithiothreitol or beta-mercaptoethanol. Active reducing agents react with metal ions, which are present on

the IMAC support. The consequences of using of the active reducing agents are therefore reduced yields of biologically active G-CSF.

In case of secretion of G-CSF directly into the medium, the process for isolation of biologically active G-CSF of the present invention enables effective and direct concentration of biologically active G-CSF from the diluted media. In the eluate from the IMAC column biologically active G-CSF of high purity is obtained.

Due to the effective separation of biologically active G-CSF from biologically inactive G-CSF and other impurities is obtained either by the isolation of biologically active G-CSF from the inclusion bodies and or by the isolation of biologically active G-CSF directly from the solution, the economy of the isolation of G-CSF is much improved if compared with other methods.

G-CSF purity of more than 95% is thus obtained by using only one purification step.

Only two chromatographic steps are further needed for the final purification (polishing) of biologically active G-CSF after eluting from IMAC. These chromatographic steps comprise:

1. Cationic exchange chromatography for removal of traces of host nucleic acids, lipopolysaccharides, proteins, ionic isomers of G-CSF and changed (damaged) forms of G-CSF with lower pI values.
2. Gel filtration for removal of traces of dimers and other aggregated forms of G-CSF.

The two final purification steps result in a purity of G-CSF greater than 99%.

The advantages of the entire process for isolation of biologically active G-CSF, which additionally comprises the cationic exchange chromatography and gel filtration are: it comprises only three steps, between the three chromatographic steps there are no intermediary steps (e.g. concentration, dialysis, precipitation...) and the native conditions are maintained all along the isolation process.

The intermediary concentration steps would cause the formation of dimers and other forms of aggregates, what would lead to reduced yields. The entire process for isolation can be transferred to industrial scale and to the production of biologically active G-CSF with a purity of at least 99%.

The biologically active G-CSF obtained by the entire process for the isolation of the present invention is suitable for the preparation of pharmaceutical composition, which comprises the therapeutically effective amount of biologically active G-CSF and is suitable for clinical use.

The term ' therapeutically effective amount' used herein refers to the amount of biologically active G-CSF which has the therapeutic effect of biologically active G-CSF.

Biologically active G-CSF which was obtained by using IMAC and additional steps of cationic exchange chromatography and gel filtration can be used for preparation of medicaments, which are indicated for the indications selected from the group, which comprises: neutropenia and neutropenia-related clinical sequelae, reduction of hospitalisation for febrile neutropenia after chemotherapy, mobilisation of hematopoietic progenitor cells, as alternative to donor leukocyte infusion, chronic neutropenia, neutropenic and non-neutropenic infections, transplant recipients, chronic inflammatory conditions, sepsis and septic shock, reduction of rist, morbidity, mortality, number of days of hospitalisation in neutropenic and non-neutropenic infections, prevention of infection and infection-related complications in neutropenic and non-neutropenic patients, prevention of nosocomial infection and to reduce the mortality rate and the frequency rate of nosocomial infections, enteral administration in neonates, enhancing the immune system in neonates, improving the clinical outcome in intensive care unit patients and critically ill patients, wound/skin ulcers/burns healing and treatment, intensification of chemotherapy and/or radiotherapy, pancytopenia, increase of anti-inflammatory citokines, shortening of intervals of high-dose chemotherapy by the prophylactic employment of filgrastim, potentiation of the anti-tumour effects of photodynamic therapy, prevention and treatment of illness caused by different cerebral disfunctions, treatment of thrombotic illness and their complications and post irradiation recovery of erythropoiesis.

It can be also used for treatment of all other illnesses indicated for G-CSF.

The process of isolation of biologically active G-CSF of the present invention starts with loading of the sample and binding of the protein to the IMAC support.

Several IMAC supports can be used: Zn(II) iminodiacetate (IDA), Ni(II)-IDA, Ni(II)- nitrilotriacetic acid (NTA), it is also possible to use other combinations, as for

example: M(II)-IDA, M(II)-NTA, M(II)-carboxymethylaspartate etc., where M presents Zn, Cu, Co, Ni etc.

The inclusion bodies solution in the presence of low detergent concentrations (e.g. 0,2% sarcosyl, 0,005% sodium dodecil sulfate and others) or in the presence of solubilizing agents (e. g. sulfobetain) can be loaded to the IMAC column. These solutions are still under native conditions, either when the detergents remain in the solutions or when they are removed by using ion exchangers, dialysis, precipitation, etc...

