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Heat inactivation of native plasmin, plasminogen and plasminogen activators in bovine milk: a revisited study

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Abstract — Thermal inactivation, at temperatures between 60 °C and 140 °C, of native plasmin, plasminogen and plasminogen activators were studied in bovine milk using improved enzymatic assays. While measured heat inactivation kinetic of plasmin and plasminogen were in line with previously reported values, plasminogen activators were, surprisingly, found to be as heat sensitive as plasmin and plasminogen in a milk system containing proteins with free SH groups. Activation energies (*Ea*) for the heat denaturation of plasmin, plasminogen and plasminogen activators were 29, 35 and 24 kJ·mol^{-1}, respectively, in the temperature range 95–140 °C , and 244, 230 and 241 kJ·mol^{-1}, respectively, in the temperature range 70–90 °C. The heat inactivation of the whole plasmin system in milk appeared to be directly influenced by the presence of β-lactoglobulin. The rate of plasmin inactivation during long heat treatments decreased rapidly, probably because of the disappearance of available β-lactoglobulin for S-S linking.

plasmin / plasminogen / plasminogen activator / heat inactivation / milk

Résumé — Étude approfondie de la dénaturation thermique de la plasmine, du plasminogène et des activateurs du plasminogène natifs du lait bovin. La dénaturation thermique entre 60 °C et 140 °C de la plasmine, du plasminogène et des activateurs du plasminogène natifs du lait bovin a été étudiée à l'aide de méthodes enzymatiques optimisées. Alors que les cinétiques d'inactivation de la plasmine et du plasminogène sont globalement en accord avec la littérature, les activateurs de plasminogène apparaissent aussi thermosensibles que la plasmine et le plasminogène dans un vrai système lait contenant des protéines à groupements thiols libres. Les énergies d'activation pour la dénaturation thermique de la plasmine, du plasminogène et des activateurs du plasminogène étaient respectivement de 29, 35 et 24 kJ·mol^{-1} entre 95 et 140 °C , et de 244, 230 et 241 kJ·mol^{-1} entre 70–90 °C. La dénaturation thermique de l'ensemble du système plasmine dans le lait apparaît directement liée à la

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présence de β-lactoglobuline. La vitesse de dénaturation thermique du système plasmine diminue rapidement pendant les traitements thermiques prolongés, probablement à cause de la disparition de la β-lactoglobuline disponible pour établir des ponts disulfures.

plasmine / plasminogène / activateur du plasminogène / dénaturation thermique / lait

1. INTRODUCTION

The conversion of milk into a processed food generally implies some form of heat treatment to insure the microbiological safety of the product. Those thermal treatments also affect the activities of indigenous milk enzymes [11], such as the plasmin system. Plasmin (serine proteinase, EC 3.4.21.7) is naturally present as the active part of a complex enzymatic system. Its inactive precursor plasminogen is present in large amounts, usually between three and five times the quantity of plasmin in healthy milks [9, 16, 24, 34]. Tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) are serine proteinases responsible for the conversion of plasminogen into plasmin [18, 27, 36, 44, 45] by hydrolysis of the Arg^{557}-Ile^{558} peptide bond [42]. Some specific inhibitors regulate this system [35]. Plasmin, which is the major native proteinase in bovine milk [4, 14], can have large effects, generally resulting in reduced quality of dairy products. These negative effects include appearance of unclean and bitter off-flavors [29], age gelation in UHT milks [13, 17, 22] and decrease in cheese yield and degradation of casein-based products during storage [1, 4, 30]. Plasmin is also involved in the maturation of some cheese varieties [12].

Several studies describe the kinetics of heat inactivation of plasmin and plasminogen in milk or in model systems [2, 7, 8, 15, 20, 31, 32, 39]. The heat inactivation of both plasmin and plasminogen appears to follow a first order process with similar rates of inactivation; Arrhenius plots show two step inactivation kinetics. However, kinetic data on heat inactivation of plasmin are not very consistent, as reported by Farkye and Imafidon [11]. Plasmin and plasminogen are more heat stable in the presence of casein, whereas the presence of β-lactoglobulin, and specially free –SH groups, accelerates the thermal inactivation of the enzyme and its proenzyme due to thiol-disulphide interactions [2, 15, 31, 39].

In model systems, plasminogen activators (PA) from bovine milk are more heat stable than plasmin and plasminogen between 60 °C and 140 °C [28]. Kennedy and Kelly [23] have reported evidence of considerable plasminogen activator activity after heat treatment of a high somatic cell count milk containing KIO_3. Inhibitors of plasminogen activators (PAI) are thought to be heat labile in milk [36]. However, the behavior of milk PA under industrial heating processes is largely unknown.

Many of the previous studies were conducted either on whey-protein-free model systems or with exogenous sources of plasmin, plasminogen and plasminogen activators. In contrast, the objective of this work was to determine the thermal inactivation of indigenous plasmin, plasminogen and plasminogen activators in a real milk system, over a wide range of temperature and time conditions, with the help of an improved and sensitive enzymatic assay [41].

2. MATERIALS AND METHODS

2.1. Biochemical and chemical reagents

Human serum plasminogen was obtained from Boehringer Mannheim (GmbH, Germany), urokinase (Urokinase

Choay) from Sanofi Winthrop (94258 Gentilly Cedex, France), plasmin from bovine blood, and β-lactoglobulin and aprotinin from Sigma Chemicals Co. (St Louis, MO 63178, USA).

