Presence in Bovine Milk of Two Protease Inhibitors of the Plasmin System[1]

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ABSTRACT

Proteolysis, caused by the serine proteinase plasmin (EC 3.4.21.7) that is present in milk, influences the quality of dairy products. Within the plasmin system, activators and inhibitors control plasmin activity. This study investigated the presence in bovine milk of two serine proteinase inhibitors of the plasmin system, α_2-antiplasmin and plasminogen activator inhibitor-1, and an isolation procedure used for partial purification of them from milk. Two colorimetric assays were used to detect either plasmin inhibitor activity or plasminogen activator inhibitor activity. Two inhibitors were partially purified from milk using a combination of ammonium sulfate fractionation and concanavalin A affinity chromatography. Plasminogen activator inhibitor-1 and α_2-antiplasmin antigens, which were associated with the inhibitory activities from bovine milk, were visualized by Western blot using commercial polyclonal antibodies raised against the corresponding human inhibitors. Both inhibitors were present in milk as several forms, possibly from the formation of complexes with other milk proteins. The predominant forms of the inhibitors in milk exhibited an approximate molecular mass of 60 kDa for α_2-antiplasmin and 55 kDa for plasminogen activator inhibitor-1.
(**Key words**: serine proteinase inhibitor, bovine plasmin, α_2-antiplasmin, plasminogen activator inhibitor-1)

Abbreviation key: **α_2-AP** = α_2-antiplasmin, **Con A** = concanavalin A, **PA** = plasminogen activator, **PAI** = plasminogen activator inhibitor, **PI** = plasmin inhibitor, **rPAI** = recombinant PAI.

INTRODUCTION

The serine proteinase plasmin (EC 3.4.21.7) that is naturally present in bovine milk is involved in the aging phenomena of dairy products, such as protein gelation in UHT milk during storage (9) and the development of flavor during cheese ripening (5). Investigation of the ways to control plasmin activity in dairy products is important to improve dairy product quality. The plasmin system of milk must be thoroughly understood before innovative methods can be designed to increase plasmin activity, to improve the sensory attributes of products in which proteolysis is desirable, or to limit the action of plasmin in products in which proteolysis causes quality defects.

Plasmin is a fibrinolytic proteinase that is present in blood and milk and is generated upon activation of its zymogen, plasminogen. The primary physiological role of plasmin in blood is to dissolve blood clots. In bovine milk, plasmin is present mostly as plasminogen, and the plasmin system is controlled by activators and inhibitors. Plasminogen activators (**PA**) mediate the activation of plasminogen to plasmin. Plasmin inhibitors (**PI**) inhibit plasmin activity directly. Plasminogen activator inhibitors (**PAI**) inhibit the conversion of plasminogen to plasmin (19). The physiological role of the plasmin system in milk is not clear.

Milk PA have been investigated in great detail (6, 16). Much less information is available on the inhibitors that control the plasmin system in milk. Because the source of the plasmin system of milk is the blood stream and, to some extent, the mammary gland, the inhibitors controlling the system can be assumed to have the same origin. Obvious candidates for protease inhibitors of the plasmin system in milk are the PI and PAI in blood. A number of serine proteinase inhibitors have been studied in milk and colostrum from various sources, but most of them were trypsin inhibitors (8, 13, 18, 21).

The principal inhibitor of plasmin in vivo is α_2-antiplasmin (**α_2-AP**) (12). Human α_2-AP is a 70-kDa, single-chain glycoprotein (23). Bovine α_2-AP is very similar to human α_2-AP in amino acid composition and sequence, particularly sequences at the N-terminal and reactive site (3). Bovine α_2-AP has a molecular mass that is low enough to cross from the blood stream into milk and is therefore likely to be the major PI of the plasmin system in milk.

