Potential Sources For The Large Scale Production of Human Protein C

by

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(ABSTRACT)

The vitamin K-dependent family of proteins (VKDs) include prothrombin, factors VII, IX, and X, and protein C (hPC) is synthesized in the liver and act to maintains normal hemostasis. such as properly regulated clotting. An imbalance of any of these pro- or anti-clotting proteins result in hemophilia or disseminated intravascular clotting diseases. Therefore, these proteins have a significiant therapeutic value. Many of these proteins are not available in sufficient quantity due to the trace amounts found in plasma and limitations encountered with downstream recovery.

Protein C, a major regulatory protein of thrombosis and hemostasis, has a potent anticoagulant activity and can be used as an anti-thrombotic agent. The technology for isolating hPC from human plasma is challenged by; (1) its low concentration in plasma, (2) the limited availability of plasma, (3) similar physicochemical characteristics among VKD plasma proteases, and (4) the risk of transmitting viruses such as the human immunodefeciency virus (HIV).

This work focuses on the isolation of protein C from alternative sources for the large-scale production and downstream recovery of highly purified and biologically active hPC. The partial characterization of the protein with respect to post-translational modifications which are essential for functionally active, was also evaluated. Several studies were undertaken:

1. Cohn Fraction IV-I, an off-line discard stream during traditional plasma fractionation process is introduced as an affordable starting material for the large-scale production of hPC. More than 90 percent of the total protein C antigen detected in the various Cohn fractions was found to reside in fraction IV-I. The protein C isolated from Cohn IV-I paste using a metal-dependent monoclonal antibody to hPC was found to be biologically active.

2. Recombinant production of hPC in the milk of transgenic pigs, achieved by targeting the synthesis of the protein to the mammary gland, is presented as a model bioreactor system for the synthesis and downstream recovery of complex human proteins. Two major populations of biologically active recombinant hPC (rhPC) were detected and immunopurified by employing conformation specific metal-dependent monoclonal antibodies in the immunopurification process. A high performance thin layer chromatography method was also developed for the detection of total carbohydrate compositions in protein C.

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Last but not least, thanks are for my dear husband Göktuğ and my dear son Taylan for sharing all the burden of my Ph.D. studies during all these years. This dissertation is dedicated to them with love.

TO TAYLAN AND GÖKTUĞ

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CHAPTER ONE

BACKGROUND

INTRODUCTION

Applications of the advanced techniqus in biotechnology have faciliated the production of many therapeutic proteins with high product purity, safety, and maximum recovery even reducing the product cost in many cases. Two main developments in biotechnology; development of monoclonal antibodies and genetic engineering, have allowed a rapid development in other areas of biotechnology such as the culture of plant and animal cells, protein structural modifications, and new bioreactors including transgenic animals. Biotechnology is not a single but a multidiciplinary subject requiring a wide range of science and engineering inputs. The work presented here searching for the possible potential sources for the production of an vitamin K-dependent (VKD) human therapeutic, protein C covers most of the multidiciplinary requirments and the facination and excitment of biotechnology.

The protein of interest, protein C, is a VKD zymogen of serine protease that functions in the regulation of natural anticoagulant pathway and is an important protein in hemostasis. Due to the limitations encountered with biosynthesis and downstream processing, protein C is not available in large quantities like many other trace plasma proteins. The problems associated with large-scale production of protein C to use as a therapeutic product include its low concentration in plasma, similar physicochemical characteristics among VKD plasma proteases, and risk of viral contamination. Although the development of immunoaffinity chromatography techniques made it possible to achive maximum recovery and purity of the trace plasma proteins, testing the potential clinical

applications of protein C is restricted because of the lack of availability of sources other than plasma would give highly purifed and biologically active protein C concentrates in large quantities.

The pupose of this research is to investigate the alternative starting materials for the large-scale production of biologically active protein C using the developments in immunoaffinity chromatography and transgenic animal bioreactor systems. As the charcaterization of this highly complex protein requires a good understanding of the structure-function relationships, a brief description of the following topics are included in this introductory section;

- Biochemical characteristics of VKD proteins.
- Protein C and regulation of coagulation.
- Clinical importance of protein C.
- Recombinant production of protein C.

BIOCHEMICAL CHARACTERISTICS OF VKD PROTEINS

Vitamin K-dependent family of proteins (VKDs) including Prothrombin (Factor II), Factors VII, IX, and X, protein S, and protein C are synthesized in the liver as inactive zymogens and require vitamin K for their bisynthesis (1, 2). They are necassary for the regulation of blood coagulation (see Fig. 1).

VKD proteins show distinct squential and structural similarities, and posses common physicochemical features (2, 3). During their biosynthesis, vitamin K functions in a posttranslational step in which glutamic acid (Glu) residues in a small segment of polypeptide near the amino-terminal end are modifed to gamma-carboxyglutamic acid (Gla) residues. Presence of Gla-domain distinguishes VKD proteins as a separate group from other plasma proteins and is required for the Ca^{2+}-dependent binding of VKD proteins to negatively charged membrane phospholipids and biological activity (4-10). Several of VKD proteins also have homology to the epidermal growth factor precursor (EGF domain) and squential homology in their serine protease domains (11) (see Fig. 2).

β-hydroxyaspartic acid is formed by the posttranslational hydroxylation of aspartic acid residues in VKD proteins including protein C (12) and Factors X and IX (13). No evidence has been found that this modification is a vitamin K dependent process (14, 15). Originally it was thought metal binding occurs only in the Gla domain of VKD proteins.

However it has been found that Gla-domainless protein C and Factors IX and X all bind Ca^{2+} with high affinity indicating the presence of additional binding sites which may include β-hydroxyaspartic acid (13-14, 16-21).

An imbalance of any of these pro- or anti-clotting proteins result in hemophilia or disseminated intravascular clotting diseases (22-23). Other disease states, such as heart attack, toxin shock syndrome (blood poisoning), and plumonary embolism resulting from surgical or other trauma are all complications arising from an imbalance of one or more of these proteins. Therefore, proteins of these type have a very vital therapeutic value.

PROTEIN C AND REGULATION OF COAGULATION

Structural Features of Protein C

Protein C, a member of VKD family of plasma proteins exists in plasma as an inactive zymogen at a concentration of 3-4 mg/lt (2, 24). It is synthesized in the liver as a 461-amino acid precursor protein (see Fig.3). Removal of the signal squence and the 42-amino acid propeptide occur by multiple proteolytic cleavages of the polypeptide backbone (25). The single polypeptide chain ends up as a two-chain molecule by the removal of Lys^{156}-Arg^{157} dipeptide by an unknown proteinase (26). A single disulfide bond holds the heavy chain of apperant molecular weight (Mr) 41 kDa (kilo dalton) and the light chain of Mr 21 kDa together (27-28). However this posttranslational step in the

production of two-chain protein C molecule is not complete and 5-15 % of plasma protein C circulates as single chain (29). The amino acid squence of the amino terminus (NH2-terminal) of the light chain is homologous with the NH2-terminal squence of the other VKD plasma proteins (28) including Factors VII (30), IX prop), X (31), protein S (31-32), and protein Z (33).

The three complex N-linked (aspargine) glycosylation sites occur during posttranslational modifications of aspargine residues of Asn-X-Ser/Thr squence at postions 97, 248, 313 (see Fig.PC molecule). At the fourth N-glycosylation site of Asn329, a cystein replaces the usual Ser or Thr (34-35). The O-linked carbohydrates (serine, threonine) have not been detected. Carbohydrate content of protein C is reported as 19 % (36) to 25 % (34-35). The o-toluidine HPTLC method for detection of carbohydrates in protein hydrolysates has shown the reducing sugar composition of hPC as 5.2, 8.3, and, 10.0 galactose, mannose, and hexosamine respectively as mol/mol of protein. No galactosamine was detected (37).

The vitamin K-dependent gamma-carboxylation occurs at the first nine glutamic acid residues in the NH2-terminal of light chain (25, 28, 38). The Ca^{2+} binding charcteristic of Gla residues supports the binding of the protein C to membrane complexes with high affinity and they are required for the biological activity (39-40). In addition, protein C contains ane b-hydroxaspartic acid residue at position 71 (41) that is

belived as an additional Gla-independent Ca2+-binding site (42) and 12 interchain cystein (S-S) disulphide bonds.

About 30 % of human protein C appears to be smaller by 4 kDa (β-protein C) than the major population (α-protein C). The difference has been reported due to Asn^{329} not beeing glycosylated in β-protein C (43).

Protein C Activation and Regulation of Coagulation

Protein C is functions in the in the regulation of natural anticoagulant pathway once activated to a serine protease, activated protein C (APC). Rapid activation of protein C occurs on the endothelial cell surface by thrombin in complex with thrombodulin (44-46) as a result of cleavage of the Arg^{169}-Leu^{170} bond and 12 amino acid peptide is relesed from the NH2-terminal of heavy chain. Protein S, also a VKD protein acts as a cofactor in the protein C activation an is required for the high-affinity membrane interactin of activated protein C. APC has anticoagulant activity and inhibits clot formation by inactivating procoagulant factors VIIIa and Va proteolytically (2, 46, 47-49) (see Fig.1) which are essential for the function of factors IXa and Xa, two coagulation proteases. Thus, the generation of thrombin from prothrombin and fibrin clot formation process is shut down.

CLINICAL IMPORTANCE OF PROTEIN C

Clinical and biochemical observations have proved that protein C plays a central role in the regulation of blood coagulation once converted to its active form and both forms have a variety of therapeutic uses. Clinical studies have confirmed the link between hereditary protein C deficiency and thrombosis after the discovery of a family with recurrent thrombosis whose members were congentially deficient in protein C (50). After this initial discovery in 1981, a lot of families with thromboembolic diseases related to protein C deficiency have been reported (51).

There are also several reports indicating the association of decrease protein C antigen levels with disseminated intravascular coagulation (DIC) and chronic liver disease (22-23).

In healthy indivuduals, plasma protein C antigen levels and protein C functional activities are directly correlated. However, in pateints experiencing thrombotic complications, protin C antigen levels were found normal while functional actvities were half of the normal level compared to healthy individuals (52-53). In addition, patients with acute severe venous thrombosis that is a frequent complication following joint replacement surgery have been found about 50 % low in their protein C antigen levels as well as protein C activation peptide together with a two fold increase in in prothrombin activation peptide (54). This observations suggest the association of protein C deficiency with excessive generation of thrombin.

Factor IX complex, a mixture of VKD proteins rich in prothrombin and Factor X,

is the only licensed product in The United States for hemophilia B therapy in patients experiencing Factor IX deficiency (55-56). Although it causes no major problems to treat or prevent various hemorragic episodes of combined clotting factor deficiencies including protein C, when used in large quantities for long periods of time some life-threatening thrombogenic complications in addition to the transmission of viral hepatitis may occur (57). The current treatment for homozygous protein C deficiency includes the usage of plasma followed by the administration of warfarin to prevent thrombotic conditions and in many cases it also causes similar and many other complications. Replacement of present therapies with purified protein C and variety of other clotting diseases with activated protein C (see Table 1) would provide highly desirable options to the clinical medicine (58).

RECOMBINANT PRODUCTION OF PROTEIN C

Recombinant DNA technology has facilated the isolation and cloning of variety of genes encoding many medically important proteins, such as protein C (36, 41, 59) and Factor IX (60), permitting the expression of recombinant proteins in cultured mammalian cells.

Because of the highly complex post-translational modifications involve in synthesis of VKD proteins including protein C, attempts to the express functionally active recombinant protein C (rhPC) in cultured bacterial or mammalian cell lines have resulted

either partially active or inactive products in most cases (39, 41, 61). Although an adenovirus-transformed cell line was engineered to synthesize fully modified and biologically active rhPC (36), 10-20 μg/ml or less expression level is not considered to be commercially viable. Further studies to increase the production rates have resulted decrease in biological activity (39).

Protein C Production in the Mammary Gland of Transgenic Animals

By using the techinque of pronuclear microinjection (62), it is now a reality to direct therecombinant expression of many heterologous proteins to a specific body tissue in a '*transgenic animal*' producing an alternative bioreactor system.

Since most proteins of therapeutic interst are naturally secreted into renewable body fluids, harvesting them from body fluids are more desirable than the solid tissue. Although targetting the expression of a specific protein to liver or kidney of transgenic animal for the production of recombinant protein in the blood stream is possible, limited amounts of plasma available and risk of transmitting infectious diseases would make the process inconvenient for large-scale production purposes. In addition, high expression levels of active proteins may cause serious health problems to the animal (63).

Presently the best approach for recombinant production of many complex human

proteins appear to be the production of transgenic animals by targetting the synthesis of the protein of interest to the mammary gland such that the transgene protein is secreted into the milk of transgenic host. Milk is available in large quantities and mammary gland naturally express a variety of post-translationally modified milk proteins at extremely high levels and already has the enzyme machinary to process the transgenic proteins with similar modifications. For example mammary epithelial tissue naturally performs N-glyosylation (63), one of the most significant posttranslational modification in the production of therapeutically usefull recombinat protein, and has the potential to modify the heterologous proteins at high rates (64-65). In recent years it has been proven that transgenic livestock could be used for the large scale production of foreign proteins (63, 66-67).

The VPI-ARC Transgenic Animal Bioreactor Group has focused upon the synthesis and downstream recovery of hPC as a model for the production of complex human proteins in the milk of large transgenic animals generating an alternative bioreactor system.

Transgenic mice have been employed as the model system to evaluate the genetic construct. Mammary tissue specific expression of rhPC was obtained using murine whey acid protein-hPC hybrid gene (WAPPC1) (see Fig. 4) (68). After having biologically ative recombinant protein C in the milk of transgenic mice, pronucleus of fertilized pig

emryos had been microinjected (69-71) with the same construct at one-cell stage and pigs carrying the WAPPC1 gene were identified. Expression levels as high as 260 μg/ml milk were obtained and it was possible to isolate 90 % or more pure, biologically active recombinant protein at 90 % and higher production yields.

The results of the present work which is discussed in details in the prceeding chapters, thus far show that mammary gland of transgenic pig has the potential to produce highly complex proteins such as protein C in biologically active form and at commercially significant levels. Further characterization such as amino acid sequencing identification of charbohydrate structure will be required to identify the rate limitations leading the posttranslational modifications and to judge the therapeutic value of the recombinant product.

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LIST OF FIGURES AND TABLES

FIGURES

TABLES

TABLE 1
CLINICAL USES FOR PROTEIN C AND ACTIVATED PROTEIN C

Protein C	
	Purpura fulminans neonatalis [1]
	Warfarin-induced tissue necrosis [2]
Activated protein C	
	Heparin-induced thrombocytopenia [3]
	Septic shock [4]
	During fibrinolytic therapy [5]
	Angioplasty [6]
	Anstable angina [7]

1 A thrombotic condition observed in homozygous protein C deficiency charcacterized by microvascular thrombosis in the skin. Thrombotic damage occurs in the central nervous system. 2 Thrombosis in the skin venules during oral anticoagulant therapy. 3, 4, 5, 6, 7 Patient needs an anticoagulant to prevent the intravascular coagulation and the irregular consumption of clotting factors. (Adapted from Reference 58)

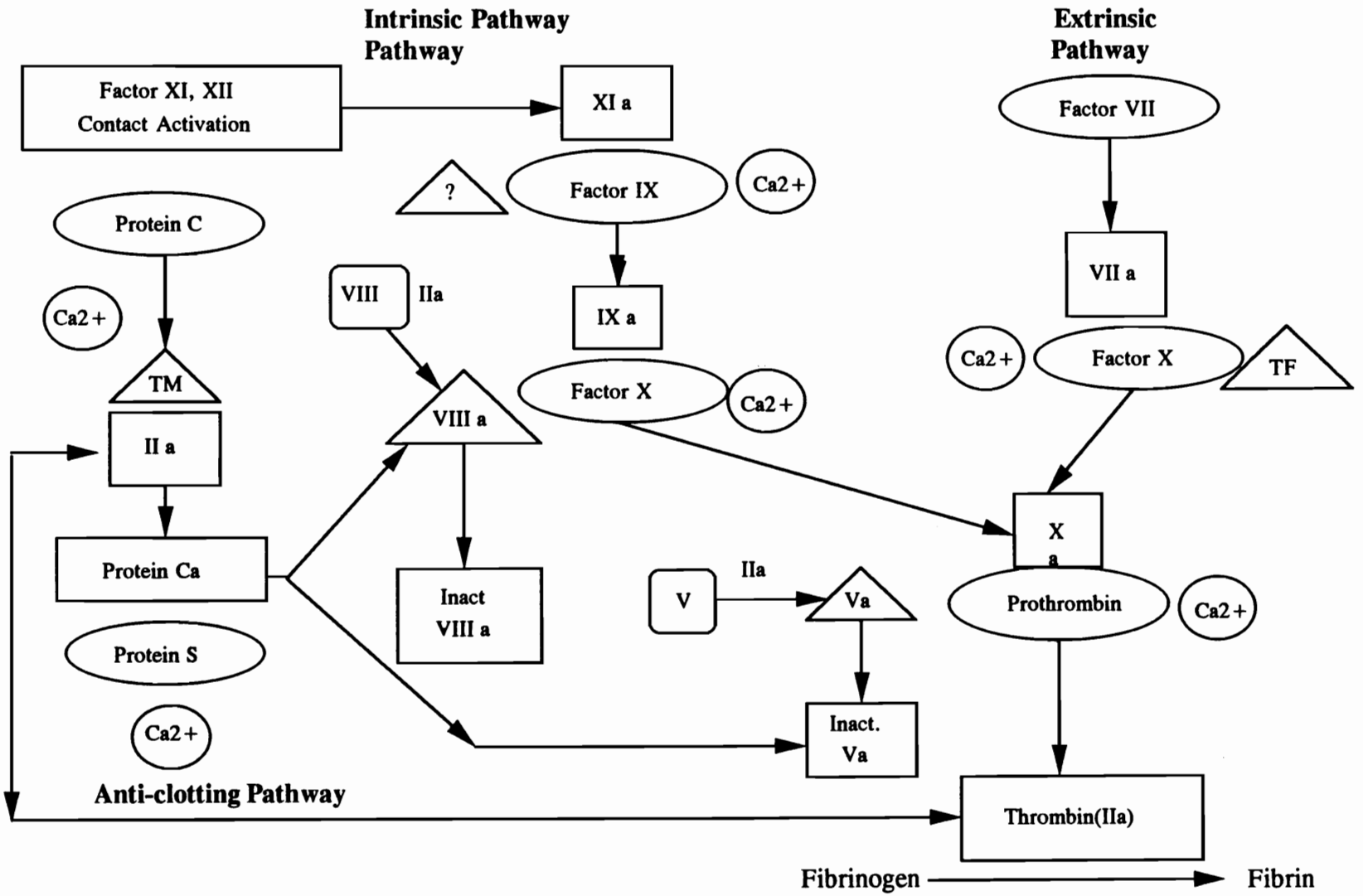

FIGURE 1
THE BLOOD COAGULATION CASCADE
a Activated form. TM Thrombomodulin. TF Tissue Factor.

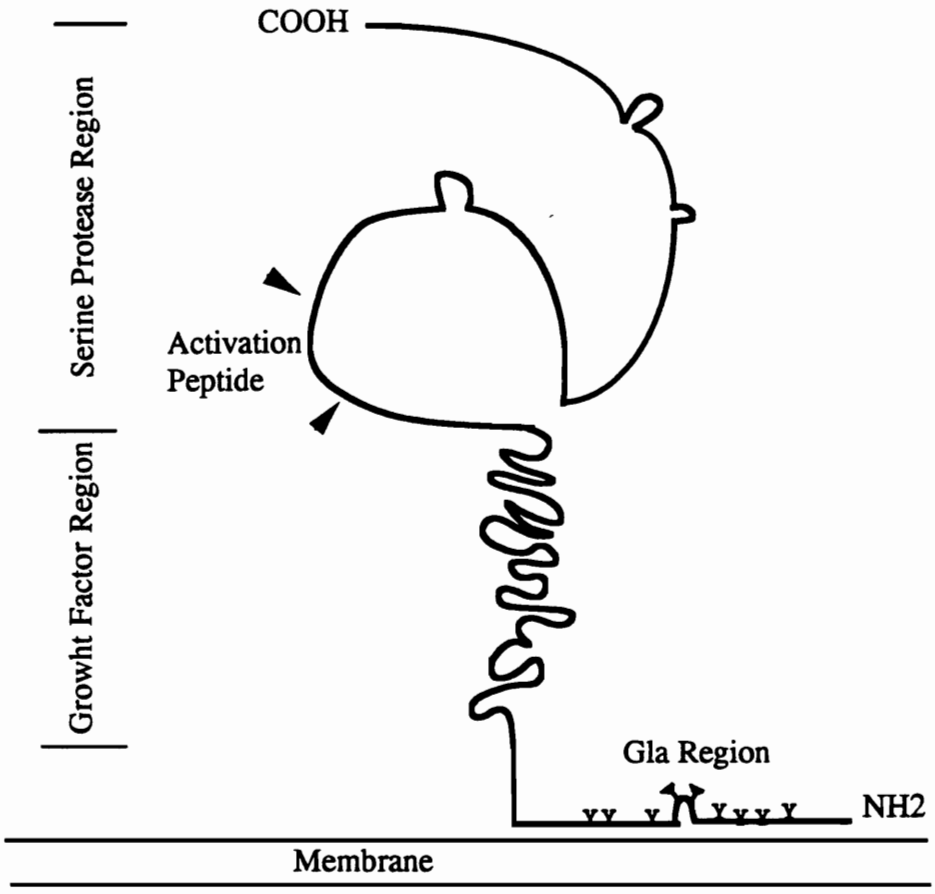

FIGURE 2
SHEMATIC REPRESENTATION OF
STRUCTURAL HOMOLOGY AMONG VKD PROTEINS
Small Y-shaped symbols represent the gamma-carboxyglutmic acid residues
(Reprinted from Reference 11)

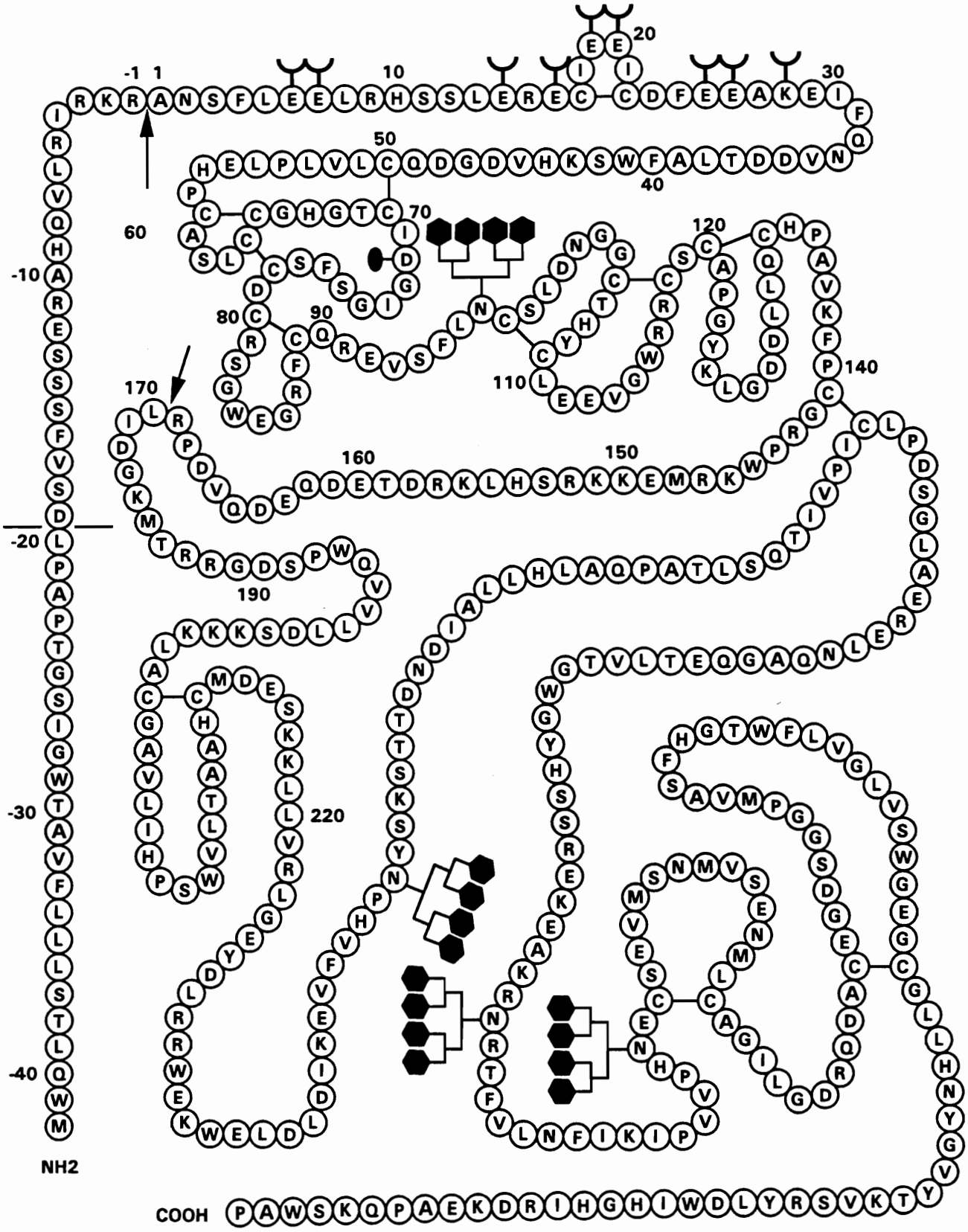

FIGURE 3
SHEMATIC REPRESANTATION OF PROTEIN C MOLECULE
⬢ : Carbohydrate moieties. ⬮ : Beta-hydroxyaspartic acid. ⋎ : Gla residues.
(Reproduced from Reference 43)

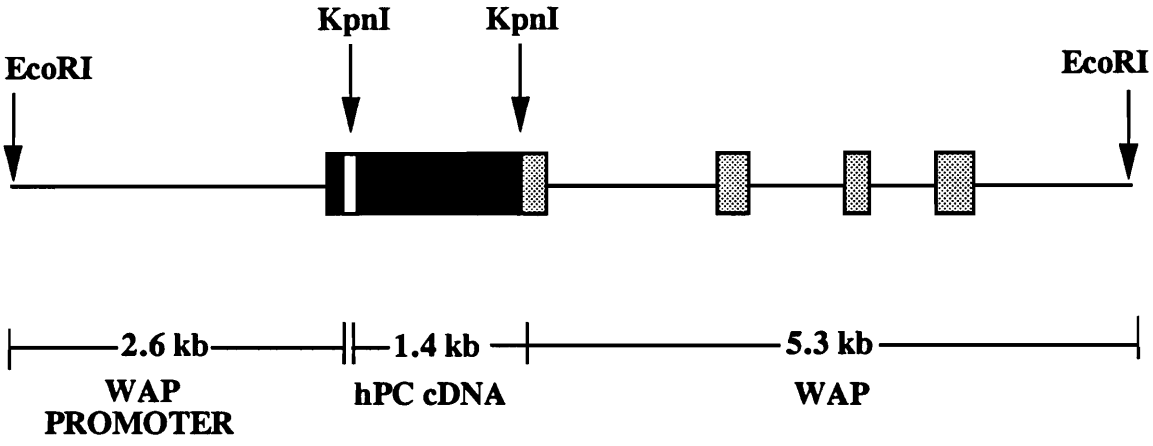

FIGURE 4
MURINE WHEY ACIDIC PROTEIN-HUMAN PROTEIN C CONSTRUCT
(WAPPC1)

CHAPTER TWO

AN O-TOLUIDINE HPTLC METHOD FOR THE DETECTION OF CARBOHYDRATES IN PROTEIN HYDROLYSATES

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ABSTRACT

The o-toluidine high performance thin layer chromatography (HPTLC) method for detection of reducing sugars has been demonstrated to be a facile method for composition analysis of protein hydrolysates with a maximum sensitivity range of 50-100 pmol. The solution phase reaction of o-toluidine with reducing sugars has been previously used for spectrophotometric detection of glucose at 480-630 nm. In contrast, the heterogeneous reaction of o-toluidine with reducing sugars resolved by thin layer chromatography produces chromophoric derivatives which have a broad absorbance at 295 nm. Detection of these chromophoric derivatives is achieved by UV diffuse reflectance scanning densitometry. It is demonstrated that detection limits of less than 10 ng can be achieved by using HPTLC plates and is therefore equal or more sensitive for some sugars than recently reported high pressure liquid chromatography (HPLC) methods using amperometric or fluorescence detection.

