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Accumulation and Localization of Cesium in Edible Mushroom (*Pleurotus ostreatus*) Mycelia

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The characteristics of Cs accumulation and localization in edible mushrooms were examined using the mycelia of *Pleurotus ostreatus*-Y1. Scanning electron microscope images revealed the existence of white spots, and energy dispersive X-ray microanalyzer analysis indicated the presence of larger amounts of Cs and P in these spots in mycelia cultured on medium containing 25 mM CsCl. The ^{137}Cs activities in the mycelia were approximately 4−6 times higher than those in water used for ^{137}Cs elution. Higher Cs concentrations in the sediment fraction including vacuolar pellets were obtained compared to the upper fractions. It was observed that yellowish spots caused by the fluorescence of 4′,6-diamidino-2-phenylindole (DAPI)-stained polyphosphate were localized in the mycelia. The higher fluorescence intensity of the yellowish-grained spots was measured in comparison with other regions in the mycelium. These results suggested that Cs in the mycelia was trapped by polyphosphate in vacuoles or other organelles.

KEYWORDS: Accumulation; localization; cesium; potassium; mushroom; mycelium; *Pleurotus ostreatus*

INTRODUCTION

After the Chernobyl accident in 1986, it was reported that wild mushrooms in European countries contained high levels of ^{137}Cs (*1*−*4*). In addition, wild mushrooms with higher ^{137}Cs contents (less than detectable-1070 Bq kg^{-1} fresh wt., <0.4−1260 Bq kg^{-1} fresh wt., and not detectable-570 Bq kg^{-1} fresh wt.) were found in Japan (*5*−*9*). Recent reports showed that the levels of ^{137}Cs in wild mushrooms were still high in Europe (*10, 11*). For example, the highest activity concentrations of ^{137}Cs were 2263 Bq kg^{-1} (dry wt.) in *Xerocomus badius* from the Czech Republic, 966 Bq kg^{-1} (dry wt.) in *Suillus luteus* from Slovakia, and 401 Bq kg^{-1} (dry wt.) in a mushroom from Turkey, and it is internationally recognized that mushrooms are capable of accumulating ^{137}Cs from the environment. In Japan, radioactivity monitoring (permissible limit of 370 Bq kg^{-1} for ^{137}Cs + ^{134}Cs) of mushrooms imported from European countries has been undertaken by the Ministry of Health, Labor and Welfare since the Chernobyl accident.

However, an explanation of why there is a high concentration and accumulation of ^{137}Cs in mushrooms has not been established. Therefore, investigations have been carried out on soil-mushroom or mushroom mycelia systems (*12*−*15*). The present studies have focused on the cultivation of the edible, saprophytic mushroom *Pleurotus ostreatus* to investigate its accumulation of ^{137}Cs and stable Cs. *P. ostreatus* is a very popular, edible mushroom in Japan, and its cultivation is relatively easy. The ease of cultivation of *P. ostreatus* enables the establishment of experimental conditions for ^{137}Cs or stable Cs tracing and allow its use as an effective tool to study the translocation characteristics and mechanisms of accumulation of Cs. Previously, the following findings using the *P. ostreatus*-Y1 strain were obtained; high ^{137}Cs contents were detected not only in mycorrhizal mushrooms in the field but also in the fruiting body of saprophytic mushrooms depending on the concentration of Cs in the culture medium (*9, 16*); Cs^+ and analogous alkali metals such as K^+ and Rb^+ were competitively taken-up by mushroom mycelia (*17*); and NMR measurement of the mushroom fruiting body and mycelia revealed the existence of Cs in ionized as well as other forms (*9, 18*).

In the present study, further experiments were conducted using the *P. ostreatus*-Y1 strain involving observations using a scanning electron microscope (SEM)−energy dispersive X-ray microanalyzer (EDX) and elemental analysis, elution of ^{137}Cs from mycelia, fractionation of Cs contents in mycelium cells by density-gradient centrifugation, and fluorescence microscopy of polyphosphate in mycelia using DAPI staining to investigate the characteristics of Cs accumulation and localization in mushroom mycelia.

