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MECHANISM OF *FUSARIUM TRICINCTUM (CORDA) SACC.* SPORE INACTIVATION BY CHLORINE DIOXIDE

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ABSTRACT

The mechanism of *Fusarium tricinctum (Corda) Sacc.* spore inactivation by chlorine dioxide (ClO_2) was investigated. During *F. tricinctum* spore inactivation by ClO_2, protein, DNA, and metal ion leakage, enzyme activity, and cell ultrastructure were examined. Protein and DNA leakages were not detected, while there were metal ion leakages of K^+, $Ca^{2+,}$ and Mg^{2+}, which were well-correlated with the inactivation rate. The enzyme activities of glucose-6-phosphate dehydrogenase, citrate synthase, and phosphofructokinase were inhibited and were also well-correlated with the inactivation rate. Electron micrographs showed the ultrastructural modifications of spores and demonstrated that spores were heavily distorted and collapsed from their regular structure. Spore surface damage and disruption in inner components was also severe. The metal ion leakage, the inhibition of enzyme activities, and the damage of spore structure were significant in *F. tricinctum* spore inactivation by ClO_2.

Keywords: Chlorine dioxide, mechanism, inactivation, *Fusarium tricinctum (Corda) Sacc.*, spore

INTRODUCTION

Chlorine dioxide (ClO_2) is an effective disinfectant that has broad and high germicidal activity. It is more stable and has a higher oxidizing capability as compared to chlorine (**Chen *et al.*, 2010**). Numerous studies have been carried out to prove the effective inactivation of various microorganisms by ClO_2 (**Gómez-López *et al.*, 2009; Chen *et al.*, 2010; Chen and Zhu, 2011a; Chen and Zhu, 2011b; Zhu *et al.*, 2012**). Nevertheless, the mechanism of microbial inactivation by ClO_2 has not been clearly clarified as lethal incidences are usually associated with complicated processes. Some authors have attempted to elucidate the biocidal mechanisms of ClO_2 and have concluded that ClO_2 could cause series of damage, such as content leakage (**Zhang *et al.*, 2007; Wei *et al.*, 2008**), protein and nucleic acid denaturation (**Hauchman *et al.*, 1986; Noss *et al.*, 1986; Li *et al.*, 2004; Cho *et al.*, 2010; Simonet and Gantzer, 2006**), and morphological alteration (**Chen *et al.*, 2002; Wang *et al.*, 2010**). However, there is still a lack of understanding on the major target site of ClO_2 interaction with microorganisms. Moreover, most of the published studies deal with inactivation mechanisms of ClO_2 on bacteria and viruses. Our previous study has showed that the metal ion leakage, the inhibition of enzyme activities, and the alteration of cell structure were critical events in *Saccharomyces cerevisiae* inactivation by ClO_2 (**Zhu *et al.*, 2012**). The objective of this research was thus to investigate the inactivation mechanism of ClO_2 on *Fusarium tricinctum (Corda) Sacc.* spore. Thorough investigations, including leakages of protein, DNA, and metal ion, enzyme activity, and spore ultrastructure, were performed to evaluate whether they were correlated with the inactivation rate. This is the first study on the inactivation mechanism of ClO_2 on fungal spores.

MATERIAL AND METHODS

Fungal spore suspension

Fusarium tricinctum (Corda) Sacc. was isolated from naturally infected chestnuts and identified based on morphological and physiological tests (**Wei, 1979**). Spores were cultivated and collected as described previously (**Chen *et al.*, 2011**). The final pellet was resuspended in sterile saline. The concentration of spores was determined in a Neubauer counting chamber and the initial concentration was adjusted to approx. 1×10^9 CFU/ml.

ClO_2 solution

A commercially available product of stabilized ClO_2 powder (Charmstar, Tianjin Charmstar Technology Development Co., Ltd., Tianjin, China) was dissolved in deionized water to prepare a stock solution (approx. 500 mg/L) according to the manufacturer's instructions. The ClO_2 concentration was accurately measured by a standard method using iodimetry right before use (**APHA, 1998**). Then the stock solution was further diluted with deionized water to prepare solutions with desired concentrations.

