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## The individual or combined action of chymosin and plasmin on sodium caseinate or $\beta$ -casein in solution: effect of NaCl and pH

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**Abstract** — Although  $\beta$ -casein appeared to be degraded by bovine plasmin slightly faster in a salt-free system than in a solution containing NaCl, increasing the concentration of NaCl had little effect on the activity or specificity of the enzyme. The activity, but not the specificity, of bovine plasmin on  $\beta$ -casein was influenced by pH; the rate of hydrolysis proceeded in the order pH 8.4 > 6.5 > 5.4. Chymosin did not appear to degrade peptides produced from  $\beta$ -casein by plasmin (i.e.  $\gamma_1$ ,  $\gamma_2$ ,  $\gamma_3$ -caseins and their corresponding proteose peptones). In sodium caseinate,  $\alpha_{s1}$ -casein was more susceptible to hydrolysis by chymosin than  $\beta$ -casein. The activity of chymosin on Na-caseinate increased with decreasing pH in the range of 6.5 to 5.4. Increasing concentrations of NaCl inhibited proteolysis to an extent that was dependent on the reaction pH. Bovine plasmin rapidly hydrolysed Na-caseinate to a wide range of peptides detectable by polyacrylamide gel electrophoresis (PAGE) and it was also capable of degrading Na-caseinate-derived peptides produced by chymosin to a similar range of peptides. © Inra/Elsevier, Paris.

**chymosin / plasmin / sodium caseinate /  $\beta$ -casein / hydrolysis**

**Résumé** — Action individuelle ou combinée de la chymosine et de la plasmine sur le caséinate de sodium ou la caséine  $\beta$  en solution : effet du NaCl et du pH. Bien que la caséine  $\beta$  soit dégradée par la plasmine bovine légèrement plus vite dans une solution sans sel que dans une solution contenant du NaCl, l'augmentation de la concentration en NaCl avait peu d'effet sur l'activité et la spécificité de l'enzyme. L'activité de la plasmine bovine sur la caséine  $\beta$  était influencée par le pH, mais pas la spécificité ; la vitesse d'hydrolyse diminuait lorsque le pH passait de 8,4 à 6,5 puis à 5,4. La chymosine ne dégradait pas les peptides obtenus par action de la plasmine sur la caséine  $\beta$  (i.e. caséines  $\gamma_1$ ,  $\gamma_2$ , et  $\gamma_3$ , et leurs protéoses peptones correspondantes). Dans le caséinate de sodium, la caséine  $\alpha_{s1}$  était plus sensible à l'hydrolyse par la chymosine que la caséine  $\beta$ . L'activité de la chymosine sur le

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caséinate de sodium augmentait quand le pH diminuait de 6,5 à 5,4. L'augmentation des concentrations de NaCl inhibait la protéolyse jusqu'à un certain point dépendant du pH de la réaction. La plasmine bovine hydrolysait rapidement le caséinate de sodium en une large gamme de peptides, détectables par PAGE, et elle était également capable de dégrader les peptides dérivés du caséinate de sodium produits par la chymosine en une gamme similaire de peptides. © Inra/Elsevier, Paris.

## chymosine / plasmine / caséinate de sodium / caséine $\beta$ / hydrolyse

### 1. INTRODUCTION

Chymosin (E.C. 3.4.23.4), a gastric aspartyl proteinase, is the principal enzyme in traditional rennets used to coagulate milk for cheesemaking. The rapid and specific hydrolysis of the Phe<sub>105</sub>-Met<sub>106</sub> bond in  $\kappa$ -casein by chymosin destabilises the casein micelles which coagulate in the presence of Ca<sup>2+</sup>. In isolated form,  $\kappa$ -casein is also hydrolysed rapidly by chymosin at this bond [7, 34]. Chymosin is also capable of hydrolysing the other major caseins in solution. The most susceptible bond in  $\alpha_{s1}$ -casein is Phe<sub>23</sub>-Phe<sub>24</sub> [22]. The proteolytic specificity of chymosin on  $\alpha_{s1}$ -casein in solution has been the subject of many studies [25, 28, 30, 32].  $\alpha_{s1}$ -Casein is optimally hydrolysed by chymosin in the presence of 5 % NaCl and considerable proteolysis occurs even in the presence of 20 % NaCl. The degree of substrate aggregation is also thought to influence the peptides produced from  $\alpha_{s1}$ -casein by chymosin [28]. The initial hydrolysis of  $\beta$ -casein in solution by chymosin yields three peptides,  $\beta$ -I,  $\beta$ -II and  $\beta$ -III, produced by cleavage of bonds 192-193 and/or 189-190, 163-164 and/or 165-166 and/or 167-168 and 139-140, respectively [6, 8, 33, 38]. Fox and Walley [16] reported that the hydrolysis of  $\beta$ -casein by chymosin was significantly inhibited by 5 %, and completely by 10 %, NaCl and that the inhibitory effect of NaCl is independent of pH and incubation temperature. However, Mulvihill and Fox [29] found that NaCl inhibited proteolysis of  $\beta$ -casein by chymosin to an extent dependent on reaction pH. They also reported that the speci-

ficity of chymosin on  $\beta$ -casein was independent of pH and the degree of substrate aggregation. The specificity of chymosin on  $\alpha_{s2}$ -casein in solution was studied by McSweeney et al. [27], who identified seven cleavage sites in the protein.

Although chymosin is capable of hydrolysing  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -caseins in solution, its activity in cheese is somewhat different [14]. In internal bacterially ripened cheese, such as Cheddar,  $\alpha_{s1}$ -casein is extensively degraded by chymosin during ripening while  $\beta$ -casein is quite resistant to proteolysis, possibly due to, at least in part, the high NaCl concentration in Cheddar cheese.  $\alpha_{s2}$ -Casein and para- $\kappa$ -casein also appear to be resistant to hydrolysis by chymosin. The coagulant (chymosin) contributes to primary proteolysis during ripening and hence affects the quality of the mature cheese [15].

