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Facile Resolution of α-Fetoproteins and Serum Albumins by Immobilized Metal Affinity Chromatography[1]

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ABSTRACT

We have explored immobilized metal affinity chromatography as a means of resolving α-fetoprotein from its homologous albumin, a problem perennially encountered in the purification of an α-fetoprotein or its detection.

Human α-fetoprotein and human serum albumin were chromatographed on immobilized iminodiacetic acid charged with either Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}. Neither human α-fetoprotein nor human serum albumin displayed any affinity for Co^{2+} and Zn^{2+}. However, both proteins were bound to Cu^{2+} and were partially resolved by affinity elution with imidazole. By contrast, human α-fetoprotein and human serum albumin were completely resolved on immobilized Ni^{2+}. Similar results were obtained using bovine α-fetoprotein and bovine serum albumin.

The resolution of an α-fetoprotein from serum albumin should aid the purification of α-fetoprotein from a biological fluid containing overwhelming quantities of albumin, for example, serum. Importantly, the separation of human α-fetoprotein from human serum albumin may improve and help maintain the accuracy of immunoassays for α-fetoprotein, making the chromatography on immobilized Ni^{2+} a valuable diagnostic tool.

INTRODUCTION

It has been recognized for some time that an increase of AFP[4] in adult serum may signal an underlying pathological condition (1–5). Therefore, monitoring of AFP levels has a diagnostic value (6, 7). However, the selectivity of immunological detection methods for AFP may be impaired by the presence of "deformed" serum albumin in clinical samples (8). The facile separation of AFP from albumin would be of great value in clinical diagnosis.

Human AFP and human serum albumin display considerable homology in their primary structures and similarity in their conformations and physicochemical properties (9, 10). Consequently, their separation presents a major obstacle and a challenge in the design of purification schemes (9, 11–13).

We now report that AFP may be readily separated from serum albumin by IMAC, a purification method hitherto unexploited for this purpose. The underlying physicochemical principles of IMAC have been discussed in considerable detail elsewhere (14, 15).

The separation of AFP from serum albumin by IMAC may aid the clinical diagnosis of AFP and its purification as well.

MATERIALS AND METHODS

Materials. Human α-fetoprotein and bovine α-fetoprotein were generous gifts of Dr. S. Nishi and Dr. E. Ruoslahti. Human serum albumin (A 8763, Lot 83F-9315) and bovine serum albumin (A 0281, Lot 63F-9350) were purchased from Sigma. Chelating Sepharose 6B was obtained from Pharmacia.

Preparation of IDA-Metal^{2+} Columns. Chelating Sepharose 6B was washed with water, degassed, and equilibrated with 0.1 M sodium acetate (1 M sodium chloride), pH 4.0. The gel was poured into a chromatographic column (1 x 6.4 cm), with a bed volume ~5 ml, and charged by equilibration with 10 volumes of a solution containing 5 mg/ml of metal salt (metal^{2+}) in 0.1 M sodium acetate (1 M NaCl), pH 4.0. Excess of metal^{2+} was washed out of the column with metal-free buffer (pH 4.0). In the case of Co^{2+}, the column charging and washing was performed at pH 6.0 rather than at pH 4.0 as done with other metals (Ni^{2+}, Cu^{2+}, and Zn^{2+}). Finally, an IDA-Ni^{2+} column was equilibrated with 0.02 M sodium phosphate (1 M NaCl), pH 6.8.

Chromatographic Procedures. Chromatography on IDA-Co^{2+} and IDA-Zn^{2+} (pH stepwise elution) was performed as follows. A sample of AFP or albumin, 10 mg, in 5 ml of column equilibrating buffer (0.02 M sodium phosphate-1 M NaCl, pH 6.8) was applied to a column (IDA-Co^{2+} or IDA-Zn^{2+}) before washing with an additional 20 ml of buffer (pH 6.8). The column was then washed, in succession, with 25 ml of 0.1 M sodium acetate (1 M NaCl), pH 5.9, and 25 ml of 0.1 M sodium acetate (1 M NaCl), pH 3.8.

