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Effects of Phosphoryl Oligosaccharides of Calcium (POs-Ca) on Mycelial Growth and Fruiting Body Development of the Edible Mushroom, *Pleurotus ostreatus*

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Abstract: Phosphoryl oligosaccharides of calcium (POs-Ca) is a calcium salt of phosphoryl maltooligosaccharides made from potato starch. POs-Ca is highly water-soluble and can supply both the calcium ion and acidic oligosaccharides in an aqueous solution. In this study, we investigated the effects of POs-Ca on the mycelial growth and fruiting body yield of *Pleurotus ostreatus*, which is one of the most widely cultivated edible mushrooms in the world. We cultivated the mushroom using both potato dextrose agar (PDA) medium and sawdust-based medium, with added calcium salts. The addition of POs-Ca into the PDA medium with a calcium concentration of 10 mg increased mycelial growth significantly ($p < 0.05$, *vs.* control). POs-Ca addition to the sawdust-based medium at concentrations of 1.0 to 3.0 g/100 g medium increased the amount of calcium in the fruiting bodies but did not affect the length of the cultivation period or the weight of the fruiting body. The calcium content in the fruiting body increased 12-fold when compared to the control. On the other hand, neither the $CaHPO_4 \cdot 2H_2O$ group nor the $CaHPO_4 \cdot 2H_2O$ with oligosaccharides group showed changes in the calcium content of the fruiting bodies. Our results indicate that the use of POs-Ca in mushroom cultivation allows for the possibility of developing new functional foods like calcium-enriched edible mushrooms. This is the first report describing the effects of POs-Ca on mushroom cultivation.

Key words: phosphoryl oligosaccharides of calcium, mycelial growth, mushroom cultivation, fruiting body, calcium accumulation, *Pleurotus ostreatus*

INTRODUCTION

Phosphoryl oligosaccharides of calcium (POs-Ca) is a highly water-soluble calcium salt prepared from potato starch hydrolysate. The phosphoryl oligosaccharides moiety consists of maltooligosaccharides with 3 to 5 glucose units and 1 phosphoryl residue at the 3- or 6-position of a glucosyl residue.[1)] Calcium phosphate has low water solubility. On the other hand, POs-Ca has high water solubility because its oligosaccharide moiety is very soluble in water. Previous studies have demonstrated that POs-Ca has various functions such as remineralization and recrystallization of tooth enamel lesions, skin barrier protection, and skin moisturization.[2)3)] Tanaka *et al.* showed that POs-Ca increased the calcium ion concentration in saliva because of its high water solubility, resulting in the enhancement of remineralization and recrystallization of tooth enamel.[2)] Moreover, chewing gum containing POs-Ca is commercially available for the prevention of dental caries.

Pleurotus ostreatus is one of the most commonly found edible mushrooms worldwide. The growth, yield, and quality of the hyphae and fruiting bodies depend on the nutrients and the physicochemical environment of the growth medium.[4)] Lignocellulosic materials, such as corn cobs, wheat straws, cotton waste, sawdust, paper, and even food industry waste have been investigated for mushroom cultivation.[5)6)7)8)] Furthermore, the effects of additives, such as nitrogen and minerals, have also been widely studied.[9)10)11)12)] Previous studies have demonstrated that calcium salts have various functions for mushroom cultivation, e.g., increasing mycelial growth, neutralizing acid in the medium during cultivation, and increasing the calcium content of a fruiting body.[13)14)15)] Therefore, we hypothesized that POs-Ca may affect the mycelial growth and fruiting body yield of edible mushrooms.

In this study, we evaluated the effects of the highly water-soluble calcium salt, POs-Ca, on the growth of *P. ostreatus*. We observed mycelial growth on both potato dextrose agar (PDA) medium and sawdust-based medium, examined the mushroom cultivation characteristics, and analyzed the calcium content of fruiting bodies. To the best of our knowledge, this is the first report on the use of POs-Ca for mushroom cultivation.