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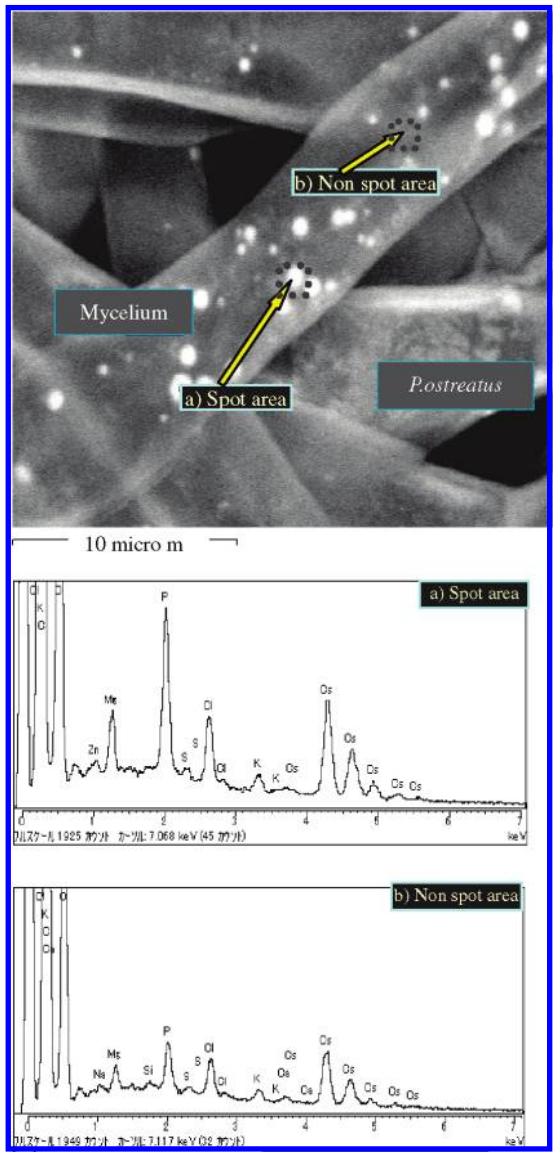

Figure 1. Image and spectra of *P. ostreatus*-Y1 mycelia using a scanning electron microscope (SEM) and energy dispersive X-ray microanalyzer (EDX). The rectangles with dotted lines indicate the scanning areas with a spot (**a**) and without a spot (**b**) for the spectra of the spot area and nonspot area.

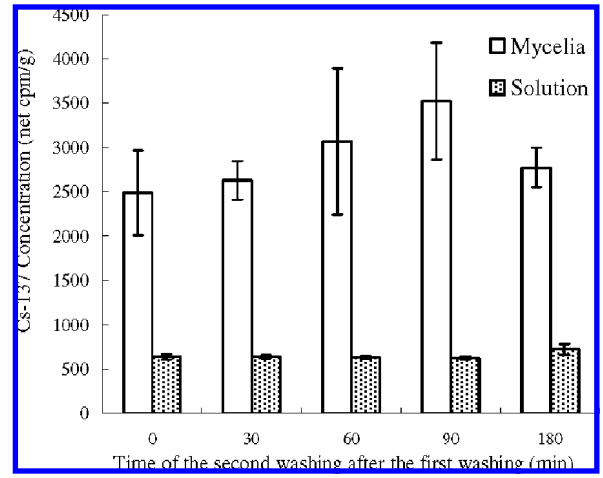

Figure 2. Concentrations of ^{137}Cs in *P. ostreatus*-Y1 mycelia and solutions after washing with distilled water. *P. ostreatus*-Y1 mycelia were cultured in YM liquid medium containing 4.4 kBq of ^{137}CsCl and 5 mM CsCl. The ^{137}Cs concentrations of the samples were determined by comparing with a ^{137}CsCl standard solution with an auto gamma-ray counter. Error bars indicates the mean value ± standard deviation of three replicates.

