Genetic polymorphism of plasminogen in dairy cattle

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Plasmin (PLM; E.C. 3.4.4.14) is the major proteolytic enzyme normally present in bovine milk. From a technological point of view, PLM activity in milk is detrimental as it increases the proteolysis of casein to proteose peptones, and this results in reduced storage time, taste defects, loss of cheese yield and quality, and changes in the physicochemical properties of milk (for review, see Fox, 1992). Therefore a reduction of PLM activity in milk would be desirable. Plasminogen (PLG), the zymogen of plasmin, and PLM content are affected by several physiological and environmental factors, and by genetic factors such as breed (Richardson, 1983; Schaar, 1985; Politis *et al*. 1989; Benslimane *et al*. 1990). We have addressed the question of PLG polymorphism in dairy cattle.

MATERIALS AND METHODS

Bovine PLG was purified from plasma, obtained from citrate-treated blood, using an affinity chromatography micro-scale method adapted from Deutsch & Mertz (1970). Plasma samples (20 μl), collected from Holstein ($n = 30$) and Ayrshire ($n = 20$) cows from the Macdonald Campus herd, were mixed and incubated with 40 μl L-Lys-Sepharose gel (Pharmacia Biotech Inc., Baie D'Urfé, Canada H9X 3V1) for 30 min at 4 °C. The L-Lys-Sepharose gel matrix, which binds PLG, was washed three times with 400 μl 0·5 M-NaCl–50 mM-Tris–10 mM-$ZnSO_4$, pH 8 and treated with 30 μl *Clostridium perfringens* neuraminidase (E.C. 3.2.1.18, 5 i.u./ml, Type V; Sigma, St Louis, MO 63178, USA) for 3 h at room temperature. Neuraminidase treatment is necessary for phenotyping PLG by isoelectric focusing polyacrylamide gel electrophoresis (IEF–PAGE) because PLG is a glycoprotein that carries varying amounts of sialic acid on oligosaccharidic side chains (Skoda *et al*. 1986); the presence of this sialic acid results in charge heterogeneity that interferes with phenotyping. Preliminary experiments demonstrated that at least three glycosylation isoforms of PLG were efficiently eliminated by a treatment using 0·15 i.u. neuraminidase in each reaction mix. The IEF pattern of PLG was not modified by increasing the amount of neuraminidase added up to 0·25 i.u. (results not shown). The gel matrix was then washed successively with 400 μl 0·5 M-NaCl–50 M-Tris–10 mM-$ZnSO_4$, pH 8 and 400 μl IEF loading buffer (containing, g/l, Ampholine pH 3·5–9·5, 48; 3-((3-cholamidopropyl)dimethylammoniol)-1-propanesulphonate (CHAPS) 2;

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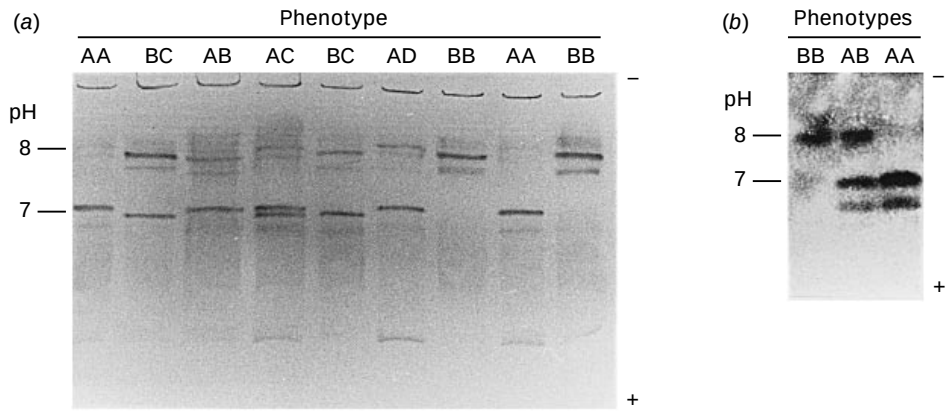

