Journal of Chromatography, 472 (1989) 422–427
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 428

Note

Immobilized metal-ion affinity chromatography of human growth hormone[a]

FEDERICO MAISANO, SILVIA ASTRUA TESTORI and GUIDO GRANDI*
Department of Genetic Engineering, ENIRICERCHE SpA, Via S. Salvo 1, 20097 S. Donato Milanese, Milan (Italy)

(First received November 22nd, 1988; revised manuscript received January 19th, 1989)

Affinity chromatography on immobilized metal ions (IMAC) is a separation technique that takes advantage of the different capabilities of proteins of interacting with metal ions mainly through their exposed histidine residues. The metal ions are immobilized on suitable supports through a chelating ligand, usually iminodiacetate, which is covalently bound to the matrix. This technique was introduced by Porath *et al.*[1] in 1975 and since then it has been adopted for the purification of many proteins[2]. Recent advances with this method include the semipreparative purification of proteins from cell culture filtrates[3] and high-performance applications[4].

We have studied the behaviour of the recombinant human growth hormone (hGH) when subjected to IMAC and found this technique to be particularly useful for hGH purification. Further, by comparing our results with those observed for some model proteins[5], we have attempted to draw some conclusions about the number and location of histidine residues exposed on the molecule surface.

EXPERIMENTAL

Sepharose 6B (Pharmacia) was activated with epichlorohydrin and coupled with iminodiacetic acid according to Porath and Olin[6]. This iminodiacetate–agarose (IDA–agarose) was used for all IMAC experiments. Biosynthetic human growth hormone was cloned and purified in our laboratory from *B. subtilis* cells[7]. Metal chlorides ($CuCl_2$, $NiCl_2$, $CoCl_2$ and $ZnCl_2$) and imidazole were from E. Merck. Diethylpyrocarbonate (DEP) was purchased from Aldrich. All other chemicals were of analytical-reagent or reagent grade.

All chromatographic experiments were performed at 4°C in an 8-ml column (10 × 1 cm I.D.); a post-column of 3 ml of the same IDA–agarose was used to trap leaking metal ions. The column was loaded with the appropriate metal ion, using 50 ml of a 50 m*M* solution of the respective chlorides in water and making it recirculate for several hours, usually overnight. It was then equilibrated with 50 m*M* sodium

[a] Part of this work was presented as a poster at the *4th International Symposium on Separation Science and Biotechnology, Gargnano del Garda, Italy, August 31st–September 3rd, 1988.*

0021-9673/89/$03.50

phosphate (pH 7)–0.8 *M* sodium chloride (buffer A) and connected to the post-column, equilibrated with the same buffer. The high concentration of sodium chloride is used to prevent any ion-exchange adsorption of proteins. The flow-rate was maintained at 12 ml/h throughout. After loading the sample (1–1.5 ml of 0.5 mg/ml hGH), the column was washed with 30 ml of buffer A; elution was accomplished either with a pH gradient to pH 5 (50 m*M* acetate buffer–0.8 *M* sodium chloride) or with an imidazole concentration gradient from 0 to 30 m*M* in buffer A. With the IDA–Cu^{2+} column, the pH gradient went from 7 to 4 and then elution was continued with 1 *M* glycine adjusted to pH 8 with 1 *M* sodium hydroxide solution.

DEP modification of histidyl residues was performed according to Miles[8] in 20 m*M* phosphate buffer (pH 6) and followed in a Perkin-Elmer 551S UV–VIS spectrophotometer by recording difference spectra between 320 and 237 nm every fifth minute after the addition of DEP; the increase in absorbance at 242 nm corresponds to the amount of modified histidyl groups ($\varepsilon = 3200\ l\ mol^{-1}\ cm^{-1}$). Concentrations of 0.025 m*M* hGH and 0.25 m*M* DEP were used. Removal of the carbethoxy group from modified histidines was achieved by treatment with 20 m*M* hydroxylamine for 30 min.

