Differential titration of Plasmin and Plasminogen in milk using sandwich ELISA with monoclonal antibodies

Didier Dupont, Jeanne Grosclaude, Christelle Bailly, Jean-Claude Collin

▶ **To cite this version:**

Didier Dupont, Jeanne Grosclaude, Christelle Bailly, Jean-Claude Collin. Differential titration of Plasmin and Plasminogen in milk using sandwich ELISA with monoclonal antibodies. Journal of Dairy Research, 1997, 64. hal-04301377

HAL Id: hal-04301377
https://hal.inrae.fr/hal-04301377

Submitted on 23 Nov 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Differential titration of Plasmin and Plasminogen in milk using sandwich ELISA with monoclonal antibodies

By DIDIER DUPONT*, CHRISTELLE BAILLY*, JEANNE GROSCLAUDE† AND JEAN-CLAUDE COLLIN*

** I.N.R.A., Station de Recherches en Technologie et Analyses Laitières, B.P.89, F 39801 POLIGNY Cedex*
† I.N.R.A., Station de Virologie et Immunologie Moléculaires, F 78352 JOUY-EN-JOSAS Cedex

Short title : *Plasmin-Plasminogen titration in milk*

Name and address for correspondence:

Didier DUPONT
INRA-SRTAL
BP 89
39801 POLIGNY Cedex
FRANCE

SUMMARY. Two sandwich ELISA have been developed for quantitation of bovine milk Plasminogen and Plasmin. The assays used two monoclonal antibodies, one specific for Plasminogen and the other specific for Plasminogen+Plasmin. Plasmin concentration was obtained by subtracting the Plasminogen concentration from the Plasminogen+Plasmin concentration. The assays were sensitive (linear range 5-75 ng/ml), repeatable (coefficient of variation = 8% and 5% for plasmin and plasminogen titration respectively), specific (no cross-reactivity with the major milk proteins) and directly applicable to skimmed milk with no particular pretreatment of the sample. However, ELISA did not allow complete titration of milk plasminogen and plasmin (only 60-90% could be quantified). This lack in accuracy was due to interference of casein with plasmin-plasminogen titration by ELISA. Results obtained with ELISA or an enzymic technique on twenty milk samples collected from individual cows milked throughout pregnancy showed that the ELISA was particularly suitable for the analysis of late lactation or mastitic milk where proteinase inhibitors interfered with enzymic quantitation.

Plasminogen (PLG) is a component of the fibrinolytic system and is the precursor of a serine-proteinase, plasmin (PLM) (EC 3.4.21.7), which displays an activity similar to that of trypsin (Wallen & Iwanaga, 1968). PLM/PLG activation systems are involved in a number of physiological and pathological processes. In particular, they take part in the involution of mammary glands at the end of lactation (Ossowski *et al*. 1979).

Activation of PLG (mol. wt~88 kDa) to PLM causes the release of 8 kDa peptide at the N-terminal end of the molecule, referred to as the preactivation peptide (Castellino & Powell, 1981). Important conformational changes are induced during activation of PLG to PLM (Wallen & Wiman, 1975).

The PLM/PLG system is exported through the mammary gland to the milk and therefore affects the properties of milk and milk products during storage (Humbert & Alais, 1979). In bovine milk, β and α_{s2}-caseins are rapidly cleaved by PLM, α_{s1}-casein is more slowly attacked, whereas κ-casein and whey proteins are resistant (Le Bars & Gripon, 1993). During UHT treatment of milk and Swiss-type cheese production, some enzymes are partially or totally inactivated as a result of heating. In contrast, PLM remains unchanged during heat treatment and its activity in milk even seems enhanced (De Rham & Andrews, 1982). Determination of potential PLM activity by differential titration of PLG and PLM is thus important in dairy technology.

PLM activity is determined by different enzymic methods, but the results found in the literature vary greatly due to the fixation of this enzyme to the caseins (Richardson & Pearce, 1981 ; Rollema *et al*. 1983). Total PLG and PLM has been titrated using cross-reacting polyclonal antibodies in an ELISA test (Collin *et al*. 1988). However, such antibodies cannot differentiate PLG from PLM. We have recently succeeded in producing monoclonal antibodies (Mabs) specific for PLG and for PLM+PLG (Dupont *et al*. 1994).

In this report, we describe two sandwich ELISA tests for the quantitation of PLG and PLM+PLG in milk. The PLM value is obtained by subtraction of PLG value from PLM+PLG value.

MATERIALS AND METHODS

Milk samples

Pre-treatments on commercial raw milk were used to define the optimal conditions for PLG and PLM titration. This milk was skimmed by centrifugation at 1000 ***g*** and 4°C for 15 min (J2-21 M/E Beckman Instruments, Gagny, France) or prepared using the method of Richardson & Pearce (1981), or treated as described by Korycka-Dahl *et al*. (1983) for dissociation of PLM/PLG from caseins.

