# Degradation of glutathione *S*-conjugates by a carboxypeptidase in the plant vacuole

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**Abstract For plants, glutathione conjugation is a major pathway to detoxify organic xenobiotic. Glutathione *S*-conjugates (SG-conjugates) are formed in the cytosol, the in vitro transport over the tonoplast has been described and a final storage in the vacuole has been postulated. We show here that alachlor rapidly accumulates as GS-conjugates in the plant vacuole and that the first step of its degradation, the formation of the respective γ-glutamylcysteinyl-*S*-conjugate, is catalyzed by a vacuolar carboxypeptidase. These results suggest the glutathione conjugate as a transport form but not a storage form of xenobiotic molecules.**

*Key words:* Detoxification; Glutathione conjugate; Xenobiotic; Carboxypeptidase; Vacuole

## 1. Introduction

Detoxification of hazardous foreign compounds (xenobiotics) is essential for survival of organisms. Numerous studies have investigated the uptake and metabolic fate of xenobiotics in plants and revealed a reaction sequence consisting of a first phase of chemical modification, a second phase of conjugation and a third phase of storage [1–3]. In most cases conjugation of a xenobiotic leads to a loss of its electrophilic properties and thereby to detoxification. The storage phase is then initiated by export of the conjugate from the cytosol either into the extracellular space followed by binding to biopolymers such as lignin and cellulose or into the large central vacuole. The transport of GS-conjugates has recently been shown to depend on an ATP driven pump in the vacuolar membrane [4]. The physiological relevance of this export pump was deduced from its high affinity and its similarities to the export pumps in the canalicular membrane of mammalian liver. Nothing is known about the metabolic activities in the plant vacuole with regard to processing and degradation of imported conjugates. However, in fluorodifen-treated cell cultures the rapidly synthesized fluorodifen-glutathione-*S*-conjugate was metabolized [9] and though the metabolites were identified, the enzyme responsible for the processing of the GS-conjugate has not yet been investigated. The present paper focusses on the elucidation of the reactions catalyzing the cleavage of GS-conjugates in plants.

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## 2. Materials and methods

### 2.1. Materials

All experiments were performed with barley (*Hordeum vulgare var. cherie*) seedlings that were grown under controlled conditions (20°C, 80% relative humidity). Plants were harvested after 10 days in the two leaf stadium (BBCH code 11).

### 2.2. In situ incubation with [$^{14}$C]alachlor

Barley leaves whose lower epidermis was removed manually were placed on a solution containing 100 mM sorbitol and [$^{14}$C]alachlor (520 kBq/410 nmol) for 3 h. The incubation medium was then exchanged for digestion medium [5] containing 0.5% (w/v) cellulase, 0.25% (w/v) macerozyme and 0.01% (w/v) pectolyase. All enzymes were from Seishin Pharmaceuticals, Tokyo. Maceration was performed for 60 min in a waterbath at 30°C. Undigested residues were the major vascular bundles and the cuticle. Mesophyll portoplasts and vacuoles were isolated by differential centrifugation on a sucrose gradient [5]. Radioactivity contained in the respective fractions was determined by liquid scintillation counting (TriCarb 2100TR, Packard).

Numbers of vacuoles and protoplasts were quantified using the vacuolar marker enzyme a-mannosidase as described previously [6].

### 2.3. Determination of carboxypeptidase activity

Carboxypeptidase activity was assayed as follows: 50 µl of the protein fractions (see section 2.2) was added to a final volume of 1 ml 0.1 M morpholinoethane sulfonic acid (MES), pH 5.0 (KOH) containing 0.5 mM *S*-(*p*-nitrobenzyl)-glutathione (Sigma Chemicals, Deisenhofen, Germany). All other GS-conjugates were synthesized as described earlier [7]. The assays were incubated at 25°C for 1 h, and the reaction was stopped by addition of 300 µl cold acetone. Precipitated protein was removed by centrifugation (5 min, 15000 rpm, Universal 30, Hettich). The supernatant was dried at 40°C under vacuum (Speedvac, Bachofer). The dried pellets were dissolved in 20% acetonitrile/80% $H_2O$ containing 0.1% trifluoroacetic acid (TFA). The samples were then analyzed by HPLC using a RP C-18 column (µ-Bondapak 25 cm × 0.5 cm). Separation was developed with a linear gradient of acetonitrile from 20 to 100%. Both substrate and degradation product were quantified from peak areas at 280 nm using authentic reference compounds for standardization.