RESULTS

hGH binding to different metal–agarose columns

We subjected biosynthetic hGH to IMAC using four different metal ions chelated to an IDA–agarose. According to what is considered to be a general rule, the extent of adsorption varies with the metal in use. These results are summarized in Fig. 1, in which the four chromatograms developed under the same conditions, except for the metal, are superimposed. The strongest retention is observed when the IMA

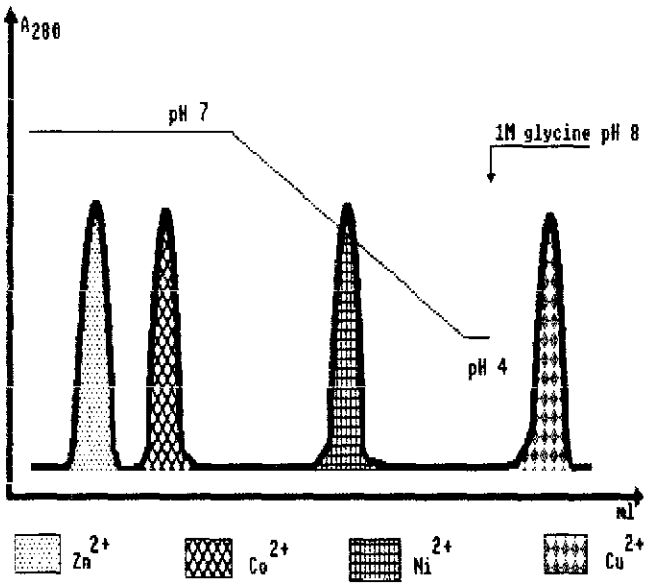

Fig. 1. Superimposition of IMA chromatograms of hGH using different metals.

column is loaded with Cu^{2+}, where the pH gradient does not elute the protein and a stronger displacer, such as 1 *M* glycine, is required. A weaker adsorption is exerted by Ni^{2+} ions and hGH can be eluted by decreasing the pH from 7 to about 5. Cobalt ions interact slightly with hGH molecules which are only retarded, but the technique may still be useful for isocratic enrichment of hGH samples. In contrast, zinc ions do not appear to have any effect and the protein is eluted in the void volume. Owing to this variety of possible behaviour, suitable conditions for IMAC purification of hGH can be chosen according to the kind of contaminants, but the most suitable adsorbent is probably IDA–Ni^{2+} gel. Fig. 2 shows the results of IMAC purification of a partially purified hGH, using an IDA–Ni^{2+} column eluted with a 2-h gradient from 0 to 30 m*M* imidazole in buffer A. Before IMAC, the crude cellular extract was purified only by ion-exchange chromatography on DEAE-cellulose. Therefore, a purity of over 90% is achieved in two steps, with no need for any sample preparation before IMAC except for the addition of sodium chloride up to 0.8 *M*.

DEP treatment

In view of the fact that in IMAC the interaction is believed to take place mainly through histidine residues, we prepared some modified hGH by attacking carbethoxy groups to the histidines. This strategy was followed by Al-Mashikhi and Nakai[9], who showed a decreased adsorption of carbethoxylated ovotransferrin on IMAC columns.

