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Purification of insulin-like growth factor-I and related proteins using underivatized silica

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Abstract

Adsorption chromatography using underivatized porous glass can be an effective capture step for the purification of recombinant proteins. Classical desorption techniques using chaotropic agents or harsh chemical solvents often result in elution of inactive material and may not be economical at the process scale. More recently, elution schemes have used tetramethylammonium chloride (TMAC) to obtain biologically active material. A TMAC elution was shown to be effective in the initial purification steps for the recovery of recombinant human insulin-like growth factor-I (rhIGF-I) from an *Escherichia coli* fermentation broth. However, TMAC also elutes other, more hydrophobic, proteins that are difficult to remove in subsequent purification steps. This paper describes the capture of IGF-I from a crude fermentation broth and a more specific elution using a combination of ethanol and NaCl rather than TMAC. This elution also can be used with other proteins including an IGF-I binding protein (BP3) expressed in mammalian cell culture.

Keywords: Growth factors; Proteins; Tetramethylammonium chloride

1. Introduction

Adsorption chromatography has been used frequently in the purification of small molecules, such as steroids and antibiotics, and is now finding increasing use for large-scale protein purification [1]. Although many of the experiments have been carried out with controlled-pore glass (CPG), underivatized silica particles can give a similar performance at a fraction of the cost. Silica also is a popular backbone for the production of chromatography media due to its economical production costs, surface chemistry, which allows it to be substituted with various functional groups, and mechanical strength, which permits good fluid mechanics relative to softer gels.

Recent advances have improved desorption of proteins bound to silica while preserving biological activity. Initially, elution from silica was obtained using a pH change [2] or chaotropic salts [3,4]. Other researchers have used combinations of solvents such as chloroform–methanol [5] or methanol–isopropanol [6] to facilitate elution of proteins bound to silica. Chadha and Sulkowski [7] pioneered the use of TMAC as a silica eluent and showed that it disrupts

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both the polar and ionic forces promoting protein interaction [8].

The use of silica chromatography as an initial capture step to recover recombinant proteins and separate them from the *E. coli* fermentation broth can be effective for purifying gram quantities of protein. In this paper we show that quantitative recovery of rhIGF-I bound to silica can be obtained using a TMAC elution scheme. More importantly, we demonstrate that a two-fold increase in purity of correctly folded IGF-I can be obtained by optimization of the biphasic action of TMAC, namely through the use of solvent and/or salt. The increase in purity at the initial purification step allows the subsequent steps to be much more effective. The solvent and salt elution scheme can also work with other proteins, including brain IGF-I [9] which lacks the N-terminal tripeptide, $Gly^1Pro^2Glu^3$, present in full length IGF-I and in an IGF-I binding protein (BP3).

2. Experimental

2.1. Materials

The recombinant proteins used in these studies were prepared and expressed at Genentech (South San Francisco, CA, USA). The proteins included IGF-I and brain IGF-I from *E. coli* and IGF-I binding protein (BP3) from a human kidney cell line, 293s.

Dry silica packing (grade 953) was obtained from Davison Chemical Division of W.R. Grace (Baltimore, MD, USA). Vydac reversed-phase analytical columns (C_{18}) were obtained from The Separations Group (Hesperia, CA, USA) and PL analytical columns were from Polymer Laboratories (Amherst, MA, USA). All other reagents used were of reagent grade or better and were obtained from a variety of suppliers.

2.2. Equipment

Small scale experiments were performed with a Rainin (Woburn, MA, USA) Rabbit peristaltic pump connected to a Pharmacia (Piscataway, NJ, USA) detector with 280 nm filters. The column eluent was fractionated using a Pharmacia Frac-300 fraction collector. Analytical chromatography was performed with a HP 1090 (Hewlett-Packard, North Hollywood, CA, USA) equipped with a ternary gradient system and a diode-array detector.

