*Journal of Chromatography*, 490 (1989) 193–197
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4676

## Note

# Elimination of contaminating *Escherichia coli* peptides in the purification of *Escherichia coli*-derived recombinant human interferon-$\beta$1 by zinc chelate affinity chromatography

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(First received October 31st, 1988; revised manuscript received January 4th, 1989)

Immobilized metal affinity chromatography (IMAC) was introduced by Porath et al. [1] and has been widely used for the purification of more than twenty proteins [2]. With the advent of polypeptide pharmaceuticals by recombinant DNA techniques, IMAC would be very useful for the purification of recombinant polypeptides produced in *Escherichia coli* cells. These polypeptides should be purified to greater than 99% purity by the removal of contaminating *E. coli* peptides (ECP) for clinical use.

We improved $Zn^{2+}$ chelate affinity chromatography for application to the purification of *E. coli*-derived recombinant human interferon-$\beta$1 (*E. coli*-rHuIFN-$\beta$1) as previously described [3]. In order to eliminate ECP from the purified preparation, we examined the chromatographic behaviour of *E. coli*-rHuIFN-$\beta$1 and ECP on a $Zn^{2+}$ chelate column. We found that the spacer length of immobilized metal gels affects the adsorption of contaminating ECP to the gels.

In this paper, we describe the contribution of the spacer length on $Zn^{2+}$ chelate column to the elimination of contaminating ECP from the purified *E. coli*-rHuIFN-$\beta$1 preparation.

0378-4347/89/$03.50 

EXPERIMENTAL

*E. coli-rHuIFN-β1 preparation*

Crude *E. coli*-rHuIFN-β1 was prepared as previously described [3]. The recombinant protein was partially purified by chromatography as follows: crude *E. coli*-rHuIFN-β1 was applied to a silica beads column (Macro Bead Silica Gel, 500 Å, 30–60 mesh, Fuji-Devison Chemical, Tokyo, Japan) at a density of 50 mg protein per ml gel. After washing with phosphate-buffered saline (pH 7.4) and 20 m*M* phosphate buffer (PB) (pH 7.4) containing 1 *M* NaCl, proteins were eluted with 20 m*M* PB (pH 7.4) containing 1 *M* NaCl and 50% ethylene glycol (EG). The eluate was applied to the $Zn^{2+}$ chelate column.

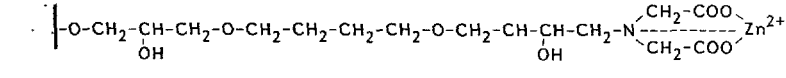
*$Zn^{2+}$ chelate gel preparation*

One of metal-chelating gels was prepared by using epichlorohydrine and iminodiacetic acid according to Hubert and Porath [4]. The other gel, which was activated by 1,4-bis(2,3-epoxypropoxy)butane and iminodiacetic acid [1], Chelating Sepharose CL-6B gel, was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). The two gels have, respectively, $C_7$ alkyl chains ($C_7$) and $C_{14}$ alkyl chains ($C_{14}$) as their spacers. The $Zn^{2+}$-chelating capacities of $C_7$- and $C_{14}$-chelating gels were 22.4 and 22.6 μmol/ml of wet gel, respectively. The structures of those ligands are represented in Fig. 1.

*Analytical methods*

IFN activity was measured by the method of cytopathic effect inhibition [5] using human FL-Amnion cells and vesicular stomatitis virus. Protein determination was performed according to Bradford [6], using bovine serum albumin as a standard protein. ECP were determined by the radioimmunoassay [7] developed using $^{125}I$-labelled anti-ECP rabbit antibody. The antibody was raised against ECP purified by chromatography on silica and was radiolabelled with chloramine T after purification with an ECP-immobilized affinity column.

**a**




**b**

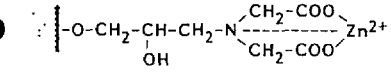



Fig 1. Structures of ligands for $Zn^{2+}$ chelate affinity chromatography: (a) coupled with 1,4-bis(2,3-epoxypropoxy)butane and iminodiacetic acid; (b) coupled with epichlorohydrine and iminodiacetic acid.

