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RAPID COMMUNICATION

Impregnation of probiotics into porous TiO_2 support for enhanced viability

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Abstract−The viability of probiotics in titania (TiO_2) support was assessed in simulated gastrointestinal environment. TiO_2 support with macropores was synthesized using titanium (IV) isopropoxide (TTIP) as a precursor and impregnated with probiotics including *Lactobacillus paracasei* and *Streptococcus salivarius*, respectively. Scanning electron microscopy analysis after impregnation with probiotics revealed that the probiotics were located inside the macropores of the support. Compared with non-impregnated free probiotics, the impregnated probiotics survived at a higher rate in a simulated gastrointestinal environment. The probiotics impregnated in the TiO_2 support exhibited low viability in the simulated stomach environment, but their viability recovered in the simulated intestinal environment. However, free probiotics did not exhibit any recovery of viability under the same conditions. These results suggest that the TiO_2 support enhanced the stability of the impregnated probiotics against environmental stress in the gastrointestinal tract.

Keywords: Probiotics, Titanium Dioxide, Impregnation, Gastrointestinal Conditions

INTRODUCTION

Probiotics, most of which are lactic acid bacteria, are defined as living microorganisms that exert a beneficial effect on the host by ameliorating the intestinal microbial imbalance when administered in appropriate amount. It has been reported that probiotics produce organic acids, such as lactic acid and acetic acid, through their metabolic cycles and that these compounds improve intestinal health. Moreover, because they also produce antibacterial compounds against pathogenic bacteria, new possibilities for their use in disease treatment are being actively studied. Because of the beneficial effects of these probiotics, they are widely used in various fields, including the food and pharmaceutical industries [1-4].

To be effective, there is the required probiotics dose of 10^8 to 10^9 colony forming unit per day depending on the type of strains. For probiotics to optimally perform their function after oral administration, they must survive until they reach the intestine even when exposed to the low pH in the stomach and digestive enzymes. However, while the pH of gastric acid is around 1.5-3.5 in healthy individuals, the optimal growth pH of probiotics is 5.0-6.5; thus, the viability of probiotics is greatly reduced when exposed to gastric acid. This insight highlights the importance of providing protection to probiotics before colonizing the human intestine for consumers. Therefore, methods to protect probiotics through the stomach are needed to increase their stability and viability [5,6]. A common way to solve these problems is to immobilize probiotics on a porous support or fix them using a process such as adsorption, fixation, or microencapsulation [7,8]. There are several reports on the encapsulation of cells using alginate, chitosan and silk protein [9-11]. However, these methods are complicated for the encapsulation, and the process causes an increase in the production cost.

The porous support used as an immobilized carrier must be non-reactive and non-toxic. It should also be economical, available in large quantity, easy to handle, and stable [12]. Considering these factors, titanium dioxide (TiO_2) is a relatively abundant and stable material, and it is used in many products such as paint, sunscreen, cosmetics, and edible pigments because it is non-toxic and economical [13]. TiO_2 nanoparticles have been shown to be toxic, but a TiO_2 porous support is non-toxic, as bulk particles and bulk TiO_2 are widely used in food. The toxicity of TiO_2 was only reported on the nano-sized particle. It was reported the bulk-sized food-grade TiO_2 had different effect on environment [14]. From a microscopic point of view, pores are classified according to size. Pores less than 2 nm are classified as micropores, between 2 nm and 50 nm are defined as mesopores, and those greater than 50 nm are classified as macropores. TiO_2 with a porous structure has a large surface area and can be selectively employed based on the pore structure [15,16]. Therefore, it can be used to easily immobilize and carry compounds, and it plays an important role in industrial processes such as gas storage, purification, and separation [17,18].

Therefore, to increase the viability of probiotics in a gastrointestinal environment, TiO_2 supports with macropores were synthesized and used for immobilization in this study. Two strains of probiotics, *Lactobacillus paracasei* and *Streptococcus salivarius*, that are mainly used in the domestic fermented milk product industry were impregnated on this porous TiO_2 support. Then, the probiotics were subjected to a simulated gastrointestinal environmentand cell viability was measured to determine the protective effect of the TiO_2 support.

MATERIALS AND METHODS

1. Bacteria and Culture Conditions

Lactobacillus paracasei (KCTC13169) and *Streptococcus salivarius*

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subsp. *thermophilus* (KCTC5098) strains were purchased from the KCTC (Korean Collection for Type Cultures, Jeongeup, Republic of Korea).