Twenty milk samples were aseptically collected, at various stages of lactation, from individual cows milked throughout pregnancy. Samples 1 to 7 (early lactation milks) were collected 2 months after calving, samples 8 to 14 (mid lactation milks) were collected between the 3rd and the 9th month of the lactation period whereas samples 15 to 20 were late lactation milks collected 1 to 6 weeks before calving i.e. during the usual dry period observed by the milk producers. These samples were defatted by centrifugation, divided into 1 ml fractions and frozen at -20°C after addition of 0.01g/l thimerosal.

Bovine plasma

Bovine blood was obtained intravenously and mixed with 0.13 M sodium citrate (9v/v) to avoid clotting. Plasma was obtained by centrifugation at 4000***g*** and 4°C for 30 min. This plasma was used for antigen purification and was submitted to plasminogen titration by ELISA.

Antigen purification

PLG was isolated from bovine plasma using the method previously described by Deutsch & Mertz (1970) slightly modified. Briefly, PLG was obtained by affinity chromatography on a Lysine-sepharose column (150 x 50 mm) by specific elution with 0.05 M Phosphate Buffer pH 7.2, 1 mM Zinc acetate, 0.2 M ε-amino caproic acid. PLG was further purified by size-exclusion chromatography on Superose 12 column (Fast-Protein Liquid Chromatography system, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with 0.05 M Phosphate Buffer pH 7.2.

PLM was obtained by activation of purified PLG with the PLG activator urokinase (EC 3.4.21.31 ; Sanofi Winthrop, Gentilly, France) at 1000 IU/ml and 37°C for 15 min, followed by size-exclusion chromatography on a Superose 12 column equilibrated with 0.05 M Phosphate Buffer pH 7.2.

Polyclonal antibody production

A rabbit was injected subcutaneously with 0.5 mg purified PLM emulsified in Freund's complete adjuvant (Difco, Detroit, MI, 48232 USA) in multiple sites. Two booster injections were given in Freund's incomplete adjuvant (Difco) at successive intervals of 21 and 15 d. The rabbit was bled 10 d after the last booster injection. After blood clotting, polyclonal serum was obtained by centrifugation at 1800 ***g*** and 4°C for 10 min. The supernatant was recovered, divided into subsamples and stored at -20°C. Specificity of the antiserum produced was assessed by double-immunodiffusion as previously described (Ouchterlony, 1949).

Monoclonal antibody production

Murine monoclonal antibodies were raised using purified PLG as antigen and characterized as described previously (Dupont *et al*. 1994). PLG specific monoclonal antibody

41(37) (Mab antiPLG) was used for PLG specific titration and the cross-reacting monoclonal antibody 1245(37) (Mab antiPLG-PLM) for PLM+PLG quantification.

Assay of plasmin and plasminogen

PLM/PLG activity in milk was determined using the method of Rollema *et al.* (1983). Briefly, a sample of skimmed milk (25 µl) was diluted to 250 µl using 40 mM Tris-HCl buffer, pH 7.4, containing 40 mM KCl, 100 mM EDTA, 50 mM aminocaproic acid and 0.6 mM chromogenic substrate H.D-valyl-L-leucyl-L-lysyl-4 nitroanilide (Serva, Heidelberg, Germany). Absorbance at 405 nm (A_{405}) was measured as a function of time. Change in absorbance per unit of time (dA_{405}/dt) was taken as a measure of PLM activity.

To determine the PLG level, 10 µl of 1000 IU/ml urokinase were added to the reaction mixture. After 15 min incubation, A_{405} was monitored again as a function of time. The urokinase-induced increase of dA_{405}/dt relative to the dA_{405}/dt due to PLM was taken as a measure of the PLG level in the sample. Absorbance measurements were made at 37°C using a DU 64 spectrophotometer (Beckman Instruments, Inc., Fullerton, USA) equipped with a thermostable cell holder. Each milk sample was analysed in triplicate.

PLM and PLG titrations of samples 15 to 20 were also carried out using the enzymic method of Richardson & Pearce (1981). Milk samples (3 ml) were diluted with 0.4 M- sodium citrate solution (to dissociate the casein micelles), centrifuged at 27000 ***g*** for 10 min and the PLM content of the clear supernatants was usually assayed without further treatment. PLM activity was determined using a modification of the N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (coumarin peptide) assay. Briefly, PLG was determined as the increase in PLM content of the samples induced by the inclusion of urokinase in the assay mixture : 0.25 ml citrate-treated milk, 0.05 ml urokinase solution (1000 IU /ml) and 0.2 ml 0.05 M-Tris-HCl buffer pH 8.5 containing 0.02 M-lysine, 0.14 M-NaCl and 500 ml/l glycerol were incubated for 1 h at 37°C, prior to assaying for total PLM activity. Care was taken to

ensure that the concentration of casein in the assay mixtures remained the same for all samples.