RESULTS

Partially purified *E. coli*-rHuIFN-$\beta$1 was applied to each $Zn^{2+}$ chelate column (2.5 cm×1.6 cm I.D.), with $C_{14}$ and $C_7$ spacers, respectively. After the protein had been loaded, the columns were independently chromatographed essentially according to Heine et al. [8]. The chromatographic profiles are shown in Fig. 2. The $Zn^{2+}$–$C_7$ column leaked a larger amount of protein in

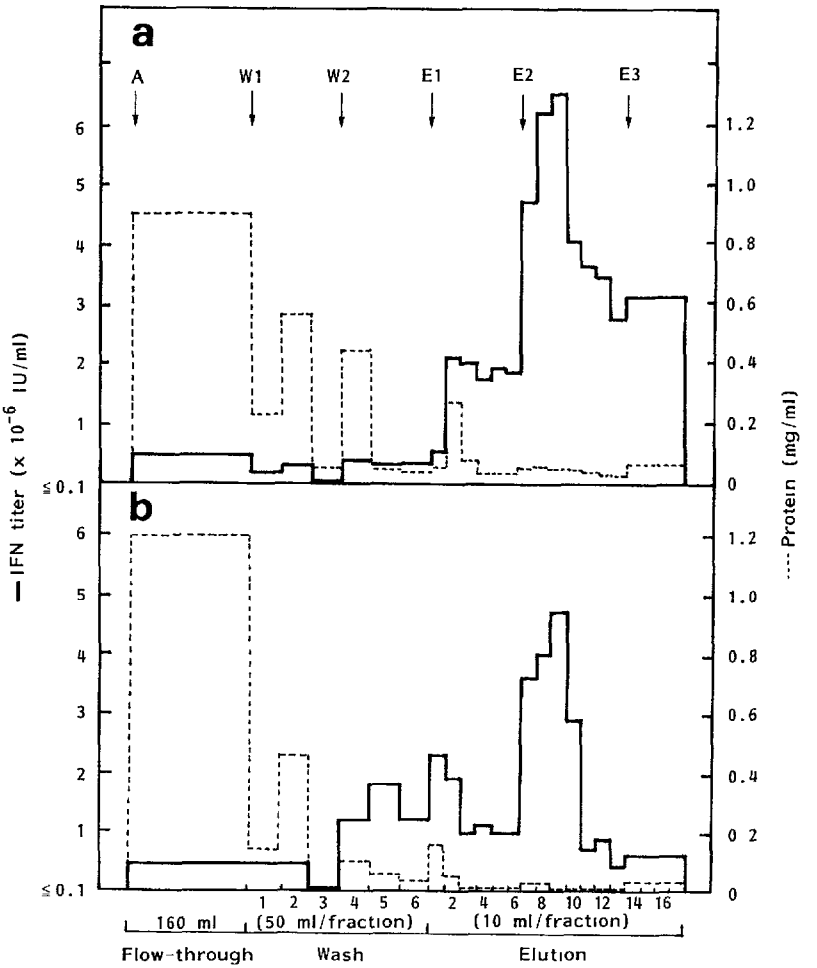



Fig. 2. Profiles of *E coli*-rHuIFN-$\beta$1 in $Zn^{2+}$ chelate affinity chromatography: (a) $Zn^{2+}$-$C_{14}$ column chromatography; (b) $Zn^{2+}$-$C_7$ column chromatography. Eluents: (A) apply partially purified *E. coli*-rHuIFN-$\beta$1 in 20 m*M* PB (pH 7.4) containing 1 *M* NaCl and 50% EG; (W1) 20 m*M* PB (pH 7.4) containing 1 *M* NaCl and 50% EG; (W2) 0.1 *M* acetate buffer (AB) (pH 5.6) containing 0.5 *M* NaCl; (E1) 0.1 *M* AB (pH 5.0) containing 0.5 *M* NaCl; (E2) 0.1 *M* AB (pH 4.0) containing 0.5 *M* NaCl; (E3) 0.1 *M* histidine (pH 7.0) containing 50% EG.

