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Immunodiagnostic analysis of lacritin in human breast milk

Veronica Christine Vassilev
James Madison University

Immunodiagnostic Analysis of Lacritin in Human Breast Milk

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by Veronica Christine Vassilev

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Accepted by the faculty of the Departments of Integrated Science and Technology & Biology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

FACULTY COMMITTEE:

Project Advisor: Robert L. McKown, Ph.D.,
Professor, ISAT

Reader: Kyle Seifert, Ph.D.,
Associate Professor, Biology

Reader: Ron Raab, Ph.D.,
Professor, ISAT

HONORS PROGRAM APPROVAL:

Barry Falk, Ph.D.,
Director, Honors Program

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Abstract

Purpose: Human breast milk and tears share a number of common proteins including lysozyme, lactoferrin, albumin, secretory immunoglobulins, and mucins. Human breast milk has been used as self-medication for conjunctivitis and has been recently reported to improve corneal healing in an animal model system. Lacritin is prosecretory, mitogenic and when cleaved, antimicrobial. Topical application promotes sustained basal tearing in rabbits. This study aims to determine if lacritin can be detected in human breast milk.

Methods: Milk fat from human breast milk was removed by centrifugation and samples were subjected to DEAE and Hydrophobic Interaction Chromatography (HIC) to separate proteins in the sample. The samples were analyzed by SDS-PAGE and Western blot using polyclonal anti-lacritin antibodies. Blots included recombinant lacritin, human tear samples, and human milk samples. In addition, a colony forming unit (CFU) antimicrobial assay was performed against *Escherichia coli*.

Results: Protein bands between 18-25 kDa on the Western blots for milk, tears, and recombinant lacritin were detected using N-terminal antibodies. C-terminal specific lacritin antibodies produced distinct primary bands between 18-20 kDa for tears and recombinant lacritin. A band at 75 kDa on the blot was detected for milk corresponding to a previously reported cross-linked lacritin complex. DEAE purification was unsuccessful, and HIC partially purified lacritin. Milk diluted 0.25X with PBS had 37% antimicrobial activity in the Colony Forming Unit (CFU) assay.

Conclusions: Anti-lacritin antibodies that detect recombinant lacritin and human tear lacritin also detect a protein in human milk after HIC purification with similar electrophoretic mobility suggesting that lacritin or lacritin-like proteins are expressed in human breast milk. These proteins are present with antimicrobial properties.

Introduction

Lacritin Discovery and Properties

Lacritin is a 12.3 kDa, 119 amino acid human tear glycoprotein that has prosecretory, mitogenic, and antimicrobial activity (McKown, *et. al.,* 2009). The gene for lacritin has five exons and four introns and is located in region 13 of chromosome 12 (Ma, *et. al.,* 2008). Lacritin is secreted by the lacrimal gland in both human and non-human primates and contains two alpha helices confirmed by circular dichroism in the C-terminal region (Ma, *et. al.,* 2008). Lacritin can undergo alternative splicing to produce different splice variant proteins. Two alternate splice variants were isolated from lacritin glands and are named lacritin-b and lacritin-c (Figure 1) (Ma, *et. al.,* 2008).

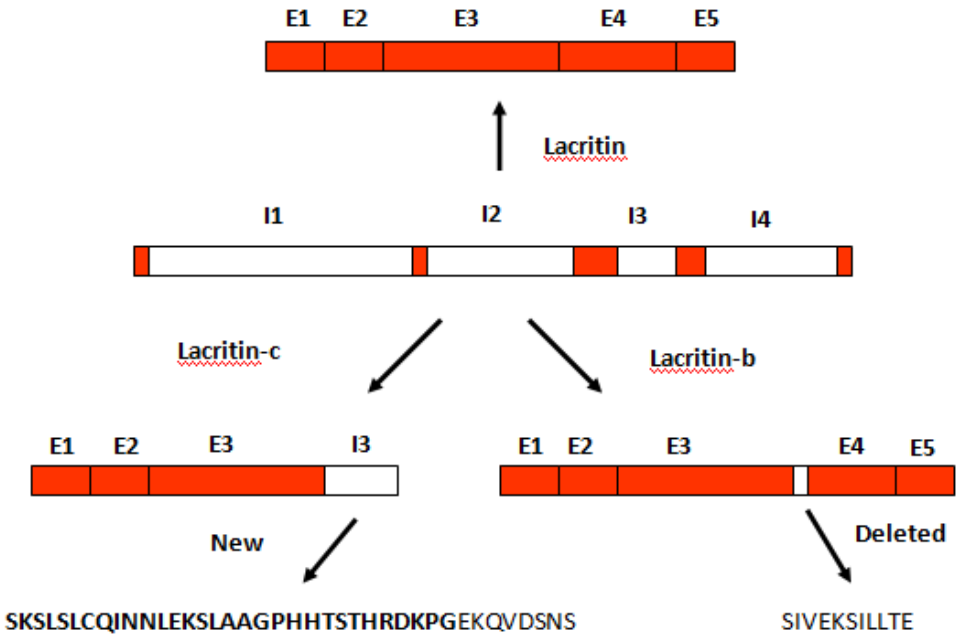

Figure 1. **Genomic lacritin and two splice variants.** The 138 amino acids of lacritin are contained within 5 exons separated by 4 introns. I1-I4 indicate the introns. E1-E5 indicate the exons. Alternative splicing results in two splice variants, lacritin-c and lacritin-b. Lacritin-c contains exons E1-E3 and its C-terminus is made up of 39 amino acids from intron 3. Lacritin-b lacks amino acids from exon 4.

Dr. Gordon Laurie first discovered and characterized lacritin in 2001 (Sanghi, *et. al.,* 2001) at the University of Virginia. The lacrimal gland above the eye generates lacritin, where it is released by acinar cell secretory granules and transported through ducts to the ocular surface (Ma, *et. al.,* 2008) (Figure 2).