ELISA assay

Flat-bottomed ELISA plates were coated with Mab antiPLG or Mab antiPLG-PLM diluted 1/3500 and 1/5000 respectively in 0.1 M bicarbonate buffer pH 9.6 (100 µl per well) and incubated for 2h at 37°C. Blocking of the remaining binding sites was performed with 10 g/l gelatin (Sigma, St Louis, 63178 USA) in 0.05 M phosphate buffer pH 7.2, 0.15 M NaCl /0.05 ml/l Tween 20 (PBS-T). Serial dilutions of purified PLG and PLM solutions (0-100 ng/ml) in 0.4 M tri-Sodium Citrate/0.5 ml/l Tween 20 (TSC-T) were used as standards (each value obtained was the result of five independent determinations). Milk or plasma samples diluted in TCS-T (4 dilutions from 1/100 to 1/1000, 75 µl), or PLM and PLG standards were incubated in test tubes with 75 µl of a 1/125 dilution of the rabbit antiserum against PLM+PLG for 1.5 h at 37°C. TCS-T caused dissociation of the casein micelles and was used for untreated as well as for treated milk samples.

The mixture (100µl) was then added to each ELISA plate well and further incubated for 1.5 h at 37°C. After washing, bound immune complexes were revealed by a Goat anti-Rabbit Immunoglobulins-alkaline phosphatase conjugate (Sigma) diluted 1/3000 in PBS-T. P-Nitrophenyl phosphate (PNPP, 100µl) at 1 mg/ml was used as substrate. Absorbance was measured at 405 nm using a Titertek Multiskan Autoreader (Life Science International, Cergy-Pontoise, France). PLG concentration was obtained directly whereas PLM concentration was determined by subtracting PLG from PLG+PLM concentration.

Accuracy and specificity of the ELISA assay

Accuracy of the method was studied by addition of different amounts of purified PLG (500, 1000 and 5000 ng/ml) to a commercial milk sample. PLG and PLM+PLG concentrations were determined before and after this addition as described above, and results were expressed as a percentage of recovery of the amount of PLG added.

Specificity of the method was studied by testing both ELISA assays for their possible cross-reactivity against five major milk proteins : purified caseins, immunoglobulins G, β-lactoglobulin, α-lactalbumin and BSA (Sigma). Each of these proteins (1 µg), or 50 ng of PLG was added to 1 ml milk samples and PLG and PLG+PLM were titrated by ELISA on these samples as described above.

Efficiency of urokinase-induced plasminogen activation

Efficiency of urokinase-induced plasminogen activation was studied by determining residual PLG in milk after urokinase-induced activation i.e. to determine whether urokinase-induced activation of PLG in milk was partial or total after a 15 min incubation. Urokinase (1000 IU/ml, 2µl) was added to 200 µl of skimmed milk and incubated at 37°C. The activation was stopped after 15 min by addition of 4 µl of 4 M acetic acid as described by Humbert (1986). Then, PLM and PLG concentrations were determined by the sandwich ELISA test described above, and the results compared to those obtained before activation.

RESULTS

Polyclonal antibody specificity

Double-immunodiffusion used for determination of polyclonal antibody specificity showed that this polyclonal serum was PLG-PLM cross-reacting (results not shown).

Sandwich ELISA

Binding of purified PLM and PLG was determined using Mab antiPLG and Mab antiPLG-PLM as capture reagents in the sandwich ELISA test. With Mab antiPLG, the standard curve obtained with purified solutions of PLG showed a linear detection range between 5 and 75 ng/ml with PLG. Only background values were observed with PLM confirming that this antibody only recognized PLG (Fig. 1). With Mab antiPLG-PLM, the standard curve showed a linear range with purified PLM as well as PLG between 5 and 75 ng/ml. The slopes of the curves obtained with these two antigens were similar showing that PLM and PLG were recognized similarly by this antibody (Fig. 1).

Specificity of the method towards the plasmin/plasminogen system

Among the six different milk proteins tested, only PLG reacted in ELISA (Table 1). No cross-reactivity with other proteins was observed.

Measurement of the efficiency of urokinase-induced plasminogen activation

Incubation of a milk sample with 1000 IU/ml urokinase caused complete disappearance of immunoreactive PLG titratable by ELISA in the sample (793 ng/ml before activation, 0 ng/ml after activation) and, in the same time, appearance of an equivalent amount of PLM (474 ng/ml before activation, 1232 ng/ml after activation). Urokinase at 1000 IU/ml allowed total activation of PLG to PLM. These results also demonstrated that ELISA only quantified the PLG-PLM system, since specific activation of PLG by urokinase caused total disappearance of any signal with Mab antiPLG.

