UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF NOVEL ANTIMICROBIAL PEPTIDE HUMAN BETA DEFENSIN 9 AND ITS EFFICACY AGAINST COMMON EYE PATHOGENIC BACTERIA IN DIFFERENT SALT CONCENTRATION

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By

NURUL ATIKAH BINTI AB HAMID

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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April 2018

Chairman : Ho Kok Lian, PhD
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Beta defensins are small cationic peptide secreted by the surface epithelium in response to microbial threat. The human beta-defensin 9 (hBD9) is a relatively new defensin and its role as antimicrobial agent has not been described previously. In this study, *Escherichia coli Mach 1* and *E.coli BL21 (DE3)* were used as cloning and protein expression hosts respectively. HBD9 gene was fused to a protein expression vector using the small ubiquitin-related modifier (SUMO) fusion system. The soluble hBD9-SUMO fusion protein expressed was purified by Ni-IDA immobolised metal affinity chromatography (IMAC) and SUMO was removed by SUMO protease. This study defined the novel antimicrobial peptide hBD9 and its efficacy against common Gram-positive and negative bacteria. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were chosen because they are common bacteria that causing eye infection. The Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) of the hBD9 were determined for these microbes. Interestingly, hBD9 showed antimicrobial activity against *Staphylococcus aureus* but not *Pseudomonas aeruginosa*. Salt sensitivity and cationic concentration were tested by incubation with NaCl and $MgCl_2$ using liquid culture containing hBD9 to analyse the effects of NaCl and $MgCl_2$ concentration on the antimicrobial activity. Antimicrobial activity against *Staphylococcus aureus* of hBD9 was not supressed by NaCl and $MgCl_2$. All the antimicrobial property of the hBD9 protein against the common bacteria was verified by using conventional methods. The results improved the current knowledge on the hBD9 and its antimicrobial properties. This will drive towards the discovery of a broad spectrum, safe by effective resistant-free antibiotic in the future. The finding of this study can be used as a reference in the future investigation and it might benefits scientific community to better by others scientist or researchers to increase understanding and find new effective antibiotics.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN ANTIMIKROBIAL PEPTIDE BETA DEFENSIN 9 MANUSIA DAN KEBERKESANANNYA TERHADAP PATOGENIK BAKTERIA MATA DALAM PELBAGAI KEPEKATAN GARAM

Oleh

NURUL ATIKAH BINTI AB HAMID

April 2018

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Defensin beta adalah peptida kationik kecil yang dirembeskan oleh epitelium permukaan sebagai tindak balas terhadap ancaman mikrob. Defensin beta manusia 9 (hBD9) adalah defensin yang agak baru dan peranannya sebagai agen antimikrobial tidak digambarkan sebelum ini. Dalam kajian ini, *E.coli* Mach 1 digunakan untuk pengklonan klon dan *E.coli* BL21 (DE3) digunakan sebagai tuan rumah ekspresi. Sistem penggabungan yang berkaitan ubiquitin kecil (SUMO) digunakan. Protein gabungan yang disahkan telah disucikan oleh IMAC Ni-IDA dan dipotong oleh protease SUMO. Kajian ini menerangkan novel antimikrobial peptide hBD9 dan keberkesanannya terhadap bakteria biasa Gram positif dan negatif. *Staphylococcus aureus* dan *Pseudomonas aeruginosa* dipilih kerana bakteria biasa yang menyebabkan jangkitan mata. Kepekatan menghalang Minimal (MIC) dan kepekatan bakterisial Minimal (MBC) hBD9 telah menentukan untuk organisma. Yang penting, hBD9 menunjukkan aktiviti antimikrobial terhadap *Staphylococcus aureus* tetapi bukan *Pseudomonas aeruginosa*. Kepekaan garam diuji dengan inkubasi dengan NaCl dan konsentrasi kationik diuji dengan inkubasi dengan MgCl ke dalam kultur cair yang terkandung hBD9 untuk menganalisis kesan kepekatan NaCl dan MgCl terhadap aktiviti antimikroba. Aktiviti antimikrobik terhadap *Staphylococcus aureus* hBD9 tidak ditekan oleh NaCl dan MgCl. Semua sifat antimikrobial protein hBD9 terhadap bakteria biasa disahkan dengan menggunakan kaedah konvensional. Hasilnya meningkatkan pengetahuan semasa mengenai hBD9 dan sifat antimikrobanya.Ini akan mendorong kepada penemuan spektrum yang luas, selamat oleh antibiotik bebas tahan yang berkesan pada masa akan datang. Maklumat baru ini boleh digunakan sebagai rujukan dalam penyiasatan masa depan. Analisis ini mungkin bermanfaat oleh saintis atau penyelidik yang lain untuk meningkatkan kefahaman dan mencari antibiotik yang berkesan baru.

