ISSN: 2586-7342
KJFHC website: http://accesson.kr/kjfhc
doi: http://dx.doi.org/10.13106/kjfhc.2024.vol10.no2.7

Potential for Efficient Synthesis of GSH Utilizing *GCS1* and *GLR1* Mutant Strains of *Candida albicans**

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Received: February 29, 2024. Revised: March 10, 2024. Accepted: March 14, 2024.

Abstract

Glutathione (GSH) is a vital compound composed of glutamic acid, cysteine, and glycine, crucial for cellular functions including oxidative stress defense and detoxification. It has widespread applications in pharmaceuticals, cosmetics, and food industries due to its antioxidant properties and immune system support. Two primary methods for GSH synthesis are enzymatic and microbial fermentation. Enzymatic synthesis is efficient but costly, while microbial fermentation, particularly using yeast strains like *Candida albicans*, offers a cost-effective alternative. This study focuses on genetically modifying *C. albicans* mutants, specifically targeting glutathione reductase (*GLR1*) and gamma-glutamylcysteine synthetase (*GCS1*) genes, integral to GSH synthesis. By optimizing these mutants, the research aims to develop a model for efficient GSH production, potentially expanding its applications in the food industry.

Keywords: Glutathione (GSH), GSH synthesis, *Candida albicans*

Major Classifications: Food industry, Food biochemistry, Probiotics, Food Science (Food Nutrition, Healthy Food)

1. Introduction

Glutathione (GSH) is a crucial intracellular compound composed of three amino acids: glutamic acid, cysteine, and glycine. GSH plays a significant role in oxidative stress and detoxification, providing protection against oxidative damage to cells by scavenging harmful chemicals such as free radicals. Additionally, it maintains the physiological functions of cells and contributes to the strengthening of the immune system. Due to its various properties, it is widely used in the pharmaceutical, cosmetic, and food industries. It is utilized in a variety of food products to preserve the taste and aroma of beer (Chen et al., 2012), as an antioxidant in food (Ye et al., 2016; Lee et al., 2015), as a dietary supplement to protect liver function (Chu, 2013), and as an anti-aging cosmetic (Wang, 2014). Due to its high

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utilization value, yeast containing GSH or GSH purified from it generated global annual sales exceeding $9 billion in 2019 (Marz, 2014). Over 200 tons of pure GSH are produced worldwide each year (Orumets et al., 2012), and research into cost-effective ways to synthesize GSH is ongoing.

There are two methods for synthesizing GSH: enzymatic synthesis and microbial fermentation. Enzymatic synthesis requires minimal inputs such as amino acids, ATP, and enzymes and is easy to purify while ensuring high yields (Li et al., 2004). However, the high cost of ATP and the lack of efficient ATP regeneration systems make it difficult to utilize on an industrial scale. Therefore, much research has been done on the production of GSH through microbial fermentative methods utilizing yeast. These strains already have high concentrations of GSH (Bachhawat et al., 2009; Li et al., 2004). Their ability to grow rapidly to high cell densities using inexpensive media increases the economic value of this method (Bachhawat et al., 2009). Therefore, research has focused on optimizing media, bioconversion, and genetic engineering for efficient industrial production of GSH. Currently, commercially, gsh synthesis is mostly carried out through fermentation of *Saccharomyces cerevisiae* (Hara et al., 2012). In this study, *Candida albicans* with characteristics similar to *S. cerevisiae* was used to suggest a wider possibility of efficient synthesis of gsh.

This study utilized genetically modified mutants of *C. albicans*, including glutathione reductase (*GLR1*) and gamma-glutamylcysteine synthetase (*GCS1*), to provide a model for efficient GSH synthesis. *GLR1* uses NADPH to catalyze a chemical reaction, in which GSSG is reduced to GSH. This process is an important part of intracellular redox reactions, contributing to maintaining the intracellular GSH/GSSG ratio and protecting cells from oxidative stress. *GCS1* is responsible for the initial step in the GSH synthesis pathway, utilizing ATP to synthesize γ-glutamylcysteine. As these two genes are closely associated with GSH, we aim to present a strain model capable of efficient GSH synthesis by utilizing a combination of mutants from both genes. This could lead to a more diverse utilization of GSH in the food industry.

2. Materials and Methods

2.1. Strains and Culture Conditions

The *C. albicans* strains and plasmids used in this study are listed in Table 1. The strains were primarily cultured at 30°C on YPD (1 % yeast extract, 2 % peptone, 2 % D-glucose) medium. Cells carrying plasmids or disrupted genes were cultured in synthetic D-glucose (SD) medium containing 0.67 % yeast nitrogen base without amino acids (Difco), 2 % D-glucose, and appropriate supplements. Solid media were prepared by adding 1.8 % agar to liquid broth. *Escherichia coli* DH5α was used for most plasmid construction and maintenance, and cells of this strain were grown at 37 °C in Luria-Bertani medium supplemented with ampicillins.

Table 1: Strains, primers, and plasmids used in this study.