Treatment of the milk sample before titration

PLG concentration was determined in triplicates on commercial whole milk and on the same milk previously skimmed or treated according to Korycka-Dahl *et al*. (1983) or to Richardson & Pearce (1981). Results were expressed as means with SD. PLG concentration determined on a commercial skimmed milk by ELISA was 0.39±0.01 µg/ml. Treatment of this sample using the methods of Korycka-Dahl *et al*. (1983) and Richardson & Pearce (1981) resulted in loss of respectively 20 and 12% of the PLG.

PLG concentration determined on the original whole milk was 0.34±0.004 µg/ml. Thus, skimmed milk showed a higher concentration of PLG than the whole milk, probably due to milk concentration by skimming.

Addition of exogenous plasminogen to a milk sample

The percentage of purified PLG recovered after addition of 0.5, 1 and 5 µg/ml to a milk sample was 60, 65 and 90% respectively. These results show that PLG titration by sandwich ELISA was partially inhibited, probably because of binding of PLG to proteins, and this interference was more sensitive on low PLG milks. In order to clarify this point, PLM and/or PLG titration was carried out on a sample of purified PLM or PLG, before and after

addition of each of the five major milk proteins (casein, β-lactoglobulin, α-lactalbumin, BSA and immunoglobulin) at their concentration in milk (26.5, 2.9, 1.3, 0.3 and 0.7 mg/ml respectively). The results obtained showed that inhibition of PLM/PLG recognition by the monoclonal antibodies was almost exclusively due to casein (Table 2).

Determination of plasmin and plasminogen concentrations in milk and plasma

A total of twenty milk samples submitted to no pre-treatment other than skimming was tested for PLM and PLG quantitation via two different techniques : enzymic measurement of PLM/PLG activity according to Rollema *et al.* (1983) chosen as the reference method, and PLM and PLG concentration measurement by ELISA. Important differences were observed between the two methods, especially for PLG titration (Table 3). PLG concentrations of the milks 15-20 determined by ELISA were much higher than those obtained with the enzymic method. PLG titration carried out again on the six late lactation milks with the method of Richardson & Pearce (1981) confirmed the results previously found with the method of Rollema *et al*. (results not shown).

Enzymic titration of PLM+PLG with the method of Rollema *et al* (1983) in bovine plasma did not allow detection of any PLG, even though plasma is known to contain very high amounts of PLG . In contrast, the PLM+PLG concentration determined by ELISA in plasma was found to be 192 μg/ml. This result is in agreement with the values found in literature (Miyashita *et al*. 1988).

Repeatability of the sandwich ELISA

Assay precision was estimated from replicates of PLG/PLM titration on 20 individual milks (Table 3). Coefficients of variation for plasmin and plasminogen titration by ELISA in milk were 8 and 5% respectively.

DISCUSSION

We have developed a new ELISA method to quantify specifically PLM and PLG in milk. A PLG specific monoclonal antibody (Mab antiPLG) used as a capture reagent allowed specific titration of PLG whereas a cross-reacting monoclonal antibody (Mab antiPLG-PLM) allowed quantification of the PLM+PLG system. Because PLM and PLG concentrations in herd milk fluctuate between 300 and 1460 ng/ml (Benslimane *et al.* 1990), the ELISA assay appeared to be of sufficient sensitivity to allow determination of PLG and PLM+PLG in milk, even though sensitivity of the assay in milk would probably be lower than in purified solutions. The low limit of detection of the ELISA test required a dilution of the milk samples to be analysed, sufficient to limit the influence of the high protein content of milk on the results, in most cases.

However, sandwich ELISA did not allow titration of the total amount of PLG and/or PLM in a milk sample. This problem might be overcome by using different dispersant buffers such as urea buffers for milk dilution, or by separating PLG and PLM from casein before titration using components such as ε-amino caproic acid or lysine. Such work is currently in progress in our laboratory. These components have been used previously by some authors to dissociate PLG and PLM from casein and their use in ELISA would probably not interfere with antigen-antibody reaction. However, some authors have recently pointed out that these compounds do not allow significant dissociation of PLG/PLM from casein (Baer *et al.*, 1994). Richardson & Pearce (1981) have shown that by using their spectrophotometric method, concentrations in dairy products could be calculated from the measured apparent activities using a correction factor of 2.5 to account for the apparent effect of casein on PLM activity. In this article, we have demonstated that caseins were not recognized in either ELISA tests but that they clearly inhibited the antigen-antibody reaction. It is then possible that binding of PLM and PLG to caseins obscured the epitope recognized by the monoclonal antibodies Mab antiPLG and Mab antiPLG-PLM. However, it should be possible to increase the percentage recovery of exogenous added PLG by producing new monoclonal antibodies with higher

affinities for PLM and PLG. It might also be possible to calculate correction factors that could be applied to the apparent PLG and PLM concentrations found by ELISA to bring them near to the real values. Anyway, milks with high concentrations of PLG and/or PLM could be further diluted for their titration so that recognition of the antigen by the antibody is not inhibited by other milk proteins.

