Fungal cleavage of thioether bond found in Yperite

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Abstract The degradation of thiodiglycol (I) and benzyl sulfide (II) was attempted using *Coriolus versicolor* and *Tyromyces palustris* to investigate the potential ability of basidiomycetes to degrade Yperite (bis(2-chloroethyl) sulfide), a mass-produced and stored chemical warfare agent. I was very rapidly degraded by both fungi. The metabolic pathway of II was elucidated, showing that the initial step was the hydrolytic cleavage of the thioether bond to yield benzyl alcohol and benzyl mercaptan. Benzyl alcohol was further oxidized and finally mineralized. Benzyl mercaptan is reversibly converted to benzyl disulfide and also converted to benzyl alcohol. Finally, the effective degradation of bis(2-bromoethyl) sulfide strongly suggests that basidiomycete would be a potential tool for Yperite degradation.

Key words: Basidiomycete; Benzyl sulfide; Bioremediation; Chemical warfare agent; Thiodiglycol; Yperite

1. Introduction

According to the Chemical Weapons Convention (effective on 29 April 1997), chemical weapons should be destroyed within 10 years. More than 65 countries, including Japan, have ratified the Treaty. Among many types of chemical weapons, the agent which has been the most produced and stored was found to be bis(2-chloroethyl) sulfide, also known as Yperite or mustard gas. Yperite is markedly cytotoxic and is also a carcinogenic and mutagenic alkylating agent [1–4]. Large-scale incineration of Yperite was started in 1990. However, during incineration and neutralization, the occurrence of several drawbacks have been pointed out [5]. As an alternative to incineration, several methods have been proposed. The bioremediation process has been thought to be advantageous over other conventional processes because of a minimal impact on the environment and of cost effectiveness [6]. Recently, a biological process was proposed to the US National Research Council, that is, the chemical hydrolysis of Yperite followed by biodegradation of the hydrolyzed products [7]. Immediately after this proposal, a possible bioremediation of thiodiglycol (TDG), a product of Yperite hydrolysis, was reported, showing that *Alcaligenes xylosoxidans*, an aerobic Gram-negative bacterium, was isolated for its ability to degrade TDG [8]. This bacterium was reported to be capable of surviving against exposure to high concentration of TDG (up to 80 mM). However, the metabolic pathway was not reported; furthermore, the accumulation of byproduct thiodiglycol sulfoxide was observed.

Basidiomycetes are the only known organisms to completely degrade wood components including lignin, one of the most recalcitrant aromatic biomaterials [9,10]. Lignin-degrading basidiomycetes are called white-rot fungi, and the others are called brown-rot fungi. Utilizing the ability of lignin degradation, fungal bioremediation has been applied to trinitrotoluene, chlorophenols, chlorinated dibenzo-*p*-dioxins, and polycyclic hydrocarbons, etc. [11–16]. In this study, we initiate the biodegradation of TDG by either white-rot or brown-rot basidiomycetes. They exhibited a strong ability to metabolize TDG either for xenobiotic elimination or for sulfur demand. Then, to elucidate reaction mechanisms, benzyl sulfide was utilized, showing effective cleavage of thioether. Finally, the fungal degradation of Yperite analog, bis(2-bromoethyl) sulfide was attempted.

2. Materials and methods

2.1. Chemicals

Thiodiglycol (**I**), benzyl sulfide (**II**), 1,4-dithiane (**III**), benzyl alcohol (**IV**), benzaldehyde (**V**), benzoic acid (**VI**), benzyl mercaptan (**VII**), and benzyl disulfide (**VIII**) were purchased from Wako Pure Chemicals Co., except **IV** which was synthesized from **V** using sodium borohydride. Bis(2-bromoethyl) sulfide (**IX**) was synthesized by bromination of **I** at 0°C using triphenylphosphine and tetrabromomethane in dry THF. **IX** was extracted with ether after water addition, then purified using silica gel chromatography (hexane/ethyl acetate).

