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Immobilized metal affinity chromatography of human growth hormone
Effect of ligand density[1]

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Abstract

Immobilized metal affinity chromatography was examined as a method for the purification of recombinant human growth hormone, somatotropin. Cellulose-based chelating supports, Chelat-Granocel, of a different content of ligand, charged with Cu(II), were assessed for their ability to bind the protein from both crude extract and solution purified by two chromatography steps. Human growth hormone was found to exhibit high affinity to chelating support charged with Cu(II). One step of immobilized metal affinity chromatography on Chelat-Granocel gives 80% purification of the protein. It was shown that the protein retention depends highly on the ligand density. By regulating sorbent ligand density a favourable desorption was achieved.

Keywords: Immobilized metal affinity chromatography; Ligand density; Growth hormones; Somatotropin; Proteins

1. Introduction

Various chromatographic techniques used for the purification of proteins exploit different characteristics of sorbate: net electrical charge (ion-exchange chromatography), molecular size (gel permeation chromatography), hydrophobicity (hydrophobic interaction chromatography), etc. Among these methods, immobilized metal affinity chromatography (IMAC) is of high selectivity because it exploits the affinity of proteins for the coordination sites of immobilized transition metal ions. As was shown [1], exposed histidine, and to a lesser extent cysteine and tryptophan residues, exhibit this affinity.

IMAC was introduced by Porath et al. in 1975 [2] and since then it has been adopted for the purification of many proteins. In all cases, the selectivity of IMAC was very high. However, for the large-scale preparative purification of proteins, IMAC has not yet been exploited to the extent it deserves. Not only effectiveness but also economic factors are very important when the process is scaled up. Therefore, when the large-scale process is developed the main problems are (i) to maximize sorption capacity and (ii) to optimize the elution process without loss in resolution.

Chelating Sepharose and its analogue for fast flow

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Chelating Sepharose FF (Pharmacia LKB Biotechnology, Uppsala, Sweden) with ligand content of ca. 34 μmol/ml are the only commercial adsorbents for large-scale IMAC of proteins. Selectivity and retention on those adsorbents are adjusted by changing the type of metal [3] or by varying the elution conditions including eluent pH [2], concentration of salt [4] and competing agents [5]. Desirable elution in a large-scale IMAC process is done by a pH gradient because the addition of competing agents complicates the process of purification and makes it more costly. However, in many cases the retention of proteins on Chelating Sepharose is stronger, requiring imidazole or glycine as an eluent.

In this paper the possibility of manipulating the retention of the proteins by changing the ligand density on the surface of adsorbent was studied. For this purpose, several chelating sorbents of different ligand density were synthesized and graduated metal concentration on the surface of the support was achieved. Granulated cellulose Granocel was used as a matrix, its suitability for large-scale chromatographic process having been shown in previous works [6]. The chelating sorbents were assessed for their ability to bind human growth hormone, somatotropin (STH). The influence of ligand density on the retention of protein was studied in order to optimize the STH separation process.

2. Experimental

2.1. Materials

STH was synthesized from *E. coli* as described [7]. Chelating Sepharose (ligand content 34 μmol/ml) was purchased from Pharmacia LKB Biotechnology.

Cellulosic matrix Granocel-500 was used for the preparation of chelating sorbents [8]. Characteristics of the matrix are as follows: fraction 100–200 μm, exclusion limit for dextrans $4 \cdot 10^5$.

All other chemicals were of reagent grade.

2.2. Preparation of chelating sorbents

2.2.1. Epoxidation of matrix

A 25-g amount of sucked matrix Granocel-500 was suspended in 30 ml of 5% NaOH, required amount of epichlorohydrin and 30 mg of sodium borohydride were added and the mixture was stirred at 40°C for 2 h. Then the activated matrix was washed carefully with water.

