Effects of *Pseudomonas fluorescens* M3/6 Bacterial Protease on Plasmin System and Plasminogen Activation

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ABSTRACT

Heat-stable proteases produced by the psychrotroph *Pseudomonas fluorescens* M3/6 have been shown to affect the plasmin system in milk, which in turn will affect the quality of processed milk. The M3/6 proteases cause dissociation of plasmin from casein in minimally processed milk. The objective of this work was to study the effect of M3/6 protease on the plasmin system, as well as its role in plasminogen activation, under commonly applied cheese-making conditions. Isolated M3/6 protease was added to raw milk, which then was pasteurized, and subjected to pH adjustments and CaCl_2 addition. Casein and whey fractions were separated by chymosin treatment then analyzed for plasmin activity. Individual and interaction effects of M3/6 protease addition, pH treatment, and CaCl_2 addition on plasmin activity were studied. Enzyme activity assays were carried out to study individually the effect of M3/6 protease on plasmin system components. Kinetic parameters were calculated to characterize the effect of M3/6 protease on plasminogen activation. Plasmin activity increased in the curd fractions of the protease-treated milk that was subjected to conditions most resembling cheese-making conditions, indicating that M3/6 protease triggered plasminogen activation rather than dissociation of plasmin from casein micelles. Results from the studies on plasminogen activation confirmed that the observed activation of plasminogen in protease-treated samples subjected to cheese making conditions was attributed to the stimulatory effect M3/6 protease had on plasminogen activators (PA). The M3/6 protease stimulated human and bovine PA by increasing their activity 4.5- and 2.5-fold, respectively. Similarly, the catalytic efficiencies of human urokinase-type PA and bovine PA were increased in the presence of M3/6 protease by 12- and 4-fold, respectively. Our research presented a basic step toward fully understanding the effect of bacterial proteases under differ-

ent processing conditions, where the gathered information can aid in better control of processing conditions based on the desired outcome.

(Key words: *Pseudomonas fluorescens*, protease, cheese, plasmin system)

Abbreviation key: MTB = modified Tris buffer, PA = plasminogen activator, PAI = plasminogen activator inhibitor, PG = plasminogen, PI = plasmin inhibitor, PL = plasmin, SpecPL = Spectrozyme PL, Sup2 = supernatant 2 bovine PA fraction, u-PA = urokinase-type plasminogen activator.

INTRODUCTION

Plasmin (PL; EC 3.4.21.7), an alkaline serine proteinase, is the principal indigenous proteolytic enzyme in milk, where it hydrolyzes mostly α_{s1} -CN, α_{s2} -CN, and β -CN (Grufferty and Fox, 1988; Bastian and Brown, 1996). Plasmin is usually associated with the casein fraction of milk; however, it can be found in whey and, under specific conditions, can shift from the casein to the whey fraction (Benfeldt et al., 1995; Fajardo-Lira and Nielsen, 1998). In fresh milk, plasminogen (PG), the zymogen of PL, is the predominant form (Nielsen, 2003). However, the levels of both PL and PG can vary significantly with the stage of lactation (Bastian et al., 1991), lactation number (Bastian et al., 1991), and mastitis (Politis et al., 1989a,b). Plasmin and PG are part of a complex system, commonly referred to as the plasmin system, including plasminogen activators (PA), plasminogen activator inhibitors (PAI), and plasmin inhibitors (PI). The interactions between PG, PL, PA, PAI, and PI, which characterize the plasmin system, have been studied by several researchers as discussed in review articles (Grufferty and Fox, 1988; Bastian and Brown, 1996; Kelly and McSweeney, 2003). The conversion of PG into PL is mediated by at least 2 types of PA, tissue-type PA, associated with casein, and urokinase-type (u-PA) associated with somatic cells (Bastian and Brown, 1996). Plasmin inhibitors and PAI are present in milk serum (Weber and Nielsen, 1991), and they are known to be heat-sensitive (Richardson, 1983).

Proteolysis induced by PL is sometimes essential and desirable for flavor development and texture changes

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during ripening of cheese, thus enhancing the product quality. The loss of PL from the casein micelle may slow down the cheese-ripening process, and consequently increase the processing expense. Conversely, uncontrolled proteolysis can have a detrimental effect on the quality, such as poor curd formation (Srinivasan and Lucey, 2002), gelation of stored UHT milk (Kohlmann et al., 1991a), and degradation in stored casein intended for use as functional enhancers in food (Nielsen, 2002). However, proteolysis in milk is not only caused by the native PL. Heat-stable metalloproteases produced by psychrotrophic microorganisms during refrigerated storage (Cousin, 1982) can also contribute to proteolysis in milk.

