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ISOLATION AND CHARACTERIZATION OF A NOVEL 39 KILODALTON WHEY PROTEIN FROM BOVINE MAMMARY SECRETIONS COLLECTED DURING THE NONLACTATING PERIOD

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A 39 kilodalton glycoprotein has been isolated from bovine mammary secretions by heparin-agarose affinity chromatography and gel filtration. It is a minor whey protein in mammary secretions collected during the nonlactating period, but is clearly detectable by affinity chromatographic and immunoblotting techniques. It is not detectable by these techniques in milk or colostrum. This protein is not immunologically related to milk proteins, serum proteins or cytoskeletal proteins. The N-terminal amino acid sequence (36 amino acids) is not similar to other known proteins. Isolating this novel 39 kilodalton protein provides a specific marker for mammary function during the nonlactating period.

Lactational function of the mammary gland occurs in cycles, beginning with mammary development and lactogenesis and concluding with mammary involution. During the period between successive lactations, the mammary gland undergoes several functional transitions, including active involution after cessation of milk removal, followed by redevelopment and colostrum formation prior to parturition (1).

The specific cellular and biochemical changes that occur in the mammary gland during the nonlactating period have not been fully defined. In the process of purifying proteins from bovine mammary secretions by an affinity chromatographic method, we isolated a 39 kilodalton protein that is found in mammary secretions during the nonlactating period in elevated concentrations. Purification and characterization of this protein are described.

MATERIALS AND METHODS

Sample collection and whey preparation

Mammary secretions for protein isolations were collected from multiparous cows at 2 weeks following cessation of milk removal. Aqueous phase of secretions and acid precipitation of casein in the aqueous phase was as described previously (2). Prior to acid precipitation, sodium azide was added to the aqueous phase (to 0.02%) to inhibit microbial growth. Immunoglobulins and residual caseins were removed by ammonium sulfate

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precipitation (33% saturation) and centrifugation. Ammonium sulfate was then added to the supernatant to 80% saturation; precipitated proteins were collected by centrifugation and the supernatant was discarded.

Protein purification

Whey proteins were dialyzed against 0.05 M NaCl in barbital buffer (0.005 M Na barbital-HCl, pH 7.4). Approximately 1600 absorbance units (280 nm) of whey protein was applied to a packed column (2.0 x 16.5 cm) of Affi-Gel heparin-agarose (BioRad, Richmond, CA) equilibrated in barbital buffer. Protein was eluted at a flow rate of 48 ml/h using a continuous gradient of 0.05 M to 1.0 M NaCl in barbital buffer. Fractions (12 ml) were collected and absorbance at 280 nm was determined. Fractions from absorbance peaks were pooled and concentrated, and then equilibrated against gel filtration buffer (0.01 M Tris, 0.5 M NaCl, 0.01 M EDTA and 0.02% Na azide, pH 8.0). The dialyzed material was filtered through a 5.0 x 87.0 cm packed column of Sephacryl S-200 superfine (Pharmacia Fine Chemicals, Piscataway, NJ) at a flow rate of 68 ml/h. Fractions (17 ml) were collected and absorbance at 280 nm was determined. Fractions from absorbance peaks were pooled and analyzed using double immunodiffusion in agarose and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; ref. 3) under reducing conditions, as described previously (4).

Antisera

All antisera were to bovine proteins. Antisera to the purified 39 kilodalton protein, to lactoferrin and to whey proteins were produced in our laboratory. Specificity of antiserum to 39 kilodalton protein was analyzed using double immunodiffusion in agarose and immunoblotting. Antiserum to secretory component was generously provided by Dr. A. J. Guidry (USDA-ARS, Beltsville, MD). Antisera for α-lactalbumin, β-lactoglobulin, colostral whey, serum proteins, and IgG1 were generously provided by Dr. B. L. Larson (University of Illinois, Urbana, IL). Antiserum for serum albumin was from Miles Scientific Laboratories (Naperville, IL). Antiserum to transferrin was from Cooper Biomedical (Westchester, PA). Antiserum to actin was from Biomedical Technologies, Inc. (Cambridge, MA), and antisera to keratin and tubulin from DAKO Corporation (Santa Barbara, CA). Antiserum for caseins was from Calbiochem (San Diego, CA). Peroxidase conjugated to the affinity purified $F(ab')_2$ fragment of goat antiserum to rabbit IgG was form Pel-Freeze Biologicals (Rogers, AR).

Double immunodiffusion in agarose and immunoblotting

Double immunodiffusion was performed as described (5). For immunoblotting, proteins were separated by SDS-PAGE and transferred overnight to nitrocellulose, as described previously (4). Protein blots were blocked with 5% bovine serum albumin in 0.05M Tris-HCl and 0.1M NaCl buffer, pH 7.6 (TBS), for a minimum of 16 h. Primary antiserum appropriately diluted in TBS with 0.5% Nonidet P-40 (TBS/NP-40) was applied to the blots and incubated at 25 C for 3 h with constant agitation. After blots were washed three times in TBS/NP-40, peroxidase conjugated to $F(ab')_2$ of goat anti-rabbit IgG, diluted 1:100,000 in TBS/NP-40, was applied to each blot and incubated at 25 C for 1 h with constant agitation. Labeled proteins were detected as described previously (4).

