Evaluation of a novel method for measurement of intracellular calcium ion concentration in fission yeast

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(Received October 24, 2016; Accepted January 5, 2017)

ABSTRACT — The distribution of metal and metalloid species in each of the cell compartments is termed as “metallome”. It is important to elucidate the molecular mechanism underlying the beneficial or toxic effects exerted by a given metal or metalloid on human health. Therefore, we developed a method to measure intracellular metal ion concentration (particularly, intracellular calcium ion) in fission yeast. We evaluated the effects of nitric acid (HNO₃), zymolyase, and westase treatment on cytolysis in fission yeast. Moreover, we evaluated the changes in the intracellular calcium ion concentration in fission yeast in response to treatment with/without micafungin. The fission yeast undergoes lysis when treated with 60% HNO₃, which is simpler and cheaper compared to the other treatments. Additionally, the intracellular calcium ion concentration in 60% HNO₃-treated fission yeast was determined by inductively coupled plasma atomic emission spectrometry. This study yields significant information pertaining to measurement of the intracellular calcium ion concentration in fission yeast, which is useful for elucidating the physiological or pathological functions of calcium ion in the biological systems. This study is the first step to obtain perspective view on the effect of the metallome in biological systems.

Key words: Intracellular calcium ion, Fission yeast, Inductively coupled plasma atomic emission spectrometry, Micafungin

INTRODUCTION

Metals and metalloids are vital for human life. Currently, about 28 elements are considered as essential or beneficial components, regulating a great number of biological processes and sustaining a healthy functional environment in living organisms (Hu et al., 2013). The “metallome” can be defined as the distribution of metal and metalloid species in the cell compartments. Haraguchi (2004) proposed the term “metallomics” to designate “metal-assisted functions in biochemistry”, suggesting an analogy with genomics and proteomics owing to the role of metalloenzymes in gene and protein synthesis (Szpunar, 2004; López-Barea and Gómez-Ariza, 2006). Metallomic information comprises the identities of individual metal species (qualitative metallomics) and their concentrations (quantitative metallomics) (Haraguchi, 2004; Szpunar, 2004; López-Barea and Gómez-Ariza, 2006). For example, zinc regulates the expression of numerous genes and acts as a cofactor for over 50 enzymes that regulate metabolism (Vallee and Auld, 1990; Jackson et al., 2008; Peter, 2014). Disruption of zinc homeostasis is associated with a variety of diseases, including diabetes, cancer, and immune and connective tissue disorders (Franklin et al., 2005; Formigari et al., 2007; Wenzlau et al., 2007; Jackson et al., 2008; Fukada and Kambe, 2011; Jeong and Eide, 2013; Peter, 2014). Selenium (Se) is an essential dietary component for animals including humans, and is considered a prospective anticarcinogen (Brozmanová et al., 2010). Narayanan reported that Se compounds have the potential to be used not just for cancer prevention but also for cancer treatment (Narayanan, 2006). Furthermore, calcium signal is an important regulator of various physiological processes in eukaryotic cells (Ma et al., 2011). Calcium ion has the potential to modulate proliferation and apoptosis of cancer cells, and at the same time, it modulates proliferation, apoptosis, and effector efficacy of immune cells (Schwarz
On that account, systematic studies on the metal uptake, trafficking, and functions in many basic and complex biological processes aid in understanding the molecular mechanisms underlying the beneficial or toxic effects exerted by a given metal or metalloid, and further elucidating their impact on human health (Hu et al., 2013).

The emergence of element-detection techniques, such as inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) make it possible to detect metabolic variations due to trace element perturbation in an organism (Szpunar et al., 2003; Shen et al., 2008). In addition, these methods have analytical capabilities, such as wide dynamic range of the working calibration curve and the capability for simultaneous multi-element detection. This progress in analytical methodology has enabled detection and quantification of almost all elements in biological, geochemical, and environmental samples (Haraguchi, 1999; López-Barea and Gómez-Ariza, 2006).

Attempts made since the 1920s to measure intracellular calcium ion concentration were mostly unsuccessful (Takahashi et al., 1999). Predictably, the interests of many researchers shifted from calcium analysis at the cellular level to that at the subcellular level. It has been found that calcium ions are not evenly distributed throughout the cell and that intracellular heterogeneity of calcium ions (such as calcium waves and calcium sparks) is observed in a variety of cells (Kasai and Augustine, 1990; Takamatsu and Wier, 1990; Rooney et al., 1990; Takamatsu et al., 1991; Camacho and Lechleiter, 1993; Girard and Clapham, 1993). However, calcium ions function as universal second messengers in different cells. Therefore, accurate detection of intracellular calcium ions is vital to exploring the pathophysiological conditions in biological systems (Szpunar, 2004).

