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PLASMINOGEN POLYMORPHISM IN DAIRY CATTLE

by

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ABSTRACT

M.Sc. Animal Science

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Plasminogen Polymorphism in Dairy Cattle

A genetic approach to lowering protease (plasmin) levels in milk, requires the presence of polymorphism of bovine plasminogen. This study was conducted to determine to what extent genetic polymorphism exists in dairy cattle. Bovine plasminogen was first purified from Holstein cow plasma by affinity chromatography on Lysine-Sepharose and antibodies to bovine plasminogen were raised by monthly intramuscular injection of the isolated bovine plasminogen into rabbits. For plasminogen phenotyping, blood samples were collected at random from 50 Holstein and Ayrshire cattle, and plasminogen was isolated from the plasma using lysine-Sepharose and then treated with neuraminidase. After separation by isoelectric focusing (pH 3.5-9.5) in polyacrylamide gels, Plasminogen polymorphs were detected immunologically using rabbit anti-bovine plasminogen antibodies. Additionally, the plasminogen isoforms were evaluated with a functional assay (caseinolytic overlay technique) after activation of the plasminogen with urokinase. Six plasminogen phenotypes were identified which represent products of 5 variant alleles. The 5 plasminogen variants were characterized based on their isoelectric points and designated PLG A_2 (pI 6.5 and 7.0), B_2 (pI 7.6 and 7.8), C_1 (pI 6.8), D_2 (pI 7.8 and 8.0), and E_2 (pI 6.8 and 7.0). PLG A_2 and PLG B_2 were the most common variants in these cattle. The 6 phenotypes were A_2A_2, B_2B_2 A_2B_2, B_2C_1, A_2D_2 and D_2E_2. The

phenotypic frequencies in Holstein and Ayrshire were very different, A_2A_2 and B_2B_2 being respectively the most frequent phenotype. In addition, DNA polymorphism at bovine plasminogen gene was detected when genomic DNA was digested with the restriction enzyme Msp I and hybridized with mouse plasminogen cDNA. This is the first description of plasminogen polymorphism reported in dairy cattle. If different variants have altered activity, the detrimental effects of milk plasmin activity could be reduced through genetic selection.

RESUME

M.Sc. Animal Science

Wei Wang

Le polymorphisme de la plasminogène chez le bovin laitier

Une approche génétique visant à diminuer l'activité protéolytique de la plasmine dans le lait implique la présence d'un polymorphisme de la plasminogène bovine. Cette étude a été menée dans le but de déterminer si un tel polymorphisme génétique existait chez le bovin laitier. La plasminogène bovine a été purifiée par chromatographie d'affinité sur Lysine-Sepharose, à partir de plasma de vaches de race Holstein, puis injectée à des lapins pour la production d'antisérum anti-plasminogène. L'analyse phénotypique de la plasminogène a été effectuée sur 50 vaches de race Holstein et Ayrshire prises au hasard. Des échantillons de sang furent prélevés et la plasminogène fut isolée du plasma par chromatographie d'affinité sur Lysine-Sepharose puis traitée à la neuraminidase. Après fractionnement par focalisation isoélectrique en gel de polyacrylamide (pH 3.5-9.5), les isoformes de la plasminogène furent détectées immunologiquement, après transfert de type Western des protéines sur nitrocellulose, en utilisant l'antisérum de lapin anti-plasminogène bovine. L'activité protéolytique de ces isoformes fut évaluée grâce à un essai fonctionnel *in situ* après activation de la plasminogène par l'urokinase. Cinq variants génétiques de la plasminogène ont été caractérisés par leur point isoélectrique et désignés PLG A_2(pI

6.5 et 7.0). B_2 (pI 7.6 et 7.8), C_1 (pI 6.8), D_2 (pI 7.8 et 8.0) et E_2 (pI 6.8 et 7.8). PLG A_2 et PLG B_2 étant les variants génétiques les plus fréquents. Au total, six phénotypes différents ont été identifiés: A_2A_2, B_2B_2, A_2B_2, B_2C_1, A_2D_2 et D_2E_2. La fréquence phénotypique est fonction de la race, le phénotype majeur chez les Holstein et les Ayrshire étant respectivement A_2A_2 et B_2B_2. De plus, nous avons pu détecter un polymorphisme au niveau de l'ADN en hybridant les fragments avec l'ADNc du gène de plasminogène de souris. C'est la première étude démontrant la présence d'un polymorphisme de la plasminogène chez le bovin laitier. S'il s'avère que certains variants génétiques ont une activité altérée, l'effet néfaste de la plasmine sur la qualité du lait pourrait être réduite par sélection génétique.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
HRP	Horse Radish Peroxidase
IEF	Isoelectric Focusing
PAGE	Polyacrylamide Gel Electrophoresis
PLG	Plasminogen
RFLP	Restriction Fragment Length Polymorphism
SDS	Sodium Dodecyl Sulphate
SSC	Sodium Chloride-Sodium Citrate
SSPE	Sodium Chloride-Sodium Phosphate, Monobasic-Ethylenediaminetetra Acetic Acid
TBS	Tris-buffered Saline
TCA	Trichloroacetic Acid
TE	Tris-Ethylenediaminetetra Acetic Acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPE	Tris-Phosphate-Ethylenediaminetetra Acetic Acid
TTBS	Tween-Tris-buffered Saline
TZN	Tris-Zinc Sulphate-Sodium Azide

1. GENERAL INTRODUCTION

Plasmin is the major proteolytic enzyme in normal bovine milk of good bacteriological quality. This alkaline serine-protease is produced from plasminogen, an inactive precursor of some 90 kD, which originates principally from blood (Kaminogawa *et al.*, 1972; Eigel *et al.*, 1979; Politis *et al.*, 1990). In freshly drawn milk, the concentration of plasmin is low, around 0.3 μg/ml, while plasminogen is the predominant form, around 1.04 to 1.30 μg/ml (Korycka-Dahl *et al.*, 1983; Richardson, 1983b; Politis *et al.*, 1989). Plasmin and plasminogen are associated with the casein micelle (Humbert and Alais, 1979). Activation of plasminogen by a plasminogen activator begins early during the milk secretion process (Schaar, 1985), and the extent of activation, as determined by the degree of hydrolysis of caseins, is related to several physiological factors such as the age of the cow, stage of lactation, somatic cell count and the season of the year (Politis and Ng-Kwai-Hang, 1989). Genetic factors also affect plasmin activity. For instance, the plasmin concentration in milk of Jersey and Ayrshire cows is typically lower than in the milk of Holstein cows (Richardson, 1983b). These differences arise from the blood plasminogen concentration as well as the extent of activation of milk plasminogen to plasmin.

The main consequence of the presence of plasmin activity in milk is the hydrolysis of caseins. The fact that plasmin activity significantly increases during cold storage, results in an increased amount of proteose-peptone in milk (Schaar, 1985). The

detrimental effects of plasmin proteolysis include: reduced storage time, presence of a bitter taste, loss of cheese yield and quality, problems in milk protein fractionation and impaired physicochemical properties (Grufferty and Fox, 1988).

An effective means of suppressing plasmin activity in milk is not currently available. For example, plasmin is very thermostable. It is largely unaffected by pasteurization conditions and significant activity remains even after ultra high temperature processing. Further, heat treatment may actually increase the net plasmin activity by inactivating certain plasmin inhibitors (Alichanidis *et al.*, 1986; Igarashi, 1990). Our approach is to use genetic selection for plasminogen variants as a means of lowering plasminogen concentration in milk, to decrease the rate of plasminogen activation to plasmin and to decrease plasmin's proteolytic activity.

The objective of this study was to determine to what extent genetic polymorphism of plasminogen existed in dairy cattle. This work represents the first attempt to characterize bovine plasminogen polymorphism at the protein, as well as at the DNA levels. As such it addresses the first stage of a long term project to reduce milk plasmin through genetic means.

2. REVIEW OF LITERATURE

2.1 Biochemistry of human plasminogen

2.1.1 Production and distribution

Plasminogen is the inactive plasma protein precursor of the proteolytic enzyme plasmin which occupies a key position within the fibrinolytic system. Among the various species, human plasminogen is characterized best. It is synthesised by many organs. The production site of this zymogen includes: the liver (Maturen and Barnhart, 1975), eosinophiles (Barnhart and Riddle, 1963) and the kidney (Highsmith and Kline, 1971). Approximately 40% of the plasminogen appears to be extravascular (Collen *et al.*, 1972) and its presence has been demonstrated in a wide variety of locations such as milk (Kaminogawa *et al.*, 1972), follicular fluids and uterine fluids (Dano *et al.*, 1985). The serum plasmin-plasminogen level is low in newborns (Rabiner *et al.*, 1969), and even lower in the premature infant (Ambrus *et al.*, 1963). In healthy human adults plasminogen is found in plasma or serum in a concentration of 200 mg/l. (Wohl *et al.*, 1979). The half-life of plasminogen is 2.24 ± 0.29 days (Collen and Verstraete, 1975).

2.1.2 Structure

Native human plasminogen is a single chain glycoprotein with 790 residues and a molecular mass of approximately 92-94 kD (Castellino, 1981), and has NH_2-terminal glutamic acid (Glu-plasminogen, Wallen and Wiman, 1970; Rickli and Cuendet, 1971). Glu-plasminogen is easily converted by limited plasmic digestion to modified forms with NH_2-terminal lysine, valine or methionine (Wallen and Wiman, 1972), which are commonly designated "Lys-plasminogen" (Robbins et al., 1967). Plasminogen is converted to plasmin by a number of activators, including tissue plasminogen activator, urokinase, or a complex of plasminogen and streptokinase. During plasminogen activation the Agr 560-Val 561 bond is opened to give the active two-chain molecule consisting of the heavy (A) and the light (B) chain (Robbins et al., 1967).

The structure of human plasminogen is illustrated in figure 1. The heavy chain portion (A-chain) of the native Glu-plasminogen (560 amino acids, Glu 1 to Arg 560) contains a preactivation peptide (Glu 1 to Lys 76) and 5 triple loop structures ("kringles", Wiman and Wallen, 1975; Sottrup-Jensen et al., 1978). These "kringles" are held in a loop structure by three disulphide bridges. The light chain, B-chain of the plasminogen (230 amino acid, Val 561-Asn 790) contains an active site composed of His 602, Asp 645 and Ser 740 (Groskopf et al., 1969; Rickli, 1984). This light chain is responsible for the catalytic activity, and its sequence is characteristic for serine protease.