2.3. Silica column preparation

One part of dry silica packing was resuspended in five-to-ten parts of water to remove fines. A slurry of approximately one part resin to two parts water was transferred to an appropriately sized glass column. The packed bed was equilibrated with water until a constant bed height was obtained. Then the top flow adapter was positioned above the packed bed and the column was equilibrated with water at a flow-rate of 200–500 cm/h. Once a stable bed height was achieved, the column was ready for use. A total of three column volumes of water was generally used to prepare a uniform packed bed and the column dimensions were generally 12.8 cm bed height×1 cm diameter. Unless otherwise specified, all of the silica chromatography steps were performed at room temperature.

2.4. Sample preparation; loading and elution from silica

IGF-I or brain IGF-I expressed by the cells was secreted directly into the fermentation broth. At the end of a 10-l fermentation, the broth was heat-inactivated at 60–70°C for 5 min and clarified by centrifugation for 20 min at 5000 *g* in a Beckman RC3B (Beckman Instruments, Fullerton, CA, USA) to remove cells and cell debris. Mammalian cell culture fluid containing BP3 (~10 l) was separated from cells by centrifugation for 10 min at 3000 *g* and was sterile filtered. The clarified fermentation broth or cell culture fluid was loaded directly to silica at a rate of 200–500 cm/h. Unless otherwise specified, all wash and elution volumes were 3–5 column volumes at a flow-rate of 60 cm/h.

2.5. Preparation and use of an IGF affinity column

The method of Martin and Baxter [10] was used to prepare an IGF-I affinity column. IGF-I was bound

to Affi-Prep 10 (Bio-Rad, Hercules, CA, USA) at a coupling density of 1 mg of IGF-I per ml of resin. All affinity column operations were performed at 4°C and a flow-rate of 50–100 cm/h. Clarified harvested cell culture fluid was adjusted to approximately 0.5 *M* NaCl, pH 7, and loaded onto a 25×1 cm IGF-I affinity column previously equilibrated with 20 m*M* sodium phosphate, 0.5 *M* NaCl, pH 7. After loading, the column was washed with five column volumes each of equilibration buffer and 20 m*M* sodium phosphate, pH 7. Bound BP3 was eluted with 0.5 *M* sodium acetate, pH 3. Fractions were assayed for BP3 by the PL-HPLC assay described below. The affinity column was regenerated using 0.5 *M* NaCl, 0.5 *M* sodium acetate, pH 3.

2.6. Analysis

2.6.1. Vydac HPLC analysis for IGF-I

Samples were analyzed at 50°C on a 25×0.46 cm Vydac C_{18} (5 μm/300 Å) reversed-phase column equilibrated with 29% acetonitrile in 0.1% TFA buffer. A modification of the method described by Canova-Davis et al. [11] was used. Protein was eluted at a constant rate of 2 ml/min with a linear gradient of 29–30% acetonitrile in 0.1% TFA buffer over 10 min, followed by a gradient of 30–40% acetonitrile over 5 min. The column was regenerated with 60% acetonitrile in 0.1% TFA prior to re-equilibration. A linear standard curve (1–100 μg) using purified IGF-I was used to quantitate samples.

2.6.2. PL-HPLC analysis for BP3

Chromatography was performed at 50°C on a 5×0.46 cm Polymer Laboratories PLRP-S column (8 μm/4000 Å). The column was equilibrated with 10% acetonitrile in 0.1% TFA. A gradient of 10–60% acetonitrile in 0.1% TFA over 9 min was used to elute the bound protein. Then the column was regenerated with 90% acetonitrile in 0.1% TFA before re-equilibration. The total time between injections was 13 min. Samples were quantitated relative to a linear standard curve of 1–100 μg of IGF-I.

2.6.3. Solid phase extraction

The quantitation of IGF-I and brain IGF-I in a crude fermentation broth required a filtration step prior to loading to the analytical HPLC column. An aliquot of the broth was adjusted to 30% acetonitrile and applied to a 1-ml Bakerbond C_{18} column (J.T. Baker, Phillipsburg, NJ, USA) previously equilibrated with 30% acetonitrile in 0.1 *M* sodium phosphate, pH 7. The column eluent was collected using a 5-ml syringe and filtered through a 0.22-μm, 25 mm Millex-GV filter (Millipore, Milford, MA, USA) prior to injection onto the analytical column. Quantitative recovery of samples enriched with purified IGF-I was obtained.