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All gratification are referred to Allah

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I certify that a Thesis Examination Committee has met on 16 April 2018 to conduct the final examination of Nurul Atikah binti Ab Hamid on her thesis entitled "Production of Novel Antimicrobial Peptide Human Beta Defensin 9 and its Efficacy Against Common Eye Pathogenic Bacteria in Different Salt Concentrations" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Signature: _____
Name of Chairman of Supervisory Committee: Dr. Ho Kok Lian

Signature: _____
Name of Member of Supervisory Committee: Associate Professor Dr. Rukman Awang Hamat

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LIST OF ABBREVIATIONS

%	Percent
>	More than
° C	Degree celcius
µl	Microliter
ϴ	Delta
µM	Micrometer
α	Alpha
β	Beta
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
BNBD	Bovine neutrophil β-defensin
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
CFU	Colony forming unit
Co^{2+}	Cobalt
Cu^{2+}	Copper
DNA	Deoxyribonucleic acid
DEFB109	Human beta defensin 9 gene
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Chemiluminescene
ECM	Extracellular matrix
et al.,	and others
E1	Ubiquitin-activating *enzymes*

FPLC	*Fast protein liquid chromatography*
FPSK	*Fakulti perubatan sains kesihatan*
GST	*Glutathione S-transferase*
HA	*Hemagglutinin antigen*
HBD1	Human beta defensin 1
HBD2	Human beta defensin 2
HBD3	Human beta defensin 3
HBD5	Human beta defensin 5
HBD6	Human beta defensin 6
HBD9	Human beta defensin 9
HCl	Hydrochloric acid
HDP	Host defence peptide
HNP	Human neutrophil peptide
HRP	Horse radish peroxidase
HSV	Herpes Simplex Virus
IL-1	Interlukien-1
IL-1β	Interlukien-1 Beta
IPTG	Isopropyl-D-thiogalactoside
KCl	Potassium chloride
kDa	Kilo Dalton
K_2HPO_4	Potassium phosphate
LB	Luria bertani
LC	Lethal concentration
M	Molar
MBC	Minimal bactericidal concentration

MBP	Maltose binding protein
MIC	Minimal inhibitory concentration
Mg^{2+}	Magnesium
MgCl	Magnesium chloride
mg	Microgram
mL	Millilitre
mM	Milimeter
mRNA	Messenger RNA
NaCl	Sodium chloride
Na_3PO_4	Sodium phosphate
Ni^{2+}	*Nickel*
NK	Natural killer cell
NLRs	NOD like receptors
Nus A	Protein Nus A
OD	Optical density
PAMPs	Pathogen associated molecular patterns
pH	Potential hydrogen
PCR	Polymerase chain reaction
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SARs-CoV	Severe acute respiratory syndrome coronavirus
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	Super optimal catabolite repression

SUMO	Small ubiquitin like modifier
TBS	Tris buffered saline
TBS-T	Tris buffered saline with detergent tween 20
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor – alpha
TRX	Thioredoxin
U	Unit
UB	Ubiquitin
UK	United Kingdom
Ulp 1	Ubl-specific protease 1
USA	United State of America

CHAPTER 1

INTRODUCTION

1.1 Background

The term antimicrobial peptides (AMPs) are descriptive for a family of peptides with antimicrobial properties. Various AMPs have been isolated from many classes of all kingdoms and they are classified based on their structures and amino acid composition (R. Koczulla & Bals, 2007). Besides having broad antimicrobial spectrum activities, AMPs are also involved in cellular processes such as immune induction, wound healing, cell proliferation, cytokine release, protease-antiprotease balance, chemotaxis and angiogenesis (Szyk et al., 2006).