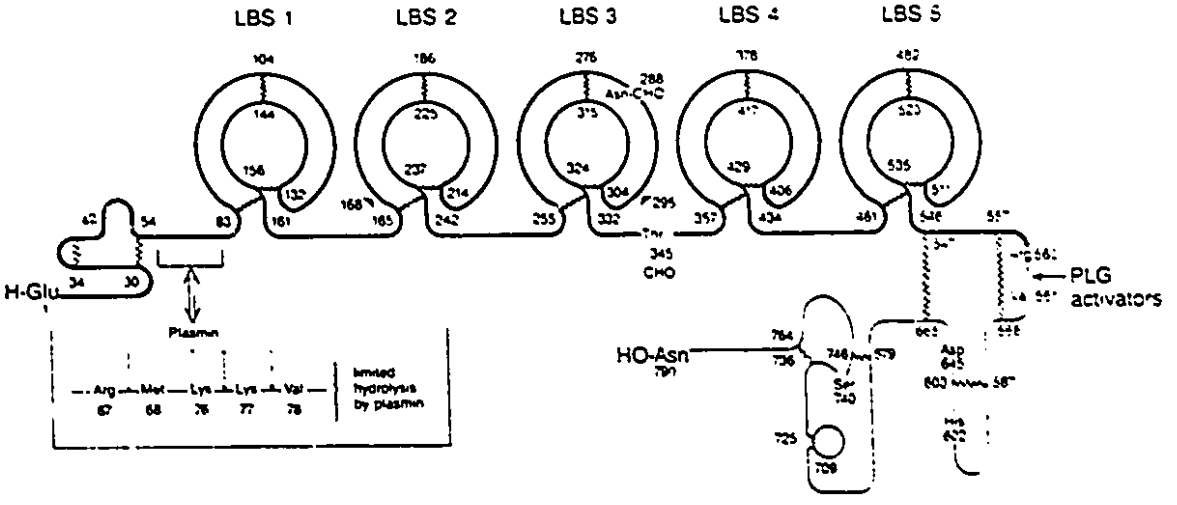

Figure 1. Scheme of the tertiary structure of human plasminogen. Attached bonds by plasminogen activators as well as by plasmin are shown. —: heavy (A) and —: light (B) chains in plasmin which is converted from plasminogen after cleavage of Arg 560-Val 561 bond by plasminogen activators.ʷʷ : disulphide bonds. -CHO: carbohydrate moeities. His, Asp, Ser: amino acid forming the active site in plasmin (from Miyashita *et al.*,1988).

2.1.3 Glycosylation

Native plasminogen occurs in two variants which differ in their extent of glycosylation (Brockway and Castellino, 1972; Hayes and Castellino, 1979a; 1979b; Rickli, 1984). Variant I is diglycosylated and carries a mannose type carbohydrate moiety with 10-11 monosaccharide units linked N-glycosidically to Asn 288 and a carbohydrate moiety of 3 to 4 residues linked O-glycosidically to Thr 345, while variant II is mono-glycosylated only to Thr 345. The two variants differ in their affinity for lysine which is higher for variant II and is decreased by the introduction of the carbohydrate moiety at Asn 288 (Hayes and Castellino, 1979a). In addition, variant I is more easily activated to plasmin than variant II (Collen and De Maeyer, 1975; Rickli, 1984). Due to their different lysine affinity the two variants can be separated by affinity chromatography on lysine substituted Sepharose (Brockway and Castellino, 1972; Hayes and Castellino, 1979a).

2.1.4 Isoelectric forms

Human plasminogen is known to possess multiple isoelectric forms, which can be separated by isoelectric focusing techniques (Summaria *et al.*, 1976). Glu-plasminogen I possesses multiple forms of pI values of 6.2, 6.3, 6.4 and 6.6. Glu-plasminogen II possesses two isoelectric forms with pI values of 6.4 and 6.6. On the other hand, Lys-plasminogen I contains three major isoelectric forms of pI 6.6, 7.2 and 7.5; whereas

Lys-plasminogen II contains three major forms of pI values 7.5, 7.8 and 8.1. These multiple pI values are caused by variations in the content of sialic acid.

2.1.5 Purification

Several methods for the purification of plasminogen have been described (reviewed by Mannhalter, 1988). The most significant advance in the purification of plasminogen was the introduction of a simple, one-step procedure using affinity chromatography on Lysine-Sepharose 4B (Deutsch and Mertz, 1970). This adsorbent has been reported to bind with plasminogen from humans as well as some animal species (Rapacz *et al.*, 1989; Humbert *et al.*, 1990). The affinity has been shown to be due to the properties of the first and fourth kringles in the protein (Rickli and Otavsky, 1975; Sottrup-Jensen *et al.*, 1978).

2.2 Bovine plasminogen and the significance of plasmin activity in dairy products

2.2.1 Bovine plasminogen

The complete amino acid sequence of bovine plasminogen has been determined by Schaller *et al.* (1985). By sequence analysis, Schaller *et al.* (1985) revealed that bovine plasminogen apparently contained the same structural and functional domains as human plasminogen. However, the bovine sequence comprises 786 residues, four

less than the human one. This difference is due to the deletion of Val 447, Val 448, Leu 449, Pro 539 and Thr 574 in the human sequence and to the insertion of Pro 65 in bovine plasminogen. Bovine plasminogen also contains two carbohydrate moieties. The N-glycosidic site was ascribed to Asn 289 in analogy to the N-glycosidic carbohydrate attachment site of human plasminogen (Asn 288), whereas the O-glycosidic site of the human sequence, Thr 345, is shifted to Ser 339 in bovine plasminogen. In addition, human plasminogen is efficiently activated by trace amounts of streptokinase. With bovine plasminogen, streptokinase fails to produce an activating effect. This difference is most probably due to differences in the amino acid sequence in certain regions of the plasminogen from the two species. The concentraion of plasminogen in plasma is unknown, while in milk, the plasminogen level is around 1.04 to 1.30 μg/ml (Korycka-Dahl *et al.*, 1983; Richardson, 1983b; Politis *et al.*, 1989).

2.2.2 Plasmin and plasminogen in milk

Normal bovine milk contains a number of indigenous proteinases (Reimerdes, 1983). The most thoroughly studied is milk alkaline proteinase, the properties and the significance of which have been reviewed by Fox (1981), Visser (1981) and (Grufferty and Fox, 1988). As a proteinase isolated from milk and plasmin isolated from bovine blood had very similar pH optima, pH stabilities, heat stabilities, sensitivity to various inhibitors, molecular weights and proteolytic specificities on caseins, it was concluded that milk proteinase and plasmin were identical (Kaminogawa *et al.*, 1972). It is now

generally accepted that the major proteinase in fresh, normal bovine milk is plasmin.

Addition of urokinase, which converts plasminogen to plasmin, increased the indigenous proteolytic activity in milk, indicating that milk also contains plasminogen (Kaminogawa *et al.*, 1972; de Rham and Andrews, 1982a). Korycka-Dahl *et al.* (1983), used a chromogenic tripeptide as substrate, to show that there was nearly 8 times more plasminogen than plasmin in bovine milk. Richardson (1983a) reported the same ratio of plasminogen to plasmin when their activities in milk were measured using the fluorogenic coumarin peptide as substrate. Plasminogen can be gradually activated to plasmin during storage or incubation (de Rham & Andrews, 1982a). The activation may also occur while milk is held in the lumen before milking, and possibly even at an earlier stage in the synthetic process (Donnelly and Barry, 1983; Schaar, 1985).

2.2.3 Plasminogen activators and inhibitors in milk

Plasminogen activators are present in many animal tissues (Wallen *et al.*, 1982; Roche *et al.*, 1983) and have also been reported in bovine and human milk (Okamoto *et al.*, 1981; Korycka-Dahl *et al.*, 1983). Milk also contains at least two trypsin inhibitors (Honkanen-Buzalski and Sandholm, 1981), which are probably identical to α1-antitrypsin and α2-macroglobulin of blood serum, and a colostrum trypsin inhibitor, all of which also inhibit plasmin. Because of the interplay between these factors the net plasmin activity observed in milk samples is variable, especially as it is also known

to be influenced by factors affecting the permeability of mammary gland membranes, such as stage of lactation (Richardson, 1983b) and particularly mastitis (de Rham and Andrews, 1982b; Andrews, 1983). The much higher levels of plasmin activity in mastitic milk are the result of increases in both active enzyme and plasminogen, but the proportion present as plasminogen is lower than in normal milk (de Rham and Andrews, 1982b). This may suggest that levels of plasminogen activator are also elevated.

2.2.4 Influence of plasmin activity on milk and milk products

Plasmin binds predominantly to casein micelles (Humbert & Alais, 1979; Rollema *et al.*, 1983), while small amounts are present on the fat globule membrane (Hofmann *et al.*, 1979). The main consequence of the presence of plasmin activity in milk is the hydrolysis of caseins. Only κ-casein is resistant to plasmin digestion, all other caseins are hydrolysed to a different extent, ß-casein being the most sensitive (Snoeren and Van Riel, 1979; Eigel *et al.*, 1979; Barry and Donnelly, 1981; Visser, 1981; Andrews, 1983). The detrimental effects of plasmin on casein integrity are amplified by storage and processing due to further activation of plasminogen to plasmin. In fact, plasmin activity increased significantly during cold storage (de Rham & Andrews, 1982a) which resulted in an increasing amount of proteose-peptone in milk (Schaar, 1985). As plasmin is very thermoresistant, its activity is largely unaffected by pasteurization (Richardson, 1983a; Alichanidis *et al.*, 1986). Further, plasmin activity often increases

due to pasteurization because of the inactivation of plasmin inhibitors. Moreover, significant activity remained even after ultra high temperature processing conditions (Richardson, 1983a; Alichanidis *et al.*, 1986; Igarashi, 1990).

Proteolytic damage to casein directly affects the processing and manufacturing properties of milk (Grufferty and Fox, 1988). Plasmin activity has detrimental effects on cold storage time, it produces peptides and amino acids that generate taste defects (Reimerdes, 1983) such as bitterness and it induces gelation in UHT milk (Kohlmann et al.,1991). Plasmin also affects cheesemaking process and cheese quality by decreasing cheese yield and modifying cheese texture and taste development because of the production of soluble peptides (Fox, 1981). Finally, the hydrolytic process affects functional properties of caseinate used as an ingredient in many food preparations. On the other hand, plasmin activity seems to be beneficial for ripening of Gouda (Visser, 1977) and Swiss-type cheese (Richardson and Pearce, 1981).

2.3 Factors affecting plasmin activity in milk

Various factors affect plasmin activity in milk. These include parity, stage of lactation, mastitis and breed of cows.