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Human α_2-AP was first isolated from plasma by ammonium sulfate fractionation and various techniques for chromatographic separation (17). Later, α_2-AP was purified from plasminogen-depleted plasma using plasminogen fragment affinity chromatography, followed by size-exclusion chromatography (22). Another purification procedure involved successive chromatographic separations on plasminogen (affinity), DEAE (anion-exchange), concanavalin A (**Con A**) (affinity), and size-exclusion columns (24). Bovine α_2-AP also was purified from plasminogen-depleted plasma using ammonium sulfate fractionation, followed by size-exclusion and affinity (plasminogen fragment and protein A) chromatography (3). In early studies, trace amounts of α_2-AP were detected in human milk by immunochemical methods (14). More recently, seven plasma-derived protease inhibitors, including α_2-AP, have been identified in bovine milk using Western blot analysis and have been quantified by ELISA (4).

Four types of PAI, which often are related to pregnancy or pathological conditions, have been identified in human blood, tissue extracts, cells or cell cultures, and urine. Among them, PAI-1 represents the majority of PAI activity in normal human plasma (7). Bovine PAI-1 is a 50-kDa, single-chain glycoprotein (20). Small molecular mass makes PAI-1 likely to migrate from blood to milk and thus participate in the control of the milk plasmin system.

Human PAI-1 (1) and bovine PAI-1 (7) were purified from various endothelial cell cultures. Purification methods involved zinc-chelate chromatography, followed by monoclonal antibody affinity chromatograph (1), and Con A affinity chromatography, followed by dye-ligand and size-exclusion chromatographic procedures (7). The presence of PAI-1 in bovine milk has been mentioned (6).

Based on the results in the literature pertaining to blood, plasma, and cell cultures, we investigated the possibility of isolating PI and PAI from bovine milk after their presence had been verified. This study is, to our knowledge, the first attempt to purify α_2-AP and PAI-1 from bovine milk. These two protease inhibitors are likely to control proteolysis by plasmin.

MATERIALS AND METHODS

Activity Assays

The assays used to quantify PI and PAI activity in bovine milk samples were adapted from colorimetric assays used previously for PA activity in bovine milk (15) or PAI-1 activity of human recombinant PAI-1 (11). The assays were based upon cleavage by plasmin (for PI activity) or urokinase (for PAI activity) of the chromogenic substrate, Spectrozyme™ PL (H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide) or Spectrozyme™ UK [carbobenzoxy-L-G-glutamyl (a-t-butoxy)-glycil-arginine-*p*-nitroanilide], respectively (American Diagnostica, Inc., Greenwich, CT). The concentration of the chromophore product *p*-nitroanilide was determined by absorbance at 405 nm.

For each purification step, the PI or PAI operating buffer in place of the sample served as one control, the 0% inhibition control. To obtain the 100% inhibition control, the sample and the plasmin (or urokinase) were replaced by the PI or PAI operating buffer. Before the activity assays were performed, Centricon™-10 microconcentrators (Amicon, Division of Grace and Co., Beverly, MA) were used to replace the sample buffer with PI or PAI operating buffer, and samples were concentrated to an appropriate volume by centrifugation (5000 × *g* at 4°C).

Assay for PI activity. All reagents were in solution in 0.05 *M* Tris, 0.1 *M* NaCl, and 0.01% Tween® (Sigma Chemical Co., St. Louis, MO) 80 (pH 7.5). A standard curve was constructed by dilution of standard human α_2-AP from plasma (Sigma Chemical Co.) and was used to quantify the amount of PI in the samples. Twenty-five microliters of 2 mU/ml bovine plasmin (Sigma Chemical Co.) and 25 μl of sample or standard (0.1 to 10 μg/ml) were combined and incubated for 5 min at 37°C; then, 50 μl of 0.8 m*M* Spectrozyme™ PL were added. The absorbance at 405 nm was read after incubation for 1 h at 37°C. The decrease in absorbance that was detected using the PI assay represented the PI activity that was present in milk samples.

Assay for PAI activity. For this assay, 0.5 *M* Tris containing 0.01% Tween®-80 (pH 8.8) was used as the buffer. The standard curve was obtained with standard human recombinant PAI-1 (**rPAI-1**) (American Diagnostica, Inc.). Fifteen microliters of 300 IU/ml high molecular weight urokinase (American Diagnostica, Inc.) were incubated for 15 min at 37°C with 35 μl of sample or rPAI-1 (0.1 to 1.0 μg/ml). Then, 25 μl of 0.2 m*M* Spectrozyme™ UK were added, and the mixture was incubated for 2 h at 37°C. To correct for turbidity occurring in samples with high protein content, absorbances at 490 nm were subtracted from the absorbances at 405 nm. The decrease in measured absorbance using the PAI assay represented the PAI activity in milk samples.