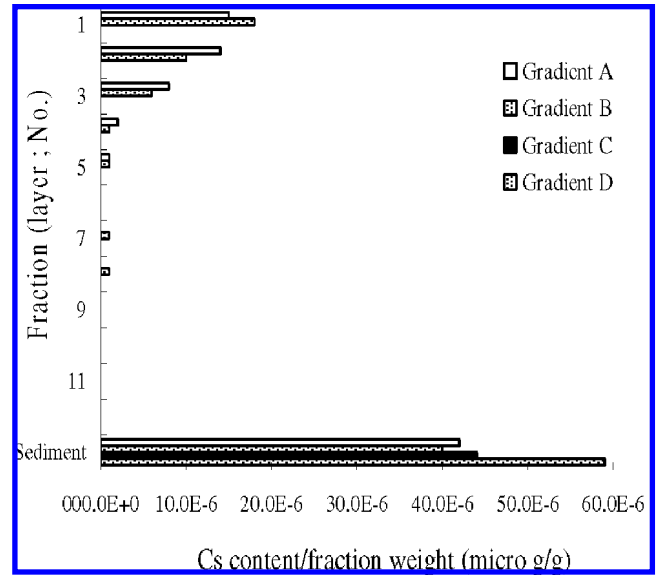

Figure 3. Distribution of Cs in each fraction of *P. ostreatus*-Y1 mycelia cultured on YM agar with 15 mM CsCl following density-gradient centrifugation.

MATERIALS AND METHODS

This study used the *P. ostreatus*-Y1 strain, a popular, edible, saprophytic mushroom in Japan.

SEM-EDX Observation of *P. ostreatus* Mycelia. *P. ostreatus* mycelia were cultivated at 27 °C for seven days on the upper side of a membrane filter (Millipore, pore size: 0.45 μm) placed on yeast extract−malt extract agar medium (0.4% yeast extract (Difco), 1% malt extract (Difco), and 0.4% glucose (Wako), pH 7.2) containing 25 mM CsCl (Wako) in a petri plate. Parts of mycelia on the filter were smeared onto double-sided carbon tape (Shinto Paint, 8W mm for Al specimen mounting; Hisco, 15φ × 6H mm for SEM without vapor deposition). Observation of images and element identification in *P. ostreatus* mycelia samples were performed by SEM-EDX (Hitachi S-3000N) in low vacuum mode (50 Pa) at 15 kV. Identification and semiquantitative analysis of elements were carried out using the spectra and peak heights of the spectra using EDX-Analysis Manager (Horiba).

Uptake of ^{137}Cs into *P. ostreatus* Mycelia and Elution. *P. ostreatus* mycelia were incubated by shaking in liquid medium (0.4% yeast extract, 1% malt extract, and 0.4% glucose, pH 7.2) containing 4.4 kBq ^{137}Cs (The Japan Radioisotope Association, ^{137}CsCl standard solution 56-CS005; ^{137}Cs 370 kBq kg^{-1}) and 5 mM CsCl using 6 baffle flasks at 27 °C for seven days. After incubation, the culture fluids were centrifuged at 500 rpm for 10 min (15 °C). Each pelleted mycelia sample was combined in a centrifuge tube, and the mycelia were suspended in 35 mL of distilled water. Approximately 5 g of mycelia was shaken in distilled water (35 mL) for the first wash using a test tube mixer (Vortex) for 1 min. For ^{137}Cs elution experiments, a total of 15 2-mL aliquots of the mycelia suspension were collected. At time points of 0, 30, 60, 90, and 180 min after the start of ^{137}Cs elution, the samples were centrifuged (3000 rpm, 10 min) in triplicate for each time point to isolate mycelia from the mycelia-washing solution. Approximately 0.3−0.4 g of fresh mycelia and 2 mL of wash solution were obtained per sample. ^{137}Cs activities in the mycelia (sediment before and after washing), solutions for elution (supernatant before and after washing), and ^{137}CsCl standard solution in 0.5 mL tubes were measured with an auto well γ-ray counter (Aloka; Auto Well Gamma System ARC-380CL) for 10 min. The ^{137}Cs contents (cpm g^{-1}) of the samples were determined on the basis of ^{137}CsCl standard solution activities and the weight of samples. Loss of ^{137}Cs from the mycelia during the first wash was about 47%.

