UPSTREAM AND DOWNSTREAM ISSUES IN THE PRODUCTION OF RECOMBINANT VITAMIN K-DEPENDENT PROTEINS IN THE MILK OF TRANSGENIC PIGS

by

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(Abstract)

The short supply and pathogen safety concerns for plasma-derived therapeutic proteins, such as protein C and Factor IX, have spurred the development of recombinant DNA technology for large-scale, economically feasible production of therapeutic proteins. The mammary gland is unique in its ability to synthesize and secrete large amounts of protein into a harvestable medium, so transgenic livestock secreting recombinant proteins into their milk can be a viable method for the large-scale production of certain proteins. Pigs offer several distinct advantages over other dairy livestock for production of certain recombinant proteins in milk: rapid herd development since pigs have a one year generation time and sows average about two litters per year (about 21 offspring per year), and lactating sows produce an average of about 10 kg of milk/day. The above factors, combined with the excellent safety record of parenteral porcine derivatives, have been the basis for our work on the development of recombinant vitamin K-dependent protein production in porcine milk. This thesis contains the first collection of articles meant to specifically address issues that will affect FDA clinical trials and approval of recombinant VKD proteins from the milk of transgenic pigs. We report on the genotypic and phenotypic stability in lines of transgenic pigs secreting recombinant protein C. In addition, this work details the first reported effects of recombinant protein production on the endogenous milk protein composition of livestock, the first reported production of high levels of biologically active recombinant Factor IX, and the purification of biologically active and inactive subpopulations of recombinant protein C. Our observations suggest that pigs may be used as transgenic bioreactors for large-scale production of protein C and Factor IX.

Acknowledgments

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Chapter 1: Introduction: Vitamin K-dependent Proteins, Regulatory Issues for Transgenic Livestock

Vitamin K-Dependent Proteins

Plasma vitamin K-dependent proteins are necessary for the regulation of hemostasis. All vitamin K-dependent (VKD) proteins are distinguished by the γ-carboxylation of glutamic acid residues in the N-terminal region of the protein, an enzymatic process requiring vitamin K as a cofactor (Wu *et al.*, 1991). This domain is known as the "Gla domain," and facilitates the VKD protein's interaction with the phospholipid membrane of the endothelial cells and platelets (Ahmad *et al.*, 1994; Jacobs *et al.*, 1994; Nelsestuen *et al.*, 1978). Vitamin K-dependent proteins in plasma include protein C (hPC), Factor IX (FIX), Factor X, Factor VII, prothrombin (Factor II), and Protein S. Factor IX, Factor X, Factor VII, and prothrombin are all pro-coagulant enzymes in the clotting cascade (See Figure 1, Coagulation cascade). Protein C and Protein S have anticoagulant activity in the cascade, inactivating Factor VIIIa and Factor Va (Figure 1). Deficiencies in active vitamin K-dependent proteins can be caused by insufficient circulating levels, by incorrect post-translational modifications (PTM), or by mutations in the amino acid sequence, and can result in serious blood coagulation disorders. The two vitamin K-dependent proteins of interest in this work are protein C and FIX.

Protein C is a serine protease synthesized in the liver and circulates in plasma at a concentration of 4 μg/ml (Stenflo, 1976; Kisiel, 1979) (Figure 2). A number of post-translational modifications are made to the immature polypeptide (McClure *et al.*, 1992): (1) removal of signal peptide and propeptide, (2) γ-carboxylation of nine glutamic acid residues, (3) β-hydroxylation of Asp^{71}, (4) removal of Lys^{156}-Arg^{157}, (5) N-linked glycosylation of 4 sites, and (6) formation of 12 disulfide bridges. Mature hPC circulates as a disulfide linked heterodimer of a heavy chain (M_r ~41kDa) and a light chain (M_r ~ 21 kDa). The light chain contains the gla domain and the epidermal growth factor domain (EGF domain). The heavy chain contains the serine protease domain. Protein C is activated in a calcium-dependent manner by a complex of Protein S, thrombin and thrombomodulin at the endothelial cell surface, releasing a dodecapeptide (Esmon 1985,

Esmon 1989). Activated hPC proteolyses Factor Va and Factor VIIIa (Esmon 1985).

Protein C has been identified as having therapeutic value for a number of thrombotic conditions. Homozygous and heterozygous human protein C deficiency has also been identified in the population (Mann and Bovill, 1990). Warfarin induced tissue necrosis, heparin induced thrombocytopenia, septic shock, fibrinolytic therapy, angioplasty, and unstable angina have all been successfully treated with protein C (Comp, 1990). Current treatments of thrombosis include 'artificial' anticoagulants such as warfarin or heparin. However, warfarin and heparin are not under the stringent regulatory control of hemostasis, and can eventually cause more thrombosis. For example, treatment of thrombosis with warfarin, which blocks the γ-carboxylation in the gla domain of plasma proteins, can lead to tissue necrosis in some patients (Comp, 1990). The low concentration in plasma (4 μg/ml) precludes the use of donor plasma as a source for meeting the anticipated clinical demand for hPC, about 100 kg/year in the US (Paleyanda *et al.*, 1991). In addition, transmission of blood-borne pathogens such as Hepatitis B, HIV, and prions is an inherent risk (Barrowcliffe *et al.*, 1993; Velander *et al.*, 1989; Velander *et al.*, 1990).

Factor IX is a serine protease homologous to hPC (Figure 3). FIX is synthesized in the liver and circulates in plasma at a concentration of about 4 μg/ml. Similar PTMs to that of hPC are made to FIX (Yoshitake *et al.*, 1985; Kurachi *et al.*, 1993): (1) removal of the signal peptide and propeptide, (2) γ-carboxylation of 12 glutamic acid residues in the light chain, (3) β-hydroxylation of Asp^{63}, (4) N-linked glycosylation of two sites in the activation peptide, (5) O-linked glycosylation of two sites in the activation peptide and two sites in the light chain, and (6) formation of 11 disulfide bridges. Factor IX circulates as a single chain protein of about 65 kDa. FIX can be activated by either Factor XIa or Factor VIIa/Tissue Factor in a calcium dependent manner to release a 35 amino acid activation peptide (Kurachi *et al.*, 1993). Activated FIX is a disulfide-linked heterodimer of a light chain and heavy chain and acts in concert with FVIIIa to activate Factor X (Kurachi *et al.*, 1993).

Factor IX deficiency results in hemophilia B, and can be caused by either low

circulating levels of the molecule or a functional deficiency of the molecule. More than 278 different mutations have been identified for being responsible for hemophilia B (Kurachi *et al.*, 1993). Hemophilia B has been treated with Factor IX concentrates or purified Factor IX derived from donor plasma (Kurachi *et al.*, 1993; Littlewood *et al.*, 1987; Kingdon *et al.*, 1975; Barrowcliffe, 1993). Episodal treatment of bleeding has been traditionally used, but early prophylactic treatment of hemophiliacs by regular infusion of purified FIX into the patient's bloodstream is a therapeutic goal (Skolnick, 1994; Manco-Johnson *et al.*, 1994). Prophylaxis requires more FIX than episodal treatment, but reduces the risk of brain hemorrhage and joint damage, enabling patients to lead a more normal lifestyle (Skolnick, 1994; Manco-Johnson et al., 1994; Nilsson *et al.*, 1992). However, the low concentration of FIX in plasma results in a shortage of available FIX for prophylaxis (Paleyanda *et al.*, 1991; Lubon *et al.*, 1996). In addition, there is also a risk of the transmission of blood-borne pathogens from plasma-derived products (Velander *et al.*, 1989; Velander *et al.*, 1990; Barrowcliffe, 1993).

Recombinant Methods for VKD Protein Production

The inadequate supply and viral safety concerns for plasma-derived proteins such as hPC and FIX have led to the use of recombinant methods for production of these proteins. Genetically engineered plants, prokaryotes, and yeast and are adequate for producing simple mammalian proteins, but they lack the cellular machinery to perform the complex post-translational processing required by human vitamin K-dependent proteins (Yan *et al.*, 1989). Mammalian cell lines have been used to produce a number of VKD proteins, including protein C and FIX. Investigators have succeeded in producing fully active recombinant hPC (rhPC) in a human kidney 293 cell line (Grinnell *et al.*, 1990) and partially active recombinant FIX (rhFIX) in CHO cell lines (Wasley *et al.*, 1993; Rehemtulla *et al.*, 1993; Kaufman *et al.*, 1986). However, the feasibility of mammalian cell culture is limited. Expensive fermentation equipment and low maximum cell densities during fermentation limit the productivity and economic viability of this method (Lubon *et al*, 1996).

In an effort to produce large amounts of complex recombinant protein, attention

was turned toward using the mammary gland of livestock as the production "bioreactor" (Pursel and Rexroad, 1993; Morcol *et al.*, 1994; Lubon *et al.*, 1996). The mammary gland is specifically designed for the synthesis and secretion of large amounts of protein, making it an ideal organ for protein expression. The generation of founder transgenic animals and transgene construct strategies have been reviewed elsewhere (Wall *et al.*, 1992). A number of different recombinant proteins have been expressed in the milk of transgenic livestock: α-1-antitrypsin in sheep (Wright *et al.*, 1991; Carver *et al.*, 1993), tissue plasminogen activator in goats (Denman *et al.*, 1991; Ebert *et al.*, 1991), rhPC in pigs (Velander *et al.*, 1992; Morcol *et al.*, 1994), and Mouse Whey Acid Protein (WAP) in pigs (Wall *et al.*, 1991). Pigs offer several advantages over other dairy livestock for rapid generation of transgenic herds and production of recombinant therapeutic proteins: pigs have short generation times of about one year, the average sow produces about 21 offspring per year, and a lactating sow produces about 10 kg milk per day. Lee *et al.* (1995), Velander *et al.* (1992), Morcol *et al.* (1994), and Van Cott *et al.* (1996), have all demonstrated that pigs are capable of producing active rhPC. Even though per-cell synthesis rates of the recombinant proteins may be equivalent to that found in mammalian cell cultures, the high cell density makes the mammary gland a more productive bioreactor (Morcol *et al.*, 1994). Rate limitations to certain PTMs have been observed in the mammary gland (Van Cott *et al.*, 1996; Drews *et al.*, 1995; Lee *et al.*, 1995), but methods of reducing certain rate limitations by making a "bigenic" animal have already been published (Drews *et al.*, 1995).

Regulatory Issues in Production of Recombinant Proteins in Transgenic Animals

One of the important remaining issues in the brief history of transgenic livestock is Food and Drug Administration (FDA) approval of a recombinant therapeutic protein from a transgenic animal. Clinical trials on anti-thrombin III, an anticoagulant protein produced in goat milk by Genzyme Transgenics, and α-1-antitrypsin, produced in sheep milk by PPL Ltd., are scheduled to begin in 1996 (Rudolph, 1995).

Regulatory guidelines have been identified by the FDA and Center for Biologics Evaluation and Research (CBER) in their document titled "Points to Consider in the

Manufacturing and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals" (Document No. 95D-0131, 1995). Upstream issues include (1) generation and characterization of the founder animal, (2) genetic stability of the transgenic line of animals, (3) stability of protein expression, and (4) maintenance of a healthy production herd. Downstream issues include (1) defining the production herd and product lot, (2) defining pathogen concerns and methods for pathogen removal, (3) purification and characterization of the product, and (4) the preparation of the product for human use.

The purpose of this thesis is to investigate some of the upstream and downstream issues in the production of recombinant human protein C and recombinant human Factor IX in the milk of transgenic pigs. Throughout this work, the concept of the mammary gland as a bioreactor is emphasized. An engineering analysis of the mammary gland leads to the identification of similar biochemical reaction processes, nutrient requirements, product harvesting, and downstream processing of a typical mammalian cell culture bioreactor. The upstream and downstream issues discussed here are important to the recovery of a stable and reproducible final product, whether it is from a transgenic animal or a traditional bioreactor. As with any engineering analysis of bioreactor design, an iterative process taking into account how upstream events interact with and affect downstream processing is necessary for optimization of the bioreactor (Figure 4). Upstream issues addressed here will include the genotypic and phenotypic stability of four lines of transgenic pigs producing rhPC and the effects of expression of recombinant proteins on endogenous milk protein composition and mammary gland function. Downstream issues addressed here will be the first-reported production and purification of active rhFIX in a transgenic animal, the purification of rhPC by scalable methods, and fractionation of biologically active and inactive subpopulations of rhPC. This work represents initial steps made in characterizing a transgenic herd of pigs with multiple founders.

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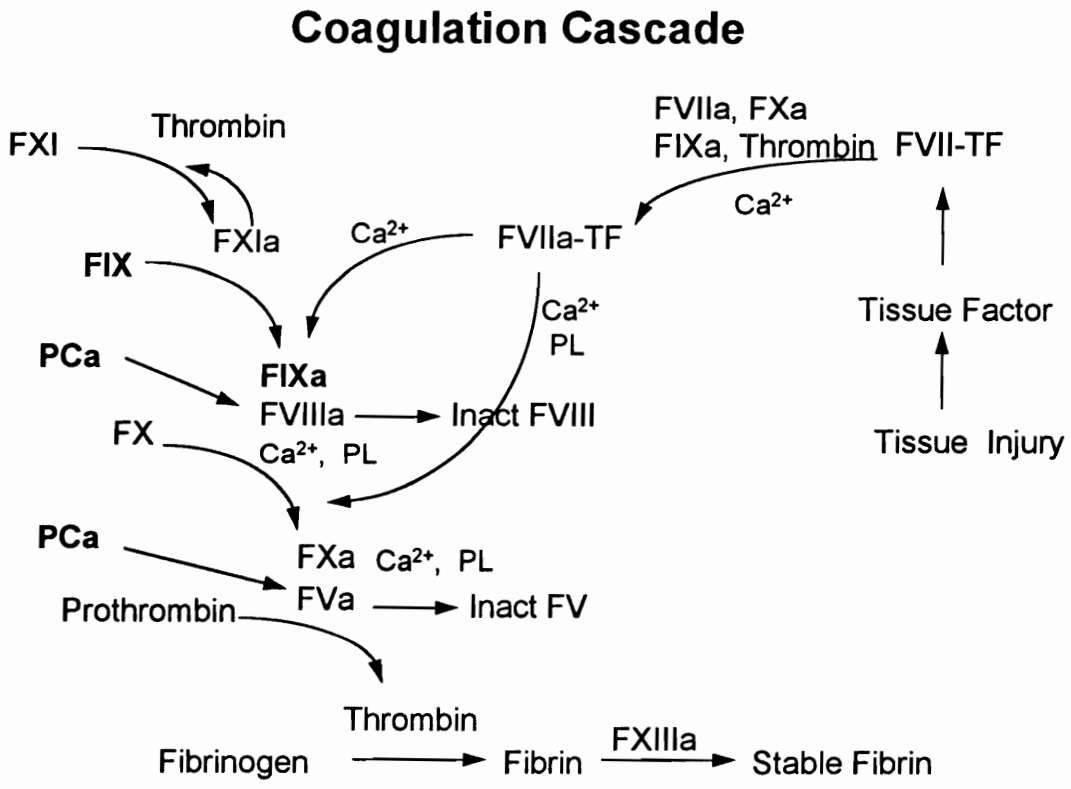

Figure 1. Schematic of Blood Coagulation Cascade

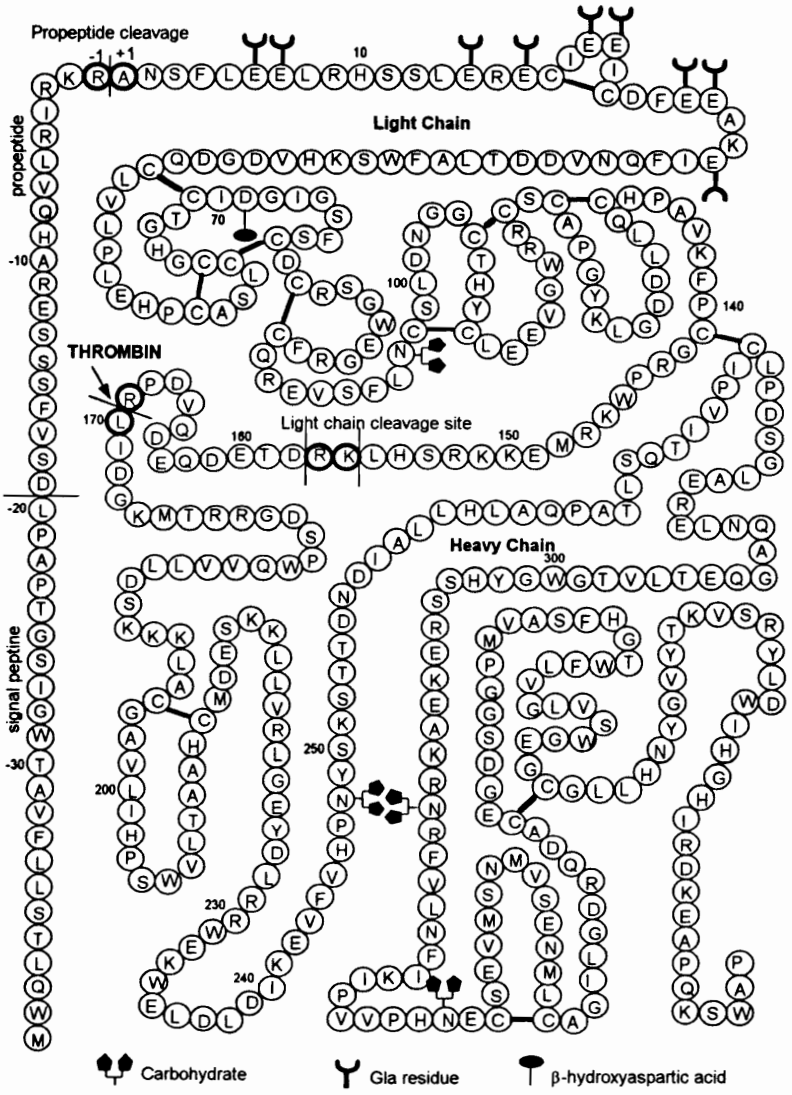

Figure 2. Schematic of Human Protein C

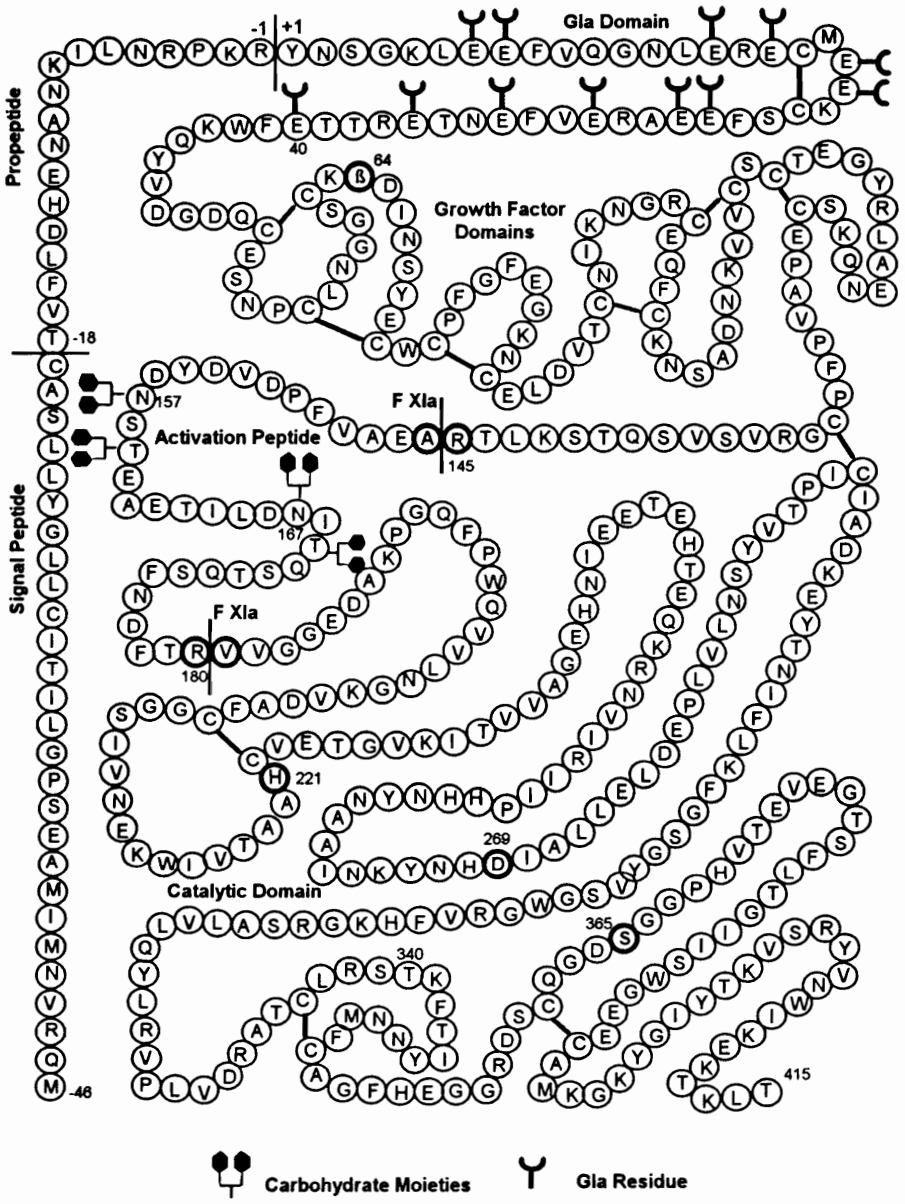

Figure 3. Schematic of Human Factor IX

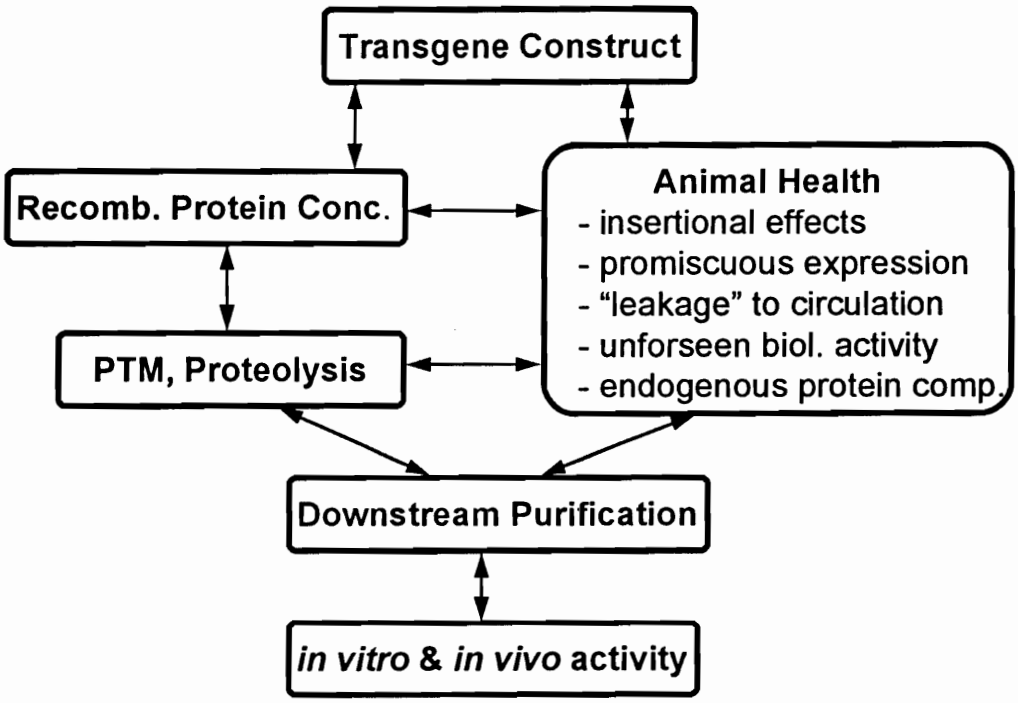

Figure 4. Iterative process of transgenic animal bioreactor design, and interactions between upstream and downstream events.