Chromatography on IDA-Ni^{2+} (pH gradient elution) was performed as follows. A protein sample, 5 to 10 mg, in 5 ml of equilibrating buffer (0.02 M sodium phosphate-1 M NaCl, pH 6.8) was applied on an IDA-Ni^{2+} column, and the column was washed with 20 ml of buffer (pH 6.8). A falling pH gradient was then developed by mixing 25 ml of 0.02 M phosphate (1 M NaCl), pH 6.8, with 25 ml of 0.02 M phosphate (1 M NaCl), pH 5.7. After pH gradient development of the column, the final wash was performed with 0.02 M sodium phosphate (1 M NaCl) adjusted to pH 5.3 (25 ml).

Chromatography on IDA-Cu^{2+} (imidazole elution) was performed as follows. An IDA-Cu^{2+} column, equilibrated with 0.02 M sodium phosphate (1 M NaCl), pH 6.8, was charged with 50 ml of 10 mM imidazole in the same buffer; next, the IDA-Cu^{2+} (imidazole) column was equilibrated with 50 ml of 1 mM imidazole solution in phosphate (1 M NaCl) buffer, pH 6.8. The column was now ready for the sample application. A protein sample, 5 to 10 mg, in 5 ml of 1 mM imidazole solution was applied on an IDA-Cu^{2+} (imidazole) column, and the column was rinsed with 20 ml of 1 mM imidazole solution in 0.02 M sodium phosphate (1 M NaCl), pH 6.8. A linear concentration gradient of imidazole was then developed by mixing 25 ml of 1 mM imidazole (0.02 M sodium phosphate-1 M NaCl, pH 6.8) and 25 ml of 10 mM imidazole (0.02 M sodium phosphate-1 M NaCl, pH 6.8). Finally, an IDA-Cu^{2+} (imidazole) column was developed with the 10 mM imidazole solution (0.02 M sodium phosphate-1 M NaCl, pH 6.8).

All IDA-Metal^{2+} columns were developed at room temperature. The flow rates were maintained at ~1 ml per 4 min by means of a peristaltic pump. Fractions, 1 ml, were collected, and their protein content was measured by spectrophotometry at a 280-nm wavelength.

RESULTS

Neither human AFP nor HSA interacted with IDA-Co^{2+} and IDA-Zn^{2+} columns when applied at near neutral pH. The same was the case with bovine α-fetoprotein and bovine serum albumin (not illustrated).

The affinity of α-fetoproteins for IDA-Ni^{2+} was very weak (Fig. 1). They were not retained on the column at neutral pH. The transient binding of human AFP is somewhat stronger than that of bovine AFP. By contrast, both HSA and BSA were

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[4] The abbreviations used are: AFP, α-fetoprotein; BSA, bovine serum albumin; HSA, human serum albumin; IDA, iminodiacetic acid; IMA, immobilized metal affinity; IMAC, immobilized metal affinity chromatography.

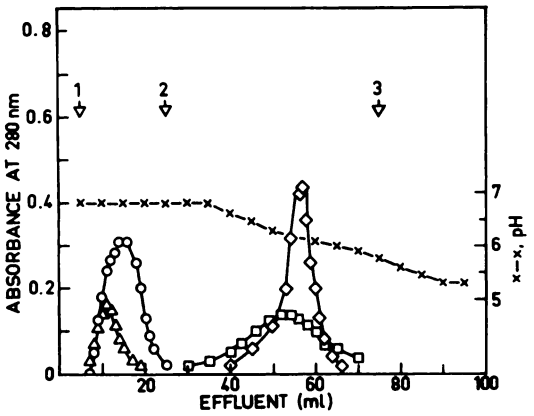

Fig. 1. Chromatographic separation of α-fetoproteins from homologous serum albumins on IDA-Ni^{2+}. ○, human AFP; △, bovine AFP; □, HSA; ◇, BSA. Elution by a falling pH gradient. All details in "Materials and Methods."

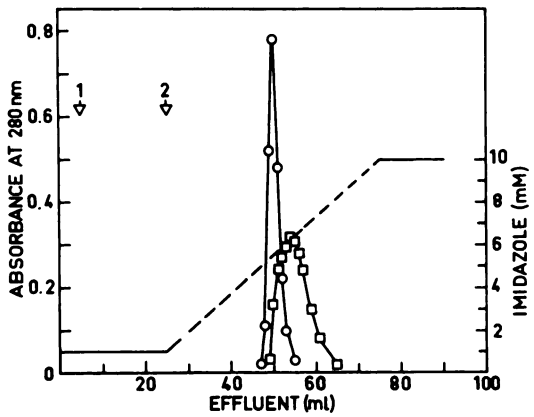

Fig. 2. Chromatography of human α-fetoprotein and human serum albumin on IDA-Cu^{2+}. ○, human AFP; □, HSA. Affinity elution with imidazole (——- - -——). All details in "Materials and Methods."

retained on IDA-Ni^{2+} and could be subsequently recovered by lowering the pH of the eluent. Thus, both α-fetoproteins can be readily separated from their respective serum albumins.