2.6.4. Other assays

Total protein was determined based on binding to Coomassie blue dye as described by Bradford [12] using purified rhIGF-I as a reference standard.

3. Results and discussion

3.1. Recovery of IGF-I from fermentation broth: TMAC and salt elution

A direct secretion process for IGF-I has been developed at Genentech. In addition to correctly folded monomer, the fermentation broth will contain other IGF species, including incorrectly folded and aggregated forms. All of these species will bind to bare silica and will be eluted by 0.7 *M* TMAC in phosphate buffer, pH 7. Attempts to optimize the TMAC elution using different isocratic steps or gradient conditions were unsuccessful (data not shown). Likewise, isocratic washes of NaCl prior to TMAC elution were not successful. Interestingly, 2 *M* NaCl in 0.1 *M* phosphate, pH 7, did not remove any of the bound IGF-I species, even though this buffer has a much greater conductivity than 0.7 *M* TMAC. This suggests that it is not solely the ionic strength that makes TMAC an effective eluent for proteins bound to silica. Since the elution power of TMAC has been ascribed to its ionic and solvent character, its elution strength must depend on disrupting both of these interactions.

3.2. Effect of solvent on IGF-I elution from silica

The effect of various solvents in eluting IGF-I from silica was investigated. The solvents tested

included isopropanol, acetonitrile, methanol, ethanol and ethylene glycol. After fermentation broth containing IGF-I was applied, each column was washed with a solvent at increments of 0–30% (v/v) in 0.1 *M* sodium phosphate buffer, pH 7. A separate column was used for each solvent tested. After the 30% solvent wash, salt (1 *M* NaCl, final) was added to see if there was any benefit in including solvent and NaCl to elute IGF-I. Finally, a buffer containing 1 *M* TMAC in 0.1 *M* sodium phosphate buffer, pH 7, was used to remove any residual hydrophobic proteins that were still on the column after the solvent wash. All samples were analyzed for IGF-I by the Vydac HPLC and the results from this experiment are summarized in Fig. 1. The elution power of the solvents could be divided into two groups. Isopropanol and acetonitrile seem to be the stronger eluents, but still required 30% solvent to remove all of the bound IGF-I. Ethylene glycol, methanol and ethanol were weaker solvents and were effective eluents only in the presence of 1 *M* NaCl at the highest solvent strength. Inclusion of 1 *M* NaCl also facilitated complete IGF-I elution following treatment with 30% isopropanol or acetonitrile. Since the solvent effects appeared to be similar, the combination of ethanol and salt on the elution of IGF-I from silica was investigated further, due to the cost, availability and safety of ethanol relative to the other solvents.

3.3. Effect of ethanol and salt on IGF-I elution

The combination of ethanol and NaCl was investigated using different increments of each component. Three silica columns were prepared and loaded, as described. Then the columns were washed with phosphate buffer containing a constant amount of sodium chloride (0.1, 0.6 or 1.5 *M*) and varying amounts of ethanol (0–30%). Following the 30% ethanol–NaCl treatment, the columns were washed with 0.1 *M* phosphate, pH 7, to remove solvent and

