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BIOSYNTHESIS OF HIGH SPECIFIC ACTIVITY ^{35}S-GLUTATHIONE

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SUMMARY

The biosynthesis of high specific activity ^{35}S- glutathione was carried out using a diploid strain of baker's yeast -'Saccharomyces cerevisiae'. Yeast cells were grown in a synthetic medium in which sodium ^{35}S-sulphate was supplemented as sole sulphur source. During growth, a major fraction of the radioactivity was incorporated into the protein as ^{35}S-methionine and ^{35}S-cysteine. After the growth of yeast, the cell wall was broken using a cell disrupter and the free ^{35}S-glutathione present was separated from protein and other sulphur containing compounds by chromatographic procedures. The specific activity of the ^{35}S-glutathione was determined by hydrolysing into its constituent amino acids and quantifying them using an amino acid analyser employing a post-column orthophthaldehyde derivatization method, followed by radioactivity assay of ^{35}S-cysteine. The overall radiochemical yield of ^{35}S-glutathione was 5 % . The radiochemical purity of the product was found to be greater than 95 % and its specific activity, greater than 1000 Ci/mmol when sodium ^{35}S-sulphate having a specific activity of 1200 Ci/mmol was used as the starting material.

Key words : Biosynthesis, ^{35}S-Glutathione, Yeast, Radio-labelling.

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INTRODUCTION

Glutathione , a tripeptide (γ-glu-cys-gly), plays a key role in many metabolic functions including removal of hydro peroxides, protection from ionising radiation, maintenance of the sulfhydryl status of the protein transport of glutamyl amino acids etc[1-6]. Many of these functions are accomplished by reaction at the cysteinyl sulfhydryl group catalysed by glutathione requiring enzymes. ^{35}S-glutathione with high specific activity can profitably be utilised as a radiotracer in these metabolic studies, especially for (i) the quantitative estimation of glutathione related enzymes, and (ii) the study on the effect of anti-oxidants on the transport of glutathione in cells. In this work, we present a method for the preparation of ^{35}S-glutathione having a specific activity of the order of 1000-1100 Ci/mmol. No report of a synthetic procedure for the preparation of this important labelled compound has been noted in the literature.

MATERIALS AND METHODS

The organism used was a diploid strain of *Saccharomyces cerevisiae*. It was received from Radiological Physics and Advisory Division, BARC as a generous gift in connection with the biosynthesis of high specific activity ^{35}S-methionine and ^{35}S-cysteine[7]. Sodium ^{35}S-sulphate having a specific activity greater than 1300 Ci/mmol was procured from NEN™ Life Science Products, USA. All the chemicals used for preparing the 'minimal medium' were of fine quality and devoid of sulphate. The 'minimal medium' employed for the growth of cells in culture contained per litre of water: glucose 10 g, diammonium hydrogen phosphate 2 g, trisodium citrate 1 g, asparagine monohydrate 2.5 g, potassium dihydrogen phosphate 875 mg, dipotassium hydrogen phosphate 125 mg, sodium chloride 100 mg, magnesium chloride hexahydrate 100 mg, zinc acetate 0.4 mg, ferric chloride 0.15 mg, copper (II) chloride 0.025 mg, biotin 0.01 mg, calcium pantothenate 0.5 mg, thiamine hydrochloride 0.6 mg, pyridoxine 1 mg and inositol 10 mg. The Yeast Extract-Peptone-Dextrose (YEPD) medium contained yeast extract 1%, peptone 2% and dextrose 2%. Purification of ^{35}S-glutathione was carried out using HPLC equipped with a GM detector.

Sulphate depletion

Yeast cells were grown in YEPD liquid broth for 24 h by incubating in a shaker water bath (120 rpm) at 30 °C. The cells were harvested and washed three times using sterile deionised water. The cells were then suspended in the minimal medium at a concentration of 10^6 cells/ml and incubated again after adding sodium ^{35}S-sulphate as tracer (about 50,000 dpm/ml of medium). The radioactivity, remaining in the supernatant after 10 h incubation was found to be less than 2500 dpm/ml indicating greater than 95% depletion of sulphate from the medium. The final cell concentration was found to be about 10^7 indicating a ten fold increase in population.

