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Preparation, Purification, and Quantitative Detection of Polyclonal Antibodies against Milk Lactoferrin: Postprint

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Abstract

This study aimed to prepare high-purity, specific polyclonal antibodies against bovine lactoferrin to provide experimental materials for identifying and quantitatively detecting lactoferrin in milk samples and bovine mammary gland tissues. Four healthy New Zealand white rabbits were immunized initially with lactoferrin, followed by booster immunizations at 4 weeks and subsequently every 2 weeks. After the serum achieved the desired antibody titer, cardiac blood was collected and serum was isolated. Antibodies were purified via saturated ammonium sulfate precipitation and protein A resin chromatography. The purity and specificity of the purified antibodies were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The antibody titer was determined, and an inhibition curve was constructed. The prepared antibodies were then applied to quantify lactoferrin in commercial liquid milk, bovine mammary gland tissue homogenates, and milk powder samples. The results demonstrated that the rabbit anti-bovine lactoferrin polyclonal antibodies exhibited high purity and strong specificity, with a concentration of 11.02 mg/mL and a titer of 1:128,000. The lactoferrin concentrations determined using these antibodies were 16.13 g/g in mammary gland tissue, approximately 0 g/g in liquid milk, and 5.28 g/g in milk powder. In summary, this study successfully obtained high-purity, high-specificity rabbit anti-bovine lactoferrin polyclonal antibodies through a two-step purification process involving saturated ammonium sulfate precipitation and protein A resin, which are suitable for lactoferrin detection in milk and related products.

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Full Text

Preparation, Purification and Quantitative Detection of Bovine Lactoferrin Polyclonal Antibody

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Abstract

This study aimed to prepare polyclonal antibodies against bovine lactoferrin with high purity and specificity to provide experimental materials for identifying and quantifying lactoferrin in milk samples and mammary gland tissues. Four healthy New Zealand white rabbits were initially immunized with lactoferrin, followed by booster immunizations at 4 weeks and subsequently every 2 weeks thereafter. Once serum antibody titers reached desirable levels, cardiac blood was collected and serum was separated. Antibodies were purified using saturated ammonium sulfate precipitation and protein A resin chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were employed to evaluate the purity and specificity of the purified antibodies. Antibody titer was determined, and an inhibition curve was established. Finally, the prepared antibodies were utilized for quantitative detection of lactoferrin in commercial liquid milk, dairy cow mammary tissue homogenates, and milk powder samples.

The results demonstrated that the rabbit anti-bovine lactoferrin polyclonal antibodies exhibited high purity and strong specificity, with a concentration of 11.02 mg/mL and a titer reaching 1:128,000. Using these antibodies, lactoferrin concentrations were determined to be 16.13 g/g in mammary tissue samples, approximately 0 g/g in liquid milk, and 5.28 g/g in milk powder. In conclusion, this study successfully obtained high-purity, high-specificity rabbit anti-bovine lactoferrin polyclonal antibodies through a two-step purification process involving saturated ammonium sulfate and protein A resin, which can be applied for lactoferrin identification in milk products.

Keywords: lactoferrin; polyclonal antibody; antibody purification

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Introduction

In recent years, consumer demands for milk quality have increased substantially. As milk protein serves as a crucial indicator of milk quality, nutritional regulation of milk protein synthesis has become a focal point in dairy cow nutrition research. Lactoferrin (Lf), also known as lactotransferrin, represents one of the important bioactive components in milk, exhibiting iron-binding properties and broad-spectrum antibacterial activity, while also modulating immune responses

and demonstrating antioxidant, anticancer, and antiviral effects. Lactoferrin concentrations are notably higher in colostrum (6-8 mg/mL in human colostrum; 1-2 mg/mL in bovine colostrum) compared to mature milk (1-2 mg/mL in human milk; 0.02-0.35 mg/mL in bovine milk). Given the limited availability of human milk, cow milk has become the most direct source for lactoferrin acquisition.

