# PE4.103 Antimicrobial Activity of Bovine Lung and Liver Sarcoplasmic/cytoplasmic Proteins Hydrolysates 375.00

*Jenny Hayes (1) jenny.hayes@teagasc.ie, Roberta di Bernardini( 1), Declan Bolton 1, Joe Kerry 2 Eileen O'Neill 2 Annemaria Mullen 1*

*(1)Teagasc, Ashtown Food Research Cente, Ashtown, Dublin 15, Ireland*

*(2)University College Cork, Cork, Ireland*

**Abstract — The aim of this work was to compare two different antimicrobial assays in examining the antimicrobial activity of liver and lung protein hydrolysates against a range of gram positive and gram negative micro-organisms. Cytoplasmic/sarcoplasmic proteins from bovine lung and liver proteins were hydrolyzed for 0, 4 and 24 hrs with a commercial enzyme. Antimicrobial activity was investigated using the well plate antimicrobial assay and showed that the liver and lung protein hydrolysates had no antimicrobial activity against *E coli Salmonella enterica Typimurium DT104*, *Yersinia entercolita Listeria Monocytogenes* or *Staphlococcus aureus*. A liquid growth inhibition assay was then employed to assess the antimicrobial activity of liver protein hydrolysates at 0 to 24 hr hydrolysis time against *E. coli* and *Listeria Monocytogenes*. The results showed that liver protein hydrolysates inhibited the growth of *E. coli* from 60 to 98%. No inhibition was recorded against *Listeria Monocytogenes*.**

**Keywords:** antimicrobial, bioactive peptides, bovine by-products, hydrolysate, lung, enzymatic hydrolysis

J. Hayes is with the Teagasc, Ashtown Food Research Centre, Dublin 15, Ireland (e-mail: jenny.hayes@teagasc.ie).

R. Di Bernardini is with the Teagasc, Ashtown Food Research Centre, Dublin 15, Ireland (corresponding author to provide phone: +35318059500; fax: +35318059550; e-mail: roberta.dibernardini@teagasc.ie).

D. Bolton is with the Teagasc, Ashtown Food Research Centre, Dublin 15, Ireland (e-mail: declan.bolton@teagasc.ie).

J.P. Kerry is with the Department of Food and Nutritional Sciences, University College Cork, Ireland (e-mail: joe.kerry@ucc.ie).

E.E. O'Neill is with the Department of Food and Nutritional Sciences, University College Cork, Ireland (e-mail: e.oneill@ucc.ie).

A.M. Mullen is with the Teagasc, Ashtown Food Research Centre, Dublin 15, Ireland (e-mail: anne.mullen@teagasc.ie).

## I. INTRODUCTION

Bioactive peptides are described as food-derived components (genuine or generated) that in addition to their nutritional value exert a physiological effect in the body [1]. In recent years there is a growing interest on the food industry to try to reduce its reliance on synthetic chemical preservatives. As a result, manufacturers are urged to develop alternative preservatives that are based on natural compounds.

It has been recognized that dietary proteins provide a rich source of biologically active peptides. Diverse protein sources including milk, soya, fish and beef have been utilised for the production of peptides ([2], [3], [4], [5] and [6]).

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health [7]. Such peptides maybe inactive within the sequence of the parent protein and can be activated through hydrolysis by endogenous enzymes, through hydrolysis by proteolytic microorganisms and by the hydrolytic action of commercial enzymatic proteolysis. The activity is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to reveal multifunctional properties [8]. They are generally low molecular weight peptides (<5 kDa).

It is generally accepted that peptides released from food proteins may exhibit different biological activities including antimicrobial activity. Antimicrobial peptides are naturally occurring molecules that have shown promise as effective antimicrobial agents. In the past it has been shown that food proteins could also act as microbial peptide precursors and, in this way may enhance the organism's natural defenses against invading pathogens and as a result food proteins can be considered as components of nutritional immunity [9].

Recently, antimicrobial peptides have received much interest due to their potential uses as new generation antibiotics. Due to the fact that antimicrobial peptides are active against a wide range of pathogenic microorganisms makes them attractive candidates for possible use as antibiotics [10][11], bio-preservatives [12], anticancer agents [13], and for

enhancing disease resistance in aquaculture [14]. Antimicrobial peptides have been identified from many protein hydrolysates such as bovine milk and hen eggs [15][16][17]. Bioactive peptides may be used as components in functional foods due to their therapeutic potential for treatment or prevention of diseases [18].

Recovery of value from meat by-products is an increasing problem for the meat industry, hence there is potential to produce functional peptides from bovine offal such as lung where the cost of the raw material is inexpensive and the amount of waste is reduced.

The objective of the present work was to optimize a method to investigate the antimicrobial activity of enzymatic hydrolysates of bovine lung and liver hydrolyzed at several time points with a commercial enzyme.

## II. MATERIALS AND METHODS

*Materials and chemicals:* Organ tissue liver and lung samples were collected at time of slaughter from the abattoir at Ashtown Food Research Centre, Dublin, Ireland and stored at -80°C until analysis. All other chemicals were of reagent grade or purer.