The concentration of epoxy groups was determined as follows: 1 g of epoxycellulose was immersed in 10 ml of 1.3 *M* sodium thiosulphate and the obtained sodium hydroxide was titrated with 0.1 *M* HCl keeping the pH near 7.0. 1 ml of HCl corresponds to 100 μmol of epoxy groups.

2.2.2. Introduction of chelating groups

Freshly activated matrix (25 g of sucked material) was suspended in 25 ml of 1 *M* Na_2CO_3. The required amount of iminodiacetic acid (IDA) was dissolved in 6 ml of 5% NaOH and the solution was added to matrix suspension. The mixture was stirred at 60°C for 3 h. After the reaction was finished, the sorbent material was washed with water. The amount of IDA groups was calculated from the nitrogen content determined by the Kjeldal method. The IDA group content was evaluated by adsorbed Cu content at a low degree of substitution, when the Kjeldal method gives a remarkable error.

2.3. Chromatographic methods

2.3.1. Equipment

Chromatography was carried out using a Model 2132 LKB Perspex pump, a Model 11300 LKB Ultrograd gradient mixer, a Model 2195 LKB pH/Ion monitor, single path monitor UV-1, a Model 2111 LKB Multisac, LKB potentiometric recorder. The protein concentration was measured with LKB Ultrospec II.

Sodium dodecyl sulphate (SDS)-electrophoresis was performed on Protean II Slab Cell Bio-Rad and scanned on a Model CS-930 Shimadzu dual-wavelength thin-layer Chromatoscanner.

All chromatographic experiments were performed at 4°C.

2.3.2. Samples

Two STH samples of different grade were used.

Crude STH was prepared as follows: *E. coli* extract was mixed with polyethyleneimine (PEI) to precipitate the nucleic acids and membrane com-

ponents. The proteins remaining in PEI supernatant were fractionated by precipitation in 50% ammonium sulphate and the ammonium sulphate pellet was dissolved in 10 m*M* sodium phosphate buffer, pH 7.8. The concentration of proteins was 2.7 mg/ml.

Partly purified STH was prepared as follows: the ammonium sulphate pellet was dissolved in 20 m*M* sodium phosphate buffer containing 0.6 *M* NaCl, pH 6.8 (buffer A) until the concentration of proteins was ca. 10 mg/ml. The solution was purified by two chromatography steps. The first step was hydrophobic interaction chromatography (HIC) on Phenyl-Granocel-500 performed on a column 7.5×2.6 cm I.D. The solution (80 ml) was applied to the column equilibrated with buffer A. After saturation, the column was washed with buffer A, containing 1 *M* ammonium sulphate to remove the unbound material. Bound proteins were eluted with 50 m*M* glycine–NaOH buffer, pH 9.2. After this step the concentration of STH in eluted proteins was approximately 15%. The second step was ion-exchange chromatography on DEAE-Granocel. STH was dialyzed against 50 m*M* glycine–NaOH buffer, pH 8.7 and loaded (10 mg of protein/ml sorbent) onto the column, equilibrated with the same buffer containing 0.028 *M* sodium chloride. Bound proteins were eluted with a linear gradient of 0.028–0.26 *M* NaCl in the same buffer at a flow velocity of 20 cm/h. The purity obtained after this step was 60%.

2.3.3. Immobilized metal affinity chromatography of STH

Saturation of column with metal ions

A column (6.5×1.0 cm I.D.) packed with IDA-cellulose was first conditioned with 50 m*M* sodium acetate buffer, pH 4.0, and then loaded with the copper(II) by injection of 20 m*M* solution of copper(II) sulphate in the same buffer until saturation. The excess unchelated metal was subsequently removed from the column by washing it with starting buffer. The amount of immobilized Cu(II) was calculated as the difference between the total amount of Cu(II) applied to the column and the amount which was washed out as excess. For the determination of the Cu(II) content in the solution 0.2 ml of analyzed solution was added to 3 ml of 1 *M* KCNS and the absorbance was measured spectrophotometrically at 354 nm.

