Inhibitory Activity Against Plasmin, Trypsin, and Elastase in Rennet Whey and in Cheese Fortified with Whey Protein

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ABSTRACT

The inhibitory activity against trypsin, elastase, and plasmin was determined in samples of Danbo 45+ that were manufactured from milk pasteurized at 72, 80, and 90°C for 15, 30, and 60 s; the corresponding rennet wheys; and Havarti 45+ manufactured from milk concentrated 1.8-fold, 2.7-fold, and 4.6-fold by ultrafiltration. A sensitive colorimetric assay demonstrated that the incorporation of thermally denatured whey proteins into the cheese curd by pasteurization resulted in a decreased proteinase inhibitory activity against trypsin and elastase in Danbo 45+ and against trypsin, elastase, and plasmin in the corresponding rennet wheys. However, incorporation of native whey proteins into Havarti 45+ by ultrafiltration of the cheese milk resulted in an increased inhibitory activity against trypsin and elastase in the cheeses. Cheese manufactured from milk concentrated 1.8-fold, 2.7-fold, or 4.6-fold displayed trypsin inhibitory activity that was 1.8, 2.9, and 5.1 times, respectively, that of the reference cheese. Similarly, the elastase inhibitory activity in the cheeses increased 2.2, 3.2, and 7.8 times. The increased inhibitory activity in cheese fortified with native whey protein likely contributes to the decreased proteolysis and altered ripening characteristics of the resulting cheeses, and, further, the method can be adapted to detection of other inhibitors if sufficiently sensitive substrates are available.

(**Key words**: proteinase inhibitors, cheese, pasteurization, ultrafiltration)

INTRODUCTION

The proteolytic digestion of casein during cheese ripening is thought to be of great importance to the development of body and texture as well as flavor in the cheese. The digestion of casein is a result of the concerted action of residual rennet, the major indigenous milk proteinase, plasmin (EC 3.4.21.7), and microbial proteinases and peptidases from the starter culture, the secondary starter, and the nonstarter bacteria (7, 8, 23).

The importance of the proteolytic digestion of the caseins during cheese ripening (7, 8, 23) leads to the theory that the presence of proteinase inhibitors could affect the cheese ripening process. Proteinase inhibitors normally reside in milk serum (4) but can be incorporated into the cheese curd by three methods that can be used to increase the yield of cheese from a given quantity of cheese milk: pasteurization of the cheese milk at elevated temperatures, use of UF, and addition of heat-denatured whey protein concentrate to the cheese milk. The use of UF leads to the incorporation of native whey proteins in the cheese, and the other two methods result in the incorporation of at least partially denatured whey protein in the cheese curd. The resulting cheeses display atypical ripening characteristics, including the slower digestion of casein during ripening (15).

Milk contains a large number of proteinase inhibitors that have a broad range of specificity. Bovine colostrum has been shown to contain some colostrum-specific proteinase inhibitors, including trypsin inhibitory activity, and cysteine proteinase inhibitory activity, which provide protection for the immunoglobulins and other biologically active proteins against proteolysis by gastrointestinal enzymes in the newborn (11, 13, 17). In addition to these inhibitors, a number of plasma-derived proteinase inhibitors have been detected in bovine milk: α_1-proteinase inhibitor, α_2-antiplasmin, C1 inhibitor, antithrombin-III, α_2-macroglobulin, inter-α-trypsin inhibitor, and two inhibitors with homology to human α_1-antichymotrypsin, mainly possessing inhibitory activity against trypsin and elastase, respectively (3, 4, 13, 16, 19, 24). The inhibitory activity is strongly elevated in mastitis milk because of an elevated leakage of blood proteins, including α_1-proteinase inhibitor, from the intravascular compartment into the milk

(12, 13). Determination of the trypsin inhibitory activity in milk samples, therefore, was suggested to be useful for the diagnosis of mastitis in cows (21). Conversely, normal bovine milk is relatively low in proteinase inhibitory activity, and the majority of the activity is suggested to reside in the trypsin inhibitor and elastase inhibitor, which resembles human α_1-antichymotrypsin (4). Because plasmin is thought to be important to the primary proteolysis of caseins during cheese ripening (6), plasmin inhibitory activity incorporated in the cheese curd may affect this process. Potential candidates for this inhibition are α_2-antiplasmin and the trypsin inhibitor, which possess plasmin inhibitory activity (2, 3), and α_1-proteinase inhibitor, α_2-macroglobulin, C1 inhibitor, and inter-α-trypsin inhibitor, which are expected to possess plasmin inhibitory activity because of resemblance to the human proteinase inhibitors that inhibit human plasmin.