MATERIALS AND METHODS

Microorganism and chemicals. *Pleurotus ostreatus* was

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Abbreviations: POs-Ca, phosphoryl oligosaccharides of calcium; PDA, potato dextrose agar

purchased from Kagawashiitake Co. (Miyagi, Japan). The stock culture was inoculated on PDA medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), incubated at 25 °C for 7 days, and used throughout the experiments. Petri plates were stored at 4 °C. Calcium hydrogen phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), POs-Ca (POs-Ca 50, calcium content approximately 5 % (w/w), Glico Nutrition Co., Ltd., Osaka, Japan), and Oligotose (Sanwa Starch Co., Ltd., Nara, Japan) were used as additives. To evaluate the effects of calcium, the additive amount was adjusted to the concentration of elemental calcium in calcium salts.

Mycelial growth on the PDA medium supplemented with calcium salts. The calcium salts tested were POs-Ca and $CaHPO_4 \cdot 2H_2O$. *P. ostreatus* was cultivated with the PDA medium containing 20.2, 101.1, 202.2, 606.7, and 1011.1 mg/dish of POs-Ca and 4.3, 21.5, 42.9, 128.8, and 214.7 mg/dish of $CaHPO_4 \cdot 2H_2O$. In both groups, the calcium concentration was adjusted to 1, 5, 10, 30, and 50 mg/dish. Control dishes contained PDA medium only. The medium was autoclaved (121 °C, 15 min) and poured into petri dishes (20 mL/dish). *P. ostreatus* stock culture was previously grown on PDA medium in a dish, as described above. The precultured *P. ostreatus* was cut with a sterile cork borer (7 mm in diameter) and inoculated onto the center of each dish (1 plug/dish). The inoculated dishes were incubated at 25 °C for 7 days in the dark, and then the colony diameter was measured. Due to deviations from absolute circularity, both the largest and smallest diameters of the colonies were measured, and the mean was calculated. The measurements of mycelial growth were determined on Days 4, 5, and 7. All experiments were conducted in quintuplicate.

Mycelial growth on the sawdust-based medium supplemented with calcium salts. Sawdust of Japanese beech (2 × 2 mm) and wheat bran (4:1, v/v) were mixed, and the moisture content of the medium was adjusted to 63 % (w/w) with tap water. The calcium salts, POs-Ca and $CaHPO_4 \cdot 2H_2O$, were added at concentrations of 1.0, 2.0, 3.0, 4.0, and 6.1 g/100 g of medium and 0.2, 0.4, 0.6, 0.9, and 1.3 g/100 g medium, respectively. In both groups, calcium concentrations were adjusted to 50, 100, 150, 200, and 300 mg/100 g medium, and the mediums were mixed well for homogeneity. Thirty-five grams (in fresh weight) of the substrate was tightly packed into a test tube (24 mm in diameter and 22 cm in length) and then capped with a silicone cap. The test tubes were autoclaved at 121 °C for 30 min. After cooling to room temperature, precultured *P. ostreatus* were cut with a sterile cork borer (7 mm in diameter) and inoculated onto the center of each test tube (1 plug/tube). The inoculated test tubes were incubated at 25 °C for 21 days in the dark. Both the longest and shortest lengths from inoculated plug to tip of hyphae were measured, and the mean was calculated. The measurements of mycelial growth were taken on Days 7, 14, and 21. All experiments were conducted in quintuplicate.

