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ISOLATION OF PROTEINS FROM CRUDE MIXTURES WITH SILICA AND SILICA-BASED ADSORBENTS

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SUMMARY

Silica and silica-based adsorbents have been used to isolate specific proteins from crude mixtures. Acid-activated Nugel silica and Nugel SP-Silica were used to extract human immune interferon from the conditioned medium of mixed lymphocyte cultures. Human interleukin-2 was extracted from the conditioned medium of Jurkat cells with LiChroprep RP-8. In each case, batch adsorption was accomplished by gentle stirring in a microcarrier vessel. The adsorbate was collected by sedimentation, and either batch-eluted or packed into a column for gradient elution. A high degree of concentration and purification was achieved with a high recovery of biological activity.

INTRODUCTION

Many proteins and peptides of interest are present in trace amounts in tissue extracts or in the conditioned medium of cells. An approach to the extraction, concentration and partial purification of proteins is presented. It is based on the use of inexpensive silica-based adsorbents for batch extraction, followed by either batch or column elution. Details of this procedure, as well as specific applications are discussed.

MATERIALS AND METHODS

Microcarrier tissue culture vessels and micro-carrier magnetic stirrers were obtained from Bellco Glass (Vineland, NJ, U.S.A.). One-inch diameter stainless-steel tubing was obtained from Components and Controls (Carlstadt, NJ, U.S.A.) and mirror-polished by Steel Brite Polishing (Elizabeth, NJ, U.S.A.). End-fittings (Swagelock or Parker) and frits (10 μm) were obtained from Alltech Assoc. (Deerfield, IL, U.S.A.). LiChroprep RP-8 (25-40 μm, 100 Å pore) is a product of E. Merck and was obtained from Ace Scientific Supply (Linden, NJ, U.S.A.). Nugel acid-activated silica and sulfopropyl (SP) silica (37–44 μm, 200 Å) were obtained from Diagnostic Specialities (Metuchen, NJ, U.S.A.). Pyridine, acetic acid, formic acid and 1-propanol were distilled from ninhydrin prior to use. High-purity water was obtained with a system

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from Hydro Service and Supplies (Durham, NC, U.S.A.). All other reagents were of the highest grade commercially available and were used without further purification. The column effluents were monitored for amino acids, peptides, and proteins with fluorescamine (Hoffmann-La Roche, Nutley, NJ, U.S.A.) by a stream splitting technique previously described[1]. Aliquots of the column effluents were assayed for biological activity in systems based on previous methods. Immune interferon (IFN-γ) was monitored by a cytopathic inhibition assay[2]. Interleukin-2 (IL-2, T-cell growth factor, TCGF)-dependent incorporation of [^{3}H]thymidine into an IL-2-dependent line of T-cells was measured as previously described[3].

Crude human IFN-γ was produced by human peripheral lymphocyte cultures induced with phytohemagglutinin-L (Cal-Med, San Francisco, CA, U.S.A.) and 12-O-tetradecanoylphorbol 13-acetate (Consolidated Midland, Brewster, NY, U.S.A.)[4]. Crude human IL-2 was obtained from cultures of the Jurkat cell line[3].

Immune interferon is a glycoprotein that can be purified by affinity chromatography with concanavalin A–Sepharose (Pharmacia, Piscataway, NJ, U.S.A.)[4,5]. After the cells are removed from the crude IFN-γ preparation by low-speed centrifugation, the material must still be passed through a filter with a maximum pore size of 0.2 μm. Otherwise it will rapidly foul the chromatographic frits and resins. A large-scale Millipore (Bedford, MA, U.S.A.) filtration apparatus [three PL-1 filter housings with sequential CP20 (0103), CP06 (022W3), and CP03 (022W3) filter cartridges], followed by a Millipack 0.2-μm filter is used for this purpose. The filtered preparation is applied to a 200-ml Con A column, which is then washed with phosphate-buffered saline (MA Bioproducts, Walkersville, MA, U.S.A.) and eluted with α-methyl mannoside (Sigma, St. Louis, MO, U.S.A.) in phosphate-buffered saline.

Proteins in the conditioned medium of the Jurkat cells (grown under serum-free conditions) were precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1 *M* sodium bicarbonate (*ca.* pH 8.0). About one-tenth the original volume of conditioned medium was required for resolubilizing. It is possible that the conditioned medium could have been processed directly by the procedures described below. This possibility was not tested, since the precipitate was a convenient and stable storage form of the IL-2.

