PHYSIOLOGIA PLANTARUM 99: 271–278. 1997
Printed in Denmark – all rights reserved

ISSN 0031-9317

Peroxidase-mediated conjugation of glutathione to unsaturated phenylpropanoids. Evidence against glutathione S-transferase involvement

John V. Dean and Timothy P. Devarenne

Dean, J. V. and Devarenne, T. P. 1997. Peroxidase-mediated conjugation of glutathione to unsaturated phenylpropanoids. Evidence against glutathione S-transferase involvement. – Physiol. Plant. 99: 271–278.

A number of plant species are thought to possess a glutathione S-transferase enzyme (GST; EC 2.5.1.18) that will conjugate glutathione (GSH) to *trans*-cinnamic acid (CA) and *para*-coumaric acid (4-CA). However, we present evidence that this activity is mediated by peroxidase enzymes and not GSTs. The N-terminal amino acid sequence of the GSH-conjugating enzyme purified from etiolated corn shoots exhibited a strong degree of homology to cytosolic ascorbate peroxidase enzymes (APX; EC 1.11.1.11) from a number of plant species. The GSH-conjugating and APX activities of corn could not be separated during chromatography on hydrophobic-interaction, anion-exchange, and gel filtration columns. Spectral analysis of the enzyme revealed that the protein had a Soret band at 405 nm. When the enzyme was reduced with dithionite, the peak was shifted to 423 nm with an additional peak at 554 nm. The spectrum of the dithionite-reduced enzyme in the presence of 0.1 m*M* KCN exhibited peaks at 430, 534 and 563 nm. These spectra are consistent with the presence of a heme moiety. The GSH-conjugating and APX activities of the enzyme were both inhibited by KCN, NaN_3, *p*-chloromercuribenzoate (*p*CMB), and iodoacetate. The APX specific activity of the enzyme was 1.5-fold greater than the GSH-conjugating specific activity with 4-CA. In addition to the corn enzyme, a pea recombinant APX (rAPX) and horseradish peroxidase (HRP; EC 1.11.1.7) were also able to conjugate GSH to CA and 4-CA. The peroxidase enzymes may generate thiyl free radicals of GSH that react with the alkyl double bond of CA and 4-CA resulting in the formation of a GSH conjugate.

Key words – Ascorbate peroxidase, corn, glutathione S-transferase, horseradish peroxidase, *para*-coumaric acid, *trans*-cinnamic acid, *Zea mays*.

J. V. Dean (corresponding author, e-mail jdean@condor.depaul.edu) and T. P. Devarenne, Dept of Biological Sciences, DePaul Univ., Chicago, IL 60614, USA.

Introduction

Several plant species have been shown to possess an enzyme that will conjugate glutathione (GSH) to either *trans*-cinnamic acid (CA) or *para*-coumaric acid (4-hydroxycinnamic acid; 4-CA; Diesperger and Sandermann 1979, Edwards and Owen 1987, 1988, Dean et al. 1991, 1995, Edwards and Dixon 1991, Dean and Machota 1993). Originally, this enzyme was thought to be a glutathione S-transferase (GST). However, this particular GST enzyme was reported to possess some very unusual properties when compared to other plant or animal GSTs (Dean et al. 1995). For instance, the enzyme was found to exist as a monomer rather than a dimer; the enzyme was able to utilize Cys as a sulfhydryl source as well as GSH; and when CA was used as the substrate the in vitro activity could be enhanced by 4-CA and 7-hydroxycoumarin (Dean and Machota 1993, Dean et al. 1995). Although sequence information regarding the structure of this enzyme was not available, it seemed reasonable to conclude that the corn enzyme was a GST since mammalian and insect tissues are known to con-

Received 13 March, 1996; revised 23 September, 1996

tain GSTs that will catalyze the conjugation of GSH to a number of α,β-unsaturated compounds (Boyland and Chasseaud 1967, Habig et al. 1974, Ålin et al. 1985, Mannervik 1985, Wadleigh and Yu 1987, Berhane et al. 1994).

In addition to the reactions catalyzed by GSTs, it has also been shown that peroxidase enzymes can catalyze the conjugation of GSH to alkyl double bonds (Stock et al. 1986, Foureman and Eling 1989). However, peroxidase-mediated GSH conjugation has not received nearly as much attention as GST-mediated GSH conjugation. Peroxidase enzymes are generally divided into two groups depending on their substrate specificities and physiological function. The guaiacol peroxidases have a very broad substrate specificity and appear to be involved in a number of physiological processes including the biosynthesis of lignin and ethylene and the degradation of indole-3-acetic acid (Asada 1992). Horseradish peroxidase (HRP) is typically described as the prototype for this first group. The second group of enzymes are typically referred to as ascorbate peroxidases (APX) because of their specificity for ascorbate. The physiological role of the second group appears to be the scavenging of hydrogen peroxide (Asada 1992). We present evidence in the present report that in addition to the functions described above, both guaiacol peroxidases and APX can also catalyze the conjugation of GSH and Cys to unsaturated phenylpropanoids, and that the corn enzyme originally described as a GST (Dean et al. 1995) is actually a cytosolic APX. In addition, we propose a mechanism for the peroxidase-mediated GSH conjugation that is dependent on the formation of thiyl free radicals.

