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Plasmin System and Microbial Proteases in Milk: Characteristics, Roles, and Relationship

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Proteolysis of milk proteins can be attributed to both native proteases and the proteases produced by psychrotrophic bacteria during storage of fresh raw milk. These proteases cause beneficial or detrimental changes, depending on the specific milk product. Plasmin, the major native protease in milk, is important for cheese ripening. Milk storage and cheese-making conditions can affect the level of plasmin in the casein and whey fractions of milk. A microbial protease from a psychrotrophic microorganism can indirectly increase plasmin levels in the casein curd. This relationship between the plasmin system and microbial proteases in milk provides a means to control levels of plasmin to benefit the quality of dairy products. This paper is a short review of both the plasmin system and microbial proteases, focusing on their characteristics and relationship and how the quality of dairy products is affected by their proteolysis of milk proteins.

KEYWORDS: Bovine milk; plasmin; psychrotrophs; proteases; cheese; whey protein concentrates

BACKGROUND

Milk Proteases. Breakdown of proteins (i.e., proteolysis) is important in a wide variety of food products. Proteolysis may have beneficial effects and may be essential for desirable qualities in food products, such as flavor development and texture changes during the ripening of cheese. However, uncontrolled or unwanted proteolysis can adversely affect the quality of foods.

Proteolyis in milk is caused by both native proteases and proteases produced by psychrotrophic microorganisms (referred to as psychrotrophs) during refrigerated storage of the milk (*1*−*3*). These proteases differ in their specificity toward milk proteins. Although native proteases were deemed previously not to have serious effects in dairy products, they now are considered to be an important factor (*3*). The major native protease in milk is plasmin (EC 3.4.21.7), which is part of a complex system. Recent research has shown that bacterial proteases affect the plasmin system, which can then affect the quality of dairy products.

Proteases Produced by Psychrotrophs. *Psychrotrophs.* Psychrotrophic microorganisms are those that grow at ≤7 °C, although their optimal growth temperature may be higher. During cold storage after milk collection, they dominate the flora and are responsible for many quality problems in dairy products. Under sanitary conditions, <10% of the total microflora are psychrotrophs, compared to >75% under unsanitary conditions (*4*). The numbers of psychrotrophs that develop after milk collection depend on the storage temperature and time. Among the psychrotrophs, *Pseudomonas* are most frequently reported in raw milk. Psychrotrophs produce extracellular enzymes, mainly proteases and lipases, that contribute to defects in dairy products. Optimal enzyme synthesis occurs in the majority of psychrotrophs at 20−30 °C, but considerable synthesis occurs at lower temperatures. For example, production of extracellular protease by *Pseudomonas fluorescens* at 5 °C was 55% of that produced at 20 °C (*5*).

Protease Classification. The proteases produced by psychrotrophs generally are metalloproteinases (i.e., they require the presence of a metal ion such as calcium for optimum activity) (*6*), whereas plasmin is classified as a serine proteinase (i.e., it has the amino acid serine at the active site) (*3*) and chymosin, commonly used in cheese-making, is an aspartic proteinase (i.e., it has the amino acid aspartic acid at the active site).

Conditions for Activity. The proteases produced by psychrotrophs have a broad pH and temperature range for activity (*6*). Temperature optima are 30−45 °C, but the proteases remain partially active at lower temperatures. These proteases are generally extremely heat stable, withstanding ultrahigh temperature (UHT) processing (140 °C for 4 s).

Specificity. Heat-stable proteases from psychrotrophs attack all forms of casein, with preferential hydrolysis for κ-casein, then β-casein, and finally α-casein (*1*, *7*). There is controversy in the literature as to whether the major whey proteins are susceptible to bacterial proteases (*1*, *8*). An extracellular protease isolated in our laboratory 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 (*9*, *10*). Hydrolysis of κ-casein can result in destabilization of the casein micelle and the production of small peptides that contribute to bitter flavors (*11*).

Plasmin System. Native milk proteases and their activators and inhibitors all must be considered to study the stability of milk and milk products. To understand the detailed description of each component as given below, it is helpful first to visualize

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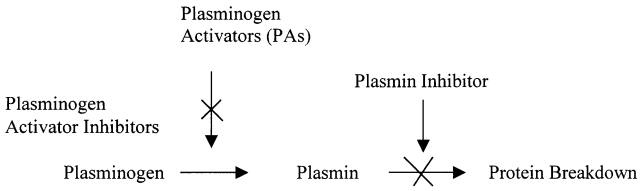
and briefly describe their relationship. The complete native enzyme/activator/inhibitor system in bovine milk has been illustrated as follows (*12*):



Plasminogen activators (PAs) convert inactive plasminogen to active plasmin, which then can cause the breakdown of milk proteins. The activator, zymogen, and active plasmin are all quite heat stable. In contrast, plasminogen activators and inhibitors and plasmin inhibitors, which also are naturally present in milk and could shut down the plasmin system, are quite heat labile.