This study, the first step to acquire a perspective view about the metallome, aimed to measure the intracellular calcium ion concentration by using ICP-AES in fission yeast, a genetically tractable model organism. The effects of HNO₃, zymolyase, and westase treatment on cytolysis of fission yeast were investigated. Moreover, we evaluated the changes in the intracellular calcium ion concentrations of fission yeast in response to treatment with/without micafungin, which induces intracellular calcium ion influx (Deng et al., 2006). Because calcium signaling is highly conserved through evolution, we consider the results of this study will provide new insights for development of a method for direct detection of intracellular calcium ion.

MATERIALS AND METHODS

Materials
Calcium [CaCO₃], zinc [Zn(NO₃)₂], iron [Fe(NO₃)₃], copper [Cu(NO₃)₂], cadmium [Cd(NO₃)₂], and manganese [Mn(NO₃)₂] standard solutions (in 0.1 M HNO₃) were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Formaldehyde, 2-mercaptoethanol, ethylene glycol-bis (2-aminoethylether)-N,N',N'-tetracetic acid (EGTA), Sodium tartrate, di-Sodium Dihydrogen Ethylenediaminetetraacetate Dihydrate (EDTA), di-sodium hydrogenphosphate, and tri-Ssodium citrate dihydrate were purchased from Nacalai Tesque Inc., Kyoto, Japan. In addition, zymolyase 20T (from Arthrobacter luteus), westase (from Streptomyces rochei DB-34), and micafungin were purchased from Seikagaku Corporation (Tokyo, Japan), Takara Bio Inc. (Shiga, Japan), and Astellas Pharma Inc. (Tokyo, Japan), respectively.

The Edinburgh minimal medium (EMM) used in the study has been described previously (Moreno et al., 1991). The wild-type HM123 cells (h-leu1-32) and pKB801, a LEU2 expression vector, were obtained from in-house stocks.

Cytolysis of fission yeast cells with different treatments
The wild-type HM123 cells harboring the pKB801 (LEU2 expression vector) plasmid were grown to saturation in liquid medium EMM for 20 hr at 27°C. Formaldehyde was added to the culture (at final concentration of 4 wt%) and maintained for 30 min at room temperature, following which the cultured cells were harvested and subsequently subjected to different treatments as detailed below.

The cultured cells (1 mL each) were centrifuged for 1 min at 15,000 rpm by MX-205 (TOMY SEIKO Co., Ltd., Tokyo, Japan). The pellets obtained were incubated with the addition of 1% HNO₃ at 100°C in a block incubator BI-516S (ASTEC Co., Ltd., Fukuoka, Japan) for 0, 5, 10, 15, 20, and 60 min, and 14 hr. All the suspensions were recentrifuged for 1 min at 15,000 rpm, and the pellets were collected (A).

The cultured cells (7 mL) were centrifuged for 2 min at 2,000 rpm, and the pellet obtained was incubated with 2-mercaptoethanol (5 mL) for 30 min at room temperature. The suspension was centrifuged again for 2 min at 2,000 rpm. The pellet obtained was resuspended in zymolyase (5 mL) and shaken for 1 hr at 120 rpm at 36°C. This suspension was recentrifuged for 2 min at 2,000 rpm; subsequently, the pellet was resuspended in 1% HNO₃.
Measurement of intracellular calcium ion in fission yeast

The wild-type HM123-pKB801 (LEU2 vector) cells were grown to saturation in liquid medium EMM for 10 hr at 30°C. Subsequently, the pre-cultured cells were also grown to saturation in liquid medium EMM for 20 hr at 30°C to achieve an OD value of 0.92 at 660 nm. Formaldehyde was added (at final concentration of 4 wt%) and the culture was maintained for 15 min at room temperature. The cultured cells were cooled on ice for 15 min, and then centrifuged for 3 min at 6,000 rpm at 4°C and the supernatant was discarded. Cell pellets were washed five-times with ice-cooled ultrapure water. The obtained pellet was dried for 4 d. The dried pellet was mixed with 60% HNO3 (3 mL) and maintained for 14 d at 4°C. The suspensions were centrifuged for 2 min at 2,000 rpm, and the pellets collected (B).

