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Low Temperature Heating-Induced Death and Vacuole Injury in *Cladosporium sphaerospermum* Conidia

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The mechanism of thermal death of mold conidia has not been understood in detail. The purpose of this study is to analyze the death kinetics of heated conidia of *Cladosporium sphaerospermum* and to ascertain the expectant cell injury responsible for the death. The death of the dormant (resting) conidia of *Cladosporium sphaerospermum* was examined at temperatures of between 43 and 54℃ with the conventional colony count method. The death reaction apparently followed the first order kinetics, but the Arrhenius plot of the death rate constant demonstrated seemingly a break. The linearity at temperatures higher than that at the break was lost at lower temperatures, suggesting the involvement of an unusual mechanism in the latter temperatures. In the cell morphology, we observed with quinacrine staining the vacuole rupture at a lower temperature but not at a high temperature. Interestingly, the vacuole rupture by low-temperature heating was found to correlate with the viability loss. Furthermore, active protease originally locating in vacuoles was detected in the cytoplasm of the conidia after heated at a low temperature. The results obtained suggest the involvement of potent autophagic cell death induced by low temperature heating of *C. sphaerospermum* conidia.

Key words：Thermal death / *Cladosporium*, conidium / Vacuole / Protease.

INTRODUCTION

Molds cause hazardous problems not only in human life but also in various industries and environments (Dijksterhuls, 2019). *Cladosporium* is one of the most commonly detected mold in home and outdoor air and causes an allergic reaction (Ledford, 1994; Dijksterhuls, 2019; Bensch et al., 2021) and also often contaminate fresh and processed foods as well as food materials (Filtenborg et al., 1996). They form sexual and asexual spores which live long and distribute freely in environmental spaces and adhesively on material surfaces. For the purpose of mold control, heat treatment has been widely used as one of the most effective methods in food pasteurization and environmental disinfection.

There has been a recent trend of minimal processing in food pasteurization, which targets mainly molds and yeasts as objective microorganisms, designing the manufacturing of high-quality foods. In this case, however, milder heating may increase a risk of survival of cells even sublethally injured. As reviewed previously (Stevenson and Graumlich, 1978; Beuchat, 1984; Wu, 2008; Horikiri and Tsuchido, 2020), injured fungi may repair their damaged sites and grow during the subsequent storage and distribution processes.

Although the thermal death of fungal spores has been studied by many researchers so far (Stevenson and

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Graumlich, 1978; Beuchat, 1984; Shearer et al., 2002; Horikiri and Tsuchido, 2020), its detailed mechanisms remain to be investigated. Although heat has a multi-target action on microbial cells, the reaction of wet heat-induced death of microorganisms including fungal spores is in general supposed to follow the apparent first order reaction and is characterized by rather high activation energy (Karel and Lund, 2003). These kinetic characteristics have also been known for the wet-heat denaturation of proteins (Eyring and Stearn, 1939; Karel and Lund, 2003; Ling et al., 2015). Based upon such a kinetic resemblance, protein denaturation is supposed to be a critical cause of thermal death of microorganisms (Rosenberg et al., 1971).

Fungal spore injury generated by exposure to mild heat stress has not been characterized yet and different spore organelles, including nucleus, mitochondria and vacuoles, and their membranes, and also cytoplasm as well as their components, such as proteins, enzymes, and nucleic acids are presumed to be damaged. Furthermore, such injuries to cellular sites caused by external stresses including heat are likely to be related to different cell death systems in fungi, i.e., apoptosis, necrosis and autophagic cell death, as well as other eukaryotic cells (Fulda et al., 2010; Falcone and Mazzoni, 2016; Gonçalves et al., 2017; Hardwick, 2018).

In this study, following our previous report (Horikiri et al., 2020), in which the evaluation method of the growth-dependent and -independent modes of thermal injury in *Cladosporium* conidia was proposed, we investigated the thermal death patterns and mechanisms of the fungal conidia. Based upon the results obtained, we suggested that low temperature heating caused vacuole injury putatively leading to cell death.

