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HIGH-PERFORMANCE IMMOBILIZED-METAL-ION AFFINITY CHROMATOGRAPHY OF PEPTIDES AND PROTEINS

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SUMMARY

The present status of the undeveloped branch of high-performance immobilized-metal-ion affinity chromatography (HPIMAC) is reviewed. As demonstrated, high resolution of peptides and protein mixtures can be obtained. Under specified conditions, more than 1000 runs can be made repeatedly on a single column without much change in pattern or recovery. Occasionally, 10^3- to 10^4-fold purification of a protein can be obtained in a single chromatographic run.

INTRODUCTION

A protein has a large indented molecular surface with solvent-exposed functionalities, many of which can participate in interactions with a contacting solid matrix and various substituents that have been deliberately attached to the matrix to act as selectively operating sorption centres. If we want to exploit, for fractionation, catalysis or other purposes, immobilized-metal-ion affinity (IMA) for proteins, we must also take into consideration all possible additional interactive contributions from the metal-ion microenvironment: matrix, spacer arm and chelating ligand, and of course the properties of the surrounding liquid medium and its dissolved solute species. The complexity renders difficult a rational development of IMA chromatography (IMAC). Hence experience obtained with one particular gel-bound chelator ligand and a specified matrix does not necessarily apply fully for the same chelator attached to another support, and *vice versa*.

Today, high-performance (HP) IMAC is based on matrices of inorganic (silica gel), biological (agarose) or synthetic organic origin. So far, only studies with iminodiacetate as chelator for HPIMAC have been published, revealing the fact that this branch of chromatography is still not well developed. The forerunner, "classical IMAC", for biomacromolecules was introduced in 1975[1] and has since become a widely accepted tool for protein fractionation, although not yet exploited to the extent it deserves. The leading principles and many applications have been described in reviews[2–10] and original papers[11–17]. The components involved and their relative positions are shown schematically in Fig. 1. The possibility of directing the interaction towards a single kind of adsorption centre on the solute-ligate molecules (on proteins, *e.g.*, imidazole or thiol side-groups, calcium-binding sites or phosphate groups) provides excellent opportunities for HP chromatography.

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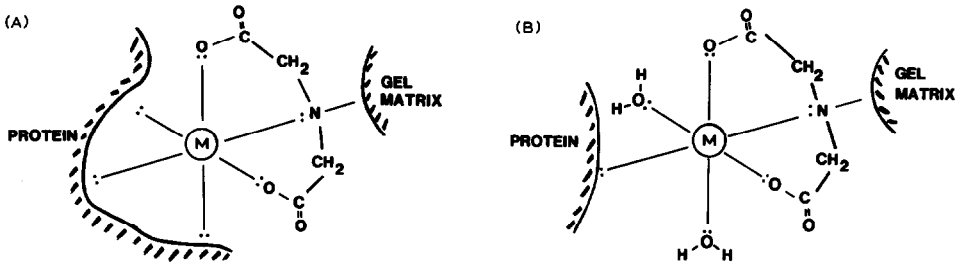

Fig. 1. Schematic illustration of IMA for the case where the chelating group consists of iminodiacetic acid and the metal ion can bind six ligands, three of which are fixed to the support and three are free to bind solutes (ligates). A bulky ligate such as a protein probably, as a rule, forms only a single coordinate bond with the metal ion (case B). Case A seems unlikely.

EXPERIMENTAL

HPIMAC on synthetic polymer

Kato *et al.*[18] described the IMAC of proteins on TSK-gel chelate-5PWE, a "hydrophilic resin-based material of large pore size". Fig. 2 illustrates the chromatographic isolation of soya bean lipoxidase from a crude enzyme sample, performed on Zn^{II}-chelate-5PW. The chromatogram in Fig. 2A compares favourably with those obtained with the same material on the ion exchanger TSK-gel DEAE-5PW (Fig. 2B) and by reversed-phase chromatography on a TSK-gel Phenyl-5PW RP column (Fig. 2C). These diagrams also illustrate that IMAC operates according to a principle that is different from those of the other two procedures. HPIMAC thus offers a complement and an alternative to the methods more frequently used at present. This will be further emphasized by the following examples of HPIMAC peptide fractionation.