AMPs play an important reaction towards pathogens. They have small endogenous cationic particles that are released by epithelial and phagocytic cells. The antimicrobial events of AMPs are focused towards bacteria, viruses, yeast and fungi. Once AMPs reached their target microbial membrane, they kill the microbial host through various mechanisms (Szyk et al., 2006). AMPs function's supported in innate and adaptive immunity by do their roles as immunomodulatory activity like leukocyte migration and direct inactivation.

Among the AMPs, defensins are the major AMPs developed in ocular surface (McDermott, 2009a). Defensins are the largest family of antimicrobial peptides and also they are the most studied. To date approximately 300 defensins have been identified from a wide range of organisms including mammals, plants, birds, invertebrates, and recently in the ebony-cup fungus. Defensins are a group of peptides that are part of the basic components of the host defense system. They can identify and neutralize microorganisms rapidly and specifically (Dhople, Krukemeyer, & Ramamoorthy, 2006). They are synthesized as preproteins and undergo post translational process before transformed into a biologically active peptide.

Defensins are divided into alpha- and beta-defensins depending on their disulfide bridging pattern. The α- and β-defensins are able to induce the release of cytokines, histamine and prostaglandin D2 from the mast cells and alter cell migration and maturation. In addition, defensins are classically described as antimicrobial peptides and are also involved in immune modulation, fertility, cell development and wound healing.

Native human α-defensin peptide also known as human neutrophil peptides (HNP) can be isolated from neutrophils. The first defensin was isolated from the rabbit alveolar macrophages and the first defensin discovered in multicellular organisms is α-defensins (Selsted, Brown, DeLange, & Lehrer, 1983).

Beta defensins, which belong to the family of mammalian defensins, form part of the defence system rendering the epithelial surfaces resistance to microbial colonization and they also play a role in the innate immune system. β-defensins genes are liable for the production of antimicrobial peptides that are found in the white blood cells such as granulocytes, macrophages, Natural Killer (NK) cells and also in the epithelial cells.

α-defensins was derived from infiltrating neutrophils. McDermott, 2009 reported that ocular surface epithelial cells synthesized and secreted the β-defensins (McDermott, 2009b). Both cornea and conjunctival expressed the β-defensins. Human beta-defensin 1 (hBD1) has been shown effectively expressed while human beta-defensin 2 (hBD2) expression is variable because it is infrequently expressed by normal tissues (Haynes, Tighe, & Dua, 1998). The expression of human beta-defensin 4 (hBD4) in cultured ocular surface of epithelial cells has been detected but in non-cultured samples it is only found irregularly (McIntosh et al., 2005). Human beta-defensin 3 (hBD3) also has been shown to be effective against *Pseudomonas aeruginosa* and *Staphylococcus* strains (Dua, Otri, Hopkinson, & Mohammed, 2014). In addition human beta-defensin 5 (hBD5) and human beta-defensin 6 (hBD6) were found to have antimicrobial activities against *Escherichia coli* but not *Staphylococcus aureus* (Huang, Ching, Jiang, & Leong, 2008).

A study on expression of a novel β-defensin gene, *DEFB 109* has been discovered in the ocular surface epithelia (Abedin, Mohammed, Hopkinson, & Dua, 2008). Human Beta Defensin 9 (hBD9) is a newly discovered defensin and has not been isolated and fully characterised. HBD9 was first reported on the ocular surface by Abedin and co-workers in 2008 (Abedin et al., 2008)(Abedin et al., 2008). Previous study demonstrated that hBD9 mRNA is expressed constitutively but subsequently down regulated in the presence of ocular surface infection and inflammation (Abedin et al., 2008). This is in good agreement with the earlier findings reported by Premratanachai et al (2004) who demonstrated that *in vitro* stimulation of gingival keratinocytes with *Candida albicans* down-regulate the hBD9 mRNA after the stimulation (Premratanachai et al., 2004). Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are two main receptor of innate immune response. These receptor have high affinity for pathogen recognition and cytokine modulation and AMP expression (Akira, Uematsu, & Takeuchi, 2006). A study on hBD9 found it has a unique mechanism. HBD9 expression can be downregulated and upregulated in a time dependent manner in response to the stimulation of different TLRs and NLRs with specific PAMs and IL-1β (Mohammed et al., 2010). Different expression of hBD9 suggest that it may have other roles, like in between innate and adaptive immune system and immunomodulation to the attributed antimicrobial response (Mohammed et al., 2010).

