Visualization of Glutathione Conjugation and Inducibility of Glutathione S-Transferases in Onion (*Allium cepa* L.) Epidermal Tissue

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Glutathione conjugation of 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, NBD-Cl, monobromobimane and monochlorobimane was found to occur in epidermal tissue of onion bulbs (*Allium cepa* L.). Conjugation required the presence of glutathione S-transferases (GST). In order to follow glutathione conjugation microscopically, bimanes were utilized. Monochlorobimane was converted to a brightly fluorescing conjugate that was shown to be transported to the nucleus before sequestration in the vacuole occurred. GST activity was stimulated as well as induced by several electrophilic xenobiotics, by cycloheximide and by several glutathione conjugates. γ-Glutamylcysteine conjugates that are formed during enzymatic cleavage of glutathione conjugates in plants, were not active as inducers of enzyme activity. In the light of the stimulating effects of xenobiotic glutathione conjugates on GST activity, it is concluded that glutathione conjugates may act as signal molecules.

Introduction

Plant metabolism of xenobiotics may be subdivided into three phases (Coupland, 1991). In phase I xenobiotic molecules are activated via oxidation, reduction or hydrolysis, and in phase II detoxification is achieved by conjugating biomolecules, e.g. sugars, amino acids or reduced glutathione (GSH) to the activated sites. Compounds with sufficiently high electrophilicity may be conjugated without activation. Phase III is characterized as cleavage, secondary conjugation and metabolization of conjugates and may include compartimentation into the vacuole, the apoplast or the cell wall (Lamoureux and Rusness, 1989; Schröder, 1997).

Glutathione S-transferases (EC 2.5.1.18) are multifunctional phase II detoxification enzymes catalyzing the nucleophilic attack of GSH to electrophilic sites of xenobiotics. GST spring from multigene families and are distributed ubiquitously amongst aerobic organisms. Most reports on plant GST are from crops and in close relation to pesticide detoxification (Lamoureux and Rusness, 1989). Few data are available for GST from weeds (Cole, 1994; Hatton *et al.*, 1996) and from non-agricultural plants. In most cases, multiple GST isozymes have been determined in the investigated plant species. The natural roles and the regulation of these enzymes have not been understood so far, but the involvement of GST in numerous plant responses to environmental conditions is well documented.

Recently evidence for the sequestration of xenobiotic and model conjugates in plant vacuoles has been presented *in vitro* (Tommasini *et al.*, 1995) and *in vivo* (Wolf *et al.*, 1996; Coleman *et al.*, 1997). In microscopic studies using the glutathione conjugate of monochlorobimane (bimane-GS), the process has been even visualized (Coleman *et al.*, 1996; Fricker *et al.*, 1997). The glutathionyl-moiety seems to act as a tag for transport of the conjugate. A physiological role for glutathione conjugates has, however, not been elucidated so far.

It was the aim of this study to visualize GST-dependent conjugate formation, the transport of the conjugates in the cells and to monitor the effect of xenobiotics and their respective glutathione

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EPNP, 1,2-epoxy-3(4-nitrophenoxy)-propane; γ-GC, γ -glutamyl-cysteinyl-residue; GS, glutathionyl-residue; GSH, glutathione, reduced; GST, glutathione S-transferase; MBBr, monobromobimane; MCB, monochlorobimane; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

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conjugates on the activity of GST in onion. In the context of pesticide application and pesticide fate in the plant, these investigations are expected to be important for agricultural practice.

Materials and Methods

Plant material

For the experiments, the inner epidermal layers of storage leaves in the bulbs of onions (*Allium cepa* L. cv. Zittauer gelbe and cv. Ailsa Craig) obtained after harvest from farms close to Munich, Germany, were peeled using a razor blade and tweezers and immersed into tap water immediately after harvest. All chemicals used for the experiments were of highest available analytical grade.

Visualization of glutathione conjugation

Freshly prepared isolated epidermal layers from onion bulbs were transferred to microscopic cover slides in approx. 150 µl of tap water. The slides were mounted as a surface in a special measuring chamber. The chamber was fixed on the stage of an inverse fluorescence microscope (Zeiss IM 35).