Using the same IDA (iminodiacetate) TSK-gel, Yip *et al.*[19] in this laboratory have recently undertaken a methodological study of peptides on immobilized Cu^{II}, Ni^{II} and Zn^{II}. The resolution is satisfactory (Figs. 3 and 4) and the column lifetime is also acceptable: the column has been used for over 900 working hours for more than 1000 injections with no appreciable deterioration in performance or recovery. As shown, elution may be effected by "chelate annihilation", by gradually lowering the pH of the eluent or by a shallow gradient of the monodentate imidazole.

A comparison between the TSK-chelate gel and IDA-agarose was made by Belew *et al.*[20]. They found the capacity of the Cu^{II} TSK adsorbent for imidazole to be 24 μmol ml^{-1} gel, and the equilibrium constant for imidazole adsorption was $8 \cdot 10^3\ M^{-1}$. The equilibrium constants for model proteins on the same immobilized Cu^{II} were found to be in the range $1.8 \cdot 10^4$–$3.7 \cdot 10^5\ M^{-1}$. Of the potential adsorption sites, on third to half seem to be involved in the formation of protein complexes.

It was also found that around neutrality, glycine and primary amines displace the immobilized metal ions, as is also the case for Zn^{II} and Ni^{II}. In terms of leakage, TSK-gel chelates appear to be inferior to those of IMA adsorbents, based on cross-linked agarose.