By using sandwich ELISA, PLM was determined by subtraction of the PLG concentration from the PLM+PLG concentration. In order to obtain direct results, a PLM specific monoclonal antibody must be produced that could give directly the amount of PLM in milk. Such work is currently in progress within our laboratory.

For this study, milk samples were collected from individual cows milked throughout pregnancy as it was thought that variations of PLM and PLG concentrations in these milks were more important than in bulk milks. But, we also demonstrated that sandwich ELISA may be applied to titration of PLM and PLG in commercial milks : PLG concentration determined on a commercial skimmed milk was in agreement with the published values (Schaar, 1985). PLG concentration in commercial milk was lower than most of the PLG concentrations obtained on individual milks by ELISA, but these values are not directly comparable because the samples were from different origins.

PLM and PLG are currently quantified in milk by measuring the PLM activity by enzymic methods (Richardson & Pearce, 1981 ; Rollema *et al.* 1983), but the results obtained are highly dependent on the proteinase inhibitors present in the sample (Schaar & Funke, 1986) and on the binding of PLM and PLG to the caseins (Richardson & Pearce, 1981). The immunochemical technique developed by Collin *et al.* (1988) is very sensitive but is unable to quantify differentially PLM and PLG. Thus, the method developed here was compared to the enzymic technique of Rollema *et al.* (1983). This procedure was chosen because it is the only one which allows PLM/PLG quantitation directly on skimmed milk without pretreatment i.e., identical conditions to these used for the ELISA assay. Results for the two methods could be directly compared without taking into consideration the different pretreatments of the milk samples. Important differences observed between the two methods for the PLG concentration of six particular samples cannot be explained by an incomplete urokinase-induced activation

of PLG. It must be noted that these six samples were collected at a very late stage in lactation when the PLM and PLG contents are supposed to increase dramatically (Schaar, 1985). One of these samples (15) was even bloody and therefore it was not surprising to find an extremely high PLG concentration, close to that found in bovine blood. In contrast, no large increase in the PLG concentration was found with these six samples using the method of Rollema *et al.* (1983). The method of Rollema *et al*. (1983) measures an enzymic activity whereas the ELISA technique quantifies directly the molecules of PLM and PLG. In milk, it is well known that PLM activity is a function of an equilibrium between PLM, PLG, PLG activators, PLM inhibitors and inhibitors of PLG activators (Grufferty & Fox, 1988). It has been reported that the presence of PLM inhibitor in milk strongly reduces the activity of the enzyme (Grufferty & Fox, 1988). It has also been demonstrated that, at the end of lactation, exchanges between the blood system and milk through the mammary glands are very important (Richardson, 1983). It is possible that some of the fibrinolytic components pass into the milk through the mammary gland and the presence of a high concentration of PLM inhibitors coming from blood would interfere with the enzymic determination of PLM activity in milk. Moreover, plasminogen quantitation in plasma was not possible in this study using the enzymic method of Rollema *et al* (1983). Mussoni *et al*. (1979) have shown that plasma has to be acidified for PLM titration in order to obtain a measurement that is unaffected by the acid-labile plasma proteinase inhibitors. However, even after acidification, PLM activity of plasma is still inhibited, proving that plasma also contains acid-stable proteinase inhibitors.

Immunochemical quantitation by sandwich ELISA allowed differential determination of PLM and PLG in a milk sample, without taking into consideration the amount of the different inhibitors and activators of the fibrinolytic system. Thus, this technique allowed analysis of milks containing high concentrations of PLG, such as late lactation or mastitic milks in which PLG is likely to be activated into PLM, causing intense proteolysis of the sample. In contrast to the enzymic assays the ELISA allowed determination of the antigenic presence of PLM and PLG without taking into consideration proteinase inhibitors. Anyway, it is in the understanding of the mechanism responsible for PLG activation that this technique could be very useful. Study of UHT milks during storage with this method could show

whether PLM is involved in age-gelation of UHT milks. If we succeed in overcoming the inhibition by casein, it is planned to apply this method to PLM and PLG quantitation in hard cheeses in order to determine the effect of PLM on cheese ripening and it could also be of great interest for PLM and PLG titration in blood.

This study received the financial support of the Regional Council of Franche-Comte (Grant 90682) and the Ministry of Agriculture (Grant P10309). The authors would like to thank B. Remond (INRA, Theix, France) for providing milk samples and are also grateful to R. Grappin (INRA, Poligny, France) and D. Levieux (INRA, Theix, France) for comments on the manuscript.