Lactobacilli MRS medium, tryptic soy medium, and agar were purchased from BD (Becton & Dickinson, Franklin Lakes, NJ, USA) and used for strain culture. Titanium (IV) isopropoxide used for the synthesis of the TiO_2 porous support was purchased from Sigma-Aldrich ($Ti[OCH(CH_3)_2]_4$; 97%; St. Louis, MO, USA).

S. thermophilus was maintained, stored, and cultured in tryptic soy medium, and MRS medium was used for *L. paracasei* for the same purposes. The strains were inoculated into 3 mL of each medium, cultured for 24 h at 37 °C and 100 rpm, and concentrated to an OD_{595}=5.0 (*S. thermophiles* 4.4×10^7 CFU, *L. paracasei* 4.0×10^8 CFU). To provide a solid medium for measuring cell viability, 1.5% agar was added to the medium.

2. Preparation of Simulated Gastrointestinal Fluids

NaCl, NaH_2PO_4, NH_4Cl, $MgCl_2$, and NaOH were purchased from Daejung Chemicals & Metals Co. (Busan, Republic of Korea); KCl and urea were purchased from Yakuri Pure Chemicals Co. (Osaka, Japan); $CaCl_2$ was obtained from Shinyo Pure Chemicals Co. (Osaka, Japan); and $NaHCO_3$ was purchased from Junsei Chemical Co. (Tokyo, Japan).

Glucose was purchased from Junsei (Tokyo, Japan); D-(+)-glucosamine hydrochloride was purchased from TCI (Tokyo Chemical Industry Co., Tokyo, Japan); D-glucuronic acid, pepsin (1 : 3,000), and pancreatin from porcine pancreas were purchased from Daejung Chemicals & Metals Co.; bile (bovine), BSA and lipase from porcine pancreas were purchased from Sigma-Aldrich; and mucin was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following the method described in a previous report [18]. Inorganic and organic compounds were dissolved in distilled water. The pH was adjusted using 1 M HCl, and the solution was then filtered through a 0.2 μm filter. All the prepared solutions were stored at 4 °C, and enzymes were added immediately before mimicking the gastrointestinal environment.

3. Synthesis of the TiO_2 Porous Support and Impregnation with Probiotics

First, 25 mL of titanium (IV) isopropoxide (TTIP) was added dropwise to 100 mL of distilled water and the mixture was shaken sufficiently. Then, it was filtered and washed three times with distilled water to obtain a precipitate. Finally, it was calcined at 400 °C to obtain the TiO_2 support with macropores.

Using the incipient wetness method, probiotics (100 μL; at an OD_{595}=5.0) were quickly impregnated into the TiO_2 support (0.5 g).

4. Survival Rate of Probiotics under Simulated Gastrointestinal Tract Conditions

The viability of TiO_2 support-impregnated probiotics and free probiotics (control) was measured in a simulated gastrointestinal environment. All reactions were conducted at 60 rpm and 37 °C.

For the simulated gastric environment reaction, 4.5 mL of phosphate-buffered saline (PBS) and 5 mL of SGF were added to 0.5 g of TiO_2 support impregnated with 100 μL of probiotics and allowed to react for 1 h. To simulate the intestinal environment, 15 mL of SIF was added to the solution after the simulated gastric environment reaction and maintained for 2 and 4 h under the same conditions [9,19,20].

After the reaction was completed, the cell pellet was obtained by centrifugation at 4,000 rpm for 20 min and then washed with 10 mL of PBS. It was then stirred for 1 min to allow the probiotics to escape from the TiO_2 porous support. After the mixture was allowed to stand for 1 min, 1 mL of the supernatant was obtained as a sample. The control group samples were obtained in the same way except for the stirring step. The collected samples were diluted to 10^{-2} to 10^{-5} and plated (100 μL) on agar media. After incubation at 37 °C for 24 to 48 , the number of colonies was determined to calculate the viable cell count. All experiments were carried out in triplicate. The values are shown in average±standard deviation.

5. Characterization

X-ray diffraction (XRD, D/MAX-2500, Rigaku, Tokyo, Japan) was used to analyze the crystalline structure of the synthesized TiO_2 using a Cu Kα source. To obtain images of the inside of the pores in TiO_2, an analytical high-resolution scanning electron microscope (Analytical HR-SEM Su-70, Hitachi, Tokyo, Japan) was used after the TiO_2 impregnated with the probiotics was subjected to freeze-drying and vacuum evaporation coating with platinum. Mercury porosimetry (UPA-150, ASAP2010, AutoporeIV, Micromeritics, Norcross, GA, USA) was performed to confirm the pore structure of TiO_2.

