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# $Zn^{2+}$-selective purification of recombinant proteins from the milk of transgenic animals

Arthur Degener[a], Makonnen Belew[b], William H. Velander[a,*]

[a]*Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA*
[b]*Pharmacia Biotech AB, S-751, Uppsala, Sweden*

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**Abstract**

The milk of transgenic livestock is becoming a viable, large-scale source of post-translationally complex, recombinant therapeutic proteins. Recombinant vitamin K-dependent proteins such as human protein C (rhPC) and Factor IX can be produced in milk. However, rate limitations in post-translational modification such as intrachain proteolytic cleavage and γ-carboxylation occur in the mammary gland. Thus, most desirable recombinant products often exist as sub-populations in milk because the mammary gland tends to secrete incompletely processed polypeptides. In general, a nonaffinity purification strategy by which to purify mature recombinant proteins from milk is desirable. $Zn^{2+}$ is used to selectively modify ion-exchange adsorption behavior of endogenous and recombinant milk proteins through conformational changes which cause aggregation and or precipitation. $Zn^{2+}$-selective precipitation of milk and recombinant proteins results in the purification of active rhPC at high yield from the milk of transgenic pigs using expanded bed chromatography. This method selects for rhPC which is both heterodimeric and properly γ-carboxylated. Due to the homology of milk proteins among different species, this same $Zn^{2+}$-selective precipitation strategy is useful for developing purification methods for other recombinant proteins from the milk of transgenic livestock. 

*Keywords:* Milk; Proteins; Zinc

## 1. Introduction

The milk of transgenic livestock can be an ample, specific pathogen-free source of recombinant proteins to augment or replace certain therapeutics derived from human plasma [1,2]. However, both plasma and milk which can have a total protein content of about 40–60 g protein/l, have complexities which can make purification processing difficult [3,4]. For example, affinity chromatography is frequently necessary to isolate desired protein species from antagonistic protein impurities in plasma [4–6] or inactive recombinant protein sub-populations in transgenic milk [7,8]. Due in part to high protein concentrations and the reactivity of endogenous protease cascades, both plasma [6] and milk [9] can spontaneously precipitate while undergoing purification processing. Therefore, solids removal can be repeatedly required during processing.

Cohn fractionation of human plasma has been used for decades to selectively precipitate the major plasma proteins like serum immunoglobulins and albumin by using different combinations of temperature, pH, ionic strength, ethanol [10,11]. In par-

*Corresponding author.

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ticular, $ZnCl_2$ was shown to be highly selective reagent for precipitating plasma proteins such as albumin [12]. Similarly to the major blood plasma proteins, recombinant proteins can be harvested at g/l levels in the milk of transgenic livestock using precipitation techniques [13]. For example, multiple PEG precipitations in combination with ion-exchange adsorption chromatography have been use to purify recombinant α-1 antitrypsin from the milk of transgenic sheep [14] and human protein C from the milk of transgenic swine [13]. However, the addition of PEG at these high levels, the use of filtration or centrifugation to remove somatic cells and protein precipitates, and the need for affinity chromatography to achieve high resolution of desired sub-populations can significantly increase production costs for therapeutic proteins from either blood plasma or transgenic milk.

We use the milk of transgenic pigs containing recombinant human protein C as a model complex source material for therapeutic proteins and apply a novel, non-affinity purification by expanded bed adsorption chromatography (EBA). The milk of ruminant dairy livestock such as sheep, goats and cattle is similar in major milk protein composition and structure to that of swine [3]. For example, the casein protein families share much sequence homology among these livestock [15–17]. However, the milk of swine contains higher levels of non-casein proteins and is more similar in complexity to plasma than ruminant milks. EBA is normally used as a volume reduction step where solids are removed from crude feed material and the product of interest is adsorbed to the gel matrix thereby achieving a low resolution purification [18–20]. In contrast, this study combines deliberate, selective aggregation using low levels of $Zn^{2+}$ to achieve high resolution purification of major milk proteins, as well as inactive from γ-carboxylated, active sub-populations of a recombinant protein in crude transgenic whey by EBA.

## 2. Experimental

### *2.1. Materials*

Streamline DEAE, Streamline 50 glass column, butyl-*S*-Sepharose 6 FF, and Watson Marlow 505U peristaltic pumps were gifts from Pharmacia Biotech (Uppsala, Sweden). Pharmacia C-10 columns were purchased from Pharmacia Biotech. Human protein C from plasma was a gift from American Red Cross (Rockville, MD, USA). Murine metal-dependent anti-hPC monoclonal antibody, 7D7B10-mAb, was purified from cell culture supernatant. Deionized water was produced by a Nanopure Barnstead system. Sodium hydroxide, ammonium hydroxide, EDTA, sodium chloride, rabbit antiserum against protein C, anti-goat immunoglobulins conjugated to horseradish peroxidase, silver nitrate, citric acid, glycerol, 0.45 μm nitrocellulose membranes, Tris–base, bovine casein, low EEO agarose, bromophenol blue, trichloroacetic acid, magnesium chloride, calcium chloride, zinc chloride, Protein C depleted plasma, normal pooled reference plasma and ammonium sulfate were purchased from Sigma. Immulon II Plates, electrophoresis grade polyacrylamide:bisacrylamide [29:1], acetic acid, Accumet 25 pH meter, Marathon 21KR centrifuge, BRL Horizon 11×14 cm horizontal electrophoresis apparatus and FB600 power supply were purchased from Fisher Scientific. Goat antiserum to human protein C and Protac were purchased from American Diagnostica. PTT Automate 5 was purchased from American Bioproducts. *o*-Phenylenediamine–2HCl (OPD) tablets were purchased from Abbott Laboratories. Glutaraldehyde, methanol and Tris–HCl were purchased from Scientific Products. Blotting paper and the Mighty Small electrophoresis apparatus and transphor unit were purchased from Hoeffer Scientific Instruments. The Immulon II plates were analyzed at 490 nm using an EL308 Bio-Tek Microplate reader. A masterflex peristaltic pump, Knauer Spectrophotometer and a Rainin data acquisition system were used to monitor the HIC. A DynaPro-801 dynamic light scattering (DLS) instrument from Protein Solutions Incorporated was also used. The syringe filters ranging from 0.02 to 0.2 μm were Anotop10 inorganic membrane filters with a diameter of 10 mm from Whatman. The DLS data analysis was done using AutoPro software.

