Journal of Functional Foods 37 (2017) 650–657

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

# Effects of *S*-allyl glutathione disulphide and vinyl-dithiin isomers from garlic on the chronological lifespan of *Saccharomyces cerevisiae*

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ARTICLE INFO

*Article history:*
Received 21 April 2017
Received in revised form 14 July 2017
Accepted 12 August 2017
Available online 31 August 2017

*Keywords:*
*S*-allyl glutathione disulfide
2-Vinyl-4H-1,3-dithiin
3-Vinyl-4H-1,2-dithiin
2-Dithiin
Hydrogen sulphide
Garlic

ABSTRACT

*Abstracts:* The slow hydrogen sulphide ($H_2S$) releasing compound *S*-allyl glutathione disulfide (GSSA) from garlic, which can release $H_2S$ via α carbon substitution, showed anti-aging effects on the chronological lifespan (CLS) of yeast *Saccharomyces cerevisiae*. Hormesis was observed, showing lifespan extension at nanomolar concentrations (100–200 nM), whereas at high concentration (1 mM) toxicity were observed. A possible explanation therefore might be the beneficial properties of the recently discovered gasotransmitter hydrogen sulfide ($H_2S$). Yet, 2-vinyl-4H-1,3-dithiin (2-VDT) and 3-vinyl-4H-1,2-dithiin (3-VDT) from garlic appeared both inactive in terms of prolonging the lifespan of yeast. While for 2-VDT these findings seem reasonable since it cannot release $H_2S$, for 3-VDT the contrary was expected as $H_2S$ releasing properties were observed. The underlying mechanism of $H_2S$ release of 3-VDT was proposed to be a similar α carbon substitution-like observed for GSSA.



## 1. Introduction

Hydrogen sulphide ($H_2S$) is an important gaseous mediator in both, cellular physiology and pathology and shows significant regulatory functions in cardiovascular (Wang, 2010), immune (Hosoki, Matsuki, & Kimura, 1997), gastrointestinal (Abe & Kimura, 1996), endocrine (Wang, 2012), and nervous systems (Tan, Wong, & Bian, 2010). In addition to carbon monoxide (CO) and nitric oxide (NO), $H_2S$ is the third and most recent gasotransmitter reported (Wang, 2012). While its physiological concentration in plasma or tissue is likely to be fluctuating and suggested to be in between nanomolar to micromolar range (Furne, Saeed, & Levitt, 2008; Whitfield, Kreimier, Verdial, Skovgaard, & Olson, 2008). Reported health benefits of $H_2S$ range from antioxidation (Kimura & Kimura, 2004), anti-inflammation (Lee, Schwab, Yu, McGeer, & McGeer, 2009), anti-aging (Zhang et al., 2013), cardio protection (Yong et al., 2008), anti-hypertension (Yang et al., 2008) to anti-cancer (Lee et al., 2011).

The endogenous production of $H_2S$ occurs in a thiol-dependent desulfydration mechanism, catalyzed by four enzymes, namely cystathionine γ-lyase (CSE, EC 4.4.1.1), cystathionine β-synthase (CBS, EC 4.2.1.22), 3-mercapto-pyruvate sulfur transferase (3-MST, EC 2.8.1.2), and cysteine aminotransferase (CAT, EC 2.6.1.3). In mammalian tissues, L-cysteine is the predominant source of $H_2S$, which mostly gets released after hydrolysis through CBS and CSE-dependent pathways (Li, Rose, & Moore, 2011; Qu, Lee, Bian, Low, & Wong, 2008).