At the protein expression level, hBD9 is constitutively expressed in all region of normal ocular surface tissue and it is primarily localised at the basal epithelial cells of the conjunctiva but more superficially in cornea epithelium (Mohammed et al., 2010). The existing data interestingly points towards possibly different or additional roles it

could plays in the host immune system including in the ocular surface against invading pathogens.

Application of fusion technology is notably effective to enhance the solubility and expression level of recombinant proteins. With respect to their advantages, fusion partners are categorized differently. These advantages include improvement of recombinant protein solubility, recombinant protein expression level, reduction in proteolytic degradation of the recombinant protein, and simplicify the purification protocol and detection.

Traditionally, hBD9 was expressed using conventional pET fusion system. These conventional protein expression systems produced low level of recombinant hBD9, which was also associated with protein insolubility, non-specific proteolysis, and high enzyme to substrate ratio as well as narrow pH and urea tolerance for protein stability. In addition, the isolation of the target protein following cleavage required multiple chromatographical steps with variable retrieval rates. Overall, this has increased the production cost. In order to improve the protein production yield, solubility and stability, hBD9 gene has been cloned into a pET-SUMO protein expression system. The fusion protein of hBD9 and SUMO was expressed by Isopropyl-β-D-thiogalactoside (IPTG) induction and purified with immobilized metal affinity column (IMAC) attached to a Fast Protein Liquid Chromatography (FPLC) system. The SUMO protein and its 6x His-tagged was removed by SUMO protease before the cleaved hBD9 was reversed purified with another round of IMAC chromatography. Purified hBD9 was assayed for its biological activity using antimicrobial assay

1.2 Objectives

1.2.1 General objectives

The general objective was to produce antimicrobial peptide hBD9 and determined its antimicrobial efficacy against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in various salt concentrations.

1.2.2 The specific objectives of this study includes:

a. To express and purify the hBD9 protein
b. To determine antimicrobial activity of hBD9 protein against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

REFERENCES

Abedin, A., Mohammed, I., Hopkinson, A., & Dua, H. S. (2008). A Novel Antimicrobial Peptide on the Ocular Surface Shows Decreased Expression in Inflammation and Infection. *Investigative Opthalmology & Visual Science*, *49*(1), 28. http://doi.org/10.1167/iovs.07-0645

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*. http://doi.org/10.1016/j.cell.2006.02.015

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*, *48*(suppl 1), 5–16. http://doi.org/10.1093/jac/48.suppl_1.5

Befus, A. D., Mowat, C., Gilchrist, M., Hu, J., Solomon, S., & Bateman, A. (1999). Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action. *Journal of Immunology (Baltimore, Md. : 1950)*, *163*, 947–953. http://doi.org/ji_v163n2p947 [pii]

Bensch, K. W., Raida, M., Mägert, H.-J., Schulz-Knappe, P., & Forssmann, W.-G. (1995). hBD-1: a novel β-defensin from human plasma. *FEBS Letters*, *368*, 331–335. http://doi.org/10.1016/0014-5793(95)00687-5

Bharathi, Mj., Amuthan, M., Viswanathan, S., Ramesh, S., & Ramakrishnan, R. (2010). Prevalence of bacterial pathogens causing ocular infections in South India. *Indian Journal of Pathology and Microbiology*, *53*(2), 281. http://doi.org/10.4103/0377-4929.64336

Boman, H. G. (2000). Innate immunity and the normal microflora. *Immunological Reviews*, *173*(1), 5–16. http://doi.org/10.1034/j.1600-065X.2000.917301.x

Brogden, K. A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*, *3*(3), 238–250. http://doi.org/10.1038/nrmicro1098

Brown, K. L., & Hancock, R. E. W. (2006). Cationic host defense (antimicrobial) peptides. *Current Opinion in Immunology*. http://doi.org/10.1016/j.coi.2005.11.004

Delcour, A. H. (2009). Outer membrane permeability and antibiotic resistance. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1794*(5), 808–816. http://doi.org/10.1016/j.bbapap.2008.11.005

Dhople, V., Krukemeyer, A., & Ramamoorthy, A. (2006). The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochimica et Biophysica Acta - Biomembranes*. http://doi.org/10.1016/j.bbamem.2006.07.007

Dua, H. S., Otri, A. M., Hopkinson, A., & Mohammed, I. (2014). In vitro studies on the antimicrobial peptide human beta-defensin 9 (HBD9): signalling pathways and pathogen-related response (an American Ophthalmological Society thesis). *Transactions of the American Ophthalmological Society*, *112*, 50–73.