To determine the fluorescence excitation spectrum of the GSH-conjugate, aliquots of monochlorobimane were added to the cells in the measuring chamber and the cells were illuminated after 20 min with wavelengths between 320 nm and 500 nm in steps of 1 nm. The fluorescence intensity was measured by a photomultiplier for each wavelength (Fig. 1). Excitation with 380 nm wavelength led to a maximum of fluorescence emission at 510 nm.

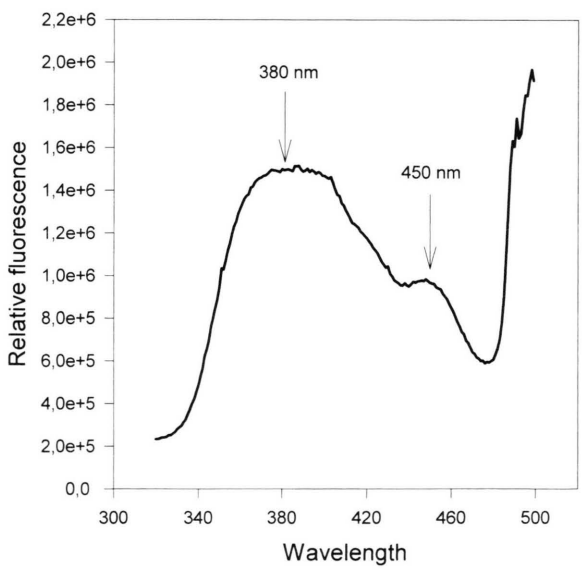

Fig. 1. Spectral properties of the glutathionyl-bimane conjugate.

To visualize and localize the development of GSH-conjugate a light intensified camera (Hamamatsu, Japan) combined with an imaging system (Applied Imaging, Sunderland, UK) was used and the fluorescence was excitated with UV-light (380 nm). The epidermal layers were prepared as described above. After addition of an aliquot of monochlorobimane (15 µM) the cells were illumiated every 10 seconds with light of 380 nm and a picture (256 × 256) was obtained. The regions of interest were defined by using a light pen and the grey levels for each area were determined.

Incubation experiments

In order to study the effects of xenobiotics on peeled onion epidermal tissue epidermal layers were placed on droplets of water in Petri dishes. Different concentrations of the xenobiotic under consideration were added to the water droplets and the epidermal tissue was allowed to rest for the respective duration of the incubation. The incubations were terminated by removing the incubation liquid and washing the tissue several times with tap water. Before freezing the tissue specimens in liquid N_2 they were blotted on lab tissue to remove excess liquid from the surface.

Enzyme extraction

Onion bulb epidermal tissue was frozen in liquid N_2 and ground to a fine powder with mortar and pestle. Ten volumes (v/w) of 100 mM potassium phosphate buffer (pH 7.8), containing 10 mM dithioerythritol, 5 mM EDTA, 1% Nonidet P40, and 10 $mg \cdot ml^{-1}$ soluble polyvinylpyrrolidone were added. After an incubation of ten minutes, the slurry was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 30,000×*g* for 30 min. Solid ammonium sulfate was added to the supernatant (designated as crude extract) to give a saturation of 40%. After stirring for 30 min, precipitated proteins were removed by centrifugation at 48,000×*g* for 25 min. The supernatant was dec-

anted and adjusted to 80% ammonium sulfate saturation. After stirring for 30 min the solution was centrifuged as described for the first step. The resulting pellet was resuspended in 2 ml 20 mM Tris[-hydroxymethyl]aminomethane-HCl, pH 7.8. The extract was desalted by gel filtration (PD 10, Pharmacia) and stored at −80 °C until use. Microsomes were extracted following published methods (Boll and Kardinal, 1990, Schröder and Belford, 1996). In short, homogenized tissue in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.4 M sucrose, 0.01 M $MgCl_2$, 0.02 M EDTA, 0.01 M DTE and 10% (w/v) PVP K40 was sonicated for 30 sec. The homogenate was centrifuged for 10 min at 4,000×*g* and the supernatant was further centrifuged for 40 min at 18,000×*g*. The pellet was washed in the above buffer and recentrifuged. The supernatant of the first 18,000×*g* step was further centrifuged for 60 min at 100,000×*g*. The pellet as well as the 18,000×*g* pellet were suspended in 0.5 ml KH_2PO_4 containing 0.01 M EDTA, 0.005 M $MgCl_2$ and 0.05 M DTE.