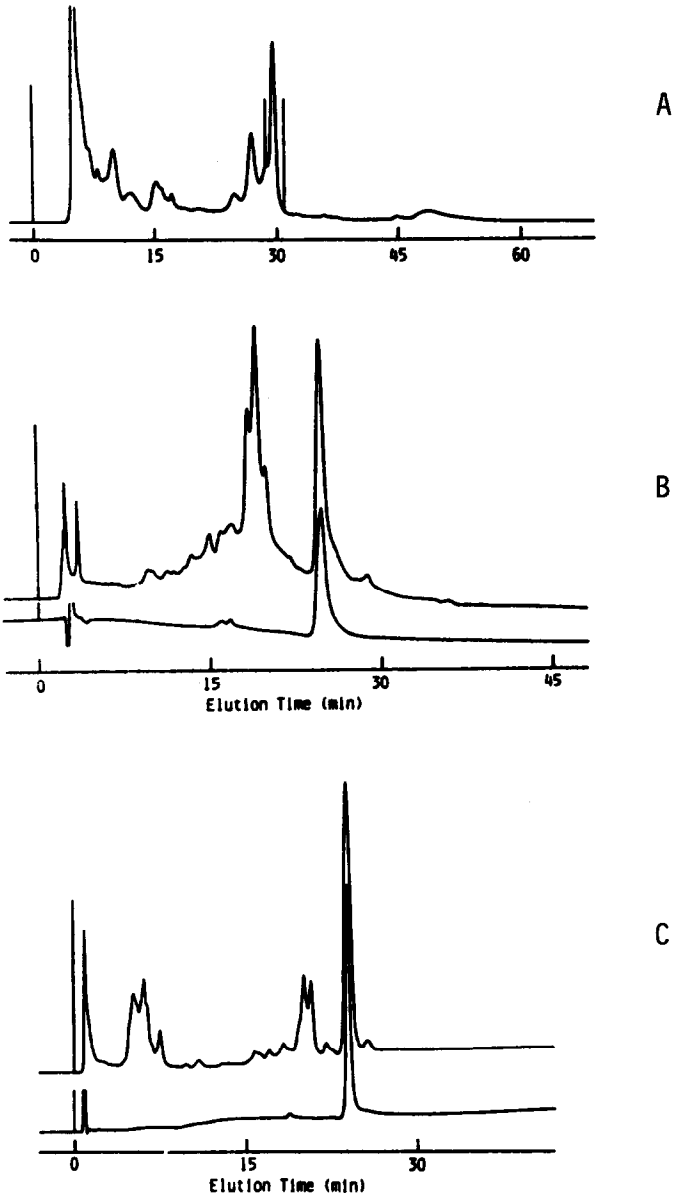

Fig. 2. (A) Chromatogram of a commercial sample of lipoxidase, fractionated on Zn^{II}-Chelate-5PW. The separation was performed in 0.02 *M* Tris–HCl (pH 8.0), containing 0.5 *M* sodium chloride, with an increasing 60 min gradient of glycine from 0 to 0.2 *M*. The upper curves in B and C are ion-exchange and reversed-phase chromatograms, respectively, of the same preparation as in A. B was obtained on TSK-gel DEAE-5PW and C on TSK gel Phenyl-5PW. The lower curves in B and C are chromatograms obtained with the indicated fraction from A (sampled after an elution time of *ca.* 30 min.)[18]. Published with the authors' permission.

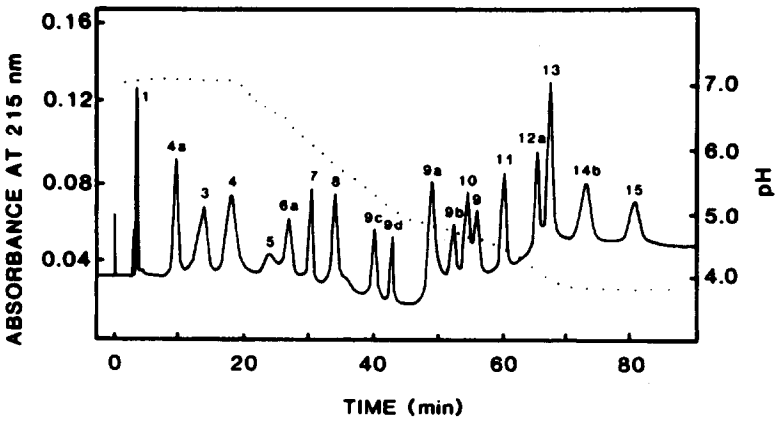

Fig. 3. Separation of 19 peptides on a Cu^{II}-loaded IDA-type TSK gel Chelate 5PW column. The sample, 20 μl containing 1–4 μg of each peptide, was applied to the column, equilibrated in 20 m*M* sodium phosphate containing 0.5 *M* sodium chloride (pH 7.0). The gradient was formed by 0.1 *M* sodium phosphate containing 0.5 *M* sodium chloride (pH 3.8). Flow-rate, 1 ml/min; UV absorption measured at 215 nm. The peptides (in order of peaks) were the following: 1 = neurotensin; 4a = sulphated [Leu^5]enkephalin; 3 = oxytocin; 4 = [Leu^5]enkephalin; 5 = mastoparan; 6a = Tyr-bradykinin; 7 = substance P; 8 = somatostatin; 9c = [$Asu^{1,7}$]eel calcitonin; 9d = eel calcitonin; 9a = [$Asu^{1,7}$]human calcitonin; 9b = human calcitonin 17–32; 10 = bombesin; 9 = human calcitonin; 11 = angiotensin II; 12a = [Trp(for)^{25,36}]human gastric inhibitory peptide; 13 = luteinizing hormone-releasing hormone; 14b = human parathyroid hormone; 15 = angiotensin I.