The possible interference of α_2-AP in the PAI activity determination was studied; various concentrations of standard α_2-AP (0.01 to 10 μg/ml) were

tested for urokinase inhibitor activity using the procedure just described.

SDS-PAGE

The SDS-PAGE was carried out by the method of Laemmli (10) using a Tall Mighty Small™ vertical gel unit (Hoefer Scientific Instruments, San Francisco, CA) with 4% stacking gel and 10% resolving gel run at 20 mA. The molecular mass references were low range standards (94, 67, 43, 30, 20.1, and 14.4 kDa; Pharmacia LKB, Piscataway, NJ) and prestained low range standards (106, 80, 49.5, 32.5, 27.5, and 18.5 kDa; Bio-Rad Laboratories, Hercules, CA). Control proteins were the commercial inhibitors α_2-AP from human plasma (Sigma Chemical Co.) and rPAI-1 (American Diagnostica, Inc.).

Western Blot

Either polyclonal rabbit anti-human α_2-AP (Celsus Laboratories, Inc., Cincinnati, OH) or polyclonal rabbit anti-human melanoma PAI-1 (American Diagnostica, Inc.) was used as the primary antibody. Goat anti-rabbit IgG alkaline phosphatase conjugate was used as the secondary antibody (Bio-Rad Laboratories). Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) for 1 h at 100 V using a Mini-Trans-Blot® cell (Bio-Rad Laboratories). The loaded membrane was blocked overnight at 4°C with 5% nonfat dry milk, incubated with primary antibodies (diluted 1:2500, vol/vol, for anti-α_2-AP or 1:250, vol/vol, for anti-PAI-1) for 20 h at 4°C, and finally incubated with secondary antibodies (1:3000, vol/vol) for 2 h at 20°C. The alkaline phosphatase color reaction was allowed to develop between 3 and 20 min until contrast was adequate.

Sample Collection and Preparation

Bovine milk was collected from the cows (Animal Sciences Research Center dairy farm, Purdue University, West Lafayette, IN) and immediately put on ice. The milk was centrifuged at 2500 × g for 20 min at 4°C to separate the cream, and the skim milk was collected through cheesecloth. The skim milk was ultracentrifuged at 90,000 × g for 1 h at 4°C to separate most of the caseins, and the milk serum was collected and filtered through cheesecloth. The milk serum was then fractionated with ammonium sulfate.

Purification Procedure

Fractionation of milk serum by ammonium sulfate. Ammonium sulfate was added to milk serum at 20°C to final, successive concentrations of 40 and 80% saturation. The serum was stirred for 30 min and centrifuged for 10 min at 10,000 × g at 20°C. Each precipitate was resolubilized in a small quantity of deionized distilled water and frozen (–20°C) until used. The milk serum thus was fractionated into the 40% precipitate, the 80% precipitate obtained from the 40% supernatant, and the final 80% supernatant. The final supernatant was dialyzed exhaustively against deionized distilled water and concentrated in an 8050 UF stirred cell on a YM3 membrane (Amicon, Division of Grace and Co.).

After SDS-PAGE and Western blot analyses, the 40% precipitate was discarded, and the 80% precipitate was selected for application onto the Con A column. The pellet was resolubilized in distilled water and dialyzed extensively against Con A column running buffer (0.02 M sodium phosphate, 1.0 M NaCl, 0.01% Tween®-80, and 0.02% sodium azide, pH 7.0) at 4°C in a Spectra/Por® tubing of 3500 molecular mass cutoff (Spectrum Medical Industries, Inc., Los Angeles, CA).

Con A affinity chromatography. The Con A-Sepharose® resin (Pharmacia LKB) was prepared according to the instructions supplied by the manufacturer and packed in a 15-mm i.d. × 200-mm column at a flow rate of 30 ml/h to a final packed volume of approximately 25 ml. The column was equilibrated with Con A column running buffer and then run at 20 ml/h. A 25-ml milk sample, prepared as described, was applied onto the column. The column was rinsed with 200 ml of running buffer and then subjected to one-step elution (0.5 M methyl α-D-mannopyranoside in running buffer).

