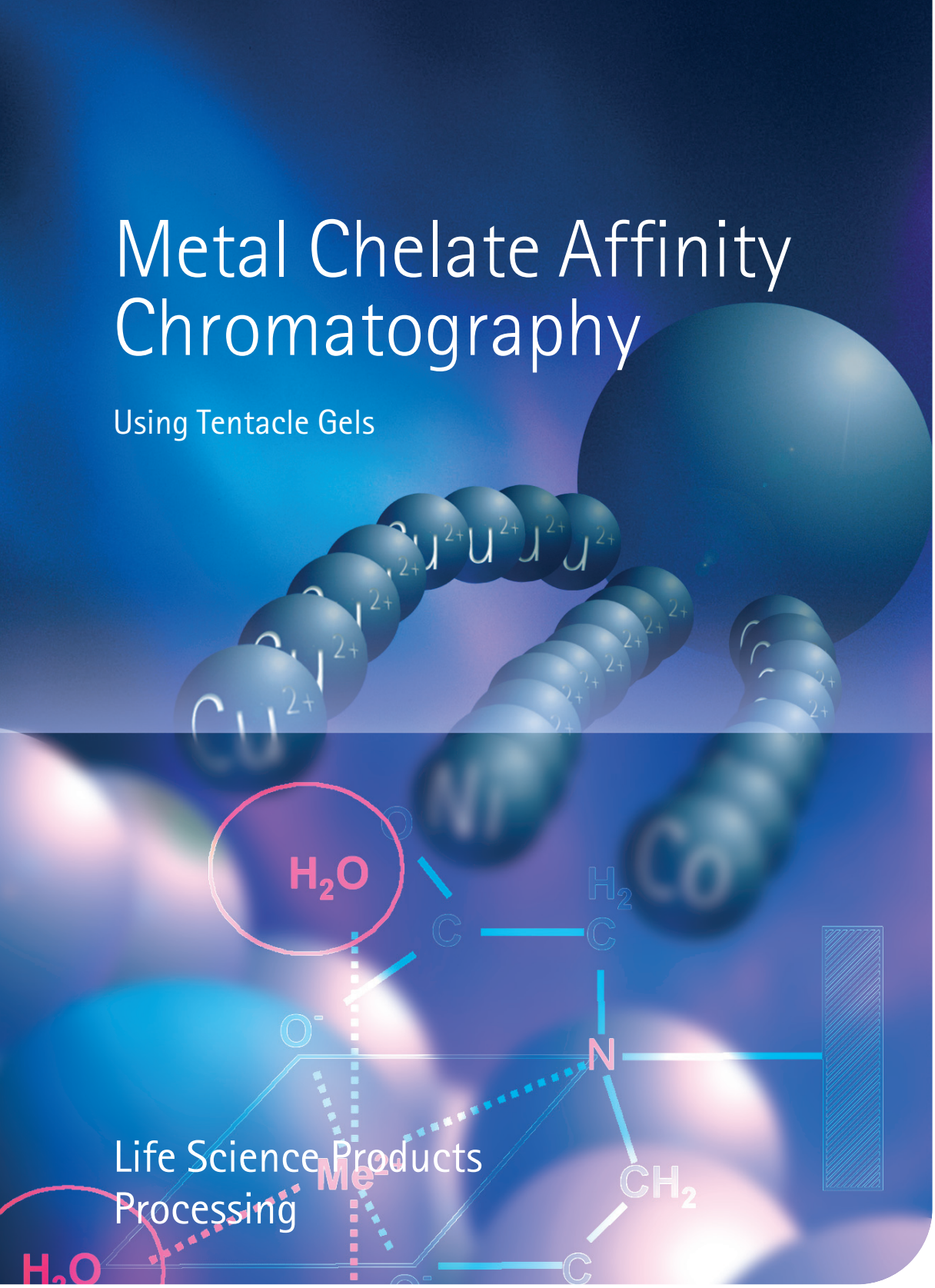
Metal Chelate Affinity Chromatography

Using Tentacle Gels



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Metal Chelate Affinity Chromatography Using Tentacle Gels

Affinity chromatography represents one of the most effective methods for the purification of proteins. The separation principle is based on the characteristics of many proteins to enter into specific interaction with certain molecules. In addition to the biospecific ligands (e.g. enzyme substrates, antibodies, receptors), so-called pseudo-biospecific ligands (e.g. lectines, dyes, sulfur containing groups, etc.) are also used. Pseudo affinity chromatography on immobilized metal ions becomes more and more important for the separation of proteins and peptides. Special advantages of this pseudo-biospecific technique is the stability of the used ligands, easy coupling to the chromatography support and low prices compared to most of the bio-ligands. For Fractogel® EMD Chelate iminodiacetic acid has been chosen as the metal chelating ligand. The iminodiacetic acid residue is very suitable as an immobilized chelating agent, since a bidentate chelating moiety remains free after immobilization, to which a metal ion can be coordinated. Various metal ions can be immobilized on the stationary phase via this immobilized chelating agent. Free coordination sites of the metal ions are used to bind different proteins and peptides.