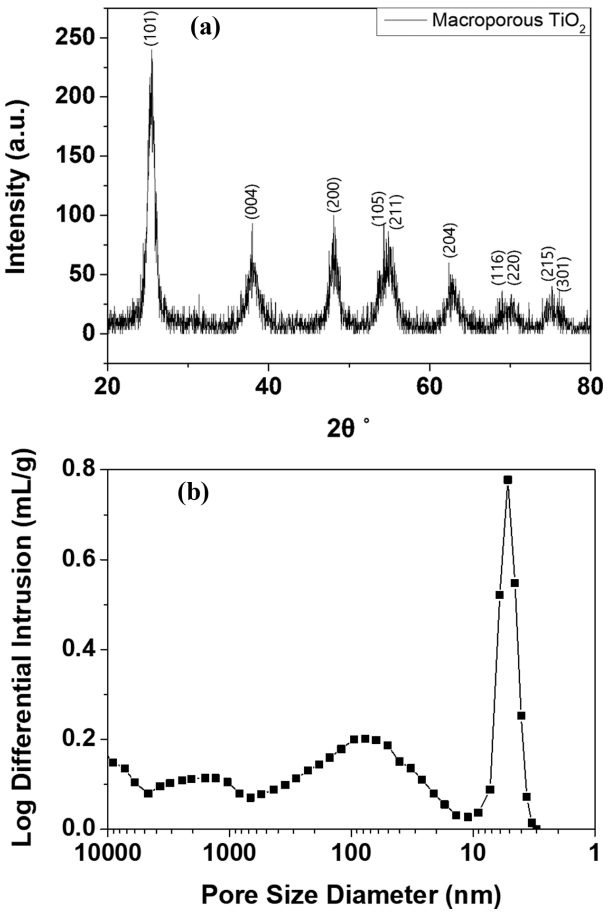

Fig. 1. (a) X-ray diffraction patterns of the TiO_2 support. (b) Pore size distribution of the TiO_2 support obtained with mercury porosimetry.

RESULTS AND DISCUSSION

1. Physical Properties Analysis of the Porous TiO_2 Support

Fig. 1(a) shows the XRD patterns of the TiO_2 synthesized in this study. The results indicate an anatase structure (JCPDS No. 21-1272) having a corresponding (1 0 1), (0 0 4), (2 0 0), (1 0 5), (2 1 1), (2 0 4), (1 1 6), (2 2 0), (2 1 5), (3 0 1) plane at 2θ of 25.0°, 37.6°, 47.7°, 54.3°, 62.4°, 68.8°, 74.9°, 76.2°. These peaks demonstrate that TiO_2 with an anatase phase was formed with clear crystallinity.

Fig. 1(b) shows the pore size distribution of TiO_2 determined by mercury porosimetry. These results indicate that this sample has well-developed macropores. Among these pores, macropores with size ranging from 1 to 10 μm are suitable for immobilizing the probiotics. Thus, the TiO_2 contains suitable macropores and is expected to be an appropriate support for probiotics.

2. Analysis of the Porous TiO_2 Support after Probiotic Impregnation

SEM (Fig. 2) was performed to determine whether the probiotics were properly located inside the macropores in the TiO_2 support. The freeze-dried TiO_2 porous support was found to have a broken glass structure, which is a common characteristic of freeze-dried powders, showing irregular shapes and various pore sizes [21]. *L. paracasei* has been reported to have a bacillus shape with a width of about 0.5-1.0 μm and a length of 2.0-4.0 μm, and *S. thermophilus* has been reported to have a coccus shape with a length of 1.07-1.21 μm [22,23]. SEM images revealed that the cells appeared to be smaller than these reported sizes following the freeze-drying and dehydration process, which is one of the pretreatment procedures for SEM analysis. Therefore, taking this into account, it can be expected that before the freeze-drying, the cells were of an appropriate size to fit in the macropores of the TiO_2 support.

3. Survival of Probiotics under the Simulated Gastrointestinal Tract Conditions

L. paracasei and *S. thermophiles* are the main probiotics in yogurt product, and its benefits by daily uptake were well investigated. To investigate the effect of the TiO_2 support on the survival rate of probiotics, the survival rate of the bacteria was measured in a simulated gastrointestinal environment and compared with those of the control group. *L. paracasei* impregnated in the porous TiO_2 support exhibited a low survival rate in the simulated gastric environment (Fig. 3). In the simulated intestinal environment, however, the survival rate of *L. paracasei* impregnated in the TiO_2 supports recovered to 9.7%. However, in the control group, non-impregnated free *L. paracasei*, no viable cells were observed in the simulated gastric environment. Then, even in the intestinal environment, free *L. paracasei* showed a low survival rate of 0.06%, exhibiting little recovery.