Chapter 2: Phenotypic and Genotypic Stability of Multiple Lines of Transgenic Pigs Expressing Recombinant Human Protein C

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Key words: protein C, transgenic livestock, recombinant, mammary gland

Abbreviations: hPC, human protein C; rhPC, recombinant human protein C; WAP, whey acid protein; EDTA, ethylene diamine tetra-acetic acid; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay; HRP, horseradish peroxidase; TBS, Tris buffered saline

Abstract

The genotypic and phenotypic stability of four lines of transgenic pigs expressing recombinant human protein C in milk was examined. Two lines were established with a construct consisting of a 2.6 kb mouse WAP promoter and a 1.5 kb human protein C cDNA. Two lines were established with another construct consisting of a 4.1 kb mouse WAP promoter and a 11.1 kb human protein C genomic DNA. Genotypic stability was measured by transgene copy number transmission. Outbred offspring having a single transgene integration locus were established from a founder having three independent, multicopy loci. Phenotypic stability over multiple lactations was defined by the combination of recombinant human protein C expression levels and the isoform signature of recombinant human protein C in western blots. Both cDNA and genomic human protein C transgenes gave similar ranges of expression levels of about 100-1800 μg/ml. Within a given outbred lineage having a single loci for the cDNA transgene, the expression levels ranged from about 100-400 μg/ml. Western blots of reduced recombinant protein C revealed that single chain content was not dependent on expression level and was consistent within each transgenic line, but varied between transgenic lines. This suggests that native swine genetics may play a role in selection of production herds with optimal post-translational proteolytic processing capability. Although swine are not conventional dairy livestock, we have shown that the short generation times, multiple offspring per litter, stable paternal transmission of the transgene, and milk production capabilities of swine offer distinct advantages over conventional dairy livestock for the establishment of a herd producing a therapeutic recombinant protein.

Introduction

Pigs are a well-defined livestock species that have been bred and developed for meat production for centuries. Crude extracts and purified proteins from pigs have been used for treatment of various diseases in human patients. In the last 40 years, diabetes and hemophilia A have been treated by parenteral administration of porcine insulin and porcine Factor VIII, respectively, from slaughtered pigs (Lollar *et al.*, 1988; Brettler *et al.*, 1989; Gatti and Mannucci, 1984). The short supply of human organs and blood has stimulated genetic engineering of pigs for xenotransplantation of animal organs into humans (Fodor *et al.*, 1994) and production of recombinant human hemoglobin in porcine erythrocytes (Sharma *et al.*, 1994). Porcine intestinal submucosa also has been studied for its potential in xenograft applications (Sandusky *et al.*, 1992; Lantz *et al.*, 1993). The development of specific pathogen free (SPF) pig herds, the apparent resistance of pigs to transmissible spongiform encephalopathy (Dawson *et al.*, 1990), and the general resistivity of pathogen transmission between humans and pigs (Weiss *et al.*, 1984; Wattanavijarn *et al.*, 1985; Joklik *et al.*, 1988) all contribute to making the pig a strong candidate for production of parenteral therapeutics.

Transgenic livestock secreting recombinant proteins into their milk can be a feasible method for the large scale production of certain proteins. The mammary gland is notable in its ability to synthesize and secrete large amounts of protein into a harvestable medium, and thus can be an ideal production system for large amounts of complex recombinant proteins (Lubon *et al.*, 1996; Pursel and Rexroad, 1993; Wilmut *et al.*, 1990; Wall *et al.*, 1992). Although pigs are not thought of as dairy animals, they offer several distinct advantages over sheep, goats, and cows for large scale production of certain recombinant proteins in milk: sows have a relatively short generation time of one year, sows average about two litters per year and about 21 offspring per year, sows produce an average of about 10 kg of milk/day, and it is possible to induce lactogenesis in transgenic virgin sows (Whittemore, 1993; Shamay *et al.*, 1992). In our previous efforts, we have shown that biologically active recombinant human protein C (rhPC) can be produced in transgenic pig milk and purified by scalable processes (Velander *et al.*, 1992a; Lee *et al.*, 1995; Van Cott *et al.*, 1996; Degener *et al.*, 1996). Recently we have

developed transgenic pigs producing high levels of biologically active recombinant human Factor IX, Factor VIII, and fibrinogen in milk (unpublished data). The above factors, combined with the excellent safety record of parenteral porcine derivatives, have been the basis for our work on the development of recombinant protein production in porcine milk.

Future developments in genetic engineering of pigs for recombinant protein production will require the understanding of transgene transmission in multiple lines and protein expression during multiple lactations. The objective of this study was to examine the feasibility of using transgenic pigs as a genotypically and phenotypically stable source pool of recombinant human protein C (rhPC). We report on the propagation of these lines of transgenic pigs and relate genotypic stability to transgene transmission, and phenotypic stability to the concentration of rhPC and isoform signature of rhPC secreted into the milk. Our observations suggest that pigs may be used as transgenic bioreactors for large-scale production of recombinant proteins.

Materials and Methods

Generation of Transgenic Swine

Construction, purification, introduction into the swine genome and detection of the WAP-PC1 cDNA transgene has previously been described (Velander *et al.*, 1992a; Velander *et al.* 1992b). Construction, purification and detection of the WAP-genomic-PC hybrid transgene have been described earlier (Drohan *et al.*, 1994a). In this paper we study two founders (29-2 and 83-1) and outbred offspring having a WAP-PC1 cDNA construct, and two founders (110-1 and 110-3) and outbred offspring having a WAP-genomic-PC construct.

Copy Number Estimation

The 29-2 line WAP-PC1 copy number was confirmed by dot blot hybridization (described below), PCR of serial DNA dilutions and southern blot. Dot blot analysis was performed as follows. DNAs were quantitated in triplicate using a TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Successive two-fold dilutions of each sample were made, resulting in applied samples of 5, 2.5, 1.25, and 0.625 micrograms of genomic DNA per well. Nontransgenic pig DNA was used as a negative control. For a positive control, the transgene construct DNA was spiked into the nontransgenic pig DNA at molar ratios of 1:1 and 10:1 (transgene:pig DNA). DNA was denatured by using 0.4 N NaOH (final concentration) and heating to 95°C for five minutes and then applied to a Hybond N+ (Amersham Life Sciences, Inc., Arlington Heights, IL) membrane using a Bio-Dot apparatus (Bio-Rad, Inc., Hercules, CA) . The membrane was rinsed three times in 200 ml 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) and UV crosslinked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA) on Autocrosslink mode. The hybridization probe was prepared by digesting WAP-PC1 transgene DNA with EcoRI and KpnI restriction enzymes followed by agarose gel electrophoresis and selection of the 2.5 kb band, which corresponds to a portion of the promoter region of the Whey Acidic Protein gene common to both transgenes. The probe was labeled with α-^{32}P dCTP by random priming (Redi-Prime, Amersham Life Sciences) followed by gel-filtration chromatography on a NAP-5 column (Pharmacia Biotech, Uppsala, Sweden). The membrane was pre-

washed in 0.1X SSC, 0.1% SDS and rinsed in 0.1X SSC, followed by equilibration in 6X SSC and prehybridization for one hour at 65°C in a Mini-6 hybridization oven (Savant-Hybaid, Halbrook, NY). Rapid-Hyb buffer (Amersham) was used for both prehybridization and hybridization. The probe was heat-denatured (five minutes at 100°C) and added directly to the prehybridization buffer. Hybridization was continued for two hours followed by three post-hybridization washes of 1X SSC, 0.1% SDS, 65°C in the roller bottle, one wash for 10 minutes in 500 ml of the same buffer at 65°C, and a final rinse in room temperature 1X SSC. The membrane was then blotted briefly, wrapped in plastic wrap, and exposed to x-ray film (Kodak BioMax MS, NY) overnight at room temperature. After several autoradiographs, the radioactive samples on the membrane were punched out with a hole-punch and counted by liquid scintillation using ReadySafe scintillant and a Beckman LS 5801 Liquid Scintillation Counter (both Beckman Instruments, Fullerton, CA). The results were normalized for background and analyzed.

Milk collection and handling

Lactating sows were injected IM with 30-60 IU of oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk let-down. Letdown occurred 2-5 minutes after injection. Pigs were milked both by hand and by machine during the course of this study. There were no detected cases of clinical mastitis in the sows during the course of their lactations. A minimum of five daily samples were collected from each sow during lactation. Immediately after collection the milk was diluted 1:1 with 200 mM EDTA, pH 7.0 to solubilize the caseins and then frozen. Small aliquots (~1 ml) of the milk/EDTA mixture were taken and centrifuged for approximately 30 minutes at 3000g at 4°C. The fat layer was separated from the diluted milk/EDTA fraction and discarded. The milk/EDTA fraction was used for all further assays. Pools of the daily samples in each lactation were made by combining equal volumes of each daily milk/EDTA sample. In this study, all concentration values reported for milk were obtained from diluted milk/EDTA samples that were multiplied by a factor of 1.9 to account for dilution with EDTA and subsequent removal of milk fat.

rhPC Determination in Milk

The rhPC levels in milk were determined by polyclonal ELISA. Immulon II

microtiter plates (Fisher Scientific, Pittsburgh) were coated overnight with 100 μl/well of 5 μg/ml of rabbit anti-human Protein C (Sigma, St. Louis, MO) in carbonate buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6) . Triplicates of sample dilutions and hPC standard (American Red Cross, Rockville, MD) in TBS-BSA (Tris buffered saline, 20mMTris, 50 mM NaCl, 0.1% BSA, pH 7.2) were incubated at 37°C for 20 minutes. After washing the plates with TBST (TBS-0.5% Tween 20), 1:1000 goat anti-hPC (American Diagnostica, Greenwich, CT), followed by 1:1000 anti-goat IgG/HRP (Sigma) were incubated in the wells. Bound chromophore was detected with OPD substrate (Abbot, Chicago), the reaction was quenched with 3 N sulfuric acid after 3 minutes, and the plates were read at 490 nm.

Western Blot

Selected pools of milk/EDTA were directly analyzed for rhPC by western blots. Gradient SDS gels (8-16%) from Novex (San Diego, CA) were used for electrophoresis. A total of 100 ng of rhPC (determined by ELISA), 100 ng of hPC standard (American Red Cross, within the linear range for densitometric analysis), and 20 μg of total protein from a pool of nontransgenic milk were applied to the wells for each sample. After electrophoresis proteins were transferred to a PVDF (polydivinylidene difluoride) membrane (Bio Rad, Hercules, CA) according to the manufacturer's instructions. The membrane was washed with TBST for 10 minutes, followed by blocking with TBST-0.5% casein for 30 minutes. The membrane was developed with goat anti-hPC (American Diagnostica), followed by anti-goat IgG/HRP (Sigma), and the metal enhanced DAB immunostaining kit (Pierce). The developed membranes were scanned with a Shimadzu densitometer.

Results

Two lines of transgenic pigs having a 2.6 kb mouse WAP promoter and the 1.5 kb human protein C cDNA transgene (WAP-PC1) were studied. We have followed the inheritance of the WAP-PC1 transgene by copy number per genome analysis on DNA from founder, G1, and G2 pigs of the 29-2 line (Figure 1). Founder 29-2 had an estimated 10 copies of the transgene. G1 offspring inheriting either 5, 3, or 2 copies stably transmitted that same number of copies to their respective G2 offspring. Founder 83-1 had about 8-10 copies of the WAP-PC1 transgene (Table 1). One male G1 offspring, 62-2, transmitted 8-10 copies to its transgenic female offspring, 126-5 and 138-1. Transgenic G1 female 6-1, inherited the 8-10 integration site, also. G2 female 126-5 also transmitted 8-10 copies to transgenic G3 offspring (data not shown).

Two lines of transgenic pigs having a WAP-genomic-PC construct with a 4.1 kb mouse WAP promoter and 11.1 kb genomic human protein C transgene (Drohan *et al.*, 1994a) were included in this study. Genomic founder 110-3 had an estimated 30-40 copies of the transgene (Table 1). It appears that these copies segregated into a 18-28 copy locus (122-5 and two other G1 offspring), and a 7-12 copy locus, transmitted to one female offspring. Genomic founder 110-1 had 1-3 copies of the transgene (Table 1), and all transgenic G1 offspring inherited 1-3 copies.

The amount of rhPC in the daily milk samples from selected animals in each line was measured by polyclonal ELISA. The rhPC levels in the milk of pigs from founder 29-2 and her offspring are given in Figure 2A-C. The first lactation rhPC levels of 29-2 ranged from 200-500 µg/ml milk (Figure 2A). During both second and third lactations, the levels gradually increased from 200 µg/ml to 1000 µg/ml milk by the end of lactation. Both 115-6 and 115-7 had rhPC levels from 100-500 µg/ml milk in their first lactations. However, both pigs had levels of 100-200 µg/ml in their second and third lactations. G2 offspring 64-4 (from G1 115-6) had rhPC levels of about 150-250 µg/ml milk (Figure 2C).

Figure 3A-C shows the rhPC levels in the milk of founder 83-1 and her offspring. The concentration of rhPC during the first and second lactations of 83-1 varied between 100-200 µg/ml milk (Figure 3A). Midway through the third lactation, and continuing

through the fourth and fifth lactations, the rhPC expression level increased to 200-600 µg/ml milk. The G1 offspring 6-1 ranged from about 50-125 µg/ml milk during two lactations (Figure 3B). G2 offspring 126-5 and 138-1 obtained from transgenic G1 male 62-2 had rhPC levels of 400-600 µg/ml and 300-500 µg/ml milk, respectively (Figure 3C).

The concentration of rhPC in the milk of transgenic pigs with the WAP-genomic-PC construct is given in Figures 4-5. Founder 110-3 had rhPC levels between 1000-1800 µg/ml milk (Figure 4A), and we have measured rhPC levels up to 5000 µg/ml in selected daily milk samples of 110-3 (data not shown). G1 offspring 122-5 had slightly lower levels ranging from 750-1500 µg/ml milk (Figure 4B). Founder 110-1 had levels of 100-300 µg/ml milk during the first two lactations (Figure 5A). G1 offspring 114-7 had rhPC levels ranging from 150-250 µg/ml milk (Figure 5B).

Western blots of selected milk/EDTA pools were used to examine the quality of rhPC from pigs from different lines and with different expression levels (Figure 6A-C) . Western blots of serial dilutions of milk/EDTA and purified rhPC produced linear responses when analyzed by reflectance densitometry. Figure 6A shows pooled milk/EDTA from founder 29-2 (WAP-PC1 cDNA construct), G1, and G2 offspring. Figure 6B shows pooled milk/EDTA from founder 83-1 (WAP-PC1 cDNA construct), G1, and G2 offspring. Figure 6C shows pooled milk/EDTA from founders 110-1 and 110-3 (WAP-genomic-PC construct) and their respective G1 offspring. The nontransgenic control (Figure 6A, Lane 2), showed faint bands above and below the single chain form of hPC and rhPC, and no bands in the heavy and light chain regions.

Western analysis showed differences between rhPC and plasma derived hPC. We observed more single chain rhPC than hPC, lower molecular weight heavy chain isoforms (α, β, γ) of rhPC compared to plasma hPC, and an rhPC light chain doublet. Reflectance densitometry of the membranes showed that about 90% of purified plasma hPC is a disulfide-linked heterodimer: the single chain population accounted for less than 10% of the total hPC signal, which is similar to previously reported results (Heeb *et al.*, 1988). The single chain population of rhPC accounted for about 40-50% of the total rhPC signal in milk from animals in the 29-2 line, 45-55% of the total rhPC signal in milk from

animals in the 83-1 line, and 60-80% of the total rhPC signal in milk from animals in the 110-1 and 110-3 lines. These proportions were independent of the average expression level within the respective line (Table 1). Densitometric analysis of the light chain doublet indicated that about 20-40% of the total light chain doublet signal was from the higher molecular weight light chain band in all the milk samples. These proportions were not dependent on genetic line and expression level.

Discussion

Stability of transgene transmission with steady secretion of the recombinant protein is one of the requirements for transgenic animals used for production of therapeutic proteins (Docket No. 95D-0131, Center for Biologics Evaluation and Research, 1995). Stable mouse Whey Acid Protein (WAP) transgene transmission and WAP secretion in the milk of founder pigs and G1 offspring has been reported, but the agalactic phenotype associated with precocious WAP synthesis limits the feasibility of these pigs for extended analysis (Shamay *et al.*, 1991; Shamay *et al.*, 1992). Our study specifically shows that the production of recombinant human protein C in lines of transgenic pigs during multiple lactations of founders and outbred G1 and G2 offspring can be stable within the range of 100-1800 µg/ml milk.

We have used copy number to help identify various genotypes associated with the transgene. Mendelian transmission of a 2-copy locus in the 29-2 lineage and an 8-10 copy locus in the 83-1 lineage was seen in outbred G1 and G2 animals. About 50% of the offspring carried the WAP-PC1 cDNA transgene. Gene rearrangement or deletion is an important criterion for genotypic stability. PCR analysis of the WAP-hPC cDNA junctions to detect head-to-tail transgene rearrangement showed that 2-copy G1 and G2 animals in the 29-2 lineage were similar, and no deletions or rearrangements were apparent across the 500 bp region (data not shown). Thus, we have established that transmission of the WAP-hPC transgenes in pigs is similar to transmission of the α-1-antitrypsin transgene in sheep, where a founder having 12-16 copies/genome stably transmitted 8-10 copies and 2-4 copies to G1 and G2 outbred offspring (Carver *et al.*, 1993). Similar to transgene transmission data in transgenic sheep (Carver *et al.* 1993), paternal transmission of the transgene in pigs in the 83-1 lineage did not appear to affect secretion of the recombinant protein in the transgenic female offspring. Sperm banking from transgenic males can thus be a feasible method for rapid herd expansion with stable gene transmission.

In the case of the 29-2 line where we detected more than one integration loci, each locus was segregated by outbreeding, and segregation was related to the level of rhPC in the milk. The rhPC level copy number dependence within the 29-2 line appears to

be a result of the different integration loci of about 2, 3, and 5 copies, and we postulate that each locus contributed to the higher overall expression levels seen in 29-2. Thus the 29-2 lineage provides a good example of multiple, independently functioning loci. Due to the high protein expression levels achieved in founders with multiple loci (29-2 and 110-3), we postulate that the function of each locus was not grossly limited by competition for mammary tissue transcription factors which have been shown in mice to bind to the WAP promoter region (Lubon and Hennighausen, 1987). Developmental effects may have also played a role in the up-regulation of the WAP promoter near the end of lactation for founder animals 29-2 and 83-1. Up-regulation of WAP expression in mice normally occurs near the end of lactation (Burdon *et al.*, 1991). With the exception of the later lactations of founders 29-2 and 83-1, rhPC levels during the course of lactation were generally steady.

The rhPC concentration ranges we have obtained with both long and short WAP promoters and hPC cDNA and genomic transgenes are comparable to mouse WAP protein concentration ranges obtained in pigs and endogenous WAP expression in mice (Wall *et al.*, 1991; Shamay *et al.*, 1991). The range of rhPC secretion levels among transgenic pigs is similar to the range of α-1-antitrypsin concentration in sheep milk (Carver *et al.*, 1993). The 4.1 kb WAP-genomic-PC construct has been shown to greatly increase rhPC secretion up to 700 µg/ml in mice (Drohan *et al.*, 1994a), as compared to 3 µg/ml with the WAP-PC1 cDNA construct (Velander *et al.*, 1992b). However, the 11.1 kb genomic hPC and the 4.1 kb WAP promoter sequences gave similar ranges of rhPC levels in pigs (100-1800 µg/ml) to the 1.5 kb cDNA hPC and 2.6 kb WAP promoter (100-1000 µg/ml). The high number of transgene copies in genomic pigs 110-3 and 122-5 may be the cause of consistently high rhPC levels of $\geq$1000 µg/ml. Founder 110-1 had only 1-3 copies and rhPC expression levels comparable to the 2-copy offspring of the 29-2 WAP-PC1 lineage. However, the influence of positional effects of the location of the integration site found by several investigators using WAP driven transgenes should not be ruled out (Pittius *et al.*, 1988; Burdon *et al.*, 1991; McKnight *et al.*, 1994).

The significance of the range of expression in the milk of transgenic livestock can be countered by the lack of change in protein isoform signature over that range. A salient

feature of the isoform signature of rhPC is the presence of single chain rhPC. The amount of single chain rhPC population was not related to expression level within each line, nor was it related to rhPC level between different lines of transgenic pigs. This is in contrast to γ-carboxylation rate limitations observed among these four lines of transgenic pigs (Van Cott *et al.*, 1996), where the amount of properly γ-carboxylated rhPC was inversely proportional to the secretion level. Data presented in this study, in addition to previously reported results (Colman, 1995), suggests that general swine genetics may define rate limitations in proteolytic processing of recombinant proteins. For example, less than 20% of the rhPC from transgenic pigs studied here contained propeptide (Lee *et al.*, 1995), while about 50% of the rhPC produced by pigs in the study of Colman (1995) contained propeptide. Alternatively, rate limitations in proteolytic processing of rhPC propeptide and single chain cleavage can be corrected in mice by co-expression of the processing enzyme PACE, which also aids in cleavage of the propeptide (Drews *et al.*, 1995).

The presence of the WAP-protein C transgenes and secretion of rhPC in the milk did not produce adverse phenotypic characteristics in the transgenic pigs. All transgenic pigs had normal reproductive behavior, producing litters ranging from 6-15 offspring. Physiological abnormalities such as infertility, respiratory distress, and joint damage that are reported in transgenic pigs producing bovine growth hormones were not observed in our transgenic herd (Pursel *et al.*, 1989; Pinkert *et al.*, 1994).

In conclusion, we have found that a recombinant vitamin K-dependent protein can be stably secreted in the mammary gland of multiple lines of transgenic pigs over multiple lactations. Transgenic swine of this study produced a constant isoform signature over the range of about 100-1800 μg/ml over multiple lactations. Thus, the milk of transgenic pigs can be used as a stable source of recombinant therapeutic proteins. The screening of transgenic lines for stable production of therapeutic proteins can feasibly select for segregated sites with steady protein expression at levels which will best suit the purification of properly post-translationally modified protein. Comparison of the isoform signature of rhPC in this study with other transgenic swine studies suggests that the phenotypic differences between pigs, such as for proteolytic cleavage, may be specific to

the native swine genetics. Thus, native swine genetics which provide better proteolytic processing may be selected within outbred transgenic pig lines.

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Table 1. Summary of single chain as compared to amount of rhPC in the milk pools from pigs included in Figure 6.

Pig	Transgene / Generation	Copy Number	Lact.	Average rhPC [µg/ml milk]	% Single Chain Population
29-2	WAP-PC1 G0	10	2nd	450	40%
115-6	WAP-PC1 G1	2	1st	280	45%
			2nd	90	40%
			3rd	100	47%
115-7	WAP-PC1 G1	2	1st	305	49%
			2nd	150	49%
64-4	WAP-PC1 G2	2	1st	160	44%
83-1	WAP-PC1 G0	8-10	4th	300	37%
			5th	290	48%
6-1	WAP-PC1 G1	8-10	1st	70	47%
			2nd	95	58%
126-5	WAP-PC1 G2	8-10	1st	475	52%
138-1	WAP-PC1 G2	8-10	1st	340	47%
110-1	WAP-Genomic G0	1-3	1st	160	77%
			2nd	280	67%
114-7	WAP-Genomic G1	1-3	1st	200	63%
110-3	WAP-Genomic G0	30-40	1st	1200	56%
122-5	WAP-Genomic G1	18-28	1st	960	65%

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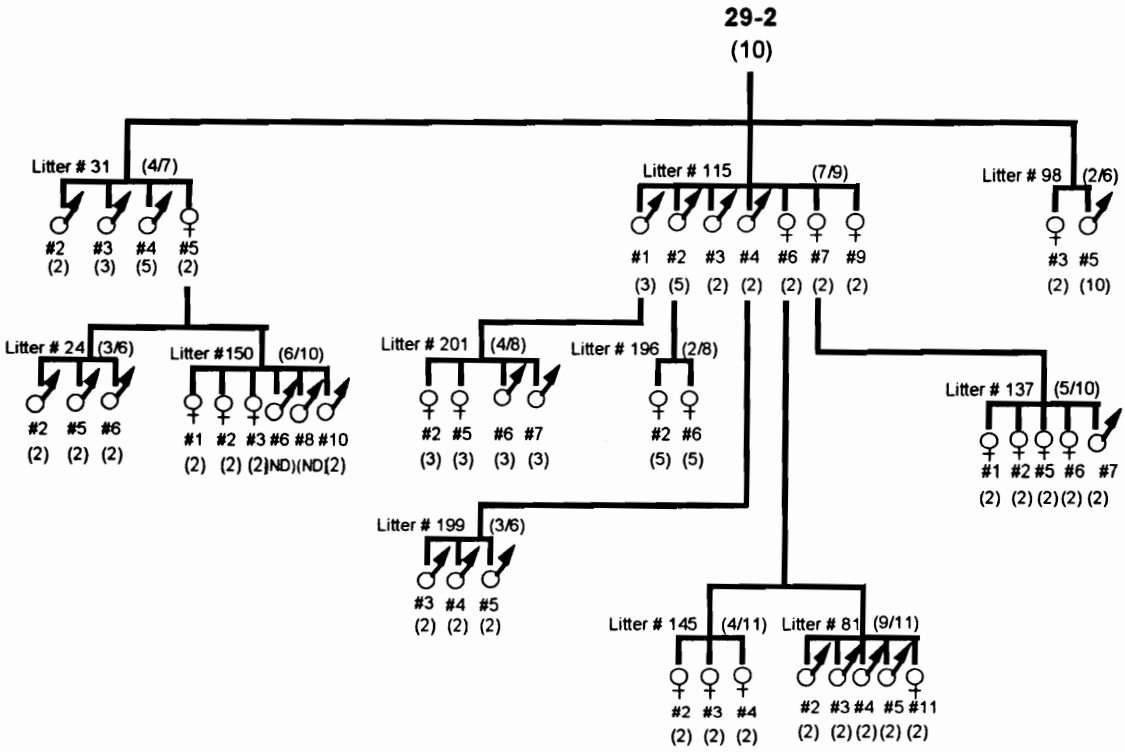


Figure 1. Family tree of the transgenic line from WAP-PC1 founder 29-2. Estimated transgene copy number of each animal is listed below the respective animal in parentheses. The number of transgenics per total pigs born is listed after the litter number (e.g., Litter #31, 4 transgenic of 7 total pigs born). ND: transgenic but copy number not determined.