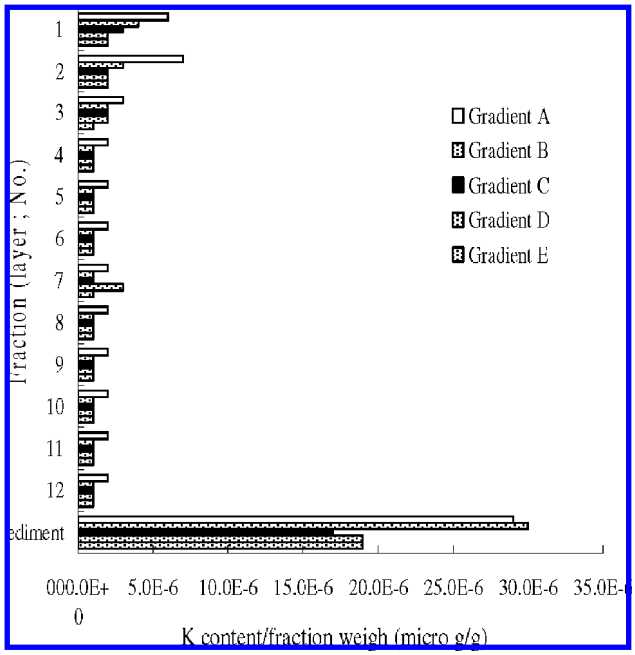

Figure 4. Distribution of K in each fraction of *P. ostreatus*-Y1 mycelia cultured on YM agar following density-gradient centrifugation. The basic K content in YM medium was 8.4 mM.

Measurement of Cs and K Contents in *P. ostreatus* Mycelia Fraction by Cell Fractionation. *P. ostreatus* mycelia incubated on YM agar medium (0.4% yeast extract, 1% malt extract, 0.4% glucose, and 1.8% agar, pH 7.2) containing 15 mM CsCl at 27 °C for 20 days were harvested and washed with distilled water. The mycelia were disrupted using a Bead-Beater (Biospec Products, Bartlesville, OK, USA). The chamber was filled with 1 M sorbitol (Sigma), and mycelia were homogenized with glass beads (diameter: 0.5 mm) in a vessel filled with crushed ice for 1 min. The homogenized mycelia were fractionated according to the revised method for isolating vacuolar and mitochondrial pellets (*19, 20*). The homogenized mycelia were centrifuged at 600*g* for 5 min (15 °C), and the supernatant was additionally centrifuged at 15,000*g* for 20 min (15 °C) to obtain the sediment. The sediment was suspended in 6 mL of 1 M sorbitol. Eighteen milliliters of 1.8 M sucrose (Sigma) − 1 M sorbitol, 12 mL of 1.8 M sucrose and 6 mL of mycelia suspension in 1 M sorbitol were added to a centrifuge tube in the order of lower to upper layers, and then centrifuged at 43,000*g* for 120 min (4 °C). In addition to the above-mentioned mycelia suspension, control samples were also prepared according to the procedures described above, except that the top layer was changed to 1 M sorbitol solution containing 5 mM CsCl or 1 M sorbitol containing 5 mM KCl (Wako). A total of 12 layers (3-mL aliquots of both mycelia suspension and controls) were pipetted into clean tubes to fractionate the liquid and sediment layers. These fractions were digested individually with approximately 20 mL of 14 M HNO_3 (Wako) and 2 mL of 30% H_2O_2 (Wako) at 160 °C for 4−12 h on a hot plate. The digested samples were diluted with up to 10 mL of 0.5 N HCl (Wako and MilliQ Element), and then the Cs and K contents were analyzed separately by flame atomic absorption spectrometry (Hitachi A-2000) using hollow cathode lamps (Hamamatsu Photonics, Cs; 852.1 nm absorption lines and Hitachi, K; 766.5 nm absorption lines) Similarly, flame atomic absorption spectrometry of the control CsCl and KCl fractions was performed without the wet digestion. The K content in the YM medium as a blank value was 8.4 mM.