Abbreviations – APX, ascorbate peroxidase; CA, *trans*-cinnamic acid; 4-CA, 4-hydroxycinnamic acid (*para*-coumaric acid); GST, glutathione S-transferase; HRP, horseradish peroxidase; *p*CMB, *para*-chloromercuribenzoate; rAPX, recombinant ascorbate peroxidase.

Materials and methods

Plant material and chemicals

Corn (*Zea mays* L. Pioneer hybrid 3906) seeds were grown in vermiculite in the dark for 3 days as described by Dean et al. (1995). [U-^{14}C]CA and [U-^{14}C]4-CA were enzymatically synthesized from L-[U-^{14}C]Phe or L-[U-^{14}C]Tyr (New England Nuclear, Boston, MA, USA), respectively, as described by Dean et al. (1995). The pea recombinant APX (rAPX) was from Patterson and Poulos (1994). HRP type VI and all other chemicals were purchased from Sigma Chemical Company.

Enzyme assays and purification

In all experiments (except during column chromatography), the GSH-conjugating activity with 4-CA was determined with the radiochemical assay as described by Dean et al. (1995), except that the time of the assay was reduced to 2 min. The GSH-conjugating activity with 4-CA during column chromatography experiments was measured with the colorimetric assay as described by Dean et al. (1995). GSH-conjugating activity with CA was determined as described by Dean and Machota (1993). APX activity for the corn enzyme was determined as described by Koshiba (1993), and APX activity with rAPX and HRP was determined as described by Mittler and Zilinskas (1991). When inhibitors were included in the assay medium, the enzyme was preincubated with various concentrations of the inhibitor for 5 min before the start of the assay. All of the inhibitors used were soluble up to 10 m*M* in the assay media used to determine the GSH-conjugating activity. However, the APX assay for the corn enzyme was conducted at pH 5.5 which limited the solubility of *p*-chloromercuribenzoate (*p*CMB) to 1.0 m*M*. In order to alleviate this problem, the pH of the APX assay medium was adjusted to 8.0 for experiments conducted in the presence of 10 m*M* *p*CMB. The inhibition seen at 10 m*M* *p*CMB was compared to control assays (absence of inhibitor) also conducted at pH 8. The corn enzyme was purified from etiolated corn tissue as described by Dean et al. (1995).

Absorption spectra

The purified corn enzyme was desalted on Sephadex G-25 columns (PD-10, Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with 50 m*M* potassium phosphate buffer, pH 7.5, for the determination of the absorption spectrum of the oxidized enzyme. The oxidized enzyme was reduced by the addition of 0.3 mg of dithionite per 1 ml of enzyme solution, and the reduced enzyme spectrum was determined. The reduced enzyme was then treated with potassium cyanide (KCN; 0.1 m*M*) and the spectrum was again determined.

Protein sequencing and sequence analysis

The purified corn enzyme was desalted on Sephadex G-25 columns equilibrated with water, concentrated to 0.3 mg ml^{-1} in a Centricon-10 microconcentrator (Amicon, Inc., Beverly, MA, USA), and submitted to the University of Wisconsin Biotechnology Center (Madison, WI, USA) for microsequencing of the amino terminus by Edman degradation using an ABI 475A automated sequencer (Applied Biosystems, Foster City, CA, USA). Protein sequence data bases were accessed through the World Wide Web at the Uniform Resource Locator address http://www.ncbi.nlm.nih.gov using the BLASTp program (Altschul et al. 1990) and the nonredundant (nr) database.

Results

N-terminal amino acid sequence

Sequence analysis revealed that the purified corn enzyme responsible for GSH conjugation to CA and 4-CA

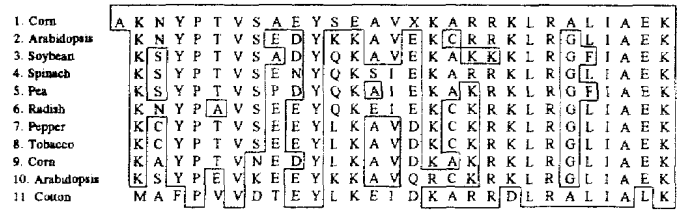

Fig. 1. N-terminal amino acid sequence of the purified corn enzyme, and its comparison to sequences of APX enzymes from various plant species. The boxed areas indicate regions of homology. Scientific names, references, and accession numbers are given as follows: (1) *Zea mays* L. Pioneer hybrid 3906, this publication, and *Zea mays* L. cv. Golden Cross Bantam 70, Koshiba 1993; (2) *Arabidopsis thaliana* [L] Heynh. cv. Columbia, Kubo et al. 1993, Q05431; (3) *Glycine max* [L] Merr. cv. Hobbit, Chatfield and Dalton 1993, L10292; (4) *Spinacia oleracea* L., Webb and Allen 1995, L20864; (5) *Pisum sativum* L. cv. Alaska, Mittler and Zilinskas 1992, A45116; (6) *Raphanus sativus* L., Lopez et al. 1996, S43157; (7) *Capsicum annuum*, Schantz et al. 1995, X81376; (8) *Nicotiana tabacum* L. cv. Xanthi, Örvar and Ellis 1995, U15933; (9) *Zea mays* L., line B73, van Breusegem et al. 1995, S49914; (10) *Arabidopsis thaliana* [L] Heynh., M. Santos, H. Gosseau, G. Creissen, C. Foyer and P. Mullineaux, unpublished, X80036; and (11) *Gossypium hirsutum*, R. N. Trelease, unpublished, U37060.