INTRODUCTION

The most sensitive methods for the assay of carbohydrates utilize derivatization to form UV absorbing compounds since sugars lack chromophoric and fluorophoric groups necessary for UV and fluorescence detection. Resolution of mixtures of sugars is possible using a variety of chromatographic techniques such as thin layer chromatography (TLC) or high performance thin-layer chromatography (HPTLC), high pressure liquid chromatography (HPLC), and gas chromatography (GC). Detection methods utilizing these resolution techniques have employed derivatization either before or after chromatographic separation. Derivatization for both HPLC and GC techniques

usually requires μg quantities of sugar and a sample preparation which is lengthy (1-4). This is problematic for the inherently small sample sizes frequently encountered in glycoprotein isolates. There are a large number of spray reagents available for TLC detection of carbohydrates. These include florumetric reagents like aniline citrate (5), dansyl hydrazine (6), and N-(1 Napthyl) ethylendiamine dihydrochloride (7) which typically yield detection limits of about 5-50 nmol. Thus, there is still a need for more facile and sensitive methods for sugar derivatization as applied to any of the above chromatographic methods.

There has been great emphasis upon improving detector instrumentation used in conjunction with HPLC to analyze underivatized sugars. Although refractive index measurement can possess detection limits as low as 1-2 ng (8), it is highly susceptible to changes of column temperature and solvent composition (9). Furthermore, detection based upon absorbance in the near ultraviolet (180-210 nm) imparts no specificity for detection of carbohydrates (10). Recently, an anion exchange HPLC method which uses pulsed amperometric detection has been demonstrated to detect carbohydrates in protein hydrolysates in the range of 100-200 picomol (11,12).

A high degree of sensitivity, precision, and linearity can be achieved with HPTLC methods employing diffuse-reflectance densitometry. Kubelka-Monk theory for light scattered by surfaces composed of defined particulate enables a linearized analysis of reflected light (13,14,15). Furthermore, the stability of the resolved and derivatized carbohydrate upon HPTLC media further enhances the precision of diffuse-reflectance densitometry.

The o-toluidine method for derivatization and subsequent detection of reducing sugars has been primarily used for spectrophotometric assay of glucose in body fluids (16). In this work, reaction of the amino group of 2 aminotoluene (o-toluidine) with the internal hemi-acetal or aldehyde group of reducing sugars, as illustrated in Figure 1, is performed on thin layer chromatographic plates. This derivatization is specific for reducing sugars and results in the formation of a UV chromophore. Because of the specifity and sensitivity of the assay, we have applied this method to analysis of carbohydrates contained in protein acid hydrolysates. Human protein C, prothrombin, and several murine IgGs are evaluated for the reducing sugars most commonly found in glycoproteins. A limit of detection comparable or greater than HPLC methods using pulsed amperometric or fluorescent detection methods (6,11,12,17,18) is demonstrated for the o-toluidine HPTLC method.

MATERIALS AND METHODS

Materials

All chemicals and sugars were commercially available materials of ACS-grade or better. O-Toluidine reagent (o-toluidine/glacial acetic acid) was purchased from Sigma (St Louis, MO). Whatman LH-K 20x10 cm HPTLC plates with pre-adsorbent strip, and disposable Preval Power Unit Sprayers were purchased from Alltech Associates (Deerfield, IL). Diffuse reflectance densitometry of HPTLC plates was performed using a Shimadzu 9000-Flying Spot Scanner. Samples were applied using a SMI quick-set Type A micropipetter. Three murine monoclonal anti-human protein C antibodies (7D7, 1H5, and 8861 Mabs) produced by mammalian-cell culture and human prothrombin derived from plasma were graciously supplied by the American Red Cross

(The Jerome H. Holland Laboratory for the Biomedical Sciences). Human protein C was immunopurified by our laboratory using methods previously described by Orthner et al. (19). The purity of protein C and prothrombin employed in this study was judged as greater than 98 % by transmission densitometry of reduced sodium dodecyl sulfate treated polyacrylamide gel (10 %) electrophoresis using the method of Laemmli (20). The extinction coefficients for protein C and prothrombin were taken to be 14.5 (21) and 15.5 (22), respectively. Bovine serum albumin (BSA) was purchased from Sigma.

Standard HPTLC procedure

Stock solutions and serial dilutions of reference sugars were prepared in "nanopure" deionized water, pH 7. Solvent mixtures were prepared fresh and allowed to equilibrate in the developing tank about 1 hr prior to use. All HPTLC plates were pre-marked for maximum solvent migration from the application line before before being placed in development tank. Application volumes of 1 μl for each sugar reference or sugar mixture were applied onto prewashed (chloroform-methanol, 2:1) plates as quadruplicates using a SMI micropipetter and completely dried using a hot air blow-dryer. The plates were then placed in the pre-equilibrated resolution tank with a solvent depth of about 0.75 cm and developed until the solvent front reached to the pre-marked migration distance. A combination of solvent System 1, n-butanol-pyridine-water (16:5:4), and System 2, ethyl acetate-methanol-acetic acid-water (4:1:1:1), was employed. The plates were first allowed to migrate 3 cm above the application line in System 1. The plates were then completely dried and subsequently allowed to migrate 7 cm in System 2. The migrated plates were then dried and sprayed uniformly with o-toluidine reagent inside a ventilated chemical hood and allowed to evaporate inside the hood before they were baked at 110 ^{0}C for 25 minutes. The same procedure was also

performed upon pre-baked HPTLC plates.

O-Toluidine-treated plates were scanned in the diffuse-reflectance mode on a Shimadzu 9000-Flying Spot Scanner; a beam width of 5 mm provided integration of signal for the entire area of each sample lane. The UV spectrum of each reference sugar was measured and maximum absorbance noted for each.

Procedure for carbohydrate analysis in protein hydrolysates

Protein samples were first dialysed in nanopure deionized water and then lyophilized. Glycoprotein-free Bovine Serum Albumin (BSA) was obtained by periodate oxidation of Sigma fraction V BSA (23). Samples containing 100-300 μg protein were hydrolyzed in 400 μl of 6 N HCl prepared from constant boiling HCl for 5, 8, 14 and 20 hrs at 110 0C (24,25) and in 400 μl 2N trifluoracetic acid (TFA) (11) for 2 and 6 hrs in sealed, nitrogen flushed glass tubes. Two sets of mannose, N-acetylglucosamine (NAG), glucosamine , fucose, galactose, and N-acetylneuraminic acid (sialic acid) references were identically treated; one set of references contained BSA and the remaining set did not. Removing all traces of acid from acid hydrolysates of proteins is also very important to prevent the possible formation of sugar-acid complexes occuring when the acid hydrolysate is taken to dryness (26). To minimize acid complex formation, all samples were evaporated to dryness at 0.1 torr. The dried samples were reconstituted (5-10 mg/ml) in nanopure deionized water and applied onto prewashed (chloroform-methanol, 2:1) silica gel HPTLC plates as 1 or 3 μl spots. Resolution of protein hydrolysates was performed in an identical manner to that described above.

RESULTS AND DISCUSSION

Chromatographic Separation

Table 1 lists the solvent systems employed to resolve 6 reducing sugars most commonly found in glycoproteins. A simultaneous resolution of 10 different reducing sugars can also be performed using this method and it has been discussed elsewhere by us (27). Several solvent systems were tried and evaluated for migration time, resolution capacity, and diffusion (spreading) of resolved components. Solvent System 1 was found to provide a slow migration time but significantly limited the spreading of resolved species thus producing highly dense spots. However, no migration of amino sugars was seen with this solvent system. System 2 provided rapid migration and high resolution capacity but decreased the density of resolved species thus reducing the sensitivity of the assay. In all cases, unknowns were determined by comparison of Rf's (distance from center of resolved spot/ distance that the solvent front was allowed to migrate). A combination of these solvent systems provided a fast migration, satisfactory resolution and increased sensitivity due to higher spot density. A typical resolution of reducing sugar hydrolysates sequentially migrated in System 1 and 2 and then derivatized by o-toluidine is shown in Figure 2. Acid hydrolysis did not affect the migration of sugar species except for the appearance of glucosamine in the N-acetylglucosamine (NAG) migration lane, which results from deacetylation of NAG.

Limits of Detection

The maximum absorbance of the o-toluidine-derivatized sugars ranged between 290-310 nm, and all reducing sugars and sugar hydrolysates showed a broad maxima at about 295 nm as shown in Figure 3. In contrast, solution phase condensation products

formed from o-toluidine and reducing sugars absorb at 480-630 nm (28). Sialic acid gave no signal thus showing the specificity of o-toluidine for the derivatization of reducing sugars. Control plates treated with o-toluidine showed little or no background absorbance.

The limits of detection for 6 reducing sugars commonly found in protein hydrolysates are given in Table 2 for 8 and 14 hrs of hydrolysis with 6N HCl at 110° C. Detection ranges of 5-15 ng and 10-25 ng were obtained for reference sugars and sugar hydrolysates, respectively, on pre-baked HPTLC plates sequentially developed in solvent Systems 1 and 2. NAG showed the lowest sensitivity of about 25 ng while fucose and glucosamine gave the highest sensitivity of 5 ng with a signal-to-noise ratio of approximately 75:1.

Method Precision

References containing 20 or 50 ng of sugar were applied onto plates as 1 μl spots and developed in solvent systems 1 and 2. Precision of the method was evaluated for HPTLC plates. A 2-4 % coefficient of variance (n=10 or 15) was obtained using this technique.

A good stability of the o-toluidine derivatized, HPTLC-resolved reducing sugars was observed during storage. The plates were stored in the dark at room temperature in sealed plastic bags. These plates uniformly lost an average of 1 % of densitometric signal over 3 months of storage. Thus, densitometry of resolved plates can be convenienty performed without significant loss of precision or sensitivity.

Linearity

All the reducing sugars hydrolyzed in 6N HCl and 2N TFA at 110° C were found to have a linear range of 0-1 μg on HPTLC plates as shown in Figure 4a, 4b, and 4c. The linear correlation coefficients were all 0.98 or higher for these evaluations.

Kinetics of acid hydrolysis and sugar decomposition

Because multiple acid catalyzed reactions are superimposed upon one another during hydrolysis, the sugar determination of glycoproteins is highly dependent upon the hydrolysis conditions. In particular, the rates at which individual sugars are freed from the protein and rates of destruction can complicate analysis. The effect of hydrolysis conditions on different classes of glycoproteins and conditions has been reviewed by Marshall and Neuberger (29). We have further detailed some of these effects using the o-toluidine HPTLC method. Table 3 shows the percent extent of destruction for various reducing sugars commonly found in glycoproteins. Here, reference sugars hydrolyzed in either 6N HCl or 2N TFA at 110° C in the presence of carbohydrate-free BSA were compared to identical samples without acid. Galactose and fucose were found to have slightly higher destruction in HCl than in TFA. Mannose appeared to be much less stable in TFA than in HCl. However, NAG is more stable in HCl system. The conversion of NAG to glucosamine, hydrolyzed in the presence of BSA, was found to be slower in 6N HCl than 2N TFA at 110° C. These conversion kinetics were also found to be nonlinear as can be seen in Figure 5. Furthermore, the decomposition of glucosamine at the same hydrolysis conditions have been previously shown to be nonlinear (30). It is noted that each time point studied yielded a linear response to o-toluidine derivatization for 0-1 μg of NAG present at the start of hydrolysis at 110° C.

Protein-Sugar Analysis

Human protein C and prothrombin, which belong to the vitamin K-dependent class of plasma proteins, were analyzed for NAG, glucosamine, galactose, mannose, and fucose content under different hydrolysis conditions. Hydrolysis was performed in 6N HCl for 8 and 14 hrs and also in 2N TFA for 2 and 6 hrs at 110° C. IgG free BSA was used as a carbohydrate free protein in sugar standards which were hydrolyzed in the same way as the IgG, protein C, and prothrombin samples. BSA alone gave no detectable presence of reducing sugars. The carbohydrate content detected by o-toluidine HPTLC analysis of protein C and prothrombin hydrolysates from either TFA or HCl for ordinary hexoses and for hexosamines are significantly lower than previously reported by Kisiel (21) and Stenflo (31) as can be seen in Table 4. The results are more comparable to those reported by Kisiel and Hanahan (32). The precision, sensitivity, and linearity of reference sugars treated in a manner identical to the protein C and prothrombin samples indicate that hydrolysis conditions are the most likely reason for sugar composition which are different than previously reported. Fucose was detected only in human protein C as 1.7 mol/mol of protein (data not shown) with 2N TFA hydrolysis conditions. Fucose has been detected in human protein C at 0.9 mol/mol by Yan et al. using HPLC with amperometric detection (12). No galactosamine was detected for either protein. The kinetics shown in Figure 5 were used to estimate NAG content based upon conversion to glucosamine. A minimum of 61 % of the hexosamine in protein C and 73 % of the hexosamine in prothrombin were calculated to result from the deacetylation of NAG.

Three murine monoclonal antibodies (Mabs) were also analyzed for their NAG,

glucosamine, galactose, mannose, and fucose content. Results obtained from 2N TFA and 6N HCL hydrolysis are shown in Table 5. O-Toluidine analysis of these IgG class antibodies indicated the presence of NAG in its deacetylated form glucosamine, as well as galactose and mannose residues. Little or no fucose was detected in any of the Mab hydrolysates (data not shown). These results compare favorably with the studies by Melchers (33) which reported the presence of glucosamine, galactose, and mannose with little or no fucose in almost all of the mouse myeloma proteins studied. The carbohydrate compositions showed appreciable differences between Mabs. The study of Mizuochi et.al. (34) indicates a heterogenous distribution of carbohydrate exists for both monoclonal and myeloma antibodies. In general carbohydrate microheterogenity is a salient feature of protein glycosylation. Furthermore, Rhotman et.al. (35) demonstrated that culturing conditions could affect the glycosylation pattern for monoclonal antibodies.

In conclusion, the reaction of o-toluidine with the aldehyde group of sugars which have been migrated on HPTLC plates provides a UV chromophore with a broad maxima at about 295 nm and permits detection of many reducing sugars at pmol levels. Because of the specificity of o-toluidine to reducing sugars, this method is not applicable to the detection of sialic acid residues. However, the o-toluidine HPTLC method provides a facile alternative for the analysis of reducing sugars in protein hydrolysates with a sensitivity which is comparable or exceeds HPLC or GC methods.

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TABLE 1

HPTLC SOLVENT SYSTEMS

System No.	Solvent Mixture	Volumetric Ratio
1	n-Butanol/Pyridine/Water	16: 5: 4
2	Ethyl acetate/Methanol/Acetic acid/Water	4:1:1:1

TABLE 2

DETECTION LIMITS OF REDUCING SUGARS ON HPTLC PLATES

Sugar References	Detection Limit (ng)		
	In 6N HCl [1]		in D.I. water
	14 hr	8 hr	
N-Acetylglucosamine	50	40	25
Fucose	20	15	5
Galactose	25	20	10
Galactosamine	15	15	10
Glucosamine	15	10	5
Mannose	25	20	10

1 Hydrolyzed in 6N HCl at 110 C for 8 or 14 hrs.

TABLE 3

DESTRUCTION OF SOME REDUCING SUGARS WITH HYDROLYSIS

Sugar	% Destroyed (at 110 ^{0}C)				
	2N TFA		6N HCl		
	2 hr	5 hr	5 hr	14 hr	20 hr
Galactose	0	20	24	40	45
Mannose	10	43	25	50	55
Fucose	27	34	40	55	70
NAG	24	37	10	35	70
Glucosamine	0	13	20	40	50
Galactosamine	14	17	10	40	45

TABLE 4

CARBOHYDRATE COMPOSITION OF HUMAN PROTEIN C AND PROTHROMBIN [1]

Protein	Galactose	Mannose	Hexosamine	Reference
Human protein C	5.2	8.3	10.0 [2]	O-toluidine HPTLC [3]
(Mr= 62,000)	n.g.	n.g.	14.8	Stenflo , (31)
	14.4	21	23.4	Kisiel, (21)
	9.3	9.1	13.8	Yan et.al. (12)
Human Prothrombin (Mr= 72,000)	3.3	5.6	7.7 [4]	O-toluidine HPTLC [3]
	11.2 [5]		10.7	Kisiel and Hanahan, (32)

1 Compositions expressed as mol/mol of glycoprotein. Molecular weight in parentheses.
2 Given as total NAG and glucosamine; 6.1 and 3.9 mol/mol protein respectively Glucosamine data from 8 and 14 hrs hydrolysis with 6N HCl.
3 Data from 2N TFA and 6N HCl for 2-14 hrs hydrolysis with an standard deviation of about $\pm$ 0.9.
4 Given as 5.6 mol NAG and 2.1 mol glucosamine/mol of protein. Glucosamine data from 8 and 14 hrs of hydrolysis with 6N HCl.
5 Given as total hexoses.
n.g. Not given.

TABLE 5

CARBOHYDRATE COMPOSITION OF SOME MOUSE MONOCLONAL ANTIBODIES [1]

IgG Hydrolysate	NAG [4] mol/mol	Galactose mol/mol	Mannose mol/mol
7D7 Mab [2]	20.6 ± 2.5	4.2 ± 1.0	7.0 ± 1.5
1H5 Mab [2]	15.9 ± 1.1	9.2 ± 1.7	3.6 ± 0.6
8861 Mab [3]	8.8 ± 1.0	6.2 ± 0.6	3.9 ± 0.7

1 Molecular weights of 150,000 for monoclonal antibodies (Mab) were assumed. Data is corrected for destruction upon hydrolysis.

2 Average values from 2 and 6 hrs of hydrolysis in 2N TFA and 14 hrs hydrolysis in 6N HCl.

3 Data from 2 and 6 hrs of hydroly sis in 2N TFA.

4 Data are corrected for NAG conversion to glucosamine.

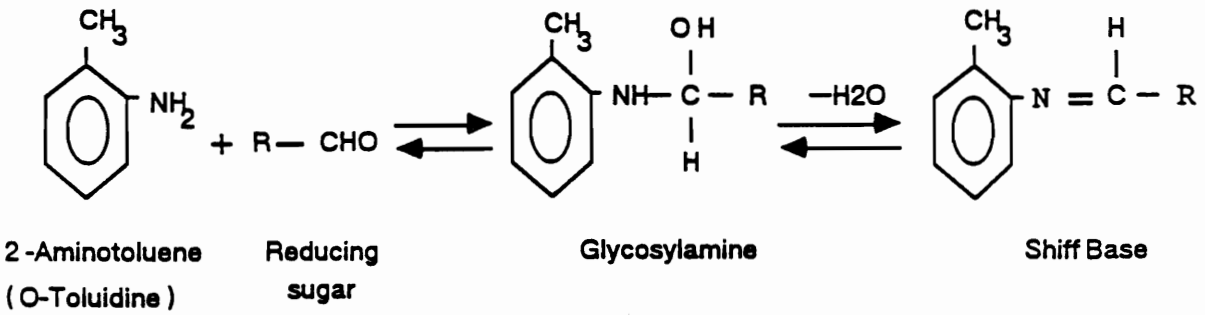

FIG.1. Reaction of reducing sugars with o-toluidine (Figure is adapted from reference 28).

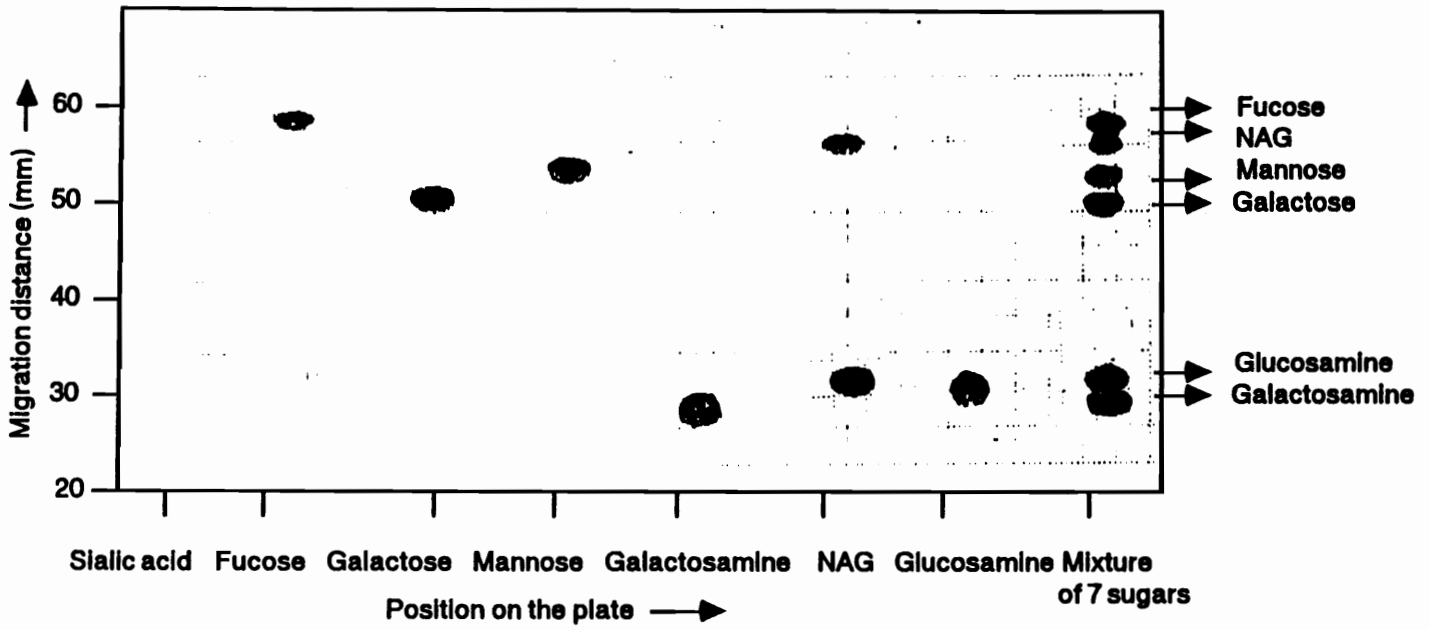

FIG.2. Resolution of reducing sugar hydrolysates (6N HCl, 5 hr at 110 C) on an HPTLC plate ("Hatch" mapping at a threshold level of -0.01 absorbance units by densitometry, $\lambda = 295$ nm) for 1 μg applications of each sugar.

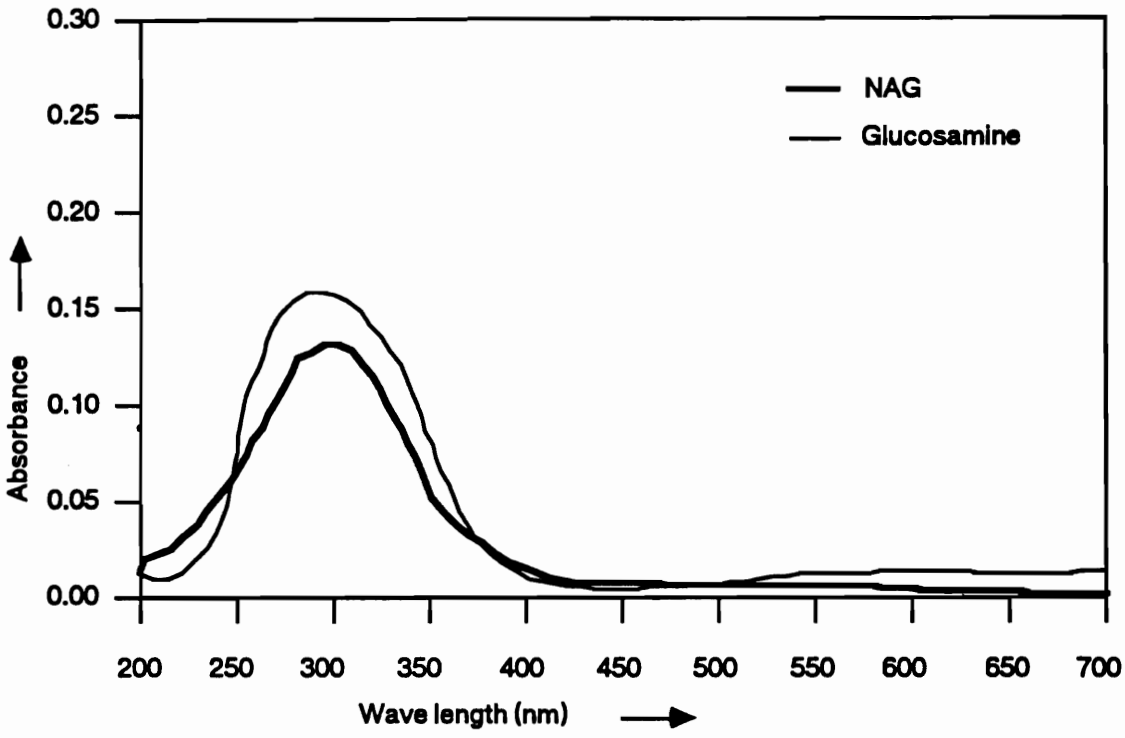

FIG.3. Typical U.V. spectral scans for sugar references hydrolyzed in 6N HCl at 110 °C, migrated using System 1-2 sequentially and sprayed with o-toluidine; NAG and glucosamine are provided as examples.