The objective of this study was to develop a method that could be used to examine the activity of proteinase inhibitors in samples of whey and cheese and that could contribute to an evaluation of the effect of whey proteins on the proteolytic digestion of casein during cheese ripening. The method has been used for comparison of the activity of proteinase inhibitors against trypsin (EC 3.4.21.4), elastase (EC 3.4.21.36), and plasmin in samples of Danbo 45+ manufactured from milk pasteurized at 72, 80, and 90°C for 15, 30, and 60 s, the corresponding rennet whey fractions, and Havarti 45+ manufactured from milk concentrated by UF 1.8, 2.7, and 4.6 times.

MATERIALS AND METHODS

Cheese Manufacture

Rindless Danbo 45+ was manufactured from standardized bulk milk pasteurized at 72, 80, and 90°C for 15, 30, and 60 s. The cheeses were produced according to conventional cheese-making procedures from 15 L of milk in a pilot-scale cheese plant, using 1% starter (Flora Danica; Chr. Hansen, Hørsholm, Denmark), 35 ml of rennet/100 kg of milk (standard 180; Chr. Hansen), and a curd cube size of 6 mm. The cheeses were salted and stored according to standard procedures. Because the heat treatment differed and in order to obtain comparable cheese characteristics, $CaCl_2$ in various concentrations was added, and the renneting time and stirring time in the vat were prolonged for cheeses manufactured from milk pasteurized at higher temperatures.

Havarti 45+ without rind was manufactured from standardized milk that had been pasteurized at 72°C

for 15 s and from milk that had been concentrated 1.8-fold, 2.7-fold, or 4.6-fold by ultrafiltration and then pasteurized at 74°C for 30 s. The traditional cheese was produced according to conventional cheese-making procedures from 180 L of milk in a pilot-scale cheese plant, using 0.8% starter (Flora Danica; Chr. Hansen), 35 ml of rennet/100 kg of milk (standard 180; Chr. Hansen), and a curd cube size of 10 mm. The concentrated milk was inoculated with 1% starter and 45 ml of rennet/100 kg of concentrate (standard 180; Chr. Hansen) and coagulated (Alcurd Pilot Plant MK IV coagulator; Tetra Pack Filtration Systems A/S, Aarhus, Denmark). The curd was cut and then stirred in the cheese vat in permeate from the UF. Twenty and 15 g of KNO_3/100 kg of milk were added to the traditional and UF cheeses, respectively. The cheeses were salted and stored according to standard procedures.

Sample Preparation

Samples were taken from the rennet whey and from the fresh cheeses before salting. Ten grams of grated cheese were mixed with 90 ml of 0.1 M trisodium citrate and dissolved by stirring for 45 min at 25°C, followed by stirring for 30 min at 45°C. The sample was centrifuged at 25,000 × g for 15 min at 5°C, and the clear supernatant was removed from under the fat by a pipette. Rennet whey was centrifuged at 25,000 × g for 15 min at 5°C. Samples were stored at –25°C until further analysis.

Determination of Proteinase Inhibitory Activity

The inhibitory activity against plasmin, trypsin, and elastase in samples of fresh cheese and rennet whey was determined by a method modified after Sandholm et al. (21). The assay was performed in microtiter plates (Certified Maxisorp, Nunc-Immuno plates; Nunc, Roskilde, Denmark) in a reaction volume of 225 μl including 75 μl of enzyme, 75 μl of substrate, and 75 μl of diluted sample. The assay buffer was 50 mM Na_2HPO_4, 100 mM NaCl, pH 7.4, and 10 mM EDTA; the reaction mixture included 0.1% (wt/vol) gelatine (food-grade quality, Merck, Darmstadt, Germany). The buffer was used for dilution of samples, enzymes, and substrates. Samples were 2-fold serially diluted (2- to 1024-fold dilution), applied to the microtiter plates, and incubated for 30 min at 37°C with the enzyme solution [0.05 μg/ml of trypsin (N-tosyl-L-phenylalanine chloromethylketone treated from bovine pancreas, 24581; Merck), elastase (type IV, from bovine pancreas, E-0258;