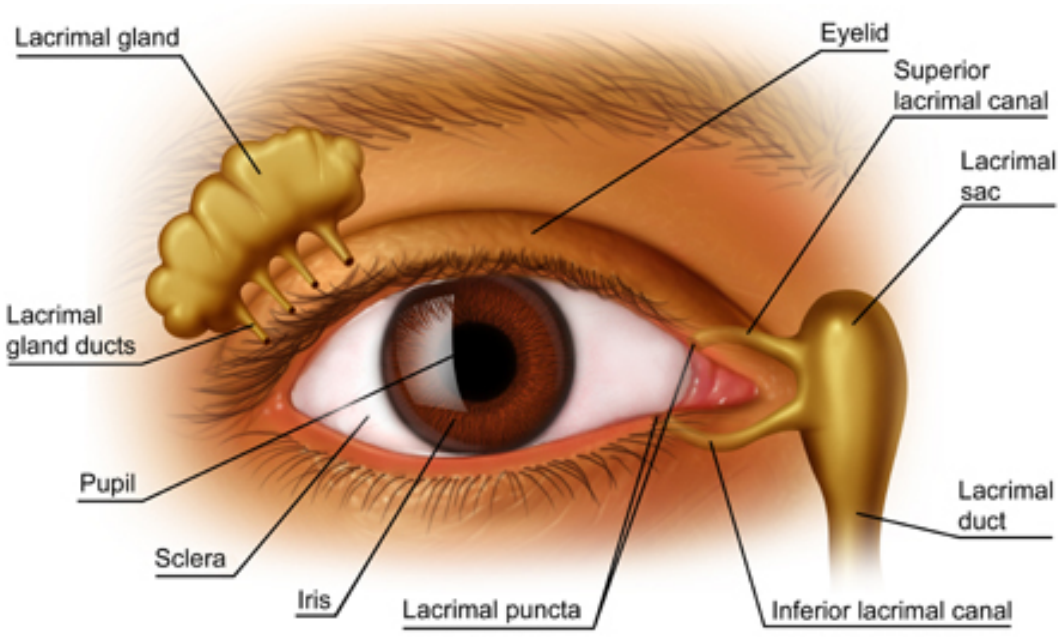

Figure 2. Diagram of the eye and associated structures including the lacrimal gland, lacrimal sac, and lacrimal duct.

Lacritin is expected to also be a product of the meibomian gland at the rim of the eyelids (Tsai, *et. al.,* 2006). It was found that lacritin was one of the eight ocular proteins down regulated in patients with chronic blepharitis, an inflammation of the eyelid associated with dry eye (Tsai, *et. al.,* 2006). Lacritin has also been shown to stimulate basal tearing in healthy rabbits upon topical application and the increased basal tearing lasted for a week after chronic application ended (Samudre, *et. al.,* 2011) making lacritin a good candidate for a new therapeutic to treat dry eye, which affects 5% of the world's population (Wang, *et. al.,* 2013). Lacritin purified from monkey

tears has also been shown to promote secretion of tear proteins from cultured monkey lacrimal acinar cells (Fujii, *et. al.,* 2013). Lacritin is predicted to play an important role in secretion as well as renewal of lacrimal and ocular surface epithelia (Tsai, *et. al.,* 2006). It was found that lacritin concentrations in tear samples from healthy males and females and individuals 18-52 years of age is approximately 4% of the total tear proteins (Seifert, *et. al.,* 2012).

The C-terminal domain of lacritin has been shown to have antimicrobial activity against bacterial species of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermis* using a colony forming unit (CFU) assay (McKown, *et. al.,* 2009). Lacritin also has potential to aid in ocular wound healing due to its mitogenic and anti-inflammatory activities.

Currently, recombinant lacritin is expressed in *E. coli*, and purified at James Madison University. Multiple tools have been developed in order to detect lacritin. The McKown lab also utilizes an enzyme-linked immunosorbent assay (ELISA) that is specific to lacritin, in order to quantitate the amount of lacritin in different tear samples using anti-lacritin antibodies produced in rabbits (Figure 3). These antibodies are designated for lacritin's N-terminus, C-terminus, and the lacritin splice variant Lac-C (Figure 4). The sandwich ELISA in Figure 3 is being developed, while the indirect ELISA has been used to determine lacritin levels in tear samples.

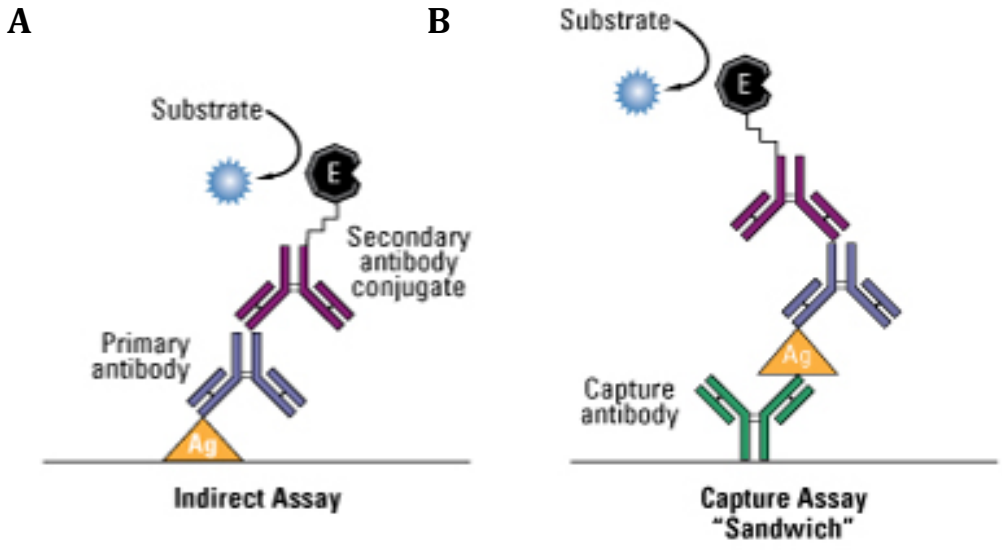

Figure 3. Indirect and Sandwich Enzyme-Linked Immunosobent Assay (ELISA) diagrams. (A) Diagram of an indirect ELISA and its components: antigen (Ag), primary antibody, secondary antibody conjugated to an enzyme (E), and substrate. (B) Diagram of a capture or "sandwich" ELISA and its components: capture antibody, antigen (Ag), primary antibody (in light purple), secondary antibody (in dark purple) conjugated to an enzyme (E), and substrate.