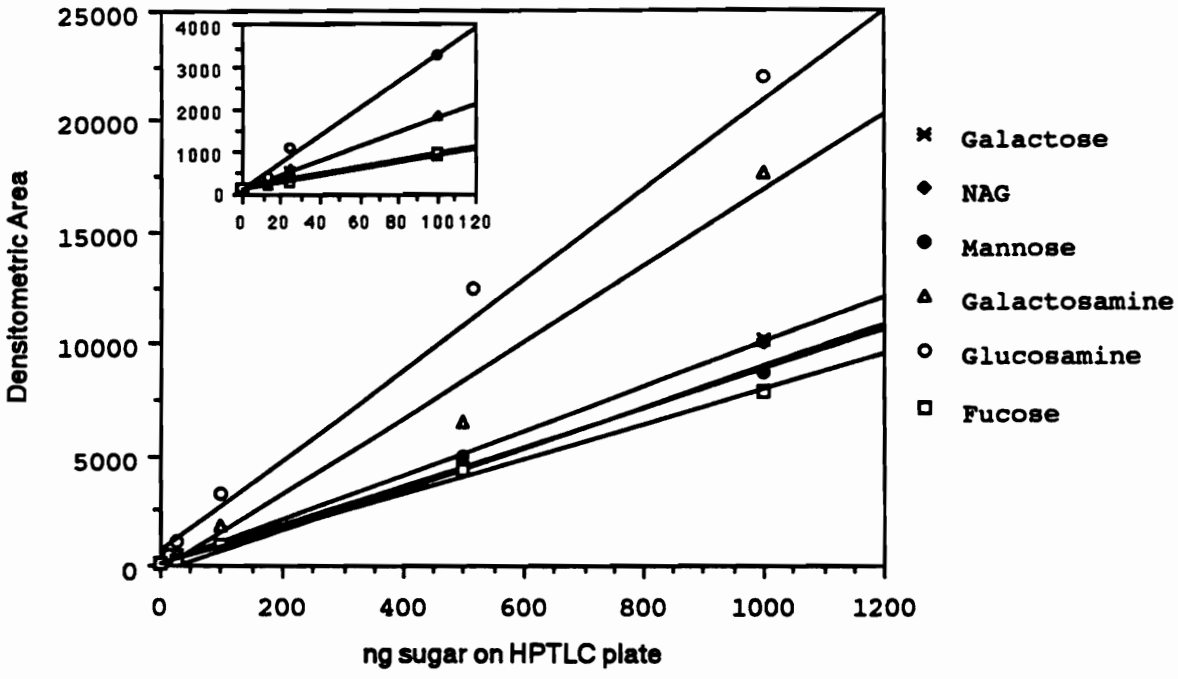

FIG.4a. Standard curves for reference sugars in 6N HCl for 14 hrs at 110 °C. Linearity in the range of 0-100 ng is inset.

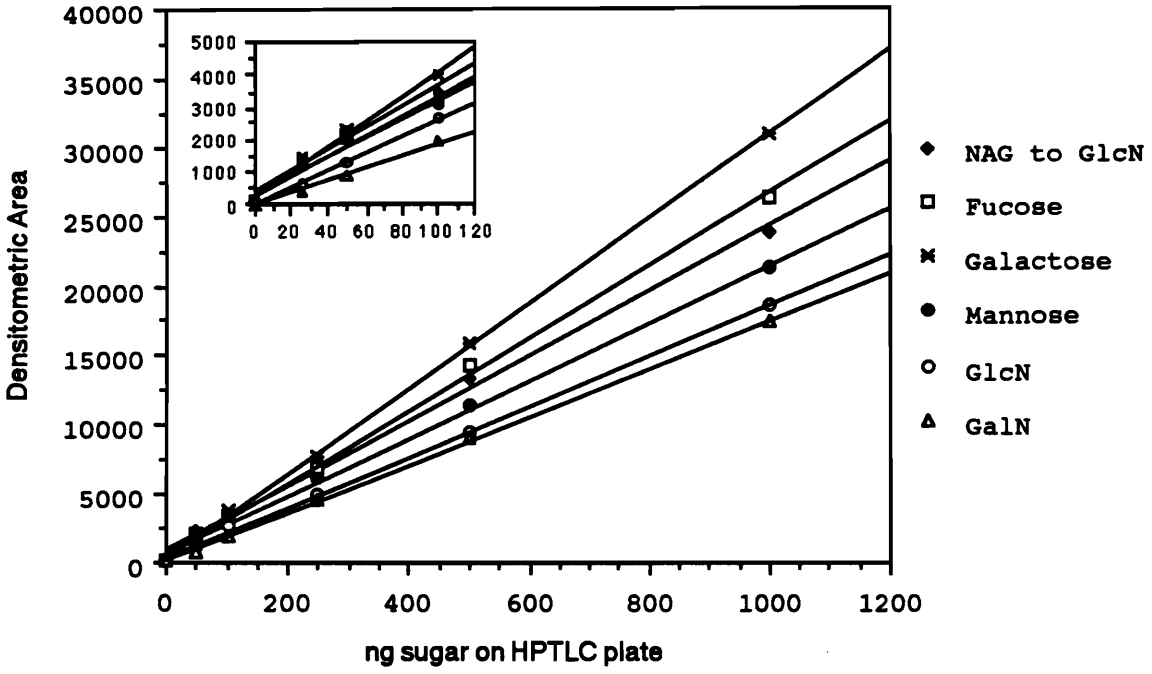

FIG.4b. Standard curves of reference sugars in 2N TFA for 2 hrs at 110 ℃. 0-100 ng range is inset.

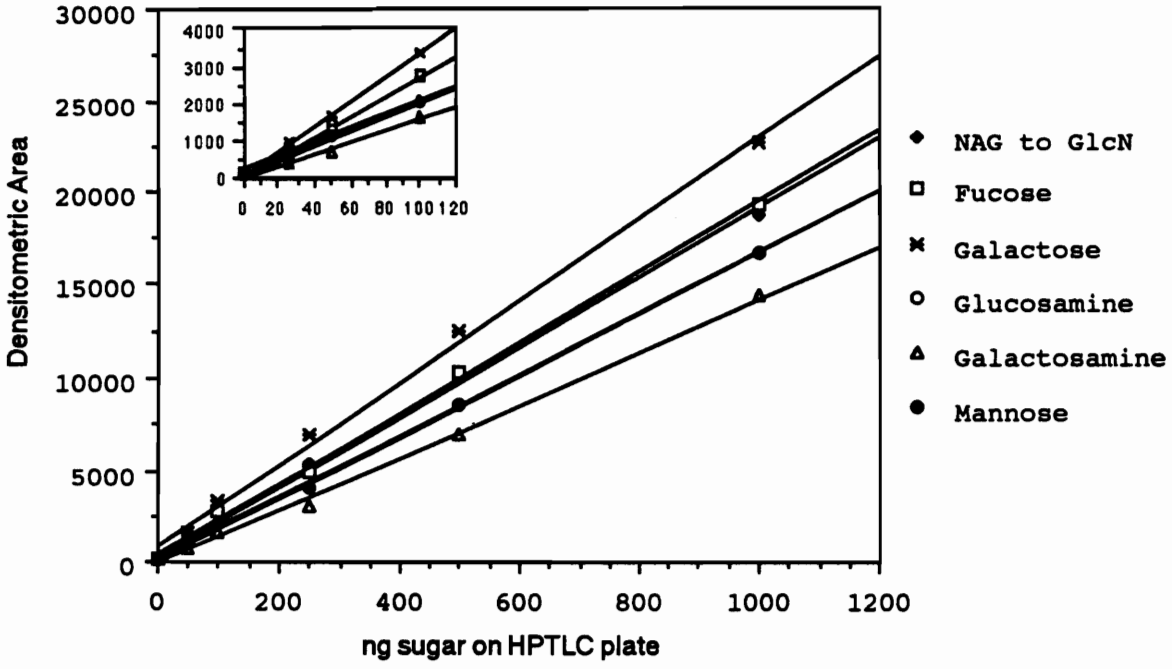

FIG. 4c. Standard curves for reference sugars in 2N TFA for 6 hrs at 110° C. 0-100 ng range is inset.

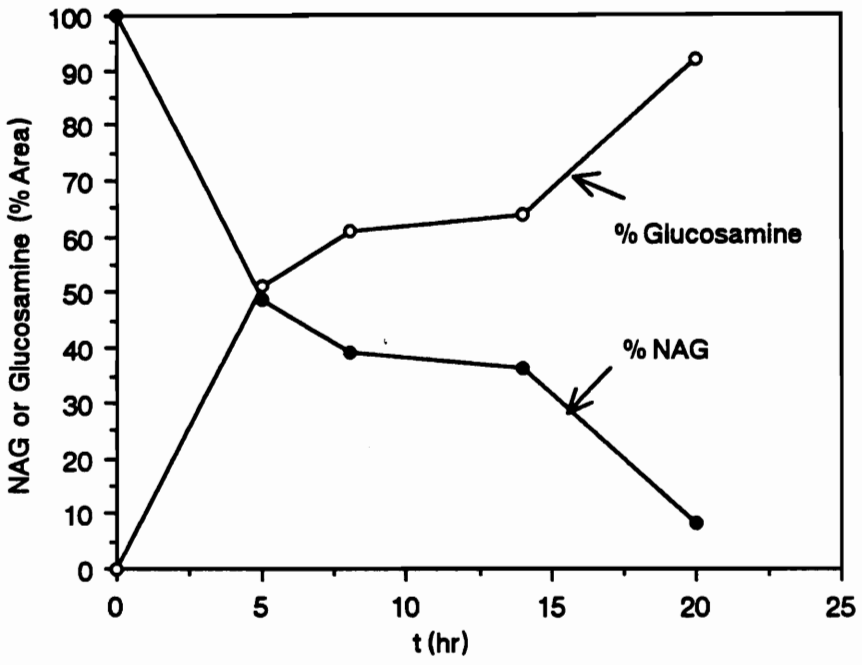

FIG.5. Kinetics of NAG conversion with 6N HCl hydrolysis at 110°C.

CHAPTER THREE

TECHNOLOGICAL CHALLENGES FOR THE LARGE-SCALE PURIFICATION OF PROTEIN C

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Plasma Derivatives

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ABSTRACT

A large-scale process for producing a highly pure, plasma-derived protein C therapeutic will likely consist of the following steps; volume reduction and prepurification by anion-exchange chromatography, viral-reduction by solvent-detergent treatment, affinity purification, and ion-exchange clean-up to remove immune globulins which have leached from the immunosorbent. Alternatively, IV-1 paste obtained from the Cohn fractionation of frozen plasma can provide a volume reduced and enriched starting material for producing pure protein C. Furthermore, the yields of protein C from fresh Cohn IV-1 paste or anion exchange chromatography of fresh frozen plasma both range from 50-70%. Upon volume reduction, viral inactivation can be efficiently performed by treatment with 0.3% (v/v) tri(n-butyl) phosphate and 1.0% (v/v) Triton X-100 at 0-2° C. The solvent-detergent can then be removed by the wash step performed during immunopurification. Finally, immunosorbents which possess a metal-dependent interaction with protein C can provide for gentle elution conditions which enable higher total protein loadings and thus more efficient processing at large-scale. The protein C produced from Cohn IV-1 paste using a metal-dependent immunosorbent was found to be biologically active as judged by clotting assay.

Challenges facing large-scale production of Protein C. The problems associated with the large-scale isolation of Protein C for use as a therapeutic product include its low concentration in plasma, the structural similarities between it and members of the vitamin K-dependent protein family, and the potential for viral contamination. In addition, any new process must be compatible with the fractionation processes for the other current products including Factors VIII and IX, antithrombin III, albumin and immunoglobulins. A difficulty arises in developing a process for purification of Protein C from plasma which both circumvents these obstacles and also provides an affordable product.

Biochemical factors. Protein C is a trace plasma protein occurring at levels of 3-4 ug/ml which belongs to the family of vitamin K-dependent (VKD) proteins associated with hemostasis (1-4). Most of the other VKD-plasma proteins also have plasma concentrations of 10 ug/ml or less with the exception of prothrombin which occurs at 120 ug/ml. The VKD-proteins share extensive sequence and structural homology as well as have similar physicochemical properties such as isoelectric point and molecular weight (6,7). The VKD-proteins are characterized by a highly conserved metal-binding domain located in the amino-terminal end of these molecules. This domain is rich in gamma-carboxyglutamic acid (gla) which is a vitamin K-dependent post-translational modification of glutamic acid residues. Protein C has nine gla residues in the first 30 amino acids of the amino-terminal end of the light chain which impart most of the divalent-metal ion binding capacity of this molecule (4,5). An additional high affinity metal ion binding site is present outside of the gla domain of the VKD-proteins, with the exception of prothrombin and Protein Z (8,9).

These biochemical similarities render separation of VKD-proteins from one another

by classical chromatographic methods extremely difficult. Even laboratory-scale electrophoretic isolations of ion exchange eluates can yield Protein C products contaminated with Factor X (4). In contrast, ion exchange methods or modified versions of these methods are useful for isolation of highly purified Protein C from recombinant sources where other VKD-proteins are not being expressed (10).

Although the clinical benefit of using highly purified plasma protein concentrates is currently controversial, in the case of the VKD-proteins purity may be quite important. Factor IX Complex, the only currently licensed product for treatment of hemophilia B (Factor IX deficiency), is a mixture of VKD-proteins containing significant amounts of prothrombin and Factor X and variable amounts of Factor VII, Protein C and Protein S in addition to Factor IX. In routine use for treatment of bleeding episodes, Factor IX Complex causes no major problems; however, when used in large amounts for extended periods of time, as for surgery, Factor IX Complex has been associated with potentially life-threatening episodes of thrombosis (11-13). This is thought to be due to overloading the patient with the other clotting factors upsetting the balance between pro- and anti-coagulation (14). The same would potentially be true of a Protein C concentrate which contained other VKD coagulation proteins.

Process factors. Because of the similar physicochemcial characteristics of the VKD-proteins, multiple chromatographic steps are generally needed when classical methods are employed to produce a purified concentrate. For example, the negatively charged VKD-proteins can be captured from plasma or a downstream plasma fraction by adsorption on anion exchange media. Further purification by removal of more positively charged contaminants can subsequently be made by cation exchange chromatography (4,15). A single anion exchange step will typically

give a 25-50 fold purification with yields of 50-70% (14-16). Even multiple, successive ion exchange steps may not result in a pure protein concentrate and yet may cause unacceptable yield losses for a protein in limited supply such as Protein C.

The low levels of Protein C present in plasma are further problematic because of the large volumes which must be processed. The chromatographic materials currently employed at large scale withstand only relatively low flow rates which lead to longer process times and increased cost. Furthermore, because these materials are easily crushed, large-scale chromatographic columns with a small bed height to diameter ratio are generally necessary. This geometric constraint does not permit efficient use of purification methods which rely upon multiple equilibration stages (e.g. gradient ion exchange, displacement, and hydrophobic interaction chromatography). Thus, conventional chromatography can not be performed without a significant loss of efficiency upon scale-up. Membranes and beaded matrices which employ perfusion rather than diffusion may provide higher processing rates for large-scale applications in the near future (17-19).

In current practice, classical chromatographic methods are being relegated more and more to pre-purification to reduce volume and extraneous protein level while the high resolution purification is performed by more specific methods such as immunoaffinity chromatography (16,20). An additional ion exchange or other chromatographic step may still be employed after immunopurification to reduce leached immunoglobulin contaminants from the product (16,20). The on-off or single equilibrium stage characteristic of affinity purification methods provides greater scalability and efficiency for purification of dilute plasma proteins, although immunosorbents which could operate at higher flowrates would greatly decrease processing costs.

In summary, a large-scale process for purification of Protein C from human plasma would consist of a volume reduction and pre-purification step, a high resolution immunoaffinity step, and a final clean-up step (16,20). This would then be followed by product formulation, sterile filtration, and lyophilization. A viral inactivation step must also be placed at an appropriate point in the process. Such a process is schematically shown in Figure 1.

Possible starting materials. The choice of plasma or a downstream plasma fraction as a starting material for a Protein C process involves a number of trade-offs. Because plasma is a limited resource, the goal is to extract as many products as possible from each unit. Thus any new process must integrate into the current fractionation scheme with as little disruption as possible to existing products. In addition, since the processes for biologicals are strictly regulated, any change to existing processes, including modification of the starting materials caused by changes in upstream processing, requires a license amendment which is supported minimally by new product stability studies (e.g. three years for albumin) or, at worst, by costly clinical studies.

Although cryopoor plasma (plasma which has been depleted of cryoprecipitate which contains Factor VIII) would provide maximal yield of Protein C, other starting materials may be more compatible with current downstream processes. For example, Factor IX Complex which is a mixture of the VKD-proteins that is produced by most fractionators for treatment of hemophilia B. Use of Factor IX Complex would take the Protein C process effectively "off-line" from the rest of the fractionation process but might cause significant yield penalties since its production has been optimized for recovery of Factor IX, not Protein C.

Another attractive starting material is Cohn fraction IV-1 paste. For the past 40 years, albumin and immune globulins have been fractionated from plasma using the method developed by Cohn (21). This method produces crude plasma protein precipitates by exploiting differences in isoelectric point and temperature-dependent solubility in aqueous-ethanol (see Figure 2). Protein C is precipitated along with other VKD-proteins in Cohn fraction IV-1 paste because of the physicochemical similarities mentioned above. Greater than 90% of the total Protein C antigen detected in the various Cohn fractions resides in fraction IV-1 (22). Cohn fraction IV-1 paste is an off-line discard stream and hence provides a source of Protein C which would not affect immune globulin or albumin production. It is noteworthy that Cohn fraction IV-1 paste has been used as a starting material for production of Factor IX Complex by some plasma fractionators (15), and that its use would also provide a convenient volume reduction and pre-purification step for a Protein C process.

Quantitation of Protein C in Cohn fraction IV-1 paste. In samples taken from one kilogram of homogenized, wet IV-1 paste the Protein C content ranged from 0.5-0.7 ug Protein C per mg total soluble protein, 0.05-0.07 weight % Protein C (22). Protein C was measured by enzyme immunoassay employing a polyclonal immunocapture of Protein C. The IV-1 paste assayed was 2 weeks old and had been stored at -25 ° C. It was about 50% solids based upon yield from lyophilized samples. Approximately 50-75% of the dry solids were soluble after being reconstituted in 25 mM EDTA at pH 6.5. The reconstituted IV-1 paste was centrifuged at 15000 xg for 20 minutes and the supernatant was filtered. To construct standard curves for the assay, reconstituted fraction IV-1 paste was depleted of Protein C by immunopurification such that no Protein C antigen was

detectable. This material was subsequently reconcentrated and dialyzed to 40 mg/ml total protein based on absorbance at 280 nm (OD_{280}), and then doped with Protein C which had been immunopurified from plasma (Reference Protein C appears in lanes 5 and 6 of Figure 4).

By a similar method, negligible amounts of Protein C antigen were detected in Cohn fractions I + II + III, IV-4, and V. Cohn Method 6 yields approximately four grams of soluble protein per 60 grams of plasma protein starting material (21). This represents a theoretical maximum of one microgram of Protein C per mg of soluble protein from IV-1 paste or 0.1 weight % Protein C. Thus, the yield of Protein C based upon antigen content ranges from 50-70% for Cohn IV-1 paste, and is comparable to yields obtained from anion-exchange adsorption of cryopoor-plasma.

Immunopurification for large-scale production of plasma-derived Protein C. The specificity and stability of antigen-antibody complexes can make immunoaffinity chromatography one of the most suitable high resolution purification methods for trace plasma proteins at large-scale. Selection criteria for affinity sorbent qualities which are desirable for large-scale applications include gentle elution conditions that do not require agents which would complicate subsequent processing and/or waste disposal, the ability to operate under conditions which decrease nonspecific adsorption during loading, and conditions which decrease the desorption of nonspecifically bound protein during product elution. Some of these criteria are often not a matter of importance for laboratory-scale applications, but can adversely impact processing costs at large-scale.

Harsh chemical agents used in elution buffers can both impair Protein C activity and increase the level of contamination of extraneous protein found in the product

eluate (16). Protein denaturation and yield losses may occur as a result of the processing associated with harsh elution conditions. In addition, the use of elements containing thiocyanate can interfere with the measurement of Protein C biological activity. Disposal of the waste associated with eluates containing chaotropic salts also increases processing costs. The use of other harsh conditions such as high or low pH, or high ionic strength suffer from similar problems.

The discovery of metal-dependent interactions between VKD-proteins and some antibodies provides a vehicle for selecting elution conditions which do not use chaotropic agents or other harsh conditions (16,20). Antibody-antigen complexes which possess metal-dependent interactions are selected by their ability to form stable complexes with Protein C only in the presence or absence of divalent metals such as calcium or magnesium. Antibodies which exhibit an avidity for Protein C in the absence of available calcium may be preferred for process applications since fibrin formation is not supported under those conditions. In addition, less nonspecific binding of proteins to chromatographic media tends to occur in a chelated environment (22,23).

Conformational changes associated with metal-containing and metal-free states of VKD-proteins have been well documented (24-31). These conformers have been characterized using metal-dependent interactions with monoclonal antibodies. In many cases, it is the conformers that result from metal-free and metal-containing forms of the VKD-protein that have been shown to be the central determinant in the stability of the antigen-antibody complex (30,31). However, at least one metal-dependent-antibody/Protein C complex has been elucidated in which the interaction is not a strong function of changes in tertiary structure associated with occupation of VKD-metal binding domains (29).

Loading and wash conditions for an immobilized Protein C-antibody complex which is stabilized in the absence of free calcium would typically utilize pH 6.5 to 8.0, 25-50 mM sodium citrate or EDTA buffers. Elution buffers would typically contain 25 mM CaCl2. For example, Figure 3 illustrates a separation performed with an immunosorbent consisting of a monoclonal anti-Protein C IgG, ester-linked to Affigel-10 which forms a stable complex with Protein C or prothrombin in the absence of available calcium. The cross-specificity of this anti-Protein C IgG was found to be due to the sequence homology in the binding epitope which was located in the gamma carboxylated glutamic acid domain (16,31). Thus, this is an example of the difficulty even immunoaffinity methods can have in discriminating between VKD-proteins. Here, fresh Cohn IV-1 paste was reconstituted in 25 mM EDTA, pH 6.5 buffer to a total protein level greater than 40 mg total protein per ml, filtered, and then loaded directly onto the immunosorbent. The column was washed with 25 mM EDTA and the immunosorbed Protein C product was then eluted with 25 mM CaCl2, 0.05 M Tris, 0.1 M NaCl, pH 6.5 buffer. The small regeneration peak seen upon elution with 1 M NaCl represents 20% of the total OD_{280} of the product peak eluted with 25 mM CaCl2, tris-buffered saline. No Protein C antigen was detected in the NaCl regeneration peak.

Typically, no significant OD_{280} is detected in the NaCl regeneration step when prepurified ion exchange fractions of less than 5 mg total protein per ml are applied to the immunosorbent. The large regeneration peak seen in Figure 3 is presumably the result of the much higher protein loading. This demonstrates a significant drawback of affinity sorbents which require harsh elution conditions. It is possible that harsh elution conditions would have co-eluted the non-specifically adsorbed impurities found in the regeneration peak with the Protein C product.

Cohn fraction IV-1 paste as an expedient source of Protein C. Protein C derived from fresh Cohn fraction IV-1 paste has been shown to be biologically active and essentially equivalent to Protein C derived from cryopoor plasma. Figure 4 shows a SDS-polyacrylamide (7.5%) gel electrophoresis stained with 2% Coomassie brilliant blue R of Protein C immunopurified from Cohn Fraction IV-1 paste. Lanes 1 and 10 show 1:40 dilutions, reduced and non-reduced, of Cohn IV-1 paste reconstituted in 25 mM EDTA, pH 6.5 at 40 mg total protein per ml which was used as starting material. Lanes 2 and 9, show the reduced and nonreduced anion exchange eluate processed from the Cohn IV-1 starting material. Lanes 3 and 8 are 20-fold concentrated, reduced and nonreduced Protein C products from a once-immunopurified product of an anion exchange eluate generated by adsorption of reconstituted Cohn fraction IV-1 paste onto DEAE-Sephacel using methods similar to those given in reference 4. Lanes 4 and 7 are reduced and nonreduced, twice-immunopurified eluate from an anion exchange processed Cohn IV-1 paste using the same procedure described in reference 16. The same EDTA-dependent immunosorbent discussed above (see Figure 3) was employed to purify the anion exchange eluate. Lanes 5 and 6 are reference Protein C derived from plasma using methods described in reference 31. Reconstituted Cohn IV-1 paste at 40 mg total protein per ml which was directly subjected to immunopurification produced a similar product to the once-immunopurified, ion exchange eluate seen in lanes 3 and 8 (data not shown).

The biological activities as judged by clotting and amidolytic assay are essentially equivalent. The amidolytic assay employed using an activator (Protac^{TM}) derived from **Agkistrodon contortrix** venom is described in references 32 and 33. Specific activity was determined by normalization of the total amidolytic activity using

identical samples which had not been activated with Protac^{TM}. The total amidolytic activity was about 300 units per mg as compared to 260 units per mg for the normalized value. Thus, the overall amidolytic activity was in better agreement with the prolonged clotting activity of 340 units per mg as measured by activated partial thromboplastin time against a normal plasma reference pool defined as 1 unit per ml. Furthermore, the presence of activated Protein C in the Cohn fraction product is indicated upon comparison of the un-normalized amidolytic activity with the activity found from a clotting time prolongation assay.

Cohn fraction paste samples which were stored at -25 ° C for several months showed widely variable yields of Protein C. The use of fresh Cohn IV-1 paste provided the highest and most consistent recovery of Protein C activity. Protein C yields are currently being evaluated with an immunosorbent which has no cross-specificity for prothrombin.

Viral inactivation using organic solvents and detergents. Chemical methods such as treatment with solvent/detergent have been developed for the reduction of viral activity associated with lipid-enveloped viruses such as HIV or hepatitis non A, non B (15,34,35). Solvent/detergent inactivation of plasma or plasma concentrates has advantages of rapid, multi-log viral inactivation kinetics (35) while having negligible effect upon the biological activities of plasma proteins such as Factor IX (20). Figure 5 presents data showing the solvent/detergent inactivation of several different lipid-enveloped viruses at 0-2 ° C . In less than five minutes of contacting (as fast as a sample could be taken and diluted to a non-viricidal concentration) with a mixture of 0.3% (v/v) tri-n-butyl phosphate and 1.0% (v/v) Triton X-100, essentially 100% of the virus introduced is found to be inactivated.

The rapid inactivation kinetics seen for solvent/detergent treatment are in contrast to the much slower rates of inactivation seen in various heat treatment methods. Typically, these methods employ the heating of products in lyophilized form ("dry heat"), in steam ("vapor heating") or in solution or suspension ("wet heat") for periods ranging from 10 to 144 hours at 60 ° C or greater (34). In addition, all heating methods may denature plasma proteins in the absence of stabilizing agents (34,39). The much slower inactivation kinetics for the dry heat treatment of a purified Factor IX concentrate are also shown in Figure 5.

Immunopurification can result in an additional multi-log reduction of viral activity (35). While solvent/detergent methods are specific for lipid-enveloped viruses, it is expected that immunopurification would result in reduction of the activity of non-lipid enveloped pathogens as well (34,35). Thus far, the only plasma-borne, non-enveloped viral particles which have been associated with pathogenicity are the hepatitis delta agent (36) and parvovirus (37,38).

