Enzymatic assays for native plasmin, plasminogen and plasminogen activators in bovine milk

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Summary. Rapid and sensitive assays for plasmin, plasminogen and plasminogen activators (PA) were developed and applied to bovine milk. The reaction medium was clarified by addition of a dissolving agent after hydrolysis of a fluorescent substrate specific for plasmin. This final step enabled the use of larger sample amount with higher substrate concentration than other methods, and avoided previous sample preparation. The use of 4 g gelatin/l in buffers preserved plasmin activity, thus avoiding risks of overestimation of the assays results. Sensitivity, detection level, repeatability and analysis run time of plasmin and plasminogen assay were improved over previous enzymatic methods with synthetic substrates. The PA assay was assessed by measuring conversion of exogenous plasminogen into plasmin. A new kinetic approach was used to enable the direct determination of global PA activities on raw milk samples without interference from indigenous plasmin.

Keywords: Plasmin, plasminogen, plasminogen activators, enzyme assay, bovine milk.

Proteolysis phenomena potentially affect all dairy products. Except during the maturation of some cheeses (Farkye & Landkammer, 1992), proteolysis generally results in a lower quality of dairy products, with appearance of unclean and bitter off-flavours and age gelation in UHT milks (MacKellar, 1981; Harwalkar, 1982; Grufferty & Fox, 1986; Kelly & Foley, 1997), decrease in cheese yield and degradation of casein-based products during storage (Andrews, 1983; Grufferty & Fox, 1988*b*; Bastian & Brown, 1996; Mara *et al*. 1998).

Plasmin (serine proteinase, EC 3.4.21.7) is the major indigenous proteinase in milk (Grufferty & Fox, 1988*a*; Bastian & Brown, 1996). Plasmin is the active part of a complex enzymic system. Its inactive precursor plasminogen is present in high quantities (Fox, 1981; Korycka-Dahl *et al*. 1983; Politis *et al*. 1989; Haïssat *et al*. 1994). Plasminogen activators (PA) are serine proteinases responsible for the conversion of bovine plasminogen into plasmin (Richardson, 1983; Zachos, 1992; Lu & Nielsen, 1993*a* & *b*; Markus *et al*. 1993; Heegaard *et al*. 1994; White *et al*. 1995) by hydrolysis of the Arg^{557}-Ile^{558} bond (Schaller *et al*. 1985). Some specific inhibitors regulate this system (Precetti *et al*. 1997).

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Several protocols have been proposed to measure the activity of plasmin and plasminogen (after its activation into plasmin) in dairy products (Richardson & Pearce, 1981; Korycka-Dahl *et al*. 1983; Rollema *et al*. 1983). All these techniques use specific synthetic substrates, which are hydrolysed to chromogenic (Rollema *et al*. 1983) or fluorogenic products (Pierzchala, 1979; Snoeren & van Riel, 1979). They suffer from interference due to the natural turbidity of milk or due to the effects of several milk constituents on the plasmin system, such as α2-antiplasmin, α-lactalbumin, bovine serum albumin and β-lactoglobulin A (Bastian, 1993; Politis *et al*. 1993). Furthermore, Bastian *et al*. (1991) have reported competitive inhibition of casein towards synthetic substrates. Either a strong dilution or a pre-treatment of the sample are necessary to avoid those interferences (Rollema *et al*. 1983; Politis *et al*. 1989, 1993). However, these steps highly reduce the sensitivity of the methods for measuring plasmin and plasmin-derived activities.

New protocols have been recently adapted from these methods to measure indirectly the activity of native PA from milk by converting added plasminogen to plasmin (Baldi *et al*. 1993; Lu & Nielsen, 1993*a* & *b*).

The aims of this study were to improve sensitivity and detection levels for plasmin, plasminogen and plasminogen activators assays, to facilitate the sample preparation and to enable direct measurements of PA activity in milk.

materials and methods

Biochemical and chemical reagents

Plasmin from bovine blood was obtained from Sigma (St Louis, MO 63178, USA), human serum plasminogen from Boehringer Mannheim (GmbH, D-68305 Mannheim, Germany) and urokinase (Urokinase Choay) from Sanofi Winthrop (94258 Gentilly Cedex, France).

Clarifying Reagent® was obtained from Prolabo (94126 Fontenay-Sous-Bois, France), N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (trifluoroacetic acid salt) from Bachem AG (4416 Bubendorf, Switzerland), 7-amido-4-methyl coumarin (AMC) and ϵ-aminocaproic acid (EACA) from Sigma.

Preparation of milk samples

To determine plasmin activities, milk samples (1 ml) were pre-incubated for 10 min at 37 °C with 1 ml of 100 mM-Tris-HCl buffer, pH 8·0, containing 8 mM EACA and 0·4 M NaCl to dissociate plasmin from casein micelles. To determine plasminogen activities, this proenzyme was converted into active plasmin, named plasmin-derived activity (Korycka-Dahl *et al*. 1983; Rollema *et al*. 1983) by a 60 min incubation at 37 °C of 1 ml milk sample in the presence of 1 ml urokinase solution (200 Ploug U/ml in 100 mM-Tris-HCl-8 mM-EACA-0·4 M-NaCl buffer, pH 8·0; 1 Ploug = 1·5 UI). To determine plasminogen activators activity, the milk sample was directly used without any preparation.

Stabilization of plasmin solution with gelatin addition

Gelatin (4 g/l) was added to 100 mM-Tris-HCl–8 mM-EACA–0·4 M-NaCl buffer, pH 8·0, as a carrier protein to stabilize diluted plasmin solutions. To verify the stabilizing effect of this gelatin buffer, the same amount of commercial plasmin was added to the same volume of four different solutions prepared as reported in Table 1.

