

Barostability of milk plasmin activity

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(Received 31 January 2000; accepted 21 June 2000)

Abstract — The influence of high pressure (HP) on the stability and activity of the milk alkaline proteinase plasmin was examined. Assays of enzyme activity following HP treatment of plasmin in phosphate buffer (pH 6.7) indicated that the enzyme was extremely pressure stable, retaining almost all activity even after treatment at 600 MPa for 20 min at 20 °C. Plasmin was also extremely stable when HP treated in buffer containing 25 mg·mL⁻¹ sodium caseinate, and in cheese. However, HP treatment in buffer containing 5 mg·mL⁻¹ β -lactoglobulin resulted in enzyme inactivation at pressures > 400 MPa, indicating that the presence of β -lactoglobulin greatly destabilises the enzyme under high pressure, which is analogous to the effect of this protein on the heat stability of plasmin. In separate experiments, hydrolysis of β -casein by plasmin at 20 °C for 30 min at various pressures (300–800 MPa) was studied. Parallel control incubations were performed at atmospheric pressure. Urea-PAGE analysis of digests showed that primary proteolysis of β -casein was decreased at $P > 400$ MPa. As judged from RP-HPLC analysis, production of 2%-TCA soluble peptides by plasmin appeared unaffected at $P < 700$ MPa, above which pressure the rates of peptide production decreased. Overall, plasmin is relatively pressure stable in most systems and can hydrolyse its preferred substrate (β -casein) at pressures up to 700 MPa, but is sensitive to destabilisation by denatured β -lactoglobulin.

plasmin / high pressure / specificity / stability

Résumé — **Stabilité de la plasmine sous l'influence de hautes pressions.** L'influence des hautes pressions sur la stabilité et l'activité de la plasmine bovine a été examinée. Les déterminations de l'activité enzymatique suivant le traitement à hautes pressions de la plasmine dans une solution tampon phosphate (pH 6,7) a indiqué que l'enzyme était très résistante à la pression, conservant presque toute son activité, même après un traitement à 600 MPa pendant 20 min à 20 °C. Lorsque la plasmine a été traitée dans une solution tampon contenant 25 mg·mL⁻¹ de caséinate de sodium ou dans le fromage, elle s'est aussi avérée très stable. Cependant, le traitement à hautes pressions dans une

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solution tampon contenant 5 mg·mL⁻¹ de β -lactoglobuline, a entraîné l'inactivation de l'enzyme pour des pressions > 400 MPa, indiquant que la présence de β -lactoglobuline déstabilise fortement l'enzyme sous hautes pressions, ce qui est analogue à l'effet de cette protéine sur la stabilité thermique de la plasmine. Par ailleurs, l'hydrolyse de la β -caséine par la plasmine a été réalisée à 20 °C pendant 30 min à différentes pressions (300–800 MPa). En parallèle, le contrôle témoin a été réalisé à pression atmosphérique. L'analyse des hydrolysats avec PAGE a montré que la protéolyse primaire a diminué lorsque la pression > 400 MPa. L'analyse avec RP-HPLC a montré que la production par la plasmine des peptides solubles dans 2 % TCA n'a pas été affectée à pression < 700 MPa, mais au-dessus de cette pression, les taux de production des peptides ont diminué. Ainsi, la plasmine est relativement stable à la pression dans de nombreux systèmes, elle est aussi capable d'hydrolyser son substrat préféré (β -caséine) à des pressions jusqu'à 700 MPa, mais est déstabilisée par la β -lactoglobuline dénaturée.

plasmine / haute pression / spécificité / stabilité

1. INTRODUCTION

The principal proteinase in bovine milk is the alkaline serine proteinase plasmin (E.C. 3.4.21.7) [5]. Most of the potential plasmin activity in milk is in the form of the inactive precursor plasminogen, which is converted to active plasmin by a heterogeneous group of plasminogen activators [2, 13].

Plasmin activity influences the quality of many dairy products [5, 13]. Plasmin is an alkaline serine proteinase with a pH optimum of 7.5, which readily hydrolyses β -casein and α_{s2} -casein and, more slowly, α_{s1} -casein [8]. Plasmin-mediated hydrolysis of casein influences milk coagulation properties and cheese yield [19] and is important in cheese ripening, where it appears to be mainly involved in primary proteolysis of caseins (particularly β -casein), through which action it may enhance the flavour of cheese [4].

