Determination of Glutathione in Biosynthesis Reaction by Paper Chromatography

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Abstract A method of separation and quantitative determination of glutathione(GSH) in biosynthesis reaction from its constituent: *L*-cysteine, glycine and glutamic acid, in a medium containing, glucose, potassium phosphate buffer and $MgCl_2$, by paper chromatography was developed. Cystine was found to play the role of *L*-cysteine in the biosynthesis of glutathione and R_f values of the 5 components: *L*-cysteine, glycine, glutamic-acid, GSH and cystine were reported. The results showed that among mixtures, phenol and water mixture in a ratio of (5 : 1) gave the best separation.

Key words Glutathione(GSH); Biosynthesis reaction; Paper chromatography; Analysis determination

INTRODUCTION

Glutathione(GSH) can be considered as an ideal, multifunctional and biologically active compound. It is widely distributed in animals, plant cells, and microorganisms[1]. In earlier papers, many analysis methods, such as titration method[2], electrophoresis method[3], and spectrophotometric method[4] were described. In those methods the separation is not good or -SH group of cysteine interfere with that of GSH during the analysis and affect the results. In this study, investigations were undertaken because of the convenience use of paper chromatography as a tool for the qualitative identification and quantitative determination of amino- acids and peptides. The most satisfactory solvents are those which are partially miscible with water. Those employed solvents provided that the water content is not too high, so that the cellulose, by a "salting out" effect allows the system to function as a partition chromatogram[5]. Different ratioes of a mixture of n-butanol, ethanol, acetic acid, water and phenol have been studied. Phenol and water in a ratio of (5:1) shows a very good result with a clear separation of the five components. Different R_f values of the five components for every ratio of the mixture of different solvents were illustrated.

The different spots were cut off and dissolved in 0.5 mol/L HCl and then treated with ninhydrine. By colorimetry method at 570 nm, the quantity of GSH, *L*-cysteine, glycine, glutamic acid and cysteine were determined refering to the standard curve.

1 Experimental

1.1 *Materials*

Glutathione (GSH) was purchased from Shanghai Biochemistry Company (specification: more than 90%). Glutamic acid (BR, TLC pu-

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rity, Shanghai Jiangda Amino Acid Company). Glycine(AR, 99.5%-100.5%, Huzhou Biochemistry Company). Cysteine and cystine(BR, TLC purity, China Scienses Institute East Instrumental Preparation Co. LTD Biochemistry Section). The paper used is Xinhua Filter Paper No 1. Colorimeter apparatus type 722 (Shanghai). Other chemicals such as ethanol and acetatic acid were used without further purification.

1.2 *Method*

1.2.1 *Cultivation of Saccharomyces cerevisiae Cells*

Cells were cultivated in 100ml of medium containing 0.5% glucose, 1.0% peptone, 1.0% yeast extract, 0.5% meat extract and 0.5% NaCl(pH 6.0) at 30℃ for 20 h with reciprocal shaking. After cooling the broth to 0℃, cells were centrifugated, and resuspended in 0.85% cold saline solution. The cell suspention was centrifugated at 3000r/min for 10 min, and weighed[6].

1.2.2 *Assay of Glutathione-producing activity* Intact cells (2.4g wet wt.) were incubated in 20ml of a mixture containing 0.5 mol/L glucose, 0.01 mol/L $MgCl_2$, 0.02 mol/L *L*-glutamic acid, 0.02 mol/L *L*-cysteine, 0.02 mol/L glycine and 0.1 mol/L potassium phosphate buffer (pH 7.0) at 30℃ for 4 h with shaking. 0.5 ml of reaction mixture was removed and heated in boiling water for 1 min, chilled immediately in ice water and centrifugated to remove cell debris. Using the supernatant, the cencentration of reactants and products were determined by paper chromatography[6].

1.2.3 *Qualitative identification* Trapezoidal strips(19cm×8cm×7cm)were cut with a paper cutter from Xinhua Filter Paper No. 1, were selected to be held in a position without touching the walls of the chomber assay except at the upper end. The solvent is added gently to the chomber without wetting the walls and then the strip is introduced later. The chomber stands for certain hours until the solvent ascends to a position of about 15mm below the upper end[7]. The strip is removed, suspended, dried, and sprayed lightly with 0.25% solution of ninhydrine in (5%) water-saturated butanol and dried at 110℃ for about 4 min. The R_f was calculated for every sample.

1.2.4 *Quantitative determination* The spots after traveling the distance were removed and cut off into small pieces, and then introduced to the assay chomber 1 ml of 0.5 mol/L HCl was added to dissolve the contents of the small pieces of filter paper. After mixing it, 1 ml of 2 mol/L acetic acid buffer(pH 5.4) is added. After blending it, 1 ml of ninhydrine solution was introduced. The solution was heated at 100℃ for 15 min. Cooling it to room temperature, and then add 2 ml 60% ethanol. After mixing and filtrating it, the color intensities of the solutions of each sample were determined at 570 nm[8].

The linear regression equations, of the 5 components, obtained from the data were, taking known absorbances on Y-axis and weight (μg) on X-axis, as follow:

Cysteine: $Y=0.0021X-0.0419, r=0.9923$;

Cystine: $Y=0.0022X+0.0177, r=0.9983$;

Glutamic acid: $Y=0.0662X-0.1717, r=0.9987$

Glycine: $Y=0.1010X-0.0586, r=0.9984$

Glutathione: $Y=0.0009X+0.0073, r=0.9979$

2 RESULT AND DISCUSSION

The differently obtained R_f values can be based on the following factors: paper, temperature, and the degree of saturation with water. The degree of saturation with water can be con-

sidered as the principal factor to reach a good separation for this research. Tab. 1 shows that the increase of n-butanol in the mixture give a slow deplacement of the components but cysteine and GSH still have a close R_f.