MATERIALS AND METHODS

Fungal strains, cultivation and conidium preparation

Cladosporium sphaerospermum NBRC 6348 strain was used throughout this study and *Aspergillus niger* NBRC 6342 strain was also employed in part of the experiment. Both strains were obtained from National Biological Resource Center (NBRC), NITE (Tokyo, Japan). The former strain was used as *Cladosporium cladosporioides* NBRC6358 in our previous study (Horikiri et al., 2020) but afterwards the species name was revised by NBRC and furthermore the strain number was misused and then corrected to the above in this study. The strain was cultivated at 28℃ for 7 d on potato dextrose agar (PDA, pH5.1; Becton Dickinson, Franklin Lakes, NJ, USA) plates containing 1.5% (w/v) agar and 2% (w/v) glucose. Resultantly formed conidia were collected from the culture plate by using sterile 50 mM phosphate buffer saline (PBS) at pH7.4 containing 2% (w/v) glucose and 0.05% (w/v) polyoxyethylene sorbitan monooleate (Tween 80), and the suspension was then filtered with sterilized gauze and then with 50 μmϕ Nylon mesh filter (Filcon S; Becton Dickinson). Conidia were washed twice with the above solution by centrifugation (3,000 g, 5 min), and the pellet was then resuspended in sterile 50 mM PBS containing 2% (w/v) glucose. The suspension contained about 10^7 conidia per ml and was used immediately after preparation. To obtain swollen conidia, the conidium suspension was incubated statically at 28℃ for 24 h in potato dextrose broth (PDB) containing 0.5 M hydroxyurea (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), which was added to synchronize the population with the swollen stage by inhibition of germ tube elongation. After incubation, conidia were harvested, washed and then resuspended as described above.

Heat treatment and viability assay

A portion (0.1 ml) of the conidium suspension was added to a 1.5 ml volume polypropylene micro tube containing 0.9 ml of 50 mM phosphate buffered saline (PBS) with 2% (w/v) glucose, which was preheated to a temperature in the range of 45 to 54℃ for *C. sphaerospermum* conidia or in the range of 54 to 59℃ for *A. niger* conidia in an incubator (MYBL-10, AS ONE Corp., Osaka, Japan). During the isothermal heating period, samples were taken out and serially diluted every 10-fold with PBS using a series of micro tubes before plating on PDA. The plates were incubated at 28℃ for up to 7 d and the resultantly formed colonies were counted to determine viability.

Kinetic analyses of thermal death

The microbial death has generally been supposed to follow the first-order reaction kinetics, which is expressed by the following equation.

$$dN/dt = -kN \quad (1)$$

where N and t are the viable conidium number (ml^{-1}) and heating time (min), respectively, and k is the death rate constant (min^{-1}), which has a relationship with the decimal reduction time, D value, of 2.303/D.

Taking integral of both sides of the Eq. (1) and expressing with common logarithm,

$$\log N = \log N_0 - (k/2.303)\, t \quad (2)$$

where N_0 is the initial viable conidium number (ml^{-1})

The dependency of k upon the heating temperature can be expressed with the Arrhenius equation as follows.

$$\log k = -(E_a / 2.303\, R)\,(1/T) + \log A \quad (3)$$

where T is the absolute temperature of heat treatment (K), A is the frequency coefficient (dimensionless), R is the gas constant (8.314 J mol^{-1} K^{-1}) and E_a is the acti-