Ganz, T. (2003). The Role of Antimicrobial Peptides in Innate Immunity. *Integrative and Comparative Biology*, *43*(2), 300–304. http://doi.org/10.1093/icb/43.2.300

Ganz, T., & Lehrer, R. I. (1995). Defensins. *Pharmacology & Therapeutics*, *66*(2), 191–205. http://doi.org/10.1016/0163-7258(94)00076-F

Ganz, T., Selsted, M. E., Szklarek, D., Harwig, S. S., Daher, K., Bainton, D. F., & Lehrer, R. I. (1985). Defensins. Natural peptide antibiotics of human neutrophils. *Journal of Clinical Investigation*, *76*(4), 1427–1435. http://doi.org/10.1172/JCI112120

Groenink, J., Walgreen-Weterings, E., Van 'T Hof, W., Veerman, E. C. I., & Nieuw Amerongen, A. V. (1999). Cationic amphipathic peptides, derived from bovine and human lactoferrins, with antimicrobial activity against oral pathogens. *FEMS Microbiology Letters*, *179*(2), 217–222. http://doi.org/10.1016/S0378-1097(99)00414-0

Hancock, R. E. W., & Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defences. *Trends in Microbiology*. http://doi.org/10.1016/S0966-842X(00)01823-0

Hancock, R. E. W., & Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*. http://doi.org/10.1038/nbt1267

Harder, J., Bartels, J., Christophers, E., & Schröder, J.-M. (1997). A peptide antibiotic from human skin. *Nature*, *387*(6636), 861–861. http://doi.org/10.1038/43088

Harder, J., Siebert, R., Zhang, Y., Matthiesen, P., Christophers, E., Schlegelberger, B., & Schröder, J. M. (1997). Mapping of the gene encoding human beta-defensin-2 (DEFB2) to chromosome region 8p22-p23.1. *Genomics*, *46*(3), 472–5. http://doi.org/10.1006/geno.1997.5074

Haynes, R. J., Tighe, P. J., & Dua, H. S. (1998). Innate defence of the eye by antimicrobial defensin peptides. *Lancet*, *352*(9126), 451–452. http://doi.org/10.1016/S0140-6736(05)79185-6

Haynes, R. J., Tighe, P. J., & Dua, H. S. (1999). Antimicrobial defensin peptides of the human ocular surface. *British Journal of Ophthalmology*, *83*(6), 737–741. http://doi.org/10.1136/bjo.83.6.737

Hiratsuka, T., Nakazato, M., Date, Y., Ashitani, J., Minematsu, T., Chino, N., & Matsukura, S. (1998). Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochemical and Biophysical Research Communications*, *249*(3), 943–947.

Hochstrasser, M. (2010). NIH Public Access, *458*(7237), 1–19. http://doi.org/10.1038/nature07958.Origin

Hoover, D. M., Chertov, O., & Lubkowski, J. (2001). The structure of human beta-defensin-1: new insights into structural properties of beta-defensins. *The Journal of Biological Chemistry*, *276*(42), 39021–6. http://doi.org/10.1074/jbc.M103830200

Huang, L., Ching, C. B., Jiang, R., & Leong, S. S. J. (2008). Production of bioactive human beta-defensin 5 and 6 in Escherichia coli by soluble fusion expression. *Protein Expression and Purification*, *61*(2), 168–174. http://doi.org/10.1016/j.pep.2008.05.016

Izadpanah, A., & Gallo, R. L. (2005). Antimicrobial peptides. *Journal of the American Academy of Dermatology*, *52*(3), 381–390. http://doi.org/10.1016/j.jaad.2004.08.026

