Evidence for the Participation of a 5-Oxo-prolinase in Degradation of Glutathione in *Nicotiana tabacum*

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During degradation of glutathione in tobacco suspension cultures substancial amounts of 5-oxo-proline are formed *in vivo* as well as in crude cell homogenates *in vitro*. The existance of a 5-oxo-prolinase that catalyzes the conversion of 5-oxo-proline to glutamic acid was demonstrated in tobacco cells, grown with glutathione as sole sulfur source.

Introduction

Tobacco cells in suspension cultures produce high amounts of glutathione and release it into their culture media [1, 2]. Surplus production of glutathione is restricted to chloroplast-containing cells and induced by high ammonium and sulfate concentrations [2, 3]. When the sulfate supply in the medium is exhausted, released glutathione is taken up again and reutilized as sulfur source for protein synthesis [2]. As tobacco cells are also able to grow with glutathione as sole sulfur source [4, 5], degradation to the constituent amino acids is supposed.

In animal cells enzymatic hydrolysis of 5-oxo-proline to glutamic acid is known to be the rate-limiting step in degradation of glutathione (*cf.* [6]). A 5-oxo-prolinase that catalyzes this reaction has recently been demonstrated in several plant species [7, 8]. Whether this enzyme participates in degradation of glutathione in higher plants, however, has not been investigated. The present experiments show evidence that during degradation of glutathione in tobacco cells 5-oxo-proline is formed and hydrolyzed enzymatically to glutamic acid.

Materials and Methods

Plant material

The tobacco suspension cultures used in the present investigation were obtained from a callus culture isolated by Bergmann [9]. Cells were subcultured in a modified liquid M+S medium [10] and grown at 25 °C and 60–70% air humidity under continuous illumination (3000 lx). Determination of 5-oxo-prolinase activity was performed with tobacco cells grown for 6–8 days in a culture medium, where sulfate was completely replaced by equimolar amounts of reduced glutathione (GSH). GSH was added to sulfur free medium as a filtersterilized solution of pH 5.6–5.8 under sterile conditions.

Incubation with [^{14}C-glu-U]GSH

[^{14}C-glu-U]GSH was prepared from [^{14}C-glu-U]-GSSG (NEN) by reduction with dithiothreitol (DTT); excess DTT was removed by extraction with ethyl acetate after acidification [11]. 9–10 mg d. w. mixotrophically grown exponential phase tobacco cells were inoculated into 3 ml sulfur free M+S medium and placed for 25 h on a shaker (100 rpm) in the light under the conditions mentioned above. Subsequently 0.1 ml of [^{14}C-glu-U]GSH (0.79 µM GSH/ml; 18.5×10^4 Bq/ml) was added and the suspension exposed to the radioactivity for 20 h. Incubation was stopped by filtering and washing the cells with GSH-containing M+S medium. Cells were homogenized for 5 min in a pre-cooled mortar with 5 ml cold, destilled water. Soluble protein was precipitated by incubation of the homogenate in boiling water (5 min) and removed by centrifugation. In controls radioactivity was added either to the suspension immediately before cells were harvested, or to the homogenate prior to protein precipitation. Deproteinized solutions were subjected to TLC directly or after fractionation on a Dowex 50 WX4 column (see: Enzyme assay). TLC was performed on cellulose MN-300 plates with BuOH/AcAc/H_2O (60/15/25) as solvent. Radioactivity on TLC-plates was analysed with a TLC-scanner

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(Berthold); co-chromatographed reference compounds were localized with an *o*-phthalaldehyde spray-reagent [2].

Preparation of crude cell-homogenates

5 g f. w. of tobacco cells, grown with GSH as sole sulfur source, were pre-homogenized together with 1 g Polyclar AT (Serva) in a 0.02 M hepes/NaOH-buffer, 5 mM in dithioerythritol (DTE) and pH 7.4, using a potter-homogenizer. The suspension was incubated in a cell disruption bomb (Parr, model 4635) at a N_2-pressure of 2000 psi for 5 min. By release to atmospheric pressure cells and organelles were completely disrupted. This homogenate was centrifuged at $20000 \times g$ for 15 min and the supernatant placed on a sephadex G-25, medium (Pharmacia) column (4×1.8 cm ∅), equilibrated with homogenisation buffer. The column was centrifuged at $800 \times g$ for 20 min and the eluate obtained from this centrifugation used for the determination of 5-oxo-prolinase activity. Protein content of the samples was measured by the Biuret-Phenol method described by Layne [12].