The inclusion bodies solution in the presence of strong denaturating agents, such as 8 M urea or 6 M GndHCl can also be loaded to the IMAC column and also the solutions in the presence of denaturating concentrations of detergents (e.g. sarcosyl, 2% sarcosyl or 1% sodium dodecil sulfate) after the previous renaturation by dilution, dialysis or removal of denaturation agents/detergent.

In case of the expression in secretory systems such as yeast, fungi or mammalian cell lines, the supernatant or concentrated supernatant with pH in the range from 6.5 to 9.0 can be loaded to the IMAC support.

The eluate resulting from the first elution from the IMAC column can also be used as loading solution for IMAC. pH of the eluate must be adjusted (e.g. by addition of NaOH solution) to the pH range from 6.5 to 9.0. The eluate can be then reloaded to the same IMAC support (e.g. Zn(II)-IDA, Ni(II)-IDA, Ni(II)-NTA (NTA – nitrilotriacetic acid) or to different IMAC supports. The combination with other immobilised metal ions is also possible (e.g. Zn(II), Cu(II), Co(II), Ni(II) itd...) and enables better separation and removal of specific host proteins.

Regardless to the preparation and the origin of the loading solution, the pH of the loading solution must be in the range from 6.5 to 9.0. Preferred pH of loading solution is between 7.0 and 8.5, mostly preferred pH is about 8.0.

Different buffers, which can maintain pH in the range from 6.5 to 9.0 can be used for loading and binding of G-CSF to the IMAC support.

Phosphate, acetate, hydroxymethylaminometan (Tris)/HCl, Tris/acetate and some other buffers can be used. Preferably, Tris/HCl is used.

Tris/HCl buffer is preferably used in the concentration range between 5 and 50 mM, most preferably in the range from 10 to 40 mM.

After binding to the support the process is continued by washing of the column and elution of proteins from the column. Elution can be performed by using either a discontinuous stepped gradient or linear gradient by lowering pH or competitive elution at high pH (e.g. with imidazole, histidine, ammonium chloride and similar)

The term 'linear gradient' used herein refers to washing of chromatographic column by a solution which composition is changed in a way that the proportion of one buffer (or one component of the buffer) is increased linearly, whereas the proportion of the other buffer (or another component of the buffer) is decreased linearly.

The term 'discontinuous stepped gradient' used herein refers to washing of chromatographic column by a solution which is composed of certain proportion of one buffer (or one component of the buffer) and certain proportion of another buffer (or another component of the buffer) for a determined time period. The proportions of both buffers are rapidly (suddenly) changed and the column is washed by a another determined time period. The composition of the solution (change of buffer proportions or component proportions) is not changed linearly.

Preferably, discontinuous stepped gradient at low pH is used. Namely, high pH could cause the activation of cysteine residues and the formation of dimers. Stability of G-CSF is also high at low pH.

Several elution buffers can be used for discontinuous stepped or linear washing and elution and are selected from the group comprising of: acetate, Tris/acetate, phosphate, citrate and some other buffers. pH range for the elution can be between 3.0 and 5.0, preferably 4.0. By the discontinuous stepped gradient pH is rapidly changed from 7.0 to 4.0 and the isoelectric point is thus jumped over, avoiding the precipitation of the protein. Namely, the isoelectric point can cause the precipitation of the protein.

In the eluate, monomeric, biologically active, correctly folded G-CSF is obtained with a purity of greater than 95%.

The eluate obtained from the IMAC column can be loaded directly to the cationic exchange chromatography column, without any additional steps. Several cationic exchange chromatography supports can be used and are selected from the group comprising of: SP Sepharose FF, SP Sepharose HP, CM Sepharose FF, TSK

gel SP-5PW. TSK gel CM-5PW, TSK gel SP-650M, TSK gel SP-550C, TSK gel CM-650M, Macro-Prep High S support, Macro-Prep S support, Macro-Prep CM support etc. Preferably, SP Sepharose FF or TSK gel SP-5PW are used.

pH range of the loading solution for cationic exchange chromatography is in the range from 3.0 to 5.8, preferably in the range from 4.0 to 5.0.