Periodic acid-Schiff reagent and endoglycosidase reactions

Detection of glycoproteins on polyacrylamide gels was accomplished using periodic acid-Schiff reagent (PAS), as described by Matthieu and Quarles (6). For endoglycosidase reactions, affinity-purified 39 kilodalton protein was incubated with endoglycosidase F (NEN Research Products, Boston, MA) or endoglycosidase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 50 units/mg of glycoprotein in 0.02 M Na phosphate and 0.15 M NaCl, pH 6.0, plus 1.0% SDS. Reactions were incubated for 72 h at 37 C and terminated by addition of 1/5 volume of 0.4% SDS/24% 2-mercaptoethanol in 0.5 M Tris buffer, pH 6.8 (sample buffer used in SDS-PAGE). Control incubations were

carried out in the absence of enzymes. Samples were boiled for 2 min and frozen at -20 C. Digested protein was analyzed on SDS-PAGE to determine molecular weight changes.

N-terminal amino acid sequence

The N-terminal amino acid sequence of bovine 39 kilodalton protein was determined by the Biotechnology Center at the University of Illinois at Urbana-Champaign from 1 nmole of 39 kilodalton protein using an Applied Biosystems Liquid Phase Protein/Peptide Sequencer.

RESULTS

Heparin-agarose affinity chromatography initially was adapted and utilized to isolate lactoferrin from bovine mammary secretions obtained during the nonlactating period (7). Analysis by SDS-PAGE of proteins in absorbance peaks eluted from the heparin column (Figure 1A) identified lactoferrin as the major protein eluted in peak PK-IV at a conductivity of about 30.0 mS (data not shown). A protein of approximately 39 kilodaltons was identified by SDS-PAGE (data not shown) as the major band in fractions

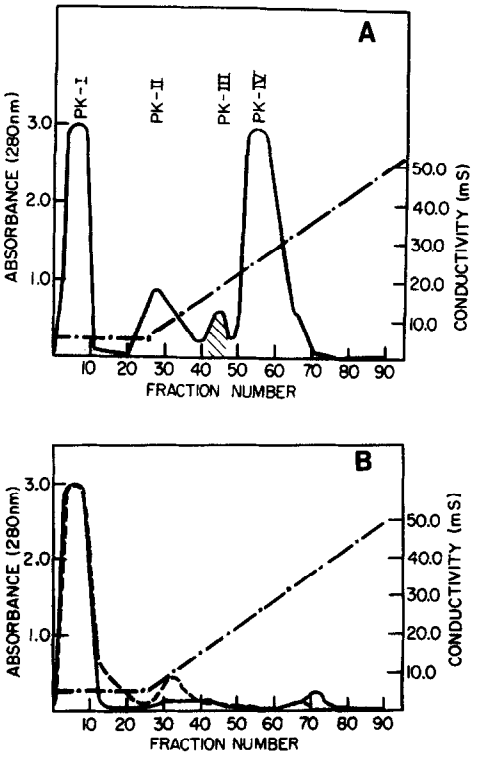

Figure 1. Protein elution profile from heparin-agarose affinity chromatography used to isolate the bovine 39 kilodalton protein from mammary secretions during the nonlactating period (A), from milk (B, single dashed line) and from colostrum (B, solid line). Chromatogram shows absorbance (280 nm) for each eluted fraction. Hatched area indicates fractions containing 39 kilodalton protein which generally elutes between fractions 40 and 50 (A, peak PK-III). Conductivity is in milliSiemans (mS).

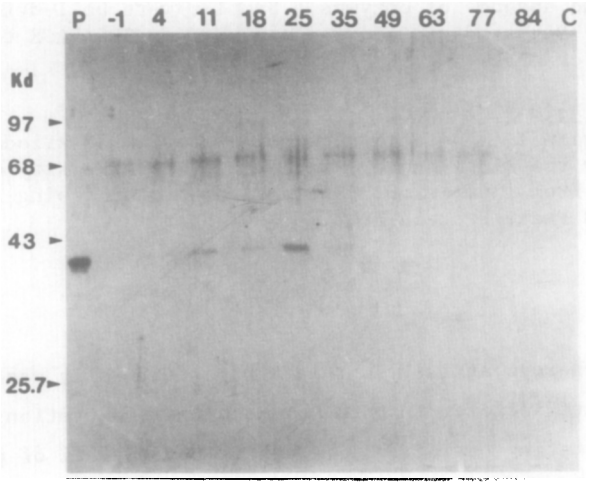

Figure 2. Immunoblot for detection of the 39 kilodalton protein in whey fractions of mammary secretions from a cow during the nonlactating period. Lane numbers represent days of the nonlactating period (day -1 is milk whey); C = colostrum (parturition on day 88); P = purified 39 kilodalton protein. Protein samples were separated by SDS-PAGE (7.5 µg protein/lane, except P at 200 ng protein/lane), blotted to nitrocellulose and 39 kilodalton protein detected as described in Materials and Methods. Molecular weight standards are in kilodaltons (Kd).

