Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 808 (2004) 57–62
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Immobilized metal-ion affinity chromatography of human antibodies and their proteolytic fragments

Daniela Todorova-Balvay [a], Olivier Pitiot [a,1], Mustapha Bourhim [a], Thamarapu Srikrishnan [b], Mookambeswaran Vijayalakshmi [a,*]

[a] *Laboratoire d'Interactions Moléculaires et de Technologies de Séparation (LIMTechS), Centre de Recherche de Royallieu, BP 20 529, Université de Technologie de Compiègne, Compiègne, France*
[b] *Department of Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Elm& Carlton Streets, Buffalo, NY 14263, USA*

Abstract

Immobilized metal-ion affinity chromatography (IMAC) performed with four different transition metal ions: copper(II), nickel(II), zinc(II) and cobalt(II), was used to study the adsorption properties of human polyclonal γ-globulines (IgG), Cohn II–III fractions, and their pepsin cleaved fragments: $F(ab')_2$ and F′c. In each case, digested products showed lower affinity for metal ions, as well by decreasing pH elution as by competition with imidazole. An explanation was proposed by the presence of a histidine (His) cluster in the F′c domain of IgGs, identified by computer calculation (accessible surface area (ASA) determination) as the more probable His 433-x-His 435 sequence presented in the CH_3 domain of human IgG heavy chain. As shown by IMAC and electrophoresis, F′c and undigested IgG have higher affinity for transition metal ions than $F(ab')_2$ fragments and could be then separated in one step by IMAC. When chelated Zn(II) or Co(II) are used as ligands, the $F(ab')_2$ fragment could be easily recovered under mild conditions (pH 7) in the non-retained fraction. This approach could be used as a powerful alternative to conventional protein A/G methods for the commercial preparation of non immunogen active $F(ab')_2$ fragments.

Keywords: Immobilized metal-ion affinity chromatography; Immunoglobulins G; $F(ab')_2$ fragment; F′c fragment; Histidine

1. Introduction

Human serum or monoclonal immunoglobulins (IgG) (generally obtained from mouse ascites fluid or from hybridoma cell culture supernatant) are of great interest for the therapeutic applications (antiserum preparation, autoimmune and autocatalytic antibodies) and for biological diagnostics (immunotest, ELISA assays). These numerous applications require efficient purification tools in regard to the quantity as well as to the integrity (activity) of the isolated immunoglobulins.

Different chromatographic methods, generally preceded by precipitation step have been developed for the purification and functional studies of IgG [1]. Various protocols, including size-exclusion [2], ion exchange [3], hydroxyapatite [4] and affinity [5] have advantages and limitations. For example, ion exchange chromatography, which is probably the most employed method, does not display sufficient selectivity and additional steps, such size-exclusion separation are necessary to achieve purification. Biological specific ligands, e.g. proteins A and G, seem to be more appropriate for IgG recovery with high purification factor. However, the protein nature of these high cost ligands, limits their large scale applications because of possible cleavage and/or denaturation during sanitization-in-place (SIP) and cleaning-in-place (CIP) industrial processes. Moreover, the affinity constants of these ligands are very high and elution step includes denaturing conditions which can lead to loss of activity of antibodies [6].

In order to overcome these different problems, low molecular weight ligands were introduced and developed. Vijayalakshmi [7] reviewed them under the term of "pseudobioaffinity". Initially reviewed by this author they are amino acids, such as tryptophan (Trp) and histidine (His), metal chelates and dyes. This ligand family could be

* Corresponding author. Tel.: +33-3-4434404; fax: +33-3-44204813.
E-mail address: viji@utc.fr (M. Vijayalakshmi).
[1] Present address: Aventis Pasteur, 1541 av. Marcel Mérieux, 69280 Marcy l'Etoile, France.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.05.034

extended to thiophilic ligands and small mimicry peptides produced by combinatorial library.

Pseudobiospecific ligands have been successfully demonstrated to be an interesting alternative to conventional chromatographic ligands, especially for antibody separation [8–12].