2.2. Culture conditions

Coriolus versicolor (IFO 30340) and *Tyromyces palustris* (IFO 0507) were utilized as the white-rot and brown-rot fungi, respectively. They were grown from hyphae-inocula at 28°C in stationary culture under air [17]. The complete medium (pH 6.0) used in this study was as previously described with 1% glucose (HC; high concentration of nutrient carbon) and either 1.2 (LN; low concentration of nutrient nitrogen) or 12 mM (HN) ammonium tartrate as the carbon and nitrogen source, respectively [18]. In the no sulfur (No-S) medium, sulfate salts used in the complete medium were substituted with chloride salts. Mycelium weights were determined as previously described [18].

After 5 days of incubation, the substrates in acetonitrile (50 mM) were added to the cultures to a final concentration of 0.5 mM. After additional incubation, the metabolic products were analyzed either by HPLC or by GCMS as reported [17]. HPLC analysis was carried out using a Shimadzu STR ODS-II column. A UV monitor was utilized at 210–240 nm. GCMS was performed at 70 eV on a Shimadzu QP 1000 equipped with a Shimadzu GC 9A (80–280°C). Products were identified by comparison of their retention times on GC and HPLC and of mass fragmentation patterns with standards. Quantification was carried out on HPLC using calibration curves obtained with standards.

3. Results

3.1. Effect of culture media on fungal degradation of thiodiglycol

Thiodiglycol (**I**) was effectively degraded by either *C. versicolor* or *T. palustris*. Fig. 1 shows the time course of TDG (**I**) degradation by both fungi under several different culture conditions. *C. versicolor* brought about the disappearance of

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55% of added **I** (0.5 mM) in 1 h in the HCLN complete medium. On the other hand, *T. palustris* caused the disappearance of 80% of added **I** in the No-S medium in 2 h. Table 1 exhibits the effect of several different nutrient sulfur sources on fungal growth. When *C. versicolor* and *T. palustris* were cultured in No-S HCHN cultures, the dry weight decreased by 52% and 45%, respectively, compared to complete HCHN culture. The fungal weights were recovered to 80% (*C. versicolor*) and 90% (*T. palustris*), upon the addition of 5.0 mM Na_2SO_4 as sulfur source. When **I** was added to the No-S cultures, the growth weight of *T. palustris* was recovered back to 93%, while that of *C. versicolor* was back to 68% (Table 1).

1,4-Dithiane (**III**) was also degraded by both fungi, albeit slowly. Again, *C. versicolor* caused the fastest degradation in the complete HCLN medium, while *T. palustris* in the No-S HCHN medium.

3.2. Degradation pathway of benzyl sulfide

Both fungi, especially *T. palustris*, seemed to be capable of utilizing sulfur in TDG, suggesting that the fungi might cleave thioether bond to generate nutrient sulfur. To better characterize fungal cleavage of thioether, benzyl sulfide (**II**) was used as a substrate. Aromatic moiety was thought to help identifying C–S cleaved products.

II was effectively degraded by both fungi. The best results were seen in the complete HCLN medium with *C. versicolor*, and in No-S HCHN with *T. palustris*. Both fungi yielded

