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Venom Peptides Lasioglossin II and Mastoparan B as Escherichia coli ATP synthase Inhibitors

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Venom Peptides Lasioglossin II and Mastoparan B as *Escherichia coli* ATP synthase Inhibitors

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by

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ABSTRACT

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The inhibitory effects on *Escherichia coli* ATPase activity by two venom peptides, lasioglossin II and mastoparan B. Membrane bound F_1F_O ATP synthase was isolated from *E. coli* strain pBWU13.4/DK8 and treated with varied concentrations of lasioglossin II and mastoparan B. Lasioglossin II caused very low inhibition of ATPase activity, but the inhibition profile of mastoparan B was suggestive of an interesting biological effect. A relatively shorter total length, a smaller net positive charge, and a reduced amphipathic character of both peptides, as compared to previously tested antimicrobial peptides, may account for the limited degree of inhibition observed in the present study.

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CHAPTER 1

INTRODUCTION

Several natural and synthetic compounds have been described as inhibitors of ATP synthase. One such group of inhibitors are Antimicrobial Peptides (AMPs). Several possible mechanisms have been described regarding the activity of AMPs. The most accepted models are membrane permeabilization and cell death (apoptosis) by either toroidal pore model, barrel stave model, or carpet model. Mechanism of activity studies suggest that AMPs seem to connect with negatively charged, anionic phospholipid layers thereby injecting itself into the bacterial cell membrane. AMPs can also extend over the cell membrane by passive transport upsetting some of the cellular activities in there. Dermaseptins seem to induce a non-pore dependent cytolytic process that results in breakdown and miscellization of the membrane bilayer (Shai 2002). A previously examined hypothesis concerning the antimicrobial effects of AMPs is based on their possible inhibitory effects on ATP synthase. This hypothesis was motivated by the observation that several other peptides which conform to basic amphiphilic α-helical structures are reported to bind at the βDELSEED-loop of *E. coli* ATP synthase (Ahmad et al. 2015).

Ahmad and Laughlin (2010) discussed the concept that amphibian AMPs with possible inhibitory effects on ATP synthase, through binding at the βDELSEED-loop, would be relatively short positively charged peptides of ~10-30 amino acid residues, with α-helical secondary structure, and has antibacterial or anticancer effects. Previous studies suggested that in a state of low adenosine-5‘-triphosphate (ATP) and high H^+ gradients concentrations, the C-terminal α-helical domain of the ε-subunit of F_1-ATPase experience enormous structural changes then join with the $\alpha_3\beta_3$ hexagon ring, where it is places in the vicinity of the βDELSEED-loop (Ahmad et

al. 2015). Electrostatic interactions between basic residues of the ε-subunit and the acidic residues of βDELSEED-motif result in inhibition of ATPase activity (Hara et al. 2001b; Tsunoda et al. 2001; Suzuki et al. 2003). Inhibition of ATP synthase has been demonstrated to defend or delay cell damage by conserving ATP during ischaemia (Vuorinen et al. 1995). Because short linear cationic α-helical peptides with antimicrobial activity would be fascinating candidates as therapeutic agents, I examined the venom peptides mastoparan B and lasioglossin II as possible inhibitors of *Escherichia coli* ATP synthase.

Hypothesis

Venom peptides Mastoparan B and lasioglossin II can inhibit *Escherichia coli* ATP synthase activity.

ATP synthase is a ubiquitous multi-subunit membrane enzyme that participate and plays a major role in cellular energy production. This membrane bound enzyme is found in almost every living cell. ATP synthase is highly conserved and structurally homologous in all organisms. In prokaryotes, ATP synthase is embedded in the plasma membrane, but it is located in the thylakoid membrane of chloroplasts of higher plants, and in the inner membrane of mitochondria in eukaryotic cells. ATP synthase is also spot on the exterior of various mammalian cell kinds, including endothelial cells, adipocytes, and keratinocytes (Hong and Pedersen 2008). ATP synthase is the terminal enzyme in the oxidative phosphorylation pathway. It activates ATP synthesis by oxidative phosphorylation or by photophosphorylation in the membranes of cells and organelles (Ahmad and Laughlin 2010). ATP synthase utilizes energy from a proton motive force generated by the electron transport chain to catalyze the production

of adenosine-5‘-triphosphate (ATP) from adenosine-5‘-diphosphate (ADP) and inorganic phosphate (Pi). In some bacteria, ATP synthase reverses this activity by functioning as an ATP-driven proton pump that generates a proton (H^+) gradient. The energy available from ATP is used for a number of cellular processes, including active transport of molecules across cell membranes, transport of nutrients, motility, nucleic acid synthesis, and protein synthesis.

Inhibitors of ATP Synthase

The inhibitory effects on ATP synthase, and the range of inhibition are fluctuating on a molar scale among various inhibitors. Inhibitors can be classified based on bond formation (Gledhill and Walker 2006) and other physical and chemical properties (Hong and Pedersen 2008).

Based on bond formation with ATP synthase, they are grouped into two types.

1. The covalent inhibitors include 4-chloro-7-nitrobenzofurazan (NBD-Cl), N,N´-dicyclohexylcarbodiimide (DCCD), 8-azido-ATP, 2-azido-ATP, 5´-p-fluorosulphonyl-benzoyladenosine and 5´-p-fluorosulphonylbenzoylinosine.
2. The non-covalent inhibitors include natural inhibitor protein IF1, amphiphilic peptides, non-peptidyl lipophilic cations, polyphenolic phytochemicals, non-hydrolysable substrate analogues, aurovertins and efrapeptins.

Based on other physical and chemical properties of inhibitors, they are grouped into nine types.

1. Peptide inhibitors include:
    - α-Helical basic peptide inhibitors: include the natural inhibitor protein IF1, bacterial/chloroplast ε subunit, antimicrobial peptides (AMP) like melittin,

dermaseptin, maculatin, aurein and venom peptides such as mastoparan B and lasioglossin II

- Angiostatin and enterostatin
- Leucinostatins and efrapeptins
- Tentoxin and its derivatives

Efrapeptin is an effective strong inhibitor of soluble ATP synthase compared to other compounds known to inhibit this enzyme like aurovertin and citreoviridin, efrapeptin is capable of causing maximium inhibition (Jackson et al. 1979). The binding site for the F_1 targeting inhibitor efrapeptin is found in α, β and γ subunits (Abrahams et al. 1996) while that of aurovertin B is primarily confined to the β subunit (van Raajj et al. 1996).

2. Polyphenolic phytochemicals, estrogens and structurally related compounds include:
    - Flavones and isoflavones: including quercetin, kaempfero, thymoquinone, morin apigenin, genistein, biochanin A and daidzein.
    - Stilbenes: including resveratrol, piceatannol, diethylstilbestrol (DES), diisothiocyanatostilbene- 2,2-disulfonic acid (DIDS) and 4-acetamido-4-isothiocyanostilbene 2,2 -disulfonate (SITS).
    - Steroidal estradiols and estrogen metabolites: including 4-hydroxyestradiol, 2-hydroxyestradiol, 17-α-estradiol, 17-β-estradiol α-zearalenol and β-zearalenol.
    - Other polyphenolic phytochemicals: including epicatechin gallate (ECG), epigallocatechin gallate (EGCG), curcumin, phloretin, theaflavin and tannic acid.

Oestradiol and several other oestrogens have been shown to inhibit the activity of rat brain F_OF_1-ATPase (Zheng and Ramirez, 1999). A synthetic oestrogen, diethylstilbestrol has also been reported to inhibit rat liver F_OF_1-ATPase (McEnery and Pedersen 1986; McEnery et al. 1989).

Flavones like quercetin, which are weak oestrogenic phytochemicals, have been reported to inhibit bovine and porcine heart F_OF_1-ATPase (Lang and Racker 1974; Di Pietro et al. 1975). Thymoquinone, a major phytochemical compound found in the black seeds of the medicinal plant *Nigella sativa*, was shown to strongly inhibit ATPase activity and bacterial growth (Ahmad et al. 2015). Piceatannol inhibits ATP synthase by targeting the F_1 sector and exhibits mixed inhibition of F_1-ATPase activity (Zheng and Ramirez 1999). Resveratrol was found to show a non-competitive inhibition of F_1-ATP synthase activity (Zheng and Ramirez 2000).

