
Note

ShellCoat, a Calcinated Calcium Solution, Effectively Inactivates SARS-CoV-2

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Received 21 September, 2021/Accepted 6 November, 2021

SARS-CoV-2, an acute respiratory syndrome-causing virus, suddenly emerged at the end of 2019 in China, and rapidly spread all over the world. In this study, we examined whether a calcinated calcium solution (ShellCoat), which has been approved as a food additive in Japan can inactivate SARS-CoV-2. Furthermore, antiviral activity of ShellCoat against SARS-CoV-2 was also evaluated in the presence of organic matter, namely, fetal bovine serum (FBS). When concentrated SARS-CoV-2 were treated with ShellCoat for 10 sec in presence or absence of FBS as organic matters, the viral titer was decreased more than 4 logs 50% tissue culture infective dose per mL (TCID₅₀/mL) but use of ShellCoat for 20 sec or more under similar experimental conditions the viral titer was below the detection limit (≤ 2.1 logs TCID₅₀/mL). These results clearly indicate that the ShellCoat is a powerful antiviral agent against SARS-CoV-2 even in the presence of organic matters.

Key words : Calcinated calcium / Antivirus activity / SARS-CoV-2.

At the end of 2019, a novel coronavirus was isolated from patients with pneumonia of unknown cause in Wuhan, China and the virus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu et al., 2020). This virus has spread all over the world causing the pandemic coronavirus disease 2019 (COVID-19), which is still continuing. In Japan 1,434,370 persons were infected and 15,896 died as of August 29th, 2021 (<https://www.mhlw.go.jp/stf/covid-19/kokunainohasseijoukyou.html>) and as of August 27, 2021, more than 214 million confirmed cases with 4.47 million deaths were reported from several countries to WHO (World Health Organization, 2021).

SARS-CoV-2 is transmitted through aerosols, droplets and fomites. Therefore, it is important to inactivate

the viruses on different surfaces, which could be contaminated by droplets from infected persons, by using appropriate disinfectant. Calcinated calcium, a known disinfectant, could reduce the bacterial number on several vegetables such as cabbage and tomato (Bari et al., 2002; Fukuyama et al., 2009). Yamashita has reported that the ShellCoat containing 10% sodium lactate, 9.9% ethanol, 0.26% calcium hydroxide and 0.01% lactic acid, has a more potent antimicrobial activity compared to 150 ppm sodium hypochlorite against food poisoning causing bacteria, such as *Vibrio* species, *Staphylococcus aureus* and *Salmonella enterica* under dirty condition by modified Kelsey-Sykes method (Yamashita, 2016). Furthermore, the National Institute of Health Sciences, Japan reported that the ShellCoat has anti-Norovirus activity and its antiviral activity was almost same as 1,000 ppm sodium hypochlorite.

WHO has recommended 1,000 ppm sodium hypo-

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chlorite or 70% ethanol for efficiently disinfecting SARS-CoV-2 contaminated surfaces (World Health Organization, regional office for the western pacific, 2020). However, sodium hypochlorite solution cannot be used for human and some places where metals are present because of toxicity to human and oxidation of metals (rust formation). Furthermore, 70% ethanol is an inflammable item and may cause skin irritation in human. Therefore, alternative disinfectants, which are safe and side effects free, are urgently needed.

In this study, therefore, we have examined whether the ShellCoat, an approved food additive in Japan, can efficiently inactivate SARS-CoV-2. For this purpose, the antiviral activity of ShellCoat against SARS-CoV-2 was evaluated in the presence and absence of organic matters, namely, fetal bovine serum (FBS).

VeroE6/TMPRSS2 cell line was purchased from Japanese Collection of Research Bioresources (Osaka, Japan) for cultivation of SARS-CoV-2. One hundred forty thousand cells of VeroE6/TMPRSS2 cells were cultured in a 25 cm² cell culture flask containing Dulbecco's modified Eagle medium, low glucose, pyruvate (DMEM; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% heat inactivated FBS (Thermo Fisher Scientific Inc.) and 1 mg/mL of geneticin (G418, Nacalai Tesque, Inc., Kyoto, Japan) at 37°C for 16 h under 5% CO₂ in air. Cells were infected with MOI=0.001 of SARS-CoV-2 JPN/TY/WK-521 strain and incubated at 37°C for 48 to 96 h. After cytopathic effect was observed, spent culture medium was harvested, centrifuged at 1,600 xg for 5 min, and supernatant fraction containing virus particles was used for further experiment. One gram of polyethylene glycol 6,000 and 233 mg of NaCl (Nacalai Tesque, Inc.) were added to 10 mL of collected virus solution and incubated at 4°C for 16 h. After that, virus solution was centrifuged at 20,000 xg at 4°C for 10 min, supernatant was discarded and pellet was suspended in 1 mL of PBS (-) at pH 7.4.

