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Note

Purification of commercial human albumin on immobilized IDA-Ni^{2+}

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Human serum albumin commonly used for therapeutic purposes is prepared by the cold-ethanol precipitation procedure [1]. The purified albumin is further processed to inactivate hepatitis virus by heat treatment, for 10 h at 60°C, in the presence of octanoic acid and N-acetyl-DL-tryptophan as albumin stabilizers [2]. The final product is used with a high degree of safety even though there are some side-effects due, presumably, to trace impurities [3, 4]. These impurities result, in part, from the imperfection of the standard purification procedure, i.e. carry-over of proteins other than albumin. Artifacts due to the harshness of the procedure (ethanol) are also introduced, e.g. albumin oligomers.

Human albumin was purified from plasma by affinity chromatography [5, 6] and affinity partition [7] on fatty acid adsorbents. Dye–ligand chromatography was also successfully applied to the isolation of human albumin [8, 9]. The isolation of human albumin from plasma on IDA-Cu^{2+} [10] and IDA-Ni^{2+} [11] has also been reported.

In contrast to these reports, we describe here the further purification of human albumin after its initial purification by the Cohn's procedure. We report that many of protein impurities resident in commercially available albumin can be readily removed by immobilized metal affinity chromatography (IMAC); specifically, by percolating albumin solutions through a chromatographic column containing immobilized nickel ion (IDA-Ni^{2+}).

EXPERIMENTAL

Human serum albumin, crystallized and lyophilized, was purchased from Sigma (St. Louis, MO, U.S.A.) (A 8763, Lot 83F-9315). Human serum albumin, for-

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mulated for therapeutic use (25% solution), was obtained from the American Red Cross (Armous Pharmaceutical, Kankakee, IL, U.S.A.). Chelating Sepharose 6B was obtained from Pharmacia (Pharmacia Biotechnology International, Uppsala, Sweden). Tris(carboxymethyl)ethylenediamine (TED)-agarose was prepared in this laboratory [5]. Electrophoresis was performed on polyacrylamide gradient gel slabs (4–30%) obtained from Pharmacia, after an initial concentration of protein samples in Amicon (Danvers, MA, U.S.A.) Minicon cells. Protein standards (molecular mass) were obtained from Pharmacia. The content of nickel in protein samples was determined, after wet combustion, in a Perkin-Elmer 5000 atomic absorption spectrometer.

Chelating Sepharose 6B was washed thoroughly with water, degassed and equilibrated with 0.1 *M* sodium acetate (pH 4.0), which was made 1 *M* in sodium chloride (final pH 3.8). The gel was poured into a chromatographic column (6.4×1 cm I.D.), bed volume ca. 5 ml, and charged with a solution of nickel sulphate, 5 mg/ml in 0.1 *M* sodium acetate, pH 4.0 (1 *M* sodium chloride) until saturated. The excess Ni^{2+} was washed off the column with the equilibrating buffer (pH 3.8). Finally, the column was re-equilibrated with 0.02 *M* sodium phosphate (pH 7.4), which was made 1 *M* in sodium chloride (final pH 6.8). TED-agarose, used to scavenge Ni^{2+} from the albumin solutions, was equilibrated with 0.02 *M* sodium phosphate (1 *M* sodium chloride, pH 5.7). The IDA-Ni^{2+} column was regenerated with 50 m*M* EDTA (1 *M* sodium chloride, pH 7.0) before re-use.

RESULTS

As shown in Fig. 1 (open circles) human serum albumin (Sigma), when applied on the IDA-Ni^{2+} column at pH 6.8, is retained only transiently: it starts to appear in the eluate at fraction 15, i.e. after only three column volumes of the equilibration buffer have passed through. This elution is accelerated by lowering the pH of the eluent to 6.5–6.0. Additional traces of the material absorbing at 280 nm can be recovered at still lower pH, 5.7 and 3.8. The chromatography of human serum albumin especially formulated for therapeutic use (American Red Cross) gives essentially the same experimental outcome (Fig. 1, closed circles) except for the conspicuous presence of fractions 7–15, which contain N-acetyl-DL-tryptophan (added as a stabilizer by the manufacturer).