REFERENCES

BAER, A., RYBA, I. & COLLIN, J.-C. 1994 Binding of bovine plasminogen to immobilized casein and its activation thereon. *International Dairy Journal* **4** 597-616

BENSLIMANE, S., DOGNIN-BERGERET, M.J., BERDAGUE, J.-L. & GAUDEMER, Y. 1990 Variation with season and lactation of plasmin and plasminogen concentrations in Montbeliard cow's milk. *Journal of Dairy Research* **57** 423-435

CASTELLINO, F.J. & POWELL, J.R. 1981 Human plasminogen. *Methods in Enzymology* **80** 365-378

COLLIN, J.C., COMPAGNONE, P., RYBA, I. & BAER, A. 1988 Determination of plasmin (alkaline milk proteinase) and chymosin in milk products by the ELISA assay. *Le Lait* **68** 235-240

DE RHAM, O. & ANDREWS, A.T. 1982 The roles of native milk proteinase and its zymogen during proteolysis in normal and bovine milk. *Journal of Dairy Research* **49** 577-585

DEUTSCH, D.G. & MERTZ, E.T. 1970 Plasminogen : purification from human plasma by affinity chromatography. *Science* **170** 1095-1096

DUPONT, D., LABIAU, S., GROSCLAUDE, J. & COLLIN, J-C. 1994 Production and characterization of specific bovine plasminogen monoclonal antibodies. *Hybridoma* **13** 543-547

GRUFFERTY, M.B. & FOX, P.F. 1988 Milk alkaline proteinase. *Journal of Dairy Research* **55** 609-630

HUMBERT, G. & ALAIS, C. 1979 Review of the progress of dairy science. The milk proteinase system. *Journal of Dairy Research* **46** 559-571

HUMBERT, G. 1986 [The milk alkaline protease (plasmin) : titration, purification and dairy technology applications]. *Thesis* Université de Nancy, France

KORYCKA-DAHL, M., RIBADEAU-DUMAS, B., CHENE, N. & MARTAL, J. 1983 Plasmin activity in milk. *Journal of Dairy Science* **66** 704-711

LE BARS, D. & GRIPON, J.-C. 1993 Hydrolysis of α_{s1}-casein by bovine plasmin. *Le Lait* **73** 337-344

MIYASHITA, C., WENZEL, E. & HEIDEN, M. 1988 Plasminogen : a brief introduction into its biochemistry and function. *Haemostasis* **18** (suppl.1) 7-13

MUSSONI, L., RACZKA, E., CHMIELEWSKA, J., DONATI, M.B. & LATALLO, Z.S. 1979 Plasminogen assay in rabbit, rat and mouse plasma using the chromogenic substrate S 2251. *Thrombosis Research* **15** 341-349

OSSOWSKI, L., BIEGEL, D. & REICH, E. 1979 Mammary plasminogen activator : correlation with involution, hormonal modulation and comparison between normal and neoplastic tissue. *Cell* **16** 929-940

OUCHTERLONY, O. 1949 Antigen-antibody reaction in gels. *Acta Pathologica et Microbiologica Scandinavica* **26** 507

RICHARDSON, B.C. & PEARCE, K.N. 1981 The determination of plasmin in dairy products. *New Zealand Journal of Dairy Science and Technology* **16** 209-220

RICHARDSON, B.C. 1983 Variation of the concentration of plasmin and plasminogen in bovine milk with lactation. *New Zealand Journal of Dairy Science and Technology* **18** 247-252

ROLLEMA, H.S., VISSER, S. & POLL, J.K. 1983 Spectrophotometric assay of plasmin and plasminogen in bovine milk. *Milchwissenschaft* **38** 214-217

SCHAAR, J. 1985 Plasmin activity and proteose-peptone content of individual milks. *Journal of Dairy Research* **52** 369-378

SCHAAR, J. & FUNKE, H. 1986 Effect of subclinical mastitis on milk plasminogen and plasmin compared with that on sodium, antitrypsin and N-acetyl-β-D-glucosaminidase. *Journal of Dairy Research* **53** 515-528

WALLEN, P. & IWANAGA, S. 1968 Differences between plasmic and tryptic digests of human S sulfo-fibrinogen. *Biochimica et Biophysica Acta* **154** 414-417

WALLEN, P. & WIMAN, B. 1975 On the generation of intermediate plasminogen and its significance for activation. *Cold Spring Harbor Conferences on Cell Proliferation* **2** 291-303

Table 1. *Cross-reactions with five milk proteins of sandwich ELISA for plasminogen and plasmin+plasminogen titrations*

	Absorbance at 405 nm	
Proteins	PLG[❼] titration	PLG+PLM[❼] titration
Plasminogen	1.32	1.36
Immunoglobulin G	0.04	0.01
β-lactoglobulin	0	0.04
α-lactalbumin	0	0.02
BSA	0	0
Caseins	0.01	0

[❼]PLM, plasmin ; PLG, plasminogen

Table 2. *Percentage of reduction of plasminogen and plasmin+plasminogen concentrations measured after addition of different milk proteins*