Radiolabelling of yeast cells

Sodium ^{35}S-sulphate was added to the above culture medium at a radioactive concentration of about 50 mCi/ml and incubated at 30 °C. Maximum incorporation of radioactivity in yeast cells was obtained after about 10 h incubation. Further incubation showed no improvement in the incorporation which is attributed to the cell death and lysis possibly due to radiolytic effect.

Isolation and purification of ^{35}S-glutathione

The ^{35}S labelled yeast cells were separated and collected by centrifugation. 1 ml water containing 0.1% 2-mercaptoethanol and 1% SDS was added and the cells were disrupted using a cell disrupter and centrifuged at 10000 rpm for 10 min. The supernatant containing ^{35}S-glutathione was collected and purified by means of HPLC using Whatman Partisil 10 SCX column in Na^+ form. Isocratic elution of glutathione was carried out using 0.05 M sodium citrate buffer, pH 3.5. An on-line GM detector was used to monitor and collect radioactive effluent fractions separately. The radiochemical purity of the product was determined by paper chromatography in n-butanol : acetic acid : water (4 : 1 : 5, v/v/v) system coupled with autoradiography. The non-radioactive authentic glutathione spot present in the chromatogram was characterized by reaction with ninhydrin.

Specific activity assay

Several methods have been described in the literature for the determination of glutathione, especially using HPLC in conjunction with OPA derivatisation. [8-12] The specific radioactivity assay of ^{35}S-glutathione was carried out by a post-column OPA derivatization method using an automated amino acid analyser to determine its concentration and its radioactivity using a liquid scintillation analyser. The result obtained was confirmed by converting an extract of the radioactive sample of the purified ^{35}S-glutathione into its corresponding amino acids by acid hydrolysis in a Pierce vacuum hydrolysis tube and then by analysing the individual amino acids, especially, glycine and glutamic acid. The quantitative conversion of glutathione to the corresponding amino acids on hydrolysis was confirmed by carrying out experiments with known amounts of non-radioactive authentic samples of glutathione and analysing the individual amino acids formed therefrom.

RESULTS AND DISCUSSION

High specific activity ^{35}S-glutathione was obtained by the biological depletion of residual sulphate present in the minimal medium before adding radioactive sulphate and by incubation of yeast cells at a concentration of 10^6 cells/ml before allowing for 10 hours' growth. A radioactive concentration of 50 mCi/ml of nutrient medium was selected as optimum, as a compromise between maximum radiochemical yield and high specific activity. Increasing the radioactive concentration and incubating with less number of cells will be useful for getting a higher specific activity product with a lower yield. β-mercapto ethanol was added during vacuum hydrolysis to maintain a reducing atmosphere and to avoid oxidation. All operations after incubation were carried out under an inert atmosphere to minimise the oxidation of glutathione to its dimer (GSSG). The specific activity obtained by the direct OPA method was confirmed by converting glutathione into its individual amino acids. The specific activity obtained was >1000 Ci/mmol by both the above methods for the product prepared using sodium ^{35}S-sulphate of specific activity ≥1200 Ci/mmol. The radiochemical yield and specific activity of ^{35}S-glutathione

obtained with respect to the starting material, sodium ^{35}S-sulphate, are summarised in Table 1.

Table 1 : Specific activity and radiochemical yield of ^{35}S-glutathione with respect to $Na_2{}^{35}SO_4$

Sodium ^{35}S-sulphate used		^{35}S-Glutathione obtained		
Radioactivity mCi (GBq)	Specific activity (Ci/mmol)	Radioactivity mCi (GBq)	Specific activity (Ci/mmol)	yield (%)
100 (3.7)	310	5.0 (0.185)	200	5
100 (3.7)	1350	6.2 (0.229)	1120	6.2
100 (3.7)	1200	5.5 (0.203)	1085	5.5

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