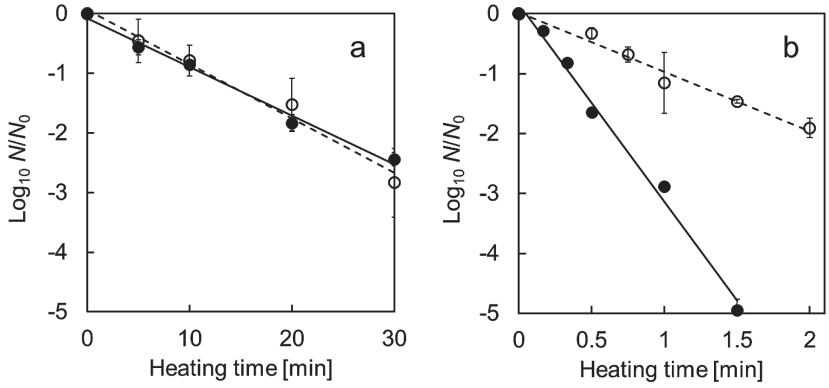

FIG. 1. Survival curves of the resting (closed symbols) and swollen conidia (open symbols) of *C. sphaerospermum* heated at 45℃ (a) and 52℃ (b) in PBS with glucose. The mean values with SDs were demonstrated with bars.

vation energy of the reaction (J mol^{-1} K^{-1}).

Vacuole staining

An acidic fluorescent dye quinacrine dihydrochloride dihydrate (Wako, Osaka, Japan) was used for vacuole staining (Ho et al., 2015). It was added to conidium suspension at a final concentration of 0.5 mM and then the suspension was incubated in a water bath at 30℃ for 1 h. The conidia were washed twice and resuspended in PBS containing 2% (w/v) glucose. The fluorescent imaging analysis was performed with a fluorescence microscope (BX-50; Olympus, Tokyo, Japan) using a B/G filter set. As many as fifty conidia were inspected for counting the numbers of the whole stained conidia as well as the total conidia including only vacuole-stained ones.

Protease reaction assay

Conidium suspension was prepared in PBS as described above. After heat treatment at 45℃, a fluorogenic substrate for protease cleavage, CMAC-Ala-Pro ((7-amino-4-chloromethylcoumarine)-Ala-Pro, Molecular Probes Inc., Eugene, OR, USA), was used for the protease assay (Devenish et al., 2008). A portion of PBS solution containing 2% (w/v) glucose and this substrate was added to the conidium suspension at a final concentration of 100 μM, and then the suspension was incubated for 6 h for staining. After that, conidia were washed twice with 2% (w/v) glucose aqueous solution and resuspended in the fresh buffer containing 2% (w/v) glucose. To verify the type of involving protease, a serine protease inhibitor 4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride (AEBSF HCl, Wako Pure Chemical Corp.) at a final concentration of 1 mM) and a cysteine protease inhibitor E-64 (Wako Pure Chemical Corp.) at a final concentration of 10 μM were also used. They were also used for in vitro inhibition assays of intracellular proteases involved in autophagy in the plant *Arabidopsis* or *Plasmodium falciparum* (Inoue et al., 2006; Navale et al., 2014). The fluorescent image was obtained via a charge-coupled device (CCD) camera (Orca, Hamamatsu Photonics K. K., Hamamatsu, Shizuoka, Japan) attached to the fluorescence microscope. A fluorescence spectrophotometer (JASCO, Hachioji, Tokyo, Japan) was employed to measure the fluorescent intensity of the conidium suspension at wavelengths of excitation and emission of 353 and 465 nm, respectively.

Chemicals

Chemicals described above without manufacturer's name are in reagent grade and purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan), except for Difco™ PDA and PDB, which were obtained from Becton Dickinson and Co. (Franklin Lakes, NJ, USA).

Statistical analysis

All assays were performed at least three times and the mean with SD was determined. To check statistical significance, the Student *t*-test was performed at $p < 0.05$. Calculations were carried out using the Microsoft Excel 2010 software.