2.3.1 Parity

In 1977, Davies and Law suggested that the plasmin concentration in milk was affected by the age of the cow. The increase in τ-casein and the decrease in ß-casein which occur in late lactation milk were especially marked in milks from older cows. Plasmin activity in milk appears to increase with increasing lactation number (Schaar, 1985). This was supported by Politis and Ng-kwai-Hang (1989) who showed that plasmin activity was relatively low during the first three lactations and increased significantly during the fourth and subsequent lactations.

2.3.2 Lactation period

In late lactation, the ß-casein content of milk decreases while the level of τ-casein increases (Davies and Law, 1977; Barry and Donnelly, 1980; Donnelly and Barry, 1983). These changes have been attributed to a higher concentration and/or activity of plasmin in late lactation milk. Support for this suggestion included work by Donnelly and Barry (1983) who showed that similar compositional changes occurred when bulk milk was treated with plasmin at 30^0C. They also showed that the rate of conversion of ^{14}C-labelled ß-casein to ^{14}C-labelled τ-casein was much greater in milk from cattle in late lactation (200-300 days) than in mid lactation (100-200 days). The concentrations in milk of both plasmin and plasminogen increased in late lactation (Schaar, 1985; Benslimane et al., 1990). However, Korycka-Dahl et al. (1983) reported

that the plasmin concentration in late lactation milk was not significantly different from that in mid-lactation milk but the plasminogen concentration was 2-fold higher in the former. The increase in plasmin activity coincides with the process of gradual involution which is characterized by a loss of milk synthetic capacity by bovine mammary epithelial cells at late lactation. Plasmin has been implicated as the key enzyme in mammary gland involution in rodents (Ossowski *et al.*, 1979) and the role of palsmin in bovine mammary gland involution has been recently reviewed by Turner and Huynh (1991).

2.3.3 Breed

The plasmin content of milk is related to the breed of cow. The τ-casein content of Friesian milks (Barry and Donnelly, 1980) is more than twice that of milk from Ayrshire cattle (Davies and Law, 1977) which may indicate a higher plasmin content in the former. Richardson (1983b) reported that the plasmin content of milk from Jersey cattle was lower than that from Friesian cattle while Schaar (1985) found the lowest milk plasmin content in Jersey cattle, with highest levels in the milks of cross-bred Swedish Feiesian-Swedish Red and White cows; milk from pure-bred Swedish Friesian and Swedish Red and White had intermediate values. Recently, Benslimane *et al.* (1990) reported that the levels of plasmin and plasminogen in milk of Montbeliard cows (a French breed) were significantly higher than those of other commercial breeds.

2.3.4 Mastitis

Mastitic infection of the mammary gland results in increased milk proteolytic activity that is related to a higher concentration of plasmin (Barry and Donelly, 1981; Andrews, 1983; Donnely and Barry, 1983; Schaar, 1985; Schaar and Funke, 1986). Schaar and Funke (1986) reported that plasmin and plasminogen content of milk increased by 82 and 21%, respectively, during subclinical mastitis. Politis and Ng-Kwai-Hang (1989) found that an increase of somatic cell count from 100,000 to 1,300,000/ml was associated with a 2.3 fold increase in milk plasmin activity.

2.4 Genetic polymorphism of plasminogen

The genetic polymorphism of human plasminogen was first demonstrated independently by Hobart (1979) and Raum *et al.*(1979). Since then, a large number of rare variants have been found in many population studies (Skoda *et al.*, 1988a; Yamaguchi *et al.*, 1989; Spinetti *et al.*,1990; Sebetan, 1991).

2.4.1 Methodology

Plasminogen phenotypes have been identified using agar gel-diffusion, agarose gel electrophoresis, isoelectric focusing (IEF) in agarose and polyacrylamide gels. Suitable detection methods have also been developed, such as *in situ* immunofixation,

immunoblotting on nitrocellulose (NC) sheets from acrylamide and agarose IEF gels, and functional detection techniques as plasmin-mediated lysis with substrate overlays using exogenous plasminogen activators (Hobart, 1979; Raum *et al.*, 1980; Skoda *et al.*, 1986)

2.4.2 Nomenclature

In 1984, at the 18th Congress of the International Society of Blood Transfusion in Munich, a symposium dealing with plasminogen polymorphism took place, and a standardized technology for the determination of the plasminogen phenotypes and a uniform nomenclature for the designation of the plasminogen alleles was proposed (Skoda *et al.*, 1986). Recommendations included the desialization of serum samples, separation by IEF with subsequent functional or immunological methods for the detection of genetic variability of plasminogen (Skoda *et al.*, 1986). The nomenclature included common alleles called PLG*A and PLG*B; the known variants with acid pI: PLG*A1 to *A3; intermediate variants: PLG*M1 to *M5; and basic variants: PLG*B1 to *B3 (Skoda *et al.*, 1986).

Isoelectric focusing of plasminogen with the presently recognized human variants are presented in figure 2 (Skoda *et al.*, 1986). Homozygous plasminogen phenotypes in the caseinolytic overlay usually show two major bands and one or two anodal minor bands. Additional anodal and cathodal faint minor bands are sometimes observed with

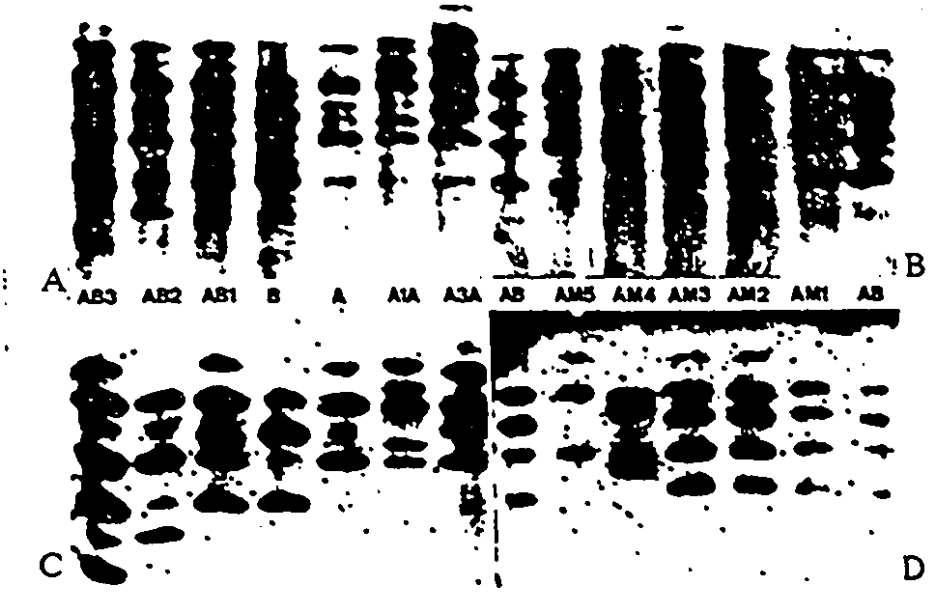

Figure 2. Isoelectric focusing of human plasminogen phenotypes with the presently recognized variants. A,B. Detection by "Western blotting". C,D. Caseinolytic overlay. Anode at top (from Skoda *et al.*, 1986)

Western blots. Heterozygous individuals exhibit the addition of the two homozygous plasminogen patterns, major or minor bands becoming enhanced by superimposition in some phenotypes.

2.4.3 Inactive human plasminogen variant

Clinical importance of plasminogen inactivity was first demonstrated by Aoki *et al.* (1978) in a Japanese patient with recurrent thrombosis caused by inadequate serum plasmin due to the variant PLG*M5. This abnormality was found to include the replacement of Ala by Thr at position 601 (Sakata, 1980; Miyata *et al.*, 1982). The PLG*M5 allele, which leads to the production of an abnormal plasminogen molecule with little or no activity, is polymorphic in Japanese people (Nishimukai *et al.*, 1981; Nakamura and Abe, 1982; Nishigaki and Omoto, 1982; Yamaguchi *et al.*, 1986). Manabe and Matsuda (1985) reported a patient with a congenital homozygous deficiency of protein C combined with a heterozygous molecular abnormality of plasminogen, probably also identical with PLG*M5. Recently, Yamaguchi *et al.* (1989) found a new variant designated PLG*Mosaka which had a very low activity and appears to be an abnormal plasminogen with functional defect similar to that of PLG*M5. The presence of a silent allele (which does not code for a protein) in the PLG system (PLG*QO) has been demonstrated by family data and quantitative genetic investigations (Scherz *et al.*, 1986; Brandt-Casadevall *et al.*, 1987; Dykes and Polesky, 1988; Skoda *et al.*, 1988b).

2.4.4 Plasminogen allelic frequencies in human populations

Plasminogen allelic frequencies in various human populations have been reported (Skoda *et al.*, 1988a). Table 1 lists the allelic frequencies of PLG*A, PLG*B and PLG*R (all rare PLG alleles) in various populations (Skoda *et al.*, 1988a). PLG*A is the most common allele in all populations, having its highest frequency in Mongoloids, Amerindians and Eskimos, the lowest in Caucasoids. Some populations seems to be characterized by specific PLG variants. The functionally inactive plasminogen protein M5 first described by Aoki *et al.* (1978) so far was only seen in Japanese individuals. Basic variants such as PLG*B1, B2, or B3, seems to be typical for Negroid population. Silent PLG alleles were only observed in the heterozygous state.