Plasmin (fibrinolysin, E.C. 3.4.21.7), the principal indigenous proteinase in milk, is a serine proteinase with trypsin-like activity and a pH optimum of ~7.5; it cleaves bonds of the type Lys-X and to a lesser extent, Arg-X. The properties and significance of plasmin have been extensively reviewed [4, 21, 37]. In milk,  $\alpha_{s2}$ - and  $\beta$ -caseins are hydrolysed rapidly by plasmin [21, 36];  $\alpha_{s1}$ - is degraded more slowly while  $\kappa$ -casein is very resistant [10]. Plasmin activity results in the formation of  $\gamma$ - and  $\lambda$ -caseins and proteose peptones.  $\gamma$ -Caseins result from the hydrolysis of  $\beta$ -casein by plasmin [19] while the  $\lambda$ -casein fraction consists of peptides produced from  $\alpha_{s1}$ -casein [1]. The proteose peptone fraction consists mainly of peptides produced from caseins by the action of plasmin [3].

The specificity of plasmin on  $\beta$ -casein is well documented [10, 11]. As well as producing  $\gamma_1$ - ( $\beta$ -CNf29-209),  $\gamma_2$ - ( $\beta$ -CNf106-209),  $\gamma_3$ - ( $\beta$ -CNf108-209) caseins and their complementary N-terminal peptides, plasmin can also cleave bonds Lys<sub>113</sub>-Tyr<sub>114</sub> and Arg<sub>183</sub>-Asp<sub>184</sub> in  $\beta$ -casein fairly rapidly [18].  $\alpha_{s2}$ -Casein contains more potential plasmin sites than any other casein; however, only eight of them appear to be hydrolysed in solution [23, 39]. The specificity of plasmin on  $\alpha_{s1}$ -casein was reported by Le Bars and Gripon [24] and McSweeney et al. [26].

Since plasmin is strongly associated with the casein micelles in milk [21], it is incorporated into the curd during cheesemaking and is active in cheese during ripening. The level of plasmin activity depends on the cheese variety [21]. In internal bacterially ripened cheeses, such as Cheddar, plasmin activity is evident by the limited hydrolysis of  $\beta$ -casein with a concomitant increase in the  $\gamma$ -caseins. The extent to which plasmin contributes to proteolysis during ripening is ill-defined and it was thought for a long time to be limited to the slow hydrolysis of  $\beta$ -casein. However, Farkye and Fox [12, 13] found that plasmin contributes to both the level and type of water-soluble peptides formed from caseins during Cheddar cheese ripening.

It is difficult to assess the individual contributions of chymosin and plasmin to proteolysis during cheese ripening, but it seems possible that chymosin may degrade peptides derived from casein by the action of plasmin and vice versa. This study was undertaken to investigate the activity of chymosin on a plasmin hydrolysate of  $\beta$ -casein and that of plasmin on a chymosin hydrolysate of Na-caseinate. The conditions used in both experiments (i.e. pH 5.4 and 5%, w/v, NaCl) mimicked those prevailing in Cheddar cheese. The effects of pH and NaCl on the proteolysis of  $\beta$ -casein by plasmin or Na-caseinate by chymosin were also studied.

## 2. MATERIALS AND METHODS

### 2.1. Enzymes

Recombinant chymosin (Maxiren, expressed in *Kluyveromyces marxianus* var. *lactis*) was obtained from Gist Brocades Ltd., Delft, the Netherlands. It was dialysed against three 50-volume changes of distilled H<sub>2</sub>O at 4 °C for 72 h and freeze-dried. The freeze-dried enzyme was redissolved in 100 mmol·L<sup>-1</sup> sodium phosphate buffer, pH 6.5 (22.8 mg·mL<sup>-1</sup>). This solution had an activity of approximately 53 chymosin units (CU) mL<sup>-1</sup>, where 1 CU is the activity required to coagulate 10 mL of bovine milk, pH 6.5, in 100 s at 30 °C. Plasmin (fibrinolysin, E.C. 3.4.21.7, from bovine or porcine plasma) was obtained from the Sigma Chemical Co., St. Louis, MO, USA. It was dissolved in distilled H<sub>2</sub>O (38.46 mg·mL<sup>-1</sup>); this solution had 1.3 units of plasmin activity, where 1 unit is the amount of enzyme that will produce a  $\Delta A_{275}$  of 10 from  $\alpha_{s1}$ -casein in 20 min at pH 7.5 and 37 °C.

### 2.2. Substrates

Whole casein was prepared from bovine skimmed milk by isoelectric precipitation, according to the method of Mulvihill and Fox [28]. Crude  $\beta$ -casein was prepared by the method of Fox and Guiney [17] and purified by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) using 10 mmol·L<sup>-1</sup> imidazole buffer, pH 7.0, containing 4.5 mol·L<sup>-1</sup> urea and 0.1% (v/v) 2-mercaptoethanol. Proteins were eluted from the column by a linear NaCl gradient (0–0.5 mol·L<sup>-1</sup>). Fractions corresponding to  $\beta$ -casein were pooled, dialysed against three changes of distilled H<sub>2</sub>O and freeze-dried. The purity of  $\beta$ -casein was assessed by urea-polyacrylamide gel electrophoresis (PAGE).