Column effluents from Con A were collected in 5-ml fractions. Prior to preparation for SDS-PAGE or Western blot, the wash and elution peaks were concentrated (20×) by UF using Centricon™-3 microconcentrators (Amicon, Division of Grace and Co.). The microconcentrators were centrifuged at 5000 × g at 4°C until the desired retentate concentration was reached.

The Con A elution peak was pooled, freeze-dried, and resolubilized in size-exclusion column running buffer (0.02 M Tris and 0.48 M NaCl, pH 8.0). The prepared sample was applied onto a 1.5-cm i.d. × 100-cm size-exclusion column (Sephacryl® S-100 HR). The column was run at 10 ml/h.

RESULTS AND DISCUSSION

Activity Assays

Control experiments were performed on the activity assays, particularly to test the possible inhibition of plasmin by β-LG (2). Neither BSA nor β-LG

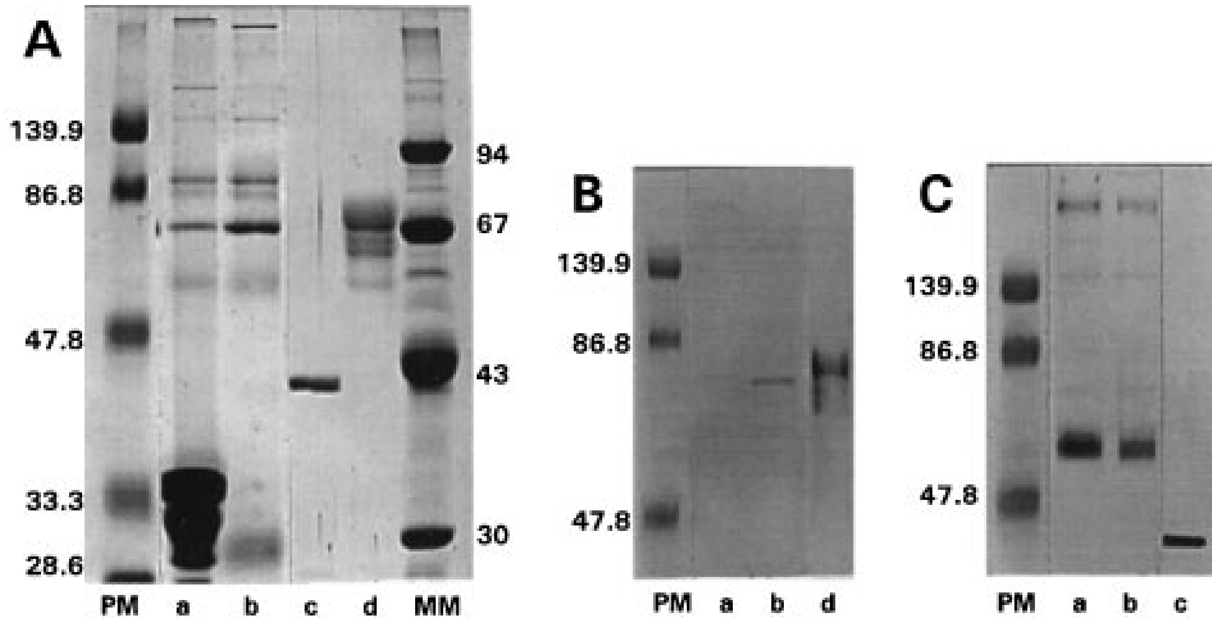

Figure 1. Comparison of the bovine milk inhibitors with human inhibitors by SDS-PAGE (A) and by Western blot for α_2-antiplasmin (B) and plasminogen activator inhibitor-1 immunodetection (C). Milk (lane a), 80% ammonium sulfate precipitate from milk serum (lane b), recombinant plasminogen activator inhibitor-1 (A and C, lane c), and α_2-antiplasmin (A and B, lane d). Prestained (PM) and SDS-PAGE (MM) molecular mass standards are expressed in kilodaltons.