### *2.2. Milk collection and preparation*

Milk from transgenic swine was collected and

handled by the method described in Subramanian et al. [21].

### 2.3. Determination of rhPC

The method used for determining the level of rhPC in the milk of transgenic swine is described in Velander et al. [7].

### 2.4. PAGE

Silver-stained 12% SDS-PAGE was done by the method described in Velander et al. [7].

### 2.5. Western blot analysis

Western blot analysis was done by the method described in Velander et al. [7].

### 2.6. Protein solubility

Frozen whey was thawed in a water bath at 4°C for 4 h. A 2 ml sample of whey was aliquoted into each of 11 test tubes, and 1 ml of 50 m*M* Tris–HCl, pH 7.2. Each of the tubes were rendered 0–100 m*M* $Mg^{2+}$ with 500 m*M* $MgCl_2$ and a Tris concentration of 25 m*M* by dilution with deionized water to a total volume of 4 ml and then were mixed by rotary agitation for 30 min at RT. Each tube was then centrifuged at 4500 *g* for 30 min at 23°C. The pH and $A_{280}$ of the decanted, clear supernatant was measured. Each supernatant was also analyzed by silver-stained 12% PAGE. The experiment was replicated using $CaCl_2$ or $ZnCl_2$ instead of $MgCl_2$.

### 2.7. Electrophoretic mobility

Electrophoretic mobility was done on α- and β-pcasein (purified by free flow electrophoresis; Velander et al., unpubl. methods) and rhPC (purified by immunoaffinity chromatography [8]). A 1% low EEO agarose solution was prepared with 25 m*M* Tris–acid pH 7.2 and heated to 100°C, and poured onto a glass plate until a thickness of 3 mm. Approximately 10–15 μg of total protein with 60% glycerol was loaded into each well with the last two lanes being filled with bromophenol blue at 1 mg/ml. The proteins were horizontally electrophoresed in 25 m*M* Tris–acid–acid, pH 7.2, at 100 V until the tracking dye was 1 cm from the end. The gel was fixed in a aqueous, 20% trichloroacetic acid for 20 min, preserved by drying and then stained with 0.125% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) deionized water for 15 min, destained with 50% (v/v) methanol, 10% (v/v) acetic acid and 40% (v/v) deionized water and redried. Protein mobility was recorded. The experiment was replicated but using 2, 4 and 10 m*M* solutions of $MgCl_2$, $CaCl_2$ or $ZnCl_2$ added to the gel, samples and buffer solutions.

### 2.8. Dynamic light scattering (DLS) measurements

Light scattering measurements were done on pure α-pcasein, β-pcasein and rhPC. The DLS instrument was calibrated with BSA at 5 mg/ml in 25 m*M* Tris buffer, pH 7.0 using a 0.1 μm syringe filter. Samples were prepared with $MgCl_2$ ranging from 0–10 m*M* in increments of 1 m*M*. The DLS was washed with 5 ml of dionized water with a syringe filter of 0.02 μm until a photon count rate of 9000 was obtained. A 500 μl was injected into the DLS instrument. AutoPro software was used to estimate hydrodynamic radius, molecular weight and the sampling error. The experiment was replicated using 0–10 m*M* $CaCl_2$ in 1 m*M* increments and with 0–10 $ZnCl_2$ in 1 m*M* increments.

### 2.9. Expanded bed adsorption (EBA)

The following buffers were used during Streamline DEAE chromatography: Buffer A (loading buffer) consisting of 25 m*M* Tris–HCl, pH 7.2; Buffer B–D (elution buffers) having increased NaCl in Buffer A where Buffer B contained 125 m*M* NaCl, Buffer C contained 250 m*M* NaCl, and Buffer D contained 500 m*M* NaCl.

A 300 ml Streamline DEAE chromatography column was operated 4°C at a three-fold, expanded bed mode of 50 cm bed height by pumping Buffer A at a flow-rate of ca. 300 cm/h (6 l/h).

EDTA-treated whey (300 ml of skim) was applied to the expanded bed followed by washing with about 5 expanded-bed volumes of Buffer A until an $A_{280}$ of the top effluent returned to baseline. Further washing with Buffer A was done in top down until

the bed was packed. The packed bed was eluted with a sequence of 5 column volumes (C.V.) of Buffer B, 3 C.V. of Buffer C and 3 C.V. of Buffer D. The column was regenerated using a sequence of 2 C.V. of 4 *M* NaCl, 0.5 *M* NaOH, deionized water, and Buffer A. Each of the four eluate pools obtained above were separately analyzed by SDS-PAGE, Western Blot and ELISA.

The above chromatography was repeated using 300 ml of skim, EDTA-treated whey samples containing 2 m*M* and 4 m*M* $ZnCl_2$.

### *2.10. Hydrophobic interaction chromatography (HIC)*

Butyl-*S*-Sepharose 6 FF medium was packed into a Pharmacia C-10 (15×1 cm, I.D.) column to a bed height of 10 ml. The bed was equilibrated using 4 C.V. of Buffer E (25 m*M* Tris–HCl, 0.8 *M* ammonium sulfate, pH 7.0). Pooled eluates from Buffer C and D were rendered 0.8 *M* $NH_4SO_4$ and pH 7.0, and then applied to the HIC column at a flow-rate of 90 cm/h followed by washing with 2 C.V. of the equilibration buffer, and then was then eluted with 25 m*M* Tris–HCl buffer, pH 7.0. The unabsorbed and eluted pools were analyzed by SDS-PAGE using silver stain and western blotting and ELISA.

The column was regenerated by a sequence of 3 C.V. 30% isopropanol in 25 m*M* Tris–HCl, pH 7.5, 3 C.V. 0.5 *M* NaOH, 4 C.V. deionized water and 5 C.V. Buffer E.

### *2.11. Purification of rhPC by immunoaffinity chromatography*

rhPC was purified from transgenic swine whey by immunoaffinity chromatography as described in Van Cott et al. [8].

### *2.12. Conformational ELISA*

The presence of native γ-carboxylated rhPC was analyzed as described in Subramanian et al. [21]

### *2.13. Activated partial thromboplastin time (APTT) of rhPC from pigs*

The delay in clotting time is described in Morcol et al. [22].