### 2.3. Hydrolysis conditions

#### 2.3.1. Proteolysis of $\beta$ -casein by bovine or porcine plasmin

$\beta$ -Casein (5 mg·mL<sup>-1</sup>) was dissolved in 100 mmol·L<sup>-1</sup> sodium phosphate buffer, pH 5.4, containing 5% (w/v) NaCl. Sodium azide (NaN<sub>3</sub>; 0.05%, w/v) was added to the solution (and all other solutions) to inhibit bacterial growth.

Bovine or porcine plasmin ( $0.25 \text{ U}\cdot\text{mL}^{-1}$ ) was added and the solution incubated at  $30^\circ\text{C}$ . Samples were taken periodically over 120 min and heated ( $100^\circ\text{C} \times 5 \text{ min}$ ) to inactivate the enzyme.

### **2.3.2. Effect of NaCl on the proteolysis of $\beta$ -casein by bovine plasmin**

Solutions of  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) containing 0, 2, 5 or 10 % (w/v) NaCl were adjusted to pH 5.4. Bovine plasmin ( $0.10 \text{ U}\cdot\text{mL}^{-1}$ ) was added and the solutions incubated at  $30^\circ\text{C}$ . Samples were taken after 5, 15 or 30 min and heated ( $100^\circ\text{C} \times 5 \text{ min}$ ) to inactivate plasmin.

### **2.3.3. Effect of pH on the proteolysis of $\beta$ -casein by bovine plasmin**

Solutions of  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) were individually adjusted to pH 8.4, 6.5 or 5.4 and treated with bovine plasmin ( $0.05 \text{ U}\cdot\text{mL}^{-1}$  at pH 8.4;  $0.075 \text{ U}\cdot\text{mL}^{-1}$  at pH 6.5;  $0.10 \text{ U}\cdot\text{mL}^{-1}$  at pH 5.4) at  $30^\circ\text{C}$ . Samples were taken after 5, 15 or 30 min and heated at  $100^\circ\text{C} \times 5 \text{ min}$ .

### **2.3.4. Proteolysis of $\beta$ -casein or a plasmin hydrolysate of $\beta$ -casein by chymosin**

A solution of  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) containing 5 % (w/v) NaCl was adjusted to pH 5.4. Bovine plasmin ( $0.10 \text{ U}\cdot\text{mL}^{-1}$ ) was added and the solution incubated at  $30^\circ\text{C}$  for 15 min. The solution was heated at  $100^\circ\text{C}$  for 5 min to inactivate plasmin. Solutions of  $\beta$ -casein and the plasmin hydrolysate of  $\beta$ -casein were treated with chymosin ( $0.1 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$ . Samples were taken periodically between 0 and 9 h and heated ( $100^\circ\text{C} \times 5 \text{ min}$ ) to inactivate chymosin.

### **2.3.5. Effect of NaCl and pH on the proteolysis of sodium caseinate by chymosin**

Na-caseinate ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) was dissolved in  $100 \text{ mmol}\cdot\text{L}^{-1}$  sodium phosphate buffer at pH 6.5, 5.8 or 5.2, containing 0, 2, 5 or 10 % (w/v) NaCl. Chymosin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) was added and the solutions incubated at  $30^\circ\text{C}$  for 1 h. Chymosin was then inactivated by heating at  $100^\circ\text{C}$  for 5 min.

### **2.3.6. Proteolysis of Na-caseinate or a chymosin hydrolysate of Na-caseinate by bovine plasmin**

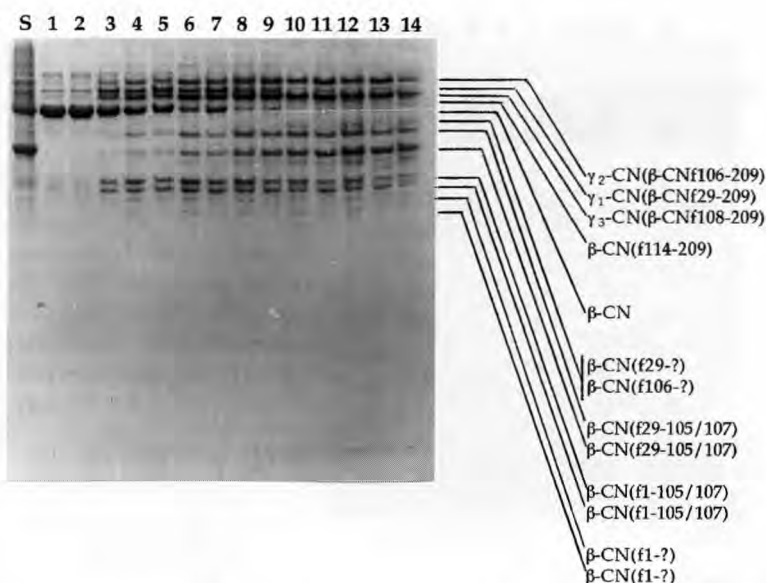
A solution of Na-caseinate ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) containing 5 % (w/v) NaCl was adjusted to pH 5.4. Chymosin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) was added and the solution incubated at  $30^\circ\text{C}$  for 3 h. The solution was heated at  $100^\circ\text{C}$  for 5 min to inactivate chymosin. Solutions of Na-caseinate and the chymosin hydrolysate of Na-caseinate were treated with bovine plasmin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$ . Samples were taken periodically between 0 and 24 h and heated at  $100^\circ\text{C}$  for 5 min.

### **2.4. Urea-polyacrylamide gel electrophoresis (urea-PAGE)**

Samples of hydrolysates were prepared for urea-PAGE by the addition of an equal volume of double-strength sample buffer [25]. Urea-PAGE was performed in a Protean II XI (Bio-Rad Laboratories Ltd., Watford, Herts, UK) vertical slab-cell according to the method of Andrews [2], with direct staining using Coomassie Brilliant blue G 250 [5].