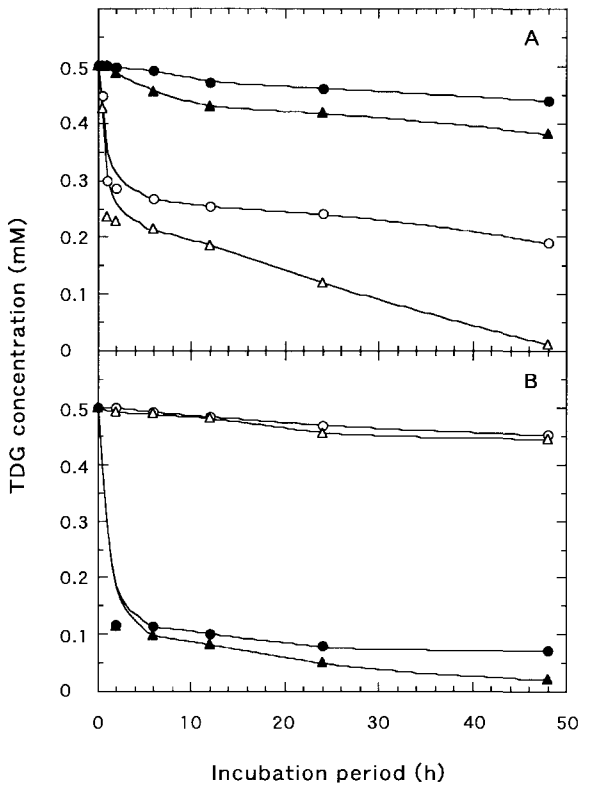

Fig. 1. Effect of culture conditions on fugal degradation of thiodiglycol (**I**). After 5-day pre-incubation, **I** was added to cultures at a final concentration of 0.5 mM. **I** was quantified as described in the text. A: Degradation of **I** in the complete media (HCHN; circle, HCLN; triangle) by either *C. versicolor* (open symbol) or *T. palustris* (closed symbol). B: As in (A) except cultured in the No-S media.

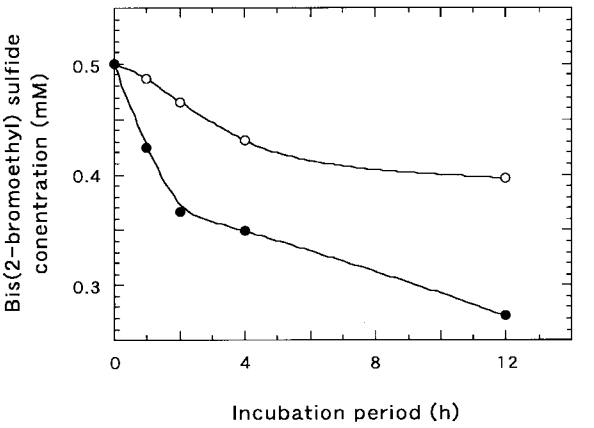

Fig. 2. Fungal degradation of bis(2-bromoethyl) sulfide (**IX**). *C. versicolor* (circle) was incubated in the complete HCLN medium, while *T. palustris* (triangle) was in the No-S HCHN medium.

essentially the same products (Table 2). Five aromatic products were identified from the fungal metabolism of **II**: benzyl alcohol (**IV**), benzaldehyde (**V**), benzoic acid (**VI**), benzyl mercaptan (**VII**), and benzyl disulfide (**VIII**). When **IV** was added exogenously to the cultures, **V** and **VI** were detected as reported previously [15]. **VII** was also added exogenously, showing that very rapid formation of **VIII** and trace formation of **IV** and **V** were observed. Finally, **VIII** was added exogenously to the cultures. A time course showed a decrease of the substrate (**VIII**), but only trace formations of **IV**, **V**, and **VII** were confirmed. Disappearance rates of the substrate and metabolic intermediates are listed in Table 2.

3.3. Degradation of Yperite analog, bis(2-bromoethyl) sulfide

Both fungi exhibited the ability to degrade bis(2-bromoethyl) sulfide (**IX**), analog for Yperite. A higher initial rate and a greater amount of degradation of **IX** were observed with *T. palustris* under No-S conditions (Fig. 2). Product analysis was achieved using HPLC equipped with refractive index detector (Toso, RI-8022), showing no accumulation of either TDG (**I**) or bromoethanol (data not shown). Although some more study is required, the rate limiting step of fungal degradation of **IX** seemed to be the hydrolytic dehalogenation to yield **I**, which is further metabolized rapidly (Fig. 1). A slow conversion of bromoethanol (data not shown) also suggested the occurrence of the initial dehalogenation.