exhibited nearly 100% homology to the corn cytosolic APX described by Koshiba (1993) and a great deal of homology to the cytosolic APX enzymes found in a number of other species (Fig. 1). The enzyme did not exhibit any significant sequence similarities to any of the GST enzymes present in the database nor did it exhibit any sequence similarities to the chloroplastic APX or HRP as described by Koshiba (1993).

Co-purification of APX and GSH-conjugating activity

In order to determine if the GSH-conjugating and APX activities would co-purify, the purification procedure of Dean et al. (1995) was repeated and every fraction was assayed for both GSH-conjugating activity and APX activity. Only one peak of GSH-conjugating activity and APX activity was observed in the elution profile of the hydrophobic-interaction column, and both of these peaks appeared to coincide (Fig. 2A). The same was true for the elution profiles from both the anion-exchange column (Fig. 2B) and the gel filtration column (Fig. 2C). In addition, only one A_{403} peak was observed at each stage of the purification and this peak coincided with both the GSH-conjugating and APX activities. The absorbance at 403 nm is commonly used to detect the presence of hemoproteins (including peroxidase enzymes) in eluants from chromatography columns (Koshiba 1993). The results shown in Fig. 2 are representative of results obtained on three different trials of the experiment. The results were the same regardless of whether the colorimetric assay or the radiochemical assay was used to determine the GSH-conjugating activity.

Spectral analysis

The spectrum of the purified corn enzyme exhibited a Soret band with an absorption maximum at 405 nm (Fig. 3; oxidized enzyme). When the enzyme was reduced with sodium dithionite, the peak was shifted to 423 nm and an additional peak at 554 nm was observed (Fig. 3). The spectrum of the reduced enzyme in the presence of 0.1 *M* KCN exhibited peaks at 430, 534, and 563 nm (Fig 3). These spectra are nearly identical to those reported by Koshiba (1993), and indicate that the enzyme contains a heme moiety (Dalton et al. 1987, Chen and Asada 1989, Mittler and Zilinskas 1991).

Inhibitor studies

The GSH-conjugating activity of the purified corn enzyme was strongly inhibited by KCN with only 37.5% of the activity remaining in the presence of 0.1 m*M* KCN (Tab. 1), with concentrations of 1 and 10 m*M* KCN resulting in nearly complete loss of activity. Significant inhibition of the GSH-conjugating activity was observed at only the highest concentration examined (10 m*M*) for sodium azide (NaN_3; 31.6% of the activity remaining), *p*CMB (0.8% of the activity remaining), and iodoacetate (45.7% of the activity remaining; Tab. 1). The APX activity of the enzyme was inhibited by low concentrations (0.1 m*M*) of *p*CMB (16.7% of the activity remaining) and KCN (86.4% of the activity remaining), but higher concentrations of NaN_3 and iodoacetate were required for inhibition (Tab. 1). When the sensitivity of the GSH-conjugating activity to the inhibitors was compared to that of the APX activity, the GSH-conjugating activity was more sensitive to KCN, the APX activity was more sensitive to NaN_3 and *p*CMB, and the pattern of inhibition observed in the presence of iodoacetate was nearly identical for both activities. In regards to the results with *p*CMB, the decreased sensitivity of the GSH-conjugating activity compared to the APX activity may indicate that a sulfhydryl group of the enzyme is more critical for the APX activity than for the GSH-conjugating activity.

The GSH-conjugating and the APX activities of the enzyme were unaffected by the presence of 10 µ*M* of either sulphobromophthalein or tridiphane (data not shown). These two compounds have been described as GST specific inhibitors (Lamoureux and Rusness 1986, Edwards and Dixon 1991).