IMAC on silica-based stationary phase

Karger's and Horváth's research groups have described HP separations of proteins on microparticulate, wide-pore silica gels[21,22]. El Rassi and Horváth[22] made an extensive study of the importance of many parameters involved in the interaction of proteins with IDA gel, loaded with Cu^{II}, Zn^{II}, Fe^{II} and Fe^{III}. They confirmed earlier findings and also presented new and interesting results with methanol in the mobile phase. Some deviations from the behaviour of the model substances observed with other IMA adsorbents probably reflect differences in the affinity contributions made by the respective matrices. Mixed-mode interactions are indicated. "Silanophilic" interactions may be especially prominent at low ionic strength[23].

Figueroa *et al.*[21] using the IDA ligand coupled to the silica support via an amphiphilic diether spacer, compensated for slow metal leakage by including in the buffer $2 \cdot 10^{-6}$ *M* Cu^{II}. Fig. 5, taken from their work, illustrates that a sharp separation of a crude protein extract may be obtained within 15 min.

IMAC on cross-linked agarose

Exploratory work has been initiated by Belew and co-workers[20,24], and further developments are under way. As in gels of low to moderate rigidity, IMA for proteins in these highly rigid gels follows the order $Cu^{II} > Ni^{II} > Zn^{II}$. The efficiency of IMAC was demonstrated in the separation of monoclonal antibodies from mouse ascites fluid.

Hutchens *et al.*[25] have recently shown by Scatchard analyses based on batch adsorption experiments and frontal chromatography that, for ovalbumin and lyso-

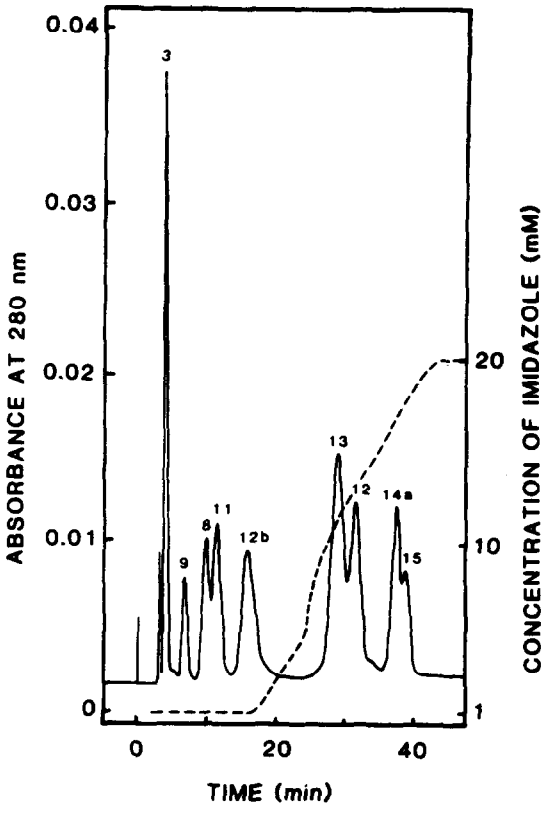

Fig. 4. Separation of nine peptides on a Zn^{II}-loaded TSK gel Chelate-5PW column. Column size, 754 × 7.5 mm I.D. Sample size, 35 μl with 5–25 μg of each peptide. The starting buffer consisted of 20 m*M* sodium phosphate containing 0.5 *M* sodium chloride and 1 m*M* imidazole adjusted to pH 7.0. The chromatogram was developed with an imidazole gradient from 1 to 20 m*M*, keeping the sodium phosphate and sodium chloride concentrations constant. The eluate was monitored by absorbance at 280 nm. The peptides (in order of peaks) were the following: 3 = oxytocin; 9 = human calcitonin; 8 = somatostatin; 11 = angiotensin II; 12b = bovine gastric inhibitory peptide; 13 = LH–RH; 12 = human gastric inhibitory peptide; 14a = human parathyroid hormone (13–34); 15 = angiotensin II.

zyme as model proteins, the apparent equilibrium dissociation constants, K_d, are located in the range 10^{-5}–10^{-6} mol of proteins per ml of gel. Nearly 20% of the immobilized copper may be involved in protein binding. The capacity is therefore very high and can be further increased by water-structuring salts[11,22].