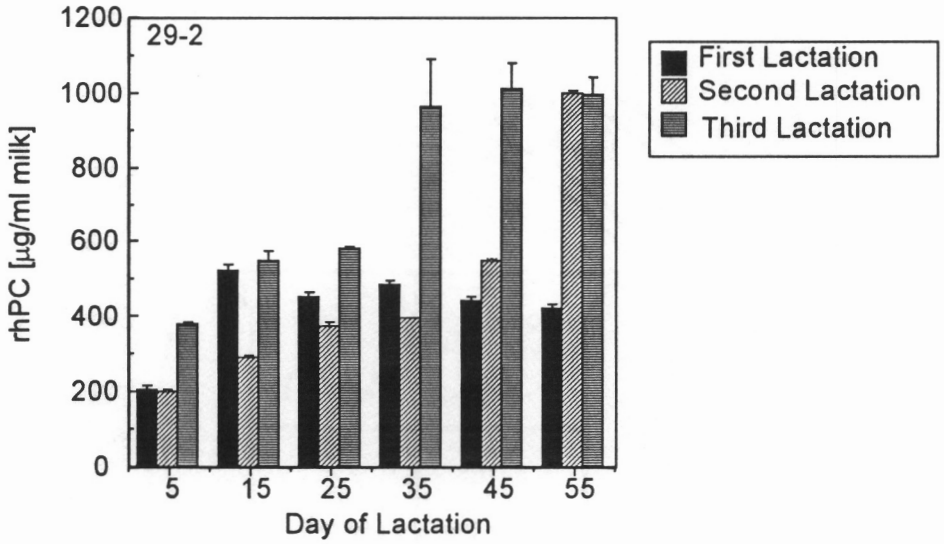
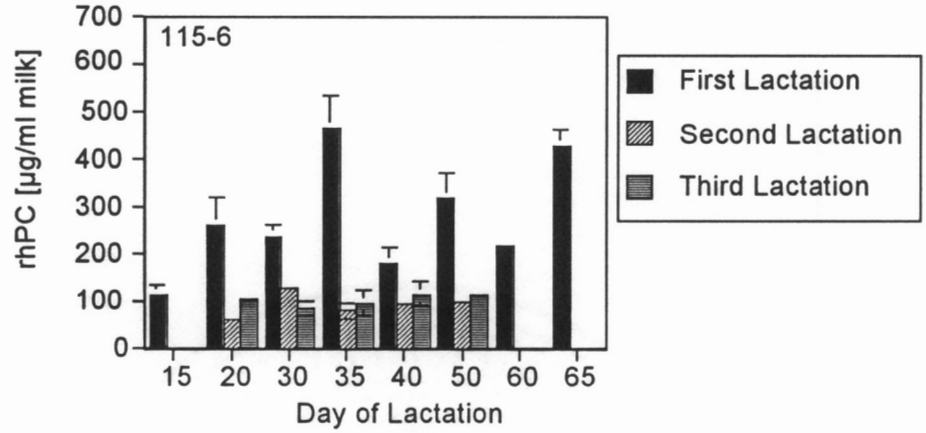


Figure 2A. rhPC levels in the milk of WAP-PC1 founder 29-2.



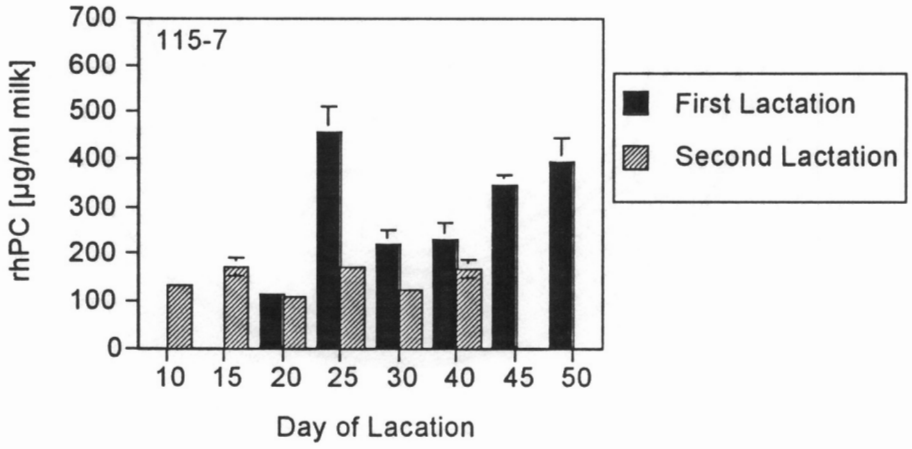


Figure 2B. rhPC levels in milk of 29-2 G1 outbred offspring 115-6 and 115-7.

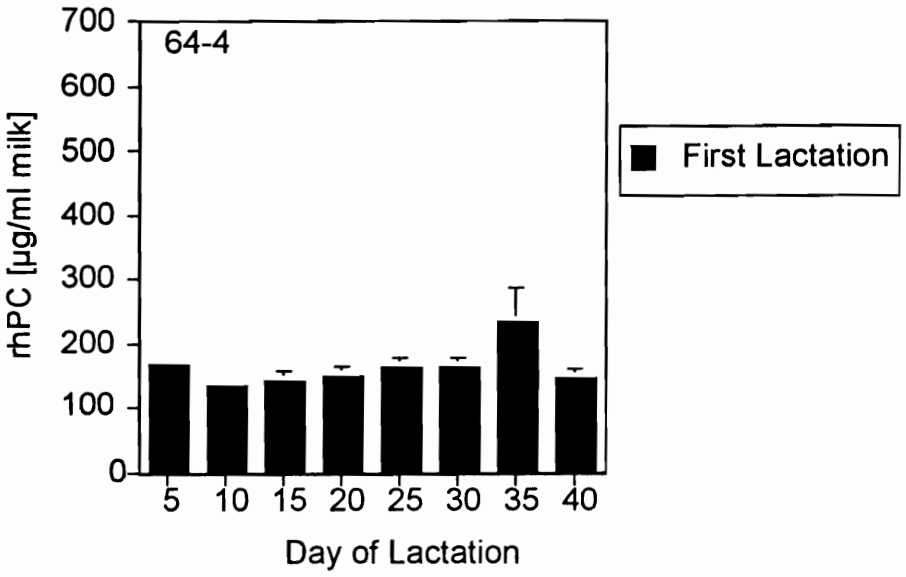


Figure 2C. rhPC levels in the milk of 29-2 G2 outbred offspring 64-4.

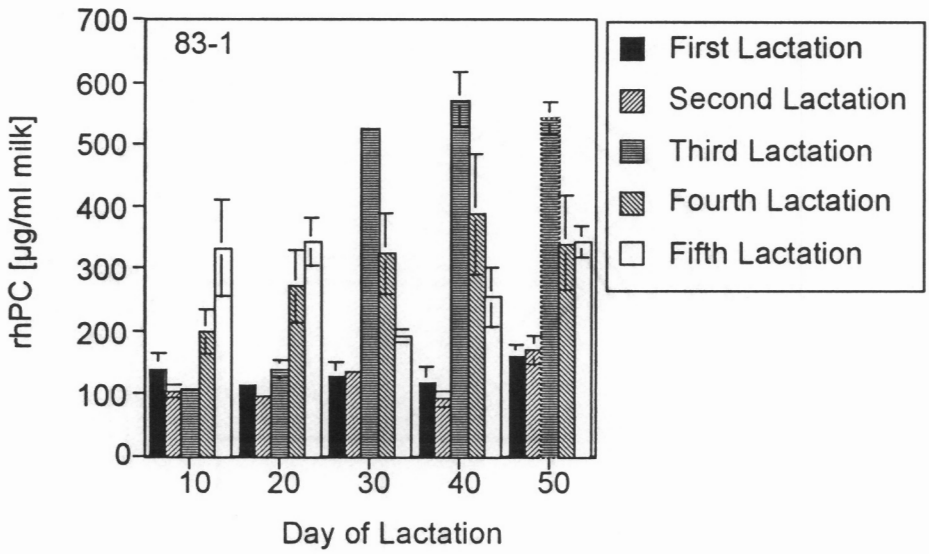


Figure 3A. rhPC levels in the milk of WAP-PC1 founder 83-1.

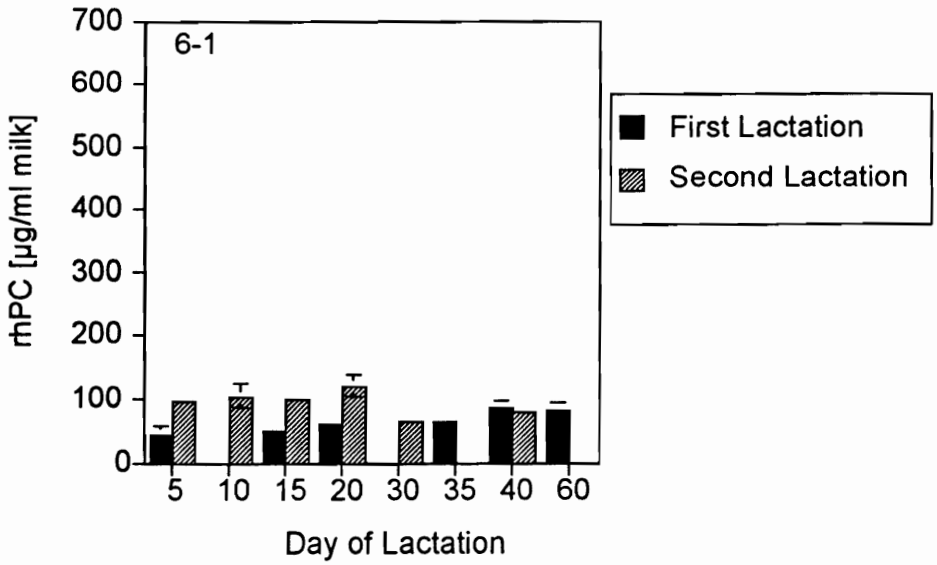


Figure 3B. rhPC levels in the milk of 83-1 G1 outbred offspring 6-1.

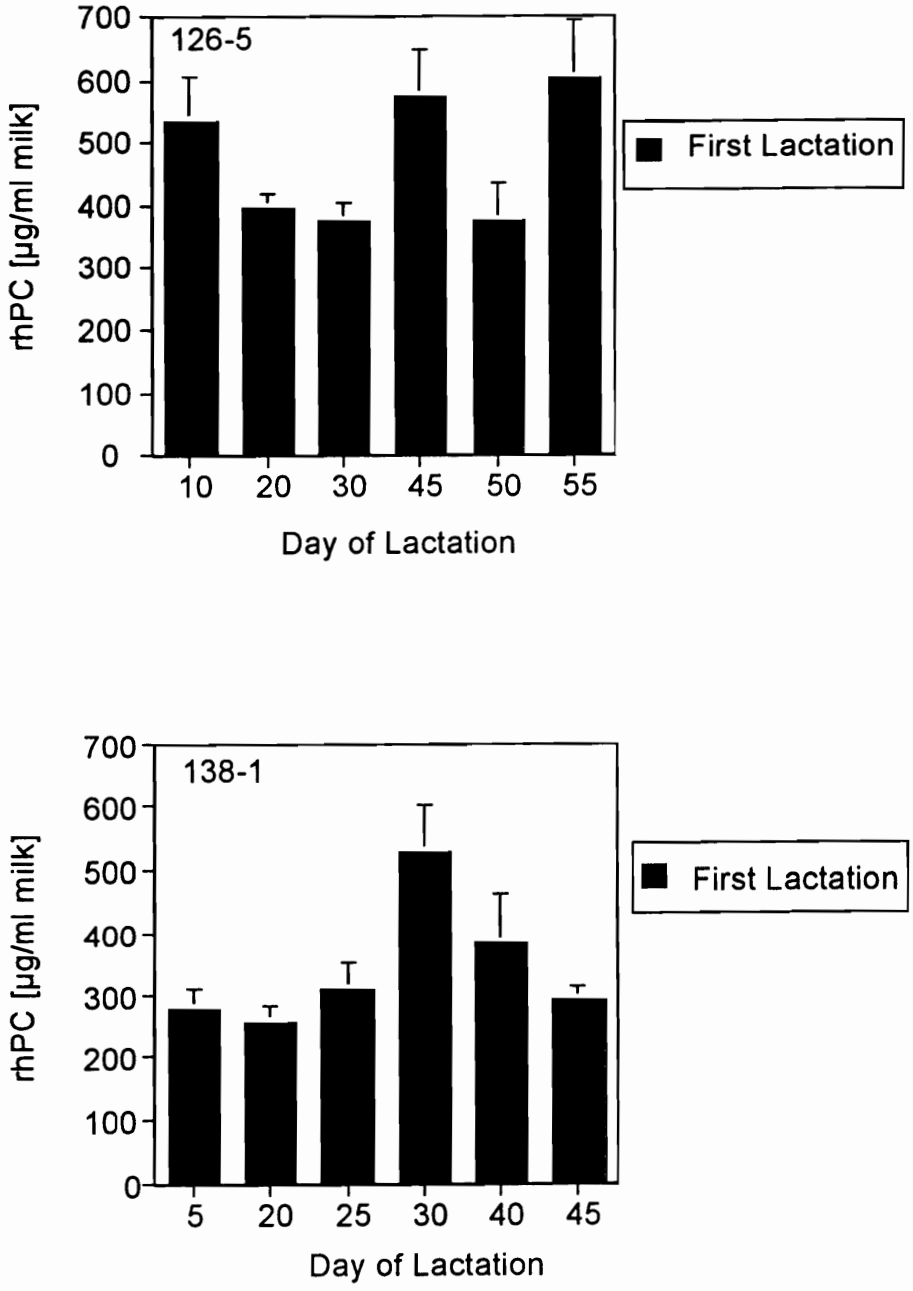


Figure 3C. rhPC levels in the milk of 83-1 G2 outbred offspring 126-5 and 138-1.

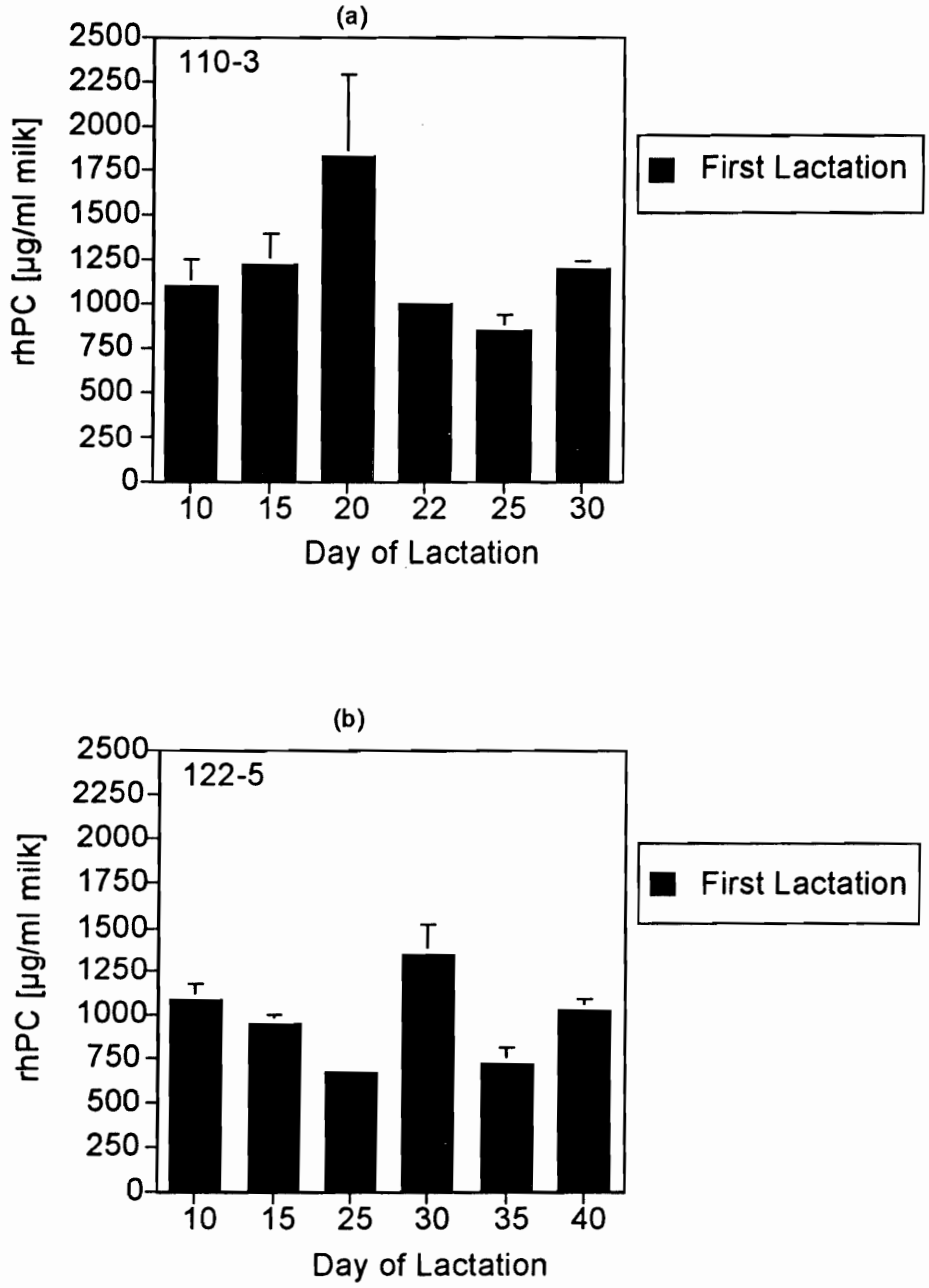


Figure 4A-B. rhPC levels in the milk of **(A)** WAP-genomic-PC founder 110-3, and **(B)** outbred G1 offspring 122-5.

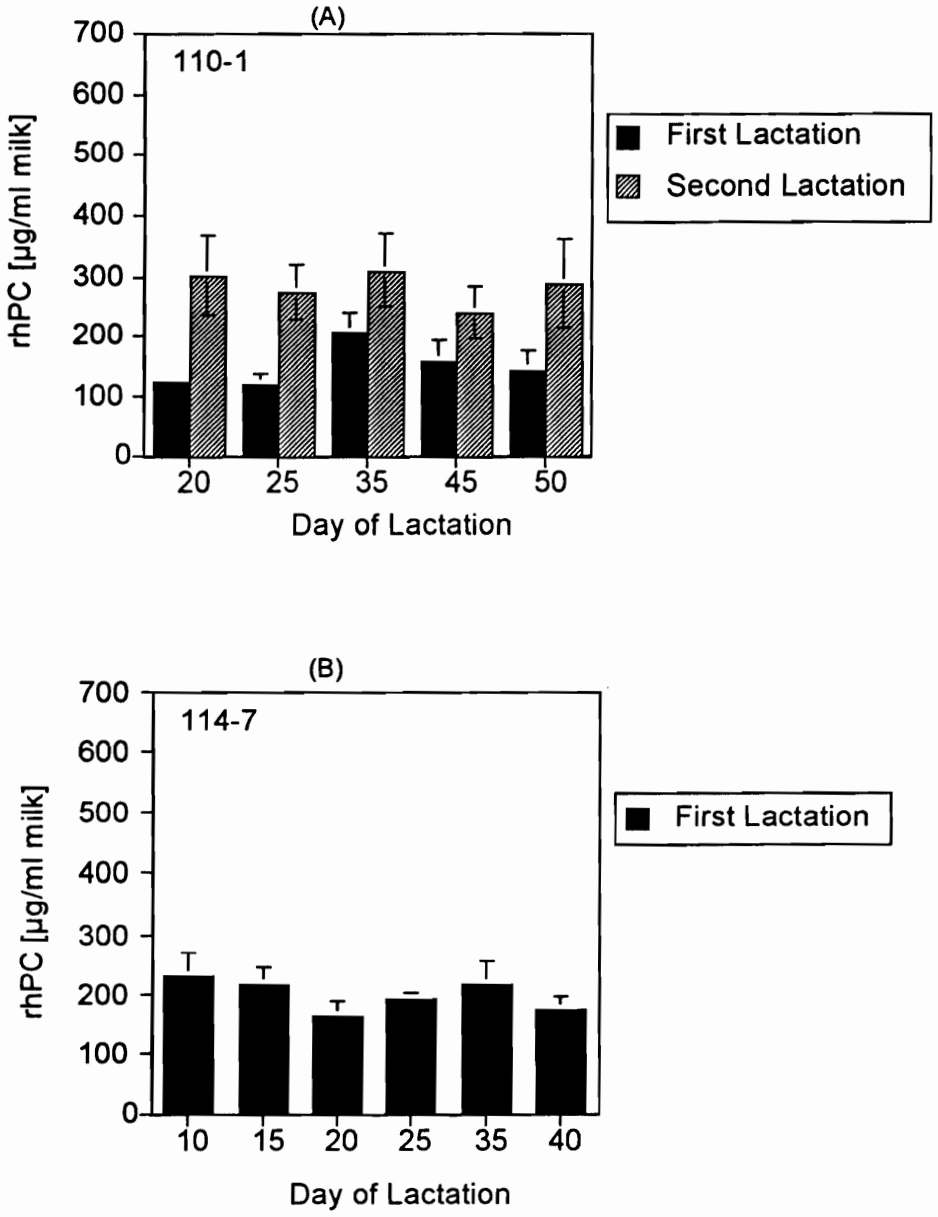
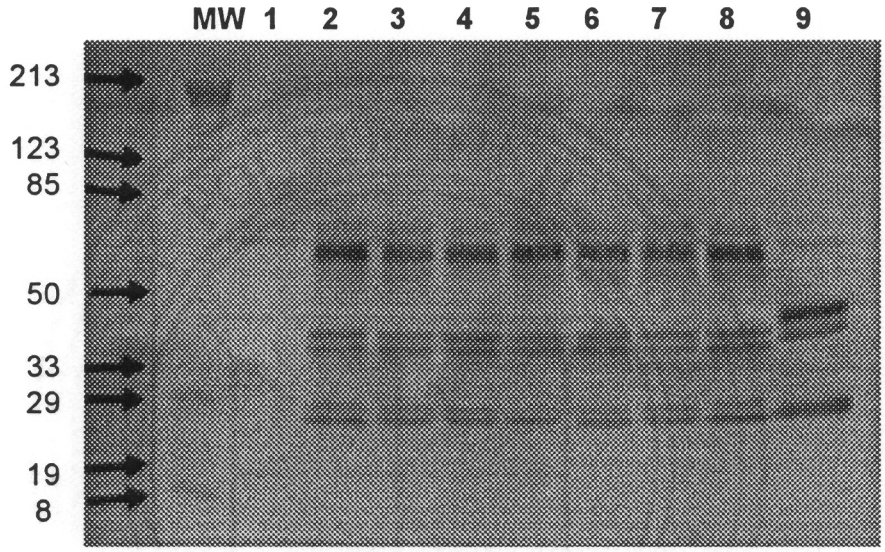
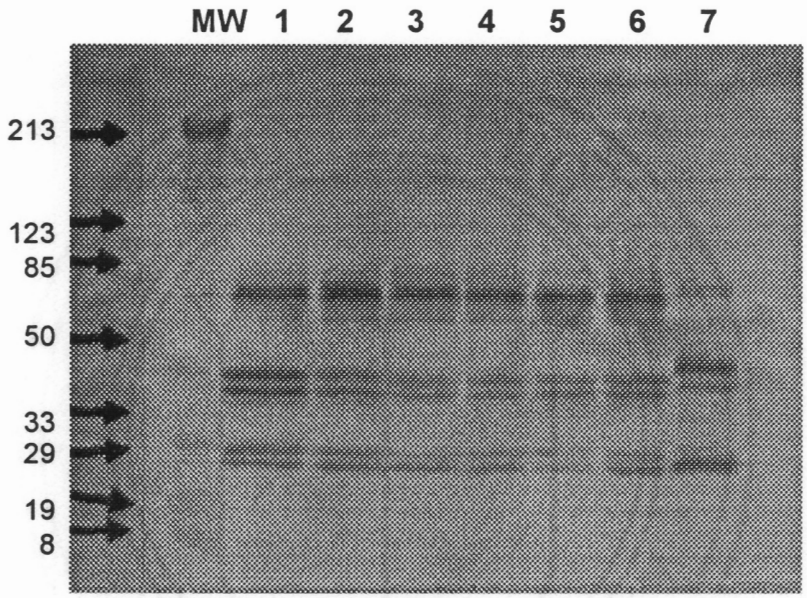


Figure 5A-B. rhPC levels in the milk of **(A)** WAP-genomic-PC founder 110-1, and **(B)** outbred G1 offspring 114-7.



