Detection of Histatherin: a Potential Antimicrobial Peptide

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Abstract: Human health has advanced significantly since the discovery and development of antibiotics in the early 20th century. In recent times, due to a combination of overusing, misusing and declining research and development of antibiotics, resistance among pathogens has become endemic. Since the 1950's antimicrobial peptides produced by higher order organisms such as cows, sheep and goats have been investigated. However studies into large-scale development of using antimicrobial peptides as a form of antibiotic have not become significant until recent years.

One potential antimicrobial peptide identified in bovine milk and saliva is Histatherin. Studies into the synthetic version of Histatherin have shown antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida*. The aim of this research was to identify the presence of the native Histatherin in bovine tissue samples, produce a purified fraction of Histatherin for antibody generation and to test the true antimicrobial activity of the native peptide as opposed to the synthetic.

Initial studies were carried out using High Performance Liquid Chromatography (HPLC) to fractionate samples of lactoferrin eluate, 30 – 50 kDa permeate and bovine saliva. Western blots were conducted to detect the presence of Histatherin in these samples. Western blots conducted on lactoferrin eluate and bovine saliva fractions indicated positive results. However, larger starting volumes of lactoferrin eluate and saliva, using higher capacity columns may be needed to produce a more purified fraction of Histatherin. Due to some bands on the western blot being larger than 6 kDa in size it is thought that post-translational modifications or binding to other factors may be an explanation for this. Immunohistochemistry was finally carried out to identify the presence of Histatherin in bovine tissue samples, however the results are inconclusive at this stage.

1. Introduction

One of the central themes of success in human therapeutics in the 20th century was the discovery and development of antibiotics and antimicrobial agents. Bacterial infections have been a known cause of death throughout human history and when antibiotics were first introduced they were regarded as miraculous compounds. The introduction of antibiotics reduced the death rate caused by infectious diseases from 797 per hundred thousand in 1900 to 36 per hundred thousand in 1980[1]. Over the years the rate of approved antibiotics has declined significantly. The current development of novel antibiotics is insufficient to combat growing bacterial resistance. The majority of the second, third and fourth generation antibiotics have come from alterations of natural and synthetic first generation antibiotics.

To date there are no systematically administered antimicrobials in advanced development that have antimicrobial activity against either a purely Gram-negative spectrum or bacteria already resistant to all currently available antimicrobials[2]. The search continues for new antibiotics that are active in vivo, are fast acting and broad spectrum and also have limited side effects[3]. A potential solution to this global problem being investigated is antimicrobial peptides produced by higher order organisms such as cattle.

Antimicrobial Peptides

Antimicrobial peptides provide domesticated animals with an innate immunity to protect them against microbial infections. Antimicrobial peptides have the ability to protect against a broad-spectrum of pathogenic bacteria without inducing resistance among them.

Antimicrobial peptides are usually small molecules approximately 1-5 kDa, cationic or contain regions of positive charge of 10-25 amino acids and when placed in non-polar solvents they tend to form amphipathic structures with separate hydrophobic and hydrophilic domains. There are two families of antimicrobial

peptides: Defensins and Cathelicidins. Defensins are small cationic peptides widely distributed in mammalian immune cells, whereas Cathelicidins are cationic antimicrobial polypeptides found in lysosymes of the host cell[4].

The exact mechanism for destroying bacteria using anionic peptides is unknown. It is thought anionic peptides require zinc for maximum activity and form a complex with it. It is thought that the zinc complex forms a cationic salt bridge that overcomes the negative charge. The zinc complex creates a net positive charge that can then penetrate the microbial surface and once inside the cytoplasm, anionic peptides may attach to ribosomes and inhibit ribonuclease activity.

Cationic antimicrobial peptides are very common in domesticated animals. There are three categories of cationic peptides: linear helical, linear rich in proline and cysteine-stabilised peptides with β-sheets. Linear helical peptides become amphipathic, i.e. able to permeate bacterial cell walls. Linear rich in proline peptides inhibit protein and DNA synthesis in Gram-negative bacteria. Cysteine-stabilised peptides aggregate the bacterial membrane forming a larger pore where the cationic peptide disrupts the cell membrane[3].