The current trend in the dairy industry is to reduce the frequency of milk collection, thus the refrigerated storage of milk has been lengthened, allowing the psychrotrophic bacteria to dominate the microflora. The heat-stable proteases produced by the psychrotrophic bacteria can destabilize the casein micelles by hydrolyzing κ -CN (Ewings et al., 1984; Mitchell and Marshall, 1989; Cromie, 1992), resulting in reduced cheese quality, production of small peptides that contribute to bitter flavor, UHT gelation, and fouling of heat exchangers (Grufferty and Fox, 1988; Champagne et al., 1994). An extracellular protease from *Pseudomonas fluorescens* M3/6, produced after incubation in reconstituted nonfat dry milk stored at 7°C, was characterized and shown to have activity on α -, β -, and κ -caseins (Kohlmann et al., 1991a,b).

Several studies have shown that bacterial proteases affect the PL system, which in turn will affect the quality of dairy products. Plasmin activity has been reported to decrease with microbial growth and storage time. Decreased PL activity was observed in fresh raw milk after 4 d of storage at 4°C, with psychrotrophic bacterial count reaching 10^6 to 10^7 cfu/mL (Guinot-Thomas et al., 1995). The PL decrease was attributed to psychrotrophic bacterial protease activity and PL autolysis (Guinot-Thomas et al., 1995). Studies with reconstituted nonfat dry milk (Fajardo-Lira and Nielsen, 1998) and fresh milk (Fajardo-Lira et al., 2000) stored under refrigerated conditions indicated that proteases produced by *Pseudomonas fluorescens* M3/6 affected PL location by disrupting the casein micelle to release enzymes of the PL system into whey fractions. Reduced PL activity in the casein fraction and increased activity in the whey fraction were observed with the growth of psychrotrophic microorganisms and the presence of proteases they produced (Fajardo-Lira and Nielsen, 1998; Fajardo-Lira et al., 2000). The 2 studies demonstrated clearly the effect of the bacterial protease on PL activity in the casein and whey fractions, when casein was separated from whey by acid treatment (Fa-

jardo-Lira and Nielsen, 1998) and by centrifugation (Fajardo-Lira et al., 2000).

No research has been done to examine the effect of proteases produced by psychrotrophic bacteria on the PL system under typical cheese-making conditions (pasteurization, calcium chloride addition, and chymosin treatment) or any other dairy production condition. Thus, to complement the work done by Fajardo-Lira and Nielsen (1998) and Fajardo-Lira et al. (2000), research is needed to study the effect of proteases produced by psychrotrophic bacteria on PL location (i.e., curd vs. whey) and PL system components under commonly applied processing conditions. Furthermore, research is still deficient in studying the effect of proteases produced by psychrotrophic bacteria on PG activation, in particular. Preliminary research showed that in the presence of proteases produced by psychrotrophic bacteria, PL activity increased in casein curds prepared under cheese-making conditions. These results led to the hypothesis that PG can be activated in the presence of proteases produced by psychrotrophic bacteria that stimulate PA under specific cheese-making conditions. Understanding how the PL system, as well as location in the presence of psychrotrophic bacteria, is affected under commonly applied processing conditions, is beneficial for better control of the quality of dairy products utilizing refrigerated milk. Therefore, our objective was to determine the effect of *Pseudomonas fluorescens* M3/6 protease under cheese-making conditions on PL system, in general, and on PA, in particular.

MATERIALS AND METHODS

Materials

Bovine PL was purchased from Roche Diagnostics (product #602 370; Indianapolis, IN). Human Urokinase-Type Plasminogen Activator (u-PA, product # U-5004) was purchased from Sigma Chemical Company (St. Louis, MO). Bovine PG (product # 416) and Spectrozyme PL (**SpecPL**, product #251) were purchased from American Diagnostica (Greenwich, CT). All above reagents were diluted to appropriate concentrations in modified Tris buffer (**MTB**; 0.05 M Tris, 0.1 M NaCl, 0.01% Tween 80, pH 7.6). *Pseudomonas fluorescens* M3/6 strain was provided by M. Griffiths from the University of Guelph, Ontario, Canada. Micro bicinchomic acid protein assay kit (Micro BCA protein kit, product #23235) was purchased from Pierce (Rockford, IL). Chymosin (product # 73863) was purchased from Chr. Hansen (Milwaukee, WI). Polyethylene glycol (product # P-2139) was purchased from Sigma Chemical Co. Laemmli buffer (product #161-0737), precast 12.5% acrylamide gel (product #161-0737), prestained low range molecular weight standards (product #161-0305), and

10× Tris/glycine/SDS (product #161-0732) were purchased from BioRad Laboratories (Richmond, CA).

