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Enhancement of paraben-fungicidal activity by sulforaphane, a cruciferous vegetable-derived isothiocyanate, via membrane structural damage in *Saccharomyces cerevisiae*

Running headline: Sulforaphane boosts paraben action

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Significance and impact of the study

Sulforaphane, a natural and beneficial cruciferous vegetable-derived isothiocyanate, increased the ability of parabens to disrupt fungal cell membranes. Paraben-containing products have been reported to cause allergic contact dermatitis and drug hypersensitivity; therefore, methods to preserve organic products that may reduce the concentrations of parabens are both timely and necessary. In this study, we found that the combined antimicrobial effects of sulforaphane and parabens had the potential to reduce the paraben concentration needed to preserve organic products, thereby indicating that paraben toxicity may be reduced without affecting its activity as a preservative.

Abstract

Parabens have been widely used as antimicrobial preservatives in cosmetics, pharmaceuticals, foods, and beverages. Commonly, methyl-, ethyl-, propyl-, and butylparaben are used independently or in combination to maintain the quality of industrial products, and they are considered to have low toxicity. However, recent evidence has suggested that parabens are toxic in mammalian cells, and parabens have been associated with allergic-contact dermatitis, breast cancer, and changes in testosterone levels. Sulforaphane, a cruciferous vegetable-derived isothiocyanate, was effective in decreasing the growth inhibitory concentrations of ethyl-, propyl-, butyl-, and methylparaben in the yeast *Saccharomyces cerevisiae*. The sulforaphane-enhanced fungicidal effects of methylparaben were deemed to be caused by drastic cell membrane damage and the leakage of internal substances, such as nucleotides, from *S. cerevisiae* cells. Moreover, sulforaphane markedly decreased the minimum concentration of methyl- and ethylparaben required to inhibit the growth of various microbes, such as the pathogenic yeast that causes severe mycosis, *Candida albicans*; the filamentous fungi *Aspergillus niger*; and the gram-negative bacterium *Escherichia coli*. Enhanced antimicrobial activity from the beneficial components of edible plants may increase paraben efficacy at low concentrations and minimize preservative-induced side effects in consumers.

Keywords: parabens, sulforaphane, amplification, antimicrobial activity, preservatives

Abbreviations: Sulforaphane (SFN)

Introduction

Parabens are alkyl ester homologues of *p*-hydroxybenzoic acid and are commonly used as antimicrobial preservatives in cosmetics, pharmaceuticals, foods, and beverages (Andersen 2008). Despite widespread use, paraben antimicrobial mechanisms remain unclear; however, their antimicrobial activity and water solubility depend on the molecular weight and length of the alkyl side chain (Elder 1984). Paraben combinations may produce synergistic inhibitory effects on bacteria (Doron *et al.* 2001). Methylparaben and combinations of methyl-, ethyl-, propyl-, or butylparaben (Figs 1A, B, C, and D) are commonly used as preservatives and have low toxicity (Golden *et al.* 2005; Soni *et al.* 2005; Charnock *et al.* 2007). Nonetheless, paraben toxicity has been reported in mammalian cells. Paraben-containing products have caused allergic contact dermatitis (ACD) and drug hypersensitivity (Mowad 2000). Patients with long-standing dermatitis and leg ulcers may become sensitized when topical paraben preparations are applied (Cashman and Warshaw 2005). Furthermore, parabens present weak estrogenic activity and have caused reductions in testosterone levels and sperm production in rats (Routledge *et al.* 1998; Pedersen *et al.* 2000). In addition, parabens have been shown to induce MCF-7 human breast cancer cell growth *in vitro* (Byford *et al.* 2002) and have been suggested to potentially contribute to endocrine disruption by altering hormone metabolism (Fujino *et al.* 2019). On the basis of these studies, reductions in the toxicity of these preservatives are necessary to minimize human health risks.

Sulforaphane (SFN; Fig. 1E) is a dietary isothiocyanate compound found as the precursor glucosinolate in cruciferous vegetables, such as broccoli, and is a potent inducer of phase 2 enzymes, which inactivate electrophiles and are strong oxidants (Fahey and Talalay 1999). In addition, SFN is a potent preventive agent against breast (Zhang *et al.* 1994; Cheng *et al.* 2019), colon (Chung *et al.* 2000; Kassie *et al.* 2003), stomach (Fahey *et al.* 2002; Kiani *et al.* 2018), and lung cancer (Hecht *et al.* 2002) and

modulates carcinogen metabolism by the inhibition of the metabolic activation of phase 1 enzymes and the induction of phase 2 detoxification enzymes and glutathione levels (Brooks *et al.* 2001; Lampe and Peterson 2002).