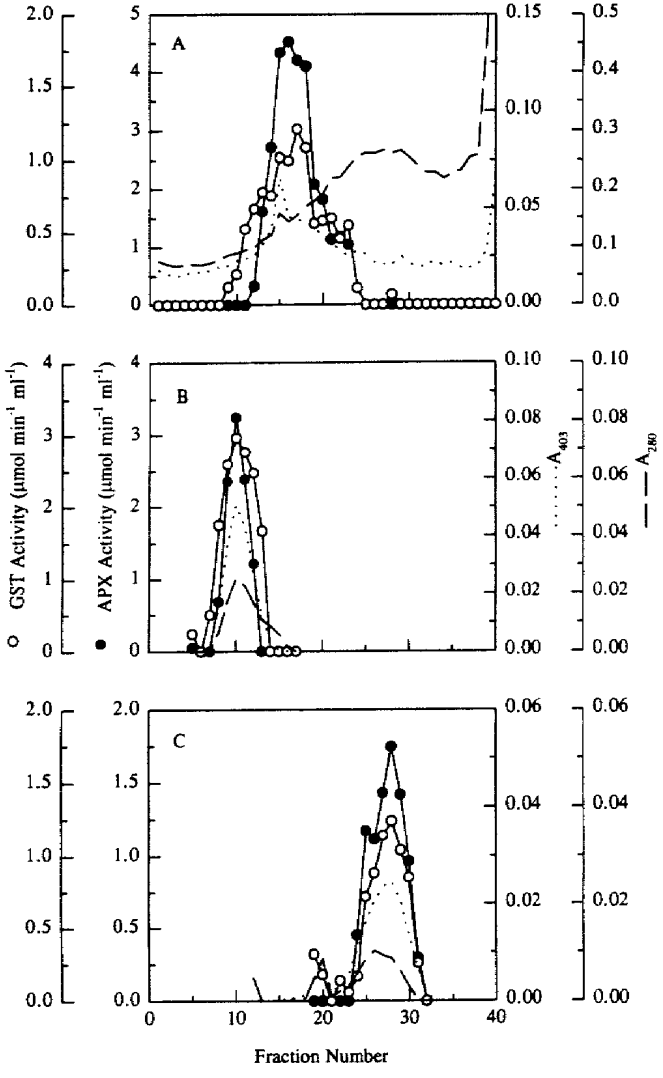

Fig. 2. Chromatographic purification of the GSH-conjugating and APX activities from corn shoots. Chromatography was performed on phenyl-Sepharose hydrophobic-interaction (A), Q-Sepharose FF anion-exchange (B), and Sephacryl S-200 gel filtration (C) columns. The GSH-conjugating activity with 4-CA was determined with the colorimetric assay.

Comparison of the GSH-conjugating and APX activities of various peroxidase enzymes

The APX activity of the purified corn enzyme was 1.5-fold greater than the GSH-conjugating activity with 4-CA (Tab. 2). The corn enzyme could also catalyze the conjugation of GSH to CA but the activity with CA was only 0.003% of the activity observed with 4-CA. The pea rAPX exhibited approximately the same ratios of APX activity to GSH-conjugating activity as observed for the corn enzyme; however, on the average, the corn enzyme was 1.7-fold more active than the pea rAPX for all three activities (Tab. 2). The GSH-conjugating activity of HRP with 4-CA was less than the activity observed for both the pea and the corn enzyme. However, HRP was substantially more active with CA than the corn or the pea enzyme. Yet even for HRP, the activity with CA was only 0.053% of the activity observed with 4-CA (Tab. 2). HRP is described as a guaiacol peroxidase (Asada 1992) and not an ascorbate peroxidase, therefore, as expected, the activity of HRP with ascorbate was substantially less than the APX activity of both the pea and the corn enzyme (Tab. 2).

Other properties of the peroxidase enzymes

The reaction products formed by the pea rAPX and HRP during the GSH-conjugating reactions in the presence of [^{14}C]4-CA had the same retardation factor value (data not shown) as the main product formed by the corn en-

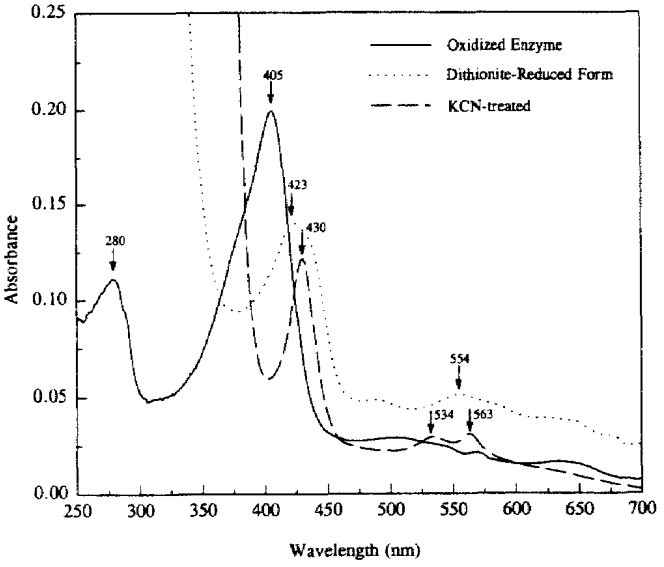

Fig. 3. Absorption spectra of the purified corn enzyme. Absorption spectra were determined for the purified corn enzyme in 50 m*M* potassium phosphate buffer, pH 7.5.

zyme in the TLC system described by Dean et al. (1995). The pH optimum of both the pea rAPX and HRP for the GSH-conjugating activity was 8.0 (data not shown). This is the same pH optimum observed for the corn enzyme (Dean et al. 1995). The pH optimum for the APX activity of the corn enzyme was 5.5. The K_m for H_2O_2 was 52 µ*M*, and the K_m for ascorbate was 0.33 m*M*. The pH optimum and the K_m values for the APX activity of the corn enzyme are nearly identical to those described by Koshiba (1993). Characterization of the corn enzyme in regards to the GSH-conjugating activity has previously been be reported by Dean et al. (1995).