Plasmin is relatively heat stable in simple buffer systems, but is greatly destabilised when heated in the presence of β -lactoglobulin, due to thiol-disulphide interchange reactions between the disulphide bonds within the enzyme structure and the exposed thiol group of denatured β -lactoglobulin [12, 16]. This leads to greatly reduced plasmin activity in, for example,

ultra-high temperature (UHT) milk [15]. Casein in milk increases the thermal stability of plasmin [1]. The mechanisms and kinetics of thermal inactivation of plasmin have been extensively studied [1, 16, 22].

The sensitivity of plasmin to high pressure (HP), however, is far less well defined. Some preliminary reports suggest that indigenous plasmin activity in milk can survive pressure treatments at 400 Mpa [10, 18]. HP treatment of milk is known to affect many properties of milk, for example, improving gelation properties during rennet or acid-induced coagulation in manufacture of yoghurt and cheese [3]. However, as plasmin activity has a wide range of effects on the functional properties of dairy products, including the acid and rennet coagulation properties of milk [11, 19], it is important to understand the stability of plasmin in HP-treated systems.

The objective of the present study was to perform a fundamental study of the barostability of plasmin activity. Two sets of experiments were performed. Firstly, the activity of plasmin against a fluorescent substrate after pressurisation in buffers containing various milk constituents, such as caseins and whey proteins, was determined. Secondly, the activity of plasmin against its preferred substrate, β -casein, while under high pressure, was also examined.

2. MATERIALS AND METHODS

2.1. Enzymes and substrates

For the study of enzyme activity remaining after high pressure (HP) treatment, bovine plasmin was prepared by activation of bovine plasminogen with urokinase. A 0.1 mL aliquot of plasminogen (from bovine plasma, Sigma-Aldrich Chemie GmbH, Germany, 1.176 units·mL⁻¹, where one unit is the amount of enzyme that will produce a ΔA_{275} of 1.0 from α -casein in 20 min at pH 7.5 at 37 °C) was mixed with 0.1 mL urokinase solution (Sigma-Aldrich Chemie GmbH, Germany, 1 000 Ploug units·mL⁻¹) and the volume made up to 2.0 mL by the addition of 50 mmol·L⁻¹ Tris-HCl buffer, pH 8.5 (containing 20 mmol·L⁻¹ lysine, 140 mmol·L⁻¹ NaCl and 50% (w/v) glycerol). The mixture was incubated at 37 °C for 1 h, to give a maximum yield of plasmin. Pure β -casein and β -lactoglobulin were also purchased from Sigma. The purity of the former was determined to be very high by urea-PAGE analysis (see, for example, Fig. 3, lane 1), while the latter gave a single peak on RP-HPLC analysis, also indicating a high purity.

Cheddar cheese was manufactured using a standard protocol in the Processing Hall of University College Cork and sodium caseinate was obtained from the Irish Dairy Board. For the specificity experiments, porcine plasmin (Sigma-Aldrich Chemie GmbH, Dublin, Ireland), which is known to have an identical cleavage specificity to bovine plasmin [17], was used.

2.2. Pressure inactivation studies

Pressurisation of samples (in 700 μ L plastic Eppendorf tubes) was carried out at room temperature in a Stansted Fluid Power high pressure vessel (Stansted Fluid Power Ltd, 70 Bentfield Road, Essex, CM24 8HT, England). The pressurisation vessel had a maximum pressure limit of 900 MPa,

chamber dimensions of 37 mm \times 300 mm, and an immersion fluid of 15% castor oil in ethanol. The maximum temperature increase during pressurisation was 20 °C at 800 MPa, and pressurisation times quoted refer to holding times at constant target pressure, excluding pressurisation and depressurisation times.

For enzyme inactivation studies bovine plasmin was added, immediately prior to pressurisation, to different buffers (at dilutions from 1:13 to 1:20) and subjected to treatment at a range of pressures at 20 °C for different time intervals (1, 10, 20 and 30 min). Buffers used were:

- 100 mmol·L⁻¹ phosphate buffer, pH 6.7;
- 100 mmol·L⁻¹ phosphate buffer, pH 6.7 containing 5 mg·mL⁻¹ β -lactoglobulin;
- 100 mmol·L⁻¹ phosphate buffer, pH 6.7 containing 25 mg·mL⁻¹ sodium caseinate.