2.4.5 Plasminogen polymorphism in domestic animals

Studies on plasminogen polymorphism in domestic animals are very limited. In 1989, Rapacz *et al.* reported plasminogen polymorphism in swine. When whole plasma were separated on non-denaturing polyacrylamide gradient gel, transferred to NC paper, and immunoblotted with either anti-human or porcine plasminogen immune sera, a total of 11 different phenotypic patterns of plasminogen have been observed. The patterns were characterized by varying in number and intensity of bands from one individual to another and have been designated alphabetically from A to K (Rapacz *et al.*, 1989). Recently, plasminogen polymorphism has been detected in red deer

Table 1. Plasminogen allelic frequencies in various human populations (from Skoda et al., 1988a)

Population	N	Allele frequencies PLG*A	PLG*B	PLG*R[a)]
Amerindians				
Pima-Indians	200	0.993	0.007	–
US-Indians	100	0.860	0.125	0.015
Black Caribs (Guatemala)	189	0.677	0.262	0.061
Mexican Americans	295	0.854	0.143	0.003
Mexicans[b)]	197	0.882	0.111	0.007
Mexicans[c)]	123	0.858	0.110	0.033
Caucasoids				
Danes	1664	0.639	0.303	0.058
Germans				
North Germans	516	0.776	0.224	–
North Germans	604	0.713	0.270	0.018
West Germans	222	0.698	0.261	0.041
West Germans	713	0.675	0.307	0.018
Hessians	345	0.701	0.257	0.033
Hessians	118	0.69	0.26	0.02
South Germans	527	0.676	0.295	0.029
South Germans	957	0.717	0.278	0.005
South Germans	315	0.727	0.267	0.006
West Germans	1330	0.702	0.277	0.021
English	327	0.708	0.292	–
Italians				
Venetians	1325	0.840	0.158	0.002
South Italians	287	0.766	0.214	0.021
Polish	230	0.706	0.294	–
Swiss	308	0.688	0.281	0.031
Mennonites	183	0.666	0.303	0.036
White Americans	146	0.74	0.26	–
White Americans	102	0.686	0.299	0.015
White Americans	1501	0.665	0.304	0.031
White Americans	2187	0.677	0.293	0.030
Eskimos	273	0.989	0.011	–
Mongoloids				
Japanese	258	0.958	0.020	0.022
Japanese	795	0.944	0.019	0.037
Japanese	400	0.969	0.031	–
Japanese	750	0.956	0.011	0.033
Japanese	373	0.958	0.004	0.038
Japanese	585	0.969	0.003	0.028
Negroids				
Gambians	89	0.859	0.141	–
US Negroids	139	0.81	0.18	–
US Negroids	127	0.795	0.193	0.012
US Negroids	203	0.741	0.214	0.045
South African Negroes	1252	0.698	0.274	0.029

a) all rare variants

(Tate *et al.*, 1992) where 5 codominant alleles of plasminogen were observed when plasma was separated on native alkaline PAGE followed by immunoblotting with antisera to human plasminogen. However, no distinction was made between glycosylation and genetic variations in both studies. To date, no studies have addressed the question of plasminogen polymorphism in dairy cattle.

3. PLASMINOGEN POLYMORPHISM IN DAIRY CATTLE

3.1 Introduction

Plasminogen is a component of the fibrinolytic system and is the precursor of the serine protease plasmin, which is formed by the action of specific activators. It is a single-chain glycoprotein, consisting of 790 amino acids with a molecular weight of approximately 92 to 94 kD (Castellino, 1981).

The genetic polymorphism of plasminogen in humans was first demonstrated independently by Hobart (1979) and Raum *et al.* (1979). Since then, a large number of rare variants have been found in many population studies. Two main genetic variants, PLG*A and PLG*B and 14 minor variants have been identified so far, using isoelectric focusing and subsequent caseinolysis or immunodetection methods (Skoda *et al.*, 1986). The phenotypes are expressed by two-codominant alleles on a single autosomal locus. Interestingly, the nature of genetic variants of plasminogen has been shown to affect not only the plasmin activity but also the activation process of plasminogen (Wohl *et al.*, 1979). Certain human plasminogen variants display important clinical defects such as completely inactive plasmin. An abnormal plasminogen with an inactive molecule was found in a patient with recurrent thrombosis (Aoki *et al.*, 1978), and this plasminogen variant is characterized by a single substitution of an Ala by Thr at position 601 from the NH_2 terminal end

(Ichinose *et al.*, 1991).

Studies on plasminogen polymorphism in domestic animals are few in number. Recently, plasminogen protein polymorphism has been detected in swine (Rapacz *et al.*, 1989) where 11 plasminogen phenotypic patterns were detected, as well as in red deer (Tate *et al.*, 1992) where 5 codominant alleles of plasminogen were observed. However, no distinction was made between glycosylation and genetic variation in both studies.

In the present study, plasminogen polymorphism was investigated in Holstein and Ayrshire cows, using isoelectric focusing with subsequent immunoblotting or caseinolyis. This work represents the first attempt to characterize bovine plasminogen polymorphism at the protein level.

3.2 Materials and methods

3.2.1 Isolation of bovine plasminogen

Preparation of blood plasma

Approximately 300 ml of fresh bovine blood was collected at slaughter. The blood was chilled and mixed to a final concentration of 130 mM trisodium citrate, then

centrifuged (4000 g, 10 min) at 4^0 C. The citrated plasma was removed and centrifuged again at 3000 g for 10 min. The plasma thus obtained was then used for the isolation of plasminogen.

Affinity chromatography

Bovine plasminogen was purified from the citrated plasma by affinity chromatography on Lysine-Sepharose according to the procedure of Deutsch and Mertz (1970). The chromatography was carried out at 4^0 C. A volume of 100 ml of plasma was loaded onto a lysine-Sepharose 4B (Pharmacia, Uppsala, Sweden) column (2.5 x 15 cm) equilibrated with TZN (50 mM Tris, 10 mM $ZnSO_4$ and 0.02 % NaN_3, pH 8) buffer. The flow rate was 60 ml/h. The column was next washed with 20 ml of TZN buffer and washed again with 100 ml of 0.5 M NaCl-TZN at 60 ml/h. The plasminogen was then eluted as a single peak with 100 ml of 0.2 M ϵ-aminocaproic acid (Sigma, MO, USA)-TZN buffer. The ϵ-aminocaproic acid was removed from the plasminogen by gel filtration on a 2 x 10 cm Sephacryl-300 (Pharmacia) column in TZN buffer at a flow rate of 30 ml/h. The plasminogen fraction was collected and concentrated to 1 ml by ultrafiltration using a stirred cell (10 ml, Amicon, MA, USA) with membrane size of 10,000 NMWC (nominal molecular weight cut-off).

Determination of plasminogen concentration

The protein concentration was determined using the Bradford protein assay kit (Bio-Rad, CA, USA) with bovine serum albumin (BSA, Bio-Rad) as a standard. The assay was performed as follows. A serial dilutions of known concentration BSA solution was first prepared and a number of different dilutions of purified plasminogen was also made. The dye reagent (Coomassie Brilliant Blue G-250, provided with the kit) was diluted 5 times with distilled water and filtered through Whatman No.1 filter paper. To each 5 ml of diluted Dye Reagent, 60 μl of different dilutions of BSA solution or purified plasminogen was added and mixed by gentle inversion of test tube several times. The mixture was incubated at room temperature for 5 min and then measured at 595 nm vs. reagent blank. The concentration of purified plasminogen was determined using the BSA standard curve.

3.2.2 Purity examination of the isolated plasminogen

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the isolated plasminogen was characterized by SDS-PAGE (Laemmli, 1970) on vertical gel slabs using Mini protean II electrophoretic apparatus (Bio-Rad). The SDS-PAGE used 12 % acrylamide resolving and 4.8 % acrylamide stacking gels. The resolving gel (10 ml) was made by mixing the following:

30 % acrylamide-N',N'-methylenebisacrylamide (29:1, Bio-Rad)	4.0 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml
H_2O	3.5 ml
N,N,N',N' tetramethylenediamine (TEMED, Bio-Rad)	6 μl
10 % ammonium persulphate (Bio-Rad)	60 μl

The stacking gel (5 ml) was made from a mixture of:

30 % acrylamide-N',N'-methylenebisacrylamide	0.8 ml
0.5 M Tris-HCl (pH 6.8)	1.25 ml
H_2O	3.0 ml
TEMED	6 μl
10 % ammonium persulphate	60 μl

Seven μg of purified plasminogen dissolved in 10 μl of H_2O were mixed with 10 μl of 2x sample buffer (4 % SDS, 4 % ß-mercaptoethanol, 20 % sucrose, 0.2 % bromophenol blue and 0.06 M Tris-HCl, pH 6.8) and boiled for 2 min. Samples were then loaded using a Hamilton microliter syringe. Low molecular weight markers (Bio-Rad) were used as standards which include phosphorylase B (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,500 Da) and lysozyme (14,400 Da). Electrophoresis was

conducted in a 0.05 M Tris-0.4 M glycine buffer (pH 8.3) containing 0.1% SDS at 100 V until the tracking dye reached the bottom of the gel.

Coomassie Blue staining

After completion of the electrophoresis, the gel was carefully removed from between the glass plates and then fixed and stained in 0.25 % Coomassie Brillant Blue R250 (Bio-Rad), 50 % methanol, 7 % acetic acid for 30 min. The background colour was removed by destaining in 30 % methanol, 7 % acetic acid for a total of 3 hr with several changes of the destaining solution. The destained gel was finally wrapped in a piece of cellophane membrane (Bio-Rad) and dried on a gel dryer for 60 min at 80^0 C.

3.2.3 Preparation of plasminogen antibodies

Immunization

To prepare polyclonal antibodies, purified bovine plasminogen was used to immunize New Zealand white rabbits. Each rabbit was injected intramuscularly with 3 mg of plasminogen dissolved in 1 ml of 1/1 mixture of physiological saline and complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for the other ones. The rabbits were given monthly injection for 4 months. A small

amount of blood was collected from the ear vein before immunization, 10 days after the second and third immunization. Ten days after the last injection, a large quantity of blood was collected by cardiac puncture. The blood was allowed to clot at room temperature for 30 min and to retract overnight at 4^0 C and then separated from the serum by centrifugation at 2000 g for 10 min at 4^0 C. The serum was removed, aliquoted and stored at -20^0 C.

Antibody titration

To determine the titration of the rabbit anti-bovine plasminogen antibodies, a serial dilutions of antiserum were prepared and reacted to a fixed amount of bovine plasminogen on nitrocellulose membrane. The titration was carried out using the Bio-Dot dot blotting apparatus (Bio-Rad). One μg of plasminogen dissolved in 200 μl of TBS (25 mM Tris, 150 mM NaCl, pH 7.5) was applied to each well and the plasminogen was bound to the membrane by vacuum drying of the TBS solution. The wells were rinsed 3 times with TBS (200 μl each time) and the membrane was blocked by incubation with 200 μl (each well) of 1 % BSA-TBS for 15 min. Antisera obtained after second, third and fourth immunization, as well as serum taken before immunization were diluted 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800 in TBS. Two hundred μl of each diluted serum were added to each well and incubated for 15 min. The wells were then washed 2 times with TTBS (0.05 % Tween 20-TBS) and once with TBS. Two hundred μl of 1:3000 diluted goat-anti-rabbit-IgG

conjugated to horseradish peroxidase (Bio-Rad) were added to each well and incubated for 15 min. After washing away the unbound antibodies, the membrane was freed from the metal blocks and developed in HRP colour development solution (Bio-Rad).

3.2.4 Plasminogen phenotyping

Animals and blood sampling

Blood samples were collected in citrated tubes from 50 randomly selected Holstein and Ayrshire cows from the Macdonald Campus herd. Plasma samples were obtained by centrifugation (4000 g, 10 min) and stored at -20^0C.