3. Polyketide inhibitors that are polymers of two carbon ketide units synthesized by the enzyme polyketide synthases. These types include natural macrolides like apoptolidin, cytovaricin, oligomycin, peliomycin, ossamycin, and venturicidin.

Oligomycin is the inhibitor that led to the name 'F_O' for the intramembrane hydrophobic region of ATP synthase. Oligomycin inhibits the apoptosis incited by Bax, a pro-apoptotic Bcl-2 family member protein found on the mitochondrial outer membrane, and ATP synthase activity is needed for the killing of cells by Bax (Matsuyama et al. 1998). Oligomycin also activate apoptosis in tumour cells (Wolvetang et al. 1994).

4. Organotin compounds and structural relatives: These are tin containing organic compounds. They are grouped into R_4Sn, R_3SnX, R_2SnX_2, and $RSnX_3$. Examples include; tributyltin chloride, tricyclohexyltin hydroxide, triethyltin sulfate, triphenyltin chloride and triethyllead, etc (Von Ballmoos et al. 2004).
5. Polyenic α-pyrone derivatives: α-Pyrone (a six membered cyclic unsaturated ester) and its derivatives are known to inhibit ATP synthase. Derivatives include aurovertin, citreoviridin and asteltoxin (Hong and Pedersen 2008).
6. Cationic inhibitors:

- Amphiphilic cationic dyes containing a basic amine group and a lipophilic portion. They include rhodamine 123, acridine orange, rhodamine 6G, rosaniline, rhodamine B, malachite green, quinacrine, pyronin Y, safranin O, nile blue A and ethidium bromide (EtBr).
- Tertiary amine local anesthetics (TALAs) which consist of an aromatic group, an middle chain, and a terminal amine group. They include tetracaine, dibucaine, procaine, lidocaine, chlorpromazine, trifluoperazine, trifluoperazine and propranolol.
- Other organic cations such as octyl guanidine, spermidine, atrazine, PDT-ferrous chelate and DPBP-ferrous chelate (Hong and Pedersen 2008).

7. Substrates and substrate analogs that include phosphate analogs, divalent metal ions, purine nucleotides and nucleotide analogs.
8. Amino acid modifiers that include amino group modifiers, carboxyl group modifiers, cysteine and tyrosine residue modifiers and histidine residue modifiers
9. Miscellaneous inhibitors that include dicarbopolyborate, almitrine, n-butanol, 4,4´-dichlorodiphenyltrichloroethane (DDT) and 4,4´-dichlorodiphenyltri- chloroethane (DMSO) (Hong and Pedersen 2008).

Antimicrobial Peptides

Antimicrobial peptides are a unique and diverse group of active biological molecules that are produced by different organisms as a valuable and crucial constituent of their innate immune system. Most AMPs have similar features such as cationicity and amphipathicity. They are generally comprised of 12 to 50 amino acids, but differ in several characteristics including their

primary structure, the presence of disulphide bonds, and in a number of posttranslational changes like amidation of C-terminal residues, pyroglutamation of N-terminal glutamine residues, sulphation of tyrosine, hydroxylation of proline, and racemisation of certain L-amino acids to their D-isomers (Bevins and Zasloff 1990; Erspamer 1994). Based on their secondary structure AMPs follow four structural themes: α-helical peptides, β-stranded peptides, extended peptides and β-hairpin or loop peptides (Dhople et al. 2006). Previous studies have demonstrated that they often have wide spectrum activity against viruses, fungi, bacteria, protozoans, parasites and cancerous cells.

The basic function of AMPs is host protection by making use of cytotoxicity to pathogenic organisms. AMPs bind to negatively charged residues of microbial membranes where peptide complexes form pores that break down the cell membrane or disturb metabolic processes in the target. They also play immune modulators in more advanced organisms (Zanetti 2004). In addition to their antimicrobial function, AMPs may be used as antitumor agents, contraceptive agents, mitogenic agents, drug delivery vectors, and signaling molecules in signal transduction pathways (Kamysz et al. 2003). Laughlin and Ahmad 2010 report the first candid experimental proof of amphibian antimicrobial peptides as potential *E. coli* ATP synthase inhibitors testing samples from both purified F_1 - ATPase and membrane bound F_1F_O - ATP synthase. Currently, there are more than 2700 entries into the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/main.php Wang et al. 2015), out of which 2255 (83.14%) are known to have antibacterial property, 988 (36.43%) have antifungal property, 196 (7.22%) are anticancer peptides, and 177 (6.52%) are identified as having antiviral activity. The diversity of antimicrobial peptides apparently reflects adaptation to the particular microbial communities associated with the environments of different species (Boman 2000).

Linear cationic α-helical Peptides; Mastoparans and Lasioglossins

Linear cationic α-helical peptides are small peptides rich in hydrophobic and basic amino acid residues. They are usually 10 to 45 amino acid residues long. They can adopt α-helical amphipathic secondary structure in the cell membrane environment, or in the presence of such membrane, and thereby have effects similar to substances such as trifluoroethanol (TFE) or sodium dodecyl sulfate (SDS). They are often found in insects, and amphibian skin, as well as arthropod venoms, and possess antimicrobial activity against a wide range of bacteria (Cerovsky et al. 2008). Venoms are a highly evolved and rich source of natural cocktails of biochemically active enzymes, proteins, peptides and other molecules, each of which plays a defined role through interaction with highly specific molecular targets. Venom peptides have very strong and selective effects, and may serve as potential drugs or scaffolds for drug design. Many venom peptides are used in proof-of-concept studies *in vivo*, with many undergoing preclinical or clinical development for use in the treatment of diabetes, epilepsy, multiple sclerosis, cardiovascular disorders, pain, cancer and other neurological disorders. Captopril (Capoten; Anakena) from the Brazilian arrowhead viper (*Bothrops jaracusa*), was the first model of a successful venom-based drug. This drug inhibits angiotensin converting enzyme (ACE), a vital enzyme in the production of angiotensin, which is in turn a vasoconstrictor affiliated with hypertension (Cushmann and Ondetti 1991). Chlorotoxin, a peptide derived from the venom of the desert scorpion *Leiurus quinquestriatus*, has been established as an effective treatment for malignant glioma (Soroceanu et al. 1998). Exenatide, a synthetic analogue of exendin-4, the venom peptide of the gila monster lizard *Heloderma suspectum*, is ready for use as a prescription medicine in the treatment of Type 2 diabetes and related metabolic disorder (Nielsen et al. 2004).

Mastoparans are the mast cell degranulating peptides from the venoms of vespid wasps (Vespidae), has been identified as the dominant peptide found in the venoms of several species of hornets (Nakajima 1984). They are tetradecapeptides characterized by 7 - 10 hydrophobic amino acid residues and from 2 - 4 lysine residues in their basic sequence (Nakajima 1986). Mastoparans also form the most abundant class of peptides in the venoms of social wasps (Hirai et al. 1979). Their biological characteristics include the activation of enzymes like phospholipase A_2, phospholipase C, guanylate cyclase and G proteins (Higashijima et al. 1990; Dong-Li et al. 1993). Mastoparan is an effective stimulator of exocytosis in many mammalian cell types. Mastoparan causes the secretion of histamine from mastocytes, catecholamines from pheochromocytes, serotonin from thrombocytes, and prolactin from the adenohypophysis (Hirai et al. 1979; Kuroda et al. 1980; Kurihara et al. 1986). When mastoparan interact with the phospholipid bilayer it creates an α-helical structure that aligned to the surface of the membrane, the hydrophobic region is inside the bilayer with its four positive charges (three Lys residues and a terminal amino group) lining the exterior (Higashijima 1983; Wakamatsu et al. 1983).