Upon release of pressure, plasmin activity in each of the samples was measured using the fluorescent assay method of Richardson and Pearce [28]. Denaturation of β -lactoglobulin after HP treatment of buffer (b) was determined by diluting samples 1:10 with deionised HPLC grade water (containing 0.1% v/v trifluoroacetic acid), centrifuging at 16 000 *g* for 10 min (after which step a precipitate of insoluble protein was clearly apparent) and analysing the supernatant by RP-HPLC.

2.3. Activity of plasmin against β -casein under high pressure

To examine the activity under high pressure of plasmin on β -casein, isolated β -casein (2 mg·mL⁻¹) was dissolved in 50 mmol·L⁻¹ ammonium bicarbonate buffer (pH 8.4), in the presence of sodium azide (0.05% w/v) to inhibit bacterial growth, and porcine plasmin was added (0.0625 units·mL⁻¹, where 1 unit is the amount of enzyme that will produce a ΔA_{275} of 1.0 from α -casein in 20 min at pH 7.5 at 37 °C). The solution was immediately incubated at 20 °C at a range of

pressures (300–800 MPa) for times up to 30 min. Hydrolysis reactions were terminated after removal from the HP chamber by boiling for 5 min. Rates and products of hydrolysis were examined using urea-PAGE and RP-HPLC.

2.4. Analytical methods

Samples of hydrolysates of β -casein were prepared for urea-PAGE by the addition of an equal volume of double strength sample buffer [20] and electrophoresis conditions were as described by [15]. Hydrolysates were prepared for RP-HPLC analysis by the addition of an equal volume of 4% trichloroacetic acid (TCA), followed by centrifugation at 16 000 *g* for 10 min and analysis of a sample of supernatant (175 μ L). Reversed-phase HPLC was performed using a Beckman HPLC system (Beckman, San Ramon, Ca., USA), consisting of a model 506 autosampler, a model 126 programmable solvent module and a model 166 programmable detector module (interfaced with a personal computer using Beckman Gold Nouveau software). A nucleosil C₈ column (250 \times 4.6 mm, 5 μ m particle diameter, 300 Å pore size) was used for all separations (JVA Analytical, Unit 1, Longmile Business Centre, Longmile Road, Dublin 12, Ireland). Solvent A consisted of 0.1% (v/v) TFA in deionized HPLC grade water and solvent B consisted of 0.1% TFA in HPLC grade acetonitrile, and the flow rate used was 0.75 mL·min⁻¹. The elution profile consisted of initially increasing solvent B from 0 to 30% over 25 min, increasing to 50% B over 45 min and finally holding at 50% B for 6 min. The column was washed by increasing to 95% B over 2 min, holding for 10 min and returning to 100% A over 2 min. Final re-equilibration was at 100% A for 15 min before injection of the next sample. The peak areas of separated peptides were determined by integration. The gradient described by [12] was used to measure β -lactoglobulin denaturation, using the same HPLC system.

3. RESULTS

3.1. Pressure inactivation of plasmin

After pressurisation for 20 min at 300–600 MPa, plasmin was extremely pressure sensitive in the presence of β -lactoglobulin, as compared to plasmin pressure treated in the presence of sodium caseinate or in buffer alone (Fig. 1). In buffer, plasmin was, in fact, more pressure sensitive than β -lactoglobulin, although both β -lactoglobulin denaturation and plasmin inactivation were almost complete (> 90%) after treatment at 500 MPa for 20 min. Inactivation of indigenous plasmin activity present in Cheddar cheese showed that plasmin was very pressure resistant in such a product.

Since plasmin was significantly inactivated at 400 MPa in the presence of β -lactoglobulin, samples of enzyme in the same buffers were treated at 400 MPa for different time periods (Fig. 2). Plasmin inactivation in buffer containing β -lactoglobulin increased almost linearly with duration of treatment and, after 30 min at 400 MPa, it was largely inactivated (> 90%). Inactivation of plasmin and denaturation of β -lactoglobulin, in the buffer where both were present, appeared to be, in both cases, first-order reactions. Again, plasmin was far more stable in phosphate buffer, in cheese and in the presence of sodium caseinate than in the presence of β -lactoglobulin.