Protease Isolation and Activity Measurement

An extracellular protease from *Pseudomonas fluorescens* M3/6 was isolated according to the method described by Kohlmann et al. (1991a), with the modifications outlined by Fajardo-Lira et al. (2000). Isolated protease was kept frozen in Tris-HCl buffer (0.05 M, pH 7.5). To determine the amount of M3/6 protease required for addition to milk, the specific activity of the enzyme was measured. The M3/6 protease samples were thawed and analyzed for specific activity in triplicate. The specific activity of the M3/6 protease was measured with an azocasein assay (Kohlmann et al., 1991a), and expressed as a function of protein content, which was determined using a Micro BCA protein assay kit following the manufacturer's instructions. One unit of azocasein activity (proteolytic activity) was defined as the amount of enzyme required to produce an increase in absorbance of 0.01/h at 366 nm. The specific activity of M3/6 protease was determined to be $1.22 \times 10^4 \pm 62.9$ units/mg of protein.

Cheese Study

Milk collection and treatment. Fresh milk was obtained from the Purdue University Dairy Research Center and processed the same day. Milk was drawn from 5 nonmastitic, antibiotic-free cows characterized as midlactation and in second calving. The milk was transported in sterile containers on ice to maintain low bacterial counts. Upon arrival, the milk was pooled and samples were drawn to obtain total and psychrotrophic plate counts according to procedures outlined by Cousin et al. (1992). Then, in triplicate, milk was divided into two 1.2-L samples; one was treated with 17.6 mL of isolated M3/6 protease (318 units/mL), and another was left untreated. All milk samples were incubated at 4°C for 5 h. The treatment and incubation conditions were based on results from Fajardo-Lira et al. (2000). After incubation, milk samples (protease-treated and untreated) were pasteurized at 72°C for 15 s using a laboratory-scale, dual-coil tubular heat exchanger (DHTC 55-4, Parker Hannipin Co., Brookfield, WI) with a positive displacement pump (Dayton model 4Z369; Emerson Electric, St. Louis, MO).

Curd and whey production. Protease-treated pasteurized milk (200 mL) was placed in each of four 500-mL beakers. The pH of milk samples in 2 beakers was left unadjusted (pH 6.6); however, the pH of the other 2 milk samples was decreased to pH 5.3 (while maintaining the milk at room temperature) with lactic acid

(85%), to mimic the pH decrease that would result from the growth of cheese cultures. Then 2 milk samples of pH 6.6 and 5.3 were left with no further treatment, and calcium chloride (CaCl_2) was added to the remaining 2 samples at a level of 0.02% (wt/vol), to simulate the level of application in many cheese-making procedures (Bylund, 1995). Finally, chymosin (25 μL) was added to all the milk samples, and the milk was incubated in a waterbath (Tecator 1024, Höganäs, Sweden) at 37°C for 30 min. The whole procedure was carried out on all 3 protease-treated pasteurized milk replicates, and was repeated for the untreated pasteurized milk samples. After incubation, the soft curd was cut with a spatula across the diameter of each beaker into 6 pieces (as a pie). Then the casein curd (henceforth referred to only as curd) was separated from the whey by centrifugation ($5000 \times g$, 21°C, 10 min; Beckman Instruments, Palo Alto, CA), and both fractions were frozen at -20°C until further analyses were performed.

Curd and whey preparation for analysis. Curd (3 g) was mixed with purified water (Barnstead/Thermolyne model D4631, Dubuque, IA) to a total volume of 30 mL. To disrupt the ionic bridges formed by calcium ions, trisodium citrate was added to obtain a 0.1 M solution. To help dissociate PL from the casein micelle, ϵ -amino caproic acid (0.1 g), a lysine analog, was added. The curd slurry was stirred in a waterbath (Precision model 260, GCA Co., Chicago, IL) for 15 min at 45°C. Samples then were cooled to 22°C and the pH was adjusted to 4.6 with concentrated hydrochloric acid to remove caseins. After holding for 15 min at 22°C, samples were transferred to 50-mL tubes and centrifuged ($1000 \times g$, 21°C, 15 min). The supernatant was recovered and the pH was adjusted to 7.5 using 8 M sodium hydroxide. The resultant supernatant was used for PL analyses.

Liquid whey samples were thawed and mixed 1:1 (vol/vol) with sodium citrate buffer (pH 3.2) then centrifuged ($15,600 \times g$, 21°C, 5 min). The supernatant whey (20 mL) was pipetted into a 12 cm long segment of pretwetted dialysis tubing (one end securely closed) with a 12,000 to 14,000 Da molecular mass cut-off (SpectraPor, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The remaining end of the dialysis tubing was closed and the pouch was placed on a layer of polyethylene glycol in a glass tray, then another layer of polyethylene glycol was applied to completely cover the sample. Samples were incubated at 22°C with occasional replacement of the saturated polyethylene glycol. Once concentrated to approximately 5 mL, the whey was quantitatively transferred to a beaker. The tubing was rinsed twice with 0.5 mL of MTB and the final volume was recorded. Collected samples were immediately used for PL analyses.