Chromatographic procedure

The column was equilibrated with 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. The STH dialyzed against 10 m*M* sodium phosphate buffer, pH 7.7, containing 0.5 *M* NaCl was used in the chromatographic process. After loading the column was washed with 0.5 *M* sodium acetate buffer, pH 6.0. Elution conditions are described in Section 3. A flow velocity of 20 cm/h was maintained throughout. The purity of the fraction was checked by SDS-electrophoresis.

Capacity of the sorbent for STH was determined by frontal analysis.

2.3.4. Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out in 7% gel by application of the technique of Laemli and Farre [9]. The proteins were denaturated in 1% SDS solution containing 1% of 2-mercaptoethanol at 100°C for 5 min and then loaded onto the gel. After electrophoresis the gel was scanned on a Chromatoscanner and the concentration of STH was calculated.

3. Results and discussion

The use of recombinant DNA technology for the production of human growth hormone, STH, is nowadays a well established procedure. The purification of recombinant STH has been developed in some works [10–13]. It consists of a series of conventional chromatography steps, including ion-exchange chromatography (usually two or three times), HIC and gel permeation chromatography. Flodh [10] compared some of the purification schemes. It was shown that two ion-exchange steps and one gel filtration step result in *E. coli* polypeptide (ECP) purity exceeding 0.2 g/l. ECP levels were somewhat reduced by the introduction of an additional ion-exchange step and further reduced by introduction of a precipitation step. Lefort et al. [11] used HIC on Octyl-Sepharose or Phenyl-Sepharose as the initial step for the concentration and partial purification of STH. Concentrated STH solution was

further purified by ion-exchange and gel filtration chromatography. The STH obtained was homogeneous, based on polyacrylamide gel electrophoresis, N-terminal sequence and finger-print analysis.

Maisano et al. [12] showed that introduction of IMAC into the STH purification scheme remarkably simplifies this process. The separation of STH by IMAC was studied using four different metal ions chelated to IDA–agarose. The strongest retention was observed on IDA–Cu(II), where the pH gradient did not elute the protein and a stronger displacer, such as 1 *M* glycine, was required. The most suitable adsorbent was IDA–Ni(II) from which STH was eluted with a gradient from 0 to 30 m*M* imidazole in acetate buffer. A purity of 90% STH was achieved. Before IMAC the crude cellular extract was purified only by ion-exchange chromatography on DEAE-cellulose. In this report the concentration of the metal ions in adsorbents, however, is not reported.

In order to investigate the influence of the ligand density on the binding properties of the chelating sorbents, a different content of ligand was introduced into the matrix Granocel-500. The adsorbents were prepared by the coupling of iminodiacetic acid to the epoxy-activated cellulosic matrix Granocel-500. The graduated ligand density was achieved (i) by changing the content of epoxy groups in the matrix (Table 1) or (ii) by altering the concentration of IDA in the reaction mixture (Table 2).

Table 3 shows the results of IMAC experiments with partly purified STH (see Section 2.3.3) on Chelat-Granocel–Cu(II) with different ligand content as well as on Chelating Sepharose. Chromatography of proteins on metal charged chelating sorbent is usually performed in the presence of an electrolyte in order to quench ordinary electrostatic interactions [1,2]. In most cases 0.5 *M* sodium chloride is used. However, STH precipitates from such solution when the pH is lower than 5. The sorption of STH on chelating sorbent was performed at pH 7.7, therefore 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer was used for the column loading. 50 m*M* sodium acetate buffer without salt was used for the washing of the column and for the elution experiments.

As shown by Maisano et al. [12], STH exhibits high affinity for chelating support charged with Cu(II). However, retention of the protein depends highly on the ligand density. From the sorbents containing 3.5–8 μmol Cu(II)/ml sorbent STH could be recovered by lowering the pH of the eluent stepwise. By increasing the content of immobilized Cu(II) the desorption pH decreases. Thus, from Chelat-Granocel with ligand density 3.5 μmol/ml 94% of STH was eluted in a relatively sharp peak with the maximum at pH 6.0 (Fig. 1), and from the sorbent containing 7 μmol Cu(II)/ml the major peak of STH was given at pH ca. 5.7 (Fig. 2).