40-50 (PK-III) eluted at a conductivity of about 23 mS (Figure 1A). The 39 kilodalton protein was purified further by gel filtration (Sephacryl S-200). Three absorbance peaks were eluted from the S-200 column (Data not shown). The third peak contained purified 39 kilodalton protein. Double immunodiffusion in agarose, against rabbit antiserum for bovine whey proteins obtained during the nonlactating period, resulted in a single precipitin line (data not shown).

Double immunodiffusion in agarose was also used to identify the protein. Antisera used to analyze the 39 kilodalton protein were against bovine proteins found in mammary tissue or proteins identified in mammary secretions. No precipitin lines were observed for any of the antisera tested, including those specific for keratin, tubulin, actin, α-lactalbumin, β-lactoglobulin, caseins, IgG1, secretory component, lactoferrin and serum proteins.

Analysis of the protein elution profiles obtained by separation of colostral whey and milk whey (mid-lactation) by heparin-agarose affinity chromatography (Figure 1B) demonstrated the absence of a defined peak around fraction 40-50 where the 39 kilodalton protein was detected in secretions from the nonlactating mammary gland (Figure 1A). Using immunoblotting, the 39 kilodalton protein was detected in whey proteins from the nonlactating period (Figure 2, lanes 4, 11, 18, 25 and 35), but not in milk (lane -1) or colostrum (lane C). These data suggest that the 39 kilodalton protein is specific to the nonlactating period.

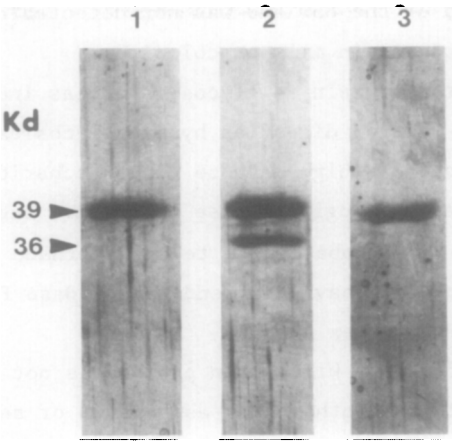

Figure 3. Endoglycosidase digestion of purified 39 kilodalton protein. Protein was incubated for 72 h without enzyme (lane 1), with endoglycosidase H (lane 2), or with endoglycosidase F (lane 3), as described in Materials and Methods. Molecular weight changes (Kd = kilodaltons) were demonstrated by SDS-PAGE (200 ng protein/lane) and silver staining.

The purified 39 kilodalton protein stained positive using periodic acid-Schiff reagent on SDS-PAGE, indicating the presence of carbohydrate (data not shown). Incubation of purified protein with endoglycosidase H (cleaves high-mannose oligosaccharides) for 72 h at 37 C resulted in a reduction of the molecular weight of the protein from 39 to 36 kilodalton, indicating removal of carbohydrate (Figure 3, lane 2). Endoglycosidase F (cleaves complex oligosaccharides) did not result in a molecular weight change of the glycoprotein (Figure 3, lane 3).

The 36 amino acid N-terminal sequence of bovine 39 kilodalton protein (Figure 4) did not have significant similarity to any known amino acid sequence, as analyzed using NBRF Pro Data Base consisting of over 5000 known sequences.

DISCUSSION

During the purification of proteins from bovine mammary secretions by heparin-affinity chromatography, a 39 kilodalton protein was isolated. Presence of this protein in mammary secretions was associated primarily with

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1                                                    12
TYR-LYS-LEU-ILE-X-TYR-TYR-THR-SER-TRP-SER-GLN-
13                                                   24
TYR-ARG-GLU-GLY-ASP-GLY-SER-X-PHE-PRO-ASP-ALA-
25                                                   36
ILE-ASP-PRO-PHE-LEU-X-THR-HIS-VAL-ILE-TYR-SER-
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Figure 4. N-terminal amino acid sequence of purified 39 kilodalton protein. Numbers indicate number of amino acids. X = residue not identifiable by sequence procedure, probably cysteine.

the nonlactating period of the cow and was not detectable by chromatographic or immunoblotting techniques in milk or colostrum.

The 39 kilodalton protein is glycosylated, as indicated by periodic acid-Schiff staining. Partial digestion by endoglycosidase H indicates that the purified protein contains high-mannose oligosaccharides. Incomplete cleavage of the protein by endoglycosidase H may have been due to decreased enzyme activity during the incubation or to an alternate form of glycosylation. The lack of cleavage by endoglycosidase F suggests that the protein does not contain complex sugars.

The identity of the 39 kilodalton protein is not known. It is immunologically distinct from other milk-associated or serum proteins and from cytoskeletal proteins. The lack of amino acid similarity with sequences of known proteins suggests that it may have a novel role in the mammary gland. While the role of the 39 kilodalton protein remains obscure, its particular presence in mammary secretions during involution and the nonlactating period provides an important marker protein for mammary function during that period. Further characterization of the origin and role of this protein in the bovine mammary gland will provide insights into the function of the gland as it undergoes involution.

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