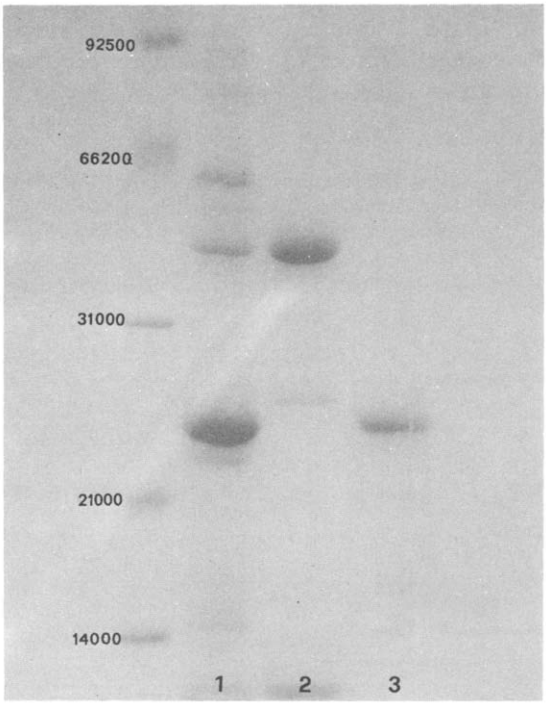

Fig. 2. Purification of hGH on IDA–Ni^{2+} gel. Lane 1 = partially purified hGH; lane 2 = unretained fraction; lane 3 = hGH eluted with 0–30 m*M* imidazole gradient. Numbers on vertical scale indicate molecular weights.

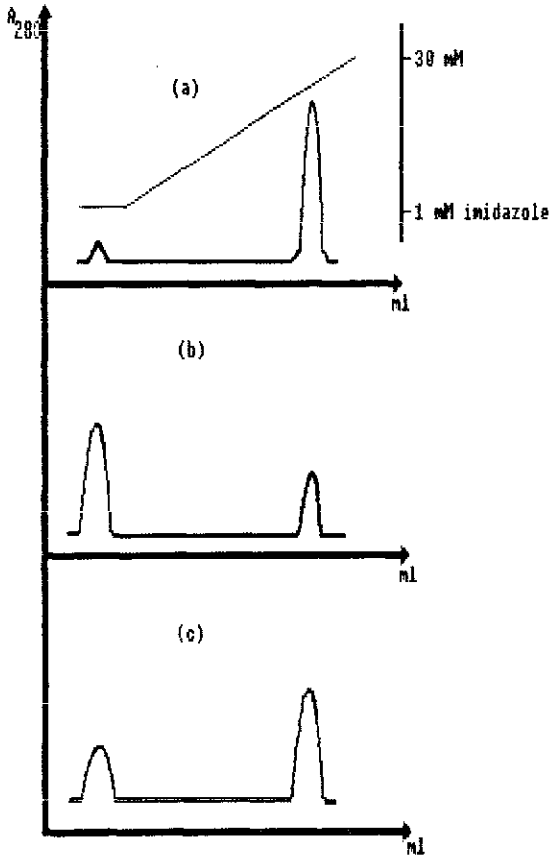

Fig. 3. IDA–Cu^{2+} chromatograms of modified hGH. (a) Unmodified hGH; (b) DEP-modified hGH; (c) DEP–hGH after removal of carbethoxy groups.

Diethyl pyrocarbonate (DEP) was ten times more concentrated than hGH, but we observed the modification of only about two of the three hGH histidines; this can be attributed to the poor accessibility of the third histidine. When subjected to IDA–Cu^{2+} chromatography, the amount of carbethoxylated hGH retained was only 30%, and it could be recovered with an imidazole concentration gradient. This residual adsorption was probably due to incomplete carbethoxylation of exposed histidines. Proper adsorption was almost fully restored by removing the carbethoxy groups by treatment with hydroxylamine (Fig. 3).

DISCUSSION

IMAC is a very flexible technique because the degree of retention can be modulated not only by changing external parameters such as pH, ionic strength or concentration of competitive molecules, but also by varying the intrinsic strength of the adsorbent, using different metal ions. Among the metals explored, nickel appears

to be the best candidate for hGH purification, owing to its moderate adsorption. hGH can be released from IMA–Cu^{2+} column by a decrease in pH or an imidazole concentration gradient. The results are satisfactory in terms of selectivity and degree of purity, as in this work where none of the traditional low-pressure chromatographic techniques (cation-exchange, hydrophobic interaction, size-exclusion chromatography) could achieve the same degree of purification (data not shown).