3.2. Hydrolysis of β -casein by plasmin under high pressure

The effect of high pressure on primary proteolysis of β -casein by plasmin in 50 mmol·L⁻¹ ammonium bicarbonate buffer, pH 8.4, was studied by Urea PAGE (Fig. 3). Primary proteolysis of β -casein appeared unaffected relative to an unpressurised control at 300 MPa, with extensive degradation of this protein being visible in lanes 2 and 3. However, increasing residual intact β -casein was apparent after incubation relative to the

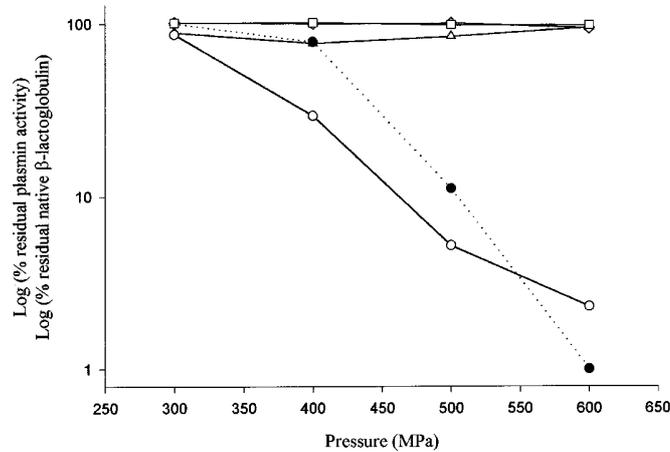


Figure 1. Inactivation of plasmin activity (whole lines, open symbols) and denaturation of β -lactoglobulin (dotted lines, closed symbols) in the following systems: $0.1 \text{ mol}\cdot\text{L}^{-1}$ phosphate buffer, pH 6.5 (Δ); $0.1 \text{ mol}\cdot\text{L}^{-1}$ phosphate buffer, pH 6.5, containing $5 \text{ mg}\cdot\text{mL}^{-1}$ β -lactoglobulin (\circ , \bullet); $0.1 \text{ mol}\cdot\text{L}^{-1}$ phosphate buffer, pH 6.5, containing $25 \text{ mg}\cdot\text{mL}^{-1}$ sodium caseinate (\square) and cheese (\diamond), over a range of pressures (treatments in each case were for 20 min).

Figure 1. Inactivation de l'activité de la plasmine (lignes pleines, symboles ouverts) et dénaturation de la β -lactoglobuline (lignes pointillées, symboles fermés) dans les systèmes suivants : solution tampon phosphate, $0,1 \text{ mol}\cdot\text{L}^{-1}$, pH 6,5 (Δ) ; solution tampon phosphate, $0,1 \text{ mol}\cdot\text{L}^{-1}$, pH 6,5, avec $5 \text{ mg}\cdot\text{mL}^{-1}$ β -lactoglobuline (\circ , \bullet) ; solution tampon phosphate, $0,1 \text{ mol}\cdot\text{L}^{-1}$, pH 6,5, avec $25 \text{ mg}\cdot\text{mL}^{-1}$ caséinate de sodium (\square) et fromage (\diamond), pour une série de pressions (traitements de 20 min dans chaque cas).

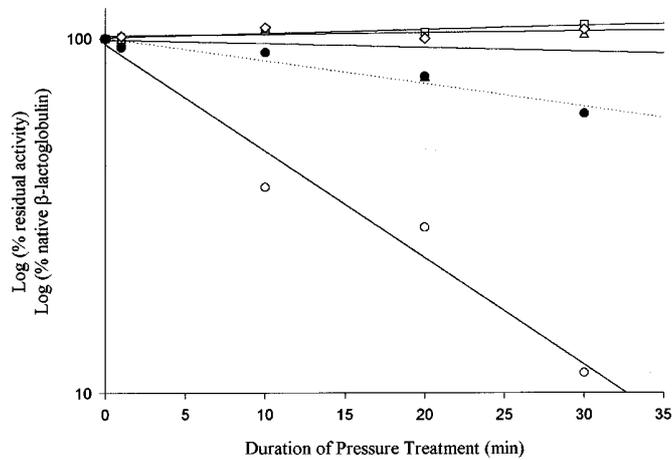


Figure 2. Inactivation of plasmin activity (whole lines, open symbols) and denaturation of β -lactoglobulin (dotted lines, closed symbols) in a range of systems (see Fig. 1), for a range of times, at 400 MPa pressure (except sodium caseinate-containing buffer, which was treated at 800 MPa).