To detect lactoferrin concentrations in milk and dairy products and minimize processing losses, establishing a rapid and accurate detection method is essential. Enzyme-linked immunosorbent assay (ELISA) represents an effective approach for specific protein detection, offering advantages of speed, sensitivity, simplicity, and standardized carriers. When combined with SDS-PAGE, ELISA enables both qualitative and quantitative detection of target antigens. However, this approach requires specific lactoferrin antibodies, and commercially available antibodies from Sigma are prohibitively expensive for large-scale sample analysis. Therefore, this study immunized New Zealand white rabbits to prepare rabbit anti-bovine lactoferrin polyclonal antibodies, aiming to provide effective experimental materials for investigating lactoferrin synthesis regulation in dairy cows. To obtain antibodies with high purity and specificity, antiserum was purified through saturated ammonium sulfate precipitation and protein A resin chromatography. The purified antibodies were subsequently evaluated by ELISA for titer determination and inhibition rate assessment, providing valuable research materials for quantitative detection of lactoferrin in milk and mammary tissues.

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1. Materials and Methods

1.1 Experimental Materials Materials included lactoferrin standard (Sigma, USA, purity 85%), Freund' s complete adjuvant (Sigma, USA), Freund' s incomplete adjuvant (Sigma, USA), protein A resin (Genscript, USA), and goat anti-rabbit IgG-horseradish peroxidase (IgG-HRP) (Wuhan Boster Biological Engineering Co., Ltd.). Mammary tissue samples were collected from the Changping Experimental Base of the Institute of Animal Science, Chinese Academy of Agricultural Sciences. Liquid milk samples were commercial ultra-high temperature sterilized milk, and milk powder samples were commercially available regular brands.

1.2 Experimental Animals and Management Four healthy purebred New Zealand white rabbits were selected and housed individually. Animals were fed pelleted feed (casein-free) ad libitum with free access to water. Rabbit housing was thoroughly cleaned and disinfected prior to the experiment. After a one-week acclimation period, ear vein blood was collected to verify the absence of pre-existing reactivity against lactoferrin. Rabbits showing no serum reactivity were considered qualified and subjected to the immunization protocol.

1.3 Immunization Protocol and Antiserum Preparation Lactoferrin antiserum preparation followed the method described by You et al. During week 1, Freund' s complete adjuvant was thoroughly emulsified with lactoferrin solution at a 1:1 ratio, and each rabbit received 1 mL of the emulsion (containing 500 g lactoferrin) via multi-point subcutaneous injection. Four weeks later, booster immunization was administered using Freund' s incomplete adjuvant emulsified with lactoferrin (containing 250 g lactoferrin) via the same multi-point subcutaneous injection method on the back. This booster immunization was repeated every 2 weeks thereafter. Ear vein blood was collected 7 days after each immunization to monitor antiserum titer by ELISA. Cardiac blood collection was performed 7 days after the fourth immunization. After blood coagulation, serum was separated by centrifugation at 940×g for 20 minutes, aliquoted, and stored at -80°C until use.

1.4 Antiserum Purification by Saturated Ammonium Sulfate Saturated ammonium sulfate purification was performed according to the method described by Pang et al. Briefly, 500 L of serum was mixed with an equal volume of phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4). Saturated ammonium sulfate solution (250 L) was added dropwise on ice with vortex mixing, followed by incubation at 4°C for 30 minutes. The mixture was centrifuged at 1,550×g for 15 minutes at 4°C, and the supernatant was collected. An additional 1,000 L of saturated ammonium sulfate solution was added dropwise to the supernatant, vortex mixed, and incubated at 4°C for 30 minutes. After centrifugation, the supernatant was discarded, and the precipitate was dissolved in 500 L PBS. This process was repeated by adding 250 L saturated ammonium sulfate solution dropwise with stirring, incubating at 4°C for 30 minutes, and centrifuging to retain the precipitate. The final precipitate was dissolved in PBS, transferred to a dialysis bag, and dialyzed overnight at 4°C.