Plasmin and Plasminogen. (a) Blood versus Milk. Bovine versus Human. Plasmin and plasminogen in bovine milk are essentially identical to those found in blood, as suggested by their similar heat stabilities, pH optima and stabilities, specificities for casein hydrolysis, and inhibition patterns (*13*−*15*) and as indicated by amino acid sequence (*16*). The comparisons between human and bovine plasmin and plasminogen and the activation of human and bovine plasminogen have been reviewed (*17*, *18*).

(b) Interaction of Components of Plasmin System. Plasmin is an important part of the clotting mechanism in blood, with its activity controlled by interactions between plasmin, plasminogen, PAs, and inhibitors of plasmin and PAs (*19*). Likewise in milk, plasminogen must be activated to plasmin by PAs present, before plasmin can degrade milk proteins (*20*). Plasmin exists primarily in its inactive form, plasminogen (*21*), which indicates the importance of PAs in the plasmin system. The ratio of plasminogen to plasmin in milk has been reported to be from 50:1 to 2:1 (*21*). Plasmin inhibitor and PA inhibitor could potentially control the plasmin system in milk, but they are heat labile (*12*), whereas plasmin, plasminogen, and PAs are extremely heat stable (*18*, *22*).

(c) Specificity. Plasmin (EC 3.4.21.7) is a serine proteinase, similar to trypsin in its activity and characteristics. Plasmin cleaves proteins on the carboxyl side of L-Lys and L-Arg residues (*23*), with a preference for L-Lys. Plasmin hydrolyzes α_{s1}-, α_{s2}-, and β-caseins but has little or no activity on the whey proteins β-lactoglobulin and α-lactalbumin (*3*, *24*, *25*). Reports are conflicting on whether plasmin can hydrolyze κ-casein (*26*, *27*), but differences in results may be due to environmental conditions and/or the concentrations of enzyme and substrate used (*3*). Degradation of α_{s2}-casein and β-casein occurs at the same rate, and α_{s1}-casein is hydrolyzed at a slower rate (*3*). In kinetic studies using a synthetic substrate, casein has been shown to be a competitive inhibitor of plasmin. However, the interferences can be overcome by the use of appropriate concentrations of substrate and longer incubation periods (*28*). Denatured β-lactoglobulin inhibits plasmin (*29*), probably by thiol−disulfide interchange between the disulfide bonds of plasmin and the activated sulfhydryl groups of β-lactoglobulin (*30*). Plasmin inhibition by the whey proteins β-lactoglobulin, bovine serum albumin, and α-lactalbulin at concentrations found in milk has been investigated (*31*).

(d) pH and Temperature Stabilities. Bovine milk plasmin is most active at pH 7.5−8.0 and at 37 °C (*32*), but it is stable and active over a broad pH range as indicated in part by its activity in various cheeses (*18*). Bovine milk plasmin and plasminogen are both quite heat-stable, especially in the absence of the whey protein β-lactoglobulin (*33*). Plasmin in milk fully survives pasteurization conditions at pH 6.8 (*34*) and is somewhat resistant to certain high-temperature, short-time heat treatments (*35*, *36*). The heat stability of plasminogen is similar to that of plasmin (*12*). Plasminogen activation actually is increased by pasteurization (*12*, *37*). The significant increases in plasmin-attributed proteolytic activity of milk by pasteurization treatments is likely attributable to inactivation of an inhibitor of plasminogen activator (*12*).

(e) Other Characteristics. The molecular mass of bovine milk plasmin is 48000 as determined by size exclusion chromatography (*14*), but much higher molecular weights also have been reported for plasmin. Plasmin is a glycoprotein, exists as a dimer held by disulfide bonds, and readily autodigests (*38*). Recently, it has been shown that plasmin must be partially unfolded (e.g., by heat treatment) prior to any autodigestion (*36*).