Mastoparan B from the venom of the hornet species *Vespa basalis* has high mast cell degranulation activity, a mastocytotropic peptide, with well-defined amino-acid sequence different from the other vespid mastoparans. Explicit differences in amino acid sequence were present at positions 1, 2, 5, 8 and 9, where mastoparan B has less hydrophobic amino acids like Leu^1, Lys^2, $Ser^{5,8}$ and Trp^9, rather than Ile^1, Asn^2, $Ala^{5,8}$ and Leu^9 or other amino acids as seen in many other *Vespa* mastoparans (Hirai et al. 1979). When compared to the sequence of the prototypic mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) (Hirai et al. 1979), mastoparan B may present a more hydrophilic surface, potentially causing differences in its biological activity.

Lasioglossins are members of a distinct group of AMPs that are found in the venom of the wild eusocial bee *Lasioglossum laticeps*. These peptides exhibit strong antimicrobial activity, low haemolytic activity, and cytotoxic activity against various cancer cells *in vitro* (Cerovsky et al. 2008). Lasioglossins has a net charge of +6 fitting within the broad class of essential AMPs with net charge varying from +4 to +6, which symbolize the maximum charge for antimicrobial activity (Tossi et al. 2000). They exhibit low mast cell degranulation activity. The VNWK sequence structure of lasioglossins is similar to the INWK sequence structure of a few mastoparans obtained from other species of social wasps (Čeřovský et al. 2008).

Vespa basalis Mastoparan B sequence

H-Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH_2

Lasioglossum laticeps Lasioglossin II (LL-II) sequence

H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2

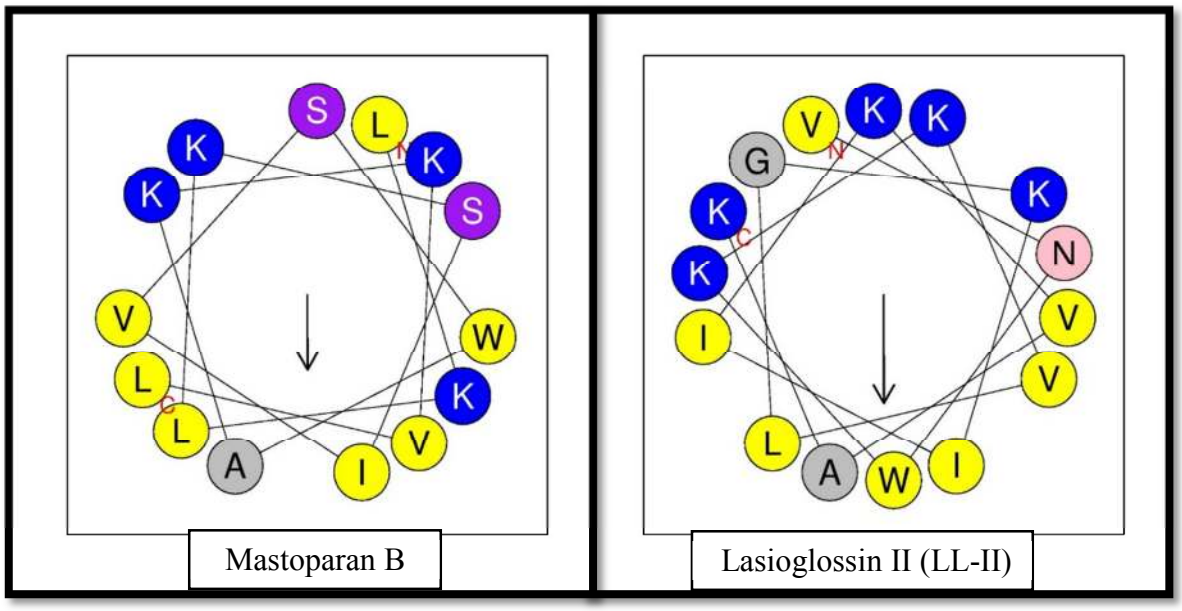

Figure 1. Helix-wheel plots of venom peptides sequence mastoparan B and lasioglossin II (LL-II). The size of the downward arrow ↓ reflects the extent of hydrophobicity. Hydrophobic and hydrophilic faces are shown in yellow and blue colors respectively. (http://heliquest.ipmc.cnrs.fr/ Gautier et al. 2008).

Potential Mechanism of Action of Antimicrobial Peptide

Antimicrobial peptides are thought to be structured such that they interact with a biomembrane. The initial mechanism by which AMPs target pathogens occurs by means of an electrostatic synergy between positive charged antimicrobial peptides and the negatively charged lipid membranes of bacteria. In receptor-mediated membrane interactions, some antimicrobial peptides expressly bind to bacterial lipid II, a negatively charged membrane bound constituent tangled in peptidoglycan synthesis (Yeaman and Yount 2003).

After initial membrane binding, AMPs may go into a second phase of membrane connection known as the threshold concentration. Peptides infiltrate and crisscross the lipid

bilayer by mean of several probable methods, amplifying their antimicrobial activity to focus on the inner cell membrane. Theoretically, the threshold concentration needed to propel such occurrence is caused by a build-up of peptides on the cell membrane. A number of factors including tendency to self-associate or multimerization, peptide concentration, fluidity, phospholipid membrane constitution, and head group size likely influence this threshold (Yang et al. 2000). After membrane binding, another major event happening is the peptide structural or conformational phase transformation. The peptides immediately adopt a well-structured amphipathic α-helical conformation in membrane mimetic solvents, or upon interaction with phospholipid bilayers. β-sheet antimicrobial peptides are consistently organized in membrane environments and aqueous solution, because of constraints dictated by cyclization of the peptide backbone or disulfide bonds. Self-assemble or multimerize of antimicrobial peptides may occur following initial binding with the target cell membranes. Peptide-lipid and peptide-peptide interactions inside membranes apparently form elaborate structures affiliated with individual antimicrobial peptide mode of action (Yeaman and Yount 2003).

Membrane permeation by amphipathic α-helical or β-sheet peptides is thought to proceed via one of the following mechanisms:

1. In the Barrel Stave Mechanism, the ‘stave’ caption alludes to individual transmembrane rods within this barrel, which consist of single peptides or peptide complexes. Here, varying amount of channel building peptides are placed in a barrel like loop about the aqueous pores. The hydrophobic planes of α-helical or β-sheet peptide faces outside towards the acyl chains of the membrane, while the hydrophilic planes make the pore lining (Breukink and Kruijff 1999).

2. In the Toroid Pore or Wormhole Mechanism, peptides in the outer cellular environment assume the α-helical form as they correspond with the negatively charged hydrophobic bacterial membrane. Lipids are inserted with peptides in the transmembrane channel, forming a structure known as the supramolecular complex, which epitomize a membrane-spanning pore lined with the polar surfaces of peptides and the phospholipid head groups. The hydrophobic residues of the connected peptides dislocate the polar head groups, generating a gap in the hydrophobic plane and activating positive curvature strain in the cell membrane (Hara et al. 2001a). The commencement of strain and thinning appears to further destabilize the membranes surface quality making it also liable to change with subsequent peptide connections. Some peptide may become displaced to the cytoplasmic leaflet of the membrane upon fragmentation of the pore. Uematsu and Matsuzaki (2000) suggest that toroid pore break down might be the major mechanism through which peptides move into the microbial cytoplasm to affect possible intracellular destinations.
3. The Carpet Mechanism model, some peptides oppose microorganisms in a comparably diffuse mode. This is a model of nonspecific membrane permeabilization by AMPs which includes widespread results that have been compared to detergents. However, peptides in this mechanism are not indiscriminate membrane detergents. Here, a high mass of peptide aggregates on the target membrane surface (Yeaman and Yount 2003). Phospholipid dislocation, decline in membrane barrier properties and/or alterations in membrane fluidity may consequently leads to membrane disruption.