Growth and fruiting of* P. ostreatus *on the sawdust-based medium supplemented with calcium salts. Mushroom cultivation was performed with a slightly modified method of the approach described by Obatake *et al.*[16] A mixture of Japanese oak sawdust (2 × 2 mm) and wheat bran (4:1, v/v) was supplemented with calcium salts: POs-Ca, $CaHPO_4 \cdot 2H_2O$, and $CaHPO_4 \cdot 2H_2O$ with Oligotose. The main component of Oligotose is maltotriose. POs-Ca was added at concentrations of 1.0, 2.0, and 3.0 g/100 g medium. To compare with the POs-Ca groups, the concentrations of $CaHPO_4 \cdot 2H_2O$ in the $CaHPO_4 \cdot 2H_2O$ group and $CaHPO_4 \cdot 2H_2O$ in the $CaHPO_4 \cdot 2H_2O$ with oligosaccharides group were 657.9 mg/100 g medium. These calcium salts were added at an elemental calcium concentration of 150 mg/100 g medium. The calcium level was set based on the result of mycelial growth on the sawdust-based medium described in the preceding paragraph. With regard to the group of $CaHPO_4 \cdot 2H_2O$ with oligosaccharides, the amount of oligosaccharides was adjusted to be equivalent to the oligosaccharide moiety of POs-Ca (95 % of the weight of POs-Ca). Then tap water was added to the mixture to give a moisture content of 63 % (w/w), and it was mixed well for homogeneity. Twenty grams (in fresh weight) of the substrate was tightly packed into a test tube (length, 98 mm; inner diameter, 22 mm). The test tubes were capped with a polypropylene cap and autoclaved at 121 °C for 30 min. After cooling, precultured *P. ostreatus* were cut with a sterile cork borer (7 mm diameter) and inoculated onto the center of each test tube (1 plug/tube). The cultures were incubated for 14-15 days at 25 °C with 70 % relative humidity. The surface of the culture was then removed to induce fruiting body formation. The cultures were transferred to another incubator maintained at 15 °C with 90 % relative humidity and continuously illuminated (500–600 lx) by fluorescent lamps. The harvested fruiting bodies were dried in a conventional air oven for 48 h at 100 °C. After drying, the weights were measured. The dried fruiting bodies were powdered with a conventional mill. Next, the dried fruiting bodies obtained from 10 or 11 tubes in each group were combined and mixed well.

Determination of calcium content in fruiting body. The content of calcium in the dried fruiting bodies from the sawdust-based medium was determined using an inductively-coupled plasma (ICP) emission spectroscopy (ICPS-8100, Shimadzu Co., Kyoto, Japan). The sample (0.2 g) was digested with a mixture of sulfuric acid and nitric acid and diluted with water. The diluted solution was applied to the ICP emission spectroscopy. These chemical analyses were performed at Kobelco Research Institute, Inc. (Hyogo, Japan).

Statistics. Statistical analysis of all experimental data was performed using the statistical software package JMP13.2. Analysis of variance was followed by the comparison of means using Tukey's honest significance test. Differences were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

The effects of calcium salts on the mycelial growth on PDA medium.

Mycelial growth was observed within 7 days post-inoculation on the PDA medium. Table 1 shows the effects