Jentsch, S., & Pyrowolakis, G. (2000). Ubiquitin and its kin: How close are the family ties? *Trends in Cell Biology*, *10*(8), 335–342. http://doi.org/10.1016/S0962-8924(00)01785-2

Johnson, E. S., Schwienhorst, I., Dohmen, R. J., Blobel, G., & Ju, R. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *Embo Journal*, *16*(18), 5509–5519. http://doi.org/10.1093/emboj/16.18.5509

Kaushal, A., Gupta, K., & Van Hoek, M. L. (2016). Characterization of Cimex lectularius (bedbug) defensin peptide and its antimicrobial activity against human skin microflora. *Biochemical and Biophysical Research Communications*, *470*(4), 955–960. http://doi.org/10.1016/j.bbrc.2016.01.100

Keymanesh, K., Soltani, S., & Sardari, S. (2009). Application of antimicrobial peptides in agriculture and food industry. *World Journal of Microbiology and Biotechnology*. http://doi.org/10.1007/s11274-009-9984-7

Kimple, M. E., Brill, A. L., & Pasker, R. L. (2013). Overview of affinity tags for protein purification. *Current Protocols in Protein Science*, (SUPPL.73). http://doi.org/10.1002/0471140864.ps0909s73

Koczulla, A. R., & Bals, R. (2003). Antimicrobial peptides: Current status and therapeutic potential. *Drugs*. http://doi.org/10.2165/00003495-200363040-00005

Koczulla, R., & Bals, R. (2007). CHAPTER 10 MODULATE ANGIOGENESIS Abstract :, *49*(0), 191–196.

Kunt, H. (2016). Investigating Science Student Teachers ' Ideas about Function and Anatomical Form of Two Human Sensory Organs , the Eye and the Ear. *International Journal of Environmental & Science Education*, *11*(5), 535–542. http://doi.org/10.12973/ijese.2016.406a

Lan, W., Petznick, A., Heryati, S., Rifada, M., & Tong, L. (2012). Nuclear factor-κB: Central regulator in ocular surface inflammation and diseases. *Ocular Surface*, *10*(3), 137–148. http://doi.org/10.1016/j.jtos.2012.04.001

Lehrer, R. I., Daher, K., Ganz, T., & Selsted, M. E. (1985). Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. *Journal of Virology*, *54*(2), 467–72. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=254818&tool=pmcentrez&rendertype=abstract

Liu, A. Y., Destoumieux, D., Wong, A. V., Park, C. H., Valore, E. V., Liu, L., & Ganz, T. (2002). Human ??-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *Journal of Investigative Dermatology*, *118*(2), 275–281. http://doi.org/10.1046/j.0022-202x.2001.01651.x

Malakhov, M. P., Mattern, M. R., Malakhova, O. A., Drinker, M., Weeks, S. D., & Butt, T. R. (2004). SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. *Journal of Structural and Functional Genomics*, *5*(1–2), 75–86. http://doi.org/10.1023/B:JSFG.0000029237.70316.52

Marblestone, J. G. (2006). Comparison of SUMO fusion technology with traditional gene fusion systems: Enhanced expression and solubility with SUMO. *Protein Science*, *15*(1), 182–189. http://doi.org/10.1110/ps.051812706

Marblestone, J. G., Edavettal, S. C., Lim, Y., Lim, P., Zuo, X. U. N., & Butt, T. R. (2006). Comparison of SUMO fusion technology with traditional gene fusion systems : Enhanced expression and solubility with SUMO, 182–189. http://doi.org/10.1110/ps.051812706.for

Marx, W. (2012). Tracking historical papers and their citations. *European Science Editing*, *38*(2), 35–37. http://doi.org/10.1023/B

McDermott, A. M. (2004). Defensins and other antimicrobial peptides at the ocular surface. *Ocular Surface*. http://doi.org/10.1016/S1542-0124(12)70111-8

McDermott, A. M. (2009a). The role of antimicrobial peptides at the ocular surface. *Ophthalmic Research*, *41*(2), 60–75. http://doi.org/10.1159/000187622

McDermott, A. M. (2009b). The role of antimicrobial peptides at the ocular surface. *Ophthalmic Research*. http://doi.org/10.1159/000187622

