Downstream Processing of Recombinant and Endogenous Proteins from Livestock Milk

by
Arthur W. Degener, Jr.

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William H. Velander, Chairman

Richey M. Davis	William L. Conger
Kimberly Foresten	William N. Drohan

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(ABSTRACT)

With the increased demands of therapeutic proteins, there is going to be a need for new purification technologies which have high throughput, high yield and high resolution. Three purification technologies were explored as potential new technology to isolate recombinant and endogenous milk proteins: Expanded bed adsorption chromatography(EBAC) combined with hydrophobic interaction chromatography(HIC), Recycle continuous flow electrophoresis(RCFE) and Free flow isoelectric focusing(FFIEF). The first process(EBAC/HIC) used with Zn^{2+} as a selective precipitating agent, purified recombinant human protein C(rhPC) and IgG(contaminated with less than 1% IgA) from swine milk with high resolution and high yield while processing about 10-20 grams in a single operation. The second process(RCFE) was able to isolate the active sub-populations of rhPC from major milk contaminants(α- and β-pig casein) as wells as from the inactive sub-populations of rhPC. RCFE was able to process 1.5g total protein per hour on a small scale and is currently being researched to process 1kg total protein per hour. The third and final purification process(FFIEF) sub-fractionated 100mg of immuno-purified rhPC into 50 fractions. The FFIEF was able to produce a linear pH gradient over the range of 3-10 using 2% ampholytes. The fractionated rhPC showed differing degrees of activity that resulted from the γ-carboxylated glutamic acids and the sialic acids.

Dedication

This work is dedicated with love and affection to my wife, Laurel, and my two children Jessica and Bradley.

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Table of Contents

List of Illustrations

Chapter 1

Chapter2

Chapter 3

List of Tables

Appendices

Chapter 1: Introduction: Therapeutic Proteins and Purification

1.1 Recombinant Therapeutic Proteins

Genetic engineering has produced recombinant therapeutic alternatives In the wake of viral safety concerns and clinical demand of human plasma-derived therapeutics. Several production systems that utilize prokaryotes, yeast and plants have been used to produce recombinant proteins, but they lack the ability to produce proteins with complex biochemical modifications such as those found in vitamin K dependent (VKD) proteins[1]. While mammalian cell culture can be used to make post-translationally complex proteins, low cell densities and therefore low protein concentrations and high culture equipment costs have made these products less available and less economical[2]. These systems all provide a challenging and complex feedstock for purification technology. In particular, there are a number of natural and recombinant sources of therapeutic proteins which generate the need for high resolution technology which is more economically desirable.

An alternative source to recombinant mammalian cell culture may be the mammary gland of livestock. The mammary gland is naturally capable of producing and secreting large quantities of protein. In fact, several proteins have already been expressed in the milk of transgenic livestock such as tissue plasminogen activator in goats[3], α-1-antitrypsin in sheep[4] and recombinant human protein C (rhPC)in pigs[5]. Milk is also a source of recombinant[6] and natural therapeutic proteins such as immunoglobulins[7]. While goats and cows

are more classical dairy animals, pigs produce almost as much milk per year as do sheep. The use of pigs offers several advantages over other dairy livestock such as short generation times, approximately 20 offspring per year and about 10kg milk per day.. Thus there are a number of natural and recombinant sources of therapeutic proteins which generate the need for economical high resolution purification technology.

There are many important classes of therapeutic proteins which are challenging to purification process technology. For example, VKD plasma proteins are a part of the blood coagulation cascade which maintains hemostasis. The family of VKD-plasma proteins characteristically contain γ-carboxylated glutamic acid residues which arise from a vitamin K dependent intracellular post-translational processing step that occurs prior to secretion into plasma from the liver[8]. VKD plasma proteins are: Protein C (hPC), Protein S (hPS), Factor VII (FVII), Factor IX (FIX), Factor X (FX) and prothrombin. A notable purification complexity is that these proteins can have antagonistic activities. For example, FVII, FIX, FX and prothrombin have pro-coagulant activities while hPC and hPS have anticoagulant activity (Figure 1). In addition, the clinical demand for therapeutics is high due to insufficient amounts which can be isolated from human plasma. For example, FIX and protein C occur at only about 4µg/ml in plasma. Thus, there is a need for improved high resolution technology to separate proteins having antagonistic activity at high yield to make

efficacious and more economical therapeutic products.

Human protein C (hPC) may be useful as a therapeutic to treat many thrombotic conditions. Currently, heparin and warfarin are used as anti-coagulants to treat thrombosis. Because these are used as artificial inhibitors of coagulation, a more unnatural hemostatic regulation is usually achieved and could lead to serious life threatening side effects[9]. At the heart of the anticoagulant process is hPC. Protein C is a serine protease synthesized by the liver and circulates in plasma as the natural heterodimer this is a zymogen(inactive precursor) at a concentration of 4 µg/ml[10-12]. The complexity of hPC arises from post-translational modifications such as removal of pro-peptide and signal peptide, removal of Lys^{156} -Arg^{157} to form a heterodimer consisting of a light chain(Mr~ 22kDa) and a heavy chain(Mr~41kDa), 12 disulfide bridges, 4-N linked glycosylation sites and 9-γ carboxylation sites within the first 21 amino acids of the light chain(Figure 2). Upon the onset of pro-coagulation activation, hPC undergoes a feedback activation that consists of the cross-formation of a calcium-dependent complex of Protein S, thrombin and thrombomodulin at the endothelial cell surface where vascular damage and thrombosis(clot formation) is generated. Thrombosis is controlled by the proteolytic inactivation of Factors V_A and $VIII_A$ by activated hPC[13,14].

1.2 Native Milk Therapeutic Proteins

The mammary gland of dairy livestock is capable of producing proteins in large quantities to nourish young offspring. The major milk proteins are α_{s1}-, α_{s2}- and β-casein, but there are other proteins at low concentration such as albumin, transferrin, α-lactalbumin, β-lactoglobulin and immunoglobulin(Table I). Transferrin and immunoglobulins impart passive immunological protection to young. The proteolytic degradation products of caseins serves a nutritional function and also antibiotic activity[15]. Neonatal health of livestock is of major concern and can be mitigated by dietary immunoglobulin augmentation. Currently, 10-20% of the young die as a result of diarrheal disease and this could be negated[16]. Human applications can include using immunoglobulins from appropriately sensitized animals to treat recurring infections[17,18].

As discussed above, the milk of dairy live stock may be a viable feed source of recombinant and natural immunoglobulins(IGs). IGs are molecules having affinity for foreign molecules of various types including those bound to cells as well as those of greater molecular weight of about 200 kDa. These target species are called antigen. There are at least three types of IGs that are found in milk: IgG, IgA and IgM(Figure 3), at concentrations of 2, 10 and 5 mg per ml respectively. IgG is a complex protein having 4 chains, 2 glycosylated heavy chains with molecular weight around 60kDa and 2 non glycosylated light chains with molecular weights around 25kDa. IgG is a symmetric molecule

having two identical binding sites for the same antigen. The amino terminus contains a region which has a variable primary structure(amino acid sequence), while the carboxyl terminal domain remains constant. IgG contains 4 disulfide bonds, 2 on the heavy chain and 2 on the light chain. There exist at least 4 subclasses of IgG all with similar physiochemical properties, but differing amino acid compositions within the constant domain. IgG is the major antibody (80% of the serum IGs) that circulates in the blood, but can also enter tissue space to help antigen uptake. The primary structure of IgA (12% of the serum IGs) is very similar to IgG in structure, but usually exists as a dimer and is found in secretions such as tears, saliva and bronchial mucus to protect body fluids from antigen. IgM (8% of the serum IGs) is also very similar to IgG in primary structure, but exists as a pentamer. IgM class antibodies are the first products of the immune response. The pentameric nature of IgM gives it a deca-valency for the same antigen that enables rapid and high capacity adsorption of antigen[19].

1.3 Protein Purification

The milk of transgenic livestock can be an ample, specific pathogen-free source of recombinant and natural proteins to augment the source of certain therapeutics such as those derived from human plasma. However, both plasma and milk can have a total protein content of about 40 to 60 g protein/liter. Thus, both are complex mixtures which make purification processing a difficult challenge [20,21]. The downstream processes which can be used to isolate proteins like serum IGs and hPC from human plasma as well as rhPC and IGs from the milk of transgenic livestock is described below:

Precipitation

The solubility of aqueous proteins is largely determined by the tertiary(3-dimensional) conformation which is dependent on ionic strength, pH, dielectric and entropic driving forces of hydrogen binding. Aqueous soluble proteins which are typically non-membrane proteins can be reversibly denatured and precipitated using an organic solvent(ethanol) or salt(Ammonium Sulfate). The lowered dielectric provided by the organic solvents reduces aqueous solvation of hydrophilic domains. This leads to aggregation and precipitation. High salt concentrations can also decrease the solvation of proteins due to charge shielding of hydrated ionic groups which enables hydrophobic interactions between proteins. Aggregation and eventually precipitation result from protein-protein hydrophobic interaction[22]. A single precipitation step can be done to

reduce the concentration of background proteins while keeping the target protein in solution. Ammonium sulfate has been used to isolate IgG from serum at about 40-45% saturation[23], and total protein concentration can be reduced by one third to one half the initial value while keeping certain targeted proteins in solution. For example, Cohn used a combination of organic solvent and salt to precipitate plasma into many fractions[24,25]. From each of these precipitate and supernatant fractions, immunoglobulins, albumin and VKD rich protein fractions therapeutics have been isolated in a commercial process at greater than 95% purity.

Chromatography

Solution conditions such as pH and ionic strength can be altered to favor the selective adsorption of proteins onto appropriately derivatized solid or gel matrices. The derivatization of matrices with ionic groups, hydrophobic and affinity ligands is used to make selective matrices for ion-exchange, hydrophobic and immunoaffinity adsorption chromatography. The adsorption matrices are typically packed within tubular vessels optimized for plug flow of an aqueous mobile phase. The aqueous mobile phase is commonly passed over a packed bed stationary phase and proteins adsorb to the surface. The matrix is then washed free of background contaminants. Finally, the specifically adsorbed species is then desorbed from the washed matrix [22,23,26,27].

Ion-exchange chromatography is the most common type of adsorption chromatography and can be either selective for cationic or anionic interaction with the target species. Anion exchange with diethlyaminoethyl(DEAE) moieties covalently attached to the adsorption matrix is more commonly used at pH 6-8. Cationic exchange is more commonly used at pH 4.5 to 6. The binding of proteins to the DEAE depends on the charge of the protein and the charge density of binding matrix where most proteins have a net negative charge at pH above 6. Many proteins precipitate when adsorbed from solutions of high concentration to matrices with high charge densities. This can be a draw back for proteins which aggregate and self denature like fibrinogen. There are several factors which can influence the matrix adsorption, but the two most important are pH and salt concentration (ionic concentration). Once the target protein is adsorbed to the matrix, varying salt concentration will elute both the target protein and background proteins. The best approach to determining the effects of binding and elution are to bind the protein around pH 7-8.5 on a small column and elute with a gradient. Typical binding of proteins is about 20-40mg total protein per ml of matrix. Once the small column operations have been optimized, the scale up to a larger column is straightforward. Typically, the packed bed column height is kept constant and the width increased. The elution for a larger column is typically done in step gradients and in 1-2 column columns[22,26,27].

Hydrophobic interaction chromatography(HIC) separates proteins based upon their hydrophobicity. Typically, the hydrophobic areas of an aqueous soluble protein are shielded from the aqueous surrounding due to entropic driving forces. Changes in conformation induced at high salt concentration or lowered dielelctric by the presence of organic solvents can expose these domains for interaction with the matrix. A common hydrophobic matrix consists of alkyl chains ranging from 4 to 18 carbons in length. Desorption can be done using low ionic strength or aqueous miscible organic solvents. The separation power of HIC for complex protein mixtures can be similar to ion-exchange, but it frequently results in irreversible denaturation of the target protein. Thus, it is sometimes used as a final step to help remove the remaining background proteins[22,26,27].

Affinity chromatography is increasingly being used as a high resolution processing step. In particular, large scale immunoaffinity chromatography(IAC) has become a useful tool because of technological advances in making monoclonal antibodies. Monoclonal antibodies(MAbs) can be selective for binding sites in proteins which are typically only 4 to 6 amino acids in length. Furthermore, MAbs have been selected for highly specific adsorption and desorption properties under differential changes in pH or even divalent metal concentration[22,23,26,27]. The ability to select MAbs for specific adsorption and desorption conditions for the target protein makes IAC a powerful technique

for purification of proteins from a complex protein mixture such as milk or blood plasma. Adsorption capacity of IAC primarily depends upon antibody orientation and local ligand density[22,26,27]. Synthetic ligands have also been developed to replace MAbs as immobilized affinity ligands[28-30].

Free flow electrophoresis

For decades, electrophoretic techniques with high resolution have been used as analytical tools to separate proteins and protein isoforms based upon their mass and charge [31,32]. While electrophoresis does not have the resolution capacity of affinity chromatography, it can be used to potentially separate problematic contaminants and sub-populations. In addition, there is a need for high resolution, process scalable techniques which can provide barriers to pathogen transmission in pharmaceutical applications which provide separation physics that are orthogonal to current process steps [2]. Recently, a scalable, continuous electrophoretic technique called Recycle Continuous-Flow Electrophoresis (RCFE) has been developed. The RCFE device uses convective flow through a high aspect ratio rectangular duct upon which an electrophoretic field is applied [33,34]. At large scale, the aspect ratio width to depth can be as great as 100:1 and a length to depth ratio of 100:1. These high aspect ratios are needed to dissipate heating by current flow up to 125 mAmps. Two convective streams are introduced to the chamber: the feed mixture and crossflow buffer which is recycled. The recycled buffer remains at the original

conductivity so as to not promote heating due to an increase in resistance and thus buffer usage can be minimized. A membrane barrier prevents proteins from reaching either electrode and causing protein loss or fouling.

A crossflow velocity is necessary to render the anode directed fluxes of selected species of the protein mixture to zero or less to achieve separation. Those species which have a net flux towards the anode are separated from those species which are washed out as cathode efflux due to weaker mobility. As a preliminary test of the RCFE resolution, a binary mixture consisting of coomassie blue dyed BSA and bovine azo-casein, as well as a binary mixture of coomassie blue dyed BSA and hemoglobin were separated. A resolution of >95% and a yield >90% were obtained for both at an aqueous feed concentration of 30g of protein per liter of each species at a feed flow rate of 20 ml per hour [33,34].

A bench scale RCFE device having a chamber gap of 2mm and that can process up to 1.5 g total protein per hour with an aqueous feed concentration of 60 g total protein per liter at a feed flow rate of 25 ml per hour is currently being used. It is estimated to process 100 g total protein per hour at a feed flow rate of 1 liter per hour for a feed concentration of 100g total protein with a chamber gap of 8 cm. Fouling of the electrode membrane due to concentration polarization has not been evaluated for complex mixtures. A major disadvantage

of the initial RCFE prototype is the dilution of the products streams due to the introduction of crossflow buffer. As a result of dilution, applications of the RCFE for high resolution of therapeutic proteins from complex mixtures such as human plasma or the milk of transgenic livestock have not been done.

The net charge of any given protein is dependent upon pH. Most proteins are negatively charged at pH 6 or above. Hence, under an electric field at pH 6, most proteins will move towards the anode electrode with a velocity that correlates with their charge to mass ratio and charge to hydrodynamic ratio. Since these proteins are moving through a liquid medium, they will have several forces acting upon them such as buoyancy (gravitational) and fluid drag(resistant to direction of flow). It is assumed that the buoyant forces are negligible, but the fluid drag forces however are not. “Stokes Law”, can be used to estimate these forces by assuming that proteins are spherically shaped. Thus, “Stokes Law” predicts that the fluid drag forces for a sphere are directly proportional to the radius. The larger the radius, the larger the surface area and hence larger the fluid drag forces. These differences in mobility and fluid drag are the chief separation forces in electrophoresis. However, since most proteins will migrate towards the anode, RCFE introduces a crossflow to direct the less mobile proteins towards the cathode. The crossflow is used at a velocity which renders selected anode directed proteins with a flux towards the cathode.

Free flow iso-electric focusing

Iso-electric focusing is commonly used as an analytical tool to resolve complex proteins and purified protein subpopulations based upon their isoelectric point. These mixtures are typically applied to cross-linked, polymeric gels that act as a viscous, carrier phase. The viscous gel helps to minimize the dispersion loss of resolution due to diffusion and convection in applications which use a relatively strong electric field (about 30-40 volts/cm) to focus proteins within a pH gradient (about 0.1 to 0.5 pH units/cm)[32]. The separation of protein subpopulations is based upon their pI, the point at which the protein has a neutral charge. A pH gradient is electrophoretically established within the gel due to the presence of selected sub-populations of relatively low molecular weight, amphoteric molecules at concentrations which impart significant buffering capacity relative to protein which are introduced to the gel matrix. These aqueous soluble molecules are termed ampholytes and each ampholyte sub-population has a specific isoelectric point (pI) that has been engineered through the relative degree of substitution of acidic and basic chemical moieties. A static pH gradient occurs after a steady-state, electrophoretically induced, spatial distribution of each ampholyte is established. This steady-state exists due to the simultaneous phenomena of specific self-buffering and extinguished electrophoretic mobility that onsets each ampholyte upon reaching a field location where its respective pI occurs. In the presence of sufficient buffering capacity by ampholytes, protein sub-populations can be electrophoresed from

locations of lower to higher pH until the pI of the protein is reached and its mobility is halted along with that of the respective ampholyte at that field position.

Under a sufficiently strong electric field, the dissipation of the pH gradient due to diffusion can be prevented even in a purely aqueous, low viscosity media. However, once the electric field is removed, the device must somehow prevent the loss of separation by the diffusion that would rapidly occur in solution environments. Thus, isoelectric focusing can be extended to an ordinary purification processing environments only if the focused samples can be isolated prior to removing the electric field. To that end, the multiple recycle design feature of a recycle crossflow electrophoresis chamber (RCFE) can make possible the isolation sub-populations of recombinant proteins on a larger scale [35]. The recycle flow along a multiple sequence of proximal recycle streams enables sufficient residence time within the separation chamber to achieve electrophoretic separation across the duct to which the electric field is applied. Furthermore, because the recycle lines can be scaled to any volume, the bulk of the resolved protein can be sequestered within the recycle lines (relative to that remaining the resolution chamber), once the electric field is removed. However, resolution time must be optimized relative to recycle line volume and product yield from each of the recycle lines.

The chief design constraint upon free-flow electrophoresis devices for large-scale applications is material throughput as restricted by the resistive heating effects caused by the combination of large current and poor heat transfer. Several designs for recycle flow within various thin rectangular or cylindrical ducts have been tried for general free-flow, electrophoretic applications. For example, the RCFE design provides a scalable unit operation for maintaining isothermal conditions within a rectangular duct (30 volt/cm). Most importantly, the residence time outside the chamber needed to cool the fluid from the N^{th} stream and maintain isothermicity and separation from the proximal N-1 and N+1 streams is achieved in a fashion scaleable to high throughput. This device has not previously been adapted to iso-electric focusing applications because it is primarily a steady-state feed device while iso-electric focusing is most amenable to batch processing. However, when set to 100% recycle, a balance of upward, laminar flow to minimize dispersion by free convection, high voltage operation to minimize diffusive dissipation of the pH-dependent resolution, and adjustable residence time within the resolution chamber to achieve steady-state focusing along with efficient heat transfer through the lines outside of the resolution chamber makes many aspects of the RCFE design desirable for isoelectric focusing in either a batch or fed-batch mode.

Commercial Free-Flow Iso-electric Focusing

Throughout this research, one goal was to use free flow iso-electric focusing to sub-fractionate a pure recombinant protein. Being able to produce a product from a sub-population that is biologically active and that is isolated from the inactive species is both commercially and clinically important. Currently, there are at least 2 types of commercial free-flow apparatii being used to purify proteins from complex mixtures by iso-electric focusing at preparative scale. The first has a cylindrical annular flow geometry and is called the Rotofor sold by BioRad. This device can sub-fractionate the protein mixture into 20 fractions with a linear pH gradient using 1-2% ampholytes[36]. The minimum process volume is only 60ml, and it has been shown to batch process at least 100mg. The second device has a rectangular duct geometry and has several commercial names such as: ATIsolator by Ampholife Technologies, Iso-Prep by Ionics Inc. and RF3 by Protein Technologies. These devices have 8 fractionating channels, 12 fractionating channels and 30 fractionating channels respectively[37-39]. Each of these can produce a linear pH gradient using 1-2% ampholotyic solutions. Of the 3 rectangular duct configurations, the RF3 is able to process the greatest amount of material: 500mg total protein in 106 ml has been processed by the RF3[39]. The focusing chamber holds only 6% of the total process volume with the remainder of the volume in the recycle tubes.

The preparative free-flow isoelectric focusing(FFIEF) device used in this

research showed that a rectangular duct device could operate at 100mg loadings and an average of 50 watt power consumption with no significant buildup of heat. In addition, 50 channels provided extended resolution over that of RF3. The configuration of the FFIEF used here had a focusing chamber of 40ml and recycle line volume of 3.2 ml. However, the chamber accounted for approximately 20% of the total recoverable IEF fractionated volume.

Summary

The purpose of this thesis is to investigate the downstream processing of both endogenous and recombinant therapeutic proteins from the milk of transgenic livestock. The milk of transgenic pigs is used as a model source material for rhPC. The focus was to use Zn^{2+} as an agent to change protein conformation resulting in selective aggregation and precipitation of certain milk proteins and combine this with adsorption chromatography to produce a high resolution purification technique. This technology must be scalable to large volumes. In particular, the purification of rhPC(recombinant protein model) by a Zn^{2+} selective chromatography is used to combine 2-3 process steps into a single step and to fractionate the active subpopulations from the inactive subpopulations. Pig milk is also used as a model complex mixture from which to isolate IGs by combining 2-3 process steps into a single step combining Zn^{2+} selective precipitation with chromatography.

In addition, two different types of electrophoresis techniques are applied to milk to evaluate the application to large scale high resolution processing of recombinant protein subpopulations. A novel modification of cross free-flow electrophoresis unit to 100% recycle is used to convert it to the isoelectric focusing mode. The potential for large scale isoelectric focusing is evaluated for high resolution processing.

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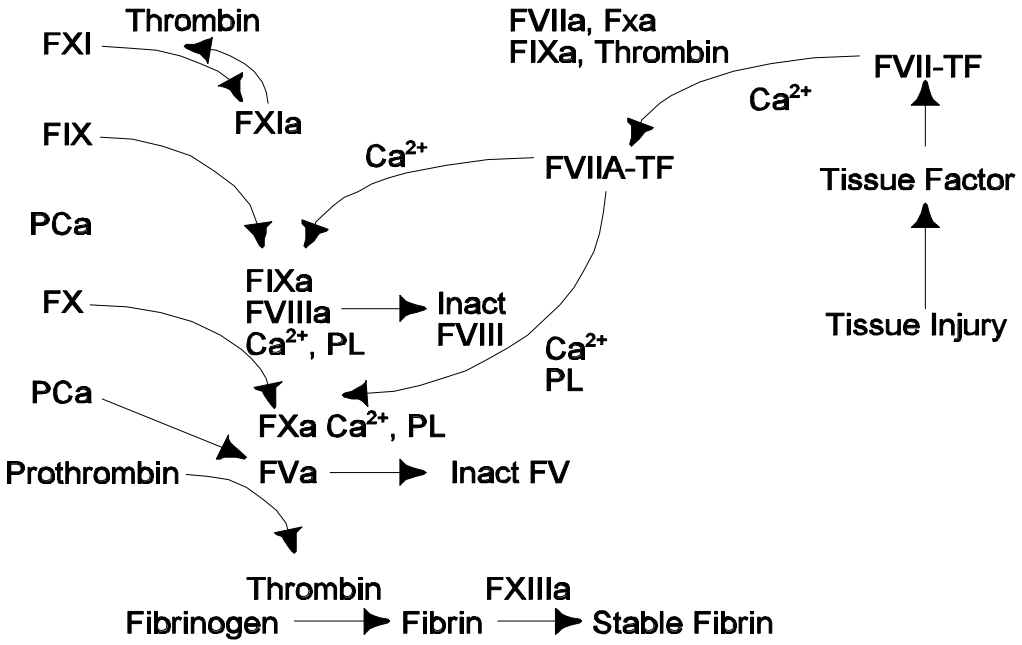

Figure 1: Blood coagulation cascade showing regulatory control of a blot clot. The letter **F** in front of the Roman numerals stands for Factor and **TF** stands for Tissue Factor.

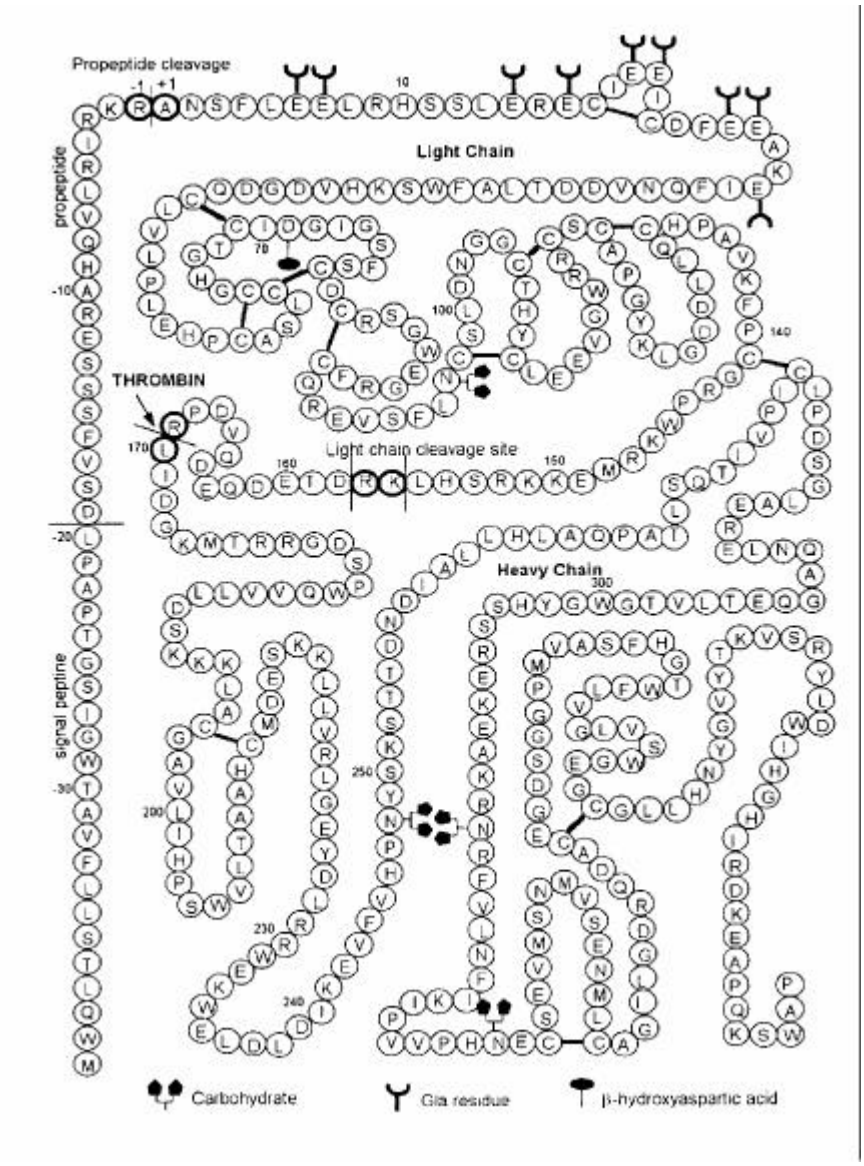

Figure 2: Schematic of Protein C

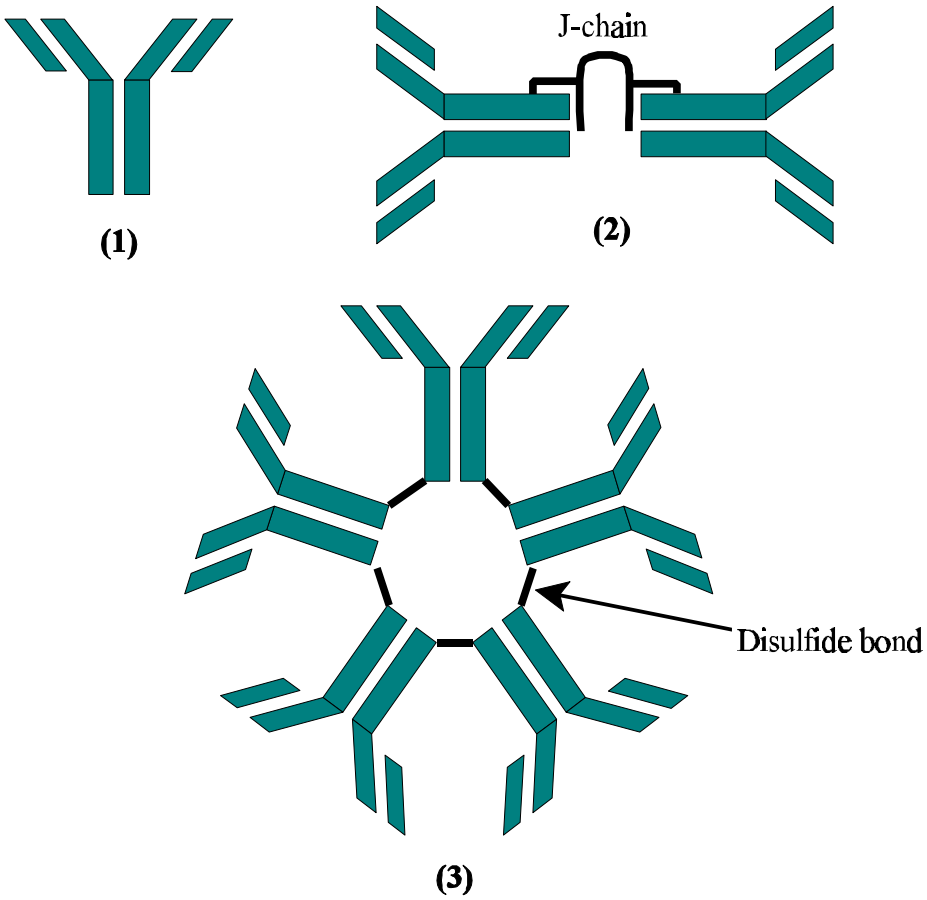

Figure 3: Schematic of the major immunoglobulins present in serum and milk. (1) is IgG, (2) is IgA and (3) is IgM. IgG is a monomeric unit while IgA is a dimer and IgG is a pentamer of IgG. Each IgG unit has 2 light chains and 2 heavy chains that are held together by disulfide bonds.

Table I: Major pig milk proteins

Protein Composition of Pig Milk		
Protein	Concentration (mg/ml)	Molecular Weight (kDa)
α-casein	15 to 20	40
β-casein	12 to 20	27
κ-casein	6 to 9	19
transferrin	0.020 to 0.200	80
serum albumin	1.3 to 1.8	54
IgG	1 to 3	150
IgM	3 to 4	750
IgA	3 to 9	300-400
α-lactalbumin	0.2 to 0.5	15
β-lactoglobulin	0.7 to 1.3	18
lactoferrin	0.020 to 0.200	70

Chapter 2: A Zn^{2+}-Sub-variant Selective Purification of Recombinant Proteins from the Milk of Transgenic Animals

Arthur Degener and William H. Velander
Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA

Makonnen Belew
Pharmacia Biotech AB, S-751 Uppsala Sweden

Key Words: Protein C, Transgenic Animals, Ion-exchange Chromatography, Expanded Bed Adsorption Chromatography, Protein Sub-populations, Zinc interaction

Abbreviations: rhPC, recombinant human protein C; EBA, expanded bed adsorption; DLS, dynamic light scattering; HIC, hydrophobic interaction chromatography; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme linked immuno sorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; gla, gamma carboxy glutamic acid; DEAE, diethyl-amino-ethyl

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 intra chain proteolytic cleavage and gamma 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 gamma-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.

2.0 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 to 60 g protein/liter, 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 particular, $ZnCl_2$ 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 alpha-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 separating the inactive sub-population from the active sub-populations of a recombinant protein in crude transgenic whey by EBA.