The salt concentration in the loading solution for cationic exchange chromatography has to be low enough to enable the binding, which also depends on pH of the solution.

Different buffers with the pH range from 3.0 to 5.8 can be used by loading and binding to the support for cationic exchange chromatography and are selected from the group comprising: acetate, citrate, Tris/HCl, Tris/acetate, phosphate, succinate, malonate, MES and other buffers. Preferably, acetate buffer is used.

Acetate buffer can be used in the concentration range from 10 to 60 mM, preferably in the concentration range from 10 to 30 mM.

The column loading is followed by washing of the column and the elution of the proteins from the column. The elution occurs due to increased ionic strength after the addition of high concentration of salt in buffer solution. Discontinuous stepped gradient, linear gradient and a suitable combination of stepped and linear gradient can be used.

Elution buffers, which can be used for washing and elution are selected from the group comprising: acetate, citrate, Tris/HCl, Tris/acetate, phosphate, succinate, malonate, 2-(N-morfolinoethansulfonate) (MES) and other buffers with addition of salts such as NaCl or KCl. Ionic strength and salt concentration, by which the elution is achieved depends on the pH of the buffer solution. The higher is pH of the buffer, the lower ionic strength is needed for the elution of the proteins from the column.

In the eluate, monomeric, biologically active, correctly folded G-CSF is obtained with a purity of greater than 98%.

The eluate obtained from the cationic exchange chromatography column can be loaded directly to the gel filtration column, without any additional intermediary steps.

Different gel filtration supports can be used and are selected from the group comprising: Sephacryl S-200HR, Sephacryl S-100HR, Superose 12, Superose 6,

Superdex 75, TSK gel G-2500PW, TSK gel G-3000 PW, Bio-Gel P-60, Bio-Gel P-100 etc. Preferably, Superdex 75 is used.

A broad pH range of the loading solution for gel filtration can be used and the eluate directly from the cationic exchange chromatography is therefore suitable as a loading solution. Loading of the solution, binding of the protein to the gel filtration support and elution of the protein can be performed by using the same buffer. Different buffers can be used and are selected from the group comprising: citrate, acetate, Tris, phosphate and some other buffers, which can maintain pH in the range from 3.5 and 8.0. Preferably, phosphate buffers with pH from 6.0 to 7.0 are used.

Phosphate buffers (for loading) can be used in the concentration range from 2 to 100 mM, preferably in the concentration range between 3 and 10 mM.

The salt concentrations in the gel filtration buffer can be in the range from 30 to 100 mM, preferably about 50 mM.

In the eluate, monomeric, biologically active, correctly folded G-CSF is obtained with a purity of greater than 99% and biological activity of $1x10^8$ IU/mg.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

The description of the drawings

Fig. 1 shows the chromatographic separation of proteins from the solubilized inclusion bodies by using the IMAC support: Zn(II)-IDA Chelating Sepharose fast flow (Pharmacia).

The chromatogram shows the absorbance change at 280 nm (A280)( ) and the proportion of buffer P3 (-----) in dependence of time (min).

Peak A –*E. coli* proteins and aggregated G-CSF; peak B – monomeric correctly folded biologically active G-CSF and traces of *E. coli* proteins.

Fig. 2 presents the analyses with polyacrylamide gel electrophoresis in the presence of sodium dodecil sulfate (SDS-PAGE) of the starting sample and the samples represented in chromatographic peaks after the separation by using Zn-IDA Chelating Sepharose fast flow (Pharmacia), according to Fig. 1.

Legend:

1. Molecular weight standards (Bio-Rad) and G-CSF standard (Neupogen) (marked with arrow).
2. Starting sample before the chromatographic separation.
3. Proteins in peak A on Fig. 1 (aggregated G-CSF and *E. coli* proteins).
4. Proteins in peak B on Fig. 1 (monomeric, correctly folded, biologically active G-CSF and traces *E. coli* proteins).

Fig. 3 shows the chromatographic separation by using the column XK50/20, with IMAC support Zn-IDA Chelating Sepharose Fast Flow (Pharmacia).

The chromatogram shows the absorbance change at 280 nm (A280)( ) and the proportion of buffer P6 (-----) in dependence of time (min).

Peak A –*E. coli* proteins and aggregated G-CSF; peak B – monomeric correctly folded biologically active G-CSF and traces of *E. coli* proteins.