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Table 1. *Apparent plasmin activity in 5 assay mixtures. Composition of mixtures and the point of gelatin addition during the assay (i.e. before or after 45 min incubation) were designed to demonstrate the stabilization of plasmin in solution by addition of gelatin.*

	Assay 1 (reference)	Assay 2	Assay 3	Assay 4	Assay 5
UHT milk (ml)	500	500	—	—	500
Tris-HCl Buffer† (ml)	—	—	500	500	500·1
Gelatin (g/l)	—	4	4	—	4
Plasmin solution at 1 Sigma units/ml (μl)	100	100	100	100	0
Incubation period of 45 minutes at room temperature (22 °C)					
UHT milk (ml)	—	—	500	500	—
Tris-HCl Buffer† (ml)	500	500	—	—	—
Gelatin (g/l)	—	—	—	4	—
Apparent PL-AMC Units‡	**31·2** ± 1·0	**31·1** ± 1·0	**31·2** ± 1·0	**23·5** ± 0·9	**0**

† 100 mM-Tris-HCl–0·4 M-NaCl–8 mM-EACA buffer, pH 8·0.
‡ Determined using the plasmin assay (described in Materials and Methods). Values are means ($n = 3$) ± SD.

Spectrofluorometric assay for plasmin, plasminogen and plasminogen activators in bovine milk

The plasmin and plasminogen assays were derived from the method of Richardson & Pearce (1981). Plasmin activity in dairy products and plasmin-derived activity after plasminogen activation by urokinase were determined by measuring the concentration the fluorescent product AMC (7-amido-4-methyl coumarin) released by plasmin from the non-fluorescent coumarin peptide N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (Pierzchala, 1979).

PA activity was assessed by measuring the conversion of exogenous plasminogen into active plasmin following the principle of methods previously described (Baldi *et al*. 1993; Lu & Nielsen, 1993*a* & *b*). The plasmin produced was quantified with the coumarin peptide N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin.

Fluorescence measurements

In proposed protocols, measurement of AMC fluorescence was carried out after complete dissolution of an aliquot of the reaction mixture by using a Clarifying Reagent® (Linden *et al*. 1987). This made possible the use of greater sample volumes for analysis, which consequently increased sensitivity of the assay. Fluorescence measurements were carried out on a Shimadzu Spectrofluorometer RF5000 (Ets ROUCAIRE 91943 Courtaboeuf Cedex, France). Excitation and emission wavelengths were respectively set to 370 nm and 440 nm, with both excitation and emission bandwidths at 3 nm. Standard curves were prepared by plotting the fluorescence intensity versus concentration of AMC (up to 10^{-9} M) to calculate the rate of AMC release.

Kinetics determination of plasmin and plasminogen activities

Incubation were performed at 37 °C in 400 μl volumes in a V-bottom microtube. The reaction mixture consisted of 200 μl of prepared milk samples mixed with 200 μl

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of 2·0 mM-N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (dissolved in 20% v/v dimethyl sulfoxide and 80% v/v 60 mM-Tris-HCl-0·25 M-NaCl buffer, pH 8·0). After 10 min pre-incubation to stabilize the temperature at 37 °C, the rate of peptide hydrolysis was determined by measuring the fluorescence of released AMC during incubation, at 3 time points over an interval of 5 to 90 min, depending on the plasmin or plasmin-derived activity in the sample. For each measurement, 100 μl of reaction mixture was mixed in a cuvette with 1 ml of distilled water and 1 ml of Clarifying Reagent® to stop any enzymatic reactions. These steps made it possible to perform direct spectrofluorometric measurements without interference of milk turbidity. Each sample was analysed in duplicate. The increase in fluorescence intensity during incubation was linear up to 4 h. A similar reaction mixture without milk sample was used as a control for spontaneous hydrolysis of the coumarin peptide, which was negligible in all experiments. Plasminogen content was calculated by subtracting native plasmin activity from the total plasmin activity after plasminogen activation by urokinase.

One PL-AMC unit of plasmin activity was defined as the amount of enzyme that produced one nanomol of AMC per liter of reaction mixture per minute at 37 °C under the assay conditions (1 nmol AMC.min^{-1}.l^{-1})

A standard with a known plasmin content of commercial origin or purified at the laboratory was used to express real concentrations of plasmin instead of apparent PL-AMC units. Plasmin standards were prepared in 100 mM-Tris-HCl-8 mM-EACA-0·4 M-NaCl buffer, pH 8·0 with 4 g gelatin/l. Apparent plasmin activity of a raw milk sample (A) was compared to apparent plasmin activity of the same sample with a known amount of added plasmin standard (B) and compared to activity of the same amount of plasmin standard added in 100 mM-Tris-HCl buffer, pH 8·0, containing 4 g gelatin/l (C). True plasmin or plasmin-derived activities (PL-AMC units) of the sample were calculated by dividing measured apparent plasmin activities by the factor ([B-A]/C). Only one Tris-buffer plasmin standard assay (C) per batch of sample is required.

