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Abstract of Thesis/Dissertation

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Title : Research on the virucidal activity of theaflavins-enriched tea leaf extract against influenza A virus and human norovirus surrogates

(A型インフルエンザウイルスおよびヒトノロウイルス代替ウイルスに対する茶葉由来テアフラビン濃縮 抽出物の殺ウイルス効果)

Abstract

Influenza is one of the contagious diseases caused by influenza viruses that infect humans on a worldwide scale. Among several species of influenza viruses, influenza A virus (IAV) is the causative agent of all the past influenza pandemics. Within the past 100 years, the world witnessed 4 pandemics by novel strains of IAV, lifting 500,000–50 million deaths. Vaccination has been recognized as one of the most common and effective measures for preventing IAV infection. Nevertheless, because of the genetic mutations which induce the antigenic drift and antigenic shift, mutated IAV sometimes evade the neutralizing activity of previously established antibodies, causing vaccines to become ineffective during the following seasons. On the other hand, several antiviral drugs developed are therapeutically effective against IAV. However, the emergence of resistant viral strains is a matter of concern. Therefore, preventative measures other than vaccination and therapeutic interventions are needed to combat influenza, such as an application of disinfectants or virucidal agents.

Human norovirus (HuNoV) is one of the most important viruses causing acute gastroenteritis outbreaks worldwide, leading to over 200,000 deaths per year in all age

categories. HuNoV is globally identified as the food-borne virus of highest importance, with significant economic losses estimated at tens of billions of dollars. Currently, no approved vaccines or antiviral drugs are available for HuNoV. However, several virucidal chemical compounds have been confirmed to effectively disinfect HuNoV on environmental surfaces. Nevertheless, the residual property in the environment and the adversative health impacts of some of these virucidal compounds accounted for their limited application. Therefore, it is necessary to develop effective and environmentally-friendly strategies to prevent HuNoV infection. The difficulty of propagating HuNoV in major cell culture systems is an issue that hindering the research on this virus. Accordingly, the assessment of inactivation activities of virucidal agents against HuNoV remains mainly dependent on using readily available cultivable surrogates with comparatively close genetic and structural similarities to HuNoV, such as feline calicivirus (FCV) and murine norovirus (MNV).

Medicinal plants have been widely investigated as alternatives to conventional treatments for several diseases. Among these medicinal plants, tea polyphenols have gained research attention as safe and natural virucidal agents for multiple viral infectious diseases. Therefore, in this research, a polyphenol-enriched tea leaf extract named TY-1 (Yokoyama Food Co., Ltd. Sapporo, Hokkaido, Japan) that was produced from the raw leaves of tea tree, *Camellia sinensis*, was evaluated for its potential virucidal activity. The findings in this research are described in 2 chapters. Specifically, studies in Chapter I was focused on evaluating the virucidal efficacy of TY-1 against IAV, as a representative of enveloped-RNA viruses. Thereafter, the virucidal mechanism of action of TY-1 against IAV was analyzed. In Chapter II, the virucidal efficacy of TY-1 against HuNoV surrogates, FCV and MNV, as representatives of non-enveloped-RNA viruses was evaluated. In addition, the virucidal mechanism of TY-1 against FCV and MNV was further analyzed. The virucidal activities of the individual chemical components contained in TY-1 against IAV, FCV, and MNV were also individually evaluated.

To achieve the objective studies, a stock solution from TY-1 powder was prepared as follow: 1 g of TY-1 powder was dissolved in 100 mL of phosphate-buffered saline and centrifuged. Thereafter, the water-soluble layer obtained was filtered and stored at −80°C until use. As dextrin represents 50% of chemical composition of TY-1 powder, 50% dextrin solution was used as a solvent control in all the experiments. Thus, the concentrations of TY-1 and dextrin stock solutions prepared were 10 mg/mL and 5 mg/mL, respectively.

In Chapter I, the virucidal efficacy of TY-1 against IAV was evaluated as follows: First, the viral solution was mixed with 5 different concentrations of TY-1 and 1 concentration of

dextrin solutions. The final concentrations in the mixtures ranged between 0.3 to 5.0 mg/mL for TY-1 and 2.5 mg/mL for dextrin. Second, these mixtures were incubated at 25°C for various reaction times (10 min–24 h). Finally, the virucidal activity of each concentration of TY-1 solution was measured by comparing the viral titer between the test solution-treated group and the dextrin-treated group. As a result, TY-1 was found to exhibit a concentration- and time- dependent virucidal activity against IAV. Specifically, 5.0 mg/mL TY-1 induced a 1.33 and ≥5.17 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$)/mL reduction of the viral titer compared to dextrin group within 30 min and 6 h reaction time, respectively.