Fig. 4 shows the chromatographic separation by using the column XK16/20, loaded with cationic chromatography support Toyopearl SP-5PW (TosoHaas).

The chromatogram presents the absorbance change at 280 nm (A280)( ) and the proportion of buffer P8 (-----) in dependence of time (min).

Major peak – monomeric correctly folded biologically active G-CSF; smaller peaks - G-CSF isoforms and traces of *E. coli* proteins.

Fig. 5: Chromatographic separation by using the column XK26/70, loaded with gel filtration support Superdex^{TM} 75 prep grade (Pharmacia).

The chromatogram shows the absorbance change at 280 nm (A280) in dependence of (min).

Major peak – monomeric correctly folded biologically active G-CSF.

Examples

Example 1: The isolation of biologically active G-CSF by using IMAC: Zn-IDA (Chelating Sepharose fast flow)

The chromatographic column (h=10 cm, d=10 mm) was loaded with Chelating Sepharose fast flow (Pharmacia) support, on which Zn^{2+} ions were bound, and the column was equilibrated with 5 column volumes of buffer P2 at constant flow of 2 ml/min. Inclusion bodies were solubilized in buffer P1 (0,2 % sarcosyl, 40 mM

Tris/HCl, pH 8,0) and sarcosyl was removed by using the ion exchanger. The column was loaded with 10 ml of loading solution (17 mg total proteins), which comprised biologically active G-CSF, at constant flow of 1 ml/min. The separation was performed by using the discontinuous stepped gradient of buffer P3 at constant flow of 2 ml/min (Fig. 1). In the first chromatographic peak (peak A) the *E. coli* proteins and most of incorrectly folded and aggregated G-CSF were found. At 100% of buffer P3 (0% buffer P2) only monomeric and biologically active G-CSF was eluted (7 mg) with a purity of greater than 95 % (Fig. 2).

Biological activity of the sample from the peak A was measured and was about 1×10^6 IU/mg proteins, the measured biological activity of the sample from the peak B was 8-9 x 10^7 IU/mg proteins, whereas the biological activity of the standard was 1 x 10^8 IU/mg proteins. One-step IMAC by using the starting inclusion bodies solution leaded to the isolation of monomeric form of G-CSF, with a purity of greater than 95 % and the biological activity which is comparable with the standard.

Example 2: Isolation of biologically active G-CSF by using IMAC: Zn-IDA (Fractogel EMD Chelate)

The chromatographic column (h=2 cm, d=10mm) was loaded with Fractogel EMD Chelate (Merck) support on which Zn^{2+} ions were bound. The column was washed with water and equilibrated with five column volumes of buffer P2 at constant flow of 1 ml/min. The inclusion bodies were solubilized in buffer P1 and sarcosyl was removed by using the ion exchanger. The column was loaded with 3,3 ml solubilized inclusion bodies in total amount of 4 mg. The separation was performed by discontinuous stepped gradient of buffer P3 at constant flow of 1 ml/min (Gradient: 0% buffer P3 (100% buffer P2) 13 min, 25% buffer P3 (75% buffer P2) 12 min, 100% buffer P3 (0% buffer P2) 14 min, 0% buffer P3 (100% buffer P2) 11 min). At 100 % of buffer P3, the monomeric biologically active G-CSF (0,7 mg) was eluted.

Example 3: Isolation of biologically active G-CSF by using IMAC: Ni-NTA (Superflow)

The chromatographic column (h=10 cm, d=10 mm) was loaded with Ni-NTA Superflow (Qiagen) support and was equilibrated with five column volumes of buffer P4 at constant flow of 2 ml/min. The inclusion bodies were solubilized in buffer P1

and the sarcosyl was removed by using the ion exchanger. The column was loaded with 10 ml of solubilized inclusion bodies (10 mg total proteins) at constant flow of 1 ml/min. The separation was performed by using the discontinuous stepped gradient of buffer P5 at constant flow of 2 ml/min (the same gradient as by separation by using Zn-IDA Chelating Sepharose fast flow, presented on Fig. 1). At 100 % of buffer P5 (0% buffer P4) the monomeric biologically active G-CSF (3,6 mg) with a purity of greater than 95% was eluted. In the first chromatographic peaks only incorrectly folded and aggregated G-CSF was found in addition to *E. coli* proteins. The monomeric forms of G-CSF from the second peak after several chromatographic separations by using Ni-NTA Superflow were pooled and the final purification (polishing) was performed by using the cationic exchange chromatography and gel filtration.