2.1 Materials

2.1.1 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, Uppsala, Sweden. Human protein C from plasma was a gift from American Red Cross, Rockville Maryland, 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, trichloro-acetic acid, magnesium chloride, calcium chloride, zinc chloride, Protein C depleted plasma, normal pooled reference plasma and ammonium sulfate were purchased from Sigma Chem Co. Immulon II Plates, electrophoresis grade polyacrylamide:bisacrylamide [29:1], acetic acid, Accumet 25 pH meter, Marathon 21KR centrifuge, BRL Horizon™ 11x14 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 490nm 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. DynaPro-801^{TM} Dynamic Light Scattering (DLS) Instrument from Protein Solutions Incorporated was used to analyze size distribution by both a monomodal and bimodal analysis. The syringe filters ranging from 0.02-0.2µm were Anotop10 inorganic membrane filters with a diameter of 10 mm from Whatman. The DLS data analysis was done using AutoPro^{TM} software.

2.2 Methods

2.2.1 Milk collection and preparation: Milk from transgenic swine was collected and handled by the method described in Subramanian et. al [21].

2.2.2 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].

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

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

2.2.5 Protein solubility: Frozen whey was thawed in a water bath at 4^{o} C for 4 hours. A 2 ml sample of whey was aliquoted into each of 11 test tubes, and 1 ml of 50 mM Tris-HCl, pH 7.2. Each of the tubes were rendered 0-100 mM Mg^{2+} with 500 mM $MgCl_2$ and a tris concentration of 25 mM by dilution with deionized H_20 to a total volume of 4 ml and then were mixed by rotary agitation for 30 minutes at RT. Each tube was then centrifuged at 4500xg for 30 minutes at 23^{o} 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 $MgCl_2$.

2.2.6 Electrophoretic mobility: Electrophoretic mobility was done on α and β-pcasein (purified by free flow electrophoresis; Velander et. al unpublished methods) and rhPC (purified by immuno-affinity chromatography; see ref 8). A 1% low EEO Agarose solution was prepared with 25 mM Tris-acid pH 7.2 and heated to 100^o 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 mM 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% Trichloro-acetic acid for 20 minutes, 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 minutes, 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 mM solutions of $MgCl_2$, $CaCl_2$, or $ZnCl_2$ added to the gel, samples and buffer solutions.

2.2.7 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 5mg/ml in 25 mM Tris buffer, pH 7.0 using a 0.1µm syringe filter. Samples were prepared with $MgCl_2$ ranging from 0-10 mM in increments of 1mM. The DLS was washed with 5ml of deionized water with a syringe filter of 0.02 µm until a photon count rate of 9000 was obtained. A 500µl 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 mM $CaCl_2$ in 1 mM increments and with 0-10 $ZnCl_2$ in 1 mM increments.

2.2.8 Expanded Bed Adsorption (EBA): The following buffers were used during Streamline™ DEAE chromatography: Buffer A (loading buffer) consisting of 25 mM Tris-HCl, pH 7.2; Buffer B-D (elution buffers) having increased NaCl in Buffer A where Buffer B contained 125 mM NaCl, Buffer C contained 250 mM NaCl, and Buffer D contained 500 mM NaCl.

A 300 ml Streamline™ DEAE chromatography column was operated at 4^{o}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 a A_{280} of the top effluent returned to base-line. Further wash 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 (CV) of Buffer B, 3 CV of Buffer C and 3 CV of Buffer D. The column was regenerated using a sequence of 2 CV 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 mM and 4 mM $ZnCl_2$.

2.2.9 Hydrophobic Interaction Chromatography (HIC): Butyl-S-Sepharose 6 FF medium was packed into a Pharmacia C-10 (15x1cm, i.d.) column to a bed height of 10 ml. The bed was equilibrated using 4 CV of Buffer E (25 mM Tris-HCl, 0.8M Ammonium Sulfate, pH 7.0). Pooled eluates from Buffer C and D were rendered 0.8 M NH_4SO_4 and pH7.0, and then applied to the HIC column at a flow rate of 90 cm/h followed by washing with 2 CV of the equilibration buffer, and then was then eluted with 25 mM 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 CV 30% iso-propanol in 25 mM Tris-HCl, pH 7.5, 3 CV 0.5 M NaOH, 4 CV deionized water and 5 CV Buffer E.

2.2.10 Purification of rhPC by Immuno-affinity Chromatography: rhPC was purified from transgenic swine whey by immunoaffinity chromatography as

described in Van Cott et. al. [8].

2.2.11 Conformational ELISA: The presence of native gamma-carboxylated rhPC was analyzed as described in Subramanian et. al. [21]

2.2.12 Activated Partial Thromboplastin Time (APTT) of rhPC from Pigs: The delay in clotting time is described in Morcol et. al. [22].

2.3 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. Figure 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 of 1 to 100 mM Mg^{2+} for pure solutions of porcine α-casein (Figure 1A; initial protein concentration of 15 g/l) and β-casein(Figure 1B; initial protein concentration of 10 g/l). The concentration of pure rhPC was also unaffected by treatment with Mg^{2+} (Figure 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 mM and 3-10 mM Zn^{2+} for pure porcine α-and β-casein solutions, respectively (Figure 1A and 1B). The rhPC concentration decreased from about 360 µg/ml to less than 200 µg/ml over the range of 0-10 mM Zn^{2+} for pure protein C solutions (Figure 1C). Figure 1D shows that the A_{280} of whey samples decreased less than 10% and 50% over the range of 0-20 mM Mg^{2+} and Ca^{2+}, respectively, while a greater than 90%

decrease in the A_{280} occurred for whey treated with about 4-10 mM 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 mM Mg^{2+} and Ca^{2+} (data not shown). However, SDS-PAGE of the 0-10 mM Zn^{2+} treated samples clearly show a selective loss of α-and β-casein from the whey in the range of about 4 to 10 mM Zn^{2+}. Figure 1E shows that essentially 100% of the rhPC detected in whey by ELISA is precipitated over the range of 0-10 mM Zn^{2+}. Western Blot analysis also confirms these ELISA results(data not shown).

Figures 2A, B and 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 mM Mg^{2+} or Ca^{2+} ions and 0-1.0 mM Zn^{2+} ions, respectively. Figure 2A shows a sharp decrease in mobility of α and β-casein and of rhPC over the range of 0-2 mM Mg^{2+}. The mobility of β-casein is seen to be reversed towards the anode at about 1 mM Mg^{2+}. Figure 2B shows a similar decrease in mobility for these same proteins towards the cathode over the range of 0-2 mM Ca^{2+}. In contrast, the presence of only 0.0-0.2 mM Z^{2+} ions caused a sharp decrease in the electrophoretic mobility of pure α- and β-casein and rhPC as shown in Figure 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 mM Zn^{2+}. An increase in hydrodynamic radius of about 3 to 36 nm and 3 to 24 nm for α-and β-casein, respectively occurs over the treatment range of 4 to 10 mM Zn^{2+} while that of rhPC remained nearly constant at about 4 to 6 nm. An estimated molecular weight of about 31 and 42 kDa for β- and α-caseins and 71 for rhPC was obtained at 0 mM Zn^{2+}, based upon modeling these proteins as spheres. These molecular weight estimates are in approximate agreement with the apparent molecular weight of α and β casein (about 30 kDa) as estimated by SDS-PAGE (Figures 5A and 5B) and also for rhPC (about 62 KDa) . The molecular weights of the 10 mM Zn^{2+}-induced aggregates not clarified by centrifugation were estimated to be greater than 10^3 kDa for the porcine caseins and about 160 kDa for rhPC.

EDTA-clarified pig whey treated with 0 mM, 2 mM, and 4 mM $ZnCl_2$ were processed by Streamline™ DEAE EBA and typical chromatograms are shown in Figures 3A, B and C, respectively. Figure 4 shows a photomicrograph of the milk solids typically present in whey treated with 4 mM $ZnCl_2$. The solids present in 2 mM $ZnCl_2$ (Figure 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 (Figure 3A). Treatment of the whey with 2 mM $ZnCl_2$ resulted in the

elution of 27% of the rhPC and 56% of the total whey proteins in the unbound fraction (Figure 3B). The loading of whey treated with 4 mM $ZnCl_2$ resulted in 51% of the total rhPC and 85% of the total whey protein passing through the expanded bed column as unabsorbed effluent (Figure 3C). The adsorbed protein was eluted with step gradients of 125 mM(Buffer B), 250 mM(Buffer C), and 500 mM NaCl(Buffer D). Table 2 shows the total protein and rhPC content of each eluate pool. The Buffer C and Buffer D 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 mM $ZnCl_2$ treated-whey loading. The 2 mM $ZnCl_2$ 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 mM $ZnCl_2$ 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.

Figure 5A shows samples from the purification train of 4 mM Zn^{2+} treated whey analyzed by silver-stained SDS-PAGE. Immuno-affinity 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% to 94% for salt eluate pools and about 86% to 89 % for unabsorbed effluent from the Streamline™ DEAE expanded bed chromatography. All immuno-affinity 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 immuno-affinity or HIC.

Figure 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 mM Zn^{2+} -treated whey processed by Streamline™ DEAE EBA chromatography has greater than 50% of the population existing as single chain. The adsorbed 4 mM 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 immuno-affinity purified rhPC as a percentage of immuno-purified hPC derived from human plasma. Immuno-affinity purified rhPC material obtained from the unabsorbed effluents of Streamline^{TM} DEAE EBA of loadings with 2 mM and 4 mM $ZnCl_2$-treated wheys, showed no anticoagulant activity by APTT assay. Essentially no unabsorbed rhPC was obtained from loadings of Streamline^{TM} DEAE EBA at 0 mM $ZnCl_2$. Immuno-affinity purified rhPC from pooled Buffer C and D eluates of Streamline^{TM} DEAE EBA loadings with 0 mM, 2 mM, and 4 mM $ZnCl_2$-treated wheys showed 43%, 58%, and 75% anticoagulant activity by APTT assay, respectively relative to immuno-purified plasma derived hPC. HIC purified rhPC from the salt eluate pool of Streamline^{TM} DEAE EBA from loadings of 4 mM $ZnCl_2$-treated wheys 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 gamma-carboxylated glutamic acid (gla) domains [23]. Figure 6 shows the results of conformational ELISA analysis of immuno-affinity 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 immuno-affinity chromatography from Buffer C and D eluate pools of 4 mM $ZnCl_2$-treated whey processed on Streamline™ DEAE gave a half-maximal inhibition by Ca^{2+} of the ELISA signal of about 2 mM. In contrast, rhPC purified by immuno-affinity chromatography of Buffer C and D eluate pools from 0 and 2 mM Zn^{2+} treated wheys processed on Streamline™ DEAE gave half-maximal inhibitions of the ELISA signal by Ca^{2+} of about 6 and 10 mM, respectively. Immuno-purified rhPC from unabsorbed effluents of Streamline™ DEAE expanded bed chromatography from loadings of 2 mM and 4 mM $ZnCl_2$-treated wheys gave no half-maximal inhibition of ELISA signal by Ca^{2+}.

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 millimolar 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 with bovine caseins have found a similar sensitivity to the presence of low levels of Zn^{2+} ion [28]. The present study is the first report of the sensitivity of porcine caseins to Zn^{2+} as well as the contrasting behavior of the sub-populations of the 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 mM 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 to 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 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 gamma-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 mM Ca^{2+} and these unabsorbed rhPC populations from 2 and 4 mM $ZnCl_2$-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^{TM} DEAE expanded bed when 4 mM $ZnCl_2$ was present.

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 [35]. 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 immuno-affinity 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 [36] can also be done after HIC for further purification (Figure 5; Lane 11). It is noteworthy that this calcium elution strategy was ineffective when applied to crude whey or Streamline^{TM} 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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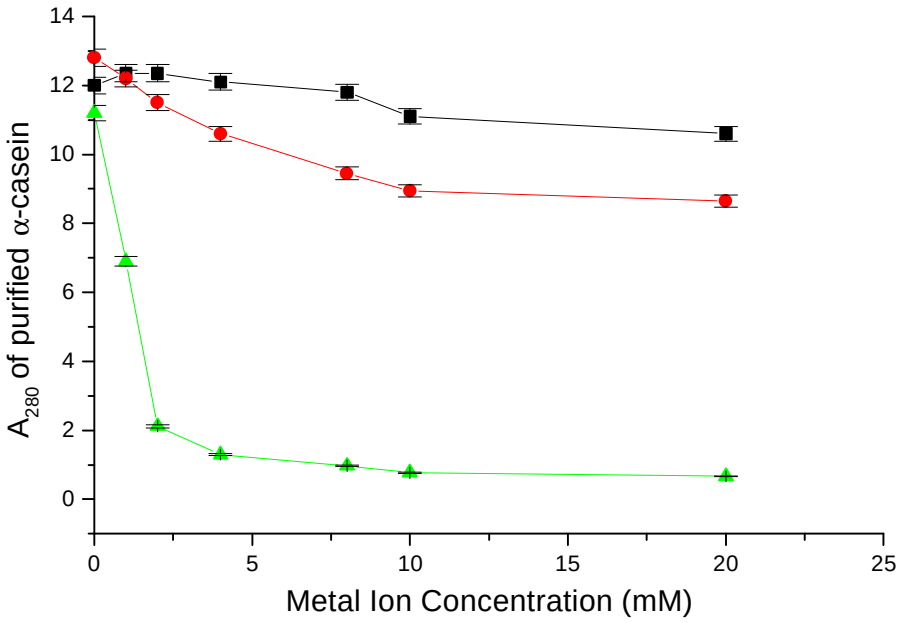

Figure 1A: The solubility of pure α-pcasein as measured by A_{280}. (■-Magnesium, ●- Calcium, ▲-Zinc). The samples after the addition of the metal were centrifuged at 4500g to remove any precipitates and the supernatant absorbance was measured.

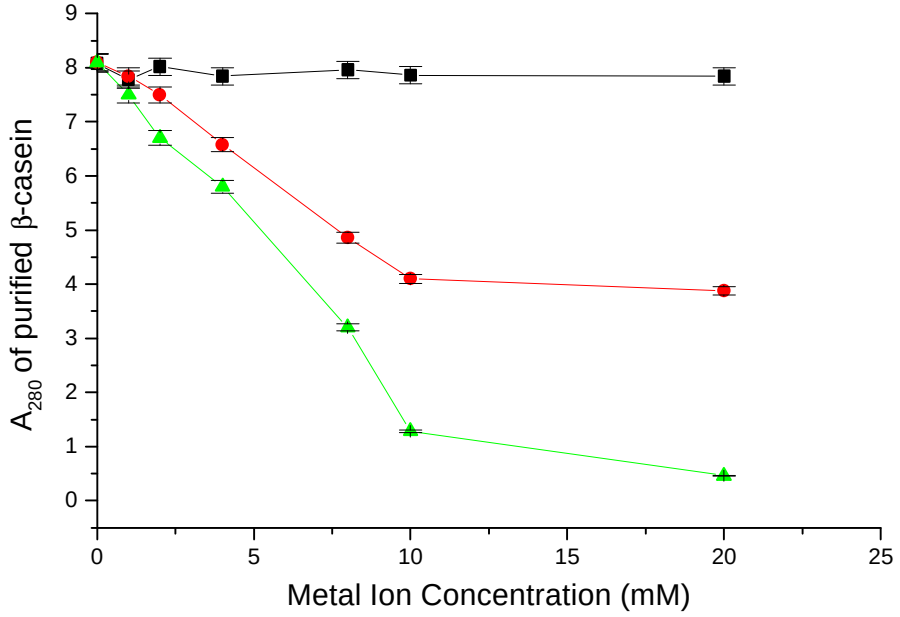

Figure 1B: The solubility of pure β-pcasein as measured by A_{280}. (■-Magnesium, ●- Calcium, ▲-Zinc). The samples after the addition of the metal were centrifuged at 4500g to remove any precipitates and the supernatant absorbance was measured.

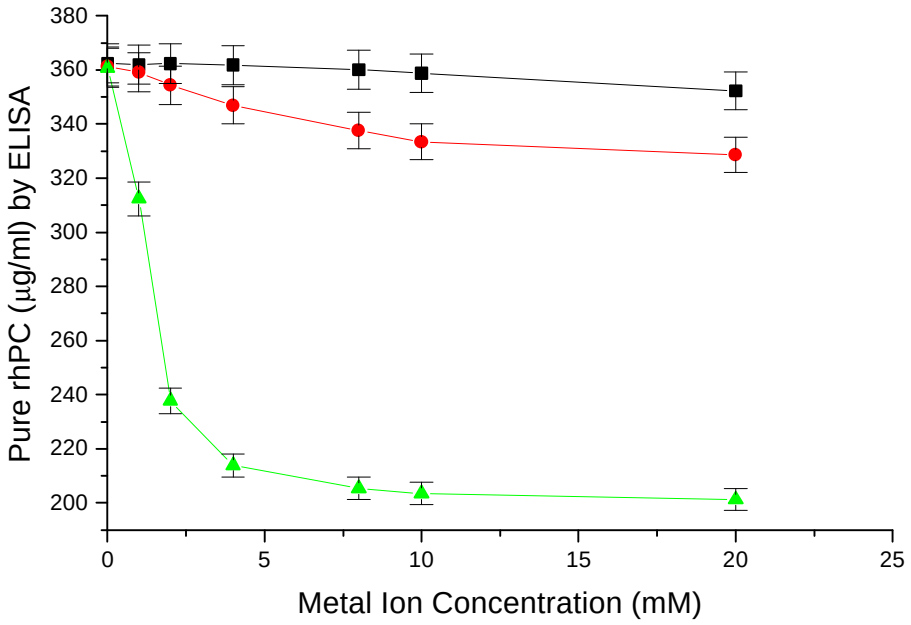

Figure 1C: The solubility of pure rhPC as measured by ELISA. (■-Magnesium, ●- Calcium, ▲-Zinc). The samples after the addition of the metal were centrifuged at 4500g to remove any precipitates and the supernatant rhPC concentrations were measured by ELISA.

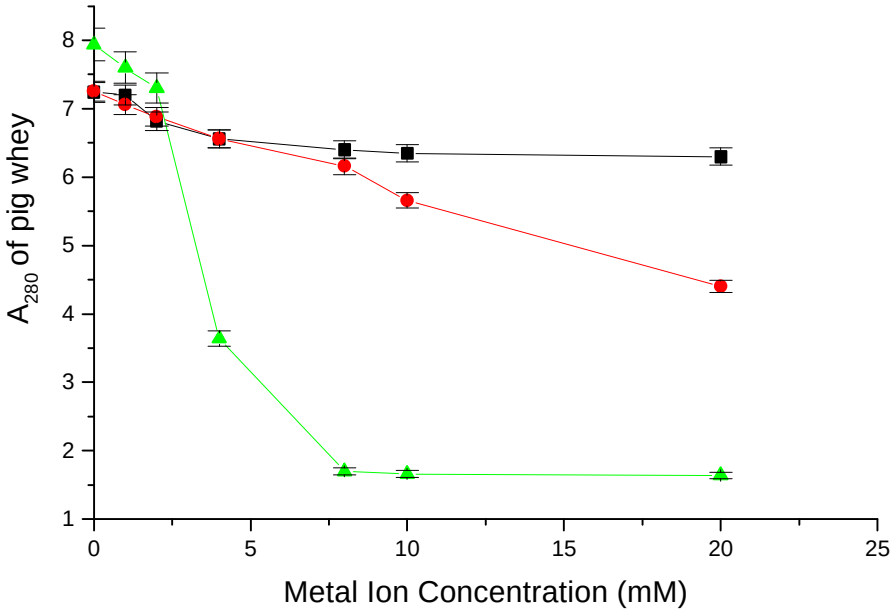

Figure 1D: The solubility of pig milk proteins as measured by A_{280}. (■-Magnesium, ●- Calcium, ▲-Zinc). The samples after the addition of the metal were centrifuged at 4500g to remove any precipitates and the supernatant absorbance was measured.

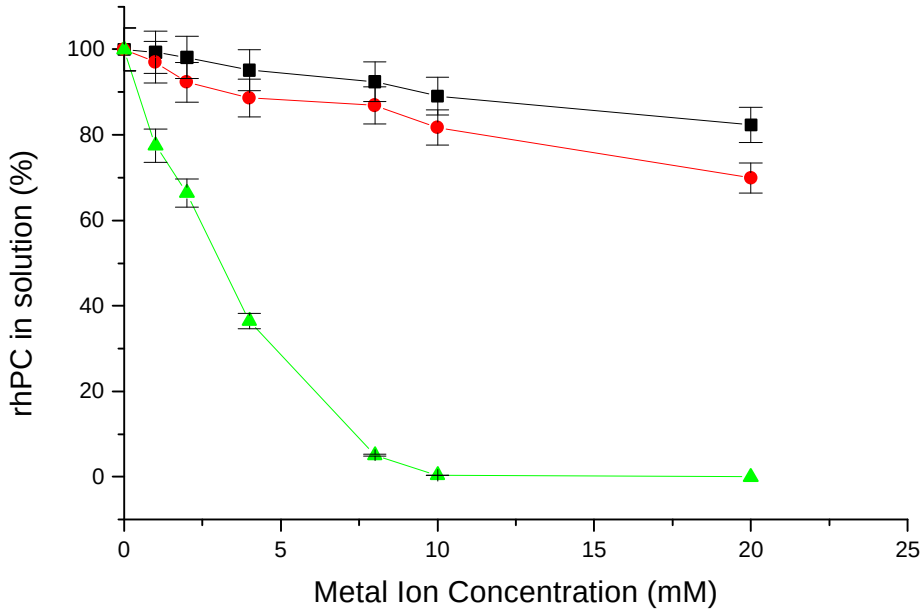

Figure 1E: The solubility of rhPC in whey treated with metal ion measured by ELISA. (■-Magnesium, ●- Calcium, ▲-Zinc). The samples after the addition of the metal were centrifuged at 4500g to remove any precipitates and the supernatant rhPC concentration was measured by ELISA.

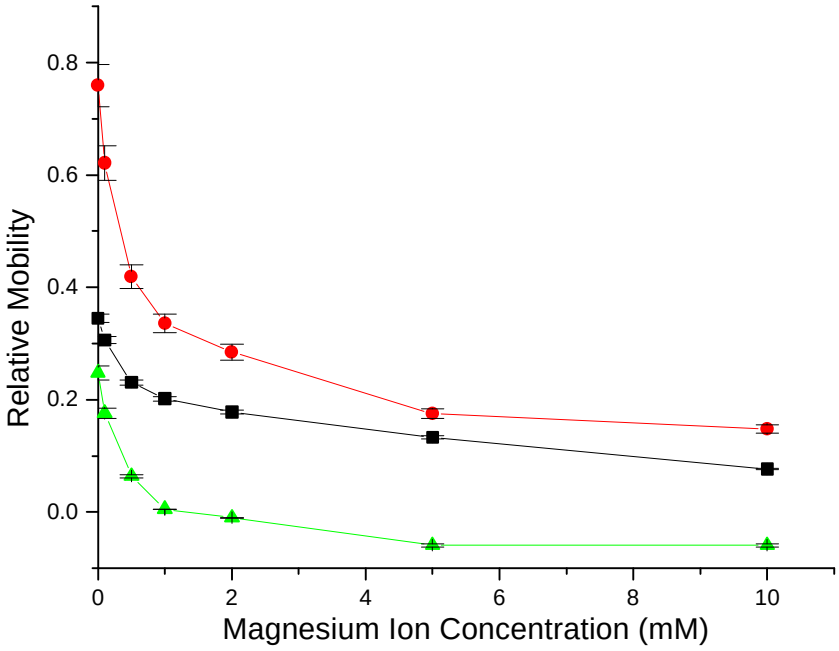

Figure 2A: Protein mobility over the range of 0-10 mM Mg^{2+}. (■-rhPC, ●- α-pcasein, ▲-β-pcasein). The mobility was determined by measuring the distance a protein moved relative to the dye front which was 1cm from the end of the gel in low EEO 1% agarose with 25 mM Tris-acid pH 7.2 at 100V.

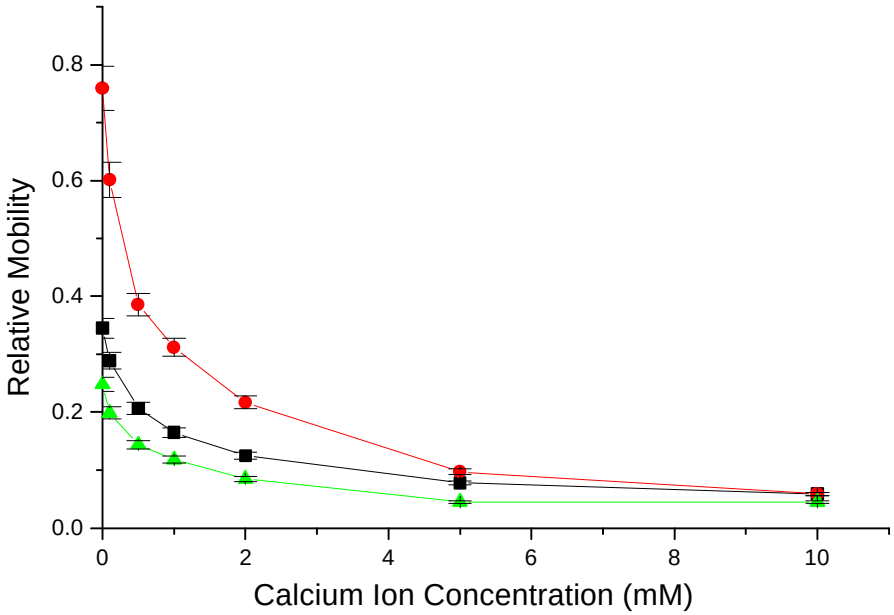

Figure 2B: Protein mobility over the range of 0-10 mM Ca^{2+}. (■-rhPC, ●- α-pcasein, ▲-β-pcasein). The mobility was determined by measuring the distance a protein moved relative to the dye front which was 1cm from the end of the gel in low EEO 1% agarose with 25 mM Tris-acid pH 7.2 at 100V.

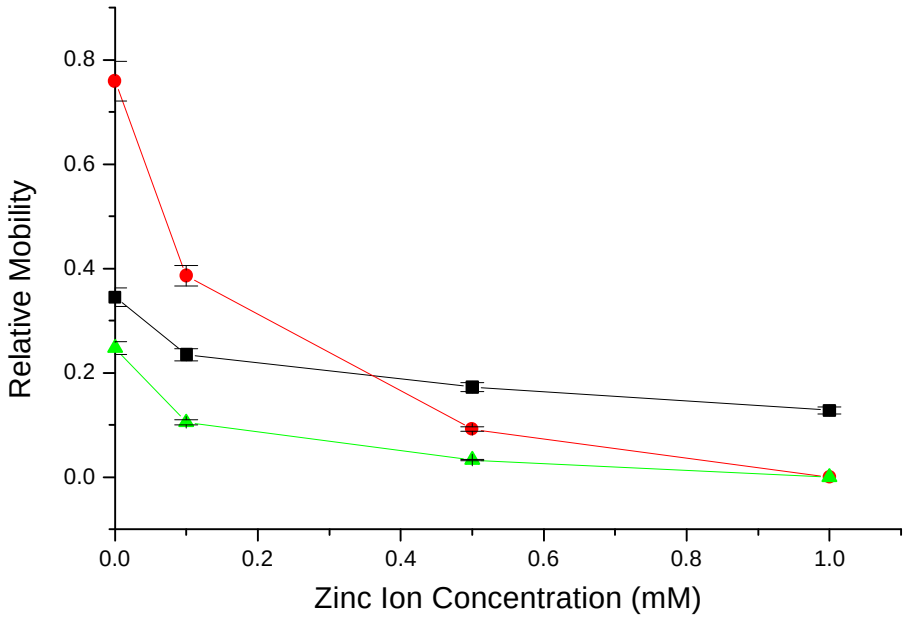

Figure 2C: Protein mobility over the range of 0-1 mM Zn^{2+}. (■-rhPC, ●- α-pcasein, ▲-β-pcasein). The mobility was determined by measuring the distance a protein moved relative to the dye front which was 1cm from the end of the gel in low EEO 1% agarose with 25 mM Tris-acid pH 7.2 at 100V.

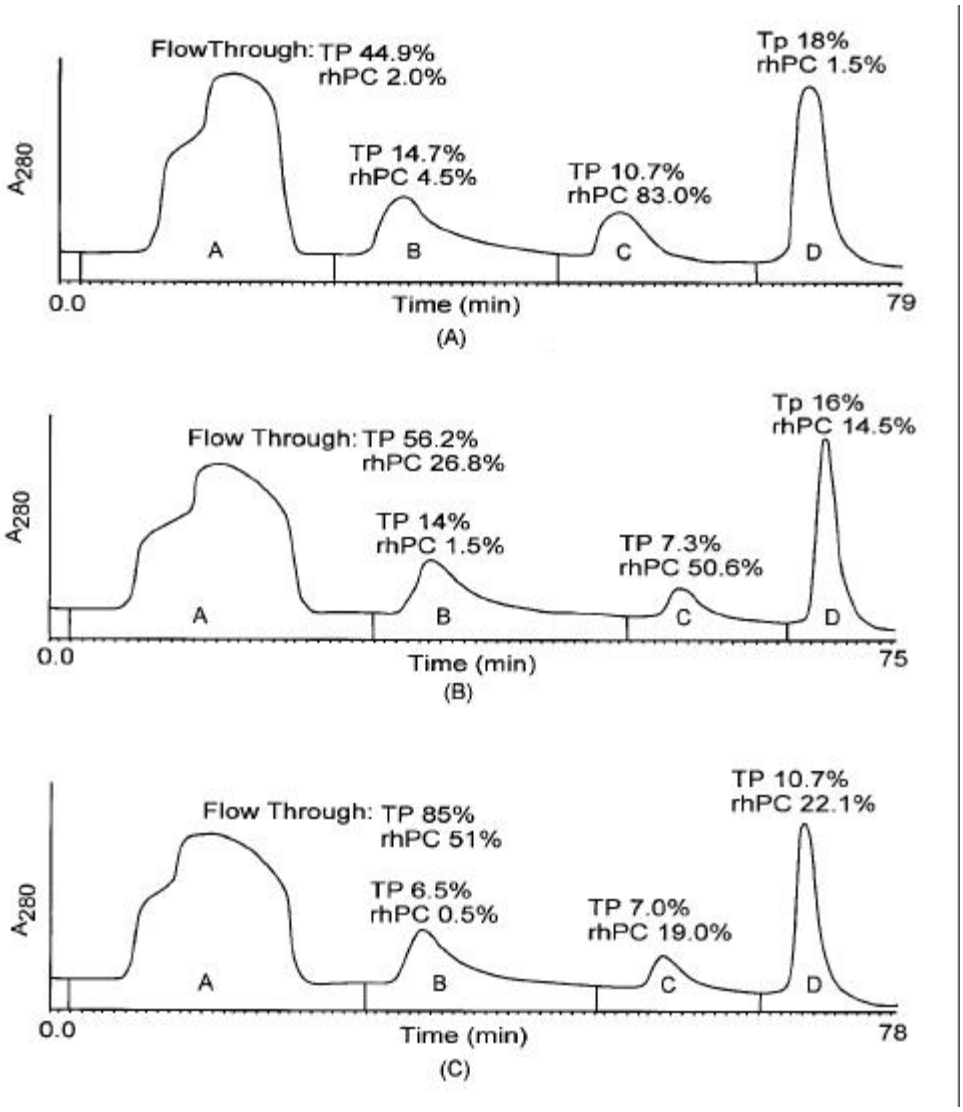

Figure 3: The effect of Zn^{2+} on Streamline™ DEAE chromatography of pig whey. a). Streamline™ DEAE chromatography of whey with no metal ion. b). Streamline™ DEAE chromatography of whey with 2 mM $ZnCl_2$. c). Streamline™ DEAE chromatography of whey with 4 mM $ZnCl_2$. (**TP**-Total Protein, **rhPC**-Recombinant Human Protein C)

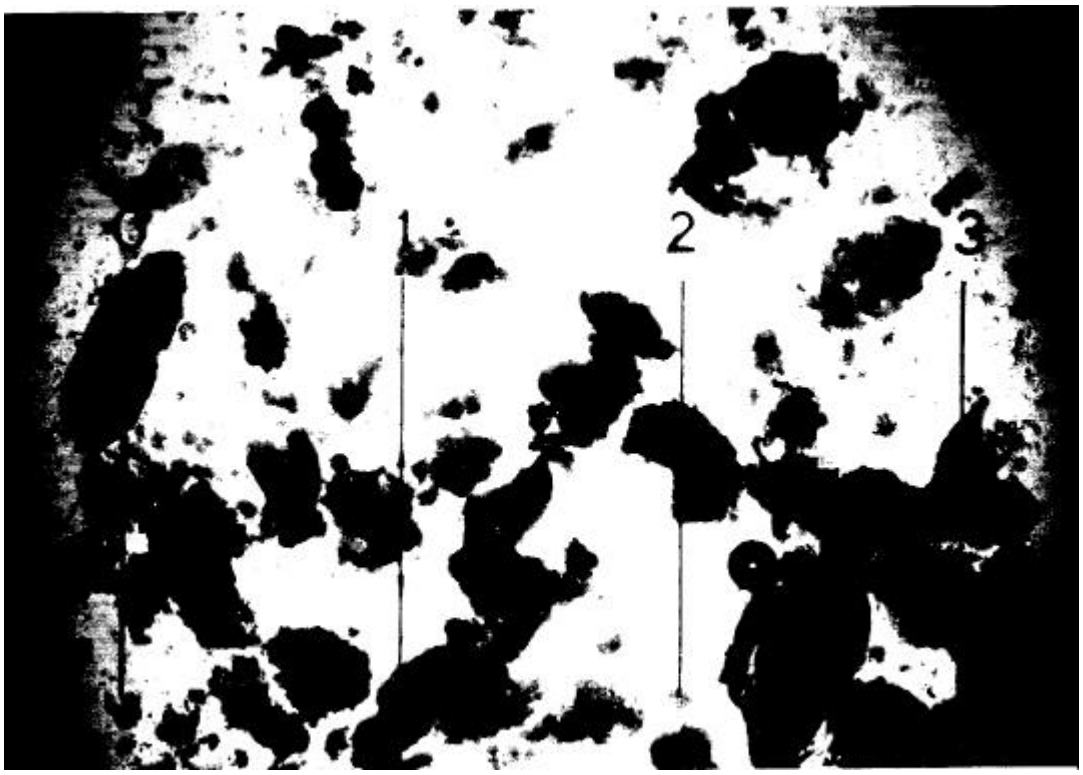

Figure 4: Photomicrograph of milk protein aggregates in the presence of 4 mm $ZnCl_2$. Each scale division is 1000 µm (1 mm) in length and the total field spans 4000 µm.