## 3. Results

The effects of the $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ ions on the physicochemical properties of solubility, electrophoretic mobility in agarose gels, and the extent of aggregation of porcine α- and β-casein and rhPC in pure and whey mixtures were studied. Fig. 1 shows the aqueous solubility of milk proteins in the presence of $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ ions as measured by $A_{280}$ or rhPC concentration by ELISA of the supernatants which were clarified by centrifugation. The $A_{280}$ was unaffected by treatment with $Mg^{2+}$ over the concentration range 1–100 m*M* $Mg^{2+}$ for pure solutions of porcine α-casein (Fig. 1a; initial protein concentration of 15 g/l) and β-casein (Fig. 1b; initial protein concentration of 10 g/l). The concentration of pure rhPC was also unaffected by treatment with $Mg^{2+}$ (Fig. 1c; initial protein concentration of 0.36 g/l). In the presence of $Ca^{2+}$ the $A_{280}$ of porcine α- and β-casein solutions was decreased by about 15% and 50%, respectively, while the concentration of pure rhPC is decreased by less than 15%. In contrast to $Ca^{2+}$ or $Mg^{2+}$, $Zn^{2+}$ ions resulted in greater than 90% decrease in solubility over the ranges of 0–3 m*M* and 3–10 m*M* $Zn^{2+}$ for pure porcine α-and β-casein solutions, respectively (Fig. 1a,b). The rhPC concentration decreased from about 360 μg/ml to less than 200 μg/ml over the range 0–10 m*M* $Zn^{2+}$ for pure protein C solutions (Fig. 1c). Fig. 1d shows that the $A_{280}$ of whey samples decreased less than 10% and 50% over the range 0–20 m*M* $Mg^{2+}$ and $Ca^{2+}$, respectively, while a greater than 90% decrease in the $A_{280}$ occurred for whey treated with about 4–10 m*M* $Zn^{2+}$. Silver-stained SDS-PAGE of these samples showed that essentially no change in protein content had resulted from the presence of 0–20 m*M* $Mg^{2+}$ and $Ca^{2+}$ (data not shown). However, SDS-PAGE of the 0–10 m*M* $Zn^{2+}$ treated samples clearly show a selective loss of α- and β-casein from the whey in the range ca. 4–10 m*M* $Zn^{2+}$. Fig. 1e shows that essentially 100% of the rhPC detected in whey by ELISA is precipitated over the range 0–10 m*M* $Zn^{2+}$. Western Blot analysis also confirms these ELISA results (data not shown).

Fig. 2a–c shows change in electrophoretic mobility of pure porcine α- and β-casein and of rhPC in 1% agarose gels in the presence of 0–10 m*M* $Mg^{2+}$ or $Ca^{2+}$ ions and 0–1.0 m*M* $Zn^{2+}$ ions, respectively. Fig. 2a shows a sharp decrease in mobility of α- and

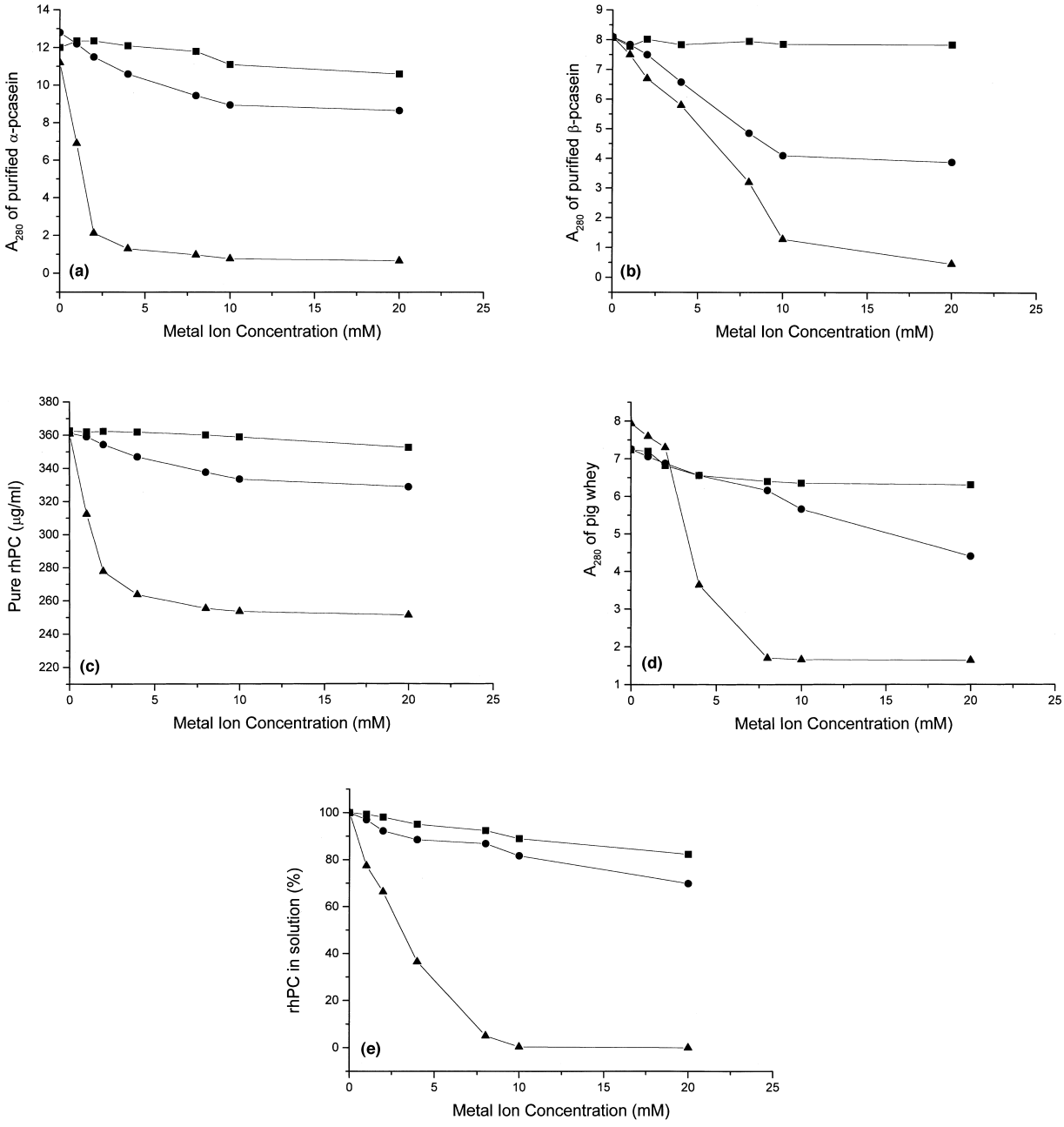


Fig. 1. Effect of $Mg^{2+}$, $Ca^{2+}$ and $Zn^{2+}$ on the solubility of α-pcasein, β-pcasein and rhPC and total milk protein. (a) The solubility of pure α-pcasein as measured by $A_{280}$. (b) The solubility of pure β-pcasein as measured by $A_{280}$. (c) The solubility of pure rhPC as measured by ELISA. (d) The solubility of pig milk proteins as measured by $A_{280}$. (e) The solubility of rhPC in whey treated with metal ion measured by ELISA; (■) magnesium, (●) calcium, (△) zinc.

