Chelating Sepharose™ Fast Flow

Chelating Sepharose Fast Flow is a BioProcess™ medium (resin) for immobilized metal ion affinity chromatography (IMAC) of native and histidine-tagged fusion proteins (Fig 1). The medium is suitable for both laboratory-scale purification and large-scale use, and is well-established in several production processes.

- Wide range of purification applications
- Choice of a variety of chelated metal ions
- High salt concentrations can be used
- BioProcess medium used in approved industrial processes
- Straightforward scale-up to production columns
- Withstands effective and rigorous cleaning-in-place (CIP) procedures

Fig 1. Chelating Sepharose Fast Flow purifies proteins and peptides with affinity for chelated metal ions, including many proteins and peptides of commercial interest.

Introduction

Immobilized metal ion affinity chromatography

Certain amino acids, for example, histidine, cysteine, and tryptophan can form complexes with transition metal ions, such as zinc, copper, and nickel. If any of these metal ions are immobilized onto a chromatographic medium by chelation with a suitable ligand, the medium will selectively retain proteins that expose these amino acids. This technique can thus be used to separate and purify proteins with affinity for chelating ions and is also the principle of IMAC, for which Chelating Sepharose Fast Flow has been designed.

IMAC is a versatile purification technique (1, 2). The possibility to select metal ion may permit control of selectivity. IMAC can be operated under a wide range of conditions, such as high concentrations of salt as well as in the presence of chaotropic salts or denaturing agents.

A wide range of biomolecules including interferons, serum and plasma proteins, peptides, peptide hormones, lectins, and nucleotides can be purified using IMAC. Fusion proteins with affinity tails are commonly used to confer specific binding properties and allow simple, one-step purification protocols.

Characteristics

Chelating Sepharose Fast Flow consists of iminodiacetic acid groups coupled to Sepharose 6 Fast Flow by stable ether linkages and sufficiently long spacer arms (Fig 2). The Sepharose 6 Fast Flow base matrix is a highly cross-linked, 6% agarose with excellent chemical and physical stability. The cross-linking of the matrix has been optimized to give process-adapted flow characteristics with typical linear flow velocities of 300 to 400 cm/h through a 15 cm bed height at a pressure of 1 bar (0.1 MPa, 14.5 psi).

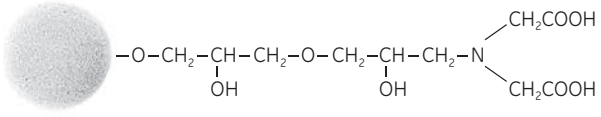

Fig 2. Partial structure of Chelating Sepharose Fast Flow. The rigid base matrix permits very high flow velocities.

Table 1 presents the basic characteristics of Chelating Sepharose Fast Flow.

Chelating Sepharose Fast Flow has excellent chemical stability under both acidic and alkaline conditions. The functional stability of the medium has been tested after storage at 40°C for seven days in 0.1 M HCl or 1.0 M NaOH and no significant change in performance was observed.

Table 1. Characteristics of Chelating Sepharose Fast Flow

Metal ion capacity	30–37 µmol Cu^{2+}/mL medium
Matrix structure	Highly cross-linked 6% agarose
Particle size	45–165 µm
Mean particle size	Approx. 90 µm
Chemical stability	Stable in all commonly used aqueous buffers: 1.0 M NaOH, 20% ethanol, 6 M guanidine hydrochloride, 8 M urea, and 0.01 M HCl
Physical stability	Negligible volume variations due to changes in pH or ionic strength
Autoclavable	121°C for 25 min in 20% ethanol
pH stability	long term: 3–13, short term: 2–14, working pH range: 4–8.5
Linear flow velocity at 25°C, 1 bar, 15 cm bed height, XK 50/30 column	> 300 cm/h

Experimental procedure

Recommendations and comprehensive instructions for packing and use are included in the information supplied with Chelating Sepharose Fast Flow. The following details highlight some key aspects of the experimental procedure.

Immobilization

Chelating Sepharose Fast Flow is supplied free of metal ions and thus needs to be charged with a suitable ion before use. When choosing the desired metal ion, consider the structural requirements underlying the basis of metal chelate-protein recognition.