RESULTS

Thermal death characteristics of conidia

How *C. sphaerospermum* conidia were killed by heat was examined in PBS at different temperatures between 43 and 54℃ and the results obtained at two representative temperatures of 45 and 52℃ were depicted in Fig. 1a and 1b, respectively. Supposed any possible time-dependent changes during the germination process, the heat resistance of the dormant (resting) conidia

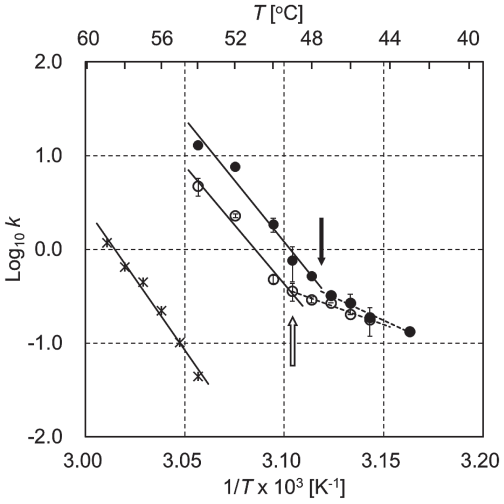

FIG. 2. Arrhenius plots for the thermal death reaction of the resting (closed circle) and swollen conidia (open circle) of *C. sphaerospermum*. The data of the resting conidia of *Aspergillus niger* were also plotted (asterisk). The mean values with SDs were demonstrated with bars.

was compared with that of germinated (swollen) spores obtained after incubation in PDB with hydroxyurea added at 28℃ for 24 h. For both conidia, the thermal death reaction was confirmed to follow apparently the first order reaction kinetics and the swollen conidia were more resistant to heat than the resting spores. When the resting spores were heated at 45 and 52℃, the death rate constants were 0.187 and 7.60 min^{-1} (12.2 and 0.30 min as *D* values), respectively, while for the swollen spores the rate constants were 0.181 and 2.85 min^{-1} (12.7 and 0.80 min as *D* values), respectively. Although the curve of the resting conidia heated at 47℃ showed a slight tailing character, the data was obtained from five replicates of the sample, assuming the linearity of the curve (data not shown). We also examined the thermal death pattern of the resting conidia of *A. niger* and compared it with those of the *C. sphaerospermum* resting conidia. The results obtained indicate that the heat resistance of the former conidia was definitely higher than that of the latter (data not shown but described later).

Based upon the survival data of the heated conidia, the Arrhenius plot of the death rate constant (*k* values) was depicted in Fig. 2. As a result, with both resting and swollen conidia of *C. sphaerospermum*, a break was found to appear on the plot, while there was no such a break with *A. niger* conidia at comparable levels of the death rate constant. In addition, the temperatures at those break points for *C. sphaerospermum* conidia were calculated to be 47.2 and 49.1℃ for resting and swollen conidia, respectively. Above these boundary temperatures, the thermal death reaction of both conidia demonstrated high activation energy (E_a) values (501 and 482 kJ mol^{-1} deg^{-1}, respectively), while did low E_a values (190 and 148 kJ mol^{-1} deg^{-1}, respectively) below the specific temperature. Such low E_a values in the low temperature range seemed to be a specific property of this mold strain used in this study.

Vacuole injury by low temperature heating in conidia

Although the mechanism of the abovementioned low temperature heating-induced death of *C. sphaerospermum* conidia is still unclear, we have found a fatty acid- and its diglycerin ester-induced death and vacuole disruption of *Saccharomyces cerevisiae* cells in our separate experiment (unpublished). In addition, several investigators have reported the vacuole disruption or fragmentation by exposure to external stresses in fungi (Corson et al., 1999; Matsuura and Takagi, 2005; Suzuki et al., 2012). Therefore, we assumed vacuole injury, which is involved in the crucial conidial death, in particular, focusing on whether any differences can be observed with the resting conidia heated at temperatures below and above the boundary temperatures.

