EFFECT OF MELITTIN ON INFLUENZA-INFECTED CHICKEN EMBRYOS

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Abstract: Antimicrobial peptides are peptides isolated from a wide range of organisms that exert microbicidal activity against a wide spectrum of targets, including bacteria, viruses, fungi, and parasites. These peptides are aimed directly on the phospholipid bilayer and do not target on the cellular or metabolic activities of the cells as antibiotics and other drugs do. But thanks to many vital proteins associated with the membrane, these peptides can also influence these structures and therefore facilitate and accelerate their death. Melittin is a well-characterized pore-forming lytic amphiphilic peptide (consisting of 26 amino acids) found in bee venom. The amphiphilic property of this peptide makes it water-soluble and yet it spontaneously associates with natural and artificial membranes. This integration leads to the distortion and permeabilization of the membrane. In this study melittin has been utilized to study antiviral properties against influenza A virus. Melittin was used in the mixture with the influenza A virus and together were inoculated into chicken embryo. Living conditions were monitored during infection. After death of chicken, biochemistry of allantoic fluid was performed.

Key Words: Melittin; Influenza virus; Chicken embryos

INTRODUCTION

Antimicrobial peptides (AMPs) are small peptides found in all living organisms, where they are major players in the innate immune response against, providing their hosts rapid non-specific defense against parasitic invaders (Wachinger et al. 1998). Interestingly, AMPs have been put forward as one potential class of novel antivirals. Their antimicrobial activity has been reported against both enveloped and non-enveloped viruses, blocking viral infection in different ways (Klotman and Chang 2006): they can directly inactivate the virion through disruption of its envelope, where they spontaneously induce transmembrane pores in lipid bilayers under certain conditions. It is commonly believed that pore formation is the mode of action of these peptides (Huang 2000, Yang et al. 2001). Some peptides exhibit interaction with viral glycoproteins so they can act on infected cells possibly through interactions with cell receptors resulting in alterations in cell signaling pathways required for virus binding or replication, as they can block the fusion of the viral membrane with the endosome from the host cell (Krajewski et al. 2004). Therefore, these peptides can specifically bind to the viral hemagglutinin (HA) protein as an entry blocker. Therefore, HA-binding peptides are promising candidates for antiviral drugs as well as anti-HA antibodies (Jones et al. 2006, Shen et al. 2013). Finally, they can interact with and inhibit viral enzymes essential to the virus replication (Krajewski et al. 2004).

One of antimicrobial peptide which can act against influenza virus could be melittin (Meenakshisundaram et al. 2009). It has been reported that melittin has multiple effects, including antibacterial and anti-inflammatory in various cell types (Raghuraman and Chattopadhyay 2007). Replication inhibition of murine retroviruses, tobacco mosaic virus and herpes simplex virus has been

observed and therefore is suggested that melittin also displays antiviral activity (Meenakshisundaram et al. 2009). Melittin binds to membranes as monomers but acts on the membrane collectively. Even at concentrations as low as a few nanomoles per liter, melittin can induce transient pores that allow transmembrane conduction of atomic ions but not leakage of glucose or larger molecules, whereas at micromolar concentrations, melittin induces stable pores allowing transmembrane leakage of molecules up to tens of kilodaltons (Lee et al. 2013, Wachinger et al. 1992). Melittin functions via the carpet model (lipid destabilization) at low (below 0.5 μM) and high concentrations (above 3 μM), or via the toroidal model at intermediate concentrations forming partially or completely lipidic pores with average diameter of ~1.3−2.4 nm (Chen et al. 2007, Lee et al. 2013, Olaru et al. 2009). Due to the presence of a single tryptophan residue, Trp-19, melittin is intrinsically fluorescent, which makes it a sensitive probe to study the interaction of melittin with membranes (Raghuraman and Chattopadhyay 2007).

In this study effect of synthetic melittin against the H7N7 influenza virus was studied on embryonated chicken eggs.

MATERIAL AND METHODS

Preparation of the synthetic melittin

Melittin, which is the main fraction of bee venom, was synthetically prepared by the automated peptide synthesizer Liberty Blue (CEM Corporation, Matthews, NC, USA). The sequence of the melittin was GIGAVLKVLTTGLPALISWIKRKRQQ.