was present in the purified fractions in quantities that were large enough to interfere with the PI assay. Assays of PI and PAI activity exhibited linear responses ($r = -0.99$ and $r = -0.98$, respectively) when the standards were tested in the ranges 0.1 to 0.8 μg of α_2-AP/ml and 0.1 to 0.7 μg of rPAI-1/ml, respectively. In the PAI assay, standard α_2-AP within the concentration range 0.01 to 10 μg/ml did not inhibit urokinase (data not shown). Because the estimated amount of PI in our samples was within the concentration range of standard α_2-AP tested, milk PI did not appear to interfere with the PAI assay.

Purification Procedure

In a preliminary experiment, the milk serum obtained from different cows exhibited some variability in the number, position, and intensity of bands that were visible in SDS-PAGE and on a Western blot for α_2-AP detection (data not shown). To compensate for possible physiological or genetic differences (or both), milk serum pooled from five cows was used as the sample.

A Western blot showed that strong antigenicity bands for both inhibitors were present in bovine blood serum. The Con A affinity elution fractions and milk serum also exhibited antigenicity bands, although those bands were weaker (data not shown). The antigen bands that were obtained for both inhibitors on Western blots demonstrated the crossreactivity of the bovine milk inhibitors with antibodies raised against human inhibitors, thus allowing for further screening for bovine inhibitors.

Pooled milk serum was fractionated with ammonium sulfate before application to the Con A affinity column, using successive saturation concentrations of 40 and 80% ammonium sulfate. The fractionation reduced the protein load and concentrated the sample. The samples of milk serum that had been fractionated by ammonium sulfate were visualized by SDS-PAGE, and the α_2-AP and PAI-1 antigens were detected on Western blots (data not shown). On the Western blot, the majority of the α_2-AP antigen bands was found in the 80% precipitate. The PAI-1 antigen bands were present in both the 40 and 80% precipitates. The loss of some PAI antigen in the 40% precipitate, which was discarded, was justified because of the large amount of nonantigenic protein that was also present in that precipitate. Therefore, the 80% precipitate was used as the sample for Con A affinity chromatography. We compared the 80% ammonium sulfate precipitate containing bovine milk inhibitors with human inhibitors (α_2-AP and rPAI-1) by electrophoresis and Western blot (Figure 1). The main bands that were elicited by human α_2-AP on a Western blot did not match the molecular mass of proteins attributed to the bovine PI (Figure 1B).

Similarly, the strong band elicited by human rPAI-1 on a Western blot did not match the molecular mass of the major proteins attributed to the bovine PAI (Figure 1C).

The inhibitory activities were minimal in the peak that was eluted by the wash of the Con A column (Figure 2). Western blots showed that the inhibitor antigens were almost exclusively in the Con A elution peak (Figure 3) and not in the wash peak (data not shown). The Con A elution peak was loaded onto a size-exclusion column to study the suitability of this type of chromatography to purify the inhibitors further. The eluted material from the Con A column was resolved into two protein peaks after size-exclusion chromatography. Western blots for α_2-AP and PAI-1 showed the presence of both antigens in the protein peak of highest molecular mass. We concluded that size-exclusion chromatography, under the conditions used, was not suitable for further purification of the inhibitors because of their similar molecular masses.

As visualized from the Con A elution peak (Figure 3B), the α_2-AP antigen was distributed between a major band of approximately 60 kDa in molecular mass, another band in the vicinity of 55 kDa, and a cluster of minor bands between 70 and 90 kDa. A recent study of bovine milk by Christensen et al. (4) reported the presence of PI in two forms: a 60-kDa protein (possibly cleaved PI) and a 120-kDa protein (possibly a complexed form of PI). Those researchers (4) postulated that neither of these PI was active against plasmin in milk. In the present study, we not only visualized the presence of PI in the 60 kDa range, but we also detected inhibitory activity by a colorimetric assay for PI.