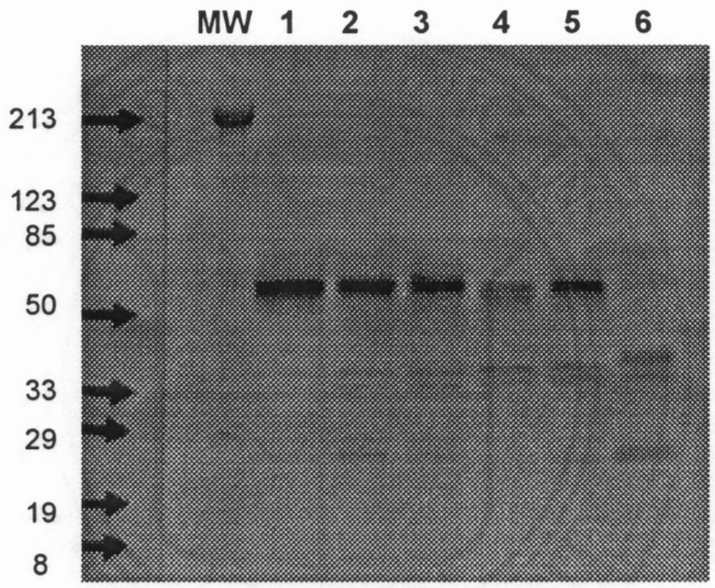
(a)

Figure 6A. Western blot of rhPC in the milk/EDTA pools of transgenic pigs from the WAP-PC1 29-2 lineage. **Lane 1:** Nontransgenic milk/EDTA pool; **Lane 2**: 29-2 second lactation; **Lane 3:** 115-6 first lactation; **Lane 4:** 115-6 second lactation; **Lane 5:** 115-7 first lactation; **Lane 6:** 115-7 second lactation; **Lane 7:** 64-4 first lactation; **Lane 8:** purified hPC.



(b)

Figure 6B. Western blot of rhPC in the milk/EDTA pools of transgenic pigs from the WAP-PC1 83-1 lineage. **Lane 1:** 83-1 fourth lactation; **Lane 2:** 83-1 fifth lactation; **Lane 3:** 6-1 first lactation; **Lane 4:** 6-1 second lactation; **Lane 5:** 126-5 first lactation; **Lane 6:** 138-1 first lactation; **Lane 7:** purified hPC.



(c)

Figure 6C. Western blot of rhPC in the milk/EDTA pools of transgenic pigs with the WAP-genomic-PC transgene. **Lane 1:** 110-1 first lactation; **Lane 2:** 110-1 second lactation; **Lane 3:** 114-7 first lactation; **Lane 4:** 110-3 first lactation; **Lane 5:** 122-5 first lactation; **Lane 6:** purified hPC.

Chapter 3. Milk Protein Composition of Pigs Transgenic for Human Protein C

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Abstract

To date, there are no reports in the literature on the effects of the expression of recombinant proteins on the endogenous milk protein composition of transgenic livestock. Previous reports have indicated that small changes in alveolar and epithelial cell structure and endogenous milk protein composition of mice may be caused by production of about 1 mg/ml of recombinant human protein C (rhPC) in milk. Four lines of transgenic pigs producing recombinant human protein C (rhPC) in milk were studied to discover possible effects of the presence of rhPC on the endogenous milk protein composition. We found that three of the four lines of pigs having average rhPC concentrations of about 400 µg/ml or less had milk protein composition profiles similar to nontransgenic pig milk samples. One line of transgenic pigs (from G0 110-3) with average rhPC concentrations of about 1 mg/ml had slightly altered milk protein composition, with elevated IgG, IgA, IgM, and transferrin levels. In addition there appeared to be more proteolysis of caseins in the milk. However, overall casein, whey protein, and albumin levels in the milk of pigs in the 110-3 line indicate that the elevated levels of immunoglobulins and transferrin were not due to low grade infection or disrupted epithelial tight junctions. We postulate that the presence of consistently high levels of rhPC may have slightly altered the mammary gland to increase transcytosis of plasma proteins. Increased levels of plasma proteins in milk could have harmful effects on the production of recombinant plasma zymogens. High expression levels of recombinant proteins may therefore not always be the optimum, and case-by-case studies on the effects of recombinant vitamin K-dependent proteins homologous to protein C are warranted.

Introduction

Human therapeutic proteins produced in the milk of transgenic livestock have the potential to complement other recombinant methods for the large-scale production of proteins such as Factor IX, Factor VIII, protein C, and other blood coagulation factors (Paleyanda *et al.*, 1991; Wall *et al.*, 1992; Lubon *et al.*, 1996). The mammary gland is an effective bioreactor, capable of synthesis and secretion of several biologically active recombinant proteins in the milk of pigs, sheep, and goats (Velander *et al.*, 1992; Wright *et al.*, 1991; Carver *et al.*, 1993; Ebert *et al.*, 1991; Denman *et al.*, 1991). While primary efforts in developing the mammary gland of transgenic animals for recombinant protein production were devoted to defining milk gene regulatory sequences and characterizing the recombinant protein, other issues, such as effects of the synthesis and secretion of a heterologous protein on the mammary gland and milk protein composition, should be considered (Center for Biologics Evaluation and Research, Docket No. 95D-0131, 1995). Milk protein composition and patterns of protein secretion are indicative of the physiological status of the mammary gland (Klobasa and Butler, 1987). Thus, analysis of milk protein composition is a useful method for following phenotypes of transgenic animals, and can be a secondary diagnostic of mammary gland function.

Although there are many cases reported in the literature where secretion of a heterologous protein does not seem to affect mammary gland function, adverse phenotypes have been associated with the expression of mouse whey acid protein (mWAP, Burdon *et al.*, 1991), bovine β-casein (Bleck *et al.*, 1993), α_1-antitrypsin (Bischoff *et al.*, 1992), tissue plasminogen activator variant (H. Meade, personal communication), human growth hormone (Reddy *et al.*, 1991), and transforming growth factor β1 (Jhappan *et al.*, 1993). Specifically, agalactia in mice and pigs has been linked with precocious synthesis of mWAP, and subsequent study has suggested that the homology of mWAP with protease inhibitors involved in tissue development may be responsible for underdevelopment of the mammary gland (Shamay *et al.*, 1992; Hennighausen *et al.*, 1994). The unexpected biological activity of a mouse milk protein in the porcine mammary gland emphasizes the need for extensive study of the possible effects of expression of a heterologous protein on the structure and function of the mammary gland.

To date, there are no reports in the literature describing the effects of recombinant proteins on the endogenous milk protein profile of livestock; detailed milk protein composition analysis of milk from transgenic animals has been reported only for β-lactoglobulin expression in transgenic mice (Wilde *et al.*, 1992; McClenaghan *et al.*, 1995). We have previously reported that the production of high levels of rhPC in the milk of mice caused slight changes in the alveolar and epithelial cell structure (Paleyanda *et al.*, 1994). Additionally, changes in the endogenous murine milk protein composition were observed. In this study, we focus on measuring the concentrations of endogenous milk proteins to determine if the presence of recombinant human protein C (rhPC) is affecting milk protein composition in four lines of transgenic pigs (Table 1). We found that a line of transgenic pigs with consistently high expression levels of rhPC ($\geq$1 mg/ml) showed an increase in the concentration of milk immunoglobulins and transferrin, but imperceptible changes in concentrations of caseins, whey proteins and serum albumin levels.

Results

rhPC Levels in Milk

The averages of the daily expression levels of rhPC measured by polyclonal ELISA from a minimum of five different days in each sow's lactation are given in Table 2. Sow 29-2 (WAP-PC1 founder) averaged about 410 µg rhPC/ml milk, with daily expression levels ranging between 200-1000 µg/ml. Offspring 31-5 had an average rhPC expression level of about 30 µg rhPC/ml during lactation, with daily values ranging from 15 µg/ml to 60 µg/ml. Offspring 115-6 averaged 90 µg rhPC/ml and 100 µg/ml for both second and third lactations, respectively, with daily values ranging from 60-120 µg/ml. Sow 83-1, another WAP-PC1 founder animal, averaged about 300 µg rhPC/ml milk in both fourth and fifth lactations, with daily expression levels ranging from 200-400 µg/ml in both lactations. One outbred offspring, 6-1, had a lower average rhPC expression level of 70 µg rhPC/ml, with daily expression levels of 40-90 µg/ml. Sows with the WAP-PC1 transgene had average lactation lengths of about 50-60 days.

Founder 110-1 averaged 160 µg rhPC/ml during her first lactation, with daily expression levels of 110-220 µg/ml. The average expression level during the second lactation increased to 280 µg rhPC/ml, with daily values of 240-310 µg/ml. One outbred offspring of 110-1, 114-7, had an average expression level of 200 µg rhPC/ml, with daily values of 160-230 µg/ml. Founder 110-3 and G1 offspring 122-5 had the highest average expression levels of 1200 µg rhPC/ml and 960 µg/ml, respectively. Daily samples of 110-3 ranged from 840-1800 µg/ml. Daily samples of 122-5 ranged from 670-1400 µg/ml. The length of lactation of sows in the 110-1 lineage was about 50-60 days. Sows 110-3 and 122-5 were able to maintain their lactations for about 40 days.

Milk Protein Analysis

Silver stained SDS PAGE and western blots were used to measure qualitative differences in milk protein composition (Figures 1-3). Relative to nontransgenic pigs, the major milk proteins such as the caseins (Figure 1, Lane 1, 30-40 kDa) do not show significant variability in concentration levels in the milk of pigs with low and high rhPC expression levels. The levels of the lower molecular weight proteins such as α-

lactalbumin (MW ~ 16 kDa) and β-lactoglobulin (MW ~ 18 kDa) are also consistent among all the milk pools. Comparison between nontransgenic and transgenic milk pools suggests that pigs 110-3 and 122-5 (Lanes 9, 10) secrete higher levels of IgG (Lane 13) and transferrin (Lane 14) in their milk. Albumin levels (Lane 15) appear to be constant among all the pools.

Differences in the protein composition of minor milk proteins are also evident between the WAP-genomic-PC pigs with average rhPC expression of 1 g/l and the other transgenic and nontransgenic pigs (Figure 1): (1) milk from 110-3 and 122-5 has a doublet of bands of about 80 kDa that is not visible in milk from other pigs; (2) 110-3 and 122-5 have higher levels of another doublet above 97 kDa; and (3) 110-3 and 122-5 have a number of bands in the 10-30 kDa range that are not visible in the other milk samples.

The daily milk samples and pools were further analyzed by ELISA, rocket immunoelectrophoresis, and radial immunodiffusion (RID) to better quantify differences in protein composition. Porcine serum albumin (PSA) levels in milk were measured by rocket immunoelectrophoresis, and the values are given in Table 2. The PSA levels of transgenic milk ranged from 0.9 to 2.2 mg/ml milk. The PSA levels of nontransgenic milk included in this study ranged from 1.3 to 1.8 mg/ml milk.

Porcine transferrin (Trf) levels in the milk pools were measured by RID (Table 2). Transferrin levels in nontransgenic milk ranged from 35-115 µg/ml milk, within the 20-200 µg/ml range reported by Masson and Heremans (1971). Transferrin levels in milk from animals with the WAP-PC1 construct and the WAP-genomic-PC pigs with low rhPC concentrations ranged from 25-115 µg/ml milk. Trf levels measured in the milk of pigs 110-3 and 122-5 were 350 µg/ml and 305 µg/ml, respectively.

Averages of the daily IgG levels in the milk measured by ELISA during a lactation are given in Table 2. The average values range from 0.8 mg/ml milk to 4.6 mg/ml milk for all animals. Founder 110-3 and G1 offspring 122-5 had average IgG levels in their milk of greater than 4 mg/ml milk, with both having daily highs between 6-7 mg/ml milk at about day 30. All other transgenic and nontransgenic pigs had average IgG levels of about 0.8-

2.5 mg/ml milk. Previously reported IgG levels in pig milk have ranged from 1-3 mg/ml (Jenness, 1982; Klobasa *et al.*, 1987; Bourne, 1973).

IgM levels in the milk pools of the transgenic and non-transgenic pigs were measured by ELISA (Table 2). The nontransgenic pigs had average IgM levels of about 3-4 mg/ml milk, and the transgenic pigs ranged from 2-9 mg/ml milk. Genomic pigs 110-3 and 122-5 had the highest IgM levels of about 9 mg/ml milk, and transgenic pigs 6-1 and 110-1 had IgM levels between 6-7 mg/ml. Previously reported IgM levels in pig milk have ranged from 0.3-2 mg/ml (Jenness, 1982; Klobasa *et al.*, 1987; Bourne, 1973).

IgA levels in the pools were measured by RID with a polyclonal antibody to the alpha chain (Table 2). IgA levels in the milk of 115-6 and 114-7 were between 6-8 mg/ml. WAP-genomic-PC pigs 110-3 and 122-5 had the highest milk IgA levels of 27 mg/ml and 23 mg/ml, respectively. Milk IgA levels in the remaining transgenic and nontransgenic pigs ranged from 12-20 mg/ml. Previously reported IgA levels in pig milk have ranged from 3-9 mg/ml (Jenness, 1982; Klobasa *et al.*, 1987; Bourne, 1973).

Relative levels of secretory IgA (sIgA) were measured by RID with an antibody to the non-covalently attached secretory component on sIgA. sIgA values from RID were normalized with respect to nontransgenic pig 102 (Table 3). sIgA levels were highest in the milk of transgenic pigs 6-1, 110-3, and 122-5. The milk of these pigs also had the highest total IgA levels. Normalized sIgA levels in the milk were roughly proportional to total IgA levels. In addition 110-3, 122-5, and 6-1 also had the highest values for the ratio of normalized sIgA:normalized IgA.

Discussion

This study seeks to detail changes in milk protein composition that may occur with the presence of a recombinant protein. Protein C is a plasma protein that should come in contact with the lumenal side of the mammary epithelial cells only in cases of extensive tissue damage. We have investigated whether the presence of rhPC or up-regulation of post-translational machinery associated with synthesis of the mature molecule are linked to changes in the endogenous milk protein profile. In addition, the pigs included in this study had a range of rhPC concentrations of about two orders of magnitude, enabling us to investigate whether expression level of this heterologous recombinant protein affects mammary gland function.

The structure and function of the mammary gland and the gross composition of milk have been extensively reviewed (Pitelka and Hamamoto, 1983; Davies *et al.*, 1983; Mercier and Gaye, 1983; Klobasa *et al.*, 1987). Caseins, α-lactalbumin, β-lactoglobulin, lactoferrin, and lipid-associated proteins are the major porcine milk proteins synthesized by the mammary epithelial cells (Klobasa *et al.*, 1987). Milk also contains a number of plasma proteins (Davies *et al.*, 1983; Jenness, 1982; Klobasa *et al.*, 1987). The major plasma proteins present in the milk include the immunoglobulins, serum albumin, and transferrin. The majority of the milk immunoglobulins are produced locally in the mammary gland by monocytes located on the basal side of the epithelial cells (Bourne, 1973).

Comparison of silver stained SDS PAGE and western analysis of milk samples from transgenic and nontransgenic pigs showed that the presence of rhPC did not cause any significant differences in the levels of caseins and major whey proteins synthesized by the mammary epithelial cells. It is noted that nontransgenic pigs, WAP-PC1 pigs, and the WAP-genomic-PC pigs with lower rhPC concentrations (110-1 and 114-7) had similar milk protein composition as judged by SDS PAGE and western analysis. In addition, histological evaluation of 29-2 has revealed no detectable anomalies in alveolar structure (Morcol *et al.*, 1994). However, the presence of the minor bands in the 10-30 kDa range in the milk of 110-3 and 122-5 are attributed to partial proteolysis of caseins. Casein

proteolysis has been reported to be caused by plasmin, a plasma serine protease of 80 kDa (Eigel *et al.*, 1979). Given that the milk of 110-3 and 122-5 have higher levels of a protein of about 80 kDa (Figure 1), we postulate that limited proteolysis of the porcine caseins in the milk was caused by elevated levels of plasmin.

Plasma proteins can be transported into the milk by two mechanisms: leakage from the interstitial fluid through disrupted tight junctions between the epithelial cells, or receptor-mediated transcytosis of the protein from basal extracellular space to the lumen on the apical side of the epithelial cell. Elevated levels of plasma proteins in the milk, especially albumin and immunoglobulins, simultaneous decreases in milk proteins (caseins, β-lactoglobulin, and α-lactalbumin), and extensive proteolysis of caseins (up to 56%, Saeman *et al.*, 1988) have been used as indicators of mastitis and/or leaky tight junctions between the epithelial cells in the mammary gland (Davies *et al.*, 1983; Klobasa and Butler, 1987; Jenness, 1982; Jenness, 1985). The most abundant plasma protein, serum albumin, which is present at approximately 40 mg/ml in pig serum, was measured at relatively constant levels in all transgenic and nontransgenic milk samples. However, elevated levels of transferrin, IgG, IgM, and IgA were measured in the milk of pigs having rhPC expression levels of about 1 mg/ml (110-3 and 122-5). We have utilized western analysis, countercurrent immunoelectrophoresis, and indirect ELISA on milk/EDTA samples and protein A-concentrated immunoglobulins to see if any soluble porcine antibody to rhPC or antibody/antigen complexes of porcine antibodies and rhPC exist in the milk. No signal indicative of an immune response to rhPC appeared in any of the milk samples studied (data not shown).

Transferrin, IgG, IgA, and IgM are specifically transported across epithelial cells by receptor-mediated transcytosis (Apodaca *et al.*, 1991, Seddiki, *et al.*, 1992). Changes in the transcytosis of proteins in transgenic and nontransgenic epithelial cells were studied by comparison of the relative levels of IgA and sIgA subpopulations in the milk. sIgA is structurally differentiated from IgA by the non-covalent attachment of a 60-70 kDa polypeptide known as "secretory component" (SC) to IgA. SC is synthesized by the mammary epithelial cells, and is part of the transmembrane polymeric immunoglobulin receptor (Apodaca *et al.*, 1991; Solari and Kraehenbuhl, 1987). During the transcytosis

of IgA through the epithelial cell, the SC is non-covalently attached to the IgA molecule. Of transgenic pigs with rhPC expression levels less than 410 µg/ml, only 6-1 had elevated sIgA and sIgA:IgA levels. However, 6-1 had normal transferrin and IgG levels. As seen in Table 3, 110-3, 122-5 had about 1.6 - 8 times higher sIgA levels in their milk, and higher sIgA:IgA ratios than other transgenic and nontransgenic animals. If the tight junctions between the epithelial cells were disrupted, the high concentration driving force of serum albumin would favor the leakage of albumin into the milk. For example, we have measured albumin levels of 4-12 mg/ml in the milk of a pig with symptoms of mastitis (data not shown). Since albumin is not present at elevated levels, and noticeable changes in the levels of other milk proteins such as the caseins and whey proteins are not apparent in the milk of 110-3 and 122-5, it is hypothesized that increased general transcytosis activity, and not the presence of infection or disrupted tight junctions, is the primary cause of increased levels of transferrin, IgG, IgM, and IgA in the milk. We also postulate that the failure of 110-3 and 122-5 to maintain their respective lactations for more than 40 days may be indirectly caused by elevated rhPC levels. However, because of the limited sample size, further study in transgenic pigs must be done to verify the above hypotheses. The value of performing experiments with mammary epithelial cell cultures is questioned because of the intimate relationship of structure and function of the mammary gland and the artifacts that may be introduced by the use of immortalized cell lines.

IgM and IgA levels measured in the milk for most pigs in this study were higher than what has been previously reported. In the literature, IgA has been considered to be the most abundant immunoglobulin in the milk, followed by IgG, and then IgM (Jenness, 1982; Klobasa *et al.*, 1987; Bourne, 1973). This study confirms that IgA is the most abundant immunoglobulin in pig milk, but that IgM is more abundant than IgG. We note that in all the previous studies the milk was pretreated with a casein precipitation step before any assays were conducted. We suggest that a portion of the IgM and IgA in the milk was co-precipitated at the low temperatures, high g-forces, or non-selective precipitation techniques used in these previously reported procedures. We propose that our method of solubilizing the caseins with EDTA and centrifuging at lower g-forces (3000 x g) for 30 minutes resulted in more accurate measurement of milk proteins.

Changes in the endogenous milk protein composition can have a significant impact on upstream and downstream events in the production of a recombinant plasma zymogen, especially if the levels of endogenous plasma enzymes in the milk are elevated. The lumen of the mammary gland is a conducive environment for many of the calcium-dependent and phospholipid membrane-dependent reactions of the plasma coagulation cascade. For example, Clark *et al.* (1989) have reported that recombinant human Factor IX is proteolytically activated during upstream or downstream processing. The specific activation mechanisms of zymogens in the coagulation cascade suggest that this may have been caused by the presence of activated sheep plasma enzymes in the milk. In fact, we have detected elevated levels of activated porcine Factor XI and Factor VII in pig milk with mastitic characteristics (data not shown), and porcine FXIa has been shown to have similar substrate specificity of human FXIa (Mashiko and Takahashi, 1994). Thus, whether milk protein composition is affected by the presence of a recombinant protein, by subclinical mastitis, or by milk collection methods (Stelwagen *et al.*, 1994), the presence of activated plasma proteases can cause significant problems in recovery of a recombinant plasma zymogen.

In conclusion, we have found that production of protein C in the mammary gland of transgenic pigs does not appear to alter the milk protein composition profile of pigs with average rhPC expression levels of about 400 µg/ml or less. In contrast, one line of transgenic pigs with average rhPC expression levels of about 1 mg/ml had a slightly altered milk protein composition profile with incremental increased proteolysis of caseins and elevated levels of transferrin, IgG, IgM, and (s)IgA. Limited proteolysis of caseins is consistent with sightly elevated plasmin levels in milk, and does not necessarily indicate up-regulation of proteolytic post-translational enzymes. Albumin, whey protein, and casein levels were normal in this line, which is not consistent with the presence of infection, so we hypothesize that increased transcytosis of plasma proteins is primarily responsible for these protein composition changes. The limited sample size precludes a definitive differentiation between inherent phenotypic characteristics and effects of high levels of rhPC among the pigs in this study, but we postulate that the consistent rhPC concentrations above 1 mg/ml in the milk of pigs may be causing slight but specific changes in the endogenous milk protein composition that can further affect the length of

lactation. Until more information is known, we suggest that arbitrary development of transgenic pigs secreting high levels (>1 mg/ml) of recombinant vitamin K-dependent proteins in milk may not be an optimal design, and the effects of each protein on endogenous milk protein composition should be evaluated individually.

Materials and Methods

Transgenic pigs Generated at Virginia Tech

Four lines of transgenic pigs were used in this study. The founder transgenics were generated by the methods given in Velander *et al.* (1992) using the WAP-PC1 cDNA construct and the WAP-genomic-PC construct (Drohan *et al.*, 1994). The two founders with the WAP-PC1 cDNA construct were 29-2 and 83-1. All transgenic offspring are the result of outbreeding. Two G1 offspring of 29-2 were included in this study: 31-5 and 115-6. One G1 offspring of 83-1 was included: 6-1. The two founders with the WAP-genomic-PC construct were 110-1 and 110-3. Sows 114-7, G1 offspring of 110-1, and 122-5, G1 offspring of 110-3, were included in this study. Four nontransgenic sows were also included in this study, and are denoted as 101, 102, 104 and B30.