ClO_2 treatment

In concentration-dependent inactivation experiment, 5 ml of spore suspension was treated with equal volumes of ClO_2 with final concentrations of 1, 2, 4, 5, 10, 20, 50, and 100 mg/L ClO_2 for 1 min; in time-dependent inactivation experiment, 5 ml of spore suspensions was treated with equal volumes of ClO_2 with final concentration of 4 mg/L for 1, 5, 10, 15, 20, 25, 30, and 60 min. Immediately after each treatment, 5 ml of cell suspensions was transferred to a 5 ml of sterile neutralizing phosphate buffer (containing 0.5% sodium thiosulfate, pH 7.2) to neutralize the residual ClO_2 to terminate the inactivation process. The spores treated by sterile deionized water were used as the control.

Determination of inactivation rate

After neutralization, serial 10-fold dilutions were prepared in sterile saline. Diluents were spread on dichloran glycerol (DG-18) agar and colonies were counted and results expressed as log cfu/ml after incubation at 30ºC for 3 d. The initial spore populations before treatments were also counted. Inactivation rate was then determined. The minimal fungicidal ClO_2 concentration and treatment time were defined as the minimal ClO_2 concentration and treatment time producing 99.9% inactivation rate, respectively (**Zhu *et al.*, 2012**).

Measurement of intracellular content leakage

After neutralization, the mixture was centrifuged at 3,000×*g* for 10 min at 4ºC. Afterwards, the supernatants were collected for the determination of intracellular content leakage. The protein content was measured at 595 nm by Bradford method using bovine serum albumin as the standard (**Bradford, 1976**), while the DNA content was measured at 280 nm using bovine thymus DNA as the standard (**Chen *et al.*, 2002**). The metal ion (K^+, Ca^{2+}, and Mg^{2+}) contents were

determined by using ICP-MS (VISTA-PRO, Varian, Varian Medical Systems, Inc., Palo Alto, CA, USA).

Measurement of enzyme activity

After neutralization, the mixture was centrifuged at 3,000×g for 10 min at 4ºC and the pellets suspended in 5 ml of 0.05 mol/L Tris-HCl buffer (pH 7.5). Suspended spores were then disrupted by submitting them to a vortex for 5 min, in the presence of 5 g of glass beads (0.5 mm diameter). The disruption period was 1 min separated by 30 s interval in an ice bath. Cell debris and glass beads were then removed by centrifugation at 3000×g for 10 min at 4ºC and the supernatant was used for the determination of enzyme activities.
The glucose-6-phosphate dehydrogenase (G6PD) activity was measured by spectrophotometry at 30ºC and 340 nm using $NADP^+$ as cofactor (**Gurpilhares *et al.*, 2006**). G6PD activity was determined in a medium composed by: 500 µl Tris-HCl buffer (0.05 mol/L, pH 7.5), 100 µl $MgCl_2$ (0.035 mol/L), 5 µl $NADP^+$ (0.131 mol/L), 10 µl G6P (0.5 mol/L) and 100 µl of sample. The citrate synthase (CS) activity was determined at 30ºC and 412 nm using 520 µl buffer (50 mmol/L Tris-HCl containing 100 mmol/L KCl and 1 mmol/L EDTA, pH 7.5), 20 µl DTNB, 20 µl acetyl coenzyme A (2.5 mmol/L in distilled water), 20 µl oxaloacetate (5.0 mmol/L in distilled water), and 20 µl of the sample (**Lemos *et al.*, 2003**). The phosphofructokinase (PFK) activity was assayed spectrophotometrically by monitoring the change of absorbance at 340 nm and 30ºC (**Gancedo and Gancedo, 1971**). The assay was performed in 1 ml mixture of the following composition: 0.05 mol/L Tris-HCl buffer, pH 7.5, 0.05 mmol/L ATP, 5 mmol/L $MgCl_2$, 0.125 mmol/L NADH, 0.25 mmol/L F6P, 0.5 units of aldolase, glycerophosphate dehydrogenase, and triosephosphate isomerase. One unit of enzyme activity (U) was defined as the quantity of enzyme catalyzing the conversion of 1 µmol of substrate per min. Specific activity was expressed as units per milligram of total protein. Total protein concentration was measured by Bradford method using bovine serum albumin as the standard.