1.5 Antiserum Purification by Protein A Resin Following the method of Pang et al., protein A resin chromatography was employed for further purification. One milliliter of binding/washing buffer was added to the purification column, followed by 1 mL of resuspended protein A resin. The column was equilibrated with 5 mL of binding/washing buffer at a flow rate of approximately 1 mL/min. Serum samples diluted with binding/washing buffer were loaded onto the column at a flow rate of about 1 mL/min. The column was washed with 30 mL of binding/washing buffer at approximately 2 mL/min, and antibodies were eluted with 15 mL of elution buffer at about 1 mL/min. Eluted antibody fractions were collected in a beaker containing neutralization buffer to adjust pH to 7.4. The eluted antibody solution was transferred to a dialysis bag and dialyzed overnight at 4°C.

1.6 SDS-PAGE Analysis of Purified Antibody Purity A 12% separating gel and 5% stacking gel were prepared. Eighty microliters of antibody sample were mixed with 20 L of 5× loading buffer and boiled for 5 minutes. Twelve

microliters per well were loaded for electrophoresis (PowerPac 3000, Bio-Rad, USA) at 80 V initially. When the tracking dye entered the separating gel, voltage was increased to 120 V and electrophoresis continued until the dye reached approximately 1 cm from the bottom of the gel. The gel was fixed, stained with Coomassie brilliant blue, and destained until bands became clearly visible. Images were captured using a gel imaging system.

1.7 ELISA Determination of Purified Antibody Titer Antibody titer was determined by ELISA following the method described by Hei. Lactoferrin standard was diluted to 0.31 g/mL in coating buffer, and 100 L was added to each well of a microtiter plate, which was incubated overnight at 4°C. PBS served as negative control. After discarding the liquid, 200 L of blocking solution [1% ovalbumin (OVA)] was added to each well and incubated at 37°C for 1 hour. Wells were washed four times with phosphate-buffered saline containing Tween (PBST), with 3-minute incubations between washes, and then blotted dry. Purified antibodies at various dilutions (1:2,000, 1:4,000, 1:8,000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000) were added in triplicate and incubated at 37°C for 1 hour. After washing, 100 L of goat anti-rabbit IgG-HRP antibody (diluted 1:5,000) was added to each well and incubated at 37°C for 1 hour, followed by PBST washing. One hundred microliters of freshly prepared tetramethylbenzidine (TMB) substrate solution was added to each well and incubated at room temperature for 20 minutes. The reaction was stopped by adding 50 L of stop solution (2 mol/L sulfuric acid), and absorbance at 450 nm (OD450) was measured after 5 minutes using a microplate reader. Samples with OD450 values 2.1 times that of the negative control were considered positive, from which antibody titer was determined.

1.8 Lactoferrin Standard Curve for Quantification A competitive ELISA was employed to determine the inhibition rate between antibody and lactoferrin. Antibodies were appropriately diluted and mixed with an equal volume of serially diluted lactoferrin standards (40,000, 20,000, 10,000, 5,000, 2,500, 1,250, 625, and 312.5 ng/mL). After 15 minutes of reaction at room temperature, the mixture was added to pre-coated microtiter plates. Subsequent steps followed the procedure described in section 1.7. A standard curve was generated based on OD450 values to calculate the inhibition rate between antibody and lactoferrin.