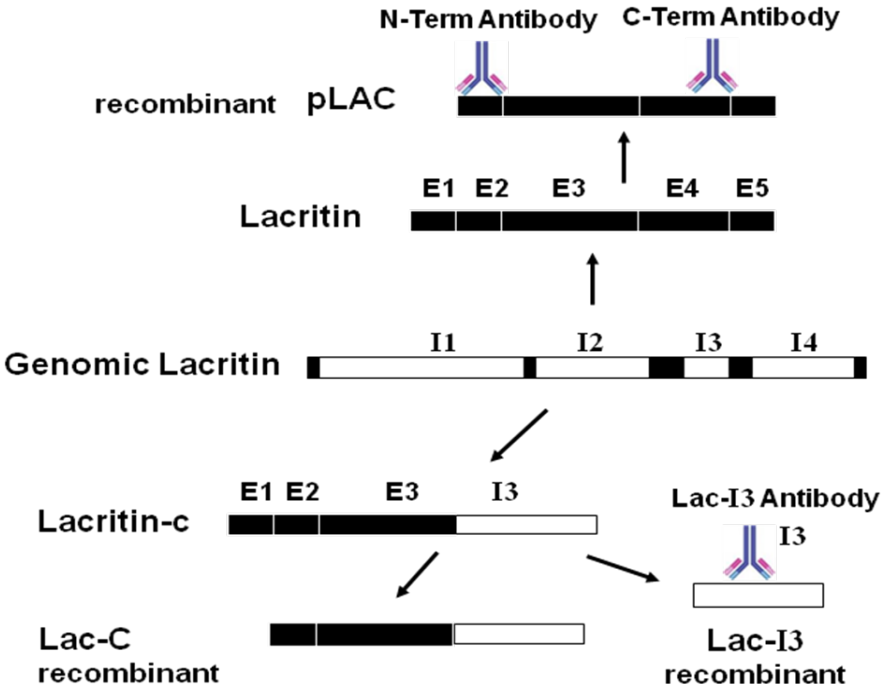

Figure 4. Diagram of lacritin binding sites for antibodies Lac-I3, C-terminal, and N-terminal. I1-I4 indicate the introns in genomic lacritin and E1-E5 indicate the exons in mature lacritin. Exon 1 codes for the signal peptide and is cleaved during secretion. Recombinant lacritin designated as pLAC codes for the 119 amino acids within E2-E5. The splice variant lacritin-c encodes sequences for 39 new amino acids derived from intron 3 fused to exons 2 and 3.

Breast Milk

Human breast milk consists of many proteins secreted from the mammary gland including lactoferrin, lysozyme, albumin, immunoglobulins, caseins, and muscins; proteins that are also found in human tears (McKown, *et. al.,* 2012). Human breast milk is advantageous to infants in protecting them from different infectious diseases (Hennart, *et. al.,* 1991), enhancing immune function, and in development of the gut (Lonnerdal, 2003). Antimicrobial activity in human breast milk has been correlated with lysozyme and lactoferrin. Human breast milk has been used for self-medication against conjunctivitis, also known as pink eye, the inflammation of the membrane lining the eyelids (Baynham, *et. al.,* 2013). The purpose of this study was to determine if antibodies created to detect lacritin in human tear samples can also detect lacritin in human breast milk and if so can the breast milk lacritin be purified or enriched from breast milk and analyzed for antimicrobial activity.

Materials and Methods

Processing

Breast milk samples were collected from volunteers, frozen, shipped to JMU, and stored at -60 ° C. For immunoanalysis and chromatography of breast milk, samples were thawed and centrifuged at 8,000 rpm for 10 minutes. Using a 10 cc syringe, the bottom layer of fluid was removed leaving the top fatty layer. The supernatant cleared of milk fats was designated Fraction 1 or F1.

DEAE & Hydrophobic Interaction Chromatography (HIC)

In an attempt to purify lacritin in human breast milk, DEAE chromatography was utilized. The F1 fraction cleared of milk fats was dialyzed against 14 mM NaCl Phosphate Buffer (PB) at pH 7.4. A 5 mL bed volume of DEAE sepharose beads was equilibrated with 10 column volumes of 14mM NaCl PB, pH 7.4, and 10 mL of the dialyzed F1 sample was loaded onto the column. The unbound material was collected in 1 mL fractions followed by a 10 column volume wash at 14 mM NaCl PB, pH 7.4. Fractions were eluted with three differing concentrations from DEAE sepharose with 70, 140, and 280 mM NaCl PB, pH 7.4.

In a further attempt to purify lacritin in human breast milk, Hydrophobic Interaction Chromatography (HIC) was utilized. The F1 milk sample was dialyzed against 280 mM NaCl PB, pH 7.4 and loaded onto a 5 mL bed volume HIC column equilibrated with 280 mM NaCl PB, pH 7.4. Following a wash with the same buffer, the column was eluted with 140 mM NaCl PB, pH 7.4 and 1 mL fractions were collected.

SDS-PAGE and Western Blot Analysis

Fraction 1 milk samples were loaded onto multiple any kD™ Mini-PROTEAN® TGX™ Precast Gels (BioRad) and electrophoresed at 200 volts in order to separate milk proteins. One gel was stained using Coomassie Blue, de-stained, and imaged using the Gel Doc™ XR+ System (Bio-Rad Laboratories; Hercules, CA).

Another gel was transferred to a nitrocellulose membrane at 34 V overnight and used for Western blotting. The membrane was washed 4 times for 15 minutes each in Phosphate Buffered Saline with 0.3% tween 20 (PBS-Tween 20), and incubated with a 1:1,000 dilution of antibody in PBS-Tween 20 for 1 hr. The membrane was then washed 4 times for 15 minutes each in PBS-Tween 20, and incubated with a 1:5,000 dilution of HRP-conjugated goat affinity purified antibody to rabbit IgG (MP Biomedicals; Solon, OH) in PBS-Tween 20 for 1 hr. Following incubation, the membrane was washed twice for 15 minutes each in PBS-Tween 20 and then twice more in PBS for 15 minutes each. The membrane developed by chemiluminescent development and exposure to x-ray film.