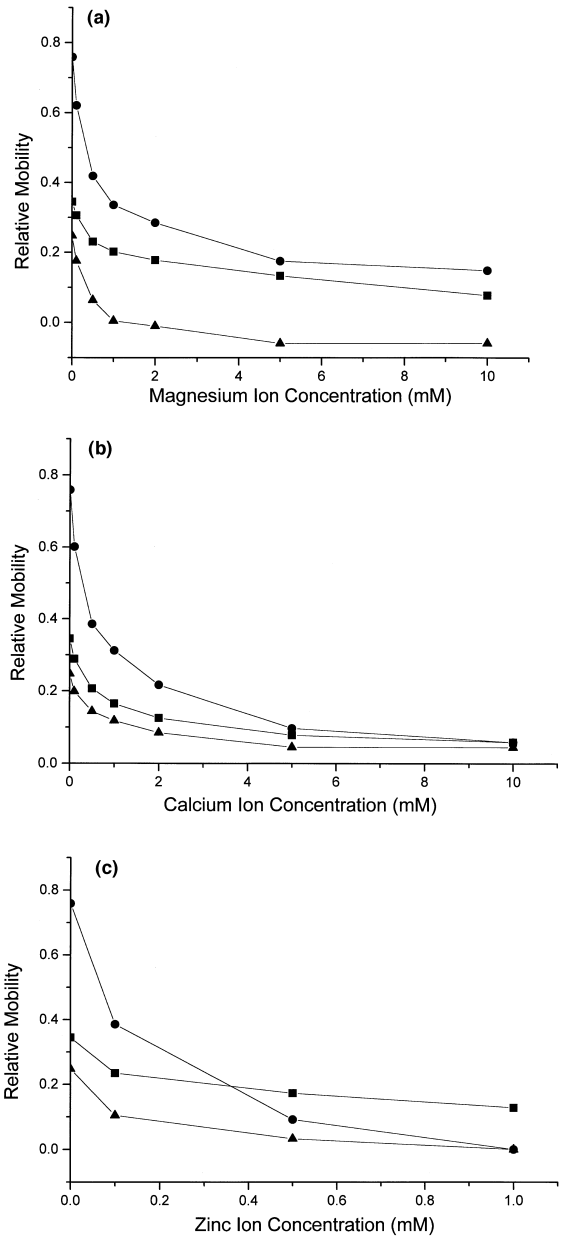


Fig. 2. Effect of $Mg^{2+}$, $Ca^{2+}$ and $Zn^{2+}$ on mobility of the major milk proteins, α and β-pcasein and rhPC. (a) Protein mobility over the range 0–10 m*M* $Mg^{2+}$. (b) Protein mobility over the range 0–10 m*M* $Ca^{2+}$. (c) Protein mobility over the range 0–1 m*M* $Zn^{2+}$; (■) rhPC, (●) α-pcasein, (△) β-pcasein.

β-casein and of rhPC over the range 0–2 m*M* $Mg^{2+}$. The mobility of β-casein is seen to be reversed towards the anode at about 1 m*M* $Mg^{2+}$. Fig. 2b shows a similar decrease in mobility for these same proteins towards the cathode over the range 0–2 m*M* $Ca^{2+}$. In contrast, the presence of only 0.0–0.2 m*M* $Zn^{2+}$ ions caused a sharp decrease in the electrophoretic mobility of pure α- and β-casein and rhPC as shown in Fig. 2c.

Table 1 shows the changes in the hydrodynamic radius as estimated by dynamic light scattering of pure solutions (5 g/l) of α- and β-casein and rhPC in the presence of 0, 4 and 10 m*M* $Zn^{2+}$. An increase in hydrodynamic radius of about 3–36 nm and 3–24 nm for α- and β-casein, respectively, occurs over the treatment range 4–10 m*M* $Zn^{2+}$ while that of rhPC remained nearly constant at about 4–6 nm. An estimated molecular mass of about 31 000 and 42 000 kDa for β- and α-caseins and 71 000 for rhPC was obtained at 0 m*M* $Zn^{2+}$, based upon modeling these proteins as spheres. These molecular mass estimates are in approximate agreement with the apparent molecular mass of α- and β-casein (about 30 000) as estimated by SDS-PAGE (Fig. 5a,b) and also for rhPC (about 62 000). The molecular masses of the 10 m*M* $Zn^{2+}$-induced aggregates not clarified by centrifugation were estimated to be greater than $10^6$ for the porcine caseins and about 160 000 for rhPC.