Fig. 2 illustrates an experiment in which 50 ml (1.25 g) of human serum albumin (American Red Cross) was chromatographed on a 5-ml column of IDA-Ni^{2+} under the experimental conditions described earlier (Fig. 1). Most of the applied material is eluted in the breakthrough fractions and in those fractions eluted with the equilibrating solvent 1 (pH 6.8). Further development of the column with solvents of decreasing pH yielded additional protein fractions (pools 2, 3 and 4) in amounts permitting electrophoretic analysis. As shown in Fig. 3, these fractions contain many protein impurities in addition to small and decreasing amounts of albumin.

The question then arose as to whether a single passage of commercial albumin (American Red Cross) through an IDA-Ni^{2+} column results in the complete removal of the protein impurities (Fig. 2) found in fractions 2, 3 and 4. Therefore

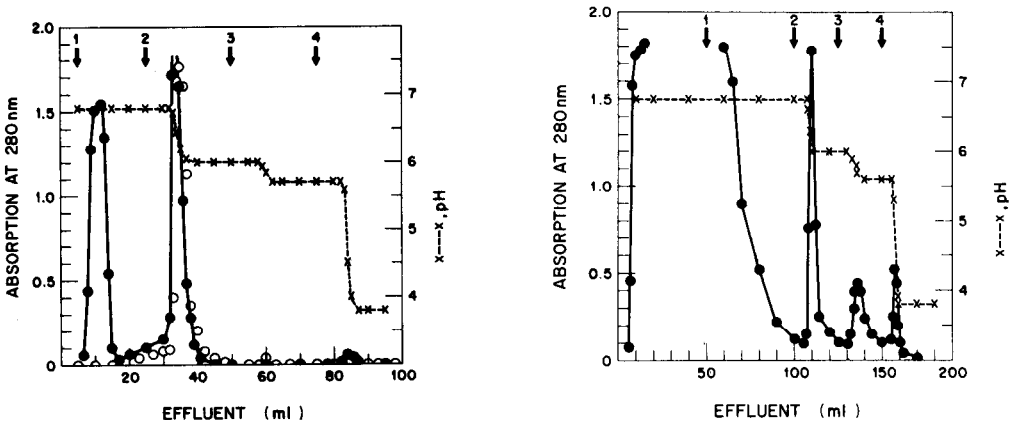

Fig. 1. Chromatography of commercial human serum albumin preparations on IDA-Ni^{2+}. The open circles show the A_{280} elution profile of albumin from Sigma, and the closed circles the A_{280} elution profile of albumin from the American Red Cross. A sample of albumin solution, 5 ml, containing 25 mg of protein in 0.02 *M* sodium phosphate (1 *M* sodium chloride, pH 6.8), was applied to an IDA-Ni^{2+} column (6.4×1 cm I.D.), bed volume ca. 5 ml. The column was washed in succession as indicated by arrows with: 1 = 0.02 *M* sodium phosphate (1 *M* sodium chloride, pH 6.8); 2 = 0.02 *M* sodium phosphate (1 *M* sodium choride, pH 6.1); 3 = 0.02 *M* sodium phosphate (1 *M* sodium chloride, pH 5.7); 4 = 0.1 *M* sodium acetate (1 *M* sodium chloride, pH 3.8). Fractions of 1 ml were collected at a flow-rate of 1 ml per 4 min at room temperature.

Fig. 2. Isolation of protein impurities from human serum albumin (American Red Cross) on IDA-Ni^{2+}. Sequential elution was performed as described in Fig. 1. The closed circles show the A_{280} elution profile.

the breakthrough fractions (5–105) were combined and re-chromatographed on a freshly prepared IDA-Ni^{2+} column (5 ml bed volume). After two cycles of re-chromatography, fractions 3 and 4 were substantially (ca. ten-fold) diminished although not completely eliminated (not illustrated).

Fig. 4 compares the electrophoretic purity of human serum albumin (American Red Cross) with that after three cycles of chromatography on IDA-Ni^{2+} (breakthrough fractions). As a result of IDA-Ni^{2+} chromatography the polymeric material (Fig. 4, lane 1, top of the gel) was removed below the level of detection (Fig. 4, lane 2,top of the gel); the amount of albumin dimer was also much less.