(f) Levels Present in Milk. Factors have been identified that influence the amount of plasmin and plasminogen activity in bovine milk (*21*, *39*, *40*), with higher plasmin activity being attributed to plasminogen activation (*40*). Plasmin activity is increased in milk at the end of lactation, in older cows, and in mastitic milk (*18*). Concentrations of plasmin and plasminogen in milk are estimated to be 0.3 and 2.5 μg/mL, respectively (*21*).

Plasminogen Activators. Plasminogen is activated to plasmin by PAs during storage of normal milk (*35*, *41*) and even while milk is held in the mammary lumen before milking (*39*). Tissue-type PAs (t-PA) (EC 3.4.21.68) and urokinase-type PAs (EC 3.4.21.73) are present in bovine mammary tissue (*42*) and in milk (*43*, *44*). The urokinase-type PA is associated with somatic cells, and t-PA is asssociated mainly with casein (*45*, *46*). Casein (specifically, α_s-casein) enhances plasminogen activation by t-PA and urokinase (*45*, *47*, *48*). The PAs are serine proteinases that activate plasminogen to plasmin by cleaving an Arg^{557}−Ile^{558} bond in bovine blood plasminogen (*17*) and an Arg^{560}−Val^{561} bond in human plasminogen (*49*).

Plasminogen activators are seemingly even more heat stable than plasmin and plasminogen. The decimal reduction times (*D* values) of PAs isolated by Lu and Nielsen (*44*) were 109 min at 70 °C and 32 s at 140 °C. This indicates that PAs in bovine milk are not affected by pasteurization processes and largely are not inactivated by UHT processing conditions used in the dairy industry. Reported *D* values for plasmin are 7 s (*35*) and 10 s (*41*) at 142.5 °C and 35.7 min (*35*) and 12.4 min (*41*) at 72.5 °C. Plasminogen is reportedly less heat stable than plasmin, but their rates of inactivation are similar (*33*).

Inhibitors of Plasmin System. Because plasmin, plasminogen, and PAs are not readily inactivated by heat treatment, inhibitors of plasmin and PAs are one potential means to control plasmin-related activities. However, whereas plasmin, plasminogen, and PAs are heat stable and are associated with the casein fraction, inhibitors of plasmin and PAs in milk are heat labile and are found only in milk serum (*12*, *50*, *51*). Inhibitors of plasmin and PAs have been isolated from blood (*52*, *53*). Numerous researchers reported protease inhibitor activity in bovine milk (*50*, *54*, *55*) before a putative α_1-antitrypsin was isolated and partially characterized (*56*). The search in bovine milk for α_2-antiplasmin (a plasmin inhibitor, PI) and plasminogen activator inhibitor 1 (a PA inhibitor, PAI) led to their identification in milk and their partial purification (*57*). These two inhibitors are important in regulating the plasmin system in blood and

are of the size most likely to cross mammary membranes to enter milk (*57*).

Association of Plasmin-Related Activities with Casein Micelle. The enzymes plasmin, plasminogen, and PAs in fresh milk all are known to be associated with casein micelles (*3*, *11*, *46*, *58*). The amino acid lysine in the enzymes seems to have some role in the association between the enzymes and the proteins in the casein micelle (*59*). Plasminogen interaction with immobilized casein has been shown to involve lysine binding and, to a lesser extent, electrostatic forces (*59*). At concentrations of up to 50 mM, ϵ-amino-*n*-hexanoic acid, a lysine derivative, dissociates plasmin from casein micelles, but higher concentrations inhibit plasmin (*51*).

Properties of casein micelles (including self-association of individual caseins, metal ion binding to the caseins, properties and models for the casein micelle) have been reviewed recently by Rollema (*60*). Casein micelles are spherical aggregates made of casein proteins and calcium phosphate, likely organized into submicelles, with κ-casein on the surface (*61*). The casein micelle is quite stable but aggregates when the κ-casein is enzymatically cleaved by treatment with the enzyme chymosin (also called rennet) or when dissolution of the calcium phosphate occurs due to acid treatment.

Temperature, pH, and ionic strength have been studied as possible factors that influence plasmin dissociation from casein micelles. Evidence on the effect of temperature is conflicting (*3*, *62*, *63*). However, pH clearly influences plasmin dissociation from casein micelles. Although it is unclear at what pH the release begins below the normal pH 6.6−6.8 of milk, most or all of the plasmin activity is dissociated at pH 4.6−4.7 (*64*, *65*). Addition of 1 M NaCl to milk causes complete loss of plasmin activity from casein micelles (presumably due to dissociation, because controls showed no inhibition of plasmin activity up to 2.5 M NaCl) (*65*).