Colony Forming Unit (CFU) Antimicrobial Assay

Approximately 50 mL LB broth was inoculated with 0.5 mL of an overnight *E. coli* culture. The culture was grown at 37°C with shaking, until an optimal OD_{600} of ~0.5-0.6 was obtained. Approximately 1 mL was removed, and centrifuged at 13K rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in 1 mL PB 10 mM NaCl. The cells were washed two more times and the pellet resuspended in 1 mL PB 10 mM NaCl. Two 10-fold dilutions were made with PB 10 mM NaCl and used in the CFU assay. Fraction 1 milk samples were diluted with PBS, pH 7.4, for concentrations ranging from 0.125-1X. CFU reaction

mixtures were prepared according to Table 1, incubated at 37°C for 1.5 hr, and 100 µL was spread onto LB agar plates in triplicate. The agar plates were incubated overnight at 37°C and individual colonies counted. Percent antimicrobial activity was calculated as the average number of colonies in the milk samples divided by the average number of colonies on the PBS reactions. A lacritin truncation mutant with 65 amino acids removed from the N-terminus previously determined to have antimicrobial activity was used as a positive control.

Table 1. Mixtures made in order to perform the colony forming unit (CFU) assay.

	PBS	**N-65**	**Milk Samples**
10mM Sodium PB	350 µL	350 µL	350 µL
PBS (filtered)	100 µL	----	----
***E. coli* (C-2)**	50 µL	50 µL	50 µL
N-65	----	100 µL	----
Milk (1X-0.125X)	----	----	100 µL
TOTAL:	500 µL	500 µL	500 µL

.

Results

SDS-PAGE of Breast Milk Samples

Breast milk samples from two volunteers were processed to remove the fats (Fraction 1), loaded on an SDS gel, separated by electrophoresis, and visualized by coomassie staining (Figure 6). The most abundant milk proteins were identified in this gel. The predominant bands were determined to be lactoferrin (LF), casein (CN), and lactalbumin (LA) based on similarities in molecular weights from published data (Figure 5) (Iver, 1995).

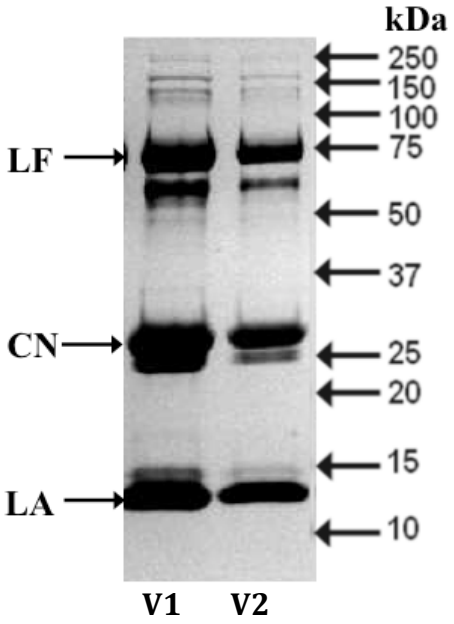

Figure 5. SDS PAGE of Human Breast Milk Samples. Human milk samples from volunteer 1 (V1) and volunteer 2 (V2) were electrophoresed on 4-20% gels and coomassie stained. The predominant bands in human milk have been identified as lactoferrin (LF), casein (CN), and lactalbumin (LA) as described in previously published gel profiles (Iver, 1995).

Western Blot of Breast Milk Samples

Breast milk samples were analyzed using N-terminal antibodies that bind to lacritin present in tears. N-terminal antibodies bound to recombinant lacritin (pLAC) at approximately

18 kDa. The same antibodies produced two predominant bands of 25 kDa and 75 kDa in both fraction 1 milk samples (Figure 6).

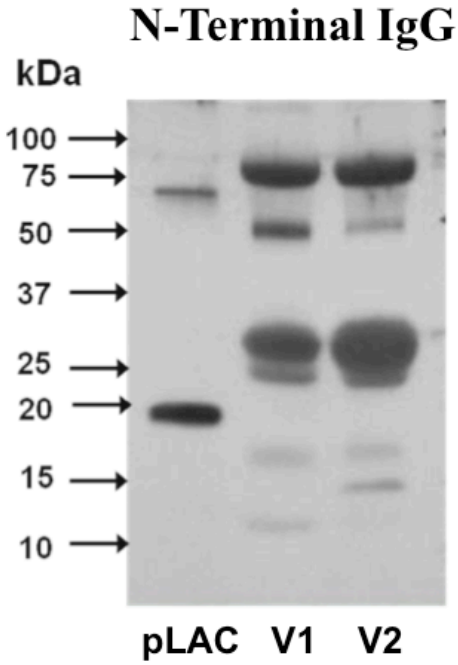

Figure 6. Western Blot Analysis of Human Milk Samples. pLAC and human milk samples from volunteer 1 (V1) and volunteer 2 (V2) were electrophoresed and transferred to nitrocellulose. Blots were incubated with anti-Lac N-term and HRP-conjugated goat anti-rabbit IgG and developed with chemilumenscence.

DEAE Chromatography

An attempt was made to purify lacritin from human breast milk by chromatography on DEAE sepharose. Following DEAE chromatography and elution, an elution profile was created to visualize the different protein peaks (Figure 7). Four different peaks were produced in the elution profile. The first peak was detected in fraction 3 at 3.4 mg/mL during the 14 mM NaCl wash, the second was in fraction 14 at 1.6 mg/mL during the 70 mM NaCl elution, the third was in fraction 24 at 3.75 mg/mL during the 140 mM elution, and the fourth was in fraction 34 at 8.41 mg/mL during the 280 mM NaCl elution (Figure 7).

Two SDS-PAGE gels were run in order to visualize relative protein sizes present in different selected fractions. There is a predominant band present in the first gel around 10-15 kDa and a doublet at 20-25 kDa in fractions 2-6 (Figure 8A). There is more protein detected in the second gel containing fractions 21-24 with similar bands visualized in the first gel except the doublet is not as distinguishable (Figure 8B).