Milk Proteins

Milk is a rich source of bioactive components that positively influencing host health. Milk can be considered the ultimate food, being a complete food for new born and the sole food during early stages of mammalian development. Milk contains components that provide critical nutrients, immunological protection and biologically active material to both neonates and adults. Analyses of milk fractions have indicated that milk proteins exhibit antimicrobial properties, which is presumed to contribute to suppression of pathogens either in the mammary gland itself or in the digestive tract of new born[5].

The major protein fractions in bovine milk consist of caseins, whey proteins and immunoglobulins. Table 3 below gives the concentration and biological activity of major bovine milk proteins.

Table 3: Major proteins in milk with concentration and function[6].

Protein	Concentration (g/L)	Function
Total Caseins	26.0	Ion carrier (Ca, Fe, Zn, Cu), Precursor of bioactive peptides
α-casein	13.0	Precursor of bioactive peptides
β-casein	9.3	Precursor of bioactive peptides
κ-Casein	3.3	Precursor of bioactive peptides
Total Whey	6.3	Anti-carcinogenic, weight management
β-lactoglobulin	3.2	Retinal carrier, binding fatty acid
α-lactalbumin	1.2	Lactose synthesis in mammary gland
Immunoglobulin (A, M, G)	0.7	Immune Protection
Serum Albumin	0.4	
Lactoferrin	0.1	Antimicrobial, iron adsorption
Lactoperoxidase	0.003	Antimicrobial
Lysozyme	0.0004	Antimicrobial, synergistic effect
Miscellaneous	0.8	
Proteose-peptone	1.2	Function unknown
Glycomacropeptide	1.2	Antiviral

Whey derived proteins, in particular, have proven beneficial in the reduction of the risk of many chronic human diseases[7]. Bovine milk is an abundant product, which makes it a promising starting point for antimicrobial bio prospecting.

Histatherin

Histatherin was identified from a bioinformatics survey of AgResearch's 200,000 bovine EST collection as a peptide sequence similar to known antimicrobial peptides. Histatherin is a chimera of the known peptides histatin and statherin. Histatins are salivary cationic peptides, which have antimicrobial and antifungal activities and are involved in innate immunity. Statherin is a small cationic peptide found in saliva that supersaturates salivary calcium, binds to teeth and maintains tooth enamel[8].

Associate Professor M. Rijnkels observed that the Histatherin gene is not present in all animals. The genome of mice, rats, humans, cows, dogs and opossums revealed that the gene is only present in dogs and cattle shown below in Figure 1.
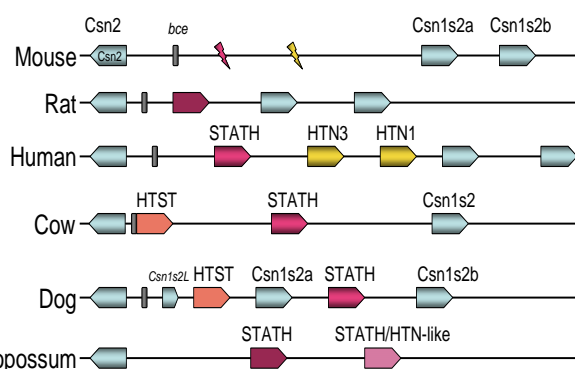
Figure 1: Genomic analysis illustrating the presence of Histatherin gene[8].

Tissue samples of the mammary, brain, heart, kidney, trachea, lung, spleen, skin, bladder, ovary, parotid, pancreas and 13 other locations were investigated by northern blot analysis. This study documented high levels of Histatherin RNA expression occurs in lactating mammary and parotid salivary tissue. Histatherin has been detected in milk fractions of lactoferrin eluate, whey retenate and acid whey. It is thought the peptide may undergo post-translational modification such as glycosylation or phosphorylation. Post-translational modification may be the reason for bands appearing at sizes larger than 6 kDa or 12 kDa in the western blot protocol. Amino acid analysis revealed that human

Histatin and Statherin are 21% and 37% respectively similar to bovine Histatherin. The peptide was found to be high in proline, arginine and tyrosine. An innovagen analysis identified Histatherin has a molecular weight of 4995 g/mol, with an isoelectric point of 10.23. Histatherin has an amino acid sequence:

(DSSEEKRHRKRKKHHRGYFQQYQPYQRYPLNYP PAYPFP)

A synthetic version of Histatherin was developed exhibiting antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* (<3.1ug/ml) and *Candida* (<6.25 ug/ml). Peptides such as Histatherin could have many potential applications similar to Lactoferrin and other known AMP's found in animals[8].

