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The determination of plasmin and plasminogen-derived activity in turbid samples from various dairy products using an optimised spectrophotometric method

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ABSTRACT

A spectrophotometric assay for plasmin and plasminogen-derived activity in dairy products was optimised and extended to determine plasmin and plasminogen-derived activity in turbid samples of dairy products. The method was validated by assessing reproducibility, repeatability, level of detection and recovery of plasmin activity in different sample matrices. Plasmin activity in raw milk was not affected by skimming, but decreased by 30% in pasteurised and homogenised whole milk, leading to an underestimation of plasmin activity. The effects of dissociation of plasmin and caseins by ε-aminocaproic acid (EACA) plus NaCl on the plasmin activity were investigated. Comparison of pasteurised milk with a micellar casein solution showed that the dissociation of plasmin and caseins on adding EACA and NaCl decreases interference by caseins, but increases inhibition of plasmin with serum-based inhibitory components. The level of detection and repeatability of this method for plasmin activity analysis were improved compared with previous spectrophotometric assays.

1. Introduction

The major indigenous proteinase in milk, plasmin (PL; EC 3.4.21.7), is the active part of a complex enzyme system. Its zymogen, plasminogen (PLG), is present in raw, bovine milk at a 2–30 times higher concentration than PL (Ismail & Nielsen, 2010), and is activated by two families of plasminogen activators (PA), urokinase-type PA (uPA) and tissue-type PA (tPA). The activity of PL and PA is regulated by plasmin inhibitors (PIs), such as α_2-antiplasmin, and plasminogen activator inhibitors (Grufferty & Fox, 1988; Ismail & Nielsen, 2010; Korycka-Dahl, Dumas, Chene, & Martal, 1983; Lu & Nielsen, 1993; Precetti, Oria, & Nielsen, 1997). The level of PL in milk can vary and depends on environmental factors, such as stage of lactation and somatic cell count (Larsen et al., 2010; Nicholas, Auldist, Molan, Stelwagen, & Prosser, 2002). PL readily hydrolyses β- and α_{S2}-casein, and, to a lesser extent, α_{S1}-casein (Grufferty & Fox, 1988). PL activity may be linked to the formation of unclean and bitter off-flavours in milk, and age gelation of UHT milk (Harwalkar, Cholette, McKellar, & Emmons, 1993; Kelly & Foley, 1997). On the other hand, hydrolysis of caseins by PL plays an important role in the initial ripening of Swiss cheese types (Bastian & Brown, 1996).

Various methods to measure PL and PLG-derived activities have been described (Politis, Zavision, Barbano, & Gorewit, 1993; Richardson & Pearce, 1981; Rollema, Visser, & Poll, 1983; Saint-Denis, Humbert, & Gaillard, 2001). The determination of PL activity in these assays is based on the hydrolysis of a specific substrate, which upon cleavage releases a chromogenic (Rollema et al., 1983) or fluorogenic product (Richardson & Pearce, 1981; Saint-Denis et al., 2001). The different sample preparations used in these assays, however, cause varying results and different interfering effects have been reported. Two substrates (S-2251 and Spectrozyme PL) have been reported to enhance PLG activation (Kolev, Owen, & Machovich, 1995), while ε-aminocaproic acid (EACA), a lysine

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derivative often used to enhance PL activity, inhibits activation of human PLG (Alkjaersig, Fletcher, & Sherry, 1959; Cesarman-Maus & Hajjar, 2005). EACA dissociates PL and PLG from caseins by binding to the lysine-binding sites on PL and PLG (Korycka-Dahl et al., 1983).

Furthermore, it has been shown that the dissociation of human PLG by EACA also inhibits activation of PLG by tPA and uPA (Cesarman-Maus & Hajjar, 2005; Collen, 1987; Sun et al., 2002). On the other hand, the natural substrate of PL in milk, the caseins, can act as competitive inhibitors towards a chromogenic substrate used in the assays (Bastian, Brown, & Ernstrom, 1991a) and affect the activation of PLG (Heegaard, Andreasen, Petersen, & Rasmussen, 1997; Markus, Hitt, Harvey, & Tritsch, 1993). Whey proteins are also able to inhibit PL activity (Hayes, McSweeney, & Kelly, 2002; Politis et al., 1993), but the exact mechanism has not been described so far; β-lactoglobulin and bovine serum albumin have shown a higher inhibitory effect than α-lactalbumin (Politis et al., 1993).

Another consideration in terms of assay suitability and performance is that, especially for fluorescence assays, the turbidity of milk can severely interfere with the measurement. In most assays described, an extensive sample preparation protocol is needed to avoid these interferences (Politis et al., 1993; Rollema et al., 1983). In case of fluorescence methods, a high dilution of the sample (Richardson & Pearce, 1981) or an additional clarification treatment (clarifying reagent™) before measurement (Saint-Denis et al., 2001) is required. These preparations may not only reduce the sensitivity of the assay, but could also further complicate the comparison of PL activities measured by different sample preparations, substrates and detection methods (Kelly, O'Flaherty, & Fox, 2006).

The aim of this study was to combine the spectrophotometric assay for PL and PLG-derived activity described by Rollema et al. (1983) with the sample preparation proposed by Saint-Denis et al. (2001) and expand it to turbid, fat-containing milk types and different sample matrices including cheeses. A further aim was to study the effect of skimming on PL activity in different milk types and to characterise the inhibitory effects of casein and serum -based components on the assay, especially the effect of dissociation of PL and caseins by EACA and NaCl on PL and PLG-derived activity in different sample types.