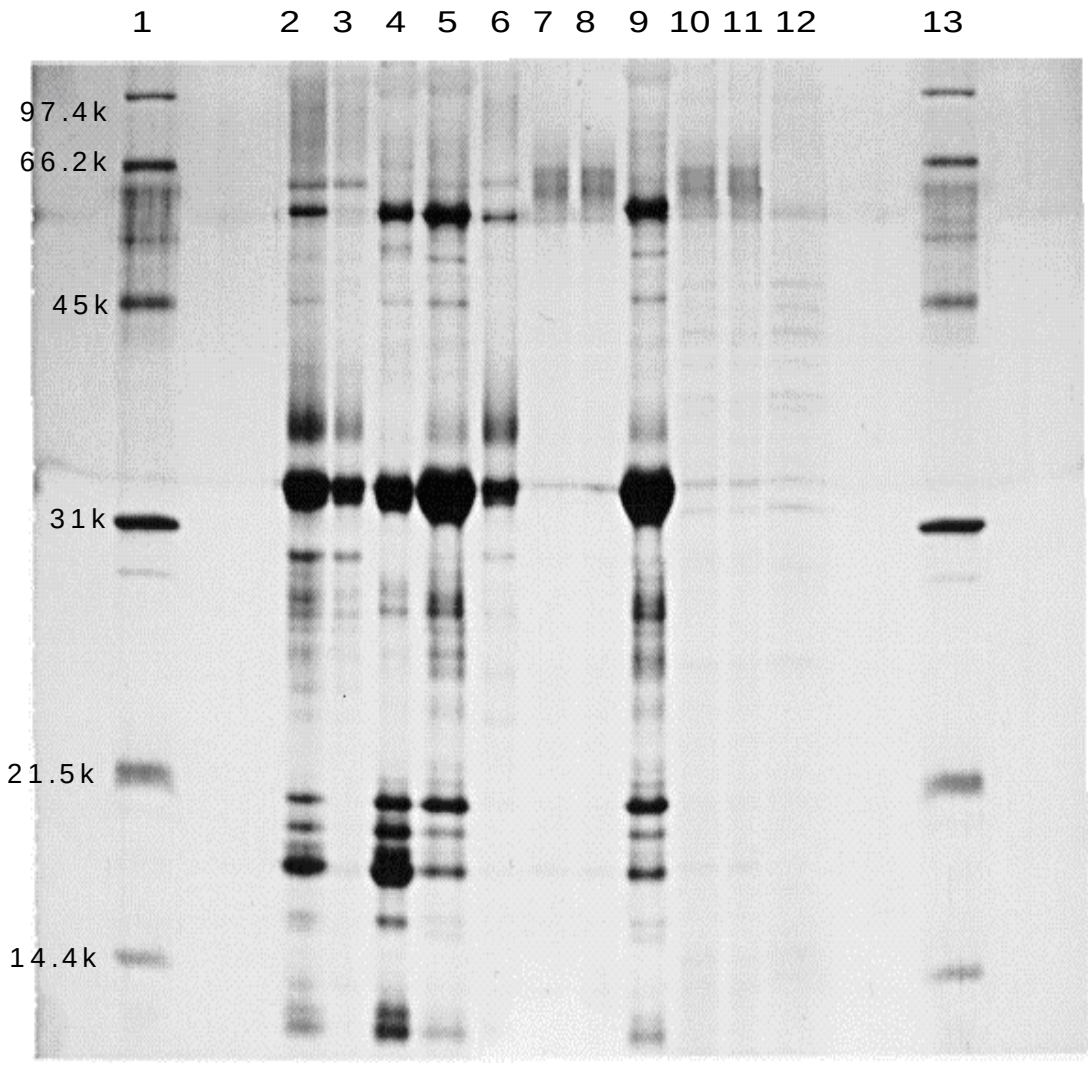

Figure 5A: A 12% silver stained SDS-PAGE of whey fractions from Streamline™ DEAE chromatography in the presence of 4 mm Zn^{2+} with molecular weight markers ranging from 14.4 kDa to 97 kDa present in lanes 1 and 13. Lane 2 is the starting material, lane 3 is the unabsorbed protein from Zn^{2+} treated 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 immuno-purified rhPC form 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 25mM $CaCl_2$ and lane 12 is the 1M NaCl elution of proteins from DEAE Sepharose FF that did not elute with 25mM $CaCl_2$. There was 1-2 µg of total protein applied to each lane except for lanes 7 and 8 which only have .25 to 0.50 µg of total protein.

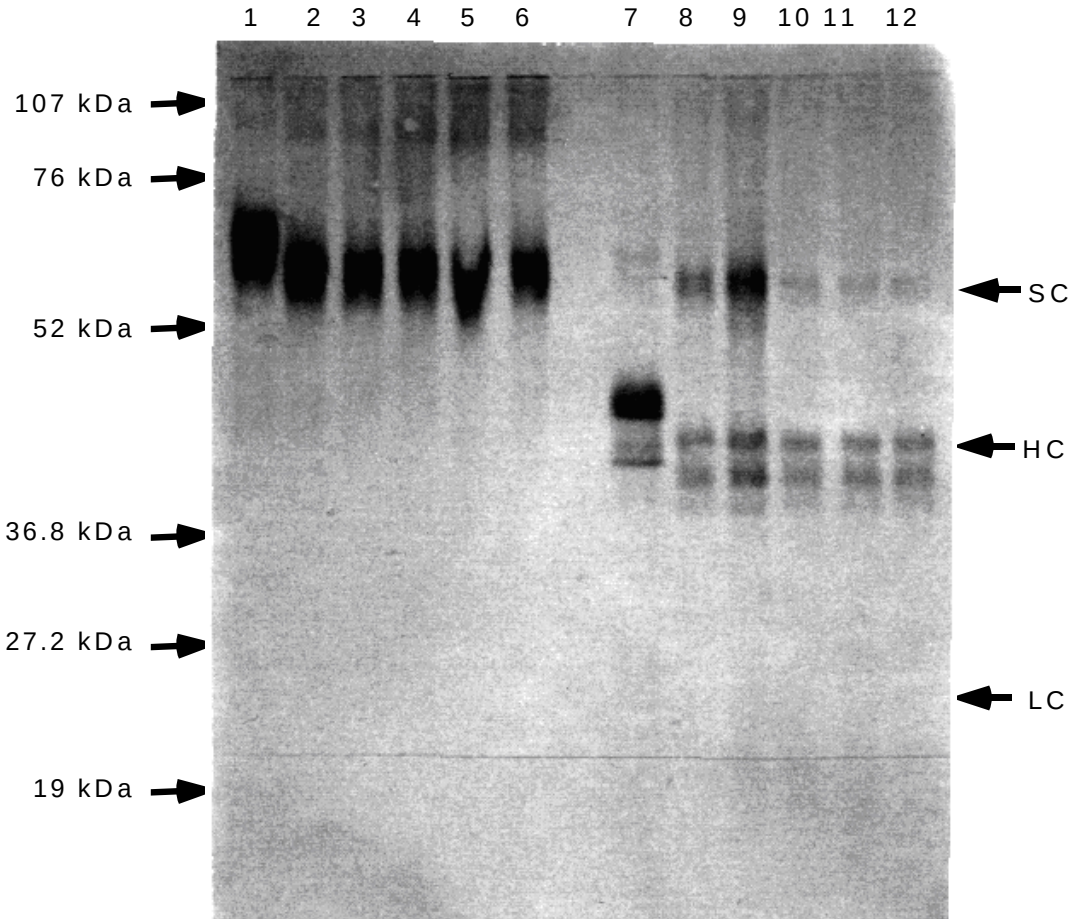

Figure 5B: A non-reduced and reduced westerns of the rhPC purification process. Prestained molecular weight markers ranging from 19 KDa to 107 Kda were used, but are not shown. Lanes 1 and 7 are non-reduced and reduced reference human Protein C from plasma respectively, lanes 2 and 8 are non-reduced 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 ηg of rhPC applied to each lane for the non-reduced lanes and 500 ηg of rhPC applied to each lane for the reduced lanes

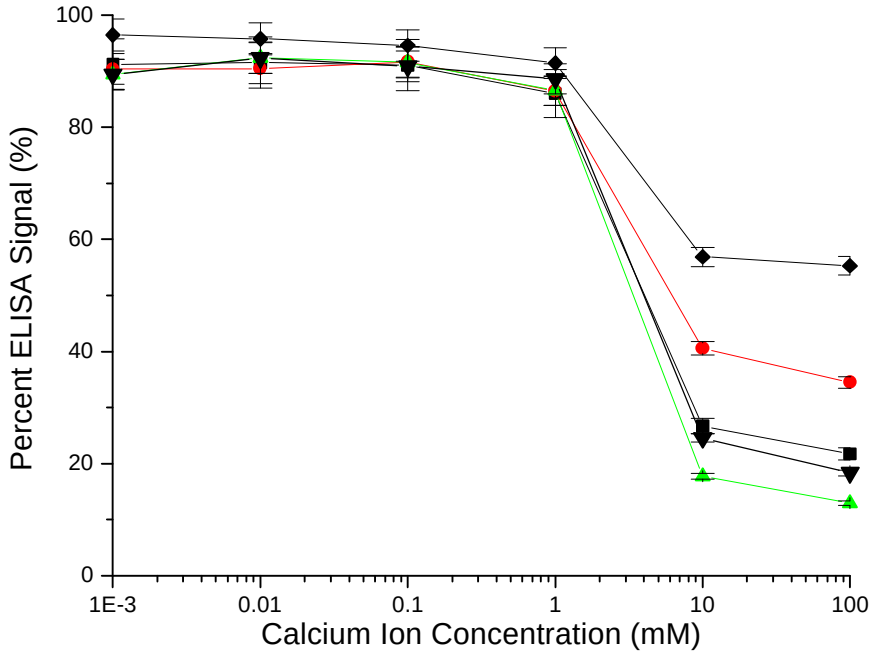

Figure 6A: The 250 mM NaCl fractions from the Streamline™ DEAE with and without Zn^{2+} purified by immuno-affinity or hydrophobic interaction chromatography(HIC). (■-MAb-affinity product of hPC from human plasma, ●-MAb-affinity product of 2 mM $ZnCl_2$ Streamline™ eluate, ▲-β-MAb-affinity product of 4 mM $ZnCl_2$ Streamline™ eluate, ◆-MAb-affinity product of no $ZnCl_2$ Streamline™ eluate, ▼Butyl isolation of rhPC from 4 mM $ZnCl_2$ Streamline™ eluate)

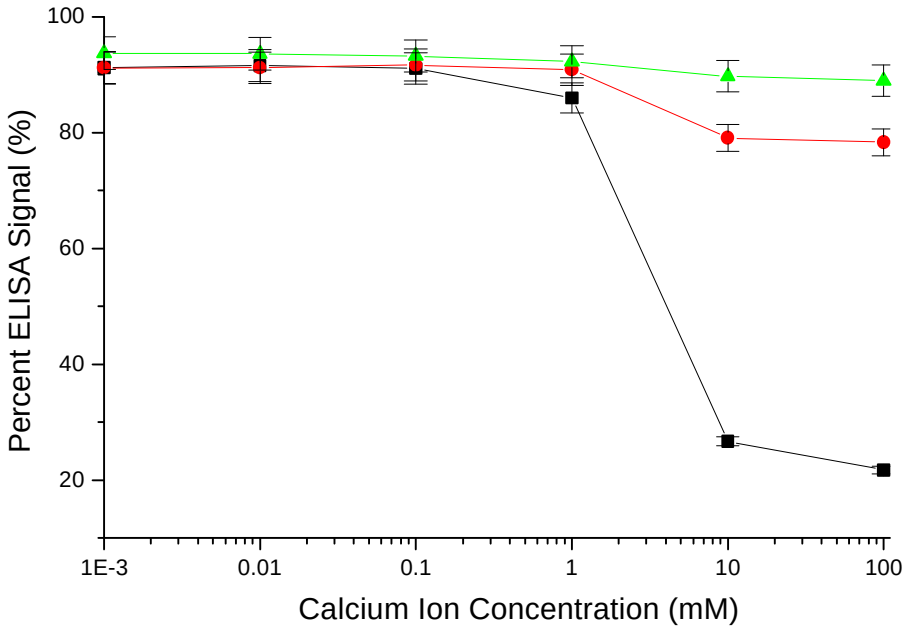

Figure 6B: The unabsorbed material from the Streamline^{TM} DEAE with Zn^{2+} purified by immuno-affinity chromatography. (■-MAb-affinity product of hPC from human plasma, ●-MAb-affinity product of 2 mM $ZnCl_2$ Streamline^{TM} flow through, ▲-β-MAb-affinity product of 4 mM $ZnCl_2$ Streamline^{TM} flow through)

Table I: Dynamic Light Scattering of pure α- and β-casein and rhPC in the Presence of Zn^{2+}. At least 10 measurements were recorded for each sample at a temperature of 22C. The samples were analyzed using a monomodal size distribution.

Protein	Zn^{2+} Concentration (mM)	R_h (nm)	Estimated Molecular Weight (kDa)
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

Table II: Purification and Yield from HIC and Immuno-purified rhPC Purification Processes.

Purification of rhPC with No Zinc					
Samples	Total Protein (%)	Percent rhPC Yield (%)	Purification Factor	Purity (%)	Activity by APTT (%hPC ref)
Start	100	100	1.0	NA	NA
Fall Through (FT)	44.9	2	0.1	NA	NA
125mM NaCl Peak	14.7	4.5	0.3	NA	NA
250mM NaCl Peak	10.7	83.1	8.0	NA	NA
500mM NaCl Peak	18.1	1.3	0.1	NA	NA
MAb-Affinity Products-Streamline FT	NA	NA	NA	NA	NA
MAb-Affinity Products-Streamline	100	89	200	>95	43
Purification of rhPC with 4mM Zinc					
Samples	Total Protein (%)	Percent rhPC Yield (%)	Purification Factor	Purity (%)	Activity by APTT (%hPC ref)
Start	100	100	1.0	NA	NA
Fall Through	85	51.1	0.6	NA	NA
125mM NaCl Peak	6.5	0.5	0.1	NA	NA
250mM NaCl Peak	7	19	3.0	NA	NA
500mM NaCl Peak	10.7	22.1	2.0	NA	NA
MAb-Affinity Products-Streamline FT	100	89	200	>95	0
MAb-Affinity Products-Streamline	100	94	200	>95	75
HIC Products-Streamline Eluates	100	93	150	>80	71

Chapter 3: A Zn^{2+}-Selective Purification of Immunoglobulins from the Milk of Dairy Livestock

Arthur W. Degener and William H. Velander
Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA

Key Words: Expanded Bed Adsorption Chromatography, Protein A, Immunoglobulins, Zinc Interaction

Abbreviations: IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgM, Immunoglobulin M; DLS, Dynamic Light Scattering; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme linked immuno sorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abstract

Milk of dairy livestock has been genetically engineered to produce recombinant therapeutic plasma proteins, but the endogenous proteins may also be of therapeutic value. There are currently 10-20% neonatal deaths due to diarrheal diseases, but this can be controlled through the use of immunoglobulins. IgG is commonly used to treat many types of infections and is used to aid people who are immuno deficient. However, IgG purified by precipitation, centrifugation and affinity chromatography is contaminated with IgA. EBAC-SPA processing of zinc induced precipitation of clarified pig milk did not reduce the IgA contamination typically seen, it did remove two time consuming processes: filtration and centrifugation. The presence of zinc also enhanced the adsorption of IgA and IgM unto SPA.

3.0 Introduction

The milk of livestock is a complex mixture of proteins and lipids. In the case of pigs, the milk resembles plasma in composition and makes protein isolation a challenging task[1,2]. Neonatant health of livestock is of major concern and can be mitigated by dietary immunoglobulin augmentation, the 10-20% deaths that results from diarrheal disease would ne negated. The current methods for processing proteins from blood plasma or milk typically involve multiple precipitation steps combined with filtration or centrifugation followed by packed bed chromatography[3-10]. Thus, these processes may be lengthy operations and have low overall yields. More recently, the milk of transgenic pigs has been processed at preparatory scale using Zn^{2+}-assisted precipitation of major milk proteins combined with anion exchange, expanded bed adsorption chromatography (EBAC) using Streamline DEAE [11]. This technique resulted in a selective adsorption of the target species in the presence of protein precipitates while simplifying processing and providing a high recombinant protein yield (>90% of active populations)[11].

Transition metals such as $Zn^{2+,}$ Cu^{2+}, Fe^{3+} and Ni^{2+} are more traditionally used in immobilized metal affinity chromatography (IMAC) to isolate proteins from complex mixtures with high yield and selectivity[12,13]. These metal ions

can damage protein structure through metal catalyzed oxidation (MCO)[14,15]. However, Zn^{2+} is a physiologically occurring, non-transition metal which binds through ionic and coordinate covalent bonds, but has not been shown to cause MCO of proteins[12,13]. Thus, it can be used with metal chelating techniques to selectively isolate proteins from complex mixtures without risk of oxidation. Importantly, the coordinate covalent complexes formed with Zn^{2+} are sufficiently strong to cause proteins to undergo profound conformational changes which can cause precipitation at concentrations less than 5 mM $ZnCl_2$ [8,16,17].

The clinical demand for immunization therapy for passive immunization is great but milk derived immunoglobulins may be a future source. Today, treatments typically consist of IgG fractions made from human plasma, but impurities can cause life threatening reactions. For example, some autoimmune disorders result in IgA, deficiency due to the presence of anti-IgA[18-19]. However, when the IgG fractions are purified by methods such as precipitation by Cohn fractionation or affinity chromatography using Protein G and Protein A, impure fractions containing IgA can cause anaphylaxis [20-22]. These methods generally will not selectively adsorb IgA or IgG.

We present a novel technique for Zn^{2+}-assisted precipitation used to increase the purity of the immunoglobulin products made from milk Specifically,

we describe the use of $ZnCl_2$ combined with Streamline Protein A (SPA) EBAC for purifying immunoglobulins from pig milk. The major milk immunoglobulins are IgG from IgA and IgM. Each of these milk immunoglobulins bind to SPA with similar adsorption kinetics making separation from each other difficult. We have examined the potential for the selective formation of Zn^{2+}-immunoglobulin complexes to alter the relative adsorption kinetics of the immunoglobulin classes to selectively enhance adsorption onto SPA.

3.1Materials

3.1.1 Materials: Streamline™ Protein A(SPA) and 4-15% gradient phast gels were gifts from Pharmacia Biotech, Uppsala, Sweden. Pharmacia C-20 columns, Silver Stain Kit, PhastSystem™ control and separation unit and PhastSystem™ Developer unit were purchased from Pharmacia Biotech, Uppsala, Sweden. Anti-swine IgG from rabbit, anti-swine IgG HRP conjugated from goat, anti-swine IgA from rabbit, anti-swine IgM HRP conjugated from goat, Anti-swine IgM from rabbit and anti-swine IgM HRP conjugated from goat were purchased from Betyl Laboratories Inc. Bovine IgG, Bovine IgA and Bovine IgM used as primary reference were purchased from Accurate Chemical & Scientific Corporation. The IgA and IgM primary references were further purified with SPA to increase the purity. Sodium hydroxide, Tris-HCL, sodium chloride, EDTA, zinc chloris, glycine and acetic acid were purchased from Sigma Chemical Co.. Immulon II Plates were purchased from Fisher Scientific. DynaPro 801 TC light scattering device with AutoPro and Dynamic software(Protein Solution, Charlottesville VA) was used to analyze the size distribution by both a monomodal and bimodal analysis. O-phenylenediamine-2HCl (OPD) tablets were purchased from Abbott Laboratories. The Immulon II plates were analyzed at 490nm using an EL308 Bio-Tek Microplate reader. A Knauer Spectrophotometer from Rainin and a Rainin data acquisition system were used to monitor the Streamline™ Protein A chromatography.

3.2 Methods

3.2.1 Milk collection and preparation of clarified whey: Milk from pigs was collected, EDTA added to a final concentration of 100mm and then the pH was adjusted to 7.2. The milk-EDTA solution was centrifuged at 4500RPM(3500 g force) for 90 minutes to remove the fat, creating skim milk with EDTA. The whey was dialyzed at 4^{o}C with a 10kDa MW cut off membrane against a 10:1 vol/vol ratio of de-ionized (5µmho conductivity) water to pig whey where the water was exchanged 4 times within 24 hours. Dialyzed, chelated whey mix is termed “clarified whey”.

3.2.2 Determination of Immunoglobulins concentration: Enzyme Linked Immuno sorbent Assay(ELISA) was used to quantify immunoglobulin content of all samples. To detect pig IgG, Immulon II plates were coated with 100µl of 5µg/ml rabbit anti-swine IgG(coating antibody) in 0.1M $NaHCO_3$, 0.1M NaCl at a pH of 9.6 at 4^{o}C for 12 hours. Affinity purified pig IgG was used as a reference and was diluted to a range of 0-125ηg/ml with Buffer A(25mM Tris-HCL, 50mM NaCl, 0.05 vol/vol% Tween 20 and 1mg/ml BSA at a pH of 7.2). The Immulon II plates were washed 4 times with Buffer B(25mM Tris-HCL, 50mM NaCl and 0.05 vol/vol% Tween 20 at a pH of 7.2). The plates were blocked for 20minutes at room temperature(RT) with Buffer A. 100 µl of reference IgG or samples were applied to the plate in triplicate and incubated at 37^{0}C for 20 minutes. The

plates were then washed 4 times with Buffer B. 100µl of a 1:1000 dilution of goat anti-swine IgG HRP conjugated(detecting antibody) was added to each well and then incubated at 37^0C for 20 minutes. The plates were then washed 4 times with Buffer B. Detection was done using OPD and the reacted chromaphore was read at 490nm on the EL308 Bio-Tek Microplate reader. The analogous procedure was used for detecting pig IgA and pig IgM. To detect pig IgA, the coating antibody used was rabbit anti-swine IgA, the detecting antibody used was goat anti-swine IgA HRP conjugated. To detect pig IgM, the coating antibody used was rabbit anti-swine IgM, the detecting antibody used was goat anti-swine IgM HRP conjugated.

3.2.3 Phast gels: Samples were resolved using sodium-dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) 4-15% Phast gels and developed using silver staining by the method described in the Pharmacia operators manual for Phast gel systems.

3.2.4 Zn^{2+}-sensitive Immunoglobulin solubility: "Clarified whey" was thawed in a water bath at 4^oC for 4 hours. A 2 ml sample of clarified whey was aliquoted into each of 11 test tubes containing 2 ml of 50 mM Tris-HCl, pH 7.2. Each of the tubes were treated with $ZnCl_2$ and rendered from 0 to100 mM Zn^{2+} in 10mM $ZnCl_2$ increments, then agitated for 10 minutes at 4^oC. After centrifugation at

4500RPM for 30 minutes at 4 C, the pH and immunoglobulin (IgG, IgA and IgM) concentration by ELISA of the decanted, clear supernatant was measured. Each supernatant was also analyzed by silver-stained, 4-15% Phast gel.

3.2.5 Expanded Bed Adsorption (EBA): The following buffers were used during SPA chromatography: Buffer A (loading buffer) consisting of 25 mM Tris-HCl/50mM NaCl, pH 7.2 and Buffer B (elution buffer) consisting of 0.1M glycine/2% acetic acid pH 2.2.

A small scale, 10ml EBAC-SPA chromatography column was operated 4 ^{o}C at a three-fold, expanded bed mode of 30 cm bed height by pumping Buffer A at a velocity of 300 cm/h (600 ml/h). “Clarified whey” with 25 mM Tris-HCl/50mM NaCl, pH 7.2 (10 ml of skim milk) was applied to the expanded bed followed by washing with about 5 expanded-bed volumes of Buffer A until the A_{280} of the top effluent returned to base-line. Further wash with Buffer A was done in top down mode until the bed was packed. Elution of SPA was done with 1 column volumes (CV) of Buffer B in a packed bed column mode. The column was regenerated using a sequence of 2 CV of 2M NaSCN, 4 M NaCl, 0.5 M NaOH, and 5 CV of Buffer A. All process materials(start, flow through and elution materials) were analyzed by 4-15% Phast gel and by ELISA . The above chromatography was repeated using 10 ml “clarified whey” containing 5 mM, 10

mM, 15 mM, 20 mM, 30 mM and 40 mM $ZnCl_2$. Reprocessing of the 40mM $ZnCl_2$ treated whey was repeated on the flow through until the IgA and IgM were removed to help estimate preparative scale dynamic loading capacity. Approximately 3 runs were needed at a ratio of 3mg swine IgA per ml SPA and 2mg swine IgM per ml SPA . After the 40mM $ZnCl_2$ column operation, the flow through was resolubilized with 100mM EDTA at pH 8 and loaded onto the STREAMLINE™ Protein A at 300cm/h. The flow through was collected. Elution of loaded SPA was done with 2 column volumes (CV) of Buffer B in a packed bed column mode. The samples(flow through number 1, flow through number 2 and elution) were analyzed by 4-15% Phast gel and by ELISA.

A preparative scale, 300ml Streamline™ Protein A chromatography column was operated 4 ^{o}C at a three-fold, expanded bed mode of 45 cm bed height by pumping Buffer A at a velocity of 300 cm/h (6 l/h). “Clarified whey” with 25 mM Tris-HCl/50mM NaCl, pH 7.2 (100 ml of skim milk) was applied to the expanded bed followed by washing with about 5 expanded-bed volumes of Buffer A until the A_{280} of the top effluent returned to base-line. Further wash with Buffer A was done in top down mode until the bed was packed. Elution of SPA was done with 2 column volumes (CV) of Buffer B in a packed bed column mode. The column was regenerated using a sequence of 2 CV of 2M NaSCN, 4 M NaCl, 0.5 M NaOH, and 5CV of Buffer A. All process materials(start, flow through and elution

materials) were analyzed by 4-15% Phast gel and by ELISA. The above chromatography was repeated using 100 ml “clarified whey” containing 40 mM $ZnCl_2$. After the 40mM $ZnCl_2$ column operation, the flow through was resolubilized with 100mM EDTA at pH 8 and loaded onto the STREAMLINE™ Protein A at 300cm/h. The flow through was collected. Elution of loaded SPA was done with 2 column volumes (CV) of Buffer B in a packed bed column mode The samples(flow through number 1, flow through number 2 and elution) were analyzed by 4-15% Phast gel and by ELISA.

3.2.6 Light Scattering: IgG aggregation and precipitation analysis was done by dynamic light scattering using a Protein Solution DynaPro DL801 device. Test tubes were filled with 1 ml of 1mg of purified swine IgG(>95% purity by silver stained SDS-PAGE) per ml solution in 25mM tris pH 7.0. Zinc chloride ranging from 0-50 mM in 10mM increments from a 1M stock solution were added to each test tube and agitated for 10 minutes at 4^oC. Samples were diluted to the linear range of the dynamic light scattering device(500 ng/ml). Approximately 250µl of each sample was injected with a 1cc syringe through a 0.2 µm syringe filter. A minimum of 10 hydrodynamic radius sampling intervals were taken over 1 hour for each sample. The light scattering data was analyzed by both mono-modal and bimodal distribution analysis(Protein Solution Dynamics software). An analogous aggregation and precipitation analysis was done for IgM basically

using the same procedure. Test tubes were filled with 1ml of 3mg swine IgM per ml solution in 25mM tris pH 7.0. Zinc chloride ranging from 0-50 mM in 10mM increments from a 1M stock solution were added to each test tube and agitated for 10 minutes at 4^{o}C. Samples were diluted to the linear range of the dynamic light scattering device(50 ng/ml). Approximately 250µl of each sample was injected with a 1cc syringe through a 0.45 µm syringe filter.

Several replicates of Zn^{2+}-treated whey samples were subsequently brought to a concentration of 100mM EDTA to test the effects of chelating conditions upon the presence of Zn^{2+}-induced aggregates. EDTA was added to chelate the zinc in each of the IgG and IgM samples (containing 50mM $ZnCl_2$). This sample was diluted to the linear range of the dynamic light scattering device(ng/ml). Approximately 250µl of each sample was injected with a 1cc syringe through a 0.2 µm syringe filter. A minimum of 10 hydrodynamic radius sampling intervals were taken over 1 hour for each sample. The light scattering data was analyzed by both mono-modal and bimodal distribution analysis(Protein Solution Dynamics software).

3.2.7 Immunoglobulin Binding to Protein A: A 1ml SPA column was used to study the binding capacity effects of affinity purified IgG and IgM from mouse, bovine, swine and rabbit with and without the addition of 40 mM $ZnCl_2$. The IgG

from mouse was batch loaded for 16 hours at 4^{o}C in Buffer A. After batch loading with immunoglobulin, the SPA was put into a 1cm diameter x 1.3 cm packed bed length. The loaded SPA was washed column wise with Buffer A until absorbance of less than 0.01 was detected at 280 nm. Column wise elution of washed, immunoglobulin loaded SPA was done with 2CV of Buffer B in a packed bed mode This batch process for mouse IgG was repeated using 40mM $ZnCl_2$. All loading and elution samples were analyzed by OD 280nm to determine the binding capacity.