2. Methodology and Materials

In previous studies conducted at AgResearch samples of lactoferrin eluate, bovine saliva and 50 kDa permeate from acid whey were separated using HPLC with a Superdex 75 peptide column to isolate a fraction of Histatherin. Fractions were taken and a western analysis performed. It was found that larger initial volumes of lactoferrin eluate and 30 – 50 kDa permeate using higher capacity columns may be required to detect Histatherin. Bovine saliva fractions gave results with the

western blotting, however as previously observed elsewhere, the size of the bands detected are larger than 6 kDa. The reasons for this are unclear but could be due to post-translational modifications or binding to other salivary factors.

Unfortunately saliva was no longer available to conduct further research on and lactoferrin eluate was proving difficult to separate. However the 50 kDa permeate from acid whey was in supply to continue research. To obtain more concentrated samples, 50 kDa acid whey was passed through a 1 kDa filter. Western blots could then be carried out to detect the presence of Histatherin.

Ultra Filtration Technique

One technique to concentrate a sample is by ultra filtration.

Method:

2.5 L of 50 kDa permeate of acid whey was thawed out and ultra filtrated through a 1 kDa membrane. Histatherin has been identified as a 6 kDa peptide that can also be found as a 12 kDa dimer. The membrane used will allow any particle in the 50 kDa permeate smaller than 1 kDa to pass through as permeate, and any particle larger than 1 kDa to be recycled to the feed as retenate. The 2.5 L sample was ultra filtrated down to 200 mL, using the MILLIPORE gyrostatic pumping system. This gave an initial 12.5 fold increase in concentration.

The 200 mL retenate was then ultra filtrated through another 1 kDa membrane using a smaller pumping system to give a final volume of 30 mL. This gave another 7 fold increase in concentration giving a total of an 84 fold concentration increase.

The 30 mL sample was then diafiltrated with Milli-Q water to dilute the salt concentration. The sample was then tested for antimicrobial activity via western analysis. The figure below shows a simple flow diagram for an ultra filtration set up in the laboratory.
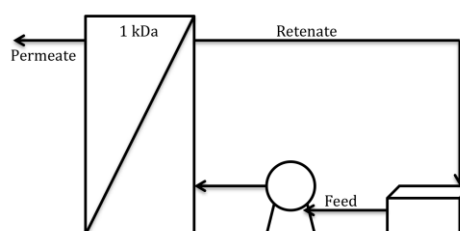

Figure 2: Ultra Filtration flow diagram.

Western Analysis Technique

Commonly used to detect the presence of a particular protein using Polyacrylamide Gel Electrophoresis

(PAGE) transferred onto either Nitrocellulose (NC) or Polyvinylidene Fluoride (PVDF) membrane. Identification of the particular antimicrobial peptide is due to the binding of a specific set of primary and secondary antibodies targeted to that peptide followed by Enhanced Chemiluminescence (ECL). The result can be captured using XAR film due to the short emission of light caused by the ECL application (Dr A. Molenaar, personal communication, 2013).

Method:

1. Determine the protein concentrations of collected samples.
2. Combine a defined amount with Lamelli buffer and boil.
3. Run samples on a 10% Bis Tris gel.
4. Transfer using an iBlot device (Invitrogen semi-dry transfer) onto polyvinylidene fluoride (PVDF) membrane, or transfer using Bio-Rad Trans blot cell apparatus (wet transfer) with PVDF membrane.
5. Fix with 1/500 dilution of 25% glutaraldehyde in phosphate buffer saline and Tween 20 without movement.
6. Rinse once with MQ water and Ponceau stain the membrane, de-stain with MQ water and capture an image.
7. With Roti-block or 4% non-fat milk, block with rocking.
8. Wash once with TBT with rocking
9. Apply primary antibody at 1/10 k (Roti Block) or 1/ 2.5 k (4% non-fat milk powder) with 40 ml TBT with rocking.
10. Wash with TBT while rocking, repeat three times.
11. Apply secondary antibody at 1/15 k (Roti Block) or 1/10 k (4% non-fat milk powder) with 40 ml with rocking.
12. Wash with TBT while rocking, repeat three times.
13. Conducted ECL detection. Repeat until an acceptable signal to noise ratio is achieved.