Thereafter, the virucidal mechanism of action of TY-1 against IAV was analyzed. Western blotting (WB) analyses revealed that TY-1 treatment reduced the band intensity of the viral spike protein, hemagglutinin, and induced the appearance of additional high molecular mass ladders/bands. TY-1 treatment also reduced the band intensity of another spike protein, neuraminidase (NA). A hemagglutination assay revealed that TY-1 reduced the hemagglutination activity, and NA assay revealed that TY-1 also reduced the NA activity. These results indicated that TY-1 caused structural abnormalities and loss of function in IAV spike proteins, possibly leading to their destruction. Reverse transcription polymerase chain reaction (RT-PCR) targeting the gene of IAV treated with TY-1 revealed the significant reduction of PCR products, which indicates that TY-1 damaged the IAV genome. In addition, observation of viral particles by transmission electron microscopy (TEM) showed that TY-1 treatment drastically reduced the number of intact viral particles.

To evaluates the contribution of theaflavins (TFs) and catechins, which are the main components contained in TY-1, to IAV inactivation, the viral solution was mixed with TFs, catechins, TFs+catechins, TY-1, or dextrin solutions. The final concentrations of the TFs, catechins, TFs+catechins, TY-1, and dextrin solutions were 0.083, 0.034, 0.116, 5.0, and 2.5 mg/mL, respectively in the mixture. The concentrations of TFs, catechins, and TFs+catechins solutions were equal to their concentrations in the 5.0 mg/mL TY-1 solution. Then, these mixtures were incubated at 25°C for 3 h and 24 h. The virucidal activity of each test solution represents the difference in viral titer between each test solution-treated group and the dextrin-treated group. The obtained results revealed that TFs partially contributed to the virucidal activity of TY-1. Meanwhile, the contribution of catechins to IAV inactivation was limited. The enhanced IAV-inactivation was observed under the TFs+catechins treatment compared to that under TFs treatment alone. These results suggested that TFs are active components of TY-1 in its virucidal activity, but other multiple virucidal polyphenolic compounds such as catechins contribute to the comprehensive

virucidal activity of TY-1.

In Chapter II, the virucidal efficacy of TY-1 against HuNoV surrogates FCV and MNV was evaluated in solution and on a dry surface. The viral solutions were mixed with TY-1 or dextrin solutions. The final concentrations of TY-1 and dextrin in the mixtures were 0.3 to 5.0, or 25.0 mg/mL, and 2.5 or 12.5 mg/mL respectively. Thereafter, all the mixtures were incubated at 25°C for 10 s to 24 h, and the viral titers ($log_{10}TCID_{50}$/mL) were measured. As a result, TY-1 exhibited a concentration- and time- dependent virucidal activity against FCV and MNV. More potent and rapid TY-1-mediated inactivation was observed against FCV compared with MNV, where 2.5 mg/mL TY-1 effectively inactivated FCV in 10 s, while 5.0 mg/mL TY-1 required the time longer than 10 min for the inactivation of MNV. However, at 1 min contact time, the higher concentration of TY-1 (25.0 mg/mL) exhibited a statistically significant virucidal activity against MNV. Moreover, TY-1 reduced the viral titers of FCV and MNV on a dry surface within 10 min. Thereafter, the effect of TY-1 on viral proteins and genomes of FCV and MNV was analyzed via WB and RT-PCR. TY-1 was found to induce the profound disruption of virion components including the capsid proteins and viral genomes. The TEM analysis revealed that TY-1 treatment caused abnormalities in virion morphology. Moreover, it was observed that a limited but effective FCV/MNV-inactivation was accomplished under catechins treatment, as well as TFs treatment. These findings indicated that both TFs and catechins may contribute to the overall virucidal activity of TY-1 against FCV and MNV, although other components seem to be involved in these activities.

In conclusion, this study demonstrated a tea leaf extract TY-1 enriched with polyphenols such as TFs and catechins as a possible natural-derived virucidal agent. TY-1 might be applied as troche, mouthwash, nasal mask, and food additive in various health-care settings, or as a virucidal spray on environmental surfaces for preventing and controlling IAV and HuNoV infection.