Enzyme assays and protein determination

GST activity was determined spectrophotometrically using CDNB and DCNB as model substrates according to Habig *et al.*, (1974) using the assay method of Schröder *et al.*, (1990). GST assays with EPNP as electrophilic substrate were carried out as described by Fjellstedt *et al.*, (1973). For the determination of GST activity with NBD-Cl the method of Ricci *et al.*, (1994) was slightly modified: To sodium acetate buffer (100 mM; pH 4.5), 0.05 mM NBD-Cl dissolved in ethanol and 0.5 mM GSH were added. The reaction was started by addition of varying amounts of enzyme extract to give a final volume of 1200 µl. Product release was calculated by detecting the increase of the absorption at 270 nm (ε = 5.0 $mM^{-1} \cdot cm^{-1}$). Conjugation of MCB was followed by the development of fluorescence at 385 nm excitation and 485 nm emission in a fluorescence photometer in an enzyme assay consisting of 100 mM potassium phosphate buffer, pH 6.4, 5 mM chlorobimane in EtOH and 1 mM GSH. The reaction was started by addition of enzyme to the assay. Enzyme activity (= U) is expressed as µmol product formation min^{-1}, specific activity as U (mg protein)$^{-1}$. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Conjugate synthesis

Glutathione and γ-glutamylcysteine conjugates of CDNB and chlorobimane were synthesized in 100 mM potassium phosphate buffer, pH 7.8 in the presence of excess glutathione or γ-glutamylcysteine. After several days, when the reaction was complete, the mixture was vacuum evaporated and resuspended in acetonitrile/water. Aliquots were then chromatographed via semipreparative HPLC on C18-columns following standard procedures (Schröder *et al.*, 1990) and monitored by UV-Vis detection. Collected peaks co-chromatographing to authentic standards of GS-conjugates were again vacuum evaporated to drieness and stored dark until use.

Results

GST activity for several xenobiotic substances was detected in protein extracts from onion epidermal tissue. The activities were not constant throughout the day but followed a diurnal rhythm connected to the general diurnal protein turnover (data not shown). Activity was present in cytosolic as well as in microsomal fractions (Table I).

Table I. Specific activity of glutathione S-transferase [µmol min^{-1} mg^{-1}] in onion epidermal tissue with different xenobiotic substrates. Data are means of 2 to 3 independent determinations ± S. D.

	Substrate				
	CDNB	DCNB	EPNP	MCB	MBB
Crude extract	279.8 ± 21.9	0.1 ± 1.7	132.3 ± 51.2	21.8 ± 1.9	709.9
Ammonium sulfate, 40–80% saturation	892.3 ± 47.3	7.8 ± 2.6	47.1 ± 21.1	36.7 ± 5.9	1775.7
Microsomes	98.6 ± 15.7	85.8 ± 33.3	794.0 ± 148.6	nil	nil

CDNB and MBB conjugation occurred at highest rates, the latter with extremely high nonenzymatic rates. However, in contrast to CDNB, MBB conjugation was only found in the cytosol. Unlike to the conditions in the cytosol, activity for the conjugation of CDNB and DCNB was equal in microsomal fractions extracted from the same tissue. EPNP was conjugated at highest rates by microsome-associated GST under the conditions applied. MCB activities were moderate and solely cytosolic, but resulted in the formation of a brightly fluorescing conjugate. Under the conditions applied *in vitro*, conjugate formation was only slowly proceeding in assay systems whithout GST present, but significant nonenzymatic formation of a glutathione conjugate occurred at pH values above 7.6 (Fig. 2).