Kinetics determination of plasminogen activators activities

PA activity was assessed at 37 °C at pH 8·0, as described by Lu & Nielsen (1993*a* & *b*). Incubation was performed at 37 °C in a V-bottom microtube (450 μl total volume). The incubation mixture consisted of 75 μl of 0·363 mg.ml^{-1} human plasminogen in 100 mM-Tris-HCl buffer, pH 8·0, 75 μl of 2·0 mM coumarin peptide substrate (dissolved in 20% v/v dimethyl sulfoxide and 80% v/v 100 mM-Tris-HCl buffer, pH 8·0) and 150 μl of 100 mM-Tris-HCl buffer, pH 8·0. The reaction was started by adding 150 μl milk or urokinase standard (known as a type u-PA, White *et al*. 1995) without any preliminary sample preparation. After 10 min pre-incubation, the kinetics of reaction was monitored by measuring 5 times, at 15 to 30 min interval (depending on PA activity level), the fluorescence of released AMC in aliquots of the reaction mixture. For each measurement, 70 μl of reaction mixture was mixed with 1 ml of distilled water and 1 ml of Clarifying Reagent®, to stop any enzymic reactions, and put in a cuvette. These steps enabled direct spectrofluorometric measurements without interference of milk turbidity. Each sample was analysed in duplicate.

One PA-AMC unit of PA activity was defined as the amount of PA that indirectly produced (by plasminogen activation and subsequent coumarin peptide hydrolysis by the plasmin produced) one nanomol AMC per minute per liter of reaction mixture in one minute at 37 °C under the assay conditions (1 nmol AMC.min^{-2}.l^{-1}).

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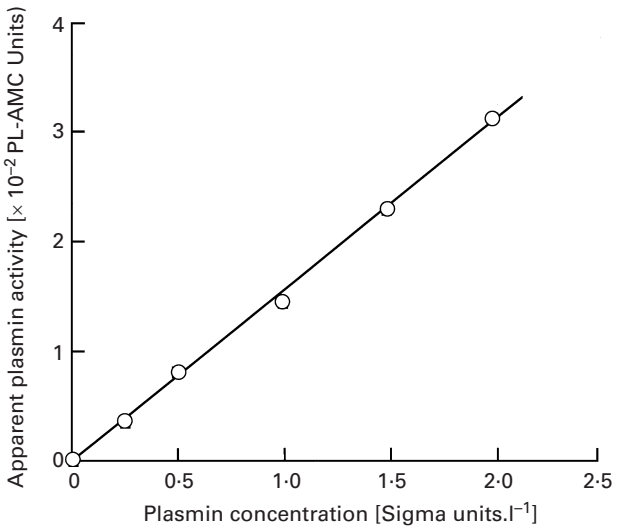

Fig. 1. Calibration curve of the plasmin assay using commercial UHT milk (without detectable indigenous plasmin) as a function of the commercial added plasmin concentration (Sigma Chemical) from 0 to 2·0 Sigma units by liter (y = 155x, n = 2, $R^2 = 0{\cdot}999$).

Calibration curves for plasmin, plasminogen and plasminogen activators assays

Dilutions of commercial plasmin were added to UHT milk with undetectable indigenous plasmin content in order to establish calibration curves for the plasmin and plasminogen assay. Two calibration curves for the PA assay were prepared, one by adding commercial urokinase to UHT milk without detectable PA activity, and the other by diluting a pasteurised milk with native PA in UHT milk.

RESULTS

Spectrofluorometric assay for plasmin and plasminogen in bovine milk

Experimental conditions were chosen in order to maximise apparent plasmin activity (data not shown). Factors tested were buffer composition (NaCl from 0 to 0·5 M and EACA from 0 to 6·0 mM in 100 mM-Tris-HCl buffer, pH 8·0) and coumarin peptide concentration (from 0·5 to 2·0 mM in the reaction mixture). The amount of Tris-HCl buffer pH 8·0 in the reaction mixture was sufficient to maintain overall pH from 7·85 to 7·95 depending on the milk samples studied. Measured plasmin activity remained constant within this range of pH (data not shown).

The plots of PL-AMC units versus relative commercial plasmin concentration (Fig. 1) were linear (y = 155x, $R^2 = 0{\cdot}999$).

Stabilization of plasmin solution with gelatin addition

Plasmin standards were prepared from commercial plasmin powder. However, those standards rapidly lost activity at room temperature when diluted in 100 mM-Tris-HCl buffer, pH 8·0. Lost of activity of the plasmin standards will lead to overestimation of plasmin contents in milk samples. Richardson and Pearce (1981) solubilized purified plasmin in Tris-HCl buffer, pH 8·5, with glycerol (25 % v/v) and bovine serum albumin (BSA, 0·1 % w/v) as a carrier protein, and reported that a one

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thousand fold dilution of this standard has lost 70 % of its initial activity after 24 h at 4 °C. Moreover, those conditions appear to be inappropriate since BSA is a plasmin inhibitor (65 % activity lost for 1·0 mg BSA/l, Politis *et al.* 1993).

In this study, gelatin was used to stabilize plasmin activity (Table 1). The final milk content was the same in each assay in order to compare plasmin activities independently of the interference of milk components. Assay 1 with UHT milk (without detectable indigenous plasmin activity as shown by assay 5) was used as reference since no loss of plasmin activity was observed within dairy products (Richardson & Pearce, 1981). After 45 min at room temperature in 100 mM-Tris-HCl buffer, pH 8·0 (assay 4), more than 25 % of the initial plasmin activity was lost compared to the reference (assay 1), confirming the instability of plasmin outside of an adequate environment. Gelatin did not have inhibitory effect because activities measured with (assay 2) or without (assay 1) gelatin were identical. Moreover, 4 g gelatin/l in 100 mM-Tris-HCl buffer, pH 8·0, acted as a protective medium equivalent to UHT milk since plasmin activities were the same in assay 1 and 3. Gelatin was suitable as a carrier protein for preserving plasmin activity of prepared standards and avoided possible overestimation of the real plasmin content of milk samples.