McIntosh, R. S., Cade, J. E., Al-Abed, M., Shanmuganathan, V., Gupta, R., Bhan, A., … Dua, H. S. (2005). The spectrum of antimicrobial peptide expression at the ocular surface. *Investigative Ophthalmology & Visual Science*, *46*(4), 1379–1385. http://doi.org/10.1167/iovs.04-0607

Melchior, F. (2000). SUMO--nonclassical ubiquitin. *Annual Review of Cell and Developmental Biology*, *16*, 591–626. http://doi.org/10.1146/annurev.cellbio.16.1.591

Miller, J. H. (1972). Experiments in molecular genetics. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*, *433*, 352–355. http://doi.org/papers2://publication/uuid/28F839D6-A272-4D50-9114-D2769DE72B38

Mohammed, I., Suleman, H., Otri, A. M., Kulkarni, B. B., Chen, P., Hopkinson, A., & Dua, H. S. (2010). Localization and gene expression of human beta-defensin 9 at the human ocular surface epithelium. *Investigative Ophthalmology & Visual Science*, *51*(9), 4677–4682. http://doi.org/10.1167/iovs.10-5334

Mossessova, E., & Lima, C. D. (2000). Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Molecular Cell*, *5*(5), 865–876. http://doi.org/10.1016/S1097-2765(00)80326-3

Müller, S., Hoege, C., Pyrowolakis, G., & Jentsch, S. (2001). Sumo, ubiquitin's mysterious cousin. *Nature Reviews Molecular Cell Biology*. http://doi.org/10.1038/35056591

Mygind, P. H., Fischer, R. L., Schnorr, K. M., Hansen, M. T., Sönksen, C. P., Ludvigsen, S., … Kristensen, H.-H. (2005). Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature*, *437*(7061), 975–980. http://doi.org/10.1038/nature04051

Panja, S., Saha, S., Jana, B., & Basu, T. (2006). Role of membrane potential on artificial transformation of E. coli with plasmid DNA. *Journal of Biotechnology*, *127*(1), 14–20. http://doi.org/10.1016/j.jbiotec.2006.06.008

Patil, A. A., Cai, Y., Sang, Y., Blecha, F., & Zhang, G. (2005). Cross-species analysis of the mammalian beta-defensin gene family: presence of syntenic gene clusters and preferential expression in the male reproductive tract. *Physiological Genomics*, *23*(1), 5–17. http://doi.org/10.1152/physiolgenomics.00104.2005

Premratanachai, P., Joly, S., Johnson, G. K., McCray, P. B., Jia, H. P., & Guthmiller, J. M. (2004). Expression and regulation of novel human β- defensins in gingival keratinocytes. *Oral Microbiology and Immunology*, *19*(2), 111–117.

Raj, P. A., & Dentino, A. R. (2002). Current status of defensins and their role in innate and adaptive immunity. *Science*, *206*, 9–18.

Riedl, S., Zweytick, D., & Lohner, K. (2011). Membrane-active host defense peptides - Challenges and perspectives for the development of novel anticancer drugs. *Chemistry and Physics of Lipids*, *164*(8), 766–781. http://doi.org/10.1016/j.chemphyslip.2011.09.004

Schibli, D. J., Hunter, H. N., Aseyev, V., Starner, T. D., Wiencek, J. M., Mccray, P. B., … Vogel, H. J. (2002). The Solution Structures of the Human ᴺᴸ -Defensins Lead to a Better Understanding of the Potent Bactericidal Activity of HBD3 against Staphylococcus aureus *, *277*(10), 8279–8289. http://doi.org/10.1074/jbc.M108830200

Schibli, D. J., Hunter, H. N., Aseyev, V., Starner, T. D., Wiencek, J. M., McCray, P. B., … Vogel, H. J. (2002). The solution structures of the human beta-defensins lead to a better understanding of the potent bactericidal activity of HBD3 against Staphylococcus aureus. *The Journal of Biological Chemistry*, *277*(10), 8279–89. http://doi.org/10.1074/jbc.M108830200

Selsted, M. E., Brown, D. M., DeLange, R. J., & Lehrer, R. I. (1983). Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. *Journal of Biological Chemistry*, *258*(23), 14485–14489.