The development of fluorescence in onion epidermal preparations *in vivo* was followed microscopically. Fluorescence development occurred rapidly within a few minutes after incubation of epidermal strips floating on droplets of tap water with MCB and was found to begin almost simultaneously in the cytosol close to the cell boundary and the nuclear envelope of the epidermal cells (Fig. 3b). When fluorescence development was monitored in nuclear, vacuolar and cytosolic positions (Fig. 4), cytosolic and vacuolar fluorescence showed the same increase, which is most probably due to an overshining of cytosolic fluorescence into the vacuole. After 3 min, nuclear fluorescence exceeded cytosolic fluorescence significantly (Fig. 4). Interestingly, cytosol and nuclear fluorescence seemed to be levelling off after 10 min and after 15 min, bright fluorescence in the vacuole appeared. This is characterized by a steep increase of the fluorescence curve in Fig. 4. At the end of

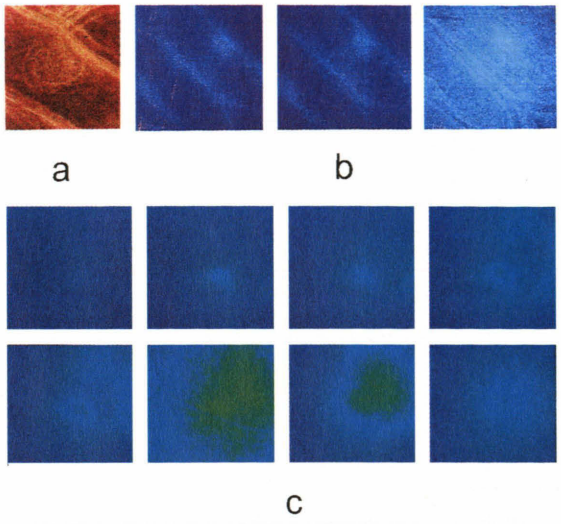

Fig. 3. Images of fluorescence development in onion epidermal cells.
a: light microscopy of the investigated tissue, b: with overshining fluorescence, c: time course of fluorescence development corresponding with Fig. 4.

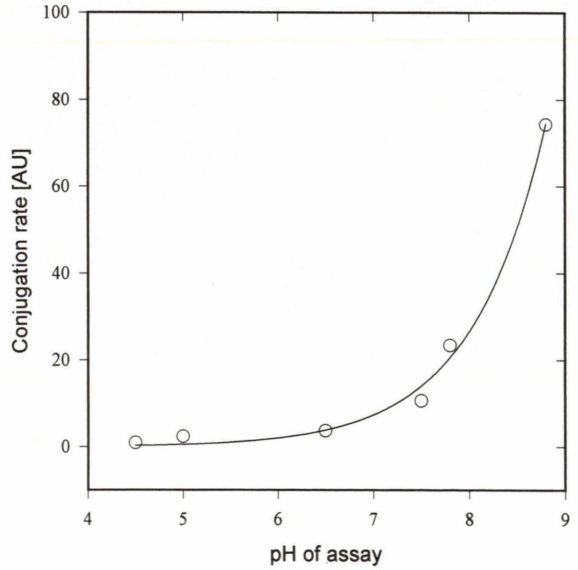

Fig. 2. pH dependent non-enzymic conjugation of monochlorobimane (AU = arbitrary unions) with reduced glutathione. 5 mM MCB was incubated with surplus GSH in different buffers.

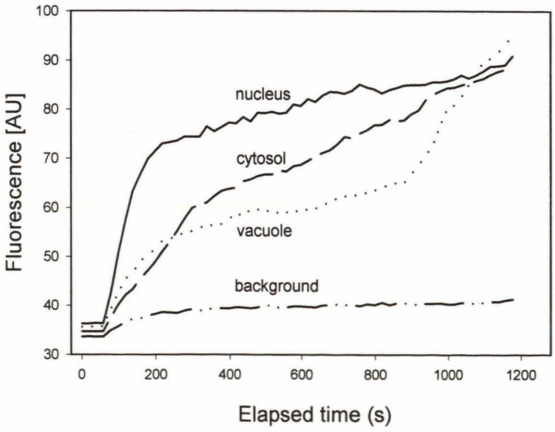

Fig. 4. Typical development of fluorescence (AU = arbitrary unions) in onion epidermal cells as monitored microscopically in a single cell (see Fig. 3).

Table II. Concentration-dependent changes of glutathione S-transferase activities [μmol min^{-1} mg^{-1}] in extracts from onion epidermal tissues after 45 min of chlorobimane (IIA) or transstilbeneoxide (IIB) incubation with the concentrations given in the table. Data are means of 3 determinations ± SD from 3 independent experiments.