RESULTS

Batch adsorption on silica-based adsorberts

Recently, several culture vessels have been developed for the large-scale culture of anchorage-dependent cells on microcarrier beads. These vessels were designed to keep the 10–100-μm diameter negatively charged beads in suspension without damaging the beads or the cells growing on them. Similarly, we found that 25–50-μm diameter silica beads could be suspended by gentle stirring for at least 48 h without noticeable damage. Beads modified for either reversed-phase or ion-exchange chromatography are now commercially available. With a 4-position stirrer, the active material contained in as much as 64 l can conveniently be adsorbed on the modified beads.

In the batch adsorption procedure, 50 ml of acid-activated silica beads were suspended in 2 l of conditioned-cell culture medium. In the case of IFN-γ, the cells were removed by centrifugation and the medium was degassed by bubbling helium

through the solution. The vessel is designed to hold the gas mixture necessary for the cultured cells, and therefore, a low oxygen tension is maintained throughout the incubation. This is important because many biologically active polypeptides are relatively unstable and marked loss of activity can occur due to oxidative aggregation. Temperature can be controlled by performing the adsorption in a cold-room or an incubator.

After the protein is adsorbed on the beads (1–24 h, depending on bead concentration, functional group and protein) the stirrer is turned off and the beads are allowed to settle. This requires only a few minutes due to the large size and density of the protein-laden beads. A compact and fairly solid layer of adsorbate is formed on the bottom of the vessel. It is recommended that the paddle be removed as soon as stirring is halted so as not to disturb the layer of beads. The supernatant can be decanted easily. The beads are then washed by resuspending them in a large volume of buffer for a few minutes. The washing procedure can be repeated, or alternatively, the following procedure is recommended for ion-exchange beads. The beads are suspended in 10 volumes of a solution barely capable of eluting the protein from the support (*e.g.* 500 ml of 0.2 *M* sodium chloride, 10 m*M* potassium phosphate pH 5.0, for 50 ml cation-exchange substituted support). After a short incubation (15–30 min), the suspension is diluted with 1 l of 10 m*M* phosphate buffer. The adsorbate is kept in suspension until all the biological activity is again bound to the beads (1–3 h). The purpose of this process is to remove the relatively large amount of protein which is non-specifically bound to the adsorbent.

After the beads are washed, they are transferred to either a column or a smaller microcarrier flask for elution. Good recoveries (90–100%) are routinely observed with both column and batch elution. Since the batch-elution procedure is simpler and requires no attention, it may be advantageous in the early stages of a purification scheme where volume is unimportant. Typically, 90% of the bound activity is recovered in 4 volumes of eluent buffer. In contrast, less than one volume can be sufficient for column elution (*cf.* Fig. 3 legend).

Isolation of IFN-γ

IFN-γ is a cationic protein that is readily bound by sulfopropyl-substituted silica at pH 5.5. 400 ml of con-A-purified IFN-γ ($2.0 \cdot 10^7$ anti-viral units, $1 \cdot 10^4$ U/mg) was further purified by the batch method discussed above. This material was diluted with an equal volume of 50 m*M* potassium phosphate pH 5.5 and incubated with 50 ml of the sulfopropyl beads for 3 h at 4°C. The beads were washed once and transferred to a stainless-steel column. The beads were further washed and eluted as shown in Fig. 1. Tetramethyl ammonium chloride (TMAC) was used for elution, as others have reported that IFN-γ is also adsorbed on acid-activated silica and can be eluted in stable condition with good recovery by TMAC[6]. Thus, TMAC serves two functions: it elutes the cations from the negatively charged groups on the adsorbent and prevents undesired interactions of the IFN-γ on incompletely glycophased areas of the silica bead (or regions newly exposed due to damage to the beads during the batch process). As shown, 90% of the original IFN-γ activity was recovered in the column effluent. Fractions 30–53 were pooled, and this material had a specific activity 120 times that of the original material ($1.2 \cdot 10^6$ U/mg). It should be noted that two-third of the total biological activity was eluted with even greater purity in fractions 45–52.