Microscopic analysis of spore ultrastructure

Spore ultrastructures of control, spores treated with 5 mg/L ClO_2 for 1 min, and spores treated with 4 mg/L ClO_2 for 10 min were examined using a transmission electron microscope (JEM-1200EX, JEOL, JEOL Ltd., Tokyo, Japan).

Statistical analysis

All experiments were carried out in three trials. Data were analyzed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA).

RESULTS

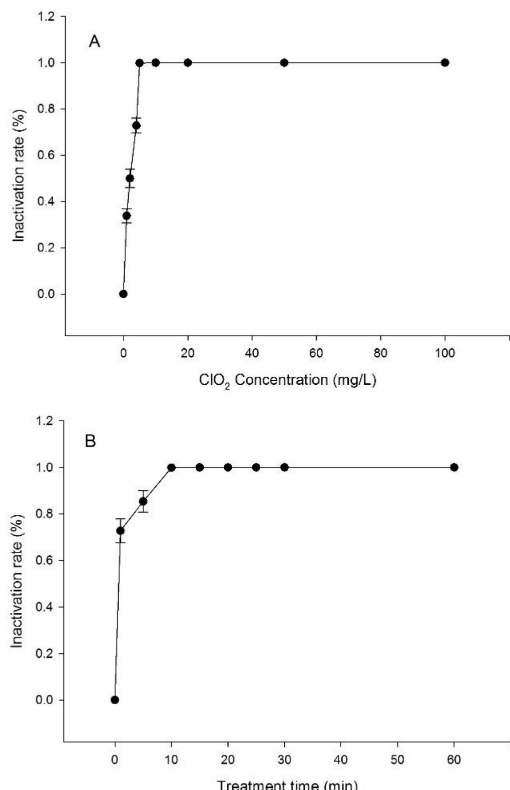

Figure 1 Inactivation rates of *F. tricinctum* spores treated by ClO_2 with different concentrations (A) and treatment times (B). Vertical bars indicate standard deviation.

As shown in Fig. 1, the inactivation rate of *F. tricinctum* spores increased gradually, as ClO_2 concentration increased from 0 to 5 mg/L and treatment time extended from 0 to 10 min. And when ClO_2 concentration increased to 5 mg/L and treatment time prolonged to 10 min, the inactivation rate reached up to 99.9%. Afterwards, the inactivation rate maintained at 100%. The minimal fungicidal ClO_2 concentration and treatment time were 5 mg/L and 10 min, respectively.

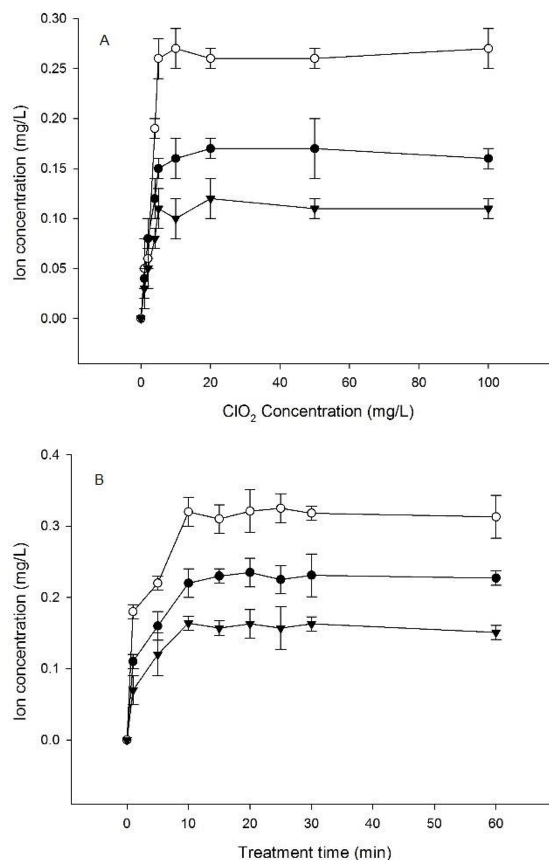

Figure 2 Metal ion leakages of *F. tricinctum* spores treated by ClO_2 with different concentrations (A) and treatment times (B). Vertical bars indicate standard deviation. (▼), K^+; (○), Ca^{2+}; (●), Mg^{2+}.