*Preparation of cytoplasmic/sarcoplasmic enzymatic hydrolysates:* Cytoplasmic/sarcoplasmic proteins were extracted from the bovine tissues using 0.02 M phosphate buffer [5], using a SPEX Prep freezer mill grinder. The extraction procedure and enzyme hydrolysis (see below) was monitored using 1D SDS polyacrylamide gel electrophoresis using the method of [19] using 12.5% resolving gels and 4% stacking gels, with some modifications. Briefly, the running buffer contained 1.92 M glycine and 1% sodium dodecyl sulphate. The resolving gel contained Protogel (41.62%), protogel buffer (25.01%), water (32.81%), ammonium persulphate (0.5%), and N,N,N',N'-tetramethylethylenediamine (TEMED) (0.05%).

A modified version of Biuret assay [20] was used for the determination of protein content of the hydrolysates. The Biuret reagent was prepared as followed: 9.0 g of sodium potassium tartrate were dissolved in 500 ml of 0.2N NaOH. Three g of $CuSO_4.5H_2O$ were added and dissolved with stirring. Five grams of potassium iodide were added and diluted to 1000 ml with 0.2N NaOH. Three dilutions of samples were prepared. Biuret reagent (250 µl) was added to 150 µl of diluted sample. The reaction mixtures were incubated at 37°C for 10 min and samples were read at 540 nm in UV-Cuvettes Micro (Plastibrand 7592-20) using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Milton Keynes). Bovine serum albumin was used as standard. The beef extracts were hydrolyzed in triplicate with a commercial enzyme at three hydrolysis time points, with a substrate to enzyme ratio of 100:1 w/w. The enzymatic hydrolysis was stopped by boiling samples at 99°C for 10 minutes. Samples were then centrifuged at 14,000 RPM for 10 minutes and the supernatants were collected and stored at -80°C until analysis.

*Ultrafiltration*: Beef protein hydrolysates were separated into a large molecular weight fraction and a low molecular weight fraction by ultrafiltration at 4°C by PM-10 membrane (MWCO: 10,000; Amicon Co., Beverly, MA, USA). Prior to use, the membrane was activated by spinning 15 ml of distilled water, and the remaining liquid was carefully removed. The antimicrobial analysis was carried out on the <10 kDa ultrafiltrated fraction only.

*Determination of antimicrobial activity*: Two approaches were taken in the development of a method for determining antimicrobial of bovine protein hydrolysates (<10 kDa).

Firstly, the well plate inhibition assay was employed. Three hydrolysis time points were examined (0, 4 & 24 hr) for both liver and lung protein hydrolysates. Five bacterial species were used as test micro-organisms for determination of antimicrobial activity: three Gram-negative (*E coli* (NCTC 09001), *Salmonella enterica Typimurium DT104* (CTC 13348) and *Yersinia entercolita* (NCTC 11599) and two Gram-positive *Listeria Monocytogenes* (NCTC 11994) and *Staphlococcus aureus* (NCTC 07428). A bacterial suspension of each bacteria was adjusted to give a final concentration of $10^{-4}$ to $10^{-6}$ were added to the petri dish then spread evenly. The enzymatic hydrolysate was diluted in 0.02M phosphate buffer (pH 7.4) to give a standard protein concentration of 2.5 µg/µl and was added (20µl) to wells punched in the Muller-Hinton agar (Oxoid CM0337). All plates were allowed to diffuse in the layer and incubated at 37°C for 24 hr except *Yersinia entercolita* which was incubated at 30 °C for 24 hr. The antimicrobial activity was measured as the diameter of the zone of growth inhibition by comparison to a negative sodium phosphate buffer (Method 1).

Secondly, the liquid growth inhibition assay was employed. The antimicrobial activity of liver protein hydrolysates following enzymatic

hydrolysis at 0, 2, 4, 6, 8, 10 and 24 hrs against *E coli* (NCTC 09001) and *Listeria Monocytogenes* (NCTC 11994) and grown up over night at 37°C in nutrient agar (1ml:19ml hydrolysate:Muller hinton agar per plate) and then 20 ml was poured into each petri dish and allowed to set. A bacterial suspension of each bacterium was adjusted to give a final concentration of $10^{-6}$. *E coli* and *Listeria Monocytogenes* (0.1 ml) was evenly spread over the agar and incubated for 24 hr at 37°C. The results for were expressed as % inhibition for each bacterium.

## III. RESULTS AND DISCUSSION

Using the well plate inhibition assay bovine liver and lung protein hydrolysates (<10 kDa) showed no zones of inhibition, hence showing no microbial activity against *E coli, Salmonella enterica Typimurium DT104* and *Yersinia entercolita Listeria Monocytogenes* and *Staphlococcus aureus.* The sample volume was increased from 10 µg/µl to 20 µg/µl and the well diameter reduced from 5 mm to 2 mm but no difference in results were recorded.

