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Simple single-step single-enzyme synthesis of [^{14}C]-GSH

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The tri-peptide [^{14}C]-glutathione ([^{14}C]-GSH) was synthesized in a single step by GSH synthetase catalyzed reaction of L-γ-glutamyl-L-cysteine and [^{14}C]-glycine. Preparative reverse phase HPLC afforded [^{14}C]-GSH in 30% yield and 98% purity. Preparation of GSH synthetase from E. coli via recombinant DNA and the interconversion of [^{14}C]-GSH to the disulfide [^{14}C]-GSSG for storage are discussed.

Keywords: [^{14}C]-glutathione; [^{14}C]-GSH; [^{14}C]-glycine; L-γ-glutamyl-L-cysteine; GSH synthetase

Introduction

The tri-peptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH) participates in a number of cellular functions including antioxidant activity, cell signaling, and drug detoxification. GSH concentration has been related to the pathogenesis of many human diseases including aging, AIDS, cancer, cystic fibrosis, ischemia, liver and heart disease, metabolic diseases (diabetes and obesity), neurodegenerative diseases, seizure, sickle cell anemia, and stroke.[1,2] In a preclinical setting, GSH is used to evaluate the formation of reactive drug metabolites. Formation of a GSH adduct is often associated with those reactive metabolites that can cause covalent binding (CVB) of drug-related material to cellular proteins and associated adverse effects. Because the GSH adducts are novel metabolites and not readily available synthetically, their quantitation with standard LC/MS/MS methods is not possible. Therefore, the standard procedure for quantifying reactive metabolite formation remains the ^{14}C (or ^{3}H) labeling of the drug and subsequent measurement of ^{14}C CVB to protein.[3]

Masubuchi[4] has reported the use of [^{35}S]-GSH in the evaluation of non-labeled drug candidates and has reported a good correlation between CVB results using both labeled drug candidates as well as [^{35}S]-labeled GSH. Because of its long-term stability, the use of [^{14}C]-labeled GSH would allow for long-term storage of this compound. It could be used as needed in CVB studies thus alleviating the need to individually label molecules of interest and should allow for rapid determination of CVB in a preclinical setting.

Though [^{14}C]-GSH is a useful tool for CVB studies, it is not a commercial item. Methods for the [^{14}C]-labeling of GSH fall into two categories: chemical and enzymatic. A reported multi-step synthesis of [^{14}C]-GSH from K^{14}CN has an overall yield of 1%.[5] Although yields have been significantly improved, all chemical routes involve a number of protection and deprotection steps and have the possibility of racemization. Hydrolysis and derivatization have been used for the determination of optical purity.[2] The enzymatic coupling of L-γ-glutamyl-L-cysteine and glycine catalyzed by GSH synthetase was first demonstrated by Block and Snoke in 1952 using a [^{14}C]-gly assay.[6] However, this was not a preparative method to isolate [^{14}C]-GSH and the GSH synthetase was a part of a complex pigeon liver cell matrix containing other enzymes.

More recently, enzymatic routes have relied on two enzymes (γ-glutamylcysteine synthetase and glutathione synthetase) to couple the three amino acids: L-glutamate, L-cysteine, and glycine.[6–10] It is known that these enzymes act sequentially.[6–7] Thus, it was expected that in a cell-free reaction, L-γ-glutamyl-L-cysteine should couple with glycine catalyzed by GSH synthetase.

Results and discussion

All necessary components for a single-step single-enzyme synthesis of [^{14}C]-GSH are commercial items. An in-house preparation of GSH synthetase provided a more active enzyme than the commercial source (Figure 1).

In Table 1, the first three experiments were designed to compare two versions of the in-house Glutathione Synthetase: GSS-1 (his-tagged, no thrombin cleavable sites) and GSS-2 (his-tagged, thrombin cleavable site), with commercial GSS-3. The cocktail (pH 6.5) used was adapted from a cocktail reported by Murata *et al.*[8] for a three amino acid two enzyme matrix synthesis of [^{14}C]-GSH. The pH was adjusted from ~6.5 to ~7.5 for the remaining sets of experiments 4–10 and only the more active in-house synthetase GSS-1 was used.