Fig. 1. Bovine plasminogen phenotyping by detection of plasminogen isoforms after isoelectric focusing–PAGE fractionation. (*a*) Immunofixation of plasminogen with a rabbit serum anti-bovine plasminogen and visualization by staining with a horseradish peroxidase-conjugated goat Ig anti-rabbit IgG and 4-chloro-1-naphthol as substrate. The capital letters indicate the type of isoforms. Plasminogen was prepared from plasma from three different Holstein (plasminogens AA and AD) and six different Ayrshire (plasminogens AB, AC, BB and BC) cows. (*b*) Caseinolytic overlay method: after activation of plasminogen to plasmin with an exogeneous plasminogen activator, clear proteolysed areas in a milky casein background that can be seen after precipitation with trichloroacetic acid of the casein overlay are produced by active plasmin.

bromphenol blue, 10). All materials for IEF–PAGE were from Bio-Rad Laboratories (Mississauga, Canada L4Z 1N9). PLG was eluted from L-Lys-Sepharose with 40 μl ϵ-aminocaproic acid (26 g/l, Sigma) in IEF loading buffer. IEF–PAGE was carried out essentially as described by Robertson *et al.* (1987). For immunostaining, the proteins were electrotransferred on to a nitrocellulose membrane for 60 min at 100 V in acetic acid (7 ml/l) using a Trans-Blot electrophoretic transfer cell. PLG protein bands were visualized using a rabbit serum anti-bovine PLG (diluted 1:3000) and a goat anti-rabbit immunoglobulin–horseradish peroxidase conjugate assay kit (Bio-Rad). The antiserum was generated in rabbits by four monthly intramuscular injections of 3 mg PLG in physiological saline mixed 1:1 with Freund's complete adjuvant (Sigma) (first injection) or Freund's incomplete adjuvant (all other injections). The caseinolytic overlay technique described by Skoda *et al.* (1986) with urokinase (E.C. 3.4.21.31; Sigma) as PLG activator was used for *in situ* analysis of proteolytic activity.

RESULTS

Four isoforms of PLG were found in our population, differing by the pI of the main protein band on IEF–PAGE (PLG A, pI = 7·0; PLG B, pI = 7·8; PLG C, pI = 6·8; PLG D, pI = 8·0; Fig. 1*a*). A minor band was usually present with a pI $\sim$ 0·2 lower than the pI of the main band, possibly corresponding to an only partly desialized PLG. The *in situ* proteolytic activity test for PLG A and PLG B, the most common isoforms, demonstrated that the main protein bands on IEF–PAGE had PLM activity (Fig. 1*b*); similar results were obtained for the other isoforms. Minor protein bands can also be proteolytically very active as was the case for PLG A (Fig. 1*b*). We detected six different phenotypes (Table 1).

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Table 1. *Distribution of bovine plasminogen phenotypes among a population of Holstein and Ayrshire cows*

	Phenotypes						
	AA	BB	AB	BC	AD	AC	Total
No. of cows							
Holsteins	17	3	3	4	2	1	30
Ayrshires	1	10	4	4	0	1	20

DISCUSSION

Genetic polymorphism of PLG was first described by Hobart (1979) and Raum *et al*. (1980) in humans. Moreover, the nature of these polymorphic forms can affect both the PLM activity and the activation process of PLG (Wohl *et al*. 1979). The present study is the first to demonstrate PLG polymorphism in dairy cattle. Our results also suggest that there may be important differences in the frequency of distribution of phenotypes between breeds. Of the PLG AA phenotypes, most were Holstein (17 out of 18), while 10 out of 13 Ayrshire cattle exhibited the PLG BB phenotype (Table 1). Further research should determine whether PLG phenotype can be associated with PLG content and/or PLM activity.

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