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Progress report to the Waikato Medical Research Foundation.
Grant # 211.

Histatherin, a potential new antimicrobial protein from milk.

June 2013

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Progress report on WMRF grant # 211; Histatherin, a potential new antimicrobial protein from milk.

Report prepared for the Waikato Medical Research Foundation

June 2013

Author/s **A Molenaar**, on behalf of Drs Molenaar, Harris and Wheeler, and **Chanelle Gavin** – Student.

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Summary for the WMRF committee.- A. Molenaar

The specific aims of the project were to;

1. Establish the true amount of histatherin present in milk and milk fractions by
    a. Removing carbohydrate epitopes from milk-derived histatherin that may mask the peptide antigen from antibody detection methods.
    b. Purify milk-derived histatherin and generate new antibodies that recognise native peptide and all its potential glyco-forms.
2. Test the native histatherin for antimicrobial activity against a range of bacteria in both antimicrobial assay buffer, and in milk.

From the outset the plan was to use the majority of the grant to support a student over several years if possible by way of a student stipend at the rate recommended by Waikato University. A highly recommended engineering student, Chanelle Gavin, was recruited and the project started in November 2012 as part of her summer project and training period. Chanelle has become proficient in the required techniques to date (western blotting and protein chromatography to name some). She has examined point 1a and determined that the glycosylation state of Histatherin does not appear to be the reason for the low detection as deglycosylation of milk fractions containing Histatherin did not improve the signal. Several attempts to enrich and purify histatherin have been attempted both at Ruakura and this work is continuing more conveniently for her at her university supervisors lab. Point 2 is dependent on point 1. Progress to date is detailed in the Engineering Work Placement below. Chanelle is now in a good position to advance the project as part of her Bachelor of Engineering project.

To date approximately $9,000 of the WMRF grant has been spent on the first year's stipend and a deglycosylation kit with the rest being held over for stipend payments in future years. Other costs have been met by AgResearch. This represents good value for the WMRF investment.

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Executive Summary, Chanelle Gavin.

The number of effective antibiotics available to the medical profession is declining. As a result drug resistant bacteria are becoming increasingly problematic. The emergence of these resistant microorganisms requires the development of new and novel antibiotics. Screening of a bovine expressed sequence tag database for homologs of known antimicrobial proteins identified a small 6 kDa and 39 amino acid peptide. The peptide was named Histatherin and was patented by AgResearch in 2006. Since its discovery, research conducted into the detection and isolation of this peptide has proven challenging.

Over the summer period, attempts have been made to improve the recognition of Histatherin and to isolate a pure fraction from bovine milk. Investigation into glycosylation, a potential masking factor, produced conflicting results. While, for a number of reasons discussed in the main report, the glycosylation state of the protein could not be determined conclusively, the western blot of deglycosylated fractions containing Histatherin did not improve its detection. In fact is appears to have hindered it.

Experiments were also conducted to precipitate Histatherin from milk fractions, in particular lactoferrin. This method had been previously determined to be effective for this fraction as it isolates Histatherin from lactoferrin thereby generating a fraction with increased purity. However, this method may prove limiting if native Histatherin occurs in multiple forms as it may selectively capture only one variety.

Native gels were conducted in an attempt to produce a purified band of Histatherin for antibody generation. A new antibody to the native form may improve the western blotting method. However, the technique of native gels requires refining before this can be achieved.

Finally, liquid chromatography was employed on skim and trim milk to determine where Histatherin may be isolated from and to develop a suitable method for its purification. Hydroxyappatite columns have been previously employed due to calcium sites on the column. However, neither pH nor NaCl elution have generated the desired result to date. An alternative approach is now being taken; whole milk is being treated to obtain a fraction between 1 and 30 kDa. This should contain only a few proteins and be highly concentrated in Histatherin.

This work is now a starting point for continued research at an honours level occurring later this year. While many of the techniques have been recently learnt they have been employed correctly. Even so, this peptide seems determined to appear elusive at this stage.

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Progress since the summer work term period.

Currently another chromatography method for the isolation of Histatherin is being investigated. Lactoferrin eluate a milk fraction known to contain Histatherin is being evaluated as a feed stock for the production of a purified fraction. So far a size exclusion peptide column has been used to determine the original profile of the milk fraction. The chromatograph showed a large peak which is believed to correspond to the lactoferrin content. On the side of this peak was a small shoulder which could be Histatherin based on the elution volume. Further studies are being conducted to determine a method of separation for these two peaks. These methods currently involve the chloroform-methanol precipitation, and cation exchange at pH 10. While they have shown promising results, further studies will be conducted to confirm these results and to isolate the supposed Histatherin fraction to test against current western blotting protocol. Upon confirmation of a pure fraction antimicrobial properties can be tested for the native protein and new antibodies generated for the recognition of Histatherin.

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ENGG372-12C Engineering Work Placement 2

Summer work term - 2012/2013

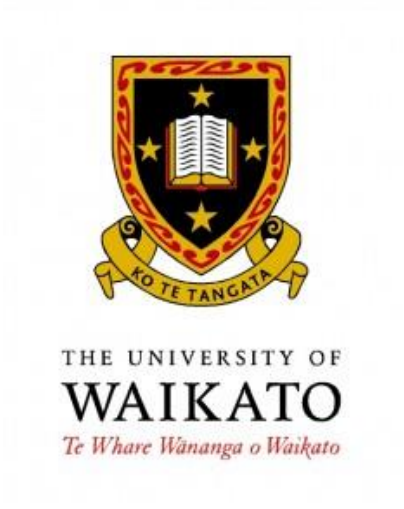
Improving the Detection of Histatherin in Bovine Milk

Chanelle Gavin
Research Assistant

Supervisors
Adrian Molenaar
Paul Harris
Tom Wheeler
AgResearch Limited

Mark Lay

University of Waikato



Acknowledgements

I would like to recognise the dedicated work of my supervisors, Dr Adrian Molenaar (primary), Dr Paul Harris (secondary) and advisor Dr. Tom Wheeler, who have guided me through this research project thus far and whose input is essential

Everyone in the dairy science building here at AgResearch who has made me feel welcome during my summer here. In particular Marita, Robert and Blake whose advice and patience is greatly appreciated.

Thank you to Grant Smolenski for predicting the glycosylation of Histatherin and Paul Harris for hands on help with the chromatography.

"The protein you are investigating is like a zebra on a pedestrian crossing"-Robert

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Introduction:

As the efficacies of current antibiotics dwindle, drug resistant bacteria are becoming increasingly problematic for the medical field (NIAID, 2006). To combat the emergence of immune microorganisms such as methicillin-resistant *staphylococcus aureus* (MRSA) constant development of novel antibiotics is required. Pharmaceutical companies however, are often more concerned with producing new remedies through complex modifications of existing antibiotics, rather than investigating previously unexploited sources (Keller, 2012).

Bovine milk is known to contain many bioactive compounds, some of which are antimicrobial in nature (Lemay, et al., 2009). The abundance of this source and reduced likelihood of acquired resistance to bovine derived medications, make milk an excellent candidate for antimicrobial bio prospecting.