The metal ions predominantly used are Cu^{2+}, Zn^{2+}, and Ni^{2+}. Cu^{2+} ions bind stongly to a wide range of proteins and some proteins have a selective affinity for Cu^{2+}. Zn^{2+} ions generally give a weaker binding and, in some cases, this can be exploited to achieve selective elution of a target protein. Ni^{2+} is a frequently used metal ion when working with proteins containing a histidine-tag. In some applications, also Co^{2+}, Fe^{3+}, and Ca^{2+} have been used advantageously.

It is recommended that the medium is charged with the selected metal ion in distilled water to avoid precipitation of metal salts on the medium.

Binding and elution

Protein binding to an immobilized metal ion usually occurs in the pH range 5.5 to 8.5. Binding is often strongest at the upper end of this range. The choice of binding buffer depends on the chelated metal ion and on the binding properties of the sample molecules. Sodium acetate and sodium phosphate are recommended buffers.

Proteins may be desorbed from Chelating Sepharose Fast Flow by:

- Reducing pH, either by a continuous gradient or step-wise change. Most proteins are eluted between pH 6 and 4. A final pH in the range of 3 to 4 is often suitable.
- Competitive elution with a gradient or step-wise increasing concentration of imidazole, histidine, ammonium chloride, or other substances with higher affinity for the chelated metal ion.
- Chelating agents such as EDTA, which will strip the metal ions from the medium and cause the proteins to co-elute. This method does not resolve different proteins.

Eluting with imidazole or reducing pH suspends interaction of the protein with the chelated metal ion while the metal ion itself remains bound to the column. Histidine and ammonium chloride displace the metal:protein complexes from the iminodiacetic acid ligand by the chelating capacity of their primary amino functions.

Regeneration and cleaning

Before Chelating Sepharose Fast Flow can be recharged with a new metal ion, it should be regenerated by stripping the previously used metal ions from the medium in the packed column using EDTA.

In some applications, substances such as denatured proteins or lipids are not eluted during regeneration. Impurities can be removed by CIP procedures.

Recommended CIP procedures include:

- Removing ionically bound proteins by washing the column with 0.5 column volumes of a 2 M NaCl solution.
- Removing precipitated proteins, hydrophobically bound proteins, and lipoproteins by washing the column with 1 M NaOH solution.
- Removing strongly hydrophobically bound proteins, lipoproteins, and lipids by washing the column with 70% ethanol, 30% isopropanol, or detergents in a basic or acidic solution.

More detailed recommendations for cleaning are included in the instructions that are enclosed with each pack of medium.

Sanitization

Sanitization reduces microbial contamination of the medium. A recommended sanitization procedure is treatment with 0.5 to 1 M NaOH.

Equipment

Chelating Sepharose Fast Flow is well-suited for use with most equipment commonly employed for affinity chromatography, from laboratory to production scale. For optimized performance, we recommend bed heights from 10 to 20 cm.

Table 2 lists recommended columns from GE Healthcare Life Sciences for laboratory, pilot, and production scale.

Applications

The versatility of IMAC as a purification technique has attracted the attention of many researchers, not least those in the industry whose task it is to develop processes suitable for the commercial manufacture of biopharamaceuticals. As well as demanding high purity, such processes must also be easy to scale up, robust, and economic. The three applications summarized below highlight several of these attributes.

Table 2. Recommended columns for Chelating Sepharose Fast Flow at different scales of operation

Column	Inner diameter (mm)	Bed volume	Bed height max (cm)
Lab scale			
Tricorn™ 5/20	5	up to 0.55 mL	2.8
Tricorn 5/50	5	up to 1.1 mL	5.8
Tricorn 10/20	10	up to 2.2 mL	2.8
Tricorn 10/50	10	up to 4.5 mL	5.8
Tricorn 10/100	10	up to 8.5 mL	10.8
XK 16/20	16	up to 30 mL	15
XK 16/40	16	up to 70 mL	35
XK 26/20	26	up to 80 mL	15
XK 26/40	26	up to 190 mL	35
XK 50/20	50	up to 275 mL	15
XK 50/30	50	up to 510 mL	25
Production scale			
BPG 100/500	100	up to 2.0 L	26
BPG 140/500	140	up to 4.0 L	26
BPG 200/500	200	up to 8.2 L	26
BPG 300/500	300	up to 18.0 L	26
BPG 450/500	450	up to 36.0 L	23
Chromaflow™ 400/100-300	400	13–37 L	30
Chromaflow 600/100-300	600	28–85 L	30

Recombinant human interferon gamma (rhIFN-γ)

Interferons are one of many protein families that are well-suited for purification by IMAC on Chelating Sepharose Fast Flow. Furthermore, their proposed therapeutic efficacy against diseases such as cancer and rheumatoid arthritis has also increased the interest for large-scale purification and characterization of interferons.