3.3 Results

Figure 1 panel A shows a silver stained, 4-15% SDS-PAGE of typical samples obtained from Zn^{2+}-treated pig whey which were processed by a small scale(10ml) EBAC-SPA. Lanes 1-3 of figure 1, panel A shows reference samples consisting of affinity purified swine IgG, IgA and IgM respectively. Lane 4 shows a sample from the pH 2 elution peak of EBAC-SPA, loaded with "clarified whey" having no $ZnCl_2$. There are about 60%IgG, 15%IgA, 15%IgM and 10% other milk proteins present in lane 4. Lanes 5, 6 and 7 are also pH 2 eluate samples of EBAC-SPA loaded with "clarified whey" having 5, 10 and 20 mM $ZnCl_2$ respectively. Each of these eluate samples showed a similar composition as indicated by SDS-PAGE analysis where about 60% IgG, 15% IgA, 15% IgM and 10% other milk proteins. Lane 8 is the pH 2 elution peak of EBAC-SPA "clarified whey" loaded at a final concentration of 40mM $ZnCl_2$. In contrast to lanes 4 through 7, lane 8 shows an eluate containing <1%IgG, >45%IgA, >45%IgM and 5% other milk proteins.

Figure 1 panel B shows a silver stained, 4-15% SDS-PAGE of material from Zn^{2+}-treated clarified whey that was re-processed by a small scale(10ml) EBAC-SPA. Panel B lane 1 shows the unabsorbed material that was treated with a final concentration of 100mM EDTA to resolubilized any precipitates and aggregates that formed from the 40 mM Zn^{2+} treatment. Panel B lane 2 shows

the pH 2 eluate from EBAC-SPA loaded with EDTA-treated, unabsorbed material from clarified whey containing 40mM $ZnCl_2$. The composition of this eluate obtained from IgA, IgM depleted whey was approximately 98% IgG, <1% IgA, <1% IgM and <1% other milk proteins.

Figure 2 shows a comparison of silver stained 4-15% SDS-PAGE of Zn^{2+} treated clarified whey samples processed by both a small scale(10ml, 1 cm diameter column x 12 cm packed bed length with a linear velocity of 5 cm/min for both loading and elution) EBAC-SPA and a preparative scale(300ml, 5 cm diameter column x 15 cm packed bed length with a linear velocity of 5 cm/min for both loading and elution) EBAC-SPA. Lanes 1-3 of figure 2 shows reference samples consisting of purified swine IgG, IgA and IgM respectively. Lane 4 shows the starting material that was loaded onto EBAC-SPA for both the small and preparative scale processes. Lanes 5 and 6 show the pH 2 eluate samples of EBAC-SPA loaded with “clarified whey” rendered to a final concentration of 40 mM $ZnCl_2$ for both small and preparative scale processes respectively. Each of these samples showed a similar composition as indicated by SDS-PAGE analysis where about <1%IgG, >45%IgA, >45%IgM and 5% other milk proteins. Lanes 7 and 8 show the pH 2 eluate from EBAC-SPA loaded with unabsorbed material from the 40 mM Zn^{2+}-treated whey for both small and preparative scale processes respectively. This unabsorbed material was treated with EDTA to

resolubilized any precipitates and aggregates that formed from the 40 mM Zn^{2+} treatment. The composition of each eluate for lanes 7 and 8 from IgA, IgM depleted whey was approximately 98% IgG, <1% IgA, <1% IgM and <1% other milk proteins.

Small scale(10ml) EBAC-SPA adsorption of immunoglobulins of clarified whey treated with different levels of $ZnCl_2$ were done to obtain dynamic binding capacities(DBC) of large scale runs(300ml). The DBC was <1, 3.1 and 2.7 mg/ml for IgG, IgA and IgM respectively at 40mM $ZnCl_2$. A yield of 30% for IgA and 50% for IgM was obtained for a single pass. The unabsorbed material was recycled until all IgA and IgM was depleted. The DBC for 300ml runs in the presence of $ZnCl_2$ was estimated to be <1, 3 and 3 mg/ml for IgG, IgA and IgM respectively from this data.

A preparative scale(300ml) EBAC-SPA was done at 6 mg total immunoglobulin per ml SPA. Table I shows the yield, purity and purification of swine IgA and IgM from clarified whey in the presence of 40mM $ZnCl_2$. The DBC of swine IgA was 3.4 mg/ml SPA which gave a yield of 90%, a purity of >45% and purification of 8 fold. The DBC of swine IgM was 1.8 mg/ml SPA which gave a yield of 90%, a purity of >45% and purification of 8 fold. In contrast, the DBC of swine IgG was <0.1 mg/ml SPA which gave a yield <1%, a purity of <1% and

a purification of <1 fold. The IgA and IgM depleted clarified whey (unabsorbed material) was treated with 100mM EDTA to resolubilize aggregates and precipitates caused by the addition of 40mM $ZnCl_2$. This chelated unabsorbed material was recycled onto the preparative scale EBAC-SPA. Table II shows the yield, purity and purification factor of IgG from IgA and IgM depleted pig whey. The DBC of IgG was 1mg/ml SPA which results in a yield of 90%, purity >98% and a 25 fold purification.

Dynamic light scattering was used to estimate the extent of immunoglobulin aggregation affected by the presence of $ZnCl_2$. Table III and IV show estimates of aggregate sizes in terms of hydrodynamic radius for pure samples of swine IgG and IgM, respectively. All aggregates are assumed to be spherical. Based upon spherical geometry, the molecular weight estimates of $ZnCl_2$-induced IgG and IgM aggregates are also given in tables III and IV respectively. Mono-modal analysis shows a 81% increase in the hydrodynamic radius of swine IgG over the range of 0-40 mM $ZnCl_2$ corresponding to a 98% increase in molecular weight over the same range. Furthermore, there is a 63% increase in hydrodynamic radius of swine IgG corresponding to an increase of 91% in molecular weight when the $ZnCl_2$ concentration was increased from 30 to 40mM. When 100mM EDTA is added to the precipitated pig IgG containing 50mM $ZnCl_2$, both the hydrodynamic radius and corresponding molecular weight are

8% and 19% above the initial value at 0mM $ZnCl_2$. Bi-modal analysis shows a similar trend to that of monomodal size distribution. Like that of monomodal estimates, bimodal estimates of size distribution show an increase in hydrodynamic radius corresponding to an increase in molecular weight estimation as the $ZnCl_2$ concentration is raised from 0 to 40mM. The extent of aggregation by both monomodal and bimodal distribution analysis of $ZnCl_2$ induced aggregation clarified by EDTA is similar to a zinc free environment.

Mono-modal analysis shows a 50% increase in the hydrodynamic radius of swine IgM over the range of 0-50 mM $ZnCl_2$ corresponding to a 81% increase in molecular weight over the same range. Furthermore, there is a 30% increase in hydrodynamic radius of swine IgM corresponding to an increase of 58% in molecular weight when the $ZnCl_2$ concentration was increased from 40 to 50mM. When 100mM EDTA is added to the precipitated pig IgM containing 50mM $ZnCl_2$, both the hydrodynamic radius and corresponding molecular weight are 2% and 5% above the initial value at 0mM $ZnCl_2$. Bi-modal analysis shows a similar trend to that of monomodal size distribution. Like that of monomodal estimates, bimodal estimates of size distribution show an increase in hydrodynamic radius corresponding to an increase in molecular weight estimation as the $ZnCl_2$ concentration is raised from 0 to 50mM. The extent of aggregation by both monomodal and bimodal distribution analysis of $ZnCl_2$

induced aggregation clarified by EDTA is similar to a zinc free environment.

A Zn^{2+}-assisted SPA binding study with immunoglobulins(IgG and IgM) from different species was done to determine the batch loading equilibrium binding capacities. Table V shows the SPA binding effects of adding varying concentrations of $ZnCl_2$ to mouse IgG and IgM, bovine IgG and IgM, swine IgG and IgM and rabbit IgG and IgM. Without $ZnCl_2$ added to the starting material before loading, the binding capacity of IgG for all species was around 3 mg per ml SPA. With 40 mM $ZnCl_2$ added, IgG binding capacity reduced to 1, 0.5, 0.5 and 0.2 mg per ml SPA for mouse, bovine, swine and rabbit respectively. Without $ZnCl_2$ added to the starting material before SPA loading, the binding capacity of IgM was around 2 mg per ml SPA for mouse, bovine and swine; while rabbit IgM bound to SPA at 0.5 mg per ml SPA. With the addition of 40 mM $ZnCl_2$ added to the starting material before SPA loading, the binding capacity of bovine and swine IgM increased 2 fold to about 4 mg per ml SPA. However, both mouse and rabbit decreased in their binding capacities to <0.1 mg per ml SPA.

3.4 Discussion

Blood transfusions and immunoglobulin treatment of viral or recurring infections can result in adverse side effects which are life threatening[24-26]. For example, it has been shown that some immuno-deficiencies result from pre-existing antibodies to IgA. Thus, immunoglobulin therapy containing trace amounts of IgA can lead to an immune response where by the native antibodies will aggressively react to the foreign IgA resulting in a significant incidence of anaphylaxis[26]. However, the current methods for purifying IgG include precipitation, centrifugation and packed-bed affinity chromatography(Protein A)[27], can result in IgG products contaminated with <0.1% IgA[28]. While not completely optimized, the EBAC-SPA Zn^{2+}-assisted precipitation process still yielded > 98% pure IgG with <1% IgA, and eliminated the filtration and centrifugation of precipitates which can drastically reduce processing times. Conformational changes in IgG and protein-protein interaction caused by the presence of Zn^{2+} induced precipitation allowed the IgG to pass through the column. In contrast, IgA and IgM bound to SPA with greater affinity also likely due to conformational changes caused by the presence of Zn^{2+} or the inability of IgG to compete for reactive sites.

Protein A affinity matrices specifically adsorb immunoglobulins, changes in pH or the addition of a denaturing agent such as $ZnCl_2$ the conformation of the

target immunoglobulin can be reversibly altered to prevent or enhance adsorption. According to Goding, IgA and IgM of most species have weak affinity to Protein A adsorbents[27], but our screening methods with SPA showed that porcine(p-) IgA, p-IgM and p-IgG bind at about equal molar concentrations from clarified whey. Zinc was used to precipitate p-IgG. At a 40mM concentration, the hydrodynamic radius of p-IgG as measured by dynamic light scattering increased 400-500% over that without Zn^{2+}. In contrast, p-IgM showed only a 25% increase in hydrodynamic radius. The increased hydrodynamic radius of p-IgG suggests that a combination of protein aggregation and or precipitation prevented adsorption onto SPA.

The Zn^{2+}-induced p-IgG precipitation prohibited adsorption on to EBAC-SPA upon initial loading, but p-IgA and p-IgM adsorption was enhanced thus enabling separation of p-IgG from p-IgA and p-IgM. After depletion of p-IgA and p-IgM from clarified pig whey by SPA adsorption, the p-IgG and other milk protein precipitates were resolubilized with the addition of EDTA and p-IgG was isolated with SPA. Dynamic light scattering of EDTA-treated zinc-induced precipitates showed that Zn^{2+}-induced p-IgG precipitation was reversible with the addition of a chelating agent. Thus, the quality of the recovered p-IgG was physiochemically similar to starting p-IgG in clarified whey.

Monoclonal antibodies are typically IgG and IgM class antibodies that are derived from mouse and rabbit hybridoma cells[27]. The purification of IgM is very difficult due to its high molecular weight. Thus, no highly efficient purification methods exist for IgM class antibodies[27]. Thus, the effect of $ZnCl_2$ on both polyclonal IgG and IgM class antibodies derived from rabbit and mouse was analyzed because these are potentially very useful as diagnostic reagents and immuno-therapeutics agents. However, the ability of Zn^{2+}-assisted EBAC-SPA to differentially enhance the adsorption of IgG and IgM appears to be animal species specific. For example, mouse, rabbit, bovine and swine IgG adsorption decreased by 66%, 90%, 85% and 80% respectively in the presence of zinc. Also, both swine and bovine IgM adsorption increased by about 200% with the presence of zinc, but mouse and rabbit IgM adsorption decreased by about 100%. Therefore, zinc-induced conformational changes in immunoglobulins can prevent or enhance adsorption onto SPA appears most useful for bovine and swine IgM class antibodies and less useful for rabbit and mouse.

In summary, zinc induced precipitation combined with EBAC-SPA is an effective method for a two step process for the purification of IgG from IgA and IgM. It also appears that bovine IGs will behave similarly.

3.5 References

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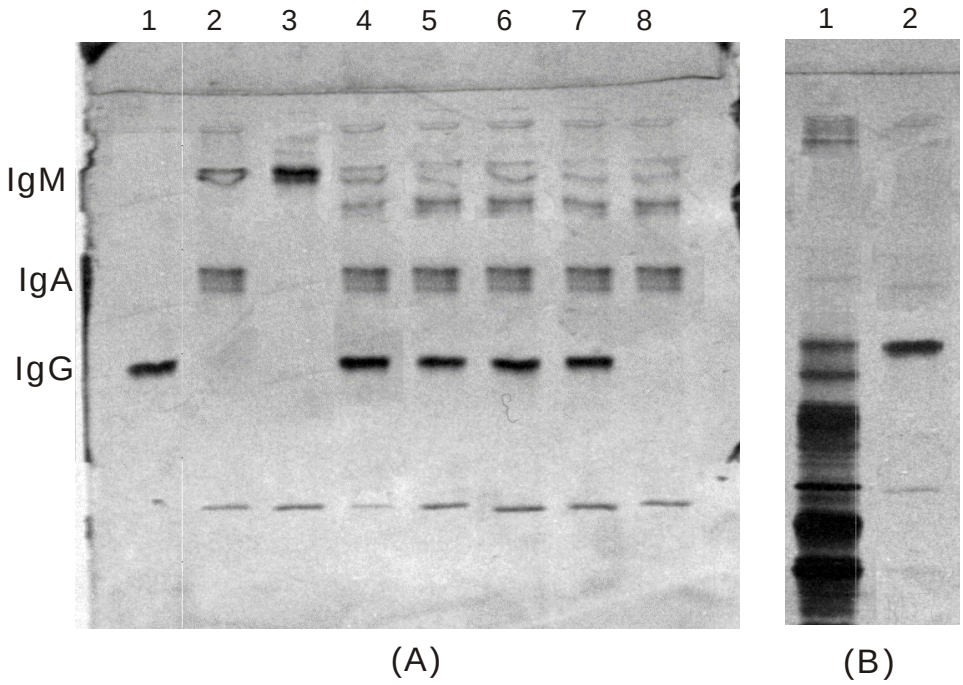

Figure 1 Panel A: Silver Stained 4-15% SDS-PAGE of samples from $ZnCl_2$ treated pig whey processed by small scale(10ml) expanded bed adsorption chromatography onto Streamline Protein A. Lanes 1-3 are purified swine IgG, IgA and IgM respectively. Lanes 4, 5, 6, 7 and 8 are pH 2 eluate samples of EBAC-SPA loaded "clarified whey" but with 0, 5, 10, 20 and 40 mM $ZnCl_2$ respectively. **Panel B:** Lane 1 is the unabsorbed IgA and IgM depleted EDTA-treated clarified whey that is reloaded onto EBAC-SPA. Lane 2 is the pH 2 eluate from EBAC-SPA of the unabsorbed material from 40mM $ZnCl_2$-treatment treated with 100mM EDTA and reloaded onto SPA.

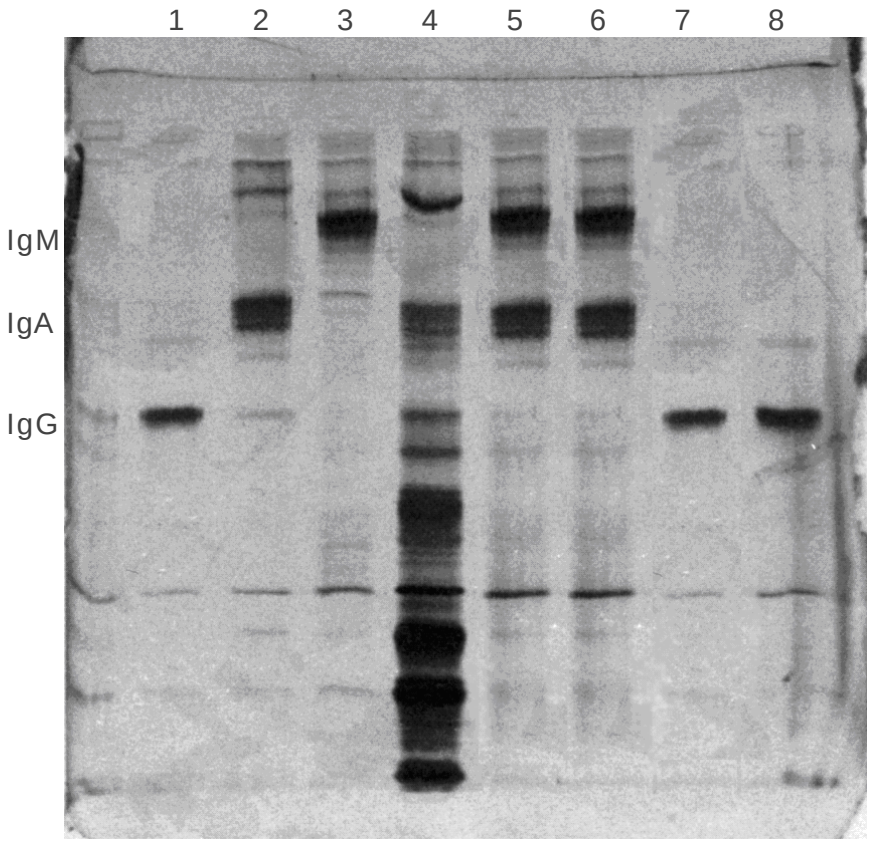

Figure 2: Silver Stained 4-15% SDS-PAGE of samples from $ZnCl_2$ treated pig whey processed by both small scale(10ml) and preparative scale(300ml) expanded bed adsorption chromatography onto Streamline Protein A. Lanes 1-3 are purified swine IgG, IgA and IgM respectively. Lane 4 is the starting material for both small and preparative scale processes. Lanes 5 and 6 are the pH 2 eluate samples of EBAC-SPA loaded with dialyzed EDTA-treated whey but with 40 mM $ZnCl_2$ for both small and preparative scale processes respectively. Lanes 7 and 8 are the pH 2 eluate from EBAC-SPA loaded with EDTA-treated unabsorbed material from the 40 mM Zn^{2+}-treated whey for both small and preparative scale processes respectively.

Table I: Yield, purity and purification factors for EBAC-SPA separation of IgA and IgM from pig whey.

Column loaded at pH 7.0 with 40 mM Zinc added to the starting material, eluted pH 2.2,							
Sample	Yield (%)			Purity (%) [Purification Factor]			
	IgG	IgA	IgM	IgG	IgA	IgM	Other
Start	100 ± 9	100 ± 8	100 ± 9	- [1]	- [1]	- [1]	-
Flow Through	90 ± 8	4 ± 1	5 ± 1	- [<1]	- [1]	- [1]	-
pH 2.2 Elution	<1 ± <1	94 ± 9	93 ± 8	<1 ± <1 [<1]	>45 ± 1 [8]	>45 ± 1 [8]	<5 ± 1

Table II: Yield, purity and purification factors for EBAC-SPA separation of pig IgG from IgA and IgM depleted pig whey.

Column loaded at pH 9 with EDTA chelated IgA and IgM depleted pig whey eluted pH 2.2							
Sample	Yield (%)			Purity (%) [Purification Factor]			
	IgG	IgA	IgM	IgG	IgA	IgM	Other
Start	100 ± 9	5 ± 1	4 ± 1	- [1]	- [1]	- [1]	-
Flow Through	6 ± 9	3 ± 1	3 ± 1	- [<1]	- [1]	- [1]	-
pH 2.2 Elution	92 ± 9	<1 ± <1	1 ± <1	>98 ± 1 [25]	<1 ± <1 [<1]	<1 ± <1 [<1]	<1± <1

Table III: Hydrodynamic radius of Swine IgG estimated by dynamic light scattering in the presence and absence of $ZnCl_2$. At least 10 measurements were recorded for each sample at a temperature of 22C. The samples were analyzed using a monomodal and bimodal size distribution.

Experimental $ZnCl_2$ Concentration (mM)	Monomodal estimates of Hydrodynamic Radius (nm)	Bimodal estimates of Hydrodynamic Radius[1] (nm)	Monomodal Theoretical Molecular Weight (MDa)	Bimodal Theoretical Molecular Weight[a,b] (MDa)
0	5.4 ± 0.5	5.1 and 10.4 (97% and 3%)	0.2 ± 0.03	0.1 and 0.8
10	6.5 ± 0.5	5.5 and 15.6 (95% and 5%)	0.3 ± 0.02	0.2 and 2
20	7.2 ± 0.4	6.3 and 18.2 (97% and 3%)	0.4 ± 0.02	0.3 and 3
30	10.9 ± 1	7.2 and 22.6 (88% and 12%)	1 ± 0.1	0.4 and 6
40	29.1 ± 2	20.3 and 38.1 (62% and 38%)	10 ± 1	4 and 20
50 mM $ZnCl_2$/100mM EDTA[2]	5.9 ± 0.6	4.9 and 13.7 (95% and 5%)	0.2 ± 0.03	0.1 and 2

Footnote:

1. The values in parentheses are estimated percentages minimum(a) and maximum(b) immunoglobulin hydrodynamic radius determined by bimodal distribution.
2. This is a step sequence sample treatment: 50mM $ZnCl_2$ was first added to induce aggregation/precipitation followed by addition of 100mM EDTA for clarification.

Table IV: Hydrodynamic radius of Swine IgM estimated by dynamic light scattering in the presence and absence of $ZnCl_2$. At least 10 measurements were recorded for each sample at a temperature of 22C. The samples were analyzed using a monomodal and bimodal size distribution.

$ZinCl_2$ Concentration (mM)	Monomodal estimates of Hydrodynamic Radius (nm)	Bimodal estimates of Hydrodynamic Radius[1] (nm)	Monomodal Theoretical Molecular Weight (MDa)	Bimodal Theoretical Molecular Weight[a,b] (MDa)
0	26.4 ± 2	17.1 and 51.0 (86% and 14%)	8 ± 1	3 and 40
10	35.3 ± 2	20.1 and 69.7 (85% and 15%)	16 ± 1	4 and 86
20	35.9 ± 2	20.8 and 73.2 (86% and 14%)	17 ± 1	5 and 96
30	36.7 ± 3	21.2 and 71.5 (85% and 15%)	18 ± 2	5 and 91
40	37.4 ± 3	21.7 and 75.9 (86% and 14%)	19 ± 2	5 and 105
50	53.6 ± 3	30.4 and 98.1 (82% and 18%)	45 ± 3	11 and 196
50 mM $ZnCl_2$/100mM EDTA[2]	26.9 ± 1	18.5 and 62.0 (92% and 8%)	9 ± 1	3 and 64

Footnote:
1. The values in parentheses are estimated percentages minimum(a) and maximum(b) immunoglobulin hydrodynamic radius determined by bimodal distribution.
2. This is a step sequence sample treatment: 50mM $ZnCl_2$ was first added to induce aggregation/precipitation followed by addition of 100mM EDTA for clarification.

Table V: Batch loading binding capacities of IgG and IgM on Streamline™ Protein A. The loading and elution were done at 4C.

Immunoglobulin	**Metal**	**Binding Capacity** (mg Ig/ml proteinA)
Mouse IgG	None	3 ± 0.2
	40 mM ZnCl	1 ± 0.2
Bovine IgG	None	3.5 ± 0.1
	40 mM ZnCl	0.5 ± <0.3
Swine IgG	None	3 ± 0.2
	40 mM ZnCl	0.5 ± <0.1
Rabbit IgG	None	2.5 ±0.2
	40 mM ZnCl	0.2 ± <0.1
Mouse IgM	None	1.5 ± 0.1
	40 mM ZnCl	<0.1 ± <0.1
Bovine IgM	None	1.5 ± 0.1
	40 mM ZnCl	3.5 ± 0.3
Swine IgM	None	2 ± 0.3
	40 mM ZnCl	4 ± 0.2
Rabbit IgM	None	0.5 ± <0.1
	40 mM ZnCl	<0.1 ± <0.1

Chapter 4.0: Selective free flow electrophoresis purification of rhPC sub-populations

Arthur Degener and William H. Velander
Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA

Cornelius Ivory
Department of Chemical Engineering, Washington State University
Pullman, WA

Key Words: Protein C, Transgenic Animals, Free Flow Electrophoresis, Protein sub-populations, Ion-exchange chromatography

Abbreviations: rhPC, recombinant human protein C; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme linked immuno sorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; gla, gamma carboxy glutamic acid; DEAE, diethyl-amino-ethyl; RCFE, recycle crossflow electrophoresis

Abstract

The milk of transgenic livestock is a source for complex post-translationally modified recombinant therapeutic proteins. Because of mammary gland rate limitations for these non-endogenous proteins, the most desirable proteins typically exist as subpopulations. Immunoaffintiy has shown the ability to isolate active subpopulations of a recombinant protein from a complex mixture, but in general nonaffinity purification is more desirable. A method called recycle crossflow electrophoresis(RCFE) has been developed to isolate proteins based upon their electrophoretic mobilities. There is typically a 10-25 fold dilution affect associated with purifying proteins using RCFE, but combined with adsorption chromatography this has been negated. It has demonstrated the ability to isolate a recombinant protein from the major milk proteins as well as the ability to isolate broad active subpopulations from the inactive.

4.0 Introduction

For decades, electrophoretic techniques with high resolution have been used as analytical tools to separate proteins and protein isoforms based upon their mass and charge [1,2]. While electrophoresis does not have the resolution capacity of affinity chromatography, it can be used to potentially separate problematic contaminants and sub-populations. In addition, there is a need for high resolution, process scalable techniques which can provide barriers to pathogen transmission in pharmaceutical applications which provide separation physics that are orthogonal to current process steps [3]. Recently, a scaleable, continuous electrophoretic technique called Recycle Continuous-Flow Electrophoresis (RCFE) has been developed. The RCFE device uses convective flow through a high aspect ratio rectangular duct upon which an electrophoretic field is applied [4,5]. At large scale, the aspect ratio width to depth can be as great as 100:1 and a length to depth ratio of 100:1. These high aspect ratios are needed to dissipate heating by current flow up to 125 mAmps. Two convective streams are introduced to the chamber: the feed mixture and crossflow buffer which is recycled. The recycled buffer remains at the original conductivity so as to not promote heating due to an increase in resistance and thus buffer usage can be minimized. A membrane barrier prevents proteins from reaching either electrode and causing protein loss or fouling.

A crossflow velocity is necessary to render the anode directed fluxes of

selected species of the protein mixture to zero or less to achieve separation. Those species which have a net flux towards the anode are separated from those species which are washed out as cathode efflux due to weaker mobility. As a preliminary test of the RCFE resolution, a binary mixture consisting of coomassie blue dyed BSA and bovine azo-casein, as well as a binary mixture of coomassie blue dyed BSA and hemoglobin were separated. A resolution of >95% and a yield >90% were obtained for both at an aqueous feed concentration of 30g of protein per liter of each species at a feed flow rate of 20 ml per hour [4,5].

A bench scale RCFE device having a chamber gap of 2mm and that can process up to 1.5 g total protein per hour with an aqueous feed concentration of 60 g total protein per liter at a feed flow rate of 25 ml per hour. It is estimated to process 100 g total protein per hour at a feed flow rate of 1 liter per hour for a feed concentration of 100g total protein with a chamber gap of 8 cm. Fouling of the membrane due to concentration polarization has not been evaluated for complex mixtures. A major disadvantage of the initial RCFE prototype is the dilution of the products streams due to the introduction of crossflow buffer. As a result of dilution, application of the RCFE for high resolution of therapeutic proteins from complex mixtures such as human plasma or the milk of transgenic livestock has not been done.

Classical purification techniques such as precipitation and ion-exchange chromatography that are easily scaled to about 10^3 liters per day processed feed are normally used in the isolation of therapeutic proteins from recombinant sources. [6,7] However, these techniques typically have a limited capacity to resolve fully mature proteins which differ in several posttranslational modifications. For example, while Ca^{2+}-dependent elutions from DEAE-chromatography at large scale can elute fully carboxylated rhPC, it can not resolve other species such as single and two-chain forms at anything but analytical scale. For example, recombinant human protein C must undergo several posttranslational modifications to become a mature and biologically active protein. Some of these modifications include the formation of disulfide bonds, multiple cleavage of amino acids, N-linked glycosylation and conversion of the first 9 glutamic acids of the amino terminus to γ-carboxy glutamic acid (gla). In addition to the presence of 7 or more gla residues, the cleavage of both propeptide and dipeptide sequences to form heterodimeric rhPC is needed for biological activity [8]. Because even synthesis of rhPC by mammalian cells results in a diverse population of mature and immature rhPC species, purification of rhPC is difficult[9,10].

The mammary gland of transgenic animals has become a viable source for production of recombinant therapeutic proteins in milk with concentrations ranging from 0.5 to 10 g/l. As is true for products from large scale mammalian

cell culture, purification of the target therapeutic protein from the milk of transgenic animals can be made difficult not only due to the presence of endogenous milk proteins, but also due to inactive sub-populations of the target protein. In this study, we use the whey of transgenic pigs containing recombinant human protein C (rhPC) as a model mixture to test separability and yield of RCFE on rhPC sub-populations and milk proteins. A novel combination of RCFE and ion-exchange chromatography of the RCFE effluent is used to resolve these proteins without dilution.

4.1 Materials

4.1.1 Materials: The free flow electrophoresis apparatus was loaned by Washington State University and was designed and built by Dr. Cornelius Ivory and Dr. Bill Gobie. Human protein C from plasma was a gift from American Red Cross (Rockville, MD). Transgenic swine milk containing rhPC was collected at the VPI&SU Swine Center (Blacksburg VA). DEAE sepharose FF was purchased from Pharmacia Biotech (Uppsala, Sweden). Deionized water was produced by a Nanopure Barnstead water filtration system. Murine metal-dependent anti-hPC monoclonal antibody, 7D7B10-MAb, was purified from cell culture supernatant. The following items were purchased from Sigma Chemical Co.: tris-base, 0.45 µm 20x20 cm nitrocellulose membranes, low EEO agarose, sodium phosphate dibasic, sodium phosphate tribasic, silver nitrate, magnesium chloride, calcium chloride, zinc chloride, sodium hydroxide, ammonium hydroxide, EDTA, rabbit antiserum against protein C, anti-goat immunoglobulins conjugated with horseradish peroxidase, protein C depleted plasma, normal pooled reference plasma and citric acid. Polyacrylamide:bisacrylamide(29:1), Immulon II plates, acetic acid, HCl, Accumet 25 pH meter, Marathon 21KR centrifuge, BRL Horizon^{TM} 11x14 cm horizontal electrophoresis apparatus, refrigeration unit and the FB600 power supply were purchased from Fisher Scientific. Goat antiserum to human protein C and protac were purchased from American Diagnostica. PTT Automat 5 was purchased form American Bioproducts. DynaPro 801 TC light scattering device with AutoPro and Dynamic

software(Protein Solution, Charlottesville VA). O-phenylenediamine-2HCl (OPD) kits were purchased from Abott Labatories. Gluteraldehyde, methanol and tris-HCl were purchased form Scientific Products. Blotting paper and Biorad Electrophoresis apparatus were purchased from Biorad. Oxytocin was purchased form Vedco Inc.. The Immulon II plates were read at 490nm using an EL308 Bio-Tek Microplate reader. The absorbance of the protein solution was read at 280nm on a Milton Roy Spectronic spectrometer. A Masterflex peristaltic pump was purchased from Cole-Parmer.

4.2 Methods

4.2.1 Milk Collection and Preparation: Milk from transgenic swine was collected and prepared by the method described in Subramanian et. al. The milk collected was treated with EDTA to chelate Ca^{2+} and to clarify the pig whey.