1.9 Determination of Lactoferrin Concentration in Mammary Tissue and Dairy Products Mammary tissue samples collected from slaughterhouses were immediately preserved in liquid nitrogen. For analysis, frozen tissue samples were ground under liquid nitrogen, weighed, and thoroughly dissolved in 5 volumes of PBS containing radioimmunoprecipitation assay (RIPA) lysis buffer and protease inhibitors. After centrifugation at 12,000×g for 10 minutes, the supernatant was collected and total protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. For liquid milk,

1 mL samples were diluted with distilled water and centrifuged at 3,000×g for 10 minutes to remove the upper fat layer. Casein was precipitated by adjusting pH to 4.6 with 1 mol/L HCl, yielding a crude lactoferrin preparation. For milk powder, 3 g samples were reconstituted to 100 mL, and fat and casein were removed by centrifugation to obtain crude lactoferrin. Lactoferrin concentrations in mammary tissue, liquid milk, and milk powder were determined using the established standard curve.

1.10 Western Blot Analysis of Purified Antibody Specificity and Reactivity Lactoferrin standard was dissolved in PBS. Mammary tissue, liquid milk, and milk powder samples were prepared as described in section 1.9. SDS-PAGE was performed using 12% separating gel and 5% stacking gel based on total protein concentration. After electrophoresis, proteins were transferred to methanol-activated polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 0.5% chicken serum and incubated with lactoferrin polyclonal antibody solution (diluted 1:6,000) for 2 hours at room temperature. After PBST washing, goat anti-rabbit IgG-HRP antibody (diluted 1:5,000) was added. Following PBST washing, bands were visualized using diaminobenzidine (DAB) substrate and scanned.

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2. Results

2.1 Purity of Lactoferrin Polyclonal Antibody SDS-PAGE results [Figure 1: see original paper] revealed a clearly visible heavy chain band for the lactoferrin polyclonal antibody after two-step purification with saturated ammonium sulfate and protein A resin, indicating successful preparation of high-purity lactoferrin antibody.

2.2 Titer of Lactoferrin Polyclonal Antibody The concentration of lactoferrin polyclonal antibody was determined to be 11.02 mg/mL using the BCA protein assay kit. The antibody titer curve [Figure 2: see original paper] demonstrated that after purification, ELISA detection yielded an antibody titer of 1:128,000.

2.3 Inhibition Rate Between Antibody and Lactoferrin The inhibition rate between antibody and lactoferrin is shown in [Figure 3: see original paper]. The lactoferrin concentration causing 50% inhibition of antigen-antibody binding (IC50) was 24.55 g/mL. For detection limit determination, lactoferrin was serially diluted in 10 two-fold steps from 1,000 g/mL to 1.95 g/mL, with six replicates per concentration. The linear range was 11–40 g/mL, and the detection limit was 11.48 g/mL.

2.4 Lactoferrin Concentration in Mammary Tissue and Dairy Products Using the established standard curve, lactoferrin concentrations were

measured in mammary tissue and dairy products. Five mammary tissue samples showed lactoferrin concentrations ranging from 14.72 to 18.64 g/g, with a mean of 16.13 g/g and relative standard deviation (RSD) of 11.05%. Lactoferrin concentration in liquid milk was approximately 0 g/g, while milk powder samples ranged from 4.91 to 5.73 g/g, with a mean of 5.28 g/g and RSD of 6.42%.

2.5 Specificity of Lactoferrin Antibody Western blot results [Figure 4: see original paper] demonstrated that the purified antibody bound specifically to lactoferrin standard, confirming its specific recognition capability. [Figure 5: see original paper] further showed that the purified antibody reacted with lactoferrin extracted from dairy cow mammary tissue and exhibited specific reactivity with lactoferrin in milk powder, enabling detection in both mammary tissue and dairy products. Band intensity roughly reflected lactoferrin concentration differences among samples, indicating higher lactoferrin concentration in milk powder compared to mammary tissue and liquid milk samples.

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3. Discussion

Milk contains various bioactive substances, including components with antitoxic, antibacterial, antiviral, and immune-stimulating properties. Lactoferrin is a multifunctional protein that not only promotes iron absorption but also exhibits antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant, and immunomodulatory activities. Habing et al. reported that lactoferrin could reduce mortality in pre-weaned calves with diarrhea. Current detection methods for lactoferrin primarily include high-performance liquid chromatography (HPLC), radial immunodiffusion, and ELISA.