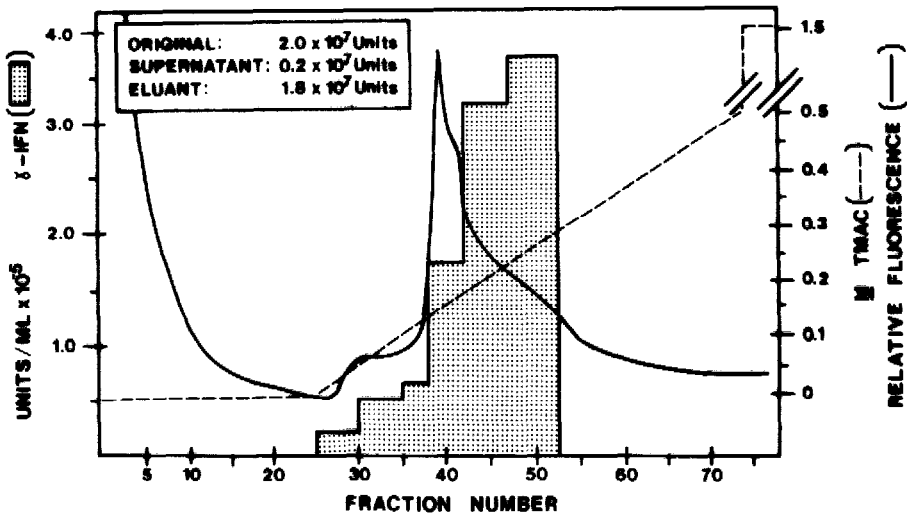

Fig. 1. Preparative cation-exchange chromatography of immune interferon. 50 ml of the 200–400-mesh sulfopropyl-substituted adsorbent were suspended in 800 ml of con-A-purified INF-γ in a microcarrier vessel. The suspension was stirred for 3 h at 4°C, and then the beads were transferred into a 50-ml (13.5 × 2.2 cm I.D.) stainless-steel column. The column was eluted with a gradient of TMAC in 25 m*M* potassium phosphate, pH 5.5. The effluent was monitored for protein by fluorescamine reactivity, and aliquots of the fractions were assayed for anti-viral activity.

Adsorption chromatography of IFN-γ can also be performed by this procedure in an alternate purification scheme. In this case, a 50- to 100-fold purification of crude material along with a 100-fold reduction in volume was usually obtained. The cells are removed from the crude IFN-γ preparation by centrifugation; filtration of conditioned media is not necessary prior to batch adsorption. A volume 50 ml of acid activated silica was suspended in 12.5 l of crude IFN-γ. Aliquots of the suspension were removed at various times and centrifuged. The supernatant was assayed for antiviral activity. The results of this study are shown in Fig. 2. Maximal adsorption of IFN-γ activity was observed only after prolonged incubation with the activated silica. In this case, approximately 70% of the biological activity was adsorbed after 20 h and further incubation did not reduce the titer of the supernatant to any appreciable degree. The beads were harvested, washed with phosphate-buffered saline, and batch-eluted with 1 *M* TMAC (65 ml, twice). A yield of $2.3 \cdot 10^7$ units of IFN-γ (86% of original biological activity) with a specific activity of $4.6 \cdot 10^4$ U/mg (128-fold purification) was obtained.

Isolation of IL-2

LiChroprep RP-8 was suspended in 1-propanol. After centrifugation, the excess propanol was decanted. An equal volume of water was added and after mixing, the adsorbent was allowed to settle and the excess liquid was decanted. This procedure was required to "wet" the adsorbent. About 1–2 l of concentrated IL-2 was mixed with 25 ml of beads at room temperature for 2–3 h. The extraction of IL-2 was found to be complete within 2 h.

When the stirring was stopped, the beads settled rapidly, and the supernatant was easily decanted without loss of adsorbent. The beads were suspended in 100 ml