Clarifying Reagent® was obtained from Prolabo (94126 Fontenay-Sous-Bois, France), N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin in trifluoroacetic salt from Bachem Feinchmikalien AG (4416 Bubendorf, Switzerland), and 7-amido-4-methyl coumarin (AMC) and ε-aminocaproic acid (EACA) from Sigma Chemicals Co. (St Louis, MO 63178, USA).

2.2. Origin of milk samples

The milk samples used in this study were either commercial pasteurized milk or fresh raw bovine milk kept at 4 °C.

For the experiments on a simulated milk system without whey proteins, the pH of milk was adjusted to 4.6 with HCl 0.1 mol·L^{-1} and casein micelles were separated from whey proteins by centrifugation at 5000 *g* for 20 min at 4 °C followed by one subsequent washing of the casein pellet with Jenness and Koops buffer [21]. Casein was then adjusted to the volume of the original milk sample with the milk salt solution [21] and the pH of this suspension was reajusted to 6.7 with NaOH (2.0 mol·L^{-1}).

For the experiments on a milk system with denatured whey proteins, a commercial UHT milk (without detectable native plasmin activity) was used with the addition of exogenous bovine plasmin (final concentration of 0.5 Sigma units·mL^{-1}).

Native β-lactoglobulin was dissolved (from 0 to 5 g·L^{-1} final concentration) in both milk systems prior to heat treatment at 90 °C.

2.3. Heating of milk samples

For the heat inactivation study, 1.5 mL milk samples were sealed in glass tubes (disposable glass pasteur pipette, 6.8 mm external diameter, 5.6 mm internal diameter) and heated in a thermoregulated and stirred oil bath (IKA HBR4 Digital, IKA Labortechnik, 79219 Staufen, Germany) from 60 to 140 °C ± 1 °C. After the desired heating time, the glass tubes were immediately cooled in an ice water bath, and plasmin, plasminogen and PA activities were measured. Residual activities were expressed as a percentage of the initial activities (without heat treatment). The lag phase (about 15 s) needed by the milk sample to reach the desired temperature was not taken into account in determination of rates of inactivation.

2.4. Enzymatic assays for plasmin and plasminogen in bovine milk

2.4.1. Reference assay for plasmin and plasminogen

Plasmin activity in milk samples and plasmin-derived activity after activation of plasminogen by urokinase were determined by measuring the concentration of the fluorescent product AMC (7-amido-4-methyl coumarin) released by plasmin from the specific non-fluorescent coumarin peptide N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin [33]. Plasmin and plasminogen assays were carried out as previously described by Saint Denis et al. [41]. One millilitre of milk sample was pre-incubated for 10 min at 37 °C with 1 mL of 100 mmol·L^{-1} Tris-HCl buffer, pH 8.0, containing 8 mmol·L^{-1} EACA and 0.4 mol·L^{-1} NaCl to dissociate plasmin from casein micelles. Plasminogen was previously converted into active plasmin [24, 37, 40] 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^{-1} in 100 mmol·L^{-1}

Tris-HCl buffer, pH 8.0, with 8 mmol·L^{-1} EACA and 0.4 mol·L^{-1} NaCl). Incubations were performed at 37 °C in a V-bottom microtube. The incubated reaction mixture consisted of 200 µL of prepared milk samples mixed with 200 µL of 2.0 mmol·L^{-1} N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (dissolved in 20% v/v dimethyl sulfoxyde and 80% v/v 60 mmol·L^{-1} Tris-HCl buffer, pH 8.0, with 0.25 mol·L^{-1} NaCl). 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® [25] to stop any enzymatic reactions. These steps enabled direct spectrofluorometric measurements (λ_{ex} = 370 nm, λ_{em} = 440 nm) without interference of milk turbidity. Plasminogen content was calculated by subtracting native plasmin activity from the total plasmin activity after plasminogen activation by urokinase. Each sample was analyzed 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 to determine spontaneous hydrolysis of the coumarin peptide, which was negligible in all experiments.

2.4.2. Modified plasmin assay

A modified protocol was used to measure the very low plasmin activities remaining after long heat treatments. Volumes of 2.5 mL of 10 mmol·L^{-1} N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin (dissolved in 20% v/v dimethyl sulfoxyde and 80% v/v 100 mmol·L^{-1} Tris-HCl buffer, pH 8.0) and 0.02% NaN_3 were added to 47.5 mL of pasteurized milk sample, for 0.5 mmol·L^{-1} final plasmin substrate concentration, with no noticeable effect on milk pH. This milk sample was aliquoted into 9 series of 4 sterile glass tubes (1.0 mL each) that were sealed, heated at 90 °C for respectively 0, 1, 2, 3, 5, 7, 10, 12.5 or 15 min and then immediately cooled in ice water. The first glass tube of each series was immediately frozen and kept as the blank; the remaining tubes were stored at 37 °C. One glass tube of each series was regularly collected at 1 h to 10 d interval depending on the plasmin activity, and deep-frozen to stop any enzymatic reaction. In order to measure the fluorescence of AMC released by cleavage of the coumarin peptide by plasmin, a 100 µL aliquot of each glass tube sample was mixed with 1 mL of Clarifying Reagent® and 1 mL of distilled water and was put into a cuvette for measurement of fluorescence. Each sample from a glass tube was analyzed in triplicate. Plasmin activity of each series was determined by calculating the rate of fluorescence appearance in the 4 glass tubes frozen after increasing incubation times. Control for spontaneous substrate hydrolysis was performed with distilled water instead of the milk sample. Hydrolysis of the coumarin peptide substrate by other proteolytic activities in milk was determined in a parallel experiment with the addition of 10 mmol·L^{-1} final concentration of aprotinin, a serine proteinase inhibitor [10], before the heat treatment at 90 °C. Sterility of the milk samples was checked at the end of the experiments by incubating 0.5 mL of sample on agar for 48 h at 37 °C.