A previous study conducted by AgResearch investigated the existence of antimicrobial compounds in bovine milk. As a result Histatherin, a small peptide was discovered through a bioinformatic study of AgResearch's bovine EST database and patented by AgResearch. Northern and western blot studies showed that Histatherin is expressed in the lactation phase and is secreted into milk from the mammary gland. The linkage of this expression to lactation as opposed to the defence phase theoretically increases the amount of Histatherin available and makes milk a promising source material.

However, the current level of Histatherin detected via a modified western blotting protocol is significantly lower than expected based on its relatively abundant RNA expression. The antibody used in detection was raised to a chemically synthesised and unmodified version of the antigen. Therefore, detection discrepancies may be caused by post translational modifications (PTMs) such as glycosylation. Other factors may include other proteins or poor transfer onto the membrane. These issues must be investigated and/or a pure fraction of native Histatherin obtained in order to improve the detection of Histatherin in bovine milk.

Research into this peptide may lead to industrial scale engineering based approaches for its purification. However if following the quantification, the amount of Histatherin present in milk too low for an economically viable process, Dairy companies such as Tatua will still be able to claim the quantified abundance of Histatherin in their milk and the benefit of its corresponding nutraceutical properties.

As a part of a larger investigation into the Histatherin during the coming year, a three month research placement was conducted with AgResearch this summer. During this time an improved understanding of this peptide and biological research techniques have been developed. Additionally, through experimentation, some previously unknown results have also been obtained. This peptide is of particular importance to the medical profession so this research is currently funded by the Waikato Medical Research Foundation. The aim of this project is to quantify the abundance in bovine milk and obtain a pure fraction of Histatherin to determine the true antimicrobial potential of the native peptide and whether purification is achievable and economic.

Background:

Histatherin was identified by AgResearch through screening of their bovine expressed sequence tag (EST) database for homologs of known antimicrobial proteins. Histatherin is a small 6 kDa chimera of a protein called Statherin and group of proteins called Histatins. Histatins are cationic proteins, found in saliva, which are involved in innate immunity. Statherin, which is also small and cationic in structure, is responsible for the super-saturation of calcium in saliva and protection of tooth enamel (Molenaar, et al., n.d.).

Amino acid analysis revealed that the similarity of bovine Histatherin to human Histatin is 21% and that it is 37% similar to human Statherin. There is also a 39% similarity to the bovine form of Statherin. However, the Histatherin gene is not found in all species. Analysis of the genome of mice, rats, humans, cows, dogs and opossums discovered that of these, the Histatherin gene is present only in the genome of dogs and cattle (Figure 1), (Molenaar, et al., n.d.).

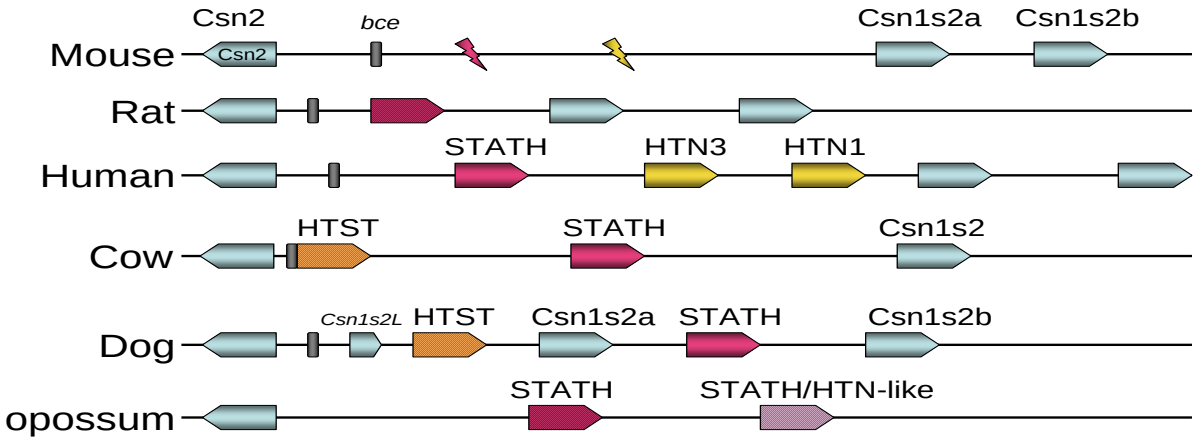


Figure 1: Genomic analysis illustrating the presence of Histatherin – performed by Ass. Prof. M. Rijnkels

The current theory as to how the gene arose is by the fusion of the genes of the two previously mentioned proteins and the associated deletion of a casein gene (Csn1s2) on the BTA6 allele (Elsik, Tellam, & Worley, 2009). The Histatherin sequence is controlled by a casein promoter and is located near the bce element which regulates beta casein expression. Due to the method of regulation Histatherin is expressed in mammary tissue during lactation

Tissues from the mammary, brain heart, kidney, trachea, lung, spleen, skin, bladder, ovary, parotid, pancreas and 13 more locations were investigated by northern blot analysis. From this study it was documented that a high level of Histatherin RNA expression occurs in lactating mammary and the parotid salivary tissue. Histatherin can also be found in bovine milk. Process fractions such as whey retentate, acid whey and lactoferrin all show detectable amounts of Histatherin under western blotting conditions. (Molenaar, et al., n.d.)

Finally the antimicrobial properties of Histatherin are inherent to its value. These abilities were tested with a synthetic form of Histatherin. The synthetic peptide demonstrated antimicrobial properties against *S.aureus, E.coli*, (<3.1µg/ml) and *Candida* (<6.25 µg/ml). (Molenaar, et al., n.d.)

Aims:

The aims of this placement were as follows:

1. Improve my understanding of the peptide Histatherin, its abilities, and the factors that may affect its detection.
2. Master the western blotting protocol for this peptide.
3. Investigate the possibility of glycosylation as a masking effect.
4. Obtain a reasonably pure fraction of Histatherin via liquid chromatography.
5. Explore other techniques which may help to generate or isolate a pure Histatherin band when subjected to gel electrophoresis under native conditions.

Experimental Work:

Western Blot Protocol:

The aim of a western blot is to identify the presence of a particular protein. The protocol is conducted following polyacrylamide gel electrophoresis (PAGE) of the samples and subsequent transfer to an appropriate membrane. The identification occurs through the specificity of a particular antibody for the target protein. Such antibodies are usually raised by injecting animals such as rabbits with a small amount of the antigen (protein/peptide) and allowing their natural immune system to generate antibodies to this foreign entity.

The use of serum from a non-immune rabbit, i.e. a rabbit without exposure to the target antigen, is incorporated as control into the method. The samples are run on the gel in duplicate in two distinct groups. By cutting the membrane in half, the primary antibodies, one to the target protein, one control, can be applied separately. Later in the protocol a secondary antibody is applied to both membranes. The role of this antibody is to recognise and bind to the primary antibodies. This is achieved by specificity of the secondary antibody for the species in which the primary antibody was generated. An example of a secondary antibody is Goat Anti Rabbit (GAR). This antibody is generated through the immune system of a goat and is specific for rabbit generated antibodies. Through the attachment of the secondary antibody to the primary an antibody complex is formed.