4. Discussion

Basidiomycetes are eukaryotic microorganisms, causing complete mineralization of wood components, which is a key step for the carbon cycle in a biosphere [9,10]. They are usually categorized into two classes, white-rot and brown-rot fungi [19]. They are taxonomically very similar, but white-rot fungi are the only known organisms to be responsible for the degradation of lignin, one of the most recalcitrant aromatic biopolymers [9,10]. We have utilized both white-rot and brown-rot basidiomycetes for biotransformation of environmentally persistent compounds such as sulfur containing heterocyclic compounds [20]. 2-Hydroxymethyl-thiophene was converted to γ-thiobutyrolactone via several redox steps, which is finally and effectively utilized as a sulfur source by fungi, strongly suggesting that fungi are capable of cleaving

Table 1
Mycelium dry weight (mg) of *C. versicolor* and *T. palustris* grown with several different nutrient sulfur sources

Fungus	Complete HCHN	No-S HCHN	No-S HCHN+			
			SO_4^{2-}	TDG (mM)		
				1.0	2.0	5.0
C. versicolor	54.5	26.1	43.8	37.2	36.9	31.4
T. palustris	25.9	14.1	23.3	22.5	24.1	20.0

Both fungi were incubated for 5 days under culture conditions shown in the table. SO_4^{2-} was added as Na_2SO_4 (5.0 mM). Complete medium contains 5.0 mM nutrient sulfur.

Table 2
Fungal metabolism of benzyl sulfide (**II**)

Fungus(culture conditions)	Substrate	Rate(μmol/h/l)	Products found[a]
C. versicolor complete HCLN	**II**	13.9	**IV, V, VI, VII, VIII**
	VII	333	(**IV, V**) **VIII**
	VIII	263	(**IV, V**) **VII**
T. palustris No-S HCHN	**II**	5.6	**IV, V, VI, VII, VIII**
	VII	312	(**IV, V**) **VIII**
	VIII	250	(**IV, V**) **VII**

[a]Compounds shown in parentheses were formed with a trace amount.

C–S bond[1]. It was then conceived that fungal cleavage of the thioether bond would be applied to that found in Yperite, the most produced and stored chemical weapon in the world. White-rot fungi have also been reported to degrade chlorinated phenols, polychlorinated dibenzo-*p*-dioxins, and nitrated benzenes including trinitrotoluene [11–16]. These results are very supportive for the biodegradation of Yperite, since it is chlorinated and sometimes contaminated with trinitrotoluene, especially in an old and rotten shell. We have begun to examine two possible strategies for fungal degradation of Yperite; one is a recently proposed method [7,8], that is, fungal degradation of thiodiglycol which is a hydrolyzed product of Yperite; and, the other is a one-step fungal degradation of Yperite.

Thiodiglycol (**I**) was rapidly degraded by either *C. versicolor* (white-rot) or *T. palustris* (brown-rot). *C. versicolor* metabolized **I** under ligninolytic conditions (Fig. 1A), suggesting that this fungus degrades **I** as a xenobiotic metabolism. On the other hand, *T. palustris* metabolized **I** when the fungus was grown in the No-S medium, where **I** was the sole sulfur source (Fig. 1B). The growth of fungi was examined under several culture conditions, supporting that the brown-rot fungi utilizes **I** as a sulfur source more effectively than the white-rot fungus does (Table 1). Albeit the effectiveness was different, both fungi utilize **I** as a sulfur source, which strongly suggests that the thioether bond should be cleaved. To better understand the mechanism, benzyl sulfide (**II**) was used as a substrate. **II** was degraded to yield benzyl alcohol (**IV**) and benzyl mercaptan (**VII**) as initial reaction products by both fungi (Table 2). It has been reported that either *C. versicolor* or *T. palustris* rapidly oxidize **IV** to benzaldehyde (**V**), then to benzoic acid (**VI**) [17]. Finally, **IV**, **V**, and **VI** are mineralized by these fungi, confirmed by using radio labeled substrates [17]. Most likely, the initial step of fungal metabolism of **II** is the hydrolytic cleavage of thioether. The reversible metabolic process between **VII** and benzyl disulfide (**VIII**) was observed (Table 2). Without fungi, **VII** was autooxidized to form **VIII** within 12 h under air (data not shown). Intracellular conversion of **VIII** back to **VII** was suggested. When either **VII** or **VIII** were fed to the fungi, a trace formation of **IV** and **V** were observed with GCMS analysis. Since only a trace formation was observed, kinetic analysis of the C–S cleavage of **VII** could not be achieved. Further study is required to determine that **V** is formed directly from **VII** or via the formation of **IV**. This data allowed us to propose the metabolic pathway of benzyl sulfide (**II**) by both fungi (Fig. 3). Kinetic analysis indicated that the rate determining step of this pathway is the initial cleavage of thioether (Table 2). Probably, the fungal metabolism of thiodiglycol may undergo a similar pathway. Both fungi are able to grow in the medium containing thiodiglycol (50 mM) as a sole carbon and sulfur source. This may assist the possible application of basidiomycetes to thiodiglycol degradation.