Importance of Plasmin Activity in Dairy Products. The reported advantages and disadvantages of plasmin activity in various dairy products indicate the necessity to control the activation of plasminogen and the activity of plasmin. The major dairy products studied in this regard are UHT milk and cheeses, but casein and whey protein products are likely affected as well. [The properties and importance of plasmin in bovine milk and dairy products have been reviewed by Grufferty and Fox (*3*) and Bastian and Brown (*18*).]

(a) UHT Milk Products. Plasmin is quite heat stable and survives pasteurization and many UHT treatments. There is conflicting evidence about the role of plasmin in gelation upon storage of UHT milk, with results seemingly dependent on factors such as processing conditions, storage conditions, levels of plasmin, concentration of milk, and other ingredients in the product (*18*, *30*, *66*). However, UHT milk samples with added plasminogen and low levels of plasmin gelled more rapidly than milk with no added enzyme (*67*, *68*). In UHT custard, plasmin activity was associated with thinning of the product (*69*). The role of plasmin in causing defects in UHT dairy products likely will remain speculative until mechanisms of thinning and gelation during storage are resolved (*18*).

(b) Cheeses. Proteolysis in cheese during ripening results in texture modifications, an increase in pH through NH_3 formation, and the production of flavor compounds (*70*, *71*). In fact, McGoldrick and Fox (*71*) have suggested that cheeses can be classified by their proteolysis patterns using reversed-phase high-performance liquid chromatography and/or urea−PAGE. The importance of plasmin in cheese ripening is still under debate and probably depends on the cheese variety (*18*). Increased plasmin activity, as a result of either plasminogen activation (*28*, *72*) or addition of plasmin (*73*, *74*), has been shown to improve the flavor and overall quality of certain cheeses. Results of numerous studies with various cheeses indicate that the importance of plasmin in cheese ripening depends on the cooking temperature during cheese-making and the pH during ripening (*75*, *76*). Plasmin is more active and more important in the ripening of high-pH cheese (e.g., Camembert) than in low-pH cheeses (e.g., Cheshire) (*18*). However, even for a fairly low-pH cheese such as mozzarella (pH 5.2), there is evidence that plasmin contributes to the aging process.

Results of numerous studies suggest that plasmin could be a valuable enzyme for accelerated ripening and improved flavor development in natural cheese (*18*). Because cheese ripening is a time-consuming and expensive process (*77*), there are considerable benefits to be gained in accelerating ripening and considerable costs associated with slow ripening. In cheeses for which plasmin is important for ripening, reduced levels of plasmin in the casein micelle presumably would lead to reduced cheese quality and/or increased costs of production because ripening time must be extended.

(c) Milk Protein Products. The amounts and effects of plasmin activity in various milk protein products generally are unknown but very likely important. Both casein and whey protein products (e.g., caseinates from the casein fraction, whey protein concentrate, and whey protein isolate from the whey fraction) are produced in large quantities and are important functional ingredients in formulated food products (*78*−*80*). These protein products are utilized in numerous bakery, dairy, beverage, dessert, pasta, confectionery, and meat products. In addition to food applications, caseinates have numerous industrial applications including use in glues, adhesives, paints, rubber products, lubricants, and cleaning agents. Both casein and whey protein products are used in animal feeds. Casein and whey protein products are used in foods and other products because of their solubility, gelation, coagulation, hydration, viscosity, emulsifying, and foaming properties. These functional properties are controlled largely by the chemical composition and physicochemical properties of the protein products, which in turn are determined by the composition of the milk and the processing conditions used for their isolation (*79*). Plasmin content of the protein products certainly would be included in these determining factors. The functional properties of β-lactoglobulin are reportedly improved with 4% degree of hydrolysis caused by plasmin (*24*).

In both casein and whey protein products, it may be desirable to have little or no plasmin present that would hydrolyze other proteins in a food system to which they are added as functional ingredients. Thinning has been reported in industrial products to which caseinates are added, possibly due to plasmin activity (*3*). Such observations suggest the necessity to control plasmin activity in milk protein products, which is possible only by understanding the factors that determine the level of active plasmin in the casein and whey fractions.