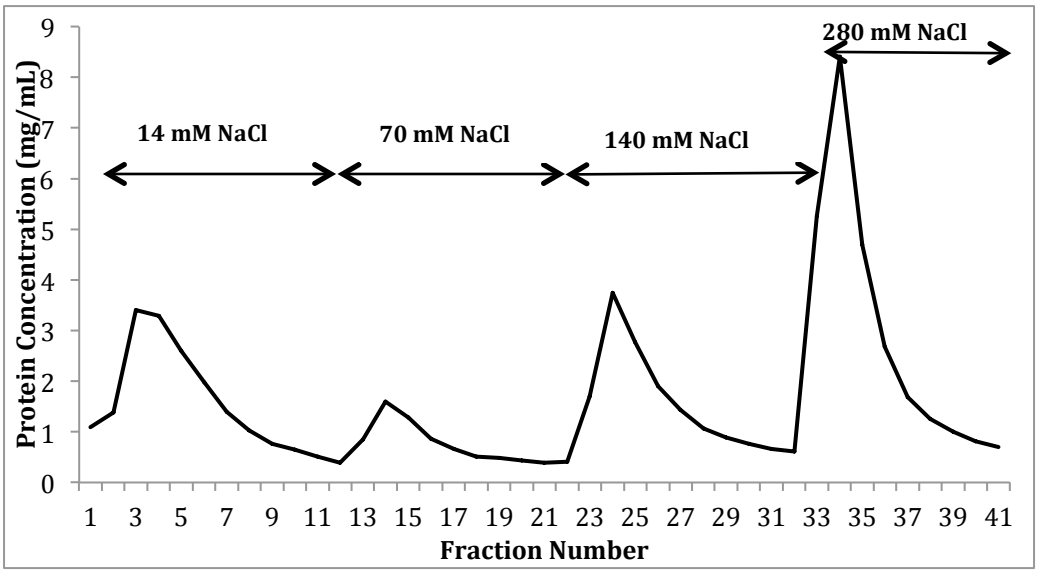

Figure 7. Elution profile of breast milk fractions following DEAE chromatography. Different salt concentrations of 14, 70, 140, and 280 mM NaCl were used on the column in order to elute different proteins. Protein concentrations were determined using ThermoScientific Nanodrop1000 Spectrophotometer.

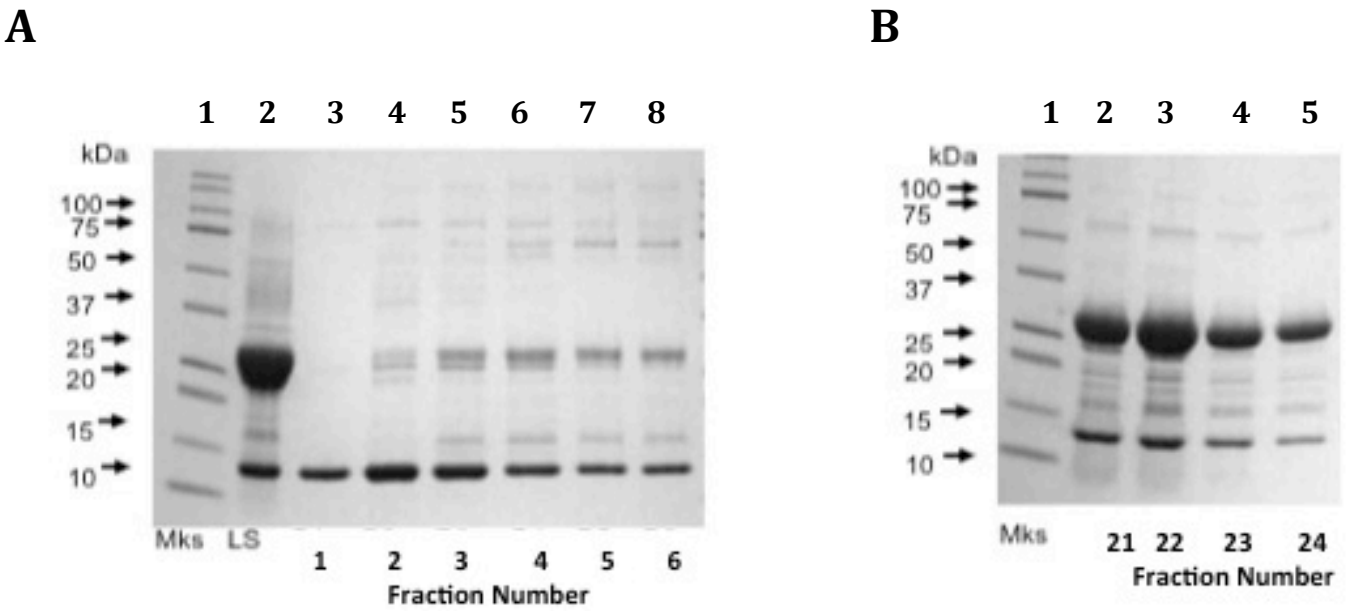

Figure 8. Two SDS-PAGE analyses of DEAE fractions. There is a molecular weight marker in lane 1 (mks). A. LS in lane 2 is the cleared milk sample loaded onto the column, lanes 3-8 are DEAE fractions 1-6. B. Lanes 2-5 are fractions 21-24 and lanes 6-10 are fractions 11-15.

A Western Blot was also performed on selected DEAE fractions using anti-lacritin antibodies, N-terminal, C-terminal, and Lac-I3. The cleared supernatant (F1) reveals two bands at 15 and 25 kDa, but fraction 2 (D2) only has one band at 25 kDa in all three blots (Figure 9).

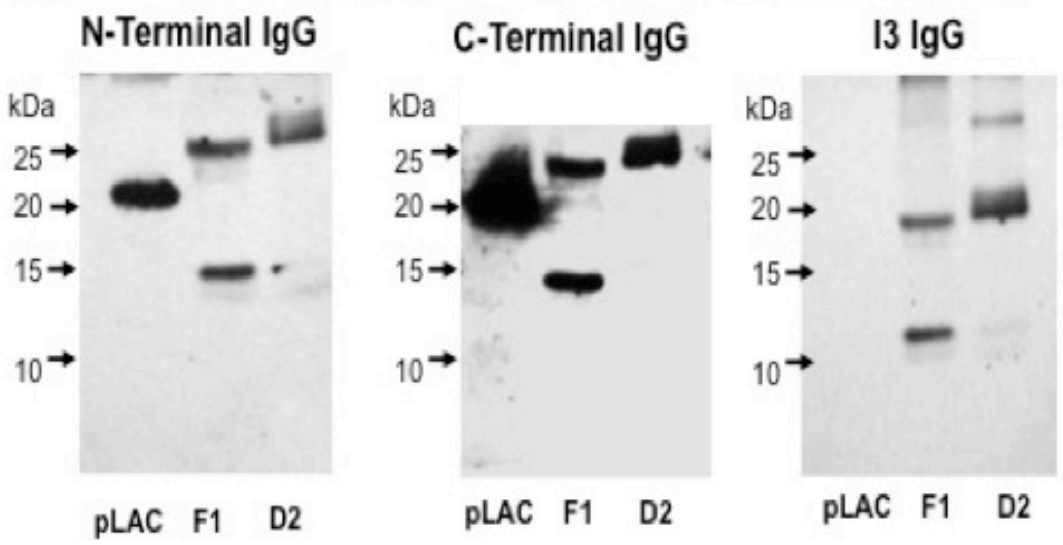

Figure 9. Western blot analysis of pLAC, Fraction 1 milk sample (F1), and DEAE fraction # 2 (D2) using C-terminal, N-terminal, and Lac-I3 antibodies**.**

Hydrophobic Interaction Chromatography (HIC)

HIC chromatography was used in order to further purify lacritin out of human breast milk, and an elution profile was produced to visualize the different protein elution peaks. Two different peaks were observed in the elution profile. The first elution peak was in fraction 8 at 4.88 mg/mL during the 280 mM NaCl wash, and the second peak was in fraction 26 at 0.37 mg/mL during the 140 mM NaCl elution (Figure 10).