Example 4: Entire process of the isolation for production of biologically active G-CSF

The entire process of the isolation of biologically active G-CSF started from the inclusion bodies solution, which was obtained after the expression of G-CSF in *E. coli*. 4,5 g washed inclusion bodies were resuspended in 225 ml of buffer P1 and were left to solubilize at 20°C 18 hours under smooth shaking using the linear shaker. After 15-min of centrifugation the sarcosyl was removed by using the ion exchanger. The sample was diluted with water to reach the double volume and ~ 430 ml of inclusion bodies solution was obtained; with the protein concentration of ~ 1,4 mg/ml (after Bradford according to G-CSF as a standard). The protein solution was loaded in two equal volumes to the chromatographic column XK50/20 (Pharmacia), loaded with Chelating Sepharose fast flow (45-165 μm, Pharmacia) support to the height of 10 cm (h=10 cm, d=5 cm, V=200 ml), on which Zn^{2+} ions were bound. The loading of the sample and the elution were performed at constant flow of 7 ml/min. After loading the column was washed with discontinuous stepped (Fig. 3): 15 min with buffer P2, then 45 min with mixture of buffers P2 and P6 in volume rate of 75:25 and 86 min with buffer P6. Monomeric form of biologically active G-CSF was eluted at 100% buffer P6. All the fractions (two separations), which comprised the monomeric biologically active G-CSF were pooled and 271 ml of solution with protein

concentration of ~ 0,7 mg/ml was obtained. EDTA was added to this solution to the final concentration of 2 mM. The solution was diluted three times with 20 mM CH_3COOH, pH 4,0 and was used as a loading solution for cationic exchange chromatography.

The IMAC eluate was loaded in two aliquots to the chromatographic column XK16/20 (Pharmacia), loaded with chromatographic support SP-5PW (30 μm; TosoHaas) to the height of 16 cm (h=16 cm, d=1,6 cm, V=32 ml). The loading of the sample and the elution from the column were performed at constant flow of 5 ml/min. After the loading of the sample the column was washed 11 min with buffer P7, followed by the elution with linear gradient with buffer P8 in 30 min from 0% to 25 % buffer P8 (from 100% to 75% buffer P7). The column was washed again for 16 min with the mixture of buffers P7 and P8 in a volume rate of 75:25 and then 22 min with buffer P8 (Fig. 4). The fractions of the main chromatographic peak, which were eluted in a linear part of the linear gradient at ~ 18% of buffer P8 and belonged to the correctly folded G-CSF (with a purity of greater than 98%), were pooled and used directly as a loading solutions for the gel filtration column.

The eluate from the cationic exchange chromatography (V= 46 ml, protein concentration ~ 2,4 mg/ml), was loaded in 5 aliquots to the chromatographic column XK26/70 (Pharmacia), loaded with chromatographic support Superdex 75 (prep grade, 34 μm) (Pharmacia) to height 57 cm (h=57 cm, d=2.6 cm, V=300 ml). The separation was performed in buffer P9 at constant flow of 2,5 ml/min. Peak, which represents the dimer protein, is clearly separated from the main peak which represents the monomeric protein (Fig. 5). The main chromatographic peak fractions were pooled, the buffer was changed and 100 mg of pure monomeric form of G-CSF with a purity of greater than 99% and biological activity 1 x 10^8 IU/mg, which corresponds to the biological activity of the standard, was obtained.

Example 5: *In vitro* G-CSF biological activity assay

Biological activity of G-CSF was determined by the method based on stimulation of cellular proliferation (NFS-60 cells) using the known method

(Hammerlingn, U. et al. v *J Pharm Biomed Anal* **13**, 9-20 (1995)) and the use of international standard Human recombinant G-CSF (88/502, yeast cell derived; NIBSC Potters Bar, Hertfordshire, UK); (Mire-Sluis,A.R. et al. v *J Immunol Methods* **179**, 117-126 (1995)).