## **3. RESULTS AND DISCUSSION**

The results showed that  $\beta$ -casein was degraded more rapidly by porcine than by bovine plasmin at  $30^\circ\text{C}$  during the 120-min incubation period (*figure 1*). No major qualitative differences were evident between the electrophoretograms of the bovine and porcine plasmin hydrolysates of  $\beta$ -casein at any stage of incubation (*figure 1*), indicating that the specificity of plasmin from these two sources on  $\beta$ -casein was similar. These results agree with the findings of de Rham and Andrews [9] and Andrews and Alichanidis [3]. The hydrolysis products of  $\beta$ -casein (labelled in *figure 1*) were previously isolated and identified by Singh [35]. It should be noted that some of the (small) peptides produced by the action of plasmin and/or chymosin on  $\beta$ -casein or sodium caseinate may be soluble under the conditions used during this study (i.e. urea-PAGE) and,



**Figure 1.** Urea-polyacrylamide gel electrophoretograms of bovine  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) in  $100 \text{ mmol}\cdot\text{L}^{-1}$  sodium phosphate buffer, pH 5.4, containing 5% (w/v) NaCl, hydrolysed by bovine (lanes 1, 3, 5, 7, 9, 11 and 13) or porcine (lanes 2, 4, 6, 8, 10, 12 and 14) plasmin ( $0.25 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$  for 0 (lanes 1 and 2), 10 (lanes 3 and 4), 20 (lanes 5 and 6), 30 (lanes 7 and 8), 60 (lanes 9 and 10), 90 (lanes 11 and 12) and 120 min (lanes 13 and 14). S: sodium caseinate.

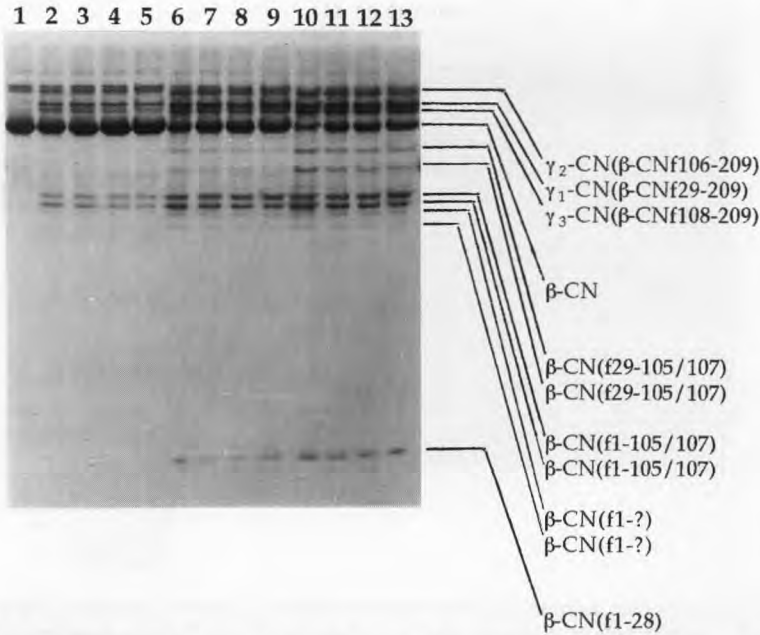
**Figure 1.** Électrophorégramme en gel de polyacrylamide-urée de caséine  $\beta$  bovine ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) dans un tampon phosphate de sodium à  $100 \text{ mmol}\cdot\text{L}^{-1}$ , pH 5,4, contenant 5% (p/v) de NaCl, hydrolysés par  $0,25 \text{ U}\cdot\text{mL}^{-1}$  de plasmine bovine (lignes 1, 3, 5, 7, 9, 11 et 13) ou porcine (lignes 2, 4, 6, 8, 10, 12 et 14) à  $30^\circ\text{C}$  pendant 0 min (lignes 1 et 2), 10 min (lignes 3 et 4), 20 min (lignes 5 et 6), 30 min (lignes 7 et 8) 60 min (lignes 9 et 10), 90 min (lignes 11 et 12) et 120 min (lignes 13 et 14). S : caséinate de sodium.

therefore, may not be detected on the gels. The initial hydrolysis of  $\beta$ -casein by plasmin (bovine or porcine) produced  $\gamma_1$ -,  $\gamma_2$ - and  $\gamma_3$ -caseins and their corresponding proteose peptones,  $\beta$ -CN f1-105/107 and  $\beta$ -CN f29-105/107 (*figure 1*, lanes 3 and 4). The band corresponding to  $\gamma_1$ -casein began to disappear after 60 min (particularly in the porcine plasmin hydrolysate; *figure 1*, lane 10) with a concomitant increase in a band with slightly faster mobility than  $\gamma_3$ -casein, identified by Singh [35] as  $\beta$ -CN f114-209.  $\beta$ -Casein was degraded almost completely by bovine or porcine plasmin after 120 min (*figure 1*, lanes 13 and 14, respectively).

The hydrolysis of  $\beta$ -casein by bovine plasmin was inhibited slightly by 2% (w/v)

NaCl (*figure 2*, lanes 3, 7 and 11); however, increasing the concentration to 10% (w/v) NaCl had little effect on the rate of hydrolysis of  $\beta$ -casein (*figure 2*, lanes 4–5, 8–9 and 12–13). Qualitatively, the electrophoretograms of the bovine plasmin hydrolysates of  $\beta$ -casein without or with 2, 5 or 10% (w/v) NaCl were similar at all stages of incubation (*figure 2*, lanes 2–13), indicating that the specificity of bovine plasmin was not influenced by NaCl.