Treatment of plasma samples

To remove plasminogen variants caused by glycosylation, the plasma samples were treated with neuraminidase before electrophoretic analysis. Forty μl of Lysine-Sepharose were mixed with 20 μl of citrated plasma in a Eppendorf tube and stored on ice for 30 min. The mixture was then centrifuged at 12,000 g for 2 min in an Eppendorf centrifuge. The supernatant was removed and the pellet was washed 3 times with 0.5 M NaCl-TZN buffer (400 μl each time). The plasminogen bound to the Lysine-Sepharose was then desialized by incubation with 30 μl of *Clostridium*

perfringens neuraminidase (5 U/ml, type V, Sigma) for 3 hr at room temperature. The neuraminidase was removed by centrifugation and the pellet was washed with 400 μl of 0.5 M NaCl-TZN and again washed with 1x washing buffer containing 4.8 % Ampholine (pH 3.5-9.5, Bio-Rad), 2% CHAPS (Bio-Rad) and 0.1 % bromophenol blue. The treated plasminogen was finally eluted by addition of 40 μl of 1x IEF loading buffer (2.6 % ε-aminocaproic acid in 1x washing buffer) and centrifugation.

Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE)

The desialized plasma was separated by IEF-PAGE in the pH range of 3.5-9.5 (2.9 % Ampholine), using a Mini Protean II electrophoretic apparatus (Bio-Rad). The IEF gel (10 ml) was prepared by mixing 2.4 ml of 30 % acrylamide-N',N' methylenebisacrylamide (29:1), 0.29 ml of Ampholine (pH 3.5-9.5, Bio-Rad), 7 ml of H_2O, 250 μl of 4 % CHAPS (Bio-Rad), 20 μl of TEMED, and 25 μl of 10 % ammonium persulphate. IEF standards (Bio-Rad) were used which include Phycocyanin (pI 4.65), ß-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrase (pI 6.5), equine myoglobin (pI 7.0), human haemoglobin A (pI 7.1), human haemoglobin C (pI 7.5), lentil lectin (pI 7.8, 8.0, 8.2) and cytochrome c (pI 9.6). The electrode solutions were 20 mM H_3PO_4 for the anode and 20 mM NaOH for the cathode. The IEF was carried out at 300 V for 30 min and then increased to 500 V for 2 hr.

Western blotting and immunodetection

After IEF, the separated plasma proteins were transferred onto a nitrocellulose membrane (Bio-Rad) by Western blotting (Towbin *et al.*, 1979) using a Mini Trans-blots Transfer cell (Bio-Rad). The IEF gel was equilibrated in 0.7 % acetic acid for 30 min to remove electrolytes or other interfering substance and then placed against a piece of prewet nitrocellulose membrane. The blotting was carried out either at 100 V for 60 min or 30 V overnight in 0.7% acetic acid buffer. A magnetic stirrer in the transfer tank was used to counteract ion migration and the transfer system was cooled by sitting the tank in an ice bath.

When the blotting was completed, the membrane was placed in 3 % gelatin-TBS for 30 min at 37^0 C with gentle agitation. The membrane was then washed twice with TBS. After removing the wash solution, the membrane was incubated with 1:3000 diluted rabbit anti-bovine plasminogen antiserum for 1 to 2 hr at 37^0 C with gentle agitation and the excess of antibodies were washed away with TTBS. The membrane was then incubated in 1:3000 diluted goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) for 1 to 2 hr at 37^0 C with gentle agitation and washed twice with TTBS and rinsed in TBS to eliminate residual detergent. Finally, the membrane was developed in HRP colour development solution (Bio-Rad) for about 5 min and the development was stopped by placing the membrane in distilled water when satisfactory signal was generated.

Caseinolytic overlay

Alternatively, the plasminogen polymorphism were detected by a functional assay (caseinolytic overlay technique, Skoda *et al.*, 1986). After completion of IEF, the polyacrylamide gel with one glass plate attached was covered with 1 ml of 100 U/ml urokinase (Sigma). The gel was incubated at 37^0 C for 30 min to allow urokinase to activate plasminogen to plasmin, and then overlaid on a premade casein/agarose gel. The casein-agarose gels were 1 mm thick and made from 1:1 mixture of 1 % casein (Sigma) in 0.05 M Tris-HCl, pH 8.3 and 1.2 % agarose (ICN) in 0.01 M Tris-HCl, pH 8.2. Overlaid gels were incubated for 4 hr at 37^0C in a water saturated atmosphere and then developed in 10 % trichloroacetic acid for 30 sec. Plasminogen patterns consisted of clear proteolyzed areas in a milky casein background and were immediately discernible. The trichloroacetic acid was finally replaced with distilled water and the gel was left in water overnight for sharping of the bands.

3.3 Results

3.3.1 Characteristics of bovine plasminogen

Using Lysine-Sepharose affinity chromatography, bovine plasminogen was purified from Holstein cow plasma. A typical elution profile of plasminogen from lysine Sepharose 4B is shown in figure 3. The plasminogen fraction eluted from the column

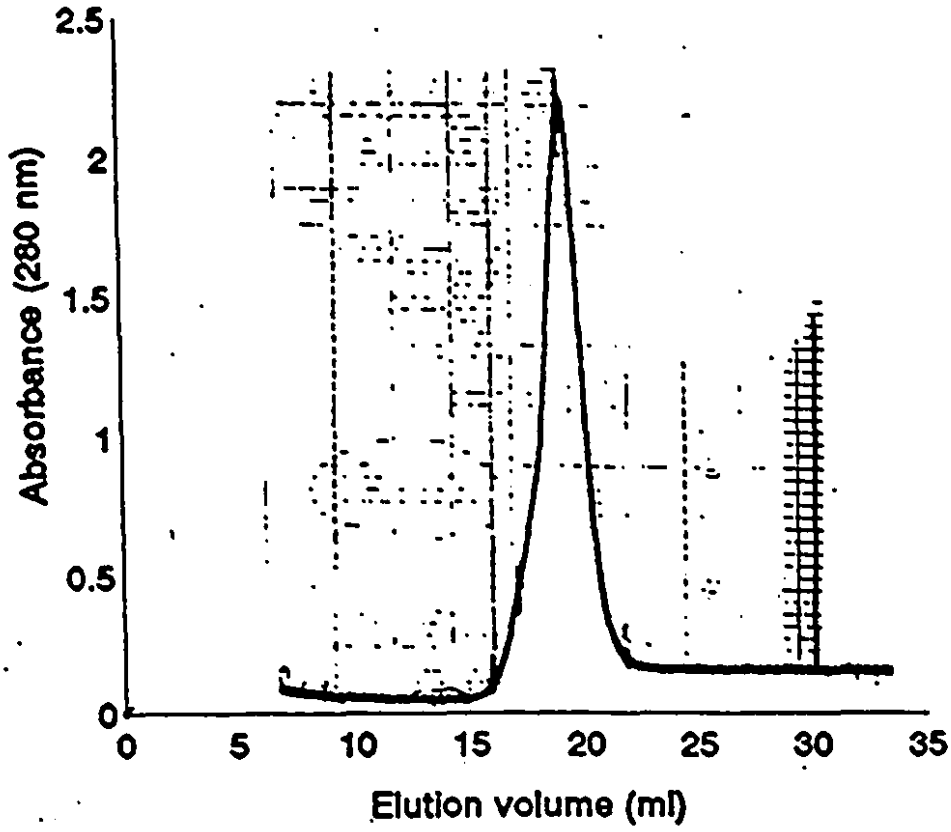

Figure 3. Isolation of bovine plasminogen by affinity chromatography on Lysine-Sepharose 4B. Column dimensions: 2.5x15 cm (bed volume 40 ml). Flow rate: 60 ml/h. A typical elution profile of plasminogen is shown.

shows a sharp peak when monitored using single path monitor UV-1 (Pharmacia). From each 100 ml of plasma, 8 ml of plasminogen fraction were collected, which were then concentrated to 2 ml by ultrafiltration. After removal of ϵ-aminocaproic acid by gel filtration, the plasminogen fraction was collected and concentrated again by ultrafiltration to a final volume of 1 ml. Using Bradford protein assay, the concentration of the purified plasminogen was determined. From a total of 300 ml of whole plasma, 34 mg of plasminogen was obtained.

After protein determination, the isolated plasminogen was analyzed by SDS-PAGE. Coomassie Brilliant Blue staining after separation of the isolated plasminogen on SDS-PAGE demonstrated a single major band corresponding to plasminogen with an apparent molecular weight of 90 kD (Figure 4). A minor band of 60 kD which corresponds to plasmin was also noted (the 25 kD light chain cleavage product was not identified). In contrast, a commercially available bovine plasminogen (Sigma) preparation showed both bands with the same intensity, indicating that approximately half of the plasminogen had been converted to plasmin. Therefore, this preparation was not used for antibody production.

3.3.2 Rabbit anti-bovine plasminogen antibodies

For immunological detection of plasminogen variants, antibodies to bovine plasminogen were raised by monthly intramuscular injection of the isolated bovine

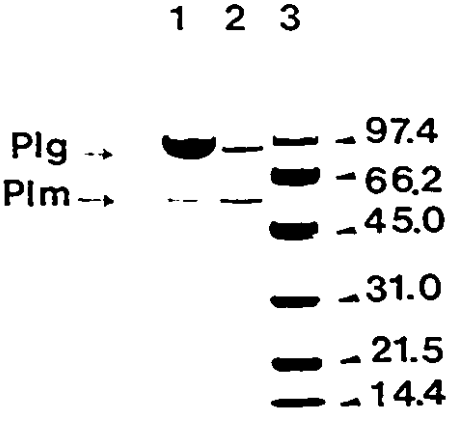

Figure 4. A preparation of purified bovine plasminogen (plg) shows a single major band of approximately 90 kD and a minor band corresponding to plasmin (plm) when separated on a 12 % SDS-PAGE under reducing conditions and stained with Coomassie Blue. Lane 1, 7 μg of purified plasminogen, Lane 2, Sigma bovine plasminogen and Lane 3, standards with molecular weights indicated in kD.

plasminogen into rabbits. The titrations of the antisera obtained after the second, third and last immunization were determined. As shown in figure 5, serum collected before immunization did not react to plasminogen, while all antisera specifically bound to plasminogen. The titration of the antibodies after the second immunization was already rather high as the antibody-antigen reaction signal was still visible even when the antibodies were diluted 1: 12800 times. The titration of the antibodies did not increase significantly after the third and fourth boost since the same dilutions of the 3 collections (antisera) showed approximately the same reaction signals.