Purification of the melittin from bee venom

Aliquots of venom (1 mg) were resuspended in 50 mM Tris buffer (1 mL) and fractionated on fast protein liquid chromatography (FPLC) system Biologic DuoFlow (Biorad, Philadelphia, PA, USA). As the mobile phase was used 50 mM Tris-HCl adjusted to pH 7.4. Flow rate of mobile phase was set to 0.5 ml · min^{-1}. Separation of melittin was done using isocratic elution. Before separation started, column was washed with mobile phase for 15 minutes. UV detection was carried out at 280 nm. Fractions were collected approximately in volume of 1 mL. After purification, the fractions were lyophilized and stored (-80°C) for further experiments.

Melittin peptide characterisation

Differential pulse voltammetry coupled with adsorptive transfer technique (AdT DPV) was employed, utilizing Brdicka reaction. In Brdicka reaction catalytic signals of hydrogen evolution, provided by peptides/proteins on the mercury electrode in the presence of ammonium buffer with content of the cobalt salt, were evaluated. Voltammograms were obtained by measurements of samples, standardized on the same concentration of total proteins (200 μg · ml^{-1}).

The mass spectrometry characterisation was performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany) using reflector positive mode, HCCA as a matrix, laser gain of 45% with 2500 averaged subspectra evaluated on one spot.

Embryonated chicken eggs incubation and inoculation

Embryonated specific pathogen-free (SPF) chicken eggs were incubated 9 days at 37°C and 55% humidity before inoculation. 200 μl of virus diluted with phosphate buffer saline (PBS pH; 7.2) to hemagglutation units (HAU) 128/25 μl, was then inoculated into embryo's allantois. Control eggs were inoculated with PBS.After 24 hours, melittin was inoculated into allatoic fluid to obtain final concentration approximately at 0.05, 0.5, 1, 2 and 4 μM as the amount of allantois varies among each egg. 11-days old embryos were cooled to 4°C overnight and then allantoic fluid was collected and centrifuged to remove blood and debris. Also, observations of the embryo were made to evaluate the effect of the virus and melittin on the embryonated chicken.

RESULTS AND DISCUSSION

Melittin is well characterized, pore-forming lytic peptide amphiphile (consisting of 26 amino acids) found in bee venom. Melittin amphiphilic properties facilitate its solubility in water and the ability

to react with both natural and artificial membranes. This integration leads to the ability of disruption and permeabilization of membranes.

Melittin used in this study was prepared synthetically, therefore characterization and comparision with the native one was done to prove identical features. Curves from differential pulse voltammetry coupled with adsorptive transfer technique (AdT DPV) (Figure 1) show similar results, where four distinct signals - Co1 (- 0.9 V), RS_2Co (approx. - 1.15 V), Cat1 (approx. - 1.3 V) and Cat2 (- 1.55 V) were measured.

MALDI-TOF (Figure 2) exhibited approximately equal values of calculated mass (2846.46 Da + H^+) in both samples.

Figure 1 Electrochemical characterization of melittin, (A) synthetic and (B) obtained from FPLC fractionation using differential pulse voltammetry coupled with adsorptive transfer technique (AdT DPV).