Peptides can develop membrane pores in microbe cells as explained earlier, this results in the discharge of metabolites and ions, biopolymer synthesis and loss of membrane coupled respiration, subsequent depolarization, and eventually cell death. However, evidence also

supports complementary mechanisms including inhibition of intracellular processes and inhibition of extracellular biopolymer synthesis as mechanisms involved in cell death (Yeaman and Yount 2003).

History of ATP Synthase Research

A brief history of ATP synthase written by Boris A. Feniouk (2010) and published on http://www.atpsynthase.info/History.html is summarized in the following. Karl Lohmann, a biochemist at Kaiser Wilhelm Institute for Experimental Medicine in Heidelberg, Germany discovered ATP from muscle and liver extract in 1929. A Russian biochemist Vladimir Engelhardt in 1935 noted that ATP was part of a coenzyme complex associated with respiration and is required for muscle contraction. ATP had been thought-out to be the final product of catabolic reactions. In 1937, Herman Kalckar entrenched that ATP synthase is connected to cell respiration. The caption oxidative phosphorylation was coined in 1939 by Belitser and Tsibakova. From 1939 to 1941, Fritz Lipmann demonstrated that ATP is the principal carrier of chemical energy in the cell. He also invented the catchphrase "energy rich phosphate bonds". Slater developed a hypothesis involving chemical intermediates to clarify the mechanism of oxidative phosphorylation in 1953. Coupled oxidative phosphorylation was reported to be triggered by the soluble factor in the bacterial particulates in 1956. In 1960, Penefsky and his group reported that soluble adenosine triphosphatase (ATPase) participated in oxidative phosphorylation, it was isolated and termed factor 1 or F_1 by Ephraim Racker in 1961. In 1961, Robert J.P. Williams proposed that there are no energy rich intermediates however protons help to transfer energy to ATP synthase from the photosynthetic proteins and respiration chain enzymes. Also in 1961, Peter Mitchell reported his chemiosmotic hypothesis, affirming the

basic function of membranes that divide the two sections, and suggesting that the membrane proteins provide a gradient of protons created by the respiration chain enzymes, which are then used by ATP synthase but not by the chemical intermediates. In 1964, Paul D. Boyer suggested that ATP molecule is synthesized owing to structural adjustments in the ATP synthase enzyme and in 1973 Boyer's group proposed that during ATP synthesis the phase which needs energy, is the releasing of ATP from the enzyme. Boyer and his group conceptualized the binding change mechanism that describes changes in the catalytic subunit of the F_1F_O during ATP synthesis and hydrolysis. The DNA sequence of genes encoding the proteins in ATP synthase was concluded by John E. Walker's lab. The initial X-ray crystallography configuration of the F_1 subunit (without epsilon, delta, and part of the gamma subunits) analyzing individual amino acid residues was completed by Walker and his group in 1994. In 1997, Kinosita and his group reported direct observance of the rotation of F_1 during hydrolysis of ATP. The Nobel Prize in chemistry in 1997 was awarded to Paul D. Boyer and John E. Walker for their illustration of the enzymatic mechanism fundamental to the synthesis of ATP and also to Jens C. Skou for first discovering ion transporting enzymes, Na^+, K^+ ATPase. In 2004, the initial demonstration of an endergonic chemical reaction being propelled by the direct precise input of mechanical energy was accomplished by H. Itoh and his group.

Structure and Function of ATP Synthase

ATP synthase is an F-Type ATPase. ATPases are membrane-bound enzymes capable of coupling ion movement through a membrane with the hydrolysis or synthesis of ATP. Based on structural and functional differences, ATPases can be grouped into F-, V-, E-, P- and A-ATPases. All these ATPases catalyze ATP synthesis or hydrolysis. The F-ATPases for

'*phosphorylation **F**actor*' are also referred to as H^+-transporting ATPases or F_1F_O-ATPases. F-ATPases provide a kind of transporter for H^+ ions to move through the membrane and possess a unique rotary motor-like mechanism that is coupled with the flow of ions. ATP synthase from diverse taxa have amino acid sequence similarity and appear to be remarkably conserved throughout evolution. F_1F_O-ATP synthase is also structurally and functionally similar among different organisms. The simplest form, and the most used model, is the *Escherichia coli* ATP synthase. The enzyme has a total molecular mass of approximately 530 kDa. *E. coli* ATP synthases have two functional rotary sectors: the intramembrane hydrophobic region called F_O sector, and the catalytic hydrophilic F_1 sector (Figure 2) (Boyer 1997).

The F_O sector consist of three subunits *a*, *b* and *c*, in the stoichiometry of $ab_2c_{10\text{-}14}$ with masses of about 30, 17 and 8 kDa respectively. The F_1 sector has five subunits, α, β, γ, δ, and ε, in the stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ with masses of about 55, 50, 31, 19 and 14 kDa respectively (Boyer 1997; Ackerman and Tzagoloff 2005). ATP synthesis and hydrolysis occur in the F_1 region, whereas proton pumping occurs through the intramembrane hydrophobic F_O region (Senior et al. 2002, Abrahams et al. 1994). There are six possible substrate binding sites on the F_1 sector. The binding sites on the three β-subunits are active catalytic sites, but those on the three α-subunits are noncatalytic regulatory sites. ATP synthase has two rotational motors, one in the F_1 (γ and ε subunits) driven by ATP hydrolysis, the other in F_O (c subunit) driven by the H^+ gradient, which are joined together so that the two motors undergo rapid continuous 360º rotation as catalysis proceeds (Noji et al. 1997). The two motors are physically connected by two stalks. The γ subunit consists of three α-helices, two of which form a coiled coil configuration that insert into the central stalk of the $\alpha_3\beta_3$ hexagon. The δ and b subunits form the peripheral stalk. Proton gradient driven clockwise rotation of γ leads to ATP hydrolysis, and anticlockwise

rotation of γ results in ATP synthesis. Under mild dissociation conditions the F_1F_O-ATP synthase dissociates into the F_1 and F_O sectors which can be isolated and studied individually. In summary, the rotor consists of $\gamma\varepsilon c_n$, while the stator consists of $\alpha_3\beta_3\delta ab_2$, and the function of the stator is to prevent co-rotation of catalytic sites with the rotor (Itoh et al. 2004; Weber 2006). ATP synthase structure and function detailed studies can be found in (Noji and Yoshida 2001; Weber and Senior 2003; Ahmad and Senior 2005; Pedersen 2007; Senior 2007).

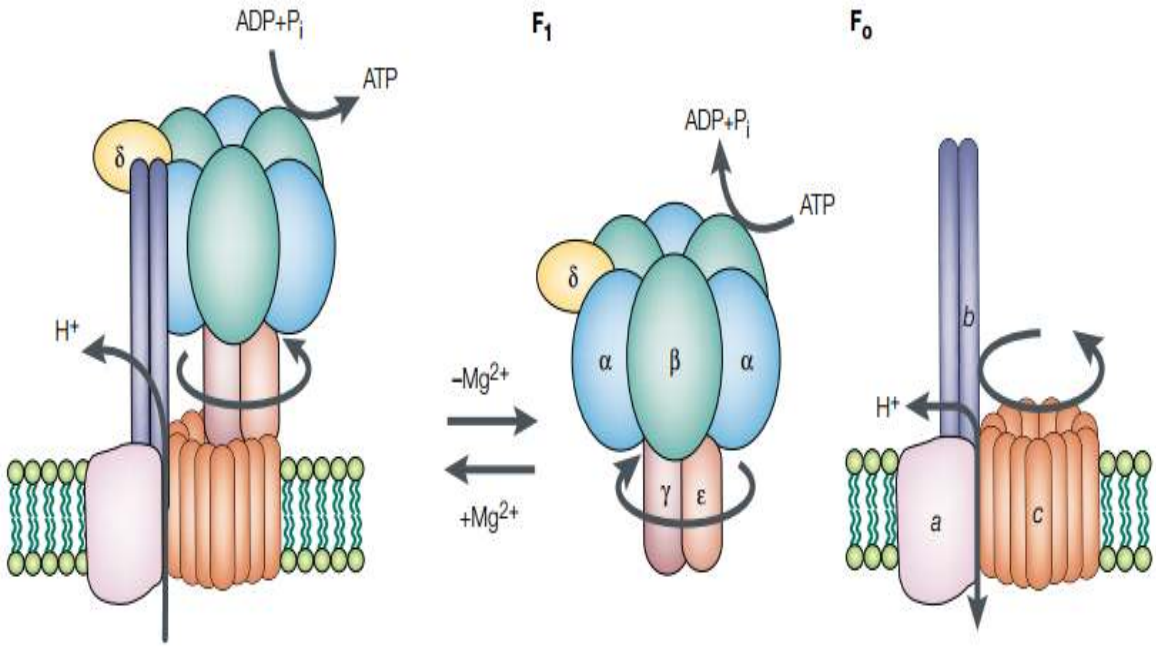

Figure 2. Structure of F_1F_O ATP synthase. F_1 sector contains five types of subunit with a stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_O contains three types of transmembrane subunits with a stoichiometry $a_1b_2c_{10-14}$. (Reproduced with permission from Yoshida et al. 2001).