Table 1. Reagents and volumes (μL) used to investigate the effect of *Pseudomonas fluorescens* M3/6 protease on plasmin system activities.

Sample	Reagent ¹						Spec
	MTB	M3/6 protease	PL	Sup2	u-PA	PG	
1	300	0	0	0	0	0	450
2	150	150	0	0	0	0	450
3	150	0	150	0	0	0	450
4	0	150	150	0	0	0	450
5	300	0	0	0	0	150	150
6	150	150	0	0	0	150	150
7	150	0	0	0	150	150	150
8	0	150	0	0	150	150	150
9 (E) ²	150	0	0	0	150	150	150
10 (E) ³	0	150	0	0	150	150	150
11	150	0	0	150	0	150	150
12	0	150	0	150	0	150	150
13 (E) ⁴	150	0	0	150	0	150	150
14 (E) ⁵	0	150	0	150	0	150	150

¹MTB = Modified Tris buffer; PL = plasmin; Sup2 = bovine plasminogen activator (PA) fraction from milk; u-PA = human urokinase-type PA; PG = plasminogen; Spec = SpectrozymePL.

²Sample 7 mixed 1:1 with EDTA.

³Sample 8 mixed 1:1 with EDTA.

⁴Sample 11 mixed 1:1 with EDTA.

⁵Sample 12 mixed 1:1 with EDTA.

Curd and whey moisture and protein analyses.

Moisture content of the curd samples was measured using a vacuum oven method as described by Richardson (1985a). For moisture determination of liquid whey, samples were predried in crucibles on a heating plate, and then placed in a vacuum oven at 70°C for 3 h to complete the drying process. To measure protein content, the Kjeldahl method (Richardson, 1985b) was followed for the curd fractions, and the Micro BCA protein assay kit was used for the liquid whey samples.

Curd and whey chromogenic PL assays. Methods used to measure PL activity in both curd and whey samples were performed as described by Fajardo-Lira et al. (2000). The PL assay for curd was carried out the same as for whey samples, with the exception of volumes used. A whey sample of 100 μL was mixed with 300 μL of SpecPL, whereas a curd sample of 150 μL was mixed with 450 μL of SpecPL.

Effect of M3/6 Protease on PL System Components

Bovine PA source. Plasminogen activator was fractionated from milk following the procedure described by Lu and Nielsen (1993), generating a PA-containing sample referred to as supernatant 2 (**Sup2**) that was used as the source of bovine PA for all analyses. Supernatant 2 was reacted with bovine PG to estimate the amount of PA present in this fraction. A control for inherent PL activity of Sup2 was used to correct for possible overestimation of PG and PA activity. After

correcting for PL activity, bovine PA concentration was estimated to be 1.42×10^{-6} mM.

Effect of M3/6 protease on PL system activities.

To identify the components of the PL system that were affected by the M3/6 protease, several samples were prepared for chromogenic assay. The amounts and reagents used to prepare each assay are presented in Table 1. For all control solutions, MTB was used to bring the total volume to the level appropriate for each assay. The MTB volume was replaced with M3/6 protease for protease-added treatments. Effects of the M3/6 protease were observed on SpecPL, PL, PG, human u-PA, and bovine Sup2. Appropriate blanks were included for all treatments. Solution mixtures containing EDTA, a metalloproteinase inhibitor, were prepared by mixing 1:1 (vol/vol) EDTA (10 mM) with reaction mixture. In triplicate, all samples were prepared in microcentrifuge tubes and incubated at 37°C for 1 h. Following incubation, samples (100 μL) were placed in a 96-well microtiter plate and absorbance was read at 405 and 490 nm using an ELISA plate reader (Vmax Kinetic Microplate Reader, Molecular Devices Co., Menlo Park, CA). A stimulation factor (i.e., x-fold) was calculated by dividing the absorbance of the sample with M3/6 protease by the absorbance of the sample without the M3/6 protease.