Sigma Chemical Co., St. Louis, MO), or plasmin] prior to the addition of the substrates (1.0 mM S-2406 for plasmin, 0.05 mM S-2222 for trypsin, and 1.0 mM S-2536 for elastase, all from Chromogenix, Mölndal, Sweden). Plasmin was obtained by activation of plasminogen [isolated from bovine blood essentially as described by Deutsch and Mertz (5)] with 148 PU (Plough units) of urokinase plasminogen activator (thrombolysin; Immuno Danmark, Copenhagen, Denmark)/mg of plasminogen by incubation at 37°C for 15 min. The activation was performed immediately before the enzyme was applied to the plates. The proteolytic activity in the reaction mixtures was determined by measuring the formation of p-nitroaniline during cleavage of the substrates at 405 nm using a Bio-Tek EL 340 Bio Kinetics Reader (Bio-Tek Instruments Inc., Wisconsin, IL). Readings were performed immediately after the addition of the substrate, after 1 h (trypsin only), and after approximately 4, 8, 20, 25, and 40 h of incubation at 37°C (elastase and plasmin). Plates containing samples to be compared included identical standard samples. In addition, all plates included a blank row (with no enzyme and no sample) and an enzyme blank with no sample. The method was optimized with respect to concentration of enzymes and substrates to obtain the most sensitive assay for all three enzymes. The linearity of reactions over time was confirmed prior to the evaluation of the results. The trypsin inhibitory activity was determined after 1 h of incubation, and the inhibitory activity against plasmin and elastase was calculated as the mean of the results after approximately 20 and 25 h of incubation.

Dose-response curves were made by subtraction of the reading at time of initiation from the reading used for evaluation. This process resulted in sigmoid curves with parallel linear parts from which the inhibitory activities were determined. Traditional Havarti 45+, the sample from Danbo 45+ manufactured from milk pasteurized at 72°C for 15 s, and the corresponding rennet whey were used as standards for the UF cheeses, pasteurized cheeses, and rennet whey, respectively. The inhibitor activities were determined by the Kineti-Calc™ software (Bio-Tek Instruments Inc.) using a four-parameter logistic model to obtain the best possible fit for the standard curve and expressed in percentage of the activity in the standard sample.

RESULTS AND DISCUSSION

Examples of dose-response curves from the determination of inhibitory activity against trypsin, elastase, and plasmin in samples of Danbo 45+ manufactured from pasteurized milk, the corresponding rennet whey, and Havarti 45+ manufactured from concentrated milk are presented in Figure 1. The inhibitory activities in samples calculated in percentage of the activity in the reference samples are presented in Figures 2 and 3.

Generally, the results display sigmoid curves with parallel linear parts for all three inhibitors in samples of rennet whey and in cheese samples for trypsin and elastase (Figure 1). However, the results do not provide information about the presence of inhibitory activity against plasmin in cheeses samples, which is thought to result from plasmin activity in the cheese samples interfering with the assay. The available plasmin inhibitory activity is thus likely to have reacted with plasmin in the cheese samples, leaving excess plasmin activity to participate in the proteolytic digestion of protein during cheese ripening. As expected, the samples are low in proteolytic activity with specificity similar to that of trypsin and elastase, which enables the determination of excess inhibitory activity against those enzymes in samples of cheese and rennet whey.

