Preliminary Study on the Contribution of Plasmin to Proteolysis in Cheddar Cheese: Cheese Containing Plasmin Inhibitor, 6-Aminohexanoic Acid

Nana Y. Farkye† and Patrick F. Fox*

Department of Food Chemistry, National Food Biotechnology Centre, University College, Cork, Ireland

Plasmin inhibitor, 6-aminohexanoic acid (AHA) was added to a portion of stirred curd Cheddar cheese at salting. Proteolysis in the AHA-treated cheese and a control cheese from the same vat was monitored through ripening for 6 months. Differences were noted in the electrophoretic patterns of control and AHA cheeses of similar age throughout ripening. Also, electrophoretic patterns of the water soluble nitrogen fractions of the cheeses were different, suggesting that plasmin plays a role in proteolysis during Cheddar cheese ripening.

INTRODUCTION

Plasmin, the principal indigenous proteinase in milk, is thought to contribute to an ill-defined extent to the ripening of certain cheese varieties (Visser and de Groot-Mostert, 1977; Noomen, 1978; Lawrence et al., 1983, 1987; Ollikainen and Nyberg, 1988). Plasmin is present in Cheddar cheese (Richardson and Pearce, 1981; Lawrence et al., 1983; Farkye and Fox, 1990), where it has been suggested to contribute little, if anything, to ripening because the pH of Cheddar cheese is unfavorable for its activity (Grappin et al., 1985). However, Noomen (1978) reported plasmin activity in Meshanger-type cheese (pH 5.1) after 6 weeks of ripening at 13 °C, and proteolysis of β-casein, the casein most susceptible to plasmin activity, occurs in Emmental (Lawrence et al., 1983), Gouda (Visser and de Groot-Mostert, 1977), and Cheddar cheeses (Creamer, 1975), suggesting that plasmin activity in Cheddar cheese has been underestimated. This paper is a preliminary report on the contribution of plasmin to proteolysis in Cheddar cheese suggested through the use of a noncompetitive inhibitor of plasmin, 6-aminohexanoic acid (Iwamoto et al., 1968).

MATERIALS AND METHODS

Cheesemaking. Stirred-curd Cheddar cheese was made by conventional methods (Kosikowski, 1982) from two lots of pasteurized (72 °C × 15 s) whole milk (90 L) obtained from a commercial dairy plant. The starter used was 2% (v/v) *Lactococcus lactis* subsp. lactis UC 317 (Department of Food Microbiology, University College, Cork, Ireland) propagated (22 °C × 16 h) in autoclaved (115 °C × 10 min) 10% reconstituted nonfat dry milk. Standard strength calf rennet (Chr. Hansen's Laboratory) was added to milk at the rate of 25 mL/100 L. At pH 5.4, curds were divided into two batches. NaCl (2.5% w/w) was added to the control batch. In addition to NaCl (2.5% w/w), 1.5% w/w AHA (Sigma Chemical Co., London) was added to the experimental batch. After overnight pressing, the cheeses were vacuum-packed and ripened at 10 °C for up to 6 months.

Analyses. The cheeses were sampled for analyses immediately after pressing (1 day) and after 1, 3, and 6 months of ripening. Moisture, pH, and total nitrogen were determined on day-old cheeses only. Moisture was determined by measuring the weight loss after 2–3 g of finely grated cheese was dried in an oven (105 °C) for 16 h, and pH was determined in a slurry obtained after maceration of 1 part cheese and 1 part water (O'Keeffe et al., 1976). Total nitrogen was determined by the standard macro-Kjeldahl method (AOAC, 1980). Protein was calculated as Kjeldahl N × 6.38. NaCl was determined by the potentiometric method of Fox (1963).

Protein breakdown in the cheese was followed by measuring the water-soluble nitrogen (WSN) and by electrophoresis of cheese and WSN thereof. WSN was extracted by the method of Kuchroo and Fox (1982) and N content of the extract determined by Kjeldahl. Discontinuous polyacrylamide gel electrophoresis (PAGE) in urea was performed according to the method of Andrews (1983). Stacking gels consisted of 4% total acrylamide (T) with 4% cross-linking (C) with bis(acrylamide) and 4.3 M urea in 62 mM Tris-HCl buffer (pH 6.8). Resolving gels consisted of 12% T, 4% C, and 4.3 M urea in 380 mM Tris-HCl buffer (pH 8.8). Running buffer was 24.8 mM Tris and 194.4 mM glycine (pH 8.3). Gels were prerun at 200 V prior to sample loading and were run at 280 V when sample was loaded. Finished gels were stained overnight by using Coomassie blue G250 according to the method of Blakesley and Boezi (1977) and destained in distilled water.