The PAI-1 antigen was present as a major band of 55 to 60 kDa (Figure 3C), which is in agreement with the reported molecular mass for bovine plasma PAI (20). Also, a minor band of high molecular mass (>110 kDa), possibly a complexed form of PAI-1, was visualized. Further work is needed to verify this assumption and to evaluate the proportions of free PAI-1 and of complexes of PAI-1 and PA.

The possibility of nonspecific recognition was tested with Western blots (data not shown). Both the anti-α_2-AP and anti-PAI-1 antibodies exhibited no reactivity against α-LA or β-LG, but recognized BSA. However, the band that was elicited by BSA was distinctively different from those attributed to the milk inhibitors. Using a different primary antibody, a control Western blot for possible nonspecific binding of antibody and antigen was also performed, and no bands were visible.

Typical purification factors and yields for milk PI and PAI are given in Table 1. In milk serum, no PI or PAI activities could be detected at the sample concentrations used. Serum concentrations that were higher than those assayed resulted in protein precipitation that interfered with the activity assays. The lack of PI and PAI activities that were detected in the wash peak of the Con A chromatography indicates the high efficiency of this step. From a first set of serum samples, 194 μg of PI and 73 μg of PAI were partially purified, as measured by the colorimetric assay. When the same purification scheme was performed with a second set of milk serum samples, the yield and purification for PI and PAI values were similar to those reported in Table 1 (data not shown). However, compared with the first set of samples, 1.5 times more PI and 2.7 times more PAI were obtained after Con A

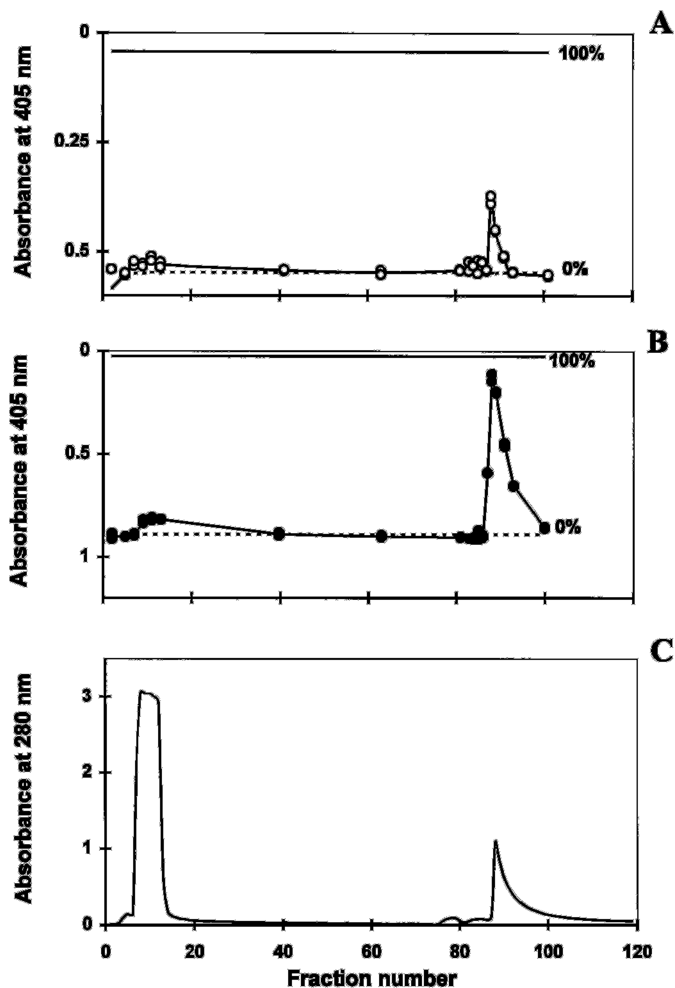

Figure 2. Chromatogram for concanavalin A affinity chromatography showing plasminogen activator inhibitor activity (A, ○), plasmin inhibitor activity (B, ●), and protein (C, —). Inhibition (0 and 100%) for each assay is shown. Sample applied was the ammonium sulfate 80% precipitate.