Milk collection and handling

Lactating sows were injected IM with 30-60 IU of oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk let-down. Letdown occurred 2-5 minutes after injection. Pigs were milked both by hand and by machine during the course of this study. There were no cases of clinical mastitis observed in any of the pigs during the course of their lactations. A minimum of 5 different days during lactation were used for each pool. Immediately after collection the milk was diluted 1:1 with 200 mM EDTA, pH 7.0 to solubilize the caseins and then frozen. Small aliquots (~1 ml) of the milk/EDTA mixture were taken and centrifuged for approximately 30 minutes at 3000g at 4°C. The fat layer was separated from the diluted milk/EDTA fraction, and the diluted milk/EDTA fraction was used for all further assays. Equal volumes of diluted milk/EDTA from each day that was collected in the lactation were combined for the lactation pool. In this study, all concentration values reported for milk were obtained from diluted milk/EDTA samples that were multiplied by a factor of 1.9 to account for dilution with EDTA and subsequent removal of milk fat.

rhPC Determination in Milk

The rhPC levels in milk were determined by polyclonal ELISA. Immulon II microtiter plates (Fisher Scientific, Pittsburgh) were coated overnight with 100

μl/well of 5 μg/ml of rabbit anti-human Protein C (Sigma, St. Louis, MO) in carbonate buffer (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6). Triplicates of sample dilutions and hPC standard (American Red Cross, Rockville, MD) in TBS-BSA (20mMTris, 50 mM NaCl, 0.1% BSA, pH 7.2) were incubated at 37°C for 20 minutes. After washing the plates with TBS-Tween (TBS-0.5% Tween 20), 1:1000 goat anti-hPC (American Diagnostica, Greenwich, CT), followed by 1:1000 anti-goat IgG/HRP (Sigma) were incubated in the wells. Bound chromophore was detected with OPD substrate (Abbot, Chicago), the reaction was quenched with 3 N sulfuric acid after 3 minutes, and the plates were read at 490 nm.

IgG Detection in Milk

Immulon II plates were coated overnight with 100 μl/well of 5 μg/ml rabbit anti-pig IgG (Nordic Immunology, Tilburg, The Netherlands) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6 at 4° C. The wells were washed with TBS-Tween and then blocked for 30 minutes with TBS-PEG at room temperature. Samples and standard in the dilution buffer were added in triplicate to the wells (100 μl/well) and incubated at 37°C for 30 minutes. The wells were then washed and blocked for another 15 minutes at room temperature. Rabbit anti-pig IgG HRP conjugate (Sigma) was then incubated in the wells for 30 minutes at 37°C. Bound chromophore was detected with OPD substrate at 490 nm using an EL308 Bio-Tek Microplate reader.

IgM Detection in Milk

Immulon II plates were coated overnight with 100 μl/well of 5 μg/ml rabbit anti-pig IgM (μ chain specific) (Pel Freeze Biologicals, Rogers, AR) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6 at 4° C. The wells were washed with TBS-Tween and then blocked for 30 minutes with TBS-PEG at room temperature. Samples and standard in the dilution buffer were added in triplicate to the wells (100 μl/well) and incubated at 37°C for 45 minutes. The wells were then washed and blocked for another 15 minutes at room temperature. Goat anti-pig IgM (Nordic Immunology) (Fc specific, 1:1000 dilution in TBS-PEG) was then incubated in the wells for 30 minutes at 37°C. The plates were then washed and 100 μl/well of 1:1000 rabbit anti-goat IgG HRP conjugate was incubated 30 minutes at

37°C. Bound chromophore was detected with OPD substrate at 490 nm using an EL308 Bio-Tek Microplate reader. The goat anti-pig IgM was also used in a western blot to detect pig IgM in the milk and test for any cross-reactivity in the transgenic milks. No evidence of non-specific cross-reactivity of the ELISA antibodies with other milk proteins was seen when a western blot of the milk/EDTA pools from B30 (nontransgenic), 83-1 fourth lactation (WAP-PC1 cDNA construct), and 122-5 (WAP-genomic-PC construct) was detected with same antibodies used in the ELISA (data not shown), and the elevated IgM level in the milk of 122-5 was also confirmed qualitatively.

Detection of PSA in Milk

Levels of porcine serum albumin (PSA) and in the milk/EDTA pools were determined by rocket immunoelectrophoresis based on the method of Catty and Raykundalia (1988). Precipitating antisera to PSA was purchased from Nordic Immunology. A 1:100 ratio of antisera to agarose solution was used for each plate. A PSA standard (Sigma) was used. A reference of nontransgenic pig serum (Sigma) consistently gave 40 mg/ml PSA. Dilutions of samples and standards were applied to the plates in duplicate. Antigen levels were quantitated by measuring precipitin arc heights on coomassie stained plates.

Determination of Porcine Transferrin, IgA, and sIgA in Milk

Levels of porcine transferrin (Trf), IgA, and secretory IgA (sIgA) in the milk/EDTA pools were determined by radial immunodiffusion based on the methods given in Catty and Raykunkalia (1988). Precipitating antisera to porcine Trf (RASw/Trf), and sIgA (RASw/sIgA) were purchased from Nordic Immunological Laboratories. Goat anti-IgA (alpha chain specific) was purchased from Bethyl Laboratories (Montgomery, TX). A 1:100 ratio of antisera to agarose solution was used for each plate. A Trf standard was purchased from Accurate Chemical, a swine IgA standard (serum-derived) was purchased from InterCell Technologies (Hopewell, NJ) for determining total IgA levels, and a swine serum reference was purchased from Sigma for measuring relative sIgA levels. Duplicates of sample and standard dilutions were applied to the wells, and incubated at 4°C for 72 hours. Antigen levels were quantitated by measuring precipitin diameters (D) on coomassie stained plates, and plotting a standard curve of

concentration vs. D^2.

A value of 4 mg/ml IgA was measured in the nontransgenic pig serum control for all the plates. Testing of the anti-IgA used with purified pig IgG and IgM standards gave no detectable precipitin bands, indicating that the antibody used is not non-specifically cross-reacting other immunoglobulin heavy chains. A western blot of the milk samples under denaturing, reduced conditions developed with the anti-sIgA antibody resulted in only a single band at approximately 60-70 kDa, indicating no non-specific cross-reactivity with other milk proteins. A western blot of the same samples under denatured but non-reduced conditions showed that no free secretory component existed in the milk; all the signal was localized at ~400 kDa in the blot.

Gel Electrophoresis and Western Blots of Milk Pools

The protein composition of pools of EDTA-expanded milk from each pig's lactation were visualized by silver stained SDS PAGE. Gradient gels of 9-18% were poured in Bio Rad Protean IIxi 16x20 cm gel casters (Bio Rad, Hercules, CA). The total protein concentration of each pool was estimated by measuring the $OD_{280\ nm}$ and assuming an extinction coefficient of 1 ml/mg. (The Bio Rad dye binding assay using bovine casein in 0.16 M lactose as a standard gave identical results for total protein concentration). A total of 4 µg of total protein was applied to the wells for all the milk samples. Standards of purified rhPC, porcine IgG (Sigma), porcine serum albumin (Sigma), porcine transferrin (Accurate Chemical and Scientific, Westbury, NY), and porcine caseins (derived from acid precipitation and ion exchange chromatography of nontransgenic porcine milk) were also loaded (0.5 µg/well) for reference. Molecular weight markers were purchased from Bio Rad.

In the western blots for detection of whey and serum proteins 10 µg of total protein were added to the wells for each milk pool. Porcine transferrin, porcine IgG, purified rhPC, porcine caseins, and porcine serum albumin were loaded at 1µg/well for reference. The proteins were transferred overnight in Towbin's Buffer/0.1% SDS onto PVDF membranes (Bio Rad) using the Bio Rad TransBlot apparatus. The blocking buffer used was TBS/0.05%Tween/0.5% casein. The westerns were then developed with

the indicated polyclonal antibodies (1:1000 dilution in TBS/Tween/casein, incubated 30 minutes at 37°C) and the metal-enhanced DAB kit (Pierce, Rockford, IL). Rabbit anti-pig serum (Pel Freeze Biologicals) followed by goat anti-rabbit IgG HRP conjugate was used in the western to detect overall serum protein levels in the milk samples. Rabbit anti-pig whey (Pel Freeze Biologicals) followed by goat anti-rabbit IgG HRP conjugate was used in the western to detect overall whey protein levels. Goat anti-pig IgM (Fc specific) followed by rabbit anti-goat IgG HRP conjugate was used in the western to detect potential cross-reactivity with proteins in transgenic milks. In the western to detect possible rhPC antibodies, 0.5 µg of rhPC standard was added to the well. The blot was then developed by incubating the membrane in 2 µg/ml rhPC in the dilution buffer, followed by 1:1000 rabbit anti-hPC, 1:1000 anti-rabbit IgG HRP, and DAB development. Bio Rad prestained broad range molecular weight markers were used in western blots.

Protein A Chromatography

Large samples of the milk/EDTA pools were diluted with five parts chilled deionized water and adjusted to pH 8 with Tris. The diluted milk feed was loaded at 1 cm/min on a protein A Emphaze column (a gift from 3M Bioapplications, St. Paul, MN). The column was washed with PBS, pH 8 until a steady baseline was achieved. Antibodies were eluted using 0.1 M glycine, 2% acetic acid, pH 2.2. The column was then cleaned with 2 M NaSCN, 8 M urea, and 4 M NaCl. The pH 2.2 fractions were immediately neutralized with 1.5 M Tris and stored frozen.

Immunoelectrophoresis (IEP)

Agarose gels were prepared using molten agarose [1.2% high EEO agarose, 50 mM tris-tricine, pH 8.6, 14 ml] poured onto glass plates [8x10 cm] into which 5 or 10 µl wells were vacuum punched approximately 1 cm apart. Wells on the cathode side (-) were filled with 5 or 10 µl of antigen to be detected; wells on the anode side (+) were filled with 5 to 10 µl antibody to be detected. Dilutions of antigen and antibody were tested to ensure optimal conditions for precipitation. Electrophoresis was performed at constant current at 1 mA/cm at 4°C for 1 h. After electrophoresis, plates were soaked in 200 ml of saline (0.9%) overnight at room temperature. The gels were dried and then stained with Coomasie Blue R-250.

ELISA for Detection of Possible rhPC Antibodies

Milk from transgenic pigs and IgG purified from the milk were analyzed for anti-protein C antibody by an indirect ELISA. Immulon II 96-well plates were coated with 50 ng/well recombinant protein C in carbonate buffer, pH 9.6 overnight at 4°C. Wells were washed twice with TBST, and blocked with TBS/BSA for 1 h at 37°C. Samples were diluted 1:1 in TBST and 100 µl was added to the wells. Samples were incubated in the wells for 1h at 37°C and washed seven times in TBST. The wells were developed using a 1:1000 dilution of rabbit anti-pig IgG-HRP conjugate or human anti-protein C IgG-HRP in TBST containing 0.1% BSA. The visualization reaction was stopped after 5 min with 3 N sulfuric acid and the plates were read on a Biotek ELISA reader at 490 nm.

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Table 1. Family trees of transgenic pigs in this study.

Founder	G1 Offspring	rhPC Construct
29-2	115-6, 31-5	WAP-PC1 cDNA
83-1	6-1	WAP-PC1 cDNA
110-1	114-7	WAP-Genomic-PC
110-3	122-5	WAP-Genomic-PC

Table 2. Average rhPC, porcine serum albumin (PSA), transferrin (Trf), IgG, IgA, and IgM levels in the milk of pigs in this study.

Pig	Lactation	rhPC** [μg/ml]	PSA* [mg/ml]	Trf* [μg/ml]	IgG** [mg/ml]	IgM* [mg/ml]	IgA* [mg/ml]
29-2	2nd	410	1.2 ± .02	40 ± 9	1.7	2.6 ± 0.3	14 ± 1.4
31-5	1st	30	1.6 ± 0.2	30 ± 5	1.5	4.0 ± 0.5	13 ± 0.2
115-6	2nd	90	0.93 ± 0.06	25 ± 4	0.80	2.1 ± 0.1	8.2 ± 0.6
115-6	3rd	100	1.3 ± 0.09	40 ± 7	0.83	2.3 ± 0.3	6.0 ± 0.2
83-1	4th	300	1.6 ± 0.05	70 ± 15	1.4	4.3 ± 0.5	12 ± 1.3
83-1	5th	290	2.1 ± 0.2	115 ± 10	2.2	4.7 ± 0.5	14 ± 1.8
6-1	1st	70	1.7 ± 0.2	90 ± 15	1.5	6.4 ± 0.6	19 ± 0.4
110-1	1st	160	1.6 ± 0.1	30 ± 5	1.7	6.9 ± 0.7	13 ± 1.3
110-1	2nd	280	1.4 ± 0.09	90 ± 10	1.2	6.0 ± 0.1	14 ± 1.3
114-7	1st	200	1.7 ± 0.1	60 ± 5	1.8	4.4 ± 0.3	5.7 ± 0.6
110-3	1st	1200	1.7 ± 0.1	350 ± 40	4.6	8.5 ± 0.8	27 ± 2.1
122-5	1st	960	2.2 ± 0.3	305 ± 40	4.3	9.2 ± 0.6	23 ± 2.5
101		NA	1.8 ± 0.1	55 ± 8	1.1	3.2 ± 0.2	14 ± 3.2
102		NA	1.3 ± 0.1	70 ± 10	1.9	4.0 ± 0.4	20 ± 2.8
104		NA	1.5 ± 0.1	35 ± 3	2.4	3.3 ± 0.2	14 ± 1.3
B30		NA	1.8 ± 0.1	115 ± 15	2.0	3.3 ± 0.4	21 ± 2.8

* Value of pool made from daily samples

** The daily samples were assayed for rhPC and IgG levels, the values given in the table are the average of all the daily samples assayed

NA Not applicable

Table 3. Normalization of total IgA and sIgA values of from the pooled milk samples. Nontransgenic pig 102 was used to normalize both sIgA and total IgA levels. Pigs are listed in order of normalized sIgA levels. Both total IgA and sIgA levels in the milk were determined by RID.

Pig ID#	Total IgA [mg/ml]	Normalized Total IgA	Normalized sIgA	$\frac{\text{[Normalized sIgA]}}{\text{[Normalized Total IgA]}}$
NTG 102	20	1.00	1.00	1.00
110-3	27	1.35	1.60	1.19
122-5	23	1.15	1.60	1.39
6-1	19	0.95	1.40	1.47
NTG B30	21	1.05	1.00	0.95
29-2	14	0.70	0.48	0.69
NTG 104	14	0.70	0.46	0.66
31-5	13	0.65	0.44	0.68
110-1 2nd Lact	14	0.70	0.44	0.63
110-1 1st Lact	13	0.65	0.42	0.65
83-1 5th Lact	14	0.70	0.40	0.57
83-1 4th Lact	12	0.60	0.38	0.63
NTG 101	14	0.70	0.30	0.43
115-6 3rd Lact	6.0	0.30	0.26	0.87
115-6 2nd Lact	8.2	0.41	0.24	0.59
114-7	5.7	0.29	0.18	0.62

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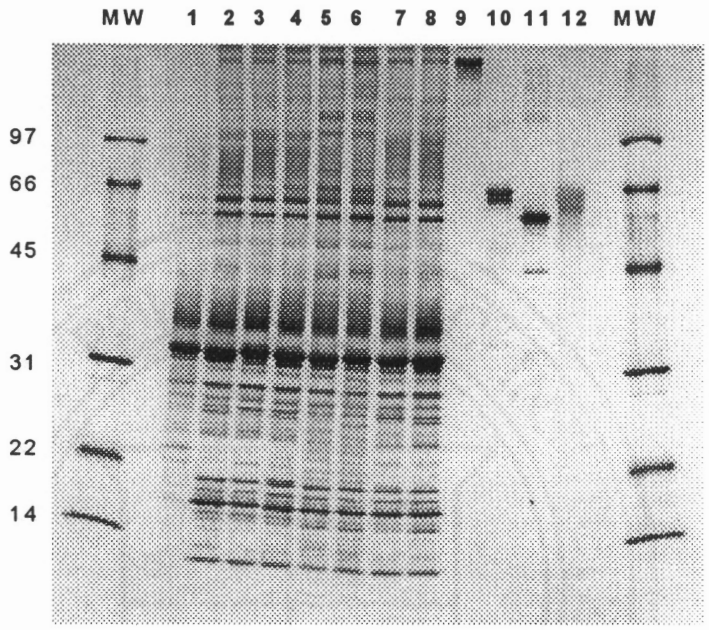

Figure 1. Silver stained SDS PAGE of pooled milk samples
Pools of selected lactations from the study were applied to 9-18% SDS PAGE. 4 µg of total protein (as measured by OD 280nm) was applied for all milk/EDTA pool samples. Standards of porcine casein, porcine IgG, porcine transferrin, porcine albumin, and rhPC were all applied at 1 µg for reference. **Lane 1**, porcine caseins. **Lane 2**, 31-5 first lactation. **Lane 3**, 115-6 third lactation. **Lane 4**, 29-2 second lactation. **Lane 5**, 110-3 first lactation. **Lane 6**, 122-5 first lactation. **Lane 7**, nontransgenic 104. **Lane 8**, nontransgenic 102. **Lane 9**, porcine IgG. **Lane 10**, porcine transferrin. **Lane 11**, porcine serum albumin. **Lane 12**, purified rhPC.

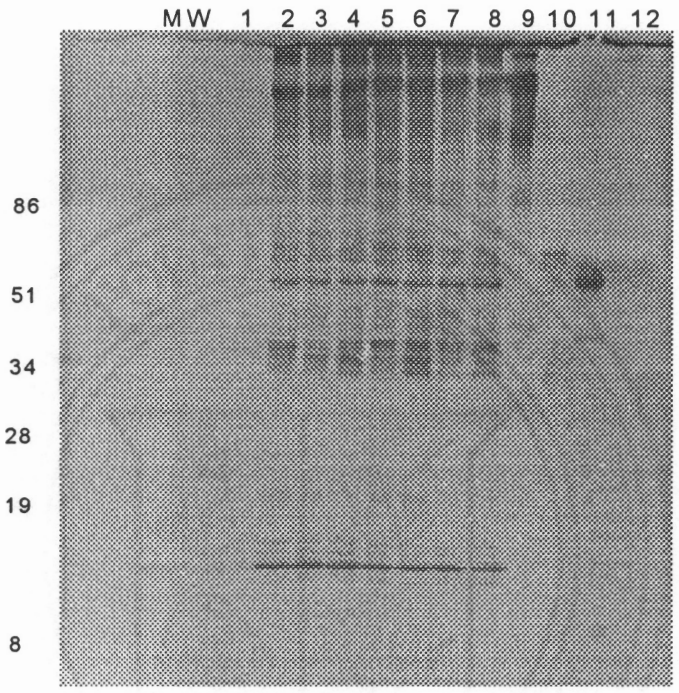

Figure 2. Western analysis of pooled milk samples, developed with anti-porcine whey protein antisera.
Pools of selected lactations from the study were applied to 9-18% SDS PAGE. 10 µg of total protein (as measured by OD 280nm) was applied for all milk/EDTA pool samples. **Lane 1**, porcine caseins. **Lane 2**, 31-5 pooled first lactation. **Lane 3**, 115-6 third lactation. **Lane 4**, 29-2 second lactation. **Lane 5**, 110-3 first lactation. **Lane 6**, 122-5 first lactation. **Lane 7**, nontransgenic 104. **Lane 8**, nontransgenic 102. **Lane 9**, porcine IgG. **Lane 10**, porcine transferrin. **Lane 11**, porcine serum albumin. **Lane 12**, purified rhPC.

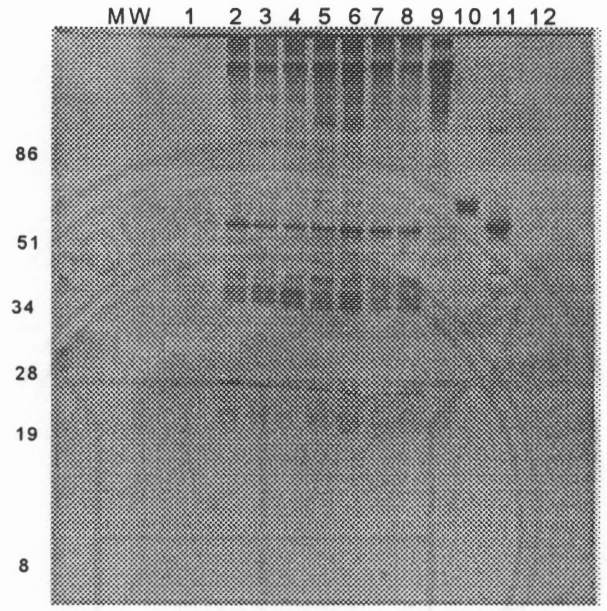

Figure 3. Western analysis of pooled milk samples, developed with anti-porcine serum protein antisera.
Pools of selected lactations from the study were applied to 9-18% SDS PAGE. 10 µg of total protein (as measured by OD 280nm) was applied for all milk/EDTA pool samples. **Lane 1**, porcine caseins. **Lane 2**, 31-5 pooled first lactation. **Lane 3**, 115-6 third lactation. **Lane 4**, 29-2 second lactation. **Lane 5**, 110-3 first lactation. **Lane 6**, 122-5 first lactation. **Lane 7**, nontransgenic 104. **Lane 8**, nontransgenic 102. **Lane 9**, porcine IgG. **Lane 10**, porcine transferrin. **Lane 11**, porcine serum albumin. **Lane 12**, purified rhPC.

Chapter 4. Affinity Purification of Biologically Active and Inactive Forms of Recombinant Human Protein C Produced in the Porcine Mammary Gland

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Abstract

We have studied the recombinant human protein C (rhPC) secreted in the milk of transgenic pigs. Transgenes having different regulatory elements of the murine milk protein, whey acidic protein, were used with cDNA and genomic human protein C (hPC) DNA sequences to obtain lower and higher expressing animals. The cDNA pigs had a range of expression of about 0.1-0.5 g/l milk. Two different genomic hPC pig lines have expressed 0.3 and 1-2 g/l respectively. The rhPC was first purified at yields greater than 60% using a monoclonal antibody to the activation site on the heavy chain of hPC. Subsequent immunopurification with a calcium-dependent monoclonal antibody directed to the γ-carboxyglutamic acid domain of the light chain of hPC was used to fractionate a population having a higher specific anticoagulant activity *in vitro*. The higher percentages of Ca^{2+}-dependent conformers isolated from the total rhPC by immunopurification correlated well with higher specific activity and lower expression. A rate limitation in γ-carboxylation of rhPC was clearly identified for the higher expressing animals. Thus, transgenic animals with high expression levels of complex recombinant proteins produced a lower percentage of biologically active protein.

Introduction

Human protein C (hPC) is a plasma serine protease important to the regulation of hemostasis. The potential therapeutic value of hPC has been well documented (Mann and Bovill, 1990, Comp, 1990), but an adequate supply of hPC is limited by its low concentration in human plasma (4 µg/ml). The short supply of hPC from human plasma, coupled with the risk of the transmission of viral pathogens, results in hPC being a prime candidate for replacement by recombinant human protein C (rhPC) (Drohan *et al.*, 1994b).

Human Protein C is synthesized as an inactive precursor. A number of co- and post-translational modifications are made to the precursor (Grinnell *et al.*, 1990, McClure *et al.*, 1992): removal of a 42 amino acid prepropeptide; vitamin K dependent γ-carboxylation of nine glutamic acid residues in the first 29 amino acids of the light chain (gla domain); glycosylation of four possible N-linked glycosylation sites; β-hydroxylation of Asp^{71}; formation of twelve disulfide bridges; and a Lys^{156}-Arg^{157} dipeptide is removed, resulting in a disulfide-linked heterodimer. Most of circulating hPC therefore consists of a heavy chain (M_r~41 kDa) connected by a single disulfide bond to a light chain (M_r~21 kDa). Activation of hPC is catalyzed by a complex containing thrombin and thrombomodulin at the endothelial cell surface, with a dodecapeptide being clipped from the N-terminal end of the heavy chain of hPC. Activated hPC degrades factors VIIIa and Va, inhibiting clot formation (Esmon, 1985, Esmon, 1989).

Recombinant human Protein C has been produced in several mammalian cell lines (Grinnell *et al.*, 1990, Yan *et al.*, 1990) and by transgenic animals (Drohan *et al.*, 1994a, Velander *et al.*, 1992a, Velander *et al.*, 1992b, Morcol *et al.*, 1994). Morcol *et al.* (1994) have shown that the primary advantage of production of recombinant proteins in the mammary gland of transgenic pigs *versus* cell culture is the significantly higher cell density of the mammary gland compared to the maximum cell density possible in cell culture medium. The rhPC produced in transgenic animals appears to have a number of different subpopulations (Drohan *et al.*, 1994a, Velander *et al.*, 1992a), as does plasma-derived hPC (Gelfi *et al.*, 1985, Heeb *et al.*, 1988, Miletich and Broze, 1990)

In this work, four transgenic pig lines having steady but different average rhPC expression levels during a lactation ranging from 90-1000 μg/ml milk were studied. We have purified the rhPC by ion exchange and immunoaffinity chromatography to capture the majority of the total population. The purified rhPC was subsequently fractionated with a calcium-dependent monoclonal antibody, 7D7B10, that recognizes the γ-carboxyglutamic acid domain of hPC (Orthner *et al.*, 1989). Our purpose in this study was to demonstrate a facile, immunoaffinity-based purification procedure of a recombinant protein from milk, and also to demonstrate that immunoaffinity chromatography can be a useful tool for isolating biologically active from inactive fractions of a complex recombinant protein population. This information is also useful to determining rate limitations in the biosynthesis of certain post-translational modifications.