The simulated gastric and intestinal environments were previously developed to mimic human digestion and used as a tool to

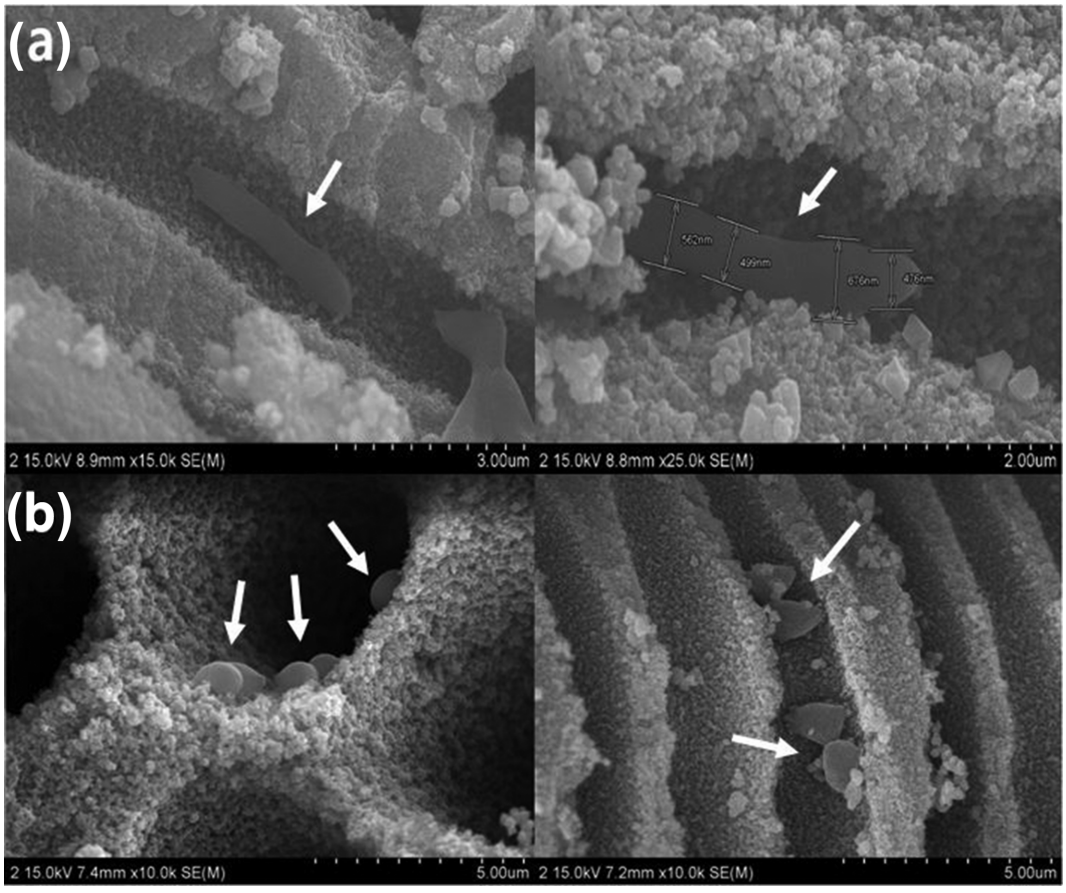

Fig. 2. SEM images of probiotics impregnated in TiO_2 porous beads. (a) *Lactobacillus paracasei* (b) *Streptococcus salivarius* subsp. *thermophiles*.