EDTA-clarified pig whey treated with 0 m*M*, 2 m*M* and 4 m*M* $ZnCl_2$ were processed by Streamline DEAE EBA and typical chromatograms are shown in Fig. 3A–C, respectively. Fig. 4 shows a photomicrograph of the milk solids typically present in whey treated with 4 m*M* $ZnCl_2$. The solids present in 2 m*M* $ZnCl_2$ (Fig. 4) had an average particle length of about 400 μm. No solids were present in the whey not treated with $ZnCl_2$ and about 2% of the rhPC and 45% of the total whey proteins were not absorbed by the expanded bed column (Fig. 3A). Treatment of the whey with 2 m*M* $ZnCl_2$ resulted in the elution of 27% of the rhPC and 56% of the total whey proteins in the unbound fraction (Fig. 3B). The loading of whey treated with 4 m*M* $ZnCl_2$ resulted in 51% of the total rhPC and 85% of the total whey protein passing through the expanded bed column as unabsorbed effluent (Fig. 3C). The adsorbed protein was eluted with step gradients of 125 m*M* (Buffer B), 250 m*M* (Buffer C) and 500 m*M* NaCl (Buffer D). Table 2 shows the total protein and rhPC content of each eluate pool. The Buffer C and Buffer D

Table 1
Dynamic light scattering of pure α- and β-casein and rhPC in the presence of $Zn^{2+}$

| Protein | $Zn^{2+}$ Concentration (m*M*) | $R_h$ (nm) | Estimated molecular mass ($\times 10^{-3}$) |
|---|---|---|---|
| α-pcasein | 0 | 3.5±0.4 | 42±0.2 |
| | 4 | 17.8±1.1 | 2719±2 |
| | 10 | 36.3±2.8 | 16899±24 |
| β-pcasein | 0 | 3.1±0.4 | 31±0.2 |
| | 4 | 5.2±0.7 | 116±1 |
| | 10 | 23.7±1.4 | 5664±4 |
| rhPC | 0 | 4.3±0.5 | 71±0.3 |
| | 4 | 5.2±0.5 | 116±1 |
| | 10 | 5.9±0.6 | 160±1 |

eluates were pooled for subsequent immunoaffinity or hydrophobic interaction chromatography. The purification factors of each individual eluate pool ranged from less than about 1 to 8. A total of 29% of the original total whey protein and 84% of the rhPC was contained in the pooled Buffer C–D eluate from the 0 m*M* $ZnCl_2$ treated-whey loading. The 2 m*M* $ZnCl_2$ treated-whey loading gave a pooled Buffer C–D eluate containing 23% of the original total whey protein and 66% of the rhPC. The 4 m*M* $ZnCl_2$ treated-whey loading gave a pooled Buffer C–D eluate which contained of 18% of the original total whey protein and 41% of the rhPC.

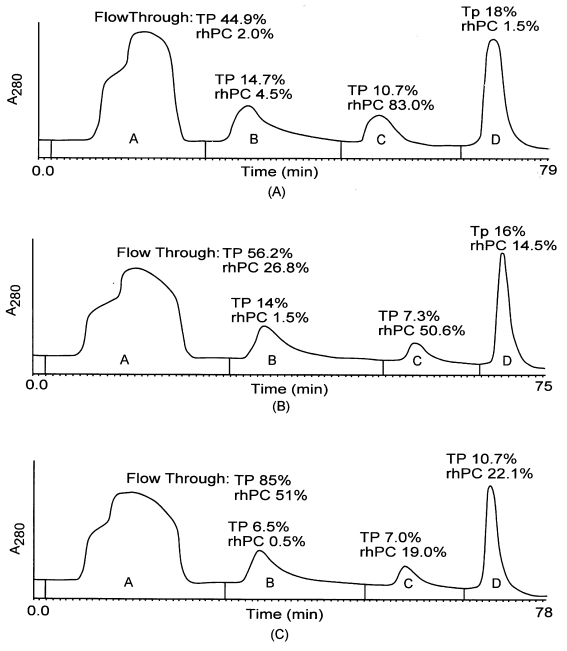


Fig. 3. The effect of $Zn^{2+}$ on Streamline DEAE chromatography of pig whey. (A) Streamline DEAE chromatography of whey with no metal ion. (B) Streamline DEAE chromatography of whey with 2 m*M* $ZnCl_2$. (C) Streamline DEAE chromatography of whey with 4 m*M* $ZnCl_2$; TP: total protein, rhPC: recombinant human protein C.

Fig. 5a shows samples from the purification train of 4 m*M* $Zn^{2+}$ treated whey analyzed by silver-stained SDS-PAGE. Immunoaffinity chromatography of unabsorbed effluents and pooled salt eluates from the Streamline DEAE expanded bed chromatography was done to recover rhPC at high yield from either unabsorbed or adsorbed Streamline DEAE expanded bed chromatography streams. The rhPC yield from the immunoaffinity step was 90–94% for salt eluate pools and about 86–89% for unabsorbed effluent

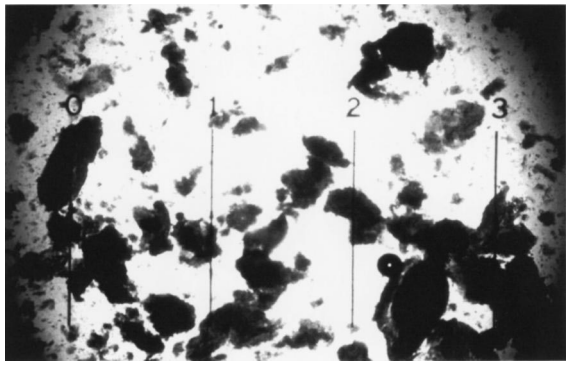


Fig. 4. Photomicrograph of milk protein aggregates in the presence of 4 m*M* $ZnCl_2$. Each scale division is 1000 μm (1 mm) in length and the total field spans 4000 μm.

Table 2
Purification and yield from HIC and immunopurified rhPC purification processes

| Samples | Total protein (%) | Percent rhPC yield (%) | Purification factor | Purity (%) | Activity by APTT (%hPC ref) |
|---|---|---|---|---|---|
| Purification of rhPC with no zinc | | | | | |
| Start | 100 | 100 | 1.0 | N.A. | N.A. |
| Fall through (FT) | 44.9 | 2 | 0.1 | N.A. | N.A. |
| 125 m*M* NaCl peak | 14.7 | 4.5 | 0.3 | N.A. | N.A. |
| 250 m*M* NaCl peak | 10.7 | 83.1 | 8.0 | N.A. | N.A. |
| 500 m*M* NaCl peak | 18.1 | 1.3 | 0.1 | N.A. | N.A. |
| Mab-affinity products–Streamline FT | N.A. | N.A. | N.A. | N.A. | N.A. |
| Mab-affinity products–Streamline Eluates | 100 | 89 | 200 | >95 | 43 |
| Purification of rhPC with 4 m*M* zinc | | | | | |
| Start | 100 | 100 | 1.0 | N.A. | N.A. |
| Fall through | 85 | 51.1 | 0.6 | N.A. | N.A. |
| 125 m*M* NaCl peak | 6.5 | 0.5 | 0.1 | N.A. | N.A. |
| 250 m*M* NaCl peak | 7 | 19 | 3.0 | N.A. | N.A. |
| 500 m*M* NaCl peak | 10.7 | 22.1 | 2.0 | N.A. | N.A. |
| Mab-affinity products–Streamline FT | 100 | 89 | 200 | >95 | 0 |
| Mab-affinity products–Streamline Eluates | 100 | 94 | 200 | >95 | 75 |
| HIC products–Streamline eluates | 100 | 93 | 150 | >80 | 71 |