RESULTS OF RECENT STUDIES

Several studies have been done to examine the relationship between the native plasmin system and heat-stable proteases produced by psychrotrophic microorganisms. Studies with reconstituted nonfat dry milk (*81*) and fresh milk (*82*) suggested that proteases produced by a psychrotrophic microorganism (*P. fluorescens* M3/6) can cause disruption of the casein micelle to release enzymes of the plasmin system into the whey fraction. Results showed a reduced level of plasmin in the casein fraction

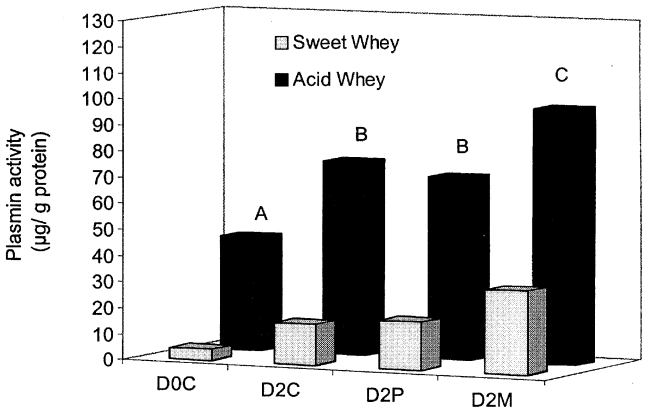


Figure 1. Plasmin activity (micrograms per gram of protein) of sweet and acid whey protein products produced from fresh milk, uninoculated or inoculated with *P. fluorescens* M3/6 and held for 2 days at 7 °C. DOC = day 0 control, D2C = day 2 control, D2M = day 2 M3/6, and D2P = day 2 pasteurized. Different capital letters represent significant differences with 95% confidence. (Reprinted with permission from ref *85*. Copyright 2000 American Dairy Science Association.)

and an increased level in the whey fraction with the growth of psychrotrophic microorganisms and the presence of proteases they produced (*81*, *82*). However, when milk treated with microbial proteases was handled as is typical of cheese-making conditions (pasteurization, pH decrease, calcium chloride addition, and chymosin treatment) and with an improved method for recovering plasmin from a casein curd, microbial proteases did not reduce the level of plasmin in the casein fraction (*83*). Results showed that microbial protease, calcium chloride, and pH decrease each had significant effects on plasmin activity under specific conditions. The microbial protease produced by *P. fluorescens* actually increased the level of plasmin in the casein curd under certain conditions (*83*). This result was consistent with another study in which milk was inoculated with *P. fluorescens* M3/6 culture, held at 7 °C for 2 days, and then pasteurized and treated with chymosin or acid to create an acid curd or a sweet curd (*84*). Both the sweet and acid curds from inoculated milk had significantly higher plasmin levels than did the appropriate controls without inoculation. In both studies to create sweet and acid curds and whey (*83*, *84*), pH had the greatest effect on the plasmin level of whey. Acid wheys had significantly greater plasmin levels than did sweet wheys (*83*, *85*) (**Figure 1**). This is consistent with reports of plasmin dissociation from casein micelles with a decrease in pH (*64*, *65*).

Recent studies pointed to the complexity of the plasmin system and the necessity of studying it in milk under realistic milk storage and cheese-making conditions. Questions were raised about the effect of microbial proteases and cheese-making conditions on the activity of PAs, which would increase the level of active plasmin by converting plasminogen to plasmin. These questions led to a study to determine the effect *P. fluorescens* M3/6 protease on the activity of bovine PAs (*86*). Results showed a 355% increase in u-PA activity in the presence of the isolated protease, whereas t-PA activity was increased by only 55%. The PA stimulation would explain at least in part the observed high levels of plasmin in pasteurized cheese curd made from milk inoculated with a bacterial culture (*84*) or the protease isolated from the culture (*83*).

SIGNIFICANCE

Implications of Plasmin Levels in Casein and Whey Fractions. A significant change from the typical levels of plasmin in the casein or whey fractions of milk could greatly affect the quality of dairy products and other food products that utilize dairy ingredients. A release of plasmin from the casein micelle could leave cheese and casein products with reduced levels of plasmin and whey protein products with increased levels. In contrast, enhanced conversion of plasminogen to plasmin by microbial proteases could result in increased levels of plasmin in the casein fraction. Plasmin activity impacts the quality of cheese and milk protein products, as described below.