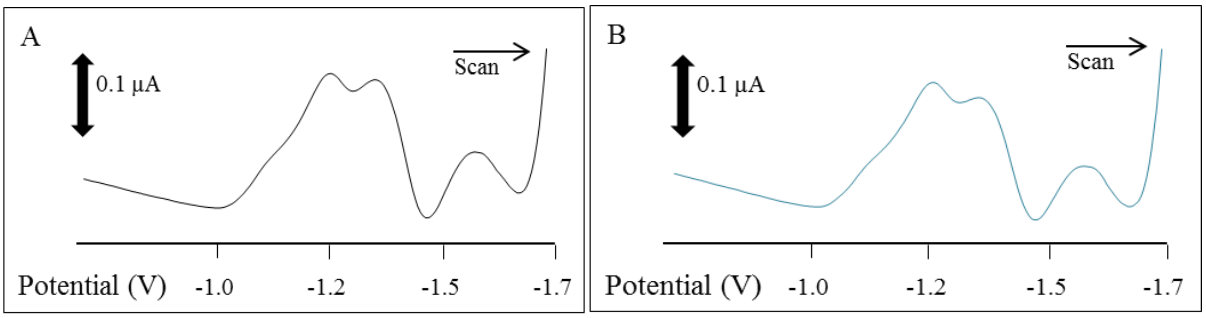
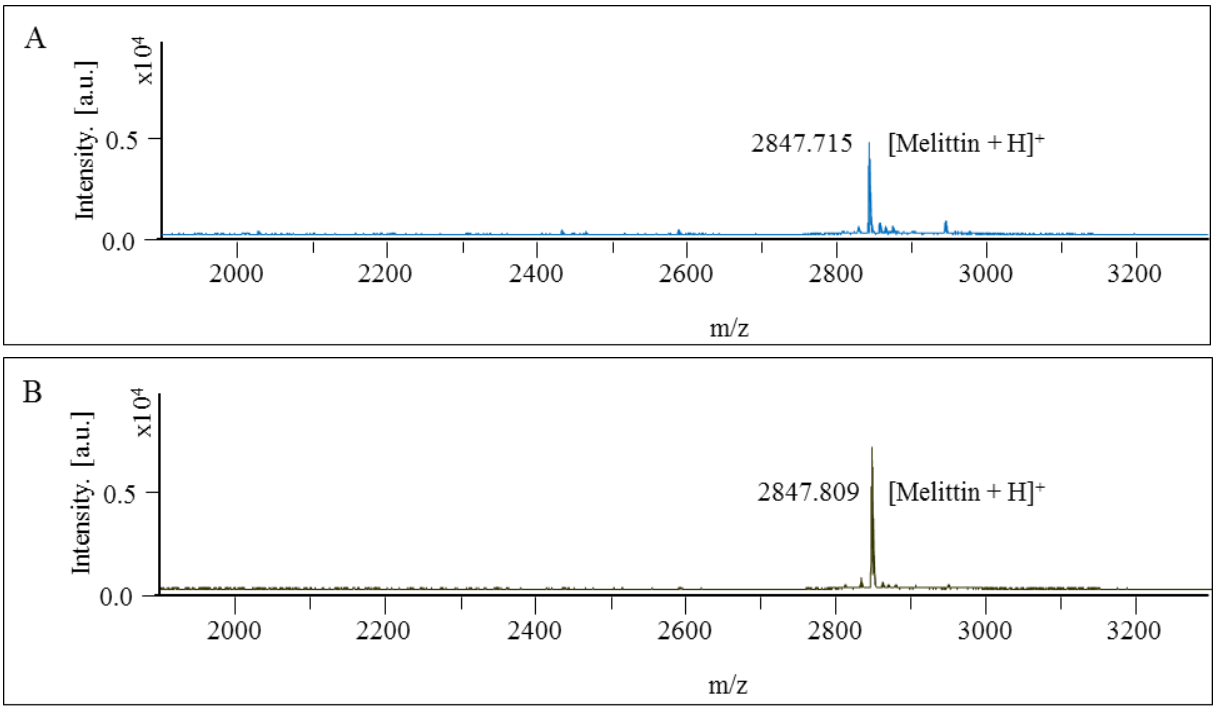


Figure 2 Expression of MALDI-TOF spectra of (A) synthetic melittin and (B) honey bee venom fraction of melittin obtained after FPLC isolation.



Conditions of chicken embryos were evaluated after harvesting the allantoic fluid to observe effect of influenza virus and melittin. Images of embryos (Figure 3) show negative effect of influenza virus (Figure 3B) on chicken embryo. Most of the embryos inoculated with the virus itself died with severe congestion and hemorrhages. Compared to other testing conditions (Figure 3A; C; D; E and F) no similar findings were observed even when they died before the end of the experiment.

As shown on Figure 4, the survival of chicken embryos from eggs inoculated solely with H7N7 influenza virus reached only 40%. This clade of virus therefore exhibit high pathogenicity for embryos.