Detection of Polyphosphate by Staining of *P. ostreatus* Mycelia. *P. ostreatus* was inoculated onto a YM agar plate (0.4% yeast extract, 1% malt extract, 0.4% glucose, and 1.8% agar, pH 7.2) containing 15 mM CsCl and incubated at 27 °C for seven days. The control sample (without Cs) was also incubated under the same conditions. Cultivated mycelia were carefully scraped from the plate and stained with 50 μg mL^{-1} of DAPI (ABD Bioquest) for 10 min. Stained mycelia were washed with distilled water and observed by fluorescence microscopy using a confocal laser scanning microscope (Carl Zeiss LSM 510 META). By DAPI staining, the fluorescence of polyphosphate (polyphosphate-DAPI) and DNA-DAPI can be observed around 526 nm (yellowish fluorescence) and 456 nm (bluish fluorescence), respectively (*21*). The presence of fluorescence corresponding to polyphosphate in mycelia and its morphology were observed.

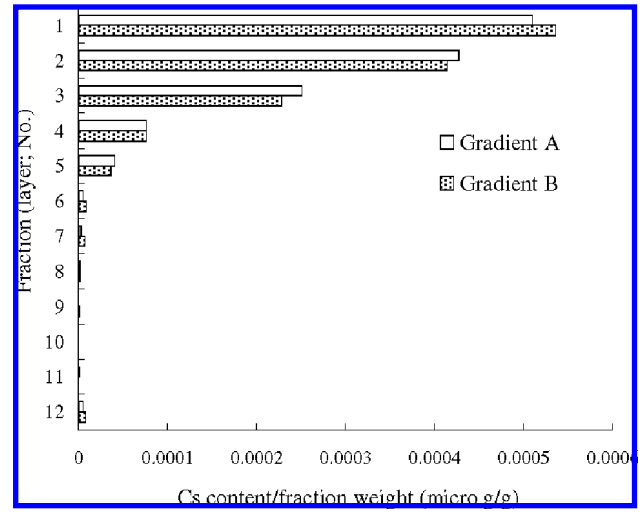

Figure 5. Distribution of Cs in each fraction of the control solution containing 5 mM CsCl following density-gradient centrifugation.

RESULTS AND DISCUSSION

SEM Observation and Qualitative Elemental Analysis of *P. ostreatus* Cultivated Mycelia. SEM equipped with EDX, which can detect the presence of elements in the microstructure of plant tissue, is one of the most effective techniques in elemental analysis. As shown in **Figure 1a**, a nonuniform distribution of circular white spots was observed in *P. ostreatus* mycelia cultivated in the presence of 25 mM CsCl. In addition, the spectra obtained from elemental analysis revealed that the Cs, P, and Mg concentrations of these spots were clearly higher compared to that of other regions without spots (**Figure 1b**). By SEM-EDX analysis, it was shown previously that the Cs content of the mycelial root (old regions of the hyphae) of *P. ostreatus* is approximately 5-times higher than that of the mycelial tip (growing regions of the hyphae) (*22*). In addition, in the previous study on Cs accumulation by soil microorganism (*23*) concerning the transfer of Cs from soil to mushroom (*24*), the presence of white spots at regular intervals was also confirmed using the mycelia of a soil filamentous bacterium (*Streptomyces lividans*) by SEM, and semiquantitative elemental analysis by EDX revealed that the Cs and P contents in these spots were as high as in *P. ostreatus* mycelia used in the present study. K, a chemical analogue of Cs, and P, an essential element transported by arbuscular mycorrhizal fungi (AMF), have been suggested to influence the uptake, translocation, and transfer of radiocesium by AMF (*25*). It was demonstrated that the transport of ^{134}Cs was affected by P independently of the concentration of K, and increasing the concentration of P in the hyphal compartment resulted in higher uptake and accumulation of ^{134}Cs in roots and shoots of *M. truncatula* plants (*26*). From these results, it was considered that Cs trapped in *P. ostreatus* mycelia was accumulated in specific regions with higher levels of P and concentrated. As specific regions where higher levels of P are present in mycelia, vacuoles accumulating polyphosphate have been suggested (*27*). The white spots where