Cheese samples for electrophoresis were prepared by dissolving 0.2 g of cheese in 10 mL of stacking gel buffer (pH 6.8) containing 8 M urea. Two drops of 2-mercaptoethanol was added, and samples were incubated at 40 °C for 45 min. After the samples were filtered through glass wool to remove fat, tracking dye (1% bromophenol blue in 10% sucrose) was added and 10 μL loaded onto gel. PAGE of WSN fraction was run as for cheese except that lyophilized WSN extract was dissolved in stacking gel buffer at the rate of 0.1 g/mL.

RESULTS AND DISCUSSION

Effect of AHA on Starter and Rennet Activities. The effect of AHA on starter activity in milk was examined by adding 0, 0.5, 1.0, 1.5, or 2.0 g of AHA to separate 100-mL portions of milk, followed by addition of 2% (v/v) starter. The pH values of the samples after incubation for 5 h at 30 °C were 4.7, 4.8, 4.9, and 5.0, respectively. Cell-free extracts, prepared according to the method of Kaminogawa et al. (1984), were added to portions of a 2% solution of sodium caseinate (pH 5.5) containing 0–2 g of AHA/100 mL, and the mixtures were incubated for 3 days at 30 °C. Thimerosal (0.02%) was added to prevent bacteria growth. Aliquots of the samples were analyzed by PAGE before and after incubation to determine the inhibitory effect of AHA on the activities of starter proteinases. The results showed that AHA, within the range used, had little or no effect on the activities of starter proteinases. Also, at levels used in this study, AHA did

* Author to whom correspondence should be addressed.
† Present address: Dairy Products Technology Center, California Polytechnic State University, San Luis Obispo, CA 93407.

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Table I. Composition of Fresh Cheeses

cheese	total N, %	moisture, %	NaCl, %	pH (1 day)
control	3.98 ± 0.02	36.34 ± 0.19	1.64 ± 0.08	5.22 ± 0.04
exptl	4.19 ± 0.03	33.90 ± 0.10	1.58 ± 0.12	5.26 ± 0.06

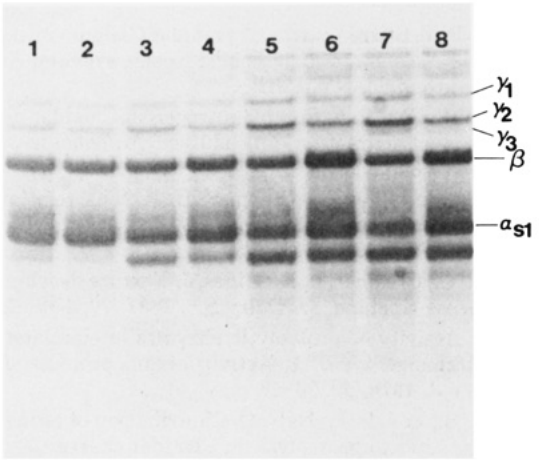

Figure 1. Urea–alkaline PAGE (12% T, 4% C, pH 8.3) of cheeses. Shown are control (lanes 1, 3, 5, and 7) and experimental (lanes 2, 4, 6, and 8) cheeses after ripening for 1 day and 1, 3, and 6 months, respectively.

not inhibit the peptidase activity of a cell-free extract of the starter bacteria on lysine *p*-nitroanilide (A. Ryan, personal communication).

In a similar experiment, the inhibitory effect of AHA (0–2%) on the proteolytic activity of rennet (1 μL/mL) on 2% sodium caseinate (pH 5.5) was assessed by PAGE after incubation for 16 h at 30 °C in the presence of thimerosal (0.02%). The electrophoretic patterns of aliquots of all the incubated samples were identical, suggesting that AHA, at concentrations used in this study, did not affect rennet activity.

Cheese Composition. As expected, the average total N content of the experimental cheeses was higher than that of the control (Table I) owing to the N contributed by AHA. Empirically, the level of N added as AHA to the cheese was 0.27%. This was reflected in the difference 0.21% in total N between control and experimental cheeses. The lower moisture levels in the experimental cheeses compared to the control cheeses (Table I) was due to the higher level of total salt (NaCl plus AHA) added to the experimental cheese. Average pH (1 day) and NaCl contents in the experimental cheeses were similar to those in the control cheeses.