Advantages of Tentacle Affinity Chromatography

An important feature of all kinds of affinity supports is not only the absolute number of ligands, but especially their accessibility. Since proteins often represent larger molecules than the ligand itself, steric inhibition can occur which prevents good binding of the protein to the ligand. Therefore a coupling of the pseudo specific bio-ligand via linear polymer chains (= tentacles) is very desirable. When using conventional spacer molecules for the manufacturing of affinity supports, the amount of ligands is limited and separation results are often negatively influenced by non-specific, hydrophobic interactions. With the newly developed Fractogel® EMD Chelate gel the polymer chains carry the functional group in high density providing an optimal steric accessibility.

In addition, due to the hydrophilic properties of the tentacle molecule, the non-specific side effects are reduced. Since each polymer chain possesses several ligands, a high protein binding capacity of the tentacle material for metal chelating affinity chromatography is obtained. Compared to conventional affinity gels or media synthesized with spacer molecules, Fractogel® EMD Chelate allows binding of double the amount of proteins. Because the functional groups are flexibly located via the polymer chains, the immobilized metal ion can be arranged in the best position relative to the binding sites on the protein's surface. As a result proteins can be bound more tightly to Fractogel® EMD Chelate than comparable conventional gels.

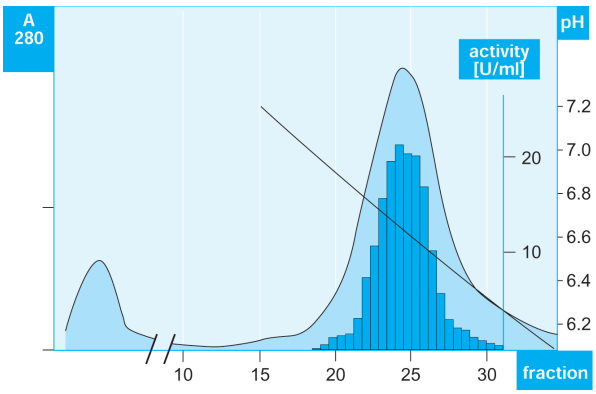

Figure 1: Purification of glucokinase from yeast using Fractogel® EMD Chelate (Superformance® Column 50 - 10 mm). The metal chelate chromatography was performed on immobilized cobalt ions. The purified enzyme can be eluted in a peak during a decreasing pH gradient. 20 mM phosphate buffer with 1 M KCl and 10 mM glucose at pH 7.5 was used as buffer A. Buffer A, which was adjusted to a pH value of 6.0, was used for elution.

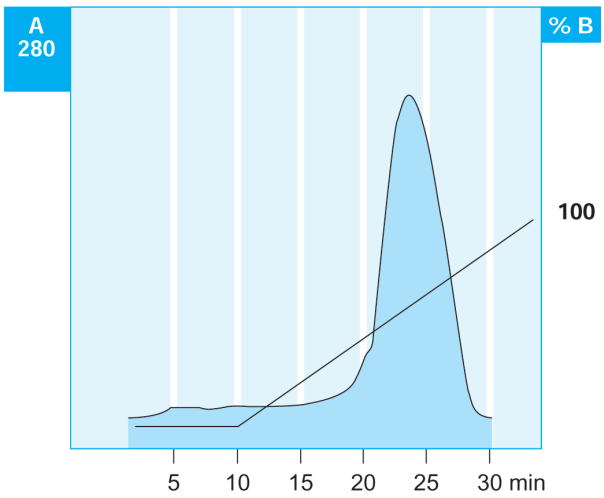

Figure 2: Metal Chelate Chromatography of a prepurified antibody sample on a Fractogel® EMD Chelate column loaded with nickel ions (Superformance® column 50 - 10 mm) equilibrated with 20 mM sodium phosphate buffer/0.5 M NaCl, pH 6.8. Elution was performed with a linear imidazole gradient, which was generated with 100 mM imidazole in buffer A (Flow rate: 1 ml/min). The antibody elutes in one peak. Any contamination can effectively be separated.