The combined results of Figs. 3 and 4 show that the passage of a commercial albumin through an IDA-Ni^{2+} column greatly enhances its purity.

As human serum albumin is known to have one high-affinity binding site per molecule for a free Ni^{2+} ion [12], it was of interest to know if albumin binds this metal during passage through the IDA-Ni^{2+} column. Consequently, the nickel content of the albumin samples was analysed. Table I shows that human serum albumin efficiently scavenged Ni^{2+} from IDA-Ni^{2+}. While this observation is itself of interest, the nickel has to be removed if albumin is to be used for therapeutic purposes. Therefore, we passed the albumin breakthrough fractions (10-ml sample containing 100 mg of protein) through an uncharged TED column (3×1 cm I.D.). A TED column rather than an IDA column was used because the

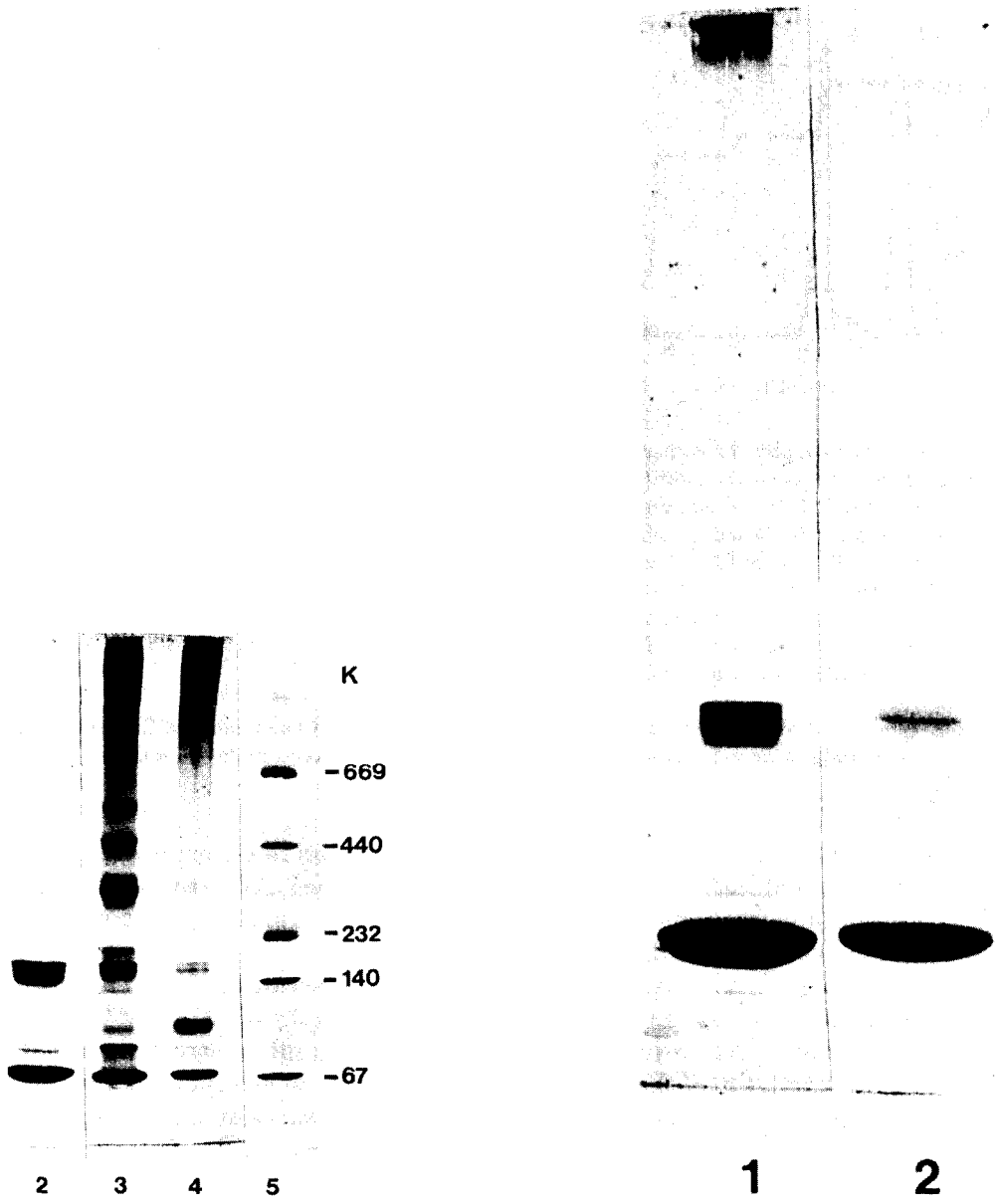

Fig. 3. Electrophoresis of chromatographic fractions obtained from an IDA-Ni^{2+} column. Chromatographic fractions (2, 3 and 4, see Fig. 2) were concentrated and electrophoresis was performed with ca. 100 μg of protein. The numbers of the lanes correspond to the numbers of chromatographic fractions (pools). The protein standards were: thyroglobulin, 669 000; apoferritin, 440 000; catalase, 232 000; lactic dehydrogenase, 140 000, and bovine serum albumin, 67 000.

Fig. 4. Electrophoresis of albumin before and after chromatography on IDA-Ni^{2+}. American Red Cross albumin before (lane 1) and after (lane 2) purification on an IDA-Ni^{2+} column.

TABLE I

ANALYSIS OF THE NICKEL CONTENT OF ALBUMIN

Albumin	Content (ng/mg)	Saturation* (%)
American Red Cross	0.4	0.05
Breakthrough (IDA-Ni^{2+})	790.0	90
Breakthrough (TED)	9.0	1.0

*Gram atom/mol of albumin.

binding of divalent metal ions (M^{2+}) is much tighter on TED than on IDA function [5]. The results (Table I) show that the level of Ni^{2+} was substantially decreased.

DISCUSSION

The selection of IDA-Ni^{2+} for the additional purification of commercially available human serum albumin has been dictated by the results of our comprehensive study of the chromatographic behavior of albumin on IDA-M^{2+} (Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}). We have shown recently that human serum albumin, at near-neutral pH, is not retained on immobilized Co^{2+} and Zn^{2+}; it binds weakly to IDA-Ni^{2+} and strongly to IDA-Cu^{2+} [13].