In this study, we focused on the fungicidal and enhancement effects that SFN presents when added to parabens. The investigation of enhanced antimicrobial activity due to a beneficial component of edible plants could increase paraben efficacy at low concentrations and, as a result, may be able to minimize preservative-induced side effects.

Results and discussion

Effect of SFN on the fungicidal activity of methylparaben against *S. cerevisiae*

Although toxic side effects have been found in humans, the use of preservatives remains indispensable to prevent manufactured organic products from being damaged by microorganisms. Finding ways to reduce the preservative concentrations used in organic products will improve human health. In a previous study, we found that allicin, an allyl sulfur compound from garlic, increased fungicidal activity due to vacuole disruptive damage by amphotericin B, a typical polyene macrolide antifungal antibiotic used in the treatment of serious fungal infection (Ogita *et al.* 2012). Allicin-amphotericin B combinations are thus predicted to reduce side effects of the antibiotic as a lower dose of amphotericin B may be administered. Such ideas that use synergy for clinical applications may also be applicable to the reduction of preservative toxicity.

S. cerevisiae cells were resistant to 0.125 mg ml⁻¹ methylparaben but were subjected to lethal damage within 24 h with 5.0 mg ml⁻¹ methylparaben (Fig. 2A). On the other hand, yeast cells were rendered susceptible to the fungicidal activity of methylparaben at 0.125 mg ml⁻¹ (Fig. 2B) when combined with SFN at a nonlethal concentration of 1 mmol l⁻¹ (Fig. 2C). The addition of SFN allows for a reduction in methylparaben dosage, which may consequently reduce toxic side effects.

Cell growth inhibitory activity: SFN-paraben combinations

Parabens are frequently used in mixtures in order to ensure sufficient antimicrobial activity and water solubility (Charnock and Finsrud 2007; Błędzka *et al.* 2014). We examined whether SFN could amplify the growth inhibitory activity of each paraben with a different alkyl chain length (see Fig. 1). Methyl- and ethylparaben were able to independently inhibit the growth of yeast cells at 2.5 mg ml⁻¹ (Figs 3A and B).

Furthermore, as the alkyl side chain became longer, its inhibitory activity increased, as may be observed for propyl- and butylparaben at 0.5 mg ml⁻¹ and 0.25 mg ml⁻¹, respectively (Figs 3C and D). Although SFN alone could not inhibit yeast cell growth at a concentration of 1 mmol l⁻¹, SFN decreased the minimum growth inhibitory concentration (MIC) of methyl-, ethyl-, and propylparaben to 6.25% of the inhibitory concentration required when each paraben was used independently (see Figs 3A, B, and C). Butylparaben, which had the longest alkyl side chain in this study, had a low MIC value when used independently but the required growth inhibitory dose when combined with 1 mmol l⁻¹ SFN was 12.5% of the dose required when butylparaben was used independently (see Fig. 3D). The combined SFN-paraben effects were synergistic, indicating that the growth inhibitory activity of the paraben, except that of methylparaben, was also amplified by the presence of SFN, regardless of the degree of water solubility or the length of the alkyl side chain.

Effect of SFN and methylparaben on cell membrane damage in *S. cerevisiae*

Although the exact paraben antibacterial mechanism has not been elucidated (Kosova *et al.* 2015), paraben antimicrobial effectiveness is strongly connected to the composition of lipid cell membranes and paraben physical properties (Flasiński *et al.* 2018). We examined the effects of methylparaben, SFN, and their combination on K⁺ ion and UV-absorbing material leakage from intact cells (Nakayama *et al.* 2002; Ogita *et al.* 2006^a). Methylparaben alone scarcely induced the leakage of intracellular K⁺ ions (Fig. 4A). However, intracellular K⁺ ion leakage was observed when SFN was present independently, and leakage was not affected by the addition of methylparaben (Fig. 4A'). Methylparaben and SFN both caused a slight leakage of UV absorbing materials from cells (Fig. 4B); however, the addition of SFN to methylparaben drastically amplified this leakage and the disruption of plasma membrane integrity (Fig. 4B'). Yeast mortality caused

by cell membrane disruption is strongly associated with nucleotide leakage rather than the leakage of K⁺ ions (Ogita *et al.* 2006^a; Ogita *et al.* 2012). These results suggest that the increase in fungicidal activity from the addition of SFN-paraben combinations may be related to a disruption of cellular plasma membrane integrity and nucleotide leakage.