4.2.2 Mobility of Major Milk Protein and rhPC: Electrophoretic mobility was determined for α- and β-pcasein (purified by free flow electrophoresis) and rhPC (purified by immuno-affinity chromatography). A 1% agarose (low EEO) solution was prepared with 5mM sodium phosphate pH 5.0 and heated to 100^{o}C to dissolve the agarose, and poured onto a glass plate until a thickness of 3mm was obtained. Approximately 10-15 µg of total protein with 60% glycerol was loaded into each lane with the last two lanes being filled with bromophenol blue at 1 mg/ml as a tracking dye. The proteins were horizontally electrophoresed in 5mM sodium phosphate, pH 7.2, at 100V until the tracking dye was 1 cm from the end. The gel was fixed in an aqueous solution of 20% trichloro-acetic acid for 20 minutes, preserved by drying with forced hot air and then stained with 0.125% w/v coomassie brilliant blue R-250, destained with methanol/acetic acid/deionized water(40:10:50) and redried. The experiment was repeated over the pH range of 5.0 to 8.5 in 0.5 increments

4.2.3 Steady State Operation of RCFE: Pig whey was thawed at 4^{o}C for 5 hours, then 5 mM tris-base added and adjusted to pH 8.5. The apparatus and

electrolyte chamber (See Figure 1) were filled with 5mM tris-base pH 8.5. All of the air entrapped within the apparatus during filling was removed by venting to the atmosphere. The recycle flow, crossflow and feed flow rates and voltage were at 3.5 ml/min, 4.8-5.2 ml/min, 0.2 ml/min and 1000V respectively. Samples were initially taken 1 hour after startup and every 30 minutes for 12-15 hours. After 8-10 hours, the feed was turned off, but the electrophoresis was continued for an additional 4-5 hours to reduce any losses. Any air collected during the run was removed by venting to atmosphere. The buffer was recycled after 1 hour to limit the amount of buffer required. To ensure that the buffer contained no contaminants, the anion exchange column effluents at both the anode and cathode were measured for protein content by absorbance at 280 nm and by PAGE. The anion exchange column buffer effluents from both the anode and cathode were pooled and the pH and conductivity checked. There was no need to re-pH the pooled buffer as the pH remained at the operating pH. The conductivity did rise by less than 1% of the starting conductivity of 125 mMho. The samples collected every hour were measured for protein by absorbance at 280nm, ELISA and PAGE. The anode product was re-electrophoresed at pH 7.0.

4.2.4 Purification of rhPC by immuno-affinity chromatography: Both the anode and cathode products containing rhPC were purified by immuno-affinity chromatography using 12A8 an monoclonal anti-hPC antibody that is attached to

Emphaze™ chromatographic matrix.[11]

4.2.5 ELISA: The method used to determine the level of rhPC in the milk of transgenic swine and in the collected samples is described in Velander et. al. This is a sandwich ELISA using rabbit anti-human protein C as the capture antibody and using goat anti-human protein C as the detecting antibody followed by rabbit anti-goat horseradish peroxidase conjugate.

4.2.6 Conformational ELISA: To determine if the rhPC from the cathode and anode products were fully γ-carboxylated we used the method of Subramanian et.al[12]. A monoclonal antibody from mouse, 7D7-B10 (5µg/ml) was bound to Immulon II plate and 100µl of samples were added with increasing levels of $CaCl_2$ over the range of 0-100mM. Rabbit anti-human protein C was used as the detecting antibody, followed by goat anti-rabbit horseradish peroxidase. In the presence of calcium, the monoclonal antibody will release the partially to fully γ-carboxylated rhPC.

4.2.7 PAGE and Western: The 12% SDS-PAGE and western/immuno-blot were used to detect total protein and rhPC by the method described in Velander et. al[13]. The gels were stained with silver for greater sensitivity and the westerns were stained using two antibodies. The first antibody for the western, rabbit anti-human protein C was used as the capturing antibody. The second antibody,

goat anti-rabbit horseradish peroxidase was used as the detecting antibody.

4.2.8 Activated Partial Thromboplastin Time(APTT) of rhPC: The rhPC in the cathode and anode products were added to hPC depleted plasma to determine if the clotting response time was delayed and is described by Morcol et. al [14] Normal plasma reference pool with 4 µg/ml of hPC was used as the standard. Protac and Ca^{2+} were added to both the standard and the samples, then the time for a clot to appear was measured.

4.2.9 Dynamic light scattering: rhPC single chain, two chain and broad population was analyzed by dynamic light scattering using a Protein Solution DynaPro DL801 device. The rhPC samples were diluted to the linear range of the light scattering device(1 mg/ml) and 250µl of sample injected through a 0.1 µm syringe filter. A minimum of 10 hydrodynamic radius measurements were recorded over 1 hour from a single injection.

4.3 Results

The electrophoretic mobility of pure major milk proteins, α and β-pcasein and of pure rhPC in 1% w/v agarose gels under native conditions over the pH range of 5 to 8.5 in increments of 0.5 pH are shown in figure 2A. The pH-dependent mobilities of the proteins increased linearly with a slope of 0.19 for rhPC, 0.13 for α-pcasein and 0.10 for β-pcasein measured to the center of the coomassie dyed blue spots as the pH increased from 5.0 to 8.5 in increments of 0.5 pH. Both β-pcasein and rhPC showed similar mobilities in the pH range 5.0-6.0 as a narrower band relative to the much broader band obtained for rhPC at pH 8.5. In contrast, the mobility of at least 50% of the rhPC population was almost 2-fold greater that of β-pcasein in the pH range 6.0-8.5. The hydrodynamic radius measured by dynamic light scattering was measured as a function of pH for α and β-pig casein and shows that as the pH is decreased from 8 to 6, the hydrodynamic radius increases by 20 and 10 for α and β-pig casein respectively (Figure 2B). The proteins (α and β-pig casein) were assumed to be symmetrical and solid spheres for the purposes of estimating surface area and fluid drag. The surface area and fluid drag ("Stoke's Law") are shown in Figures 2C and 2D respectively and as the solution pH is decreased from 8 to 6, the surface area and fluid drag of α-pig casein increases by 20% and β-pig casein increases by 10 %. The charge to mass ratio for α and β-pig casein was calculated and shows that there is 10% difference in the ratios at the highest pH and a 15% change at the lowest (Figure 2E). Finally, the relative mobilities of

α-pig casein compared to β-pig casein shows that the electrophoretic mobility of α-pig casein is higher than β-pig casein for both theoretical and for experimental values (Figure 2F).

A sequence of two steady state RCFE operations were done, where the first processed crude pig whey at pH 8.5 with an optical density at 280nm of 22. Steady state operation of the RCFE was achieved for both total protein and rhPC concentration in the product streams by 4 hours and was maintained for an additional 6 or more hours. No fouling of the 10k molecular weight cutoff permeable membrane at either electrode was observed. Figure 3A and 3C show OD at 280nm and rhPC concentration measured by ELISA of RCFE anode and cathode product streams from pig whey feed at pH 8.5. About 50% of the total OD 280nm and approximately 50% of the rhPC emerged as cathode effluent and was bound and eluted from the DEAE-Sepharose ion-exchange column. A total of 20 mg total protein per ml of DEAE-sepharose was loaded over 14 hours without breakthrough. This material was eluted using a 1 M NaCl buffer

The pH 8.5 anode-ion exchange effluent was then electrophoresed after dialysis to a pH of 7.0 and a conductivity of 125 mMho. Figure 3B and 3D show absorbance at 280nm and rhPC concentration measured by ELISA of anode and cathode RCFE product streams of the pH 8.5 anode product stream which was re-electrophoresed at pH 7.0. About 40% of the total OD 280nm, but about

100% of the re-electrophoresed rhPC emerged from the cathode product stream was captured by the DEAE-sepharose ion exchange column. An average yield of about 50% and a purification factor of about 4-fold of the rhPC was obtained for the twice electrophoresed cathode-ion exchange effluents(pH 8.5 then pH 7.0).

Silver stained 12% SDS-PAGE from RCFE-anion exchange processing was used to evaluate for general protein content and western blot analysis was used to evaluate rhPC content in products. Figure 4A shows silver stained proteins resolved by SDS-PAGE from the cathode (lanes 8 through 12 are samples from hours 2, 4, 6, 8 and 10 respectively) and anode (lanes 3 through 7 are samples from hours2, 4, 6, 8 and 10 respectively) products of RCFE processed whey at pH 8.5. Approximately 50% of the total stainable protein and 100% of the α-pcasein in the anode product stream and 100% of the β-pcasein appears in the cathode product stream. Steady state protein composition was achieved for the both the anode and cathode sides after 2 hours lanes 3 through 7 and lanes 8 through 12 respectively. The stainable protein of the anode (lanes 3 through 7 are samples from hours 2, 4, 6, 8 and 10 respectively) and cathode (lanes 8 through 12 are samples from hours 2, 4, 6, 8 and 10 respectively) products from RCFE processed at pH 7.0 are shown in Figure 4B. Steady state protein composition was achieved for the both the anode and cathode sides after 2 hours lanes 3 through 7 and lanes 8 through 12 respectively. The anode

contained 60-70% of the total stainable protein and 100% of the α-pcasein. Figure 5, the western blot shows the amount of stained rhPC species that emerged from both the anode (lanes 2 through 6 are samples from hours 2, 4, 6, 8 and 10 respectively) and cathode (lanes 7 through 11 are samples from hours 2, 4, 6, 8 and 10 respectively) of the RCFE operated at pH 8.5. Steady state protein composition was achieved for the both the anode and cathode sides after 2 hours lanes 2 through 6 and lanes 7 through 11 respectively. Approximately 50% of the rhPC is in the cathode product stream (Figure 5, lanes 7 through 11), about 80% of the two-chain, heterodimeric rhPC is in the anode (figure 5, lanes 2 through 6) product stream, and about 80% of the single chain rhPC was in the cathode (figure 5, lanes 7 through 11) product stream of the RCFE done at pH 8.5. Steady state composition of rhPC was achieved in hours 2 through 10 lanes 2 through 6. The amount of single chain present in the anode product stream accounted for 10% of the total rhPC (figure 5, lanes 2 through 6), but the single chain present in the cathode product stream accounted for 40-50% of the total rhPC. Protein C from human plasma normally contains 5% single chain (data not shown).

A Ca^{2+}-dependent rhPC conformational ELISA was done to determine the extent of γ-carboxylation of rhPC appearing in the cathode and anode-ion exchange effluents. The rhPC populations from both effluents were purified by MAb-affinity chromatography to > 90% yield and >90% purity as determined by

ELISA and by silver stained 12% SDS-PAGE respectively prior to analysis by conformational ELISA. The affinity purified rhPC recovered from the cathode effluent gave no half-maximal inhibition by Ca^{2+} which is similar to reference plasma protein C that has been de-carboxylated [15] However, the rhPC that emerged from the anode effluent gave a half-maximal inhibition of 3mM $CaCl_2$ which was similar to biologically active reference human plasma protein C. (See Figure 6).

The activity, delay in clotting, of MAb-affinity purified rhPC was measured by APTT and is shown in Table I. The affinity purified rhPC obtained from the cathode product stream showed no delay in clotting. However, the affinity purified rhPC obtained from the anode product stream gave a delay in clotting time that was 75% of the reference human plasma protein C corresponding to a specific activity of 190 U/mg. The specific activity of plasma derived protein C is defined as 250 U/mg.

Dynamic light scattering was used to estimate the hydrodynamic radius of single chain, two chain and broad population of rhPC. Table II shows the estimated sizes of single chain, two chain and broad population of rhPC for 99% pure rhPC. All of the rhPC sub-populations are assumed to be spherical and the estimated molecular weights are also presented in Table II. The hydrodynamic radius for single-chain, two-chain and broad population were about the same

value of 4.2 nm with an estimated molecular weight of 65 kDa. The distribution was found to be monomodal.

4.4 Discussion

The purification of recombinant proteins from endogenous milk proteins can be manipulated from the perspective of relative population heterogeneity. The heterogeneity of some milk protein populations is likely to be less broad than rhPC due to a lower degree of complex post-translational modifications. Proteolytic degradation can increase the heterogeneity of all species as milk contains a broad spectrum of proteins[16]. Indeed, both α(α_{s1} and α_{s2})and β-pig casein appeared as relatively narrow populations in native agarose gel electrophoresis (Appendix B) while rhPC had a more broad population with very broad mobilities, all with differing mobilities over the pH range of 5 to 8.5. Based upon these differences, we have used pH to selectively alter the relative electrophoretic mobilities of α-pcasein, β-pcasein, inactive and active populations of rhPC in RCFE. For example, a flux of the active rhPC populations and the α-pcasein towards the anode of the RCFE was achieved at pH 8.5. Conversely, the majority of the inactive rhPC population and β-pcasein were washed out as cathode effluents.

The pH dependant ratios of charge to mass and charge to effective molecular radius ratio are coupled and these physiochemical properties influence electrophoretic mobility and hence resolution. The differences in relative mobility estimated by the method of Compton [17] between rhPC, α_{s1} and α_{s2} relative to β-pcasein were in agreement with our experimental observations

using free flow and native gel electrophoresis. In general, both theoretical predictions and experimental measurements show that greater than 15% differences in relative mobility will exist between rhPC, α_{s1} and α_{s2} relative to β-pcasein at a pH higher than about 6. This constitutive phenomena can be related through the balance of fluid drag and electromotive forces exerted by the electric field. For example, an increase in relative charge to mass ratio between species would increase the relative electrophoretic mobility at constant fluid drag. However, while the charge to mass ratio has a first order effect on mobility, the ratio of charge to radius more directly characterizes changes in mobility due to fluid drag. While most proteins have an asymmetric tertiary structure, a solid spherical model can be assumed to help estimate the fluid drag effects. For example, “Stoke’s Law” estimates the force to be directly proportional to particle diameter and viscosity for a spherical model. In summary, at equal charge to mass ratio the species having a greater overall surface area would have a lesser mobility due to higher fluid drag.

While milk proteins such as α(α_{s1} and α_{s2}) and β-pcasein with very similar molecular weights and charge to mass ratio, the have greatly different mobilities. While these proteins are similar, the molecular weights of α_{s1}, α_{s2} and β-pcasein are 27kDa, 29.7kDa and 24.9kDa, respectively. The charge to mass ratio of α_{s1}, α_{s2} and β-pcasein at a pH of 7 are -1.5, -1.5 and -1.3, respectively. Indeed, α_{s1}, α_{s2} and β-pcasein can e separated by RCFE with

high yield > 90%. The different mobilities could be due to the differences in hydrodynamic radius stemming from conformations unrelated to charge such as internal hydrophobic interaction. Reasons for the difference is conformational and therefore size can be give by DNA sequence and Hopp-Woods analysis. For example, there are about 10% more negatively charged amino acids, 5% more turns and more hydrophobic domains in α-pcasein as compared to β-pcasein. There are 92, 95 and 76 charged amino acids for αs1-pig casein, αs2-pig casein and β-pig casein, respectively. In addition, the casein's are post-translationally phosphorylated to contain 8, 17 and 8 for αs1-pig casein, αs2-pig casein and β-pig casein respectively [18-20]. The differences in relative mobility can also be qualitatively estimated by the method of Compton(Appendix C) and these differences between α_{s1}, α_{s2} and β-pcasein were in agreement with our experimental observations using free flow and native gel electrophoresis[17].

The most active sub-populations of rhPC can be resolved from the inactive in an electric field at about pH 5 or above because there are extensive post-translational modifications that introduce ionic residues in native hPC. For example, in post-translationally mature, biologically active protein C, there are 9 glas present on the first 30 amino acids of the light chain. At pH 5.5 or higher, the net charge of protein C becomes more negative due to the presence of glas, sialic acids and other ionic amino acids such as asparagine and glutamic acid[21]. The pH dependent conformational changes in rhPC that are gla-

dependent have been observed using calorimetry[22]. As a result, the highest biological activity occurs in the rhPC sub-population that is fully carboxylated and these sub-species have a higher electrophoretic mobility than incompletely carboxylated rhPC. Furthermore, some rhPC sub-populations have a common amino acid sequence but will have differences in the number of N-linked glycosylated sites and also the glycosylation structure within each site. In general, properly mature N-linked carbohydrates will have a higher sialic acid content [23-24]. We have found that the most active rhPC species having the highest sialic acid also possessed a higher electrophoretic mobility towards the anode.

The formation of heterodimer is another post-translational processing step which affects both the biological activity and conformation of rhPC, therefore the hydrodynamic radius. Heterodimer results from the proteolytic removal of a dipeptide to form 2-chain hPC. However, the hydrodynamic radius of 70% single chain and 20% two-chain rhPC were both 4.3nm from a monomodal analysis. The difference in electrophoretic separation are likely due to charge to mass ratio from differences in sialic acid and gla content.

The resolution power of RCFE for applications to complex mixtures was demonstrated by our results. However, typical RCFE product streams have been observed to result in dilutions of 10 to 25 times the starting feed

concentration. We have demonstrated that combining adsorption chromatography with RCFE can effectively concentrate RCFE effluent. In our example, the feed dilution effect of RCFE was negated by using anion-exchange adsorption chromatography to capture rhPC from the product streams of RCFE. About 95% of the proteins from both the anode and cathode were continuously captured and then eluted in a single column volume upon completion of the electrophoretic process. In addition, the buffer effluent for the anion exchange maintained a steady state conductivity of 125mMho and a pH of 7 so that buffer usage was minimized due to recycle.

In summary, a combined RCFE-adsorption chromatography is a facile and efficient process step for high resolution purification from a complex mixture. This was demonstrated by the resolution of the active sub-populations of proteins from the inactive in the milk of transgenic animals.

4.5 Reference List

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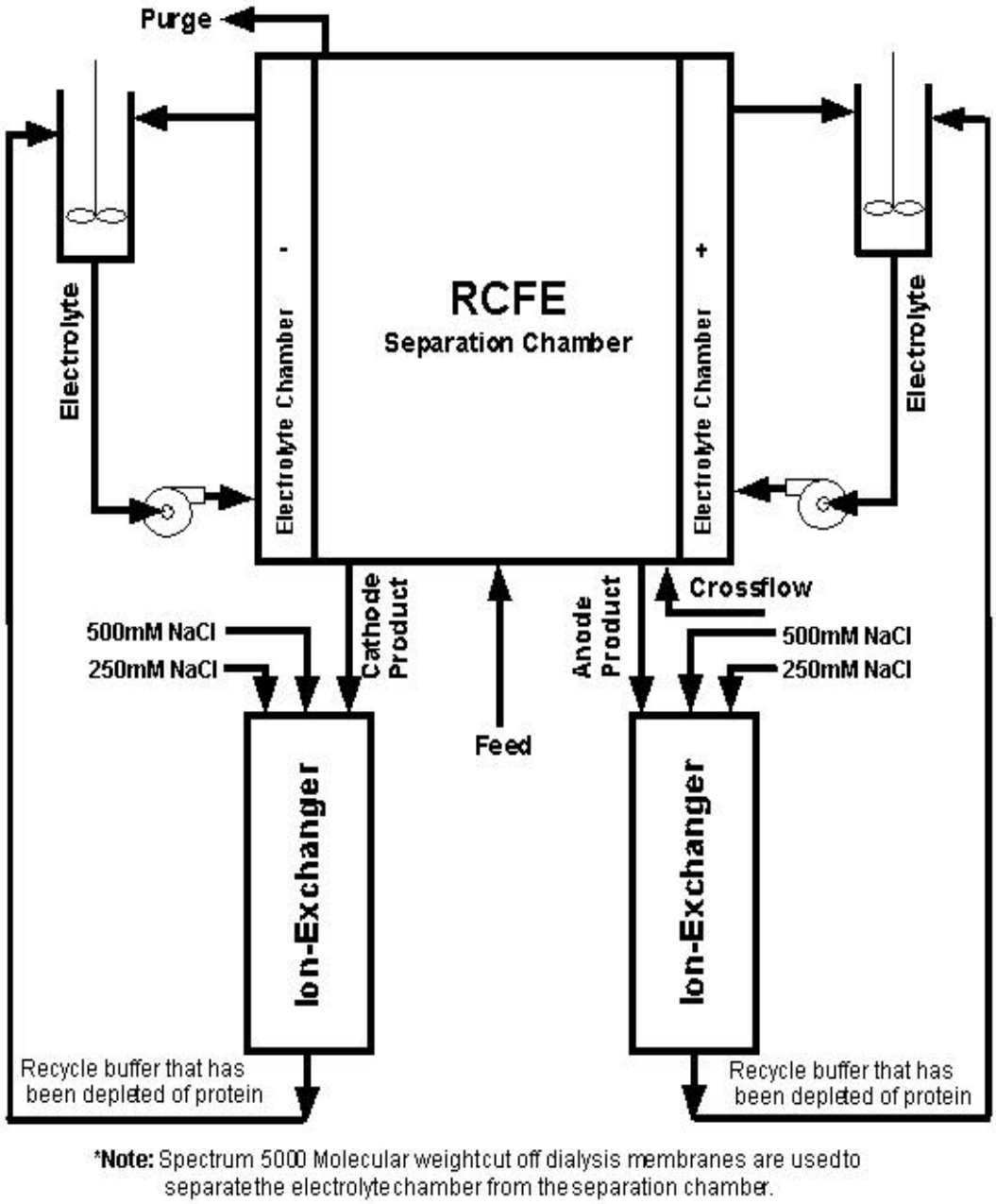

Figure 1: Diagram of the recycle crossflow electrophoresis apparatus

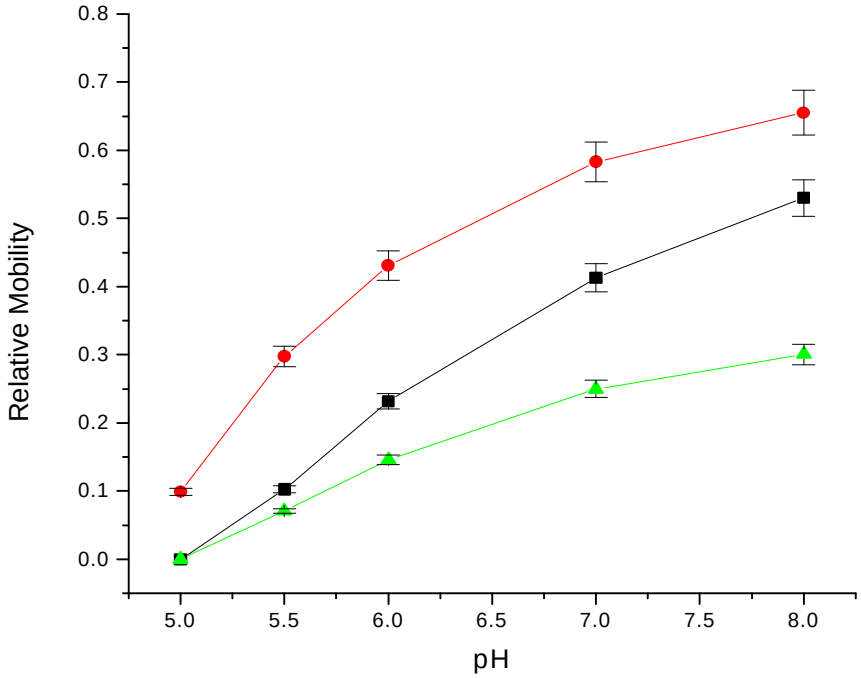

Figure 2A: The effect of pH(5.0 to 8.5) on the mobility of major milk proteins, α and β-pcasein and rhPC.(■-rhPC, ●-α pig casein, ▲-β pig casein). The mobility was determined by measuring the distance a protein moved relative to the dye front which was 1cm from the end of the gel in low EEO 1% agarose with 10 mM phosphoric acid ranged from 5-8.5 at 100V.

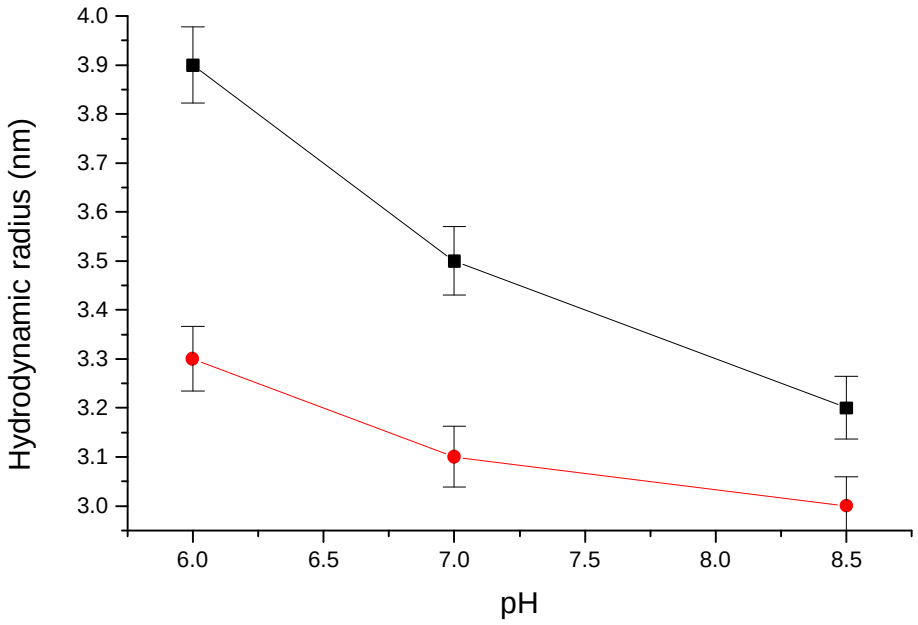

Figure 2B: The effect of pH(5.0 to 8.5) on the hydrodynamic radius of major milk proteins, α and β-pcasein.(■-α pig casein, ●-β pig casein). A 250µl sample was injected through a 0.2µm filter and 10 measurements were recorded for a single injection. This was repeated 3 times with a monomodal size distribution used each time for analysis.

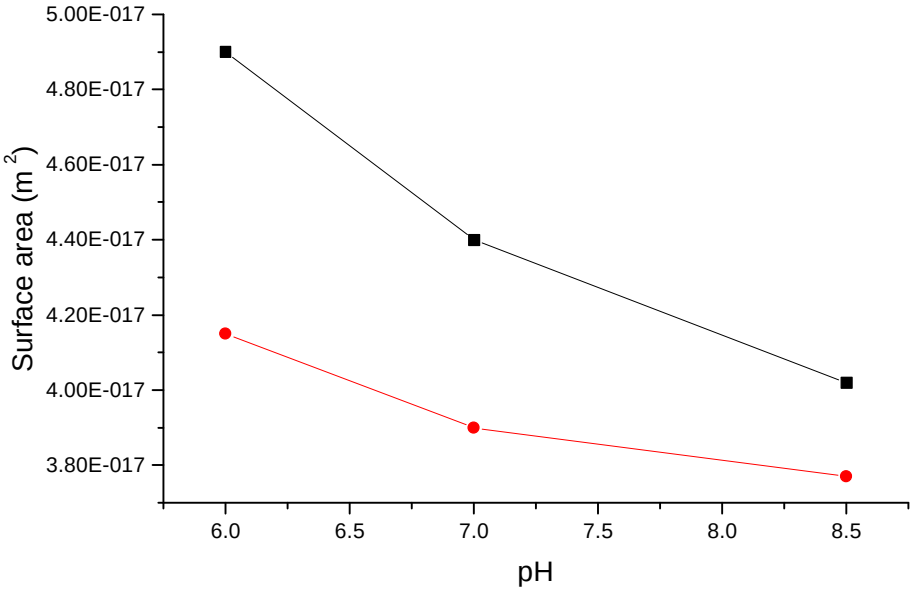

Figure 2C: The effect of pH(5.0 to 8.5) on the surface area of major milk proteins, α and β-pcasein rhPC.(■-α pig casein, ●-β pig casein). The proteins were assumed to be spherical and from the hydrodynamic radius in *Figure 2B*, a surface area was calculated.

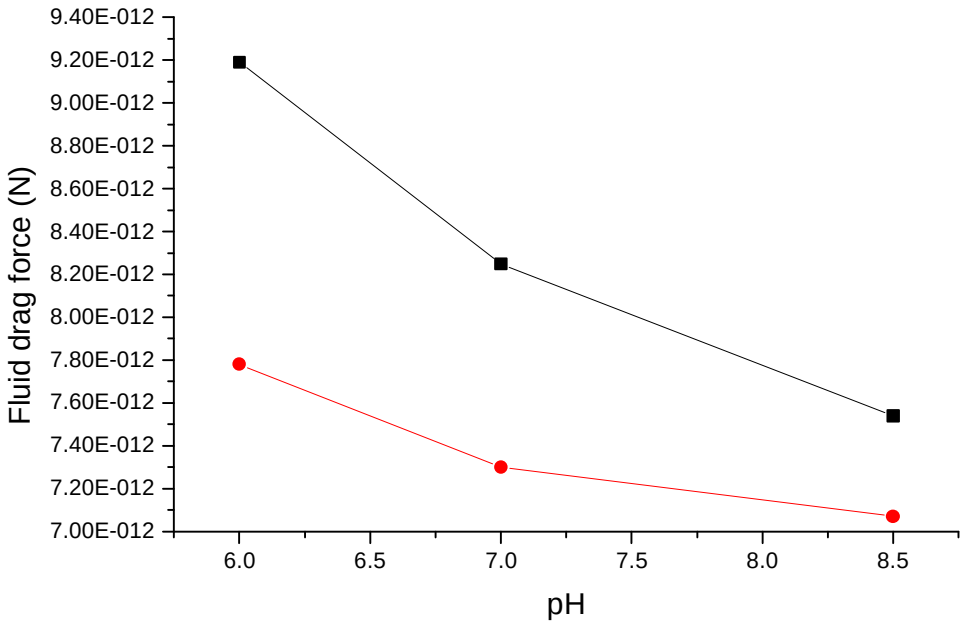

Figure 2D: The effect of pH(5.0 to 8.5) on the fluid drag(Stokes Law) of milk proteins, α and β-pcasein.(■-α pig casein, ●-β pig casein). The proteins were assumed to be spherical and from the hydrodynamic radius in *Figure 2B*, a fluid drag was calculated.

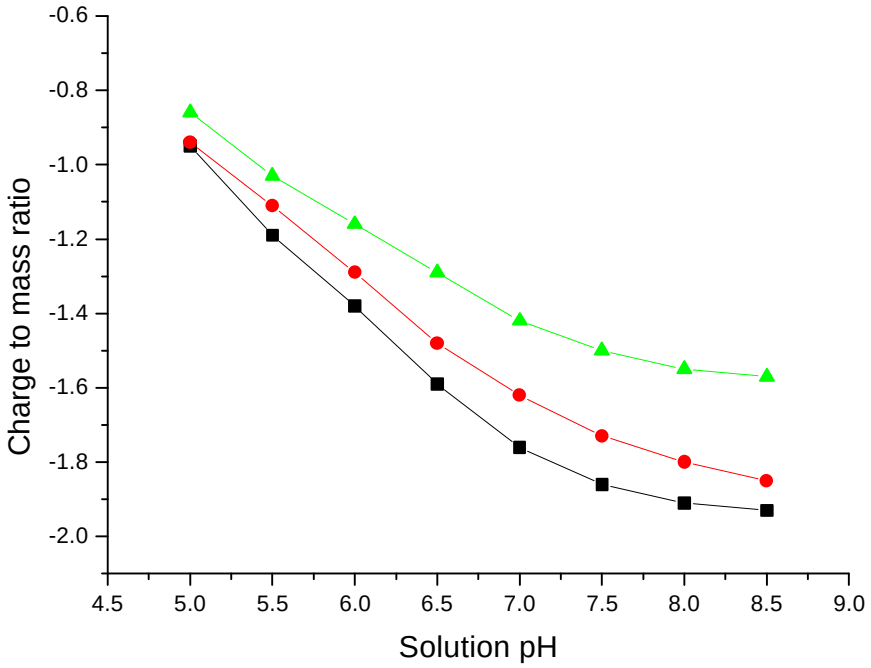

Figure 2E: The effect of pH(5.0 to 8.5) on the charge to mass ratio of milk proteins, α and β-pcasein.(■-α_{s1} pig casein, ●-α_{s2} pig casein and ▲-β pig casein). See Appendix C for charge calculations.