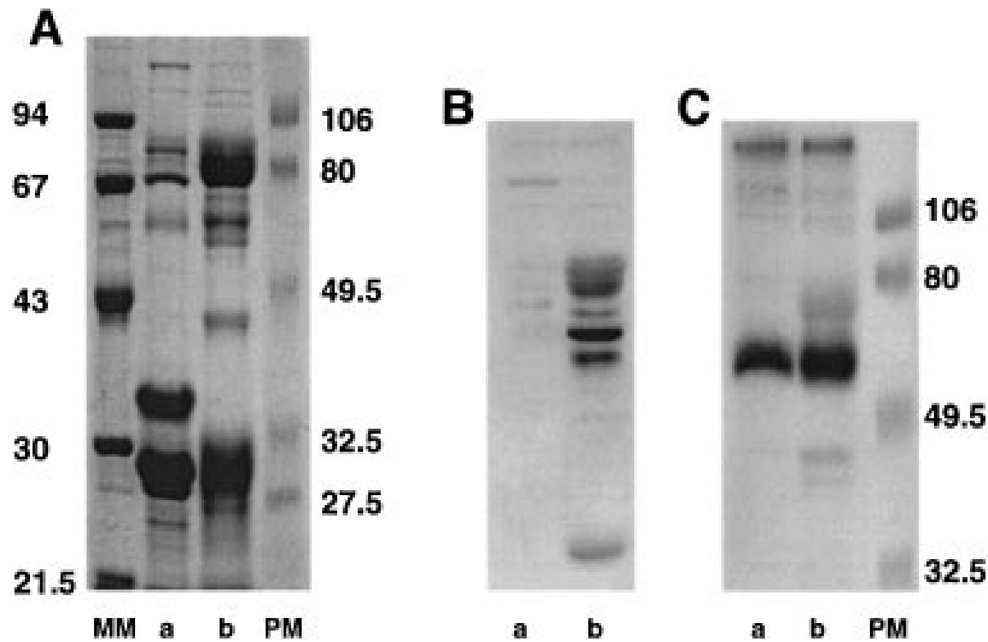

Figure 3. The SDS-PAGE (A) and Western blots for immunodetection of α_2-antiplasmin (B) and plasminogen activator inhibitor-1 (C) in milk serum (lane a) and elution peak from concanavalin A (lane b). Prestained (PM) and SDS-PAGE (MM) molecular mass standards are expressed in kilodaltons.

chromatography, indicating higher concentrations of PI and PAI in the milk. As stated, genetic and physiological differences among the animals may account for this variation.

Because of their closeness in molecular mass, the inhibitors could not be separated by the size-exclusion technique used. Further work is needed to complete the purification of the inhibitors detected, to determine their specific activity, and to measure their actual concentration in milk.

CONCLUSIONS

Two types of serine proteinase inhibitor activity were detected in bovine milk and were partially purified. The purification scheme was highly efficient for the two inhibitors, although their complete isolation has yet to be achieved. The PI and PAI activities were concomitant with the presence of α_2-AP and PAI-1 antigens. The antigens existed in more than one form, suggesting the formation of complexes with other pro-

TABLE 1. Partial purification of plasmin inhibitor (PI) and plasminogen activator inhibitor (PAI) from 500 ml of milk serum.

Samples	Total protein[1]	PI				PAI			
		PI[2]	Ratio[3]	Yield	Purification	PAI[4]	Ratio	Yield	Purification
	(mg)	(μg)		(%)		(μg)		(%)	
Serum	6753	ND				ND			
AS[5]	2443	353	0.14			85	0.04		
Con A									
Wash	2016	ND				ND			
Elution	160	194	1.20	57	9	73	0.46	85	13

[1]Total protein was calculated with the bicinchoninic acid method. Means of three determinations.

[2]Means of three determinations calculated from a standard curve using human α_2-antiplasmin and PI activity assay; ND = not detected.

[3]Ratios calculated as micrograms of PAI or PI per milligram of total protein.

[4]Means of three determinations calculated from a standard curve using human recombinant PAI-1 and PAI activity assay; ND = not detected.

[5]AS = 80% Ammonium sulfate precipitate fraction.

teins. This study contributes to a better understanding of the native plasmin system in bovine milk. The presence of α_2-AP and PAI-1 in milk makes them potential major factors in the control of proteolysis caused by the plasmin system in dairy products.

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