Dose-dependency of M3/6 protease effect on PG activation. Reactions were carried out to monitor a dose-dependent response of M3/6 protease on u-PA. In triplicate, reaction mixtures were prepared to a total

volume of 600 μL . A blank containing MTB (450 μL) and 3.2 mM SpecPL (150 μL) was prepared, as well as a control containing MTB (225 μL), 1.91×10^{-6} mM human u-PA (75 μL), 3.2 mM SpecPL (150 μL), and 1.81×10^{-4} mM bovine PG (150 μL). As in the control, the same amounts and concentrations of human u-PA, SpecPL, and bovine PG were mixed with the isolated M3/6 protease (50, 100, 150, or 225 μL) and a volume of MTB to make up a total volume of 600 μL , in each of 4 tubes representing the treatment mixtures. All solutions were prepared in microcentrifuge tubes, vortexed, and transferred to polystyrene cuvettes, which were then incubated at 37°C in a Beckman DU-640 spectrophotometer using a temperature-controlled cuvette holder (product #523415; Beckman Instruments, Palo Alto, CA). The reactions were monitored by reading absorbance at 405 and 490 nm every 4 min for up to 1 h.

Hydrolysis of human u-PA in the presence of M3/6 protease. Samples of M3/6 protease, human u-PA, and M3/6 protease with human u-PA in a mixture were placed separately in microcentrifuge tubes and incubated for 1 h at 37°C. Proteins then were visualized using SDS-PAGE following the procedure outlined by Fajardo-Lira et al. (2000) with some modifications. Samples (10 μL) were mixed 1:1 (vol/vol) with Laemmli buffer under nonreducing conditions and placed in a boiling waterbath for 5 min. Cooled samples were loaded into wells of a precast 12.5% acrylamide gel. Prestained, low-range molecular weight standards (8 μL) were also loaded on the gel. Running buffer used was 10 $\times$ Tris/glycine/SDS diluted 1:10 (vol/vol) with purified water according to manufacturer's instructions. Running conditions of the gel were 200 V for 50 to 55 min.

Effect of M3/6 protease on the kinetic parameters of PG activation. An assay was carried out to calculate the Michaelis-Menten constant (K_m) for activation of bovine PG. The following scheme describes the reactions that took place in this assay:

where pNA is the p-nitroanilide group released from the chromogenic substrate, K_m is the Michaelis-Menten constant for PA, and k_{cat} is the rate constant for PG activation.

Analysis of the reaction and calculation of the desired kinetic parameters were completed following the model

described by Nishino et al. (2000), with the modifications outlined by Ripple et al. (2004). For control reactions, 75 μL of human u-PA (1.91×10^{-6} mM) was mixed with varying volumes (50, 100, 150, and 200 μL) of bovine PG (1.81×10^{-4} mM), a constant volume (150 μL) of 3.2 mM SpecPL, and the necessary amount of MTB to make up a volume of 600 μL . The M3/6 protease (150 μL ; 89 $\mu\text{g}/\text{mL}$ protein content as measured by the Micro BCA protein assay kit) replaced an equal amount of MTB in each of 4 tubes representing the protease-added treatments. Kinetic reactions were carried out at 37°C in a spectrophotometer equipped with a temperature-controlled cuvette holder. The reaction progress was monitored by reading the absorbance at 405 and 490 nm every 2 min for up to 1 h. All kinetic reactions were carried out in triplicate and were repeated using 1.42×10^{-6} mM bovine PA instead of human u-PA (1.91×10^{-6} mM).

Statistical Analyses

Analyses of variance were carried out utilizing SAS for Windows, version 6.0 (SAS Institute, 2000) to determine effects of different treatments on PL activity in the cheese study. Data were analyzed as a factorial experiment in a completely randomized design with protease, pH, and calcium chloride treatments as factors. When a factor or an interaction had a significant effect on results, indicated by a significant F -test ($P \leq 0.05$), significant differences between the means were determined ($P \leq 0.05$) using the Least Squares Difference test. Regression analysis and ANOVA were used to investigate the effect M3/6 protease had on stimulation of human u-PA and bovine PA in the PA enhancer study. Significant differences between the means were determined ($P \leq 0.05$) following PROC GLM and Tukey-Kramer multiple means comparison test.

RESULTS AND DISCUSSION

Cheese Study

Milk quality. The milk used was of excellent quality with a low microbial count. The total and psychrotrophic plate counts were each <250 cfu/mL (estimated).