This complex is completed by the addition of the enzyme horse radish peroxidase which allows enhanced chemiluminescence (ECL) detection to be conducted. This method of detection is based on the oxidation reaction that occurs when horse radish peroxidase and hydrogen peroxide react with luminol (Biocompare, 2007). The result is a short period of light emission which can be captured through the use of XAR film which is then developed. This method is excellent for determining the presence of a given protein in a qualitative or semi-quantitative capacity and provides information about protein sizes. However this

method is limited by the optimisation of the protocol. Factors such as the gel type, gel running time, method and length of transfer and blocking as well as antibody concentration and length of application need to be optimised to produce the best results. This process also relies heavily on the specificity and ability of the primary antibody to bind to the target protein.

As the level of expression in the mammary tissue of cattle is so much greater than the detection of Histatherin on a western blot it is suspected that some factor may be masking detection. The generation of the primary antibody for Histatherin (J-11) was to a synthetic version of the Histatherin peptide generated based on bioinformatics data. It was initially predicted that the peptide would be phosphorylated at two sites. However, the native protein may have undergone other post translational modifications and acquired further masking factors such as glycosylation. Other things to be considered are the level of optimisation of the western blot and the possibility that the peptide is in fact in low abundance.

The western blotting protocol for Histatherin was established by Kwang Kim, a previous researcher, here at Ruakura in 2006. The key step to detecting Histatherin is the use of a glutaldehyde fixation step prior to blocking. This binds the low molecular weight Histatherin to the membrane and prevents it from being washed off during the procedure.

Method:

1. Ran samples on a 10% Bis Tris gel
2. Transfered using iBlot (dry transfer) onto polyvinylidene fluoride (PVDF) membrane
3. Fixed for 20 mins with glutaraldehyde in phosphate buffer saline and Tween 20
4. Ponceau stained the membrane, destained with water and capture image
5. Blocked with Roti block or 4% non-fat milk (preferred) for 1 hour at 37 °C
6. Washed once with Tris Buffer Saline- Tween (TBT/TBST) for 5 to 10 minutes with rocking
7. Applied primary antibodies at 1/10 k, 20 ml for mini gels, 40 ml for regular at 4°C overnight with rocking
8. Washed with TBT three times for 5 to 10 minutes with rocking
9. Applied secondary antibody at 1/15 k, 15 ml for mini gel, 30 ml for regular gels at room temperature with rocking for 1.5-2.0 hours depending on temperature
10. Repeated three TBT washes for 5 to 10 minutes with rocking
11. Conducted ECL detection

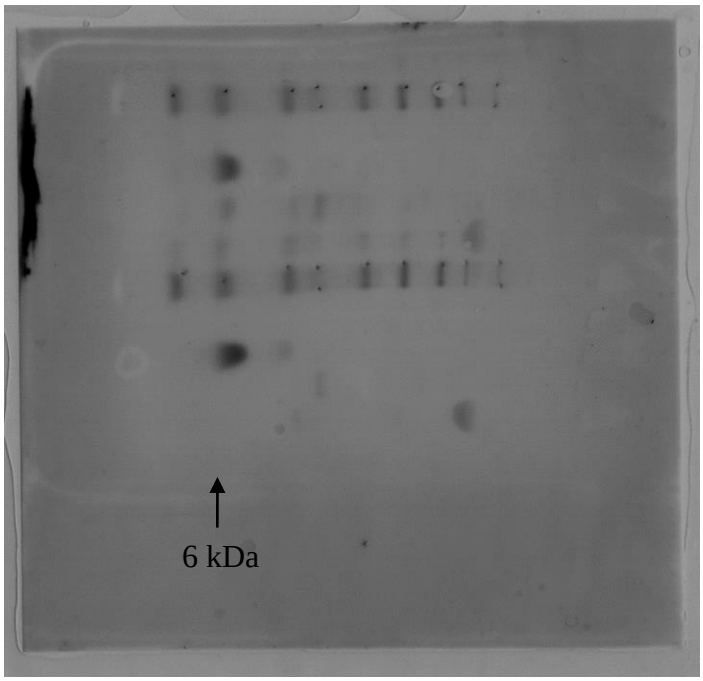
Results and Discussion:

The results obtained from this method include a ponceau stained membrane demonstrating the level of transfer that has occurred, a coomassie brilliant blue stained gel to demonstrate the level of protein not transferred by this method and the developed film results. As this is the only method used to detect Histatherin and it is used throughout all experiments the following results are only exemplary, other results will be explained when appropriate.

Gel electrophoresis was conducted with two samples of BtStath (the synthetic peptide), a salivary protein BSP30 and a sample of Lactoferrin Eluate. 6.25 ng of each of the BtStath

samples was loaded and 15 µg of each of the other samples. 5 µl of a molecular weight marker was also included to help identify the appropriate band.

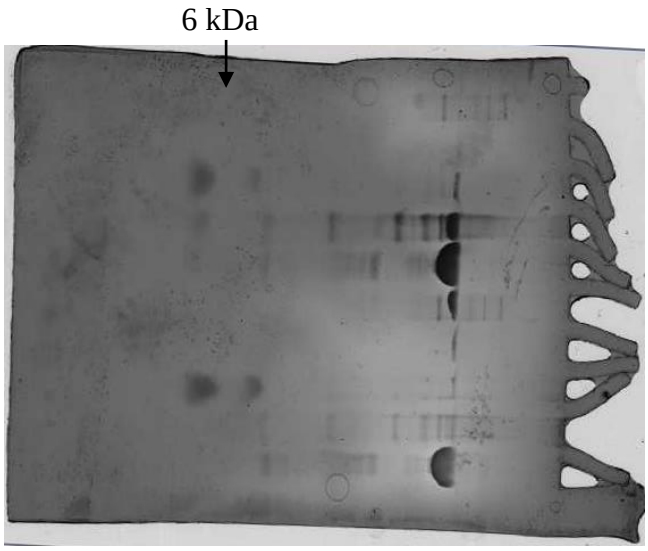
The ponceau stain binds to positively charged amino groups and through covalent bonds in non-polar regions (Sigma, 1998). This stain can therefore detect protein as demonstrated with bands locatable in the new BtStath, sample (due to overloading), ignoring marker transfer, in the 6 kDa region, (Figure 2).



1. See Blue Marker (5 µl)
2. BtStath old 6.25 ng (5 µl)
3. BtStath New 6.25 µg (5 µl)
4. BSP30 15 µg (15 µl)
5. Lactoferrin 15 µg (15 µl)
6. See Blue Marker (5 µl)
7. BtStath old 6.25 ng (5 µl)
8. BtStath New 6.25 µg (5 µl)
9. BSP30 15 µg (15 µl)
10. Lactoferrin 15 µg (15 µl)

Figure 2: Ponceau stained membrane after dry transfer nine minutes

A coomassie brilliant blue stain is then conducted on the gel after transfer. This stain binds to arginine, histidine and aromatic amino acids, enabling proteins to be detected (Citizendia.org, 2009). Once again detectable amounts of Histatherin are present in the same lanes illustrating that complete transfer may not be occurring, (Figure 3)



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8. BtStath New 6.25 ng (5 µl)
9. BSP30 15 µg (15 µl)
10. Lactoferrin 15 µg (15 µl)