For the Danbo 45+ cheeses manufactured from pasteurized milk and the corresponding rennet wheys, the majority of the inhibitory activity against trypsin and elastase resides in the rennet whey. Figure 2 shows that the inhibitory activities in rennet whey and in cheese manufactured from pasteurized milk decline as temperature and holding time increase for all three enzymes. The change in inhibitory activity in rennet whey as holding time increases is most pronounced at 72°C, at which temperature the elastase and plasmin inhibitory activity drops to about 50% at a holding time of 60 s. The same treatment results in the loss of only 25% of the trypsin inhibitory activity, but pasteurization at 80°C for 15 s results in the loss of 65% of the activity. After pasteurization at 90°C for 60 s, approximately 15, 40, and 20% of the residual inhibitory activity against trypsin, elastase, and plasmin, respectively, remains active in the whey. The inhibitory activity against trypsin and elastase in the cheese produced from milk pasteurized at 90°C for 60 s is approximately 53 and 27% of the activity in the control, respectively. This result demonstrates that the proteinase inhibitors in question are gradually inactivated by the pasteurization and that the increased amount of whey protein retained in the cheese curd upon renneting adds no proteinase inhibitory activity directed toward trypsin or elastase to the activity already present in the cheese. The present results demonstrate that the inhibitory activity against trypsin, elastase, and plasmin in rennet whey and against trypsin and elastase
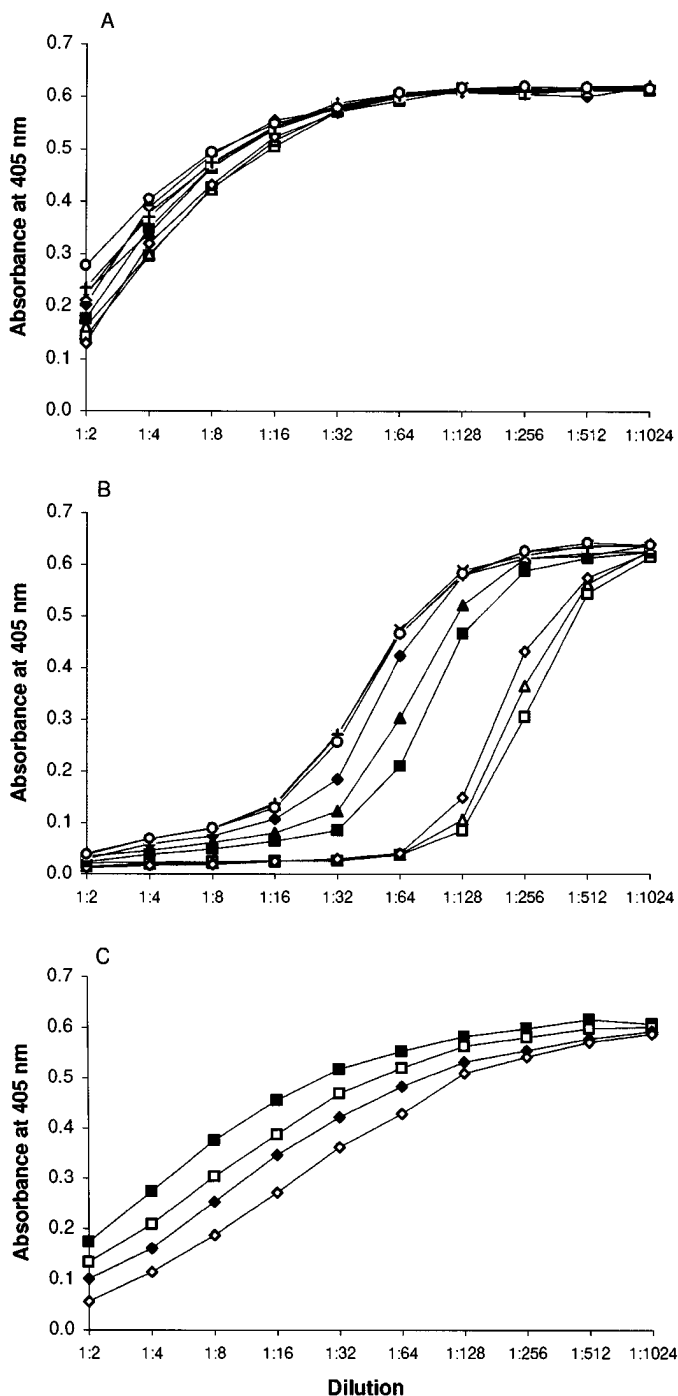
in Danbo 45+ declines as the heat treatment becomes more vigorous, which rules out the possibility of the transfer of proteinase inhibitor to the cheese upon pasteurization, leading to decreased proteolytic activity in the cheese during ripening and, thus, a slower ripening process. The drop in plasmin activity in cheeses manufactured from pasteurized milk as temperature and holding time increase and the subsequent slower proteolysis during cheese-ripening (C. Benfeldt, J Sørensen, K. H. Ellegård, and T. E. Petersen, 1998, unpublished data) must therefore have another explanation. Whether this explanation includes thermal inactivation of the enzyme, or interactions with other whey proteins of which β-LG has been suggested to be involved in the thermal inactivation of plasmin (1, 10, 14, 20), or both, still remains to be elucidated.