Figure 2. Inactivation de l'activité de la plasmine (lignes pleines, symboles ouverts) et dénaturation de la β -lactoglobuline (lignes pointillées, symboles fermés) pour une série de systèmes (Fig. 1), pour une série de temps, pour une pression de 400 MPa (excepté pour la solution tampon de caséinate de sodium, qui a été traitée à 800 MPa).

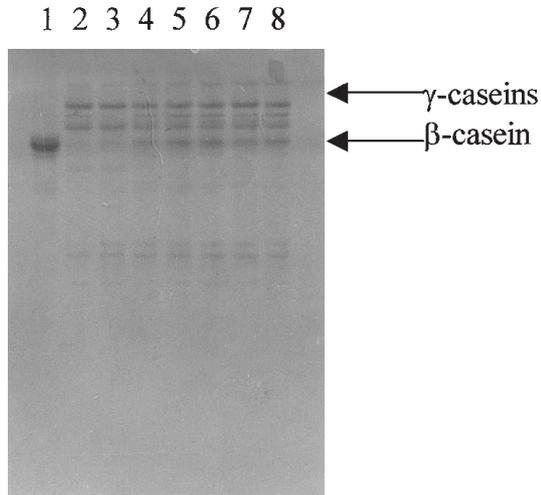


Figure 3. Urea-polyacrylamide gel electrophoretograms (pH 8.9; 12.5% T; 4% °C) of bovine β -casein ($2 \text{ mg}\cdot\text{mL}^{-1}$, lane 1) in $50 \text{ mmol}\cdot\text{L}^{-1}$ ammonium bicarbonate buffer (pH 8.4), hydrolysed by porcine plasmin ($0.0625 \text{ units}\cdot\text{mL}^{-1}$) for 30 min at 20°C at atmospheric pressure (lane 2), or at 300, 400, 500, 600, 700 or 800 MPa (lanes 3–8).

Figure 3. Électrophorégramme en gel de polyacrylamide-urée (pH 8,9 ; 12,5 % T ; 4 % C) de caséine β bovine ($2 \text{ mg}\cdot\text{mL}^{-1}$, ligne 1) dans une solution tampon de $50 \text{ mmol}\cdot\text{L}^{-1}$ de bicarbonate d'ammonium (pH 8,4), hydrolysée par la plasmine porcine ($0,0625 \text{ unités}\cdot\text{mL}^{-1}$) pendant 30 min à 20°C à la pression atmosphérique (ligne 2), ou à 300, 400, 500, 600, 700 ou 800 MPa (lignes 3–8).

unpressurised control sample at pressures $\geq 400 \text{ MPa}$. The pattern of products of β -casein hydrolysis (production of slower-moving γ -caseins and faster-moving proteose peptones) was unaffected by pressure, suggesting that plasmin had a similar proteolytic specificity but showed decreased activity at high pressures compared to atmospheric pressure.

The production of 2% TCA-soluble peptides from hydrolysis of β -casein by plasmin was monitored by RP-HPLC (Fig. 4). In general, peptide patterns produced at high and atmospheric pressures were similar, suggesting that pressure induced quantitative, but not qualitative, differences in the hydrolysis reaction although a new peak (of retention time approx. 36 min) appeared in the digest performed at 800 MPa. Peptide production was unaffected at pressures $\leq 700 \text{ MPa}$, but decreased relative to unpressurised control samples at 800 MPa (Fig. 5).

4. DISCUSSION

In this study the barostability of plasmin was examined in simple protein-containing

buffer systems, to elucidate the survival of plasmin after high pressure treatment. Plasmin was much more pressure sensitive in the presence of β -lactoglobulin than in other buffers studied. The results of the kinetic study verified that plasmin was relatively barostable in buffer alone, in cheese and in the presence of sodium caseinate. The results concerning the effects of milk proteins on plasmin stability are similar to previous observations on the heat inactivation mechanism of plasmin. Specifically, several studies have reported significant protection of plasmin in the presence of sodium caseinate, and enhanced inactivation in the presence of β -lactoglobulin [1, 22, 29]. Heat inactivation of plasmin in the presence of β -lactoglobulin is thought to be linked to the formation of thiol-disulphide bonds with unfolded β -lactoglobulin [13, 16]. Pressurisation also induces unfolding of β -lactoglobulin [9, 18], and consequently exposes the highly reactive internal thiol group, which may be able to undergo thiol-disulphide interactions with disulphide groups linking structural subunits in the plasmin molecule.