Figure 3: Coomassie brilliant blue stain conducted on gel after transfer

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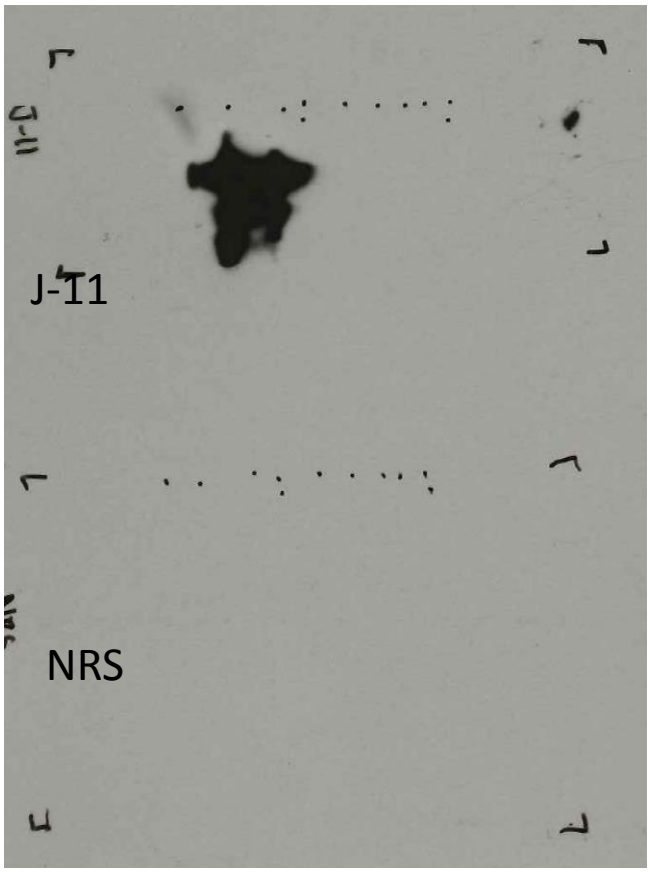

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See Blue Marker (5 µl)
BtStath old 6.25 ng (5 µl)
BtStath New 6.25 ng (5 µl)
BSP30 15 µg (15 µl)
Lactoferrin 15 µg (15 µl)

Figure 4: Developed results of example western blot

Following the western blotting protocol as previously described the results were developed using ECL detection. The results of an ideal western blot produces dark bands on regions containing Histatherin in the J-11 probed membrane and no bands (protein) evident on the normal rabbit serum (NRS) probed membrane (Figure 4). This ensures that the result seen on the J-11 sample is truly Histatherin.

Chloroform and Methanol Precipitation:

Previously, methods for concentrating Histatherin via precipitation from milk fractions such as lactoferrin, skim milk and acid whey were investigated. It was determined that a method involving chloroform and methanol could precipitate Histatherin from lactoferrin but not acid whey or skim milk.

The following precipitation method was used for conducting experiments where concentrated samples were required. It was used twice to obtain samples for further analysis. The first was to generate a concentrated sample to be run on native gels. The second was to create a sample that could be treated with a deglycosylation kit.

Method:

1. 1 ml of lactoferrin sample was added to 4 ml of methanol and mixed well using a vortex

2. 1 ml of chloroform was added to the tube, then the sample was again mixed using a vortex
3. 3 ml of MQ water was added and the tube was mixed using the vortex
4. The sample was centrifuged for one minute at 14,000g
5. The top aqueous layer was removed (Supernatant one- SN1)
6. 4 ml of methanol was added to the tube and mixed in using a vortex
7. The sample was centrifuged a second time for two minutes at 14,000 g
8. The aqueous layer was then removed without disturbing the pellet (Supernatant two- SN2) and the pellet was collected
9. The samples were then dried using the freeze dryer and re-suspended in 100 µl of MQ water
10. The concentration of the protein in each fraction was then determined using Nanodrop and Qubit™, (Invitrogen) analysis

Results and Discussion:

Precipitation One:

The first precipitation was conducted from lactoferrin and generated three fractions: a 0.2 µg/µl SN1, 0.2 µg/µl SN2 and a 2 µg/µl pellet sample. These samples were then tested using the western protocol to verify which fractions Histatherin was present in. 15 µl of the SN1 and SN2 samples were loaded while only 10 µl of the pellet was included. From the developed results it was determined that through this precipitation, Histatherin was present only in the SN1 fraction and the original starting material. The visible difference in intensity between the lactoferrin sample and the SN1 sample illustrated that the precipitation achieved the desired effect of increasing the concentration of Histatherin in the sample. Faint shadowing was also visible around 12 kDa range. This is most likely due to a dimer of the Histatherin protein being detected by the antibody. The SN1 sample was then used to conduct native gels.

Precipitation Two:

The second precipitation was conducted from lactoferrin and skim milk to investigate the effect of glycosylation. 4 ml of each sample was precipitated using the above method to generate SN1, SN2 and pellet fractions. Following the quantification of the protein concentration in each sample, two western blots were conducted, one on the lactoferrin fractions and one on the skim milk fractions.

For the lactoferrin gel 12.75 µg of SN1, 20 µg of SN2, 20 µg pellet and 50 µg of lactoferrin were loaded onto the gel with the appropriate control (BtStath). The ponceau stain showed a clearly visible band of Histatherin in the SN1 fraction only. However Histatherin was proven to be present in the lactoferrin, SN1 and pellet fractions. During this particular western blot while the J-11 or secondary antibody detected a lot of non-specific protein; the presence of a Histatherin band was still evident.

For the skim milk gel 20 µg of SN1, SN2, pellet and pasteurised skim milk was loaded onto the gel with BtStath and See Blue Marker. While the ponceau stain showed no detectable

band in the 6 kDa range the western detected Histatherin in the pellet fraction but not in the SN1sample indicating that in these conditions the HSTN precipitated in the pellet. Therefore the concentration of Histatherin in skim milk without precipitation was below the level of detection via the western blotting method in this instance

Native Gels:

Experiments were conducted with samples under non-denaturing (native) conditions as sodium dodecyl sulphate (SDS) containing samples are not suitable for direct antibody generation. Native gels were conducted to determine the behaviour of Histatherin under non-denaturing conditions. This behaviour was previously undetermined.

The first system which was investigated continued to use the NuPage 10% Bis Tris gels. A buffer solution containing 2-N-morpholinoethanesulfonic acid (MES) but no SDS or EDTA was used to ensure no denaturing agents were added to the system. This most important aspect of this experiment was that two gels were run simultaneously one under normal polarity and one under reversed polarity as without denaturing conditions the movement of the protein is unknown. However due to its theoretical high pI it was expected to run in the opposite direction to many proteins. During this experiment a colloidal coomassie stain was also conducted on a proportion of the gel to help determine where the proteins run.

Methods:

Gel and Western Blot Method:

1. Prepared all samples in non-reducing laemlli (15 ml glycerol, 1.13g Tris at pH 6.8 with bromophenol blue grains)
2. Ran 10% Bis Tris gel for 2.75 hrs at 200V using 1 x native MES buffer for 1 hour followed by 3 x native MES buffer.
3. Transfered the gels in iBlot dry transfer system with PVDF membrane on both sides of the system.
4. Fixed the membranes using the glutaldehyde fix for 20 minutes
5. Blocked with 1 x roti block for 1 hour at 37 °C
6. Washeed once with TBT
7. Cut membrane in two identical pieces
8. Applied primary antibody- 20 ml of 1 in 10 k J-11 and 20 ml of 1 in 10 k NRS to the separate parts of the membrane for 2 hours at room temperature with rocking
9. Washed three times with TBT with rocking for at least 5 minutes.
10. Applied secondary antibody- 15 ml of 1 in 15 k GAR to both membranes for 1.5 hours at room temperature with rocking
11. Repeated three TBT washes
12. Detected using ECL reagents.