C. sphaerospermum conidia were heated either at 45℃ for 20 min or at 52℃ for 2 min and then stained with quinacrine to detect microscopically conidial vacuoles (Fig. 3). As a result, unheated conidium demonstrated to possess acidic vacuole stained greenish but its cytoplasm remained unstained, whereas in conidia heated at 45℃ for 20 min the whole conidia containing vacuoles were found to be stained (Fig. 3b). This result indicates the acidification of cytoplasm resulting from vacuole injury after low temperature heating. In case of conidia heated at 52℃ for 2 min, on the other hand, almost conidia were not stained totally (namely, with keeping cytoplasm unstained) (Fig. 3c). To confirm this further, after heating for different periods at both temperatures, the conidia with unstained cytoplasm were counted microscopically, while the viable conidia were enumerated by colony formation on PDA plates. In other words, whether the microscopic count ratio of the conidia with unstained cytoplasm to the total number corresponds to the viability was examined with conidia heated at both temperatures (Fig. 4). The results indicated that 45℃-heating decreased both ratios nearly in parallel, whereas 52℃-heating decreased the CFU ratio much faster than did the count ratio of conidia with unstained cytoplasm. In fact, the ratio of the relative ratio of the cytoplasm-unstained conidia count to that of CFU at each heating time tested averaged to 1.28 at 45℃, indicating the approximate equivalence between both, whereas at 52℃ that was a deviated value of 0.086, suggesting no correlation. This result reinforces that the vacuole injury proceeds in parallel with the viability reduction during low temperature heating but

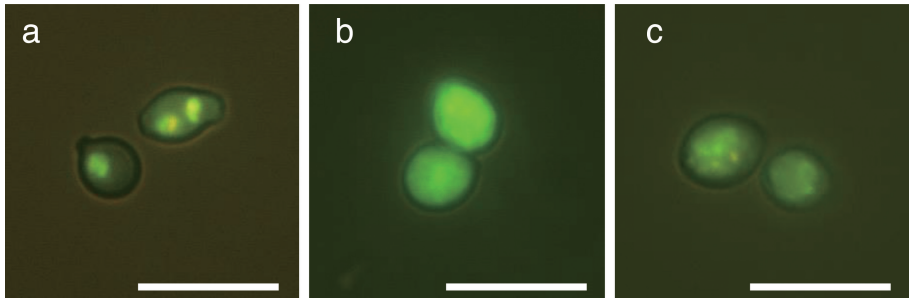

FIG. 3. Fluorescent images of *C. sphaerospermum* conidia stained with quinacrine. The resting conidia were heated at 45℃ for 20 min (b) and 52℃ for 2 min (c). The images of the unheated conidia (a) were also indicated. Scale bars represent 10 μm.

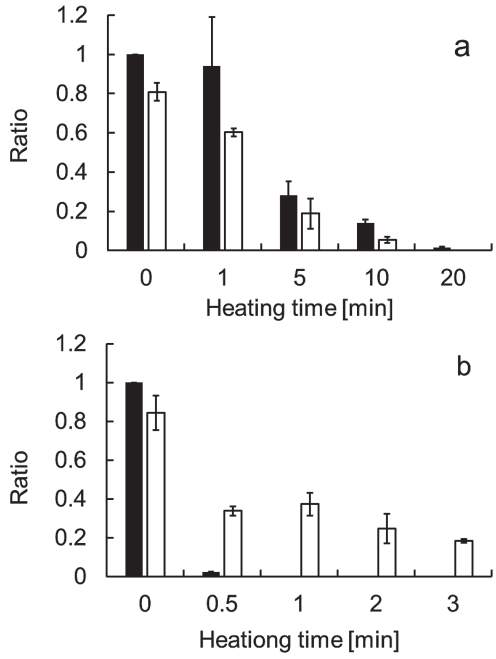

FIG. 4. Time courses of the reductions in the CFU ratio (black bar) and in the ratio of the conidium with quinacrine-unstained cytoplasm to total (white bar) during heat treatment at 45 (a) and 52℃ (b). The mean values with SDs were demonstrated.