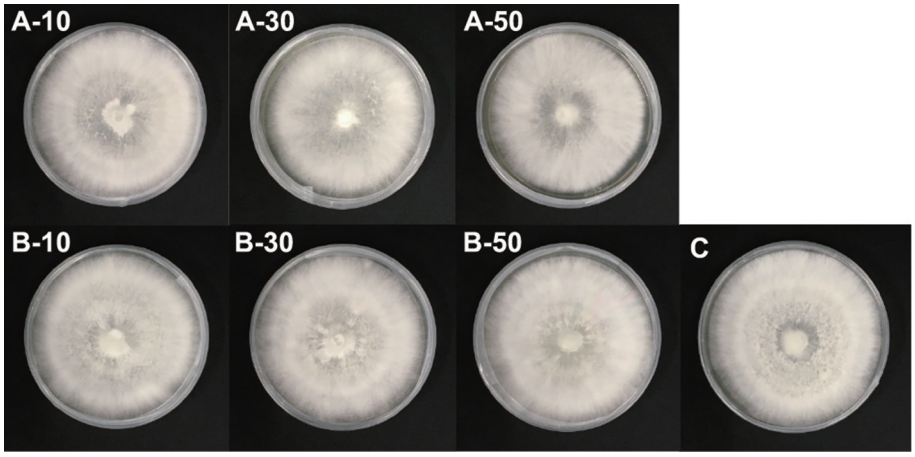

Fig. 1. Mycelial growth of *P. ostreatus* on PDA medium supplemented with POs-Ca (**A** group), supplemented with $CaHPO_4 \cdot 2H_2O$ (**B** group), and without calcium salt (control, **C**).
For **A** and **B** groups, the calcium level was adjusted to 10, 30, and 50 mg/dish, respectively. The cultivation was performed for 8 days at 25 °C.

Table 1. Mycelial growth of *P. ostreatus* on PDA medium supplemented with calcium reagents.

Calcium reagent	Additive content of calcium (mg/plate)	Mycelial growth (mm)		
		Days 4	Days 5	Days 7
Control	0	38.1±0.7^{b}	50.1±1.2^{cde}	76.8±1.5^{de}
POs-Ca	1	39.3±1.6^{ab}	39.3±1.6^{ab}	73.6±4.3^{e}
	5	40.1±0.7^{ab}	52.4±1.5^{bcd}	79.5±2.6^{bcd}
	10	37.5±2.4^{b}	48.7±2.1^{de}	82.8±1.7^{abc}
	30	34.1±0.8^{c}	47.7±1.0^{e}	80.6±1.8^{abcd}
	50	27.9±0.7^{d}	40.9±1.0^{f}	76.5±2.6^{de}
$CaHPO_4 \cdot 2H_2O$	1	37.4±1.4^{b}	50.4±1.7^{cde}	78.6±1.5^{cd}
	5	39.9±1.7^{ab}	53.4±2.2^{abc}	82.4±2.6^{abc}
	10	39.8±0.9^{ab}	54.8±1.9^{ab}	83.0±1.3^{abc}
	30	41.6±0.8^{a}	57.3±1.6^{a}	84.6±0.6^{a}
	50	40.0±1.1^{ab}	55.3±1.8^{ab}	83.6±1.4^{ab}

Each value is expressed as mean ± SD (n = 5). Mean values followed by the same letter(s) are not significantly different at the 5 % level (Tukey-Kramer's HSD test).

Table 2. Mycelial growth of *P. ostreatus* on sawdust medium supplemented with calcium reagents.

Calcium reagent	Additive content of calcium (mg/100 g of medium)	Mycelial growth (mm)		
		Days 7	Days 14	Days 21
Control	0	31.7±1.9^{a}	83.6±3.2^{ab}	129.8±2.5^{bc}
POs-Ca	50	31.2±1.6^{a}	83.4±1.4^{ab}	132.4±2.5^{abc}
	100	29.9±1.7^{a}	81.5±1.4^{b}	127.7±2.0^{c}
	150	26.4±1.3^{b}	74.6±1.1^{c}	117.7±1.6^{d}
	200	23.3±1.0^{b}	67.7±1.2^{d}	108.1±1.8^{e}
	300	17.7±1.7^{c}	58.3±2.8^{e}	96.8±3.9^{f}
$CaHPO_4 \cdot 2H_2O$	50	31.8±1.7^{a}	84.2±1.9^{ab}	132.7±1.9^{abc}
	100	31.7±1.3^{a}	83.9±1.8^{ab}	131.1±4.5^{abc}
	150	31.3±1.5^{a}	84.4±2.1^{ab}	133.8±1.5^{ab}
	200	32.8±0.9^{a}	87.3±2.4^{a}	135.8±2.0^{a}
	300	31.9±1.1^{a}	86.6±2.2^{a}	134.4±2.9^{ab}

Each value is expressed as mean ± SD (n = 5). Mean values followed by the same letter(s) are not significantly different at the 5 % level (Tukey-Kramer's HSD test).