Materials and Methods

Generation of Transgenic Pigs

Four different lines of transgenic pigs were used in this study. The founder transgenics were generated by the methods given in Velander *et al.* (1992a). Two females having a cDNA hPC construct (animals 115-6 and 83-1) and two females having a genomic hPC construct (110-1 and 122-5) were included in this study. Both the cDNA-hPC genetic construct and the genomic construct cloning have been previously detailed (Velander *et al.*, 1992a, Drohan *et al.*, 1994a).

Milk collection and handling

Lactating sows were injected IM with 30-60 IU of oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk let-down. Letdown occurred 2-5 minutes after injection. Three to six different days during lactation were used for each pool. Immediately after collection the milk was diluted 1:1 with 200 mM EDTA, pH 7.0 to solubilize the caseins and then stored frozen. The milk/EDTA mixture was centrifuged for approximately 30 minutes at 3000 x g at 2°C. The fat layer was skimmed from the diluted milk/EDTA fraction, and the milk/EDTA mixture was centrifuged once more and filtered through Whatman #3 filter paper (clarified milk/EDTA). All milk concentration values reported were measured from clarified whey samples using a dilution factor of 1.9 to account for EDTA addition and removal of milk fat.

rhPC Detection in Milk

An ELISA was used to measure the concentration of rhPC in the milk samples. Immulon II microtiter plates (Fisher Scientific, Pittsburgh) were coated overnight with 100 µl/well of 5 µg/ml of rabbit anti-human Protein C (Sigma, St. Louis, MO) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6 at 4° C. Wells were washed with 25 mM Tris, 50 mM NaCl, 0.05% Tween 20, pH 7.2 (TBS-Tween), and blocked for 20 minutes with 25 mM Tris, 50 mM NaCl, 0.1% BSA, pH 7.2 (TBS-BSA) at room temperature. Human protein C standard (American Red Cross, Rockville, MD) and samples in the dilution buffer (TBS-BSA) were added in triplicate (100 µl/well) and incubated at 37°C for 20 minutes. Wells

were washed and blocked with TBS-BSA for 10 minutes at room temperature. The wells were washed and 1:1000 Goat anti-human protein C (American Diagnostica, Greenwich, CT) in TBS-BSA was incubated at 37°C for 20 minutes. After washing, 1:1000 rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate (Sigma, St. Louis) was incubated at 37°C for 20 minutes. Bound chromophore was detected with OPD substrate (Abbot, Chicago) at 490 nm using an EL308 Bio-Tek Microplate reader.

Purification of rhPC from Milk

Clarified milk/EDTA was diluted with two parts chilled deionized water and loaded columnwise at 1 cm/min onto a DEAE Sepharose FF (Pharmacia, Uppsala, Sweden) column. The column was washed with TBS (25 mM Tris-HCl, 50 mM NaCl, .02% sodium azide, pH 7.2) until a steady baseline absorbance (A_{280nm}) was reached. The rhPC was then eluted from the DEAE column with TBS-0.25 M NaCl (25 mM Tris-HCl, 0.25 M NaCl, 0.02% sodium azide, pH 7.2), and directly loaded onto the 12A8-8861-mAb immunoaffinity column (at 1 cm/min) connected in tandem to the DEAE column. The murine mAb 12A8-8861 was produced from hybridoma cells in bioreactors (Kang *et al.*, 1992). The 12A8-8861-mAb was anchored on Emphaze (a gift from 3M Bioapplications, St. Paul, MN) according to the procedure given in Subramanian *et al.* (1994). The 12A8-8861-mAb binds to the activation region of hPC. After loading, the 12A8-8861-mAb immunoaffinity column was washed with TBS-0.25 M NaCl and bound rhPC was eluted with a pH 10 buffer (0.1 M Na_2CO_3, 0.15 M NaCl, 0.02% sodium azide). The pH 10 fraction was immediately brought to neutral pH by the addition of Tris-HCl. The immunoaffinity column was then regenerated with 2 M NaSCN and 4 M NaCl. Bound whey proteins remaining on the DEAE column were eluted with 1M NaCl, and the column was regenerated with 4 M NaCl. All chromatographic fractions were analyzed by ELISA for rhPC.

7D7B10 Fractionation

The pH 10 elution products were further fractionated using a 7D7B10-mAb immunoaffinity column. The mAb 7D7B10 binds to the light chain of hPC in the presence of EDTA and elutes fully γ-carboxylated hPC in the presence of calcium (Orthner *et al.*, 1989). The immunosorbent was made by anchoring the mAb to Emphaze at a final

density of 1 mg mAb/ml swollen gel. The column was equilibrated in TBS-25 mM EDTA before each purification. The neutralized pH 10 products were mixed 1:1 with TBS-25 mM EDTA, pH 7.2, and loaded on the 7D7B10 column at 1 cm/min. The columns were loaded with pH 10 products having five times the maximum capacity of rhPC, washed with TBS, and bound rhPC was eluted with TBS-25 mM $CaCl_2$, followed by regeneration with 2 M NaSCN and then 1 M NaCl. The calcium and thiocyanate peaks were analyzed by ELISA for rhPC, and the percentage of the total rhPC bound eluted in the calcium peak was determined from the sum of calcium, thiocyanate, and NaCl products.

SDS PAGE and Western Blots

Silver-stained SDS PAGE was used to visualize the proteins in the chromatographic fractions. Gradient gels of 9-18% were poured in Bio Rad Protean IIxi 16X20 cm cassettes. The total protein concentration of the crude chromatographic fractions was estimated by measuring the $OD_{280\ nm}$ and assuming an extinction coefficient of 1 ml/mg. The Bio Rad dye binding assay using bovine casein in 0.16 M lactose as a standard was also used and similar results for total protein concentration were found relative to that obtained using the $OD_{280\ nm}$ method. A total of 4 µg of total protein was applied to the wells for all crude fractions. Purified fractions of rhPC were loaded at 0.5 µg (non-reduced) and 1 µg (reduced). Molecular weight markers were purchased from Bio Rad.

For western blots, the proteins were electrophoresed in 9-18% gradient gels as above. Nonreduced samples were loaded at 0.25 µg/well; reduced samples were loaded at 0.5 µg/well. The proteins were transferred to PVDF membranes (Bio Rad). The blocking buffer used was TBS/0.05%Tween/0.5% casein. The westerns were then developed with goat-anti-hPC (American Diagnostica) (1:1000 dilution in TBS/Tween/casein, incubated 30 minutes at 37°C), rabbit-anti-goat IgG HRP conjugate (Sigma), and the metal-enhanced DAB kit (Pierce, Rockford, IL). Bio Rad prestained broad range molecular weight markers were used in western blots.

APTT Activity Assay

The biological activity of the purified rhPC fractions was measured by the ability of

the rhPC to prolong the activated partial thromboplastin time (APTT) of protein C immunodeficient plasma (Martinoli and Stocker, 1986). Samples of the pH 10 and calcium peaks were diafiltered in Centricon 10 diafiltration units. Four times the original sample volume of 10 mM imidazole, pH 7.2, was added to each sample followed by a reconstitution with APTT assay buffer (50 mM Imidazole, 0.1 M NaCl, 0.1% BSA, 0.01% Tween 20, pH 7.4) to approximately 2 units rhPC/ml. hPC activator (Protac, American Diagnostica, Greenwich, CT) was reconstituted to 1 U/ml and 5 ml was added to each vial of PTT Automate 5 (American Bioproducts). Protein C depleted plasma (PCDP, Sigma) and normal pooled reference plasma (NPRP, Sigma) were used in the assay. Briefly, 0.1 ml of PCDP, 0.1 ml of sample in assay buffer, and 0.1 ml of PTT/Protac were added to the assay tubes. The tubes were incubated at 37°C for 3 minutes, and then 25 mM calcium chloride was added, and the clot formation time was measured using an Elektra 750. Each sample was run in duplicate, with the average of clotting times for 2 or 3 dilutions of each sample reported. A standard curve (hPC Units *vs.* time) was constructed using NPRP doped into PCDP to a total volume of 0.1 ml, followed by the addition of 0.1 ml of assay buffer and 0.1 ml PTT/Protac. A value of 1 U/ml plasma (4 µg/ml) in NPRP was assumed. Relative specific activities of rhPC to that of hPC were calculated by regression analysis of APTT clotting times.

Results

Table 1 shows the average daily expression levels of rhPC in the milk of the transgenic pigs. The two females transgenic for the cDNA-hPC (animals 115-6 and 83-1), had average expression levels of rhPC in milk of about 90 µg/ml and 390 µg/ml, respectively. The two females transgenic for the genomic-hPC (animals 110-1 and 122-5), had average expression levels of rhPC in milk of about 280 µg/ml and about 1 mg/ml, respectively.

A SDS-PAGE of a representative purification process of rhPC from pig 110-1 clarified whey is shown in Figure 1. Initial total protein levels in the transgenic milk samples ranged from 60-80 mg/ml (as measured by OD_{280nm}). The caseins (M_r~ 27-30 kDa) are the most difficult proteins to remove from the pH 10 product. Contamination by caseins is significant (up to approximately 20% as judged by silver stain SDS PAGE) if the salt concentration of the feed to the immunoaffinity column is less than 0.25 M NaCl (data not shown). By directly loading the 0.25 M NaCl eluate from the DEAE column, weak ionic interactions between rhPC, caseins, and/or the immunoaffinity column are lessened, and a purer product is obtained (Figure 1, Lane 5). The yields of rhPC in the pH 10 fraction ranged from 65-80% of the total rhPC detected in the product fractions. The majority of rhPC not eluted in the pH 10 product was accounted for in the flow-through of the 8861-mAb column and the 2 M NaSCN fraction.

An SDS PAGE and western blot of all the purified pH 10 eluates are given in Figures 2 and Figure 3, respectively. The purity of the rhPC in the pH 10 eluate is estimated to be >98%, as judged by densitometric analysis of the silver stained SDS PAGE of the nonreduced samples. The longer gradient gels used in this work significantly improve the resolution of the different populations of rhPC and hPC. The nonreduced samples in Figures 2A and 3 all show heterogeneity in the total rhPC population, with three to five major subpopulations visible as distinct bands in the purified products of 115-6, 83-1, and 110-1(Figure 2A, Lanes 1,2, and 3, respectively). This heterogeneity ranges in M_r from about 50 kDa to about 66 kDa for the rhPC populations and about 55 kDa to about 68 kDa for hPC. Two major populations are seen in the nonreduced products from the milk of the higher rhPC expressing animal, 122-5. In all

cases, the apparent M_r of the majority of the rhPC populations is about 2-5 kDa lower than the analogous forms of hPC. The reduced products in Figures 2B and 3 show that, unlike hPC, about 40 to 50% of the rhPC produced in all the pigs is of the single chain form. Only about 5 to 10% of the hPC appears as single chain form (Figure 2B and 3; Lane 5 and 10, respectively). All three gross forms of the putative heavy chain appear in all the rhPC products; alpha, beta, and gamma heavy chains. The light chain of the rhPC products also ran at a lower M_r of about 25 kDa for rhPC relative to about 25 to 28 kDa for hPC. More heterogeneity was seen in the light chain of hPC.

The percentage of the total rhPC bound that eluted in the 25 mM $CaCl_2$ from the 7D7B10 column is given in Figure 4. Approximately 33% of the rhPC bound from the pH 10 product from the milk of animal 115-6 eluted in the calcium fraction from the 7D7B10 column. About 22% of rhPC from the pH 10 product of animal 110-1, 20% from animal 83-1, and 10% from animal 122-5 eluted in the calcium fraction from the 7D7B10 immunosorbent. Figure 4 also shows the average daily rhPC expression level and the percentage of calcium-dependent conformer population is roughly inversely proportional to the average daily expression level in milk.

The relative specific biological activities for the majority of the total rhPC population (pH 10 fraction from the 12A8-8861 mAb column) and the calcium subpopulation (calcium fraction from the 7D7B10 mAb column) as measured by APTT are given in Table 2. The relative specific activity of rhPC is expressed as a percentage of the specific activity of hPC. For the pH 10 fractions, pig 115-6 had the highest relative specific activity of 35%, followed by 110-1 at 28%, 83-1 at 16%, and 122-5 at 13% of hPC. The relative specific activities of the rhPC from the pH 10, 12A8-8861-mAb column products were about inversely proportional to the average expression levels. The activities of the calcium fractions for all the pigs were higher than the activities from the respective pH 10 fractions. The relative specific activities of the rhPC from the Ca^{2+}-7D7B10 mAb column products ranged from 67% for rhPC from animal 115-6, 75% for rhPC from animal 122-5, 81% for rhPC from animal 110-1, to 160% for rhPC from animal 83-1.

Discussion

We have included in this study four pigs representing four different genetic lines of transgenic pigs, each having a steady expression level of rhPC throughout a normal and healthy lactation. The range of rhPC expression in these pigs covers a full order of magnitude: 100-1000 µg/ml milk. The rate limitations for post-translational modifications can be studied here due to the wide range of expression levels in the porcine mammary gland coupled with the numerous post-translational modifications of fully processed hPC.

The first objective of this study was to purify the majority of the rhPC population from the milk without significant yield losses. Porcine milk is a complex, multiphase mixture, and a challenging feedstock to process (Wilkins and Velander, 1992). Ultracentrifugation and other casein precipitating steps were not used, as this results in a significant loss of rhPC in the casein pellet (Drohan *et al.*, 1994b, and unpublished observations). Instead, the casein micelles were solubilized with EDTA, and the milk/EDTA mixture was centrifuged to remove the milkfat. The rhPC was eluted from the ion exchange column with a 0.25 M NaCl buffer. In previous studies, this salt eluate was diluted to a lower ionic strength and then loaded on a 12A8-8861 mAb column (Morcol *et al.*, 1994). However, silver stain SDS-PAGE of the resulting pH 10 product showed about 10-20% contamination by caseins. In an effort to discover what type of nonspecific interactions were causing this contamination, the ionic strength of the 0.25 M NaCl eluate was left unchanged and loaded directly on to the 12A8-8861 mAb column. Apparently, ionic interactions between the caseins and the rhPC and/or the immunoaffinity column were responsible for the casein contamination, as the increased ionic strength resulted in a much more pure pH 10 product (Figures 1-2). However, increasing the ionic strength of the immunoaffinity feed appeared to yield slower binding kinetics, as about 10-20% of the rhPC population passed through the column. The remaining yield losses for the process (~10%) were from rhPC that remained tightly bound to the immunosorbent and eluted in the 2 M NaSCN cleaning step of the column. No further characterization of the rhPC in the 12A8-8861 immunoaffinity fall-through or the NaSCN fractions was done for this study.

The 7D7B10 mAb was developed for gentle elution conditions needed for large

scale purification of hPC from plasma (Velander *et al.*, 1990). Nonspecific protein-protein interactions between rhPC, milk proteins, and/or the 7D7B10 immunosorbent resulted in prohibitively slow adsorption kinetics and very low yields of rhPC when loading a crude feed (whey/EDTA and the 0.25 M NaCl DEAE eluate) on the 7D7B10 column. In contrast, the adsorption kinetics of rhPC on to the 12A8-8861 mAb immunosorbent were much faster and greater yields of rhPC were obtained. The rhPC was therefore first purified using the 12A8-8861 mAb immunosorbent, and then further fractionation of active from inactive populations was done using the 7D7B10 mAb immunosorbent.

A pure, biologically active fraction of the pH 10 population was obtained by immunofractionation with the calcium-dependent monoclonal antibody 7D7B10 (Table 2). The 7D7B10 binds protein C in the presence of EDTA and releases protein C *via* a conformational change of the epitope (residues 1-15 of NH_2-terminus of the light chain) in the presence of calcium. Fully γ-carboxylated hPC from plasma was shown to have a calcium-dependent interaction with 7D7B10 (Orthner *et al.*, 1989). The percentage of rhPC eluted in a calcium dependent manner was inversely proportional to the average rhPC expression level during the respective lactations (Figure 4). These data indicate that at higher synthesis rates (beginning at about 1 mg/ml milk), the biosynthetic machinery of the mammary epithelial cells is unable to fully γ-carboxylate Glu^6, Glu^7, and/or Glu^{14} in as much as 90% of the secreted rhPC. This corresponds to a per cell synthesis rate of about 10-20 pg/cell/24 hr (Morcol *et al.*, 1994). Furthermore, the calcium-dependent fractions of rhPC from each pig either approached 70-80% of the functional activity of hPC from a plasma reference pool (for rhPC from animals 115-6, 110-1, 122-5), or were hyperactive at about 160% of reference hPC activity (for rhPC from animal 83-1). Thus, the calcium-dependent mAb immunosorbent was capable of selectively eluting the most active population of rhPC.

The yields and specific activities of the rhPC from the immunopurification process and the previously reported multistep precipitation/ion exchange process (Drohan *et al.*, 1994b) were similar for the same source milk. However, this immunoaffinity chromatographic process is more amenable to scale-up than the process based on multiple precipitation, centrifugation, filtration, and solubilization steps. An affinity

chromatographic based purification process such as presented here eliminates the need for multiple time consuming, labor intensive, low resolution steps.

A number of studies have investigated the contributions of various domains of hPC to its anticoagulant activity (Mesters *et al.*, 1991, Mesters *et al.*, 1993a, Mesters *et al.*, 1993b). In this work we focus on the "gla domain," located in the first 29 residues of the NH_2-terminal of protein C. In fully γ-carboxylated hPC, all nine glutamic acid residues in this region are γ-carboxylated. The gla residues bind Ca^{2+} ions, enabling hPC to interact with endothelial phospholipids. An hPC molecule with a nonexistent gla domain (Esmon *et al.*, 1983), or a site-specific mutated gla domain (Glu^6-Glu^7 → Asp^6-Asp^7, Zhang and Castellino, 1990) all show greatly reduced activation rates and biological activities. The mAb 7D7B10 binds to residues 1-15 of the light chain NH_2-terminus of hPC in the presence of EDTA, and releases hPC *via* a conformational change of the epitope in the presence of calcium (Orthner *et al.*, 1989). This epitope contains three gla residues, including the critical gla residues Gla^6-Gla^7, identified by Zhang and Castellino (1990) as being essential to obtaining fully functional hPC. Calcium dependent elution of protein C is not observed if the gla residues are not present, and harsh elution conditions (e.g., 2 M NaSCN) must then be used to remove bound protein C.

Silver stain SDS-PAGE and analogous western analysis of the purified pH 10 products show that a number of different populations of rhPC were produced, the majority having a lower M_r than hPC (Figures 1-3). Human protein C contains about 14% carbohydrate (Yan et al., 1990), and differences in the M_r of hPC subpopulations have been shown to be caused by different glycosylation patterns (Miletich and Broze, 1990). The reduced gel and western blot indicate that glycosylation patterns of the heavy chain of the rhPC samples is likely responsible for the heterogeneity seen in the nonreduced samples. This has been confirmed by silver stain SDS-PAGE and analogous western analysis of de-glycosylated rhPC (data not shown). Inspection of the gel and western blot of the reduced samples also reveals that the β and γ heavy chains for both rhPC and hPC migrate with very similar M_r. As would be predicted from the greatest permutation in glycoform variants due to the presence of three potential N-linked glycosylation sites, it is the putative α-chain of rhPC having that shows the greatest difference in M_r to that of

hPC.

Heterogeneity in the rhPC light chains is visible, but is not as significant. Propeptide cleavage, differing glycosylation, and the extent of γ-carboxylation can be causes of these differences between light chain forms. Amino terminal sequencing shows that propeptide is not removed in about 20% of the rhPC forms (data not shown). The majority of the rhPC light chains migrate with a M_r of that of the smallest hPC light chain population, possibly due to different glycosylation of the light chain.

The products from this immunopurification scheme show the same amount of single chain form: about 40 to 50%, as was found for differently immunopurified rhPC reported in Drohan *et al.*, (1994a). Immunofractionation using a different monoclonal antibody to the activation peptide domain was able to separate some of the single chain populations from mixed heterodimeric and single chain populations (Velander *et al.*, 1992a). A rate limitation is indicated for post-translational cleavage of the dipeptide Lys^{156}-Arg^{157} at all of the expression levels studied here.

In conclusion, immunoaffinity chromatography using monoclonal antibodies can be a useful method of fractionating biologically active populations from the gross population of a recombinant protein. A purification process including a step such as this may be of great importance once recombinant proteins gain clinical approval for use as therapeutics. In addition, the use of a monoclonal antibody directed toward an epitope containing a post-translationally modified portion of the protein can help identify rate limitations in the synthesis and secretion process. We postulate that the rate of γ-carboxylation is a key rate limiting step in the synthesis of biologically active rhPC in the porcine mammary gland. This hypothesis is supported by the inverse relationship between the rhPC expression level and the calcium-dependent rhPC-7D7B10 interaction and the high biological activity of the calcium-dependent fraction obtained from the 7D7B10 column. Further study will be done to characterize the carbohydrate moieties, amino acid sequences, and gla-domains of rhPC from pigs with varying expression levels. This study indicates that while transgenic livestock can secrete complex recombinant proteins in milk at gram/liter levels, a significant fraction of the protein may

be biologically inactive due to rate limitations in key post-translational modifications.

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Table 1. Average rhPC expression levels of transgenic pigs in this study. Protein C values were measured by polyclonal ELISA. The average of at least three daily samples was used for each pool.

Pig	Gene Construct	Lactation	Average rhPC Expression Level [µg/ml milk]
115-6	cDNA	2nd	90
83-1	cDNA	2nd	390
110-1	Genomic	2nd	280
122-5	Genomic	1st	960

Table 2. Summary of APTT activities measured for pH 10 and calcium fractions. Activities are reported assuming values of 1 U hPC/ml NPRP (250 U/mg hPC).

Pig	APTT Activity of pH 10 Fraction [% of hPC in NPRP]	APTT Activity of Calcium Fraction [% of hPC in NPRP]	Average rhPC Expression Level [µg/ml]
115-6	35 ± 1%	67 ± 3%	90
110-1	28 ± 2%	81 ± 7%	280
83-1	16 ± 2%	160 ± 26%	390
122-5	13 ± 1%	75 ± 3%	960

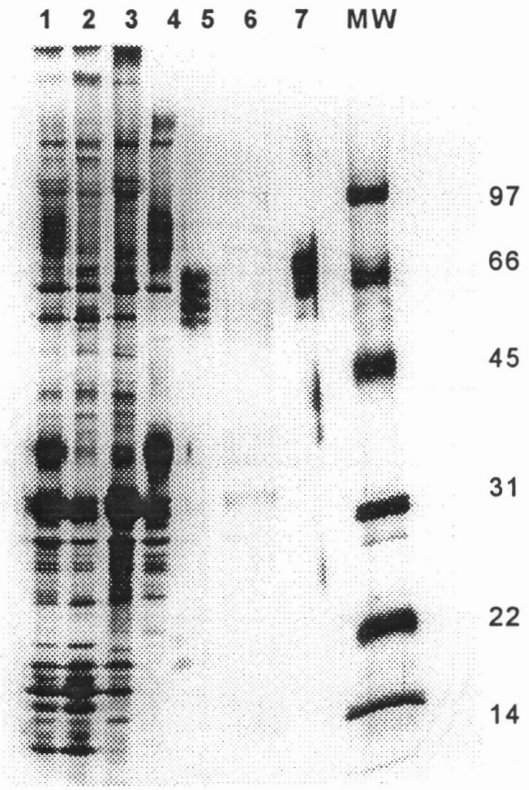

Figure 1. 9-18% Silver stained SDS PAGE of the purification process for transgenic pig 110-1. **Lane 1:** clarified milk/EDTA feed; **Lane 2:** DEAE column flow through; **Lane 3:** 12A8-8861 column flow through; **Lane 4:** 1 M NaCl DEAE eluate; **Lane 5:** pH 10 product; **Lane 6:** 12A8-8861 column 2 M NaSCN; **Lane 7:** purified hPC reference.