Tab. 1. Effect of inhibitors on the GSH-conjugating and APX activities of the purified corn APX enzyme. The enzyme was preincubated with each inhibitor for 5 min before the start of the assay.

Inhibitor	Inhibitor concentration (m*M*)	Relative activity (%)	
		GSH-conjugating activity with 4-CA	APX activity
None	–	100	100
KCN	0.1	37.5	86.4
	1.0	4.1	35.7
	10.0	1.3	6.3
NaN_3	0.1	100	113
	1.0	91.9	69.1
	10.0	31.6	8.1
*p*CMB	0.1	109	16.7
	1.0	91.3	6.2
	10.0	0.8	4.4
Iodoacetate	0.1	102	119
	1.0	100	101
	10.0	45.7	43.4

Discussion

The results described above provide strong evidence that the enzyme from corn that conjugates GSH to CA and 4-CA is a cytosolic APX enzyme and not a GST. This conclusion is based on the following information: (1) Strong homology exists between the N-terminal amino acid sequence of the corn enzyme and APX enzymes from a number of other plant species (Fig. 1). (2) In all of the elution profiles only one peak of GSH-conjugating activity and APX activity was observed, and these two peaks coincide at every step of the purification procedure (Fig. 2). (3) Spectral analysis of the protein (Fig. 3) revealed that the enzyme appeared to be a heme protein and that the spectra were similar to those reported for other APX enzymes (Dalton et al. 1987, Chen and Asada 1989, Mittler and Zilinskas 1991, Koshiba 1993). (4) Though the sensitivities differed, the GSH-conjugating activity of the enzyme was inhibited by the same compounds as the APX activity (Tab. 1). We have also shown that the GSH-conjugating activity was not unique to the corn cytosolic APX enzyme since it was demon-

Tab. 2. Comparison of the GSH-conjugating and APX activities of the purified corn APX, pea rAPX, and HRP enzymes. Values are means of at least three replicates ± SD.

Enzyme	GSH-conjugating activity		APX activity (µmol mg^{-1} min^{-1})
	with 4-CA (µmol mg^{-1} min^{-1})	with CA (nmol mg^{-1} min^{-1})	
Corn APX	261.2 ± 16.1	8.3 ± 0.6	400 ± 26.6
Pea rAPX	169.6 ± 10.4	4.30 ± 0.18	227.8 ± 32.6
HRP	46.4 ± 1.9	24.4 ± 0.42	0.54 ± 0.02

strated that this activity was also associated with a pea rAPX and HRP (Tab. 2).

Based on the reactions that are known to be catalyzed by peroxidase enzymes, it is possible to propose a mechanism for the peroxidase-mediated GSH conjugation of 4-CA. In the presence of oxygen, peroxidase enzymes can catalyze the oxidation of GSH (reaction 1), with the subsequent formation of H_2O_2 (Olsen and Davis 1976).

(1) $2GSH + O_2 \rightarrow GSSG + H_2O_2$

(2) $2GSH + H_2O_2 \rightarrow 2GS^{\cdot} + 2H_2O$

(3) $2HOC_6H_4CH{=}CHCO_2H + H_2O_2 \rightarrow 2^{\cdot}OC_6H_4CH{=}CHCO_2H + 2H_2O$

(4) $^{\cdot}OC_6H_4CH{=}CHCO_2H + GSH \rightarrow HOC_6H_4CH{=}CHCO_2H + GS^{\cdot}$

(5) $GS^{\cdot} + HOC_6H_4CH{=}CHCO_2H \rightarrow HOC_6H_4C^{\cdot}H\underset{\displaystyle SG}{\underset{|}{C}}HCO_2H$

(6) $GSH + HOC_6H_4C^{\cdot}H\underset{\displaystyle SG}{\underset{|}{C}}HCO_2H \rightarrow GS^{\cdot} + HOC_6H_4CH_2\underset{\displaystyle SG}{\underset{|}{C}}HCO_2H$