Effect of cheese making conditions on PL. Plasmin activity in the curd fractions of the protease-treated samples was significantly higher than the PL activity observed in corresponding untreated samples (Figure 1A). The ANOVA (Table 2) indicated that, overall, the dominating treatment effect on PL activity in curd fractions was M3/6 protease addition. The observed increase in PL activity in the curd fractions of the protease-treated milk samples was the result of PG activation. However, the decrease of pH did not seem to exert

Figure 1. Plasmin activity for the curd (A) and whey fractions (B) produced from pasteurized milk subjected to different treatments. Pasteurized milk treatments: 1 = M3/6 protease was added; 2 = No M3/6 protease was added, in both treatments (1 and 2) pH was not adjusted (6.6), and CaCl_2 was not added; 3 = M3/6 protease was added; 4 = No M3/6 protease was added, in both treatments (3 and 4) pH was adjusted to 5.3, and CaCl_2 was not added; 5 = M3/6 protease was added; 6 = No M3/6 protease was added, in both treatments (5 and 6) pH was not adjusted (6.6), and 0.02% CaCl_2 was added; 7 = M3/6 protease was added; 8 = No M3/6 protease was added, in both treatments (7 and 8) pH was adjusted to 5.3, and 0.02% CaCl_2 was added. ^{A,B,C,D}Different letters above the bars indicate significant differences ($P = 0.05$).

much effect on the PL activity in curd. Grufferty and Fox (1988) found that between pH 6.6 and 4.8, micelle-associated PL activity was not reduced, but at 4.6, most of the activity was dissociated from the micelles. Our results also suggested that the effect of pH could have been counteracted by the effect of the protease. The ANOVA showed that, in the presence of chymosin, CaCl_2 had a significant effect on PL activity, where in CaCl_2 -treated samples increased PL activity was observed in the curd fractions. It has been suggested that CaCl_2 stimulates PA (Richardson and Elston, 1984); however, this assumption still needs to be pursued fur-

Table 2. Analysis of variance of plasmin activity in curd prepared from pasteurized milk subjected to M3/6 protease, pH, and calcium chloride treatments.

Source of variation	Degrees of freedom	Mean square	Significance
Corrected model	7	0.147	0.000
Intercept	1	14.007	0.000
pH	1	0.002	0.759
Protease	1	0.652	0.000
CaCl_2	1	0.264	0.001
pH × protease	1	0.023	0.256
pH × CaCl_2	1	0.007	0.540
Protease × CaCl_2	1	0.006	0.554
pH × protease × CaCl_2	1	0.078	0.047
Error	16	0.017	
Total	24		
Corrected total	23		

ther. Another explanation could be that, in the presence of chymosin, CaCl_2 allows for stronger coagulation of the micelles.

In curd fractions, M3/6 protease had the most influence on PL, but in whey fractions, pH had the most influence (Table 3), and CaCl_2 did not seem to exert much effect (Figure 1B). When studying the pH as a single treatment effect, there was significantly more PL activity in all treatments that had a pH of 5.3. The drop of pH affected the results of PL activity in whey more than it did in curd. This can be attributed to the small amounts of PL present in whey as compared with curd (curd PL content was 200 to 1000 times higher than that of whey) where a small change can be significant. Whey fractions of lowered pH and added CaCl_2 had higher PL activity when M3/6 protease was in the sample (Figure 1B, bars 7 and 8). This was consistent with the fact that the curd fractions of the same samples had higher PL activity when M3/6 protease was present (Figure 1A, bars 7 and 8). Although other factors could

Table 3. Analysis of variance of plasmin activity in whey prepared from pasteurized milk subjected to M3/6 protease, pH, and calcium chloride treatments.

Source of variation	Degrees of freedom	Mean square	Significance
Corrected model	7	0.096	0.000
Intercept	1	1.269	0.000
pH	1	0.523	0.000
Protease	1	0.021	0.056
CaCl_2	1	0.004	0.369
pH × Protease	1	0.025	0.039
pH × CaCl_2	1	0.031	0.025
Protease × CaCl_2	1	0.029	0.028
pH × Protease × CaCl_2	1	0.037	0.016
Error	16	0.005	
Total	24		
Corrected total	23		

have contributed to this observation, it seemed that when pH is lowered, more PL could be released from casein in a sample that had more total PL activity. The amount released might not have been large, but in whey, a small increase would be significant.

Results of studies carried out on reconstituted nonfat dry milk (Fajardo-Lira and Nielsen, 1998) and fresh milk (Fajardo-Lira et al., 2000) showed reduced PL activity in the casein fraction and an increased activity in the whey fraction with the growth of *Pseudomonas fluorescens* M3/6 and the presence of proteases they produced. However, conditions of these studies, including storage time, pH treatment, pasteurization of the milk, and separation technique of curd from whey were all different from the conditions of the present study. It was observed that cheese-like conditions, including pasteurization of milk, which inhibits PAI and PI, as well as chymosin addition, resulted in a microbial protease effect different than that reported previously. The conditions used were in favor of the protease triggering activation of PG rather than dissociating PL from the casein micelles. The CaCl_2 seemed to prevent PL dissociation and pH treatment did not exert much effect on the PL activity of curd fractions of protease-treated milk.