An SDS-PAGE and Western blot analysis of human breast milk samples was performed on selected samples following hydrophobic interaction chromatography in order to visualize the relative protein sizes in the samples examined (Figure 11). Common bands are detected with flow through 5 (FT5) in lane 3, and fractions 25-27 in lanes 4-6 at 25-35 kDa (Panel A). The cleared supernatant sample loaded onto the HIC column (loading sample) in lane 2 has similar bands visualized along with the addition of bands at ~12 and ~80 kDa (Panel A).

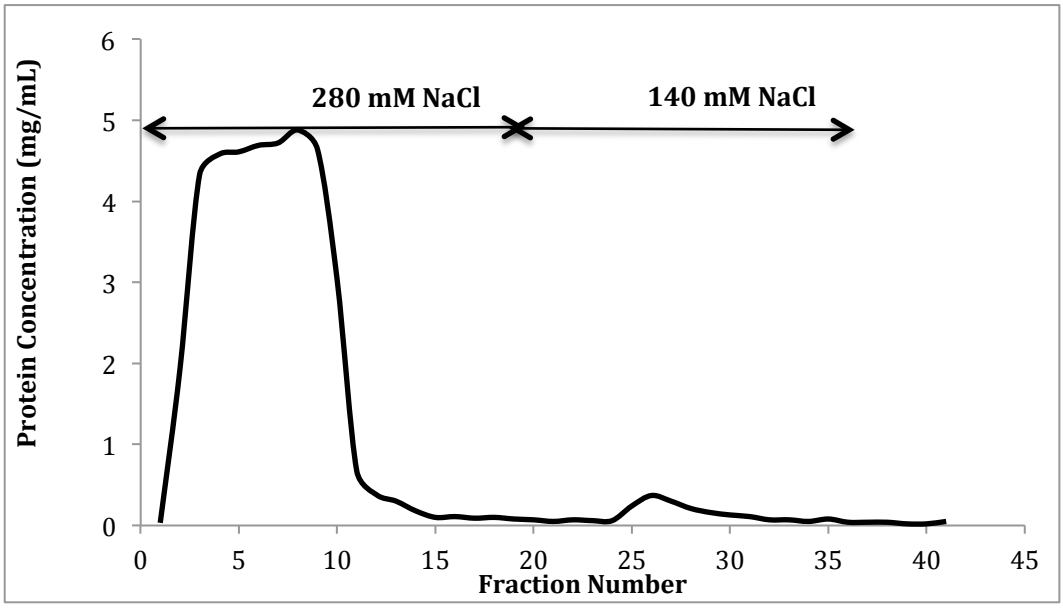

Figure 10. Elution profile of breast milk fractions following HIC chromatography. Different salt concentrations of 280 and 140 mM NaCl were used on the column in order to elute different proteins. Protein concentrations were determined using ThermoScientific Nanodrop1000 Spectrophotometer.