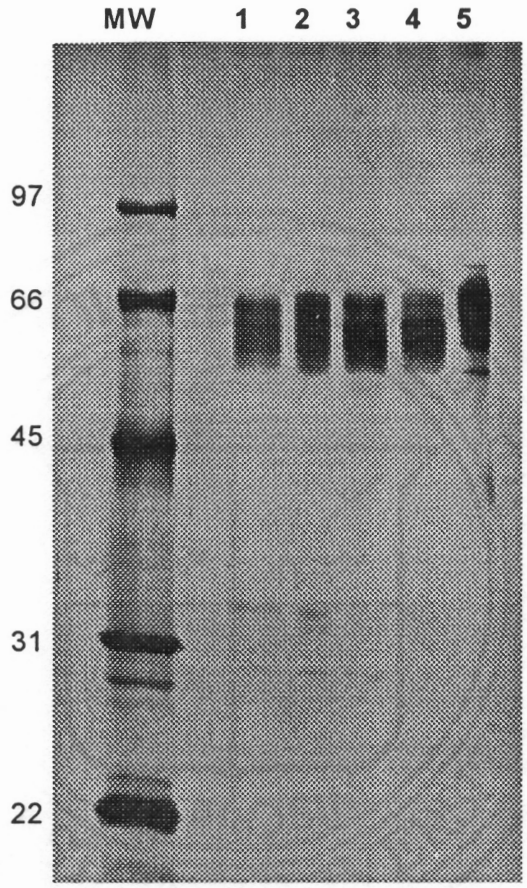

Figure 2A. 9-18% Silver stained SDS PAGE of purified pH 10 products, nonreducing conditions. **Lane 1**: 115-6; **Lane 2**: 83-1; **Lane 3**: 110-1; **Lane 4**: 122-5; **Lane 5**: purified hPC reference

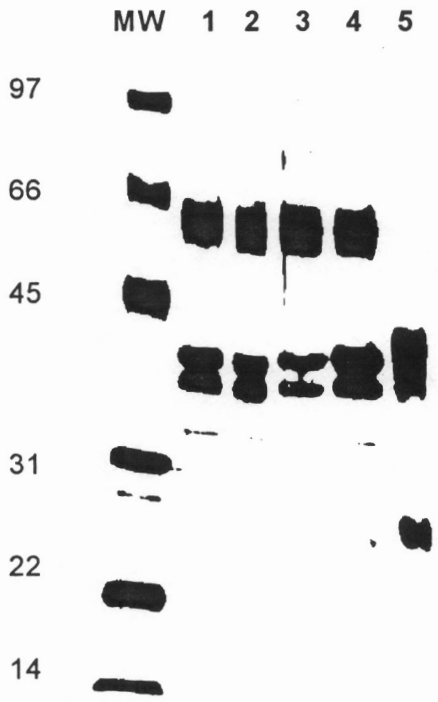

Figure 2B. 9-18% Silver stained SDS-PAGE of purified pH 10 products, reducing conditions. **Lane 1:** 115-6; **Lane 2:** 83-1; **Lane 3:** 110-1; **Lane 4:** 122-5; **Lane 5:** purified hPC reference.

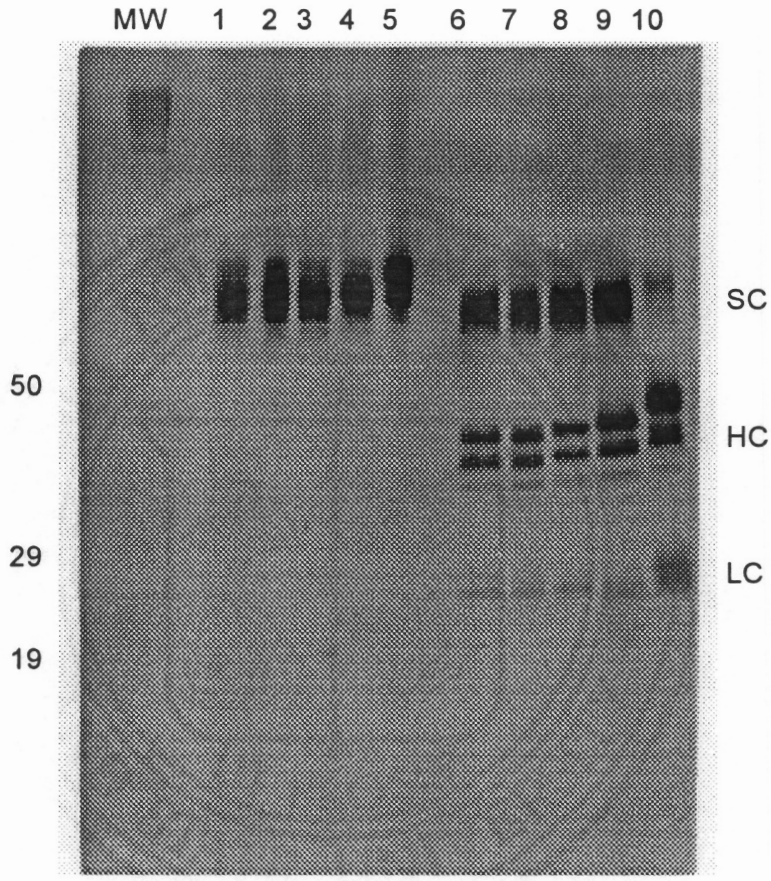

Figure 3. Western blot of non-reduced (Lanes 1-5) and reduced (Lanes 6-10) pH 10 products. Lanes 1 & 6: 115-6; Lanes 2 & 7: 83-1; Lanes 3 & 8: 110-1; Lanes 4 & 9: 122-5; Lanes 5 & 10: purified hPC reference.

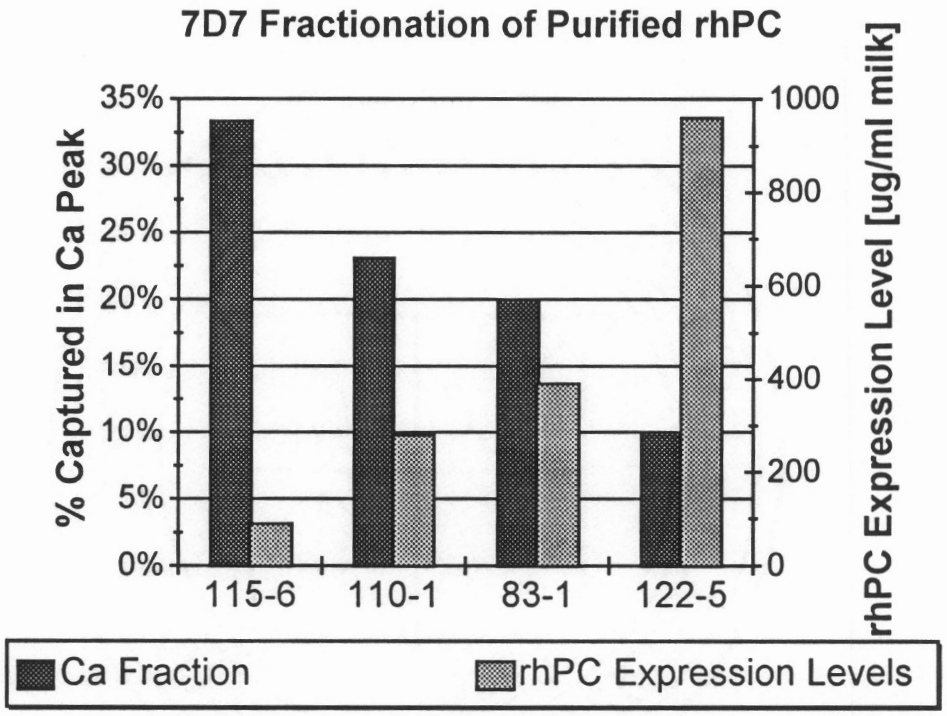

Figure 4. 7D7B10 fractionation of the pH 10 products. A 7D7B10 immunoaffinity column was overloaded with purified rhPC from the pH 10 fractions. The amounts of rhPC that eluted in the calcium peak and the 2 M NaSCN peak were quantitated by ELISA. The percentage of bound rhPC eluting in the calcium peak is reported here.

Chapter 5: High Level Expression of Active Recombinant Human Factor IX in the Milk of a Transgenic Pig

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Abstract

Synthesis of high levels of biologically active recombinant human Factor IX (rhFIX) in the milk of transgenic livestock has resulted in very low rhFIX levels and minimal biological activity. We report here that a transgenic pig with a mouse whey acid protein (WAP) promoter region and the human FIX cDNA sequence secretes up to 200 μg rhFIX/ml milk. The rhFIX was purified by a combination of ion exchange chromatography and immunoaffinity chromatography. The monoclonal antibody used recognizes properly γ-carboxylated FIX with proper propeptide processing. Amino acid sequencing of the immunopurified product confirmed that the majority of purified rhFIX was properly γ-carboxylated and did not contain propeptide. One-stage activated partial thromboplastin time (APTT) showed that the rhFIX had full biological activity. This is the first reported case of a transgenic livestock animal producing high levels of biologically active rhFIX in milk.

Introduction:

A schematic of the human FIX molecule is given in Figure 1. FIX is homologous to other vitamin K dependent (VKD) proteins, consisting of a N-terminal gla domain, an EGF domain, and the enzymatic serine protease domain. FIX circulates in plasma as a zymogen at a concentration of about 4 µg/ml. FIX is activated by a two-step removal of the activation peptide (Ala^{146}-Arg^{180}) from the molecule (Bajaj and Birktoft, 1993). The first cleavage is made at the Arg^{145}-Ala^{146} site by either FXIa or FVIIa/tissue factor. The second, and rate limiting cleavage, is made at Arg^{180}-Val^{181}. The activation pathways involving FXIa and FVIIa/tissue factor are both calcium dependent. However, the FVIIa/tissue factor pathway requires tissue factor that is released from damaged endothelial cells. Activated hFIX thus exists as a disulfide linked heterodimer of the heavy chain and light chain. For full biological activity, hFIX must also have the propeptide removed and must be fully γ-carboxylated (Kurachi *et al.*, 1993).

To date, there is no recombinant human FIX (rhFIX) product commercially available to patients with hemophilia B. Production of biologically active recombinant hFIX in both mammalian cell culture and in the milk of transgenic animals has been problematic. Mammalian cell culture systems are limited in their secretion rates of rhFIX, and incomplete γ-carboxylation and propeptide removal reduce the activity of the secreted rhFIX (Anson *et al.*, 1985; Busby *et al.*, 1985; Jallat *et al.*, 1990; Wasley *et al.*, 1993; Kaufman *et al.*, 1986). Production of rhFIX in the milk of transgenic sheep resulted in extremely low levels of about 25 ng/ml milk, caused in part by cryptic splicing in the 3' region of mRNA in the sheep mammary gland (Clark *et al.*, 1989; Yull *et al.*, 1995). Cryptic splicing also occurs in transgenic mice expressing FIX having 3' FIX UTR sequences. Even using gene rescue, only 5 µg/ml rhFIX with a low specific activity was produced in sheep milk (Colman, 1995).

Results

We have made two founder transgenic pigs (one male and one female) having a 2.6 kb WAP-FIX cDNA construct (Table 1). The male, 57-7, did not transmit the transgene. Founder 58-1 has produced one female offspring having the FIX cDNA transgene. Founder 58-1 has produced six additional offspring, three females and three males, from her second litter. The three females were not transgenic. Two of the males from the second litter tested positive for the FIX transgene. We have evaluated two lactations of the female founder 58-1.

Daily levels of rhFIX in the milk of transgenic pig 58-1 were measured by polyclonal ELISA (Figure 2). Daily levels of 100-220 µg/ml milk were maintained throughout the 10-day lactation. Daily expression levels in the second lactation are estimated to be in the same 100-200 µg/ml milk range, based on western blot analysis of the whey samples (Figure 3).

Figure 3 shows a western blot of the milk/EDTA samples from 58-1's first (Figure 3A) and second (Figure 3B) lactations under reducing conditions. There are three sub-populations of rhFIX: the major population migrating at a M_r of about 60-65 kDa, which is a slightly lower M_r than hFIX (Lane 9), and minor sub-populations migrating at about 40-45 kDa, and at about 25 kDa. Purified hFIX also possessed a subpopulation at about 45-50 kDa.

Purification of rhFIX from a pool of the first lactation from the milk of 58-1 was accomplished by ion exchange chromatography followed by metal-dependent immunoaffinity chromatography (MAb 1H5). The yields from the anion exchange and immunoaffinity steps were quantitative, and no rhFIX was detected in the flow-through chromatographic fractions by polyclonal ELISA. Using this two-step chromatographic procedure, we were able to purify the rhFIX to about 98% purity, as judged by the silver stained 9-18% SDS PAGE. The N-terminal amino acid sequence of immunopurified rhFIX is given in Table 2. The N-terminal amino acid sequence of the purified rhFIX corresponded to the N-terminal sequence of human FIX (Yoshitake *et al.*, 1985), and matched the sequence of purified human FIX standard obtained from the American Red

Cross. The lack of a strong glutamic acid signal in cycles 7 and 8 of the purified rhFIX sample is noted.

The biological activity of the purified rhFIX was measured by a one-stage APTT clotting assay. The immunopurified rhFIX had a specific activity of 337 U/mg, the immunopurified hFIX from plasma had a specific activity of 230 U/mg, with the normal plasma reference pool activity defined as 250 U/mg (Table 3). Plots of $\ln\{time^{-1}\}$ *vs.* $\ln\{[rhFIX^{-1}]\}$ resulted in similar intercepts for NPRP, purified hFIX, and the immunopurified rhFIX. The intercept is related to the K_m and V_{max} for a simplified Michaelis-Menten analysis of the *in vitro* coagulation process. The slope indicates the relative enzymatic activity.

Discussion

We have demonstrated that transgenic pigs can synthesize and secrete high levels (100-200 µg/ml milk) of active rhFIX in milk. Based on reduced western blots, the majority of the rhFIX population appears to be a single chain polypeptide having a post-translationally modified structure similar to hFIX. The rhFIX secreted into pig milk is biologically active and is able to initiate clotting in FIX-deficient human plasma. This is the first reported production of high levels of fully active rhFIX in the milk of transgenic livestock. By using a combination of metal-dependent immunoaffinity chromatography selective for the native Gla-domain, amino acid sequencing, and coagulation assay we have shown that at these high expression levels sufficient γ-carboxylation has occurred in the majority of secreted rhFIX. This is in contrast to transgenic pigs secreting similar levels of another VKD protein, recombinant human protein C, where about 60-70% of the secreted rhPC was not fully γ-carboxylated (Van Cott *et al.*, 1996). Although we have a limited sample size, we postulate that rhFIX may be a better substrate for propeptide removal and γ-carboxylation in the porcine mammary gland. The N-terminal region of the propeptide of protein C has been identified as necessary for γ-carboxylation (Foster *et al.*, 1987). Differences in the propeptide amino acid sequences of protein C and FIX affecting tertiary structure may be responsible for making rhFIX a better substrate for γ-carboxylation in the porcine mammary gland.

This same WAP-FIX construct produced no detectable levels of rhFIX in the milk of transgenic mice. Furthermore, rhFIX produced in sheep with gene rescue was only at 5 µg/ml and had low biological activity (Colman, 1995). Thus, the proper synthesis of active, single chain rhFIX appears to be species specific in livestock. In addition, it appears that human VKD proteins can be sufficiently γ-carboxylated by the mammary gland of transgenic pigs at levels of at least 100-200 µg/ml. Interestingly, a native FIX mRNA from mammary gland biopsies at day 11 in the first lactation was detected; no cryptic splicing was observed, as was seen in sheep (Clark *et al.*, 1989; Yull *et al.*, 1995).

Materials and Methods

Milk collection and handling

Lactating sows were injected IM with 30-60 IU of oxytocin (Vedco Inc., St. Joseph, MO) to stimulate milk let-down. Letdown occurred 2-5 minutes after injection. Pigs were milked by hand during the course of this study. Immediately after collection the milk was diluted 1:1 with 200 mM EDTA, pH 7.0 to solubilize the caseins and then frozen. Small aliquots (~1 ml) of the milk/EDTA mixture were taken and centrifuged for approximately 30 minutes at 16000 x g at 4°C. The fat layer was separated from the diluted milk/EDTA fraction, and the diluted milk/EDTA fraction was used for all further assays. In this study, all concentration values reported for milk were obtained from diluted milk/EDTA samples that were multiplied by a factor of 1.9 to account for dilution with EDTA and subsequent removal of milk fat.

Determination of Recombinant Factor IX in Milk

Immulon II microtiter plates (Fisher Scientific, Pittsburgh) were coated overnight with 100 µl/well of 1:1000 rabbit anti-human FIX (Dako) in 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 9.6 at 4° C. The wells were washed with TBS-Tween (TBST, 25 mM Tris, 50 mM NaCl, 0.2% Tween 20, pH 7.2), and then blocked for 30 minutes with TBS/ 0.1% BSA at room temperature. Samples and human FIX standard (a gift from the American Red Cross) in the TBS-BSA dilution buffer were added in triplicate to the wells (100 μl/well) and incubated at 37°C for 30 minutes. The wells were then washed and blocked for another 10 minutes at room temperature. Goat anti-human FIX (American Diagnostica, Greenwich, CT), 1:1000 in TBS-BSA, was then incubated in the wells for 30 minutes at 37°C, followed by anti-goat IgG/HRP (Sigma). Bound chromophore was detected with OPD substrate (Abbot, Chicago) at 490 nm using an EL308 Bio-Tek Microplate reader.

Purification of rhFIX from Milk/EDTA

All columns and buffers were kept at 4°C. A pool of daily milk/EDTA samples was diluted to OD 280 nm of 5.0 with TBS, pH 7.2, then loaded at 1 cm/min on DEAE FF Sepharose. The column was washed with TBS, pH 7.2, and then eluted with 0.25 M

NaCl in TBS. This fraction was diluted 1:1 with 40 mM $MgCl_2$ in TBS to a final concentration of 20 mM $MgCl_2$ and loaded on a 1H5 MAb column. The column was washed with TBS containing 20 mM $MgCl_2$, and the product was eluted with 20 mM citrate, 0.15 M NaCl, pH 6.8. The product was dialyzed overnight against 10 mM imidazole, pH 7.2.

APTT Coagulation Assay

The protocol given by the American Red Cross Plasma Derivatives Laboratory (Procedure for Factor IX Coagulation Assay, March 1992) was used for measuring the APTT activity of the purified rhFIX from 58-1. Standards of normal plasma reference pool (Sigma) and hFIX (American Red Cross Plasma Derivatives Laboratory) were used in the assay. Duplicates of 58-1 rhFIX, hFIX, and normal plasma reference pool were run at each dilution.

SDS PAGE of and Western Blots of rhFIX

Daily samples of milk/EDTA from 58-1 were electrophoresed on 8-16% SDS gels (Novex, San Diego). Approximately 125 ng of rhFIX (as determined by polyclonal ELISA) and hFIX standard (American Red Cross), were loaded in each lane. A total of 25 µg of total protein from a pool of non-transgenic (NTG) whey was loaded. After electrophoresis, proteins were transferred overnight to PVDF membranes (Bio Rad). The membranes were washed for 30 minutes in TBST, blocked with TBS/0.05% Tween 20/0.5% Casein (TBST-Casein). The membranes were developed with rabbit anti-FIX (Dako) (1:1000 in TBST-Casein for 45 minutes at 37°C), followed by anti-rabbit IgG/HRP (Sigma) (1:1000 in TBST-Casein for 45 minutes at 37°C), and the DAB metal enhanced staining (Pierce). Molecular weight markers were purchased from Bio Rad.

Protein Sequencing

Immunopurified rhFIX and hFIX (American Red Cross) were electrophoresed on 16 x 20 cm Bio Rad Protean IIxi 9-18% SDS PAGE, and then blotted onto PVDF membranes (Bio Rad) for sequencing per the manufacturers instructions. The bands on the membranes were excised and then sequenced at the Virginia Tech University Protein Sequencing Laboratory using an Applied BioSystems 477A protein sequencer and 120A

analyzer.

Table 1. Inventory of rhFIX Transgenic Pigs

Pig ID	Construct	Sex	DOB	Comments
57-7	WAP/FIX	M	5/19/64	Founder, PCR* positive for WAP and FIX
58-1	WAP/FIX	F	5/21/94	Founder, PCR positive for WAP and FIX
63-1	WAP/FIX	F	7/14/95	G^1 from 58-1, positive for WAP and FIX
63-2	WAP/FIX	F	7/14/95	G^1 from 58-1, positive for WAP and FIX (dead)
litter#10 to 58-1	WAP/FIX	3F, 3M	1/22/96	2 transgenic males

DOB Date of birth

M/F Male/Female

WAP Whey acid protein

* Detection of hFIX transgene carried out by PCR method.

Table 2. Amino acid sequencing results of immunopurified rhFIX from the milk of 58-1.

Cycle	rhFIX Sequencing Results		hFIX Sequence
	Amino Acid	[pmol]	
1	Y	94	Y^1
2	N	74	N^2
3	S	34	S^3
4	G	108	G^4
5	K	62	K^5
6	L	90	L^6
7	E	3	E^7
8	E	3	E^8
9	F	64	F^9
10	V	72	V^{10}

Table 3. APTT coagulation assay of immunopurified rhFIX from the milk of 58-1. A one stage clotting assay was performed according to the protocol given by the American Red Cross Plasma Derivatives Laboratory (Procedure for Factor IX Coagulation Assay, March 1992).

Sample	Slope	Slope Ratio	Equation	% Activity	Specific Activity
NPRP	0.094	1.0	y=0.094x - 3.7	100%	250 U/mg
hFIX (ARC)	0.086	0.92	y = 0.086x - 3.6	92%	230 U/mg
58-1 rhFIX	0.127	1.35	y = 0.127x - 3.4	135%	337 U/mg

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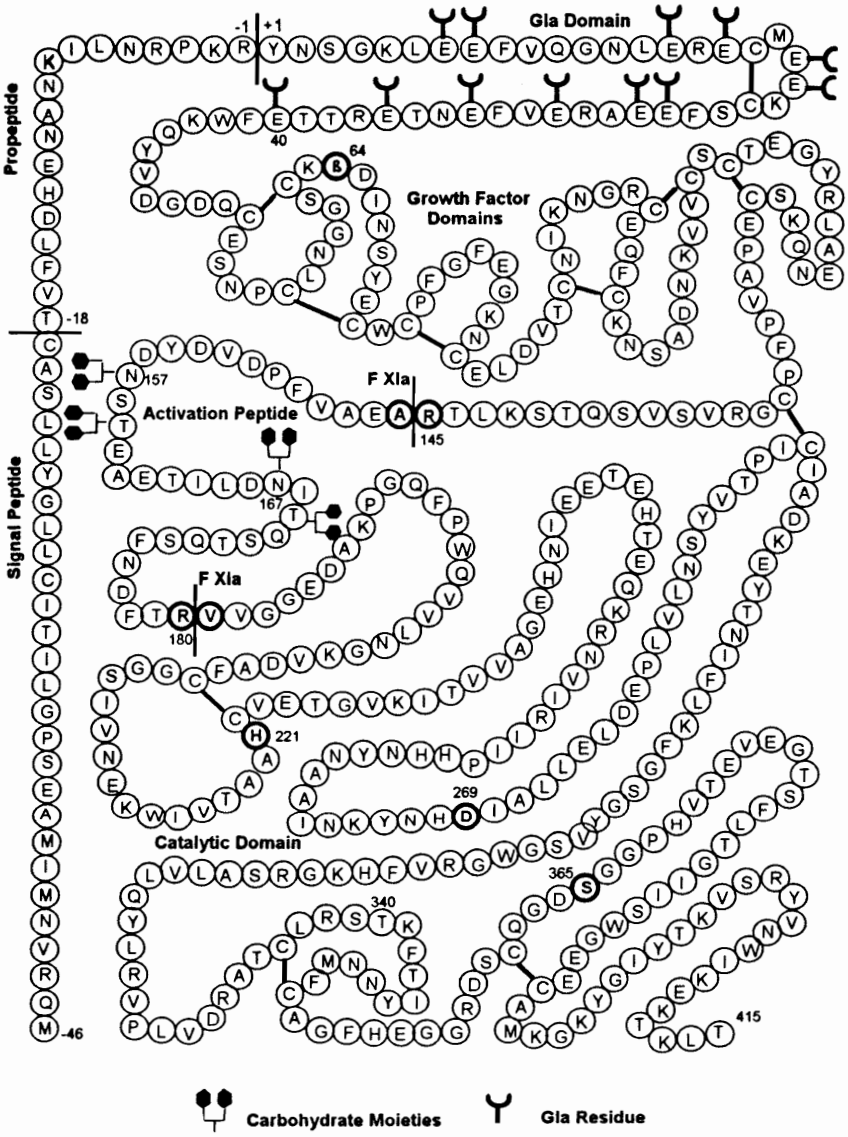


Figure 1. Schematic of human Factor IX.