Colloidal Coomassie Blue:

The colloidal coomassie blue stain was conducted on a portion of the gel which had not been transferred.

1. Fixed the gel with 1 ml orthophophate and 250 ml methanol in a 500 ml volume for 1 hour changing the solution twice.
2. Conducted two millipore water washes
3. Stained in colloidal coomassie blue stain overnight.
4. Destained with 10% methanol

Results and Discussion:

The western blotting protocol did not yield any usable results in this case, as Histatherin was not detected on either of the membranes. However the colloidal coomassie stain illustrated some interesting behaviour of the synthetic peptide under the native running conditions.

The synthetic peptide BtStath made to the amino acid sequence derived from the mRNA sequence runs into the gel under reverse polarity and out of the gel under normal conditions, (

Figure 5). This means that the native form of Histatherin is likely to exhibit the same behaviour. As a result all further native analysis should include reverse polarity conditions and this may help to separate out the Histatherin peptide from other components in milk due to this property.

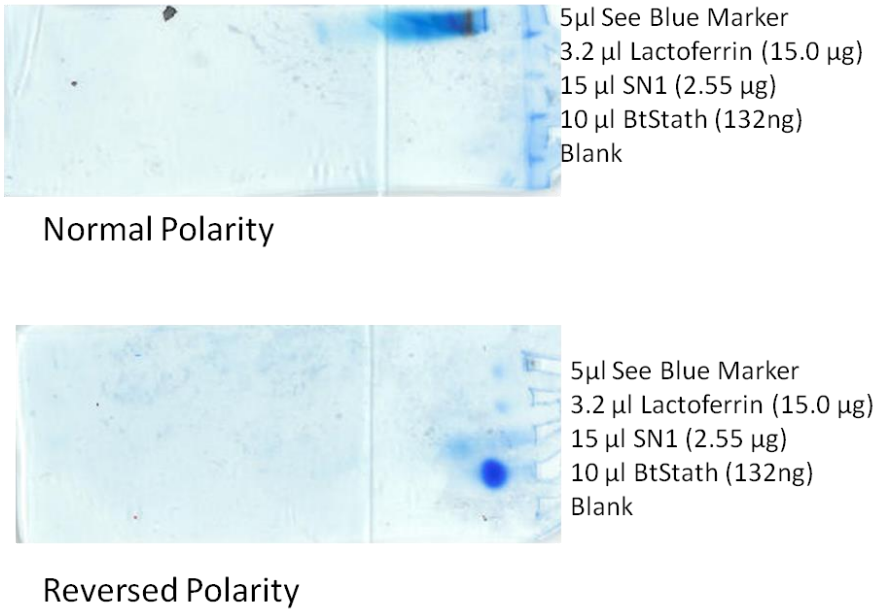


Figure 5: Colloidal coomassie blue stain on native gels under different polarities

Glycosylation:

Research:

Due to the difference between the amount of Histatherin detected and the amount of RNA expression, it is expected that some form of post translational modification may be acting as a masking factor. Modifications such as phoshorylation, methylation, acetylation, lipidation, proteolysis, and glycosylation all affect the structure of the peptide and thus its behaviour. Glycosylation, the addition of sugar residues to the protein, appeared to be a likely candidate for modifications to Histatherin as it is a secreted protein. It has previously been reported that

a similar protein histatin, can be found in a glycosylated state therefore as Histatherin has potential glycosylation sites, this was investigated through the use of deglycosylation enzymes and glycoprotein stains.

However, glycosylation does not only occur in one form (Scientific, 2013): `

- N- linked glycosylation occurs when glycans bind to asparagine residues in the endoplasmic reticulum.
- O- linked glycosylation occurs when monosaccharides bind to hydroxyl groups , throughout any phase of protein synthetic via serine or theronine residues.
- Glypiation occurs where a glycan links a phospholipid to the protein.
- C- linked glycosyaltion occurs when mannose binds to tryptophan.
- Phosphoglycosylation is the last form and occurs when glycans bind to serine through phophodiester bond.

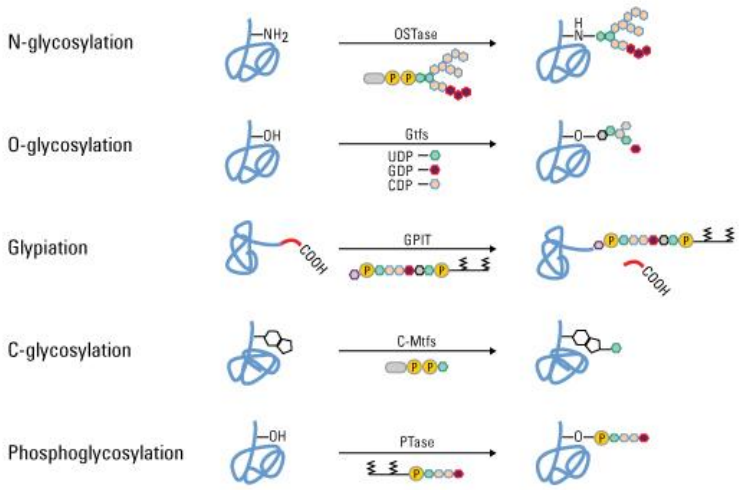


Figure 6: Summary of different forms of glycosylation

The possible form of glycosylation for Histatherin was predicted using a computer model for the predicted amino acid code of Histatherin:

DSSEEKRHRKRKKHHRGYFQQYQPYQRYPLNYPPAYPFP

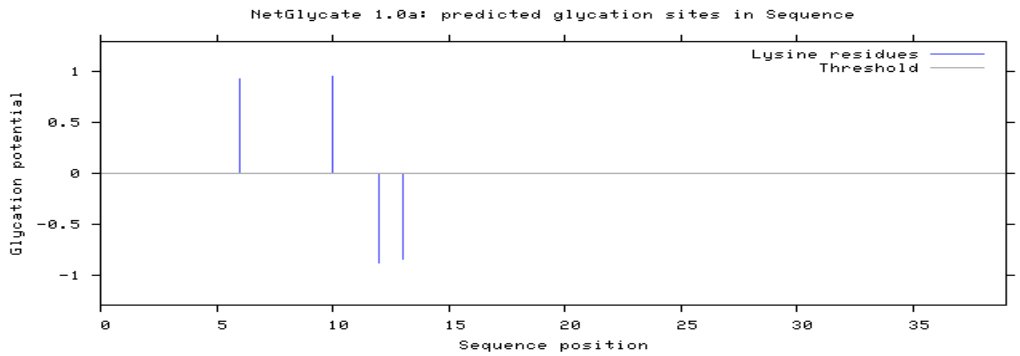


Figure 7: Results of predicted glycosylation of lysine residues

The predicted result was glycosylation of hydroxylysine residues at positions 6 and 10 on the peptide (Figure 7). This form of glycosylation can occur through the O-linked strategy so an enzymatic deglycosylation kit was purchased from QAbio which contained enzymes capable of breaking O and N linked glycosylation (QAbio, n.d). The kit contains a number of enzymes and explains their modes of action, Figure 8.