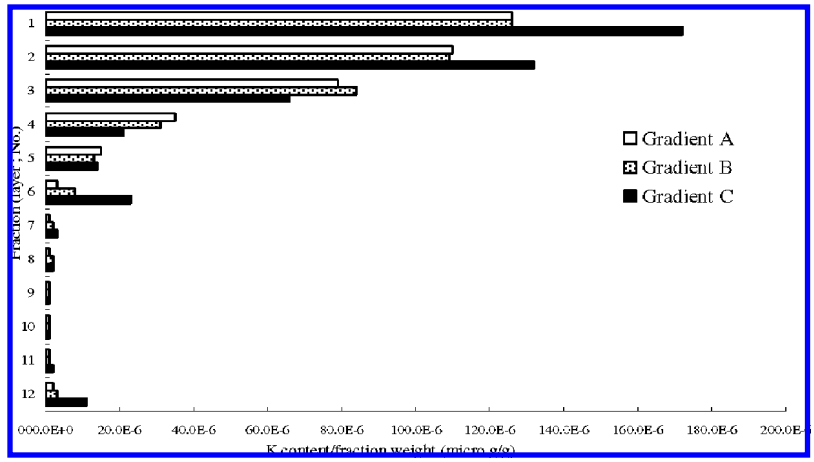

Figure 6. Distribution of K in each fraction control solution containing 5 mM KCl following density-gradient centrifugation.

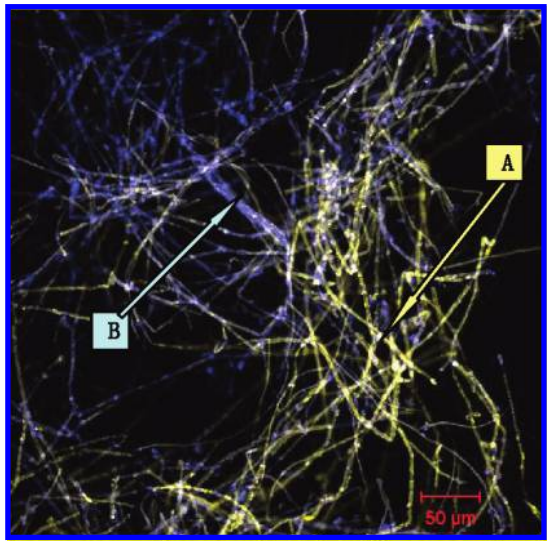

Figure 7. Image of 4′,6-diamidino-2-phenylindole (DAPI)-stained *P. ostreatus*-Y1 mycelia cultured on a YM agar plate containing 15 mM CsCl with laser-scanning confocal microscopy. A, polyphosphate-DAPI (yellowish granular fluorescence) around 526 nm; B, DNA-DAPI (bluish granular fluorescence) around 456 nm.

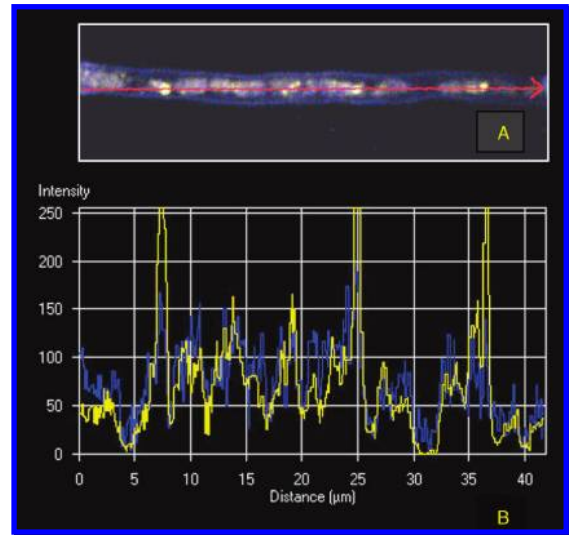

Figure 8. Image and fluorescence intensity caused by polyphosphate-4′,6-diamidino-2-phenylindole (DAPI)-stained *P. ostreatus*-Y1 mycelia cultured on a YM agar plate containing 15 mM CsCl by laser-scanning confocal microscopy (526 nm). (**A**) Image of fluorescence from polyphosphate-DAPI in the mycelium. (**B**) Fluorescence intensity of the mycelium. The scale of the horizontal axis of the graph (**B**) is the same scale as the fluorescence microscopy image of *P. ostreatus* mycelia (**A**).