Effect of casein and other milk components on the measured apparent plasmin activity

To investigate the possible effects of diverse components of milk with the proposed protocol, several dilutions of a pasteurised milk with 100 mM-Tris-HCl buffer pH 8·0 were assayed for native plasmin activity. In a parallel experiment a constant amount of a commercial plasmin standard was added to the dilutions of the pasteurised milk, then the total (native plus added) plasmin activity was determined. Fig. 2*a* shows that apparent plasmin activities of the dilutions of pasteurised milk (containing native plasmin) with Tris-HCl buffer were not proportional to the dilution rate. Thus, the sample diluted 10 fold with Tris-HCl buffer showed about one third of the non-diluted sample activity. Moreover, apparent activity of the added plasmin (calculated by subtracting native plasmin activity to the total measured activity) greatly increased with the dilution rate. In contrast, dilutions of the pasteurised milk with UHT milk having an equivalent casein content resulted in a linear decrease in measured apparent activities with increasing the dilution rate ($R^2 = 0{\cdot}999$).

Percentage activity of added plasmin, relative to the activity in 100 mM Tris-HCl buffer, pH 8·0 with 4 g gelatin/l measured without interfering milk components, decreased when the milk amount increased in the reaction mixture (Fig. 2*b*). The apparent plasmin activity in undiluted milk was only 30 % of the real plasmin activity.

In the present methodology, instead of removing casein (Bastian *et al.* 1991) or whey proteins (Politis *et al.* 1993) from samples to get rid of interfering milk components and to obtain an absolute quantification of plasmin and plasmin-derived activities, real plasmin activity of the raw sample was calculated from measured apparent value (A) corrected by the values of a known amount of added plasmin standard determined in milk (B) and in Tris-HCl buffer containing gelatin (C). In raw and pasteurised milks, added plasmin standard produced only 30 % to 40 % of its activity in Tris-HCl buffer with gelatin. Assuming that the added plasmin interacts with milk components in the same way as the indigenous plasmin, true plasmin or plasmin-derived activities (PL-AMC units) of the sample can be calculated by

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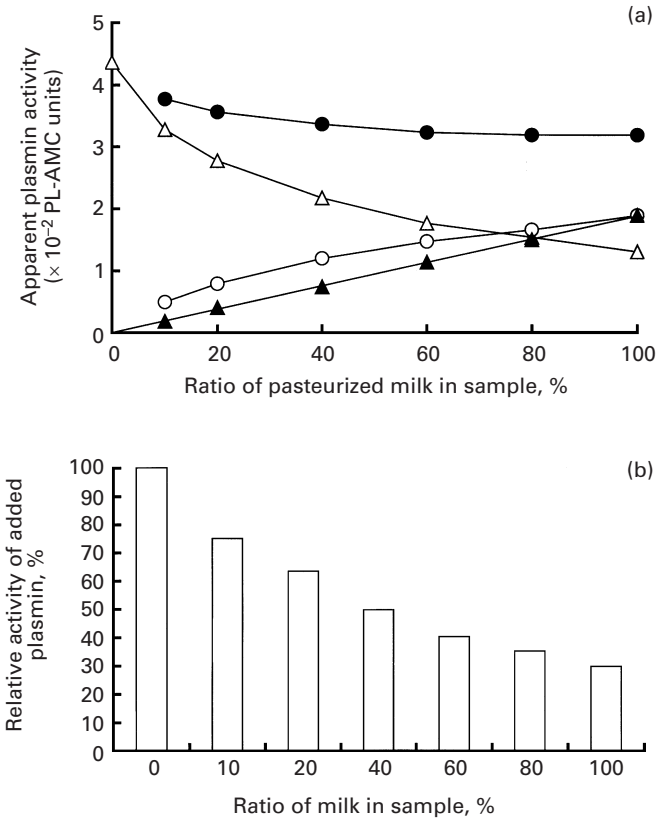

Fig. 2. Interference from milk components in the sample with the plasmin assay. (*a*) Pasteurised milk with native plasmin was diluted in 100 mM-Tris-HCl buffer, pH 8·0 without (○) or with (●) addition of a constant amount of commercial plasmin. Apparent plasmin activity of the added plasmin (△) was calculated by subtracting native plasmin activity (○) from total plasmin activity (●). Apparent native plasmin activity of pasteurised milk sample diluted in UHT milk with an equivalent casein content (▲). (*b*) Percentage of activity of the added plasmin (relative to its activity measured in absence of milk) in samples with variable amount of milk, calculated from values in Fig. 2*a*.

dividing measured apparent plasmin activities by the factor ([B-A]/C). A standard with a known plasmin content of commercial origin or purified at the laboratory can be used to express real concentrations of plasmin instead of PL-AMC units.

Spectrofluorometric assay for plasminogen activators in bovine milk

Fig. 3 shows the increase of fluorescence of a reaction mixture containing native milk PA with an excess of added plasminogen and the coumarin peptide substrate used for plasmin determination. The rate of increase of fluorescence over time increased with incubation time, which may be related to continuous plasmin production in the mixture assay due to plasminogen activation by PA. With an excess of fluorogenic substrate, the rate of substrate hydrolysis by plasmin depends only on plasmin concentration [*PLM*]. At time (t), the fluorescence variation is:

$$\frac{dFU}{dt} = K.[PLM]_{(t)},$$

where K is a constant and FU are fluorescence units.