Selsted, M. E., Tang, Y. Q., Morris, W. L., McGuire, P. A., Novotny, M. J., Smith, W., … Cullor, J. S. (1996). Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *The Journal of Biological Chemistry*. http://doi.org/10.1016/S0167-7799(00)88947-7

Sternsdorf, T., Jensen, K., & Freemont, P. S. (2003). Sumo. *Current Biology*, *13*(7), R258–R259. http://doi.org/10.1016/S0960-9822(03)00193-3

Suresh, A., & Verma, C. (2006). Modelling study of dimerization in mammalian defensins. *BMC Bioinformatics*, *7*(SUPPL.5). http://doi.org/10.1186/1471-2105-7-S5-S17

Szyk, A., Wu, Z., Tucker, K., Yang, D. E., Lu, W., & Lubkowski, J. (2006). and HD6, (Zasloff 2002), 2749–2760. http://doi.org/10.1110/ps.062336606.)

Tang, Y. Q., Yuan, J., Osapay, G., Osapay, K., Tran, D., Miller, C. J., … Selsted, M. E. (1999). A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science (New York, N.Y.)*, *286*(5439), 498–502. http://doi.org/10.1126/science.286.5439.498

Valore, E. V, Park, C. H., Quayle, a J., Wiles, K. R., McCray, P. B., & Ganz, T. (1998). Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *The Journal of Clinical Investigation*, *101*(8), 1633–1642. http://doi.org/10.1172/JCI1861

Veldhuizen, E. J. A., Rijnders, M., Claassen, E. A., van Dijk, A., & Haagsman, H. P. (2008). Porcine β-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. *Molecular Immunology*, *45*(2), 386–394. http://doi.org/10.1016/j.molimm.2007.06.001

Wang, Z., Li, H., Guan, W., Ling, H., Wang, Z., Mu, T., … Fang, X. (2010). Human SUMO fusion systems enhance protein expression and solubility. *Protein Expression and Purification*, *73*(2), 203–208. http://doi.org/10.1016/j.pep.2010.05.001

Weber, K., Pringle, J. R., & Osborn, M. (1972). Measurement of Molecular Weights by Electrophoresis on SDS-Acrylamide Gel. *Methods in Enzymology*, *26*(C), 3–27. http://doi.org/10.1016/S0076-6879(72)26003-7

Willoughby, C. E., Ponzin, D., Ferrari, S., Lobo, A., Landau, K., & Omidi, Y. (2010). Review Anatomy and physiology of the human eye : effects of mucopolysaccharidoses disease on structure and function – a review, (May), 2–11. http://doi.org/10.1111/j.1442-9071.2010.02363.x

Xu, Z., Zhong, Z., Huang, L., Peng, L., Wang, F., & Cen, P. (2006). High-level production of bioactive human beta-defensin-4 in Escherichia coli by soluble fusion expression. *Applied Microbiology and Biotechnology*, *72*(3), 471–479. http://doi.org/10.1016/j.reactfunctpolym.2005.10.008

Zanetti, M., Gennaro, R., & Romeo, D. (1995). Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Letters*. http://doi.org/10.1016/0014-5793(95)01050-O

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, *415*(0028–0836 (Print)), 389–395. http://doi.org/10.1038/415389a

Zelezetsky, I., & Tossi, A. (2006). Alpha-helical antimicrobial peptides-Using a sequence template to guide structure-activity relationship studies. *Biochimica et Biophysica Acta - Biomembranes*. http://doi.org/10.1016/j.bbamem.2006.03.021

Zhao, L., & Lu, W. (2014). Defensins in innate immunity. *Current Opinion in Hematology*, *21*(1), 37–42. http://doi.org/10.1097/MOH.0000000000000005

Zimmerman, G. R., Legault, P., Selsted, M. E., & Pardi, A. (1995). Solution structure of bovine á-defensin-12: the peptide fold of the á-defensins is identical to that of the classical defensins. *Biochemistry*, *34*, 13663–13671.

Zuo, X., Li, S., Hall, J., Mattern, M. R., Tran, H., Shoo, J., … Butt, T. R. (2005). Enhanced expression and purification of membrane proteins by SUMO fusion in escherichia coli. *Journal of Structural and Functional Genomics*, *6*(2–3), 103–111. http://doi.org/10.1007/s10969-005-2664-4