from the Streamline DEAE expanded bed chromatography. All immunoaffinity products were greater than about 95% pure as judged by silver-stained SDS-PAGE. The pooled salt eluates were also purified by hydrophobic interaction chromatography (HIC) using a butyl-Sepharose FF column. All HIC products were greater than about 95% pure as judged by silver-stained SDS-PAGE. The rhPC yield from the HIC was 90% or greater. The primary remaining protein impurity was identified as porcine serum albumin (data not shown). Table 2 shows that a purification factor of about 200 was achieved by combination of Streamline DEAE expanded bed chromatography and immunoaffinity or HIC.

Fig. 5b is a non-reduced and reduced western (immuno-blot) of rhPC purified by both EBA-immunoaffinity and EBA-HIC. There was no apparent difference between the rhPC products under non-reducing conditions, but under reducing conditions differences in single chain content were apparent. The starting whey material has about 50% of the rhPC population existing as single chain. The unabsorbed material from 4 m*M* $Zn^{2+}$-treated whey processed by Streamline DEAE EBA chromatography has greater than 50% of the population existing as single chain. The adsorbed 4 m*M* $Zn^{2+}$-treated whey material eluted from Streamline DEAE EBA chromatography and the subsequent product from EBA-HIC has less than 10% of the rhPC existing as single chain, which was similar to immunopurified-hPC.

Table 2 shows the anticoagulant activity of immunoaffinity purified rhPC as a percentage of immunopurified hPC derived from human plasma. Immunoaffinity purified rhPC material obtained from the unabsorbed effluents of Streamline DEAE EBA of loadings with 2 m*M* and 4 m*M* $ZnCl_2$-treated wheys showed no anticoagulant activity by APTT assay. Essentially no unabsorbed rhPC was obtained from loadings of Streamline DEAE EBA at 0 m*M* $ZnCl_2$. Immunoaffinity purified rhPC from pooled Buffer C and D eluates of Streamline DEAE EBA loadings with 0 m*M*, 2 m*M* and 4 m*M* $ZnCl_2$-treated wheys showed 43%, 58% and 75% anticoagulant activity by APTT assay, respectively, relative to immunopurified plasma derived hPC. HIC purified rhPC from the salt eluate pool of Streamline DEAE EBA from loadings of 4 m*M* $ZnCl_2$-treated wheys