2. Materials and methods

2.1. Materials

PL from bovine serum was obtained from Roche Diagnostics (Hvidovre, Denmark) and urokinase (Thrombolysin) was from Immuno Danmark A/S (Copenhagen, Denmark). The ε-aminocaproic acid (EACA) was obtained from Sigma–Aldrich Denmark ApS (Brøndby, Denmark) and H-D-valyl-L-leucyl-L-lysyl-4-nitroanilide (S-2251) from Haemochrom Diagnostica GmbH (Essen, Germany).

2.2. Origin of samples

The pasteurised (minimum of 72 °C for 15 s) skim milk (SM), homogenised and pasteurised whole milk with 3.5% fat (WM), and UHT milk (0.5% fat) used in this study were commercially available milk products obtained from a local supermarket, stored at 5 °C and analysed before expiry date. Raw bulk milk (RM) was obtained from Arla Foods (Brabrand Dairy Plant, Brabrand, Denmark). A whey-protein-free milk serum (UF-permeate) was prepared by ultrafiltration of SM using 5-kDa spiral wound membranes (Koch Membrane Systems HFK-328). A micellar casein solution (MCS) was prepared by ultracentrifugation of SM at 100,000 × *g* for 60 min at 25 °C. The casein pellet was reconstituted with UF permeate to original volume and gently stirred at 4 °C overnight. Samples of Swedish Herrgård cheese (>28% fat, >6 months) and parmigiano Reggiano (>32% fat, >6 months) were obtained from a local supermarket. Danish Havarti cheese (>60% fat, 1 week) was produced in the pilot plant at Arla Foods Strategic Innovation Center (Brabrand, Denmark).

2.3. Basic sample preparation and measurement of PL and PLG-derived activity

Milk samples (1 mL) were mixed with 250 μL 0.4 M tri-sodium citrate buffer, pH 8.9, and shaken for 15 min to dissociate the casein micelles. Sample preparation for cheese was adapted from that of Richardson and Pearce (1981) with modifications as follows. A mass of 5 g of grated cheese was dissolved in 45 mL 0.4 M tri-sodium citrate buffer, pH 8.9, and stirred for 30 min at room temperature, followed by holding for 30 min at 45 °C, and finally, 15 min at room temperature to separate the fat phase. The solution was then centrifuged at 7500 × *g* for 15 min, and the supernatant below the cream phase was removed and centrifuged again under the same conditions.

The citrate-treated milk or cheese samples were diluted 1:1 (v/v) with an assay buffer as described by Saint-Denis et al. (2001) containing 0.1 M Tris–HCl, 8 mM EACA, 0.4 M NaCl, pH 8, and mixed for 15 min to dissociate PL and PLG from the caseins.

For determination of PLG-derived activity, PLG in the citrate-treated samples was activated by a 1:1 (v/v) dilution with an assay buffer containing 200 Plough units mL^{-1} urokinase and incubated for 60 min at 37 °C (Korycka-Dahl et al., 1983; Rollema et al., 1983; Saint-Denis et al., 2001).

PL and PLG-derived activity were determined by measuring the rate of hydrolysis of the chromogenic substrate S-2251 (Rollema et al., 1983) and release of *p*-nitroaniline. To compensate for lower dilution of 1:5 v/v the sample in the final mixture compared with 1:10 (v/v) in the assay described by Rollema et al. (1983) and, to increase the sensitivity of the assay, the substrate concentration was increased from 0.6 mM S-2251 (Rollema et al., 1983) to 2 mM in the final mixture. The substrate, S-2251, was dissolved in 0.1 M Tris–HCl buffer, pH 8 to a concentration of 4 mM.

The sample solution (50 μL) was transferred to a microtitre plate and the reaction was initiated by addition of 50 μL of the substrate solution. Absorbance was read at 405 nm and 490 nm at 37 °C using an ELISA plate reader (Bio-Tek EL 808 and Bio-Tek Synergy Mx, BioTek Instruments Inc., Wisconsin, IL, USA) at intervals of 2–10 min for 60–180 min depending on the level of PL activity in the sample. Substrate volumes per sample were minimised through use of Costar 3695 clear half-area polystyrene 96-well plates (Corning Inc., New York, NY, USA). To correct for turbidity, the background absorbance values (490 nm) were subtracted from the absorbance values at 405 nm, corresponding to colour development due to release of *p*-nitroaniline. Correction for turbidity using the absorbances at 540 and 630 nm was also tested. The increase in absorbance, indicated as $\Delta A_{405\ nm-490\ nm}$ as a function of time, d*A*/d*t*, was converted into PL units using a standard curve prepared with commercial PL solution from a PL stock solution (0.5 U in Tris buffer, pH 8) with assay buffer additionally containing 4 mg mL^{-1} gelatine to stabilise PL. The correlation between PL activity and absorbance was linear in the range 15 μU mL^{-1} to 1 mU mL^{-1} ($R^2 = 0.999$). No increase over time in absorbance was observed in a blank sample containing assay buffer with 4 mg mL^{-1} gelatine only.

2.4. Method validation

The linearity of the assay was evaluated by measuring PL activity in undiluted SM as well as 1:1 and 1:10 (v/v) dilutions of SM and 3