### 2.4. Purification procedure

30 g of primary leaves were pulverized in liquid nitrogen with mortar and pestle and suspended in homogenization buffer containing 100 mM MES (KOH, pH 5), 3 mM ethylenediamine tetraacetic acid and 0.5% Nonidet K40. The homogenate was filtered through two layers of Miracloth (Calbiochem) and was spun at 20000 × *g* for 30 min. The supernatant was subjected to differential $(NH_4)_2SO_4$ fractionation. The precipitate obtained between 35% and 80% $(NH_4)_2SO_4$ saturation was desalted by passage through a PD 10 column (Pharmacia, Uppsala, Sweden) and thereby transfered into 0.1 M MES, KOH, pH 5 supplemented with 1 mM DTT.

Desalted protein fractions were subjected to gel filtration, performed on a Superose 12HR 10/30 (Pharmacia) with a flow rate of 0.5 ml/min and a fraction size of 1 ml. Fractions containing the nitrobenzyl-GS carboxypeptidase activity were pooled and applied to a Resource S-column for cation exchange chromatography (Fig. 2). Proteins were eluted with a linear gradient of NaCl (0–0.5 M). Chromatographic seperations were performed on an FPLC system (Pharmacia).


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Table 1
Uptake and compartmentation of [$^{14}$C]alachlor in barley leaves

| Fraction | Amount [nmol] | Percent of total | Percent in vacuole |
|---|---|---|---|
| 1. Total amount in assay | 410 | 100 | – |
| 2. Incubation medium | 120 ± 10 | 28 | – |
| 3. Digestion medium (cell wall) | 23 ± 3 | 6 | – |
| 4. Major bundles and cuticle | 3 ± 0.5 | 1 | – |
| 5. Mesophyll protoplasts | 279 ± 16 | 66 | 100 |
| 6. Mesophyll vacuoles | 262 ± 17 | 64 | 97 |

Radioactivity contained in fractions 1–6 was determined. Vacuoles and protoplasts were quantified using a vacuolar marker enzyme (see section 2.2). [$^{14}$C]Alachlor content of protoplasts was set at 100%; 97% of this was contained in the vacuoles. (1) Total amount of [$^{14}$C]alachlor in the incubation solution. (2) Remaining amount of [$^{14}$C]alachlor in the incubation medium after 3 h. (3) Digestion medium (see section 2.2) to obtain protoplasts. (4) Undigested residues. (5) Mesophyll protoplasts. (6) Vacuoles were isolated as described in section 2.3. Data are means of three independent experiments ± standard deviations.

Protein was determined photometrically, using the method of Bradford [8] and BSA as a standard.

## 3. Results and discussion

Alachlor, a chloracetamide herbicide, has been described as being detoxified via glutathione conjugation [7]. We administered [$^{14}$C]alachlor to barley leaves after removal of their lower epidermis. 72% of the radioactivity applied with the incubation medium was incorporated into the leaves within 3 h (Table 1). The cellular and subcellular compartmentation of [$^{14}$C]alachlor was determined after enzymatic maceration of the leaves, followed by fractionation in intact mesophyll protoplasts and vacuoles. 97% of the radioactivity associated with the protoplasts was recovered within the vacuoles demonstrating the efficacy of the first steps of the detoxification sequence. The vacuolar contents were then analyzed by thin layer chromatography. The radiolabel separated as one peak distinct from alachlor and exhibited an identical Rf (0.4) with the authentic alachlor-glutathione-*S*-conjugate (alachlor-GS). Based on the amount of tissue incubated and the specific activity of alachlor, transport rates of the conjugate into the vacuole were calculated to be 0.4 nmol per $10^7$ vacuoles each min. Martinoia et al. [3] using isolated vacuoles reported maximal transport rates for *N*-ethylmaleimide-GS and metolachlor-GS of 1.6 and 1.4 nmol per $10^7$ vacuoles per min, respectively. It is clear from our data that the vacuolar pump for GS-conjugates also operates in vivo and efficiently transports GS-conjugates into the vacuoles.