A

	Monochlorobimane [μM]						
	Control	10		50		100	
Substrate	mean	mean	x-fold	mean	x-fold	mean	x-fold
CDNB	25.40 ± 10.7	50.21 ± 1.9	2.0	155.9 ± 13.1	6.1	26.6 ± 5.0	1.0
DCNB	3.80 ± 1.8	1.99 ± 0.5	0.5	1.38 ± 1.1	0.4	9.9 ± 0.6	2.6
NBD-Cl	35.33 ± 10.3	39.5 ± 2.4	1.1	101.7 ± 10.4	2.8	13.9 ± 3.0	0.4

B

	Transstilbenoxide [μM]						
	Control	10		50		100	
Substrate	mean	mean	x-fold	mean	x-fold	mean	x-fold
CDNB	106.7 ± 6.4	183.8 ± 11.0	1.7	259.2 ± 18.1	2.4	65.99 ± 4.5	0.6
DCNB	2.3 ± 0.08	3.33 ± 0.5	1.4	2.52 ± 0.8	1.1	0.94 ± 0.5	0.4
NBD-Cl	175.6 ± 6.8	274.8 ± 0.5	1.6	326.1 ± 13.1	1.9	122.1 ± 6.7	0.7

the experiments, the epidermal cells were still alive, showing plasma circulation, and exhibited a slight total fluorescence with bright halos at nuclear and vacuolar positions. The general fluorescence is thought to be attributable to a transport and preferential distribution of the conjugate into the vacuole of the cells, as could be shown with isolated vacuoles.

On the enzymological level, the incubation of onion epidermal cells with MCB (Table II) led to rapid and drastic changes in glutathione S-transferase activity. The greatest effects were observed with concentrations between 10 and 50 μM of the xenobiotic. After 45 min of incubation with 10 μM and 50 μM MCB, GST activity for CDNB was 1.3 and 5.1fold higher, respectively, than in control tissues. Similarly, activity for the conjugation of NBD-Cl increased strongly. However, the inductive effect of the conjugate donation disappeared at concentrations above 50 μM. DCNB conjugation was affected inversely (Table II). Transstilbenoxide, added in concentrations of 10 and 50 μM caused low induction of CDNB, DCNB and NBD-Cl conjugation rates. DCNB conjugation rate dropped already at 50 μM and inhibition of all activities by 50% occurred at 100 μM concentrations (Table II B).

In time studies using the bimane-GS and -γGC or chloronitrobenzene-GS and -γGC conjugates as inducers of onion GST, the described effects could be clarified. Fig. 5 displays the consequences of the donation of xenobiotic conjugates to peeled onion epidermal cell layers. The glutathione conjugate of chlorobimane influenced GST activity strongly within 1 hr and led to doubling of the conjugation rates for CDNB and NBD-Cl (Fig. 5A,B). Two hrs after application, however, the effect was lost. DCNB conjugation was inhibited strongly and irreversibly by the donation of the bimane-GS conjugate. Higher concentrations of the conjugate interestingly did not cause the same or stronger effects on CDNB and NBD-Cl conjugation, however, DCNB activity was inhibited by 100% (Fig. 5B).

The chloronitrobenzene-GS caused an almost 200% induction of NBD-Cl conjugation (Fig. 5C, D), whereas at the same time CDNB conjugation remained unchanged and DCNB conjugation was inhibited by 50%. Donation of 20 μM conjugate caused only smaller effects (Fig. 5D). Generally, γ-GC-conjugates of the bimane as well as of CDNB did not lead to the dramatic effects observed after donation of the respective glutathione conjugates, except for some significant inhibition of DCNB conjugation (Fig. 5 E-H).

The transient effects on GST activity shown in Fig. 5 were studied further. When onion epidermis was exposed to 100 μM cycloheximide, which was