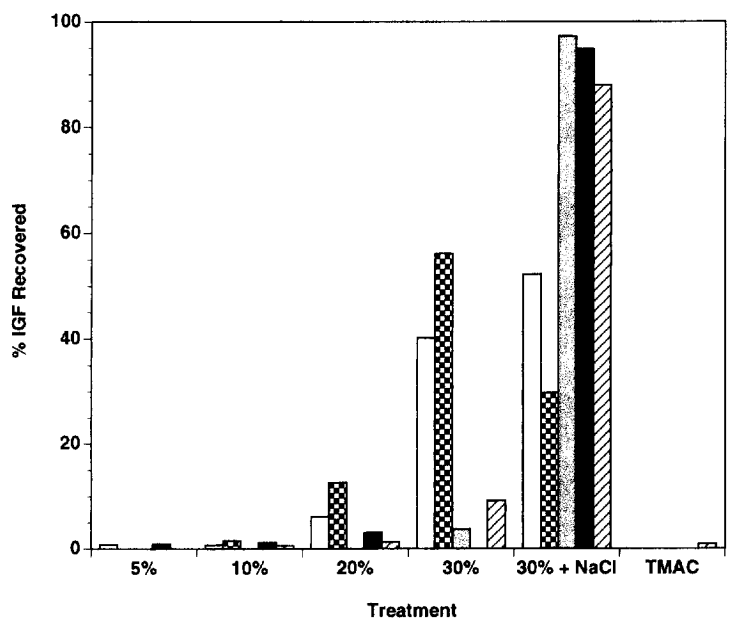

Fig. 1. Effect of solvent on IGF-I elution from silica. The open blocks are isopropyl alcohol (IPA), checkered blocks are acetonitrile (ACN), stippled bars are methanol (MeOH), solid blocks are ethanol (EtOH) and the widely spaced diagonals are ethylene glycol (EG). Increments of each solvent were evaluated for the amount of ICF-monomer by the Vydac HPLC assay. Sodium chloride (1 *M*, final) was added to this buffer after the 30% solvent treatment. After all of the solvent washes, the column was washed with phosphate buffer followed by a wash containing 1 *M* TMAC.

the column was regenerated with 1 *M* TMAC in phosphate buffer to ensure that all of the IGF-I had been eluted. The results from the HPLC analyses of these treatments are shown in Fig. 2. The elution trends seen with each column are similar. Lower concentrations of NaCl required higher levels of ethanol to remove the IGF-I completely. For example, there were still appreciable amounts of IGF-I eluted by the combination of 0.1 *M* NaCl and 30% ethanol. By increasing the level of salt, the elution profile shifted so that more IGF-I monomer was obtained at the lower ethanol concentration. In fact, if salt was absent from the buffer, no IGF-I monomer was eluted. It is important to note that the combinations of NaCl and ethanol were effective in removing all of the IGF-I from the resin, since regenerating each column with TMAC removed no additional IGF-I. The recovery of IGF-I monomer in each treatment exceeded 90% of the total amount loaded onto each column. Other experiments in which the amount of ethanol (10, 20 or 30%) was held constant in three columns and increments of 0–2 *M* NaCl were added gave the same trend. Eventually, the most effective wash and elution scheme included an ethanol wash (20±5%) to remove hydrophobic impurities (but not IGF-I monomer). Then, salt (1±0.5 *M* NaCl) was added to the ethanol buffer to elute the IGF-I monomer.

3.4. Direct comparison of TMAC and ethanol–NaCl elution

Two silica columns were used for this experiment and loaded with fermentation broth containing approximately 20 mg of IGF-I monomer. All wash treatments were buffered with 20 m*M* phosphate, pH 7. The chromatogram showing the TMAC gradient elution is shown in Fig. 3. After washing with the phosphate buffer, the column was eluted with a linear gradient of 0–1 *M* TMAC over ten column volumes. At the end of the gradient, the 1 *M* TMAC wash was extended to ensure that no additional protein would elute. The silica chromatogram using a combination of ethanol and NaCl, to elute bound IGF-I, is shown in Fig. 4. After loading and washing as described above, this column was eluted with the following sequence; 20% ethanol, 20% ethanol+1 *M* NaCl, phosphate buffer, and 1 *M* TMAC. The phosphate wash was included to minimize the effect of ethanol and salt prior to regeneration with TMAC buffer.

Analytical HPLC analysis of the pooled fractions from the TMAC elution (panel B) or the combination of ethanol and salt (panel C) is shown in Fig. 5. In both cases, greater than 90% of the IGF-I monomer was obtained in the silica pool. However, the purity of the IGF-I pool obtained by these two