According to the proposed mechanism for IMAC interaction[5], the number of histidine residues and their location on the protein molecule should determine the extent of adsorption in this kind of chromatography, and other amino acids, such as tryptophan and cysteine, could play a secondary role. Our carbethoxylation studies of the histidine residues in hGH suggest that only two of the three histidines present in the molecule are exposed to the solvent and therefore readily accessible. The hydropathic profile[10] of the protein, which shows that His-151 is located within a highly hydrophobic region whereas His-18 and His-21 are present in a more hydrophilic environment, indicates that His-151 is the least reactive histidine. This prediction is consistent with the work of Fukushima *et al.*[11], who found that in bovine growth hormone, a protein which shares 66% homology with hGH, His-19 and His-21 are exposed to the solvent.

Predictions based on the study of model proteins suggest that protein retention by Zn^{2+} and Co^{2+} takes place when two proximal histidines located in an α-helical structure are present in the molecule[5]. Although proximal histidines are present in hGH (His-18 and His-21), the molecule does not bind to such sorbents. A plausible conclusion is that the two residues are in a conformational status that does not constitute a strong binding site for the metals.

It is interesting that a recently published three-dimensional model of hGH[12] predicts that His-18 and His-21 are not located in an α-helical structure. If this is so, the two histidines are not likely to behave as "proximal" in metal binding. On the other hand, analysis according to Chou and Fasman[13] does predict an α-helical conformation between residues 11 and 21 in hGH.

We tend to favour the prediction of the three-dimensional model on the basis of the consideration that α/α proteins such as hGH could not be particularly suitable for Chou and Fasman analysis. For example, this analysis would also predict about a 17% β-structure conformation in hGH, in contrast with the circular dichroism spectrum of hGH[14] which indicates only the presence of α-helices.

In conclusion, IMAC adsorption of hGH is consistent with the model of a protein containing two histidine residues that interact with the metals bound to the matrix in a non-cooperative fashion. The third histidine of the molecule (His-151) probably does not take part in the interaction, even with the strong IDA–Cu^{2+} gel for which a single exposed histidine would determine the binding.

To our knowledge, this is the first attempt to elucidate the topological distribution of histidine residues in hGH by means of IMAC and, as has already been envisaged by Sulkowski[5], this could be a major application of the technique in the future.

REFERENCES

1 J. Porath, J. Carlsson, I. Olsson and G. Belfrage, *Nature (London)*, 258 (1975) 598.
2 B. Lonnerdal and C. L. Keen, *J. Appl. Biochem.*, 4 (1982) 203.

3 S. Krishnan, M. A. Vijayalakshmi and I. Geohel, *J. Chromatogr.*, 397 (1987) 339.
4 M. Belew, T. T. Yip, L. Andersson and R. Ehrnström, *Anal. Biochem.*, 164 (1987) 457.
5 E. Sulkowski, *Trends Biotechnol.*, 3 (1985) 1.
6 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621.
7 E. Franchi, F. Maisano, S. Astrua Testori and G. Grandi, in preparation.
8 E. W. Miles, *Methods Enzymol.*, 43 (1977) 431.
9 S. A. Al-Mashikhi and S. Nakai, *Agric. Biol. Chem.*, 51 (1987) 2881.
10 J. Kyte and R. F. Doolitle, *J. Mol. Biol.*, 157 (1982) 105.
11 J. G. Fukushima, M. J. Biscoglio De Jimenez Bonino, O. Cascone and J. A. Santomé, *Int. J. Pept. Protein Res.*, 21 (1983) 451.
12 F. E. Cohen and I. D. Kuntz, *Proteins: Structure, Function and Genetics*, 2 (1987) 162.
13 P. J. Chou and G. D. Fasman, *Adv. Enzymol.*, 47 (1987) 45.
14 L. A. Holladay, R. G. Hammonds, Jr. and D. Puett, *Biochemistry*, 13 (1974) 1653.