H_2O_2 might also be formed by the autoxidation of GSH. In the presence of H_2O_2, peroxidase enzymes can catalyze the oxidation of thiols (reaction 2) resulting in the formation of the thiyl free radical (Wefers et al. 1985, Harman et al. 1986, Svensson 1988, Chen and Asada 1992). In addition, peroxidase enzymes are known to oxidize phenolic compounds (reaction 3), including 4-CA and other hydroxycinnamic acid derivatives, in the presence of H_2O_2 which results in the production of the phenoxyl radical (Takahama and Oniki 1992, 1994, Takahama 1993, 1995). The phenoxyl radical can be reduced nonenzymatically by thiols (reaction 4) producing additional thiyl free radicals (Rao et al. 1990). Although a number of different reactions with the thiyl free radical are possible, it is well known that thiyl free radicals will react nonenzymatically with alkenes (Oswald et al. 1962), as described for 4-CA in reaction 5. The thiyl radical would attack the less hindered carbon atom of the alkene, forming a carbon-centered radical around the other carbon (reaction 5). The carbon-centered radical would then be available for the abstraction of a hydrogen atom from another molecule of GSH (reaction 6). The overall result would be a chain reaction that results in the formation of additional thiyl free radical and GSH adducts of 4-CA. This same scheme could also be used to explain Cys conjugation to 4-CA (Dean et al. 1995). Support for the H_2O_2 dependent GSH conjugation mechanism, described in reactions 1–6, comes from some of our preliminary experiments that have shown that the addition of catalase (1 mg ml^{-1}; EC 1.11.1.6) to the incubation mixture completely inhibits GSH conjugation to 4-CA, while the addition of H_2O_2 (1 m*M*) increases the rate of conjugation 3-fold (data not shown).

Although it has been shown that the alkyl double bond of CA and 4-CA is involved in adduct formation, the exact location of the addition has not been determined (Diesperger and Sandermann 1978, Dean et al. 1995). In reaction 5, the thiyl free radical would attach itself to the less-hindered carbon atom in a way that yields the more stable carbon-centered free radical. If this is the case, then we might predict that the GSH adduct occurs at carbon number 2 and that the carbon-centered radical is formed at carbon 3 as shown in reaction 5. This reaction mechanism would be very different than a GST-mediated reaction. Since it is generally believed that GST enzymes catalyze the removal of the proton from the thiol of GSH (Armstrong 1991) generating a reactive thiolate anion (GS^-), adduct formation would occur as an ionic addition. An ionic addition would be more likely to occur at carbon 3 as described by Diesperger and Sandermann (1979), Edwards and Dixon (1991), and Dean et al. (1995). Since the reaction appears to involve peroxidase enzymes and free radical addition, the structure shown in reaction 6 is probably more accurate. However, the exact structure of the adduct remains to be determined.

Peroxidase-mediated GSH conjugation of styrene, polycyclic aromatic hydrocarbon-diols, insecticides (precocene I and II) and steroids (β-estradiol) has previously been described by Stock et al. (1986) and Foureman and Eling (1989). The reaction mechanism described also involved the initial oxidation of GSH to a thiyl radical by the peroxidase and the subsequent reaction of this thiyl with alkene bonds. However, the carbon-centered radical was thought to react with molecular oxygen to form a peroxyl radical that was eventually reduced to the corresponding alcohol (for the reaction scheme, see Stock et al. 1986). We do not know if a similar reaction with oxygen occurs during the peroxidase-mediated GSH conjugation of 4-CA and CA. However, at least two GSH conjugation products are formed when 4-CA is used as the substrate (Dean et al. 1995), and it is possible that one product may be the result of a reaction between a carbon-centered radical and molecular oxygen.

In terms of the GSH-conjugating activity, the corn cytosolic APX enzyme does appear to exhibit some substrate specificity in regards to the phenylpropanoid (Dean et al. 1995). This specificity probably relates to the ability of the enzyme to catalyze the oxidation of the hydroxycinnamic acid derivatives (reaction 3). Ferulic acid, coniferyl alcohol, and 4-CA have been shown to be good reducing substrates for HRP (Takahama 1995), and these three compounds were also good substrates for GSH conjugation in the presence of the corn APX (Dean et al. 1995). Sinapic acid has been shown to be oxidized very slowly, if at all, by HRP (Takahama 1995), and this phenylpropanoid is also a very poor substrate for the GSH-conjugating activity of the corn enzyme (Dean et al. 1995). Since CA does not contain a phenolic group, GSH conjugation to this compound would only involve

reactions 1, 2, 5 and 6. Therefore, the amount of thiyl free radical (and subsequent GSH conjugate) formed by the enzyme in the presence of CA would be much less than observed for 4-CA. This would explain why the GSH-conjugating activity of the corn enzyme is several orders of magnitude greater with 4-CA than with CA (Tab. 2). The mechanism described can also be used to explain why 4-CA appears to serve as an activator of GSH conjugation to CA (Dean and Machota 1993, Dean et al. 1995). In the presence of 4-CA, additional thiyl free radical would be formed in reactions 3 and 4. These additional thiyl free radicals would then be available to form conjugates of CA via reactions 5 and 6. A similar phenomenon was observed by Stock et al. (1986) and Foureman and Eling (1989) where the addition of reducing cosubstrates (aminopyrine, phenol, uric acid, phenylbutazone, and acetaminophen) to the incubation mixture greatly increased adduct formation.