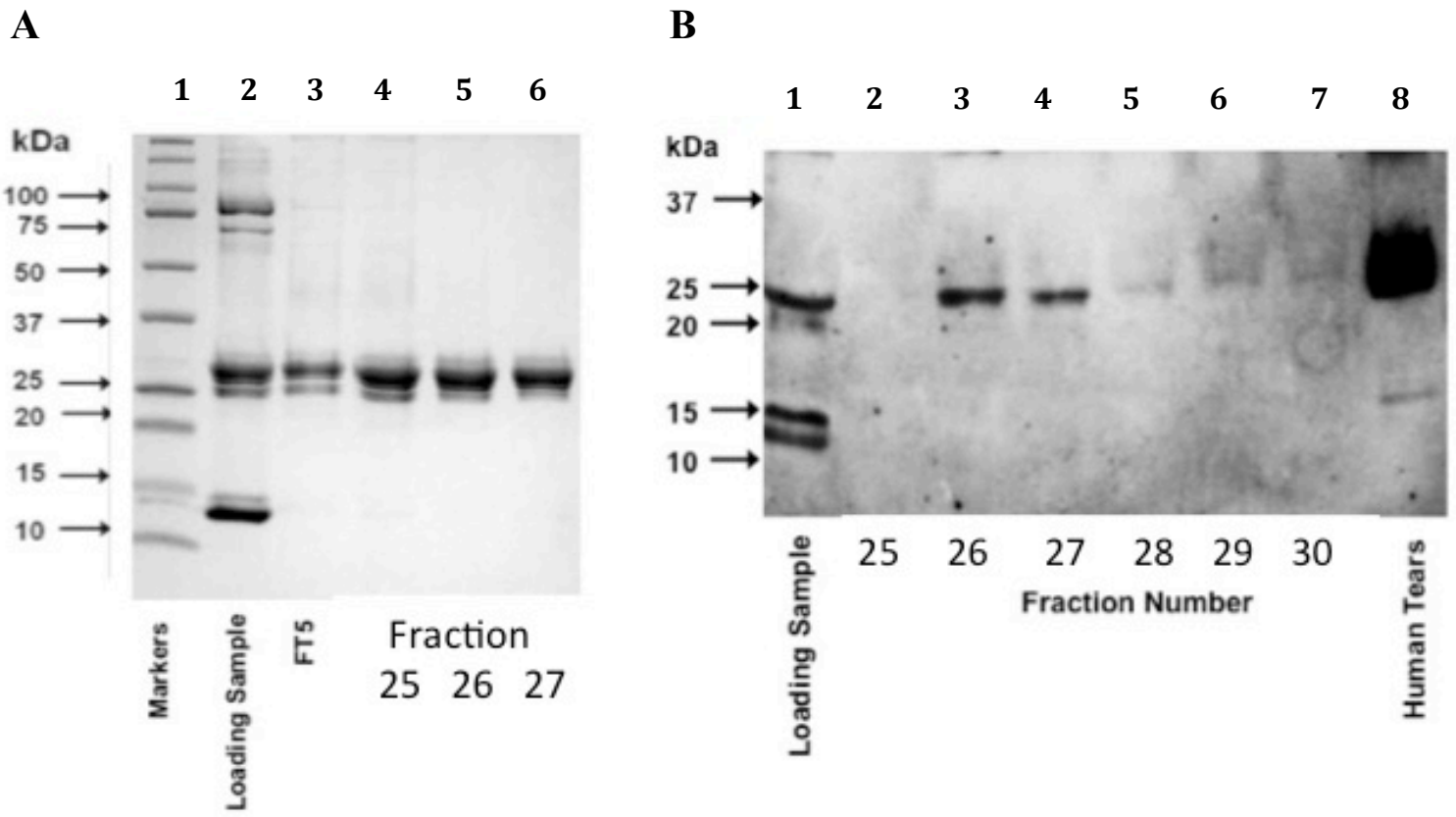

Figure 11. SDS gel and Western Blot following Hydrophobic Interaction Chromatography (HIC). A. SDS Gel following HIC. Lane 1: molecular weight markers, lane 2: cleared supernatant (loading sample), lane 3: flow through sample 5 (FT5), and lanes 4 - 6 fractions 25-27. B. Western blot using C-terminal antibody following HIC. The Western blot the loading sample in lane 1, fractions 25-30 in lanes 2-7, and human tears in lane 8.

A Western blot was also performed using the C-terminal anti-lacritin antibody on selected fractions from HIC. The cleared supernatant sample loaded onto the HIC column (loading sample) reveals bands at 10-15 kDa in addition to the 25 kDa band. Fractions 26 and 27 have one band at 25 kDa. This band is approximately the same size as the band in human tears in lane 8 (Figure 11B).

Colony Forming Units (CFU) Antimicrobial Assay

A CFU assay was performed in order to determine the antimicrobial activity of the breast milk samples against *Escherichia coli* (Figure 12). N-65, a lacritin deletion mutant lacking 65

amino acids from the N-terminus, was used as a positive control with the highest antimicrobial activity (99%). PBS was a negative control with 0% antimicrobial activity. All milk samples tested had antimicrobial activity ranging from 1-37%. Milk diluted 0.25X had the highest activity at 37% (Figure 12).

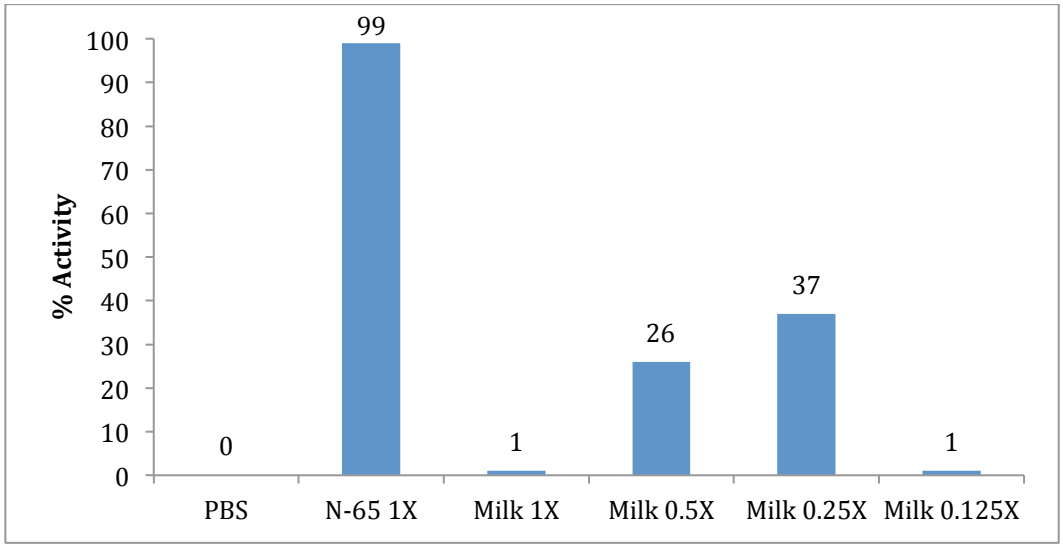

Figure 12. Colony forming units (CFU) antimicrobial assay results from human breast milk samples against *Escherichia coli*. Phosphate buffered saline (PBS) was used as a negative control. N-65 (an antimicrobial variant of lacritin) was used as a positive control. Milk 1X was a milk sample that was not diluted, milk 0.5X-0.125X are samples that were diluted respectively with PBS.

Discussion

Lacritin is a human tear protein with prosecretory, mitogenic, and antimicrobial activity (McKown, *et. al.,* 2009). Anatomically, it can be found in the lacrimal gland above the eye. Lacritin has been purified using DEAE chromatography previously (Samudre, *et. al.,* 2011), and its antimicrobial activity has been determined with colony forming unit (CFU) assays using *Escherichia coli* and other bacteria known to cause ocular disease (McKown, *et. al.,* 2009). Different variants of lacritin have been discovered, and polyclonal antibodies have been generated to detect them. These antibodies include C-terminal, N-terminal, and Lac-I3 antibodies. All of them have been used in Enzyme-Linked Immunosorbent Assays (ELISA) in order to quantify the amount of lacritin and its variants in human tears. This study aimed to determine if lacritin was present in human breast milk considering similarities between proteins found in human tears and breast milk.

Multiple proteins are secreted from the mammary gland during the production of breast milk including lactoferrin, casein, and lactalbumin (Iver, 1995). These proteins are also found in human tears (McKown, *et. al.,* 2012). In order to visualize the presence of these different proteins secreted by the mammary gland, an SDS-PAGE was performed on two samples of breast milk obtained from two volunteers. The predicted milk proteins lactoferrin, casein, and lactalbumin presence was putatively determined by their molecular weights (Figure 5) (Iver, 1995). Due to the abundance of these proteins in milk samples, these proteins were determined to be the predominant bands present on the SDS gel (Figure 5).