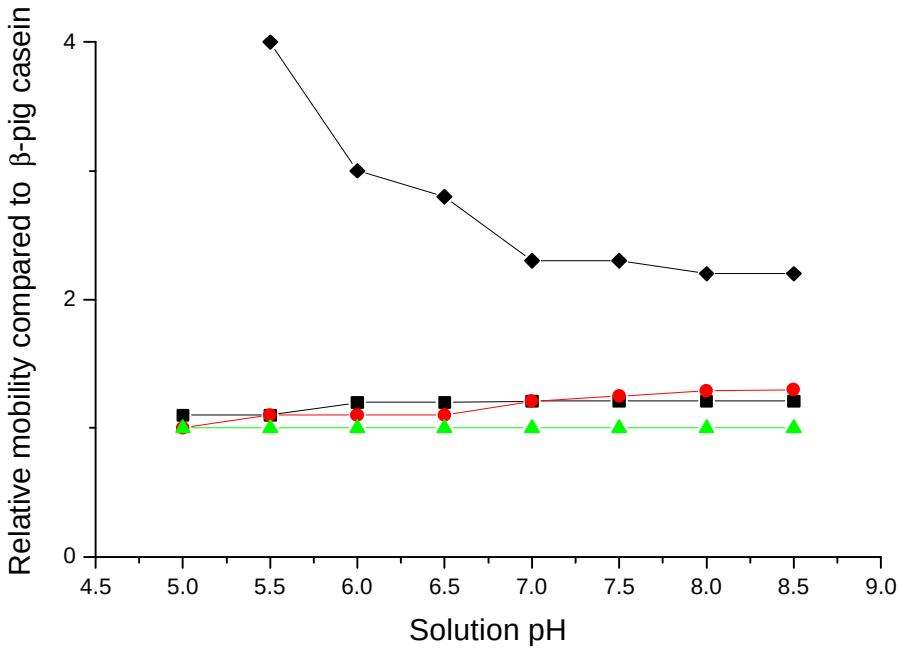

Figure 2F: The effect of pH(5.0 to 8.5) on the relative mobility of milk proteins(Comptons Approach), α and β-pcasein. (■-α_{s1} pig casein, ●-α_{s2} pig casein, ▲-β pig casein and ◆-experimental relative mobility of α-pig casein's). See Appendix C for charge and mobility caculations.

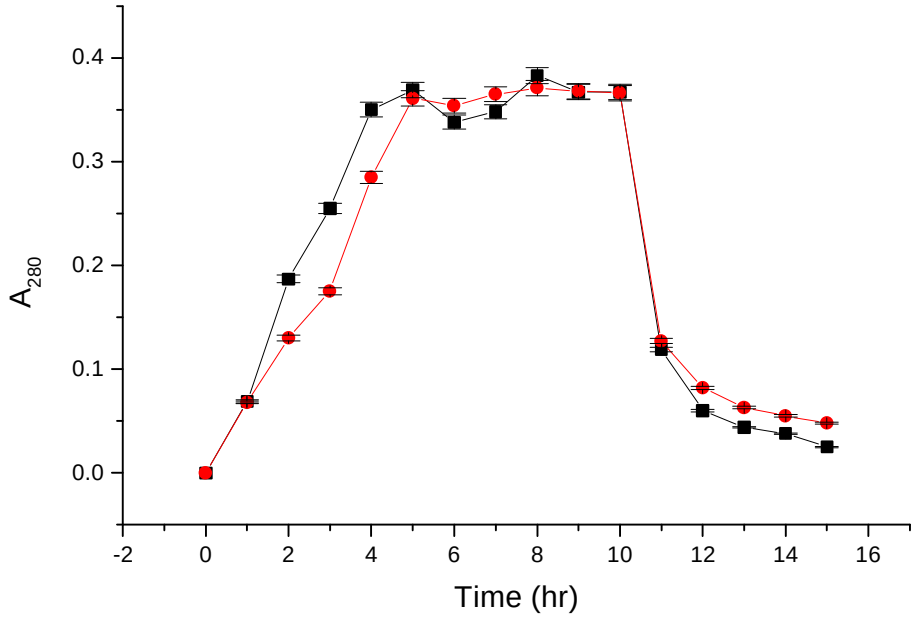

Figure 3A:. The absorbance at 280nm of both the anode and cathode products at pH 8.5. (■-anode products ●-cathode products). Dialyzed pig milk was loaded at 12ml per hour using 1000V and a crossflow of 5.0ml per minute. Steady state operation was maintained for 8 hours.

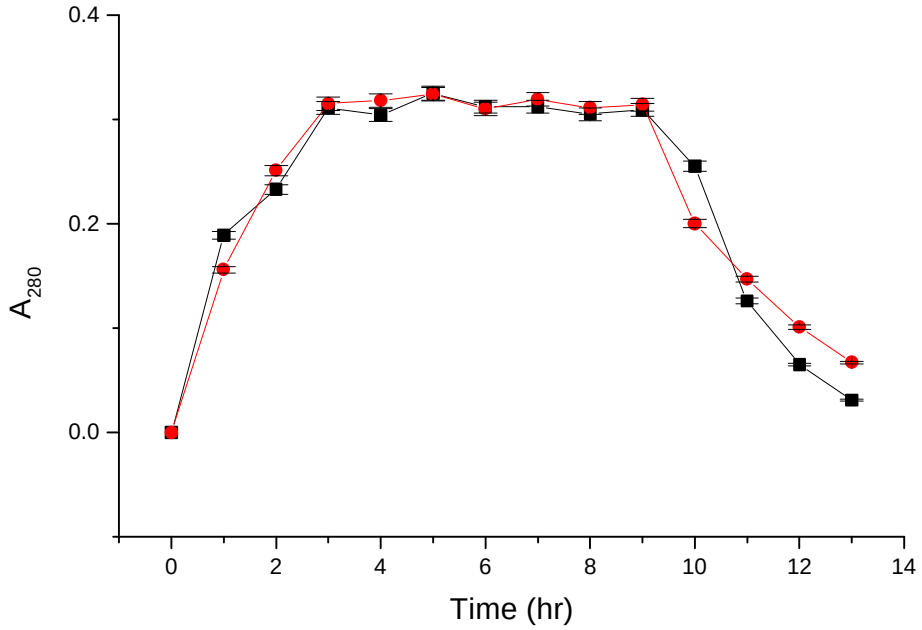

Figure 3B: The absorbance at 280 nm of both the anode and cathode products at pH 7.0. (■-anode products ●-cathode products). The pH8.5 anode fraction was loaded at 12ml per hour using 1000V and a crossflow of 5.0ml per minute. Steady state operation was maintained for 6 hours.

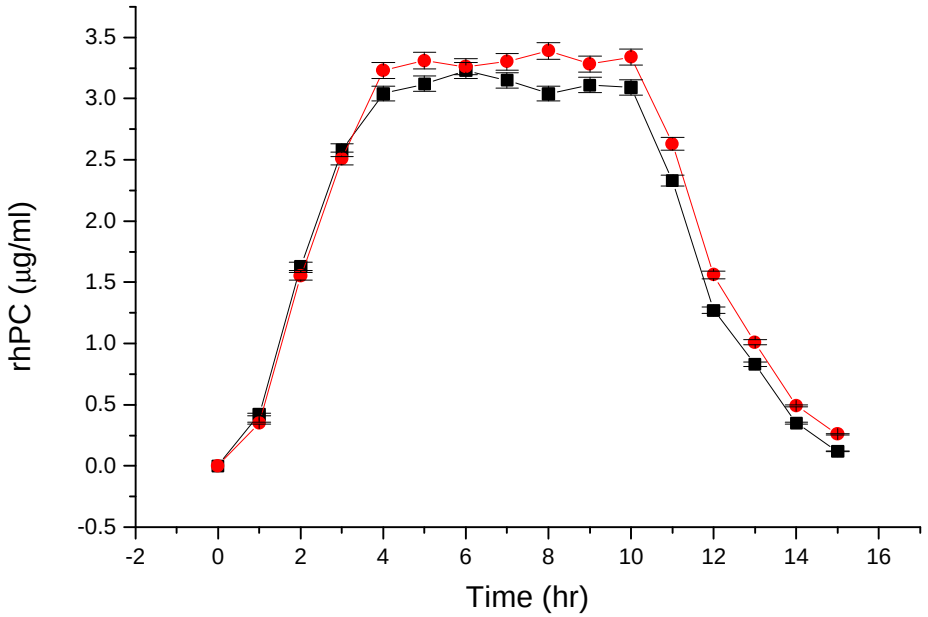

Figure 3C: The rhPC level(µg/ml) of both the anode and cathode products at pH 8.5. (■-anode products ●-cathode products). The rhPC levels were measured by ELISA.

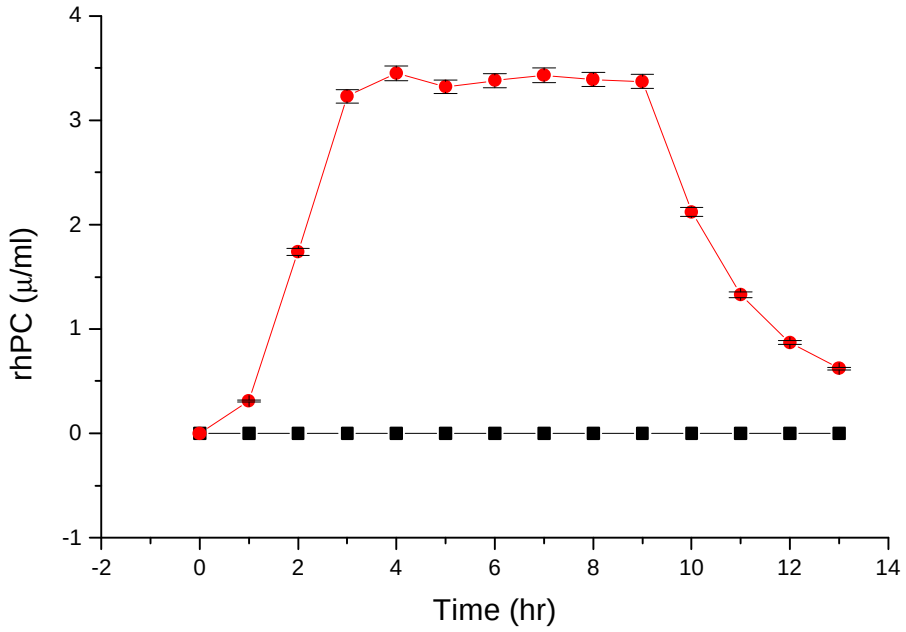

Figure 3D: The rhPC level (µg/ml) of both the anode and cathode products at pH 7.0. (■-anode products ●-cathode products). The rhPC levels were measured by ELISA.

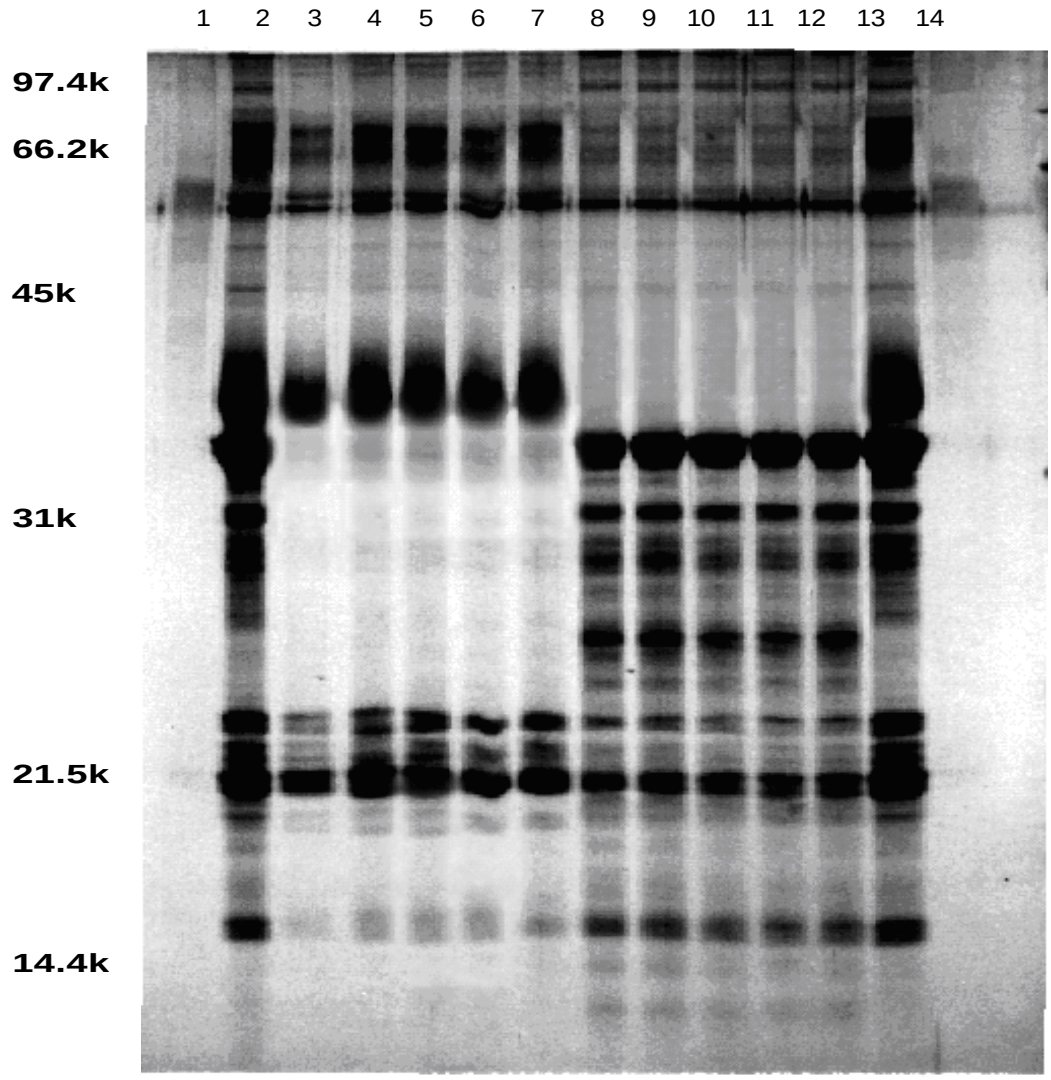

Figure 4A: A 12% silver stained SDS-PAGE of the RCFE operated at pH 8.5 with 8 µg of total protein added to each lane except for lanes 1 and 14 which have 1 µg of total protein. Lanes 1 and 14 are rhPC purified by immuno-affinity chromatography. Lanes 2 and 13 are starting material for the RCFE. Lanes 3-7 are the anode products at 2, 4, 6, 8 and 10 hours respectively. Lanes 8-12 are the cathode products at 2, 4, 6, 8 and 10 hours respectively.

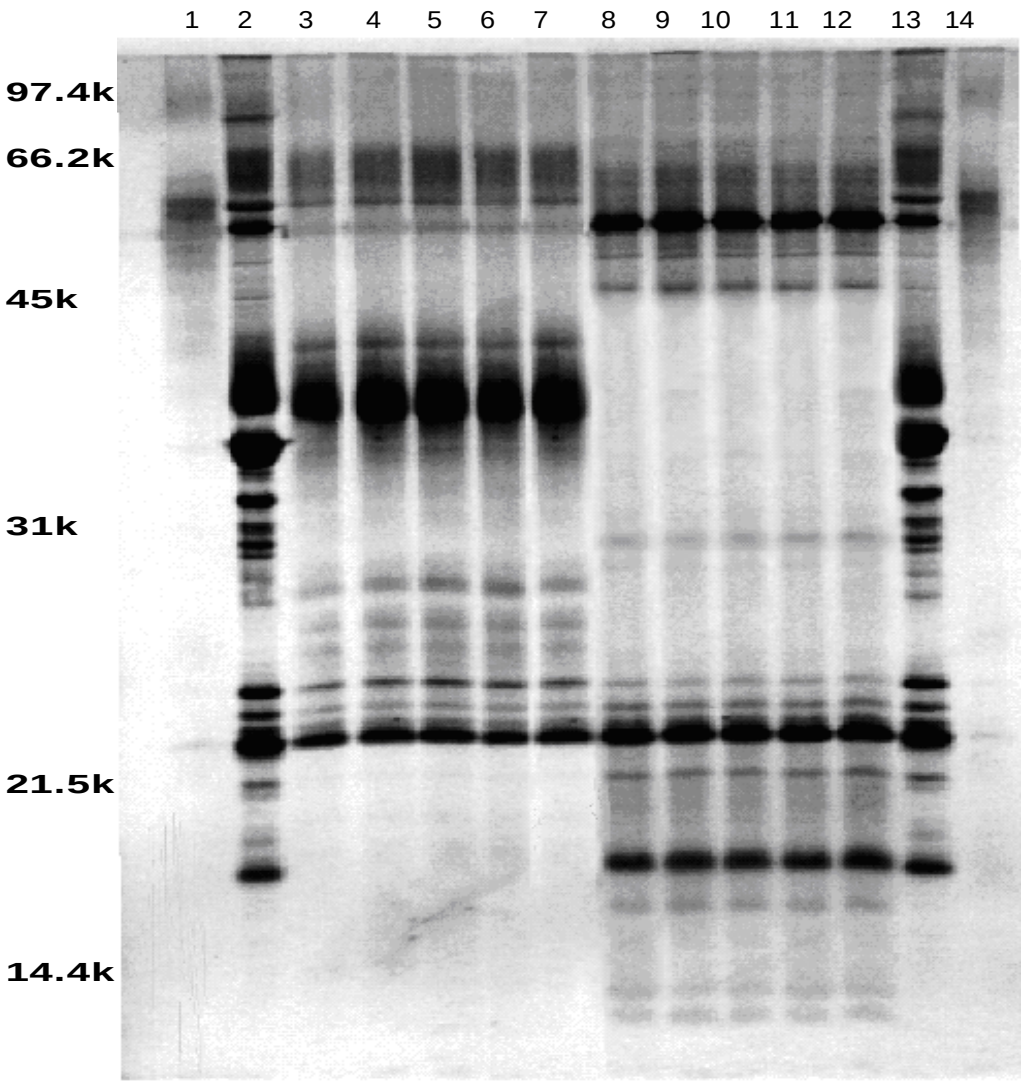

Figure 4B: A 12% silver stained SDS-PAGE of the RCFE operated at pH 7.0 with 6 µg of total protein added to each lane except for lanes 1 and 14 which have 1 µg of total protein. Lanes 1 and 14 are rhPC purified by immuno-affinity chromatography. Lanes 2 and 13 are starting material for the RCFE. Lanes 3-7 are the anode products at 2, 4, 6, 8 and 10 hours respectively. Lanes 8-12 are the cathode products at 2, 4, 6, 8 and 10 hours respectively.

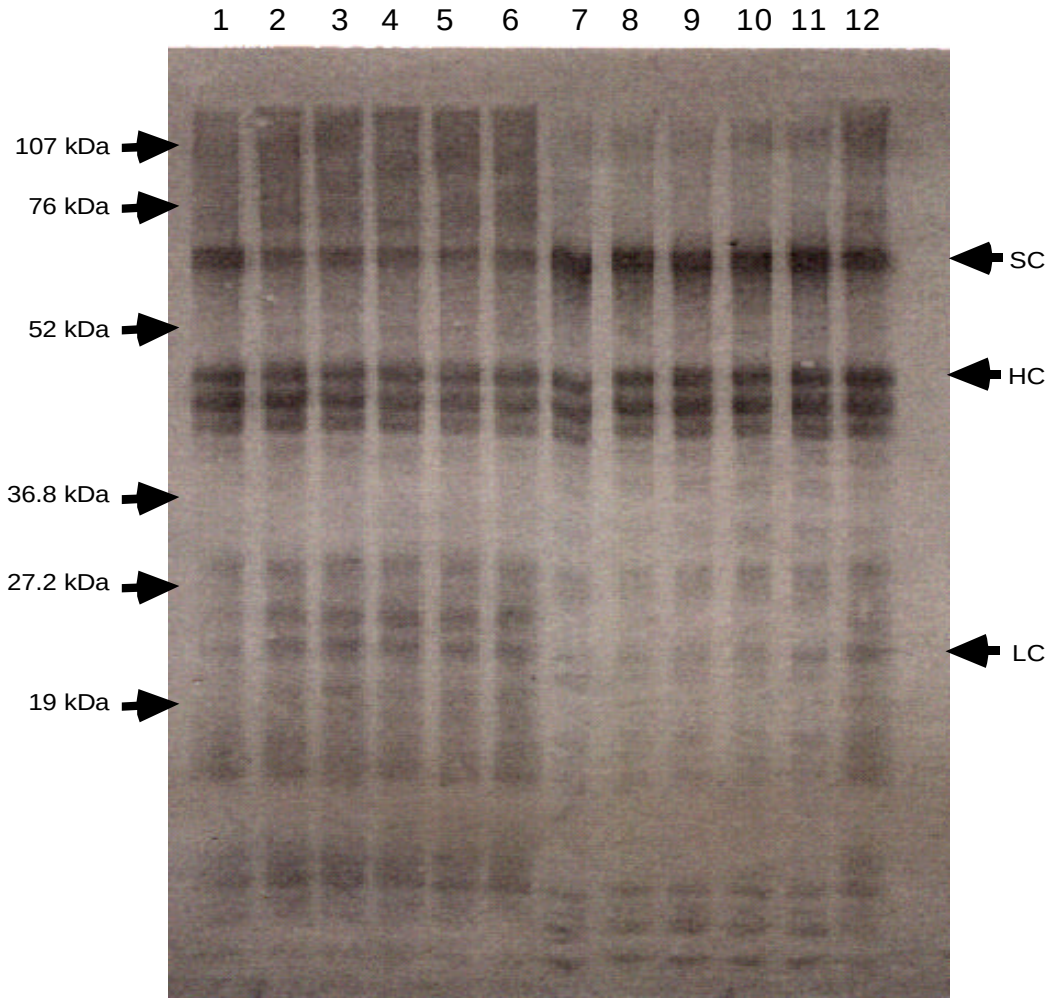

Figure 5: A western of rhPC for both the anode and cathode products from the RCFE operated at pH 8.5. There is a total of 1 µg of rhPC applied to each lane. Lanes 1 and 12 are the starting material for the RCFE. Lanes 2-6 are the anode products at 2, 4, 6, 8 and 10 hours respectively. Lanes 7-11 are the cathode products at 2, 4, 6, 8 and 10 hours respectively.

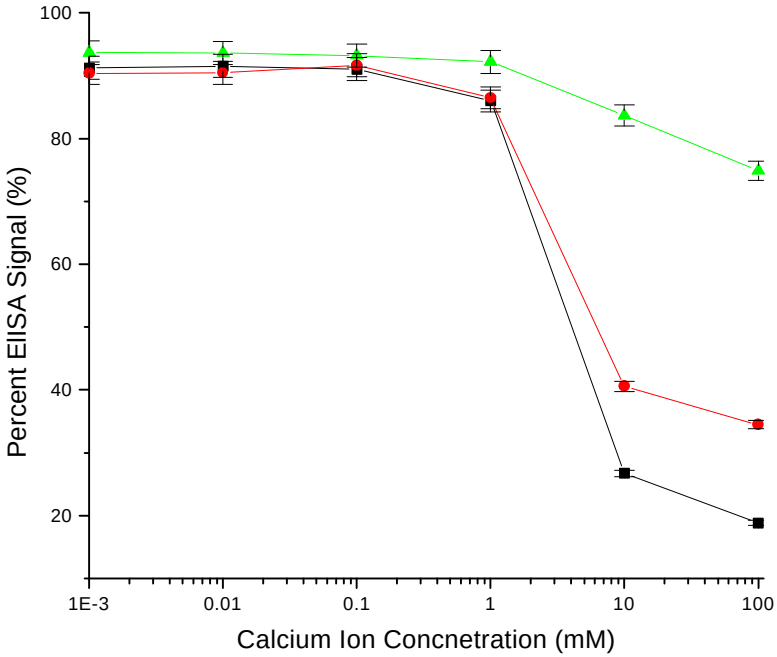

Figure 6: Conformational ELISA of rhPC of the anode and cathode products separated by RCFE at pH 8.5, but purified by immuno-affinity chromatography. (■-human plasma ●-rhPC that migrated towards the anode ▲-rhPC that migrated towards the cathode)

Table I: Purification, yield and activity of rhPC that migrated towards the anode and cathode at pH 8.5.

Purification of rhPC at pH 8.5 by RCFE						
Samples	Total Protein (%)	Percent rhPC Yield (%)	Dilution of Start	Purification Factor	Purity (%)	Activity by APTT (%hPC ref)
Start	100 ± 9	100 ± 9	NA	1	NA	NA
Cathode product stream	49.6 ± 3	55 ± 4	NA	1	NA	NA
Anode product stream	47.3 ± 5	43 ± 5	NA	1	NA	NA
MAb-Affinity Products-Cathode	100 ± 7	94 ± 8	NA	200	>95 ± 1	0 ± 2
MAb-Affinity Products-Anode	100 ± 9	93 ± 9	NA	200	>95 ± 1	72 ± 6

Purification of rhPC at pH 8.5 by RCFE and Ion-exchange Chromatography						
Samples	Total Protein (%)	Percent rhPC Yield (%)	Dilution of Start	Purification Factor	Purity (%)	Activity by APTT (%hPC ref)
Start	100 ± 8	100 ± 9	NA	1	NA	NA
Cathode product stream	51.8 ± 5	56 ± 4	NA	4	NA	NA
Anode product stream	45.4 ± 4	42 ± 4	NA	1	NA	NA
MAb-Affinity Products-Cathode	100 ± 8	92 ± 10	NA	200	>95 ± 1	0 ± 3
MAb-Affinity Products-Anode	100 ± 7	95 ± 8	NA	200	>95 ± 1	75 ± 8

Table II: Dynamic light scattering of single chain, two chain and broad populations of rhPC. At least 10 measurements were recorded for each sample at a temperature of 22C. The samples were analyzed using a monomodal size distribution.

rhPC population	Rh (nm)	Estimated Molecular Weight (kDa)
single-chain	4.0 ± 0.5	60 ± 2
two-chain	4.2 ± 0.3	68 ± 1
whole population	4.3 ± 0.3	71 ± 1

Chapter 5: Preparative Scale Free Flow Iso-electric Focusing of Recombinant Protein Sub-populations from Transgenic Pigs

Arthur W. Degener and William H. Velander
Department of Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA

Key Words: Protein C, Transgenic Animals, Protein Sub-populations, Free Flow Electrophoresis, Iso-electric focusing

Abbreviations: IEF, iso-electric focusing; rhPC, recombinant human protein C; EDTA, ethylene diamine tetra-acetic acid; ELISA, enzyme linked immuno sorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; gla, gamma-carboxy glutamic acid

Abstract

The most native recombinant protein species usually exist as sub-populations within the total secreted population due to rate limitations in post-translational processing and possibly post-secretional events. For example, the large scale production of recombinant proteins in the milk of transgenic livestock will result in secreted, but incompletely processed polypeptides. Iso-electric focusing is normally an analytical tool based upon resolution by constitutive pK of proteins within a viscous gel. A method called Free-Flow Isoelectric Focusing (FFIEF) has been developed to electrophoretically focus proteins of different isoelectric point in a fed-batch or batch-wise, free flow chamber without the benefit of a viscous gel to attenuate diffusional dissipation of focused species. FFIEF was used to selectively isolate properly gamma carboxylated and glycosylated forms of mature, biologically active rhPC from the immature species made in transgenic pig milk. A linear pH gradient of 3 to 10 was established over a 17 cm separation chamber to achieve separation into 50 fractions having rhPC species of distinct structure and function. This separation technique is also applicable as a general method to isolate recombinant protein species from the endogenous proteins of complex mixtures like milk.

5.0 Introduction

Specialized modifications of conventional purification methods such as precipitation, ion-exchange and immunoaffinity can be used to greatly enhance the purification of proteins from complex mixtures. Furthermore, the resolution of closely related sub-populations of a given heterologous or naturally occurring protein can also be enhanced by using modifications of these techniques. For example, Zn^{2+}-assisted precipitation combined with expanded bed ion exchange chromatography has successfully been used in a scalable process to separate active, post-translationally mature species from inactive, immature sub-populations of recombinant human protein C (rhPC) made in the milk of transgenic pigs [1]. In addition, successive step gradient, ion exchange chromatographies which use Ca^{2+} -dependent elutions have been used to purify rhPC in a scalable process that isolates active sub-populations of rhPC [2,3]. Alternatively, immunoaffinity chromatography using a Ca^{2+}-dependent conformational monoclonal antibody [4] was also used to purify properly gamma-carboxylated sub-populations of human protein C from plasma [5] and rhPC from transgenic pig milk [6,7]. While these modified purification techniques were successful, some mixtures will not be effectively processed using these basically conventional methods. Thus, there is still a need for alternative, scalable, high resolution techniques which utilize separation physics that are independent of chromatographic adsorption.

Iso-electric focusing has been most often used as an analytical tool for the resolution of protein populations in complex mixtures. These mixtures are typically applied to cross-linked, polymeric gels that act as a viscous, carrier phase. The viscous gel helps to minimize resolution losses due to diffusion in applications which use a relatively strong electric field (about 30-40 volts/cm) to focus proteins within a pH gradient (about 0.1 to 0.5 pH units/cm) [8]. A pH gradient is electrophoretically established within the gel due to the presence of selected sub-populations of relatively low molecular weight, amphoteric molecules at concentrations which impart significant buffering capacity. These aqueous soluble molecules are termed ampholytes and each ampholyte sub-population has a specific isoelectric point (pI) that has been engineered through the relative degree of substitution of acidic and basic chemical moieties. A static pH gradient occurs after a steady-state, electrophoretically induced, spatial distribution of each ampholyte is established. This steady-state exists due to the simultaneous phenomena of specific self-buffering and extinguished electrophoretic mobility that onsets each ampholyte upon reaching a field location where its respective PI occurs. In the presence of sufficient buffering capacity by ampholytes, protein sub-populations can be electrophoresed from locations of lower to higher pH until the PI of the protein is reached and its mobility is halted along with that of the respective ampholyte at that field position.

Under a sufficiently strong electric field, the dissipation of the pH gradient due to diffusion can be prevented even in a purely aqueous, low viscosity media. However, once the electric field is removed, the device must somehow prevent the loss of separation by diffusion that would rapidly occur in pure aqueous. Thus, isoelectric focusing can be extended to an ordinary purification processing environment only if the focused samples can be isolated prior to removing the electric field. To that end, the multiple recycle design feature of a recycle crossflow electrophoresis chamber (RCFE) can make possible the isolation sub-populations of recombinant proteins on a larger scale [9-11]. The recycle flow along a multiple sequence of juxtaposed recycle streams enables sufficient residence time within the separation chamber to achieve electrophoretic separation across the duct to which the electric field is applied (Figure 1). Furthermore, because the recycle lines can be scaled to any volume, the bulk of the resolved protein can be sequestered within the recycle lines (relative to that remaining the resolution chamber), once the electric field is removed.

The chief design constraint upon free-flow electrophoresis devices for large-scale applications is material throughput as restricted by resistive heating effects caused by the combination of large current and poor heat transfer. Several designs for recycle flow within various thin rectangular or cylindrical ducts have been tried for general free-flow, electrophoretic applications. For example, the

RCFE design provides a scalable unit operation for maintaining isothermal conditions within a rectangular duct (30 volt/cm). Most importantly, the residence time outside the chamber needed to cool the fluid from the N th stream and maintain isothermicity and separation from the proximal N-1 and N+1 streams is achieved in a fashion scaleable to high throughput. This device has not previously been adapted to iso-electric focusing applications because it was designed primarily as a steady-state feed device while iso-electric focusing is most amenable to batch processing. However, when set to 100% recycle, a balance of upward, laminar flow to minimize dispersion by free convection, high voltage operation to minimize diffusive dissipation of the pH-dependent resolution, and residence time within the resolution chamber with efficient heat transfer by recycle outside of the resolution chamber makes many aspects of the RCFE design desirable for isoelectric focusing in either a batch or fed-batch mode.

In this study, we modify the RCFE design by adapting it to a 50 fraction, fed-batch or batch, free-flow isoelectric focusing (FFIEF) device. This resolution power of this design is tested by processing about 100 mg amounts of a complex mixture of sub-populations of rhPC purified from the milk of transgenic pigs. The 50 FFIEF fractions of isoelectrically focused sub-populations of rhPC are characterized with respect to resolution by rhPC structure-function evaluation and also PI analysis by 2-D gel electrophoresis.