3.3.3 Plasminogen polymorphism

Preliminary experiment

To insure that sufficient neuraminidase activity was present to remove all the sialic acid from the plasminogen when the plasma samples were treated with neuraminidase, a preliminary experiment was conducted by treating fixed amount of purified plasminogen (7 μg) with increasing amounts of neuraminidase (0.075 to 0.225 U). The treatment was carried out at room temperature for 3 hr. Treated samples were separated by isoelectric focusing, Western blotted and immunologically detected. As shown in figure 6, the untreated plasminogen gave several bands when separated on IEF, while the bands corresponding to the glycosylated plasminogen isoforms did not appear in the samples treated with neuraminidase. As little as 0.075 U of

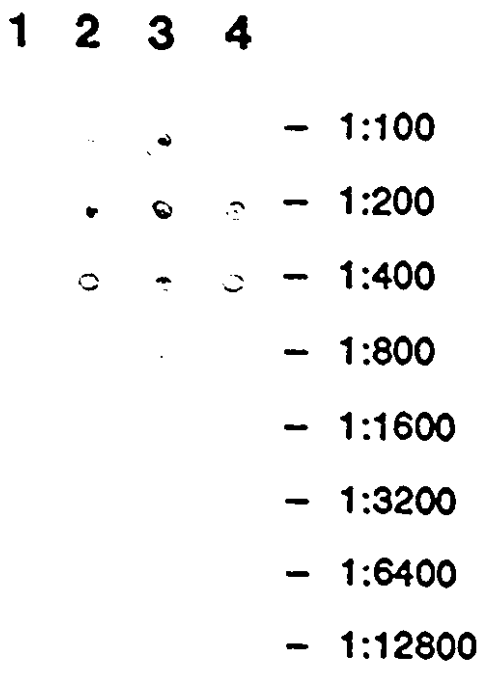

Figure 5. Titrations of rabbit anti-bovine plasminogen antibodies. Antisera obtained after second (Lane 2), third (Lane 3) and fourth (Lane 4) immunization, as well as serum (Lane 1) taken before immunization were diluted 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and 1:12800 and reacted to a fixed amount (1 μg) of bovine plasminogen on nitrocellulose membrane

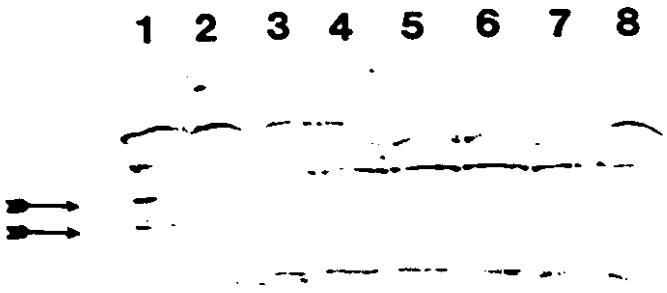

Figure 6. Neuraminidase treatment of plasma samples removes the variation of plasminogen isoforms caused by glycosylation. Such varaints, identified with arrows disappear with neuraminidase treatment. Both treated and untreated plasma samples were separated by isoelectric focusing and Western blotted. Plasminogen isoforms were immunologically detected. Lane 1, untreated plasminogen, Lane 2-8, plasminogen (7 μg) treated with increasing amounts of neuraminidase (from 0.075 U to 0.225 U with increment of 0.025 U).

neuraminidase was sufficient to remove all the sialic acid from 7 μg of plasminogen (figure 6).

Plasminogen phenotypes

When desialyzed plasma samples were separated on IEF, transferred to nitrocellulose membrane, and immunoblotted with rabbit anti-bovine plasminogen antibodies, plasminogen polymorphism was demonstrated as shown in figure 7. In the 50 samples investigated, 6 plasminogen phenotypes have been observed. The observed phenotypes represent products of 5 variant alleles which were identified based on their isoelectric points and designated PLG A_2 (pI 6.5 and 7.0), B_2 (pI 7.6 and 7.8), C_1 (pI 6.8), D_2 (pI 7.8 and 8.0), and E_2 (pI 6.8 and 7.0). The subscript represents the number of bands for each variant. The 6 plasminogen phenotypes measured to date include A_2A_2, B_2B_2, A_2B_2, B_2C_1, A_2D_2, and D_2E_2.

The 2 homozygotes PLG A_2A_2 and PLG B_2B_2 each showed 2 bands. The heterozygous types were usually characterized by 4 bands, each pair reflecting the allelic products of the respective homozygous types. Only the B_2C_1 phenotype gave 3 bands as the product of C_1 allele showed only one band.

The same samples were also analyzed for plasminogen polymorphism with the caseinolytic overlay technique (Figure 8). However, the plasminogen phenotypic

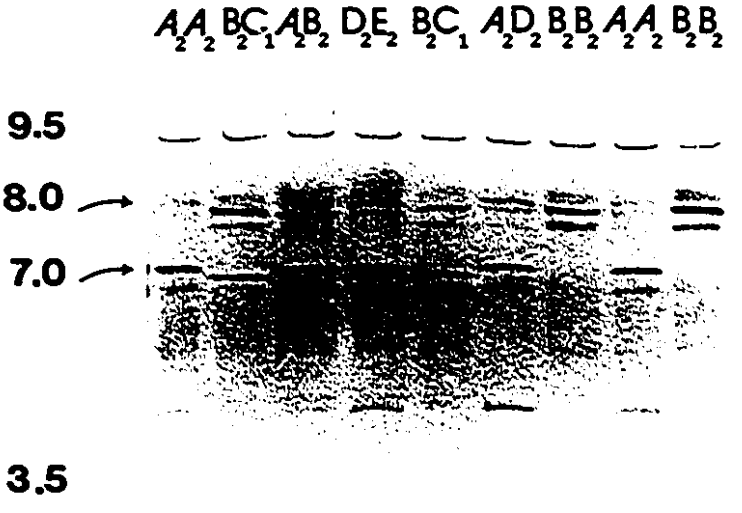

Figure 7. Plasminogen phenotypes separated with isoelectric focusing-PAGE over a range in pH 3.5-9.5 in a polyacrylamide gel with subsequent immunodetection. Capital letters indicate the variant and the subscripts represent the number of bands in that variant. Lane 1-9 plasma samples from 9 different dairy cattle.

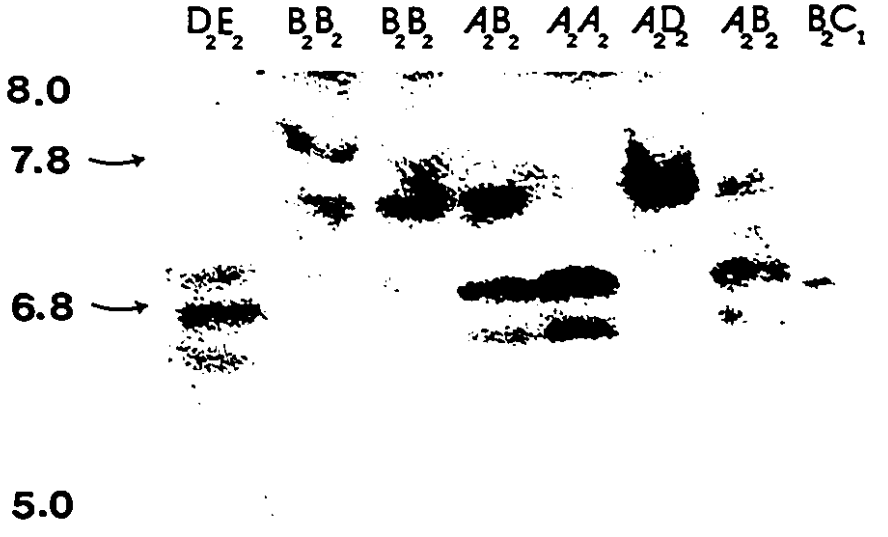

Figure 8. Plasminogen phenotypes separated with isoelectric focusing over a range in pH 5-8 in a polyacrylamide gel and detected by casein overlay. The capital letters indicate the variant and the subscripts represent the number of bands in that variant. Lane 1-8 plasma samples from 8 different dairy cattle.

patterns were not as clear as the ones revealed by immunodetection. Only the A_2A_2 and B_2B_2 phenotypes showed consistent patterns with the ones revealed by immunodetection, while the other phenotypes missed some bands on casein/agarose gel when compared to the results obtained by immunodetection. For example, the A_2B_2 individual only showed 3 bands on the casein/agarose gel. The cathodal band representing one of the two products of the B_2 allele was not detected. This probably is due to the low sensitivity of the technique.

The distributions of the plasminogen phenotypes are summarized in Table 2. PLG A_2 and PLG B_2 were the most common variants present in our herd. The estimated allelic frequencies of PLG A_2 and PLG_2 in the 50 cows investigated were 0.45 and 0.41, respectively. Among the PLG A_2A_2 phenotypes, most of them are Holsteins (17 out of 18). While most Ayrshire cattle exhibit PLG B_2B_2 phenotype (10 out of 13).

3.4 Discussion

Several methods for the purification of plasminogen have been described (Mannhalter, 1988), but the most frequently cited method is the Deutsch and Mertz (1970) which utilizes lysine-Sepharose affinity chromatography. This separation technique relies on the active site binding to the lysine exposed on the resin. The plasminogen preparation eluted from Lysine-Sepharose column contains ϵ-aminocaproic acid (a lysine analog) which was eliminated by subsequent gel filtration.

Table 2. Distributions of bovine plasminogen phenotype

Phenotype	A_2A_2	B_2B_2	A_2B_2	B_2C_1	A_2D_2	D_2E_2
No. Holsteins	17	3	3	4	2	1
No. Ayrshires	1	10	4	4	0	1
Total	18	13	7	8	2	2

This is necessary as this acid is a plasmin inhibitor as well as a competitive inhibitor of urokinase and streptokinase for the activation of the proenzyme (Ablondi *et al.*, 1959; Castellino, 1981).