Transport of GS-conjugates into the vacuole initiates phase 3 of the general detoxification scheme for xenobiotics in plants [3]. The fate of the imported conjugates has shown that in plant cell cultures fluorodifen-GS-conjugates were degraded to the corresponding γ-glutamylcysteine conjugate [9]. We therefore tested mesophyll protoplasts and different subcellular fractions for their ability to degrade nitrobenzyl-GS-conjugate (Table 2). The results revealed that extracts of chloroplasts were incapable of degrading nitrobenzyl-GS-conjugate. Rates were also very low in the cytosol as revealed in extracts of cytoplasts. Cytoplasts are plasma membrane surrounded vacuole-free cytoplasmic fractions of the protoplasts which are obtained from mesophyll protoplasts by ultracentrifugation on a continuous Percoll gradient. These compartments which have low or no peptidase activity can be contrasted with the high rates of degradation associated with extracts of intact protoplasts and isolated vacuoles. Statistically, the activities in protoplasts and vacuoles are not significantly different suggesting exclusively vacuolar localization of the carboxypeptidase. The low activity in the cytoplasm maximally accounts for 3.7% of the activity of the protoplasts and might well represent incomplete evacuolation of the cytoplasts. These results demonstrate the existence of a vacuolar carboxypeptidase which degrades glutathione-*S*-conjugates.

In an effort to further characterize this carboxypeptidase we purified the protein from barley primary leaves. Nitrobenzyl-GS was used as substrate to monitor carboxypeptidase activity during purification. Enzyme activity was 1.4 nmol nitrobenzyl-GS cleaved per mg protein per min in the crude extract. Specific activity increased 1081-fold in the course of consecutive fractionation by ammoniumsulfate precipitation, gel filtration and cation exchange chromatography (Table 3). Final yield was about 1% of initial total activity. This partially purified enzyme fraction was used for biochemical characterization of the carboxypeptidase. The stability of the enzyme was low; the enzyme activity dropped to 10–40% during a freeze-thaw cycle. The enzyme exhibited Michaelis-Menten type kinetics. The $K_m$ for nitrobenzyl-GS was 1.35 mM. $V_{max}$ was 16.5 nkat ml$^{-1}$. The apparent molecular mass of the native functional enzyme was estimated to be 55 ± 5 kDa from size exclusion chromatography. In order to analyze the reaction we then used the purified enzyme. Fig. 1 compares HPLC chromatograms of standard assays to which either boiled or purified native carboxypeptidase had been added. The substrate peak was not changed in the boiled sample after 1 h of incubation. In contrast, addition of active carboxypeptidase resulted in the appearance of the product peak. After 1 h, about 25% of the added substrate (0.5 μmol) was metabolized to this product.

The product of the reaction was identified as nitrobenzyl-γ-glutamyl-cysteine by two independent methods. (1) In HPLC

Table 2
Carboxypeptidase activity in protoplasts and subcellular fractions of the mesophyll

| Fraction | Degradation of nitrobenzyl-GS [nmol $10^{-6}$ cells h$^{-1}$] |
|---|---|
| Protoplasts | 135 ± 14 |
| Vacuoles | 147 ± 12 |
| Cytoplasts | 5 ± 4 |
| Chloroplasts | n.d. |

Protoplasts and subcellular fractions were isolated from barley leaves and quantified by markers as described earlier [6]. Carboxypeptidase activity in the respective fractions was determined by HPLC analysis and assayed as outlined in section 2.4. Data are means of three independent experiments ± standard deviations. n.d.=no degradation detectable under the conditions applied.