Among the pseudobiospecific ligand family, immobilized metal-ion affinity chromatography (IMAC), introduced by Porath et al. [8] is a very efficient tool for biomolecules purification as well as for studing the surface topography of proteins in terms of accessibility of their His residues [13–17]. IgG separation from sera or mouse ascites fluid using IMAC was previously demonstrated [8,18–20].

In this work, we performed IMA chromatographic separation of total human IgGs, polyclonal antibodies obtained from commercial Cohn fraction II–III and their pepsin cleaved fragments ($F(ab')_2$ and $F'c$). Four different transition metals(II) – Copper (Cu(II)), Nickel (Ni(II)), Zinc (Zn(II)) and Cobalt (Co(II)) were used in order to separate $F(ab')_2$ and $F'c$ fragments and study the His accessibility in human antibodies according to the ground recognition rules in IMAC advanced by Sulkowski [21]. Moreover, molecular modelling was applied in order to determine the accessibility of His residues.

2. Experimental

2.1. Materials

Acrylamide, ammonium persulfate, $CoSO_4{\cdot}5H_2O$, $CuSO_4{\cdot}5H_2O$, ethylenediamine tetraacetate disodium salt (EDTA), human γ-globulins from Cohn fractions II and III (Lot No. 76H9339), glycine, morpholino ethane sulfonic acid (MES), morpholino propane sulfonic acid (MOPS), *N*,*N*′-methylenebisacrylamide, $NiSO_4{\cdot}5H_2O$, sodium acetate, sodium chloride, sodium dodecyl sulfate (SDS), tetramethyl ethylene diamine (TEMED), $ZnSO_4{\cdot}5H_2O$ were all purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade. Ultrapure water, obtained from the Milli RO-Milli Q System (Millipore, Bedford, CT, USA), was used for the preparation of all solutions.

2.2. Pepsin digestion

Pepsin digestion was performed by incubating human IgGs (Cohn fraction II–III) with 18 units of pepsin per mg of protein in 200 mM glycine–HCl, pH 3.6 at 37 °C for 6 h. Digestion was stopped by raising the pH to 7 with 1 M NaOH. Digestion was controlled by electrophoresis and by 5% trichloracetic acid (TCA) precipitation of aliquots (100 μl) collected at different times. Buffer exchange for IMAC procedure of digested products was performed by using PD-10 Sephadex G-25 column (Amersham Biosciences, Sweden).

2.3. Chromatographic procedures

Two millilitres of chelating Sepharose Fast Flow (Amersham Biosciences, Sweden) (Iminodiacetate-Sepharose (IDA-Sepharose)) were packed in 10 cm length × 1 cm i.d. column. Experiments were carried out using Biologic system from Bio-Rad (Richmond, CN, USA). Transition metals – Cu(II), Ni(II), Zn(II) and Co(II) were loaded at flow rate of 0.5 ml min^{-1}. Non-specific bound metal ions were removed by five column volumes (CV) of water followed by five CV wash with the most acidic buffer according to the metal used in IMAC. Then, the gel was equilibrated with at least 10 CV of adsorption buffer. Mixture of 25 mM MOPS, 25 mM MES and 25 mM sodium acetate, 1 M NaCl (MMA buffer) was used for pH elution protocol according to Mrabet (1992). When competitive eluent imidazole was used, 1 mM was added to the equilibration buffer 25 mM MOPS, pH 7.2, 1 M NaCl in order to prevent the proton pump phenomenon as described by Sulkowski [22]. Tightly bound proteins were eluted by the final wash with five CV of strong chelating agent EDTA. Three minutes fractions (1.5 ml) were collected and protein content was determined by Bradford method [23], using crystalline bovine serum albumin as standard. All experiments were run in triplicate.

2.4. SDS-PAGE analysis

Chromatographic fractions were analysed by 10% SDS-PAGE gels under non-reducing conditions (sampling buffer without the reducing agent β-mercaptoethanol). Gels were silver stained [24].

