Determination of Total Glutathione in Yeasts by High-Performance Liquid Chromatography with Dansylation

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A method to determine the content of total glutathione (GSHt) was introduced based on high-performance liquid chromatography (HPLC) with dansylation. The minimum detection concentration of GSHt was 0.5 μg/mL and the measurable range 1.0–300 μg/mL. GSHt in yeasts was obtained by hot-water extraction, GSH complete autoxidation to oxidized glutathione (GSSG) in alkaline solution and purification by thin-layer chromatography (TLC). The quantitative determination of GSSG was derived by dansyl chloride at pH 9.5, 60 ºC for 60 min and assayed by HPLC. GSHt in *Saccharomyces cerevisiae* is higher than in *Candida rugosa* and *Candida utilis*. *S. cerevisiae* can be chosen as the better target for mutagenesis and industrial scale.

Key words: Total Glutathione, Yeast, HPLC

Introduction

Intracellular glutathione (GSH) is usually maintained in the reduced state which represents more than 95% of the total glutathione (GSHt), besides oxidized glutathione (GSSG) (Cereser *et al.*, 2001). Many methods were reported to determine GSH and GSSG, one of which accepted as routine is based on the 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay (Nemeth *et al.*, 2001). This method responds to both GSH and GSSG, and GSHt and GSSG must be determined separately. Furthermore, GSSG is analyzed after treatment of GSH with *N*-ethylmaleimide (NEM), which shows an inhibiting effect on the GSSG reductase activity (Araujo *et al.*, 2008).

High-performance liquid chromatography (HPLC) methods to determine GSH and GSSG with precolumn derivatization agents have been described. The amino group derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB) has the advantage of its simplicity, reproducibility, and simultaneous detection of GSH and GSSG, but pretreatment of samples with NEM can produce artifacts and additional chromatographic peaks (Giustarini *et al.*, 2003). A method described for direct measurement of both GSH and GSSG is based on a HPLC postcolumn reaction with *ortho*-phthalaldehyde at pH 12 followed by fluorescence detection which requires additional instrumentation (Lenton *et al.*, 1999).

A HPLC method was developed for simultaneous quantitative determination of amino acids and polyamines based on precolumn derivatization with 5-dimethylamino-naphthalene-1-sulfonyl chloride (dansyl chloride, DNS-Cl) (Minocha and Long, 2004; Xing *et al.*, 2001). Here, we will describe a method to determine GSHt based on dansyl chloride derivatization by RP-HPLC.

Material and Methods

Instrumentation

An HPLC instrument (Agilent Model 1200, Santa Clara, USA) with an Agilent chromatographic column (XDB-C18, 4.6 × 250 mm, 5 μm) were used for the analysis of GSH. Silica gel 60 thin-layer chromatography (TLC) plates (20 × 20 cm) were from Merck (Darmstadt, Germany).

Reagents

Dansyl chloride was obtained from Tokyo Chemical Industry Cooperation (Tokyo, Japan), and GSH and GSSG were from Amresco (Cleveland, USA). All chemicals used were of analytical reagent grade. All solutions were filtered through

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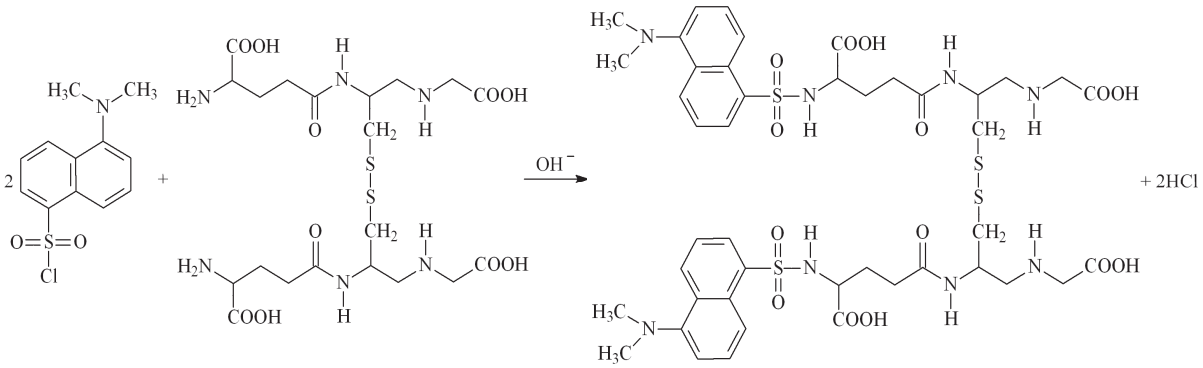