the Cs and P contents were higher than other regions, observed in the mycelia cultivated with CsCl by SEM could be a result of Cs^+ trapped by polyanionic polyphosphate.

Uptake of ^{137}Cs into *P. ostreatus* Cultivated Mycelia and Elution Fractions. The ^{137}Cs contents in mycelia and the elution fractions after washing were investigated. The rate of uptake of Cs into *P. ostreatus* mycelia cultured for seven days and elution by washing was investigated using ^{137}Cs. **Figure 2** shows the ^{137}Cs concentrations in water used for ^{137}Cs elution and mycelia at each time point in the second wash that was retained after the first wash. Mycelia of *P. ostreatus* cultivated in YM liquid medium containing ^{137}Cs with shaking were harvested by centrifugation and washed with distilled water. In **Figure 2**, error bars indicates the mean value ± the standard deviation of three replicates. The ^{137}Cs concentrations in each 2 mL aliquot of water at 0, 30, 60, 90, and 180 min after the start of elution were almost constant. These results suggested that the elution of ^{137}Cs from mycelia into distilled water occurred rapidly after the start of elution and that the increase or decrease in the amount of ^{137}Cs eluted with time was small. The ^{137}Cs activities in the water used for the ^{137}Cs elution experiment were approximately 4−6 times higher than those in the mycelia after washing. The previous ^{133}Cs NMR studies showed that the spectra exhibited two resonance signals arising from the mycelia and the fruiting bodies, whereas one signal was detected from CsCl standard solution (0 ppm) and the other lower magnetic field. This suggested that Cs existed in a different state in the samples (*9, 18*). Taking these results into consideration, part of the ^{137}Cs was concluded to be in its ionized form in the mycelia and could be eluted by washing, but the other Cs form was trapped by polyphosphate and remained in the mycelia even after washing.

Presence of Cs and K in *P. ostreatus* Mycelia Fraction as Assessed by Cell Fractionation. After density gradient centrifugation of the mycelial homogenate of *P. ostreatus*, 13 fractions were obtained. Yellowish suspensions, fractions 3 and 13 (sediment), were regarded as containing mitochondrial and vacuolar material, respectively (*19, 20*). However, confirmation of the composition by electron microscopy was not performed. The results of atomic absorption spectrometry of Cs and K in each fraction are shown in **Figure 3** (for Cs obtained from four experiments) and **Figure 4** (for K obtained from five experiments), respectively. In both graphs, the contents of Cs or K derived from *P. ostreatus* mycelia are expressed as concentra-