2.5. Molecular modelling

Computer calculations of accessible surface area (ASA) of His residues in of Fc domain of human IgG1 (1 fcc) were performed using XPLOR [25] with a probe radius of 1.93 Å, mimicking IDA-Cu(II) [16].

3. Results

3.1. IMAC behaviour of human IgGs

Twenty milligrams of human IgGs (Cohn fractions II–III) was applied on chelating Sepharose Fast Flow columns loaded with four different metals – Cu(II), Ni(II), Zn(II) or Co(II). Discontinuous pH gradient was used in order to explore the chromatographic behaviour of IgGs (Figs. 1A–4A). Protein adsorption was different depending on the metals used. IgGs were strongly retained on chelated Cu(II) and not eluted with the protonation gradient from 6 to 4. They were recovered with the strong chelating agent EDTA, 50 mM. When Ni(II), Zn(II) or Co(II) chelates were used, affinity of IgGs decreased. Moreover, the affinity of IgGs for chelated Co(II) is lower compared to the other

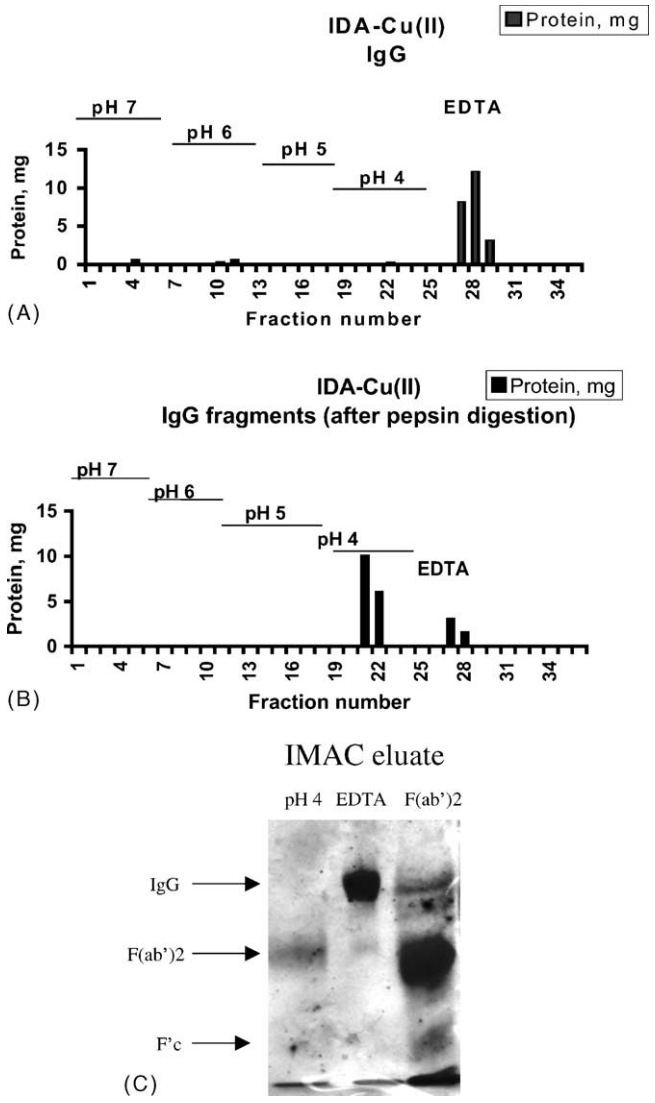

Fig. 1. IDA-Cu(II) separation of (A) IgG and (B) their proteolytic fragments $F(ab')_2$ and F′c and (C) SDS-PAGE control of separation of mixture of purified $F(ab')_2$ (pH 4) and entire IgGs (EDTA); $F(ab')_2$ – fraction after pepsin digestion. Chromatography conditions: injection: 20 mg of human IgG or 20 mg of the mixture of proteolytic fragments $F(ab')_2$ and F′c; column volume: 2 ml chelating Sepharose Fast Flow; flow rate: 0.5 ml h^{-1}, decreasing pH gradient from pH 6 to 4.

three metals – they are adsorbed but eluted under slightly acidic conditions. The affinity of IgGs for these four metals, Cu > Ni > Zn > Co, is as predicted by the conventional recognition rules in IMAC [21].