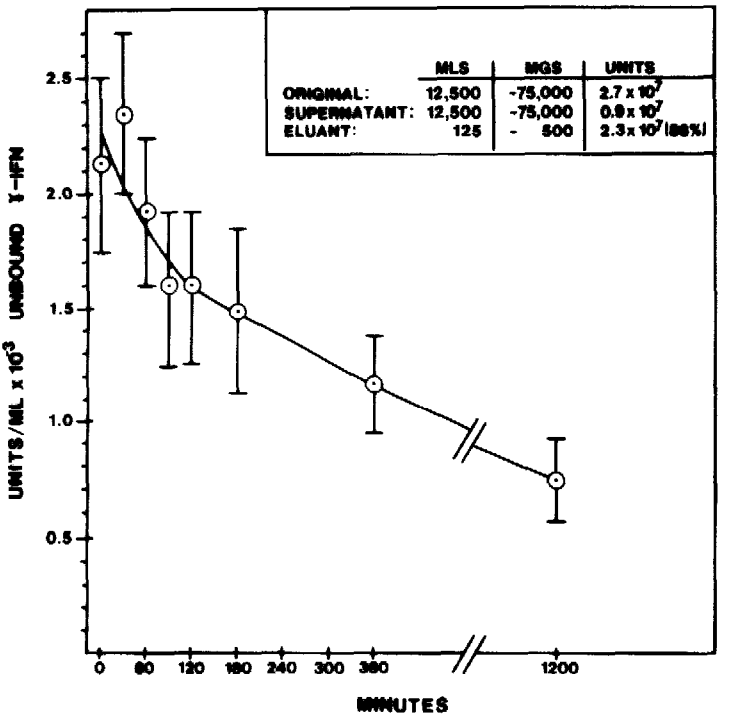

Fig. 2. Adsorption time-course study of immune interferon. A volume of 50 ml of acid-washed silica beads were suspended in 12.5 l of crude IFN-γ in a microcarrier vessel maintained at 4°C. Aliquots of the suspension were taken at various times, as indicated. The aliquots were centrifuged at 1000 *g* for 3 min, and the supernatant was assayed in triplicate for IFN-γ activity. After 20 h, the beads were harvested, washed and eluted with 1 *M* TMAC.

of 0.9 *M* pyridine, adjusted to pH 4.0 with acetic acid, and transferred to polypropylene tubes with constant swirling. They were then transferred to a 25-ml stainless-steel column (6.6 × 2.2 cm I.D.) in several portions. After each transfer, the beads were packed down by applying suction to the outlet of the column with a syringe.

The column was eluted (Fig. 3A) at 1.5 ml/min with 20% 1-propanol in the pyridine–acetate buffer. When no more protein was eluted, as indicated by a drop of the detector signal to the baseline level, gradient elution with increasing 1-propanol in pyridine–acetate was begun at a flow-rate of 1 ml/min. IL-2 was eluted after most of the other proteins as a broad peak of activity at slightly above 30% 1-propanol. By careful design of the gradient it was possible to elute the IL-2 in a much smaller volume with a similar degree of purification (Fig. 3B). Even though the adsorbed proteins are distributed throughout the entire 25-ml bed of adsorbent, it is possible to effect reasonably good chromatographic separation. Typically, about 10 μg of IL-2 was recovered per preparation with a reduction in volume of about 100-fold and with a yield close to 100%. The specific activity at this point was 10^6 U/mg, representing, 0.1% purity.