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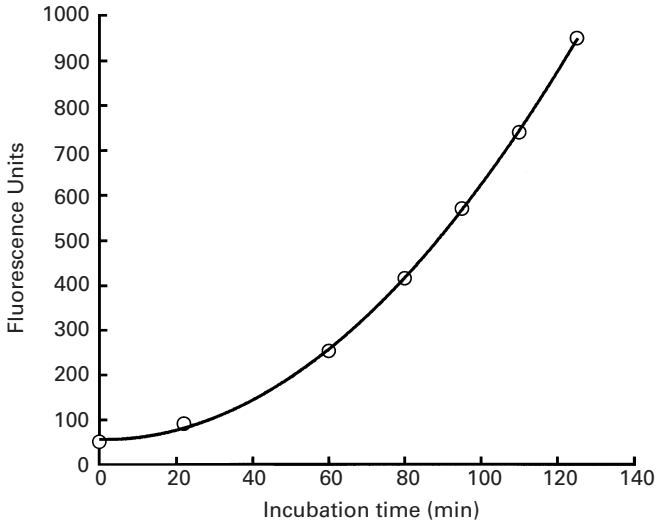

Fig. 3. Fluorescence kinetics of a reaction mixture containing pasteurised milk (with indigenous plasmin, plasminogen and plasminogen activators), exogenous plasminogen and coumarin peptide substrate N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin in 100 mM-Tris-HCl buffer, pH 8·0, at 37 °C. The curve fits well (n = 2, $R^2 = 0{\cdot}9998$) a second degree equation ($y = 0{\cdot}0583x^2 + 0{\cdot}1664x + 57{\cdot}1$).

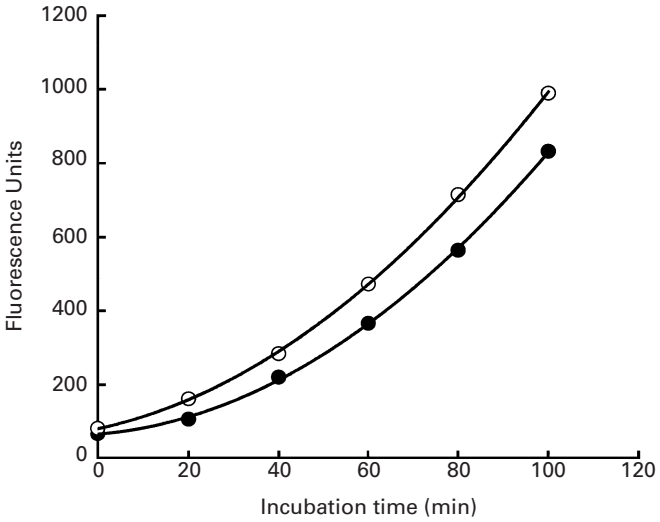

Fig. 4. Effect of added plasmin on the fluorescence kinetics for PA assay. The reaction mixture contained indigenous milk PA, exogenous plasminogen and coumarin peptide substrate in 100 mM - Tris-HCl buffer pH 8·0 with (○) or without (●) added plasmin (about $7{\cdot}0 \times 10^2$ PL-AMC units) at 37 °C. Measured fluorescence units of the reaction mixture followed a second degree equation. The amount of plasmin in the sample did not change significantly the value of the a'' factor: (●) without added plasmin $y = 0{\cdot}0663x^2 + 0{\cdot}9975x + 66{\cdot}5$ (n = 2, $R^2 = 0{\cdot}9997$), (○) with added plasmin $y = 0{\cdot}0659x^2 + 2{\cdot}5938x + 81{\cdot}2$ (n = 2, $R^2 = 0{\cdot}9998$).

When plasminogen is at saturation concentration, the quantity of plasmin produced by PA by unit of time can be considered proportional to the quantity of active PA, so the plasmin concentration at time (t) is:

$$[PLM]_{(t)} = q.[PA].t + b = a.t + b,$$

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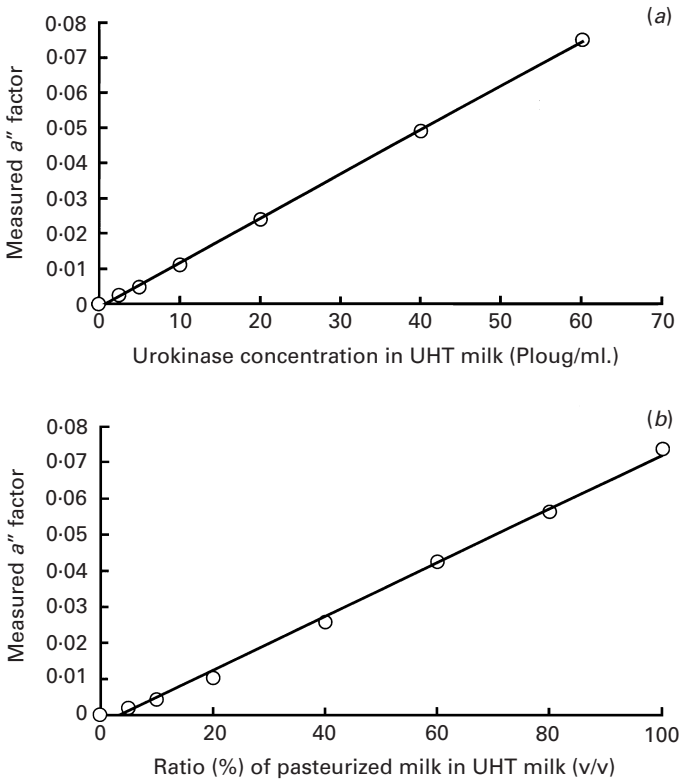

Fig. 5. Calibration curves for PA activity. (*a*) Dilutions of urokinase standard in UHT milk without detectable PA activity, linear curve (n = 2, $R^2 > 0{\cdot}999$) y = 0·0013x. (*b*) Dilutions of native PA from pasteurised milk in UHT milk without detectable PA activity, each point is the mean of two assays, linear curve (n = 2, $R^2 > 0{\cdot}999$) y = 0·0075x − 0·01.

where factor b is the initial plasmin concentration (indigenous plasmin from sample and plasmin contamination of exogenous plasminogen at t = 0) and factor $a = q.[\mathrm{PA}]$ is proportional to PA activity.