Moreover, we showed by IMAC using Ni(II), Zn(II) and Co(II) chelates, a heterogeneity in human IgGs. Two species, probably pertaining to different IgG sub-classes, were recovered by both discontinuous (Figs. 2A–4A) and linear decreasing pH gradients (data not shown). IMAC has already been reported to be an efficient tool for separation of closely related structures as sub-species, isoforms, etc. [17] and this method can contribute to distinguish between IgG sub-classes due to differences in accessibility/microenvironment of their His residues.

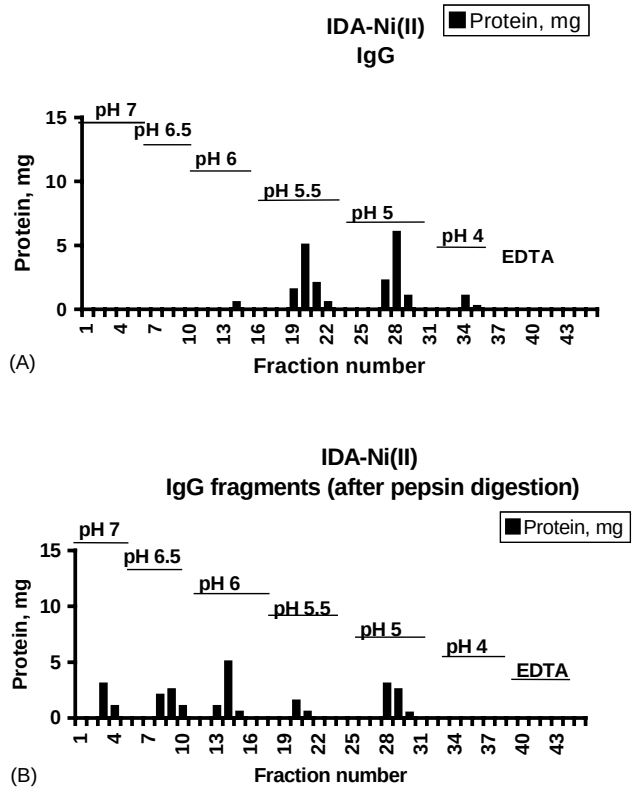

Fig. 2. IDA-Ni(II) separation of (A) IgG and (B) their proteolytic fragments $F(ab')_2$ and F′c. Chromatography conditions: injection: 20 mg of human IgG or 20 mg of the mixture of proteolytic fragments $F(ab')_2$ and F′c; column volume: 2 ml chelating Sepharose Fast Flow; flow rate: 0.5 ml h^{-1}, decreasing pH gradient from pH 6 to 4.

3.2. IMAC separation of IgG proteolytic fragments

Pepsin digested IgGs fragments, $F(ab')_2$, have lower affinity for all transition metals compared to F′c fragment and entire IgGs (Figs. 1B–4B, B versus A). The same phenomenon was observed using competitive imidazole gradient (data not shown).

When IDA-Cu(II) chelate was used, chromatographic and electrophoretic controls revealed that IgGs were fully separated from their digested $F(ab')_2$ fragments (Fig. 1B). Moreover, after IMAC of equimolar mixture of IgG and $F(ab')_2$, both species could be efficiently separated on IDA-Cu(II) chelate (Fig. 1C). Thus, $F(ab')_2$ fragments recovered from chelated Cu(II) under acidic conditions (pH 4) followed by rapid pH adjustment to neutral, can be used for immunochemical reagents.

In the case of chelated Ni(II), we probably separated different $F(ab')_2$ fragments at pH 7.0, 6.5 and 6.0 due to their slight differences in structure in terms of His residues accessibility/microenvironment (Fig. 2B). This metal chelate could be useful for fine separation of $F(ab')_2$ fragments corresponding to different IgG sub-species.