The desire for slow and steady $H_2S$ donors seems reasonable since slow $H_2S$ production may be the key to the beneficial effects of $H_2S$. We have demonstrated that diallyl trisulphide (DATS) is a fast $H_2S$ releasing agent while diallyl disulphide (DADS) is a slow $H_2S$ releasing agent. Mechanistically, DADS reacts rapidly with glutathione (GSH) to form intermediate product *S*-allyl glutathione disulfide (GSSA). Upon a very sluggish nucleophilic α carbon substitution with another molecule of GSH, GSSA is converted into GSSH and GSA. The GSSH readily release $H_2S$ upon reduction (Liang, Wu, Wong, & Huang, 2015). GSSA is also naturally produced in garlic as a product of organosulfur metabolism (Bhuiyan, Papajani, Paci, & Melino, 2015), besides, it is the direct reaction product between GSH and allylic polysulfides such as DATS, DADS, and allicin (Borlinghaus, Albrecht, Gruhlke, Nwachukwu, & Slusarenko, 2014). In addition to DADS, there are several other organosulphides formed when garlic underwent thermal processing treatment. Thermal degradation of allicin would lead to the

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http://dx.doi.org/10.1016/j.jff.2017.08.024
1756-4646/

formation of 2-vinyl-4H-1,3-dithiin (2-VDT) and 3-vinyl-3,4-dihydro-1,2-dithiin (3-VDT) (Iberl, Winkler, & Knobloch, 1990; Yu & Wu, 1989).

The anti-aging potential of $H_2S$ was firstly reported ten years ago, when researchers discovered that the nematodes *Caenorhabditis elegans* living in an $H_2S$ atmosphere were long lived and thermotolerant (Miller & Roth, 2007). Several years later, it was discovered that $H_2S$ was an endogenous regulator of aging in *C. elegans*, as worms with compromised endogenous $H_2S$ production had decreased lifespan, which could be reversed by exogenous $H_2S$ supplementation (Qabazard et al., 2014). Very recently, Hine et al. reported that $H_2S$ was key to dietary restriction benefits universally in yeast, worm, fruit fly, and rodent. They reported that restricted sulfur amino acid intake led to increased expression of the transsulfuration pathway enzyme CSE, resulting in elevated production of $H_2S$, and ultimately improved protection from environmental stress and longer lifespan (Hine et al., 2015).

Inspired by these findings, we wondered whether dietary polysulfides, which were good source of $H_2S$ precursors (Liang, Wang, et al., 2015), had similar anti-aging effect in model organisms. Previously, a high throughput screening method for determination of chronological lifespan of yeast was developed by our group (Wu, Song, Liu, & Huang, 2011). The yeast strain *Saccharomyces cerevisiae* was used in the method not only because it had very short lifespan (10–20 days), well-studied genetic background, and easiness to culture and manipulate, but also because many genetic pathways regulating aging were conserved among species, so that the conclusion obtained from yeast might also be referable in other organisms (Guarente & Kenyon, 2000). The method had been used successfully to study the effects of nutrient composition on the lifespan of yeast (Wu, Liu, & Huang, 2013), and screening of anti-aging compounds from Traditional Chinese Medicine (Wu, Song, Liu, & Huang, 2014).

In the work, we examined the effects of GSSA, 2-VDT, and 3-VDT on yeast chronological lifespan extension activity and examined the potential correlations with their $H_2S$ donating activity.

## 2. Materials and methods

### 2.1. Chemical reagents and instruments

L-amino acids were obtained from GL Biochem (Shanghai, China), yeast nitrogen base without amino acids, ammonium sulfate, peptone, YPD broth, agar, and yeast extract were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). $H_2S$ probe BCu was synthesized as previously reported (Wu et al., 2014). Glutathione (GSH), dimethyl sulfoxide (DMSO), diallyl disulfide (DADS) were purchased from Sigma-Aldrich, Singapore. Formic acid and absolute ethanol was obtained from Merck Pte. Ltd. (Singapore). Phosphate buffered saline (PBS) was purchased from Vivantis Inc., USA. HPLC grade acetonitrile was purchased from Avantor Performance Materials (USA). The two vinyl dithiin isomers, 3-VDT and 2-VDT were obtained by semi-preparative HPLC isolation from garlic.