HPLC separates proteins based on molecular weight differences, offering accurate, reliable results with high precision and reproducibility. However, it is only suitable for high-purity samples. When lactoferrin concentration is low, its similar molecular weight to other proteins results in comparable retention times on gel chromatography, making component separation difficult and affecting quantification accuracy. Additionally, HPLC requires expensive instrumentation. Gong et al. employed radial immunodiffusion to detect lactoferrin in infant formula based on antigen-antibody precipitation, though this method suffers from relatively poor accuracy. In contrast, ELISA is relatively simple to operate with good accuracy and high sensitivity for low-concentration lactoferrin in milk and dairy products, featuring low detection limits and minimal sample purity requirements, making it suitable for lactoferrin detection. Therefore, preparing lactoferrin antibodies represents the preferred approach for detecting lactoferrin in milk and mammary tissue. Compared with regular antiserum, specific antibody preparation requires more complex purification procedures but yields higher antibody titers. Previous studies have successfully prepared antisera against various milk bioactive proteins, including rabbit anti-bovine -casein, s-

casein, -casein, and lactoferrin. However, early work by Shen et al. achieved only a 1:10,000 titer for anti-lactoferrin serum from immunized rabbits, while Li obtained titers of only 1:48,000 from rabbits and high-yield Roman hens, both substantially lower than the titer achieved in this study.

Previous antibody purification studies typically employed single methods such as salting-out, ion-exchange chromatography, or affinity chromatography. Salting-out separates proteins based on precipitation in high-salt solutions, ion-exchange chromatography utilizes selective binding to ion-exchange resins, while affinity chromatography exploits specific binding between proteins and ligands on the matrix. Due to different principles, these methods yield varying purity levels: saturated ammonium sulfate precipitation typically achieves approximately 75% purity, whereas affinity chromatography can exceed 90% purity. Building upon previous methods, this study purified antibodies through sequential saturated ammonium sulfate and protein A resin chromatography, resulting in clear, distinct antibody bands with good purity and a final concentration of 11.02 mg/mL.

Antibody titer, expressed as the dilution factor, represents the highest dilution at which an antibody can still bind antigen and produce a visible reaction, or the maximum dilution where the absorbance ratio between diluted antibody and negative control exceeds 2.0. The polyclonal antiserum in this study was obtained from four New Zealand white rabbits immunized with lactoferrin. Positive reactions were still observed at a maximum serum dilution of 128,000, confirming a final antibody titer of 1:128,000. Western blot analysis confirmed that the prepared polyclonal antibodies could specifically bind lactoferrin standard and detect lactoferrin in mammary tissue and milk powder. The varying lactoferrin concentrations detected across different sample types reflect genuine differences in lactoferrin content. Research indicates that lactoferrin is secreted by exocrine glands and primarily exists in milk, while also being present in tears, saliva, plasma, bile, pancreatic juice, and neutrophils, with highest concentrations in colostrum, followed by mature milk. The relatively high lactoferrin concentration detected in milk powder suggests possible exogenous supplementation, as lactoferrin has been approved as an additive in infant formula and foods due to its beneficial biological functions and absence of toxic side effects. The low lactoferrin concentration in liquid milk may be attributed to processing and storage conditions. These results demonstrate that the prepared antibodies can effectively reflect lactoferrin concentration differences among samples.

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4. Conclusion

This study successfully prepared high-purity, high-specificity rabbit anti-bovine lactoferrin polyclonal antibodies through immunization of New Zealand white rabbits. The antibody concentration reached 11.02 mg/mL with a titer of 1:128,000. These polyclonal antibodies specifically bind lactoferrin and can

be applied for qualitative identification and quantitative detection of lactoferrin in milk and mammary tissue, establishing a foundation for future research on lactoferrin synthesis regulation in dairy cows.

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