The rate of  $\beta$ -casein degradation by plasmin proceeded in the order pH 8.4 > 6.5 > 5.4 (*figure 3*), although the amount of plasmin activity added was 1.5 and 2 times higher at pH 6.5 and 5.4, respectively, than at pH 8.4. This is not surprising since plas-



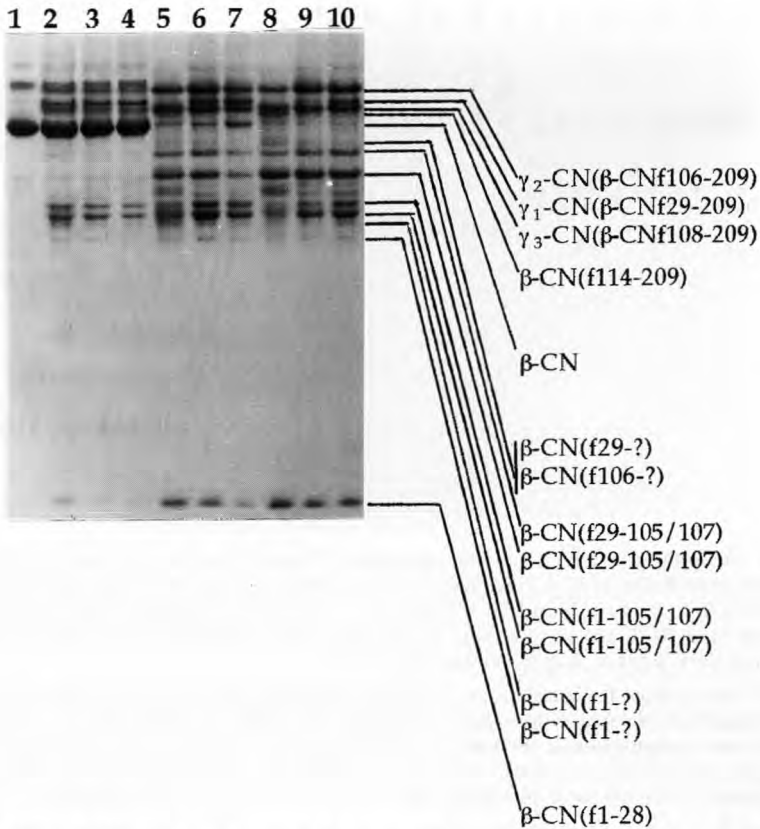
**Figure 2.** Effect of NaCl on the proteolysis of  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) by bovine plasmin ( $0.10 \text{ U}\cdot\text{mL}^{-1}$ ) at pH 5.4,  $30^\circ\text{C}$ . Lane 1:  $\beta$ -casein control; lanes 2, 6 and 10: samples containing 0 % NaCl incubated for 5, 15 and 30 min, respectively; lanes 3, 7 and 11: samples containing 2 % NaCl incubated for 5, 15 and 30 min, respectively; lanes 4, 8 and 12: samples containing 5 % NaCl incubated for 5, 15 and 30 min, respectively; lanes 5, 9 and 13: samples containing 10 % NaCl incubated for 5, 15 and 30 min, respectively.

**Figure 2.** Effet du NaCl sur la protéolyse de la caséine  $\beta$  ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) par la plasmine bovine ( $0,10 \text{ U}\cdot\text{mL}^{-1}$ ) à pH 5,4 et à  $30^\circ\text{C}$ . Ligne 1 : caséine  $\beta$  témoin ; lignes 2, 6 et 10 : échantillons contenant 0 % de NaCl incubés respectivement pendant 5, 15 et 30 min ; lignes 3, 7 et 11 : échantillons contenant 2 % de NaCl incubés respectivement pendant 5, 15 et 30 min ; lignes 4, 8 et 12 : échantillons contenant 5 % de NaCl incubés respectivement 5, 15, et 30 min ; lignes 5, 9 et 13 : échantillons contenant 10 % de NaCl incubés respectivement 5, 15 et 30 min.

min is optimally active at pH  $\sim 7.5$  [21]. Some qualitative differences were observed between the electrophoretograms of the plasmin hydrolysates of  $\beta$ -casein at pH 8.4, 6.5 and 5.4, especially after 15 and 30 min incubation (figure 3, lanes 5–7 and 8–10); these differences were probably due to the varying rates of hydrolysis of  $\beta$ -casein rather than differences in the specificity of plasmin at the different pH values.

$\beta$ -Casein was almost completely degraded by chymosin within 24 h to at least four peptides with electrophoretic mobilities faster

than  $\beta$ -casein (figure 4, lanes 1–7). The initial degradation products of  $\beta$ -casein, evident in the electrophoretogram of the 1-h chymosin hydrolysate (figure 4, lane 2), are presumably  $\beta$ -I-CN (f1-189/192) and  $\beta$ -II-CN (f1-163/57) [8, 30, 38]. The plasmin hydrolysate of  $\beta$ -casein after incubation at  $30^\circ\text{C}$  for 15 min contained residual  $\beta$ -,  $\gamma_1$ -,  $\gamma_2$ - and  $\gamma_3$ -caseins and their corresponding proteose peptones (figure 4, lane 8). Electrophoretograms of this hydrolysate after treatment with chymosin at  $30^\circ\text{C}$  for up to 9 h showed that residual  $\beta$ -casein was hydrolysed by chymosin to  $\beta$ -I-CN and



**Figure 3.** Effect of pH on the proteolysis of  $\beta$ -casein ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) by bovine plasmin  $30^\circ\text{C}$ . The enzyme levels used were  $0.05 \text{ U}\cdot\text{mL}^{-1}$  at pH 8.4,  $0.075 \text{ U}\cdot\text{mL}^{-1}$  at pH 6.5 and  $0.10 \text{ U}\cdot\text{mL}^{-1}$  at pH 5.4. Lane 1:  $\beta$ -casein control; lanes 2, 5 and 8: samples hydrolysed at pH 8.4 for 5, 15 and 30 min, respectively; lanes 3, 6 and 9: samples hydrolysed at pH 6.5 for 5, 15 and 30 min, respectively; lanes 4, 7 and 10: samples hydrolysed at pH 5.4 for 5, 15 and 30 min, respectively.