Moreover, we demonstrated that $F(ab')_2$ fragments had no affinity for chelated Zn(II) and Co(II) whereas F′c fragment was eluted at pH 5.5 (Figs. 3B,C and 4B). According to

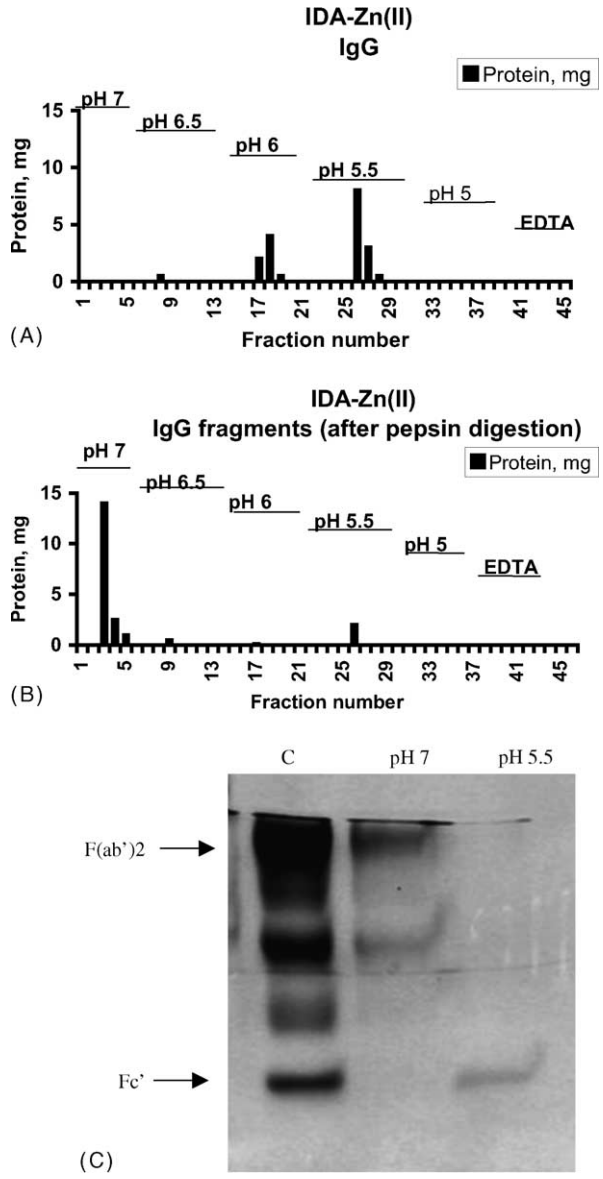

Fig. 3. IDA-Zn(II) separation of (A) IgG and (B) their proteolytic fragments $F(ab')_2$ and F′c and SDS-PAGE control of proteolytic fragments separation (C) C – IgG fragments after pepsin digestion; pH 7 – non-retained fraction; pH 5.5 –retained fraction. Chromatography conditions: injection: 20 mg of human IgG or 20 mg of the mixture of proteolytic fragments $F(ab')_2$ and F′c; column volume: 2 ml chelating Sepharose Fast Flow; flow rate: 0.5 ml h^{-1}, decreasing pH gradient from pH 6 to 4.

the ground rules in IMAC [21], the retention of F′c on both Zn(II) and Co(II) chelates is probably due to the presence of accessible His cluster in its tridimentional structure. Therefore, these two metal chelates are potentially interesting for fast recovery of the $F(ab')_2$ fragments under mild conditions which is very useful for the preparation of immunochemical reagents.

3.3. Molecular modelling of F′c fragment

F′c fragment of IgG was found to have stronger affinity for four chelated metals compared to $F(ab')_2$ (Figs. 1B–4B,