In the absence of β -lactoglobulin it was found that plasmin was quite pressure stable.

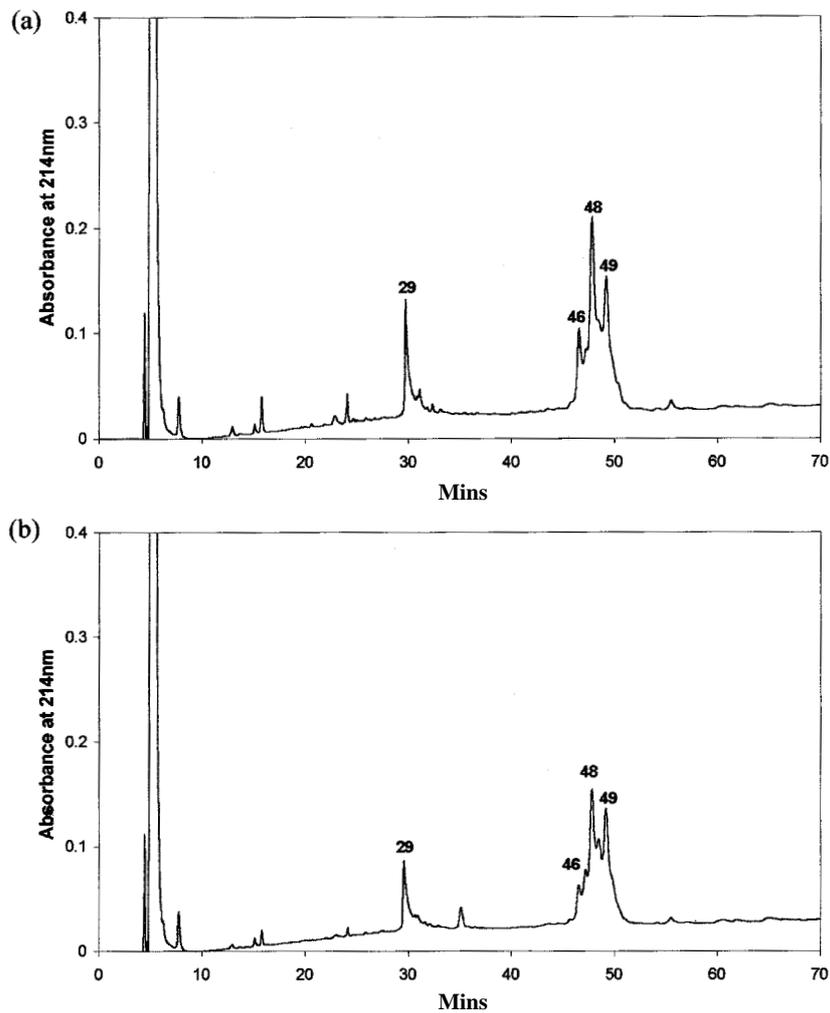


Figure 4. Reversed-phase HPLC chromatograms of 2% trichloroacetic acid (TCA)-soluble peptides produced from β -casein ($2 \text{ mg}\cdot\text{mL}^{-1}$) by plasmin ($0.0625 \text{ units}\cdot\text{mL}^{-1}$) incubated in $0.05 \text{ mol}\cdot\text{L}^{-1}$ ammonium bicarbonate buffer, pH 8.4 for 30 min at 20°C at (a) atmospheric pressure and (b) 800 MPa.

Figure 4. Chromatogrammes phase-reverse de peptides soluble dans 2 % acide trichloroacétique (TCA) produit à partir de β -caseine ($2 \text{ mg}\cdot\text{mL}^{-1}$) par la plasmine ($0,0625 \text{ unités}\cdot\text{mL}^{-1}$) incubée dans une solution tampon $0,05 \text{ mol}\cdot\text{L}^{-1}$ de bicarbonate d'ammonium, pH 8,4 pour 30 min à 20°C à (a) pression atmosphérique et (b) 800 MPa.

This indicates that, under certain conditions, plasmin molecules can refold into the correct tertiary structure when pressure is released and thus remain active. In Cheddar cheese, plasmin activity was unaffected by pressure

treatments up to 600 MPa for 20 min, although increased plasmin activity in Gouda cheese following pressurisation at 100–400 MPa for 30 min during brining has been reported [21].