Synergistic effects of SFN and methyl- or ethylparaben against other strains

We examined whether SFN enhances the capacity of methyl- and ethylparaben to inhibit the growth of a pathogenic yeast, *Candida albicans*; a filamentous fungus, *Aspergillus niger*; and a gram-negative bacterium, *Escherichia coli*. We found that SFN inhibited the growth of *C. albicans* (Figs 5A and A') and *E. coli* at 1 mmol l⁻¹ (Figs 5C and C') but did not inhibit the growth of *A. niger* at concentrations up to 1 mmol l⁻¹ (Figs 5B and B'). Both parabens were able to inhibit the growth of *C. albicans* at 1.25 mmol l⁻¹ and the growth of *A. niger* and *E. coli* at 2.5 mmol l⁻¹. The SFN-methylparaben combination showed a marked synergistic effect against *C. albicans* growth, a weak synergistic effect against *A. niger* growth, and a very weak synergistic effect (almost additive) against *E. coli* growth (Figs 5A, B, and C). On the other hand, the SFN-ethylparaben combination showed synergistic effects similar to SFN-methylparaben against *C. albicans* and *A. niger* growth (Figs 5A' and B') but only additive effects against *E. coli* growth (Fig. 5C'). Chitin is present in fungi cell walls, while bacteria cells have an outer membrane in which lipopolysaccharides may play a molecular defense role against hydrophobic compounds (Ogita *et al.* 2009). The results of this experiment strongly suggest that the inhibitory effects of SFN-paraben combinations are related to plasma membrane damage in fungal cells.

In this study, we found that the fungicidal effects of parabens were enhanced by the addition of SFN, and the isothiocyanate improved the ability of parabens to disrupt microbial cell membranes.

Combined antimicrobial SFN-paraben effects have the potential to reduce the paraben concentrations needed to preserve organic products, thereby providing an opportunity to minimize preservative-induced side effects without reducing the preservative potential of parabens.

Materials and methods

Measurement of cell viability and lethality

S. cerevisiae W303-1A cells were grown overnight in yeast-peptone-dextrose (YPD) medium (yeast extract 1%, peptone 2%, and D-glucose 2%) and diluted into freshly prepared YPD medium to a concentration of 10^6 cells ml^{-1} . Cells were incubated with vigorous shaking at 30 °C in YPD medium, and methylparaben (with or without of SFN) was added, and the viability and lethality of methylparaben and SFN were characterized. The viable cell number was also determined by counting colony-forming units after a 48-h incubation at 30 °C on YPD agar medium (1.8% agar).

Measurement of cell growth inhibitory effects

In experiments evaluating the cell growth inhibitory concentration of each component, *S. cerevisiae* W303-1A and *C. albicans* IFO1061 overnight yeast cell cultures with YPD medium were diluted with fresh YPD medium to final a concentration of 10^6 cells ml^{-1} . A filamentous fungus, *A. niger* ATCC 6275, was pre-cultivated overnight in 2.5% malt extract medium (Oriental Yeast Co., Tokyo, Japan) at 30 °C and the overnight cultures were diluted 100-fold with fresh YPD medium. *E. coli* IFO 3992 cells were grown overnight in 3.0% nutrient broth (Nissui Co., Tokyo, Japan) with vigorous shaking at 30 °C and diluted with the same medium to a final concentration of 10^6 cells ml^{-1} . The minimum growth inhibitory concentration (MIC) is the lowest concentration of a test compound that results in no visible growth.

Paraben and SFN MICs were determined by serial 2-fold broth dilution and cells were cultured in a 96-well plate at 30 °C for 48 h (bacteria) or 72 h (yeasts and filamentous fungi). Combination studies were performed by a broth checkerboard method (Ogita et al. 2006^b). A series of 2-fold dilutions for one compound were tested in combination with 2-fold dilutions of other compounds. Assays were independently performed in triplicate. The fraction inhibitory concentration (FIC) indices for these combinations were calculated as $(MIC_a \text{ combination}/MIC_a \text{ alone}) + (MIC_b \text{ combination}/MIC_b \text{ alone})$, where a and b are the two test compounds and values of <1, 1, or >1 indicated synergism, additive action, or antagonistic action, respectively (Berenbaum 1978).