The overall yield of bovine plasminogen was 11.3 mg/100 ml fresh plasma which is similar to the yield of human plasminogen (Deutsch and Mertz, 1970) but 45 % less than the value reported for swine plasminogen purification (Rapacz *et al.*, 1989). Purified bovine plasminogen applied to SDS-PAGE showed a single major band of 90 kD, which is consistent with that reported for both humans (Castellino, 1981) and swine (Rapacz *et al.*, 1989). However, the plasminogen preparation after separation on SDS-PAGE also showed a minor band of 60 kD which corresponds to plasmin, indicating only minor plasmin contamination. Since the method did not use proteinase inhibitors during the purification (although $ZnSO_4$ was used which may prevent the plasminogen activation), spontaneous activation of plasminogen to plasmin by activators present in plasma (Chibber *et al.*, 1974) may had occurred resulting in a partially degraded product of Lys-plasminogen rather than the native form of Glu-plasminogen (Castellino and Powell, 1981; Wallen and Wiman, 1970). In addition, plasmin, present in plasma at the time of collection or generated during purification procedures in the absence of proteinase inhibitor, may co-elute with plasminogen (Chibber *et al.*, 1974) thus resulting in plasmin contamination. The problem may be avoided by inclusion of proteinase inhibitors (such as Lima bean trypsin inhibitor and Trasylol) during purification and by carefully monitoring the protein absorbance

during plasminogen elution (Grant, 1990). Nevertheless, the isolated bovine plasminogen was sufficiently pure for the purpose of the present study. This was in contrast to commercially available plasminogen.

This study represents the first characterization of plasminogen polymorphism in dairy cattle. By isoelectric focusing over a pH range of 3.5-9.5 followed by an immunological detection or a functional detection method, genetic polymorphism of bovine plasminogen have been readily demonstrated. In the dairy cattle investigated, 2 common plasminogen variants (PLG A_2 and PLG B_2), as well as 3 minor variants (D_2, E_2 and C_1) were identified. With more samples from different herds, more genetic variants, as described in humans, will potentially be identified in dairy cattle.

Each of the identified variant alleles (except for C_1) produces two major forms of plasminogen. These difference are not due to glycosylation as the plasminogen was quantitatively deglycosylated prior to analysis. The reason for these two bands is not known. It is probably due to two different plasminogen forms (lysine and glutamic). It seems unlikely from the constant association of two bands with each other that the heterogeneity observed is due to more than one genetic locus for plasminogen.

The present study used two methods for the identification of plasminogen polymorphism, immunodetection via Western blotting and a functional assay, casein overlay. Immunodetection permited the recognition of all allotypes. The functional

caseinolytic overlay was more convenient and biologically meaningful, because it measures the ability of plasminogen, actually plasmin, to digest casein proteins. This assay is important as it actually measures a phenotypic trait, i.e., how well a certain isoform digests the major milk proteins, casein.

Considerable heterogeneity is observed in plasminogen due to posttranslational sialidation (Raum *et al.*, 1979). In humans, two main glycosylation patterns exist, one carrying two oligosaccharides (an O-linked and an N-linked) while the other has a single O-linked oligosaccharide. Treatment with neuraminidase desializates the plasminogen thus removing the charged sialic acid and eliminated the variation due to glycosylation. The present study used sufficient neuraminidase to remove quantitatively all the sialic acid from plasminogen, therefore the identified variations in plasminogen isoelectric point were genetic, probably due to mutations in some charged amino acids, not due to differences in glycosylation.

Plasminogen polymorphism in humans has been studied intensively since the first description by Hobart (1979) and Raum *et al.* (1979). An abnormal plasminogen variant (PLG* M5) with an inactive molecule was found in a patient with recurrent thrombosis (Aoki *et al.*, 1978), and this abnormality was found to be caused by replacement of Ala by Thr at position 601 from the NH_2 terminal end (Ichinose *et al.*, 1991). This genetic variation in the clinical setting establishes a precedent that is useful with respect to dairy cattle genetics. If such inactive plasminogen variants (or

less active variants) could be identified in bovine, it would be possible to control the proteolytic activity of plasmin in milk through genetic selection for these plasminogen variants.

The casein overlay technique is a functional assay which relies on the conversion of plasminogen into plasmin which in turn degrades casein, leaving a clear plaque. This study showed that all the bovine plasminogen variants could be converted into active plasmin. However, in a manner consistent with clinical findings (Aoki *et al.*, 1978), not all the variants detected with Western blotting could be activated within the functional assay in a consistent fashion. As the human PLG* M5 variant (completely inactive) is extremely rare (only seen in Japanese individuals), it is not surprising that an analogous variant was not detected in the present study of only 50 cows.

The present study described the presence of bovine plasminogen polymorphism in Holstein and Ayrshire. As the sample size was limited, no correlations between different plasminogen variants and plasminogen activity or plasmin concentrations in blood or milk was made. Future studies should focus on the relationship between plasminogen variants and plasminogen activity. In addition, the study only used blood samples for polymorphism determination. Further attempts should focus on the development of methodology for plasminogen phenotyping using milk samples. This is advantagous, as milk samples are more readily available than blood, so large number of cows could be typed. For example, the Dairy Herd Analysis Service has

daily access to literally thousands of milk samples taken from cows across a broad geographic area.

4. DETERMINATION OF RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) IN BOVINE PLASMINOGEN GENE

4.1 Introduction

Plasminogen is a proenzyme of the fibrinolytic system which is coded by a locus located on the long arm of chromosome 6 (Murray *et al.*, 1987). Independent investigations performed by Hobart (1979) and by Raum *et al.* (1979, 1980) demonstrated clearly the existence of a genetically defined polymorphism of human plasminogen protein. Since these first analysis, a considerable number of variants have been identified besides the proteins coded for by the common alleles (Skoda *et al.*, 1988a; Yamaguchi *et al.*, 1989; Spinetti *et al.*,1990; Sebetan, 1991). Abnormalities of plasminogen resulting in disordered haemostasis in affected individuals have been reported (Aoki *et al.*, 1978).

Molecular biology has provided techniques which permits the investigation of variations in primary gene structure. Restriction fragment length polymorphisms (RFLP) is an important tool for the identification of genetic variations (Botstein *et al.*, 1980). It occurs as a result of DNA base changes, deletions, insertions or rearrangements that either create, eliminate or translocate restriction enzyme cleavage sites. Such variations are inherited in a Mendelian fashion, and since gene expression is not required for RFLP analysis, variations in the flanking regions or introns of genes

can also be detected.

Recently, RFLPs have been found in the human plasminogen gene by using 2 genomic DNA subclones as probes and 3 restriction enzymes, MspI, SacI and RsaI (Murray *et al.*, 1987). The MspI and SacI polymorphisms were found to be highly associated with the protein variations coded for by PLG*A and PLG*B. However, the RsaI RFLP showed no significant association with MspI, SacI or PLG A/B.

This study reports the attempt to identify RFLPs in the bovine plasminogen gene using a mouse plasminogen cDNA as a probe.

4.2 Materials and methods

4.2.1 DNA extraction

The DNA was extracted from blood of Holstein cows raised on the Macdonald campus farm. Approximately 10 ml of venous blood were collected from each cow using heparin as anticoagulant. The whole blood was centrifuged at 1300g for 15 min and the "buffy coat" which contains the nucleated leucocytes was transferred to a fresh 15 ml Falcon tube, to which 5 ml of extraction buffer (10 mM Tris, pH8; 100 mM EDTA pH 8; 20 μg/ml RNAse and 0.5 % SDS) containing proteinase K (100 μg/ml) were added. After incubation at 60^0C overnight, the cell lysis was extracted with 1

volume phenol:chloroform for 2 times. After the last extraction, the supernatant was removed and mixed with 2 volume of 100 % ethanol and 0.2 volume of 10 M ammonium acetate. The precipitated genomic DNA was then collected with a glass rod and rinsed in 70 % ethanol. After air drying the DNA was dissolved in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and DNA concentration was determined by UV absorption at 260 nm.

4.2.2 Restriction enzyme digestion

Five μg of DNA were digested overnight at 37^{0}C in 5 units/μg of restriction enzyme (Pharmacia). The reaction mixture included 20 μl of DNA (250 μg/ml), 25 units of restriction enzyme and 2.5 μl of 10 x one-phor-all enzyme buffer (Pharmacia). After overnight digestion, the DNA samples were heated at 65^{0}C for 10 min to inactivate the enzymes then chilled immediately on ice to avoid possible annealing of the sticky ends.

4.2.3 Gel electrophoresis

The restricted DNA fragments were separated according to their different size by agarose gel electrophoresis in 1 x TPE buffer (80 mM Tris-phosphate, 8 mM EDTA, pH 8.0). The 1 % agarose gel was prepared by dissolving 2.13 g of agarose in 213 ml of 1 x TPE by heating in a microwave oven for several minutes. After cooling to

approximately 50^{0}C (about 30 min at room temperature), the agarose gel was poured into a 20 x 15 cm gel tray with a comb of 15 wells (the tray was balanced to be horizontal beforehand). After solidification of the gel (about 1-2 hr), the comb was removed and the gel was placed in a electrophoresis tank containing 1 x TPE buffer. The level of buffer was adjusted until the gel was 5 mm below the surface of the buffer. Five μl of 15 % Ficoll loading solution were added to each digested DNA sample. The DNA samples mixed with loading buffer were then loaded onto the wells. The gel was run at 29 V for 18 hr. After completion of the electrophoresis, the gel was stained in ethidium bromide (0.5 μg/ml) for 15 to 20 min and examined under UV light to check if the DNA samples are well digested. A polaroid picture was taken at a sec. with f 5.6 in an MP4 photographic set up.

4.2.4 Southern blotting

In order to detect specific DNA sequences, the separated DNA fragments were transferred from the agarose gel onto a Zeta-probe membrane (Bio-Rad) by Southern blotting (Southern, 1975). To do so, the gel was trimmed to desired size and soaked in 0.25 M HCl for 30 min to depurinate the DNA. After the HCl treatment, the gel was washed with distilled water and soaked in 0.4 M NaOH. To carry out blotting, a transfer tower was built up by placing the gel upside down over a wick which consists of 4 sheets of 3 MM paper and dipped in 0.4 M NaOH as transfer solution. A piece of Zeta membrane with the same size as the trimmed gel was soaked in heated 0.1

% SDS solution and placed over the gel. Four prewet sheets of 3 MM paper were placed over the membrane. Air bubbles were removed by rolling gently with a 10-ml pipet over the surface of each layer. A 10 cm stack of paper towel was placed over the sheets and finally a glass plate with some weight was put on the paper towel to keep good contact. The blotting was carried out at room temperature overnight. After transfer, the membrane was washed in 2 x SSC (20 x SSC: 3 M NaCl, 0.3 M Sodium Citrate), 0.1 % SDS.