Zhang *et al.* (3) describe an optimized purification procedure for rhIFN-γ expressed as intracellular inclusion bodies in *E. coli*. Following initial extraction and solubilization, renatured rhIFN-γ was subjected to ion exchange chromatography on S Sepharose Fast Flow. Pooled fractions from this first purification step were applied to Chelating Sepharose Fast Flow charged with Ni^{2+} ions. Figure 3 shows the elution profile of this second purification step. The second step was followed by a third and final purification step, gel filtration on Superdex™ 75 prep grade medium.

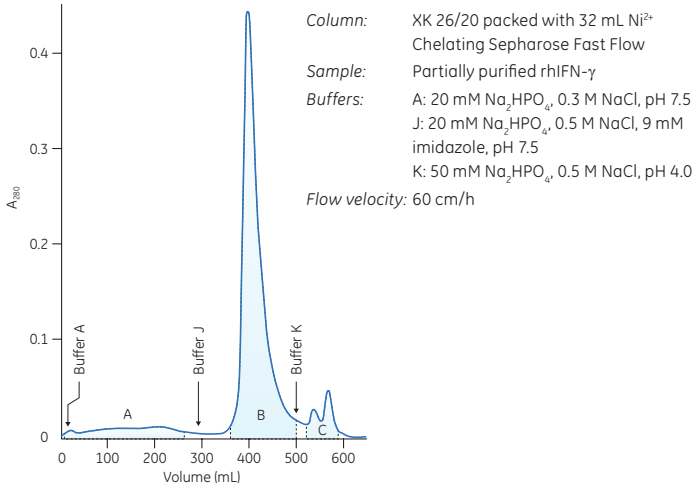

Fig 3. Partial purification of rhIFN-γ on Chelating Sepharose Fast Flow. Activity of rhIFN-γ was localized to peak B, which was pooled for final purification on Superdex 75 prep grade. Reprinted from Zhang, Z. *et al.* (3).

Chelating Sepharose Fast Flow was found to be essential for the efficient purification and high recovery of rhIFN-γ activity. For example, the medium effectively removed low molecular weight impurities and thus facilitated further purification to homogeneity in the final step. In addition, leakage of Ni^{2+} ions was extremely low, and the packed column could be used for several cycles without regeneration.

Easy scale-up

The authors concluded that, as well as giving a pure product with good recovery, the procedure was reproducible and well-suited for process-scale operation. Scaling up was straightforward, moving from XK columns at laboratory scale to BPG columns at process scale.

Human factor IX

Since the 1970's, the blood clotting disorder hemophilia B, which results from factor IX deficiency, has been treated with prothrombin complex concentrates derived from human plasma. These concentrates contain varying amounts of vitamin K-dependent proteins. In general, prothrombin is the predominant protein of the complex concentrates, while factor IX rarely exceed 2% of the total weight.

Methods have now been developed to obtain factor IX in a purer form. However, many of these methods require a large number of steps, which might challenge the physiological characteristics of the protein as well as the economy of the process. The process summarized in Figure 4, using Chelating Sepharose Fast Flow charged with Cu^{2+}, only requires two chromatographic steps without need for subsequent concentration of factor IX. In addition, the Chelating Sepharose Fast Flow step gave good clearance of model viruses (4,5).

Metal ion leakage

Metal ion leakage from the medium was investigated as this is an important issue when applying IMAC in industrial-scale pharmaceutical manufacture. It was found that levels of copper ions could be controlled and minimized to levels below those found in human plasma.

In summary, IMAC on Chelating Sepharose Fast Flow charged with Cu^{2+} ions enabled the preparation of a high-purity factor IX concentrate.