We have also shown that the distribution of adsorbate molecules in the gel phase is statistically uniform and that the intramolecular distances between ligands are large enough to preclude significant attractive or repulsive forces between adsorbed protein molecules.

By pre-washing with a buffer containing 0.1 *M* glycine, metal leakage can be avoided, and inclusion of heavy metal ions in the buffer is therefore not necessary. Improvements in gel rigidity have been achieved. The future for IMAC on modified agarose appears bright.

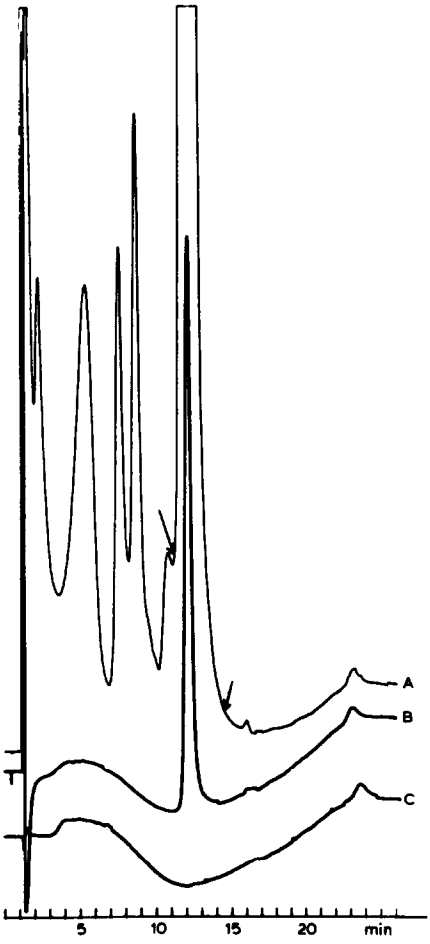

Fig. 5. Fractionation of a crude sample of dog myoglobin (A) and pure dog myoglobin (B) on a Cu^{II}–IDA column. C, blank gradient after chromatogram A, which demonstrates the absence of ghost peaks and indicates a high recovery of the myoglobin. The results were obtained by a gradient from pH 8.0 to 5.5. For a description of the liquid phase composition, the original paper should be consulted[21]. Published with the authors' permission.

DISCUSSION

HP methods imply some useful features of great value, such as savings of time or the achievement of high resolution in terms of low theoretical plate heights. The concept "high performance" should, in my opinion, also include chromatography of extremely high selectivity, as is occasionally encountered when exploiting biospecific affinity. IMAC may in fact be used for 10^3- to 10^4-fold purification in a one-step chromatogram. This kind of chromatography should perhaps be named "highly selective" or HSIMAC.

In recent years, it has become increasingly apparent that calcium ions play important roles in various regulatory processes[26]. It is therefore a challenge to re-