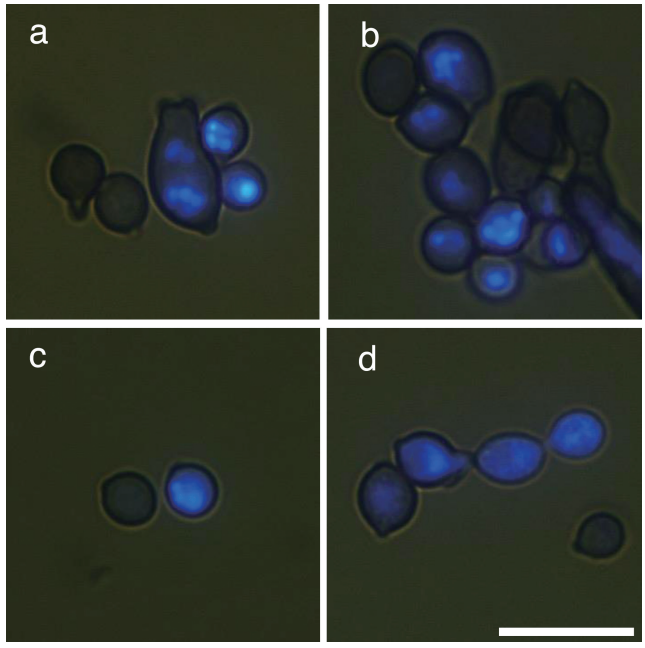

FIG. 5. Heat-induced alteration in the protease localization evaluated microscopically with a fluorogenic substrate CMAC-Ala-Pro in *C. sphaerospermum* conidia. Conidia were heated at 45℃ in PBS for 0 (a), 1 (b), 5 (c), and 10 min (d). Scale bar represents 10 μm.

both events occur independently during high temperature heating.

Protease distribution in *C. sphaerospermum* conidia exposed to low temperature heating

Because the vacuoles were suggested to be damaged by low temperature heating, intravacuolar proteases were supposed to be released into the cytoplasm to degrade proteins localizing there. To verify this, dislocation of proteases after heating at 45℃ was investigated using CMAC-Ala-Pro as a fluorescent substrate. The resting conidia were incubated with the fluorogenic substrate and the distribution of the enzyme activity in conidia was monitored microscopically (Fig. 5). In unheated conidia, the CMAC fluorescence was confirmed to be observed only in the vacuole (Fig. 5a). In conidia heated at 45℃ for 10 min, on the other hand, the fluorescence was also detected in the cytoplasm (Fig. 5d), indicating the presence of active proteases in the acidified cytoplasm after low temperature heating.

Further, to verify an increase in protease activity in the cytoplasm during the heating period, the fluorescence intensity of the conidium suspension was measured with a fluorescence spectrophotometer (Fig. 6a). The resultant intensity was increased substantially with heating time at 45℃, amounting to 1.4 times as much

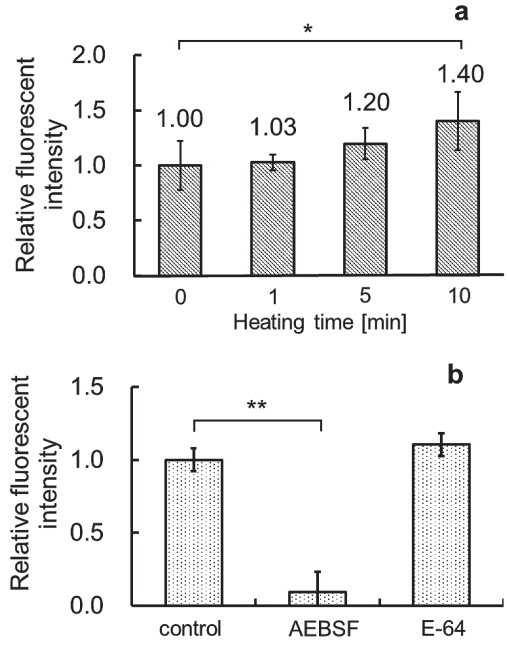

FIG. 6. Heat-induced increase in the protease activity (a) and the effects of inhibitors on the detected protease activity (b) in *C. sphaerospermum* conidia as assayed with a fluorogenic substrate CMAC-Ala-Pro. Conidia were heated at 45℃ for different periods and then incubated with the above substrate (a) or heated for 10 min and incubated with the substrate similarly and also with or without a protease inhibitor, either AEBSF or E-64 (b). Asterisks * and ** indicate $p<0.05$ and $p<0.01$, respectively.