Immunohistochemistry Technique

Tissue samples were also available to undergo immunohistochemistry techniques to identify the presence of Histatherin. The biological tissue is mounted onto sample slides, which are then subjected to a process of rehydrating, antigen retrieval, blocking, and antibody exposure followed by counter staining and dehydrating. The indication of antibody-antigen interaction is due to the antibody being bound to an enzyme, such as peroxidase, which produces a dark brown colour (Dr A. Molenaar, personal communication, 2014).

Method:

Hydrate:
1. Xylene, 100% Ethanol, 90% Ethanol, 70% Ethanol followed by Milli-Q water.
   Antigen Retrieval:
2. Heat slide in citrate buffer to reduce the formaldehyde cross-links. Let the slide cool.
3. Replace citrate buffer with TBT buffer, circle around tissue sample with PAP pen. PAP pen adheres best to dry slide but keep the tissue moistened with TBT. Figure 3 below shows where lines would be drawn over two tissue samples to divide up the negative and positive controls to allow for different concentrations of antibody to be applied.
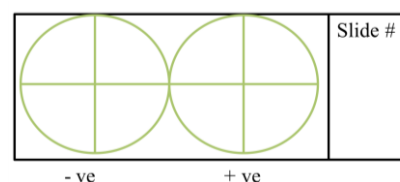

Figure 3: Slide with divisions for primary antibody dilutions, divisions drawn using a PAP pen.

Blocking:
4. Block with Avidin, rinse slide with TBT.
5. Block with Biotin, rinse with TBT.
6. Block with dual endogenous enzyme block, rinse with TBT.
7. Block in 2.5% BSA in TBT buffer, tap slide onto paper towel and dab around sides.
Primary Antibody:
8. Add primary antibody. Dilute in Amplifying Antibody Dilution Buffer. Wash in TBT three times.
9. Incubate with anti-rabbit-biotin link. Dilute 1:50 in Amplifying Antibody Dilution Buffer. Wash in TBT three times.
10. Incubate with streptavidin-HRP. Dilute 1:50 in Amplifying Antibody Dilution Buffer. Wash in TBT three times.
11. Apply DAB substrate. Rinse in Milli-Q water.
Dehydrate:
12. 70% Ethanol, 90% Ethanol, 100% Ethanol, Xylene.
13. Mount tissue in DPX

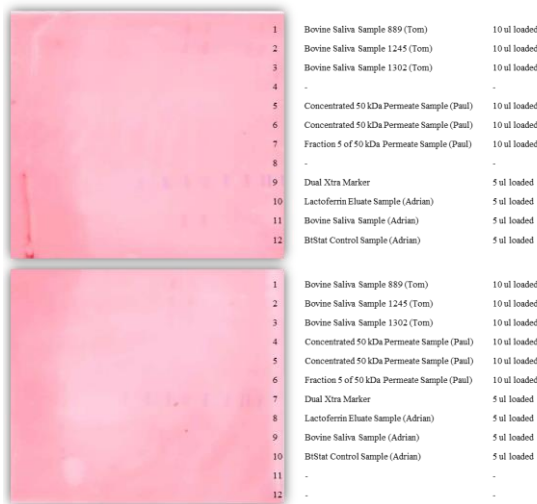## 3. Results and Discussion
Western Blot

Figure 4: Ponceau Stain of Sample (Western Blot).

Samples of bovine saliva, concentrated 50 kDa acid whey permeate, lactoferrin eluate and a control lane of synthetic Histatherin were loaded onto two electrophoresis gels (one positive control gel and one negative control gel), then transferred onto two membranes. The protein is fixed onto the membranes, which can then be stained to give a visual identification of protein present in the samples.