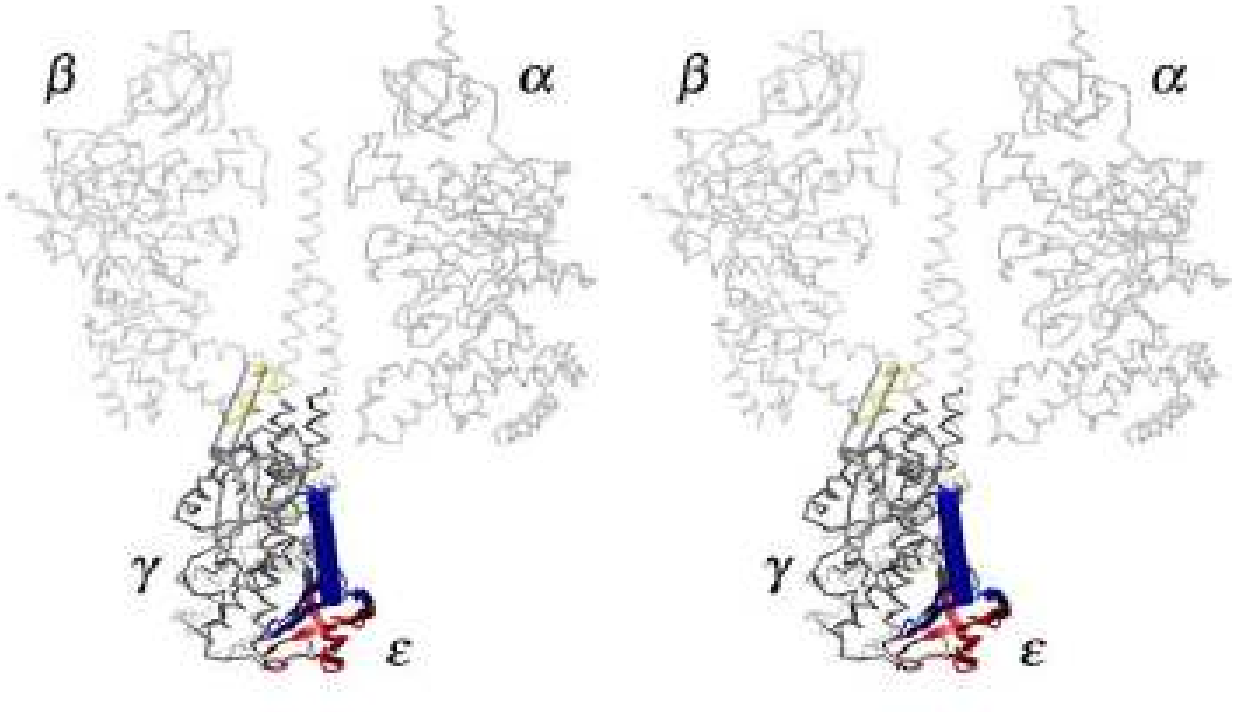

Figure 3. Cartoon representation of the structure of the isolated *E. coli* γε complex with the ε-subunit in the partially extended confirmation. A pair of α and β subunits are displayed in light-grey, subunit γ is dark-grey, subunit ε is displayed in the van der Waals representation (red is the discreet region of the β sandwich, blue is the region preserved only between bacterial and chloroplasts enzyme and the rest is white), βDELSEED-loop is displayed in yellow. (Reproduced with permission from Feniouk et al. 2006).

CHAPTER 2

MATERIALS AND METHODS

Source of Peptides and Other Chemicals

Mastoparan B and lasioglossin II were custom synthesized by Biomatik Corporation. Both peptides were received as lyophilized powder shipped on dry ice and their HPLC determined purity was greater than 97%. Once received, they were stored at -20ºC and subsequently resuspended in autoclaved deionized water when needed. Other chemicals used in this study were ordered from Fisher Scientific, Sigma-Aldrich, and Acros organics.

Preparation of *E. coli* Membrane Associate F_1F_O ATP Synthase

Membrane bound F_1F_O was isolated from the *E. coli* strain pBWU13.4/DK8 (Ketchum et al. 1998). *E. coli* membrane bound F_1F_O was prepared as follows (Senior et al. 1983). Minimal medium was inoculated with a loop full of *E. coli* strain pBWU 13.4/DK8 and was aerobically cultivated over night at 37°C at 250 rpm. The overnight starter culture was inoculated into 1 liter minimal medium and grown at 37°C at 250 rpm. Growth yield was measured at OD_{595} (optical density) every hour until late log phase was reached. Once the required growth was observed the cells were harvested. Cells were maintained as close to 0°C as possible for subsequent steps. Cells were harvested by spinning the culture at 4°C in a Sorvall RC-5B refrigerated super speed centrifuge at 9500 rpm for 15 min. The cells were then resuspended in STEM buffer containing 20mM sucrose and centrifuged at 9500 rpm for 25 min. The supernatant was discarded and the cell pellet was resuspended in 2 ml STEM/g wet cells and stored at -80°C overnight.

The frozen cells were thawed and mixed with DNAse to digest nucleic acids. Cells were then disrupted by two passages through a chilled French press cell fractionator at 20,000 psi. Cell debris were pelleted by centrifugation at 18K rpm for 20 min using Sorvall WX ultra-80, ultra-centrifuge. Membrane bound ATP synthase was subsequently pelleted by spinning the supernatant at 60K rpm for 120 min.

E. coli membranes were purified following a procedure consisting of three washes of the initial membrane pellets (Senior et al. 1984). The initial wash was performed in a buffer containing 50mM TES pH 7.0, 15% glycerol, 40mM 6-aminohexanoic acid, and 5mM p-aminobenzamidine. Two subsequent washes were performed in a buffer containing 5mM TES pH 7.0, 15% glycerol, 40mM 6-aminohexanoic acid, 5mM p-aminobenzamidine, 0.5mM DTT and 0.5mM EDTA. The final membrane bound F_1F_O ATP synthase was resuspended and stored in 50 mM TrisSO4 pH 8.0, 2.5 mM MgSO4 at -80°C (Noji and Yoshida 2001).

Measurement of Membrane Associated F_1F_O ATP Synthase Activity

ATPase activity was measured in a 1ml assay buffer containing 10mM NaATP, 4mM $MgCl_2$, and 50mM $TrisSO_4$, at pH 8.5 and 37ºC. Reactions were initiated by the addition of 1ml of assay buffer to 20 μg of membrane associated F_1F_O ATP synthase and then terminated by adding SDS to 3.3% final concentration. Inorganic phosphate (P_i) release was assayed by adding 1ml of Taussky and Shorr (T&S) reagent containing 10 mM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 250 mM $Fe(NH_4)_2(SO_4)_2\cdot 6H_2O$, and 1.176N H_2SO_4. T&S reacts with P_i to form a blue color complex. The intensity of the blue color was measured at OD_{700} using Shimadzu UV-visible recording spectrophotometer (Taussky and Shorr 1953). Here, ATPase activity was measured in terms of

ATP hydrolysis rather than the synthase activity. ATPase hydrolytic activity was measured to test ATP synthase functionality. The hydrolysis rate is correlated to the enzyme activity, and also provides useful information on possible inhibitory effects of compounds that hinder the hydrolytic process.