as that of unheated control after 10 min. Furthermore, what type of protease is involved was examined by using protease inhibitors. Consequently, the fluorescence intensity was near the basal level with AEBSF added as a serine-type carboxypeptidase inhibitor, whereas was a control level with a cysteine protease inhibitor, E64 (Fig. 6b). These results indicate that the protease involved in the heat-induced proteolysis in the cytoplasm is serine type, which is originally located in the vacuole, supporting the idea of the vacuole rupture by low temperature heating.

DISCUSSION

The heat resistance of fungal conidia depends upon the physiological state as well as the species. In this study, germinating swelling conidia of *C. sphaerospermum* were more resistant to heat than the resting conidia. A possible factor responsible for this may be the accumulation of trehalose by swelling conidia, considering the involvement in the heat resistance of bacteria as well as fungi (Argüelles, 2000; Dijksterhuls, 2017). In addition, the heat resistance of *Cladosporium* conidia is lower than that of *A. niger* conidia and comparable to those of relatively heat-sensitive molds, as demonstrated previously by other researchers (Jung et al., 2009; Dijksterhuls, 2017; Belbahi et al., 2017). A notable finding in this study was that the resting conidia of *C. sphaerospermum* demonstrated a biphasic pattern on the Arrhenius plot of the thermal death rate constant at the boundary temperature of about 47 to 48℃, whereas did *A. niger* conidia a single straight line. It is not clear at present whether such a property belongs to the *Cladosporium* strain used but a similar pattern was also observed with the germinating swollen conidia.

In previous studies, a marked temperature dependence of thermal death reaction has been indicated with high E_a values of 463 kJ mol^{-1} in *Wallemia sebi* (Beuchat and Pitt, 1990) and of between 301 to 446 kJ mol^{-1} in ascospores of four strains of *Neosartorya* spp. as converted from the original z values. (Berni et al., 2017). Such high E_a values are comparable to those obtained with molds used in this study, except for *C. sphaerospermum* conidia heated at low temperatures. At higher temperatures, the irreversible denaturation or aggregation of some vital protein may probably be responsible for the cell death (Rosenberg et al., 1971). At lower temperatures, however, it is suggested that any alternative mechanism of thermal death may be involved. Such an unusually low value of the E_a may reflect the involvement of a fatal reaction something like the deregulation or activation of any degradative enzyme induced by heat. If such a hypothesis is the case, what and how does any intracellular fatal system work?

There have recently been a number of reports describing the cell death mechanisms of eukaryotes, typically including apoptosis, necrosis and autophagic cell death (Sharon et al., 2009; Fulda et al., 2010; Falcone and Mazoni, 2016). But more recently several derivative types of apoptosis have been proposed and nowadays they were unified into the regulatory cell death including substantial autophagic cell death (Carmona-Gutierrez et al., 2018). On the other hand, necrotic cell death was categorized into another group as the accidental cell death. Even though the methods of the detection of and discrimination between those types of cell death has been listed for yeast (Wloch-Salamon and Bern, 2012), *de facto*, the discrimination between those types sometimes seems rather difficult in fungi because of different types of triggers, complicated causality, species variations and so on. Although the terminology apoptotic-like cell death has been applied to fungi (Carmona-Gutierrez et al., 2010; Shlezinger et al., 2012), this type of fungal cell death has been reported by many investigators (Corson et al., 1999). As one of such external triggers, heat has been reported to induce the apoptotic-like cell death of white-rot fungi *Pleurotus* species by Song et al. (2014). They

have demonstrated nuclear condensation, reactive oxygen species accumulation, and DNA fragmentation characteristic of apoptosis. Such fungal apoptotic-like cell death by heat has also been suggested by other researchers (Chen and Dickman, 2005; Plesofsky et al., 2008).