Figure 1. Examples of dose-response curves showing the trypsin inhibitory activity in Danbo 45+ manufactured from pasteurized milk (A), the corresponding rennet wheys (B), and Havarti 45+ manufactured from concentrated milk (C). All plates were incubated for 1 h prior to reading. Symbols in parts A and B refer to the following pasteurization conditions: 72°C, 15 s (□); 72°C, 30 s (△); 72°C, 60 s (◊); 80°C, 15 s (■); 80°C, 30 s (▲); 80°C, 60 s (♦); 90°C, 15 s (×); 90°C, 30 s (+); and 90°C, 60 s (○). Symbols in part C refer to the following concentrations of cheese milk: no concentration, traditional Havarti (■); 1.8× concentration (□); 2.7× concentration (♦); and 4.6× concentration (◊).
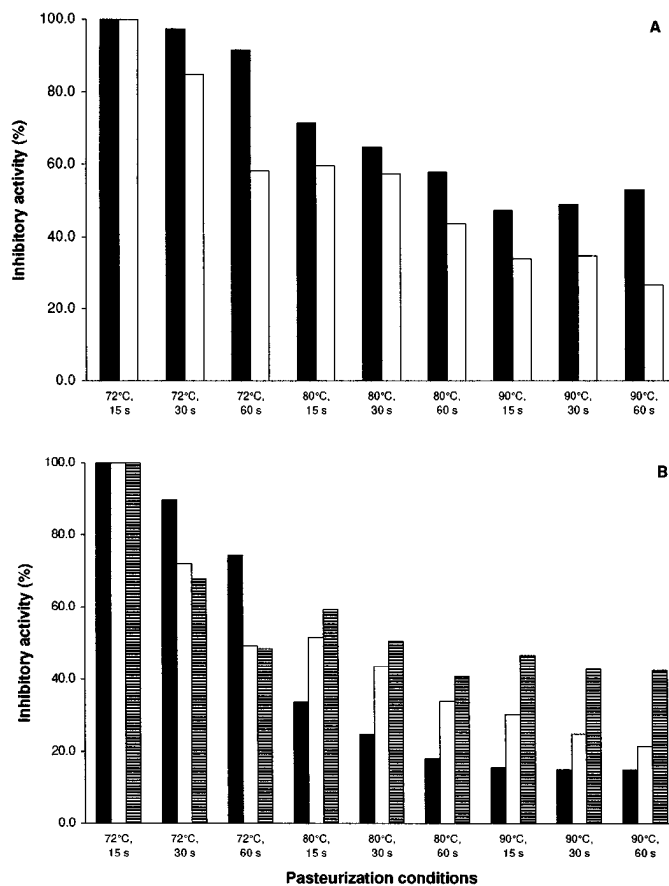
Figure 2. Inhibitory activity against trypsin (solid bar) and elastase (open bar) in samples of Danbo 45+ manufactured from pasteurized milk (A) and against trypsin (solid bar), elastase (open bar), and plasmin (patterned bar) in the corresponding rennet whey (B). The inhibitory activity is expressed as a percentage of the inhibitory activity in samples from Danbo 45+ manufactured from milk pasteurized at 72°C for 15 s.
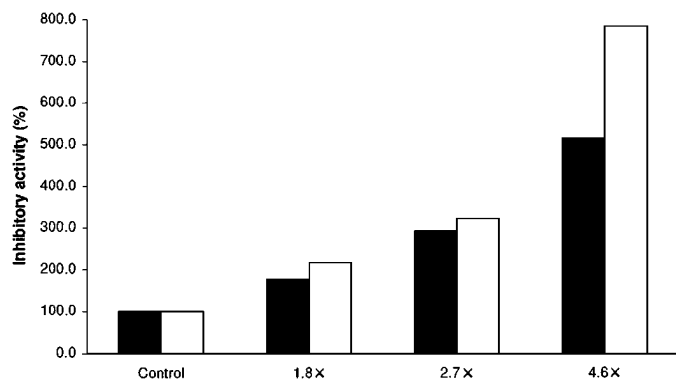
Figure 3. Inhibitory activity against trypsin (solid bar) and elastase (open bar) in samples of Havarti 45+ manufactured from concentrated milk. The inhibitory activity is expressed as a percentage of the inhibitory activity in traditional Havarti 45+.