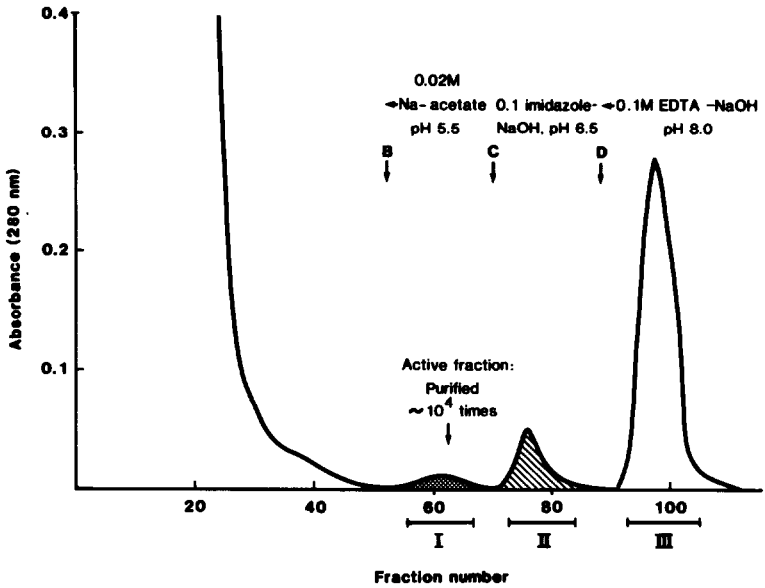

Fig. 6. Fractionation of human serum on Ca^{II}–carboxymethylated aspartic acid agarose with the aim of purifying an adhesion factor for rat liver endothelial cells (LEC). The gel was loaded with Ca^{2+} by washing it batchwise on a Büchner funnel with 50 m*M* calcium chloride solution. It was packed into a 25 × 5 cm I.D. column and equilibrated with 0.02 *M* sodium acetate (pH 5.5), containing 0.5 *M* potassium sulphate (buffer A). A flow-rate of 5 ml min^{-1} was used throughout the experiment. A 300-ml volume of dialysed human serum was applied to the column, and the unabsorbed proteins were washed away with an excess of buffer A. The adsorbed proteins were eluted with 0.02 *M* sodium acetate (pH 5.5) (buffer B), 0.1 *M* imidazole–sodium hydroxide, (pH 6.5) (buffer C) and finally with 0.1 *M* EDTA–sodium hydroxide (pH 8.0) (buffer D). The absorbance at 280 nm was measured, and the peaks were pooled as shown (pools I, II and III).

searchers in separation science to develop fractionation methods specifically directed towards calcium-binding proteins. For such purposes, "hard" metal ions could possibly be effective. (Pearson[27] has proposed the nomenclature, now widely adopted in textbooks, according to which metal ions and coordinating atoms are classified as "hard", "intermediate" and "soft".) An exploratory study was made with immobilized Group IIIB metal ions[12], coupled in tandem. We found that Tl^{III} was the only one in that group which showed, in the immobilized state, a high adsorption capacity for some serum proteins. We later discovered that Tl^{III} is similar to the dipositive ions of the later elements in the first transition series and is therefore not likely to be the metal ion of choice for calcium-binding proteins. Other tripositive metal ions are currently under study. Meanwhile, we have found that even immobilized Ca^{II} works in some instances. For good chromatographic performance, a "hard" metal ion such as Ca^{II} should be immobilized by a ligand with many "hard" coordinating atoms. Carboxymethylated aspartic acid, with three coordinating oxygens, is therefore a better choice than is iminodiacetic acid. Fig. 6, taken from the work of Mantovaara-Jönsson *et al.*[28], shows how a calcium-binding nectin (not yet chemically characterized) can be effectively concentrated and purified from human serum. In this instance, the separation was accomplished with Ca^{II}, immobilized on agarosé.

There is also a demand for methods permitting the rapid, highly group-selective isolation of phosphoproteins. Some progress toward that end has been made by Andersson and Porath[29] and Muszyńska *et al.*[30]. We are using tripositive metal ions immobilized on chelone–agarose derivatives (chelones = carboxymethylated amines).

A roundabout procedure may be useful for glycoproteins: a "soft" heavy metal ion may be used to immobilize a lectin. El Rassi *et al.*[31] recently reported the use of gel-immobilized copper to adsorb concanavalin, which in turn served as an adsorption site for carbohydrates. A "sandwich" method was also published by Wunderwald *et al.*[32], according to which α_2-macroglobulin fixed to Zn–IDA Sepharose provided adsorption centres for a large number of endoproteinases.

IMAC should also find application in other fields: for the immobilization and isolation of polysaccharides, nucleotides and nucleic acids, and also for structures of higher order, such as virus particles and cells. IMAC applied to HPLC has just begun.

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