Thirty microliters of concentrated virus solution or virus mix solution (15 µL concentrated virus and 15 µL of PBS or 5, 10, 20 or 40% FBS) was added to 570 µL of ShellCoat (10% sodium lactate, 9.9% ethanol, 0.26% calcium hydroxide and 0.01% lactic acid in water) (Kawakami. Co., Ltd, Osaka, Japan). Then, samples were incubated at room temperature for 10, 20, 40 or 80 sec. After incubation, 21.7 µL of 10% acetic acid (Nacalai Tesque, Inc.) was immediately added to neutralize the solution, followed by addition of 60 µL of 10 X DMEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 12 µL of FBS and 12 µL of 50 mg/mL G418. Next, ten-fold dilution of the solution was made using DMEM containing 2% FBS and 1 mg/mL of G418 and titration was done as described below.

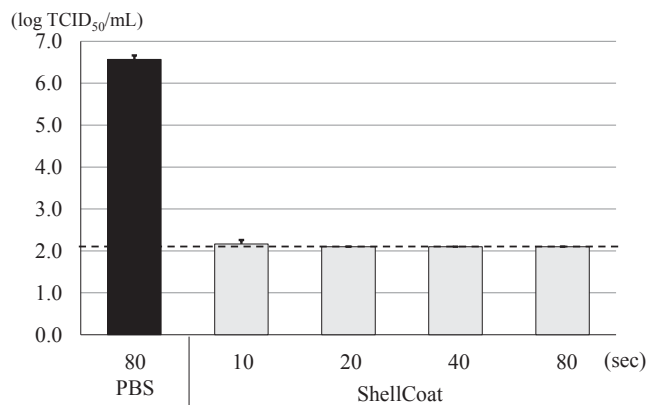


FIG. 1. Anti-viral activity of ShellCoat against SARS-CoV-2. Polyethylene glycol (6,000) concentrated SARS-CoV-2 was treated with PBS (negative control) for 80 sec or ShellCoat for 10, 20, 40 or 80 sec. All data represent the means \pm SD from three independent experiments. Dotted line indicates detection limit of the experiment (≤ 2.1 logs TCID₅₀/mL).

One hundred microliters of about 2.5×10^4 VeroE6/TMPRSS2 cells were seeded in a 96 well plate in their respective medium followed by incubation at 37°C for 16 h. After incubation, culture medium was removed and 100 µL of 10-fold serially diluted virus in DMEM containing 2% FBS and 1 mg/mL of G418 was added. The infected VeroE6/TMPRSS2 cells were cultured at 37°C for 1 h, then the virus containing culture was replaced with fresh 100 µL of DMEM supplemented with 2% FBS and 1 mg/mL of G418 followed by further incubation of plates for 72 h. Finally, cells were fixed with methanol (Nacalai Tesque, Inc.), and stained with 0.5% crystal violet stain. Then, 50% tissue culture infective dose (TCID₅₀) was determined.

In this study, we have examined the antiviral activity of ShellCoat, against SARS-CoV-2 in the presence or absence of organic matters. When concentrated SARS-CoV-2 was incubated with PBS (pH 7.4) for 10 or 80 sec, the viral titer was 6.6 ± 0.1 logs TCID₅₀/mL. However, when incubated with ShellCoat for 20 sec to 80 sec, the viral titer was found to be below the detection limit (≤ 2.1 logs TCID₅₀/mL). It is to be noted that when SARS-CoV-2 was incubated with ShellCoat only for 10 sec, the viral titer was reduced by more than 4 logs TCID₅₀ (Fig. 1). These data clearly indicate that the treatment of SARS-CoV-2 with the ShellCoat solution only for 10 sec is adequate to reduce the viral titer by more than 99.99% (Fig. 1). WHO has recommended to use 70% ethanol or 1,000 ppm sodium hypochlorite as disinfectants of surfaces contaminated with SARS-CoV-2. However, it is to be noted that both the chemicals may cause skin and mucous membrane irritations in human and sodium hypochlorite is corrosive for metals (Fukuzaki, 2006). Furthermore, the antimicrobial activity