The results from the examination of proteinase inhibitory activity in Havarti 45+ manufactured from concentrated milk reveal an image that is completely different from that discussed previously (Figure 1C and Figure 3). Contrary to the results of the former experiments, these UF cheeses included increased amounts of native whey proteins. At the same time, these cheeses display increasing inhibitory activity against trypsin and elastase as the concentration of the cheese milk increases. The trypsin inhibitory activity in the cheeses manufactured from milk concentrated 1.8-fold, 2.7-fold, and 4.6-fold is approximately 1.8, 2.9, and 5.1 times that of the traditional cheese, respectively. Similarly, the elastase inhibitory activity in the cheeses was increased approximately 2.2, 3.2, and 7.8 times that of the traditional cheese. As in the former experiment, the inhibitory activity against plasmin in the cheeses has not been elucidated. However, the UF cheeses displayed lower plasmin activity than did the traditional cheeses during ripening (C. Benfeldt, J. Sørensen, and T. E. Petersen, 1998, unpublished data). This result suggests that the elevated amount of whey protein and possibly also proteinase inhibitors present in the UF cheeses affect the plasmin activity in the cheeses during ripening. However, the nature of this plasmin inhibitory activity is unknown. The plasma-derived proteinase inhibitors in bovine milk that possess plasmin inhibitory activity may be candidates: α_2-antiplasmin, bovine trypsin inhibitor, α_1-proteinase inhibitor, α_2-macroglobulin, C1 inhibitor, and inter-α-trypsin inhibitor. The influence of other mechanisms involving constituents of the whey, however, cannot be eliminated. β-Lactoglobulin, α-LA, and bovine serum albu-

min possess inhibitory activity against plasmin in solution (1, 18). Some of the plasma-derived proteinase inhibitors possess inhibitory activity against trypsin as well as against plasmin. An increased inhibitory activity against trypsin thus also reflects an increased potential inhibitory activity against plasmin, which may explain the smaller increase in trypsin inhibitory activity than in elastase inhibitory activity in the UF cheeses and also the decrease in plasmin activity. No proteolytic enzyme with elastase-like activity has been detected in milk. However, the milk may contain some elastase activity, originating from the polymorphonuclear leukocytes (9, 22), which could be controlled by the bovine elastase inhibitor. In addition, all inhibitors might influence the proteolytic systems residing in the starter bacteria. Because the incorporation of denatured and native whey proteins into the cheese curd seems to affect the proteolytic systems in the cheese in different ways, further examination of the proteolytic systems needs to be performed to understand completely the mechanisms that are responsible for the altered ripening characteristics in cheese fortified with whey protein.

The present work clearly demonstrates that it is possible to determine the inhibitory activity against trypsin, elastase, and plasmin in samples of rennet whey and against trypsin and elastase in cheese. Although the concentration of these inhibitors is low in the samples, the use of sensitive substrates and the stability of the enzyme reaction during assay time enable the use of enzyme concentrations that are sufficiently low to detect the inhibitors. The method does not, however, provide information concerning which inhibitor or inhibitors are responsible for the inhibition observed and is thus not useful for specific characterization of the proteinase inhibitors in a sample. In combination with, for example, immunological examination such as Western blotting, the results can provide some information about whether a proteinase inhibitor is active when it is present in a sample. We suggest that the method can be adapted to inhibitors of other enzymes if sufficiently sensitive substrates are available.

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