Desorption of STH from Chelat-Granocel–Cu(II) with ligand content 17 μmol/ml was achieved by a continuous pH gradient. Total STH was eluted within a relatively wide range of pH. Desorption started at pH 5.8 and ended at pH 5.0 with the

Table 2
Effect of IDA concentration in reaction mixture on ligand density

IDA/epoxycellulose[a] (g/g)	Ligand density (μmol Cu(II)/ml)
0.04	9
0.1	17
0.2	21
0.7	28
2.0	36
2.9	42

[a] Amount of epoxy groups 575 μmol/g.

Table 1
Effect of the amount of the epoxy groups content on ligand density

Epichlorohydrin/cel (g/g)	Epoxy groups (μmol/g)	IDA/epoxycel (g/g)	IDA groups (μmol/ml)	Cu(II) ions content (μmol/ml)
0.01	144	0.28	–	3.5
0.02	375	0.28	–	7
0.03	405	0.28	22	17
0.04	490	0.28	27	26

Table 3
Purification of the partly purified STH by IMAC

Chelating sorbent	Ligand density (μmol/ml)	Capacity (mg STH/ml)	Purity (%)	Yield (%)	Elution conditions[a]
1. Chelat-Granocel–Cu(II)	3.5	2.2	93	94	Stepwise, pH 6.0
2. Chelat-Granocel–Cu(II)	7	2.7	91	94	Stepwise, pH 5.7
3. Chelat-Granocel–Cu(II)	17	3.0	90	90	Gradient, pH 5.8–5.0
4. Chelat-Granocel–Cu(II)	20	3.2	87	75	Stepwise pH 4.5
5. Chelat-Granocel–Cu(II)	26	3.5	80	99	20 m*M* Imidazole
6. Chelating Sepharose–Cu(II)	34	3.5	80	64	20 m*M* Imidazole

[a] 50 m*M* sodium acetate buffer was used. In the case of Chelating Sepharose 0.5 *M* sodium chloride was added.

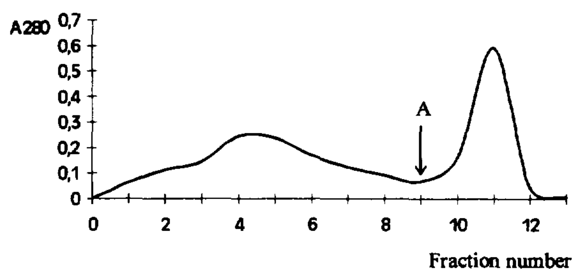

Fig. 1. Chromatography of STH on Chelat-Granocel–Cu (II). Ligand content 3.5 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 29 ml of partly purified (60%) STH containing 0.8 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with the same buffer. Elution was performed with 50 m*M* sodium acetate buffer, pH 6.0 (A). Flow velocity 20 cm/h. Fraction volume 10 ml.

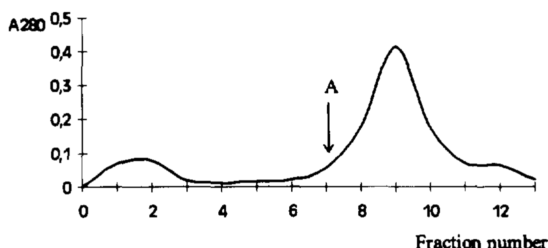

Fig. 2. Chromatography of STH on Chelat-Granocel–Cu (II). Ligand content 7.0 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 15 ml of partly purified (60%) STH containing 1.46 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0. Elution was performed with 50 m*M* sodium acetate buffer, pH 5.7 (A). Flow velocity 20 cm/h. Fraction volume 15 ml.