Proteolysis. Typical electrophoretic patterns of the control (lanes 1, 3, 5, and 7) and experimental (lanes 2, 4, 6, and 8) cheeses after 1 day and 1, 3, and 6 months of ripening, respectively, are shown in Figure 1. Major differences in electrophoretic patterns were noted in the relative intensities of the bands corresponding to γ-caseins. At all sampling times, the band corresponding to γ_2-casein in the patterns of experimental cheeses was less intense than that in the control cheese of similar age. This was particularly evident in the patterns of 3- and 6-month-old cheeses in which the relative increase in the γ_2-casein band with concomitant decrease in the β-casein band was less in the experimental cheeses compared to the control (i.e., lanes 5 vs 6 and 7 vs 8). In solution plasmin hydrolyzes all the major caseins except κ-casein (Chen and Ledford, 1971; Snoeren and Van Riel, 1979), but β-casein is most susceptible (Kaminogawa et al., 1972) and is hydrolyzed to give γ-caseins (Eigel, 1977). AHA inhibits plasmin activity noncompetitively (Iwamoto et al., 1968) by binding

Table II. Levels of Water-Soluble N in Cheese during Ripening

	water-soluble N/total N, %			
cheese	1 day	1 month	3 months	6 months
control	6.00 ± 0.14	12.86 ± 0.19	20.61 ± 0.40	27.58 ± 0.32
exptl	10.25 ± 0.41	13.90 ± 0.10	20.41 ± 0.58	23.37 ± 0.75
	increase in soluble N			
cheese	1 day	1 month	3 months	6 months
control	–	6.86	14.61	21.58
exptl	–	3.65	10.16	13.12

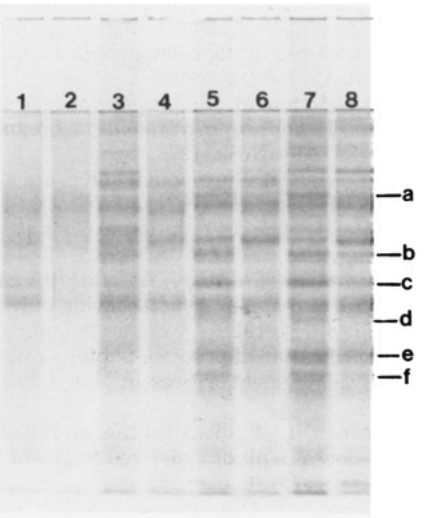

Figure 2. Urea–alkaline PAGE (12% T, 4% C, pH 8.3) of WSN fractions from cheeses. Shown are control (lanes 1, 3, 5, and 7) and experimental (lanes 2, 4, 6, and 8) cheeses after ripening for 1 day and 1, 3, and 6 months, respectively.

to the stronger of the two binding site on plasmin (Castellino and Sodetz, 1976). Thus, the effective total activity of plasmin in the experimental cheeses was reduced, resulting in the relatively slow release of its degradation products in the experimental cheese compared to the control cheese.

Average WSN levels in the control cheeses increased from 6% at 1 day to 27.58% after 6 months of ripening, while those in the experimental cheeses increased from 10.25% to 23.37% during the same period (Table II). Since AHA is water soluble, the levels of WSN in experimental cheeses did not truly reflect the actual WSN in these cheeses. Nevertheless, the slower rate of increase in WSN in the experimental cheeses compared to the control cheeses suggests that plasmin contributes to the release of water-soluble peptides in cheese. Hydrolysis of β-casein by plasmin releases hydrophobic γ-casein and complementary N-terminal peptides, propeose peptones (Eigel and Keenan, 1977; Eigel, 1981), which are water soluble.

Electrophoretic patterns of the WSN fractions from control (lanes 1, 3, 5, and 7) and experimental (lanes 2, 4, 6, and 8) cheeses (Figure 2) showed at least six bands (labeled a–f) that were more intense in the former (cf. lanes 3 vs 4 and 5 vs 6). However, the peptides corresponding to these bands remain to be chemically identified.

It is acknowledged that the low moisture levels in the experimental cheeses might result in low rates of proteolysis. However, in the present case, low moisture content is probably not exclusively responsible for the reduced rate of proteolysis since qualitative differences were noted in the electrophoretic patterns of the WSN fractions of the cheeses.

The cheeses were not examined organoleptically because of possible toxic and teratogenic effects of AHA (Sigma).

It would have been interesting to taste the cheeses to correlate plasmin inhibition in Cheddar cheese to its flavor development.

Conclusion. Noncompetitive inhibition of plasmin activity in Cheddar cheese by AHA reduced the rate of proteolysis during ripening, suggesting that plasmin plays a role in Cheddar cheese ripening.

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