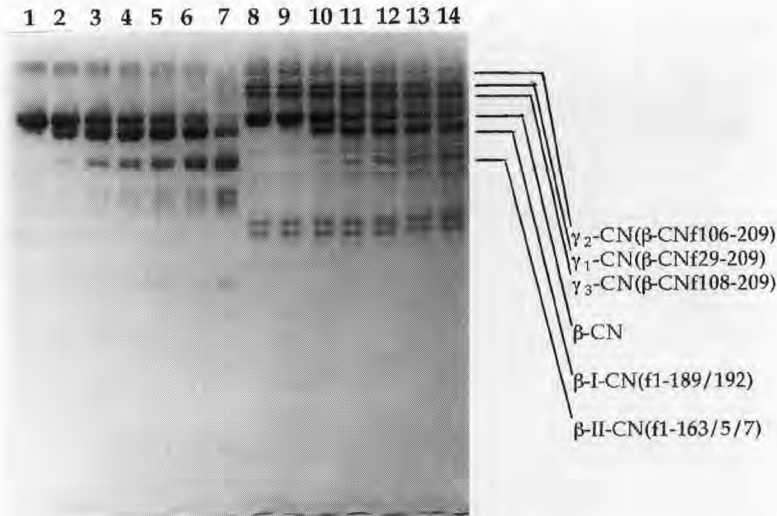
**Figure 3.** Effet du pH sur la protéolyse de la caséine  $\beta$  ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) par la plasminine bovine à  $30^\circ\text{C}$ . Les niveaux d'enzyme utilisés étaient de  $0,05 \text{ U}\cdot\text{mL}^{-1}$  à pH 8,4,  $0,075 \text{ U}\cdot\text{mL}^{-1}$  à pH 6,5 et  $0,10 \text{ U}\cdot\text{mL}^{-1}$  à pH 5,4. Ligne 1 : caséine  $\beta$  témoin ; lignes 2, 5 et 8 : échantillons hydrolysés à pH 8,4 pendant 5, 15 et 30 min respectivement ; lignes 3, 6 et 9 : échantillons hydrolysés à pH 6,5 pendant 5, 15 et 30 min respectivement ; lignes 4, 7 et 10 : échantillons hydrolysés à pH 5,4 pendant 5, 15 et 30 min respectivement.

$\beta$ -II-CN (figure 4, lanes 9–14). Although the intensity of the bands corresponding to  $\gamma_1$ - and  $\gamma_2$ -caseins decreased slightly during the latter half of the incubation period, this was probably due to some residual plasmin rather than chymosin activity, since an increase in the intensity of the bands corresponding to proteose peptones was observed at the same time (figure 4, lanes 11–14).

The primary chymosin-susceptible sites in  $\beta$ -casein are also present in the  $\gamma$ -caseins; the inability of chymosin to degrade the  $\gamma$ -caseins may be due to some conformational changes that make the susceptible sites inaccessible to chymosin.

In Na-caseinate,  $\alpha_{s1}$ -casein was more susceptible than  $\beta$ -casein to hydrolysis by chy-





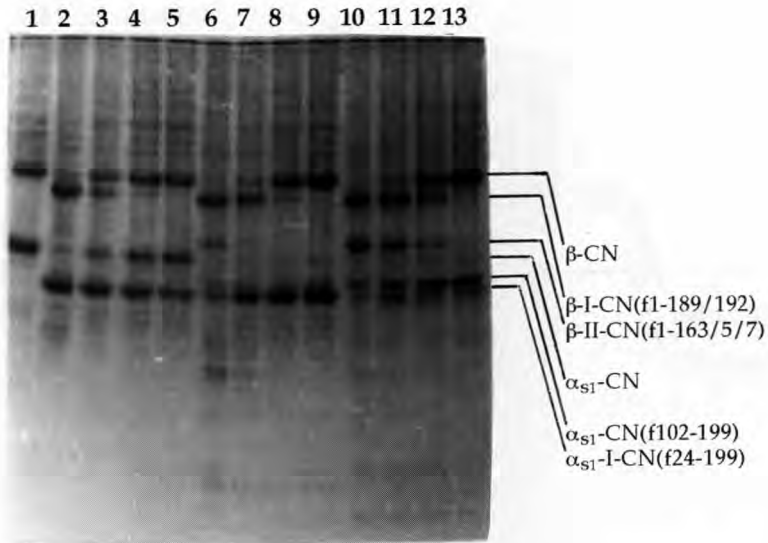
**Figure 4.** Urea-polyacrylamide electrophoretograms of bovine  $\beta$ -casein in solution in 100 mmol·L<sup>-1</sup> sodium phosphate buffer, pH 5.4, containing 5% (w/v) NaCl, hydrolysed by chymosin (0.10 U·mL<sup>-1</sup>), at 30 °C for 0, 1, 3, 4, 6, 9 and 24 h (lanes 1–7),  $\beta$ -casein hydrolysed by bovine plasmin (0.10 U·mL<sup>-1</sup>) at 30 °C for 15 min (lane 8) and the plasmin hydrolysate of  $\beta$ -casein treated with chymosin at 30 °C for 0, 1, 3, 4, 6 and 9 h (lanes 9–14).