tions among the 13 fractions (Cs or K weight/fraction weight: μg g^{-1}), which consisted of 3-mL aliquots of fractions 1−12 obtained from the upper liquid layers and the sediment layer (fraction 13). The concentrations of Cs and K in the controls without mycelia are shown in **Figure 5** (for Cs obtained from duplicate experiments) and **Figure 6** (for K obtained from triplet experiments), respectively. As shown in **Figure 3**, it was found that the Cs concentration derived from *P. ostreatus* mycelia was higher in fractions 1−3. The Cs concentrations of the sediment containing vacuolar pellets were relatively less variable with the mean among 4 samples being $46.0E^{-6} \pm 7.5E^{-6}$ μg g^{-1}. Similar to the results obtained with mycelia, a tendency toward clearly higher Cs concentrations in the upper fractions 1−3 was also observed in the control experiment of Cs. Subsequently, the Cs ratio, which compared the Cs content in each fraction to the total Cs content obtained by summing the values of all fractions and the sediment, showed that approximately 90% of Cs was present in fractions 1−3. Therefore, the Cs content in the lower nine liquid layers (fractions 4−12) was negligible. The existence of ionized Cs in the liquid layers was confirmed by comparison with the results of the Cs control experiment. It was considered that the larger amount of ionized Cs compared to the results of experiments on the uptake of ^{137}Cs into *P. ostreatus* cultivated mycelia and the water used for the ^{137}Cs elution experiment mentioned above can be attributed to the disruption of mycelia. However, the localization of Cs was also observed in the sediment-containing vacuolar pellets. It was suggested that Cs transferred into the vacuolar compartment could be trapped by polyanionic polyphosphate as mentioned above. NMR spectroscopy of both *P. ostreatus* mycelia and the fruiting body showed the existence of ionized Cs and another Cs form (*9, 28*). In addition, as the localizations of Cs and P were observed in the SEM-EDX experiment as mentioned above, polyphosphate could be related in Cs accumulation and localization in *P. ostreatus* mycelia. The distribution of K, which is a chemical analogue of Cs, derived from mycelia in each liquid fraction was slightly different from that of Cs, and no particularly high concentrations of K were observed in liquid fractions 1−12. However, the concentration of K in the sediment was higher in a similar manner to the results of Cs. Consistent with the results obtained for Cs, higher K concentrations in the upper liquid fractions 1−3 were also observed in the control experiment, showing that a different distribution of K was obtained with the results from mycelia. It was shown previously that the concentrations of K in the mycelia of *P. ostreatus* decreased with the increase in Cs concentrations in the media containing 0−15 mM Cs (*17*). Each K concentration in YM medium in the previous study and the present study was 0.02% K and 8.4 mM K (approximately 0.03% K), respectively, from the inherent content in the medium. It was considered that the above observations with K and Cs appear to show an inverse relationship, which could result from the effect of high Cs concentration (15 mM Cs) in the medium. However, it could not be clarified why the higher K concentration in the sediment was observed in a manner similar to the result of Cs distribution, and further investigation will be necessary.

Staining of *P. ostreatus* Mycelia Indicates the Presence of Polyphosphate. From the results mentioned above, it was decided to focus on the relationship between the presence of polyphosphate and Cs in *P. ostreatus* mycelia. Polyphosphate was detected by fluorescence microscopy using DAPI-stained mycelia. Fluorescence microscopy images of *P. ostreatus* mycelia cultured in medium containing 15 mM CsCl stained with DAPI are shown in **Figures 7** and **8**. As shown in **Figure 7**, yellowish granular fluorescence resulting from polyphosphate-DAPI (**A** in **Figure 7**) and bluish fluorescence resulting from DNA-DAPI (**B** in **Figure 7**) were observed in images of the mycelia. The aromatic fluorescent dye DAPI is a well-known reagent for the analysis of DNA, and the binding of DAPI to polyphosphate causes a shift in the fluorescence emission maximum from 456 to 526 nm (*29*). The fluorescence intensity around the region of yellowish granular fluorescence is shown in **Figure 8**. The scale of the horizontal axis of the graph is the same scale as the one in the fluorescence microscopy image of *P. ostreatus* mycelia. It can be noted that the fluorescent intensity is particularly strong at the granular sites. Thus, it was recognized that, in *P. ostreatus* mycelia, polyphosphate non-uniformly localizes in a granular fashion. Tijssen reported that the spectrum of yeast (*S. fragilis*)-bound DAPI had a maximum at about 526 nm, highly suggestive of a reaction with polyphosphate, and fluorescence microscopy confirmed the localization of the fluorescent DAPI-polyphosphate complex in yeast vacuoles (*21*). In this study, as shown by the results of SEM-EDX in *P. ostreatus* mycelia, Cs and P localized at the same sites as observed for the white spots, and therefore, a relationship between the fluorescence intensity of polyphosphate-DAPI and Cs in vacuoles or other organelles was inferred. A more detailed investigation of this relationship, for example, how the percentage of Cs included in polyphosphate relates to the total Cs content in mycelia, will be necessary in the future.

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