Production of Cheese and Milk Protein Products. In the United States during the past 30 years, there has been a steady 2−5% annual increase in the production of cheese and its principal byproduct, whey, with ∼8 billion pounds of cheese produced in the United States in 1999 (*87*). Numerous types of whey products are produced, but whey protein concentrates and isolates are the ones most used as food ingredients. The United States produced ∼300 million pounds of whey protein concentrate in 1999, along with numerous other whey products (*88*). High-protein (>80% protein) whey protein products sell for ∼$2.00−8.00/pound, with the price dependent on the protein concentration and method of production (G. Ward, Land O'Lakes, Arden Hills, MN, personal communication). With this volume of production and cost of whey protein products, there is great economic interest in having products of high and consistent quality that are globally competitive.

Effect on Cheese Ripening. After the manufacture of cheese, the fresh cheese curd commonly is stored for 3 weeks to 2 years, depending on the type of cheese (*70*). This ripening process is essentially an enzymatic process that slowly modifies the texture, aroma, and taste of the cheese. Plasmin plays a predominant role in the ripening of certain cheeses, such as Swiss. Reduced levels of plasmin slow the ripening process, which already is very time-consuming and expensive. With cheese ripening representing approximately one-third to half of the processing cost (*77*, *89*, *90*), manufacturers are interested in shortening the ripening period and certainly want to avoid a longer ripening time. Reduced plasmin levels in cheese curd could cause delayed ripening and reduced flavor and texture quality, leading to longer ripening time and increased production costs. Cheese ripening costs in 1989 were estimated at ∼2.8¢/pound per month (*75*). For a company storing 5 million pounds of cheese (i.e., the equivalent of three months of production for a mid-size plant), reducing the ripening time by just one month would save $125,000. The costs of increased ripening time are staggering, considering the combined production of cheeses in the United States in 1999 was nearly 8000 million pounds, of which Cheddar cheese was ∼30% (*87*).

Effect on Milk Protein Products. Although increased levels of plasmin in the casein fraction would be beneficial for most cheeses, it may be desirable to release plasmin from the casein micelle in the production of casein protein products such as sodium caseinate. The thinning observed in some products to which caseinates are added has been attributed to active plasmin. Reduced levels of plasmin may be advantageous in caseinates for some applications. Likewise, most whey protein product applications would likely require that there is little or no plasmin in the whey. Whey and casein protein products, like other food ingredients, must have a defined and uniform composition to impart consistent characteristics to food products and to compete in the global economy. The plasmin content of commercial whey protein concentrates has been shown to vary considerably

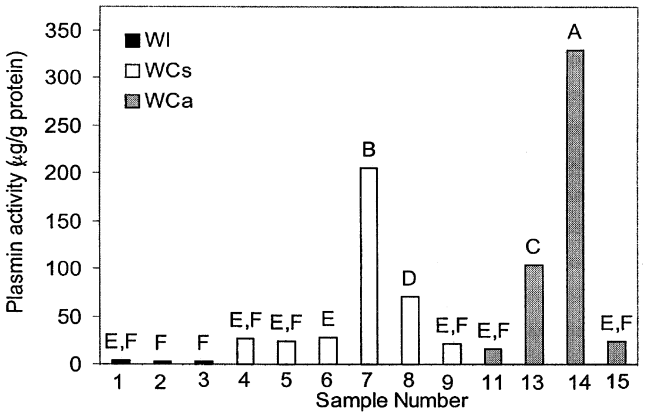


Figure 2. Plasmin activity of commercial whey protein products. WCs = sweet whey protein concentrate (~80% protein), WI = whey protein isolate (~90% protein), WCa = acid whey protein concentrate (~80% protein). Different capital letters represent significant differences with 95% confidence. (Reprinted with permission from ref *85*. Copyright 2000 American Dairy Science Association.)

(20.5−330 μg/g of protein), whereas the plasmin activity in commercial whey proein isolates is lower (2.0−2.9 μg/g of protein) (**Figure 2**) (*85*). The quality of whey protein products is jeopardized if plasmin is released from the casein fraction into the whey. Increased plasmin levels in the whey fraction could cause protein breakdown in food systems to which whey products are added as functional ingredients and thereby reduce food product quality.

Need for Further Research. Additional experiments under actual cheese-making conditions will give an increased understanding of factors that affect the level of active plasmin in the casein and whey fractions of milk, making it possible to better manage the quality of cheese, casein products, and whey protein products. The studies need to include the effect of various microbial proteases on components of the plasmin system and factors that affect the location (i.e., casein or whey fraction) and activity of plasminogen activators in bovine milk. Recommendations based on further research can be made for conditions to either enhance or minimize plasmin activity, depending on the food application. This creates the potential for improving the quality and reducing the production costs of certain dairy products.

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