For freeze-drying of samples, a SP Scientific VirTis Advantage Plus ES-53 freeze dryer was used. For rotary evaporation, a R-200 Rotavapor from Büchi (Switzerland) equipped with a heating bath (B-490), a vacuum controller (V-850), a pump (V-700), and a PolyScience temperature controller (Niles, IL, USA) were used. Optical density was measured by a Synergy HT microplate UV–VIS light absorbance reader from Bio-Tek Instruments Inc. (Winooski, VT, USA). Fluorescence intensity was determined with a HORIBA Jobin Yvon Fluoromax-4 fluorometer (Edison, NJ, USA). $^1$H NMR spectra were recorded in deuterium oxide ($D_2O$) and deuterated methanol (methanol-$d_4$) at 298 K with a Bruker AC300 spectrometer (Karlsruhe, Germany) operating at 300 MHz.

### 2.2. Preparation of GSSA

Gluthathione (GSH, 614 mg) were dissolved in double strength PBS-buffer (2 mL) added with de-ionized water (2.0 mL) in a small test tube. The pH value was adjusted to 8.0 with potassium hydroxide (KOH, 1.0 M). Dimethyl sulfoxide (DMSO, 1.0 mL) and DADS (>70% purity, 300 μL) were added. The reaction mixture was shaken for 10 min and kept at room temperature for 10 min. Then, the mixture was filtered through a 45 μm PTFE membrane filter and transferred into a 1 mL HPLC vial. A portion of the solution (200 μL) was injected into the semi-preparative HPLC. A gradient of two solvents was used to separate the reaction mixture (A: de-ionized water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid). Starting from 100% of A, it was decreased to 80% within 25 min, then to 70% in the next 5 min, and to 60% in the final 15 min. The flow rate was set to 3.0 mL/min. The separation was performed on a Waters HPLC system (Milford, MA, USA) equipped with two 515 HPLC pumps, a 717 Plus Autosampler unit, and a 2996 photodiode array (PDA) detector at a wavelength of 210 nm on an YMC-Pack ODS-AM (YMC America Inc., Allentown, PA, USA) column with dimension of 250 mm × 10 mm, S-5 μm particle size, and 120 angstrom pore size. The peak at 31.3 min was collected. By using a freeze-drier the residual solvent of the collected fraction was removed to afford GSSA.

### 2.3. Isolation of 2-VDT and 3-VDT from garlic

White garlic (1.0 kg) was purchased from a local market in Singapore and was crushed using a food homogenizer and incubated for 30 min at room temperature to allow alliinase-mediated organosulphide formation to take place. The homogenate was extracted three times with hexane and the organic layer was concentrated using a rotary evaporator. The extract was subjected to semi-preparative HPLC isolations with the same equipment as mentioned in Section 2.2. HPLC conditions were as: mobile phase, acetonitrile:water:methanol (50:41:9, v/v); flow rate, 3.0 mL/min; column temperature, 25 °C; detector wavelength, 254 nm; injection volume, 80 μL. Collections of fractions were repeated multiple times to obtain sufficient amounts of the target compounds. Each fraction obtained was combined and extracted with hexane (150 mL) three times. The organic extract was evaporated, the oil obtained was mixed with 1.0 mL acetonitrile and transferred to an HPLC vial. HPLC analysis of pure extracts was performed on a Waters HPLC system (Milford, MA, USA) equipped with a Waters 2996 PDA and Waters 2695 Separation Modules (Waters Assoc., Milford, MA, USA). The stationary phase was a Phenomenex Luna C18 (250 mm × 4.60 mm i.d., 5 μm) column (Torrance, CA, USA). The elution conditions were: flow rate, 1.0 mL/min; column temperature, 25 °C; injection volume, 10 μL; detector wavelength, 254 nm; mobile phase, acetonitrile:water:methanol (50:41:9, v/v). Pure extracts were further subjected to HPLC-MS/MS analyses, using the same HPLC conditions, for structural elucidation and identity confirmation. HPLC-MS/MS spectra were acquired using a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source.

### 2.4. Yeast chronological lifespan measurement

CLS of yeast was measured with an established high-throughput screening assay (Wu et al., 2011). *S. cerevisiae* BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0) was kindly given by Dr. Yuan Jifeng from National University of Singapore and was