In this study, on the other hand, we found vacuole rupture and viability loss in low temperature heating-treated fungal spores with a proportional relationship between those events. Vacuole has been known to play a central role in autophagy for growth and survival of fungi as well as other eukaryotes (Klionsky et al., 1990; Bryant and Stevens, 1998; Li and Kane, 2009; Pollack et al., 2009). It is also involved in autophagic cell death, although the mechanism seems to have not been well understood in fungi (Fulda et al.,2010; Carmona-Gutierrez et al., 2018). The results obtained with *C. sphaerospermum* conidia in this study indicated that vacuole rupture induced by the exposure of the conidia to low temperature heating correlated with the conidial viability, suggesting some causality between those. Although different external stresses have been reported to cause such vacuole deformation or injury as described above, whether the underlying mechanisms responsible for the cell death are identical is unclear.

In the low temperature heating-induced injury to vacuole in *C. sphaerospermum* conidia might result from the vacuolar membrane instability due to its low content of ergosterol compared to the plasma membrane, as reported for *S. cerevisiae* (Zinser et al., 1991). Such an injury may cause the permeabilization of vacuolar membrane to result in proton efflux to cytoplasm and its acidification. Imai and Ohno (1995) have reported that the reduction in viability and lowering of intracellular pH in *S. cerevisiae* cells after exposed to heat and ethanol are correlated. In a nematode *Caenorhabditis elegans*, the vacuolar H^+-ATPase (V-ATPase) playing a role for vacuolar acidification is required for its cell death under conditions leading cytoplasmic acidification including insult to an environmental injury (Syntichanki et al., 2005). In *S. cerevisiae*, Kim et al. (2012) also have reported that V-ATPase promotes vacuolar membrane permeabilization responsible for the ultimate cell death.

In conjunction with this V-ATPase activity, vacuole rupture is considered to occur to release different hydrolases inside into the cytoplasm that results in cell death. In fact, we demonstrated in the cytoplasm of *C. sphaerospermum* conidia the presence of vacuolar serine-type protease which was released from the vacuole by low temperature heating, based upon the results of the experiment of using a fluorogenic substrate CMAC-Ala-Pro and protease inhibitors. Although what kinds of proteases are present in *C. sphaerospermum* seems to have not been reported so far, in *A. oryzae* a vacuolar serine protease caroboxypeptidase O and its gene have been characterized (Morita et al., 2010) and also in *S. cerevisiae* seven vacuole proteases including a serine-type protease proteinase B have been identified (Klionsky et al., 1990; Knop et al., 1993; Van den Hazel et al., 1996). Our results obtained suggest that the low temperature heating of *C. sphaerospermum* conidia results in vacuole-dependent cell death putatively as a sort of regulated or autophagic pathway. In lysosome having a role similar to vacuole in animal cells, it has been known lysosome-dependent cell death occurs as a type of regulated cell death mediated by hydrolases releasing into cytosol after lysosomal membrane permeabilization (Tang et al., 2019). Considering these recently reported arguments (Carmona-Gutierrez et al., 2018), the vacuole-dependent cell death induced with low temperature heating in *C. sphaerospermum* conidia seems a sort of regulated cell death rather than necrotic cell death reported for yeast before (Eisenberg et al., 2010; Kim et al., 2012), although further studies are required for clarifying the underlying mechanisms. Such a cell death type may also be considered to be a lethal degradative secondary injury.

In conclusion, we present a hypothesis that low temperature heating of *C. sphaerospermum* conidia may cause the vacuolar membrane permeabilization leading to vacuole rupture and then eventually result in irreversible cell death. The findings obtained in this study may be expected to design effective conditions for thermal processing at relatively low temperatures to control mold conidia even in limited species in food and other environments.

Conflict of Interest

The authors declare no conflict of interest.

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