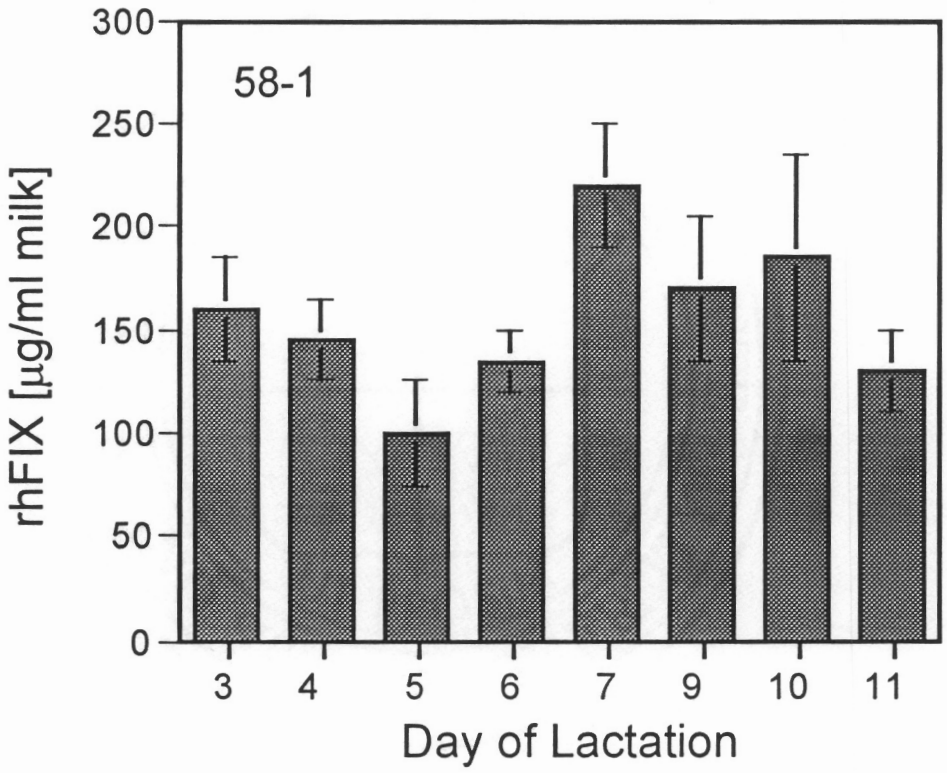
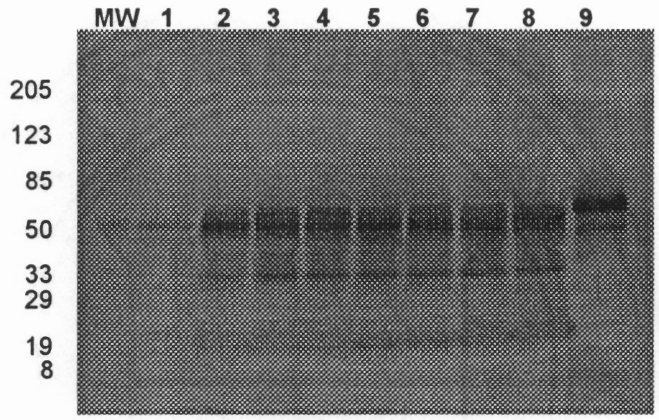
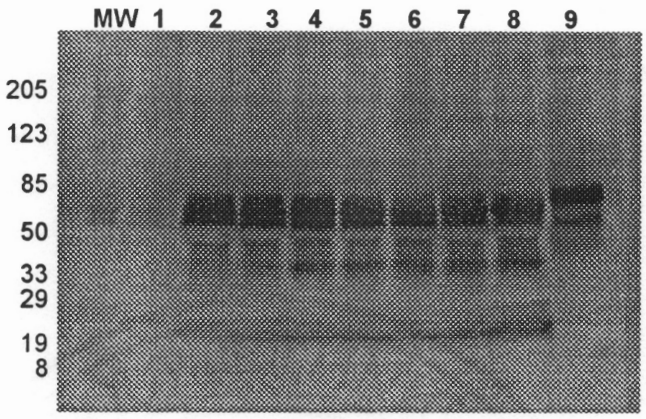


Figure 2. rhFIX levels in the milk of transgenic pig 58-1.



(a)

Figure 3A. Western blot of rhFIX produced in the milk of 58-1, first lactation. **Lane 1:** nontransgenic milk/EDTA pool; **Lane 2:** Day 3; **Lane 3:** Day 4; **Lane 4:** Day 5; **Lane 5:** Day 6; **Lane 6:** Day 7; **Lane 7:** Day 9; **Lane 8:** Day 10; **Lane 9:** purified human FIX.



(b)

Figure 3B. Western blot of rhFIX produced in the milk of 58-1, second lactation. **Lane 1:** nontransgenic milk/EDTA pool; **Lane 2:** Day 2, 0930 hr; **Lane 3:** Day 2, 1610 hr; **Lane 4:** Day 3, 0845 hr; **Lane 5:** Day 3, 1115 hr; **Lane 6:** Day 3, 1430 hr; **Lane 7:** Day 3, 1715 hr; **Lane 8:** Day 4, 0945 hr; **Lane 9:** purified human FIX reference.

Chapter 6. Conclusions and Future Work

This thesis contains the first collection of articles meant to specifically address issues that will affect FDA clinical trials and approval of recombinant VKD proteins from the milk of transgenic pigs. Although most reports in the literature on transgenic animals and bioreactors have concentrated on downstream characterization of the recombinant protein, we have characterized upstream issues such as genotypic stability, phenotypic stability, and possible consequences of the presence of a heterologous protein on the endogenous milk protein profile. Upstream issues are critical to the engineering design, control, and maintenance of all aspects of any recombinant protein production system whether it is an *E. coli* fermentation, mammalian cell culture bioreactor, or a transgenic animal. For example, Adamson (1995) has reviewed a similar systematic approach to upstream issues in the production of recombinant Factor VIII in Chinese Hamster Ovary (CHO) cell lines. Similar to this thesis, some specific issues that Adamson addresses are (1) the reasons behind selection of CHO cells over other mammalian cells, (2) media-dependent proteolysis and degradation of the product, (3) genotypic stability of the CHO cells from batch to batch, (4) phenotypic stability of the cells as determined by characterization of the product, and (5) effects of culture conditions on the downstream purification of the product.

In this work, we have shown that it is possible to select for genotypic and phenotypic stability in the outbred offspring of transgenic pigs producing rhPC. Furthermore, the extent of post-translational proteolytic processing of rhPC is stable within a given lineage and appears to be dependent on inherent swine genetics. In contrast to mice, it is not clear that the presence of hPC intronic sequences and the longer 4.1 kb mouse WAP promoter are responsible for higher rhPC levels in the milk of transgenic pigs. Further work must be done to evaluate the possible species-specific regulatory mechanisms involved in transcription, translation, and secretion of recombinant proteins by the mammary gland.

The effects of secretion of a heterologous protein into the bioreactor medium have also been addressed here. The consistent presence of rhPC at levels up to about 500

µg/ml milk has no adverse phenotypic effects on the mammary gland. However, there is preliminary evidence that consistent levels of rhPC of about 1000 µg/ml milk may cause slight but specific phenotypic changes in the mammary gland. These changes include slightly shorter lactation and elevated levels of plasma proteins that are transported into the milk by transcytosis. Given the limited sample size and physical constraints at the Virginia Tech Swine Center, more work is necessary to confirm whether or not high levels of rhPC are causing the above phenotypic response. This suggests that the goal of achieving very high expression levels of certain recombinant proteins may not always be desirable, and expression of each protein should be studied individually. In addition, changes in the endogenous milk protein composition could have profound effects on the recovery of a recombinant protein. Our research interests lie in the production of recombinant plasma zymogens (rhPC, FIX, fibrinogen, FVIII). The recovery of the recombinant zymogen is of utmost importance, as any proteolysis or specific activation of the zymogens by changes in the endogenous milk protein composition could render the product useless. The results we have presented here are a prime example of how upstream events, such as the presence of elevated levels of certain plasma proteins, could affect downstream processing and recovery.

Downstream processing of labile biological species from a multi-phase mixture such as milk is a challenging task. Using a combination of ion exchange and immunoaffinity chromatography, we have purified rhPC to >98% purity at high yields in two chromatographic steps. Further fractionation of biologically active subpopulations of rhPC was accomplished by immunoaffinity chromatography using a MAb directed to the Gla domain of rhPC. We found that the extent of γ-carboxylation and the biological activity of the whole rhPC population were inversely proportional to the concentration of rhPC in the milk. This purification process is more amenable to scale-up than previously reported processes that involved multiple precipitation, centrifugation, and resolubilization steps. Since the publication of this procedure, our lab has found that DEAE expanded bed chromatography of milk in the presence of zinc, followed by hydrophobic interaction chromatography can also fractionate the biologically active rhPC populations. Since this new procedure essentially purifies by the partial precipitation of major milk proteins (i.e., caseins), this lab is in the process of investigating this further and applying it to the

purification of other recombinant plasma proteins produced in pig milk.

Currently there are no commercially available sources of rhFIX. Past attempts to produce rhFIX in the milk of transgenic sheep have resulted in very low expression levels and very low biological activity. We have detailed here the first reported production of high levels of rhFIX in the milk of a transgenic livestock animal. During the first two lactations we measured rhFIX concentrations up to 200 μg/ml milk. Purification and characterization indicated that the majority of the rhFIX was properly γ-carboxylated and fully active in the APTT coagulation assay. This indicates that rhFIX may be a better substrate for the porcine carboxylase enzyme and propeptide processing enzyme. Further study is needed to generate multiple lines of rhFIX pigs to study rate limitations in post-translational modifications and compare them to rhPC post-translational modifications. In addition, this is also the first-reported rhFIX production process with the capability of producing enough material for prophylactic treatment of hemophiliacs.

Therefore we conclude that production of recombinant VKD proteins in the milk of pigs appears to be a feasible alternative to plasma-derived concentrates and mammalian cell culture. Although the porcine mammary gland has rate limitations for certain post-translational modifications, the productivity of biologically active populations of recombinant VKD proteins in the mammary gland of transgenic pigs is more than adequate to meet the clinical demands for protein C and prophylactic use of recombinant Factor IX.

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Appendix. Inside-Out Crosslinking (IOC) and Inside-Out Activation (IOA) of Hydrogel Beads

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The following is an excerpt from a memmorandum of invention submitted to the Virginia Tech Intellectual Properites, Inc. KVC completed this work while being supported by LigoChem, Inc. during the summer and fall of 1995.

VIRGINIA TECH INTELLECTUAL PROPERTY DISCLOSURE

NOTE: Invention disclosures are treated as confidential information. Except for individuals engaged in the evaluation and approval process, the information will not be divulged to others without the permission of the inventor(s), except as required by law.

1. TITLE OF WORK: **Inside-Out Crosslinking (IOC) and Inside-Out Activation (IOA) of Hydrogel Beads**

2. TYPE OF WORK: Invention _**X**_ Software ___ Book ___ Article ___ Video ___
Film ____ Other ____ (Describe)

3. LIST OF ORIGINATORS (writers, inventors, or other creators) of the work.

William H. Velander 340-52-4481
Kevin E. Van Cott 307-86-8203
Roger Van Tassell

4. MAILING ADDRESS OF ORIGINATORS:

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Kevin E. Van Cott	**Dept. Chemical Engineering, VPISU**	**703-231-5603**
Roger Van Tassell	**Dept. Chemical Engineering, VPISU**	**703-231-5603**

5. Was the effort that led to this work undertaken as part of a sponsored project?

Yes _X__ No ____ If yes, complete the appropriate blanks below.

a. VPI&SU Grant or Project No:

b. Name of sponsoring agency or company: **LigoChem**

c. Contract or Grant Period: Start **May 1,1995** End **December, 1996**

d. Attach copy of Grant or Contract document, if available.

6. As far as you know, does the sponsor have any claims to the invention?

Yes __X__ No _____ If yes, please indicate the nature of these commitments.

LigoChem has first rights of refusal to negotiate an exclusive contract for IOA and IOC hydrogels.

7. Based on your understanding of the VPI&SU Intellectual Property Policy, do you consider that the Intellectual Property rights to this invention belong to and are owned by:

a. __X__ VPI&SU (with royalty sharing as per Intellectual Property Policy).

b. _____ Originators, but wish to be handled under the Intellectual Property Policy as if owned by VPI&SU.

c. _____ Originators.

I. DESCRIPTION

1. Brief Description (in lay terms). If you are submitting an invention, is it a new process, composition of matter, device or one or more products for a

new use, or an improvement to an existing product or process? If this is a copyright work, describe content or attach a copy.

This invention has process implications. The process implications include a novel method of delivering a ligand to the interior of a low-solids content hydrogel for subsequent immobilization, and preventing the immobilization of excess ligand in the outer strata of the hydrogel bead. Changes in solvent conditions keep the ligand in the interior of the hydrogel so that reaction occurs in the interior of the hydrogel to achieve inside-out crosslinking (IOC) or inside-out activation (IOA)

2. If an invention, use the description above to identify and expand on the novel or unusual features. How does the invention differ from present technology, what problems does it solve, or what advantages does it possess? (This should be written so someone skilled in the art can understand it.)

Chromatographic hydrogels must be chemically crosslinked to impart chemical and mechanical robustness. Thus, leaching of polymer backbone into the purified product is prevented. Also, chemical activation is needed to facilitate attachment of ligands which provide specific adsorptive properties to the hydrogel. Conventional chromatographic hydrogels (e.g., Pharmacia Sepharose FF) are crosslinked by an "outside-in" crosslinking procedure. The crosslinking molecules are insoluble in water, the solvent used in the "outside-in" procedure. The "outside-in" crosslinking process relies on partitioning of the crosslinker into the aqueous phase of the hydrogel, and subsequent reaction with the hydrogel polymer backbone. Phase partitioning is an inefficient mass transfer operation, and this results in little penetration of the crosslinking molecule into the interior of the bead. "Outside-in" crosslinked hydrogels thus have a high degree of crosslinking in the outer strata of the beads, with little crosslinking in the interior of the

beads. Past design of chromatographic matrices also emphasized small particle sizes to reduce intraparticle diffusional mass transfer resistance. However, the high degree of crosslinking in the outer strata of the hydrogels results in minimal intraparticle penetration of average sized protein molecules (such as albumin, 66 kDa) at typical large scale processing linear velocities of 1 cm/minute.

The present invention proposes a change in the crosslinking procedure so that the interior of the hydrogel is highly crosslinked or activated, and the outer strata of the hydrogel is sparsely crosslinked or activated. The strategy we have developed delivers the solubilized crosslinker/ligand molecule to the interior of the hydrogel bead under non-reacting conditions, removes excess crosslinker/ligand from the outer strata of the hydrogel bead, entraps the crosslinker/ligand in the interior of the hydrogel, and reacts the entrapped crosslinker/ligand molecule with the interior polymer backbone. During the crosslinking reaction, the partitioning of the solvent/crosslinker phase with the external aqueous phase is visible. Gradual disappearance of the inner solvent/crosslinker phase can be visually monitored during the reaction stage. Fully crosslinked beads have a "halo" appearance, with the highly crosslinked interior of the hydrogel bead differentiated from the sparse outer strata. After IOC/IOA, further chemical derivitization attaches the protein binding ligands (e.g., DEAE, QEAE, CM, reactive dyes, antibodies) to either the activation ligand or the polymer backbone.

The sparse outer strata of the IOC/IOA hydrogel has visco-elastic fluid-like properties. Environmental electron microscopy has verified the lack of solid structure in the outer strata of the low-solids hydrogels we have developed. The combination of the fluid nature and the high charge density of DEAE ion exchange derivatized outer strata make the DEAE hydrogel's protein binding

characteristics dependent on ionic strength and charge shielding capacity of the column buffer. This aspect of IOC, low-solids content hydrogels enhances the ability to specifically choose column buffer conditions that will effect the proper binding site architecture to selectively bind a target protein, making ion exchange chromatography a more powerful purification step in the overall downstream process. A sparsely crosslinked outer strata will also allow more intraparticle penetration of proteins, resulting in higher protein binding capacity. Thus larger beaded hydrogels can be used in large scale processes to minimize pressure drops across chromatographic columns without losses in protein capacity typically associated with large beaded matrices.

Another novel application of IOC and IOA hydrogels involves our development of IOC hydrogels that are subsequently crosslinked using conventional "outside-in" crosslinking procedures. The "outside-in" crosslinking effectively blocks reactive sites on the outer edge of the hydrogel bead. This prevents reaction with activation and/or protein binding ligands on the surface of the hydrogel bead. The lack of adsorption sites on the outer edge of the bead will prevent non-specific adsorption of large particles such as virus particles and other pathogens present in a biological feed source. Thus, the combination of minimal protein binding ligands on the outer edge of the bead, and limited intraparticle diffusion of species that are much larger than the target protein molecule will result in little nonspecific adsorption of pathogens, and pathogens will be washed through the column.

3. If not indicated previously, what are the possible uses and markets of the invention? In addition to immediate applications, are there other uses that might be realized in the future?

The invention provides an alternative method for the manufacture of chromatographic hydrogels for use in large scale processes. Both ion exchange and affinity matrices can be made from IOC or IOA hydrogels. The visco-elasticity of the outer strata of ion exchange IOC hydrogels affects the protein binding selectivity and enhances the power of ion-exchange purification. IOC/IOA hydrogels will be useful in achieving significant pathogen removal, in addition to high resolution purification of the target protein.

4. Does the invention possess disadvantages or limitations? If so, can they be overcome? How?

The disadvantages and limitations are not yet well known and process development work will be necessary to reveal any limitations.

II. OTHER PERTINENT DATA

1. Has the invention been described in specific detail or in a general way in a publication?(For this purpose, "publication" includes abstracts of public presentations, news stories, etc. as well as published scientific papers.) Has the invention been described orally at public meetings? Please provide exact details, including dates and copies of any existing manuscripts, preprints, or abstracts.

No.

2. Is a publication or oral disclosure descriptive of the invention planned within the next six months? Give date (estimate, if unknown) and attach copies of any existing manuscripts, preprints, or abstracts.

No.

3. Has the invention been tested experimentally? Are experimental data available?

Yes.

4. Are there known inventions by others that are related to this one? Please describe, including information on relevant patents and publications, if

available.

No.

5. Has the material been disclosed to industry representatives? Has any commercial interest been shown in it? Do you know of any other firms that might be interested in the materials? Name companies and specific individuals and their titles, if you can.

   LigoChem, Inc. is aware of the invention. IOC and IOA have been used on hydrogels we have developed for LigoChem.

   Synthons Inc. (Corporate Research Center, Blacksburg, VA) is aware of the invention, and procedures of IOC have been given to Tom Piccarielo.

II. EVALUATION

1. Please describe briefly the technical impact this invention/discovery is likely to have on the field of endeavor (i.e., marginal improvement, significant change, revolutionary upheaval, creates new field, etc.) and why.

   The enhanced protein binding selectivity of ion exchange IOC hydrogels will make ion exchange a more powerful component of downstream processing of biological fluids. Ion exchange has been used primarily as a volume reduction step in the past. IOC/IOA hydrogels can be modified further by conventional methods and will also be used for pathogen removal from biological fluids, since nonspecific adsorption of pathogens in the outer strata of the beads will be minimized. These large-beaded IOC and IOA hydrogels have significantly less pressure drop than conventional small beaded matrices, which will enable longer columns for more efficient protein adsorption, faster throughput, and less costly capital equipment.

2. Please describe briefly the degree of technical development of the technology (i.e., theoretical design, prototype, complete product/process, ready for

commercial testing/marketing, etc.) and give an estimate of the nature and amount of work that still remains to be done before a commercial venture/product is obtained.

The visco-elastic nature of the low-solids content hydrogels suggests that IOA and IOC hydrogels can be custom designed for a particular purification process to take full advantage of the physio-chemical behavior. Testing and validation procedures of chromatographic matrices for industrial use are well established.

3. Please give your best guess as to the economic potential of this invention if successfully commercialized, in terms of annual revenues:

   Less than $10,000 ____ $10-$100,000 ____ $100,000-$1 million ____
   Over $1 million ___X.

4. If the invention is of a type on which patent(s) may be pursued, do you consider it worthwhile to spend up to $10,000 to obtain a U.S. Patent?
   Yes _X__ No ____
   Please indicate the reason(s) for your response.
   Large-scale processing using chromatographic media is about a $1 billion industry worldwide.

5. Please list individuals (both on and/or off campus) with technical or economic knowledge in the field of the invention who could be asked (under confidentially undertakings) to review, assess, or evaluate the technical and commercial potential of this invention.

6. Please give us any other pertinent comments not covered elsewhere, which may provide guidance to the decision-making process and optimal utilization of the technology.

LigoChem, Inc. should be consulted on all matters of pursuing a patent.

Signature of Originators:

a. William H. Velander _____.

b. Kevin E. Van Cott _____.

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Vita

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Education

- BS Chemical Engineering, Purdue University, West Lafayette, IN 1991.
  GPA: 5.8/6.0 Advisor: N.-H. L. Wang
- PhD Chemical Engineering, Virginia Tech University, Blacksburg, VA 1996
  GPA: 3.9/4.0. Advisor: W. H. Velander

Research Experience

- purification and characterization of recombinant proteins from transgenic milk
- health of transgenic animals and stability of protein expression
- design of process scale immunoaffinity matrices
- design of process scale ion exchange matrices
- mathematical modelling of protein separations.

Publications and Presentations

- K. E. Van Cott, B. L. Williams, F. Gwazdauskas, H. Lubon, T. Lee, W. N. Drohan, and W. H. Velander, "Affinity Purification of Biologically Active and Inactive Forms of Recombinant Human Protein C Produced in the Porcine Mammary Gland," *Journal of Molecular Recognition*, In Press.

- K.E. Van Cott, B.L. Williams, F.C. Gwazdauskas, E.T. Kornegay, J. Knight, H. Lubon, W.H. Velander, "Milk Protein Composition of Transgenic Pigs Secreting Recombinant Human Protein C" manuscript in preparation.

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- A. Subramanian, K.E. Van Cott, D.S. Milbrath, and W.H. Velander, "Role of local Antibody Density Effects on Immunosorbent Efficiency," *J. Chromatogr.* **672** (1994) 11-24.

- R. D. Whitley, K.E. Van Cott, and N.-H.L. Wang, "Analysis of Non-equilibrium Adsorption/Desorption kinetics and Implications for Analytical and Preparative Chromatography," *Ind. Eng. Chem. Res.* **32** (1993) 149-159.

- K.E. Van Cott, R.D. Whitley, and N.-H.L. Wang, "Effects of Temperature and Flow Rate on Frontal and Elution Chromatography of Aggregating Systems," *Separations Technology* **1** (1991) 142-152.

- R.D. Whitley, K.E. Van Cott, J.A. Berninger, and N.-H.L. Wang, "Effects of Protein Aggregation in Isocratic Nonlinear Chromatography," *AIChE J.* **37(4)** (1991) 555-568.

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- R.D. Whitley, K.E. Van Cott, J.A. Berninger, and N.-H.L. Wang, "Effects of Protein Aggregation in Isocratic Nonlinear Chromatography," AIChE Conference, Chicago, 1990.

- N.-H.L. Wang, K.E. Van Cott, R.D. Whitley, "Non-equilibrium Effects on Wave Propagation in Nonlinear Liquid Chromatography," AIChE Conference, Chicago, 1990.

- N.-H.L. Wang, C.Y. Lee, X. Zhang, K.E. Van Cott, J. Berninger, and R.D. Whitley, "Non-equilibrium Effects in Complex Liquid Chromatography of Proteins," The 8th International Symposium on Preparative Chromatography, May, 1991.

- K.E. Van Cott, "Effects of Slow Sorption Kinetics on Chromatographic Separations of Proteins," Undergraduate Honors Thesis, Purdue University, 1991.

Honors and Awards

- Purdue Univ. CHE Undergraduate Honors Program (1990-91)
- Purdue Univ. CHE Honors Summer Research Assistantship (1990)
- Purdue Engineering Industrial Roundtable Leadership Scholarship (1990)
- Omega Chi Epsilon CHE Honor Society (1989)
- Burmaster Scholarship (1987-91)
- DuPont PhD Fellowship (1991-95)
- NSF Graduate Fellowship Honorable Mention (1991)

Work Experience

- Undergraduate Research Assistant, Purdue CHE (Summer, 1990)
- Engineering Intern, Exxon Production Research Company (EPR), Environmental Conservation Division, Houston, TX (Summer, 1991).

Kevin E. Van Cott 9/11/96