Enzyme assay

The assay mixture used for the determination of 5-oxo-prolinase activity contained the following substances in a total volume of 1 ml: 500 mM sodium glycinate, 25 mM ATP × $3H_2O$, 12.5 mM $MgCl_2$ × $6H_2O$, 12.5 mM $MnCl_2$ × $4H_2O$, 100 mM $(NH_4)_2SO_4$, 5 mM DTE, 499.65 µM 5-oxo-proline, 0.35 µM [^{14}C-U]-5-oxo-proline (9.7×10^9 Bq/mM); pH of the mixture was 9.5. After incubation at 30 °C the reaction was stopped by addition of 0.1 ml 1 N acetic acid; protein was precipitated by incubation in boiling water (5 min) and removed by centrifugation. The supernatant was placed on a Dowex 50WX4 column (1.5×1.9 cm ∅) to separate labeled substrate (5-oxo-proline: eluated with 20 ml destilled water) and product (glutamic acid: eluated with 10 ml 10 N NH_3). TLC analysis of the NH_3-eluate on precoated cellulose plates, using BuOH/AcAc/H_2O (60/15/25) as solvent, showed that glutamic acid was the only labeled substance formed in the reaction mixture. Radioactivity of the NH_3-eluate was determined by liquid scintillation counting. 5-oxo-prolinase activity was linear with time for at least 90 min under these conditions.

Results and Discussion

Incubation of photoheterotrophically grown tobacco cells with [^{14}C-glu-U]GSH revealed that substantial amounts of 5-oxo-proline are formed during degradation of glutathione: In the water soluble fraction of tobacco cells, exposed to this labeled compound for 20 h, a radioactive substance with the same R_F-value as co-chromatographed [^{14}C-U]5-oxo-proline could be detected by TLC analysis (Fig. 1 A). Fractionation of the H_2O-extract of tobacco cells on a Dowex 50WX4 column and subsequent TLC analysis showed that a substance with

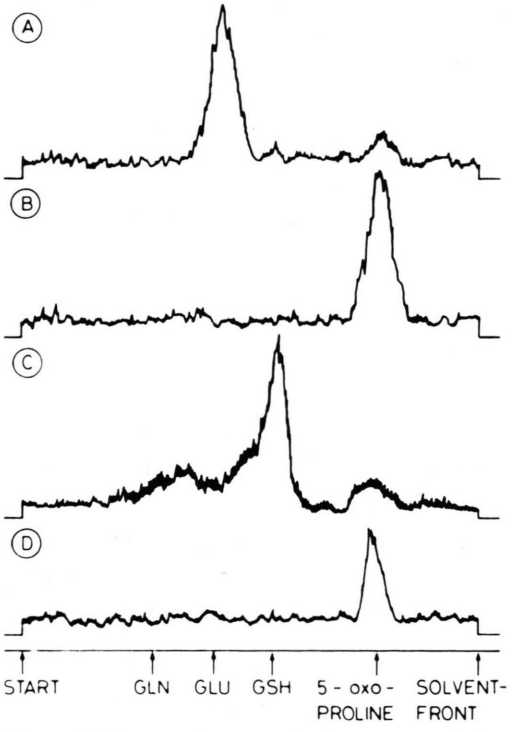

Fig. 1. TLC-scan of water-soluble compounds in tobacco cells exposed to [^{14}C-glu-U]GSH *in vivo* and *in vitro*. Photoheterotrophically grown tobacco suspension cultures were sulfur starved for 25 h and exposed to [^{14}C-glu-U]-GSH for a 20 h period. Cells were filtered, washed, and extracted with water. The deproteinized H_2O-extract was subjected to TLC either directly (A), or subsequent to a fractionation on Dowex 50WX4, using water (B) and NH_3 (not shown) for elution. *In vitro* incubation was performed with crude cell-homogenates (1 ml) and [^{14}C-glu-U]GSH (0.1 ml, 0.79 µM GSH/ml, 18.5×10^4 Bq/ml). After a 2 h exposure at 30 °C protein was precipitated by incubation in boiling water, removed by centrifugation and the supernatant analysed by TLC (C). [^{14}C]-oxo-proline (NEN), GSH, glutamic acid, and glutamine were co-chromatographed as reference compounds (D). Radioactive areas on TLC-plates were localized with a TLC-scanner (Berthold).

the R_F-value of 5-oxo-proline is eluated completely from the ion-exchange resin with water (Fig. 1 B); the other labeled substances in the H_2O extract, namely small amounts of GSH and high amounts of glutamic acid (Fig. 1 A), remained on the column under these conditions, but could be eluated with 10 N NH_3. These results demonstrate that during degradation of glutathione a compound with the same R_F-value in TLC and the same behaviour on a cation exchange resin as 5-oxo-proline is formed.