2.5. Enzymatic assay for plasminogen activators in bovine milk

PA activity was assessed by measuring the conversion of exogenous inactive plasminogen into active plasmin. The plasmin produced was quantified with the plasmin substrate N-succinyl-L-alanyl-

L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin. The kinetic approach was different from previously described methods [3, 26]. As the concentration of plasmin increased with time due to the activation of plasminogen by PA, the rate of AMC appearance and consequently the rate of fluorescence appearance in the reaction mixture increased with time [41].

Preliminary experiments showed that PA activity was optimal at 37 °C and pH 8.0, as described by Lu and Nielsen [26, 28]. Incubations were 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 mmol·L^{-1} Tris-HCl buffer, pH 8.0, 75 µL of 2.0 mmol·L^{-1} coumarin peptide substrate (dissolved in 20% v/v dimethyl sulfoxyde and 80% v/v 100 mmol·L^{-1} Tris-HCl buffer, pH 8.0) and 150 µL of 100 mmol·L^{-1} Tris-HCl buffer, pH 8.0. The reaction was started by adding 150 µL of milk sample containing PA, without any preliminary sample preparation. The kinetics of reaction were monitored by measuring five times, at 15 to 30 min intervals (depending on PA activity), the fluorescence of released AMC on aliquots of the reaction mixture. A 70 µL fraction of the reaction mixture was mixed with 1 mL of Clarifying Reagent® and 1 mL of distilled water to stop any enzymatic reactions and was put into a cuvette for direct spectrofluorometric measurements. Each sample was run in duplicate.

2.6. Fluorescence measurements

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 bandwidths at 3 nm. Standard curves were prepared by plotting the fluorescence intensity versus concentration of AMC (up to 10^{-9} mol·L^{-1} in blank tubes carried out as assays) to calculate the rate of AMC release.

3. RESULTS AND DISCUSSION

3.1. Thermal inactivation of plasmin and plasminogen in bovine milk between 60 °C and 140 °C

Figures 1A and 1B show typical first order plots for the heat inactivation of both native plasmin and plasminogen in bovine milk during heat treatment, which is in agreement with several previous studies [2, 7, 8, 15, 39]. The same pattern of heat inactivation was obtained with both raw and pasteurized milks (data not shown). Despite the reported disappearance of heat labile plasmin inhibitors [19] under the pasteurization process, low heat treatments (between 60 and 70 °C) did not seem to significantly increase the plasmin activity measured with our plasmin assay. This assay was reported to be less sensitive to plasmin inhibitors than to casein competition towards the synthetic substrate [41]. However, this observation should be verified on a wide variety of individual milks with potentially higher levels of plasmin inhibitors. In agreement with the literature there was no significant difference in the kinetic behavior of inactivation of plasmin and plasminogen in the studied temperature range (60 °C–140 °C).

Above 100 °C, the time needed to obtain 90% of enzyme inactivation (*D* values) was in agreement with that previously reported (Tab. I). *D* values below 100 °C were in line with those of Rollema and Poll [39], Alichanidis et al. [2] and Meyer [32] but different from the results of Grufferty and Fox [15] and, to a lesser extent, from those of Driessen [7, 8]. For example, the *D* value for plasmin inactivation at 85 °C was 126 s in the present study, which is close to the reported values of 120 s in a skimmed milk