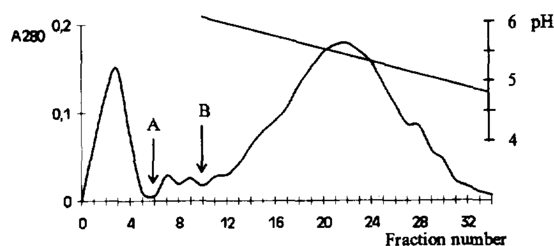

Fig. 3. Chromatography of STH on Chelat-Granocel–Cu (II). Ligand content 17 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 24 ml of partly purified (60%) STH containing 1.38 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0 (A). Elution was performed with pH gradient (6.0–4.5) in 50 m*M* sodium acetate buffer (B). Flow velocity 20 cm/h. Fraction volume 10 ml.

maximum at pH 5.3 (Fig. 3). Fig. 4 shows the results of chromatography of STH on Chelat-Granocel–Cu(II) with a ligand content of 20 μmol/ml.

With increasing ligand density, the affinity of STH for chelated Cu(II) increases. Fig. 5 shows that imidazole was required to desorb STH from the chelating sorbent with Cu(II) content ca. 26 μmol/ml. In this case STH was recovered with 99% yield but the purity of STH preparation increased insignificantly. It seems that imidazole is too strong an eluent to separate the bound proteins.

The chromatography of STH on Chelating Sepharose–Cu(II) (ligand content ca. 34 μmol/ml) indicated stronger affinity when comparing it with that on Chelat-Granocel–Cu(II) (Fig. 6). Only 64% of bounded STH were desorbed with 20 m*M* imidazole in 50 m*M* sodium acetate buffer. The purity of STH was 80%.

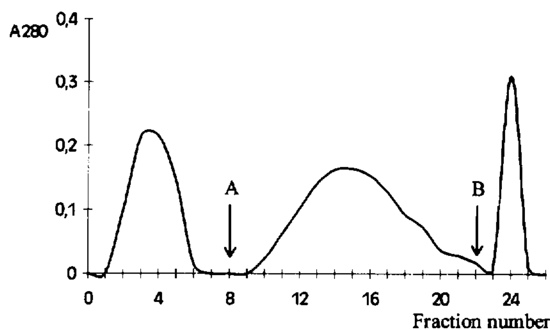

Fig. 4. Chromatography of STH on Chelat-Granocel–Cu (II). Ligand content 20 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 22 ml of partly purified (77%) STH containing 0.7 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0. Elution was performed with 50 m*M* sodium acetate buffer, pH 4.5 (A) and 20 m*M* imidazole (B). Flow velocity 20 cm/h. Fraction volume 8.6 ml.

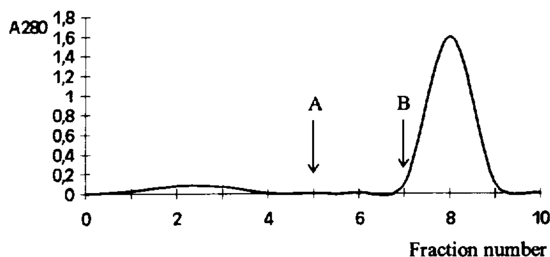

Fig. 6. Chromatography of STH on Chelat-Sepharose–Cu (II). Ligand content 34 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 33 ml of partly purified (77%) STH containing 0.7 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0. Elution was performed with 10 m*M* imidazole (A) and 20 m*M* imidazole in 50 m*M* sodium acetate buffer (B). Flow velocity 20 cm/h. Fraction volume 8.6 ml.

Based on the results obtained it may be concluded that the selectivity of IMAC is lower if a stronger eluent was used. Thus, the purity of STH was 90–93% when the desorption was achieved with the sodium acetate buffer at pH 5.3–6.0 (see Table 3, position 1–3) and the purity of 80–87% was obtained when the pH 4.5, or 20 m*M* imidazole in the same buffer was used for elution (Table 3, position 4–6).