Next, formaldehyde was added to the cells (at final concentration of 4 wt%), and maintained for 15 min at room temperature. The cultured cells were cooled on ice for 15 min to obtain an OD of 0.84 at 660 nm. The cultured cells were centrifuged for 3 min at 6,000 rpm at 4°C and the supernatant was discarded. Cell pellets were washed five-times with ice-cooled ultrapure water. The obtained pellet was dried for 9 d. The dried pellet was suspended in 60% HNO3 (3 mL) and maintained for 14 d at 4°C. The suspensions were diluted with ultrapure water (final concentration of formaldehyde is 1 wt%). The suspensions were filtered using 0.22 μm Rapid Filter Max Set (BM Equipment Co., Ltd., Tokyo, Japan). Finally, the concentrations of intracellular calcium, zinc, iron, copper, cadmium, and manganese ions in fission yeast were analyzed using ICP-AES (ICPS-7500, Shimadzu Co., Osaka, Japan). All values are presented as the mean ± standard deviation (n = 3).

Changes in intracellular calcium ion concentration of fission yeast treated with micafungin

The wild-type HM123-pKB801 (LEU2 vector) cells were grown to saturation in liquid medium EMM for 8 hr at 30°C. The pre-cultured cells were also grown to saturation in liquid medium EMM for 17 hr at 30°C. Micafungin (10 mg/mL) was added to the cultured cells (final concentration 4 μg/mL), and shaken for 45 min at 30°C. Next, formaldehyde was added to the cells (at final concentration of 4 wt%), and maintained for 15 min at room temperature. The cultured cells were cooled on ice for 15 min to obtain an OD of 0.84 at 660 nm. The cultured cells were centrifuged for 3 min at 6,000 rpm at 4°C and the supernatant was discarded. Cell pellets were washed five-times with ice-cooled ultrapure water. The obtained pellet was dried for 9 d. The dried pellet was suspended in 60% HNO3 (3 mL) and maintained for 14 d at 4°C. The suspensions were diluted with ultrapure water (final concentration of formaldehyde is 1 wt%). The suspensions were filtered using 0.22 μm Rapid Filter Max Set (BM Equipment Co., Ltd., Tokyo, Japan). Finally, the concentrations of intracellular calcium, zinc, iron, copper, cadmium, and manganese ions in fission yeast were analyzed using ICP-AES (ICPS-7500, Shimadzu Co.). Likewise, a sample solution without micafungin treatment was prepared in this experiment to be used as control.

Measurement of cytosolic free Ca2+ concentrations using aequorin

The real-time intracellular Ca2+ concentration-monitoring assay was performed according to the method described in Hagihara et al. (2013). The light emission levels expressed as relative light units (RLU) were measured using a microplate luminometer (CentroXS³ LB960, Berthold Japan K.K., Tokyo, Japan) at 1 min intervals.

RESULTS AND DISCUSSION

Differential interference contrast microscopy images of fission yeast subjected to different treatments (westase, zymolyase, and HNO3) are shown in Fig 1. The lysis of fission yeast was not confirmed in pellet (A) incubated for 0-20 min with HNO3. However, the partial cytolysis of fission yeast was observed in pellet (A) after incubation for 14 hr. These results indicated that the combined treatment with 1% HNO3 and heating (100°C) was insufficient for cytolysis of fission yeast. We also used zymolyase and westase, two well-known cell wall digesting
enzymes, and analyzed their effect on cytolysis. The lysis of fission yeast in pellet (B) was not confirmed by the images; however, in cells incubated for 14 hr, dissociation of the cell membrane from the cell wall was observed. Therefore, we could not confirm the absolute lysis of fission yeast in pellet (B) with the experimental conditions used herein. Although we could partially confirm the lysis of fission yeast in pellet (C), impurities were included by westase treatment. Consequently, we conclude that treatment with zymolyase or westase is not suitable for inducing cytolysis in fission yeast. We selected a simple treatment using HNO₃ at different concentrations for cytolysis of fission yeast. The cytolysis of fission yeast in pellet (D) was confirmed in cells treated with HNO₃ at concentration of 36% or above. Various cell disruption strategies have been used to develop an efficient, low-cost, and effective method for release of intracellular products (Middelberg, 1995; Geciova et al., 2002; Ho et al., 2008; Klimek-Ochab et al., 2011; Bzducha-Wróbel et al., 2014). Unfortunately, the method of cell wall digestion reported in previous studies might be too expensive. However, the treatment with HNO₃ is comparatively cheaper.