The fluorescence variation as function of time becomes:

$$\int_0^t \frac{dFU}{dt} = \int_0^t K.(a.t+b) = \int_0^t a'.t+b' \rightarrow FU(t) = a''.t^2+b'.t+c,$$

Where:

a'' factor $= \frac{1}{2}a' = \frac{1}{2}K.a = \frac{1}{2}K.q.[\mathrm{PA}]$ is proportional to PA activity
b' factor is initial active plasmin quantity at t = 0 in the reaction mixture
c constant is the initial reaction mixture fluorescence at t = 0

The curve obtained in Fig. 3 fitted well a second degree equation ($y = 0{\cdot}0583x^2 + 0{\cdot}1664x + 57{\cdot}12$; n = 2, $R^2 = 0{\cdot}9998$). The addition of exogenous plasmin to pasteurised milk did not change significantly the value of the measured a'' factor (Fig. 4) which validates our kinetic method and especially its independence from the unknown initial plasmin activity. On the contrary, b' factor was linked to the initial plasmin quantity.

Calibration curves for a'' factor were prepared by adding urokinase to UHT milk without detectable PA activity (Fig. 5*a*), and by diluting pasteurised milk

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with native PA in UHT milk (Fig. 5*b*). Both curves were linear, which validates this method as an assay for PA activity in milk. Standard curves prepared by plotting the fluorescence intensity versus concentration of AMC allowed correlation of the a'' factor with PA-AMC units (data not shown).

Repeatability of the plasmin, plasminogen and plasminogen activators assays

Repeatability was estimated from replicates of enzymatic assays on the same pasteurised milk. Relative Standard Deviation (RSD) for plasmin, plasminogen and plasminogen activator assays in bovine milk were 2 % (n = 33, $\bar{x} = 2{\cdot}51 \times 10^2$ PL-AMC units), 1·6 % (n = 20, $\bar{x} = 11{\cdot}14 \times 10^2$ PL-AMC units) and 8 % (n = 16, $\bar{x} = 4{\cdot}6$ PA-AMC units) respectively.

discussion

Spectrofluorometric assay for plasmin and plasminogen in bovine milk

Real plasmin and plasminogen (plasmin-derived) activities could be determined within 15 to 30 min in raw sample without the sample preparation usually required for other methods. The originality of our method lies in the use of high synthetic substrate concentration and of the Clarifying Reagent® which enables one to perform assays with large amounts of milk sample. In our reaction mixture the milk sample was diluted 4-fold as compared with the 10 to 20-fold dilution of pre-treated sample with other fluorogenic or chromogenic methods (Richardson & Pearce, 1981; Korycka-Dahl *et al.* 1983; Rollema *et al.* 1983). This improves sensitivity and detection level, with a noticeable improvement in repeatability (RSD of 2 %, n = 33, for PL-AMC units determination while Rollema *et al.* 1983, reported RSD less than 10 %, n = 10). Measured plasmin activity of a one thousand fold dilution of a crude milk containing about 0·1 mg plasmin/l was significantly higher than activity of the blank ($P < 0{\cdot}01$), suggesting a detection level of about 0·1 μg/l compared with about 15 μg/l reported by Rollema *et al.* (1983) with a chromogenic substrate. This low detection level would enable assays on low-plasmin-containing products such as heat treated milks or dairy products.

Because the higher substrate concentration (1 mM in the reaction mixture instead of 0·2 mM for Richardson & Pearce, 1981) compensated for the higher milk content in the reaction mixture, this method reached the same level (30–40 %) of apparent plasmin activities as previous enzymic methods (about 30 % reported by Richardson & Pearce, 1981; about 50 % reported by Rollema *et al.* 1983).

Effect of casein and other milk components on the measured apparent plasmin activity

Richardson & Pearce (1981) showed that the rate of hydrolysis of the coumarin peptide by purified plasmin in the presence of sodium caseinate is decreased to about 40 % of that measured in the absence of casein. Rollema *et al.* (1983) reported a similar decrease in the measured activity by increasing the milk sample ratio in the reaction mixture. Bastian *et al.* (1991) demonstrated that casein competitively inhibits plasmin in activity assays that use the chromogenic substrate (H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide) used by Rollema *et al.* (1983). Other factors like plasmin inhibitors and native whey proteins (mainly β-lactoglobulin A and α-lactalbumin) also lead to underestimation of real plasmin activity (Politis *et al.* 1993).

Fig. 2 shows that the milk ratio in the reaction mixture directly influences the apparent measured plasmin activity. Dilutions with a UHT milk having an equivalent casein content did not change this ratio and restored proportionality

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between apparent activities of the pasteurised milk and dilution rate (Fig. 2*a*). Since plasmin inhibitors and native whey proteins are largely affected by UHT treatments (Grufferty & Fox, 1986; Bastian & Brown, 1996; Kelly & Foley, 1997) this suggests that casein content would be the main agent responsible for the inhibition of the plasmin activity towards the plasmin assay as reported by Bastian *et al.* (1991). However, potential influences of other inhibition factors (plasmin inhibitors, native whey proteins) need to be verified on a wide variety of individual milks.