The buffer compositions:

P1: 0,2% sarcosyl, 40 mM Tris/HCl, pH 8,0

P2: 20 mM Tris/HCl, 150 mM NaCl pH 8,0

P3: 20 mM acetic acid, 150 mM NaCl, pH adjusted to 4,5 with addition of 1 M NaOH

P4: 10 mM Tris/HCl, 200 mM NaCl, pH 8,0

P5: 20 mM acetic acid, 200 mM NaCl, pH adjusted to 4,0 with addition 1 M NaOH

P6: 20 mM CH_3COOH, 150 mM NaCl, pH 4,0

P7: 20 mM CH_3COOH, pH 5,5

P8: 20 mM CH_3COOH, 500 mM NaCl, pH 5,5

P9: 5 mM Na phosphate, 50 mM NaCl, pH 7,0

Claims

1. A process for the purification and/or isolation of biologically active G-CSF, which comprises:
   providing a mixture which comprises a biologically active form of G-CSF in the presence of an impurity, and
   subjecting said mixture to IMAC under native conditions.

2. The process according to claim 1, which comprises the following steps:
- loading said mixture, which comprises the biologically active G-CSF in the presence of an impurity, to an IMAC support,
- selective binding of the biologically active form of G-CSF to the IMAC support and
- eluting the biologically active form of G-CSF from the IMAC support to provide the biologically active G-CSF.

3. The process according to claim 1 or 2, wherein said mixture comprises an impurity which is at least one substance among the group consisting of biologically inactive monomeric forms and incorrectly folded molecules of G-CSF, oligomeric and polymeric forms of G-CSF, denaturated forms of G-CSF, host cell proteins and other host cell impurities (components);
   and the IMAC is carried out in such a way that the impurity is substantially not bound to the IMAC support and eluted from the IMAC column, before eluting the biologically active form of G-CSF.

4. The process according to any one of claims 1 to 3, wherein the biologically active G-CSF is at least one selected from the group consisting of non-glycosylated G-CSF, glycosylated G-CSF, methionyl G-CSF, G-CSF analogues, enzymatically or chemically modified forms of G-CSF and fusion proteins which comprise G-CSF.

5. The process according to any one of the preceding claims, wherein said G-CSF-containing mixture is selected from the group consisting of:
   a mixture, medium or solution, obtained after denaturation followed by renaturation;
   a solution or suspension of inclusion bodies under native conditions;
   a mixture or solution obtained from the supernatant after the expression in secretory systems or from a culture medium of an expression system;

and an eluate which was obtained by a previous elution of G-CSF from an IMAC column or any other chromatographic column.

6. The process according to claim 1 or 5, wherein said mixture comprises an inclusion body solution or suspension under native conditions.

7. The process according to any one of the preceding claims, wherein an IMAC with chelated metal ion bound to the IMAC support is carried out, wherein the metal ion is not Hg and/or wherein the chelated metal ion bound to the IMAC support is selected from the group consisting of M(II)-iminodiacetate, M(II)-nitrilotriacetic acid and M(II)-carboxymethylaspartate, wherein M is selected from the group consisting of Zn, Cu, Co and Ni.

8. The process according to claim 7, wherein the chelated metal ion bound to the IMAC support is selected from the group consisting of Zn(II)-iminodiacetate, Ni(II)-iminodiacetate and Ni(II)-nitrilotriacetic acid.

9. The process according to any one of the preceding claims, wherein the biologically active G-CSF obtained after performing IMAC has a purity of at least 95%.

10. The process according to any one of the preceding claims, further comprising the step(s) of: cationic exchange chromatography and/or gel filtration chromatography.

11. The process according to claim 10, wherein the biologically active G-CSF obtained after performing the chromatographic steps has a purity of at least 99%.

12. A process for the purification and/or isolation of biologically active G-CSF, wherein an impure mixture containing G-CSF under native conditions is subjected to chromatographic step(s) which consist only of IMAC, and optionally at least one of the purification methods selected from ion exchange and gel filtration techniques.

13. The process according to claim 12, wherein the mixture containing G-CSF is essentially free of detergent or solubilising agent or contains detergent or solubilising agent at a concentration where the G-CSF is present under native conditions.

14. The process according to claim 12 or 13, wherein the biologically active G-CSF obtained after performing the chromatographic steps has a purity of at least 95%; preferably at least 99%.

15. The process according to any one of the preceding claims, wherein the whole process is performed under native conditions.