The unprocessed volunteer samples of human breast milk were analyzed via a Western blot in order to determine the presence of lacritin in the crude samples using N-terminal antibodies to lacritin (Figure 6). The bands around 25-30 kDa were glycosylated lacritin, which

has been reported to be around 25 kDa (Figure 6) (Velez, *et. al.,* 2013). The band around 75 kDa of the blot is presumed to be a lacritin complex, unactive forms of lacritin dimers or trimers, cross-linked by tissue transglutaminase (Velez, *et. al.,* 2013) (Figure 6).

In an attempt to purify lacritin from human breast milk, anion exchange chromatography was utilized consisting of DEAE covalently linked to sepharose beads. Anion exchange chromatography was used following previously successful purification strategies of lacritin with low salt binding and elution with Phosphate Buffer (PB) 140 mM NaCl (Samudre, *et. al.*, 2011). Three distinct protein elution peaks were obtained using various salt concentrations between 70 – 280 mM NaCl PB (Figure 7). SDS-PAGE was used to visualize the relative amount and molecular weight of proteins eluted from the DEAE column at the various elution peaks (Figures 7 and 8). The gel detects multiple proteins present in the samples, with no clear separation of lacritin (Figure 8).

A Western blot was performed to detect lacritin among selected DEAE elution fractions using lacritin specific N-term, C-term, and Lac-I3 antibodies to (Figure 9). Antibodies bound to fraction 1 milk samples of prepped breast milk, and two predominant bands were detected at 12 and 25 kDa in size (Figure 9). DEAE fraction 2 (D2) contained a band at 12 kDa but not a band present at 25 kDa, suggesting the 25 kDa protein did not bind to the column (Figure 9). There was no apparent lacritin purification without the 25 kDa band of lacritin binding to the DEAE column. These results were not expected following previous successful purification schemes of lacritin using DEAE chromatography (Samudre, *et. al.*, 2011).

A second attempt to purify lacritin from breast milk samples utilized Hydrophobic Interaction Chromatography (HIC). HIC chromatography exploits hydrophobic interactions

between amino acid side chains in proteins and hydrophobic sepharose beads (Rao, *et. al.,* 2006). Two protein elution peaks were noted in the elution profile; one during the wash and one eluted at 140 mM NaCl (Figure 10). SDS-PAGE was performed in order to determine the molecular weights of the proteins present in selected HIC fractions. Protein separation was visualized with a diminishing number of bands in comparison to the cleared supernatant loaded onto the column (loading sample), suggesting many proteins bound to the column (Figure 11A). A frequent doublet was observed in all samples analyzed using SDS-PAGE (loading sample, flow through, and PB 140 mM NaCl fractions 25-27) (Figure 11A). The doublet likely contains lacritin and another unknown protein since only one part of the doublet was visualized with Western blotting (Figure 11B). This unknown protein could be the abundant protein casein with a molecular weight of 25 kDa. The intensity of the casein sized band decreased between Figure 6 and Figure 11A, likely resulting from the alternative use of HIC to purify apart different isotopes of casein in milk (Rao, *et. al.,* 2006).

The presence of lacritin was explored using Western blotting technique with anti-lacritin C-terminal antibodies on selected HIC purified samples. HIC fractions 26 and 27 on the Western blot reveal the presence of lacritin in the sample with the observed band at 25 kDa (Figure 11B). The band present in those two fractions was consistent with the molecular weight of lacritin detected in human tear sample (Figure 11B), suggesting the band detected in human breast milk may be similar to the lacritin detected in human tears.

A Colony Forming Unit (CFU) antimicrobial assay was performed with *E. coli* and human breast milk similar to previous methods (McKown, *et. al.,* 2009). The CFU assay was performed using a positive control (N-65), a negative control (PBS), and milk supernatant diluted 0.125-1X. Compared to the positive control, the highest antimicrobial activity was 37%

with the 0.25X milk supernatant sample (Figure 12). The dilutions of milk in PBS were potentially diluting out any proteins present in the sample that were inhibiting the antimicrobial activity or binding lacritin, which resulted in an increased antimicrobial activity with each dilution. Once the milk sample was diluted too far, 0.125X, the antimicrobial properties diminish (Figure 12). Therefore, there was an optimal concentration of milk, 0.25X, which has the peak antimicrobial activity.

This works suggests that lacritin was found in human breast milk. This was determined using column chromatography purification, SDS-PAGE analysis, and Western blotting. The SDS-PAGE allowed for visualization of the various proteins present in breast milk, originally suggesting that lacritin was present along with the well-characterized, abundant proteins of human breast milk. The Western blotting procedure using anti-lacritin antibodies confirmed that lacritin was present in the HIC samples. The antimicrobial properties of human breast milk were determined with a CFU assay. The CFU assay strongly suggested that human breast milk does have antimicrobial activity. Although lacritin was not purified from breast milk using DEAE chromatography, HIC was used successfully to partially purify lacritin with the presence of a doublet visualized with SDS-PAGE analysis (Figure 11A).

Although this research suggested that lacritin was present in human breast milk, more analysis is necessary to support this preliminary data. A protein band from HIC could be sent out for mass spectrometry to confirm the presence of lacritin. Additional analysis could be performed to distinguish between the proteins that are present in the first Western blot (Figure 5) using a SDS gel that will separate the doublet present between 25-30 kDa. A gel with a more restricted molecular weight requirement would allow for the proteins that appear close on the gels used in this study to be more clearly separated. This would allow for accurate molecular

weights to assist in accurate protein identification. It would also be useful to use the HIC fractions 26 and 27, where lacritin was detected, in the CFU antimicrobial assay instead of fresh milk supernatant (F1). This would help distinguish if lacritin was the protein responsible for the antimicrobial properties. Ultimately, more analysis could be performed on other properties of breast milk like its anti-inflammatory characteristics similar to lacritin (Goldman, 1993).

There are biological and clinical implications of the findings in this study. The antimicrobial activities found in human breast milk could be used to enhance the immune support of a developing baby therefore suggesting the importance of breastfeeding (Oddy, 2002). It has been reported that breast milk has bioactive factors that activate the child's immune system (Oddy, 2002). Also, human breast milk could be used as a source to purify lacritin. Purified human lacritin could be used in clinical studies to further support that lacritin could be applied as a topical therapeutic for dry eye to stimulate basal tearing (Samudre, *et. al.*, 2011).

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