of POs-Ca and $CaHPO_4 \cdot 2H_2O$ on the mycelial growth on the PDA medium. In the experiment supplemented with POs-Ca, we observed that the mycelial growth decreased as supplementation increased on Days 4 and 5. However, after 7 days of incubation, the colony diameter for the 10 mg/dish of calcium contained in POs-Ca was significantly increased by 82.8 mm ($p < 0.05$, *vs*. control). The PDA medium supplemented with 1, 5, 30, and 50 mg/dish of calcium contained in POs-Ca did not show any effects on mycelial growth on Days 7.

For the experiment supplemented with $CaHPO_4 \cdot 2H_2O$, we observed that the mycelial growth increased, except for the 1 mg/dish of calcium contained in $CaHPO_4 \cdot 2H_2O$. On Day 7 of cultivation, the group supplemented with 30 mg/dish of calcium contained in $CaHPO_4 \cdot 2H_2O$ measured 84.6 mm ($p < 0.05$, *vs*. control). POs-Ca and calcium phosphate were shown to have positive effects on mycelial growth of *P. ostreatus* on PDA medium. Tabata and Shinohara (1995) evaluated the mycelial growth of *P. ostreatus* using potato sugar agar medium and reported that $CaHPO_4 \cdot 2H_2O$ did not affect the mycelial growth of *P. ostreatus* under 1 %.[13] Our results are consistent with their findings. As shown in Fig. 1, the appearance and density of mycelium from POs-Ca and $CaHPO_4$ groups were similar to that of the control group.

The effects of calcium salts on the mycelial growth on sawdust medium.

The growth of mycelia on sawdust-based medium supplemented with calcium salts, POs-Ca and $CaHPO_4 \cdot 2H_2O$, is reported in Table 2. In contrast to the PDA medium experiment, we observed that the mycelial growth decreased as POs-Ca supplementation increased.

In the experiment supplemented with $CaHPO_4 \cdot 2H_2O$, mycelial growth increased as supplementation increased. On Day 21 of cultivation, the mycelial growth significantly increased versus the growth of the control on the saw-

Table 3. Results of sawdust-based cultivation of *P. ostreatus*.

Calcium reagent	Additive content of calcium (mg/100 g of medium)	pH		Period from inoculation to harvest (days)	Dry weight of fruiting body (g/tube)
		Before inoculation	After harvest		
Control	0	5.3	4.6	29.3 ± 1.8[a]	0.25 ± 0.04[a]
POs-Ca	50	5.0	4.4	28.1 ± 0.8[a]	0.25 ± 0.03[a]
	100	5.0	4.3	27.5 ± 1.0[a]	0.26 ± 0.03[a]
	150	4.8	4.4	28.5 ± 0.5[a]	0.26 ± 0.04[a]
$CaHPO_4 \cdot 2H_2O$	150	5.5	4.4	29.5 ± 2.6[a]	0.24 ± 0.03[a]
$CaHPO_4 \cdot 2H_2O$ + Og	150	5.5	4.3	29.9 ± 3.3[a]	0.27 ± 0.04[a]

Og, oligosaccharide (Oligotose). Each value is expressed as mean ± SD (n = 10 or 11). Mean values followed by the same letter(s) are not significantly different at the 5 % level (Tukey-Kramer's HSD test).

