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GLUTATHIONE IN CONDITIONED MEDIA OF TOBACCO SUSPENSION CULTURES

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Key Word Index—*Nicotiana tabacum*; Solanaceae: tobacco; tissue culture; conditioned media; γ-glutamyl peptides; glutathione.

Abstract—Conditioned media of suspension cultures of *Nicotiana tabacum* var. Samsun contain 0.15–0.20 mmol/l. glutathione. These concentrations correspond closely to the intracellular content of glutathione and represent about three to four times the amount of glutathione needed to maintain the intracellular level of glutathione. In contrast to the GSH:GSSG ratio inside the cells, the amount of GSSG in the media rises to 20% of the GSH content.

In the course of our studies on substances excreted into conditioned media by liquid suspension cultures of tobacco cells [1] in addition to free amino acids, a peptide was found which inhibited the growth of soybean callus tissues in low concentrations [2]. In this paper the isolation of this peptide and its identification as glutathione are reported.

The peptide was isolated as described in Experimental from liquid media of green suspension cultures of tobacco grown at 3000 lx on a modified Murashige and Skoog [3] medium for ten days. During this time the fr. wt of the cell suspension increased from 12 to 280 g/l., and 20–40 mg of the amorphous peptide could be isolated from 1 l. culture medium. The peptide was chromatographically pure as shown by TLC and was tentatively identified as oxidized glutathione (GSSG) by its R_f-values using several solvent systems (Table 1). The purified samples contained 95 ± 3% glutathione as determined by an amino acid analyser after treatment of the compound with performic acid.

Upon hydrolysis of the peptide with 6N HCl L-glutamic acid, L-glycine and L-cystine were liberated in a ratio of 1.8:1.8:1. By treatment with carboxypeptidase A [4] only L-glycine was split from the peptide, locating L-glycine in the C-terminal position. In agreement with this result L-glutamic acid was the first amino acid detectable after incubating the peptide with aminopeptidase M [5] and therefore has to be in the *N*-terminal position. By gentle hydrolysis of the peptide with HCl, 5-oxoproline was formed, as identified by its R_f on TLC (Table 1). The formation of 5-oxoproline indicates a structure with a γ-glutamyl bond. This conclusion is supported by the chromatographic behaviour of the peptide on the amino acid analyser [6], where the following retention times (in min) were measured: GSSG 53; L-glutamic acid 69; L-glycine 102; L-cystine 147. From these data the peptide was identified as the oxidized form of γ-L-glutamyl-L-cysteinyl-L-glycine.

Table 1. TLC R_f-values of glutathione and related compounds

Substance	Adsorbent*	Solvent†	$R_f \times 100$
GSH	C	1	38
	C	2	36
	C	3	41
	S	4	50
GSSG	C	5	15
	C	1	17
	C	2	19
	C	3	13
	S	4	25
GS–Et	C	1	57
	C	2	91
5-Oxoproline	C	5	80

* Adsorbent: C = cellulose MN 300, S = Si gel. † Solvent 1: *m*-BuOH–HoAc–H_2O (12:3:5); 2: pHOH (91%)–NH_4OH (25%)—H_2O (130:1:20); 3: *iso*-ProH–EtoAc–H_2O (6:1:3); 4: *n*-BuOH–C_5H_5N–HOAc–H_2O (15:3:10:12); 5: *iso*-PrOH–HCO_2H–H_2O (20:1:5).

Apart from the oxidized form of glutathione the conditioned media also contained reduced glutathione (GSH). This was demonstrated by treatment of the media with *N*-ethylmaleimide and the subsequent demonstration of GS–Et by TLC (Table 1). In contrast to living cells which contain substantial concentrations of GSH and only small amounts of GSSG [7], the amounts of GSSG in the medium of the tobacco cells are much higher, reaching 20% of the GSH content. These high GSSG concentrations may be due to an oxidation of GSH in the culture medium. The total amount of glutathione in the culture media determined after treatment with performic acid was in the range of 0.15–0.20 mmol/l; these correspond closely to the concentrations found inside tobacco cells [8]. Together with the growth data they demonstrate that cells in suspension cultures synthesize three to four times the amount of glutathione remaining inside the cells.

In comparison with the total amount of free amino acids inside the cells and in the medium the amount of glutathione in the medium is remarkably high, too. In the present experiments the concentrations of free amino acids found in the medium were in the order of 10% of the amino acid content inside the cells [1]. These findings suggest that the release of glutathione may have a special function during growth of tobacco suspensions cultures.

EXPERIMENTAL

The tobacco tissue culture used in the present experiments was obtained from a callus culture isolated in 1959 [9]. Cells were subcultured for more than one year in a modified liquid M and S medium [1], supplemented with vitamins [10], NAA (0.2 mg/l.), and *meso*-inositol (100 mg/l.). 20 g/l. sucrose was added and the pH adjusted to 5.8. The medium was autoclaved for 5 min at 120°. Suspension cultures were grown in 3 l. Fernbach flasks with 300 ml medium at 25 ± 2° and 60–70% air humidity under continuous illumination (3000 lx). GSSG was isolated from 1.5 l. of filtered medium of 10-day-old suspension cultures. The medium was concentrated to 150 ml and pectic substances were removed by centrifugation. The supernatant was mixed with a 10-fold vol of cold MeOH, the ppt. collected by centrifugation and redissolved in cold H_2O; denaturated proteins were removed by centrifugation. The supernatant fraction was placed on a Dowex 1 × 8 column (5.5 × 20 cm) in the formate form and eluted with 2 N HOAc. After desalting on a Sephadex G-10 column (1.8 × 65 cm) GSSG was isolated on a Sephadex G-25 column (1.8 × 78 cm) by elution with H_2O. Isolated peptide was hydrolysed for 16 hr at 110° with 6 N HCl in a sealed tube under N_2. Hydrolysate was evaporated *in vacuo* to dryness and dissolved in 0.2 N lithium citrate buffer pH 2.2. The amino acid content of this soln was obtained with an amino acid analyser. Quantitative estimation of the glutathione content of the culture medium was accomplished after treatment of the samples with performic acid [11]. Enzymatic hydrolysis was carried out with carboxypeptidase A (EC 3.4.12.2) and aminopeptidase M (EC 3.4.1.2) following established procedures [4,5]. Gentle hydrolysis was performed with 1,2 N HCl at 100° for 1 hr [12]; for alkylation GSH was treated with *N*-ethylmaleimide [13]. The formation of 5-oxoproline and GS–Et was established by TLC. Chromatograms were developed on cellulose MN 300 or Si gel sheets with several solvent systems (Table 1). Amino acids and peptides were located by ninhydrin, carboxylic acids with bromocresol green–bromophenol blue–$HMnO_4$ [14].

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