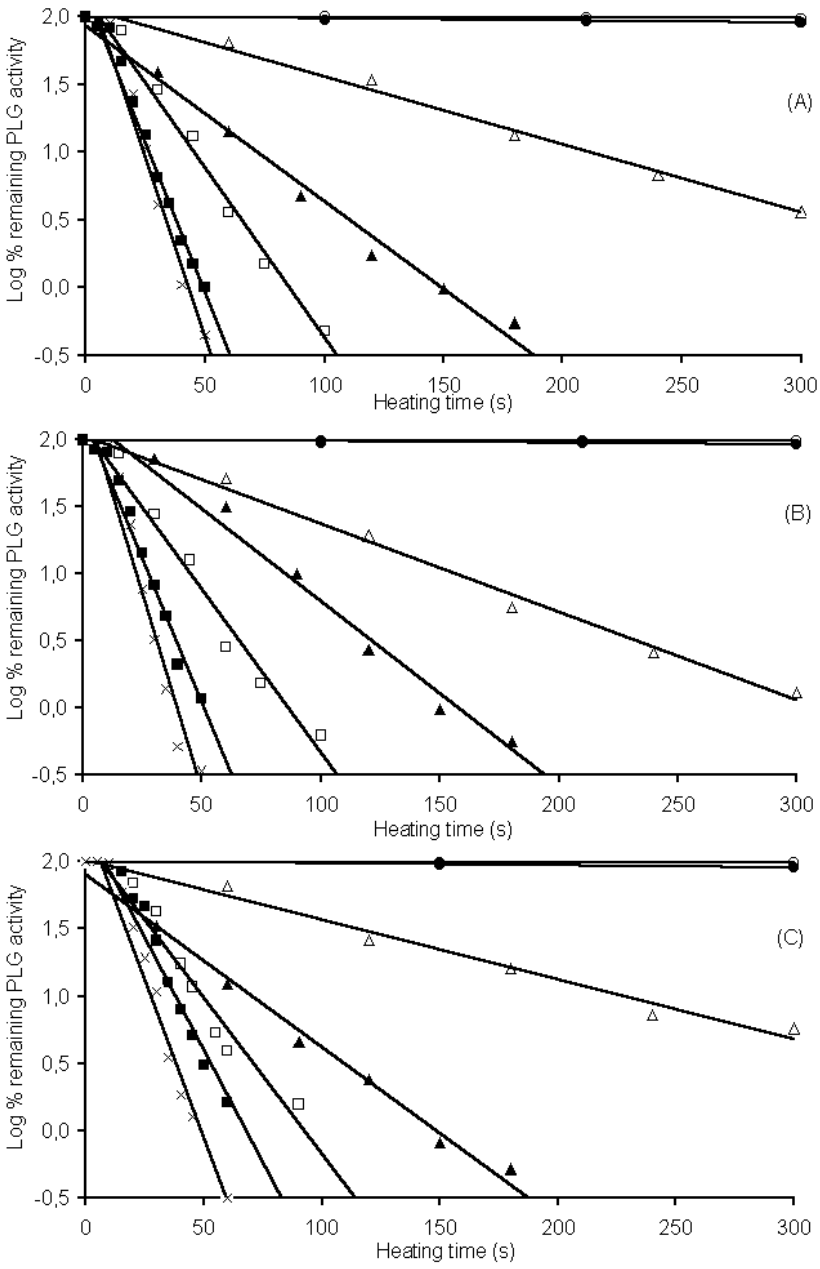

Figure 1. Heat inactivation curves for native plasmin (**A**), plasminogen (**B**) and plasminogen activators (**C**) in bovine milk. Temperatures studied were 60 ºC, ○; 70 ºC, ●; 80 ºC, △; 90 ºC, ▲; 100 ºC, □; 120 ºC, ■ and 140 ºC, ×.

Table I. *D* values (time needed for 90% inactivation) measured for indigenous plasmin, plasminogen and plasminogen activators in bovine milk. The range of validity represents the time range used to calculate those *D* values.

	D value(s)			*D* value(s)	
Temperature (°C)	*PLM*	*PLG*	Range of validity	*PA*	Range of validity
60	*59520*	*57900*	0-90 min	*39300*	0-90 min
70	*11700*	*8040*	0-90 min	*9960*	0-90 min
75	*660*	*660*	0-60 min	*840*	0-60 min
80	*187*	*161*	0-6 min	*210*	0-8 min
85	*126*	*116*	0-6 min	*129*	0-8 min
90	*71*	*73*	0-3 min	*74*	0-3 min
95	*nd*	*38*	0-3 min	*nd*	–
100	*33*	*34*	0-100 s	*36*	0-90 s
105	*nd*	*25*	0-100 s	*30*	0-90 s
110	*23*	*22*	0-90 s	*26*	0-90 s
115	*nd*	*20*	0-90 s	*nd*	–
120	*18*	*19*	0-50 s	*24*	0-60 s
130	*nd*	*13*	0-50 s	*nd*	-
140	*13*	*11*	0-50 s	*16*	0-60 s

nd = not determined.

with added porcine plasmin [2], 90 s in skimmed milk with native plasmin [39] and 150 s in a raw skimmed milk [32]. In contrast, greater heat resistance of plasmin was reported in this range of temperatures by Driessen et al. [7, 8] (384 s at 85 °C in skimmed milk) and Grufferty and Fox [15] (loss of only 45% of the original activity after 15 min at 90 °C). Those differences could result from the origin of milk samples, from the different heating setup and more probably from the specificity of the plasmin assays used. Rollema and Poll [39] and Meyer [32] used enzymatic assays with highly specific synthetic substrates while Driessen [7, 8] followed the rate of γ-casein appearance monitored by polyacrylamide gel electrophoresis, and Grufferty and Fox [15] determined the appearance of free amino groups with fluorescamine after 3 d at 37 °C. These last two methods are indirect and less specific, which could lead to overestimation of plasmin activity after heat treatment.

Figure 2A inset shows the decrease of *D* values with the temperature. Figure 2A plots the logarithm of *D* values against temperature to calculate *Z* values (temperature increase needed to reduce *D* value by 90%) from the slope of the curve. *Z* value was about 8.5 °C in the temperature range 60 °C–90 °C while it was about 80 °C between 95 °C and 140 °C. For heat treatments above 100 °C, plasmin and plasminogen inactivation thus appeared less dependent on temperature but more