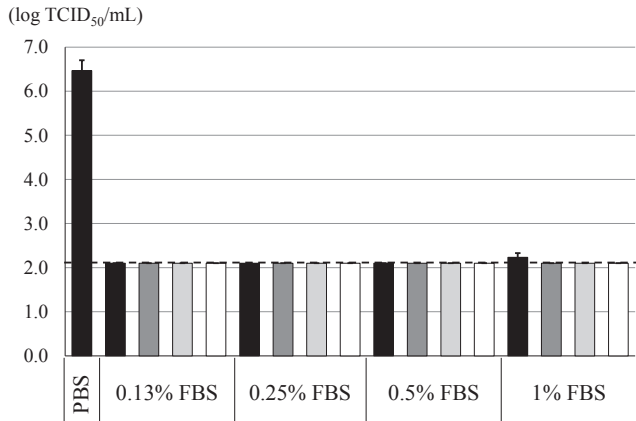


FIG. 2. Anti-viral activity of ShellCoat against SARS-CoV-2 in the presence of 0.13-1%FBS.

Polyethylene glycol (6,000) concentrated SARS-CoV-2 was treated with PBS (negative control) for 80 sec or ShellCoat for 10 (■), 20 (▒), 40 (▓) or 80 (□) sec in the presence of various concentrations of FBS and viral titer was determined as 50% tissue culture infective dose. All data represent the means \pm SD from three independent experiments. Dotted line indicates detection limit of the experiment (≤ 2.1 logs TCID₅₀/mL).

of sodium hypochlorite cannot be fully effective if organic matters are present (Yamaoka et al., 2016).

Therefore, antiviral activity of ShellCoat was evaluated under organic matter rich conditions. Saliva is the most important infection source of SARS-CoV-2. Jenzano et al. (1986) reported that protein concentration of saliva is 1.12 ± 0.39 mg/mL or 1.11 ± 0.42 mg/mL by Lowry method or by BCA protein assay, respectively. Thus, SARS-CoV-2 was incubated with ShellCoat in the presence of 0.13% to 1.0% of FBS as a final concentration. This is because 2.5% FBS (0.13% FBS as a final concentration) contains equivalent amount of protein as found in saliva i.e., 1.1 mg/mL as determined by the Bradford protein assay (data not shown). When SARS-CoV-2 was incubated with ShellCoat for 10 sec in the presence of 0.13% to 0.5% FBS, the viral titer was decreased from 6.6 ± 0.1 logs TCID₅₀/mL to below the detection limit (≤ 2.1 logs TCID₅₀/mL) within 10 sec. By incubation of SARS-CoV-2 with ShellCoat in the presence of 20% FBS (1.0% FBS as a final concentration), which contains 8 times higher concentration of proteins present in saliva, the viral titer was decreased below the detection limit (≤ 2.1 logs TCID₅₀/mL) within 20 sec and reduced by more than 4.0 logs TCID₅₀/mL within 10 sec (Fig. 2). It has been reported that about 10^5 to 10^6 copies per microliter of SARS-CoV-2 were detected in saliva of the patient by real-time PCR (IASR, 2021). Since real-time PCR could detect both RNA genomes in non-infective and infective viruses, it is not clear if these copy numbers are corresponding to

TCID₅₀ determined in this study. It is not clear how many SARS-CoV-2 are need to infect human. Nevertheless, these results clearly indicate that the ShellCoat is indeed a potent antiviral agent against SARS-CoV-2 even in the presence of organic matters and might prevent infection to human. Since ShellCoat has been approved as a food additive in Japan and it is in use in various restaurants and bars where people eat and drink, and the results of this study support the safe and wide use of ShellCoat.

In conclusion, the results of the present study suggest that ShellCoat could inactivate SARS-CoV-2 very rapidly and efficiently even in the presence of 20% FBS, which contains 8 times higher concentration of proteins present in saliva. Thus, ShellCoat can be used as a very effective and safe antiviral agent against SARS-CoV-2.

ACKNOWLEDGMENT

SARS-CoV-2 JPN/TY/WK-521 strain was kindly provided by National Institute of Infectious Diseases, Japan. We thank Dr. Rupak K. Bhadra, CSIR-Indian Institute of Chemical Biology, Kolkata, India for critically reading the manuscript.

CONFLICT OF INTEREST

This study was performed as a collaborative research of Kawakami Co., Ltd., and financially supported by this company.

ETHICAL STATEMENT

This study did not contain any human and animal subject.

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