Table 3
Purification of carboxypeptidase from barley

| Step | Activity [nmol/min/ml] | Volume [ml] | Protein [mg] | Total activity [μmol/min/mg] | Specific activity [μmol/min/mg] | Purification factor |
|---|---|---|---|---|---|---|
| Crude extract | 0.074 | 220 | 187 | 0.28 | 0.0015 | 1 |
| $(NH_4)_2SO_4$ precipitate | 2.85 | 1.2 | 1.41 | 0.068 | 0.048 | 32.3 |
| Gel filtration | 0.265 | 6 | 0.214 | 0.016 | 0.074 | 49.5 |
| Cation exchange | 0.253 | 1 | 0.0015 | 0.0025 | 1.62 | 1081 |

Barley leaves were homogenized as described in section 2.4, the crude extract was subjected to differential $(NH_4)_2SO_4$ fractionation. Desalted protein was separated on a gel filtration column, fractions containing the nitrobenzyl-GS carboxypeptidase activity were pooled and applied to cation exchange chromatography (Fig. 2).

separations on reverse phase RP C18 columns (see section 2.3), the retention time of the product was identical with the authentic reference compound *S*-(*p*-nitrobenzyl)-γ-glutamyl-cysteine. (2) Product peaks (Fig. 1) were collected from HPLC separations. Analysis by electrospray mass spectrometry (Dr. Kaudewitz, personal communication) revealed a distinct molecular ion ([M+H]⁺) at m/z 386 in the positive mode corresponding to *S*-(*p*-nitrobenzyl)-γ-glutamyl-cysteine. In addition, a fragment ion at m/z 240 was detected which corresponds to the protonated *S*-(*p*-nitrobenzyl)-cysteine. Degradation of other GS-conjugates was also tested in the purified carboxypeptidase fraction and determined by HPLC co-chromatography (Table 4). Enzyme activity for the cleavage of *S*-(*p*-nitrobenzyl)-GS was 16-fold higher than for the degradation of *S*-(alachlor)-GS or *S*-(1-chloro,4-nitrobenzyl)-GS. Cleavage of *S*-(dinitrobenzyl)-GS and *S*-(epoxynitropropane)-GS was catalyzed at intermediate rates. Under the conditions applied, a synthetic *S*-(ethacrynic acid)-GS was not a substrate for the enzyme fraction.

Our data show that the glutathione *S*-conjugate transporter efficiently secretes conjugates in vivo from the cytosol into the vacuoles of plant cells. Vacuolar accumulation of the GS-conjugates was large. 97% of the conjugate was detected in the vacuolar fraction and only 3% was associated with the cytoplasm. This accumulation exceeds that previously reported from short term uptake experiments with isolated vacuoles in vitro [4]. Theoretical calculations based on $\Delta G'_0$ of ATP hydrolysis demonstrate that a much higher degree of vacuolar accumulation should be possible by the ATP-driven GS-conjugate pump. In fact, compartmentation analysis does not allow us to distinguish between 99.9% and 95% vacuolar compartmentation [10]. The degree of vacuolar localization of both the conjugate and the carboxypeptidase is likely to be higher. The pathway of consecutive modification, conjugation and export from the cytosol detoxifies exogenously applied xenobiotics. Following transport into the vacuole, a carboxypeptidase degrades GS-conjugates by cleaving the glycine

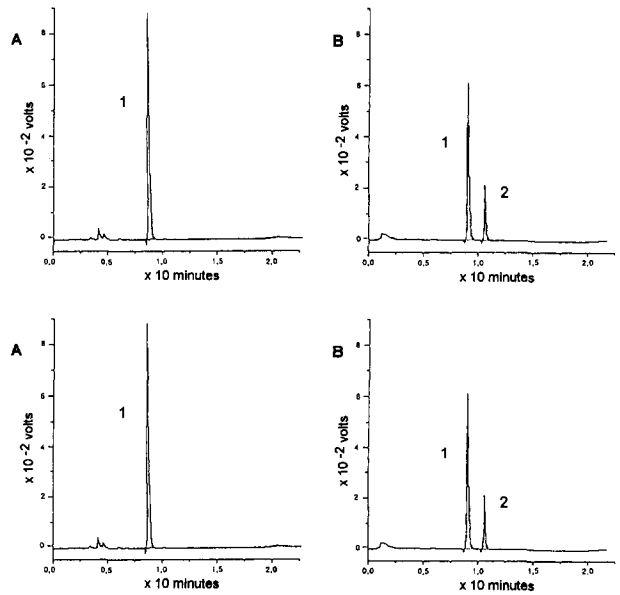


Fig. 1. HPLC separation of substrate (1: *S*-(*p*-nitrobenzyl)-glutathione) and product (2: *p*-(nitrobenzyl)-γ-glutamyl-cysteine) of the carboxypeptidase reaction. Purified carboxypeptidase (cf. Fig. 2) was added to the standard assay (see section 2.3) either after boiling (A) or in its native form (B). The sample was incubated for 1 h, followed by addition of acetone and analysis as described above.