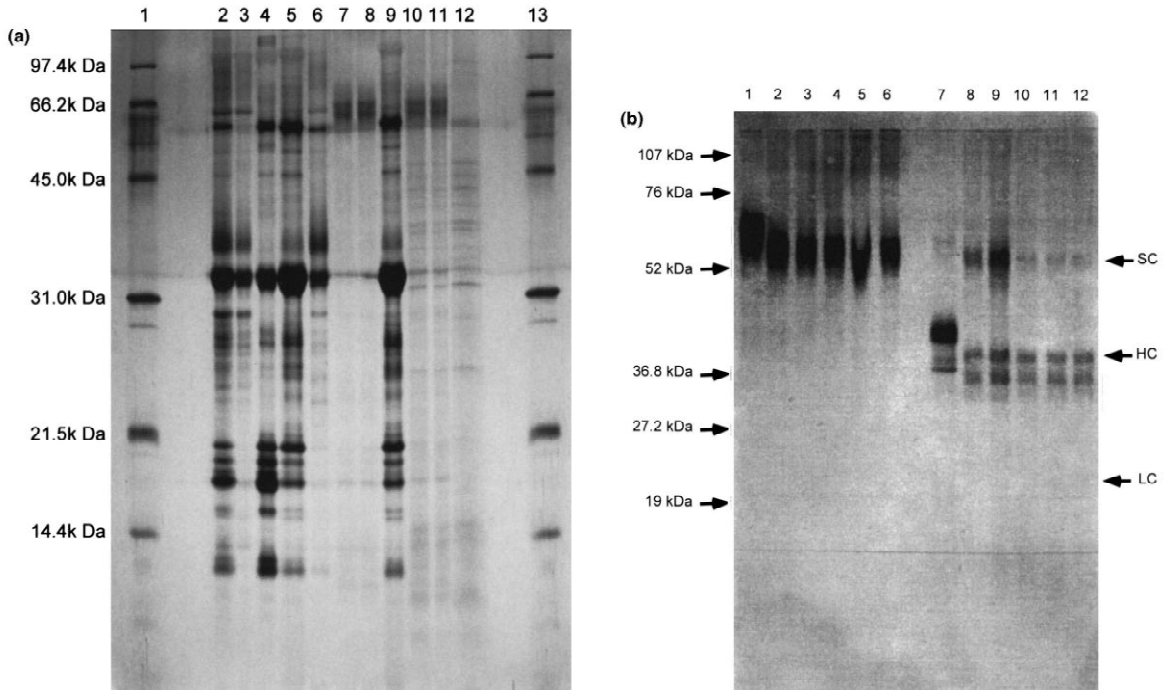


Fig. 5. Whey fractions from Streamline DEAE chromatography in the presence of $Zn^{2+}$ analyzed by SDS-PAGE, non-reduced and reduced western. (a) A 12% silver stained SDS-PAGE of whey fractions from Streamline DEAE chromatography in the presence of 4 m*M* $Zn^{2+}$ with molecular mass markers ranging from 14 400 to 97 000 present in lanes 1 and 13. Lane 2 is the starting material, lane 3 is the unabsorbed protein from $Zn^{2+}$ treated whey from EBA, lanes 4–6 are the Buffer B, Buffer C and Buffer D elution products from Streamline DEAE, respectively, lanes 7 and 8 are immunopurified rhPC from the unabsorbed protein from $Zn^{2+}$ treated whey from EBA and pooled Buffer C and Buffer D elution products, respectively, lane 9 is the unabsorbed protein from butyl-*S*-Sepharose 6 FF, lane 10 is the product purified by butyl-*S*-Sepharose 6 FF, lane 11 is the butyl-*S*-Sepharose 6 FF product shown in lane 10 loaded onto DEAE Sepharose FF and eluted with 25 m*M* $CaCl_2$ and lane 12 is the 1 *M* NaCl elution of proteins from DEAE Sepharose FF that did not elute with 25 m*M* $CaCl_2$. There was 1–2 μg of total protein applied to each lane except for lanes 7 and 8 which only have 0.25 to 0.50 μg of total protein. (b) Non-reduced and reduced westerns of the rhPC purification process. Prestained molecular mass markers ranging from 19 000 to 107 000 were used, but are not shown. Lanes 1 and 7 are non-reduced and reduced reference human Protein C from plasma, respectively, lanes 2 and 8 are non-reduced and reduced rhPC present in the starting material, lanes 3 and 9 are non-reduced and reduced rhPC from the unabsorbed protein from $Zn^{2+}$ treated whey from EBA purified by immunoaffinity chromatography, lanes 4 and 10 are non-reduced and reduced rhPC from pooled Buffer B and Buffer C elution products from $Zn^{2+}$ treated whey from EBA purified by immunoaffinity chromatography, lanes 5 and 11 are non-reduced and reduced rhPC purified by HIC then by DEAE $Ca^{2+}$ elution. There was 250 ng of rhPC applied to each lane for the non-reduced lanes and 500 ng of rhPC applied to each lane for the reduced lanes; **SC**: Single chain, **HC**: Heavy chain, **LC**: Light chain.

gave an anticoagulant activity of 71% by APTT assay.

A $Ca^{2+}$-dependent, rhPC-conformational-sensitive-ELISA was used to determine the adsorption selectivity for rhPC sub-populations having mature γ-carboxylated glutamic acid (gla) domains [23]. Fig. 6 shows the results of conformational ELISA analysis of immunoaffinity or HIC purified rhPC products that were contained in combined Buffer C and D eluates or unabsorbed effluents from Streamline DEAE EBA. Human protein C from plasma, rhPC purified by HIC or by immunoaffinity chromatography from Buffer C and D eluate pools of 4 m*M* $ZnCl_2$-treated whey processed on Streamline DEAE gave a half-maximal inhibition by $Ca^{2+}$ of the ELISA signal of about 2 m*M*. In contrast, rhPC purified by immunoaffinity chromatography of Buffer C and D eluate pools from 0 and 2 m*M* $Zn^{2+}$ treated wheys processed on Streamline DEAE gave half-maximal inhibitions of the ELISA signal by

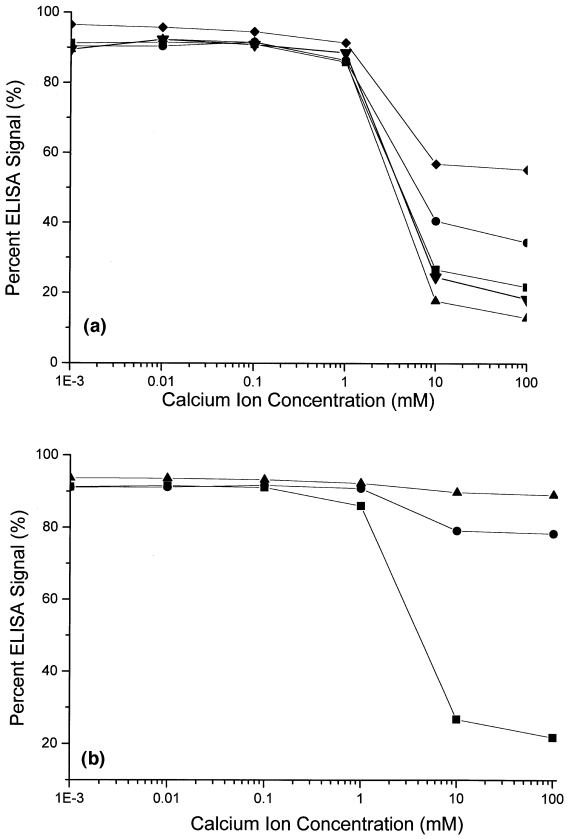


Fig. 6. Conformational ELISA of rhPC chromatographic fractions. (a) The 250 m*M* NaCl fractions from the Streamline DEAE with and without $Zn^{2+}$ purified by immunoaffinity or hydrophobic interaction chromatography(HIC); (■) Mab-affinity product of hPC from human plasma, (●) Mab-affinity product of 2 m*M* $ZnCl_2$ Streamline eluate, (△) β-Mab-affinity product of 4 m*M* $ZnCl_2$ Streamline eluate, (♦) Mab-affinity product of no $ZnCl_2$ Streamline eluate, (▼) butyl isolation of rhPC from 4 m*M* $ZnCl_2$ Streamline eluate. (b) The unabsorbed material from the Streamline DEAE with $Zn^{2+}$ purified by immunoaffinity chromatography; (■) Mab-affinity product of hPC from human plasma, (●) Mab-affinity product of 2 m*M* $ZnCl_2$ Streamline flow through, (▲) β-Mab-affinity product of 4 m*M* $ZnCl_2$ Streamline flow through.

$Ca^{2+}$ of about 6 and 10 m*M*, respectively. Immunopurified rhPC from unabsorbed effluents of Streamline DEAE expanded bed chromatography from loadings of 2 m*M* and 4 m*M* $ZnCl_2$-treated wheys gave no half-maximal inhibition of ELISA signal by $Ca^{2+}$.