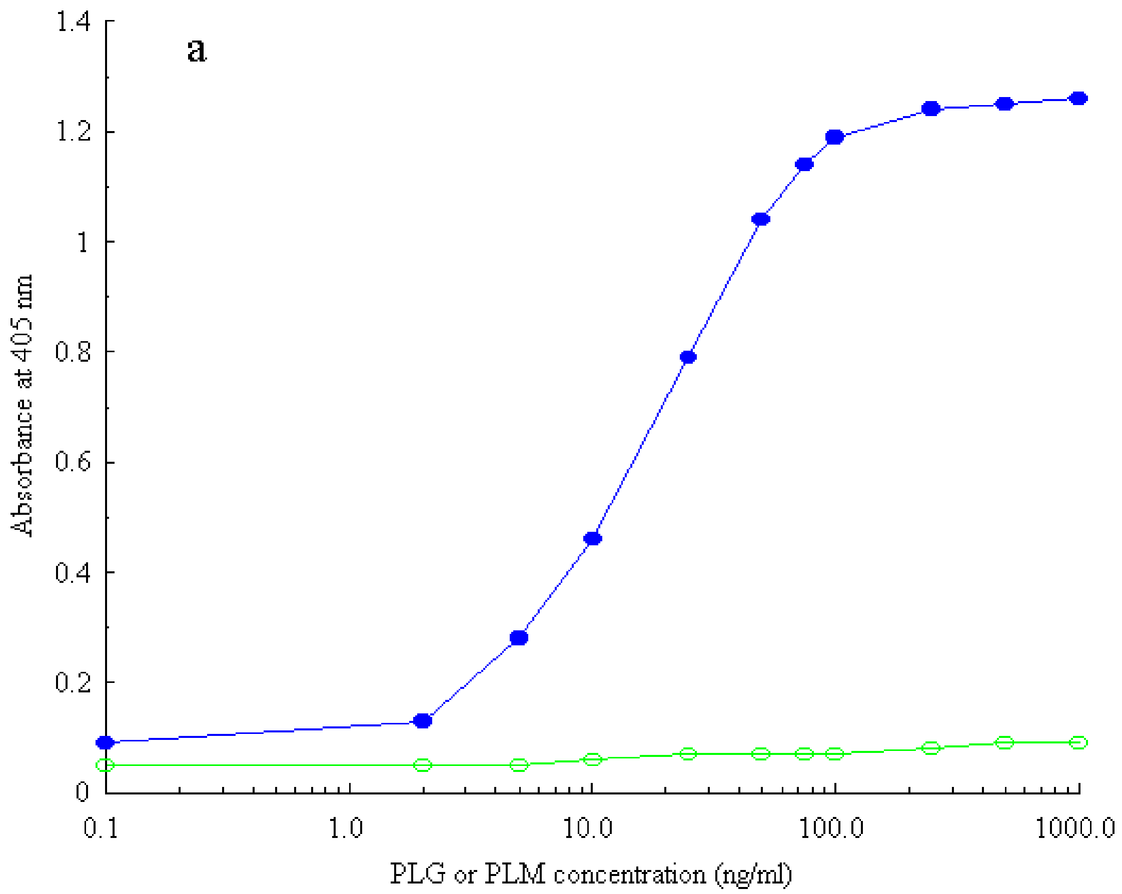
Milk proteins added to the PLG or PLM+PLG[❼] solutions (mg/ml)	Plasminogen titration	Plasmin+plasminogen titration
Caseins (26.5)	-37%	-33%
β-Lactoglobulin (2.9)	-1%	0%
α-Lactalbumin (1.3)	0%	0%
BSA (0.3)	0%	0%
Immunoglobulins (0.7)	-1%	-5%