Fig. 2 illustrates the chromatography of human AFP and HSA on IDA-Cu^{2+}. Both proteins were retained on an IDA-Cu^{2+} column equilibrated with imidazole (1 mM); upon washing with the imidazole concentration gradient, human AFP is eluted earlier than HSA, although the complete resolution of both proteins has not been accomplished. The sequence of elution of AFP and HSA is the same as that from IDA-Ni^{2+} (Fig. 1).

Bovine AFP and BSA bound on IDA-Cu^{2+} and could be recovered upon an increase in the concentration of imidazole on the column (Fractions 48 to 55). Again, bovine AFP was displaced from the column somewhat earlier than BSA (not illustrated).

DISCUSSION

Since its introduction by Porath *et al.* (14), the IMAC has been exploited with considerable success for the purification of various proteins (16). Importantly, the IMA event can be understood in terms of coordination of a limited number of amino acid side chains which can serve as electron donors (14, 17). In view of the structural relatedness of human α-fetoprotein and human serum albumin (9, 10), it was of particular interest to evaluate their behavior on immobilized metal ions (IDA-metal^{2+}).

Human serum albumin and bovine serum albumin had been shown previously not to bind to immobilized IDA-Co^{2+} and IDA-Zn^{2+} (Footnote 5). That lack of binding has been rationalized on the grounds that the presence of two histidines adjacent in space constitutes a minimum requirement for the retention of a protein on IDA-Co^{2+} and IDA-Zn^{2+} (17). The presence of a cluster of three histidines, –His 245–Val 246–His 247–Glu 248–His 249–, assigned to an α-helical configuration in human α-fetoprotein (10), would meet this requirement. However, binding to either gel requires that both histidines, His 245 and His 249, are (*a*) exposed to the solvent, (*b*) unprotonated, and (*c*) fully accessible to IDA-metal^{2+} (no steric hindrance). Apparently, in view of our findings, one or more of those caveats are not met. Perhaps, an affinity of the AFP molecule for IDA-Co^{2+}/IDA-Zn^{2+} could be observed under partially denaturing conditions (urea, guanidine-HCl) and/or with an AFP lacking carbohydrate moiety (not so distant in α-helix: Asn 232).

The chromatography of human AFP and HSA (Fig. 1) indicates a weaker affinity of AFP for chelated nickel (IDA-Ni^{2+}) by comparison to that of HSA. This finding is congruent with the observations on the association of free metal ions, Cu^{2+} and Ni^{2+}, with human AFP and HSA. It had been shown earlier that Cu^{2+} and Ni^{2+} ions bind to serum albumin with 1:1 stoichiometry (18, 19). The binding of Cu^{2+} to human α-fetoprotein occurs also with the same ratio, mol of metal per mol of protein, but the binding of Ni^{2+}, 0.62 mol of metal per mol of protein, is distinctly less (20).

At present one can only speculate about the potential causes of a weaker binding of AFP, *vis-à-vis* HSA, to IDA-Ni^{2+}. It has been proposed that His 3 constitutes a part of a β-turn in AFP, whereas it has an α-helical assignment in HSA (10). The contribution of His3 toward coordination to IDA-Ni^{2+} may be sufficiently different (pK_a value, steric accessibility) in the case of AFP and HSA, respectively, to explain our chromatographic data. However, one cannot disregard the contribution of some other histidines towards the overall affinity of AFP and HSA for IDA-Ni^{2+}.

Finally, it should be observed that the facile separation of α-fetoproteins (human and bovine) from their homologous albumins on IDA-Ni^{2+} columns may be of use in the amelioration of their present purification procedures; the same may hold true for other α-fetoproteins of interest. Plausibly, the separation of usually small amounts of an α-fetoprotein from overwhelmingly large amounts of albumin may improve the quantitation of an AFP by immunoassays. In the case of human AFP this could be of value in the diagnosis of pathological conditions.

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