Fig. 1. Derivatization of GSSG with dansyl chloride in alkaline environment.

a membrane filter (0.45 μm) before HPLC. Methanol for HPLC was chromatographic pure. The work solutions containing 2.0 mg/mL GSH and GSSG were prepared for further dilution, and 20 mg/mL methanolic dansyl chloride solution was prepared for derivatization. The solution concentration of sodium tetraborate-boric acid was 0.2 M (pH 9.5).

Derivatization procedure for determination of GSHt

0.5 mL of sodium tetraborate-boric acid buffer solution (pH 9.5) and various concentrations of GSSG solutions were placed in microfuge tubes, and then 0.2 mL of methanolic dansyl chloride solution were added to each tube and vortexed. The tubes were capped and incubated in a water bath at 60 ºC in the darkness. After 60 min, 5 μL of glacial acetic acid and 95 μL methanol were added to end the reaction.

The optimized CH_3OH/H_2O mixture had a ratio of 8:17 (v/v), containing 0.05 M ammonium acetate/acetic acid (pH 5.4) and 0.1% triethylamine. The flow velocity was 0.8 mL/min, the detection wavelength was 254 nm (Xing *et al.*, 2001).

The effects of temperature on the GSH autoxidation were evaluated by RP-HPLC. GSHt was purified by TLC.

Determination of GSHt in yeasts

Saccharomyces cerevisiae, *Candida rugosa*, and *Candida utilis* were from China Center for Type Culture Collection (CCTCC). They were inoculated in 100 mL potato liquid medium (20% potato, 2% glucose). After 2 d culture, yeast cells were obtained by centrifuging the cultivation broth at 3000 × *g* for 10 min. The samples were dried and weighed. Then the dry yeast cells were extracted with hot water (90 ºC) (Li *et al.*, 2004), and the supernatants were obtained after removing the cells by centrifugation at 3000 × *g* for 10 min. They were blended with buffer (pH 9.5) and heated (90 ºC) for 15 min. GSSG and supernatants were chromatographed on TLC plates. The eluent was composed of butanol, glacial acetic acid and water (3:2:1). After chromatography, the standard (GSSG) coloured with ninhydrin and each 0.6-cm band (R_f equal with the coloured spot) were scraped off the TLC plates, and then the compound was extracted with buffer solution (pH 9.5). The suspensions were centrifuged (3000 × *g*, 10 min). GSHt in supernatants was determined according to the derivatization procedure.

Results and Discussion

Analytical methods using colourimetric reagents and UV-Vis absorbance detection are inferior in terms of sensitivity, but simple in handling compared with electrochemical or fluorometric determinations. The ideal fluorescence reagent should be non-fluorescent, containing no fluorescence impurities, and react rapidly and specifically with GSH and other thiols to form GSH adducts with sufficient fluorescence yield (Pastore *et al.*, 2003). Dansyl chloride and its derivative can produce fluorescence. Therefore, UV detection with dansyl chloride was used.

The major weak points in GSH determination are the ease of non-enzymatic GSH autoxidation at pH > 7 and enzymatic conversion of GSH

(Monostori *et al.*, 2009). However, we can determine the GSHt by assaying GSSG after complete GSH autoxidation. Amino groups in GSSG can be derivatized with dansyl chloride in alkaline environment (Fig. 1). It is worthy mentioning that dansyl chloride and its derivative were efficiently separated by RP-HPLC (Fig. 2), which implied that the interference of substrate and product was slight, and this was the basis for our motivation to use RP-HPLC as a simple method to determine GSSG.