Figure 4 above indicated protein in the bovine saliva samples loaded into lanes 1 and 2. The visible bands are due to the concentration of protein being high enough for detection when immersed in a ponceau stain. The remaining lanes, which have no visible bands of protein, may not have a protein concentration high enough to be detected or did not transfer completely from the gel to the membrane.

Figure 5: Coomassie Stain of Sample Gel (Western Blot).

Once the samples are loaded onto electrophoresis gels they are left to run under a voltage that causes the protein to move down the wells. The gels can be used to transfer protein onto membranes, as described above, once transferred the gels can be soaked in a coomassie stain where any remaining protein in the gels becomes visible.

Figure 5 above, indicated that samples of bovine saliva and lactoferrin elute had remaining protein left in the gels. The lanes with no visible bands were either transferred completely to the membrane or do not have a high enough protein concentration to be detected.
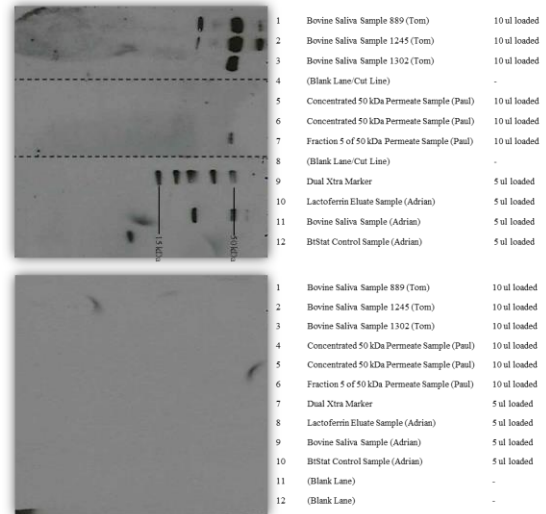

Figure 6: ECL of Sample (Western Blot).

The positive control had a primary antibody applied to the membrane. The negative control had a non-immune serum applied to the membrane. The membranes then had a secondary antibody applied to them before being subjected to ECL treatment.

The primary antibody used, was raised by injecting a rabbit with a small amount of antigen (protein/peptide) and allowing their natural immune system to generate antibodies. The use of serum from a non-immune rabbit, i.e. a rabbit without exposure to the target antigen was used as the negative control. The identification occurs through the specificity of the particular antibody for the targeted protein.

The role of the secondary 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. The secondary antibody used in this protocol 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 as shown in Figure 7.
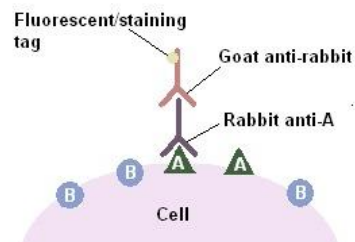

Figure 7: Antibody-Antigen Complex.

During the ECL treatment the complex is completed by the addition of the enzyme, which allows enhanced

chemiluminescence (ECL) detection to be conducted. This method of detection is based on the oxidation reaction that occurs when the enzyme and hydrogen peroxide react with luminol. The result is a short period of light emission that can be captured through the use of XAR film, which is then developed as shown in Figure 6 above.

The synthetic Histatherin sample (lane 12) gave an illuminated band around 6 kDa, which is the positive control. The bovine saliva samples in (lanes 1 – 3) gave illuminated bands around 50 kDa and smaller illuminated bands around 25 kDa. The concentrated 50 kDa acid whey permeate in (lanes 4 – 5) had no visible bands. However a fraction of the 50 kDa acid whey permeate (lane 6) that was collected after being subjected to HPLC, using a Superdex 75 peptide column, during initial studies gave an illuminated band around 50 kDa. The negative control had no illuminated bands indicating that there was no non-specific binding.
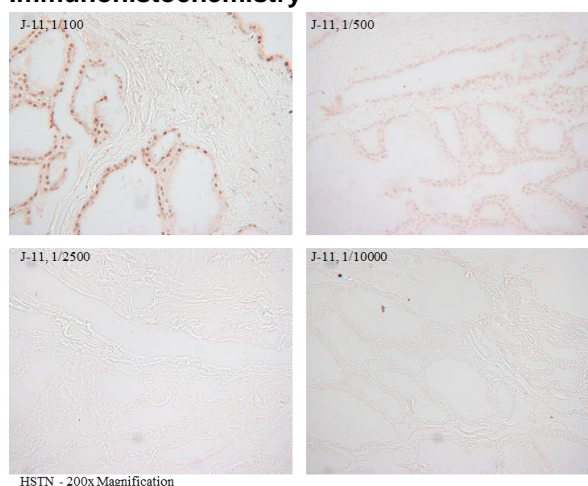
Immunohistochemistry

Figure 8: Positive Control (Immunohistochemistry).