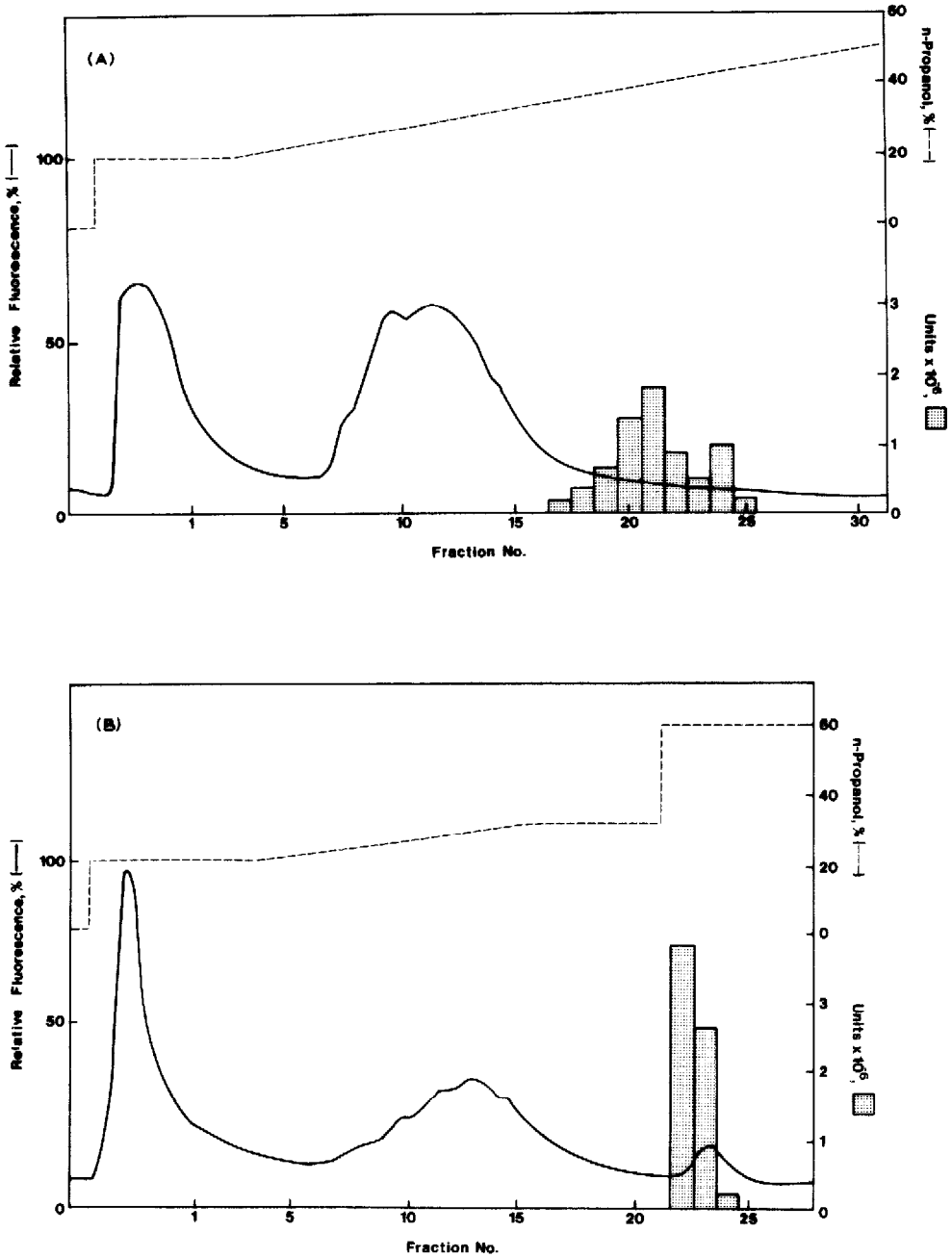

Fig. 3. Reversed-phase chromatography of human IL-2. A volume of 25 ml of LiChroprep RP-8 was suspended in 1.5 l of partially purified IL-2 for 2 h at room temperature. The beads were packed into a column which was eluted at 1 ml/min with gradient of increasing 1-propanol (panel A). By manipulating the shape of the gradient, it is possible to improve the separation and/or to concentrate the protein of interest (panel B). The volume of each fraction was 4 ml. Proteins in the column effluent were monitored by fluorescamine reactivity and aliquots of each fraction were assayed for biological activity.

DISCUSSION

The techniques presented here provide a rapid and efficient method for the isolation of proteins from crude mixtures. Furthermore, the mild, nearly physiological conditions utilized for adsorption help to prevent loss of biological activity in the case of proteins such as IFN-γ. The material to be processed does not have to be prepared for chromatography (*e.g.* 0.2-μm filtration). Biologically active proteins in suspensions of particulate matter, membranes, and/or whole cells can, in fact, be extracted (adsorbed) with this technique. Very viscous solutions, such as serum, present no difficulties. This process, when applied to suspensions of whole cells, extracts not only the biologically active material secreted by the cell, but also the materials within the cells themselves. This is due to the physical disruption of the cells by the beads.

Each batch of beads should be checked for fine particles, as is customary with agarose gels. The same beads can be used repeatedly, provided they have been properly cleaned and stored. We recommend storage of washed beads in 70% ethanol to inhibit microbial growth, followed by quick washing of the beads with at least 10 volumes of water prior to use. The washing and removal of "fines" can be performed simultaneously in a graduated cylinder. The beads are suspended by stirring with a plastic rod and then allowed to settle. After a layer of beads has settled (*ca.* 80% of total) the supernatant is discarded. The procedure is repeated as many times as necessary.

SP-silica has been used as the first step in the isolation of human PDGF in an analogous fashion to the use of CM-Sephadex previously described[7]. The procedure used was identical to that developed for IFN-γ, illustrated in Fig. 1. The PDGF was also eluted with TMAC, but greater amounts of the salt were required for its elution than for IFN-γ.

It should be added that IL-2 had previously been purified to homogeneity in this department[8], but the present procedure was not then employed. It was developed later, as an alternative method for obtaining a concentrated, partially purified preparation. A similar approach has recently been reported for the purification of gibbon IL-2[9].

These procedures have now been applied to the isolation of many different proteins. The adsorbents and equipment required for chromatography are relatively inexpensive. The silica and silica-based adsorbents have also been used to pre-deplete serum-supplemented tissue culture medium of the specific proteins that are bound by these adsorbents. This depleted medium has been useful for the production of hormones from tissue culture systems and has proven to be useful in the design of more defined bioassays.

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