Finally, the placement of a viral inactivation treatment at a particular process stage involves a number of considerations. Earlier in a process the treated intermediate may be the source of several downstream products thus eliminating the need to treat each individually. Placement of the treatment upstream of an immunoaffinity step, for example, may also eliminate the need for a separate step for removal of chemical agents (16,20). However, significant process measures are required to isolate all downstream operations to prevent subsequent re-contamination of the product (15). Finally, the solvent and detergent agents are more efficiently used after a volume reduction step such as with VKD-protein concentrates produced by anion exchange chromatography or reconstituted Cohn fractions.

Conclusions. The technology for deriving a Protein C therapeutic from human plasma is challenged by the dilute concentration and limited amounts of plasma available for fractionation. However, immunosorbent technology can be employed to isolate biologically active Protein C that is already found to be concentrated in freshly processed Cohn fraction IV-1 paste or from the eluate of an anion-exchange adsorption of cryopoor plasma. Cohn fraction IV-1 paste also serves as an expedient source because it is an off-line discard stream and therefore does not affect important plasma fractionation products such as albumin and immune globulins. Furthermore, a solvent/detergent method of viral inactivation can be incorporated into a Protein C process in a facile manner.

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TABLE 1
PURIFICATION OF PROTEIN C FROM COHN IV-I PASTE

Step	Overalla Recovery[a] (%)	Protein Cb Specific Activity[b] (units/mg)	Fold Purification
Cohn IV-I paste	100	0.12	1
Mass capture eluate	48	3	30
First immunopurification eluate	25	140	1400
Second immunopurification eluate	20	260[c] 300[d] 340[e]	2600

[a]Amidolytic activity upon chromogenic substrate S-2366 (References 32 and 33).
[b]Protein concentration based upon OD_{280}, using an absorbance conversion of 1.4 (Reference 29).
[c]Activity is normalized for Protac™ activation.
[d]Activity is not normalized for Protac™ activation.
[e]Anticoagulant activity as measured by prolongation of activated partial thromboplastin time.

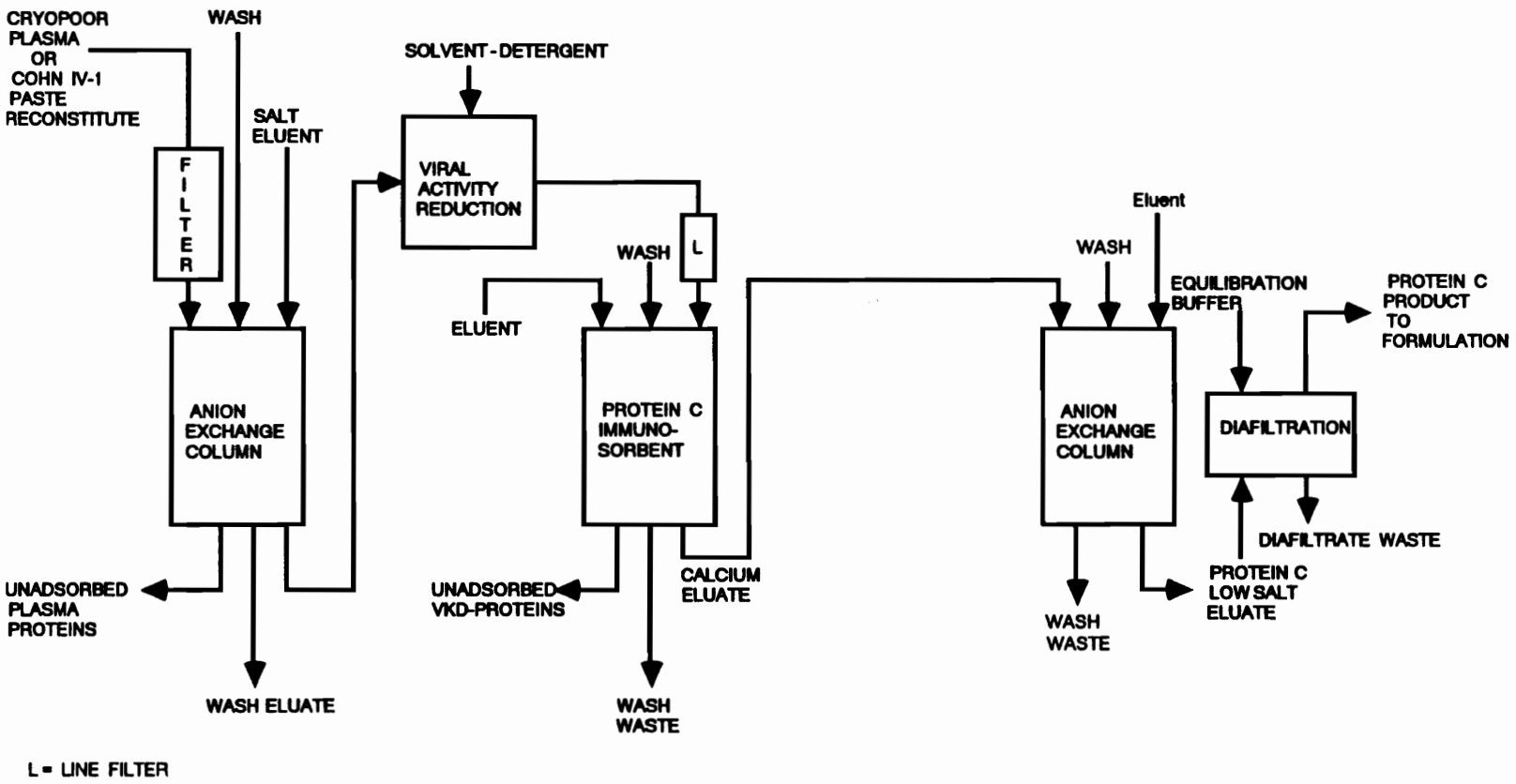

FIGURE 1
LARGE-SCALE PURIFICATION PROCESS FOR PROTEIN C

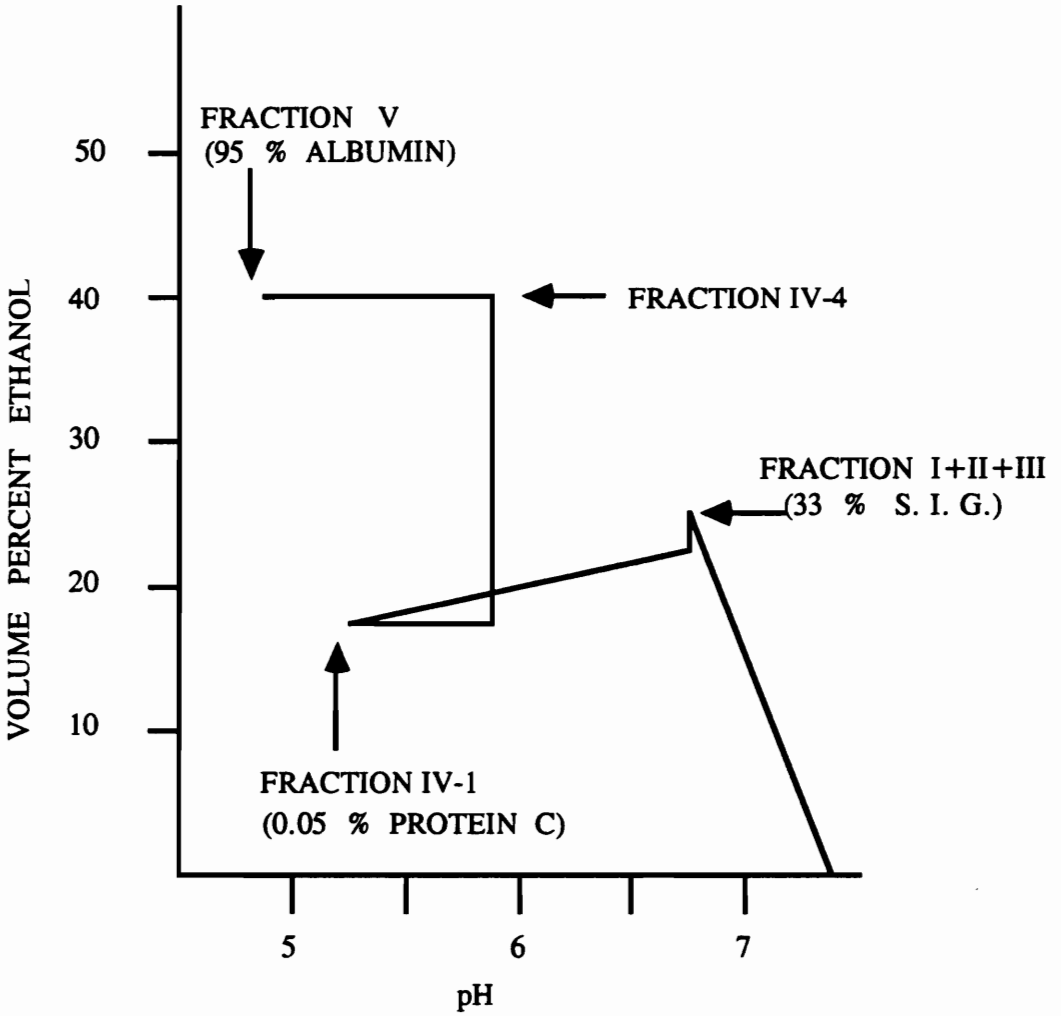

FIGURE 2
COHN PLASMA FRACTIONATION METHOD 6 AS A SOURCE OF PROTEIN C

S. I. G. = Serum Immunoglobulins. All amounts are given as weight percent of total protein in precipitate as generated by Cohn Method 6 (method 6 adopted from References 21 and 40)

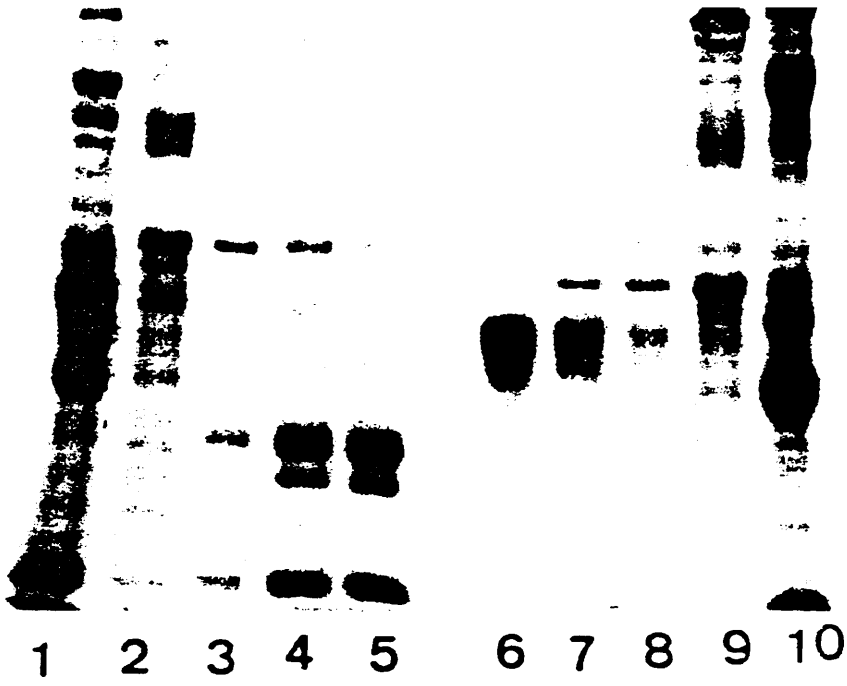

FIGURE 3
SDS-PAGE OF IMMUNOPURIFIED PROTEIN C FROM COHN FRACTION IV-I PASTE

SDS-polyacrylamide (7.5 % resolution gel, 4 % stacking gel) gel (10 cm x 8.3 cm x 0.15 cm) was loaded with 15 μl of each sample treated as in Reference 41. Lane 1, Cohn fraction IV-I paste reconstituted at 40 mg total protein/ml, 1:40 dilution (reduced); Lane 2, anion-exchange eluate of Cohn fraction IV-I paste (reduced); Lane 3, once-immunopurified product from anion-exchange eluate (reduced); Lane 4, twice- immunopurified product (reduced); Lane 5, reference protein C from immunopurified plasma (reduced); Lane 6, reference protein C from immunopurified plasma (non-reduced); Lane 7, twice-immunopurified product (non-reduced); Lane 8, once-immunopurified product from anion-exchange eluate (non-reduced); Lane 9, anion-exchange eluate of Cohn fraction IV-I paste reconstituted at 40 mg total protein/ml, 1:40 dilution (non-reduced).

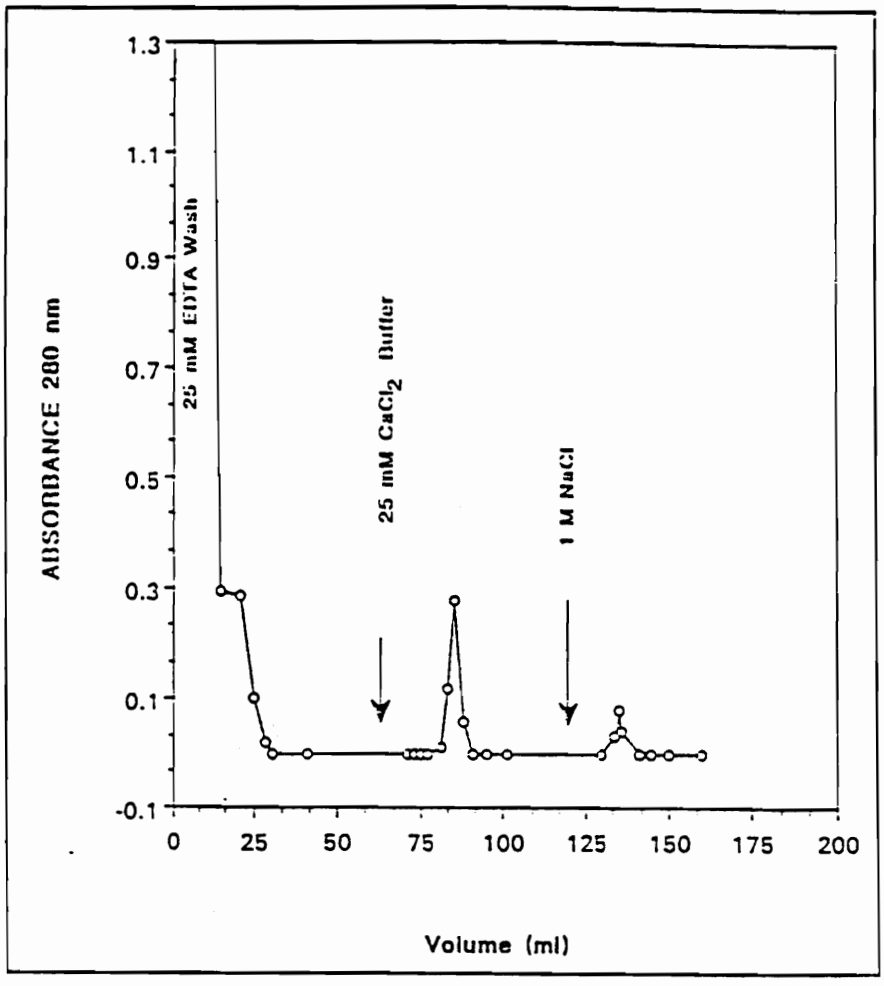

FIGURE 4
DIRECT IMMUNOPURIFICATION OF PROTEIN C FROM RECONSTITUTED COHN IV-I

Anti-protein C IgG-Affigel immunosorbent at 2 mg IgG per mililiter gel (1-cm diameter x 5-cm column height) was loaded with 40 ml of 40 mg total protein per mililiter of fresh Cohn IV-I paste reconstituted in 25 mM EDTA (sodium salt), pH 6.5. Loading, wash, and elution rates were performed at a superficial linear velocity of 1 cm per minute at 0 to 2oC.

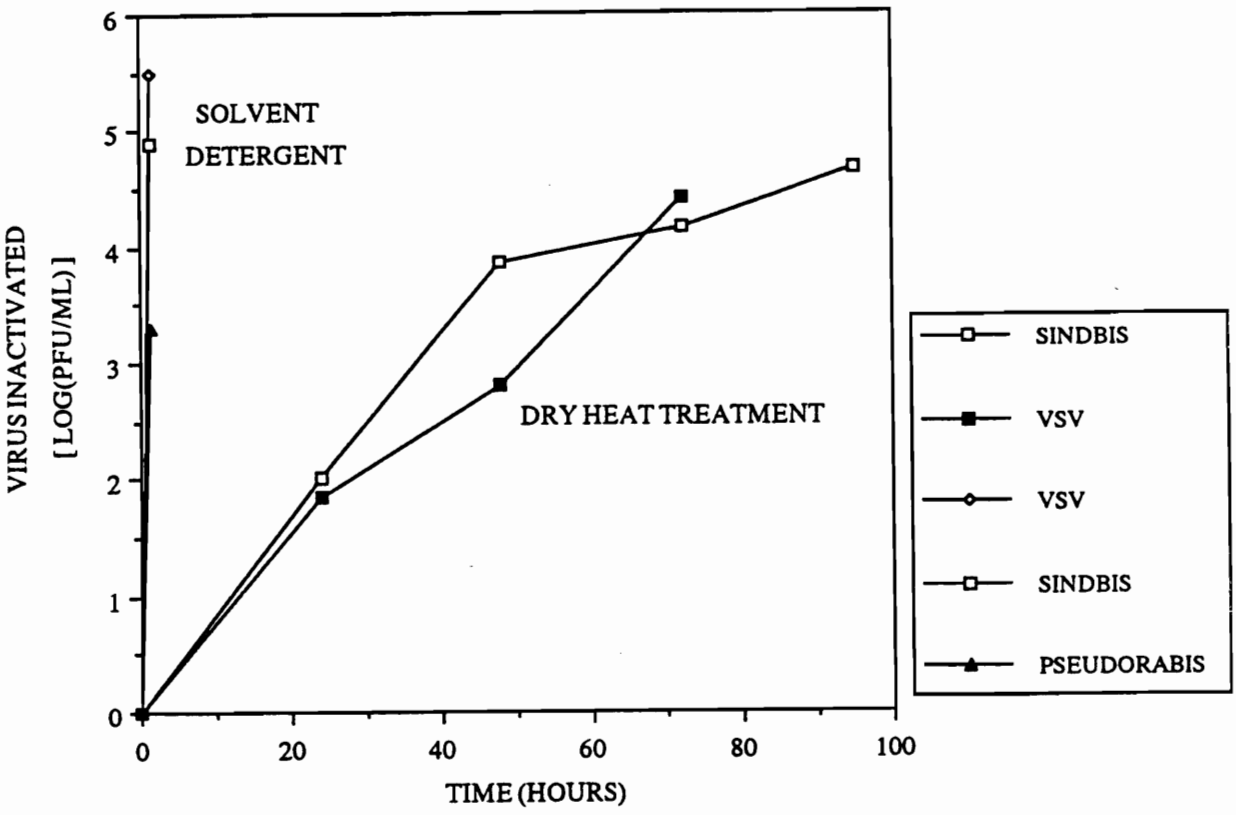

FIGURE 5
COMPARISON OF SOLVENT/DETERGENT AND DRY-HEAT VIRAL INACTIVATION METHODS FOR PLASMA

Maximum value of each inactivation data sets represents 100 % of viral titer introduced to plasma sample. Solvent/detergent treatment is 0.3 % (v/v) tri(n-butyl) phosphate and 1.0 % (v/v) Triton X-100 at 0 to 2oC. Viral components studied are listed in figure. (PFG: plague forming units, VSV: vesicular stomatitis virus)

CHAPTER FOUR

A FUSION GENE CONSISTING OF GENOMIC WHEY ACIDIC PROTEIN AND CDNA OF HUMAN PROTEIN C ENABLES HIGH LEVEL EXPRESSION IN THE MILK OF TRANSGENIC SWINE

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INTRODUCTION

Several different milk protein gene regulatory sequences have been identified which enable the expression of heterologous proteins in the milk of transgenic animals (1-7). However, expression levels from cDNAs (6, 7) and genomic sequences have been variable, with genomic sequences frequently producing much higher levels of protein (4-5, 8). For example, the cDNA encoding human Factor IX (hFIX) was expressed in sheep milk at only 25 ng/ml using a fusion gene consisting of 4.0 kbp of sheep beta-lactoglobulin (BLG) 5'-flanking sequence, 1.5 kbp hFIX cDNA, and 4.9 kbp containing the BLG transcription unit and 3'-flanking sequence (7). In contrast, secretion of human alpha-1-antitrypsin (hAAT) into sheep milk at levels up to 30 mg/ml was achieved using the same 4.0 kbp of 5'-flanking BLG sequence fused to 6.5 kbp of hAAT genomic (minigene) coding sequence (5). Additionally, there is no direct correlation between the level of expression in transgenic mice compared to livestock for a given genetic construct (3-4, 6, 9). For example, transgenic pigs expressed a 7.2 kbp genomic fragment of murine Whey Acid Protein (WAP) at a 2- to 100-fold greater level (4) than transgenic mice (3) with the same construct. Therefore, the decision to synthesize a given protein in the mammary gland of livestock can become complex with respect to the choice of employing a cDNA versus a genomic construct. We here demonstrate the expression of the cDNA sequence of human Protein C (hPC) at up to 250-fold higher concentration in transgenic pig milk over transgenic mouse milk (10) using the regulatory elements of

WAP.

Human Protein C serves as the central regulator of hemostasis (11), suggesting its potential use as a therapy for many disease states. These include: deep vein thrombosis which results from some surgical procedures (11), hereditary deficiency of hPC (12), and blood poisoning (13). Protein C exists as a zymogen of a serine protease which is activated in a species-specific manner by thrombin (14). When activated, hPC causes feedback inhibition of the formation of fibrin clots by proteolytic cleavage of Factor VIIIa and Factor Va (15).

The structure of hPC is complex and its expression in recombinant mammalian cells (16-19) and transgenic mice (10) has been limited to less than 20 μg/ml/day. Of the cell culture expression systems, only human kidney cell line 293 (20, 21) has produced functional recombinant human Protein C (rhPC), but only at levels of 10 μg/ml/day. In this report, we detail the expression of functional rhPC in transgenic swine containing a hybrid genetic construct consisting of the cDNA of hPC driven by the murine WAP gene (22).

MATERIALS AND METHODS

Transgenic swine

Crossbred gilts served as embryo donors and recipients of microinjected eggs. Estrus synchronization and surgical procedures developed by Hammer et al. (23) were used. Zygotes whose pronuclei were visible after centrifugation (24) were microinjected with 1-3 picoliters of DNA solution (3.3 μg/ml DNA in 10 mM Tris-HCl, 0.25 mM EDTA, pH 7.4) according to Brinster et al. (25). The hybrid transgene (WAPPC-1) consists of the cDNA for hPC inserted into the first exon of the murine Whey Acidic Protein gene, as illustrated in Fig. 1.

Tail tissue was biopsied from 2 day old piglets and DNA was isolated from the tissue using a modification of the procedure developed by Marmur (26). Transgenic founder animals were identified initially by Polymerase Chain Reaction (27) using hPC-specific primers, and later confirmed by Southern blotting.

Mammary gland biopsies were performed on two lactating transgenic females and a lactating control pig on day 55 of lactation. Biopsies were washed briefly in sterile saline and immediately frozen in liquid nitrogen. Total RNA was isolated from both pig mammary gland and human liver tissue samples (28). RNA samples (10 μg) were fractionated using agarose/formaldehyde gels and then vacuum-blotted onto nylon membranes (29). The membranes were probed with ^{32}P-labeled hPC cDNA.

Pig Milk Collection and Preparation

Piglets were removed from the sows for approximately 30 minutes prior to milking to allow for milk accumulation. The sows were then induced to let down milk by intramuscular administration of 20-30 I.U. oxytocin. Milk was collected directly into ice-cold Tris-Buffered Saline-EDTA buffer (2X TBS-EDTA: 100 mM Tris-HCl, 300 mM NaCl, 200 mM EDTA, pH 6.5) in a 1:1 buffer to milk ratio. Fat and precipitable caseins were removed by centrifugation at 15,000 x g for 20 min at 4 °C. The buffer-expanded whey phase was then filtered through sterile gauze and stored at -90 °C. Control pig milk was treated identically.

Analysis of recombinant human Protein C

Several milk samples from the two highest expressing pigs (29-2 and 83-1) were pooled and used for immunopurification. Expression levels in buffer-expanded whey were determined by an Enzyme-Linked-Immunosorbent Assay (ELISA), using the heavy chain epitope-specific monoclonal antibody (Mab) HPC4 that binds to hPC in the presence of Ca^{2+} (13, 30). The rhPC present in transgenic pig whey captured with HPC4 Mab was detected using horseradish peroxidase conjugated goat anti-rabbit Immunoglobulin G (IgG) following a 3 h incubation at room temperature with a rabbit polyclonal antibody to hPC (Assera C). Immunopurification of rhPC from transgenic pig whey was performed using the HPC4 Mab immobilized onto Affiprep-10 (BioRad, Richmond, CA). The whey was diluted to 15 mg/ml and then loaded onto the column

in the presence of 25 mM Ca^{2+} in TBS (pH 6.5). The rhPC that was bound to the immunosorbent was specifically eluted with 25 mM EDTA in TBS (pH 6.5).

Samples of immunopurified rhPC were analyzed using Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (10% SDS-PAGE) under reduced and non-reduced conditions according to Laemmli (31) and stained with 0.125% Coomassie Blue G-250, 50% methanol, 10% acetic acid. Identical Western blots were performed using the method of Towbin (32).

The biological activity of the rhPC was assessed using an Activated Partial Thromboplastin Time (APTT) Assay (33). Either hPC or rhPC samples, and Normal Plasma Reference Pool (NPRP) were prepared in hPC depleted plasma. The delay in coagulation time due to immunopurified rhPC samples was compared to that of reference hPC. The APTT reagent included Protac (*Agkistrodon Contortrix* venom, American Diagnostica Inc., Greenwich, CT) to specifically activate hPC or rhPC (34) prior to adding $CaCl_2$ for initiation of coagulation. NPRP was used as the standard curve assuming a theoretical specific activity of 250 units/mg for hPC.

RESULTS

A total of 26 piglets were born from recipients which had received microinjected embryos. Table 1 summarizes data on generation of founder transgenic swine. Initial screening of tail DNA using Polymerase Chain Reaction (PCR) indicated that 7 piglets had integrated the transgene. Southern blots on DNA from these pigs identified only 5 positive for the transgene. One of these transgenics was a male who died shortly after birth and is not shown on the Southern blot. Further analysis of pig 29-2 and 83-1 by quantitative PCR estimated that they had incorporated 10 and 20 copies of the transgene per genome, respectively (assuming the animals were hemizygous). Of the 2 females predicted negative by Southern and positive by PCR, one (29-1) was found to express hPC antigen in her milk (Table 2). This result suggests that PCR was more sensitive than Southern analysis. A similar observation has been made previously by Schiffman et al. (35) for the detection of human Papillomavirus DNA. Alternatively, this pig may not have been detected by Southern blotting because she was mosaic, having too few transgene copies present to be detected. Specifically, quantitative PCR showed an average of only about one copy per 100 genomes to be present in mammary gland DNA and about one copy per 1000 genomes in tail DNA for pig 29-1 (data not shown). Six of nine piglets from this sow were positive for the transgene. Southern blots of tail DNA from founder pigs are shown in Fig. 2A. For some pig DNA samples, three bands were seen which corresponded to monomers, dimers, and trimers of the transgene resulting from incomplete digestion of the construct within the chromosomal DNA (9.3 kbp, 18.6

kbp, and 27.9 kbp, respectively). Southern analysis of founder pig 29-2 and her offspring is presented in Fig. 2B. It is important to note that different signal intensities were observed for the offspring of 29-2. Overall germline transmission of the transgene to progeny is summarized in Table 3.