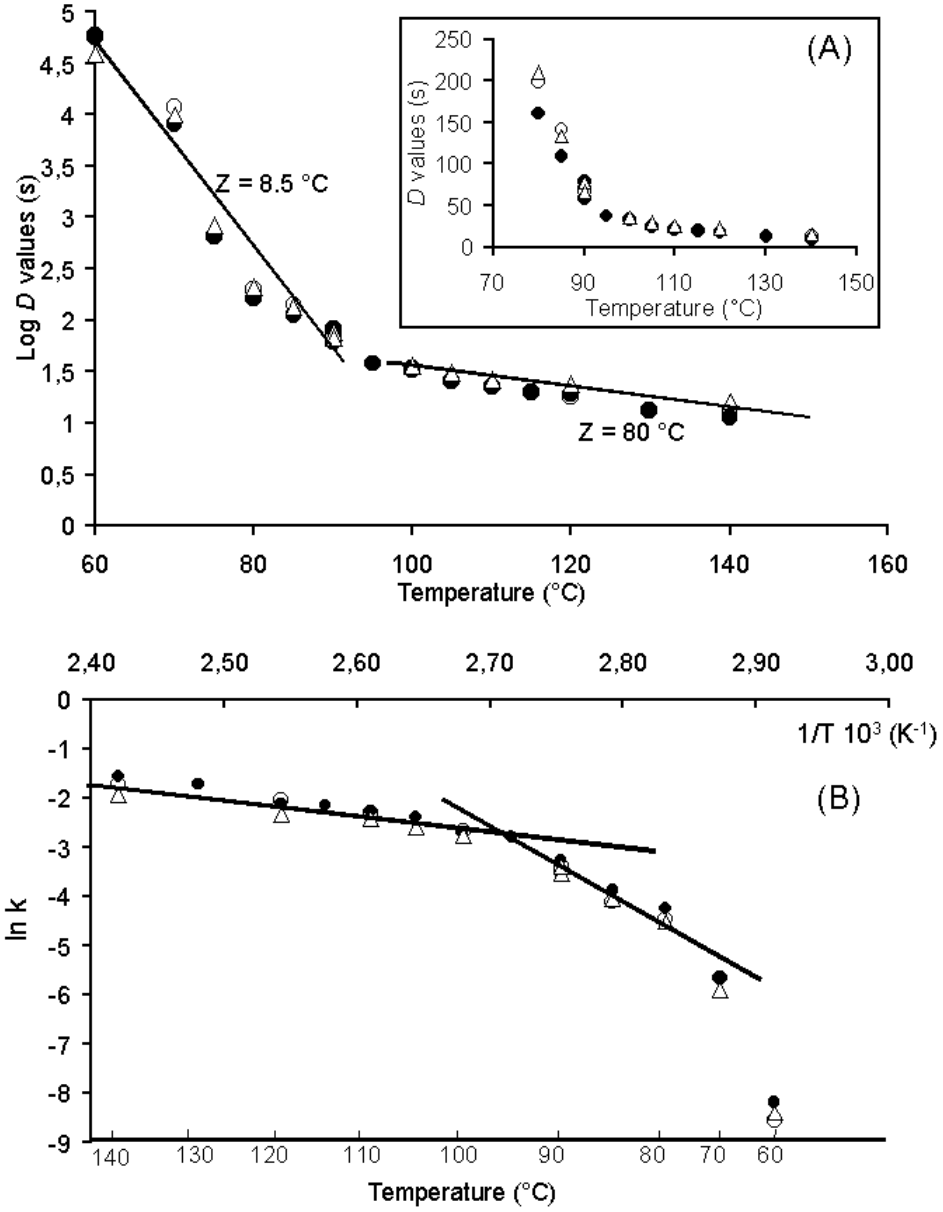

Figure 2. Heat inactivation of plasmin (○), plasminogen (●) and plasminogen activators (△) in bovine milk. **A**. Determination of *Z* values (temperature increase needed to reduce *D* values by 90%) from Fig. A inset which represents *D* values as function of heating temperatures.
B. Arrhenius plot for the heat inactivation, a plot of ln k (k, rate constant of the reaction) versus 1/T (T, absolute temperature in K) gives a slope (-*E*a/R) from which the activation energy *E*a can be calculated (R, gas constant).

dependent on the heating time. The Arrhenius plot for inactivation of plasmin and plasminogen (Fig. 2B) shows the same strong temperature dependence below 100 °C, which suggests a complex inactivation mechanism. Activation energy (*E*a) for plasmin and plasminogen inactivation, calculated from the slope of the Arrhenius plots, increased from 29 and 35 kJ·mol^{-1} between 95 °C and 140 °C respectively to 244 and 230 kJ·mol^{-1} between 70 °C and 90 °C. Those values are close to the results of Alichanidis et al. [2] who reported *E*a from 20 kJ·mol^{-1} (between 130 °C and 143 °C) to 170 kJ·mol^{-1} (72 °C–85 °C) for inactivation of plasmin.

In contrast with previous studies of either simulated milk systems [31] with added exogenous plasmin [2] or with enzymatic methods assaying only a fraction of the sample [32, 39], plasmin and plasminogen activities reported here were assayed directly in whole milk. The improved detection level and sensitivity of our plasmin assay made it possible to measure residual plasmin activities after longer heat treatments than other enzymatic methods, which extends the range of validity for measured *D* values (Tab. I). Residual plasmin activities were thus determined after heating for up to 6 min at 80 °C, to 100 s at 100 °C and to 50 s at 140 °C while previous reported data did not exceed 100 s at 80 °C, 15 s at 110 °C [39], 45 s at 100 °C or 15 s at 143 °C with a high level of added plasmin [2].