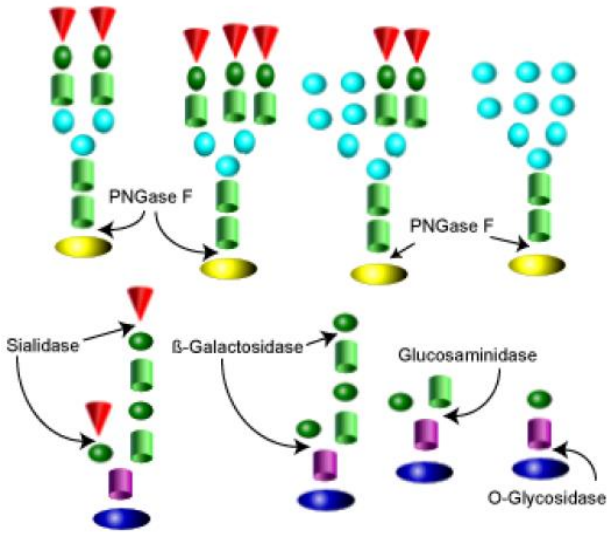


Figure 8: Enzymes contained within QAbio kit and their function

PNGase F removes all N-linked glycan groups. Sialidase and O-glycosidase then remove the O-linked and Sialic acid substituted glycan groups and β-galactosidase and glucosaminidase work to remove the larger O-linked structures. Due to the wide range of deglycosylation that this kit could achieve, it was selected as a reagent.

Methods:

Deglycosylation of Sample

The deglycosylation of a sample was achieved by following the instructions of the QAbio kit.

1. 50 µg of glycoprotein was added to 33 µl of distilled water and 10 µl of 5x reaction buffer
2. 2.5 µl of the provided denaturation solution was added. The sample was then mixed and boiled for 5 minutes before chilling on ice.
3. 2.5 µl of Triton-X was added.
4. Finally 2 µl of the DeGlycoMx (the enzymes) was added and the sample was incubated for 3 hours at 37 °C

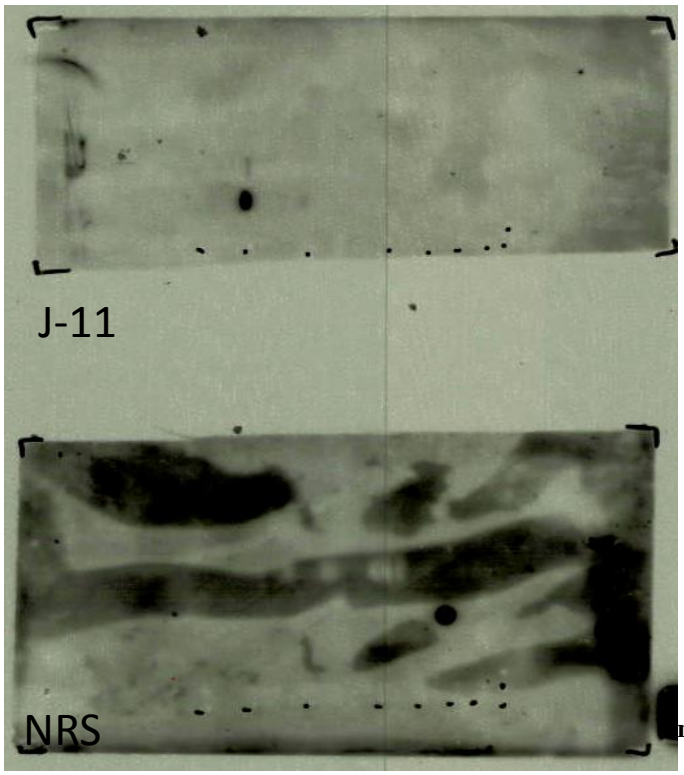
Glycoprotein Stain:

1. Performed SDS Page with dilute sample of 10-100 µg/ml, load 5-10 µl per well

2. Fixed the gel using 100 ml of fix solution. Incubated for 2 hours with mild agitation, followed by incubation overnight with the remainder of the fix solution
3. Washed the gel twice using 100 ml of wash solution. Each wash was20 min in length and conducted with mild agitation
4. Oxidized the carbohydrates by incubating the gel in 25 ml of component B (the oxidising solution) with mild agitation
5. Washed the gel three times using 100 ml of wash solution. Each wash was 20 min in length and conducted with mild agitation
6. Prepared the Pro-Q Emerald by adding 0.5 ml of DMSO to the vial of desiccated Pro-Q Emerald.
7. Diluted the Pro-Q Emerald 1 in 50 by adding 500 µl to 25 ml of component A (the staining buffer)
8. Stained the gel for 2 hours at room temperature in the dark
9. Washed three times with 100 ml of wash solution. Each wash was conducted with mild agitation at room temperature.
10. Detected the results by imaging in the 470-500 nm range.

Results and Discussion:

The second chloroform and methanol precipitated SN1 samples of lactoferrin and skim milk were tested with these protocols to determine the state of Histatherin. A sample of untreated lactoferrin and a sample of untreated skim milk were both deglycosylated using the QAbio kit as instructed. A western blot was conducted on a gel containing both untreated and deglycosylated samples of lactoferrin and skim milk. No improved detection of Histatherin was obtained. In fact Histatherin was not detected at all in the deglycosylated fractions and was only evident in the untreated lactoferrin fraction.



Skim Milk Deglycosylated 5µg (20µl)
Skim Milk Untreated 10 µg (5µl)
Lactoferrin Deglycosylated 5 µg (20 µl)
Lactoferrin Untreated 10µg (5µl)
BtStath 5 µl 6.25 ng
See Blue marker 5 ul

Skim Milk Deglycosylated 5µg (20µl)
Skim Milk Untreated 10 µg (5µl)
Lactoferrin Deglycosylated 5 µg (20 µl)
Lactoferrin Untreated 10µg (5µl)
BtStath 5 µl 6.25 ng
See Blue marker 5 µl

ns in untreated and deglycosylated states

Another western blot was conducted with increased concentration of the lactoferrin untreated and deglycosylated samples. Despite the increase in concentration the detection of Histatherin still did not improve.

Glycoprotein stains were also conducted on glycosylated and deglycosylated samples of lactoferrin and skim milk with 500 ng loading (Figure 10). This revealed no indication that Histatherin is glycosylated in any way. However, this loading may have been below the level of detection, despite the producers claims of the Pro-Q Emerald stain being able to detect as low as 4 ng of glycoprotein. The presence of bands lower than 6 kDa in the deglycosylated samples is suspected to be removed glycan groups from the deglycosylation process.