**Figure 4.** Électrophorégrammes sur gel de polyacrylamide-urée de caséine  $\beta$  bovine en solution dans un tampon de phosphate de sodium à 100 mmol·L<sup>-1</sup>, pH 5,4, contenant 5% (p/v) de NaCl, hydrolysés par la chymosine (0,10 U·mL<sup>-1</sup>) à 30 °C pendant 0, 1, 3, 4, 6, 9 et 24 h (lignes 1–7), de caséine  $\beta$  hydrolysée par la plasmine bovine (0,10 U·mL<sup>-1</sup>) à 30 °C pendant 15 min (ligne 8) et d'hydrolysats de caséine  $\beta$  par la plasmine traité par la chymosine à 30 °C pendant 0, 1, 3, 4, 6 et 9 h (lignes 9–14).

mosin, irrespective of the pH and NaCl concentration (figure 5). At pH 6.5, the activity of chymosin on both  $\alpha_{s1}$ - and  $\beta$ -casein decreased with increasing NaCl concentration (figure 5, lanes 2–5). As the pH was reduced, hydrolysis of  $\beta$ -casein and, in particular,  $\alpha_{s1}$ -casein increased but the level of chymosin activity at either pH (especially with respect to  $\beta$ -casein) was dependent on the concentration of NaCl in the solution (figure 5, lanes 6–9 and 10–13). At all three pH values, hydrolysis of  $\beta$ -casein by chymosin was strongly inhibited by 5% (figure 5, lanes 4, 8 and 12) and almost completely by 10% NaCl (figure 5, lanes 5, 9 and 13) while  $\alpha_{s1}$ -casein was extensively degraded by chymosin even in the presence of 10% NaCl, particularly at pH 5.8 and 5.4 (figure 5, lanes 9 and 13, respectively).

The effect of NaCl on the hydrolysis of isolated  $\alpha_{s1}$ - and  $\beta$ -caseins by chymosin has been reported [16, 29, 31]. Mulvihill and Fox [29] suggested that NaCl exerts its influence on the hydrolysis of  $\beta$ -casein by chymosin by modifying the substrate rather than the enzyme; the less marked influence of NaCl on the proteolysis of  $\alpha_{s1}$ -casein by chymosin tends to support this view.

Although some qualitative differences, particularly with respect to  $\alpha_{s1}$ -CN(f102-199), were evident between the electrophoretograms of the chymosin hydrolysates of Na-caseinate at pH 6.5 and the lower pH values (figure 5), it is not clear from this study whether these differences were due to variations in the rate of hydrolysis of Na-caseinate by chymosin or to differences in the specificity of the enzyme at different pH

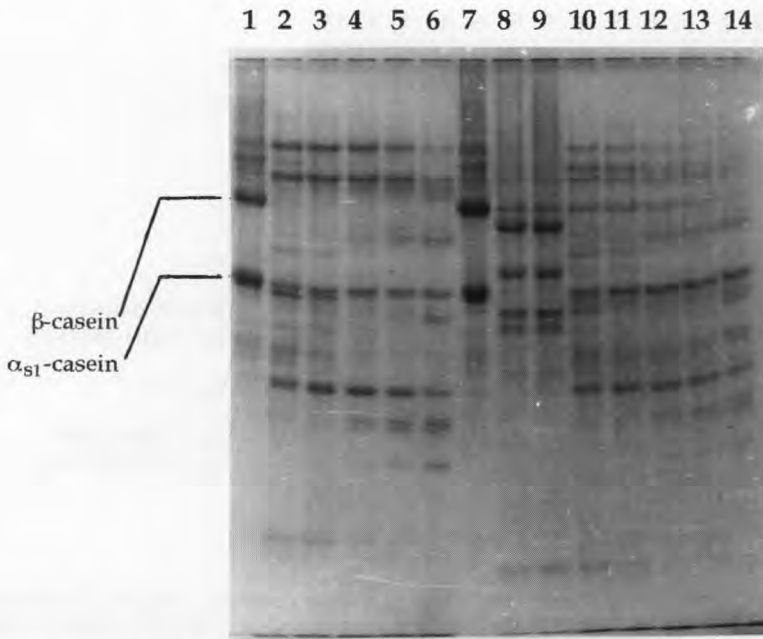


**Figure 5.** Effect of NaCl and pH on the proteolysis of sodium caseinate ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) by chymosin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$  for 1 h. Lane 1: sodium caseinate control; lanes 2–5: pH 6.5, samples containing 0, 2, 5 or 10 % (w/v) NaCl, respectively; lanes 6–9: pH 5.8, samples containing 0, 2, 5 or 10 % (w/v) NaCl, respectively; lanes 10–13: pH 5.4, samples containing 0, 2, 5 or 10 % (w/v) NaCl, respectively.

**Figure 5.** Effet du NaCl et du pH sur la protéolyse de caséinate de sodium ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) par la chymosine ( $0,5 \text{ U}\cdot\text{mL}^{-1}$ ) à  $30^\circ\text{C}$  pendant 1 h. Ligne 1 : caséinate de sodium témoin ; lignes 2–5 : pH 6,5, échantillons contenant respectivement 0, 2, 5 ou 10 % (p/v) de NaCl ; lignes 6–9 : pH 5,8, échantillons contenant respectivement 0, 2, 5 ou 10 % (p/v) de NaCl ; lignes 10–13 : pH 5,4, échantillons contenant respectivement 0, 2, 5 ou 10 % (p/v) de NaCl.

values. Mulvihill and Fox [28, 30] reported that the specificity of chymosin on isolated  $\alpha_{s1}$ -casein was dependent on pH and the state of aggregation of the substrate. In another study [29], these authors found that the specificity of chymosin on isolated  $\beta$ -casein was independent of the reaction pH and the state of aggregation of the substrate. McSweeney et al. [25] found that most of the  $\alpha_{s1}$ -casein-derived peptides produced by chymosin in solution at pH 6.5 were also produced at pH 5.2 in the presence of 5 % NaCl, while several additional peptides were formed under the latter experimental conditions, indicating that pH and/or NaCl influenced the specificity of chymosin on  $\alpha_{s1}$ -casein in solution.