4.2.5 Preparation of probe

The DNA sequence utilized as probe was a mouse plasminogen cDNA (MPLS-2000; Degen *et al.*, 1990), which was commercially obtained from American Type Culture Collection (MD, USA). The cDNA sequence is 1912 bp in length, including 16 bp of 5'-untranslated region and 1986 bp of coding region. The cDNA is inserted in the EcoRI site of the plasmid pBR322 and supplied in the form of freeze-dried E. coli containing the plasmid. To prepare the plasmid, the dried E. coli was dissolved in 0.5 ml LB media and streaked onto a LB/Amp plate. After overnight incubation at 37^0C, a single colony was inoculated in 3 ml of LB media containing 50 μg/ml ampicillin. After overnight incubation at 37^0Cwith shaking (220 revolutions/min), the cell suspension was centrifuged at 3600 rpm for 5 min and the pellet was resuspended in 100 μl of ice-cold solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0) by vortexing vigorously. Two hundred and fifty μl of freshly prepared solution II (0.2

M NaOH and 1 % SDS) was added to the cell suspension and mixed by inverting the tube several times. At this stage, the cells were lysed. Then 150 μl of ice-cold solution II (3 M potassium acetate and 1.8 M formic acid) were added to precipitate the protein and bacterial DNA. The mixture was centrifuged at 14000 rpm (Eppendorf minifuge) for 5 min and the supernatant was transferred to a fresh Eppendorf tube. The supernatant ($\pm$ 400 μl) was extracted once with one volume of phenol-chloroform-isoamyl alcohol and once with pure chloroform. The plasmid DNA was then precipitated with 2 volumes of 100 % ethanol. After 30 to 60 min air drying at room temperature, the pellet was dissolved in 40 μl of TE buffer containing 20 μg RNAse/ml.

To isolate the cDNA insert from the vector, the plasmid was digested with restriction enzyme EcoRI at 37^{0}C for 2 hr. The insert (2.0 kb) was separated from the vector (4.4 kb) by electrophoresis using a 1 % low melting temperature agarose gel (Figure 9). The gel slice containing the insert band was cut from the gel under UV light and placed in an Eppendorf tube. The tube was heated to 65^{0}C for 10 min to melt the gel and the volume was estimated. Three volumes of distilled water were then added.

Labelling of the probe was carried out by the "random primer" method using a T7 Quick Prime Kit (Pharmacia). For each reaction, a total volume of 34 μl containing 50 to 100 ng of the probe DNA was first denatured by boiling for 5 min followed

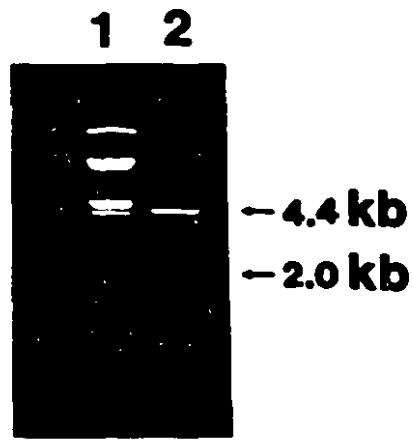

Figure 9. Purification of mouse plasminogen cDNA insert. The purified plasmid (MPL8-200) containing the insert was digested with EcoRI and the cDNA insert (2.0 kb) was separated from the vector (4.4 kb) by gel electrophoresis. Lane 1, Lambda DNA digested with EcoR I and Hind III; Lane 2, purified plasmid (MPL8-200) digested with EcoR I.

by chilling on ice. Then 10 μl of reagent mix containing dATP, dTTP, dGTP and the random primers, 5 μl of ^{32}P-dCTP and 1 μl of T7 DNA polymerase were added. The reaction mixture was incubated at 37^{0}C for 2 to 3 hr. The resulting radioactive probe was purified through a nick column (Pharmacia). The radioactivity was determined using a scintillation counter.

4.2.6 Hybridization

After washing with 2 x SSC, 0.1 % SDS, the filter was inserted into a hybridization tube and the prehybridization was carried out at 42^{0}C for at least 2 hr in 4 x SSPE (0.72 M NaCl, 40 mM sodium phosphate pH 7.4, 4 mM EDTA), 50 % deionized formamide, 1 % SDS, 5 μg/ml skim milk powder and 0.5 μg/ml herring sperm DNA. The hybridization was carried out overnight at 42^{0}C in 4 x SSPE, 10 % dextran sulfate, 50 % deionized formamide, 1 % SDS and 5 μg/ml skim milk powder with approximately 2.5-8 x 10^{6}cpm/ml ^{32}P-labelled probe (denatured before mixed with the hybridization buffer). Following the hybridization, the hybridization solution was poured off and the membrane was rinsed with 2 x SSC, 0.1 % SDS and then washed in 2 x SSC, 0.1 % SDS, 0.5 x SSC, 0.1 % SDS and 0.1 x SSC, 0.1 % SDS sequently. Each washing was at 42^{0}C for 15 min. A further washing was carried out in 0.1 x SSC, 0.1 % SDS at 52^{0}C for 30 min. After washing, the membrane was dried with paper towels and autoradiographed using Kodak X-ray film (X-OMAT. XAR-5) at - 70^{0}C with one intensifying screen for 2 to 3 days. The film was developed for 1 to 2 min

then fixed for at least 5 min followed by rinsing with tap water.

4.3 Results and discussion

A rapid method to determine restriction enzymes which can produce polymorphism with a particular probe is to pool DNA samples for 15 to 20 individuals and run the pooled sample side by side with one individual sample randomly chosen from the pool. Bands appearing in the pooled sample but missing in the individual sample is then indicative of the presence of polymorphisms in the pooled individuals. The initial experiment was carried out with this method. A pooled sample was prepared by mixing 15 DNA samples. Both the pooled sample and an individual sample out of the pool were digested with MspI, TaqI, SacI, BamHI. BglI, HincII and HinfI. The restriction patterns were shown in figure 10. Hybridization signals were observed with all the enzymes used except for HinfI. Although the signals were faint, they were still visible when the film was exposed for 72 hrs. All the bands appearing in the pooled sample also appeared in the individual sample, indicating that none of the enzymes revealed a polymorphism.

In the second experiment, 15 individual DNA samples were digested with the restriction enzymes MspI, SacI, and RsaI, respectively. After electrophoresis, hybridization and autoradiography, weak signals were observed on the MspI blot but no visible bands were observed on the SacI and RsaI blots. Interestly, the lambda

Figure 10. A pooled sample and an individual sample out of the pool each was digested with restriction enzymes MspI, TaqI, SacI, BamHI, BglI, HincII and HinfI, and hybridized with mouse plasminogen cDNA. The film was exposed at -70^{0}C for 72 hrs.

DNA marker was hybridized showing strong signals. DNA polymorphism was recognized with the MspI digestion (figure 11). All 15 individuals showed 2 low molecular size bands (±2.3 kb and ±1.2 kb) with the exception of 2 individuals which showed 2 slightly higher molecular size bands. In addition, the band intensity of the lower band in these 2 individuals was stronger than the corresponding band in the rest individuals.

The faint hybridization signals on the MspI blot and the failure of finding hybridization bands on the SacI and RsaI blots could be attributed to several factors. Low efficiency of probe labelling could be one of the reasons. However, the most probable reason could be due to the very low sequence homology between mouse and bovine plasminogen gene. Since the published sequence of the bovine plasminogen cDNA only comprise small portion of the gene, we were not able to compare the cDNA sequences of these two species. To get optimal hybridization signal, homologous probes are recommended.

Using mouse plasminogen cDNA as a probe, the present work identified DNA sequence polymorphisms in the bovine plasminogen gene with MspI digestion, although it needs to be further confirmed. The study did not show much sequence variations in the gene, but this does not exclude the possibilities of the presence of more polymorphisms in the gene. RFLPs may possibly be detected using other enzymes other than the 8 enzymes used, and also with increasing number of DNA

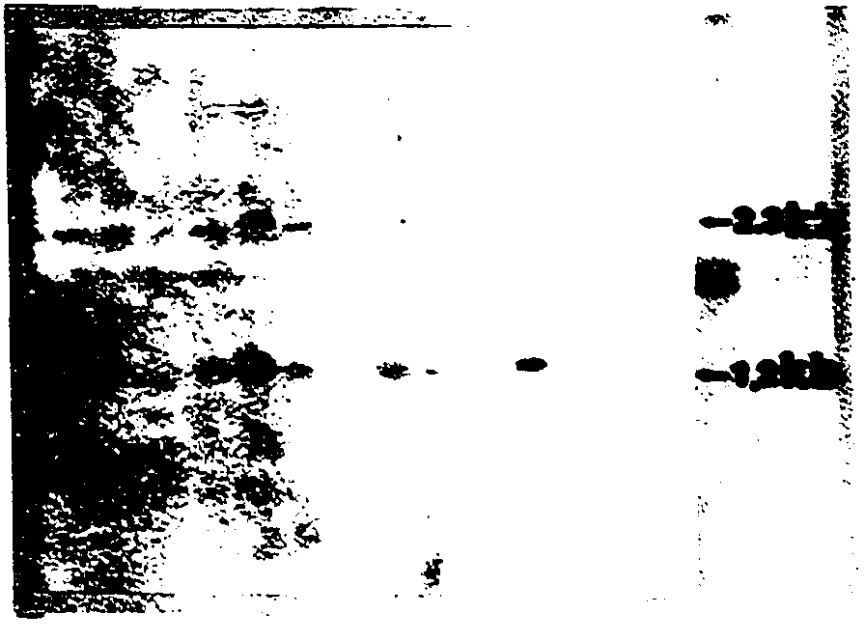

Figure 11. Fifteen individual cow DNA were digested with MspI and hybridized with mouse plasminogen cDNA. Two individuals showed different restriction pattern from the rest.

samples, the chance of finding polymorphisms will increase. In addition, the probe used is not a full length cDNA (missing 3'-UTR and part of the coding region). DNA polymorphism may be present in the missing sequence.

5. CONCLUSIONS

Plasmin degradation of milk casein can be reduced if plasminogen variants can be identified in dairy cattle which are less active. In this study, we have identified genetic polymorphism of plasminogen in dairy cattle both at protein and DNA levels. Pure bovine plasminogen was successfully isolated from cow plasma and antibodies to bovine plasminogen were prepared by immunizing rabbits with the isolated bovine plasminogen. By isoelectric focusing over a pH range of 3.5 - 9.5, followed by an immunological detection or a functional detection method, 2 common plasminogen variants (PLG A_2 and B_2), as well as 3 minor variants (D_2, E_2 and C_1) were identified in the 50 samples investigated. In addition, DNA polymorphism in the plasminogen gene was also identified with the MspI restriction enzyme digestion. This work represents the first description of plasminogen polymorphism in dairy cattle. Future research will describe the relative frequency of each variant in the population and describe their "proteolytic potential". The ability to select plasminogen phenotypes for dairy cattle with lower milk protease levels would lead to improvements in milk quality and processing attributes.

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