The results illustrated in Figs. 2 and 3 show that many impurities present in a commercial preparation of human serum albumin can be selectively retained on an IDA-Ni^{2+} column.

It should be stressed that (i) albumin is not yet homogenous on electrophoresis (Fig. 4) and (ii) it contains a considerable amount of Ni^{2+} (Table I) scavenged from the IDA-Ni^{2+} column. Further refinement of the chromatographic conditions (longer residence time of albumin on IDA-Ni^{2+} and TED; more precise calibration of the capacity of both IDA-Ni^{2+} for protein impurities and TED for Ni^{2+}; an adjustment of pH and ionic strength of the eluents) is necessary to arrive at a practical, routine protocol for a large-scale additional purification of commercial albumin. However, we propose that such an experimental refinement ought to be carried out on production line (manufacturing plant) so that one can assess, in particular, whether IDA-Ni^{2+} chromatography may be carried out before, after or instead of the heat treatment of the purified albumin solutions.

One should recall at this juncture that the heat treatment of serum albumin preparations (60°C, 10 h) was introduced as a counter-measure against dissemination of hepatitis virus. The heat treatment operation, however, requires the inclusion of stabilizing agents (N-acetyl-DL-tryptophan, octanoic acid) which, in turn, are a cause of some concern [14].

The inactivation or, better, the removal of pathogenic viruses from blood products is, of course, of unquestionable importance. Therefore, it seems appropriate to explore the possibility, in laboratories adequately equipped for such tasks, that IMAC may offer a general solution as a scavenger operation for a pathogenic virus from albumin solutions and some other blood products. A priori, it seems reason-

able to assume that a virus could be readily adsorbed on an IDA-Ni^{2+} column via its coat proteins. This notion remains, however, to be experimentally explored.

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