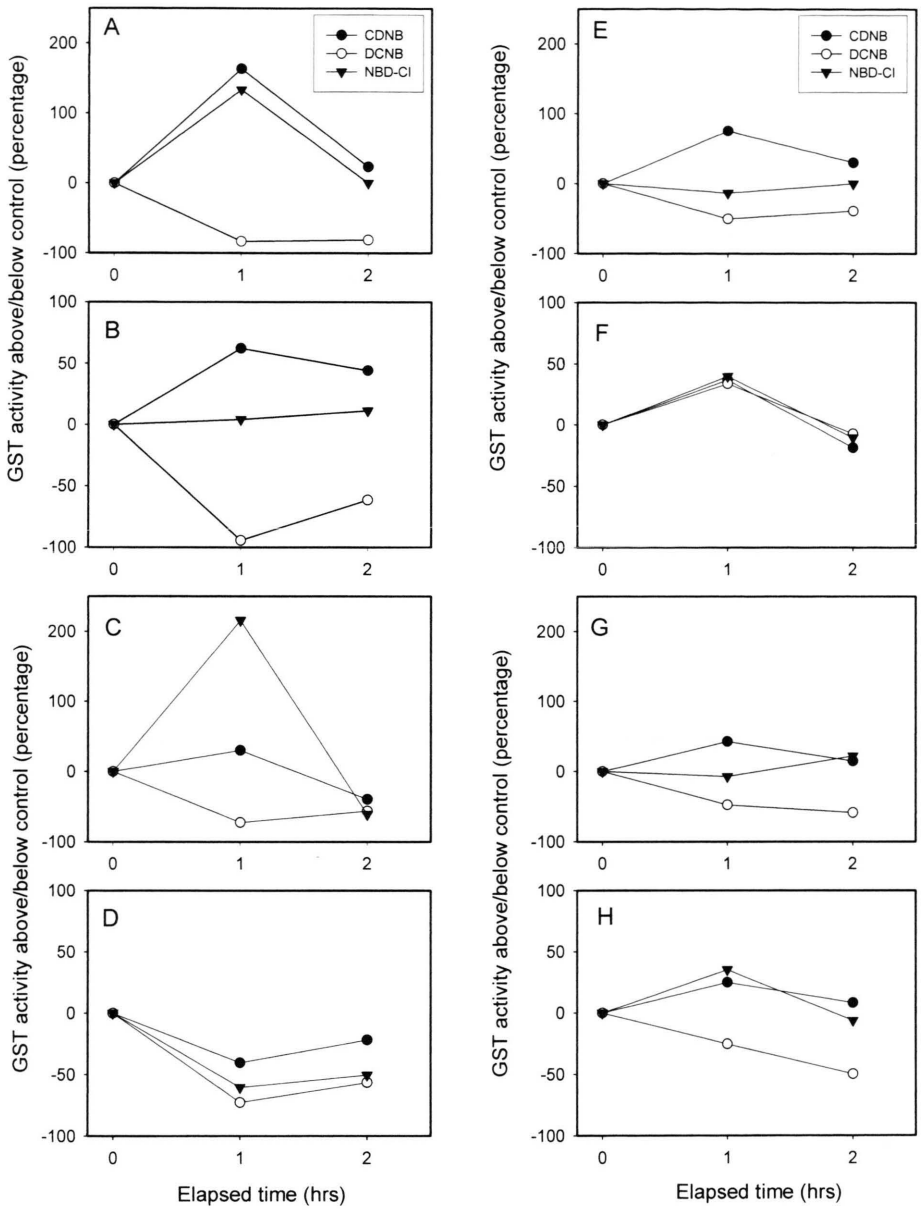

Fig. 5. Modulation of GST activity in onion epidermal tissue by xenobiotic conjugates. Data are means of 2 to 4 independent experiments. A, B: Bimane-SG conjugate at 10 μM and 50 μM concentrations, respectively; C,D: DNB-SG conjugate at 10 μM and 50 μM concentrations, respectively; E,F: Bimane-γ-GC conjugate, G,H: DNB-SG conjugate, tested under identical conditions.

originally designed as a control to inhibit *de-novo*-synthesis of GST, significant and transient increases of CDNB and NBD-Cl conjugation were observed within the first 2 hrs of the experiment. The increases made up almost 100% above control values but were lost after 3.5 hrs. After that point, losses of GST activity were observed (Fig. 6A).