Plasma membrane permeability change assay

Plasma membrane permeability was assayed by measuring the effluxes of both intracellular K⁺ and UV-absorbing materials. *S. cerevisiae* cells cultured overnight in YPD medium were harvested, washed with 50 mmol l⁻¹ succinate buffer (pH 6.0), and resuspended in the same buffer to obtain a final concentration of 10⁷ cells ml⁻¹. Cell suspensions were shaken with methylparaben at a nonlethal concentration (0.25 mg ml⁻¹) in the absence or presence of 1 mmol l⁻¹ SFN at 30 °C for 60 min. The supernatants were examined for K⁺ and UV-absorbing material content. The K⁺ content was evaluated using a K⁺ assay kit (HACH, Florissoux, Belgium) based on the tetraphenylborate method (Ramotowski and Szczesniak 1967). The concentration of UV-absorbing materials was expressed as the absorption at $\lambda=260$ nm.

Microorganisms and Chemicals

S. cerevisiae W303-1A was obtained from the National BioResource Project (NBRP, Osaka, Japan). *C.*

albicans IFO1061 and *E. coli* IFO 3992 were purchased from Institute for Fermentation, Osaka (IFO, Osaka, Japan). *A. niger* ATCC 6275 was purchased from American Type Culture Collection (ATCC, Manassas, VA). The chemicals were of reagent grade and purity, including methyl-, ethyl-, propyl-, and butylparaben (Sigma Aldrich; St Louis, MO; Figs 1A, B, C, and D) and SFN (LKT Laboratories Inc.; St Paul, MN).

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Conflict of interest

No authors of this manuscript have any financial or personal relationships with people or organizations that could inappropriately influence their work or this study.

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Figure legends

Figure 1. Paraben and sulforaphane structures.

Figure 2. Effects of methylparaben on cell viability in the absence or presence of sulforaphane (SFN). (A) Cells (10^6 cells ml^{-1}) were incubated with methylparaben alone at 0 (○), 0.125 (●), 2.5 (□), or 5 mg ml^{-1} (■). (B) Cells were incubated with methylparaben at 0 (○), 0.125 (●), or 2.5 mg ml^{-1} (□) in the presence of SFN at 1.0 mmol l^{-1} . (C) Cells were incubated with SFN at 0 (○) or 1.0 mmol l^{-1} (●). Each data point represents the mean $\pm$ standard deviation of triplicate assays.

Figure 3. Isobologram demonstrating the synergistic effects of sulforaphane (SFN) and each paraben on the growth of *S. cerevisiae*. Cells were incubated in a medium containing SFN and methyl- (A), ethyl- (B), propyl- (C), or butylparaben (D) at 30 °C for 48 h. Each data point shows the representative value of triplicate assays with similar results.

Figure 4. Promotive effects of sulforaphane (SFN), methylparaben, and a combination of SFN and methylparaben on K^+ leakage (A, A') and UV absorbing materials (B, B') from intact cells. Cells (10^7 cells ml^{-1}) were incubated in 50 mmol l^{-1} succinate buffer (pH 6.0) alone (○) or with 0.25 mg ml^{-1} methylparaben (●) in the absence (A, B) or presence (A', B') of 1.0 mmol l^{-1} SFN. Each data point shows the representative value of triplicate assays with similar results.

Figure 5. Isobologram demonstrating the synergistic effects of sulforaphane (SFN) and methyl- (A, B, C) or ethylparaben (A', B', C') on the growth of *C. albicans* (A, A'), *A. niger* (B, B'), and *E. coli* (C, C').

Cells were incubated in a medium suitable for each organism containing SFN and methyl- or ethylparaben at 30 °C for 48 h (*E. coli*) and 72 h (*C. albicans* and *A. niger*). Each data point shows the representative value of triplicate assays with similar results.