TABLE I

PURIFICATION OF HuIFN-$\beta$1 WITH $Zn^{2+}$-$C_{14}$ AND $Zn^{2+}$-$C_7$ COLUMNS

| Step | $Zn^{2+}$-$C_{14}$ | $Zn^{2+}$-$C_7$ |
|---|---|---|
| *Applied* | | |
| Protein (mg) | 256 (100%) | 256 (100%) |
| IFN ($\cdot 10^{-6}$ I.U.) | 720 (100%) | 720 (100%) |
| Specific activity (I.U./mg) | $2.8 \cdot 10^6$ | $2.8 \cdot 10^6$ |
| *Adsorbed* | | |
| Protein (mg) | 112 (44%) | 64 (25%) |
| IFN ($\cdot 10^{-6}$ I.U.) | 643 (89%) | 645 (90%) |
| *Washed* | | |
| Protein (mg) | 75 (29%) | 43 (17%) |
| IFN ($\cdot 10^{-6}$ I.U.) | 172 (24%) | 343 (48%) |
| *Eluted* | | |
| Protein (mg) | 2.9 (1.1%) | 1.1 (0.4%) |
| IFN ($\cdot 10^{-6}$ I.U.) | 310 (43%) | 172 (24%) |
| Specific activity (I.U./mg) | $1.1 \cdot 10^8$ | $1.6 \cdot 10^8$ |
| ECP (%) | 1.36 | 0.54 |

flow-through and wash fractions than the $Zn^{2+}$-$C_{14}$ column. This suggests that the adsorption and the binding strength are stronger on the latter column.

However, as shown in the material balances in Table I, the quality of purified *E. coli*-rHuIFN-$\beta$1 preparation obtained from the $Zn^{2+}$-$C_7$ column is superior to that from the $Zn^{2+}$-$C_{14}$ column. The specific activity of the preparation purified on the $Zn^{2+}$-$C_7$ column is slightly higher than the value obtained from the $Zn^{2+}$-$C_{14}$ column, and the contaminating ECP content in the preparation from the $Zn^{2+}$-$C_7$ column is reduced to 40% of that from the $Zn^{2+}$-$C_{14}$ column. These results indicate that the shorter spacer ($C_7$) contributes to the elimination of contaminating ECP from the purified *E. coli*-rHuIFN-$\beta$1 preparation.

## DISCUSSION

The interactions between ligand and protein in IMAC have been extensively examined by Sulkowski [9], who has explained that those interactions individually depend on intrinsic characteristics of proteins, except for the principal interaction that occurs between the metal and the amino acid residues such as histidine, cysteine and tryptophan. We, furthermore, clarified the effect of the spacer length in the purification of *E. coli*-rHuIFN-$\beta$1 by IMAC.

As the spacer is formed by alkyl chains, which are hydrophobic, the non-specific binding of some hydrophobic ECP to the spacer is postulated. How-

ever, in our preliminary study, metal-free chelating gels adsorbed a negligible amount of protein (unpublished results). Comparison of the behaviour of proteins during $Zn^{2+}$–$C_{14}$ and $Zn^{2+}$–$C_7$ column chromatography showed that the spacer length contributes to the adsorption and the binding strength of proteins to ligand, resulting in the elimination of the contaminating ECP from the purified *E. coli*-rHuIFN-$\beta$1 preparation (Fig. 2 and Table I). These observations must be due to the interactions between the spacers and the proteins. In the case of a short spacer, steric hindrance may well occur when a protein binds to the ligand. The $C_7$ and $C_{14}$ spacer lengths are roughly estimated from their structure models as ca. 7–9 and ca. 15–20 Å, respectively. On the basis of the relationship between the Stokes radius and molecular mass of the globular protein [10], the radii of 7–9 and 15–20 Å roughly correspond to molecular masses of proteins of ca. 2000 and ca. 20 000, respectively. Therefore, the degree of the potential steric hindrance in the matrix of $Zn^{2+}$–$C_7$ gels may be larger than that in $Zn^{2+}$–$C_{14}$ gels in the case of the adsorption of high-molecular-mass proteins. It may result in the elimination of a large amount of ECP from the purified preparation. Indeed, most ECP are greater than the *E. coli*-rHuIFN-$\beta$1 molecule (19 000), as observed from the stained protein bands on a sodium dodecyl sulphate polyacrylamide gel, as previously reported [3], so ECP were effectively removed from the preparation.

Thus, $Zn^{2+}$–$C_7$ column chromatography is useful for the purification of *E. coli*-derived recombinant proteins, because the removal of ECP is essential owing to the antigenicity of ECP against human in clinical use.

In conclusion, the consideration of the spacer length affords a good tool to the refinement of the behaviour of proteins in IMAC.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. S. Kimura for ECP assay and helpful discussion and to Dr. A. Kitai for encouragement.

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