5.1 Materials

5.1.1 Materials: A RCFE electrophoresis apparatus was designed and built by Dr's. C. Ivory and W. Gobie from Washington State University was adapted to a fed-batch FFIEF in the present study by eliminating cross-flow, moving sample injection to recycle line 50 (proximal to the cathode membrane), and using line 1 as a sample volume bleed (proximal to anode membrane). Human protein C that was immunopurified from plasma was a gift from American Red Cross (Rockville, MD). Transgenic swine milk containing rhPC was collected at the Pharmaceutical Engineering Institute, Transgenic Swine Research Center at VPISU (Blacksburg VA). Ampholytes ranging from pH 3.0 to 10 were purchased from Pharmacia Biotech (Uppsala, Sweden). Deionized water was produced by a Nanopure Barnstead water filtration system. Murine metal-dependent anti-hPC monoclonal antibody, 7D7B10-MAb, was purified from cell culture supernatant. The following items were purchased from Sigma Chemical Co.: tris-base, 0.45 µm 20x20 cm nitrocellulose membranes, low EEO agarose, sodium phosphate dibasic, sodium phosphate tribasic, silver nitrate, magnesium chloride, calcium chloride, zinc chloride, sodium hydroxide, ammonium hydroxide, EDTA, rabbit antiserum against protein C, anti-goat immunoglobulins conjugated with horseradish peroxidase, protein C depleted plasma, normal pooled reference plasma and citric acid. Polyacrylamide:bisacrylamide(29:1), Immulon II plates, acetic acid, HCl, Accumet 25 pH meter, Marathon 21KR centrifuge, BRL Horizon^{TM} 11x14 cm horizontal electrophoresis apparatus,

refrigeration unit and the FB600 power supply were purchased from Fisher Scientific. Goat antiserum to human protein C and protac were purchased from American Diagnostica. PTT Automat 5 was purchased form American Bioproducts. O-phenylenediamine-2HCl (OPD) kits were purchased from Abott Labatories. Gluteraldehyde, methanol and tris-HCl were purchased form Scientific Products. Blotting paper and Biorad Electrophoresis apparatus were purchased from Biorad. Oxytocin was purchased form Vedco Inc.. The Immulon II plates were read at 490nm using an EL308 Bio-Tek Microplate reader. The absorbance of the protein solution was read at 280nm on a Milton Roy Spectronic spectrometer.

5.2 Methods

5.2.1 Milk Collection and Preparation: Milk from transgenic swine was collected and prepared by the method described in Subramanian et. al [10]. The pig milk was treated with EDTA to chelate Ca^{2+} and solubilize casein micelles resulting in a clarified whey.

5.2.2 Purification of rhPC by immuno-affinity chromatography: The rhPC used in the iso-electric focusing experiment was purified to about 95% purity at about 90% yield from transgenic pig milk by immuno-affinity chromatography using 12A8 an monoclonal anti-hPC antibody that is attached to Emphaze™ chromatographic matrix. [5]

5.2.3 ELISA: The method used to determine the level of rhPC in the milk of transgenic pigs and in all process samples has been described in Velander et. al [6]. Briefly, a sandwich ELISA using rabbit anti-human protein C as the capturing antibody and using goat anti-human protein C as the detecting antibody followed by rabbit anti-goat horseradish peroxidase conjugate.

5.2.4 Free flow iso-electric focusing: Pure rhPC as determined by gel was mixed with ampholytes ranging in pH from 3.0 to 10. The final concentration of ampholyte was 2% when mixed and the total amount of rhPC was about 100 mg. The iso-electrophoresis chamber can hold about 300ml of protein/ampholyte

mixture. The unit was filled and the air removed through bleed valves in each recycle stream line. The recycle flow rate was set to 30% of the total pump speed which is 3.0-3.5ml/min. The unit was chilled with a cold water bath to a temperature of 5 ^{o}C for the entire run. The electric field power was set to 150 Watts for 30 minutes then readjusted to 25 Watts for 4 hours. After 4.5 hours of total run time, the power was turned off. The chamber was emptied after isolation from the recycle tubes. Finally, the 5 ml product material from each recycle tubes were emptied into samples tube and analyzed.

5.2.5 Conformational ELISA: The conformational-dependent ELISA method of Subramanian et.al [12] was used to determine the gross extent of carboxylation of the FFIEF products. A monoclonal antibody from mouse, 7D7-B10 (5 µg/ml) was bound to Immulon II plate and 100µl of samples were added with increasing levels of $CaCl_2$ over the range of 0-100mM. Rabbit anti-human protein C was used as the detecting antibody, followed by goat anti-rabbit horseradish peroxidase conjugate. In the presence of calcium, the monoclonal antibody will release the partially to properly γ-carboxylated rhPC at a characteristic half-maximal signal inhibition that occurs at about 2 mM Ca^{2+}.

5.2.6 Sialic Acid Determination: The sialic method was done according to method developed by Warren et al [13]. Before sialic acids could be determined on rhPC, the samples were initially incubated with 0.1N H_2SO_4 for 1 hour. The

standard was applied over the range of 80ng/ml to 500ug/ml. After extracting with cyclohexanone, 100 µl of the standard and sample was pipetted into an immulon II plate and read at 550ηm.

5.2.7 PAGE and Western: Two dimensional gel electrophoresis with western blot was used to analyze rhPC sub-populations with respect to molecular weight and isoelectric point. The total amount of rhPC was 10µg applied to the gel with 2% ampholytes ranging in pH from 3-10. The initial separation was done by pH was done by pH and the second direction done by molecular weight. The second direction was a 12% SDS-gel. The proteins were then transferred to a PVDF membrane at 200ma for 12-15 hours. The westerns were stained using two antibodies. The first antibody for the western, rabbit anti-human protein C was used as the capturing antibody. The second antibody, goat anti-rabbit horseradish peroxidase conjugate was used as the detecting antibody. The gel and western method are described by Velander et. al [14].

5.2.8 Activated Partial Thromboplastin Time(APTT) of rhPC: The biological activity of rhPC products was determined by APTT and is described by Morcol et. al [6]. Briefly, samples containing rhPC were added to hPC depleted plasma and the extent of delayed clotting response time was measured. Protac and Ca^{2+} were added to both the standard and the samples, then the time for a clot to appear was measured. Normal plasma reference pool with 4 µg/ml of hPC having a defined specific activity of 250 units per mg was used as the standard.

5.3 Results

Figure 1 shows a schematic of the apparatus to be used to separate the sub-populations of rhPC. The device was run either as a batch or a fed-batch unit operation. In batch mode, the 80-100 mg of immunopurified rhPC was mixed with ampholytes and inject as a mixture into the chamber at a volume of 300 ml. In fed-batch mode, 80 ml of immunopurified sample was continuously injected into a pre-focused chamber at the anode proximal port while the same volume was continually bled at the cathode proximal port over a period of 30 minutes. After sample injection, steady state operation was achieved by 2.5 hours as measured by a drop in current to a steady-state value of 50 milliamps**.** Figure 2 shows the linear pH gradient established after 2.5 hours within each of the 50 recycle lines labeled as lanes 1 through 50. Every 3 to 5 tubes were pooled and the pH was measured in the pool. Lanes 1 through 3 had a pH of 3 while tubes 46 through 50 had a pH of 10 A linear pH gradient of 3.0 through 10.0 existed in the pools from lane 1 through 50, respectively. The resolution of the pH meter was 0.05 pH units.

Figure 3 shows the percentage of total rhPC recovered from each FFIEF line as quantified by ELISA. About 90 mass% +/- 6 mass% of the rhPC was recovered from the device. The majority of the rhPC sub-populations, 83 mass% +/- 4 mass%, were extracted from the FFIEF within the pH range of 5 to 8 corresponding to lanes 10 through 40. ELISA and 2-D gel Western analysis showed that rhPC appeared in all lanes. A reduced, 2-D gel western analysis of

the immunopurified rhPC that was injected into the FFIEF is shown in Figure 4. A broad range of rhPC immunoreactive species exist that extend from a relative molecular weight (M_r) of about 18 kDa to about 65 kDA within the pH range of 3 to 5. In the pH range of 5 to 10, rhPC immunoreactive material extends from about 36 kDa to about 60 kDa M_r . The 18 to 22 kDa M_r species corresponds to light chain rhPC, the 36 to 40 kDa M_r to heavy chain rhPC, and the 56 to 65 kDa M_r to single chain species.

Figure 5A, panels 1,2 and 3 shows a non-reduced, 2-D Western Blot analyses of three different pools of samples obtained from lanes 1-3, 20-23, and 46-50, respectively. Figure 5B panels 1,2 and 3 shows a reduced, 2-D Western Blot analyses of three different pools of samples obtained from lanes 1-3, 20-23, and 46-50. These sample pools had pH values of 3, 7 and 10, respectively. The samples obtained from lanes 1-3 at pH 3 show rhPC immunoreactive populations in a tight band focused at about pH 3 within the 2-D isoelectric focusing gel/Western Blot (Figure 5A and 5B, panel 1). The samples obtained from lanes 20-23 at pH 7 show rhPC immunoreactive populations in a tight band focused at about pH 7 within the 2-D isoelectric focusing gel/Western Blot (Figure 5A and 5B, panel 2). The samples obtained from lanes 46-50 at pH 10 show rhPC immunoreactive populations in a tight band focused at about pH 10 within the 2-D isoelectric focusing gel/Western Blot (Figure 5A and 5B, panel 3). Figure 5B panels 1, 2 and 3 show that all light chain species of rhPC are focused at about pH 3 for each of the samples from FFIEF lanes 1-3, 20-23 and

46-50; while the heavy chain species of rhPC are focused at about pH 4, pH 8 and pH10 for the samples from FFIEF lanes 1-3, 20-23 and 46-50 respectively.

A conformational ELISA was done to determine the extent to which each rhPC population isolated by FFIEF was properly γ-carboxylated. Figure 6 shows the half-maximal inhibition by Ca^{2+} ($[Ca^{2+}]Max_{1/2}$) of rhPC to the EDTA-dependent 7D7B10-MAb. Fully carboxylated, reference human protein C that was immunopurified from plasma gave a $[Ca^{2+}]Max_{1/2}$ of about 2-3 mM Ca^{2+}. Samples from pH 7-8 and pH 9-10 corresponding to FFIEF lanes 33-43 and 43-50 respectively, did not give a $[Ca^{2+}]Max_{1/2}$. The rhPC recovered from lanes 33-50 represented 35 mass% +/- 3 mass% of the total rhPC as measured by ELISA. In contrast, samples from pH 5-6 and 3-4 corresponding to FFIEF lanes 16-27 and 1-10 gave a $[Ca^{2+}]Max_{1/2}$ of about 2-3 mM Ca^{2+}. The rhPC recovered from lanes 26-50 represented 48 mass% +/- 4 mass% of the total rhPC as measured by ELISA.

Figure 7 shows the sialic acid content of samples obtained from FFIEF fraction pools having pH values of 3 +/- 0.05, 4 +/- 0.05, 5 +/- 0.05, 6 +/- 0.05, 7 +/- 0.05, 8 +/- 0.05, 9 +/- 0.05, and 10 +/- 0.05 corresponding to FFIEF lanes 1-5, 7-11, 16-20, 25-26, 28-32, 34-38, 39-43 and 47-50, respectively. Reference human protein C (hPC) that was immunopurified from plasma gave a sialic acid content of 5.1 +/- 0.6 mol sialic acid /mol hPC. Recombinant protein C samples at pH 3 to 10 gave a near linear range of sialic acid contents from 3.5 +/- 0.3 to

0.7 +/- 0.1 mol sialic acid /mol rhPC, respectively.

Figure 8 shows the % specific anticoagulant activity of rhPC populations isolated by FFIEF relative to reference human protein C (hPC) that was immunopurified from plasma as measured by delay in clotting time in a APTT assay. Samples isolated from pH 3, 4 and 5 pool FFIEF fractions gave activities of 125 +/- 11%, 110 +/- 8%, and 95 +/- 7%, respectively. Samples isolated from pH 6, 7 and 8 pool FFIEF fractions gave activities of 75 +/- 6%, 40 +/- 3%, and 25 +/- 2%, respectively. Samples isolated from pH 9 +/- 0.05 and 10 +/- 0.05 pool FFIEF fractions gave no clotting activity.

5.4 Discussion

The pH gradient within the free flow recycle isoelectric focusing apparatus was maintained by ampholytes under a strong electric field (30 volts/cm). The ampholytes used in this study are commercially available and the chemical structure is proprietary. However, ampholytes are typically low molecular weight polymers or other organics having a high degree of substitution carboxylic acid and amino groups to create a broad distribution of species with different pI's [15]. While some ampholyte-polymer association is possible, the low molecular weight nature of the ampholyte makes removal by subsequent ultra filtration, affinity purification or other adsorption chromatography of the protein feasible. The biological anticoagulant activity of the native rhPC populations recovered from the FFIEF process indicates that even complex biochemical reactions are not appreciably affected by ampholytes. Extensive tabulations of toxicity data for commercially available ampholytes has not been reported.

The chief value of FFIEF lies in its ability to quantitatively isolate sub-populations of complex recombinant proteins in solution without the less selective, more highly denaturing environment often characteristic of adsorptive phenomena associated with chromatographic matrices. This is starkly shown by the comparison of the 2-D Western analysis of whole rhPC population to the narrow sub-populations isolated by FFIEF. Profound differences in structure as related to function are inherent to these rhPC sub-populations. Recombinant human protein C must undergo several post-translational modifications to

become a mature biologically active protein. Some of these modifications include the formation of 11 intra-chain disulfide bridges, three N-linked sites of glycosylation on the heavy chain and one on the light chain of hPC, 9 γ-carboxylated glutamic acid residues (gla) in the light chain, Arg-Lys dipeptide removal to make it a two chain heterodimer, and removal of both the signal and propeptide [5-6]. We have emphasized the structure -function analysis of γ-carboxylation and sialic acid content as related to native hPC conformation and anticoagulant activity [16]. Because the subpopulations could be highly resolved by FFIEF, a definitive relationship between sialic acid content and specific activity as well as between γ-carboxylation and activity was demonstrated. Although this has not been established by cell lines [17], a relationship between the post-translational processing involving sialic acid formation and γ-carboxylation may exist in mammary epithelial tissue, as the lowest specific activity and most non-native gla-dependent conformation have also the lowest sialic acid and gla content. Conversely, superactive species and those populations of rhPC with activity similar to hPC contained the most native gla-conformation and highest sialic acid content. The sialic acid content of the rhPC species with an experimental pI of 3 was similar to that of hPC. In contrast, enzymatically desialated hPC from plasma [17] showed superactivity relative to native hPC indicating that the sialic acid deficient rhPC made by mammary epithelial cells has a difference in sialo-dependent conformational structure. The particular sialic acid species contained in either rhPC and hPC has not been compared.

It is useful to evaluate the relative differences in PI's of the major milk proteins as well as the potential diversity of target sub-populations for purposes of selecting conditions which could enhance separation by FFIEF. For example, as is evident from the 2-D western and structure-function analysis of the rhPC recovered from milk, different pI's may result from a given post-translational structure. Relative differences in pI of a protein can be estimated by the group contribution to the pI by primary amino acid composition and also post-translational modification [18-19]. The differences between absolute and experimentally measured pI value would be largely due to changes in ionization potential caused by local ionic strength within the free volume of the protein [18]. We have estimated the mobility of human protein C relative to differences in post-translational modification. As discussed above, the populations of hPC that are more completely post-translationally modified will have 9 carboxy glutamic acid residues and 4 sialic acid residues which result in an estimated pI of 4.7 while those having no gla or sialic acid residues have an estimated pI of 6.5. A pI of 5.6 is predicted for the rhPC sub-population having no gla domain, but 4 sialic acid residues. Additionally, it is also estimated that a rhPC population having a pI of 5.0 would result if no sialic acid residues, but 9 gla residues were present. Estimates of charge contribution by γ-carboxylation and sialic acid to rhPC account for 6% and 8% of the overall ionizable residues, respectively. The FFIEF samples showed that 4 main groups of rhPC exist with weight fractions of 9%, 33%, 36% and 21% with pI's of 9.5, 7.5, 5.5 and 3.5, respectively. From the theoretical prediction of pI, the sialic acid assay and the APTT coagulation

assay, the two high pI fractions, 9.5 and 7.5 have no gla domain. The pI 9.5 fraction has no sialic acids, but the pI 7.5 has between 1 and 2 residues. In contrast the two lower pI fractions, 5.5 and 3.5 have partial(6-7 glas) to fully(9 glas) γ-carboxylated domain and between 3 to 4 sialic acid residues. The differences in absolute values of the pI for theoretical and those rhPC populations isolated by FFIEF are stark. This is likely due to non-ideal (solution) ionization potential that is not considered when making predictions of pI values from simple group contribution method. However, these estimates may be more valuable for comparison on a relative basis. For example, it is clear that a full complement of sialic acid and gla residues is both theoretically and experimentally resolvable from those having intermediate contents when considering the broad and linear pH gradients (about 0.5 pH units/cm) established by FFIEF.

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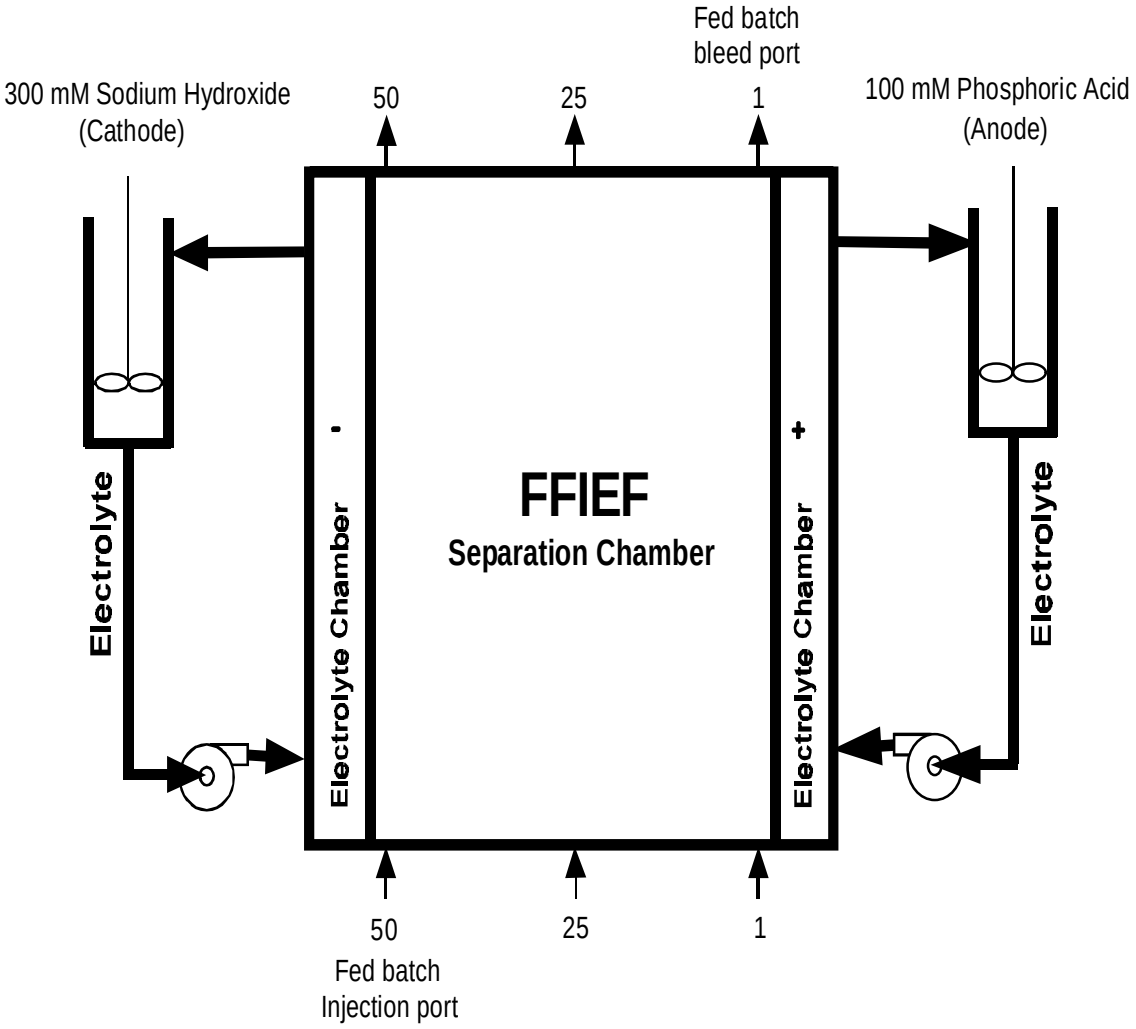

***Note:** The Cationic membrane is Neosepta and the Anionic membrane is Nafion 117. Ampholytes are added to the protein solution to a final concentration of 2%

Figure 1: Diagram of the FFIEF apparatus

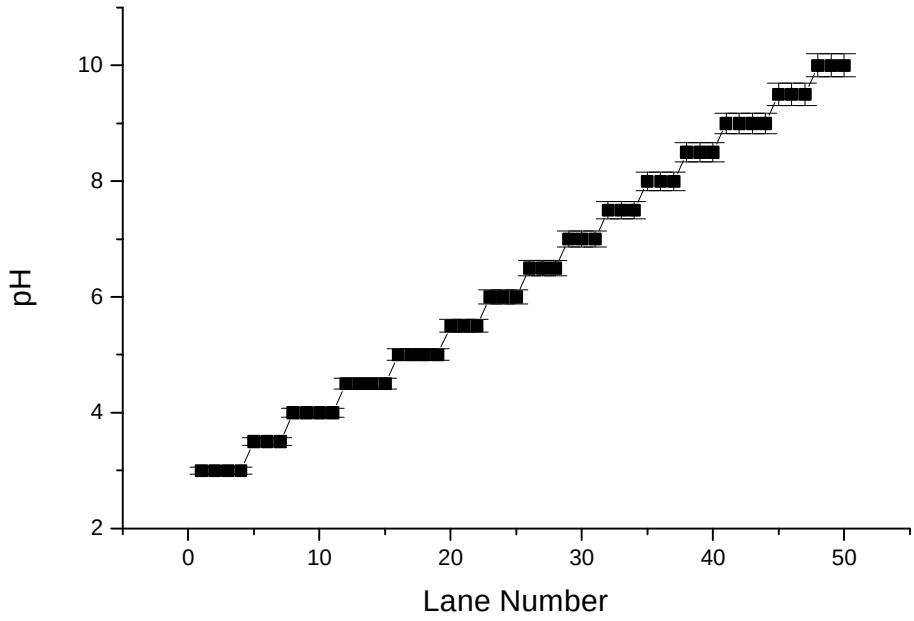

Figure 2: The pH that is associated with each lane after iso-electrically focusing rhPC sub-populations by FFIEF. The iso-electric focusing was done with 2% ampholytes at 25W for 4-6 hours at room temperature. The pH was measured using a digital pH meter.

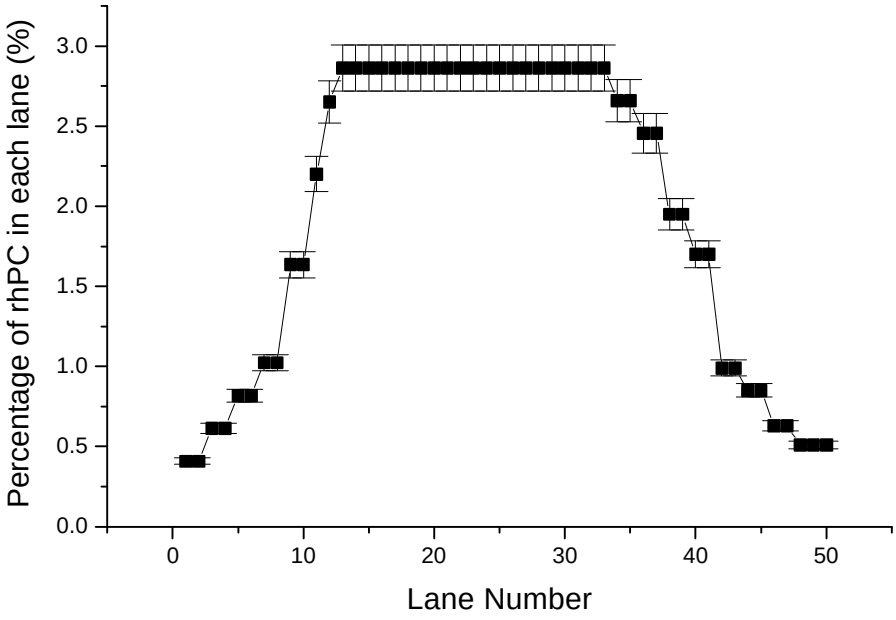

Figure 3: The percentage of the total mass of rhPC that has been iso-electrically focused by FFIEF in each lane. The iso-electric focusing was done with 2% ampholytes at 25W for 4-6 hours at room temperature. The rhPC concentration was measured by ELISA.

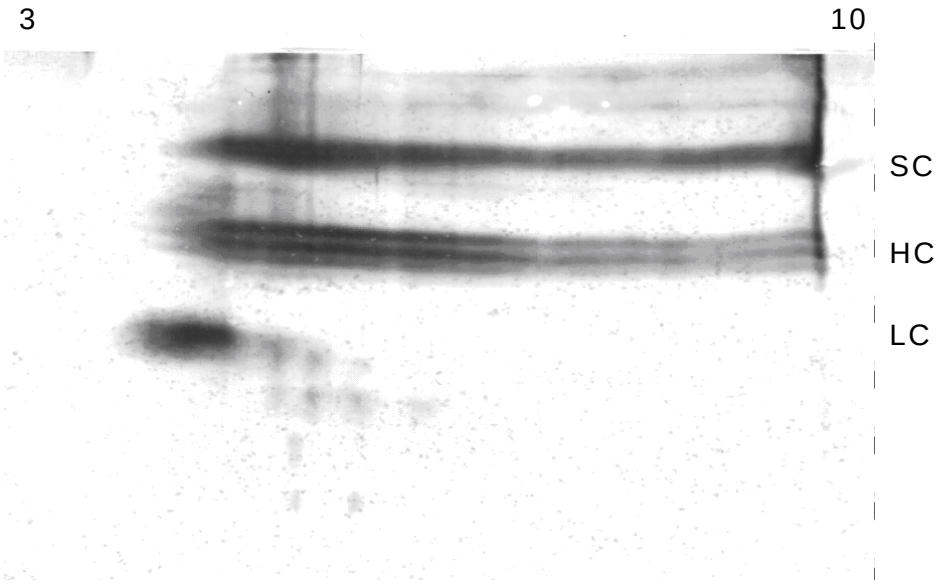

Figure 4: A 2-D reduced western of the total rhPC population over the pH range of 3 to 10. A pH gradient was used as the first dimension to separate rhPC by pI with a 4% polyacrylamide tube gel with running conditions of 400V for 3 hours. Molecular weight was used as the second dimension with a 12% polyacrylamide gel with running conditions of 20mA for 1.25 hours. The proteins were transferred to a PVDF membrane with running conditions of 200mA for 10 hours.

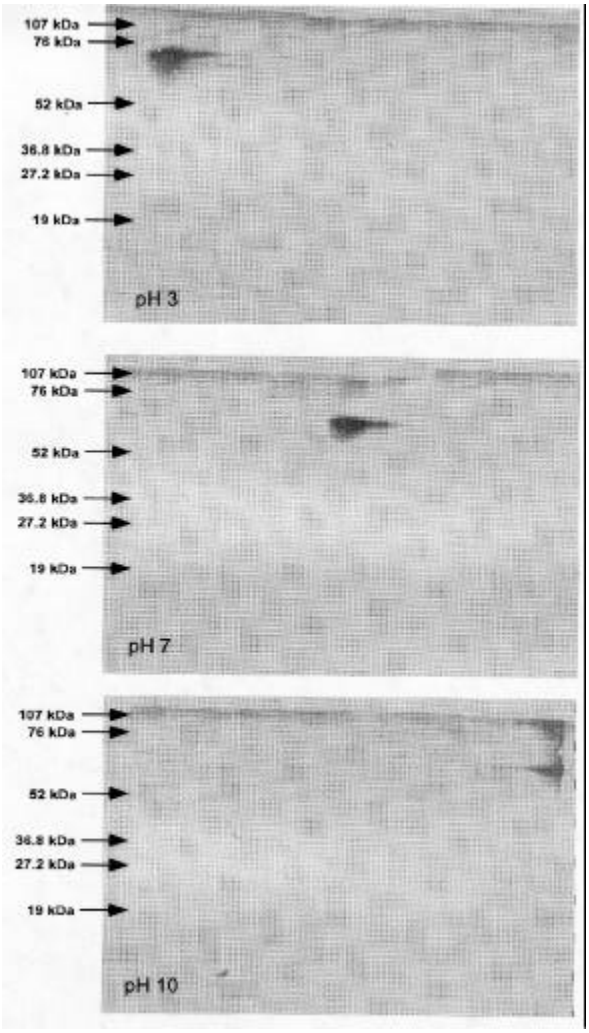

Figure 5A: Panel 1 is a non-reduced western of FFIEF purified rhPC from pooled lanes 1-5 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10. Panel 2 is a non-reduced western of FFIEF purified rhPC from pooled lanes 25-30 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10. Panel 3 is a non-reduced western of FFIEF purified rhPC from pooled lanes 45-50 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10.

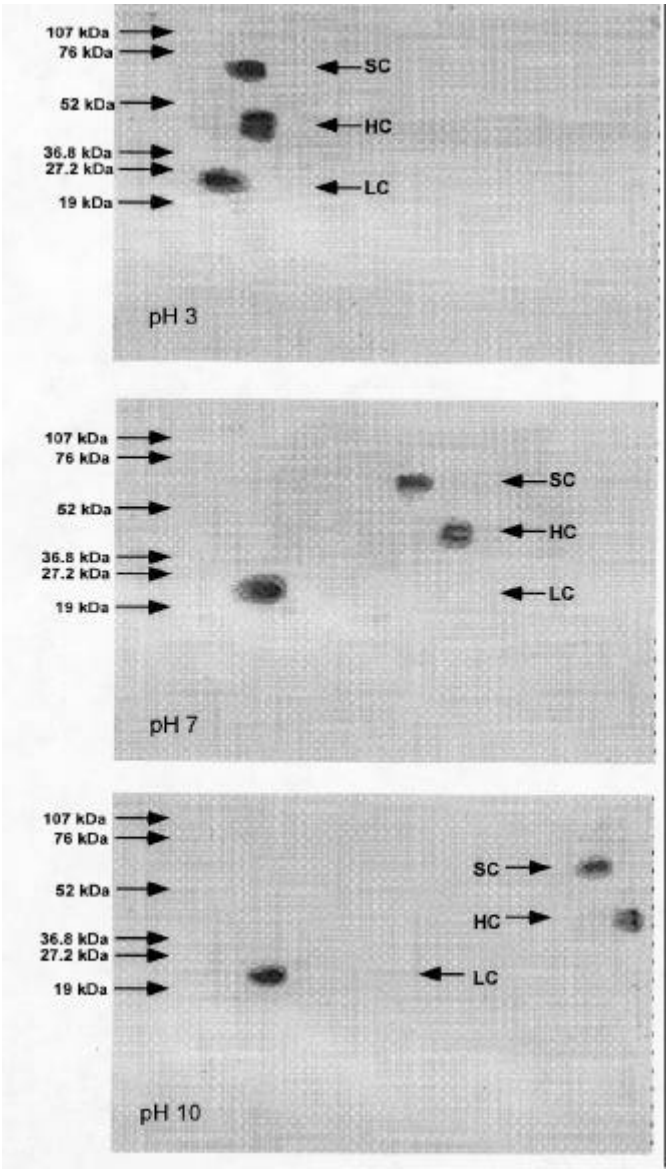

Figure 5B: Panel 1 is a reduced western of FFIEF purified rhPC from pooled lanes 1-5 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10. Panel 2 is a reduced western of FFIEF purified rhPC from pooled lanes 25-30 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10. Panel 3 is a reduced western of FFIEF purified rhPC from pooled lanes 45-50 reisoelectrically focused on a 4% polyacrylamide gel over the pH range of 3-10.