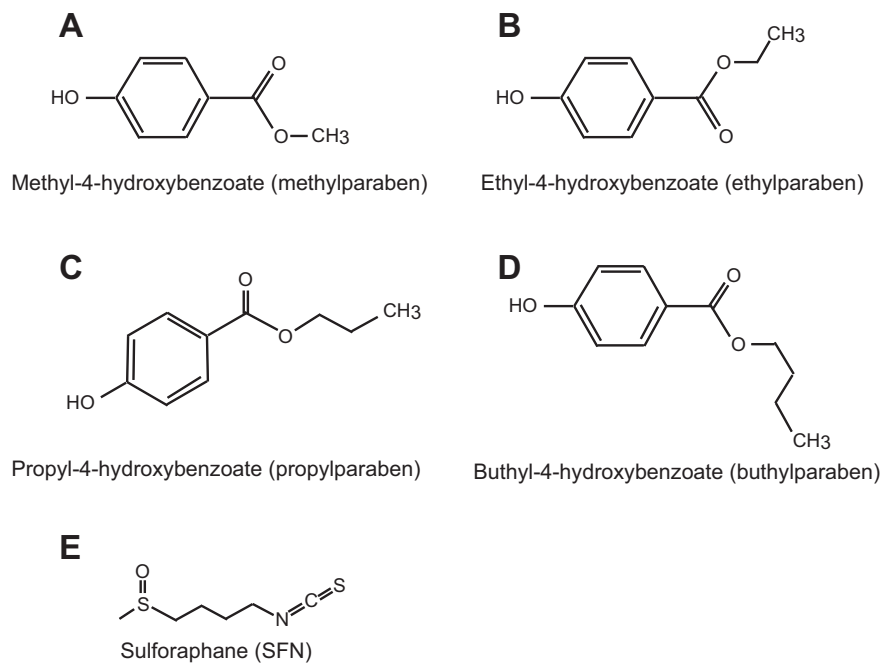


Figure 1. Murata et al.

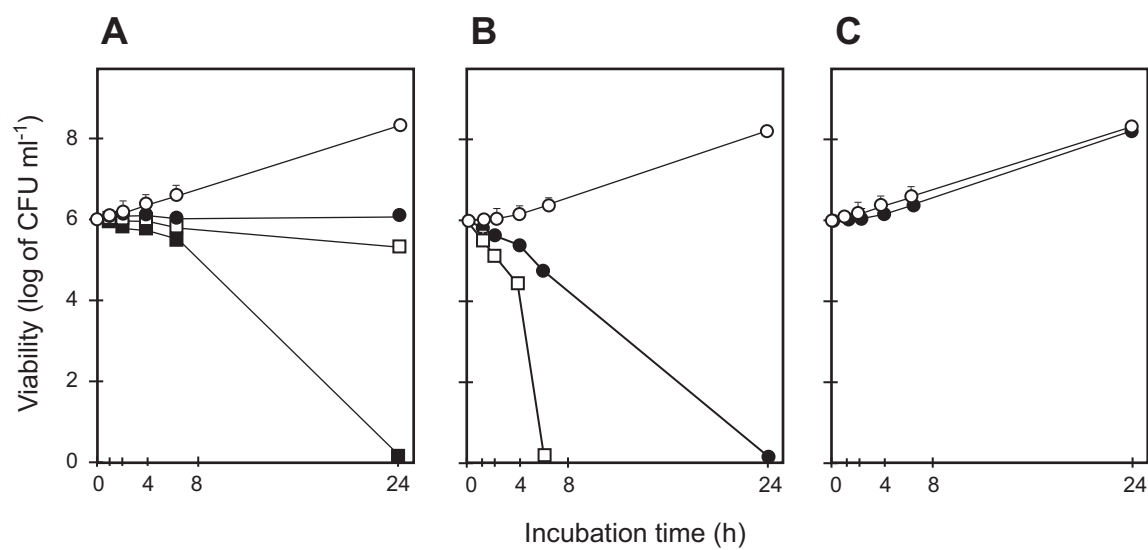


Figure 2. Murata et al.