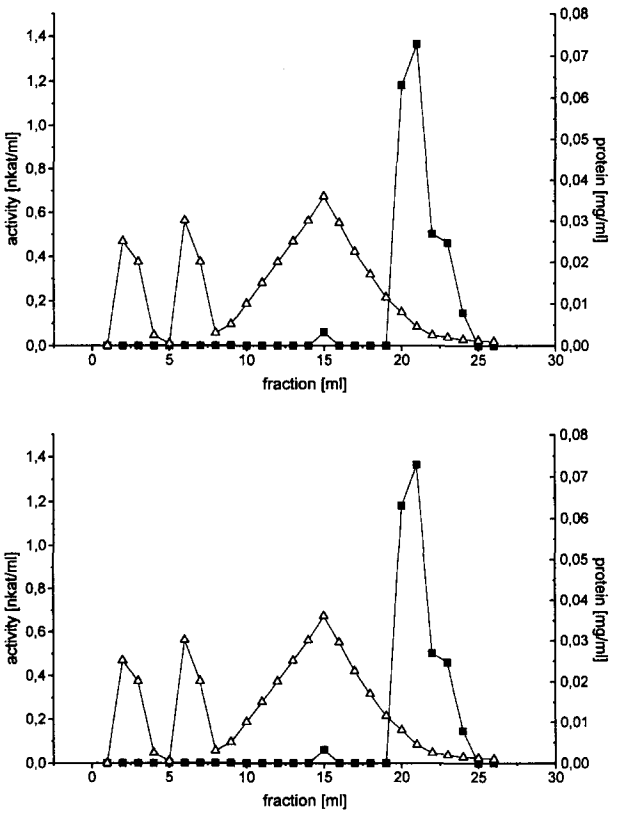


Fig. 2. Separation of a protein sample (3 mg/ml) from barley leaves (see section 2.4) on a cation exchange column (Resource S, Pharmacia). Distribution of protein (△) and carboxypeptidase activity (■) as eluted with an ascending NaCl gradient (0–0.5 M).

Table 4
Degradation of various GS-conjugates [μmol/mg]

| | |
|---|---|
| *S*-(*p*-Nitrobenzyl)-glutathione | 1.6 ± 0.1 |
| *S*-(Dinitrobenzyl)-glutathione | 0.3 ± 0.05 |
| *S*-(Alachlor)-glutathione | 0.1 ± 0.02 |
| *S*-(1-Chloro,4-nitrobenzyl)-glutathione | 0.1 ± 0.02 |
| *S*-(Epoxynitropropane)-glutathione | 0.3 ± 0.1 |
| *S*-(Ethacrynic acid)-glutathione | n.d. |

GS-conjugates were synthesized as described previously [7] and incubated with purified carboxypeptidase. Degradation products were determined by HPLC co-chromatography (see section 2.3). Data are means of three replicate measurements ± standard deviations. n.d. = no degradation detectable under the conditions applied.

residue. Evidence is at hand that the amino terminal glutamic acid residue is also cleaved successively [1]. It can be assumed that not only xenobiotic GS-conjugates but also naturally occurring GS-conjugates in the vacuole, like the recently described anthocyanin-glutathione [11] are metabolized by the carboxypeptidase. This degradation may be the important step of removing the glutathione tag from the transfer intermediate that leads to the final pigment molecule. Another function may be the recycling of amino acids that are bound to GS-conjugates or contained in oxidized glutathione and phytochelatins. Both compounds have also been shown to be transported into the vacuole [12,13], the latter as complexes of Cd or Zn and phytochelatins. This transport is part of the reactions involved in heavy metal detoxification. It may be concluded that the carboxypeptidase has important functions not only in xenobiotic metabolism but also in processing of secondary pigments as well as in glutathione and phytochelatine degradation. Our results are in line with data suggesting the plant vacuole as a metabolically active organelle and not just a compartment for permanent or transient storage.

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