When CDNB was added to the epidermal onion tissue, the same transient effect with high activity after 2 hrs and an activity drop after an additional

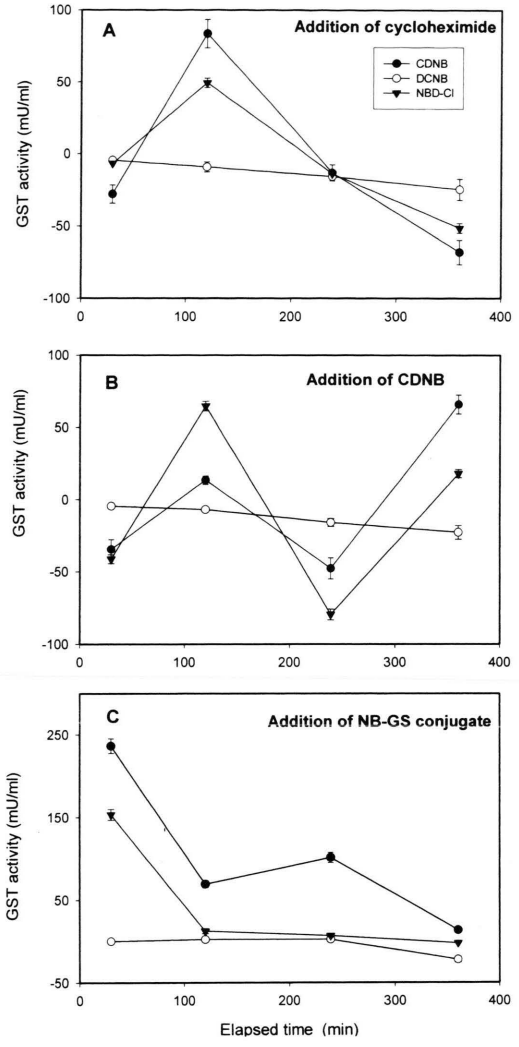

Fig. 6. Modulation of GST activity in onion epidermal tissue by xenobiotics. Data are means of 2 to 4 independent experiments. A: Addition of cycloheximide, B: Addition of CDNB, C: Addition of NB-GS conjugate.

hr was observed, but after 4 hrs, a renewed increase of GST activity could be recorded (Fig. 6B). Addition of nitrobenzyl-glutathione, a commercially available glutathione conjugate, caused strong increases of GST activity within the first 45 min of the experiment and a constantly decreasing GST activity to the control values during the following 5 hrs (Fig. 6C). In none of the different time studies, DCNB activity was altered significantly.

Administering cycloheximide together with either CDNB or nitrobenzyl glutathione led to the same changes in the patterns of GST activity with time (Fig. 7). The previously observed transient stimulation within the first phase of the experiment (0 to 2 hrs) was not observed here, but a strong transient increase of GST activities for CDNB and NBD-Cl conjugation was measurable after 4 hrs (Fig. 7 A,B).

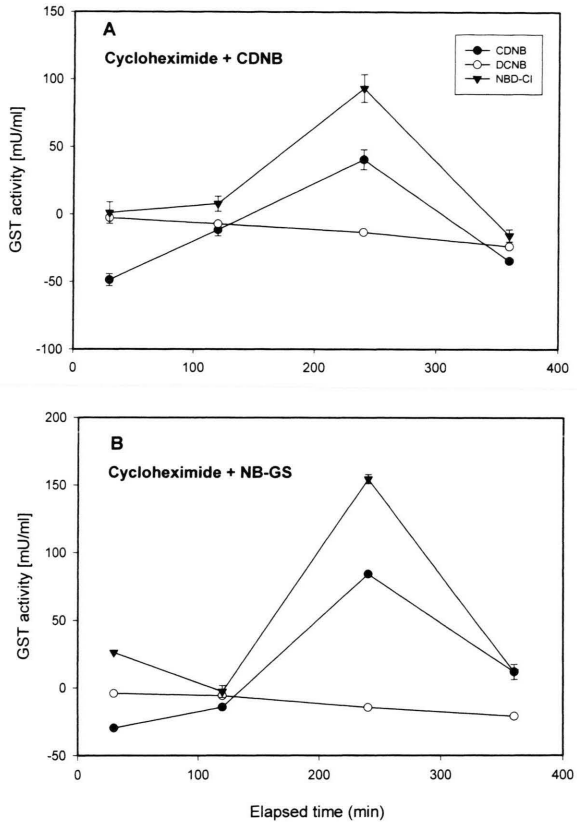

Fig. 7. Combination effects of xenobiotics on GST activity in onion epidermal tissue. Data are means of 2 independent experiments, error bars indicate standard deviations. A: Combination of cycloheximide and CDNB; B: Combination of cycloheximide and NB-GS conjugate.