Experiments 4–9 were attempts to simplify the cocktail. Experiment 4 can be considered to be the control using gly in excess (to avoid potential problems related to the separation of γ-glutamylcysteine from glutathione).[8] Experiment 5 showed

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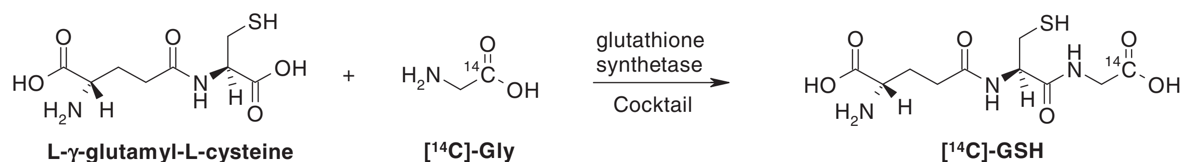

Figure 1. Single-step single-enzyme synthesis.

Table 1. Comparisons of GSS sources, and cocktail components

Expt.	Glu-cys (mmol)	Gly (mmol)	GSS (μg)	ATP (mmol)	$MgCl_2$ (mmol)	K_2HPO_4 (mmol)	LiKAcP (mmol)	EDTA (mmol)	% GSH (*h*)	Remarks
1	0.02	0.02	10	0.01	0.02	0.01	0.02	0.005	18 (1)	pH 6.5, GSS-1
2	0.02	0.02	10	0.01	0.02	0.01	0.02	0.005	14 (1)	pH 6.5, GSS-2
3	0.02	0.02	10	0.01	0.02	0.01	0.02	0.005	6 (1)	pH 6.5, GSS-3
4	0.02	0.025	10	0.025	0.025	0.025	0.025	0.005	23.4 (20)	pH 7.5, GSS-1
5	0.02	0.025	10	0.0125	0.025	0.025	0.025	0.005	49.2 (20)	pH 7.5, GSS-1
6	0.02	0.025	10	0.025	0.025	0.025	0	0.005	63.5 (20)	pH 7.5, GSS-1
7	0.02	0.025	10	0.025	0.025	0.025	0.025	0	11.6 (20)	pH 7.5, GSS-1
8	0.02	0.025	10	0.0125	0.025	0.025	0	0.005	62.0 (20)	pH 7.5, GSS-1
9	0.02	0.02	10	0.0125	0.025	0.025	0	0.005	61.7 (20)	pH 7.5, GSS-1
10	0.09	0.089[a]	23	0.056	0.113	0.113	0	0.023	30[b] (15)	pH 7.5, GSS-1

[a] [^{14}C]-gly.
[b]Isolated yield; Glu-cys (L-γ-glutamyl-L-cysteine), Gly (glycine), GSS (glutathione synthetase), LiKAcP (lithium potassium acetylphosphate), EDTA (ethylenediaminetetraacetic acid disodium salt), GSH (glutathione).

that reducing the amount of ATP improved the conversion to GSH. ATP was reported to be necessary and has been shown to be the best of several nucleotides tested to catalyze the enzyme reaction.[9] Experiment 6 showed that eliminating lithium potassium acetyl phosphate (LiKAcP) from the cocktail has a beneficial effect. Experiment 7 was based on the assumption that Na_2EDTA may not be needed in this cell-free reaction, since its use to chelate errant metal ions present in the immobilized cell matrix may not be needed. However, the elimination of Na_2EDTA resulted in a drastic reduction in the conversion of gly to GSH, thus necessitating the use of Na_2EDTA in further experiments. No attempt was made to alter $MgCl_2$ or K_2HPO_4 since literature sources claim that there is no activity in the absence of divalent cation and potassium ion was needed for maximum activity.[9] The final two non-labeled experiments 8–9 compare excess gly vs stoichiometric gly (desired for the labeled synthesis) while incorporating both beneficial effects, the reduced amount of ATP and the absence of LiKAcP. There was good reproducibility, and no apparent advantage to using excess gly. Thus, experiment 9 was used as the basis for the [^{14}C]-labeled synthesis in experiment 10.