3.2. Thermal inactivation of plasminogen activators in bovine milk between 60 °C and 140 °C

Figure 3A shows an example of determination of milk indigenous PA activity after several heating times at 90 °C. Under our experimental conditions, the increase in fluorescence followed a second degree equation, $Fluorescence\ Units\ (t) = a.t^2 + b.t + c$, where the factor a is directly proportional to PA activity and independent of contaminant plasmin interference from either the sample or the added plasminogen [41]. Plots of this factor a versus concentration of urokinase or human t-PA standards were linear (data not shown) showing that a was directly linked to PA activity and that it could be used in the calculations for kinetics of heat stability in bovine milk. Figure 3B presents the time-dependent inactivation of native PA at 90 °C with a values calculated from Figure 3A.

Plots of the logarithm of remaining PA activity versus heating time between 60 °C and 140 °C were almost linear (Fig. 1C), indicating that heat inactivation of native PA in bovine milk followed first-order kinetics as already observed by Lu and Nielsen [28]. In contrast with the previously reported heat resistance of PA in bovine milk, the calculated *D* values (Tab. I) appeared to be close to those obtained for plasmin and plasminogen. Calculated *D* values were 74 s and 16 s at 90 °C and 140 °C respectively in this study while Lu and Nielsen [28] reported values of 1440 s and 32 s respectively.

Those differences probably arise from the protocols used. Lu and Nielsen [28] tested PA purified from bovine milk in a Tris buffer (0.05 mol·L^{-1} Tris, 0.1 mol·L^{-1} NaCl, 0.01% Tween 80, pH 7.6) containing about 20 μg·L^{-1} casein while in the present study the heat stability of native PA was assessed in a real milk system. PA appear to be far less heat resistant in bovine milk than in simplified model systems.

The Arrhenius plot for PA inactivation was linear between 60 °C and 140 °C in the model system of Lu and Nielsen [28]. In the present study, the Arrhenius plot for PA inactivation shows a two stage pattern between 90 °C and 140 °C, as observed for plasmin and plasminogen (Fig. 2B). Thus, the thermal denaturation of PA in a real milk system appeared to be more complex than in a simplified model system without

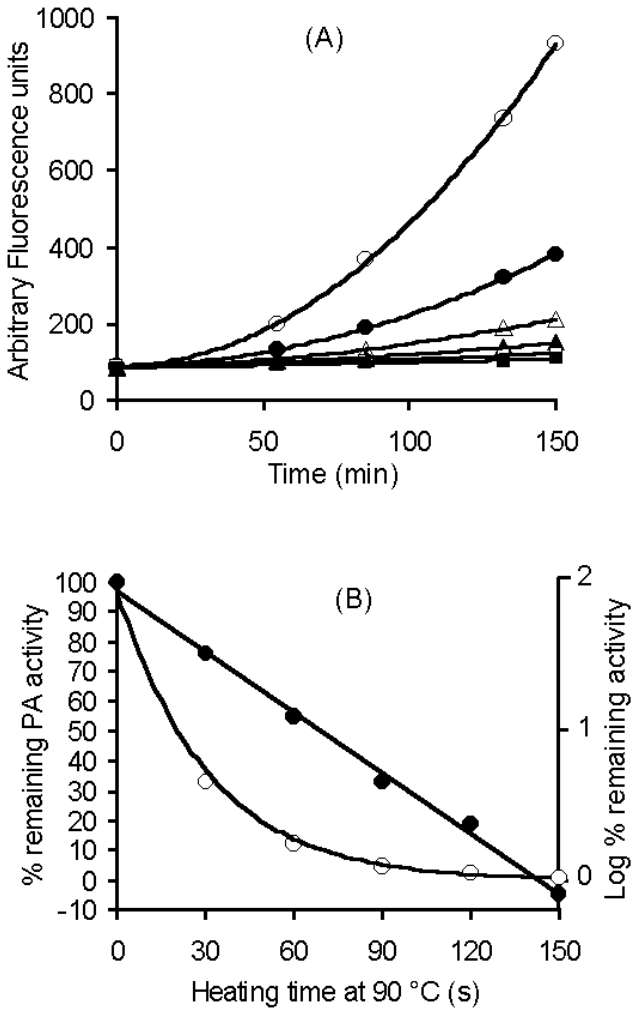

Figure 3. Kinetic determination of native PA activity during heating of bovine milk at 90 °C. **A**. Increase in fluorescence in the reaction mixture as a function of incubation time at 37 °C. The measured fluorescence fits a second degree equation $FU(t) = a.t^2 + b.t + c$, where the factor a represents PA activity. Milk sample was heated for 0 s, ○ ($y = 0.0373.x^2 + 0.0239.x + 90.0$); 30 s, ● ($y = 0.0123.x^2 + 0.1181.x + 89.6$); 60 s, △ ($y = 0.0046.x^2 + 0.1643.x + 86.2$); 90 s, ▲ ($y = 0.0017.x^2 + 0.1771.x + 85.2$); 120 s, □ ($y = 0.0009.x^2 + 0.112.x + 86.8$) and 150 s, ■ ($y = 0.0003.x^2 + 0.1058.x + 85.5$) at 90 °C.
B. Heat inactivation curve for native PA at 90 °C, drawn with the previously calculated a factor (○; decimal scale, (● Log scale).

whey proteins. Calculated *E*a for PA inactivation were 24 kJ·mol^{-1} between 95 °C and 140 °C and 241 kJ·mol^{-1} between 70 °C and 90 °C, while Lu and Nielsen [28] reported a unique *E*a of 90 kJ·mol^{-1} between 60 °C and 140 °C.