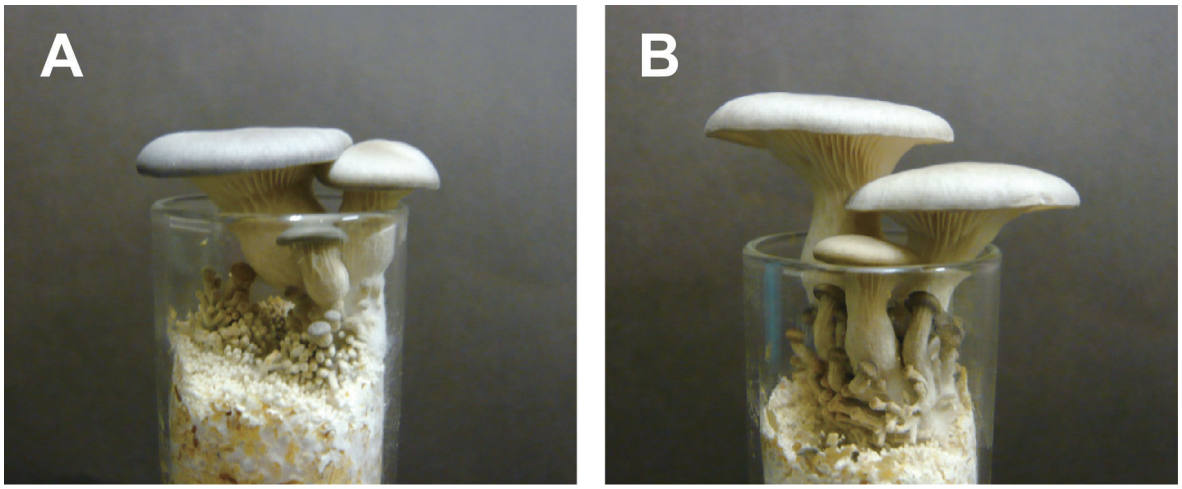

Fig. 2. Fruiting bodies of *P. ostreatus* on sawdust-based medium supplemented with POs-Ca (**A**) and without calcium salt (control, **B**). The calcium level of the sawdust-based medium of (**A**) was adjusted to 175 mg/100 g medium.

dust-based medium supplemented with 200 mg/100 g medium of calcium contained in $CaHPO_4 \cdot 2H_2O$ ($p < 0.05$). High concentrations of POs-Ca, which were greater than 150 mg/100 g medium, had adverse effects on the mycelial growth on the sawdust-based medium. Since POs-Ca contains carbohydrates, the mycelial growth may be suppressed in the higher concentration groups by the increased amount of carbohydrates. Previous research has also indicated that the supplementation of the sawdust-based medium with $CaHPO_4 \cdot 2H_2O$ did not affect the mycelial growth of *P. ostreatus*.[13]

***The effects of calcium salts on the sawdust-based cultivation of* P. ostreatus.**

Table 3 shows the results of the sawdust-based cultivation of *P. ostreatus*. Overall, the pH values decreased during cultivation for the groups grown on sawdust-based medium. The pH range was 4.8 to 5.5 before inoculation, and the pH range was 4.3 to 4.6 after harvest. It is well-known that growth medium pH decreases during mushroom cultivation due to oxalic acid secretions from the mushrooms.[17] The average cultivation period for the sawdust-based medium groups ranged from 27.5 to 29.9 days. Thus, there were no significant differences between the groups. As shown in Fig. 2, the quality of fruiting bodies cultivated from sawdust-based medium containing POs-Ca was the same as the control group. The average dry weight of the fruiting bodies from the sawdust-based medium groups ranged from 0.24 to 0.27 g/tube, and there were no significant differences between the groups. The addition of the calcium salts to the sawdust-based growth medium did not affect the cultivation period length or the amount of fruiting bodies present ($p < 0.05$). These results are confirmed by the results of Sekiya, which previously indicated that $CaHPO_4$ did not affect the cultivation period or the amount of fruiting bodies when compared to the control.[18]

Calcium content in the fruiting bodies of* P. ostreatus *from sawdust-based medium supplemented with calcium salts.