In the present study, there were no detectable protein and DNA leakages in *F. tricinctum* spore, regardless of ClO_2 concentration or treatment time. In consideration of the fact that protein and DNA are macromolecules, they might not leak out of the cell readily. In despite of no detectable protein or DNA leakage, we could not draw the conclusion that cell membranes were not permeated by ClO_2, since metal ions, including K^+, Ca^{2+}, and Mg^{2+}, showed different levels of leakage (Fig. 2). The leakages of K^+, Ca^{2+}, and Mg^{2+} displayed gradual increase before 99.9% of the spores were inactivated. Afterwards, although ClO_2 concentration and treatment time continued to increase, no significant increase in metal ion leakage was detected. Similarly, Wang *et al.* **(2010)** also found that the content of K^+, Ca^{2+}, and Mg^{2+} in *Nosema bombycis* spore was lost immediately after the 50 mg/L ClO_2 treatment. Moreover, they also indicated that a large amount of protein and DNA of the spores leaked out in a short time after the 50 mg/L ClO_2 treatment. The difference between our results and theirs may attribute to the different biological responses of different organisms to ClO_2 treatment. Hence, it is essential to investigate the biocidal mechanisms of ClO_2 on various species of organisms, since results obtained from one organism may not be generalized to others.

Table 1 Correlation between inactivation rates of *F. tricinctum* spores treated by ClO_2 with different concentrations and metal ion leakages

Correlation	Metal ion leakage		
	K^+	Ca^{2+}	Mg^{2+}
R^2	0.90	0.88	0.87

Table 2 Correlation between inactivation rates of *F. tricinctum* spores treated by ClO_2 with different treatment times and metal ion leakages

Correlation	Metal ion leakage		
	K^+	Ca^{2+}	Mg^{2+}
R^2	0.89	0.86	0.85

More importantly, the inactivation rate of *F. tricinctum* spores treated by ClO_2 with different concentrations was well-correlated with metal ion leakages (Table 1 and 2).

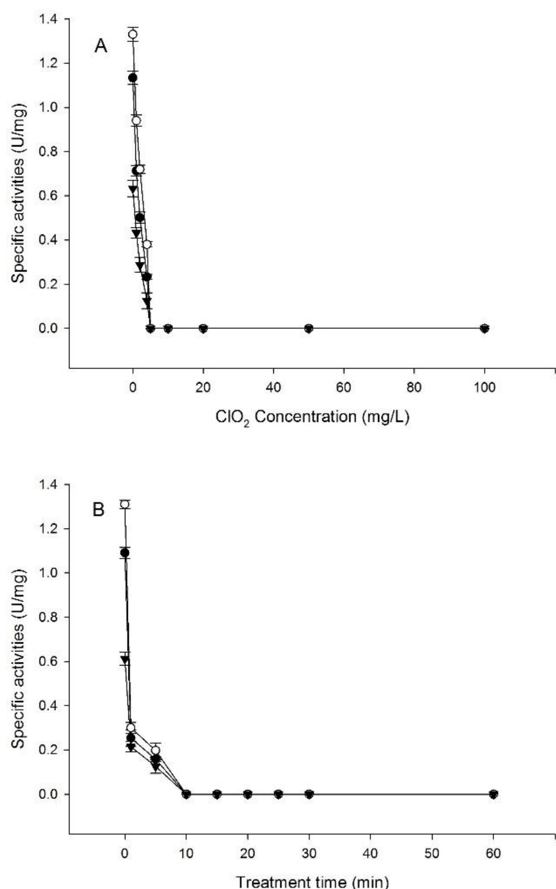

Figure 3 Enzyme activities of *F. tricinctum* spores treated by ClO_2 with different concentrations (A) and treatment times (B). Vertical bars indicate standard deviation. (●), G6PD; (○),CS; and (▼), PFK.

G6PD catalyzes the oxidation of glucose-6-phosphate to form 6-phosphoglucono-δ-lactone in the pentose phosphate pathway (PPP), which produces NADPH (**Nelson and Cox, 2012**). CS catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate, which is the first reaction of tricarboxylic acid (TCA) cycle. PFK catalyzes the transfer of a phosphoryl group from ATP to fructose-6-phosphate to yield fructose-1,6-bisphosphate. As shown in Fig. 3, ClO_2 dramatically inhibited the enzyme activities of G6PD, CS, and PFK in *F. tricinctum* spores. Inhibition of enzyme activities became more obvious when ClO_2 concentration increased from 0 to 100 mg/L and treatment time extended from 0 to 60 min. The enzyme activities of G6PD, CS, and PFK were completely inhibited when inactivation rate reached 99.9%. After enzyme activities decreased to 0 U/mg, they remained inhibited for the rest of the time.