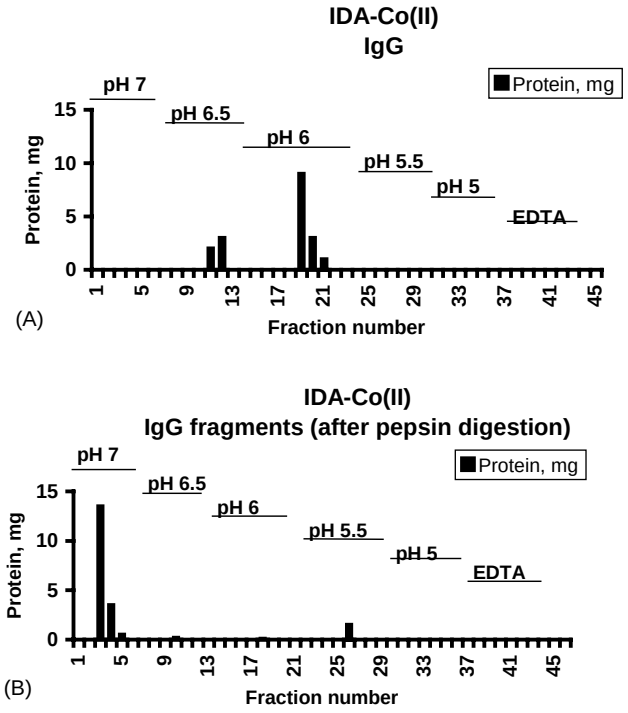

Fig. 4. IDA-Co(II) separation of (A) IgG and (B) their proteolytic fragments $F(ab')_2$ and F′c. Chromatography conditions: injection: 20 mg of human IgG or 20 mg of the mixture of proteolytic fragments $F(ab')_2$ and F′c; column volume: 2 ml chelating Sepharose Fast Flow; flow rate: 0.5 ml h^{-1}, decreasing pH gradient from pH 6 to 4.

B versus A). The difference in affinity is really obvious when Zn(II) and Co(II) chelates were used – F(ab′)2 fragment was not-retained at pH 7 whereas F′c fragment was retained and eluted at pH 5.5 (Figs. 3B and 4B, B versus A). According to the recognition rules in IMAC [21], a special His cluster structure which should be accessible on protein surface is required for binding to Zn(II) and Co(II) chelates. Consequently, we focused on the tridimensional structure of Fc fragment to study the accessibility of His residues. By computer calculations we showed that among the six His in the primary sequence of Fc, two of them His 433 and His 435 are accessible and closely situated in the tridimensional structure (Table 1 and Fig. 5). Thus, we identified His cluster signature located on Fc fragment which may be responsible for binding to chelated Zn(II) and Co(II).

4. Discussion

Immobilized metal-ion affinity chromatography is not only one of the most popular techniques for protein purification, but also it is a very efficient method for studying protein structure in terms of His residue accessibility [7,13,14,17,26]. As postulated by Sulkowski [13,14,21], the affinity of proteins for chelated Cu(II) requires at least one accessible His residue. When proteins are retained on chelated Ni(II), they have more than one His residue and

Table 1
Accessibility of solvent-exposed histidine residues (Å^2) in human IgG1 (1 fcc) calculated using XPLOR with a probe-radius of 1.93 Å

Histidine residue number	Atom number	Accessibility (Å^2)
433	N	0.00
	CA	7.50
	CB	19.70
	CG	2.39
	CD2	22.63
	ND1	0.00
	CE1	14.92
	NE2	7.80
	C	0.00
	O	0.00
435	N	–
	CA	0.00
	CB	0.00
	CG	0.00
	CD2	0.00
	ND1	0.00
	CE1	10.33
	NE2	0.00
	C	0.00
	O	0.00

the adsorption on chelated Zn(II) and Co(II) signifies a cluster of His residues accessible for coordination.

In our study, IgGs are retained on four transition metals, Cu(II), Ni(II), Zn(II) and Co(II), which signifies that at least two His and also a His cluster are accessible on their surface. Based on the recognitions rules established in IMAC [21], the lack of affinity to chelated Zn(II) and Co(II) of $F(ab')_2$ fragments and the retention of F′c fragment shows that the relevant His cluster is probably situated in F′c region. Hale and Beidler [27], described a histidine-rich region in the third constant domain of heavy chain (CH_3) of IgG1. They proposed that this domain, conserved among several immunoglobulin classes of human, mouse, guinea pig and rabbit, was the specific binding site for metal chelate.