Recently, it has been shown that a corn GST enzyme will conjugate GSH to cyanidin-3-glucoside providing the first evidence that GST enzymes may be involved in the conjugation of plant secondary compounds (Marrs et al. 1995). These glutathione conjugates appear to be transported into the vacuole by a glutathione S-conjugate pump (Martinoia et al. 1993, Marrs et al. 1995). It is reasonable to assume that the GSH conjugates of secondary compounds formed by peroxidase enzymes may also be recognized by the pump and transported into the vacuole. The peroxidase enzymes may be involved in the GSH conjugation of secondary compounds that are not good GST substrates. If this is the case, then the presence of both GSTs and peroxidase enzymes would increase the range of chemical structures that could be conjugated to GSH in vivo. However, peroxidase-mediated GSH conjugation in vivo may be limited by the presence of ascorbate. Ascorbate is an antioxidant that occurs in high concentrations in the plant cell and is generally believed to be a more efficient free radical scavenger than GSH (Rao et al. 1990). In the presence of ascorbate, the free radicals generated in reactions 2,3 and 4 may be reduced by ascorbate before the thiyl free radical is formed or before it reacts with the alkyl double bond of the phenylpropanoid. Reaction 5 would only occur if the alkyl double bond of the phenylpropanoid could effectively compete with ascorbate for the thiyl free radical. We are currently in the process of determining if GSH conjugates of phenylpropanoids are formed in vivo, and the potential role that peroxidase enzymes play in regards to GSH conjugation of secondary compounds.

Acknowledgments – The authors would like to thank Dr Thomas L. Poulos for the recombinant ascorbate peroxidase, and Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) for supplying the corn seeds. This publication is based on work supported by a National Science Foundation grant (IBN-9119296), a DePaul University URC research grant, and a grant from the DePaul University Faculty Research and Development Program.

References

Ålin, P., Danielson, U. H. & Mannervik, B. 1985. 4-hydroxyalk-2-enals are substrates for glutathione transferase. – FEBS Lett. 179: 267–270.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. – J. Mol. Biol. 215: 403–410.

Armstrong, R. N. 1991. Glutathione S-transferases: Reaction mechanism, structure, and function. – Chem. Res. Toxicol. 4: 131–140.

Asada, K. 1992. Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. – Physiol. Plant. 85: 235–241.

Berhane, K., Widersten, M., Engström, Å., Kozarich, J. W. & Mannervik, B. 1994. Detoxication of base propenals and other α,β-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferase. – Proc. Natl. Acad. Sci. USA 91: 1480–1484.

Boyland, E. & Chasseaud, L. F. 1967. Enzyme-catalyzed conjugations of glutathione with unsaturated compounds. – Biochem. J. 104: 95–102.

Chatfield, M. & Dalton, D. A. 1993. Ascorbate peroxidase from soybean root nodules. – Plant Physiol. 103: 661–662.

Chen, G.-X. & Asada, K. 1989. Ascorbate peroxidase in tea leaves: Occurrence of two isozymes and the differences in their enzymatic and molecular properties. – Plant Cell Physiol. 30: 987–998.

– & Asada, K. 1992. Inactivation of ascorbate peroxidase by thiols requires hydrogen peroxide. – Plant Cell Physiol. 33: 117–123.

Dalton, D. A., Hanus, F. J., Russell, S. A. & Evans, H. J. 1987. Purification, properties, and distribution of ascorbate peroxidase in legume root nodules. – Plant Physiol. 83: 789–794.

Dean, J. V. & Machota, J. H. 1993. Activation of corn glutathione S-transferase enzymes by coumaric acid and 7-hydroxycoumarin. – Phytochemistry 34: 361–365.

– , Gronwald, J. W. & Anderson, M. P. 1991. Glutathione S-transferase activity in nontreated and CGA-154281-treated maize shoots. – Z. Naturforsch. 46c: 850–855.

– , Devarenne, T. P., Lee, I.-S. & Orlofsky, L. E. 1995. Properties of a maize glutathione S-transferase that conjugates coumaric acid and other phenylpropanoids. – Plant Physiol. 108: 985–994.

Diesperger, H. & Sandermann, H. 1978. Evidence for an electrophilic intermediate in the microsomal hydroxylation of cinnamic acid in plants. – FEBS Lett. 85: 333–336.

– & Sandermann, H. 1979. Soluble and microsomal glutathione S-transferase activities in pea seedlings (*Pisum sativum* L.). – Planta 146: 643–648.

Edwards, R. & Dixon, R. A. 1991. Glutathione S-cinnamoyl transferases in plants. – Phytochemistry 30: 79–84.

– & Owen, W. J. 1987. Isozymes of glutathione S-transferase in *Zea mays*. – Biochem. Soc. Trans. 15: 1184.

– & Owen, W. J. 1988. Regulation of glutathione S-transferase of *Zea mays* in plants and cell cultures. – Planta 175: 99–106.

Foureman, G. L. & Eling, T. E. 1989. Peroxidase-mediated formation of glutathione conjugates from polycyclic aromatic dihydrodiols and insecticides. – Arch. Biochem. Biophys. 269: 55–68.

Habig, W. H., Pabst, M. J. & Jakoby, W. B. 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. – J. Biol. Chem. 249: 7130–7139.

Harman, L. S., Carver, D. K., Schreiber, J. & Mason, R. P. 1986. One- and two-electron oxidation of reduced glutathione by peroxidases. – J. Biol. Chem. 261: 1642–1648.