Northern analysis of mammary gland RNA (using hPC cDNA as a probe) from pigs 29-1 and 29-2 and human liver tissue was performed. The human liver RNA showed a single weak band at about 2000 nucleotides (nt). A very strong band of about 1900 nt was detected in pig 29-2, but not in the control pig. Other minor messages of 3000, 3500, and 5700 nt were detected in the RNA of pig 29-2 (Fig. 3). Similar amounts of total RNA were loaded onto the gel from both tissue types based upon 18S and 28S rRNA bands. On the Northern blot, the hPC transcript appears to be about 100-1000 fold more predominant in transgenic pig 29-2 mammary gland than in the human liver sample used. Even though a low level of hPC antigen was detected in the milk of pig 29-1, no hPC message was seen in her RNA sample. This observation may be due to the degree of mosaicism and is reflected in the very low expression level.

Identical Northern blots, using WAP cDNA as a probe, indicated the presence of several messages which have sequence similarity to WAP (Fig. 3B). A message was seen in all three pigs corresponding to about 925 nt. However, this message was much stronger for pig 29-2. In pig 29-2, two additional messages were detected at about 3500

and 5700 nt.

The hPC antigen in transgenic pig whey as measured by ELISA ranged from 12 μg/ml to 103 μg/ml for pig 29-2, and 50 μg/ml to 260 μg/ml for pig 83-1 (Table 2). The milk from four additional transgenic pigs contained hPC antigen at levels ranging from none detected to 5 μg/ml. Additional ELISA and Western blot analyses using WAP antibody showed that no WAP antigen was produced in the milk of either control or transgenic pigs. Comparison of Western analysis with Coomassie blue stained SDS-PAGE revealed that the rhPC purified from transgenic pig whey samples was greater than 90% pure (data not shown). On the Western blot using nonreduced conditions (Fig. 4A), the rhPC migrated slightly faster than hPC which had an apparent Mr of about 58 kD. When run under reduced conditions (Fig. 4B) the Western blot indicated that significantly more single chain was present in rhPC compared to hPC.

Chromatographies using control pig whey demonstrated that no hPC antigen was specifically eluted by EDTA using HPC4 Mab as determined by both ELISA and Western Blot (data not shown). Additionally, column elutions with NaCl or NaSCN in chromatographies with nontransgenic pig whey failed to yield any hPC antigen.

The rhPC purified from milk pools from swine 29-2 and 83-1 had specific amidolytic activities of 274 ± 0.5 and 130 ± 4.5 U/mg, respectively, while that of hPC

reference from human plasma was 245 ± 5.0 U/mg. Specific anticoagulant activities of 244 ± 12 and 103 ± 11 U/mg were determined for milk pools from pigs 29-2 and 83-1, respectively. The anticoagulant activity of hPC reference from human plasma was 213 ± 7 U/mg (Table 3).

DISCUSSION

WAP regulatory elements have previously been shown to direct the expression of heterologous genes in the mammary tissue of transgenic mice (3, 10) and pigs (4, 8). Here, the murine WAP gene was used to drive the expression of the cDNA for human Protein C into the milk of transgenic swine. The expression level was about 100-fold higher in transgenic pigs than in transgenic mice containing the same construct (10).

This result is notable because high level production of heterologous proteins in milk was typically detected only for transgenic animals carrying either genomic (4, 8) or minigene constructs (2, 5). In fact, the few groups that have used cDNA as the structural portion of their transgene have obtained low expression levels, ranging from 10 ng/ml to 10 μg/ml (6, 7, 9, 10). These and other data have led to conclusions emphasizing the importance of introns in allowing for efficient gene transcription (36-38).

Our data from transgenic swine suggests that the mouse WAP genetic elements used previously (4, 8) are sufficient for expression of the hPC cDNA at reasonably high

levels. These expression levels were only 2- to 5-fold lower than those obtained using the intact genomic WAP construct in transgenic pigs (8). Notably, the expression of this construct in our pigs appeared to be chromosome position dependent, as was reported previously for the intact WAP gene (4). However, other factors, such as the presence of 27 bp of linker DNA before the hPC start codon or an incomplete polyadenylation signal, may have adversely affected the expression levels of hPC cDNA. We are currently testing these hypotheses using both transgenic mice and pigs.

Analysis of Southern blots for pig 29-2 and her offspring revealed different banding patterns among the piglets. One piglet showed band intensities similar to the sow, while two others showed much less intense (but differing) signals for the transgene. This data suggests the presence of transgene insertion sites on different chromosomes. Judging from the banding patterns and intensities observed in the piglets, integration in sow 29-2 appears to have occurred on at least three different chromosomes, with different copy numbers at each insertion site. Insertion into different chromosomes seems to be the logical argument for the apparent segregation among offspring, rather than random genetic recombination. Quantitative PCR analyses performed on both the first and second litters from pig 29-2 are consistent with these conclusions.

The Northern analyses indicated the presence of messages corresponding to both hPC and WAP, which is expected considering the design of the construct (see Fig. 1).

A major message of about 1900 nt was detected (when hybridized with the hPC cDNA probe) in the RNA of pig 29-2. In the human liver RNA sample, a single band of 2000 nt was detected. The relative amounts of hPC mRNA from pig 29-2 mammary gland compared with that from human liver agrees with relative amounts of Protein C produced by the two tissues. In pig 29-2 RNA, another band of about 3000 nt was specific for hPC only. The presence of other messages at 3500 and 5700 nt (very faint) appear to be read-through transcripts containing both hPC and WAP sequences (verified below).

Using WAP cDNA as a probe for Northern blots, several bands were detected. The size of some the larger bands, which hybridized with both hPC and WAP probes, suggests they may be read-through transcripts containing WAP message in conjunction with hPC message, or processed fragments of that read-through transcript. The message seen at about 5700 nt may be a pre-mRNA containing both hPC and WAP sequences. At about 3500 nt, a message was detected which may be a spliced mRNA containing both hPC and WAP sequences. All three pig RNAs (including the control) exhibited a band at about 925 nt. This band appears to be a WAP-like message endogenous to the pig, although it is a much stronger signal in pig 29-2. The extra intensity of this band in pig 29-2 RNA may be due to a spliced mRNA containing only a WAP message. It is noted that the 925 nt species which hybridized with WAP cDNA was not seen in transgenic mammary gland RNA carrying the intact WAP gene (4). However, one would not expect any of the WAP messages on the read-through transcripts to be

translated, due to the lack of or incorrect positioning of a ribosome binding site. The absence of WAP in the milk of pig 29-2 was confirmed by both ELISA and Western analyses.

The structure of rhPC (immunopurified from pooled milk samples from transgenic pig 29-2) appeared to be similar to that of hPC, though the rhPC had slightly lower apparent Mr. Detailed biochemical analyses of the porcine rhPC have been done and are presented elsewhere (39).

Porcine rhPC from animal 29-2 had both amidolytic and anticoagulant activity values within the normal range of those values for reference hPC. This activity is evidence that the rhPC (from pooled transgenic pig milk samples of animal 29-2) possesses a functional catalytic site and phospholipid binding domain. The somewhat lower anticoagulant activity values for pooled milk samples from pig 83-1 have subsequently been attributed to differences in milk handling procedure on different days. Although variability was seen in anticoagulant activity on different days of lactation, there was no correlation between the level of rhPC present in the milk sample and the activity of that rhPC (39).

Pigs have many inherent advantages for use as bioreactors in the production of high value heterologous proteins such as rhPC. These advantages include: a short

generation time with two litters per year, multiple progeny per pregnancy, and high volume milk production. The potential of the mammary gland of transgenic pigs for synthesizing a complex heterologous protein, at concentrations much greater than have been obtained using *in vitro* systems, has been demonstrated. A comparison of the synthesis and protein processing rates of rhPC in the porcine mammary gland with the most productive of cell culture bioreactors has been evaluated elsewhere (39). It was postulated that the natural structure of the mammary gland (especially its high cell density and secretory ability) makes it much more efficient as a bioreactor than cell culture systems.

The ability to use a cDNA for obtaining high level expression is important since difficulties often arise when cloning and manipulating large genomic DNA fragments. Our results using a cDNA for the coding sequence show that intact genomic sequences are not absolutely required for high level expression of heterologous proteins in the milk of transgenic pigs.

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LIST OF FIGURES

Fig. 1. Diagram of the WAPPC-1 construct. The 1.4 kbp cDNA for hPC (including 110 bases of poly A) was inserted at the KpnI site at the first exon of WAP, using 27 bp of linker DNA. The genomic WAP gene consisted of 2.6 kbp 5'-flanking promoter sequence, 3.0 kbp coding sequence (exons and introns), and 1.6 kbp of 3'-flanking DNA. Non-coding DNA segments and introns are indicated by dark lines. The open box is 27 bp of linker DNA, the filled box is the cDNA for hPC, and stippled boxes are WAP exons. Large batches of plasmid were isolated, digested with EcoRI, and purified using HPLC to remove all traces of cloning vector DNA (10).

Fig. 2. Southern analysis of transgenic swine. DNA was isolated from tail biopsies of 2 day old piglets. Samples were digested with EcoRI, run on 0.7% agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P random primer labelled hPC cDNA. Markers indicate band sizes in kbp of DNA. (A) Founder animals. Lanes 1, 2, and 3 are 250, 25, and 2.5 pg of EcoRI digested WAPPC-1 plasmid. Lanes 4 through 12 are 5 μg EcoRI digested DNA from control pig, founder pigs 29-1, 29-2, 83-1, 83-2, 83-3, 83-4, 83-5, and 83-6. (B) Pig 29-2 and offspring.

Fig. 3. Northern analyses of pig mammary tissue RNA and human liver RNA. Left side markers indicate positions of 18S and 28S rRNA bands; and right side markers indicate band sizes in nucleotides. (A) Probed with hPC cDNA. (B) Probed with WAP

cDNA. Lanes 1 and 2 are 10 μg human liver total RNA isolated from two different liver samples. Lanes 3, 4, and 5 are 10 μg each of: control pig, transgenic pig 29-1, and transgenic pig 29-2 mammary gland total RNA, respectively. Lanes 6 and 7 are replicate samples of lanes 1 and 2.

Fig. 4. Western blot of immunopurified rhPC from pooled milk samples from transgenic pigs 83-1 and 29-2 under (A) non-reduced and (B) reduced conditions. Molecular weight markers indicated in kD. Lanes 1 and 4 are American Red Cross reference hPC (lot # 28309018). Lane 2 is from pig 83-1 and lane 3 is from pig 29-2. Membranes were probed using Assera C polyclonal antibody.

LIST OF TABLES

TABLE 1

Production Stage	Number
Embryos recovered	415
Embryos injected	332
Embryos transferred	332
Number recipients	8
Embryos per recipient	41.5
Number pregnant	3
Number piglets born	26
Number piglets transgenic*	7
Percent transgenic	26.9
Number transgenic females	6
Number assayed for expression	6
Number expressing	4

TABLE 2

Pig Number	hPC Antigen (μg/ml)	Germline Transmission*
29-1	2-3	6/9
29-2	12-103	4/7
83-1	50-260	3/6
83-2	n.d.	9/12
83-4	n.d.	6/7
83-6	0.6-1.0	0/12

TABLE 3

Source Material	Amidolytic Activity* (U/mg)	Anticoagulant Activity* (U/mg)
29-2 pool	274 ± 0.5	244 ± 12
83-1 pool	130 ± 4.5	103 ± 11
Plasma Reference**	245 ± 5.0	213 ± 7

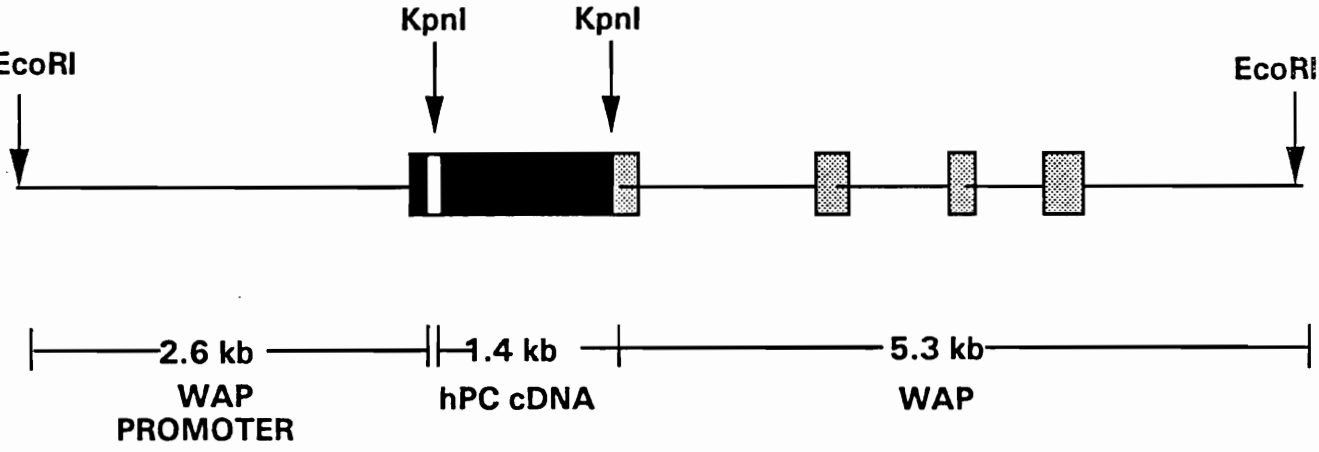
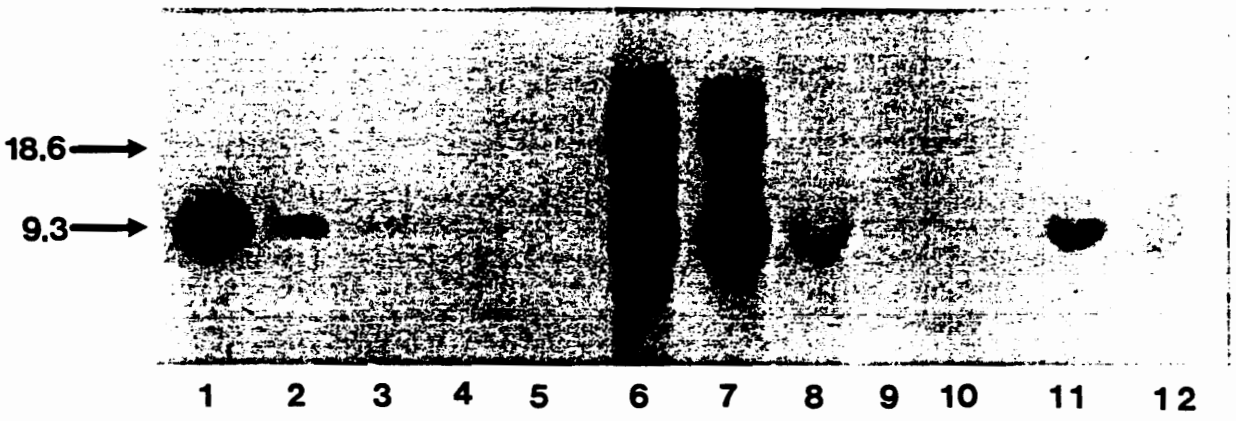


FIGURE 1

MURINE WHEY ACIDIC PROTEIN-HUMAN PROTEIN C CONSTRUCT (WAPPC1)

(A)



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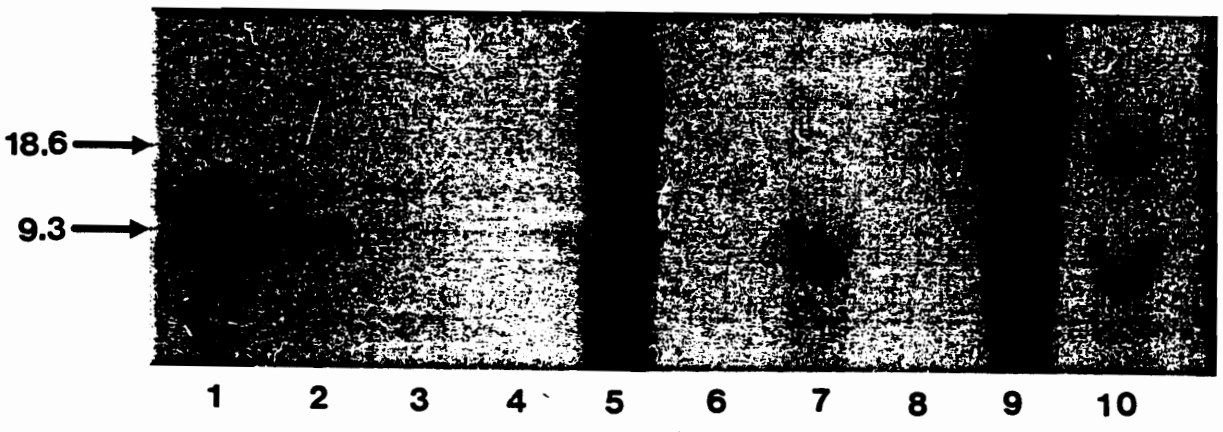


FIGURE 2

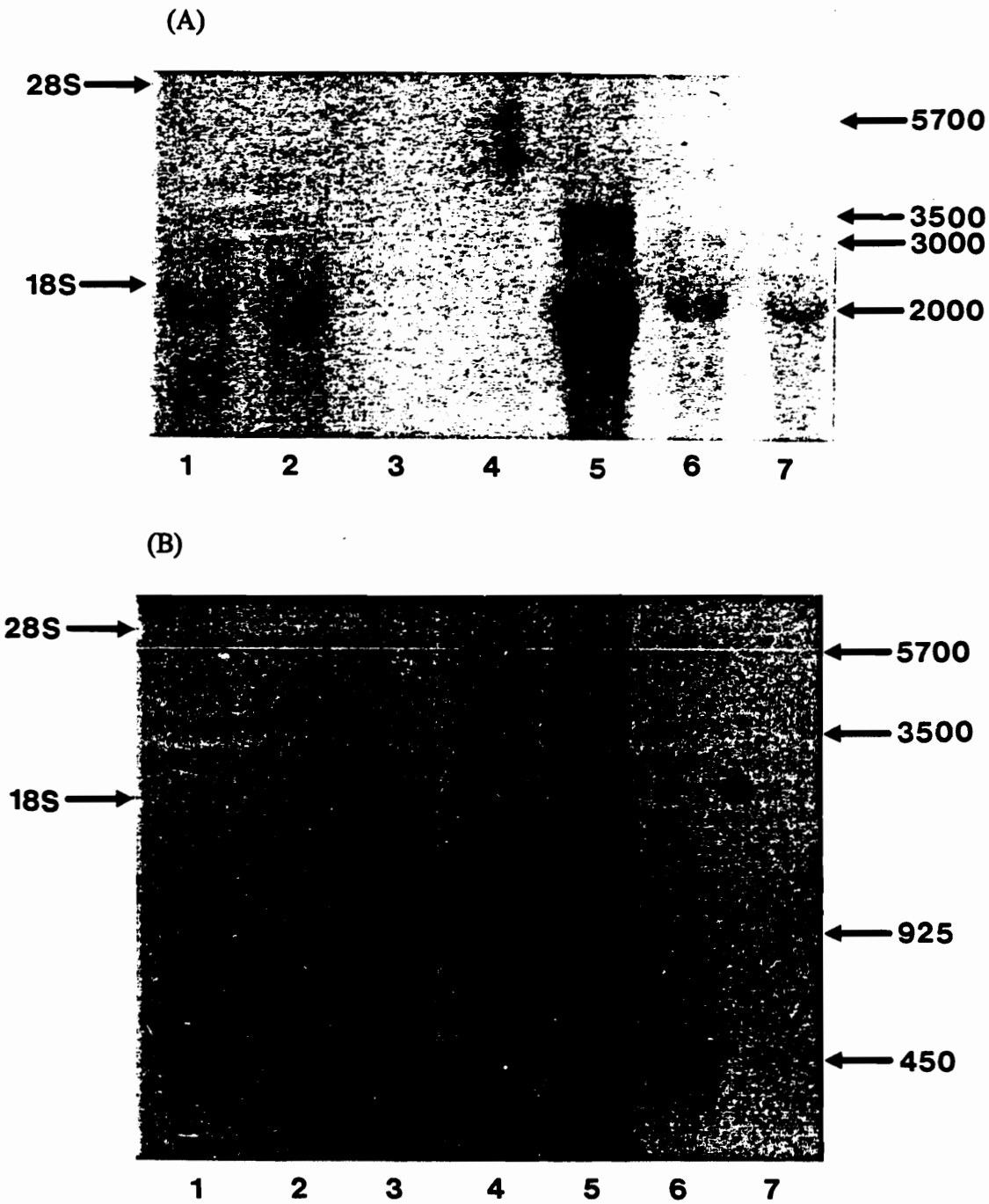
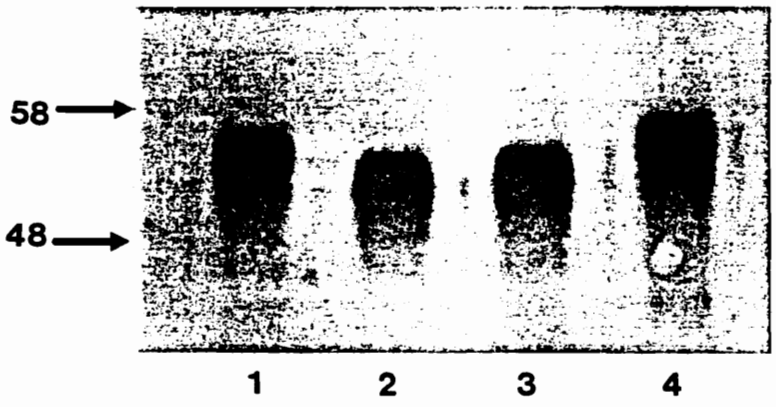

(A)
28S
18S
5700
3500
3000
2000
1 2 3 4 5 6 7
(B)
28S
18S
5700
3500
925
450
1 2 3 4 5 6 7

FIGURE 3

(A)



(B)

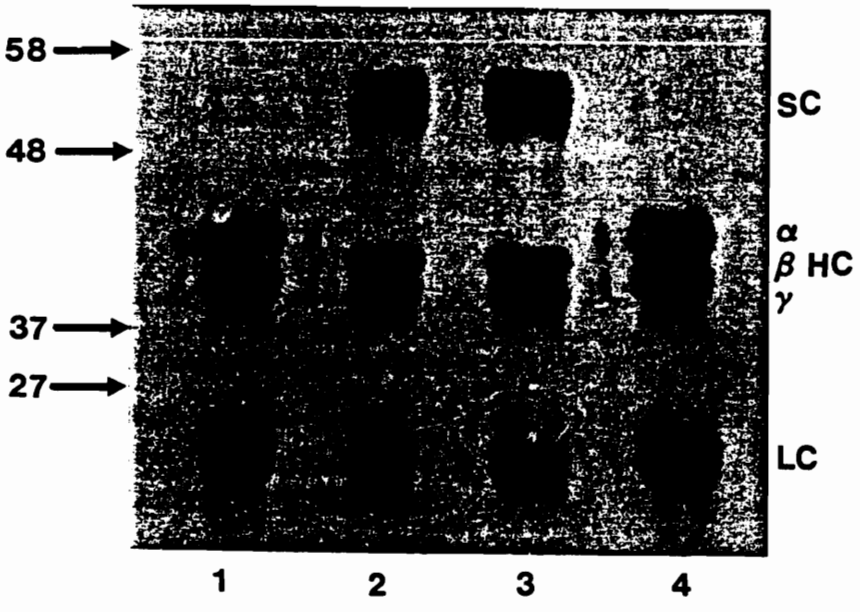


FIGURE 4

CHAPTER FIVE

COMPLEX PROTEIN PROCESSING IN PORCINE MAMMARY TISSUE

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ABSTRACT

Recent gains in the understanding of genetic regulation have enabled high levels of proteins to be expressed in cell culture (14,15) as well as in the mammary gland of transgenic animals (16-18). The potential use of *in vitro* systems as bioreactors for the production of complex proteins has been limited by their inability to perform post-translational processing at high levels of synthesis. These modifications are essential for biological activity of many proteins having therapeutic value (16-20). For example, enzymatic carboxylation of glutamic acid to form 4-carboxyglutamic acid (gla) is necessary for the membrane-mediated activities of many of the plasma proteins associated with hemostasis (3,21). These are referred to as the vitamin K-dependent (VKD) proteins because vitamin K is an essential cofactor for gla formation. We have produced transgenic swine containing the cDNA of the VKD protein, human Protein C (hPC) in an effort to utilize the porcine mammary gland as a bioreactor (13). These studies demonstrate that regulatory elements taken from the murine Whey Acid Protein (WAP) gene can govern expression of hPC in the mammary gland of transgenic pigs. In this report, we document the potential of the porcine mammary gland to perform complex post-translational modifications upon recombinant hPC (rhPC) at high rates of synthesis.

INTRODUCTION

Protein C serves as an important regulator of hemostasis and is produced by the liver (1-3). The relationship between the anticlotting function (2,3) and the structure

(1,4) of hPC makes it one of the most complex members of the VKD protein family. It is potentially indicated as a therapy for disease states where clotting must be attenuated. These indications include: treatment of sepsis (5), prevention of restenosis after myocardial infarction (3), prevention of intravascular thrombosis which results from surgical procedures such as hip and knee replacement (3), as well as replacement therapy for congenital deficiency of hPC (6). There is limited availability of hPC because it occurs naturally as a trace plasma protein at approximately 4 μg/ml (1), which makes large-scale isolation from human plasma difficult (7). Alternatively, only low levels of recombinant human Protein hPC (rhPC) have been secreted from mammalian cell lines including those derived from human kidney (8), human liver (9), and mouse mammary epithelium (10). In most cases, a population of largely dysfunctional rhPC was produced (9,10) or intracellular buildup of rhPC precursors occurred when high synthesis rates were achieved (8,11).

Protein C circulates as a 62,000 Mr zymogen (1,4) of a serine protease (Figure 1) whose species-specific activation by thrombin (1) inhibits the generation of fibrin clots by proteolytic cleavage of Factor VIIIa and Factor Va (2,3). The mature zymogen is formed from post-translational cleavages which result in removal of the signal and propeptide sequences (4). In addition, a dipeptide at amino acids 156-157 is removed in 70-95% of hPC molecules to yield a heterodimer containing a 41,000 Mr heavy chain and a 21,000 Mr light chain (4,25,26). Activation of hPC results from proteolytic

removal of a peptide consisting of the first 12 amino acids of the N-terminus of the heavy chain (1,4). A heterogeneous population of hPC occurs in plasma (25,26) because most hPC molecules possess four differently modified N-linked sites of glycosylation. In addition, hPC contains 12 intrachain disulfide bridges, one β-hydroxylated aspartic acid residue, and 9 gla residues. These gla residues occur within the first 29 amino acid residues of the light-chain (1,4) and are necessary for anticoagulant activity (8,21) which arises from the interaction of hPC with phospholipid membranes and another VKD protein, Protein S (3). Protein C is one of the most complex proteins to have been made in transgenic livestock and thus has presented a significant challenge to the porcine mammary gland in terms of protein processing (13). While there are some differences between the structure of hPC and porcine rhPC, this study demonstrates the ability of the porcine mammary gland to perform folding, disulfide bridging, proteolytic processing, glycosylation, and gamma-carboxylation which together result in a functional recombinant human protein.