The reaction is as follows:

$$ATP + ATP\ synthase \rightleftharpoons ADP + Pi$$

$$Pi + T\&S \rightarrow \text{blue color measured at } OD_{700}$$

ATPase activity in μmol/min/mg was calculated using the formula:

$$= \frac{\text{Average Sample OD - Average Blank OD}}{\text{Amount of protein (mg) x Time (min)}}$$

Inhibition of ATPase activity by Mastoparan B and Lasioglossin II

Membrane bound F_1F_O ATP synthase was preincubated with varied concentrations of mastoparan B and lasioglossin II for 60 min at room temperature in 50 mM $TrisSO_4$ pH 8.0. Then 1 ml ATPase assay buffer containing 10 mM NaATP, 4 mM $MgCl_2$, 50 mM $TrisSO_4$, and pH 8.5 was added and kept at 37ºC for 15 min to measure enzyme activity. The reaction was terminated by the addition of SDS to a final concentration of 3.3%. P_i release was assayed by adding 1ml of T&S reagent. The intensity of the blue color was assayed spectrophotometrically at OD_{700} using Shimadzu UV-visible recording spectrophotometer (Taussky and Shorr 1953). Data analysis was performed using Minitab software (Minitab 2014).

% relative activity was calculated using the formula;

$$\frac{\text{Specific activity of the test inhibitor}}{\text{Specific activity of the blank}} \times 100$$

CHAPTER 3

RESULTS

Inhibitory Effect of Lasioglossin II on Membrane Bound *E. coli* ATP Synthase

Inhibition of membrane bound *E. coli* ATP synthase was tested by measuring the mean relative ATPase activity (Figure 4) of membrane bound F_1F_O that had been preincubated with varied concentrations of lasioglossin II (10μM - 400μM) for 60 min at room temperature. The estimated mean was obtained from three independent inhibition experiments (Appendix D). The results show lasioglossin II as a very weak inhibitor (Figure 4). The maximum estimated amount of inhibition by lasioglossin II was ~ 18% with ~ 82% residual activity at a concentration of 400μM. The least amount of inhibition was at a concentration of 10μM, with ~ 2% inhibition and ~ 98% residual activity. A one-way ANOVA indicates statistically significant differences among the various concentrations of lasioglossin II tested ($P<0.000$) (Table 1). Multiple comparisons by Fisher's least significant difference method indicates significant differences in the extent of inhibition between higher and lower concentrations of peptide (Table 2).

Table 1. One – way ANOVA table for ATPase activity of membrane bound *E. coli* ATP synthase in the presence of varied concentrations of lasioglossin II

Analysis of Variance					
Source	**Degrees of Freedom**	**Adjusted Sums of Squares**	**Adjusted Mean Squares**	**F-Value**	**P-Value**
Factor	5	492.35	98.471	96.55	0.000
Error	12	12.24	1.020		
Total	17	504.59			
Standard Deviation	1.00990				
R-square %	97.57				
Adjusted R-square %	96.56				
Predicted R-square %	94.54				

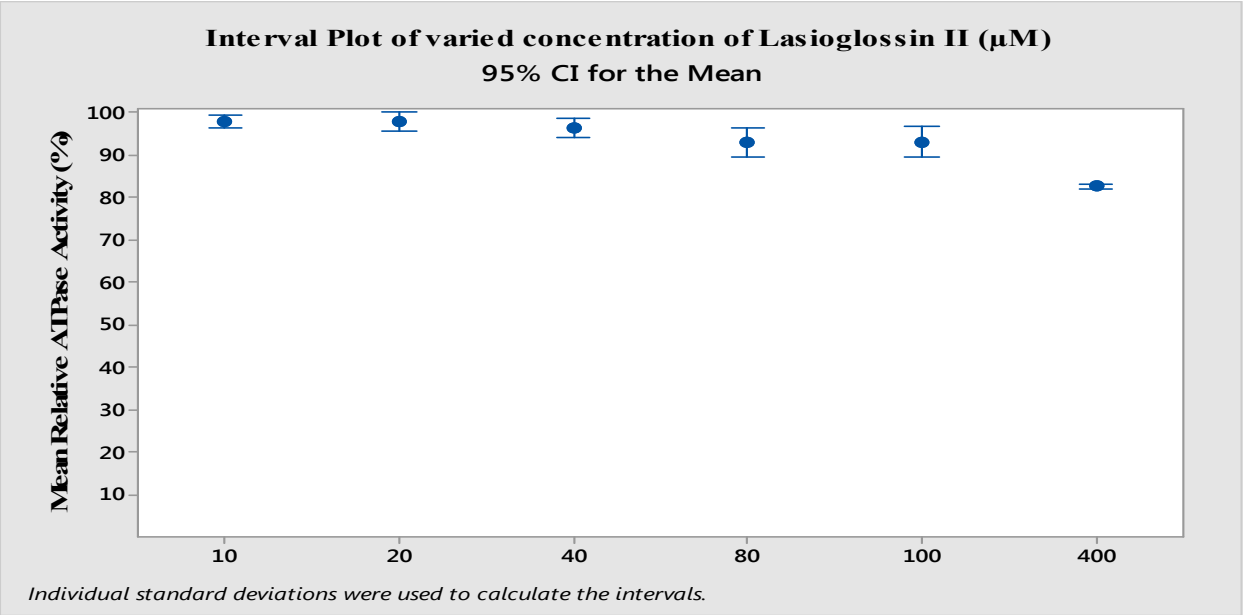

Figure 4. Inhibition of ATPase activity of membrane bound *E. coli* ATP synthase in the presence of varied concentrations of lasioglossin II

Table 2. Inhibition of ATPase activity of *Escherichia coli* ATP synthase enzyme and residual

Lasioglossin II Concentration (µM)	% Mean Relative ATPase Activity	Standard Deviation	95% Confidence Interval	Fisher Pairwise Comparisons
10	98.10	0.59	96.63 - 99.56	A
20	97.89	0.95	95.52 - 100.25	A
40	96.45	0.97	94.04 - 98.86	A
80	93.12	1.33	89.80 - 96.43	B
100	93.06	1.44	89.47 - 96.65	B
400	82.79	0.23	82.22 - 83.36	C
Means that do not share a letter are significantly different.				

ATPase activity in the presence of lasioglossin II

Inhibitory Effect of Mastoparan B on Membrane Bound *E. coli* ATP Synthase

Concentrations of mastoparan B ranged from 50 to 1000μM, with the estimated degree of inhibition varying from 0 to 55% (Figure 5). Membrane bound F_1F_O (6.1μl) was preincubated with varied concentrations of mastoparan B (50μM - 1000μM) for 60 min at room temperature, then 1 ml of ATPase assay buffer was added to measure enzyme activity. The estimated mean was obtained from three independent inhibition experiments (Appendix D). The maximum amount of inhibition estimated in the presence of mastoparan B was ~ 55%, with ~ 45 % residual activity at a concentration of 800μM (Figure 5). The least amount of inhibition estimated in the presence of mastoparan B tested was at a concentration of 50μM and was ~ 10% with ~ 90% residual activity (Figure 5). Estimates of the concentration dependent inhibition are suggestive of a partial inhibitory effect but only at very high concentrations. A one-way ANOVA indicates statistically significant ($P < 0.003$), differences among the various peptide concentrations used (Table 3). Multiple comparisons by Fisher's least significant difference method indicate significantly greater inhibition by 250μM, and higher concentrations of peptide and significantly lower inhibition at 50μM (Table 2).