## 4. Discussion

Like human plasma, milk is a relatively complex mixture containing serum passover proteins such as albumin, broadly specific proteases, and caseins [3,9]. As has been seen in plasma fractionation methods developed by Cohn et al., [11,12], the results presented here suggest that highly selective, non-affinity methods can be devised to provide a simplified process for purifying active sub-populations of complex recombinant proteins from transgenic milk. In particular, we have shown that EBA used in conjunction with purposeful, $Zn^{2+}$-selective aggregation and or precipitation can achieve both high resolution and ease of processing comparable to that of affinity chromatography. The combination of changes in electrophoretic mobility and hydrodynamic radius measured by dynamic light scattering of supernatants from $ZnCl_2$-treated whey indicate that both the formation of soluble aggregates and insoluble precipitates likely contribute to the selectivity of the $Zn^{2+}$-selective purification phenomena in milk. Specifically, aggregation can be expected to slow [24] and could also preclude chromatographic adsorption onto purification matrices to the extent that precipitation occurs. Coordinate covalent $Zn^{2+}$–protein complexes are significantly stronger than ordinary ionic metal–protein complexes [25,26]. As a result, even low m*M* concentrations of $Zn^{2+}$ provide a large free energy driving force to cause profound conformational changes in the protein which bring electron donating residues in close proximity to coordinately complex the $Zn^{2+}$ ion. Conformational changes in proteins induced by bound $Zn^{2+}$ can result in changes in tertiary structure which can initiate aggregation and precipitation [12,27,28]. With respect to the potential amenability to large-scale processing of milk and perhaps other complex mixtures, we have shown that EBA can handle crude whey containing milk protein precipitates induced by $Zn^{2+}$ ions while still selectively adsorbing active rhPC at high yield.

While immobilized metal ion affinity chromatography (IMAC) uses transition metals to achieve selective adsorption of proteins, we have found IMAC to be ineffective in the processing of crude milk and plasma derivatives (data not shown). In

addition, $Cu^{2+}$ and $Ni^{2+}$ ions have been most often applied to IMAC [25,26] and also metal affinity precipitation [27]. However, these metals have disadvantages of causing metal-catalyzed oxidation reactions which degrade proteins [29,30]. In contrast, the $Zn^{2+}$ ion is a non-transition metal and has been shown not to cause oxidative degradation of proteins [31,32].

In order to minimize the number of chromatographic conditions which were to be tried, we first examined changes in tertiary and quaternary structure of proteins reflected in a few physicochemical properties of the major milk proteins and the target protein in pure solutions, as well as in the complex mixture environment of whey. The generic approach of using selective aggregation of α- and β-caseins is of value to downstream processing of milk, since many milk proteins including the caseins have primary structure which is well conserved across livestock species [15–17]. Previous studies have shown that 2 m*M* $Zn^{2+}$ will precipitate bovine α-caseins. Bovine β-casein was precipitated by about 4 m*M* $Zn^{2+}$ [28]. Our study is the first report on $Zn^{2+}$-precipitation of α- and β-porcine casein and undercarboxylated, two-chain and single chain forms of rhPC. The interaction of $Zn^{2+}$ ion which can form coordinate covalent bonds with caseins is clearly different than that of other non-transition metals like $Ca^{2+}$ and $Mg^{2+}$ ions which can form only ionic bonds [25,26,33]. The formation of $Zn^{2+}$-casein complexes at as little as 0.1 m*M* $Zn^{2+}$ ion can be first seen the appearance of soluble aggregates which have limited electrophoretic mobility.

The selectivity of the reaction of immature populations of rhPC with $Zn^{2+}$ ion is striking and several reasons may exist for this preferential reactivity. These immature populations constitute about 40–60% of the total rhPC and have been previously shown to have under-carboxylated gla domains that have an altered $Ca^{2+}$-dependent conformation [21] relative to fully carboxylated rhPC. The immature rhPC populations in milk also predominately exist, greater than 50%, as a single chain form. The single chain form is apparently more susceptible to conformational changes resulting in aggregation and precipitation when in the presence of $Zn^{2+}$. This is consistent with the thermal melting behavior of both the gla and EGF domains of rhPC which has been associated with nonnative rhPC populations [34].

Our analysis of the gla domain of the EBA-unabsorbed rhPC populations by $Ca^{2+}$-dependent, conformational-sensitive ELISA were shown to be both inactive and have a nonnative Ca-dependent conformation. More specifically, the 7D7-MAB used to measure the Ca-dependent conformation of the gla-domain of hPC conformation recognizes an epitope in the light chain of hPC which includes γ-carboxylated glutamic acid residues at amino acid positions 6 and 7 [21]. Decarboxylated hPC is not released by the 7D7-MAb used in the ELISA in the presence of 2 m*M* $Ca^{2+}$ and these unabsorbed rhPC populations from 2 and 4 m*M* $ZnCl_2$-treated wheys behave similarly to decarboxylated hPC. In summary, while other deficiencies in post-translational processing such as carbohydrate structure and improper disulfide bridging can result in inactivity, the majority of under-carboxylated rhPC passed through, unabsorbed by the Streamline DEAE expanded bed when 4 m*M* $ZnCl_2$ was present. In addition, we have previously shown that electrophoretic mobility differences between rhPC and hPC are due to differences in glycosylation [35].

β-casein is a major milk protein and thus a predominant impurity in rhPC products from DEAE ion-exchange chromatography of transgenic whey. The selective adsorption of rhPC by HIC while leaving β-casein unabsorbed to the butyl-*S*-Sepharose FF matrix likely results from the accessability of the hydrophobic stack of hPC light chain which is independent of the gla domain [36]. Analysis of the extent of hydrophobic domains which occur in β-casein and rhPC using the method of Hopp-Woods (data not shown) indicates that β-casein is greatly more hydrophobic than rhPC. However, the β-casein did not adsorb to the HIC column and this suggests that strong nonpolar-self interactions dominates β-casein solution behavior under the conditions used to adsorb rhPC. Since, only the active population of rhPC remained in the HIC feed-stream, the use of a specialized immunoaffinity adsorption step [8,21] which recognizes only properly carboxylated rhPC can be circumvented. Further processing by calcium dependent elution of properly carboxylated rhPC from anion-exchange columns [37] can also be done

after HIC for further purification (Fig. 5; Lane 11). It is noteworthy that this calcium elution strategy was ineffective when applied to crude whey or Streamline DEAE EBA eluates, but effective on the more highly pure HIC eluate (data not shown). A similar $Zn^{2+}$-selective precipitation process has been developed for recombinant human Factor IX (data not shown) and recombinant fibrinogen expressed in transgenic milk of mice and pigs (data not shown) which helps to demonstrate the generic value of this purification strategy for recombinant proteins from milk. In summary, we have used the profound metal-dependent conformational changes associated with major milk proteins and target protein sub-populations as a tool for achieving highly selective and scaleable EBA purification processing of transgenic milk. Thus, even a complex mixture of immature recombinant proteins can be effectively resolved from transgenic milk using simple procedures making the cost-effective, large-scale transgenic production of very complex therapeutic proteins using dairy livestock more feasible.

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