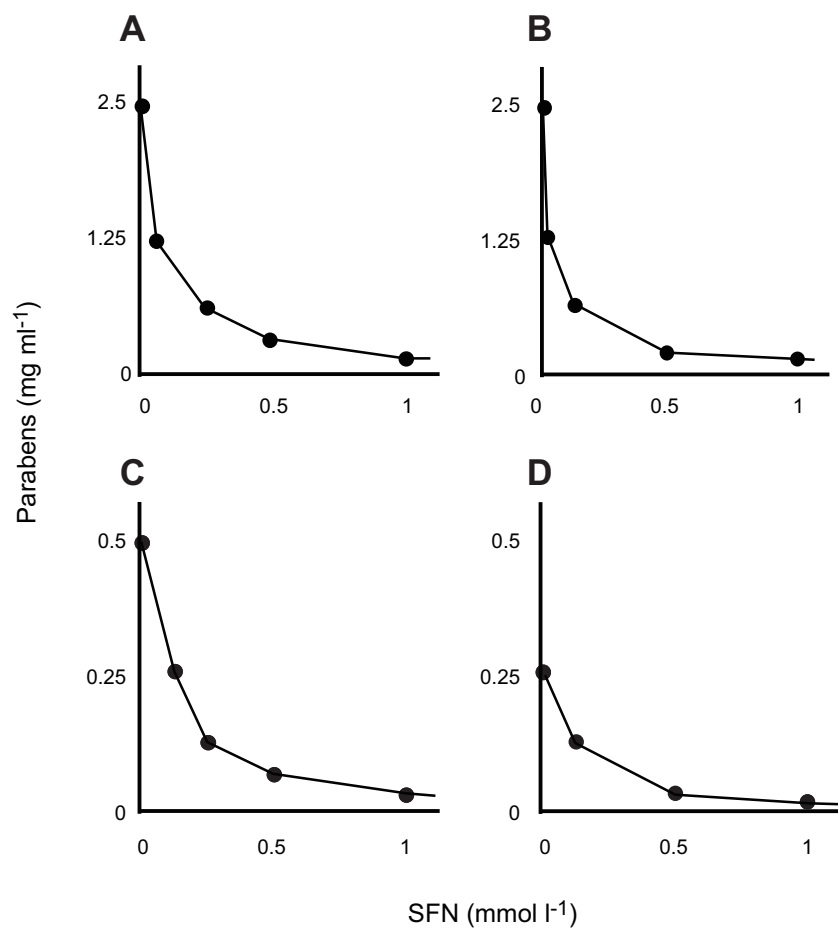


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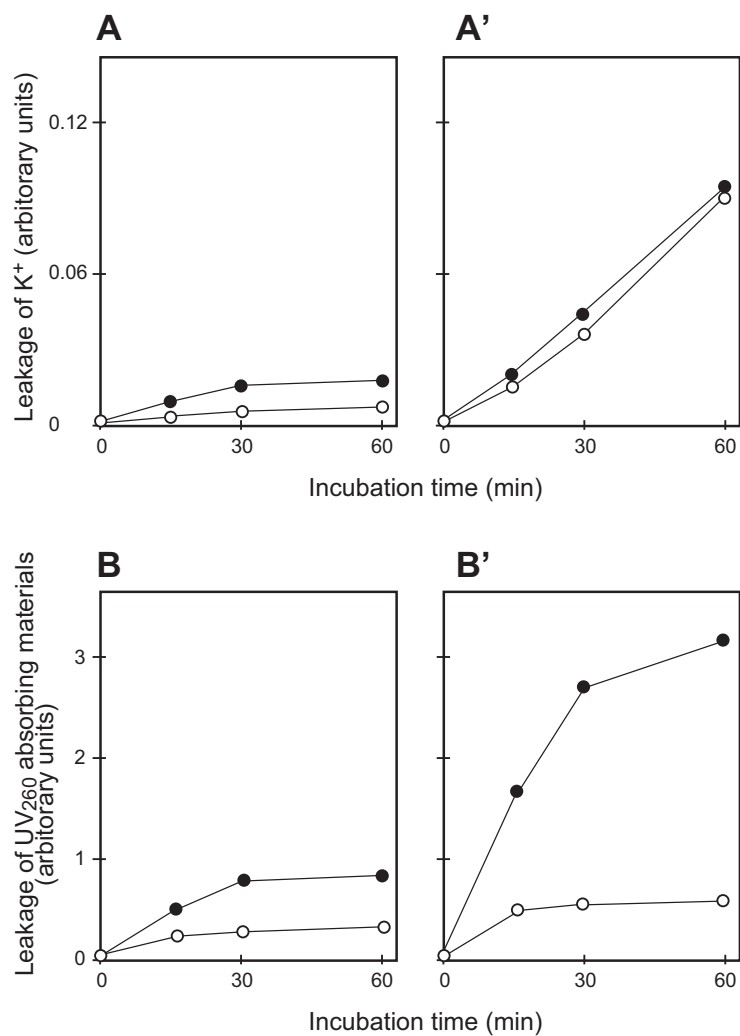