Strains, primers, or plasmids	Genotype, sequence, or description	Source or reference
Strains		
C. albicans		
SC5314	Wild type isolate	(Fonzi & Irwin, 1993)
CAI4	*Δura3::imm434/Δura3::imm434*	(Fonzi & Irwin, 1993)
SJ104, homozygous *Δgcs1*	*Δura3::imm434/Δura3::imm434 Δgcs1::hph/Δgcs1::hph*	This study
SJ201 *GLR1*^{OE}	*Δura3::imm434/Δura3::imm434*(pJY3E)	This study
SJ211 *Δgcs1/GLR1*^{OE}	As SJ104, but *Δura3::imm434/Δura3::imm434*(pJY3E)	This study
Primers		
JY1a-*Sac*I	5′-GAGCTCGAGGGCGGAAAATAAAAGATTTGG-3′, *Sac*I site of pJY1F	This study
JY1b-*Kpn*I	5′-GGTACCCCTCTTTTTGAATTATTTTCTTGA-3′, *Kpn*I site of pJY1F	This study
JY1c-*Sal*I	5′-GTCGACCGGATTTATCTTATCTTATCTTAT-3′, *Sal*I site of pJY1R	This study
JY1d-*Hind*III	5′-AAGCTTCACGACCTGCTTTTAGTTTTTCGT-3′, *Hind*III site of pJY1R	This study
JY2a-*Bgl*II	5′-AGATCTATGTTTACTAATAGTATAATATCTAAATCAACTACT-3′, *Bgl*II site of pJY3E	This study
JY2b-*Xho*I	5′-CTCGAGCTAAGTCATTGTGACCAATTCTTCAGCTGATGTAGG-3′, *Xho*I site of pJY3E	This study
Plasmids		
pQF181	pUC18 containing *hph-URA3-hph* (forward) from pQF86	(Hwang *et al.*, 2003)
pQF182	pUC18 containing *hph-URA3-hph* (reverse) from pQF86	(Hwang *et al.*, 2003)

pJY1F	pGEM-T Easy vector containing JY1a-JY1b fragment, upstream region of *GCS1*	This study
pJY1R	pGEM-T Easy vector containing JY1c-JY1d fragment, downstream region of *GCS1*	This study
pJY2F	*GCS1* deletion construct with *hph-URA3-hph* (forward)	This study
pJY2R	*GCS1* deletion construct with *hph-URA3-hph* (reverse)	This study
YPB-ADHPt	Expression vector containing promoter and terminator regions of *ADH1* gene in YPB1	(Bertram *et al.*, 1996)
pJY3E	pADHPt vector containing *GLR1* ORF	This study

2.2. Disruption of *C. albicans GCS1* and Overexpression of *GLR1*

The *GCS1* gene was disrupted as described previously (Fonzi & Irwin, 1993) with some modifications. The plasmids and primers used are shown in Table 1. The 3.5 kb *BamHI* fragment containing an *hph-URA3-hph* disruption cassette isolated from pQF86 (Feng et al., 1999) was ligated with *BamH*I, yielding pQF181 and its reverse construct pQF182, which can be excised with *Sac*I/*Hind*III (Hwang et al., 2003). *GCS1* was disrupted using the URA blaster method using pQF181 and its derivative pQF182, respectively, to remove the 2319 bp *GCS1* coding region (Feng et al., 1999; Fonzi & Irwin, 1993; Hwang et al., 2003). The 548 bp *Sac*I/*Kpn*I (JY1a-JY1b) and 394 bp *Sal*I/*Hind*III (JY1c-JY1d) digestion fragments from the pJY1F and pJY1R of the upstream/downstream flanking regions of the *GCS1* open reading frame (ORF) were inserted into the *SacI/KpnI* and *Sal*I/*Hind*III sites of *hph-URA3-hph* from pQF181 or pQF182, respectively. The completed construct was digested with *Sac*I/*Hind*III, transformed into CAI4 (Ura− derivative of wild-type strain SC5314) (Fonzi & Irwin, 1993), and selected by *Ura3* maker gene. Spontaneous *Ura3*− derivatives of the heterozygous disruptants were selected on SD medium containing 5-fluoroorotic acid (625 μg/ml) and uridine (100 μg/ml). This procedure was repeated once more to generate a homozygous *gcs1/gcs1* mutant strain (SJ104).

To overexpress *GLR1* in *Candida albicans*, we used the YPB1-ADHPt vector with the *Candida ADH1* promoter and terminator region (Bertram *et al.*, 1996). A 1551 bp *BglII/XhoI* fragment containing the *GLR1* ORF was inserted into the pGEM-T EASY vector. The *BglII/XhoI* fragment containing the entire coding sequence of *GLR1* was isolated from the pGEM-T EASY vector and ligated into the same position of YPB-ADHPt containing the *URA3* locus as the autonomous replication sequence of *C. albicans* to generate pJY3E. The resulting pJY3E construct was transformed into the homozygous Ura- CAI4 strain.