Application Areas

The existance of surface accessible histidine residues is important for the binding of a protein. With peptides the alpha amino group also plays a role so that peptides can also be retarded if no histidine residues are present. In the case of peptides, other metal binding amino acids like cysteine and tryptophane might contribute to the binding. Since the pK value of the histidine groups, contributing to the binding, lies in neutral range, the binding of the protein samples to the column should normally occur at a pH value of approximately 7. However, the actual pK value of an individual amino acid can vary strongly depending on the neighbouring amino acid residue. Various experiments show that depending on the protein's structure, the pK value of an amino acid can deviate from the theoretical pK value up to one pH unit. Therefore, an application buffer of pH 8 often achieves an improved binding. For an optimum binding of the protein to the immobilized metal complex, the buffer system can also be of influence. For example, Tris-containing buffer reduces the binding affinity and should therefore only be used if the proteins have very strong interactions with the affinity matrix. The presence of substances which also bind to the

metal ions can prevent binding of the target protein. For example, high imidazole concentrations strongly influence the protein binding characteristics of the column. At a concentration of 2 mM imidazole only 20 % of the loaded protein is retarded when an egg white solution is applied to a column charged with copper. At the same time the decrease of the pH value of the equilibration buffer results in adsorption of less protein from a complex mixture. To prevent ionic interactions between proteins and carboxy groups, which might remain uncharged with metal ions, a high ion strength should be present. The presence of 0.5 to 1 M NaCl is sufficient; sometimes also 0.1 M NaCl is enough. Because the presence of high salt concentrations in the buffer does not interfere, tentacle metal chelate chromatography is ideally used in combination with an ion exchanger column. The samples of an anion or cation exchanger column with a salt gradient, can be applied directly to a Fractogel® Chelate column (Fig. 1). Through advances in molecular biology, histidine residues can be inserted into the protein sequence resulting in improved separation by metal chelate chromatography. The modified protein has a high affinity to the immobilized metal ions due to the addition of the histidine residue in the primary sequence. The fusion part can be cleaved chemically or with a specific proteinase after purification. Due to an insufficient binding strength of proteins to bio-ligands of conventional affinity phases a desorption of the already bound protein can occur while loading the sample. Pseudo-affinity chromatography can often be used without problems on production scale since binding of proteins is better. Compared to conventional affinity supports, the pseudo affinity supports are acceptable on the preparative scale even in large quantities due to the lower costs and higher stability. Results achieved on the analytical scale can often be directly applied to the preparative scale. Metal chelate chromatography is very suitable for the isolation of antibodies and the separation of antibody conjugate complexes. Interestingly the use of Ni columns is recommended for this application area. The binding of antibodies is significantly better when immobilized nickel ions are used.

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Experimental

The following steps can be performed as standard conditions for metal chelate affinity chromatography. Fractogel® EMD chelate is packed into a column with a column dimension of 50 x 10 mm, 50 x 16 mm or 50 x 26 mm with a bed height of about 5 cm. Then the column has to be equilibrated with 20 - 50 mM phosphate buffer/0.1 - 1 M NaCl (pH 7.5). A subsequent washing step with 2 column volumes 0.1 - 1 M NaCl solution is performed to completely remove the phosphate. To load the column 250 mM $CuSO_4$ (or $CoCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$), diluted in water (MilliQ Quality), is pumped through the column. One bed volume metal salt solution is sufficient. After washing with 2 column volumes 0.1 - 1 M NaCl solution to remove unbound metal ions, the column must be re-equilibrated with 2 column volumes 20 - 100 mM phosphate buffer/0.5 - 1 M NaCl; pH 7.5. Elution can occur by displacement of the protein with a competitive molecule or by changing the pH value. In the first case, the component added to the buffer displaces the protein from the column in corresponding concentration. When lowering the pH value of the buffer the binding affinity of the protein-metal ion complex is reduced and the protein can be desorbed. If elution should be performed using imidazole, it is recommended to equilibrate the column with phosphate buffer/0.5 - 1 M NaCl to which 1 mM imidazole was added. The actual elution then occurs with a gradient of 1 to 200 mM imidazole in 0.02 M phosphate buffer/0.5 - 1 M NaCl; pH 7.5. Elution can take place either with a linear imidazole gradient or with step gradients. Alternatively, bound proteins can be eluted from the column using a descending pH gradient. For example, a phosphate buffer (0.1 M phosphate, 0.5 - 1 NaCl; pH 3.0) or an acetate buffer (0.1 M acetate, 0.5 - 1 M NaCl; pH 6.0 or pH 4.0) can be used. The declining pH gradient can be run as step gradient and/or as linear gradient. Flow rates of about 1 - 4 ml/min for columns with a diameter of 1 cm (linear flow rate: 1 cm/min) can be recommended.

Regeneration

For the regeneration of the metal chelate columns approx. 1 bed volume of a 0.1 - 1 M HCl solution at a slow flow rate (about 1.5 cm/min) has to be pumped through the column. This removes all metal ions; rinse with 2 column volumes of a 0.1 - 1 M NaCl solution and then equilibrate using 20 mM phosphate buffer. It is recommended to use a 20 % ethanol solution (prepared with buffer) for storage. Fractogel® EMD Chelate is also stable against alkali treatment, thus the column can be cleaned with 0.5 M NaOH.

Ordering information

Catalog No.	Description	Particle size	Content	Type of chromatography
1.10338	Fractogel® EMD Chelate (M)	40-90 µm	250 ml, 500 ml*	IMAC

* larger quantities on request

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