The amount of calcium contained in the fruiting body of *P. ostreatus* was determined using an ICP emission spectroscopy. Table 4 shows the results of the calcium content measurements in dried fruiting bodies from the sawdust-based medium supplemented with the calcium salts. The total amount of calcium contained in the fruiting bodies from the sawdust-based medium with no calcium salt was 0.07 mg/g. The calcium concentration of the fruiting bodies increased with the increasing calcium content, and the POs-Ca group with 150 mg calcium/100 g medium contained 0.85 mg/g calcium. The absorption ratio was 12.1 *vs*. the control. POs-Ca increased the calcium content of the fruiting bodies without affecting quality, yield, or the cultivation period. In contrast, the addition of $CaHPO_4 \cdot$

Table 4. Calcium content of the fruiting body of *P. ostreatus* harvested from the sawdust medium supplemented with calcium reagents.

Calcium reagent	Additive content of calcium (mg/100 g of medium)	Calcium content in the fruiting body (mg/g, dry weight)	Absorption ratio (*vs*. control)
Control	0	0.07	1.0
POs-Ca	50	0.15	2.1
	100	0.40	5.7
	150	0.85	12.1
$CaHPO_4 \cdot 2H_2O$	150	0.12	1.8
$CaHPO_4 \cdot 2H_2O$ + Og	150	0.11	1.5

Og, oligosaccharide (Oligotose).

$2H_2O$ did not affect the calcium content of the fruiting bodies. Tabata and Shinohara reported that the absorption ratio of calcium in the fruiting bodies of *P. ostreatus* from the sawdust-based medium supplemented with $CaHPO_4 \cdot 2H_2O$ was 1.1 *vs*. control,[13] and our results are consistent with their reported results. The primary reason for the efficiency of accumulating calcium in the fruiting bodies might be that POs-Ca is more accessible to cells of the mycelium than calcium phosphate because of its water solubility. Like POs-Ca, calcium chloride is also a highly water-soluble calcium salt. However, it was reported that calcium chloride strongly inhibited the mycelial growth of *P. ostreatus* and reduced the yield of fruiting bodies.[18] The addition of $CaHPO_4 \cdot 2H_2O$ with oligosaccharides also did not affect the calcium content of the fruiting body, which indicates that the ester bond between the oligosaccharide and phosphate is essential for accumulating calcium in the fruiting body.

Usually, mushrooms contain very little if any vitamin D2, but are abundant in the D2 provitamin ergosterol. Ergosterol is the principal sterol in mushrooms and is present in relatively high concentrations in *P. ostreatus*.[19] Numerous studies have reported that the ergosterol converts into vitamin D2 with ultraviolet (UV) irradiation or sunlight.[20][21][22] Vitamin D2 promotes the absorption of calcium from the intestine to help build and maintain bones.[23] Several studies have reported that vitamin D2 from vitamin D2-enriched mushrooms is well absorbed and quickly metabolized in rodents, and also improves bone mineralization.[24][25] Calcium is one of the most important and abundant minerals in the body. It is used in our nervous system, muscles, heart, teeth, and bones. According to the National Health and Nutrition Survey in Japan, the average daily per capita recommended dietary allowance of calcium was 650 mg for those aged 30 to 49, whereas the mean daily intake of the Japanese people was only 514 mg (National Health and Nutrition Survey, 2017). Calcium-enriched edible mushrooms using POs-Ca may help solve this nutrition problem. By combining UV irradiation technology and POs-Ca supplementation, it is possible to cultivate mushrooms that contain both calcium and vitamin D2. Calcium and vitamin D2-enriched mushrooms may have the potential to assist in bone mineralization and prevent osteoporosis. In the future, we plan to evaluate further the feasibility of edible mushrooms supplemented with POs-Ca.

CONFLICTS OF INTEREST

T.K. currently serves on the board of directors of Ezaki Glico Co., Ltd. T.K. is also the President and CEO of Glico Nutrition Co., Ltd., which is a supplier of POs-Ca. Glico Nutrition Co., Ltd. is a wholly owned subsidiary of Ezaki Glico Co., Ltd. All authors declare no other competing interests.

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