However, the accessibility of His residues is a *sine qua none* requirement for binding to metal chelates [17]. That is why we used molecular modelling to determine the accessibility of His residues located in F′c region. Computer calculations reveal the accessibility of His residues in Fc domain of IgG and show that possible cluster could be His 433-X-His 435 because: (1) both His were found accessible to probe radius of 1.93 Å mimicking metal chelate and (2) they are close in the primary sequence as well and separated only by one amino acid which also meets the requirements for binding to Zn(II) and Co(II) chelates [21]. His residues are also present in the primary structure of the $F(ab)'_2$ fragment, but there are not located near each other in the 3D structure. Consequently, the absence of His cluster could explain the non-retention of $F(ab)'_2$ on Zn(II) and Co(II) chelates. Thus, the characterisation by molecular modelling of specific His cluster signature situated on F′c domain of human IgG may contribute to the understanding of the binding mechanism of IgG to metal chelates.

Moreover, in our study human IgGs and their pepsin digested fragments, $F(ab')_2$ and F′c, can be separated on four different metal chelates (Cu(II), Ni(II), Zn(II) and Co(II)) which is an attractive approach for industrial exploitation. Two of the metal chelates, IDA-Zn(II) and IDA-Co(II), were found to be of particular interest for $F(ab')_2$ recovery in the non-retained fraction (Figs. 3B and 4B). These metal chelates could also be applied for separation of papain digested products Fab and Fc due to the cluster His signature situated on Fc fragment. This is very useful rapid one-step procedure under mild conditions (pH 7) for preparing less immunogenic commercial products of industrial relevance for immunochemical, diagnostic and therapeutic purposes.

IMAC has numerous advantages compared to the conventional methods employing protein A/G, for separation

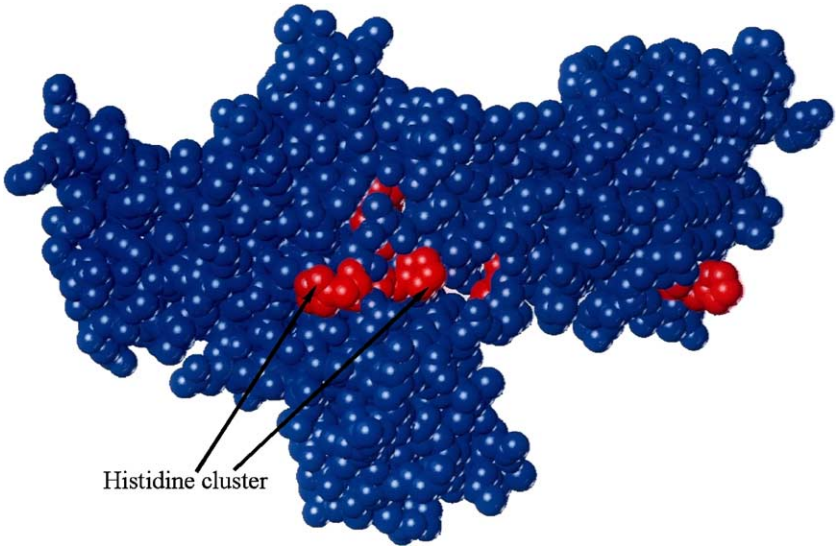

Fig. 5. Accessibility of cluster His 433-X-His 435 as calculated by computer modelling of Fc domain of human IgG1 (PDB: 1 fcc).

of proteolytic fragments of IgG. Protein A/G are obtained from microorganisms or genetically modified bacteria through complex and expensive procedures, and require time-consuming analytical controls to check the presence of contaminants which may affect the safety of the purified antibody for clinical purposes. Moreover, most protein-based affinity chromatography media are very sensitive towards alkaline treatment, a preferred method for regeneration and removal of contaminants from the purification devices in industrial applications.