Table 3 Correlation between inactivation rates of *F. tricinctum* spores treated by ClO_2 with different concentrations and enzyme activities

Correlation	Enzyme activity		
	G6PD[a]	CS[b]	PFK[c]
R^2	0.85	0.86	0.88

Legend: [a]Glucose-6-phosphate dehydrogenase.
[b]Citrate synthase.
[c]Phosphofructokinase.

Table 4 Correlation between inactivation rates of *F. tricinctum* spores treated by ClO_2 with different treatment times and enzyme activities

Correlation	Enzyme activity		
	G6PD[a]	CS[b]	PFK[c]
R^2	0.87	0.90	0.88

Legend: [a]Glucose-6-phosphate dehydrogenase.
[b]Citrate synthase.
[c]Phosphofructokinase.

More importantly, the inactivation rate of *F. tricinctum* spores treated by ClO_2 with different concentrations was well-correlated with enzyme activities (Table 3 and 4). Therefore, it can be speculated that ClO_2 penetrated into *F. tricinctum* spore and damaged the structures of enzymes, which had negative influences on some critical microbial metabolic processes. And noticeably, the inhibition of enzyme activity was correlated with the metal ion leakage. It should be due to the fact that some enzymes require an additional chemical component such as metal ion called a cofactor for activity (**Zhu *et al.*, 2012**).

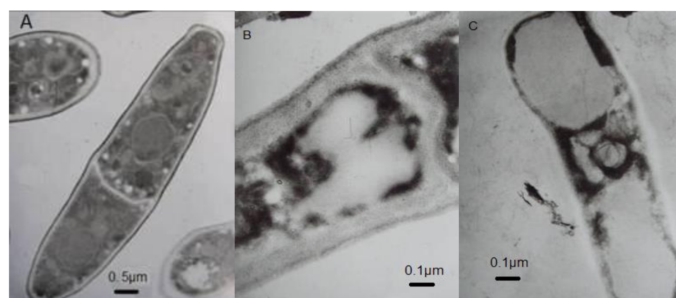

Figure 4 Ultrastructures of normal *F. tricinctum* spore (A), spore treated by 5 mg/L chlorine dioxide for 1 min (B), and spore treated by 4 mg/L chlorine dioxide for 10 min.

Fig. 4A shows a normal canoe-shaped spore structure, which had a clear observation of plasma membrane and intracellular contents divided by a cross-wall. After the treatments of 5 mg/L chlorine dioxide for 1 min and 4 mg/L chlorine dioxide for 10 min (99.9% of the cells were inactivated), membrane structure was damaged, cytoplasm was pycnotic, and organelles could not be identified (Fig. 4B and 4C). Severe damage on intracellular components, such as visible holes in the cytoplasm, were also observed. Some authors also reported that ClO_2 could cause morphological alteration for different microorganisms. Our results were consistent with Cho *et al.* (**2010**) who reported that *Escherichia coli* cell showed partial damages for both cell surfaces and intracellular components after inactivation by ClO_2. However, our findings were in contrast with Wei *et al.* (**2008**) who studied the inactivation mechanism of ClO_2 on *Candida albicans*. They observed no obvious damage on the plasma membranes and inner structures. And the cell wall was even found to be intact when the cell was inactivated. Nonetheless, they reported that the cytoplasm appeared a little 'rougher' and the cell wall became less dense after ClO_2 treatment. The differences among studies may be explained by the different responses of microbial cells to ClO_2 treatment.

CONCLUSION

The protein and DNA leakages were not correlated with the inactivation rate of *F. tricinctum* spore; however, the mechanism of *F. tricinctum* spore inactivation by ClO_2 is linked to the leakages of K^+, Ca^{2+}, and Mg^{2+}, the inhibition of key enzyme activities in some major metabolic pathways, and the damage of spore structure.

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