Implications of use of a correction factor for measured PL-AMC activities

In the present methodology, plasmin and plasminogen activities were assayed in the presence of all the milk components. The measured activities were consequently influenced by the presence of native substances that inhibit plasmin and by the presence of casein that competitively interferes with the enzymatic assay. From this point of view, this present approach is comparable to those of Richardson & Pearce (1981) and Rollema *et al.* (1983).

When casein interference remains constant among samples (for example in standardized milks or caseinates preparations), the measured apparent activity represents what could be named the plasmin-derived proteolytic potential activity of the sample. This is a result of interest for those dealing with proteolysis in dairy products, as the presence of plasmin inhibitors is taken into account in the result.

When casein content and inhibition levels may vary between samples, and more generally when the objective is to obtain the total plasmin and plasminogen activities independently of milk component interference, true plasmin or plasminogen activities (PL-AMC units) calculated by using the correction factor are to be preferred over determination of apparent plasmin activities. In this last case, additional measurements with added plasmin are necessary to calculate the correction factor for each sample. Another approach was described by Politis *et al.* (1993) in order to yield a true plasmin concentration measurement. Their method implies several sample preparation steps to remove all inhibitory components and to release plasmin from the casein. Even if the number of measurements required to determine the correction factor is greater than for the method of Politis *et al.* (1993), true plasmin and plasminogen activities can be obtained without any heavy and tedious sample preparation steps. This can substantially decrease the required analysis run time. Furthermore, a standard assay with a known plasmin content of commercial origin or purified at the laboratory can be used to directly express real concentrations of plasmin instead of PL-AMC units.

Spectrofluorometric assay for plasminogen activators in bovine milk

The conventional methodology (Baldi *et al.* 1993) indirectly determines PA activity in milk sample by measuring absorbance at 405 nm of *p*-nitro-anilide released from chromogenic substrate H-D-Valyl-L-Leucyl-L-Lysine-*p*-nitro-anilide after 2 and 3 h of incubation at 37 °C in a reaction mixture containing added plasminogen. The same reaction mixture without added plasminogen serves as control in order to remove the absorbance increase due to the native plasmin of the sample. However this method presents some limits. Firstly, the natural turbidity of milk products requires dilutions of samples or use of very low amounts of sample (1 to 5 μl in 250 μl final volume) which reduces the sensitivity of the method. Secondly, the control does not take into account the absorbance increase due to contaminant plasmin initially contained in added plasminogen, as observed in several commercial plasminogen preparations.

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In the present methodology, kinetic determination of PA activity enabled suppression of interference from indigenous and exogenous plasmin and determination of global PA activities on raw milk samples within 60–180 min without sample preparation.

The Clarifying Reagent® made it possible to use a large volume of milk in the reaction mixture (150 μl of milk sample in 450 μl final volume, compared with 1 to 5 μl of pre-treated sample in 250 μl final volume in the method of Baldi *et al.* 1993), which improved sensitivity and detection level of the method.

Our validated kinetic hypothesis implies that the plasmin produced by PA from the added plasminogen interacted with milk components as the indigenous plasmin. On this condition, the measured a'' factor is independent from milk components (casein and plasmin inhibitors) interference affecting the coumarin assay and there is no need for a correction factor.

In conclusion, the proposed methods enable the direct determination of the activities of plasmin, plasminogen and plasminogen activators in whole milk samples without any previous preparation steps. They are characterized by the presence of high synthetic substrate concentration and by the use of the Clarifying Reagent® which permits analysis of a large amount of sample in the reaction mixture. In addition, 4 g gelatin/l in 100 mM-Tris-HCl buffer, pH 8·0, is suitable for preserving plasmin activity of diluted standards, which avoids risk of overestimation of plasmin content in milk samples. Sensitivity, detection level, repeatability and analysis run time of plasmin and plasminogen assay are improved over previous enzymic methods with synthetic substrates (Richardson & Pearce, 1981; Korycka-Dahl *et al.* 1983; Rollema *et al.* 1983; Politis *et al.* 1992, 1993) despite much higher casein (and possibly inhibitors) contents in the reaction mixture. The use of a new kinetic approach makes it possible to directly determine the global PA activities within milk without interference from indigenous and exogenous plasmin contamination in the reaction mixture.

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References

Andrews, A. T. 1983 Proteinases in normal bovine milk and their action on caseins. *Journal of Dairy Research* **50** 45–55

Baldi, A., Politis, I., Barbano, D. M. & Gorewit, R. C. 1993 Primary characterization and determination of plasminogen activator in bovine milk. *Journal of Dairy Science* **76** Supp 1 92

Bastian, E. D. 1993 Inhibition of plasmin by β-lactoglobulin using casein and a synthetic substrate. *Journal of Dairy Science* **76** 3354–3361

Bastian, E. D. & Brown, R. J. 1996 Plasmin in milk and dairy products: an update. *International Dairy Journal* **6** 435–457

Bastian, E. D., Brown, R. J. & Ernstrom, C. A. 1991 Casein interference in bovine plasmin assays using a synthetic substrate. *Journal of Dairy Science* **74** 4119–4124

Farkye, N. Y. & Landkammer, C. F. 1992 Milk plasmin activity influence on Cheddar cheese quality during ripening. *Journal of Food Science* **57** 622–624