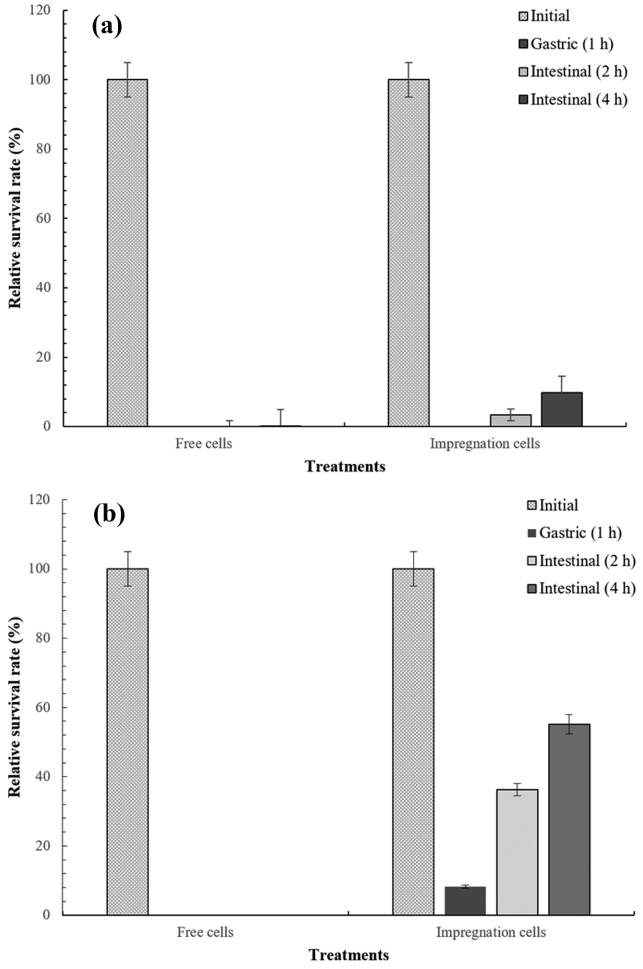

Fig. 3. Comparison of the relative survival rates of probiotics in a simulated gastrointestinal environment. (a) *Lactobacillus paracasei* (b) *Streptococcus salivarius* subsp. *thermophilus*.

access bioaccessibility [9,19,20]. The simulated gastric fluids were composed of inorganic compounds, organic compounds including glucose, glucosamine hydrochloride, urea and glucuronic acid, and enzymes such as BSA, pepsin, and mucin. The pH value of the simulated gastric fluids was adjusted to pH 1.5±0.1. In the case of the simulated intestinal fluids, the organic compound was urea, and enzymes were composed of bile, pancreatin, BSA, and lipase with pH 6.5±0.1 [9].

Similar results were obtained for *S. thermophiles*. *S. thermophiles* impregnated in the TiO_2 supports exhibited a survival rate of 8.3% in the simulated gastric environment. However, after 2 h and 4 h in the simulated intestinal environment, the survival rate of *S. thermophiles* impregnated in the TiO_2 support recovered up to 55%. However, in the control group, free *S. thermophiles*, no viable cells were observed in the simulated gastric environment, and cells remained undetectable even in the simulated intestinal environment. Moreover, the experimental results showed that *S. thermophiles* exhibited a higher survival rate than *L. paracasei* under the tested conditions. During 2 h in intestinal condition, cell concentration of *S. thermophiles* was increased with doubling time of about 1 h. The doubling time was reported to be about 20 min under favorable condition [24]. Although the growth rate was not maximal, moderate growth was observed in the simulated intestinal fluids. Therefore, these results indicate that TiO_2 represented a more effective support for *S. thermophiles* than for *L. paracasei*. The intestinal environment is more favorable for cell growth than the stomach. Therefore, the cells can grow in the intestine during the retention. *S. thermophiles* is known to have several benefits, including digestion improvement, immune enhancement and increase of HDL [25]. The higher survival rate of *S. thermophiles* was not fully understood, but the resistance to freezing and frozen storage of *S. thermophiles* would be related [26].

Impregnated probiotics that survive in the stomach environment until reaching the intestine will be able to recover their viability for 2-4 h, which is the intestinal residence time for normal food. The surviving probiotics could grow in the simulated intestinal fluids because of moderate pH. Therefore, once they recover, probiotics can exert their beneficial effects in the intestine [27,28]. This method using porous TiO_2 can be used in a variety of ways in the food and pharmaceutical industries to solve the problem of lowered cell survival rates after passing through the gastrointestinal tract.

The toxicity of TiO_2 was only reported on the nano-sized particle. It was reported the bulk-sized food-grade TiO_2 had a different effect on the environment.

CONCLUSION

TiO_2 support with macropores was synthesized using TTIP and impregnated with probiotics. X-ray diffraction and mercury porosimetry analyses revealed that the TiO_2 support exhibited an anatase phase and contained macropores suitable for the immobilization of probiotics. Then, SEM confirmed that the probiotics were located in the porous TiO_2 support. The survival rate of probiotics differed in the simulated gastrointestinal environment, depending on the probiotic strain and on whether porous TiO_2 support was used. The viability of free probiotics (not-impregnated cells) decreased to an undetectable level during the passage of the probiotics through the simulated gastric environment, and then the probiotics exhibited hardly any recovery of cell viability in the intestinal environment. However, for probiotics impregnated in the TiO_2 porous support, although the cell viability decreased greatly in the simulated gastric environment, it recovered in the simulated intestinal environment. These results indicate that probiotics impregnated in a TiO_2 porous support exhibit improved stability against gastrointestinal environmental stress. Thus, TiO_2 porous support seems to be a potential carrier to transfer probiotics to the intestine.

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