RESULTS AND DISCUSSION

Rates of Synthesis in Porcine Mammary Tissue

It was somewhat unexpected to find rhPC produced at 10-260 μg/ml in the milk of transgenic pigs (Figure 2), since mice using the same genetic construct had only 0.1-3 μg/ml detected in their milk (30). This result also presents a stark contrast in the rate of secretion of rhPC between the porcine mammary tissue (12) and cells cultured *in vitro*

(8). Much lower productivity was seen in genetically engineered cell lines which have stably secreted rhPC at levels of 10 μg/ml/day or less (8,15). The use of genetic amplification techniques to further increase the rhPC levels in cell culture resulted in decreased secretion efficiency and intracellular buildup which was attributed to limitations in post-translational processing of rhPC (8). We found no evidence for abnormal secretion as a result of the synthesis of rhPC in the porcine mammary gland. All animals which have expressed rhPC, even those who expressed at high levels, experienced normal 8-week lactations (34).

The difference in rhPC productivity between porcine (*in vivo*) and cell culture (*in vitro*) systems is curious. This prompted us to make order of magnitude estimates of the gross synthesis rates of hPC for human liver, rhPC in the porcine mammary gland, and cell culture (12). In addition, we have previously compared the levels of rhPC messenger RNA in the porcine mammary tissue to hPC message extracted from pooled samples of normal human liver (13). On a per cell basis, both the amount of hPC message detected by Northern analysis (13) and the synthesis rates estimated from antigen levels detected in the milk (12) were found to be consistent with a 10-100 fold greater hPC antigen synthesis rate in porcine mammary tissue relative to human liver.

The highest synthesis rates estimated for rhPC in porcine mammary tissue appear similar to those reported for unamplified human kidney cell 293 line (8) with both

possessing a population averaged rate of synthesis on the order of 1 μg/ 10^6 cells/day (12). However, as discussed above, stable 293 cell lines secreted at concentrations of only 10 μg/ml/day or less. Thus, the advantage of *in vivo* production of rhPC in the mammary gland appears to be a significantly higher cell density relative to milk volume than is currently possible for *in vitro* systems relative to culture media volume (8). It is noted that porcine milk contains an average of 6% protein (27) and lactation can be sustained for 60 days, with two lactations per year (28). Thus, the potential capacity of the porcine mammary gland for expressing a complex recombinant protein may be much higher than we have achieved in these early experiments.

The high endogenous protein content of porcine milk has not unduly complicated isolation of the rhPC as yields and purity (Figure 4) have been greater than 80% and 90%, respectively (51,52). However, a much higher purity will be needed for an rhPC therapeutic. We have subjected rhPC immunopurified from different days of lactation to various biochemical analyses so that protein processing could be evaluated for days with the highest levels in rates of rhPC synthesis.

Proteolytic Processing in mammary tissue

The differences between hPC and rhPC were pronounced in the extent of heterodimer formation (Figure 5b). More interestingly, although synthesis rates appeared to vary greater than ten-fold for animal 29-2 on different days, each day

consistently possessed approximately 50% single chain form (data not shown). An even greater perturbation might have been expected given the great contrast in rates of synthesis between human liver and porcine mammary tissue (12) as hPC from normal, plasma pool usually contains 5-30% single chain form (25). This observation demonstrates that *in vivo* removal of the dipeptide can occur at a much greater rate than appears necessary for hPC synthesis in human liver. Thus, we suspect that dipeptide removal is probably controlled by a more complex set of events rather than a simple kinetic limitation inherent to the proteolysis reaction.

As do many other proteins, milk proteins must undergo proteolytic cleavage to remove signal peptide sequences in order for efficient secretion to occur (29). While we have not yet completed the direct amino acid sequencing of rhPC from each day of lactation, secretion has apparently occurred in a way which is normal for a milk protein. The retention of both signal and propeptide sequences would result in an approximate 4.1 kD increase in Mr of the rhPC as judged by Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Thus, we expect that proper proteolytic removal of most signal sequences has probably occurred in light of the lower Mr of most rhPC forms. We are currently immunopurifying separate single and 2-chain forms of rhPC to enable amino terminal sequencing. Therefore, potentially different populations, which may be present as a result of incomplete proteolytic processing, will be identified.

Glycosylation in mammary tissue

The complexity of hPC carbohydrate structure is related to both the incidence that a given site is glycosylated and the nature of the branched structure at each site (8,26). The predominant forms of hPC have been attributed to tri- (alpha form), di-(beta form), and mono-substitution (gamma form) of N-linked carbohydrate at three different asparagine residues. These differences are reflected in the apparent Mr as visualized by SDS-PAGE; a triplet centered about an Mr of 40 kD occurs for those molecules having undergone dipeptide removal and an analogous triplet form occurs about an Mr of 58 kD for the single chain species (Figure 4a,b). Two different, but closely migrating, beta chains can also be identified in hPC. We propose that these species may both be beta forms but with different permutations of disubstitution at different sites. A similar triplet form occurs in the case of the transgenic rhPC with the ratio of alpha to beta chains estimated to be 1:1 compared to approximately 1.5:1 for hPC (Figure 4b). The incidence of gamma heavy chain species in rhPC is slightly higher than but similar to that of hPC.

The frequency that any given site of glycosylation occurs in rhPC appears not to be greatly perturbed from that of liver by the two-order increase in the rate of synthesis. It has been postulated that the initial transfer of high mannose core to Asn^{329} could be precluded by the presence of a disulfide bond at the distal Cys^{331}, and thus result in a di-substituted hPC species (26). This hypothesis purports competitive reactions between glycosylation site binding protein and protein disulfide isomerase within the Asn^{329}-Glu^{330}-

Cys^{331} domain of hPC. Our results would indicate that both the proposed reactions can occur at very high rates of protein synthesis in the mammary tissue.

Superimposed upon different sites of glycosylation in hPC is the variability in branched oligosaccharide structure that occurs after modification of a carbohydrate core, which is initially high in mannose content (8,40). The various rhPC forms made in transgenic swine exhibited an approximate 1-3 kD lower Mr relative to analogous forms found in hPC (Figure 5a,b). The differences in apparent size between hPC and rhPC may be due to the rate limitations which occur at each point in the maturation of the high mannose core. Alternatively, rhPC glycosylation may bear the signature of the tissue specific nature of oligosaccharide transfer reactions performed by the mammary gland (41,42). The "lighter" appearance for single, heavy, and light chains of porcine rhPC is similarly exhibited by rhPC derived from human kidney 293 cells (8). Overall, a similar amount of total carbohydrate was found for both hPC and porcine rhPC. We postulate that the differences in size of rhPC forms relative to hPC forms may be due to less branching at each of the sites which were glycosylated (32). The branched oligosaccharide structure of rhPC is currently being studied.

The carbohydrate composition of rhPC did not vary significantly for material derived from different days of lactation or from different founder animals. Thus, the kinetics which govern oligosaccharide synthesis and assembly onto rhPC do not appear

to be limiting at the rates of protein synthesis performed by either of the higher expressing founder animals. Interestingly, the carbohydrate composition of porcine rhPC is remarkably similar to that reported for the 293 cell line (Table 1). Although different techniques were used to determine carbohydrate composition, both the porcine and 293 cell rhPC possessed N-acetylgalactosamine, in contrast to none detected for hPC (32,43). Significantly higher fucose content was also found for the porcine rhPC relative to hPC. The sialic acid content of the rhPC closely resembled that of hPC. Sialic acid is important with respect to biological but not amidolytic activity of rhPC (46) and may affect *in vivo* clearance from human plasma (47). In addition, the similarity in mannose content indicates that post-translational processing from high to low mannose content was performed (8).

Evidence for Proper Vitamin K-dependent Processing in Mammary Tissue at High Rates of Synthesis

The specific anticoagulant activities of rhPC produced on the days with the highest antigen levels were similar to those found for hPC (Figure 6). Therefore, it appears that carboxylation is not rate limiting even at the highest levels of rhPC synthesis found in porcine mammary tissue. We find it intriguing to speculate about the source of the carboxylase activity. In general, milk proteins contain diverse post-translational modifications (28,29), but we have found no reports of milk proteins containing gla or evidence of significant carboxylase activity in the mammary tissue, although vitamin K

naturally occurs in milk (27). Prior to the present work, the only VKD protein produced in mammary tissue was human Factor IX which was expressed in the milk of sheep at 25 ng/ml, but specific activity was not reported (31). Perhaps there are as yet unidentified carboxylated proteins in milk or the induction of ordinarily latent carboxylase activity has occurred as a result of the translation of rhPC.

The biological and amidolytic activities of all rhPC samples analyzed were found to be specifically activated by *Agkistrodon contortrix* venom as was previously reported for hPC (33). Although amidolytic processing is independent of gla-mediated membrane binding function (39), most days exhibited specific amidolytic activity which was similar to the specific anticoagulant activity (data not shown). However, rhPC derived from day 52 milk of pig 83-1 and day 55 milk of pig 29-2 both exhibited a specific amidolytic activity (Figure 7) which was significantly lower than the corresponding specific anticoagulant activity (Figure 6). These results suggest that changes in catalytic site structure may have occurred in these later days. This change is more likely due to folding differences rather than post-translational modification of gla residues.

The availability of large quantities of rhPC from pig milk provides a novel situation for characterizing the temporal differences which occur for *in vivo* protein synthesis which can not be easily duplicated in humans. While the specific activities of rhPC changed during lactation, it is apparent that the conformational attributes of the

membrane-binding, catalytic, and activation peptide domains collectively possessed a sufficiently native structure to impart biological activity. We are currently studying the specific inactivation kinetics by rhPC upon Factor VIIIa and Factor Va in order to better understand the differences observed for amidolytic and biological specific activities between different days of lactation.

Clearly the porcine mammary gland can perform many of the complex processing reactions necessary for recombinant synthesis of functional human proteins and export them at levels suitable for therapeutic applications.

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12. The transgenic sows used in this study let down milk about every 55-60 minutes which is typical for most sows. The concentration of hPC antigen was sustained at greater than 100 μg/ml for a significant portion of the lactation period for animal 83-1 which gives an average secretion rate of 100 μg rhPC/ml/hr. Morphometric data from mammary tissue indicates that approximately 30-40% of the gland volume is composed of secretory cells (35 and see Figure 3). We estimated the average mammary gland volume of our lactating pigs to be about 250 ml. This gave a gland weight of 250 g since an average cell density of 1 g/ml was assumed for both mammary and liver tissues. Each mammary gland was estimated to secrete 50 ml of milk per hour based upon daily piglet weight gain and milk hand expression data. This translates to a minimum rate of synthesis of approximately 1600 μg rhPC/g mammary parenchyma/day or on order of 1 μg rhPC/10^6 mammary cells/day. The hPC synthesis rate in human liver was calculated to be approximately 0.3 μg hPC/ml/hr based upon an estimated circulation half-life of 7.5 hours (6) and an average steady-state hPC plasma concentration of 4 μg/ml. A ratio of 8.7 l of plasma per 1 kg of liver secretory parenchyma was calculated from the following data. There is 0.083 l blood per kg body weight, 45% hematocrit, 2.5 kg liver per 100 kg body weight, and 78% of liver volume is composed of secretory parenchymal cells (35-37). Using these data, the liver was determined to synthesize hPC at a mass- and time-averaged rate of approximately 20 μg/g liver parenchyma/day. We note that there may be zonal differences in protein synthesis for hepatocytes (38) depending upon their location within the acinus. It is likely that a similar phenomenon occurs in mammary epithelium and therefore we believe the method used here to compare rates of rhPC synthesis provides order of magnitude accuracy.

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34. Failure to adequately remove milk from the mammary gland results in progressive involution of the parenchyma such that secretory cells become congested with product, reabsorption occurs, some cells are lost, and remaining alveolar cells become non-secretory (35). This natural process accelerates during later stages of lactation. Histological evaluation of mammary tissue taken during late lactation (day 55) from either control animals or animal 29-2 revealed some localized regions of involuted secretory tissue (Figure 3). However, most alveoli possessed well-differentiated, apparently normal secretory cells.
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51. Preparation of milk samples. Approximately 5 minutes after intramuscular injection of 10-20 international units of oxytocin, 250-500 ml milk was hand expressed from transgenic or control pigs and immediately quenched into an equal volume of prechilled (0 °C) TBS-EDTA buffer (50 mM Tris-HCl, 150 mM NaCl, 200 mM EDTA, pH 6.5). The TBS-EDTA-diluted milk was centrifuged at 15,000 x g for 30 minutes at 4 °C and the resultant (middle) whey phase was separated from the (light) fat phase and the (heavy) casein phase. Most casein micelles were solubilized by the presence of EDTA (52). The whey phase was stored at -90 °C.
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to 6.5-7.0 with 5 M NaOH solution. The total protein concentration was adjusted to 10 mg/ml or less with TBS- 25 mM $CaCl_2$ (pH 6.5). The diluted whey (0.05 mg hPC antigen/ mg HPC4-Mab/ ml Affiprep) was loaded batchwise for 4 hours at 4 °C. The immunosorbent gel was settled by centrifugation for 5 minutes at 1000 xg and loaded into a 1x10 cm low pressure chromatography column (Pharmacia). After washing with TBS-$CaCl_2$ buffer, the columns were eluted with TBS-25 mM EDTA (pH 6.5) at a flow rate of 30 ml/hr. The column was regenerated with 2 M sodium thiocyanate and equilibrated with the TBS- 25 mM $CaCl_2$ wash buffer after each use.

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List of Figures and Tables

Detailed Figure and Table Captions.

Figure 1. Schematic of hPC structure, adapted from references 4, 8, and 26.

Figure 2. Human Protein C antigen levels detected in the milk of transgenic swine as a function of lactation day. ELISA screening of pig whey was done using the same monoclonal antibody (HPC4 Mab) used to purify rhPC (51). Control and transgenic whey samples were diluted to an OD_{280} of 0.5 with TBS-PEG-$CaCl_2$ (1 mg/ml Polyethylene glycol (25K), 25 mM $CaCl_2$, pH 6.5). Immunopurified hPC was doped into control milk whey and used as reference. Samples (50 μl) were pipetted into microtiter plates which were coated with HPC4 Mab. After washing with TBS-TWEEN-$CaCl_2$ (0.05 % Tween-80, 25 mM $CaCl_2$, pH 6.5), rabbit polyclonal antibody to human protein C (100 μl, 1:1000 dilution in TBS-PEG-Ca^{2+}) was added to each sample well and incubated for 3 hours at room temperature. Goat, anti-rabbit immunoglobulin G conjugated to horseradish peroxidase was added to each well, followed by the chromophoric substrate o-phenyldiamine. Immunopurified rhPC products from transgenic pigs 29-2 and 83-1 (52) were similarly assayed.

Figure 3. Histological cross-sections of transgenic and control pig mammary tissue. Tissue sections were stained with Azure II. Mammary gland biopsies were taken from founder pig 29-2 on day 55 of lactation (panel A), and control pig on day 35 of lactation

(panel B). Several alveoli, some with accumulated secretions, are present in both transgenic and control pig mammary tissues. The secretory cells are highly polarized with rounded basally displaced nuclei, darkly stained lateral/basal cytoplasm, and lacy appearing apical cytoplasm. These characteristics are all indicative of normal, actively-secreting cells (35). Photographs were taken at 250 X magnification; the total magnification incuding photograph enlargement is 800 X.

Figure 4a, 4b. SDS-PAGE analysis of the immunopurification of recombinant human Protein C from the milk of transgenic swine. Immunopurified rhPC obtained from the days of lactation with the highest expression levels for transgenic pigs 29-2 (see below) and 83-1 (data not shown) were run on 0.01% SDS-polyacrylamide (10% acrylamide) gel electrophoresis under non-reduced (panel 4a) and reduced (panel 4b) conditions according to the method of Laemmli (55). The analysis was done using milk from transgenic pig 29-2 obtained on day 18 of lactation. Molecular weight markers (kD) are indicated on the left side. **Lane 1:** Starting material prepared as detailed in (53). **Lane 2:** Fall-through fraction from the immunoaffinity chromatography (53) of starting material shown in Lane 1. **Lane 3:** Immunopurified porcine rhPC. **Lane 4:** hPC immunopurified from human plasma.

Figure 5a, 5b. Western analysis of immunopurified porcine recombinant human Protein C. Immunopurified rhPC obtained from the days of lactation with the highest expression

levels for transgenic pigs 29-2 and 83-1 were run on 0.01% SDS-polyacrylamide (10% acrylamide) gel electrophoresis under non-reduced (panel 5a) and reduced (panel 5b) conditions according to the method of Laemmli (55). Proteins were transferred electrophoretically to a nitrocellulose membrane (Bio-Rad, Melville, NY) at 4°C for 15 hours at 200 mA (56). After blocking with 0.5% BSA in TBS, the membrane was incubated with a rabbit anti-human Protein C polyclonal antisera (American Bioproducts Inc., Parsippany, NJ) for 3 hours at room temperature. Bound Protein C was detected with an anti-rabbit immunoglobulin G conjugated to horseradish peroxidase using the chromophoric substrate 4-chloro-1-naphthol (Sigma, St.Louis, MO). Molecular weight markers (kD) are indicated on the left side. The positions corresponding to α-, β-, and γ- forms of the heavy chain (HC), along with the light chain (LC), are indicated on the right side. **Lane 1:** hPC immunopurified from human plasma. **Lane 2:** rhPC immunopurified from day-52 milk of transgenic pig 83-1. **Lane 3:** hPC reference from human plasma. **Lane 4:** rhPC immunopurified from day-18 milk of transgenic pig 29-2. **Lane 5:** hPC reference from human plasma.

Figure 6. Activated Partial Thromboplastin Time (APTT) of porcine recombinant human Protein C. Dilutions of Normal Plasma Reference pool (NPRP) (American Bioproducts Inc., Parsippany, NJ) were made in Protein C depleted plasma (dhPC). NPRP was assumed to have 1 unit (U) of anticoagulant activity/ml and 4 μg hPC/ml. Thus, a theoretical specific anticoagulant activity here is defined as 250 U/mg. Sample dilutions

were made in 50 mM imidazole, 100 mM NaCl, pH 7.3, and used as reference for estimating the specific activity of immunopurified rhPC and hPC. Samples of immunopurified rhPC and hPC were diluted in dhPC. Identical stock solutions of APTT reagent (American Bioproducts) were prepared with and without 0.5 U/ml of snake venom activator (Protac™; American Diagnostics Inc., Greenwich, CT). Protac™ is an hPC-specific activator isolated from Agkistrodon Contortrix venom (33). Two sets of replicate samples of NPRP, hPC, and rhPC prepared in dhPC were combined with APTT reagent; one set with, and the other without Protac™. Protac™-specific anticoagulant activities were measured by the difference in APTT between sets with and without Protac™ (38) using an Electra 750A coagulation timer. The APTT is plotted as a function of hPC antigen content. Specific anticoagulant activities (U/mg hPC antigen ± SE) calculated using these APTT-hPC Ag curves are shown in the ( ). **Line 1-** Normal plasma reference pool (250, by definition). **Line 2-** Immunopurified hPC from Cohn Fraction IV-I paste (7) (196 ± 8). **Line 3-** rhPC from day-52 milk of transgenic pig 83-1 (183 ± 9). **Line 4-** rhPC from day-18 milk of transgenic pig 29-2 (154 ± 6). **Line 5-** rhPC from day-55 milk of transgenic pig 29-2 (136 ± 8). Essentially no anticoagulant activity was detected for samples not containing Protac™.

Figure 7. Amidolytic activity of porcine recombinant human Protein C. Protac™-specific amidolytic activity assays using the small synthetic substrate S-2366 (Kabi Diagnostics, Franklin, OH) were performed as described in reference 33. Absolute

amidolytic activity is plotted as a function of hPC antigen concentration. Specific amidolytic activities (U/mg hPC antigen ± SE) were calculated from the slopes of these curves and are given in the ( ). **Line 1-** rhPC from day-18 milk of transgenic pig 29-2 (252 ± 22). **Line 2-** Normal plasma reference pool (250, by definition). **Line 3-** Immunopurified hPC from Cohn Fraction IV-I paste (7) (223 ± 11). **Line 4-** rhPC from day-52 milk of transgenic pig 83-1 (68 ± 9). **Line 5-** rhPC from day-55 milk of transgenic pig 29-2 (45 ± 3).

Table 1. Carbohydrate content of porcine recombinant human Protein C. Reducing sugar analyses were performed by the method of Morcol and Velander (43). Warren's thiobarbituric acid assay (50) was modified for the analysis of total sialic acid in plasma-derived hPC reference (American Red Cross, Holland Labs., Rockville, MD) and immunopurified rhPC samples from transgenic pigs 29-2 and 83-1. The modified procedure is as follows. Pooled whey samples for transgenic pigs were immunopurified and antigen levels were assayed by ELISA (see Figure 2 legend). Immunopurifed products were lyophilized under reduced pressure of 0.2 torr at -100 °C and reconstituted to the desired concentrations with 10 mM sodium acetate (pH 5.0) and incubated at 37 °C for 3 hours in the presence of 0.05 units of neuraminidase (derived from *vibrio cholerae*; Sigma, St.Louis, MO). Sialic acid reference (Sigma) dilutions were prepared identically. The periodate oxidation reaction was performed in thiobarbituric acid and the resultant colored reaction product was then extracted into cyclohexanone as explained

by Warren (50). Each cyclohexanone extract was applied onto a High Performance Thin Layer Chromatography (HPTLC) plate (Whatman LH-K 20 x 10 cm; Alltec Associates, Deerfield, IL) just above the preadsorbent strip. Samples were allowed to migrate to about 3 cm above the application line in pyridine/n-butanol/water solvent system (43). The plate was then dried and scanned at 529 nm in the diffuse-reflectance mode using a Shimadzu 9000 densitometric scanner. Molar ratios for both reducing sugars and sialic acid for hPC and porcine rhPC are reported. These data are listed along with the reducing carbohydrate and sialic acid composition reported for 293 kidney cells and hPC from plasma (43).

TABLE 1

Sugar	rhPC# (transgenic pig) mol/mol	rhPC* (293 cell) mol/mol	hPC** (plasma) mol/mol
Fucose	6.1±0.4	4.6	1.9
Galactosamine	3.3±0.5	2.6	0.0
Glucosamine	8.0±0.6	13.0	11.0
Galactose	2.9±0.1	6.1	5.4
Mannose	9.9±1.1	8.8	7.9
Sialic acid	5.3±0.6	5.1	5.1±0.4
Total Carbohydrate wt%	12.8	13.4	10.7

(#) Average carbohydrate composition for pig 83-1 and 29-2 from different days of lactation.

(*) Reference # 32.

(**) Reference # 43. Total carbohydrate wt % was calculated by adding the sialic acid content obtained by the present method explained in the legend.