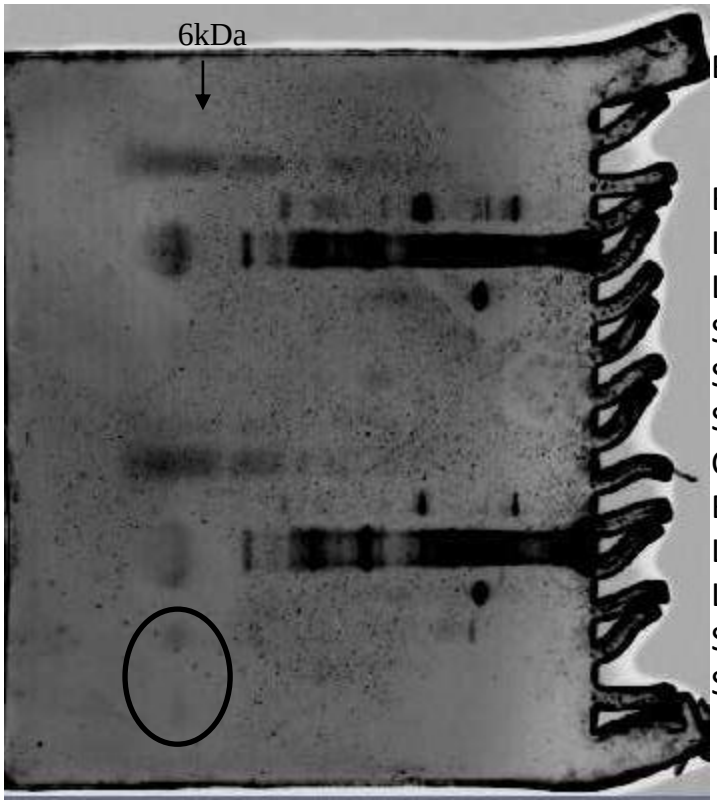


Figure 10: Glycoprotein stain of lactoferrin and skim milk untreated and deglycosylated samples

As the level of protein loaded on the gel for the glycoprotein stain was very low the process was repeated with 15 µg of lactoferrin sample and of a known glycoprotein BSP30, both samples were expected to contain Histatherin. Despite this increase in protein concentration, once again Histatherin was not detected as a glycoprotein, (Figure 11).

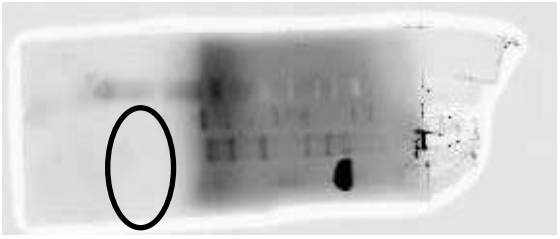


Figure 11: Glycoprotein stain of lactoferrin and BSP30

Unfortunately, these results are not definitive at this stage. The reduction in the detection of Histatherin after deglycosylation was unexpected as this suggests that some factor removed by this method, although this is not necessarily glycosylation, is aiding not masking the detection. Furthermore the results of the glycoprotein stains are not definitive as the amount of Histatherin present could still be below the level of detection. At this stage of research nothing definitively proves Histatherin is glycosylated.

Liquid Chromatography:

Liquid chromatography was conducted in an attempt to generate a purified fraction of Histatherin. The chromatography media selected was ceramic hydroxyapatite type one. This media has the ability for proteins to bind both cationically and through calcium sites. Previously enrichment has been achieved using a type two hydroxyapaptite column.

Method:

The first chromatography run was loaded with 50 ml of Tatua skim milk after column equilibration with buffer A. Elucidation occurred through the use of a gradient of 0 to 2 M NaCl over 20 column volumes (buffer B). Some protein was also eluted by the addition of buffer C (**Figure 12**).

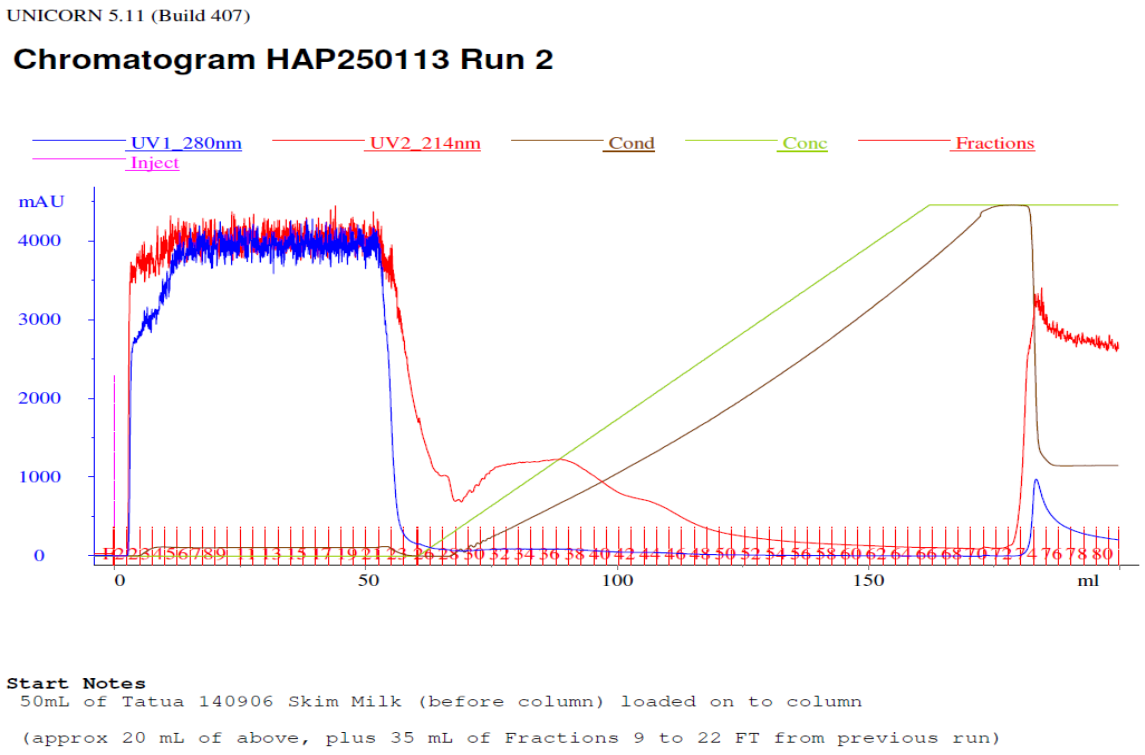


Figure 12: Chromatography run for Histatherin -Attempt two

The buffers used were as follows:

Buffer A: 10 mM sodium phosphate pH 7.0

Buffer B: 10 mM sodium phosphate + 2 M NaCl at pH 7.0

Buffer C: 500 mM Sodium phosphate pH 7.0

The column was sanitised with 1 M NaOH and stored in 0.1 M NaOH

Results and Discussion:

This chromatography run alone generated over 80 sample fractions and so some of the more likely peaks were selected for analysis by western blotting. Samples 11, 28, 34, 46, and 75 were concentrated and loaded onto a gel. Despite significant amount of protein being visible

under coomassie staining (Figure 13) none of the fractions tested contained Histatherin at a level which could be detected (Figure 14).