Fox, P. F. 1981 Proteinases in dairy technology. *Netherlands Milk Dairy Journal* **35** 233–253

Grufferty, M. B. & Fox, P. F. 1986 Potassium iodate-induced proteolysis in ultra-heat-treated milk during storage: the role of β-lactoglobulin and plasmin. *Journal of Dairy Research* **53** 601–613

Grufferty, M. B. & Fox, P. F. 1988*a* Milk alkaline proteinase. *Journal of Dairy Research* **55** 609–630

Grufferty, M. B. & Fox, P. F. 1988*b* Functional properties of casein hydrolyzed by alkaline milk proteinase. *New Zealand Journal of Dairy Science and Technology* **23** 95–108

Haïssat, S., Marchal, E., Montagne, P., Humbert, G., Béné, M. C., Faure, G. & Linden, G. 1994 Quantitative characterization of bovine plasminogen binding to caseins. *Analytical Biochemistry* **222** 472–478

Harwalkar, V. R. 1982 Age gelation of sterilized milks. In *Developments in Dairy Chemistry-1 Proteins*, pp. 229–269 (Ed. P. F. Fox). Applied Science Publisher, London

https://doi.org/10.1017/S0022029901004939 Published online by Cambridge University Press

Heegaard, C. W., Rasmussen, L. K. & Andreasen, P. A. 1994 The plasminogen activation system in bovine milk: differential localization of tissue-type plasminogen activator and urokinase in milk fractions is caused by binding to casein and urokinase receptor. *Biochimica Biophysica Acta* **1222** 45–55

Kelly, A. L. & Foley, J. 1997 Proteolysis and storage stability of UHT milk as influenced by milk plasmin activity, plasmin/β-lactoglobulin complexation, plasminogen activation and somatic cell count. *International Dairy Journal* **7** 411–420

Korycka-Dahl, M., Ribadeau-Dumas, B., Chene, N. & Martal, J. 1983 Plasmin activity in milk. *Journal of Dairy Science* **66** 704–711

Linden, G., Humbert, G., Kouomegne, R. & Guimgamp, M. F. 1987 Reagent for rendering biological fluids transparent and its analytical applications. *European Patent Application EP 0 246 978 B1, 10 pp*

Lu, D. D. & Nielsen, S. S. 1993*a* Assays for native plasminogen activators in bovine milk. *Journal of Dairy Science* **76** 3362–3368

Lu, D. D. & Nielsen, S. S. 1993*b* Isolation and characterization of native bovine milk plasminogen activators. *Journal of Dairy Science* **76** 3369–3383

MacKellar, R. C. 1981 Development of off-flavors in Ultra-High-Temperature and pasteurised milk as a function of proteolysis. *Journal of Dairy Science* **64** 2138–2145

Mara, O., Roupie, C., Duffy, A. & Kelly, A. L. 1998 The curd-forming properties of milk as affected by the action of plasmin. *International Dairy Journal* **8** 807–812

Markus, G., Hitt, S., Harvey, S. R. & Tritsch, G. L. 1993 Casein, a powerful enhancer of the rate of plasminogen activation. *Fibrinolysis* **7** 229–236

Pierzchala, P. A. 1979 A new fluorogenic substrate for plasmin. *Biochemistry Journal* **183** 555–559

Politis, I., Barbano, D. M. & Gorewit, R. C. 1992 Distribution of plasminogen and plasmin in fractions of bovine milk. *Journal of Dairy Science* **75** 1402–1410

Politis, I., Ng Kwai Hang, K. F. & Giroux, R. N. 1989 Environmental factors affecting plasmin activity in milk. *Journal of Dairy Science* **72** 1713–1718

Politis, I., Zavizion, B., Barbano, D. M. & Gorewit, R. C. 1993 Enzymatic assay for the combined determination of plasmin plus plasminogen in milk: revisited. *Journal of Dairy Science* **76** 1260–1267

Precetti, A. S., Oria, M. P. & Nielsen, S. S. 1997 Presence in bovine milk of two protease inhibitors of the plasmin system. *Journal of Dairy Science* **80** 1490–1496

Richardson, B. C. 1983 The Proteinase of Bovine Milk and the effect of pasteurisation on their activity. *New Zealand Journal of Dairy Science and Technology* **18** 233–245

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

Rollema, H. S., Visser, S. & Poll, J. K. 1981 On the determination, purification and characterization of the alkaline proteinase from bovine milk. *Netherlands Milk Dairy Journal* **35** 396–399

Rollema, H. S., Visser, S. & Poll, J. K. 1983 Spectrophotometric assay of plasmin and plasminogen in bovine milk. *Milchwissenschaft* **38** 214–217

Schaller, J., Moser, P. W., Danneger-Muller, G. A. K., Rosselet, S. J., Kampfer, U. & Rickli, E. E. 1985 Complete amino acid sequence of bovine plasminogen. *European Journal of Biochemistry* **149** 267–278

Snoeren, T. H. M. & van Riel, J. A. M. 1979 Milk proteinase, its isolation and action on αs2- and β-casein. *Milchwissenschaft* **49** 564–569

White, G. H., Zavizion, B., O'Hare, K., Gilmore, J., Guo, M. R., Kindstedt, P. & Politis, I. 1995 Distribution of plasminogen activator in different fractions of bovine milk. *Journal of Dairy Research* **62** 115–122

Zachos, T. 1992 Effect of mastitis on plasminogen activator activity of milk somatic cells. *Journal of Dairy Research* **59** 461–467

https://doi.org/10.1017/S0022029901004939 Published online by Cambridge University Press