The liquid growth inhibition antimicrobial assay was then employed to detect any antimicrobial activity against a gram positive (*E. coli*) and a gram negative microorganism (*Listeria Monocytogenes*). Results showed that in liver protein hydrolysates inhibited the growth of *E. coli* (Table 1) at the 7 hydrolysis time points examined. The inhibition of *E. coli* ranged from 60 to 98%. No inhibition was detected against the gram negative *Listeria Monocytogenes*. This positive result observed with liver protein hydrolysates against growth inhibition of *E. coli* implies that the well plate inhibition assay needs to be further developed as the liver hydrolysate showed no inhibition using well plate method and strong inhibition using liquid growth inhibition assay. In well plate assay, the sample volume may have been too small resulting in insufficient contact with the bacteria.

**Table 1: Antimicrobial activity of liver protein hydrolysates at 7 hydrolysate time points against *E. coli* & Listeria *Monocytogenes* using the liquid growth inhibition method**

| Bacteria strain | Hydrolysis time (hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 24 |
| | (% inhibition) | | | | | | |
| E. coli | 98 | 98 | 65 | 95 | 70 | 60 | 95 |
| Listeria *Monocytogenes* | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

## IV. CONCLUSION

The liquid growth inhibition assay was successful in identifying antimicrobial activity in liver hydrolysates against *E. coli.* The well plate assay was not successful in detecting antimicrobial activity in liver or lung bovine hydrolysate samples. The bovine liver and lung enzymatic hydrolysates used in this work are currently been tested for further antimicrobial activity and additional studies are also been carried out to investigate whether they have other bioactive properties such as antihypertensive, antioxidant and antithrombotic properties.


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## REFERENCES


[1] Vermeiressen, V., Van Camp, J. & Verstrate, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. British Journal of Nutrition, 92, 357-366.

[2] Amiot, J., Germain, L., Turgeona, S., Lemaya, M., Ory-Salama, C. & F.A. Augerb. (2004). Peptides from milk protein hydrolysates to improve the growth of human keratinocytes in culture, International Dairy Journal, 14 619–626.

[3] Chiang, W.D., Tsou, M.J., & Tsai Z.Y. and Tsai, T.C. (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. Food Chemistry, 98,725–732

[4] Gibbs, B.F. Zougman, A., Masse R. & Mulligan, Catherine, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Research International 37, 123–131.

[5] Jang, A. and Lee, M. (2005). Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates, Meat Science 69, 653–661.

[6] Picot, L., Bordenave, S., Didelot, S., Fruitier-Arnaudin, I., Sannier F. & Thorkelsson G. et al., (2006). Antiproliferative activity of fish protein hydrolysates on human breast cancer cell lines. Process Biochemistry 41, 1217–1222.

[7] Kitts, D.D & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Current Pharmaceutical Design 9, 1309–1323

[8] Meisel H. & FitzGerald, R.J. Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. (2003) Current Pharmaceutical Design 9, pp. 1289–1295

[9] Pellegrini, A. (2003). Antimicrobial peptides from food proteins. Current Pharmaceutical Design, 9, 1225-1238.

[10] Hancock, R.E.W. and Sahl, H.G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, Nat. Biotechnol. 24, 1551–1577.

[11] Saiman, L. Tabibi, S. Starner, T.D. Gabriel, P.S. Winokur, P.L. Jia, H.P. McCray Jr. P.B. & Tack, B.F. (2001). Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis, Antimicrob. Agents Chemother. 45, 2838–2844.

[12] Anderson, R.C., Wilkinson, B. & Yu, P.L. (2004). Ovine antimicrobial peptides: new products from an age-old industry, Aust. J. Agr. Res. 55, pp. 69–75.

[13] Cho, E., Smith-Warner, S.A., Spiegelman, D., Beeson, W.L., van den Brandt, P.A., Colditz, G.A., et al. (2004). Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies, J. Natl. Cancer Inst. 96, 1015–1022.

[14] Jia, X., Patrzykat, A., Devlin, R.H., Ackerman, P.A., Iwama, G.K. & Hancock, R.E.W. (2000). Antimicrobial peptides protect Coho Salmon from Vibrio anguillarum infections, Appl. Environ. Microbiol. 66, 1928–1932.

[15] Takahashi, M. Moriguchi, S., Yoshikawa M. & Sasaki, R. (1994). Isolation and characterization of oryzatensin - a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin, Biochem Mol Biol Int, 33, 1151–1158.

[16] Hayes, A., Ross, R.P., Fitzgerald, G.F., Hill C. and Stanton, C. (2006). Casein-derived antimicrobial peptides generated by Lactobacillus acidophilus DPC6026, Appl Environ Microbiol, 72, 2260–2264.

[17] McCann, K.B., Shiell, B.J., Michalski, W.P., Lee, A., Wan, J., Roginski H. & Coventry, M.J. (2006). Isolation and characterisation of a novel antibacterial peptide from bovine $\alpha_{S1}$-casein, Int Dairy J, 16, 316–323.

[18] Vercruysse, L., Camp, J. V., Smagghe, G. (2005). ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: a review. Journal of Agriculture and Food Chemistry, 53, 8106-8115.

[19] Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685

[20] Gornall, A. G., Bardawill, C. J., David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. Journal of Biological chemistry, 177, 751-766