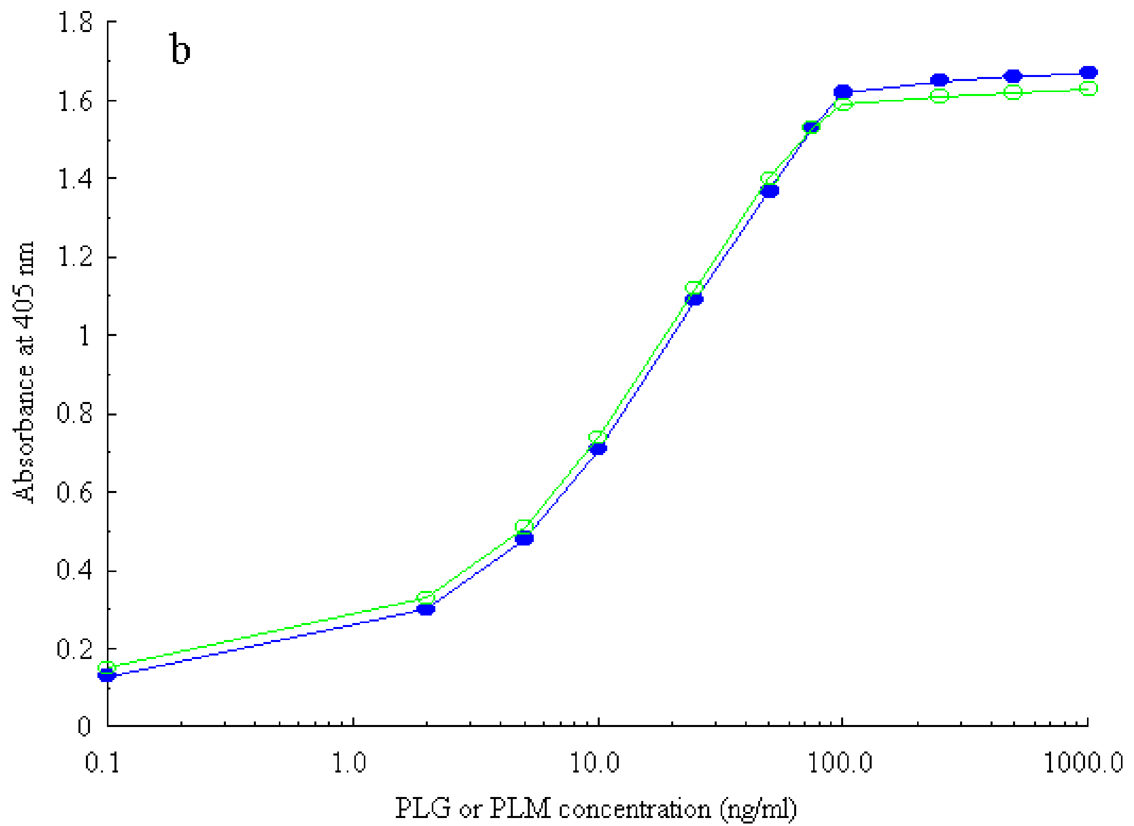
[❼] PLM, plasmin ; PLG, plasminogen

Table 3. *Plasmin and plasminogen concentrations of 20 individual milks determined by ELISA and by the enzymic method of Rollema et al. (1983)*

	Sample	**[Plasmin] μg/ml** ‡		**[Plasminogen] μg/ml** ‡	
		ELISA	Enzymic	ELISA	Enzymic
	1	0.19 ± 0.06	1.01 ± 0.08	0.71 ± 0.10	1.00 ± 0.37
	2	0.28 ± 0.04	UD†	0.21 ± 0.02	UD†
early	3	0.24 ± 0.10	0.79 ± 0.11	0.54 ± 0.27	0.75 ± 0.14
lactation	4	0.30 ± 0.05	0.68 ± 0.15	1.02 ± 0.08	0.64 ± 0.14
milks	5	0.09 ± 0.02	0.66 ± 0.17	0.34 ± 0.05	0.76 ± 0.03
	6	0.50 ± 0.04	1.20 ± 0.12	1.14 ± 0.32	0.97 ± 0.38
	7	0.38 ± 0.09	1.02 ± 0.23	0.62 ± 0.11	1.05 ± 0.17
	8	0.05 ± 0.03	0.40 ± 0.14	1.16 ± 0.04	0.48 ± 0.28
	9	0.39 ± 0.04	0.49 ± 0.08	0.78 ± 0.07	0.08 ± 0.04
mid	10	1.25 ± 0.14	0.24 ± 0.04	1.79 ± 0.25	0.39 ± 0.13
lactation	11	1.12 ± 0.14	0.34 ± 0.07	1.70 ± 0.25	0.30 ± 0.12
milks	12	0.66 ± 0.10	0.68 ± 0.01	0.49 ± 0.14	0.44 ± 0.03
	13	0.67 ± 0.03	0.30 ± 0.06	0.50 ± 0.09	0.45 ± 0.23
	14	0.80 ± 0.03	0.43 ± 0.05	0.47 ± 0.05	0.96 ± 0.21
	15	1.90 ± 0.21	0.48 ± 0.21	30.72 ± 0.34	0.58 ± 0.08
late	16	0.56 ± 0.20	0.27 ± 0.04	2.44 ± 0.30	0.44 ± 0.09
lactation	17	0.12 ± 0.06	0.21 ± 0.02	2.40 ± 0.07	0.15 ± 0.03
milks	18	0.39 ± 0.02	0.29 ± 0.14	6.43 ± 0.13	0.25 ± 0.07
	19	0.75 ± 0.06	0.07 ± 0.02	3.13 ± 0.23	0.15 ± 0.07
	20	0.99 ± 0.19	0.25 ± 0.03	2.30 ± 0.16	0.38 ± 0.18

‡ Values are means with SD for n=3
UD† = Undetectable





DUPONT *et al.*, Fig.1

Figure 1 : Sandwich enzyme-linked immunosorbent assay titration of plasminogen (PLG) (🕮) and plasmin (PLM) (☽) standards using monoclonal antibody specific for plasminogen (Mab antiPLG) (a) or plasminogen+plasmin cross-reacting monoclonal antibody (Mab anti PLG-PLM) (b) as capture reagent. Each symbol represents the mean of five independent determinations.