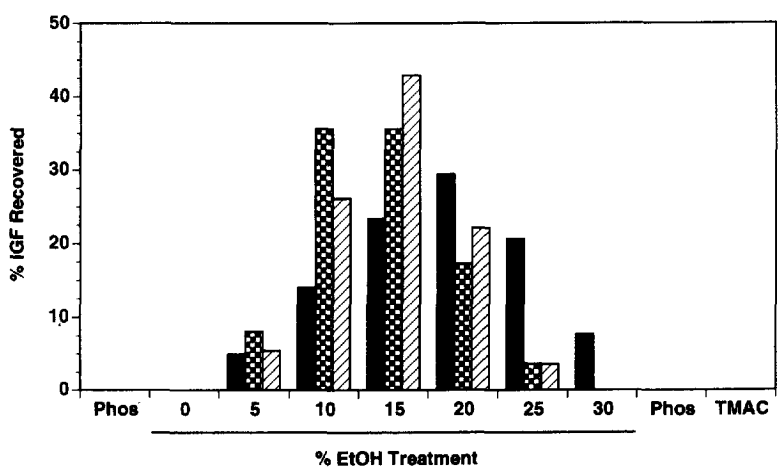

Fig. 2. Effect of ethanol and NaCl on IGF-I elution from silica. The solid blocks are 0.1 *M* NaCl, the checkered blocks are 0.6 *M* NaCl and the diagonals are 1.5 *M* NaCl. Each treatment was buffered using 0.1 *M* phosphate, pH 7.

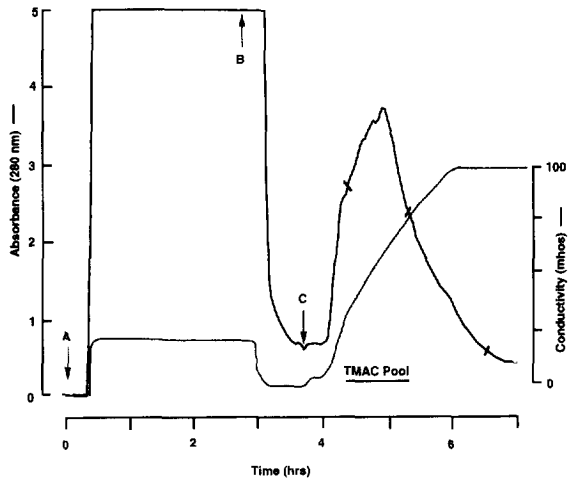

Fig. 3. Silica chromatogram using a TMAC gradient to elute bound IGF-I. The solid line is the absorbance (280 nm) and the stippled line is conductivity. Arrows indicate (from left to right): Column loading (A), phosphate wash (B) and initiation of TMAC gradient (C). Fractions were collected during elution and assayed for IGF-I by a RP-HPLC assay. Fractions containing IGF-I were eluted with 0.1–0.7 *M* TMAC (elution time 4.4–5.2 h). Fractions from approximately 5.2 to 6.5 h, corresponding to 0.7 to 1 *M* TMAC, also were pooled for analysis.

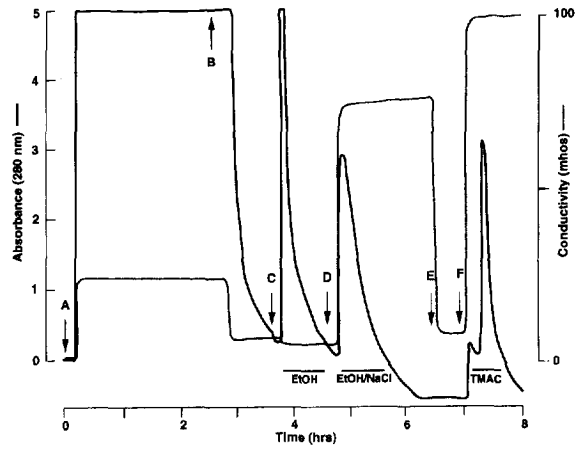

Fig. 4. Silica chromatogram using a combination of ethanol and salt to elute bound IGF-I. The solid line is the absorbance (280 nm) and the stippled line is the conductivity. Arrows indicate (from left to right): Column loading (A), phosphate wash (B), 20% ethanol wash (C), 20% ethanol and 1 *M* NaCl wash (D), phosphate wash (E) and TMAC regeneration (F). Fractions were collected during elution and were assayed for IGF-I using a RP-HPLC assay. Fractions containing IGF-I monomer, eluted by the combination of ethanol and NaCl, were pooled (4.8–5.7 h). Other pools were made based on the chromatogram absorbance peaks and include a 20% ethanol pool (3.8–4.5 h) and a TMAC pool (7.1–7.6 h).