The [^{14}C]-labeling reaction was conducted at 4.5 × scale. [^{14}C]-GSH was isolated in 30% yield and 98% purity after treatment with DTT (to convert significant [^{14}C]-GSSG to [^{14}C]-GSH)[11] followed by Prep-HPLC purification. The bulk of the activity was recovered as a single peak at the retention time and MW of [^{14}C]-glycine. A portion of the 98% pure [^{14}C]-GSH Prep-HPLC fraction was concentrated to dryness by ambient temperature rotary evaporation (releasing the vacuum with nitrogen). Dilution of this product revealed an increase in [^{14}C]-GSSG from 1% before concentration to 10% after concentration. Disulfide formation could be avoided by using lyophilization.[12] Alternatively, a DTT untreated mixture of [^{14}C]-GSH (69%) and [^{14}C]-GSSG (26%) which had been concentrated to dryness from 5% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) at ambient temperature in air and then redissolved in water was converted to 97% [^{14}C]-GSSG in 1 h by simply adjusting the pH from 2 to 7 with 1% aqueous NH_4OH. This solution could readily be converted back to 2% [^{14}C]-GSSG, 94% [^{14}C]-GSH by the addition of DTT to give a 20 mM solution. This treatment may afford a simple mode of storage in the [^{14}C]-GSSG oxidized form, which readily converts back to the reduced [^{14}C]-GSH. The DTT can be removed by extraction with EtOAc.[7]

Because of the cell-free nature of this synthesis, repeated sequential addition of reagents to convert gly to GSH was not necessary, nor was the repeated treatment with DTT to release GSH from a cell matrix.[8]

Experimental

Proton spectra were recorded on a Brucker 300 MHz NMR spectrometer. Mass spectrometric analyses were carried out on Fmoc derivatives (where needed) of the amino acids and peptides using a Finnegan LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source, a Thermo Finnigan Surveyor LC pump, a PDA detector (sheath gas flow 70 units N_2/sweep gas flow 15 units N_2; +cESi SID = 20 V, Normalized collision energy 35%), and a Packard Radiomatic 610TR Flow Scintillation Analyzer (column: Atlantis-C18, 4.6 × 150 mm, 5 μ; A: 0.1% TFA in H_2O, B: 0.1% TFA in ACN; hold 1% B for 5 min. Gradient: 1% B to 50% B 5–10 min., 50% B 15–20 min. Flow Rate: 1 mL/min, 30°C, 210 nm. Specific activity was determined by MS. TLC plates (silica gel 250 microns) were obtained from Analtech and scanned using a Bioscan System 200 Imaging Scanner. Analytical HPLC was conducted using the following conditions: Hypercarb-C18, 4.6 × 150 mm; ACN: 0.01% TFA 5:95 go to 90:10

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20–25 min; 1 mL/min at 210 nm. Prep-HPLC was performed on Hypercarb-C18, 21.2 × 150 mm; ACN: 0.01% TFA 5:95; 18 mL/min at 210 nm. The small molecules were reagent-grade and were used as received from vendors. [^{14}C]-glycine was purchased from Moravek Biochemicals, Inc. (Brea CA), L-γ-glutamyl-L-cysteine was purchased from Sigma (St. Louis, MO), and glutathione synthetase was purchased from Abnova Corporation (Taiwan) and prepared in-house by the Protein and Molecular Sciences Group.

[^{14}C]-Glutathione

L-γ-glu-cys (25.6 mg, 0.090 mmol) in 1.8 mL nitrogen purged water was combined with the cocktail: 31.4 mg ATP (0.056 mmol), 23.2 mg $MgCl_2$ (0.113 mmol), 19.7 mg K_2HPO_4 (0.113 mmol), and 8.6 mg Na_2EDTA (0.023 mmol). The pH was adjusted from 3–4 to 7–8 with 20 μL 6 M NaOH, and 2.5 mL of 2 mCi/mL aqueous [^{14}C]-gly was added. The solution was sterilized (0.45 μ filter, rinsing with 0.5 mL nitrogen bubbled water), and glutathione synthetase (23 μg in 10 μL buffer) was added. After stirring under nitrogen for 15 h at 37°C, the solution was cooled to ambient temperature, stirred for ⩾30 min with 0.50 mL 200 mM DTT. Prep HPLC purification afforded 1.5 mCi of 98% pure [^{14}C]-GSH having a specific activity of 52.9 mCi/mmol (MS ESI+, *m/z* 309.9 M+H) with a HPLC retention time of 4.63 min and 3 mCi of labeled material at the retention time (2.97 min) and MW of [^{14}C]-gly (Figure 2).