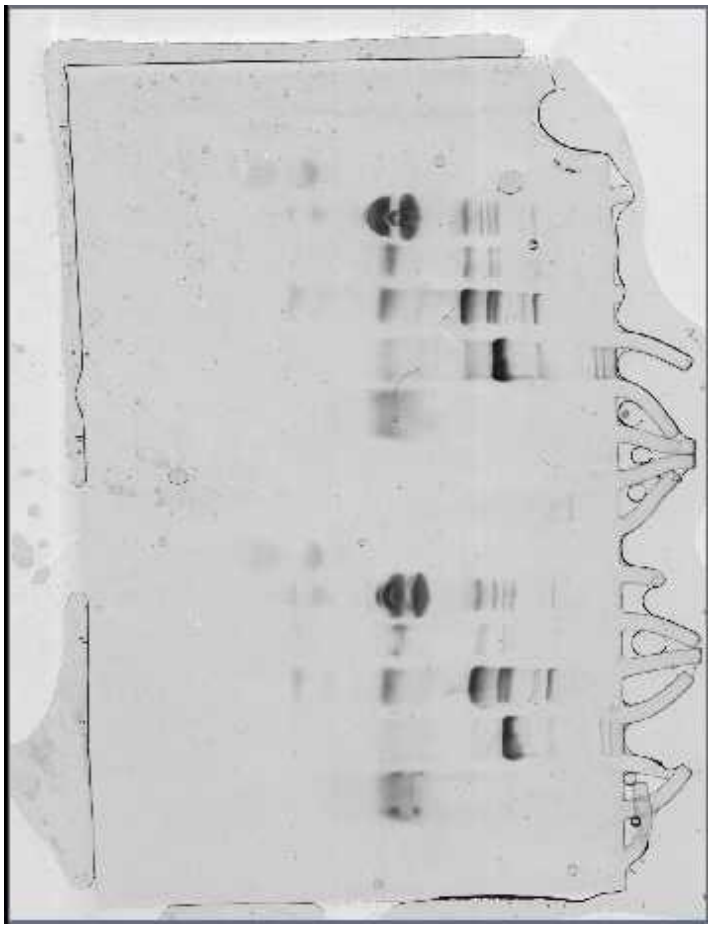
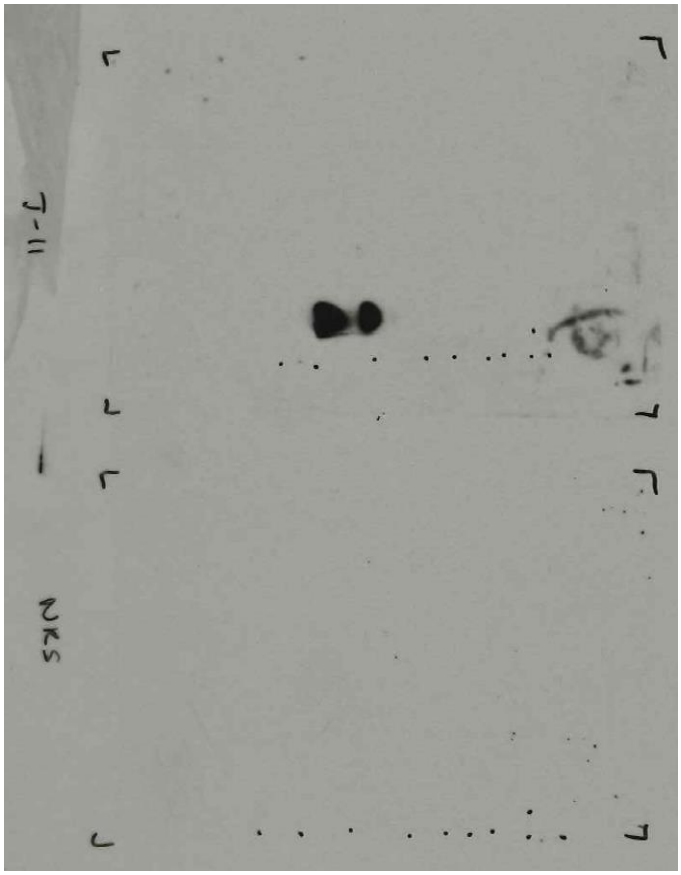


Figure 13: Coomassie blue stained gel for Histatherin chromatography run- Attempt two



Sample 75 15 µl 15 µg
Sample 46 15 µl8.1 µg
Sample 34 15 µl 8.25 µg
Sample 28 15 µl 3 µg
Sample 11 2 µl17µg
BtStath 5µl 6 ng NEW
See Blue Marker 5 µl
Sample 75 15 µl 15 µg
Sample 46 15 µl 8.1 µg
Sample 34 15 µl 8.25 µg
Sample 28 15 µl 3 µg
Sample 11 2 µl 17µg
BtStath 5µl 6 ng NEW
See Blue Marker 5 µl

Figure 14: Results of western blot for Histatherin chromatography run- Attempt two

Further chromatography runs have since been conducted with trim milk with a pH elution. All of the fractions produced by this run were tested for Histatherin however once again the western blot did not detect Histatherin in any of the fractions. As a result a new approach has been taken to generating a concentrated solution of Histatherin. Raw milk has been obtained from the Tokanui farm. It has then been subjected to centrifugation to remove the fat, acid precipitation to obtain the casein and then ultra filtration to obtain a protein fraction of 30-1 kDa for further analysis. This process is still in completion and so results as to whether this method is successful are not available at this point.

Conclusions and Recommendations:

Throughout this research placement significant progress has been made and experience gained towards quantifying the amount of Histatherin in bovine milk and obtaining a purified sample.

So far glycosylation has been investigated with unpredicted results. While no evidence has been discovered that suggests Histatherin is found naturally in a glycosylated form, it cannot be said that Histatherin is not at all glycosylated. While all glycoprotein stains conducted have failed to detect Histatherin as a glycoprotein, upon deglycosylation and western blotting, Histatherin can no longer be visualised. This suggests that some epitope removed by the deglycosylation kit is actually aiding in the detection of Histatherin. Further investigation is required, as the glycoprotein stain may not detect Histatherin if it is present at a low level or only mildly glycosylated. At this stage glycosylation does not appear to be a major factor masking the detection of Histatherin

Liquid chromatography has been used in three unsuccessful attempts to generate a purified fraction of Histatherin. Once again the main difficulty arises with ensuring that Histatherin is present at a sufficient level in the chromatography samples to be detected by western blotting. Currently research is occurring into the possibility of generating a concentrated fraction of Histatherin through fat and casein removal, acid precipitation, and ultra filtration. Further analysis of previous western blots of the chromatography fractions may also be conducted with higher protein loading to ensure the validity of these results.

A number of other techniques which may aid in the isolation of a pure form of Histatherin have also been investigated. The chloroform and methanol precipitation proves once again that Histatherin can be enriched by these means. Native gels have also been attempted to isolate a useable fraction for antibody generation in rabbits. However these attempts did not produce the desired result and so it is hoped that a more concentrated starting material may produce better results after optimisation of this method.

The research conducted so far covers just some of the possible ways to investigate this protein. To unravel the mysteries of Histatherin further investigation is still required.

On a professional front this placement has been both rewarding and enlightening. During this time at AgResearch my understanding of proteins and in particular Histatherin has exponentially grown. Every day has been a challenge to master new techniques for protein analysis. Quick uptake of these methods and the ability to work independently has been vital for this role. In particular mastering the western protocol was a huge achievement given the lack of experience I previously possessed. Doing so gave me the confidence required to tackle other technical challenges with guidance.

The work presented in this report is a personal achievement; given the elusiveness of this protein. This work will be a considerably help towards meeting the requirements of the supporting grant in the future, especially this year as I continue with this project throughout my Honours year.

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