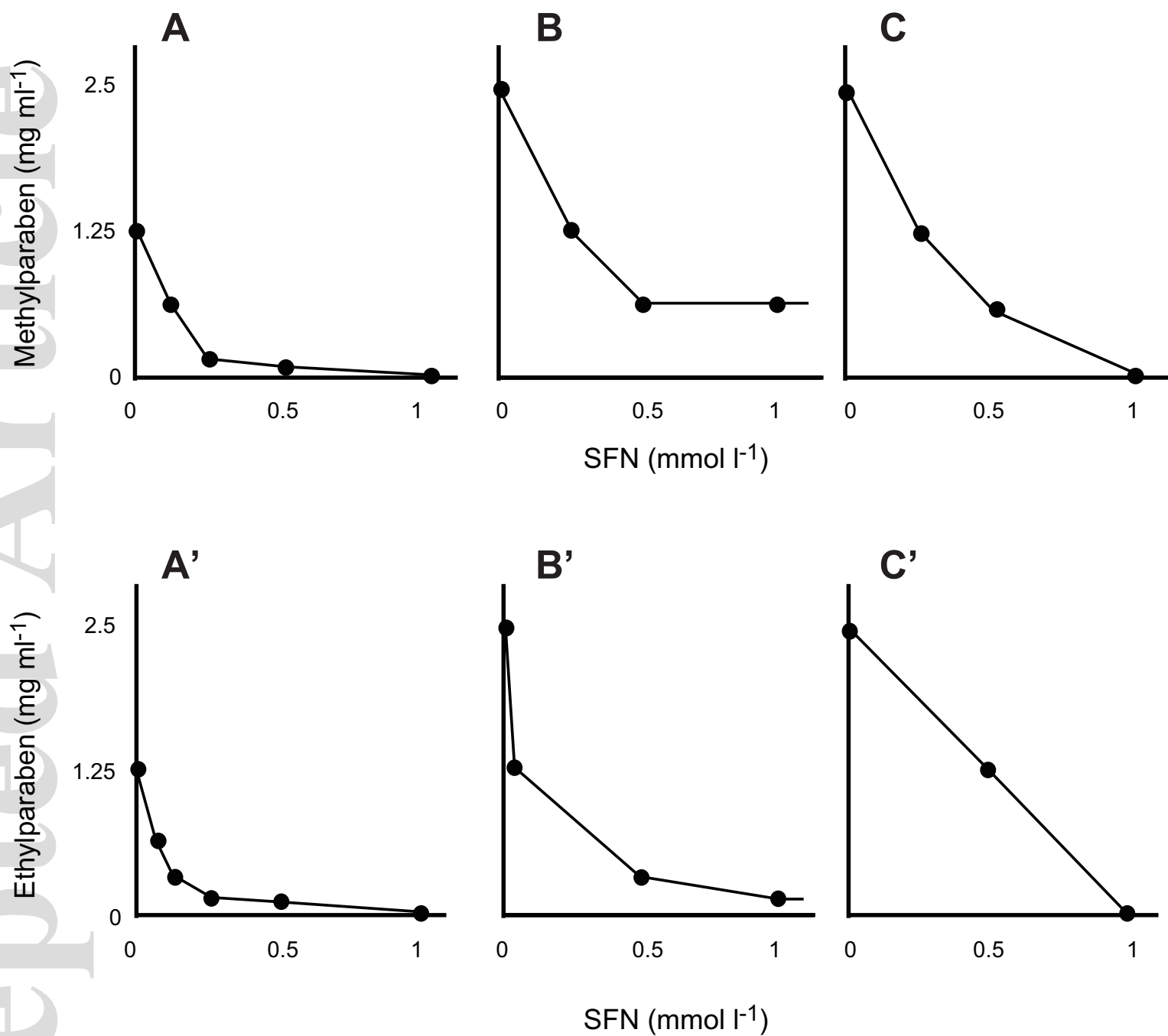


Figure 4. Murata et al.



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