methods was different. During the TMAC gradient, IGF-I eluted from approximately 0.1–0.7 *M* TMAC. Additional TMAC did not elute any IGF-I, but did elute other proteins that were predominantly more hydrophobic than IGF-I and eluted after 11.5 min (IGF-I monomer elutes at approximately 7.3 min in this Vydac analysis). It is significant to note that the use of TMAC, even in a gradient mode, was not effective in specifically removing IGF-I bound to the silica relative to the more hydrophobic impurities. Some of the more hydrophobic impurities (which include aggregated IGF-I) were eluted by the 20% ethanol wash. Then, the addition of salt to the ethanol-containing buffer is sufficient to remove the IGF-I monomer and misfolded species, but not aggregated IGF species. When a TMAC wash is used following the ethanol–salt combination, additional IGF-aggregate is removed. Analysis of the samples by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown) suggests that there is a qualitative difference in the aggregated IGF species. The combination of ethanol and salt elutes disulfide-linked aggregates which, upon reduction, migrate as IGF-monomer. The aggregate obtained from either the ethanol or TMAC treatment also contains material that does not reduce after treatment with thiol reagents. The non-reduced species migrate as dimers, trimers, tetramers, etc., of IGF-I. The different amounts of aggregated material account for a two-fold increase in the purity of the IGF-I monomer relative to the total protein observed in the ethanol–NaCl elution scheme (Table 1). In addition to full length IGF-I, the ethanol–salt elution scheme can be used to purify truncated IGF-I exactly as described above.

3.5. Capture and elution of IGF-BP3 from silica

IGF-BP3 is the predominant circulating form of the IGF-I binding proteins in adults [13]. Since BP3 is a basic protein that retains biological activity following solvent treatment, the binding of BP3 to silica and its subsequent elution using the combination of ethanol and NaCl was examined. For this experiment, harvested cell culture fluid was loaded

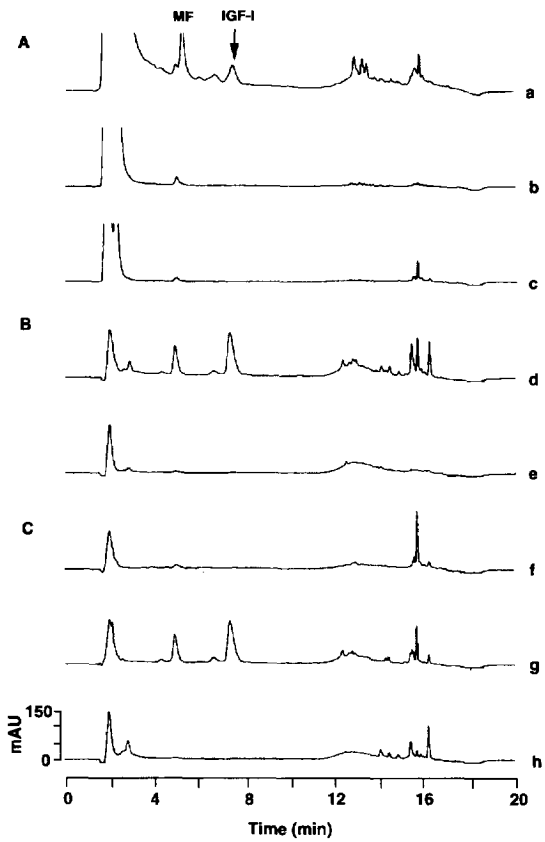

Fig. 5. Reversed-phase HPLC analysis of silica column fractions obtained from either the TMAC gradient or a combination of ethanol+NaCl treatment (refer to Fig. 3 and Fig. 4, respectively). Panel A represents the load (a), flow-through (b) and phosphate wash (c), which were equivalent in both elution schemes. Panel B shows the 0.1–0.7 *M* TMAC pool (d) and the 0.7–1.0 *M* TMAC fraction (e). Panel C shows the 20% ethanol wash (f), the 20% ethanol+1 *M* NaCl pool (g) and TMAC regeneration (h). The top chromatogram denotes the elution position of incorrectly folded (MF) and correctly folded (IGF-I) monomer in this assay.