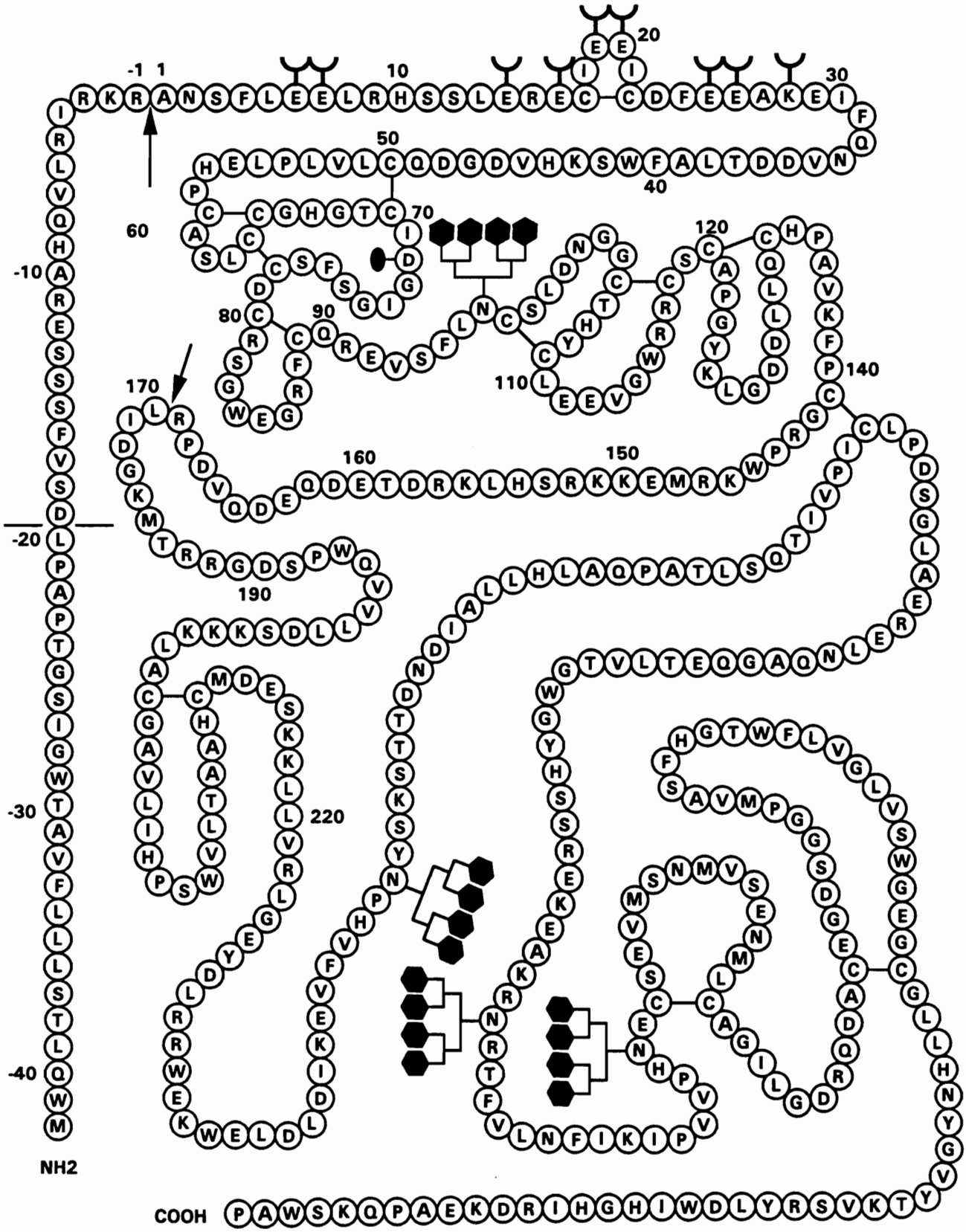


FIGURE 1

SHEMATIC REPRESANTATION OF PROTEIN C MOLECULE

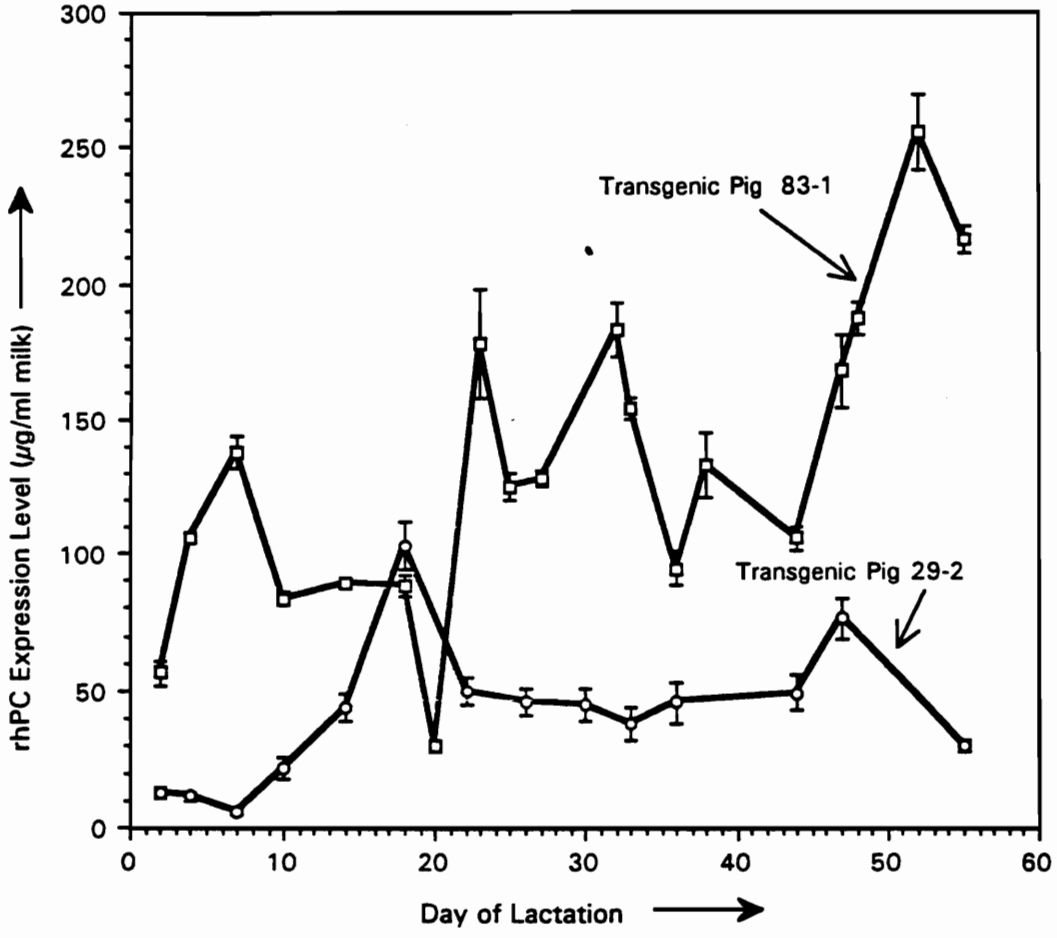
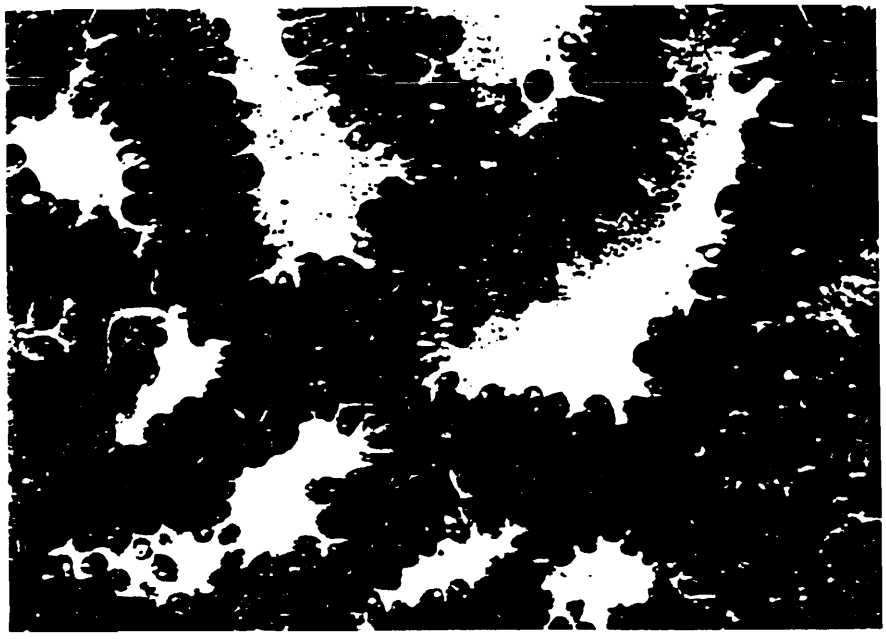

300
250
200
150
100
50
0
rhPC Expression Level (μg/ml milk)
Transgenic Pig 83-1
Transgenic Pig 29-2
0
10
20
30
40
50
60
Day of Lactation

FIGURE 2

(A)



(B)

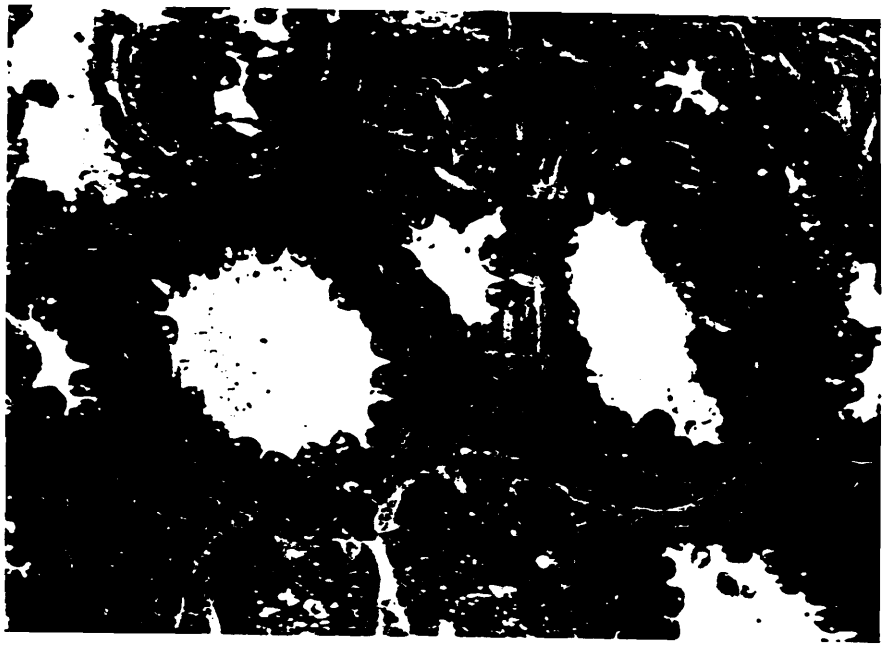


FIGURE 3

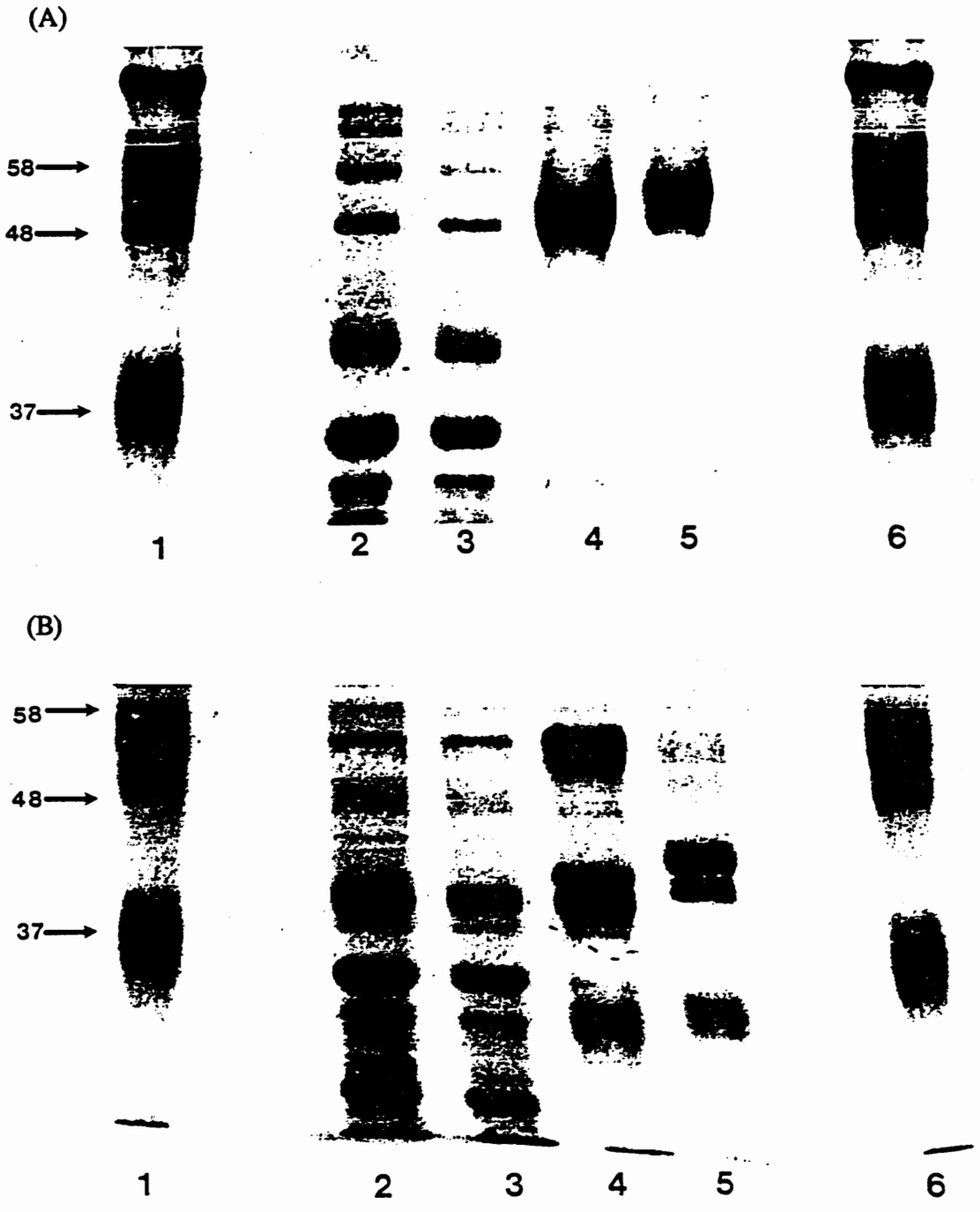
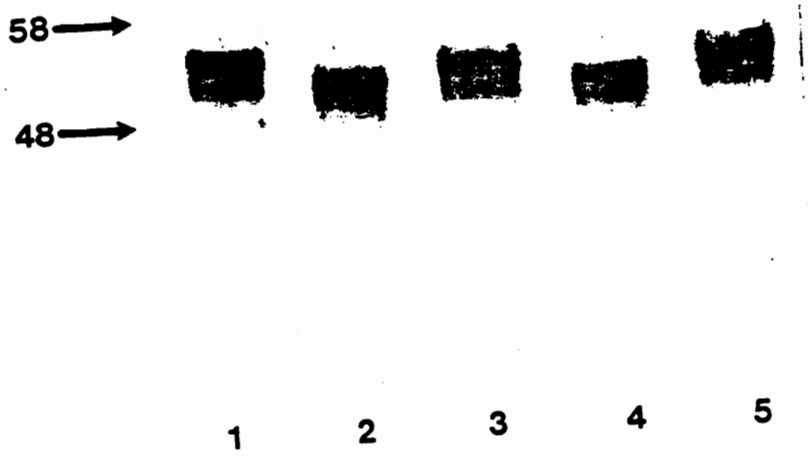


FIGURE 4

(a)



(b)

FIGURE 5

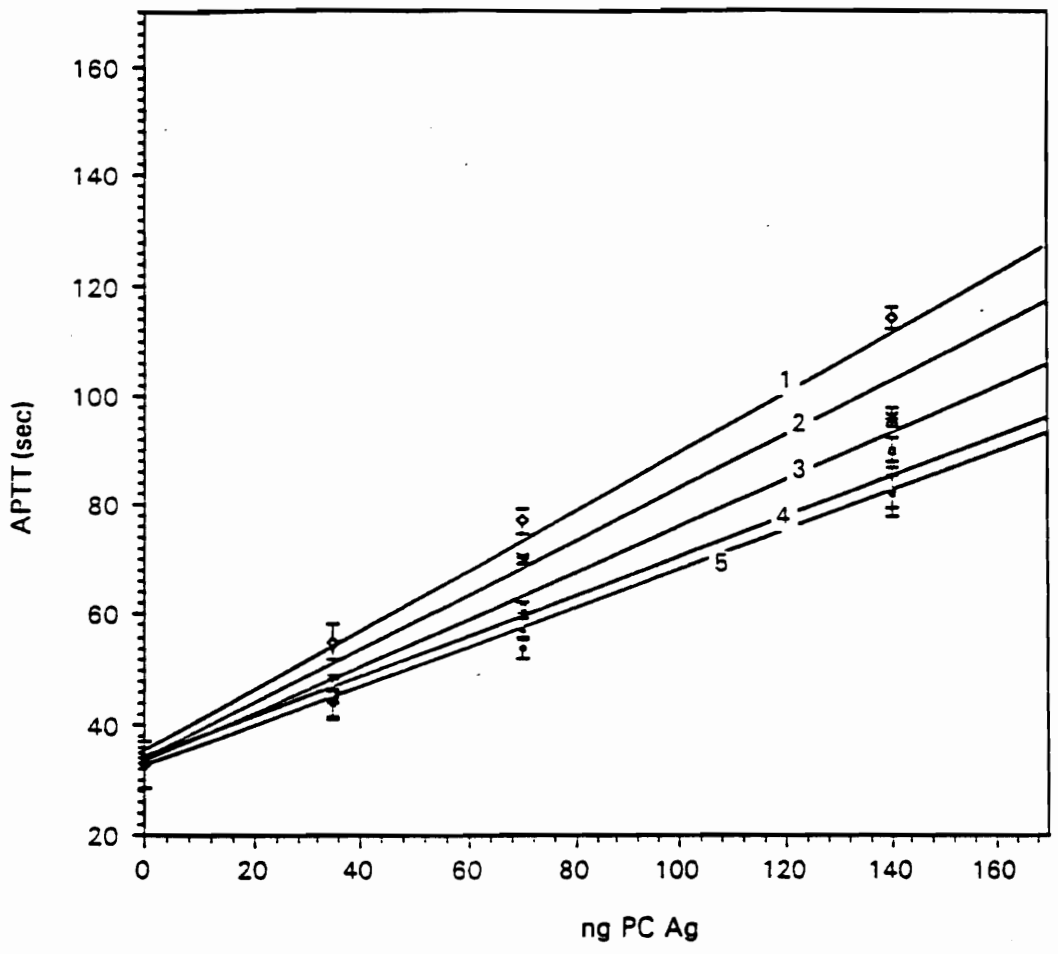

APTT (sec)
160
140
120
100
80
60
40
20
0
20
40
60
80
100
120
140
160
ng PC Ag
1
2
3
4
5

FIGURE 6

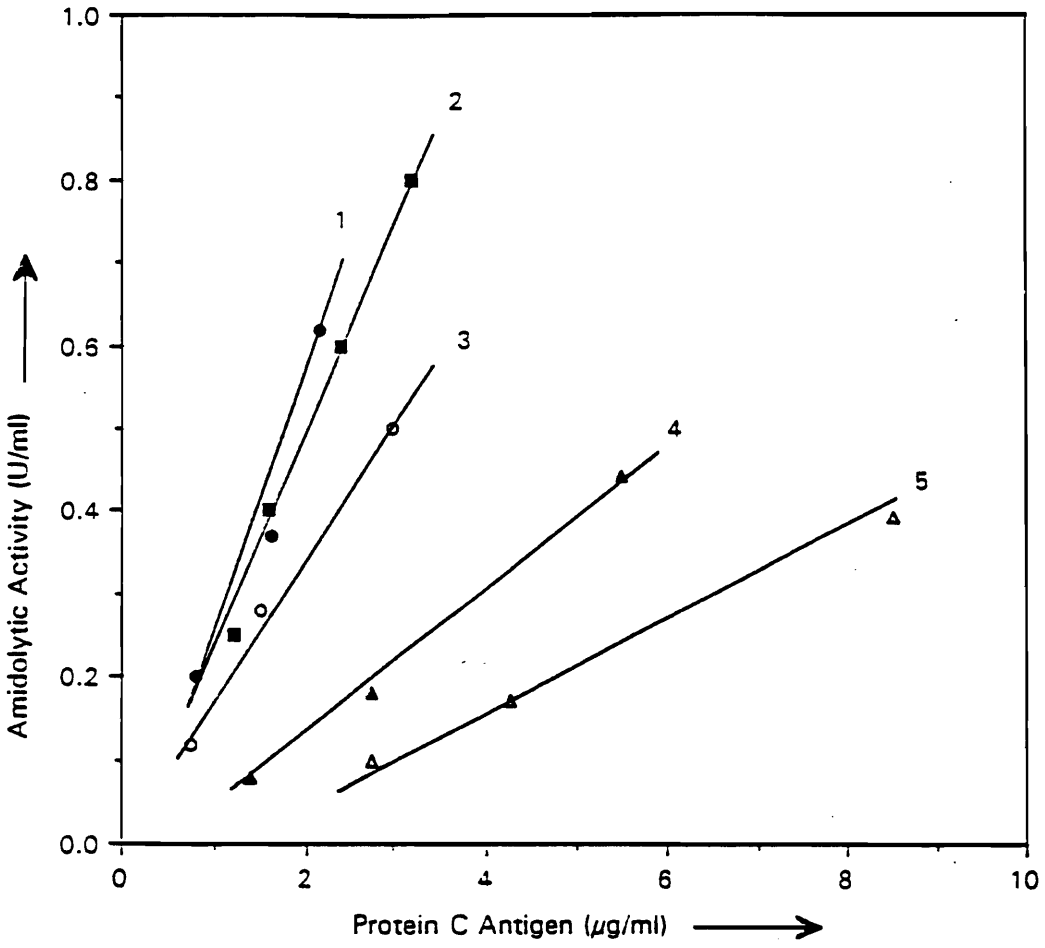

1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
1
2
3
4
5
Amidolytic Activity (U/ml)
Protein C Antigen (μg/ml)

FIGURE 7

CHAPTER SIX

RECOMBINANT HUMAN PROTEIN C FROM THE MILK OF TRANSGENIC SWINE

Evidence of Heterogenous Populations of Recombinant Protein C in the Milk of Transgenic Swine

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ABSTRACT

Mammary tissue specific expression of recombinant human protein C (hPC) was obtained in the milk of transgenic swine using murine whey acid protein-human protein C hybrid construct (WWAPPC1). Previous work has shown that the porcine mammary gland has the ability to perform complex posttranslational modifications at high synthesis rates as judged by the chemical and biochemical characterization of the recombinant hPC product (rhPC) obtained using the immobilized murine anti-hPC monoclonal antibody (HPC4-Mab) immunosorbent. In this work, the presence of a second population of rhPC that was detected in, and immunopurified from the milk of transgenic pig 29-2 using an another conformation-specific metal dependent monoclonal antibody to human protein C (7D7B10-Mab) is discussed in detail. The 7D7B10-Mab binds to the "Gla-domain" of the hPC light chain (LC) in the presence of EDTA. Immunopurified rhPC product from day-18 milk was partially characterized and found to be biologically active. Structural differences were observed between rhPC immunopurified either using the heavy chain (HC)-specific monoclonal antibody HPC4-Mab or LC-specific monoclonal antibody 7D7B10-Mab.

INTRODUCTION

The vitamin K- dependent zymogen of a serine protease, human protein C is a potent anticoagulant and plays a central role in the regulation of natural coagulation process. Several posttranslational modifications occur during its biosynthesis in the liver

such as; removal of signal and pro-peptide sequences, removal of Lys^{156}-Arg^{157} dipeptide generating the two chain protein C molecule (1), γ-carboxylation of 9 glutamic acid residues in the NH2-terminal of the light chain to γ-carboxyglutamate (Gla) residues (2-4), β-hydroxylation of Aspartic acid 71 (5), and N-glycosylation of aspargine residues at positions 97, 248, 313, and 329 (4, 6). Protein C is activated on the endothelial cell surface by thrombin-thrombodulin complex (7-9) as a result ofproteolytic cleavage at Arg^{169}-Leu^{170}. Both zymogen and the activated forms of protein C are capable of Ca^{2+}-dependent membrane binding (10).

Because of the extent and the complexities of these posttranslational modifications, biosynthesis of the protein in the mammary gland of large transgenic animals and secretion into the milk appears to be the only promising alternative for the large-scale production of biologically active recombinant protein C (rhPC) (see Chapter 5). The mammary gland produces several posttranslationally modified milk proteins at high concentrations (11-13). Therefore, it already has the necessary enzymatic machinery to process transgenic proteins with similar modifications. Mammary specific expression of rhPC has been obtained in the milk of transgenic pig 29-2 in our previous work using a murine whey Acidic protein-hPC (WAPPC1) genetic construct. The Ca^{2+} dependent monoclonal antibody (HPC4) (14) to human protein C (hPC) was used to immunopurify the immunopurification of the recombinant product. In this work, a second population of rhPC is described. This rhPC population was isolated using another metal dependent

monoclonal antibody (15, 16) (7D7B10) that binds to hPC from the epitope at the first 15 residues in the amino-terminal of light chain (see Fig. 1).

MATERIALS AND METHODS

Pig Milk Preparation and Analysis of Recombinant Protein C

Light Chain-capture ELISA

Whey fraction was prepared from the milk of transgenic pig 29-2 using an EDTA-precipitation method (see Fig. 2), (see Chapter 3). This whey was screened by an sandwich enzyme-linked-immunosorbent assay (ELISA) that was originally designed to capture the recombinant human protein C from the milk of transgenic mice (17). In this assay microttiter plates (Immulon II, Dynatec Laboratories) were coated with 100 μl of the murine monoclonal anti-human protein C (7D7B10) at a concentration of 5 μg/ml in 0.1 M sodium bicarbonate pH 9.3 overnight at 4°C. The wells were than washed with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 6.5), 0.05 % Tween-80, 25 mM EDTA, pH 6.5. The plates stored at 4°C were active over a period of 4 months. Daily whey samples collected through the lactation cycle of about 4 weeks were diluted to 0.5 mg/ml in TBS, 1 mg/ml PEG (25K), 25 mM EDTA, pH 6.5 (TBS-PEG-EDTA) and were added to the coated plates and incubated for 3 hours at room temperature. Immunopurified hPC reference was doped into identically prepared control milk whey and used for the assay's standard curve. Wells were then aspirated and washed 4 times followed by incubation of 1/1000 rabbit polyclonal antibody to human protein C (Assera C; American

Bioproducts). After an incubation time of 1 hour at 37°C wells were washed and bound protein C antigen was detected with goat, anti-rabbit IgG peroxidase-conjugate.

Polyclonal-capture ELISA

In a variation of the sandwich ELISA, microtitter plates were coated with Assera C polyclonal antibody (1/200 dilution in 0.1 M sodium bicarbonate, ph 9.3) and the whey samples prepared according to the procedure above were added. Assera C-rhPC complex was captured either with the murine monoclonal antibody 7D7B10 in the presence of EDTA or with rabbit monoclonal antibody HPC4 to human protein C in the presence of Ca^{2+}. Bound rhPC was detected either with goat, anti-mouse IgG or anti-rabbit IgG peroxidase conjugate.

Immunoaffinity Recovery of rhPC

Selection criteria for metal dependent Mabs as affinity immunosorbents for VKD proteins include their ability to operate under gentle elution conditions (16). Bound protein elution from the immunosorbents does not require harsh chemical agents or harsh elution conditions which could affect the biological activity of the final product and complicate the entire protein purification process especially for large-scale applications. We have discussed this point in detail in Chapter 2 (18).

The monoclonal antibody 7D7B10 (that binds to human protein C in the presence of EDTA) was immobilized on Affiprep-10 (Bio-Rad) at a concentration of 1-2 mg

Mab/ml gel and used as the immunosorbent for the immunopurification of rhPC from the milk of transgenic pig 29-2. Whey fraction obtained by EDTA "casein-precipitation" (see Fig. 2) stored in -90°C freezer, was defrosted at 4°C in the presence of protease inhibitors (1 mM DFP, 10 mM benzemidine, 100 ug/ml trypsin inhibitor). Whey samples containing inhibitors were diluted to 15 mg total protein/ml or less using TBS to a final EDTA concentration of 25 mM and batch-loaded onto 7D7B10/Affiprep column for 4 hours at 4°C. Following the column wash with TBS, 25 mM EDTA, pH 6.5, rhPC bound to the column was specifically eluted with TBS, 25 mM $CaCl_2$, pH 6.5 buffer at 0.5 ml/min. An additional 2 M $NaSCN^-$ wash step was employed to clean the column from the proteins nonspecifically bound to the immunosorbent. Both the Ca^{2+} eluted product peak and the SCN^- wash fraction were dialyzed against nano-pure deionized H_2O and lyophilized at -50°C under reduced pressure of 0.2 torr.

The column washes (fall through), Ca^{2+} eluates, and SCN^- fractions were assayed for rhPC antigen contents by LC-capture ELISA using the same 7D7B10 Mab, and data was used for calculating the product yields of the immunopurifications performed.

SDS-PAGE and Western Blot Analysis.

Immunopurified products from days 2, 7, 10, 18, and 55 of lactation were analyzed for their apparent molecular weights and heterogeneity on vertical slab gels (14 x 10 x 1.5 cm) using 10 % resolving polyacrylamide gel and 4 % stacking

polyacrylamide gel containing 0.1 % SDS under reduced and non-reduced conditions using the method developed by Laemmli (19). Immunopurified product from day-18 milk using the Mab-7D7B10 immunosorbent was compared with that immunocaptured by HPC4-Mab on 10 % SDS-PAGE under reduced and non-reduced conditions. Gels containing identical samples were either stained with Coomassie Blue R-250 or electrophoretically transferred to nitrocellulose in a Trans-Blot apparatus (Bio-Rad) at a constant current of 200 mA for 15-17 hours in 25 mM Tris, 190 mM glycine, 20 % methanol buffer (20) in cold room. The nitrocellulose membranes were blocked with 0.5 % bovine serum albumin (BSA) in TBS for 1 hour at room temperature and incubated in 1/1000 dilution of rabbit, anti-human protein C polyclonal antibody (Assera C). Membranes were washed with TBS for 1 hour, changing buffer 3 times and then incubated with 1/1000 dilution of goat, peroxidase conjugated anti-rabbit immunoglobulin G (IgG) for 3 hours at room temperature. Following washing with TBS, blots were developed using 0.5 mg/ml 4-chloro-1-naphthol in 1:12 (v/v) methanol-TBS including 0.1 % H_2O_2.