Glutathione synthetase

Construction of glutathione synthetase expression vector

The recombinant glutathione synthetase expression vector was PCR amplified from BL21(DE3) E. coli genomic DNA using the following primers: 5′- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAT CAA GCT CGG CAT C- 3′ (forward) and 5′- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTA CTG CTG CTG TAA ACG- 3′ (reverse). The PCR product was gel purified and cloned into an entry vector (pDONR221) followed by an expression vector (pDEST17) through recombination using the Gateway cloning system (Invitrogen). Presence of correct insert was confirmed through restriction enzyme digestion.

Expression and purification of glutathione synthetase

The expression vector pDEST17 containing the glutathione synthetase insert was transformed into BL21(DE3) E. coli cells. A single colony was used to start a 2.5 mL overnight culture in LB media containing 100 μg/mL carbenicillin and was used as the innoculum for a 250 mL culture of LB media containing 100 μg/mL carbenicillin and induced to express recombinant protein with 0.1 mM IPTG at an OD_{600} of 0.6 at 22°C overnight. Cells were harvested and lysed in 20 mL buffer A (25 mM Tris, pH 7.5, 0.3 M NaCl, 10% glycerol, 5 mM β-mercaptoethanol) plus 2 tablets of Complete Mini, EDTA-Free protease inhibitor tablets (Roche Molecular), 25 mL benzonase nuclease (Sigma), and 1x Bugbuster reagent (Novagen). Cell extracts were centrifuged at 3400 rpm at 4°C and the supernatant containing soluble glutathione synthetase was collected. The his-tagged glutathione synthetase was purified over a 1 mL NiNTA superflow column (Qiagen) in buffer A on an AKTA FPLC (GE), washed with buffer A plus 5 mM imidazole until baseline UV absorbance was reached, and eluted with buffer A plus 200 mM imidazole. The identity of the recombinant protein was confirmed by western blot using an α-penta-his monoclonal antibody (GE). Protein concentration was determined by OD_{280} ($\varepsilon = 38578\ M^{-1}$/cm) using a NanoDrop spectrophotometer (Thermo Scientific) and the purity was analyzed by SDS-PAGE. The yield was 5.5 mg of purified glutathione synthetase from 250 mL of E. coli culture. Proteins were aliquoted and stored at −80°C.

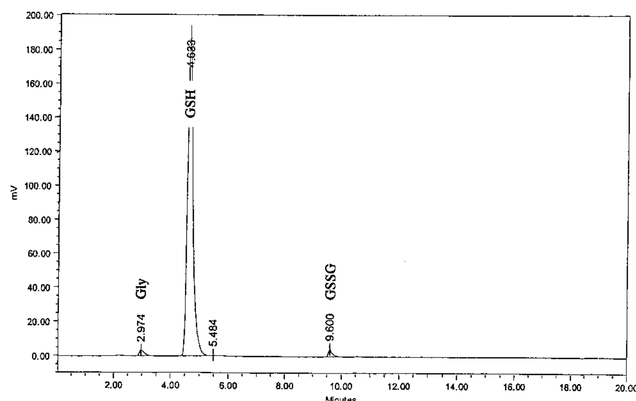

Figure 2. [^{14}C]-GSH Radio-HPLC.

Conclusion

We have developed an efficient one-step method for the cell-free preparation of [^{14}C]-GSH from readily available reagents: [^{14}C]-gly and γ-glu-cys; and catalyzed by glutathione synthetase in a simplified cocktail. The air sensitive [^{14}C]-GSH can be easily converted to the more stable oxidized form [^{14}C]-GSSG that can be readily reduced back to [^{14}C]-GSH as needed. Purification was by preparative HPLC.

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