Discussion

As in other plant tissues, glutathione S-transferase activity for the conjugation of various electrophilic substances is present in the epidermal layers of onion bulb storage leaves. Although no attempts have been made to purify isoforms of the enzyme in this study, it is apparent from the results

that at least two groups of isoforms of GST exist in onion leaf epidermal tissue. They may be distinguished by their affinity for DCNB (Habig and Jakoby, 1981, Schröder and Götzberger, 1997). Furthermore, there is considerable GST activity associated with the microsomal fraction of the cells. However, this microsomal GST does not have activity with bimanes.

GST activity in the onion tissue is not static in amount and quality, but may be subject to changes due to environmental parameters (Schröder *et al.*, 1992, Schröder and Wolf, 1996). Our results show that GST activity in onion may be both, enhanced as well as inhibited by xenobiotic substances. Furthermore, stimulation may either proceed quickly and without lag phases or it requires a longer period of time in the range of hours. Similar effects have been observed when plants had been exposed to herbicides and safeners (Fuerst and Lamoureux, 1992). Moreover, it is not clear whether the observed fast stimulation occurs via binding of the xenobiotics investigated at noncatalytic sites of the enzyme protein or whether it requires signalling and mediators. True induction is thought to proceed via gene activation and de-novo synthesis of the proteins (Schröder and Pflugmacher, 1996).

The fact that stimulation of GST activity under the influence of MCB is different from effects caused by transtilbeneoxide, a well known inducer of animal GST (Hayes and Pulford, 1996), implies that various effects may be responsible for the dynamics of enzyme activity. In any case, concentrations of 100 μM of the respective xenobiotic did not act stimulating but rather inhibitory to the enzyme activity. This may be due to a stress situation initiating a regulatory program in the tissue that is different from the short-term stimulation that has frequently been observed (see also Schröder *et al.*, 1992, Pflugmacher and Schröder, 1994). In onion tissue, even the potent inhibitor of translation, cycloheximide, transiently increased GST activity for selected substrates before general protein losses led to a decreased GST content. This is different from the findings in spruce needles, where incubation of spruce cuttings with cycloheximide or puromycin led to dramatic and irreversible losses of GST activity within 2 hrs of treatment (Schröder and Pflugmacher, 1996). It has to be pointed out that cycloheximide may also act as ionophore in elicitor treated cells (Messner and Schröder, 1999) causing effects comparable to pathogene attack to the tissue. Under these circumstances, transient GST induction is frequently observed. The mechanism is yet not understood.

Glutathione conjugates seem to play a pivotal role in the course a of the intracellular signalling process responsible for GST activation, whereas γ-GC conjugates are somewhat inactive. Similar results have been obtained when p-nitrobenzoylchloride, nitrophenylglutathione and dinitrophenylcysteine were used as modulators of GST in spruce. CDNB-conjugation was induced within 15 hrs by all compounds employed, DCNB conjugation was lost with the first and the last compound, and pNBC conjugation was sensitive to the glutathione conjugate (Schröder *et al.*, 1993).

From the above findings and also from the fact that in the present study fluorescence development due to glutathione conjugate accumulation from the xenobiotic, chlorobimane, occurred in the nuclear envelope or in the nucleus, it might be concluded, that some glutathione conjugates may serve as signal molecules for the stimulation of GST activity and for other purposes as well. The signal is deactivated by transport of the conjugate into the vacuole and a subsequent metabolism of the glutathionyl-moiety (Schröder, 1997). This working hypothesis sheds new light on the observation that glutathione conjugates seem generally and rapidly to be sequestered either into the vacuole by plants (Marrs *et al.*, 1995, Wolf *et al.*, 1996, Coleman *et al.*, 1997) or out of the cells in animal tissue (Ishikawa *et al.*, 1987). It is noteworthy that enzymes further downstream, such as the cysteine lyases, seem to be inducible by xenobiotics, glutathione conjugates and their metabolic products (Schröder *et al.*, 1993) by enhancing the velocity of conjugate breakdown.

Acknowledgements

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