The molar concentration of dansyl chloride should be at least 10 times that of GSSG, based on preliminary experiments. The minimum detection concentration of GSSG was 0.5 μg/mL (signal to noise ratio was 3). The standard curve of derivative containing 1.0–300 μg/mL GSSG was drawn, and the regression equation was $y = 39.888x + 93.563$, $R^2 = 0.9989$. The minimum detection concentration is similar to that by derivatization with FDNB, however, the method has the advantage of a wider linear range (Shen *et al.*, 2002).

Repeated experiments showed good reproducibility for peak area and transport time (derivative/dansyl chloride) with a relative standard deviation (RSD) < 3% (n = 5), and a recovery ratio of 97.1% ~ 102.5%. Table I shows that autoxidation of GSH almost completed in a water bath at 90 ºC and pH 9.5 for 15 min. Hot-water extraction disrupted the cells, resulting in the release of many water-soluble proteins (Xiong *et al.*, 2009). Proteins present in the injected samples may attenuate the performance of the analytical procedure and shorten the lifetime of the instrumentation, and therefore, proteins must be removed prior to the analysis of GSH and GSSG. Acidification and addition of organic solvents are employed as means of protein elimination, which can affect the separation, derivatization, and detection (Monostori *et al.*, 2009). Purification by TLC is another mean to remove proteins in laboratory operations.

Table I. The effect of temperature on the GSH autoxidation at pH 9.5.

Temperature	Peak area [mAU]			
[ºC]	5 min	10 min	15 min	20 min
90	651	926	1213	1216
80	562	825	986	1106
70	469	665	819	962

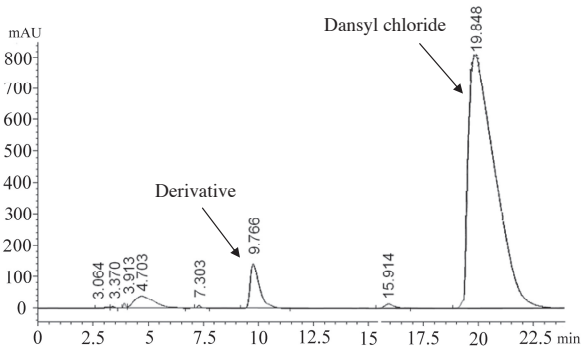

Fig. 2. Separation of dansyl chloride and the derivative by RP-HPLC.

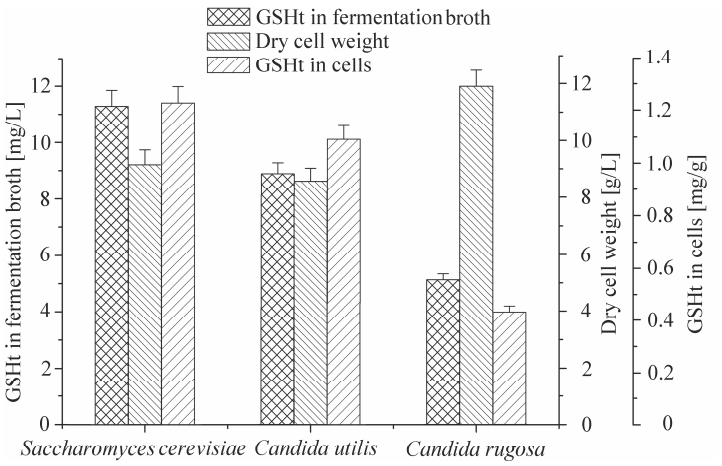

Fig. 3. The total glutathione (GSHt) in the fermentation broth of yeasts.

S. cerevisiae and *Candida utilis* are the most commonly used microorganisms on an industrial scale; the GSH contents of the wild-type strains are usually high (0.1–1.0% dry cell weight). Therefore, these two microorganisms were chosen as targets for mutagenesis (Li *et al*., 2004). In our experiment, GSHt in *S. cerevisiae* was higher than in *C. rugosa* and *C. utilis* determined by HPLC with dansylation (Fig. 3). *S. cerevisiae* can be chosen as the better target for mutagenesis and industrial scale. The intracellular content of GSHt can be increased significantly by mutagenesis and suitable cultivation strategy, and enhancement of the cell yield can be achieved by process optimization.

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