Koshiba, T. 1993. Cytosolic ascorbate peroxidase in seedlings and leaves of maize (*Zea mays*). – Plant Cell Physiol. 34: 713–721.

Kubo, A., Saji, H., Tanaka, K. & Kondo, N. 1993. Genomic DNA structure of a gene encoding cytosolic ascorbate peroxidase from *Arabidopsis thaliana*. – FEBS Lett. 315: 313–317.

Lamoureux, G. L. & Rusness, D. G. 1986. Tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane] an atrazine synergist: Enzymatic conversion to a potent glutathione S-transferase inhibitor. – Pestic. Biochem. Physiol. 26: 323–342.

Lopez, F., Vansuyt, G., Casse-Delbart, F. & Fourcroy, P. 1996. Ascorbate peroxidase activity, not mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. – Physiol. Plant. 97: 13–20.

Mannervik, B. 1985. The isozymes of glutathione transferase. – Adv. Enzymol. Relat. Areas Mol. Biol. 57: 357–417.

Marrs, K. A., Alfenito, M. R., Lloyd, A. M. & Walbot, V. 1995. A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. – Nature 375: 397–400.

Martinoia, E., Grill, E., Tommasini, R., Kreuz, K. & Amrhein, N. 1993. ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. – Nature 364: 247–249.

Mittler, R. & Zilinskas, B. A. 1991. Purification and characterization of pea cytosolic ascorbate peroxidase. – Plant Physiol. 97: 962–968.

– & Zilinskas, B. A. 1992. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. – J. Biol. Chem. 267: 21802–21807.

Olsen, J. & Davis, L. 1976. The oxidation of dithiothreitol by peroxidases and oxygen. – Biochim. Biophys. Acta 445: 324–329.

Örvar, B. L. & Ellis, B. E. 1995. Isolation of a cDNA encoding cytosolic ascorbate peroxidase in tobacco. – Plant Physiol. 108: 839–840.

Oswald, A. A., Griesbaum, K., Thaler, W. A. & Hudson, B. E. Jr. 1962. Organic sulfur compounds. VIII. Addition of thiols to conjugated diolefins. – J. Am. Chem. Soc. 84: 3897–3904.

Patterson, W. R. & Poulos, T. L. 1994. Characterization and crystallization of recombinant pea cytosolic ascorbate peroxidase. – J. Biol. Chem. 269: 17020–17024.

Rao, D. N. R., Fischer, V. & Mason, R. P. 1990. Glutathione and ascorbate reduction of the acetaminophen radical formed by peroxidase. – J. Biol. Chem. 265: 844–847.

Schantz, M.-L., Schreiber, H. & Schantz, R. 1995. Changes in ascorbate peroxidase activities during fruit ripening in *Capsicum annuum*. – FEBS Lett. 358: 149–152.

Svensson, B. E. 1988. Thiols as myeloperoxidase-oxidase substrates. – Biochem. J. 253: 441–449.

Stock, B. H., Bend, J. R. & Eling, T. E. 1986. The formation of styrene glutathione adducts catalyzed by prostaglandin H synthase: A possible new mechanism for the formation of glutathione conjugates. – J. Biol. Chem. 261: 5959–5964.

Takahama, U. 1993. Regulation of peroxidase-dependent oxidation of phenolics by ascorbic acid: Different effects of ascorbic acid on the oxidation of coniferyl alcohol by the apoplastic soluble and cell wall-bound peroxidases from the epicotyls of *Vigna angularis*. – Plant Cell Physiol. 34: 809–817.

– 1995. Oxidation of hydroxycinnamic acid and hydroxycinnamyl alcohol derivatives by laccase and peroxidase. Interactions among *p*-hydroxyphenyl, guaiacyl and syringyl groups during the oxidation reactions. – Physiol. Plant. 93: 61–68.

– & Oniki, T. 1992. Regulation of peroxidase-dependent oxidation of phenolics in the apoplast of spinach leaves by ascorbate. – Plant Cell Physiol. 33: 379–387.

– & Oniki, T. 1994. Effects of ascorbate on the oxidation of derivatives of hydroxycinnamic acid and the mechanism of oxidation of sinapic acid by cell wall-bound peroxidases. – Plant Cell Physiol. 35: 593–600.

van Breusegem, F., Villarroel, R., van Montagu, M. & Inzé, D. 1995. Ascorbate peroxidase cDNA from maize. – Plant Physiol. 107: 649–650.

Wadleigh, R. W. & Yu, S. J. 1987. Glutathione transferase activity of fall armyworm larvae toward α,β-unsaturated carbonyl allelochemicals and its induction by allelochemicals. – Insect Biochem. 17: 759–764.

Webb, R. P. & Allen, R. D. 1995. Isolation and characterization of a cDNA for spinach cytosolic ascorbate peroxidase. – Plant Physiol. 108: 1325.

Wefers, H., Riechmann, E. & Sies, H. 1985. Excited species generation in horseradish peroxidase-mediated oxidation of glutathione. – J. Free Radic. Biol. Med. 1: 311–318.

Edited by J. G. Scandalios