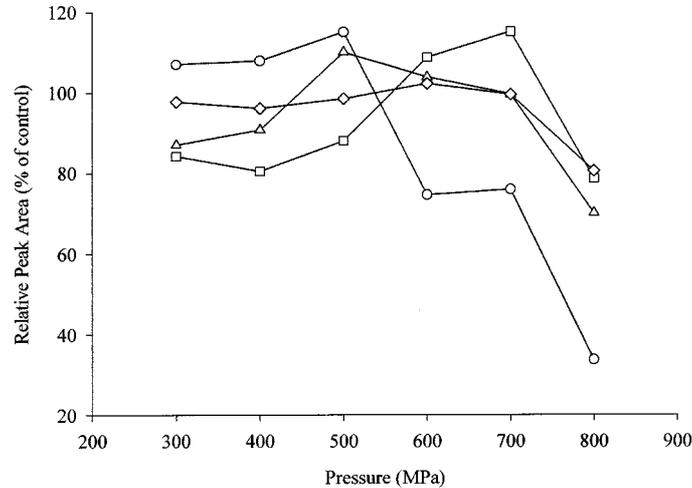


Figure 5. Effect of pressurisation on production of 2% TCA soluble peptides from β -casein by plasmin. Symbols refer to peaks in chromatograms in Figure 4, as follows: peak of retention time 29 min (Δ); peak of retention time 46 min (\circ); peaks of retention time 47–48.5 min (\square) and peak of retention time 49 min (\diamond).

Figure 5. Effet de la pression sur la production de peptides solubles dans 2 % TCA à partir de β -caséine ($2 \text{ mg}\cdot\text{mL}^{-1}$) par la plasmine ($0,0625 \text{ unités}\cdot\text{mL}^{-1}$). Les symboles réfèrent aux pics du chromatogramme de la Figure 4, comme suivant : pic de temps de rétention 29 min (Δ) ; pic de temps de rétention 46 min (\circ) ; pics de temps de rétention 47–48.5 min (\square) et pic de temps de rétention 49 min (\diamond).

Comparison of the results of this study with those of a study of the effect of HP on proteolysis in milk [30] suggest that plasmin is more pressure stable in milk than in buffer containing β -lactoglobulin, possibly due to the combination of the protective effect of casein present in milk and the destabilising effect of β -lactoglobulin.

A possible important influence on the results obtained from the inactivation experiments described which must be considered is pressure-induced pH shifts in the buffers used. In phosphate- and carbonate-based buffers, pressure-induced ionisation results in a volume change and concomitant pH shift in the acidic direction [23]. In phosphate buffer, an increase in pressure of 100 MPa results in a decrease in pH of 0.2–0.3 units [9], and thus, inactivation at increasing pressures would have occurred at decreasing pH values. Although the

effects of pH on the pressure stability of plasmin are not known, the heat stability of plasmin in micellar casein systems is increased at lower pH [14], which may suggest that the enzyme is generally more robust in increasingly acidic conditions. In phosphate buffer alone or containing sodium caseinate, at higher pressures (and hence lower pH values) plasmin activity was very barostable, which may have thus been, at least partially, due to increasing stability at lower pH.

In the more complex case of the buffer containing β -lactoglobulin, the effects of reducing pH on this protein, the enzyme and their interaction must be considered. It has been reported that, at pH values near neutral, β -lactoglobulin unfolds readily and irreversibly to expose its reactive sulphhydryl group, which can then undergo sulphhydryl-disulphide interchange reactions, while at

lower pH values, unfolding is less favoured and the protein is protected from extensive interactions [6, 9]. On heating milk, it is the sulphhydryl group that reacts with plasmin to inactivate the enzyme [16], which suggests that reducing pH should indirectly increase the stability of the enzyme in such systems, if an analogous mechanism to heat inactivation occurs. However, increasing pressure in the presence of β -lactoglobulin decreased residual plasmin activity, which indicates that the inactivation occurred despite the potentially reduced reactivity of denatured β -lactoglobulin with the enzyme at reduced pH. The magnitude of decrease in plasmin activity at 400 MPa was considerably greater than that of residual β -lactoglobulin, which may suggest that the reactions are not completely parallel, although β -lactoglobulin clearly destabilised the enzyme relative to systems where this protein was not present. Further study is necessary to elucidate the relationship between pressure-induced β -lactoglobulin denaturation and plasmin inactivation.