Table 3. One – way ANOVA table for ATPase activity of membrane bound *E. coli* ATP synthase in the presence of varied concentrations of mastoparan B

Source	**Degrees of Freedom**	**Adjusted Sums of Squares**	**Adjusted Mean Squares**	**F-Value**	**P-Value**
Factor	8	6721	840.1	4.67	0.003
Error	18	3236	179.8		
Total	26	9957			
Standard Deviation	13.4091				
R-square %	67.50				
Adjusted R-square %	53.05				
Predicted R-square %	26.87				

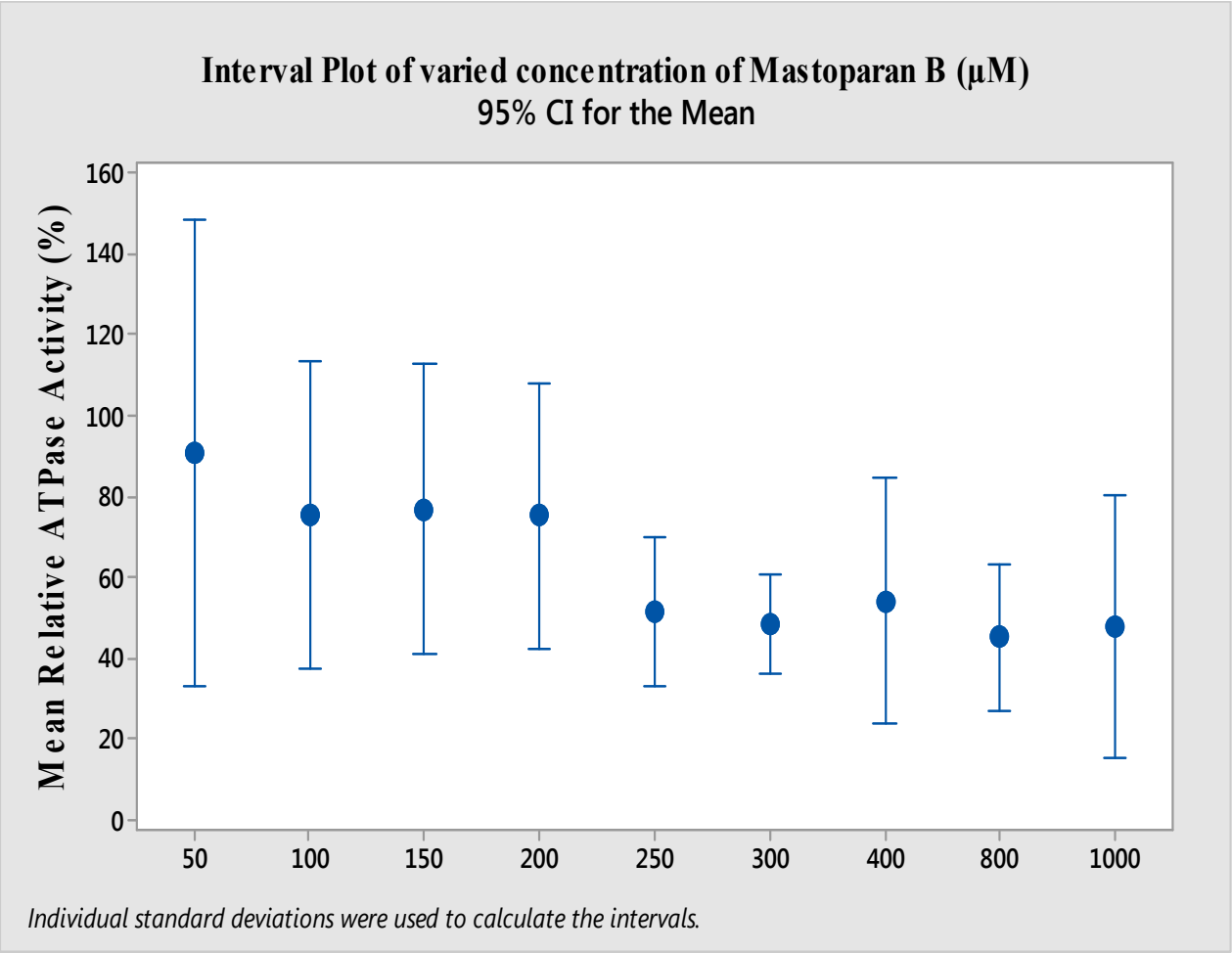

Figure 5. Inhibition of ATPase activity of membrane bound *E. coli* ATP synthase in the presence of varied concentrations of mastoparan B.

Table 4. Inhibition of ATPase activity of *Escherichia coli* ATP synthase enzyme and residual

Mastoparan B Concentration (μM)	**Mean % Relative ATPase Activity**	**Standard Deviation**	**95% Confidence Interval**	**Fisher Pairwise Comparisons**
50	90.60	23.30	32.79 - 148.41	A
100	75.17	15.33	37.09 - 113.24	A B
150	76.73	14.51	40.68 - 112.78	A B
200	75.10	13.16	42.42 - 107.78	A B
250	51.13	7.42	32.70 - 69.57	C
300	48.30	4.95	36.00 - 60.60	C
400	54.03	12.29	23.51 - 84.56	B C
800	44.97	7.28	26.87 - 63.06	C
1000	47.70	13.21	14.88 - 80.52	C
Means that do not share a letter are significantly different.				

ATPase activity in the presence mastoparan B

CHAPTER 4

DISCUSSION

In this study the inhibitory effect on *Escherichia coli* ATPase activity by the two venom peptides lasioglossin II and mastoparan B was examined. The estimation of mean inhibitory effects using ANOVA reveals that varied concentrations of both lasioglossin II and mastoparan B were statistically significant. Varied lasioglossin II concentrations from 10μM to 100μM caused very low inhibition of ATPase activity (Figure 4). At a concentration of 10μM, ~ 2% inhibition was observed, and only ~ 7% inhibition was seen at the concentration of 100μM. The maximal inhibition induced by lasioglossin II was ~18% at a concentration of 400μM. The mastoparan B inhibition profile was suggestive of an interesting biological effect with maximal inhibition of ~55% at a concentration of 800μM. Partial inhibition of ATP synthase by other potential inhibitors has been reported previously. Dadi et al. (2009) reported that the maximal inhibition by luteolin was ~20%, daidzein was ~10%, and galangin showed no inhibition of *Escherichia coli* ATPase activity. Previous studies have also shown that a variety of linear cationic α-helical AMPs inhibit ATP synthase to varying degrees. Laughlin and Ahmad 2010 reported that amphibian AMPs aurein 2.2, aurein 2.3, carein 1.9, magainin II, and magainin II-amide gave partial inhibitory effects of ~69%, 30%, 52%, 20% and 60% respectively at very low molar concentrations, while carein 1.8 did not inhibit at all (0%). The honey bee venom peptides melittin and melittin-amide inhibited ~77% and ~95% of enzyme activity respectively, while melittin related peptide (MRP) and MRP-amide inhibited at ~79% and ~96% respectively.

The results obtained from this study also contrasts with results obtained on the inhibition of ATP synthase by other venom peptides. Azim et al. (2016) documented the inhibitory effect

of four venom peptides on *Escherichia coli* ATP synthase also at much lower concentrations than the concentrations used in this study. King cobra OH-CATH (KF-34), wolf spider lycotoxin II (KE-27), banded krait cathelicidin (BF-30), and wolf spider lycotoxin I (IL-25) induced very strong inhibition of ATP synthase, as the maximum inhibition caused by KF-34, KE-27, BF-30 and IL-25 was ~90%, ~85%, ~85% and ~88% respectively.