3.3. Influence of β-lactoglobulin on thermal inactivation of the indigenous plasmin system in bovine milk

Whey proteins, and especially native β-lactoglobulin, play a major role in the thermal inactivation of plasmin in bovine milk [2, 8, 13]. Free SH-containing-proteins like β-lactoglobulin cause irreversible denaturation of plasmin by S-S/S-H interactions [31]. In order to investigate the effect of β-lactoglobulin on thermal denaturation of the plasmin system, increasing amounts of β-lactoglobulin were added to a simulated milk system without whey proteins and to a commercial UHT milk with added plasmin, prior to heat treatment at 90 °C. The simulated milk system used was acid casein resuspended in Jenness and Koops buffer. While previous studies have disagreed about the presence or absence of plasmin activity in acid casein [14, 38], in this study high residual activity (more than 70% of plasmin and plasminogen) was found in the preparation used. Those differences could be related to different ways of preparation and acidification [20]. The rate of inactivation of native plasmin in the whey-protein-free milk system (Fig. 4A) increased with the β-lactoglobulin concentration, with *D* values ranging from 833 s (no added β-lacto-globulin) to 101 s (5 $g \cdot L^{-1}$ β-lactoglobulin). The same trend was observed for inactivation of native plasminogen (data not shown).

Plasmin inactivation rates were similar in the whey protein-free milk system and in the UHT milk system (corrresponding to about 0.8 $mg \cdot mL^{-1}$ of β-lactoglobulin in the reaction mixture) in the absence of added native β-lactoglobulin (Fig. 4A). Rates of plasmin inactivation in UHT milk with added plasmin were the same as in the whey-protein-free milk system, regarding the amount of added native β-lactoglobulin (data not shown). As heat labile whey proteins, and especially β-lactoglobulin, are mainly denatured after UHT treatment [32], this suggests that the presence of already denatured whey protein had no influence on the thermal inactivation of plasmin in milk. In contrast, Bastian et al. [6] have reported that heat-denatured β-lactoglobulin leads to a greater competitive or mixed inhibition of plasmin activity than native β-lactoglobulin. However, comparisons are not readily possible since their results were obtained in very different experimental conditions: 1) β-lactoglobulin is denatured alone at 100 °C in water before being mixed with plasmin while plasmin and β-lactoglobulin were heated together at 90 °C in the present work; on the other hand, no denaturation of plasmin or β-lactoglobulin occurs during their co-incubation at 60 °C for 15 min [6], 2) absence of casein in their study with synthetic substrate. The latter is of importance since competitive inhibition from casein towards synthetic substrates is more important than that of other potential inhibitors [5, 41]. Accordingly, added native β-lactoglobulin had no effect on plasmin activity in the whey protein free milk system (Fig. 4A, t = 0) nor in the system with UHT milk (data not shown).

In the absence of β-lactoglobulin, the calculated *D* value for PA thermal inactivation was about 1180 s (Fig. 4B), which is comparable to the *D* value of about 1400 s calculated from the Lu and Nielsen data [28] obtained with a whey protein-free model system. Similarly to plasmin, the rate of PA inactivation appeared to be greatly dependent on the β-lactoglobulin concentration, with low *D* values of 160 s at 5 $g \cdot L^{-1}$ β-lactoglobulin. The exact mechanism of thermal denaturation of the serine proteinases PA with β-lactoglobulin remains unknown, but S-S/S-H interactions similar to those already described for the serine proteinase plasmin [31] could be involved. In agreement, considerable PA activity was apparent after heat treatment of a high somatic cell count milk in the presence of the oxidizing agent KIO_3 [23] which is