3. Results and Discussion

Candida albicans uses *GCS1* to initiate GSH synthesis by consuming ATP to synthesize gamma-glutamylcysteine. The resulting gamma-glutamylcysteine is then synthesized into GSH by GSH synthase. This synthesized GSH is used to detoxify hydrogen peroxide in the cell and is oxidized to GSSG. *GLR1* reduces the oxidized GSSG back to GSH by consuming NADPH (Fig. 1). This study aimed to determine the effects of *C. albicans GCS1* disruption, *GLR1* overexpression, and *GCS1* disruption + *GLR1* overexpression mutants on the GSH pathway.

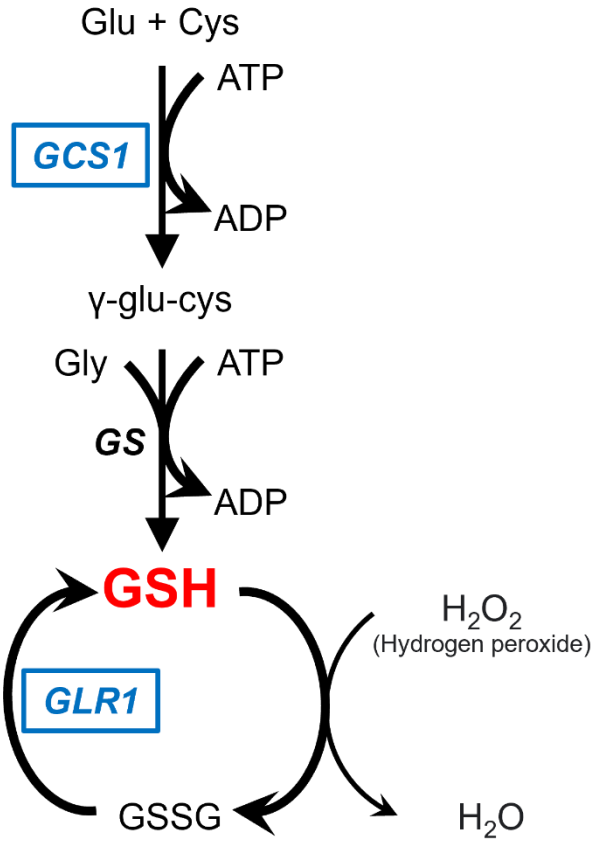

Figure 1: *C. albicans* GSH pathway

The *GCS1* disruption mutant was found to be unable to grow due to the absence of the *GCS1* gene, which is necessary for GSH synthesis within the cell. Therefore,

external addition of GSH is required for the mutant to grow. To confirm the success of yeast transformation in the mutant, we cultured the cells in medium without GSH and observed no growth (data not shown). *GLR1* activity in the overexpression mutant was compared to that of the wild type using UV spectrophotometer kinetics (data not shown).

The involvement of the mutants in the GSH pathway was determined by incubating them with the addition of GSSG but not exogenous GSH (Fig 2). The *GCS1* disruption mutant was found to grow normally, even in the absence of exogenously added GSH and the inability to synthesize GSH within the cells. The *GCS1* disruption mutant was found to grow normally, even in the absence of exogenously added GSH and the inability to synthesize GSH within the cells. The *GCS1* disruption mutant was found to grow normally, even in the absence of exogenously added GSH and the inability to synthesize GSH within the cells. A small amount of exogenous GSSG confirmed the cells' ability to reduce GSH enough for growth.

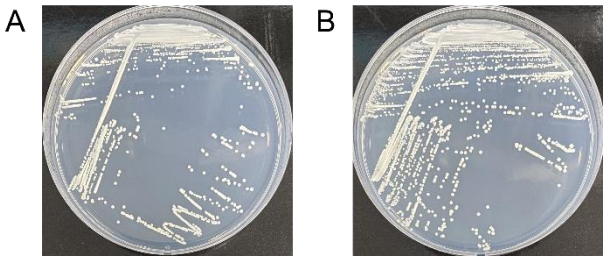

Figure 2: SD plate with 0.05 mM GSSG. (A) *GCS1* disruption mutant. (B) *GCS1* disruption + *GLR1* overexpression mutant.

Therefore, using *GLR1* overexpression mutants may increase GSH production by increasing the amount of GSSG reduced. Additionally, there are plans to combine with other genes, such as superoxide dismutase or alcohol dehydrogenase, which regulate oxidative stress in cells, to generate mutations. By comparing the amount of GSH synthesis in these mutants, the most efficient GSH synthesis method can be determined. This research can provide an opportunity to further increase the value of GSH in various industrial fields.

Funding

This work was supported by the BK21 Plus project of the National Research Foundation of Korea (NRF) Grant funded by the Korea government. This research was also supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science and ICT of the Korea government (MSIT) (NRF-2021R1F1A1061581) and by a grant from Eulji University in 2023. The funders had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Availability of Data and Materials

All supporting information including table of results and detailed methods is available upon request.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank W.A. Fonzi, M. Whiteway, and G.R. Fink for providing the *C. albicans strains* and plasmids.

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