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Development of Lipid-Modified Chitinase as a New Antifungal Reagent

プゴフ，サントソ

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氏　　名　：プゴフ サントソ
(Pugoh Santoso)

論 文 名：Development of Lipid-Modified Chitinase as a New Antifungal Reagent
（新規抗真菌薬としての脂質修飾キチナーゼの開発）

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論 文 内 容 の 要 旨

The increasing cases of fungal infection and the emergence of antifungal drug-resistant fungi have been priority health concerns globally. The annual prevalence of morbidity and mortality caused by fungal infection gradually increases due to the ineffectiveness of antifungal drugs in combating pathogenic fungi and their toxicity toward human cells. Amphotericin B (AMB), a polyene antifungal group, is a gold standard antifungal drug widely used to treat fungal infections because of its broad-spectrum activity in combating the fungal pathogen. However, AMB has been reported to possess a nephrotoxicity level in humans due to its low solubility in water. The strategy to reduce the limitation of AMB has been developed until the combination with other antimycotic agents shows promising results by decreasing its toxicity. The combination of AMB with the biologically active compound is one of the best strategies to suppress pathogenic fungi because it exhibits a low side effect on humans. Chitinase is a potential candidate that can be considered a combination agent with AMB because chitinase catalyzes the hydrolysis of chitin, of which a major component of fungal cell wall. The chitinase consists of two essential domains: chitin-binding and a catalytic domain. The chitin-binding domain, like a LysM from the fern *Pteris ryukyuensis*, and the catalytic domain have the function to bind the chitin and hydrolysis of chitinous substrate, respectively. A study showed that deletion of LysM domain could decrease the antifungal activity, and the arrangement of tandem LysM can increase the antifungal activity. In this study, I investigated the role of LysM by conjugating it with a lipid by microbial transglutaminase (MTG)-catalyzed cross-linking and found that marked increase in the antifungal activity in suppressing the fungal growth of *Trichoderma viride*.

In chapter 2, the role of chitin-binding domain (LysM) and catalytic domain (CatD) and those combination (LysM-CatD) in the antifungal activity was investigated. All the recombinant proteins were engineered by adding a glutamine-containing peptide (Q-tag) at the C-terminus to yield LysM-Q, CatD-Q and LysM-CatD-Q to facilitate the site-specific cross-linking catalyzed by MTG. I demonstrated a synergistic effect of the palmitoylated chitinase of *P. ryukyuensis* and AMB. LysM-Q, CatD-Q, and LysM-CatD-Q were successfully conjugated with a palmitoylated-peptide substrates with MTG-reactive Lys (Pal-K) by MTG. The palmitoylated chitinase domains exhibited strong antifungal activity against *T. viride* with IC_{50} values as low as 1 μM, nearly 2 orders of magnitude lower than the

previously reported IC_{50} value of LysM-CatD. The combination of AMB with these palmitoylated chitinase domains resulted in a strong enhancement of the antifungal activity. Intriguingly, the palmitoylated chitin-binding domain, LysM-Pal, showed the highest synergistic effect of the three chitinase domains despite a lack of chitin degradative activity. The palmitic acid motif of LysM-Pal probably functioned to deliver LysM to the chitin at the tip of the fungal hyphae by anchoring into the plasma membrane, which enhanced the destabilization of the chitin layer by the binding of LysM. Because both the chitin layer and the plasma membrane at the tip of the hyphae were damaged by LysM-Pal and AMB, the stability of the tip of hyphae was drastically reduced, resulting in lysis of the fungal cells. Mammalian cells do not have chitin, and therefore LysM is expected to be safe for use in mammals.

In chapter 3, based on the results with LysM-Q in the preceding chapter, I discussed the effect of the alkyl chain length of lipids such as octanoic acid (C8), dodecanoic acid (C12), and palmitic acid (C16) on the antifungal activity and their localization in the fungal cell wall. The lipid-peptide substrates with different alkyl chain length (C8-K, C12-K and C16-K) were synthesized with the addition of peptide sequence containing MTG-reactive lysine. Both LysM-Q and lipid-conjugated peptides were successfully crosslinked by MTG to yield lipidated LysMs (LysM-C8, -C12 and -C16) then tested the enhancement of antifungal activity when combined with AMB. Intriguingly, LysM-C12 and -C16 showed better antifungal activity compared with that of LysM-C8. Fluorescently labeled LysM-lipids confirmed their localization in the fungal cell wall. The confocal laser scanning microscopy (CLSM) showed that LysM-C12 and LysM-C16 exhibited much better penetration through the fungal cell wall compared with LysM-C8 and unmodified LysM-Q, suggesting the potential of protein delivery to the fungal cells through artificial lipidation. These findings were correlated with the antifungal activity of lipidated LysMs, by which its action depends on the alkyl chain length of lipids.

In chapter 4, the liposomal formulation was applied to encapsulate the Gibco™ Amphotericin B, a commercial amphotericin B solubilized into sodium deoxycholate, to prepare hybrid liposomal formulations. Furthermore, the formulation was combined with the LysM-Pal, and its antifungal activity was observed against *Trichoderma viride*. Hybrid liposomal formulations with different surface charges were prepared by combining a commercially available reagents to explore key factors in the antifungal activity. The characterization of AMB-loaded liposomal formulations (AMB-LFs), including particle size distribution and zeta potential, showed that anionic and neutral AMB-LFs could stably encapsulate AMB. The combination of either anionic or neutral AMB-LFs with unmodified LysM decreased the minimum inhibitory concentration (MIC) of AMB. The combination of neutral AMB-LF with LysM-Pal resulted in a further decrease in the MIC compared with that of the neutral AMB-LF alone. The results obtained demonstrated the potential utility of lipid-based liposomal formulations of AMB combined with lipid-modified proteinaceous binders to tackle fungal infections.