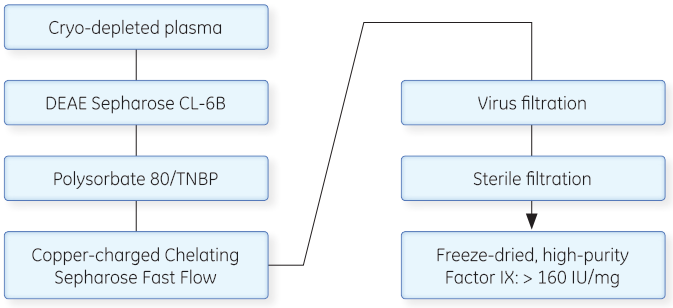

Fig 4. Purification of human factor IX using Chelating Sepharose Fast Flow.

Histidine-tagged proteins

Tagging or fusing proteins with a histidine–rich tail gives the protein affinity properties suitable for IMAC and allows simple purification of the target molecule. Histidine-tagging has today become a standard procedure and is particularly useful when valuable, but unstable, proteins require rapid capture.

One major advantage of using IMAC as a purification method for recombinant, histidine-tagged proteins is that separations can be performed under both nondenaturing and denaturing conditions. The latter can be useful for fusion proteins expressed at high levels as inclusion bodies in the host cells.

To illustrate this, recombinant protein A was expressed in *E. coli* as a fusion protein tagged with (histidine-glycine)$_4$. Isolation conditions were tested under denaturing conditions in the presence of 6 M guanidine hydrochloride as well as under nondenaturing conditions. The results show that both approaches could be used for purifying recombinant protein A. Figure 5 shows the purification under nondenaturing conditions.

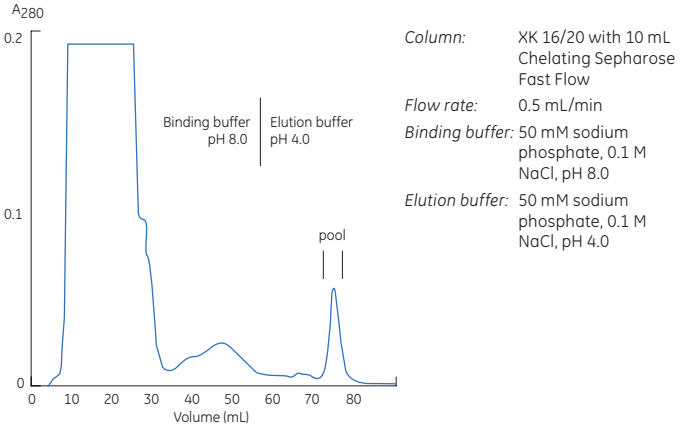

Fig 5. Purification of recombinant protein A expressed in *E. coli* as a fusion protein with (histidine-glycine)$_4$ on Chelating Sepharose Fast Flow with Zn^{2+} as chelated metal ion.

Meets criteria for industrial production

It is important to make the correct choice of chromatographic medium when purifying protein candidates for therapeutic use and vaccine applications, and to allow a straightforward transfer to production once a successful candidate has been found. As a member of the BioProcess family of media, Chelating Sepharose Fast Flow fulfils essential criteria, including validated manufacture, security of supply, scalability, and regulatory support. Evidence of the suitability of the medium for industrial applications can be found in the many regular production processes where Chelating Sepharose Fast Flow plays a reliable and well-established role.

References

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2. Chaga, G.S., Twenty-five years of immobilized metal ion affinity chromatography: past, present and future, *J. Biochem. Biophys. Methods* **49**, 313–334 (2001).
3. Zhang, Z. *et al.* Production, purification and characterization of recombinant human interferon gamma, *J. Chromatogr.* **604**, 143–155 (1992).
4. Feldman, P. A. *et al.* Preparation of a high purity factor IX concentrate using metal chelate affinity chromatography. *Colloque INSERM* **227**, 63–68 (1993).
5. Feldman, P.A. *et al.* Large-scale preparation and biochemical characterization of a new high purity factor IX concentrate prepared by metal chelate affinity chromatography, *Blood Coagulation and Fibrinolysis* **5**, 939–948 (1994).

Ordering information

Product	Pack size	Code number
Chelating Sepharose Fast Flow	50 mL	17-0575-01
Chelating Sepharose Fast Flow	500 mL	17-0575-02
Chelating Sepharose Fast Flow	5 L	17-0575-04

Note: Supplied in suspension in 20% ethanol. For long periods of storage, 20% ethanol or 0.01 M NaOH is recommended.

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