Tab 1. The R_f values of different amino acids in different solvent mixtures.

Components	Ethanol	n-Butanol-acetic acid-water					n-Butanol-acetic acid-ethanol-water			
	70%	4:1:1	4:1:2	4:1:3	7:1:2	4:2:1	4:1:1:2	4:1:2:2	4:1:4:1	4:1:2:1
Cyss	0.08	0.08	0.21	0.20	0.06	0.14	0.23	0.20	0.17	0.11
Gly	0.56	0.25	0.35	0.30	0.18	0.27	0.39	0.38	0.35	0.22
Glu	0.64	0.32	0.42	0.42	0.24	0.37	0.50	0.48	0.47	0.36
GSH	0.64	0.29	0.44	0.43	0.24	0.37	0.50	0.47	0.50	0.35
Cys	0.64	0.37	0.47	0.46	0.33	0.52	0.51	0.52	0.41	

The result shows that in *n*-butanol, acetic acid, and water in a ratio of (4 : 1 : 1), the separation of different amino acids; cystine (Cyss), glycine(Gly), glutamic acid(Glu), cysteine(Cys) and glutathione(GSH) was better.

A phenol water mixture was investigated and in the phenol saturated water at 8 C, the results were good and the separation between the components is clear reflected by a big differents between the different R_f values of different components. This saturation phenomenon is influenced by the temperature and in the phenol saturated water at 8 C 4V (volume) of phenol were calculated to contain 1V of water. The variation of water contents in phenol affects the R_f values and cystine was found to be deplaced forward by the solvent with the decreases of water contents in phenol. The data shown in Tab. 2 prove this result.

Tab 2. The R_f values of differnt Amino-acids and GSH in phenol and water solvent

Components	(Phenol-Water)V/V		
	3 : 1	4 : 1	5 : 1
Cystine	0.29	0.16	0.18
Glycine	0.35	0.26	0.28
Glutamic-acid	0.32	0.39	0.38
GSH	0.46	0.55	0.52
Cysteine	0.57	0.67	0.67

The result showed the larger is that phenol-water in a ratio of (5 : 1 V/V) have been selected as the best one.

Other factors are also having an effect on R_f values of different components, such as the normality of HCl in which amino-acids and GSH were dissolved.

The quantitative determination of GSH using ninhydrine in (5%) water-saturated butanol gives a good result with a recovery of 96%.

In this paper a separation method by the use of paper chromatography was described. Ninhydrine is used to reveal different amino-acids and GSH. Many solvent mixtures have been investigated and the phenol-water mixture (5 : 1 v/v) used as mobile phase, shows a clear separation. Other factors have been taken in consideration of the separation such as paper, pH, temperature, concentration of amino-acids, and paper contents in water. The results obtained were similar to those discribed by John M. Miller and Louis B. Rockland[9].

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纸层析法分离测定生物合成反应中的谷胱甘肽

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摘 要 报道一种分离与定量测定生物合成反应液中谷胱甘肽的方法。该反应以半胱氨酸、甘氨酸和谷氨酸为底物，在葡萄糖、氯化镁和磷酸缓冲液条件下进行，反应液用纸层析分离，实验发现胱氨酸替代半胱氨酸参与反应，并报道了五种成分的 R_f 值：*L*-半胱氨酸、甘氨酸、*L*-谷氨酸、谷胱甘肽和 *L*-胱氨酸。结果表明，在混合溶剂系统中，以酚-水（5∶1）分离效果最佳。

关键词 谷胱甘肽；生物合成；纸层析；分析测定

[文摘 017] 蝙蝠葛酚性生物碱的提取工艺研究 张 灿，张惠斌，黄文龙．中草药，1997，**28**(5)：274

报道了蝙蝠葛酚性生物碱的提取工艺，收率比文献有较大提高。采用不同碱液萃取法，适合于较大量的蝙蝠葛苏林碱的制备。

[文摘 018] 杉木根皮中的二萜类成分研究 邓京振，柳 茵，赵守训．中草药，1997，**28**(5)：267

从杉科植物杉木 *Cunninghamia lanceolata* 的根皮中分得3种半日花烷型(Labdan)二萜类成分。经理化常数和波谱分析，分别鉴定为 12，13*E*-biformen（Ⅰ，12，13*E*-ozic acid（Ⅱ）和 12，13*E*-methylozate（Ⅲ），均为首次从该植物中分得。

[文摘 019] 钾通道启开剂研究（1） 反式-4-氨基-3-羟基-3，4-二氢-2，2-二甲基-2-氢-1-苯并吡喃类化合物的合成及其心血管活性 孙宏斌，华维一，陈玲，彭司勋，王 霆，刘国卿．高等学校化学学报，1997，**28**(5)：730

通过溴醇两步环氧化法并采用三苯基膦脱氧合成了一系列新型的反式 4-氨基-3-羟基-3，4-二氢-2，2-二甲基-2-氢-1-苯并吡喃类化合物，通过 IR、NMR、MS 和元素分析等手段对其结构进行了确证。研究了其中 13 个化合物对 KCl 刺激大鼠胸主动脉条收缩抑制作用，考察了其体外血管扩张活性；还对其中 11 个化合物进行了 S. D. 大鼠体内降血压实验。结果表明，所测化合物对低钾刺激引起的收缩均有不同程度的抑制作用，其中化合物 $Ⅰ_1$、$Ⅰ_2$、$Ⅱ_5$、$Ⅱ_6$ 效果较好，部分化合物显示较好的降血压活性。

[文摘 020] 中药制剂研究进展 冯年平，张正行．中草药，1997，**28**(5)：306

综述了近年来中药制剂在剂型改革、新材料和新技术应用以及体外释放度和体内药动力学方面的研究进展。