Fig. 1. Differential interference contrast microscope images of fission yeast. The morphologies of fission yeast cells in the different pellets obtained were measured. (A) The combined treatment with 1% HNO₃ and heating (100°C) at 0, 5, 10, 15, 20, 60 min, and 14 hr. (B) The combined treatment with zymolyase and 1% HNO₃ at 0 and 14 hr. (C) The combined treatment with westase and 1% HNO₃ at 0 and 14 hr. (D) HNO₃ treatment at different concentrations (0, 0.6, 6, 12, 24, 36, 48, and 60%).
The observations yielded very useful information in relation to the cytolysis of fission yeast. Therefore, we adopted treatment with 60% HNO₃ as a method of choice for subsequent experiments.

The calibration curve of the calcium ion is illustrated in Fig. 2. We achieved a linear relationship between calcium ion concentration and peak (correlation coefficient 0.999, concentration range 0.1-1000 μg/L). In addition, concentration of intracellular calcium ion in fission yeast was measured by ICPS-7500 (Fig. 3). Concentration of intracellular calcium ion in fission yeast was in the order u10 (12 μg/L) < su19 (31.8 μg/L) < su37 (69.2 μg/L) < su55 (128.2 μg/L) < su73 (172.9 μg/L) < su91 (273.4 μg/L) < S10 (251.9 μg/L), which indicates that concentration of intracellular calcium ion is proportional to the amount of cells in fission yeast. These results suggested that the method used herein could directly measure the intracellular calcium ion concentration in fission yeast.

Deng et al. (2006) reported that micafungin, a (1,3)-β-d-glucan synthase inhibitor, caused the increase in intracellular calcium ion concentration in fission yeast, which is also dependent on the Yam8/Cch1 calcium channel. Hence, we used micafungin and analyzed the changes in calcium ion concentration induced by its treatment in fission yeast. This treatment method could be useful for measurement of intracellular calcium ion exactly in fission yeast, as we can neglect the calcium ion attached onto the cell surface. The RLU of calcium ion in fission yeast are shown in Fig. 4. The results confirmed that micafungin treatment leads to the increase in intracellular calcium ion concentration in fission yeast (Fig. 4). EGTA was used as a chelating agent, owing to its high affinity for calcium ion. Therefore, fission yeast was treated with micafungin (Fig. 4). The RLU remained unchanged in micafungin-treated cells when 1 mM EGTA was used, showing inadequacy of this concentration for chelating...
the extracellular calcium ion in fission yeast. In contrast, upon treatment with 10 mM EGTA, the RLU dramatically decreased compared to that with micafungin treatment alone. These observations indicate that 10 mM EGTA could sufficiently chelate the extracellular calcium ion in fission yeast, suggesting inhibition of calcium ion flow inside the cell by EGTA.

Lastly, to obtain a perspective view on the metallome of fission yeast, we measured the intracellular concentrations of other ions in parallel with calcium ion (Fig. 5).
Micafungin treatment induced the increase of intracellular calcium ion, but concentrations of other ions remained unchanged. Zinc, iron, copper, cadmium, and manganese ion concentrations were unchanged, and concentrations were in the order of zinc < manganese < iron < copper. On the other hand, cadmium ion was not detected in this experiment. Previous studies reported the identification of cadmium-sensitive mutants in fission yeast (Schizosaccharomyces pombe) or the cadmium uptake system by yeast (Candida tropicalis), which indicates that there is a possibility of the detection of cadmium ion in fission yeast cells at different conditions (Kennedy et al., 2008; Rehman and Anjum, 2010). Therefore, it is important to detect and exactly measure the intracellular metal ions in fission yeast. However, no other study has yet reported the absolute quantities of each metal in fission yeast. The measurement method used in this study could make it possible to confirm the absolute quantities of each intracellular metal in fission yeast. This strategy is novel and could be useful for elucidation of the physiological or pathological roles and functions of calcium ion in biological systems.

In conclusion, we elucidated the cytology condition (treatment with 60% HNO3) of fission yeast and directly measured the intracellular calcium ion concentration using ICPS-7500. In addition, we proved by using micafungin that intracellular calcium ion in fission yeast could be detected by the measurement method used in this study.

ACKNOWLEDGMENT

This work was supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2014-2018 (S1411037).

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


