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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 different 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 (Fajardo-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