None of the control embryos died during whole experiment and so it is supposed, that death of influenza-inoculated was caused by the virus and not by poor viability of the embryos. Melittin-inoculated embryos exhibited perfect viability to the concentration of 1 µM. Higher concentration (2 and 4 µM) were associated with higher losses, which could be caused by lesion of embryo's vital function by melittin itself. Samples inoculated with influenza and subsequently with melittin showed 80% survival rate in melittin concentration to 1 µM. After application of higher amount, only 40% of embryos were viable. Then, lower melittin concentration could primary affected amount of the influenza virus, whereas at larger amount could affect embryo, either alone or in conjunction with virus.

Effect of the melittin itself and its analogues could be seen in previous studies, which have been focused on some other viruses, mostly HIV (Wachinger et al. 1998) and Herpes simplex virus (Baghian et al. 1997, Matanic and Castilla 2004), where results showing ability of melittin to reduce the virus concentration were presented. One of the possible way, how to increase the melittin effectivity against viruses and simultaneously protect the host cells could be novel antiviral strategy based on the use of carriers like immunoliposomes (Falco et al. 2013) or melittin-loaded nanoparticles (Hood et al. 2013, Soman et al. 2008).

*Figure 3 Images of 11-days-old chicken embryos after 24-hour incubation with influenza and melittin. (**A**) control, (**B**) 200 µl of influenza (128 HAU), (**C**) 200 µl of influenza (128 HAU) with melittin (~2 µM) (**D**) 200 µl of influenza (128 HAU) with melittin (~4 µM), (**E**) melittin (~2 µM) (**F**) melittin (~4 µM).*

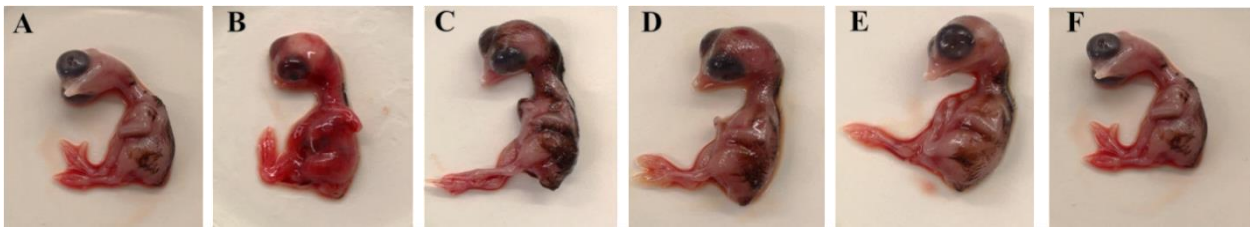
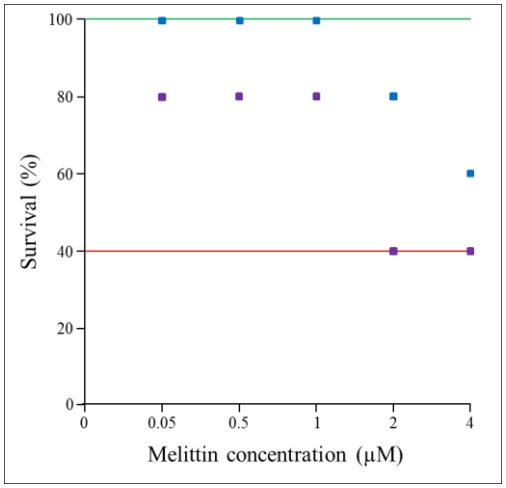


Figure 4 Estimation of survival of 11-days-old chicken embryos after application of H7N7 influenza virus (128 HAU) and melittin (different concentrations) after 24 hour effect. Control (green line), melittin without influenza (blue dots), influenza and melittin (purple dots) and influenza (red line).



CONCLUSION

Naturally antimicrobial peptide melittin has been examined for its possible effect against protective envelope of HIV. As influenza virus belongs also among enveloped virus, synthetic melittin was used to examine its features in embryonated chicken eggs. Concentrations to 1 µM exhibited highest effect against the virus with low negative influence on the embryo.

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