Protein C Activity Assays

The amidolytic and anticoagulant activities of rhPC immunopurified from day-18 milk of transgenic pig 29-2 using 7D7B10-Mab immunosorbent were determined as follows and compared either with plasma-derived hPC reference or the rhPC purified using the HPC4-Mab immunosorbent.

The amidolytic activity was performed according to the method of Odegaard et al. (21). Normal plasma reference pool (NPRP) dilutions were prepared in protein C depleted (dhPC) plasma by assuming 4 μg/ml and 1 U/ml protein C was present in NPRP. Both immunopurified rhPC and hPC dilutions were also prepared in dhPC. Protein C antigen present was specifically activated with *Agkistrodon contortix* snake venom (Protac™) (22,23). After an incubation time of 4 minutes the reaction was started with chromogenic substrate S-2366. The difference in OD_{410} (Optical Density at 410 nm)/min was recorded.

For the determination of Protac™ specific anticoagulant activities, sample and NPRP dilutions were prepared in dhPC plasma. Protac was prepared in deionized water (0.25 units/ml) and added to the APTT reagent (American Bioproducts). Purified samples (100 μl) or NPRP dilutions were combined with 100 μl APTT reagent in prewarmed polystyrene tubes and after an incubation of 5 minutes at 37°C, 100 μl of 25 mM $CaCl_2$ was added. The prolongation of the activated partial thromboplastin times (APTT) were recorded using Electra 750A coagulation timer.

RESULTS

Expression of rhPC in the Mammary Gland

The rhPC secretion levels obtained in the milk of the founder transgenic pig 29-2 averaged at a concentration of approximately 10 μg/ml milk during the 55 days of

lactation period as measured by the LC-capture ELISA. In contrast, higher rhPC antigen levels were detected by either the HC-capture or polyclonal-capture ELISA assays (see Fig. 3) predicting the presence of heterogenous populations. A minimum of 75 % product yields were obtained for prufications using 7D7B10-Mab and thus yields were in good agreement with the antigen levels predicted by ELISA (see Table 1).

Proteolytic Processing in the Mammary Gland

The immunopurified products obtained from different days of lactation migrated faster than the plasma-derived hPC reference on SDS-PAGE by about 3 kDa under non-reduced conditions as judged either by Coomassie Blue stained gels (see Fig. 4, Panel A) or Western blot analysis (data not shown). The processing of the rhPC products into 2-chain heterodimer with three sub-units of heavy chain (α, β, and γ HC) appeared to be similar to that of hPC reference (see Fig.4, Panel B), although rhPC contained higher single chain contant than hPC. The ratio of the single chain to two chain forms evaluated from the densitometric scans of Coomassie Blue gels are presented in Table 2.

There were considerable differences in terms of the dipeptide removal rates of recombinant hPC molecule during lactation. In contrast to a stable 50 % single chain formation of the rhPC population immunocaptured with the HC-specific immunosorbent (Chapter 5), the 7D7B10-Mab captured population contained about 30 %-45 % single

chain form.

A comparison of the two different rhPC populations obtained from the day-18 milk of transgenic pig 29-2 on Coomassie Blue stained 10 % SDS-PAGE and identical western blot is shown in Figure 5 and Figure 6. Under non-reduced conditions 7D7B10-Mab product migrated as a doublet similar to hPC reference while a triplet was observed for the HPC4-Mab rhPC product. In addition, the light chain of 7D7B10-Mab captured population migrated faster than both the light chains of plasma-derived hPC reference or HPC4-captured rhPC. The ratios of α, β, and γ forms of heavy chain were similar to that of HPC4-captured population.

Biological Activity

The specific amidolytic and anticoagulant activities of the rhPC produced from the milk of transgenic pig 29-2 using 7D7B10-Mab immunosorbent were approximately 50 % higher than the theoretical specific activity (defined as 250 U/mg the native plasma-derived hPC). Although the rhPC produced from 29-2 on the same day of lactation using HPC4-Mab immunosorbent showed similar amidolytic activity as the 7D7B10 captured population, its anticoagulant activity was 20 % less than the theoretical activity (see Table. 3).

DISCUSSION

Data obtained for the rhPC products immunopurified from the milk of transgenic pig 29-2 by using 7D7B10-Mab/Affiprep immunosorbent suggest several sources of differences. In vivo removal of the dipeptide to form a two chain molecule in mammary gland can occur at higher rates at the beginning of the lactation than the later days in the cycle. In the first week of lactation, mammary gland can more efficiently perform this post-translational modification. In addition, the apparent lower molecular weight of rhPC when compared to the plasma-derived hPC indicates that the proteolytic cleavages necessary to remove the signal and propeptide sequences were probably made (Chapter 5). Although the porcine mammary gland may be performing N-glycosylation differently than the human liver, the presence of three HC forms indicates a similar presence of at least three glycosylation sites on the rhPC-heavy chain as is found in hPC. Thus, the triplet formation on rhPC HC may represent the analogous mono-, di-,and tri-glycosylated forms (24). The amidolytic and anticoagulant activity obtained for rhPC derived from day-18 milk indicates that both the catalytic and the activation site are intact in both population. Although synthesis rates appeared to be stable on different days of lactation, later days in lactation possessed higher % single chain form in the rhPC population isolated using the LC-specific 7D7B10-Mab. More interestingly, the population isolated using the HC-specific HPC4-Mab has consistently produced about 50 % single chain form regardless of synthesis rates which varied widely over the 55 days

of lactation. These findings strengthen the hypothesis that dipeptide removal is controlled by more complex set of events than a simple proteolytic rate limitation kinetics (see Chapter 5).

ABBREVIATIONS

VKD	vitamin K-dependent proteins
hPC	human protein C
APC	activated protein C
rhPC	recombinant human protein C
WAPPC1	whey acid protein-human protein C construct
Mab	monoclonal antibody
HC	heavy chain
LC	light chain
Gla	gamma-carboxyglutamic acid
SDS	sodium dodecyl sulphate
kDa	kilo dalton
ELISA	enzyme-linked-immunosorbent assay
PAGE	polyacrylamide grl electrophoresis
BSA	bovine serium albumun
IgG	immunoglobulin G
TBS	tris buffered saline
DFP	diisopropyl-fluorophosphate
dhPC	protein C depleted plasma
NPRP	normal plasma reference pool
APTT	activated partial thromboplastin time

LIST OF FIGURES AND TABLES

FIGURES

TABLES

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TABLE 1

Summary of rhPC Immunopurification Data From The Milk of transgenic Pig 29-2 (7D7B10-Mab / Affiprep column)

Day of Lactation	μg rhPC Ag/ml milk (ELISA)			% total yield* of immunopurifications		% Population measured by ELISA #			
	LC (7D7B10)	HC (HPC4)	P.Clonal (Asseara C)	7D7B10	HPC4	In the milk 7D7B10	In the milk HPC4	Immunopurified * 7D7B10	Immunopurified * HPC4
2	8.5	12.6	13.5	76	80	63	93	49	75
4	7.0	11.9	10.0	77	83	70	~100	54	83
7	3.8	6.0	8.9	75	78	43	67	32	52
10	12.0	21.5	24.0	86	84	50	90	43	76
18	15.0	103.0	77.4	98	80	19	~100	19	~100
33	6.2	38.1	54.0	79	82	11	63	9	52
55	3.3	29.7	16.8	78	35	20	~100	16	62

(*) Immunopurifications using either 7D7B10-Mab/Affiprep or HPC4-Mab/Affiprep immunoaffinity column. Yields are based upon rhPC eluted from the columns measured by respective ELISA.

(#) % population that was captured either by 7D7B10-Mab or HPC4-Mab. We assume that polyclonal capture ELISA results represents the entire population.

TABLE 2

Summary of the Densitometric Scan of 10 % PAGE* (reduced) of Immunopurified rPC** from Transgenic Pig (29-2) whey

Day of Lactation	% Single chain	% Heavy chain (α+ β+ γ)	% Light chain
2	26	51	23
4	26	51	23
7	26	51	23
10	48	39	13
14	45	40	15
18	43	41	16
26	42	42	16
33	42	43	15
55	42	43	15

(*) Coomassie blue stained.

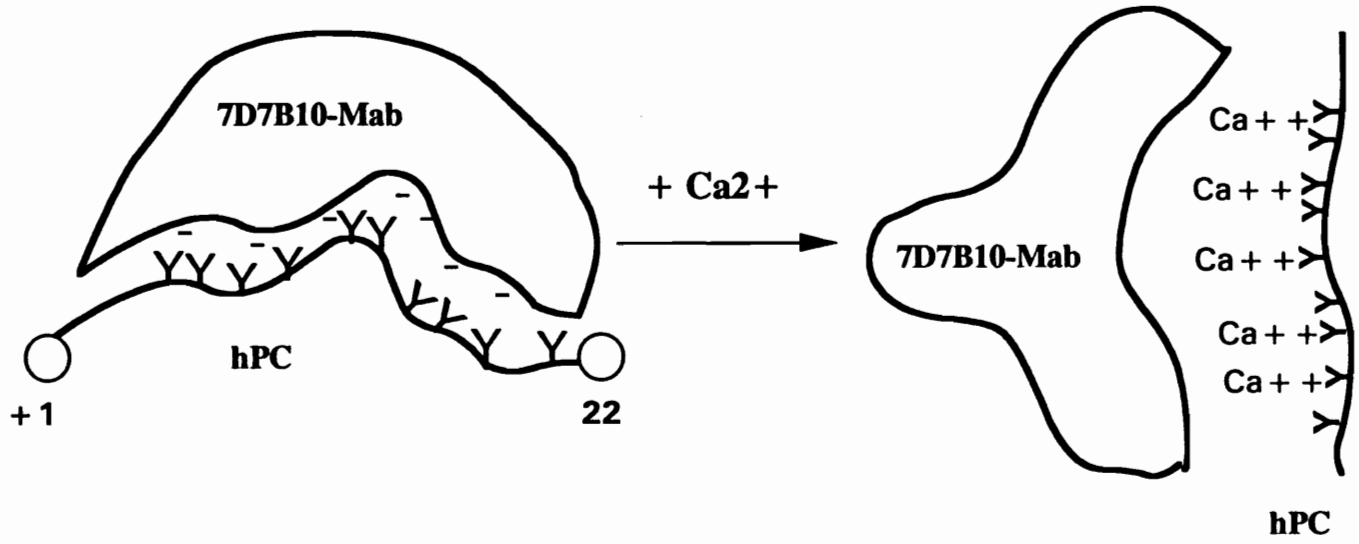
(**) Immunosorbent; 7D7B10-affiprep

TABLE 3

Comparison of immunopurified rPC from day-18 milk of transgenic pig 29-2 from LC and HC capture immunosorbents

Immunopurified PC	% Single chain *	% Heavy chain * α	β	γ	% Light chain*	Anticoagulant Activity** U/mg Ag♥	U/mg T.P.	Amidolytic activity @ U/mg Ag♥	U/mg T.P.	% Ag Yield
rPC from HPC4-affiprep	54	12	13	9	12	193 ± 7	142 ± 4	n.a.	n.a.	~80+
hPC from plasma ¤	21	41	25	2	11	230 ± 5	159 ± 4	295 ± 7	251 ± 4	
rPC from 7D7B10-affiprep	49	16	15	2	16	536 ± 37	415 ± 29	542 ± 10	419 ± 8	~98++
hPC from plasma ¤	21	41	25	2	11	289 ± 8	200 ± 8	295 ± 7	251 ± 4	

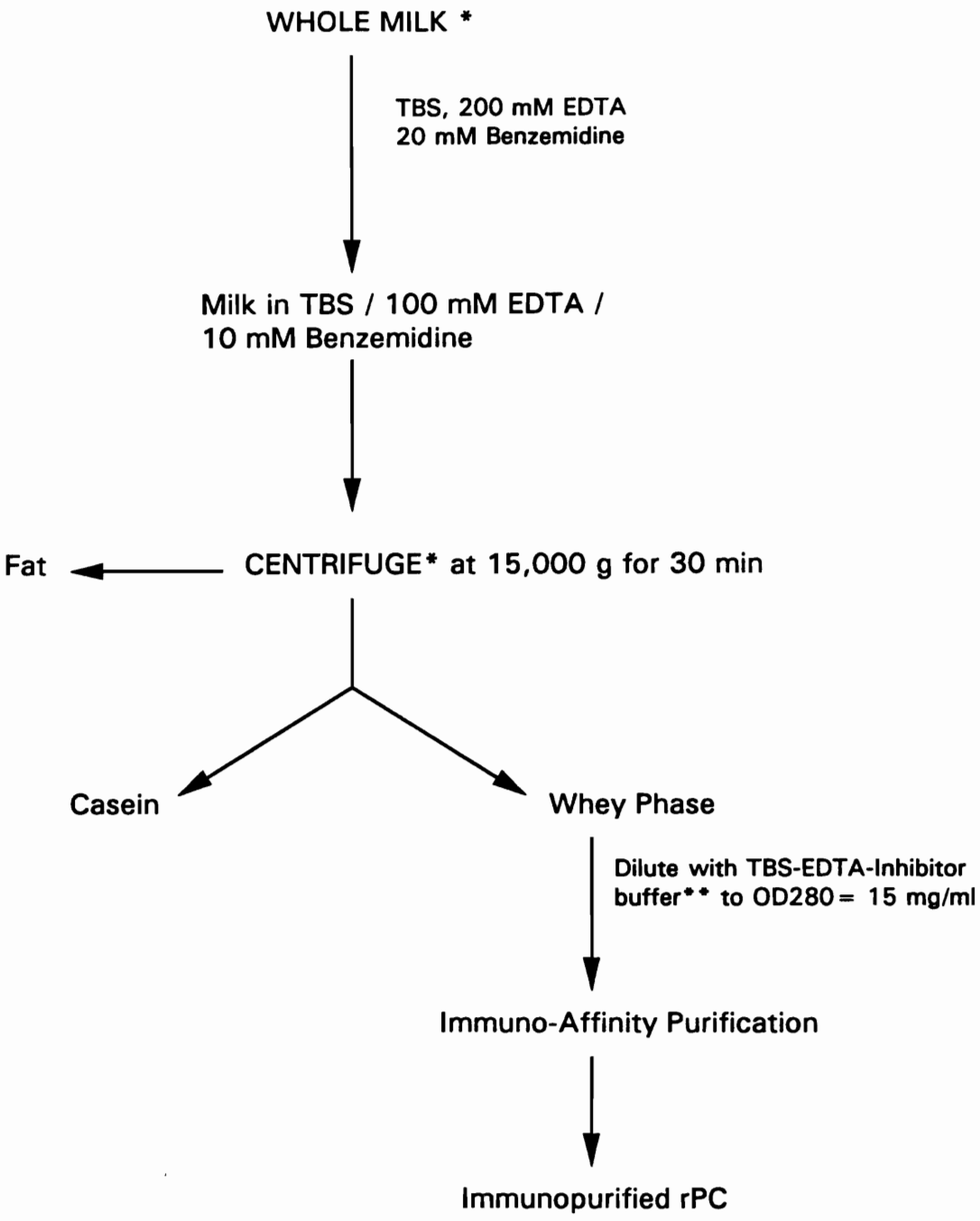
(*) Data from densitometric scan of coomassie-blue stained 10 % PAGE.
(**) Protac-specific APTT test.
(@) Protac-specific. S-2366 chromogenic substrate. Normal plasma pool as reference.
(¤) APTT assays were run at different days.
(T.P.) Total protein based on OD280 nm.
(n.a.) Not assayed yet.
(+) Yield based upon ELISA using HPC4-Mab
(++) Yield based upon ELISA 7D7B10-Mab.
(♥) Primary hPC reference for antigen used in calculating specific activities for APTT and amidolytic assay is immunopurified material (90 %)* from American Red Cross: Lot # 28309018. Theoretical activities based upon 3 μg hPC/ml and 1 U/ml normal plasma pool.



7D7B10-Mab Binding Epitope Is Located In The Gla-Domain
of Protein C Light-Chain

FIGURE 1

7D7B10-Mab Binding to Human Protein C



(*) Whole process was carried out either on ice or in cold room at 4 C

(**) Final concentrations; TBS (50 mM Tris-HCl, 150 mM NaCl), 25 mM EDTA, 1mM DFP, 10 mM benzamidine, 1 mg/ml trypsin inhibitor, pH 6.5.

FIGURE 2

Preparation of milk from transgenic pig 29-2 for the immunoaffinity isolation of rhPC.

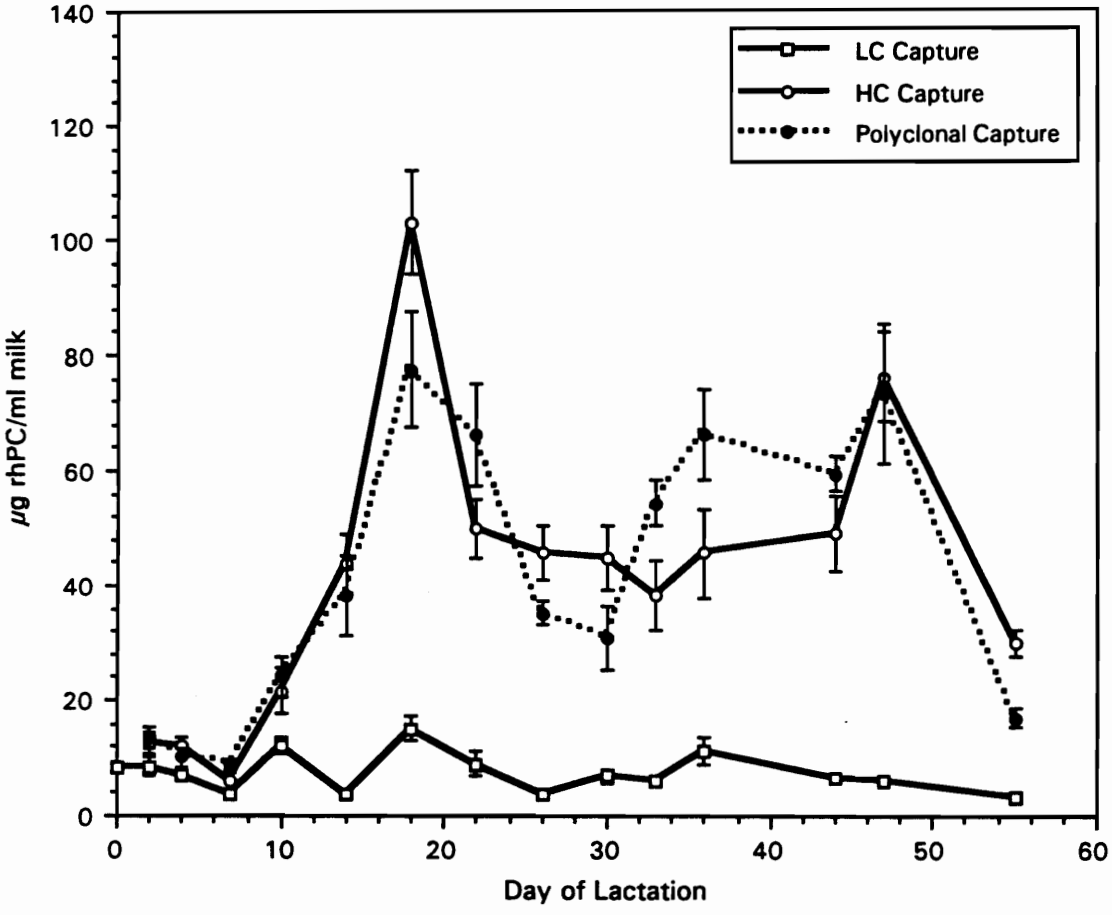


FIGURE 3

Expression of rhPC in the milk of transgenic pig 29-2 measured by HC, LC, and polyclonal capture ELISA

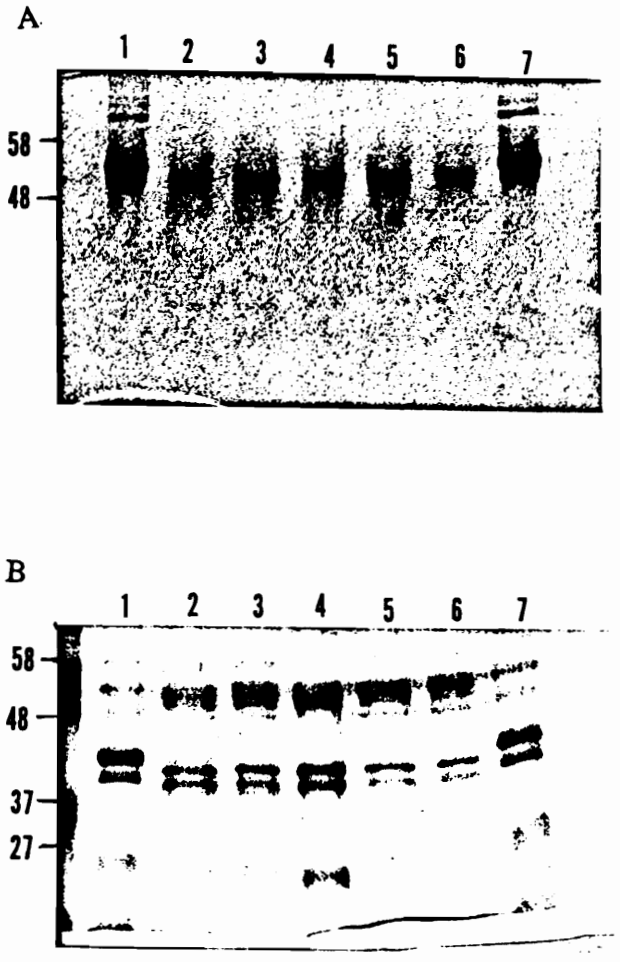


FIGURE 4
COOMASSIE BLUE-STAINED 10 % SDS-PAGE OF IMMUNOPURIFIED rHPC* FROM THE MILK OF TRANSGENIC PIG 29-2

Panel A; Under non-reduced conditions. Panel B; Under reduced conditions. Lane 1; hPC reference from human plasma. Lane 2; rhPC from day-2 milk. Lane 3; rhPC from day-7 milk. Lane 4; rhPC from day-10 milk. Lane 5; rhPC from day-18 milk. Lane 6; rhPC from day-55 milk. Lane 7; hPC reference from human plasma.

(*) 25 mM Ca2+ eluted fractions from 7D7B10-Mab/Affiprep immunoaffinity column.

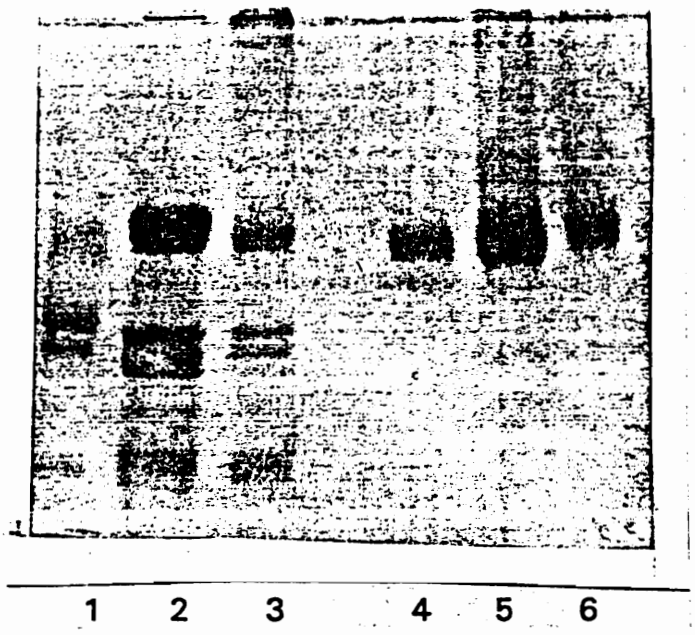


FIGURE 5
SDS-PAGE* ANALYSIS OF rhPC FROM DAY-18 MILK OF TRANSGENIC PIG 29-2

(Comparison of Different Populations)
Lanes 1 to 3; Under reduced conditions. Lanes 4 to 6; Under non-reduced conditions. Lanes 1 and 6; hPC reference from human plasma. Lanes 2 and 5; rhPC from day-18 milk (Immunopurified using HPC4-Mab/Affiprep column). Lanes 3 and 4; rhPC from day-18 milk (Immunopurified using 7D7B10-Mab/Affiprep column).

(*) 10 % SDS-PAGE; Coomassie-Blue stained.

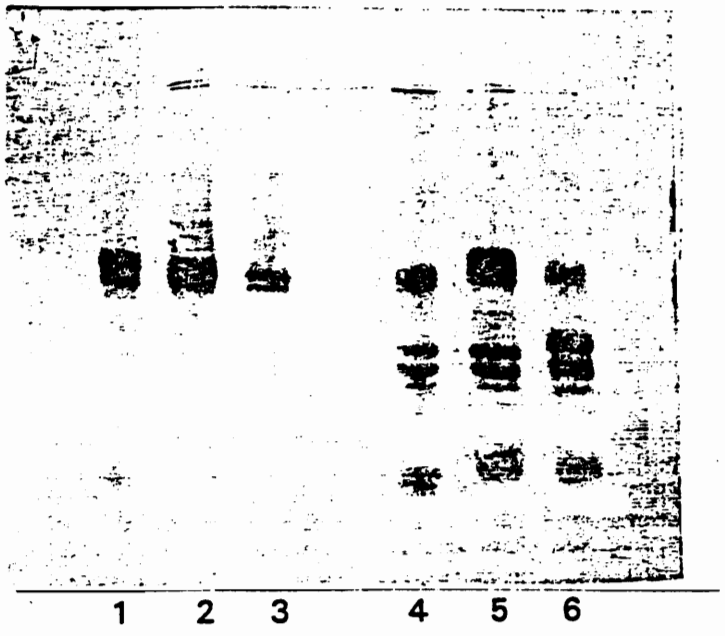


FIGURE 6
WESTERN-BLOT* ANALYSIS OF rhPC FROM DAY-18 MILK OF TRANSGENIC PIG 29-2

(Comparison of Different Populations)
Lanes 1 to 3; Under non-reduced conditions. Lanes 4 to 6; Under reduced conditions. Lanes 1 and 6; hPC reference from human plasma. Lanes 2 and 5; rhPC from day-18 milk (Immunopurified using HPC4-Mab/Affiprep column). Lanes 3 and 4; rhPC from day-18 milk (Immunopurified using 7D7B10-Mab/Affiprep column).

(*) Using 10 % SDS-PAGE.

VITA

Tülin Morçöl was born on August 30, 1955 in Kayseri, Turkey. She received her Bachelors degree in Chemical Engineering from Aegean University, Izmir, Turkey in 1981. She received her Master's degree in Chemical Engineering from Ankara University, Ankara, Turkey in 1985. She worked as a researcher at the Atomic Energy Commission of Turkey between 1982 and 1986. She was invited to Switzerland in 1982 by Hoffman La Roche Company for training in vitamin analysis. She worked as a laboratory specialist and conducted the thermal characterization of polymeric materials at Chemistry Department, Virginia Tech, Blacksburg between 1986 and 1987. She is the daughter of Mr. and Mrs. Uçar, and married with one child.