As enzymatic cyclysation of L-glutamine to 5-oxo-proline and NH_3 has been shown in plant cells [13], the finding of labeled 5-oxo-proline in tobacco cells, exposed to [^{14}C-glu-U]GSH could also be explained by this reaction, even if such an enzyme has not yet been demonstrated in tobacco cells: If glutathione is directly hydrolyzed to the constituent amino acids, synthesis of labeled glutamine from glutamic acid and subsequent cyclysation to 5-oxo-proline might occur. To exclude this possibility, crude homogenates of tobacco cells were incubated with [^{14}C-glu-U]GSH for 120 min. TLC separation of the labeled compounds in this reaction mixture showed that labeled 5-oxo-proline was formed during degradation of [^{14}C-glu-U]GSH *in vitro*, too (Fig. 1 C). As the reaction mixture did not contain any ATP, enzymatic synthesis of glutamine from glutamic acid can be excluded, indicating that the 5-oxo-proline measured was not due to cyclysation of glutamine.

The finding of labeled 5-oxo-proline in tobacco cells, grown with [^{14}C-glu-U]GSH as sole sulfur source, as well as the formation of labeled 5-oxo-proline from [^{14}C-glu-U]GSH in crude tobacco cell homogenates show that cyclyzation of the γ-glutamyl-group of glutathione takes place during enzymatic degradation of this peptide. Furthermore, these results point to the existance of a 5-oxo-prolinase that catalyzes the conversion of the 5-oxo-proline, formed during degradation of glutathione, to glutamic acid. Using a modified assay mixture of Mazelis and Creveling [8], considerable 5-oxo-prolinase activity was found in crude homogenates of tobacco cells, grown with glutathione as sole sulfur source for 6–8 days. Variation of the amount of enzyme extract in this reaction mixture revealed a saturation kinetic with a linear part up to 1.5 mg protein (Fig. 2). The specific activity of 0.35 nmol glutamic acid $\times$ min^{-1} $\times$ mg protein^{-1} is similar to

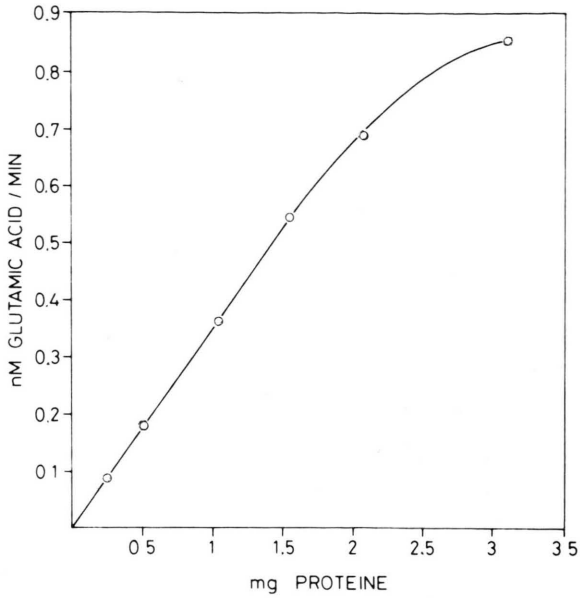

Fig. 2. Influence of different amounts of crude tobacco cell extract on enzymatic formation of glutamic acid from 5-oxo-proline. Different amounts of crude extracts of tobacco cells, grown photoheterotrophically with glutathione as sole sulfur source, were added to the reaction mixture, described in Materials and Methods, for 10, 20, and 30 min. The amount of labeled glutamic acid formed from [^{14}C-U]5-oxo-proline was determined by liquid scintillation counting after fractionation of the assay mixture on a Dowex 50 WX 4 resin.

that found in various organs of other plant species [8].

As glutathione is the main reduced sulfur compound translocated from the leaves to the roots and the growing parts of the stem of tobacco plants [14], degradation of this peptide is necessary to make reduced sulfur available for protein synthesis. If the reaction catalysed by the 5-oxo-prolinase is – as known from animal tissues (*cf.* ref. [6]) – the rate limiting step in degradation of glutathione in plant cells, too, the activity of this enzyme should be influenced by nutritional factors. Further investigations will show, whether such a regulation of 5-oxo-prolinase activity can be confirmed in tobacco suspension cultures.

Acknowledgement

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