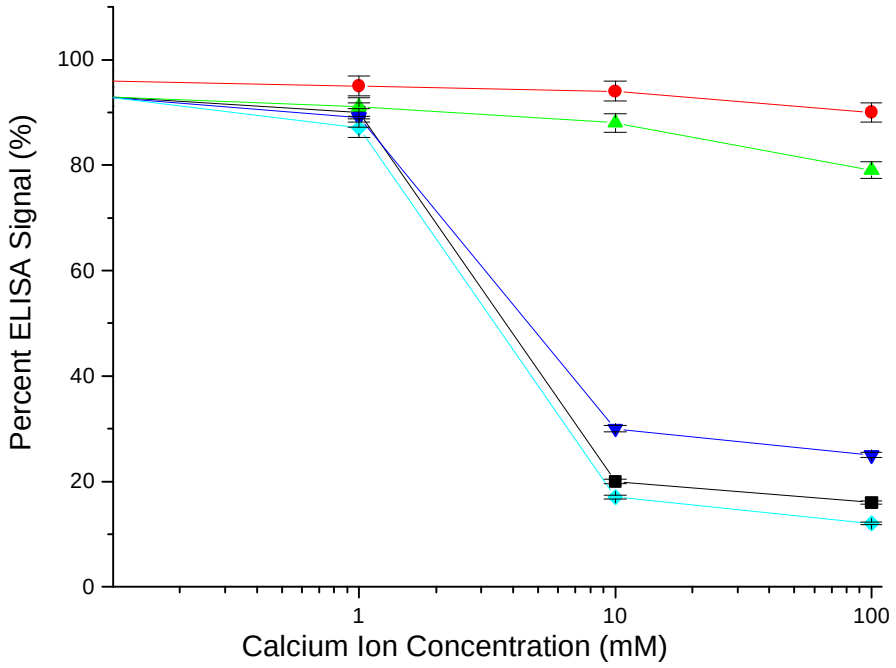

Figure 6: Conformational ELISA of rhPC sub-populations from free flow iso-electric focusing. (■-Human plasma reference, ●-pH 9 thru 10 rhPC sub-population, ▲--pH 7 thru 8 rhPC sub-population, ▼--pH 5 thru 6 rhPC sub-population, ◆-pH 3 thru 4 rhPC sub-population)

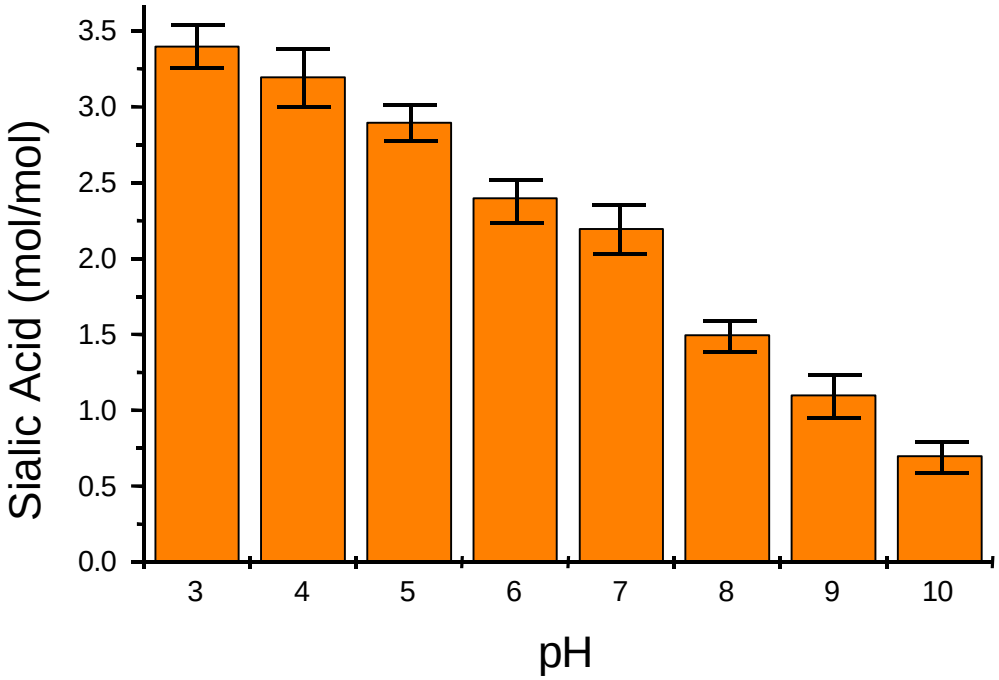

Figure 7: Sialic acid content of free flow iso-electrically focused rhPC subpopulations. The pH 3 sample was pooled lane 1-5, pH 4 sample was pooled lane 7-11, pH 5 sample was pooled lane 15-20, pH 6 sample was pooled lane 22-26, pH 7 sample was pooled lane 28-32, pH 8 sample was pooled lane 35-40, pH 9 sample was pooled lane 41-45 and the pH 10 sample was pooled lane 46-50.

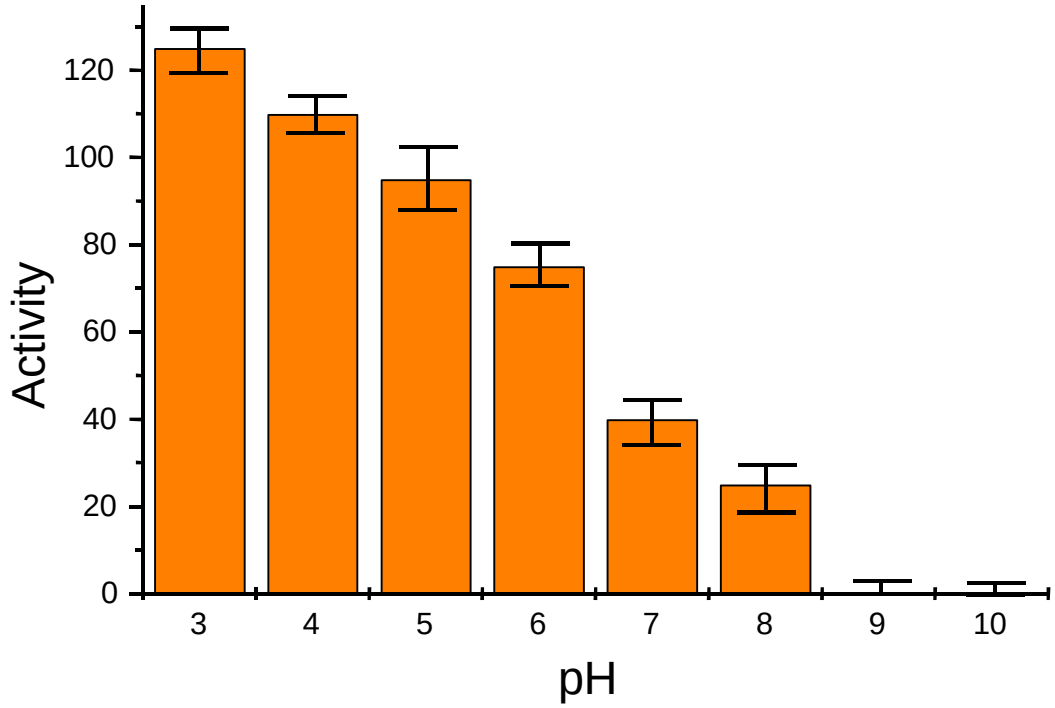

Figure 8: Activity of free flow iso-electrically focused rhPC subpopulations. The pH 3 sample was pooled lane 1-5, pH 4 sample was pooled lane 7-11, pH 5 sample was pooled lane 15-20, pH 6 sample was pooled lane 22-26, pH 7 sample was pooled lane 28-32, pH 8 sample was pooled lane 35-40, pH 9 sample was pooled lane 41-45 and the pH 10 sample was pooled lane 46-50.

Chapter 6: Conclusions and Recommendations

Conclusions

Since Chon fractionated human plasma over 50 years ago, other processes have been developed to help further fractionate plasma proteins. Currently, purification methods use ion-exchange and immuno-affinity to isolate target proteins from a complex mixture. While these methods are reliable, other purification technologies are being researched to provide more barriers to pathogen transmission. In this thesis the focus was to research several alternative purification technologies that will not only provide potential pathogen barriers, but will also have high throughput, high yield and high resolution.

Expanded bed adsorption chromatography(EBAC) was researched as a potential alternative purification technology. By using Zn^{2+} as an agent to cause conformational changes in the target protein, both biologically active rhPC and pig milk IgG(containing <1% IGA which causes anaphylaxis) were selectively isolated from a complex protein mixture. The Zn^{2+}-assisted EBAC was able to process gram quantities of total protein while maintaining high yields and high resolution and could potentially be used commercially. In addition, this process is orthogonal in separation physics to current methods and could provide a barrier to pathogen transmission.

Two electrophoresis techniques were also researched as potential alternative purification technologies. Recycle continuous flow electrophoresis combined with ion-exchange chromatography was used to isolate biologically active rhPC from the inactive as well as from a complex protein mixture. While only 1.5 g of total protein was processed in an hour, research is currently being done to increase this to 1kg per hour. Free-flow isoelectric focusing fractionated 80-100 mg of immuno-purified rhPC into 50 fractions. These fractions showed a relatively linear pH gradient over the range of pH 3-10 with the active rhPC sub-populations in the pH range of 3-6. This process is scalable by increasing the tube length and diameter to contain at least 75% of the total process volume and could potentially process gram quantities of protein. Both of these processes because of their ability to isolate biologically active sub-populations as well as process large quantities of proteins while maintaining a high resolution and high yield, could be used on a commercial scale. Finally, while pathogens were not tested, it is believed that these processes which are orthogonal to other separation processes could be used a barriers to pathogen transmission.

Recommendations

While rhPC and pig milk IgG were used as models and showed hopeful results, it is advisable to test these methods on other recombinant proteins as

well as plasma proteins. Other recombinant proteins should be used because these are going to be the therapeutic proteins of the future. Processes which can not only isolate recombinant proteins from complex mixtures but can selectively isolate the active sub-population from the inactive are needed. Plasma proteins are of interest because this is currently the major source for the therapeutics. The concentration of proteins in plasma vary greatly, but large volumes still need to be processed to meet demands. Processes that can handle high throughput and still give high yield and resolution as well as act as barriers to pathogen transmission are needed.

The free flow iso-electric focusing configuration had some limitations in being able to process proteins effectively. One of the more important issues was being able to quickly collect the sub-population protein fractions. The current apparatus has 50 tubes that have to be emptied individually. A possible recommendation is to build a non-porous polymer block which has 50 channels, that can quickly be attached to the apparatus. This block should be modular to allow for greater volumes if needed and therefore greater recovery and more total protein processing. It should also have valves at each end to prevent back-mixing and prevent product loss. Finally, it should also be built as a heat exchanger to allow for the removal of any heat generated.

Appendix A

Small scale processing is typically done to determine large scale operating parameters, with a linear scale up to large scale. To determine the dynamic binding capacities(DBC), yield and purity of immunoglobulin from pig milk for a large scale EBAC-SPA process with and without 40mM $ZnCl_2$, a small scale column containing 10ml(1cm diameter by 12cm high) of SPA was used. The whey was reloaded(6 EBAC-SPA loadings) until all the IgA (2mg IgA per ml SPA) and IgM (2mg IgM per ml SPA) was removed. The IgA and IgM depleted whey was then chelated with100mM EDTA to resolubilize precipitates that formed. This EDTA-solubilized IgA and IgM depleted whey was loaded onto SPA in packed bed mode to capture the IgG(3mg IgG per ml SPA) . This information was then used to scale-up to a 300ml(5cm diameter by 15cm high) EBAC-SPA column. The information obtained from these preliminary runs are presented in Tables IA and IIA.

From the information presented in the tables below, it appears that the presence of $ZnCl_2$ has altered the DBC of IgG, IgA and IgM. Zinc chloride appears to have lowered the DBC of IgG while raising the DBC of both IgA and IgM. Also, the addition of EDTA appears to make the process reversible as the precipitates resolubilized and the DBC of IgG returned to previous levels.

Table IA: Yield, purity and purification factor for small scale (10ml) EBAC-SPA separation of IgA and IgM from pig whey.

Column loaded at pH 7.0 with 0 mM Zinc added to the starting material, eluted pH 2.2,							
Sample	Yield (%)			Purity (%) [Purification Factor]			
	IgG	IgA	IgM	IgG	IgA	IgM	Other
Start	100 ± 8	100 ± 9	100 ± 9	- [1]	- [1]	- [1]	-
Flow Through	19 ± 2	91 ± 8	81 ± 7	- [<1]	- [<1]	- [1]	-
pH 2.2 Elution	75 ± 7	5 ± 1	13 ± 1	60 ± 2 [12]	15 ± 1 [2]	15 ± 1 [1]	10 ± 1

Column loaded at pH 7.0 with 40 mM Zinc added to the starting material, eluted pH 2.2,							
Sample	Yield (%)			Purity (%) [Purification Factor]			
	IgG	IgA	IgM	IgG	IgA	IgM	Other
Start	100 ± 9	100 ± 8	100 ± 8	- [1]	- [1]	- [1]	-
Flow Through	90 ± 10	69 ± 6	71 ± 8	- [<1]	- [1]	- [1]	-
pH 2.2 Elution	<1 ± <1	21 ± 2	19 ± 2	<1± <1 [<1]	>45 ± 1 [4]	>45 ± 1 [5]	<5 ± 1

Table IIA: Yield, purity and purification factor for EBAC-SPA separation of IgG from IgA and IgM depleted pig whey.

Column loaded at pH 9.0 with EDTA chelated IgA and IgM depleted pig whey , eluted pH 2.2,							
Sample	Yield (%)			Purity (%) [Purification Factor]			
	IgG	IgA	IgM	IgG	IgA	IgM	Other
Start	100 ± 8	1 ± <1	1 ± <1	- [1]	- [1]	- [1]	-
Flow Through	10 ± 1	<1 ± <1	<1 ± <1	- [<1]	- [1]	- [1]	-
pH 2.2 Elution	85 ± 8	<1 ± <1	<1 ± <1	>98 ± 1 [25]	<1 ± 1 [<1]	<1 ± 1 [<1]	<1 ± 1

Appendix B

Electrophoretic mobility is a direct measurement of protein separation within an electric field. Proteins with similar charge to mass and similar charge to hydrodynamic radius ratios will be difficult to separate by this technique. We were able to separate α-pcasein (α_{s1}- and α_{s2}-), β-pcasein and rhPC by an electric filed predicted by electrophoretic mobility and is presented here.

Native agarose gels: Agarose gels containing 1% low EEO agarose in 10mM sodium phosphate buffer ranging from pH 5.0 to 8.5 were used to study the electrophoretic mobility of α-pcasein (α_{s1}- and α_{s2}-), β-pcasein and rhPC. The gels were run at constant voltage (100V) until the dye front (brilliant blue) reached the edge of the gel. The gels are presented in Figures B1 through B5.

From the gels, it is observed that there is a distinct mobility for each protein present. This indicates that a separation is possible by an electric field in RCFE at a given pH. Two pH's were used to separate rhPC from α- and β-pcasein, 7.0 and 8.5. The pH 7.0 RCFE run was used to isolate α-pcasein from β-pcasein. From the gels it was also observed that the α- and β-pcasein have relatively narrow populations of protein compared to rhPC which is very broad. This suggests that we should be able to isolate a large group of rhPC sub-populations. The pH 8.5 RCFE run was used to isolate about 50% of the rhPC

from β-

pcasein.

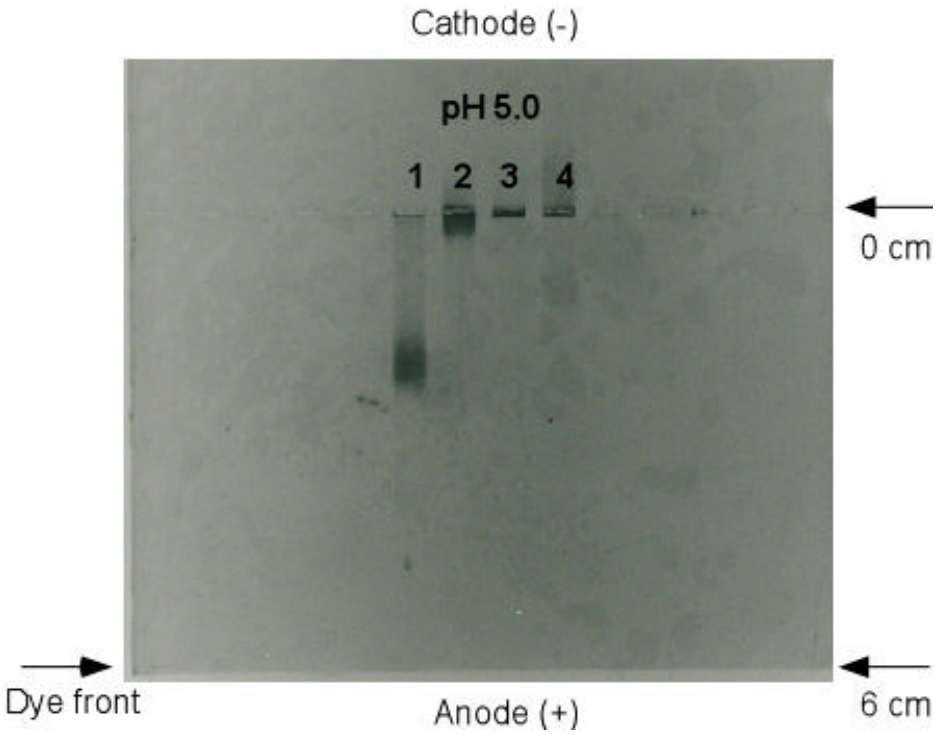

Figure 1B: Coomassie blue stained 1% agarose low EEO gel with hPC in lane 1, rhPC in lane 2, α-pcasein in lane 3 and β-pcasein in lane 4 run with 10mM phosphate buffer at pH of 5.0.

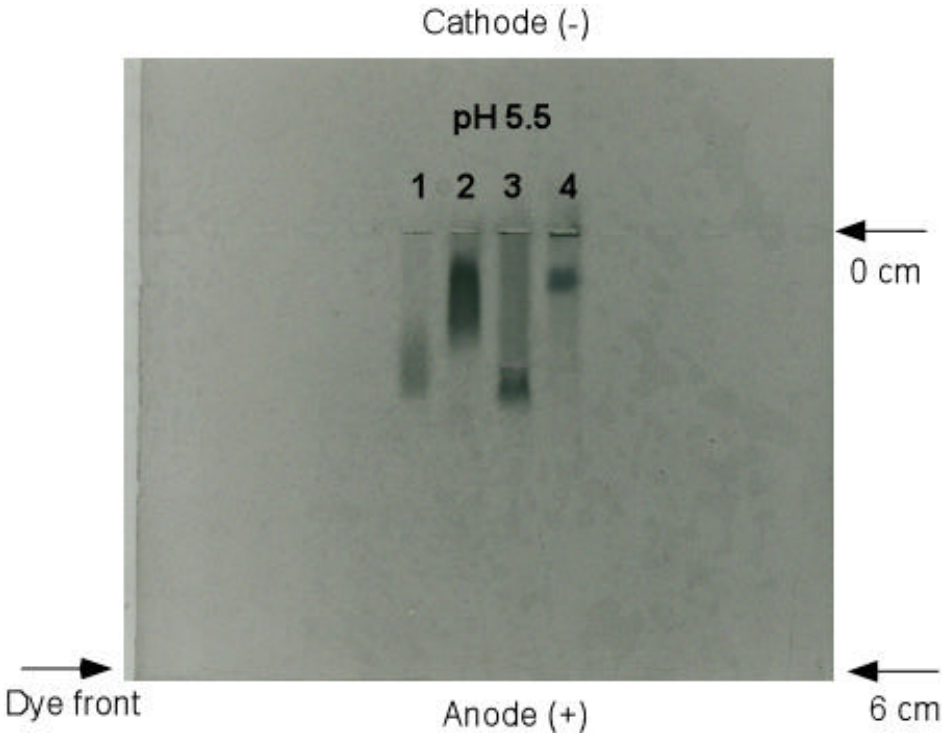

Figure 2B: Coomassie blue stained 1% agarose low EEO gel with hPC in lane 1, rhPC in lane 2, α-pcasein in lane 3 and β-pcasein in lane 4 run with 10mM phosphate buffer at pH of 5.5.

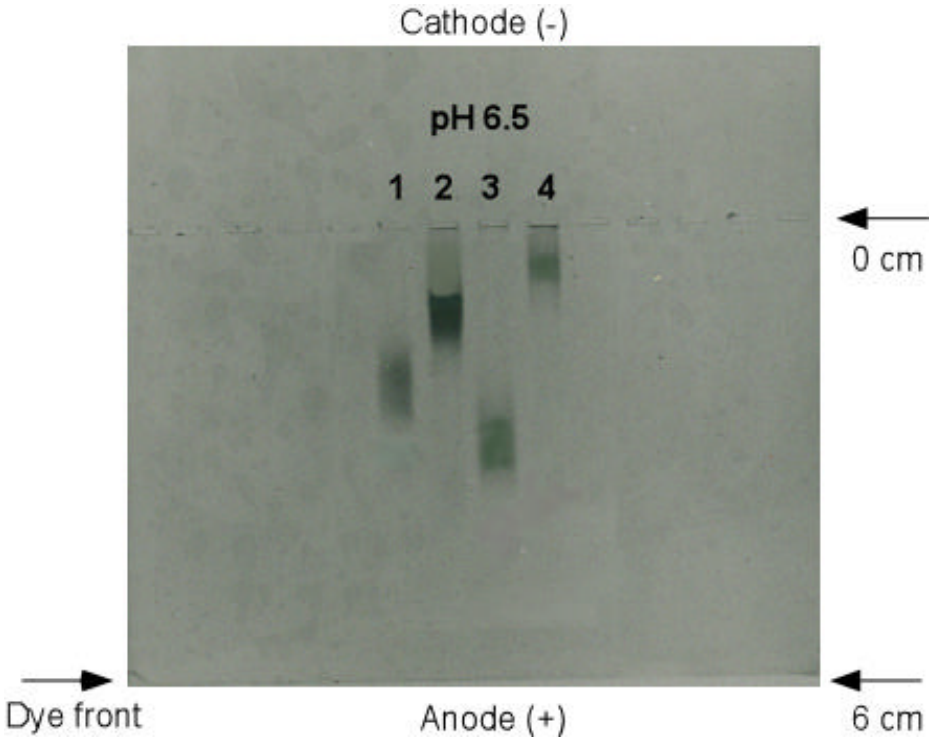

Figure 3B: Coomassie blue stained 1% agarose low EEO gel with hPC in lane 1, rhPC in lane 2, α-pcasein in lane 3 and β-pcasein in lane 4 run with 10mM phosphate buffer at pH of 6.5.

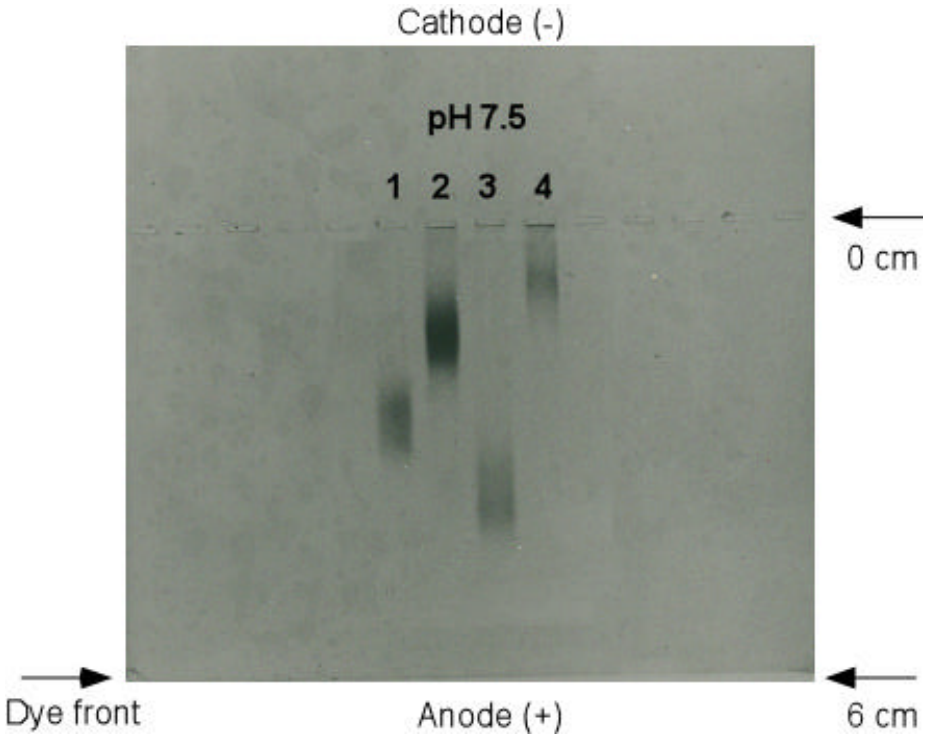

Figure 4B: Coomassie blue stained 1% agarose low EEO gel with hPC in lane 1, rhPC in lane 2, α-pcasein in lane 3 and β-pcasein in lane 4 run with 10mM phosphate buffer at pH of 7.5.

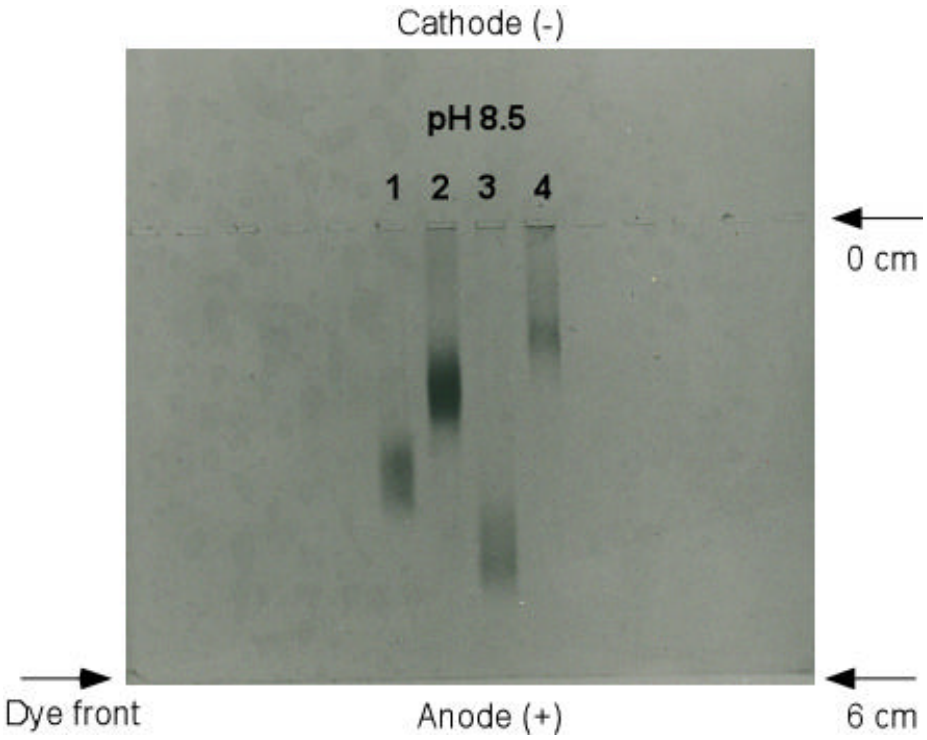

Figure 5B: Coomassie blue stained 1% agarose low EEO gel with hPC in lane 1, rhPC in lane 2, α-pcasein in lane 3 and β-pcasein in lane 4 run with 10mM phosphate buffer at pH of 8.5.

Appendix C

It was necessary to estimate the mobility of α_{s1}- and α_{s2}- relative to β-pcasein since from our experimental data we observed that the α-pcasein populations had a higher flux towards the anode than β-pcasein. The method of Compton [1] was used to make the relative mobility estimates and is presented below.:

Electrophoretic mobility model: If a protein is in an electrolyte solution with a unit filed strength, then the Debye-Huckle theory can be used to estimate the mobility.

$$m = (Ze/6\pi nr)(1/1+kr)f(kr)$$

Where μ is the electrophoretic mobility, Z is the protein valence, n is the solution viscosity, r is the stokes radius, $\phi(kr)$ is Henry's function which varies from 1.0 to 1.5 as kr goes from 0 to infinity and k is the Debye parameter

According to Oncley, the molecular weight of the protein can be related to the radius through the following expression [2]:

$$r = \left(\frac{3Mv}{4\pi n}\right)^{1/3} \frac{f}{f_0}$$

where M is the molecular weight, v is the partial specific volume ranging from 0.745 to 0.750 for natural proteins and f/f_0 is the frictional ratio ranging from 1.0 to 1.7 for globular proteins.

By substituting in the protein molecular weight for the radius, the following expression was developed:

$$m = \frac{K(1)Z}{K(2)M^{1/3} + K(3)M^{2/3}}$$

Where $K(1)$, $K(2)$ and $K(3)$ are constants depending upon solution conditions.

Relative Mobility Calculations: There were two assumptions made when estimating the mobility of α_{s1}- and α_{s2}-pcasein relative to β-pcasein. First, $K(1)$, $K(2)$ and $K(3)$ were the same for α_{s1}-, α_{s2}- and β-pcasein. Second, the molecular weights of α_{s1}-, α_{s2}- and β-pcasein varied by about 10%; therefore, they were assumed to be the same for calculation purposes. The relative mobilities are given as follows:

$$\frac{m_{a_{s1}}}{m_b} = \frac{Z_{a_{s1}}}{Z_b} \qquad \frac{m_{a_{s1}}}{m_b} = \frac{Z_{a_{s1}}}{Z_b}$$

$$\frac{m_{a_{s2}}}{m_b} = \frac{Z_{a_{s2}}}{Z_b} \qquad \frac{m_{a_{s2}}}{m_b} = \frac{Z_{a_{s2}}}{Z_b}$$

$$\frac{m_b}{m_b} = 1 \qquad \frac{m_b}{m_b} = 1$$

Protein Valence Calculation: Compton's adaptation to the method of Sillero and Ribeiro for calculating the net protein valence is given by:

$$Z = \sum_{n=1:4} \frac{Pn}{1 + 10^{(pH - pK(Pn))}} - \sum_{n=1:6} \frac{Nn}{1 + 10^{(pK(Pn) - pH)}}$$

where *pH* refers to the buffer. The values for *Pn* and *Nn* are the number of amino acids of that type. The *pK(Pn)* and *pK(Nn)* are the ionization potential of that amino acid. Table I below shows the *P* and *N* value, the amino acid type and the pK values; while Table II shows the amino acid composition used for α_{s1}-, α_{s2}- and β-pcasein to calculate the protein valence. The valence for each protein was calculated then the ratio taken with respect to β-pcasein.

A comparison between experimental and relative mobility at pH 7.0 is listed in Table III. The predicted relative mobility of rhPC with respect to β-pcasein had a 30% error compared to the experimental relative mobility. The predicted relative mobility of α-pcasein with respect to β-pcasein had a 40% error compared to the experimental relative mobility. From this we can qualitatively say that the separation of α-pcasein and rhPC from β-pcasein can be predicted by the above equations.

Table I: Ionization constant for each amino acid

Amino acid type		pK
P1	tNH_2	8.2
P2	HIS	6.4
P3	LYS	10.4
P4	ARG	12
N1	tCOOH	3.2
N2	ASP	4
N3	GLU	4.5
N4	CYS	9
N5	TYR	10
N6	Phosphate	6.8

Table II: α_{s1}-, α_{s2}- and β-pcasein amino acid composition for protein valence calculation

	Pig casein		
Amino acid type	α_{s1}	α_{s2}	β
tNH_2	1	1	1
HIS	11	6	5
LYS	11	18	14
ARG	10	10	6
tCOOH	1	1	1
ASP	14	18	10
GLU	46	43	41
CYS	0	2	0
TYR	8	11	4
Phosphate	8	17	8

Table III: Estimated and experimental relative mobility measurements at pH 7.0.

Protein	Experimental relative mobility	Estimated relative mobility
α-pcasein	2.5	1.5
rhPC	1.7	1.2
β-pcasein	1	1

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Vita

Arthur William Degener, Jr. was born in Baltimore, Maryland on May 7, 1963. Art graduated from Woodlawn High School in 1981. He received his Bachelor of Science degree in Chemical Engineering from Virginia Polytechnic Institute and State University in 1988. Art joined the doctoral program at Virginia Polytechnic Institute and State University in the fall of 1989. In the future, Art intends to work in the industrial engineering research in the field of biotechnology. Art and his wife, Laurel Hobbs Degener, have two children, a daughter, Jessica Marie Degener and a son, Bradley William Degener, and live in Blacksburg.