The capacity of the chelating sorbents Cu(II) for STH depends on ligand density and was in the range 2.2–3.5 mg/ml.

The chromatography of crude STH (purity 10%) was performed on Chelat-Granocel–Cu(II) with ligand content 17 μmol/ml. The result (Fig. 7) indi-

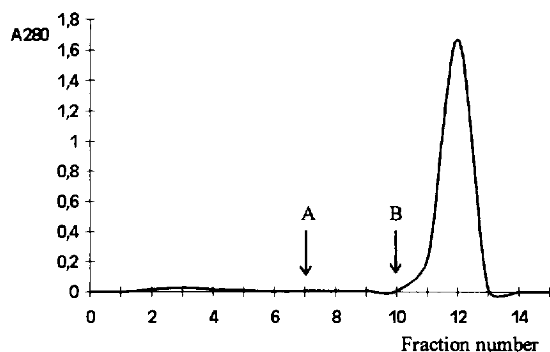

Fig. 5. Chromatography of STH on Chelat-Granocel–Cu (II). Ligand content 26 μmol/ml. The chromatography was performed on a column 6.5×1.0 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.7. 20 ml of partly purified (77%) STH containing 1.2 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0. Elution was performed with 50 m*M* sodium acetate buffer, pH 4.5 (A) and 20 m*M* imidazole (B). Flow velocity 20 cm/h. Fraction volume 12 ml.

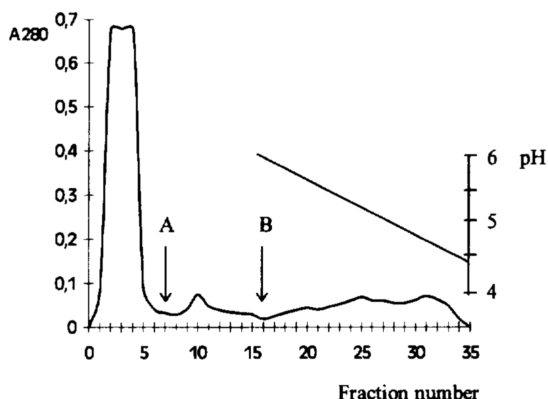

Fig. 7. Chromatography of crude STH on Chelat-Granocel–Cu (II). Ligand content 17 μmol/ml. The chromatography was performed on a column 10×1.6 cm I.D. in 0.5 *M* NaCl in 10 m*M* sodium phosphate buffer, pH 7.8. 110 ml of ammonium sulphate pellet (concentration of STH-10%) containing 2.7 mg of proteins per 1 ml of sample was applied onto the column. The column was washed with 50 m*M* sodium acetate buffer, pH 6.0 (A). Elution was performed by pH gradient (6.0–4.3) in 50 m*M* sodium acetate buffer (B). Flow velocity 20 cm/h. Fraction volume 50 ml.

cated very high selectivity. This single chromatography step gave a purity of 79.5% with a yield of STH of 55%.

4. Conclusions

IMAC has been found to be a very selective technique for the separation of human growth hormone, somatotropin. Chromatography in one step on Chelat-Granocel–Cu(II) gives better than 80% purification of the protein.

Retention of the protein on chelating sorbent depends highly on ligand density. Therefore protein elution conditions may be optimized by proper choice of the ligand concentration on the surface of the sorbent.

The ligand density of chelating adsorbents can be easily adjusted by (i) altering the amount of IDA in the reaction mixture or (ii) by changing the content of epoxy groups in activated matrix.

The sorbent of high ligand density is preferable for the initial protein purification step whereas the sorbent of low ligand density is more suitable for deep purification of the protein.

A better understanding of the effect of ligand density on the retention of proteins, could make IMAC procedures less costly and might considerably extend the use of IMAC at process scale.

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