Because there was high PL activity in the treatment most resembling cheese-making conditions with the presence of M3/6 protease (Figure 1A, bar 7), it is concluded that these conditions triggered PG activation either directly or through stimulation of PA. To investigate this effect further, experiments were conducted on PG activation in a model system in the presence of M3/6 protease.

Effect of M3/6 Protease on PL System Components

Effect of M3/6 protease on PL system activities.

The M3/6 protease did not significantly affect SpecPL, PL, or PG; however, it stimulated the activity of human u-PA and bovine PA (Figure 2). The M3/6 protease caused an increase in u-PA and bovine PA activity of about 4.5- and 2.5-fold, respectively. Apparently, the M3/6 protease effect on human u-PA was more pronounced than its effect on bovine PA, and that could be attributed to the nature of the bovine PA source. The Sup2 sample usually contains a mixture of PA (Lu and Nielsen, 1993), some of which might be in the low molecular weight or 2-chain form, which are not usually affected by protease activity (Ugwu et al., 1998). Stimulation effect of the M3/6 protease on u-PA and bovine PA decreased significantly upon addition of 5 mM EDTA, which confirmed that the protease is a metalloprotease (Kohlmann et al., 1991a) that must have caused stimulation by hydrolyzing PA.

Figure 2. Stimulation of plasminogen activators (PA) by *Pseudomonas fluorescens* M3/6 protease. Sample 1 = SpectrozymePL; sample 2 = Bovine plasmin (PL) + SpectrozymePL; sample 3 = Bovine plasminogen (PG) + SpectrozymePL; sample 4 = Bovine PG + human urokinase-type PA (u-PA) + SpectrozymePL; sample 5 = Bovine PG + human u-PA + SpectrozymePL + EDTA; sample 6 = Bovine PG + bovine PA + SpectrozymePL; sample 7 = Bovine PG + bovine PA + SpectrozymePL + EDTA. Samples were prepared once with M3/6 protease and once without. The stimulation factor (i.e., x-fold) then was calculated by dividing the absorbance of the sample with M3/6 protease by the absorbance of the sample without the M3/6 protease. A,B,C,D Uppercase letters represent statistically different values with 95% confidence.

Dose-dependency of M3/6 protease effect on PG activation. Results confirmed the dependency of u-PA stimulation on the amount of M3/6 protease (Figure 3). Considering the specific amounts of M3/6 protease tested in this study, the degree of stimulation increased linearly with higher amounts of M3/6 protease present in the reaction mixture ($R^2 = 0.9939$).

Hydrolysis of human u-PA in the presence of M3/6 protease. Sodium dodecyl sulfate-PAGE was conducted to visualize any protein breakdown that might have occurred by action of the M3/6 protease on human u-PA. The M3/6 protease cut human u-PA into 2 fragments (Figure 4, lane 2 vs. lanes 3 and 4), which were similar in molecular weight to those reported by Ugwu

Figure 3. Dose-dependency of *Pseudomonas fluorescens* M3/6 protease stimulation of human urokinase-type plasminogen activator. Values are mean of 3 determinations, with error bars representing standard deviations.

Figure 4. Sodium dodecyl sulfate-PAGE visualization of human urokinase-type plasminogen activator (u-PA) breakdown induced by *Pseudomonas fluorescens* M3/6 protease after incubation for 1 h at 37°C in MTB. Lane 1 = MW standards; lane 2 = M3/6 protease + human u-PA; lane 3 = human u-PA; lane 4 = M3/6 protease. All samples were incubated for 1 h at 37°C before being applied to the gel. Arrows indicate protein fragments resulting from hydrolysis of human u-PA by M3/6 protease.

et al. (1998) because of stromelysin-1 activity on human u-PA. The observed electrophoresis result, coupled with the EDTA data from the chromogenic assay results, confirmed that the M3/6 protease stimulated PA activity by hydrolyzing it into u-PA chains of lower molecular weight that would result in an increased specific activity (Ugwu et al., 1998).

Effect of M3/6 protease on the kinetic parameters of PG activation. A rate value was calculated for each concentration of PG, and was used to construct Lineweaver-Burk plots. These plots were used to determine kinetic parameters corresponding to the first step in the coupled PG activation scheme, which is PG activation by PA. All Lineweaver-Burk plots are slightly non-linear because of the well-characterized effect of PL, generated during the reaction, on PG (Wohl et al., 1980). When the M3/6 protease cut human u-PA, a lowering of the K_m was observed, where the x-intercept became a larger negative number for the curve with M3/6 protease (Figure 5). Similarly, the M3/6 protease caused a lowering of the K_m for bovine PA (Figure 6). Although it had no significant effect on the maximum velocity (V_{max} ; y-intercept) of the human u-PA-mediated reaction, the M3/6 protease had a pronounced effect on the V_{max} of the PG activation catalyzed by bovine PA, resulting in a lowered V_{max} , where the y-intercept got larger. The catalytic efficiency (k_{cat}/K_m), however, of human uPA and bovine PA was significantly increased in the presence of the M3/6 protease by approximately 12- and 3-fold, respectively (Table 4).