Table 1
Comparison of silica pools using TMAC or ethanol+NaCl elution

Elution pool	[Protein][a] (mg/ml)	[IGF-monomer][b] (mg/ml)	IGF–protein ratio (%)
TMAC	2.83	0.42	14.8
Ethanol+NaCl	1.24	0.41	33.1

[a] Total protein quantitated by the Bradford protein assay [12].
[b] IGF-monomer concentration quantitated by the Vydac HPLC assay.

onto either an IGF affinity resin or a silica column. Fractions obtained from both columns were analyzed by PL–HPLC assay, as shown in Fig. 6. In both cases, quantitative binding of BP3 occurred and the column flow-through contained host cell protein impurities. The BP3 purity from the affinity column was greater than that obtained from the silica column (85 vs. 65%, respectively). However, the silica pool contained less clipped BP3 species due to decreased exposure time to endogenous protease activity in the cell culture fluid (affinity column operation required one–two days, while that of silica was performed in 4 h). Since the remainder of the impurities present in the silica eluent were not related to the BP3, downstream purification was simplified in the silica elution scheme relative to that of the affinity column.

4. Conclusions

Advances in recombinant DNA technology have led to very high expression levels of the product polypeptide. The purpose of the initial capture step is to concentrate the desired product from the bulk of the host cell proteins and to facilitate downstream purification. In the case of rhIGF-I, increased expression not only resulted in increased levels of correctly folded protein but also in increased levels of incorrectly folded and aggregated IGF species. All of these species were shown to bind quantitatively to bare silica, permitting separation from the bulk of the host cell proteins. Since purification using TMAC buffer eluted significant amounts of aggregated IGF species and compromised downstream purification, elution schemes that separated the biphasic action of TMAC were investigated. By using this strategy, one could optimize the elution for interactions that are dominated by either the polar or ionic forces. Thus, it was shown that aggregated IGF species could be removed by a solvent wash. Subsequently, the addition of salt to the solvent-containing buffer eluted the remaining IGF-I species. By reducing the levels of aggregated IGF-I in the silica pool, the resulting downstream purification steps, to remove correctly folded from incorrectly folded IGF-I, could be performed more efficiently. In addition, this elution scheme was effective for removing other proteins bound to silica including brain IGF-I and an

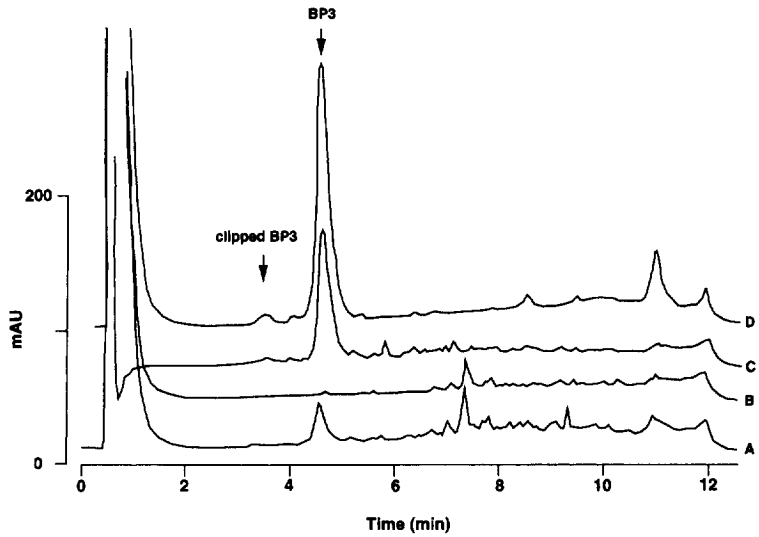

Fig. 6. Reversed-phase HPLC analysis comparing BP3 purified from an IGF affinity column and from a silica column. The chromatograms (from bottom to top) include cell culture fluid [silica- or affinity column load (A), silica flow-through (B) and silica elution pool (C)] using a combination of ethanol and NaCl. The top chromatogram (D) is cell culture derived-BP3 purified using an IGF-I affinity column. The elution of purified BP3 is designated by an arrow.

IGF-I binding protein (BP3) expressed in a mammalian cell culture system.

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