In comparison to other food enzymes reported in the literature, the results of the current study indicate that plasmin, particularly in the absence of β -lactoglobulin, is relatively pressure stable. In a similar manner to peroxidase, polyphenoloxidase and milk alkaline phosphatase, it was almost unaffected following high pressure treatment at 600 MPa for 20 min. It was more barostable, however, than other previously studied food enzymes such as catalase, phosphatase, lipase, pectin esterase, lactoperoxidase and lipooxygenase, all of which were inactivated to varying degrees by pressure treatment at 600 MPa [31]. When compared to other milk enzymes, plasmin displayed greater pressure resistance than phosphohexoseisomerase and glutamyltransferase, which were extensively inactivated following high pressure treatment at 500 MPa for 10 min [27].

The hydrolysis of β -casein by plasmin has been comprehensively studied at atmo-

spheric pressure [5, 13, 32]. However, very little is known about this reaction at elevated pressures and thus, in this study, the effect of high pressure on this digestion reaction was examined. From urea-PAGE analysis, primary proteolysis of β -casein by plasmin appeared unaffected at 300 MPa, but decreased at pressures ≥ 400 MPa, relative to unpressurised controls. The production of 2% TCA-soluble peptides by plasmin was unaffected at pressure treatments up to 700 MPa, compared to unpressurised controls. It was only at the highest applied pressure (800 MPa) that release of peptides decreased relative to control. Thus, not only is plasmin stable at high pressures in the presence of casein, but it is proteolytically active, suggesting that pressure does not induce reversible unfolding of the enzyme molecule.

Ohimya et al. [25] monitored the effect of pressure treatment on the turbidity of enzyme-treated β -casein solutions, and reported dissociation of β -casein molecules at pressures up to ~ 115 MPa, followed by re-association at higher pressures (up to ~ 290 MPa). In the present study, β -casein hydrolysis was gradually inhibited at pressures > 300 MPa, which may have been due either to modification of β -casein molecules or inactivation of plasmin. When plasmin initially cleaves β -casein at the bonds Lys₂₈-Lys₂₉, Lys₁₀₅-His₁₀₆ and Lys₁₀₇-Glu₁₀₈, the highly charged hydrophilic domain is cleaved from the remaining hydrophobic region of the protein. As a result, the enzyme treated β -casein has a greater tendency to re-associate when pressure is applied [24]. The increased association of hydrolysed β -casein molecules may result in decreased surface area available to plasmin, and consequently decreased proteolysis. Interestingly, Pitta et al. [26] reported improved foaming properties of native β -casein following pressurisation at 300, 600 and 900 MPa for various time periods, and suggested a possible change in β -casein conformation on pressurisation due to a modification of tertiary structure. However,

Dickinson et al. [7] reported no significant effect of high pressure processing on the surface activity of β -casein, and suggested that β -casein is extremely barostable as it is a non-globular protein with little tertiary and secondary structure (only ~ 5% α -helix).

Again, the effect on the enzyme reaction of decreasing pH on treating the bicarbonate buffer-based system at increasingly high pressures must be considered. At atmospheric pressure, the activity of plasmin against β -casein proceeds with similar specificity but lower rate at decreasing pH values [14]. In this study, incubation at increasing pressures resulted in decreased β -casein hydrolysis, which may thus be due to either or both of two mechanisms, enzyme inactivation or decreased pH. While these effects cannot be separated under the experimental design used, it may be acknowledged that, if the latter effect contributes significantly to the results obtained, plasmin may in fact be even more pressure stable than suggested herein.

5. CONCLUSION

The present results indicate that plasmin is a very pressure stable enzyme, except in systems incorporating β -lactoglobulin. Furthermore, it can readily hydrolyse β -casein under pressures up to 700 MPa, with a specificity identical to that observed at atmospheric pressure. As plasmin is an enzyme that has significant implications for the quality of many dairy products, the development of HP applications for such products must consider the possible effects of hydrolysis of casein by plasmin post-treatment on product quality.

ACKNOWLEDGEMENTS

The authors wish to thank C. O'Reilly and P. O'Connor of the Dairy Products Research Centre, Teagasc, Moorepark for assistance with the high pressure trials, and R. Deletang and M. Feltrin of the Department of Food Science

and Technology at University College Cork for linguistic guidance.

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