Stimulation of bovine PA caused by M3/6 protease is physiologically relevant. The concentration of bovine PG in milk, between 3.5 and 14 $\mu\text{g/mL}$, is equivalent

Figure 5. Lineweaver-Burk plot for human urokinase-type plasminogen activator (2.38×10^{-7} mM) in the presence (■) and absence (●) of 22.6 μg of *Pseudomonas fluorescens* M3/6 protease. [PG] = concentration of bovine PG. Values represent means of 2 determinations, with error bars representing standard deviations (error bars are hidden by data points).

to a range of 0.03 to 0.18 μM , assuming the molecular mass of bovine PG is 80 kDa. The K_m for bovine PA (0.35 μM , Table 2) was found to be higher than the available PG in milk, suggesting less than $\frac{1}{2}V_{max}$ activation rate. However, the K_m was reduced to 0.031 μM (Table 2) in the presence of the M3/6 protease, indicating that even in milk with low native PG, the activation reaction could run at greater than $\frac{1}{2}V_{max}$ in the presence of the M3/6 protease. This change in reaction kinetics for bovine PA in the presence of M3/6 protease largely explained the increased PL activity observed in the curd fractions of milk spiked with M3/6 protease and processed under cheese-like conditions.

Figure 6. Lineweaver-Burk plot for bovine plasminogen activator (1.77×10^{-7} mM) in the presence (■) and absence (●) of 22.6 μg of *Pseudomonas fluorescens* M3/6 protease. [PG] = concentration of bovine PG. Values represent means of 2 determinations, with error bars representing standard deviations (error bars are hidden by data points).

Table 4. Kinetic parameters of plasminogen activators in the presence and absence of *Pseudomonas fluorescens* M3/6 protease (22.6 µg).¹

Sample	K _m (mM)	V _{max} (mM/min)	k _{cat} (min ⁻¹)	k _{cat} /K _m (mM/min) ⁻¹
HuPA ²	5.4 × 10 ⁻³ (1.6 × 10 ⁻³)	2.3 × 10 ⁻⁶ (6.8 × 10 ⁻⁷)	9.55 (2.85)	1762 ^c (4.80)
HuPA + M3/6 ³	1.7 × 10 ⁻⁴ (8.6 × 10 ⁻⁵)	9.6 × 10 ⁻⁷ (4.6 × 10 ⁻⁷)	4.05 (1.95)	24,328 ^a (839)
BPA ⁴	3.5 × 10 ⁻⁴ (7.1 × 10 ⁻⁵)	1.2 × 10 ⁻⁷ (2.1 × 10 ⁻⁸)	0.686 (0.121)	1949 ^c (50.7)
BPA + M3/6	3.1 × 10 ⁻⁵ (9.0 × 10 ⁻⁷)	3.3 × 10 ⁻⁸ (6.2 × 10 ⁻¹⁶)	0.188 (0.00)	6140 ^b (180)

^{a,b,c}Different letters in a column represent statistically different values with 95% confidence.

¹Values are means and standard deviations (in parentheses).

²HuPA = Human urokinase-type plasminogen activator (2.38 × 10⁻⁷ mM), n = 2.

³M3/6 = *Pseudomonas fluorescens* M3/6 protease (22.6 µg).

⁴BPA = Bovine plasminogen activator (1.77 × 10⁻⁷ mM), n = 2.

CONCLUSIONS

Although protease can cause dissociation of PL from the casein micelles, our results showed that under cheese-like conditions, dissociation was minimized and an effect of the M3/6 protease on PG activation was pronounced. Cheese-like conditions decreased the loss of PL from curd to whey fraction and the presence of proteases produced by psychrotrophs stimulated the activation of PG. Results of the PA enhancer study confirmed that the observed activation of PG in the cheese study was attributed to the stimulation effect M3/6 protease had on PA. It seemed that the cheese-making conditions tested prompted a stimulation effect similar to the one observed in the model system, resulting in activation of PG into PL in the curd fractions. Controlling conditions to obtain or maintain the quality desired for a dairy product is possible after fully understanding the action of such proteases under different processing conditions. Therefore, our research presented a basic step toward understanding the effect of microbial proteases on the PL system, under cheese-making conditions. More research should be carried out to study PG activation as affected by various microbial proteases, under different cheese- and dairy-processing conditions.

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