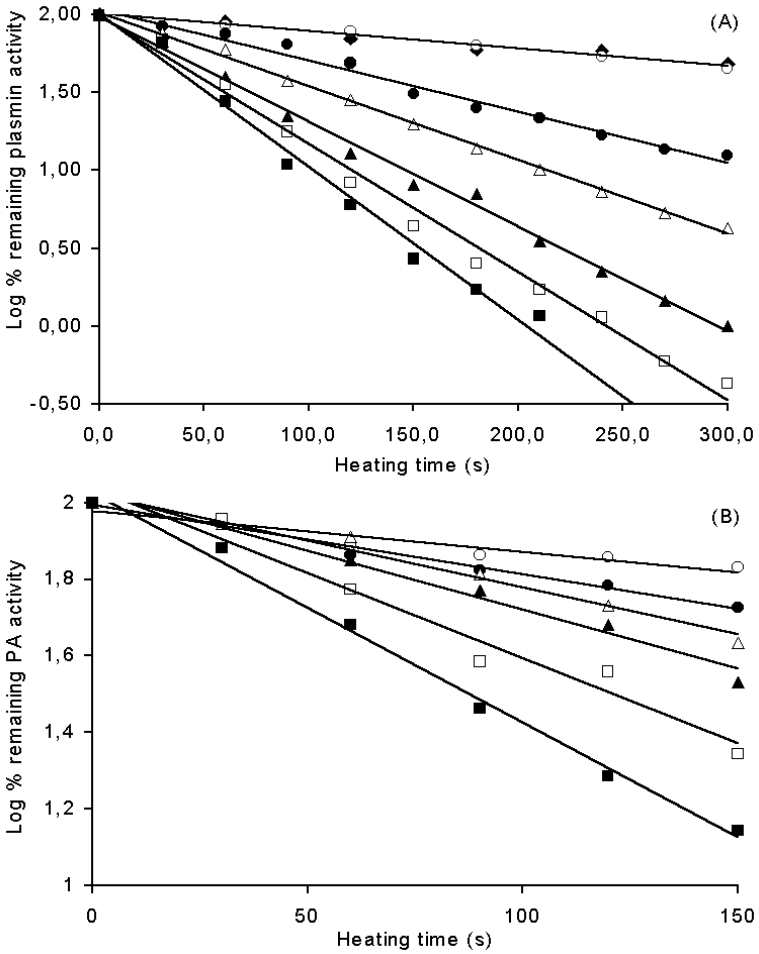

Figure 4. Effect of added β-lactoglobulin (0 g·L^{-1} ○; 1 g·L^{-1} ●; 2 g·L^{-1} △; 3 g·L^{-1} ▲; 4 g·L^{-1} □ ; 5 g·L^{-1} ■) on the thermal inactivation at 90 °C of plasmin (**A**) and plasminogen activators (**B**) in a simulated milk system (casein with Jenness & Koops buffer). Respective *D* values were 833 s, 303 s, 211 s, 148 s, 121 s and 101 s for plasmin, and 1180 s, 568 s, 373 s, 296 s, 207 s and 160 s for inactivation of plasminogen activators. Denaturation at 90 °C of added plasmin in UHT milk (A) with no added β-lactoglobulin (◆), $D = 822$ s.

known to inhibit the effect of SH-containing whey proteins like β-lactoglobulin during the heating process [13, 43].

β-lactoglobulin appeared to be an important factor influencing the rate of inactivation of the plasmin system in bovine milk. As β-lactoglobulin is heat-labile [13, 32], a decrease of the rate of plasmin inactivation would be expected during a prolonged heat treatment due to the rapid thermal denaturation of native β-lactoglobulin. In order to make possible the determination of very low plasmin activities found on those milk samples heated for

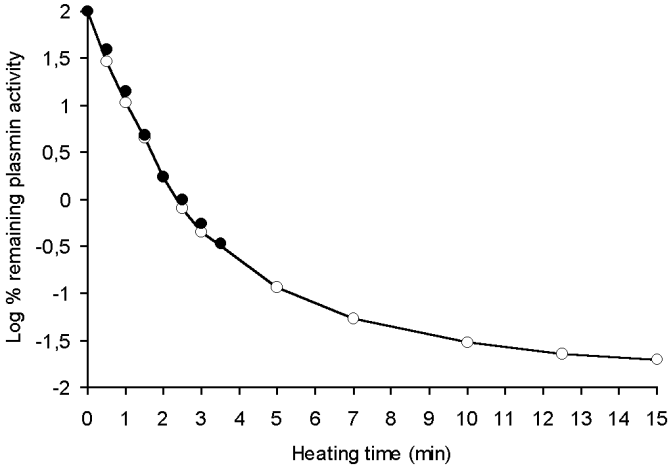

Figure 5. Heat inactivation of native plasmin in bovine milk during long heat treatment at 90 °C. Reference plasmin assay (●) could not detect remaining plasmin activity after 3.5 min at 90 °C. Modified plasmin assay (○), with up to 40 d incubation of the substrate with the milk sample kept in a sealed sterile glass tube at 37 °C, made it possible to assay remaining plasmin activity after 15 min at 90 °C.

a long time, the detection level and sensitivity of the plasmin assay were improved by directly mixing the synthetic plasmin substrate with the milk sample just before the heat treatment. This reaction mixture was kept in sealed sterile glass tubes and incubated for up to 40 d at 37 °C under sterile conditions (no CFU were detected in agarose plate after 48 h incubation at 37 °C) before the fluorescence measurements. Spontaneous hydrolysis of substrate, incubated in the same conditions with distilled water instead of milk, was apparent during such long incubations and was subtracted from fluorescence measured with milk samples. In the presence of the serine proteinase inhibitor aprotinin [10], increase in fluorescence was equal to that due to spontaneous substrate hydrolysis. Since plasmin is the major serine proteinase of bovine milk and since the synthetic substrate used is highly specific, the measured fluorescence subtracted from the spontaneous substrate hydrolysis can be considered as a good indicator of the remaining plasmin activity in the milk sample. Figure 5 presents the remaining plasmin activity found in bovine milk after a prolonged heat treatment at 90 °C. In agreement with previous findings of the present study (Fig. 1, Tab. I), plasmin was first rapidly inactivated, with a *D* value of about 75 s between 1 and 3 min at 90 °C. Then, the rate of thermal inactivation of plasmin slowed down to a *D* value of about 1000 s between 10 and 15 min heating time. This value, which is comparable to those measured in the model system without whey proteins (Fig. 4A), could reflect the disappearance of free SH β-lactoglobulin (native β-lactoglobulin) and other proteins containing free-SH during heat treatment.

4. CONCLUSION

The present study describes the behavior of plasmin, plasminogen and plasminogen activators within a real milk system under various heating conditions using new improved enzymatic assays. In contrast with the literature, native PA in bovine milk

appeared to be as heat sensitive as both plasmin and plasminogen. Those differences arise from the fact that previous studies used simple milk model systems without whey proteins containing free-SH groups, and therefore failed to properly simulate the properties of real milk. β-lactoglobulin, the major source of free-SH, increased the rate of thermal inactivation of native plasmin, plasminogen and PA in bovine milk. During heat treatment, the rate of plasmin inactivation decreased rapidly, probably because of the disappearance of β-lactoglobulin available for S-S linking. This observation could be of importance for manufacturing processes involving long heat treatments.

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