Tissue samples of sheep and cattle were subjected to immunohistochemistry techniques to identify the presence of Histatherin in the tissue cells. The biological tissue was mounted onto sample slides, which were then subjected to a process of rehydrating, antigen retrieval, blocking, and antibody exposure followed by counter staining and dehydrating.

The indication of antibody-antigen interaction is due to the antibody being bound to an enzyme, which produces a dark brown colour. This method is an indirect method involving a primary antibody that binds to the target antigen in the tissue and a secondary antibody that reacts with the primary antibody. As mentioned above, the secondary antibody must be raised against the IgG of the animal species in which the primary antibody has been raised. The primary antibodies used in this technique were the same as in the western analysis. However two secondary antibodies are applied in this technique, the first being an anti-rabbit-biotin followed by streptavidin-HRP.

Figure 8 above shows the results after applying 4 different concentrations of primary antibody. The 1/100 concentration is the highest concentration applied and indicated that there was antibody-antigen interaction due to the brown stain. As the concentrations decrease from 1/100, 1/500, 1/2500 and 1/10000 the brown stain becomes less apparent. This result indicated that for the reaction to give an observable result the concentration of antibody should be made in dilutions lower than 1/100.
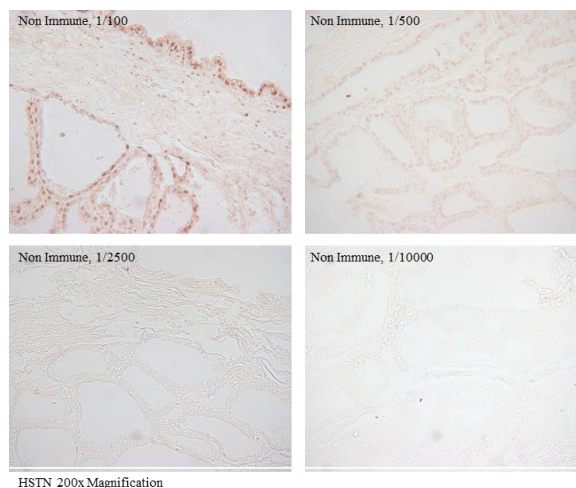

Figure 9: Negative Control (Immunohistochemistry).

Figure 9 is the result of the negative control. Due to the reaction producing a brown colour on the negative control as well as the positive control it can be concluded that there may be some non-specific binding. This protocol however was only the initial trials to become familiar with the technique and can be further optimised.

4. Conclusions

From the results achieved it would be recommended to produce higher protein concentrations of lactoferrin eluate and 30 – 50 kDa permeate to gain more accurate results. Collecting larger fraction volumes will require time dedicated to chromatography to achieve higher protein concentrations. The possibility of using a larger column could be investigated as the column used has a load limit of 500 ul.

The western analysis results have shown bands appearing at sizes larger than 6 kDa in saliva therefore an investigation into the binding of histatherin to other larger proteins or the post-translational modifications of histatherin is recommended. The saliva fractions gave clear bands, which may be investigated further. An earlier theory was that glycosylation may have accounted for the apparently poor detection of the native protein in milk compared to the synthesised protein. Previous research by Chanelle Gavin, a previous student researcher, has been conducted investigating the

glycosylation status of native histatherin and results indicated that it may not be glycosylated.

Due to time constraints the immunohistochemistry technique had not been optimised however it is a promising technique to determine the presence of Histatherin in bovine mammary cells. There were some concerns with the purity and storage of the reagents used in this protocol, it is recommended to use fresh reagents to ensure the results

5. Acknowledgements

6. References

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