Here we should emphasise that IMAC sorbents are less expensive, stable to harsh chemicals and temperatures and exhibit easy regeneration and cleaning up possibilities, longevity, resistance to proteolytic degradation, etc. Moreover, the recovery of IgG fragments in gentle, non-denaturing chromatographic conditions (pH 7) allow obtaining high yield of quality product and also retain the integrity of its tridimensional structure which is very important for immunotherapeutic applications [28]. Therefore, IMAC can be considered as a cost-effective, process-compatible alternative to affinity chromatography protein A/G sorbents for non-denaturing recovery of the proteolytic fragments of human IgG.

Acknowledgements

The authors wish to thank Professor Eugene Sulkowski for his helpful discussions and for comments on the manuscript. The authors also thank P.A.R.I.S company, Compiègne, France for providing chemicals.

References

[1] M.A. Vijayalakshmi, Appl. Biochem. Biotechnol. 75 (1) (1998) 93.
[2] J.P. Bouvet, R. Pires, A. Pillot, J. Immunol. Methods 66 (2) (1984) 299.
[3] G. Corthier, E. Boschetti, J. Charley-Poulain, J. Immunol. Methods 53 (1984) 313.
[4] B. Pavlu, V. Johanson, C. Nyghlen, A. Wichman, J. Chromatogr. 359 (1986) 449.
[5] A. Forsgren, J. Sjsquist, J. Immunol. 97 (1966) 822.
[6] J. Buchner, M. Renner, H. Lilie, H.J. Hinz, R. Jaenicke, Biochemistry 30 (28) (1991) 6922.
[7] M.A. Vijayalakshmi, Trends Biotechnol. 7 (1989) 71.
[8] J. Porath, J. Carlsson, I. Olsson, B. Belfrage, Nature 258 (1975) 598.
[9] A. El-kak, M.A. Vijayalakshmi, J. Chromatogr. Biomed. Appl. 570 (1991) 29.
[10] P.G.H. Byfield, S. Copping, R.L. Himsworth, Mol. Immunol. 21 (1984) 647.
[11] M. Belew, N. Junti, A. Larsson, J. Porath, J. Immunol. Methods 102 (1987) 173.
[12] R. Li, V. Dow, D.J. Stewart, S.J. Burton, C.R. Lowe, Nat. Biotechnol. 16 (1998) 190.
[13] E. Sulkowski, Trends Biotechnol. 3 (1985) 1.
[14] E. Sulkowski, in: Burgess (Ed.), Protein Purification: Micro to Macro, New York, 1987, p. 149.
[15] E.S. Hemdan, Y.J. Zhao, E. Sulkowski, J. Porath, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 1811.
[16] N.T. Mrabet, Biochemistry 31 (1992) 2690.
[17] P. Berna, N. Mrabet, J. Van Beeumen, B. Devreese, J. Porath, M.A. Vijayalakshmi, Biochemistry 36 (1997) 6896.
[18] J. Porath, B. Olin, Biochemistry 22 (1983) 1621.
[19] S.A. Al-Mashikhi, S. Nakai, J. Dairy Sci. 71 (1988) 1756.
[20] V. Boden, J.J. Winzerling, M.A. Vijayalakshmi, J. Porath, J. Immunol. Methods 181 (1995) 225.
[21] E. Sulkowski, BioEssays 10 (1989) 170.
[22] E. Sulkowski, J. Mol. Recognit. 9 (1996) 389.
[23] M. Bradford, Anal. Biochem. 72 (1976) 248.
[24] B.D. Hames, One-dimensional polyacrylamide gel electrophoresis, in: Hames, Rickwood (Eds.), Gel Electrophoresis of Proteins, A Practical Approach, 1990, p. 60.
[25] A.T. Brunger, XPLOR Manual, Version 3.1. Yale University, New Haven, CT, 1994.
[26] G.S. Chaga, J. Biochem. Biophys. Methods 49 (1–3) (2001) 313.
[27] J.E. Hale, D.E. Beidlem, Anal. Biochem. 222 (1994) 29.
[28] E. Nedonchelle, O. Pitiot, M.A. Vijayalakshmi, Appl. Biochem. Biotechnol. 83 (1–3) (2000) 287.