Bovine plasmin rapidly hydrolysed both  $\alpha_{s1}$ - and  $\beta$ -caseins in Na-caseinate, producing a wide range of peptides detectable by PAGE (figure 6, lanes 1–6). Plasmin was also capable of degrading peptides in a chymosin hydrolysate of Na-caseinate (figure 6, lane 8), producing peptides with similar electrophoretic mobilities (figure 6, lanes 9–14) to those produced from Na-caseinate by plasmin (figure 6, lanes 1–6). One of the plasmin-derived peptides had a similar electrophoretic mobility to that of  $\alpha_{s1}$ -casein. The fact that plasmin can hydrolyse peptides produced from  $\alpha_{s1}$ - and  $\beta$ -caseins may be important in relation to its activity in ripening cheese. The contribution of plasmin to proteolysis during ripening varies,



**Figure 6.** Urea-polyacrylamide gel electrophoretograms of sodium caseinate ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) in  $100 \text{ mmol}\cdot\text{L}^{-1}$  sodium phosphate buffer, pH 5.4, containing 5 % (w/v) NaCl, hydrolysed by bovine plasmin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$  for 0, 1, 2, 4, 8 and 24 h, respectively (lanes 1–6); sodium caseinate control (lane 7); sodium caseinate hydrolysed by chymosin ( $0.5 \text{ U}\cdot\text{mL}^{-1}$ ) at  $30^\circ\text{C}$  for 3 h (lane 8) and the chymosin hydrolysate of sodium caseinate treated with bovine plasmin at  $30^\circ\text{C}$  for 0, 1, 2, 4, 8 and 24 h, respectively (lanes 9–14).

**Figure 6.** Électrophorégrammes sur gel de polyacrylamide de caséinate de sodium ( $5 \text{ mg}\cdot\text{mL}^{-1}$ ) dans un tampon de phosphate de sodium à  $100 \text{ mmol}\cdot\text{L}^{-1}$ , pH 5,4, contenant 5 % (p/v) de NaCl, hydrolysé par la plasmine bovine ( $0,5 \text{ U}\cdot\text{mL}^{-1}$ ) à  $30^\circ\text{C}$  pendant respectivement 0, 1, 2, 4, 8 et 24 h (lignes 1–6) ; caséinate de sodium témoin (ligne 7) ; caséinate de sodium hydrolysé par la chymosine ( $0,5 \text{ U}\cdot\text{mL}^{-1}$ ) à  $30^\circ\text{C}$  pendant 3 h (ligne 8) et hydrolysats de caséinate de sodium par la chymosine traité par la plasmine bovine à  $30^\circ\text{C}$  pendant respectivement 0, 1, 2, 4, 8 et 24 h (lignes 9–14).

depending on the cheese variety but it is not considered to play a major role in Cheddar cheese because the pH of this cheese is unfavourable for its activity [20]. The degradation of  $\beta$ -casein with the concomitant increase in  $\gamma$ -caseins in Cheddar cheese is known to be due to the activity of plasmin, but little attention has been paid to the possibility that plasmin may contribute to proteolysis during ripening other than the hydrolysis of  $\beta$ -casein. Results presented by Farkye and Fox [12, 13] concerning the contribution of plasmin to Cheddar cheese ripen-

ing confirmed that the limited hydrolysis of  $\beta$ -casein during cheese ripening is due to the action of this proteinase. These authors also reported that plasmin did not contribute to the initial breakdown of  $\alpha_{s1}$ -casein during ripening but was capable of contributing to both the formation and degradation of water-soluble peptides. Mooney and Fox (unpublished) identified two peptides, produced from  $\alpha_{s1}$ -casein (i.e.  $\alpha_{s1}$ -CNf104-199 and f106-199), in the water-insoluble fraction of ripened Cheddar cheese which corresponded to the cleavage specificity of plas-

min. These results suggest that during cheese ripening, plasmin may play a role in the formation of casein-derived peptides other than the production of  $\gamma$ -caseins and proteose peptones from  $\beta$ -casein. Since the coagulant is mainly responsible for the initial degradation of casein (particularly  $\alpha_{s1}$ -casein) during ripening, it seems plausible that plasmin could hydrolyse these chymosin-derived peptides, thereby contributing to the development of water-soluble nitrogen in Cheddar cheese. The results of this study show that plasmin is capable of degrading these chymosin-derived peptides in solution.

#### 4. CONCLUSION

The results of this study show that the activity of bovine plasmin on  $\beta$ -casein was not greatly affected by NaCl but was influenced by the pH of the system. In Na-caseinate,  $\alpha_{s1}$ -casein was considerably more susceptible than  $\beta$ -casein to hydrolysis by chymosin, the activity of which (particularly on  $\beta$ -casein) was strongly dependent on both the NaCl concentration and the pH of the system. The primary products of plasmin action on  $\beta$ -casein, namely, the  $\gamma$ -caseins and their complementary proteose peptones, were not susceptible to hydrolysis by chymosin. Plasmin was capable of degrading peptides produced from both  $\alpha_{s1}$ - and  $\beta$ -caseins by chymosin, suggesting that plasmin may contribute to the degradation of casein-derived peptides produced by chymosin during cheese ripening.

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