Fungal degradation of bis(2-bromoethyl) sulfide (**IX**) was attempted, indicating that either *C. versicolor* or *T. palustris* is capable of effectively degrading Yperite analog (Fig. 2). Both fungi degrade thiodiglycol with the initial rate of 400 μmol/h/l. On the other hand, **IX** was degraded with the rate of 17.9 μmol/h/l (*C. versicolor*) and 78.1 μmol/h/l (*T. palustris*). This difference in rates might be caused by the effectiveness of the hydrolytic dehalogenation. If the C–S bond cleavage is the initial step of **IX** degradation, the rate would be more comparable to that of **I** degradation. The higher rate of *T. palustris* for **IX** degradation would suggest that the brown-rot fungi degrade chlorinated organic compound effectively.

Both fungi were capable of degrading 1,4-dithiane (data not shown), which would be important if basidiomycetes are applied to in situ clean-up at Yperite contaminated site. Not only thiodiglycol but also dithiane were usually found as contaminants derived from Yperite.

In this study, it has been shown that basidiomycetes could be a potential tool to degrade chemical weapons such as Yperite. We have initiated the degradation study of Yperite itself. The fate of chlorine in Yperite will be investigated. The identification for two hydrolytic enzymes and their encoding genes involved in dehalogenation and the thioether bond cleavage is now under way.

[1] Wariishi, H., Nakamizo, M., Ichinose, H., and Tanaka, H. unpublished results.

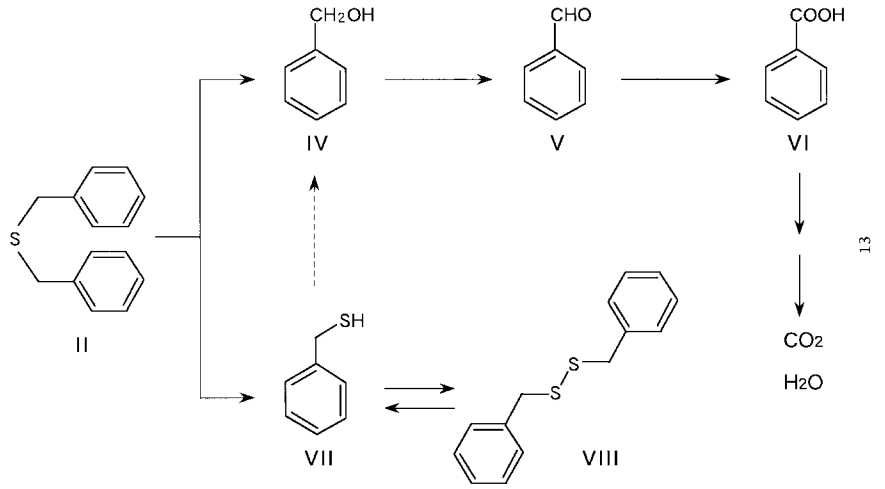

Fig. 3. Proposed metabolic pathway of benzyl sulfide (**II**). Essentially, both fungi showed the same pathway.

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