The partial or slight inhibition obtained in this study might be the result of various factors, inclusive of the sequence length of the peptides, cationicity, and amphipathic character. The sequence length of the venom peptides OH-CATH (KF-34), cathelicidin (BF-30), lycotoxin II (KE-27) and lycotoxin I (IL-25) are 34, 30, 27 and 25 amino-acids respectively, while lasioglossin II and mastoparan B have lengths of 15 and 14 amino acids. In antibacterial studies of two derivatives of esculentin-1 peptides, Esc-1a (1-21) NH_2 was two to eight fold more active against the planktonic form and exhibited a stronger anti-biofilm activity, inducing a 3 log decrease in the amount of viable biofilm cells than Esc-1b (1-18) NH_2 in many distinct strains of the bacteria *Pseudomonas aeruginosa*. This difference was attributed to the longer length and higher cationicity of Esc-1a (1-21) NH_2 (Mangoni et al. 2015). Two additional factors play vital responsibility in the action mechanism of AMPs, a net positive charge at neutral pH, because cationicity depicts essential character that supports the interaction and binding of AMPs to the negatively charged constituents of the microbial cell membrane, and amphipathic character because this parameter affects the integration of specific peptide into the hydrophobic core of the membrane lipid bilayers (Rietschel et al. 1994; Teixeira et al. 2012). The net positive charge and the hydrophobic face of OH-CATH (KF-34), cathelicidin (BF-30), lycotoxin I (IL-25) and lycotoxin II (KE-27) are also greater than that of lasioglossin II and mastoparan B.

Cytolytic peptides with high antimicrobial activity such as lasioglossins and mastoparans obtained from the venom of stinging insects have some characteristics that suggested them as candidates for the development of effective antibiotic peptides based on their potential for anti-ATP synthase activity. In this study, statistically significant differences in ATP synthase activity were observed in experiments with lasioglossin II, but the effects appear to have very limited biological relevance. The results of inhibition experiments with mastoparan B were statistically significant and were suggestive of some biologically relevant effects on ATP synthase.

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APPENDICES

APPENDIX A- Abbreviations

AMP- Antimicrobial Peptide

ATP- Adenosine triphosphate

ADP- Adenosine diphosphate

Leu [L] - Leucine

Lys [K] - Lysine

Ser [S] - Serine

Ile [I] - Isoleucine

Val [V] - Valine

Arg [R] - Arginine

Trp [W] - Tryptophan

Ala [A] - Alanine

Asn [N] - Asparagine

Gly [G] - Glycine

NBD-Cl- 4-chloro-7-nitrobenzofurazan

PAB- 4-aminobenzamidine

Pi - Inorganic phosphate

SDS- Sodium dodecyl sulphate

T&S- Taussky and Shorr

O.D- Optical density

APPENDIX B: Buffers and Reagents

50 mM Tris-SO4 buffer

To 90 ml H_2O add

0.61 g Tris

Adjust pH to 8.0 with H_2SO_4

Bring to a final volume of 100 ml with H_2O

ATPase cocktail

In 150 ml H_2O add

10 ml 1 M Tris

0.8 ml 1M $MgCl_2$

5 ml 0.4 Na ATP (Adenosine 5'-triphosphate disodium salt)

Adjust pH to 8.5 with H_2SO_4

Bring to a final volume of 200 ml with H_2O

Freeze in plastic bottles at -20^{o}C

10 % SDS

100 gm Sodium dodecyl sulfate

Bring to a final volume of 1000 ml with H_2O

Taussky and Shorr reagent (T & S reagent)

Sol A: 1.2 g Ammonium molybdate ($(NH_4)6Mo_7O_{24}\cdot 4H_2O$ in 9.8 ml 12 N H_2SO_4)

Sol B: 10 g Ferrous ammonium sulfate ($Fe(NH_4)_2(SO_4)_2\cdot 6H_2O$ in 70 ml H_2O)

Add sol A to sol B while stirring

Bring to a final volume of 100 ml with H_2O

Store at 4^oC

STEM

To 700 ml H_2O add

100 ml 1 M TES

4.29 g $Mg(CH_3CO_2)_2 \cdot 4H_2O$

85.5 g sucrose

0.0951 g EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid)

5 g EACA (6-Ainocaproic acid6-Ainocaproic acid)

Adjust pH to 6.5 with NaOH

Bring to a final volume of 1000 ml with H_2O

Freeze in plastic bottles at -20^oC

TES 50

To 700 ml H_2O add

50 ml 1 M TES

150 ml glycerol

5 g EACA (6-Ainocaproic acid6-Ainocaproic acid)

1 g PAB (4-Aminobenzamidine dihydrochloride)

Adjust pH to6.5 with NaOH

Bring to a final volume of 1000 ml with H_2O

Freeze in plastic bottles at -20^oC

TES 5 + PAB

To 700 ml H_2O add

5 ml 1 M TES

150 ml glycerol

1 ml 0.5 M DTT (Dithiothreitol)

5 g EACA (6-Ainocaproic acid6-Ainocaproic acid)

1 g PAB (4-Aminobenzamidine dihydrochloride)

2.5 ml 0.2 M EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate)

Adjust pH to 6.5 with NaOH

Freeze in plastic bottles at -20^{o}C

AET (Argenine Ent Thimine)

To 60 ml H_2O add

0.617 g 2,3 Dihydroxy Benzoic acid

16.86 g L-Arginine HcL

1 ml 20 mM Thiamine

Add just enough amount of NaOH to dissolve everything

Make final volume to 100 ml with H_2O

Filter sterile

TE (Trace Elements)

To 80 ml H_2O

0.251 g Zinc Sulfate ($ZnSO_4.7H_2O$)

0.017 g Manganese Sulfate ($MnSO_4.H_2O$)

0.029 g Boric acid (H_3BO_3)

0.012 g Calcium Sulfate ($CaSO_4.2H_2O$)

0.037 g Calcium Chloride ($CaCl_2.2H_2O$)

0.049 g Ferric Chloride ($FeCl_3.6H_2O$)

Make final volume to 100 ml with H_2O

Filter sterile

ILV (Isoleucin-Valine)

To 95 ml H_2O add

0.394 g Isoleucine

0.352 g Valine

Make final volume to 100 ml with H_2O

Filter sterile

APPENDIX C: Culture Media and Plates

LB liquid medium

12.5 g of LB broth powder

Add H_2O to bring to 500 ml

Autoclave for 30 minutes

Cool the media to ~50^{o}C

Add 500 µl of 100 mg/ml Ampicillin

Minimal Glucose

To 400 ml H_2O add

5.225 g Potassium Phosphate Dibasic Trihydrate (K_2HPO_4)

2.40 g Sodium Phosphate Monobasic (NaH_2PO_4)

0.99 g Ammonium Sulfate ($(NH_4)_2SO_4$)

Autoclave for 30 min, cool it and add the following additions

10 ml Uracil

10 ml 27 % Glucose

5 ml ILV (isoleucin-valine)

0.5 ml TE (trace elements)

0.5 ml 1 M Magnesium Sulfate ($MgSO_4$)

0.5 ml AET (Argenine Ent Thimine)

0.5 ml 100 mg/ml Ampicillin

0.312 ml 4X LB

LB-Agar plate with Ampicillin

12.5g of LB broth powder

7.5g of agar

Bring to a final volume of 500 mL

Autoclave for 30 minutes

Cool the media to ~50^{o}C

Add 500 µl of 100 mg/ml Ampicillin

Pour into sterile plates

APPENDIX D: Raw data

Result of three independent experiment

Lasioglossin II Conc. (µM)	A	B	C	% Mean Relative ATPase Activity	Standard Deviation
10	98.36	98.51	97.42	98.10	0.59
20	96.79	98.51	98.36	97.89	0.95
40	97.57	95.93	95.85	96.45	0.97
80	93.51	94.21	91.63	93.12	1.33
100	93.43	91.47	94.29	93.06	1.44
400	82.79	82.56	83.02	82.79	0.23

Mastoparan B Conc. (µM)	A	B	C	% Mean Relative ATPase Activity	Standard Deviation
50	107.7	64.1	100	90.60	23.27
100	69.6	92.5	63.4	75.17	15.33
150	61.9	77.4	90.9	76.73	14.51
200	60	81.2	84.1	75.10	13.16
250	49.6	44.6	59.2	51.13	7.42
300	52.4	49.7	42.8	48.30	4.95
400	67.7	43.9	50.5	54.03	12.29
800	52.8	38.4	43.7	44.97	7.28
1000	34.8	61.2	47.1	47.70	13.21

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