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Taxonomic distribution of plant glutathione S-transferases acting on xenobiotics

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Abstract

Soluble and microsomal glutathione S-transferase activities for five model xenobiotics (nitrobenzene derivatives), two pesticidal xenobiotics (atrazine and fluorodifen), and a natural substrate (cinnamic acid), were determined in 59 different plant species and four plant cell suspension cultures. These enzyme activities were widely distributed over the plant kingdom with certain species showing particularly high activities. Marine macroalgae had a remarkably broad substrate range that included the substrates atrazine and fluorodifen. It is concluded that the evolutionary 'green liver' concept derived for xenobiotic metabolism in higher plant species is also valid for the constitutive soluble and microsomal glutathione S-transferases of lower plant species.

Keywords: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)-propane; GSH, glutathione; GST, glutathione S-transferase; IDNB, 1-iodo-2,4-dinitrobenzene; pNBC, 4-nitrobenzoylchloride

1. Introduction

Plant xenobiotic metabolism resembles that of the mammalian liver in terms of metabolite patterns, enzyme classes and gene sequences, as summarized in the evolutionary 'green liver' concept (Sandermann, 1992, 1994). The similarity also extends to enzyme isoforms and genes that are involved in plant metabolism of natural secondary compounds. The 'green liver' concept is so far based nearly exclusively on studies with higher plant species, in particular crop plant species. The glutathione S-transferase enzymes are important for xenobiotic metabolism and antioxidative protection, and are well characterized in bacteria, insects, mammals and crop plants (reviewed: Wilce and Parker, 1994; Marrs, 1996; Vuilleumio, 1997; Armstrong, 1997; Cole et al., 1997; Droog, 1997; Dixon et al., 1998). The first report of soluble plant GST isoenzymes and of a plant microsomal GST for cinnamic acid appeared some 20 years ago (Diesperger and Sandermann, 1979). These findings have in the meanwhile considerably been extended in particular with regard to the soluble isoenzymes of crop plant species (Lamoureux and Rusness, 1993; Marrs, 1996; Cole et al., 1997). For example, maize has been shown to contain high soluble and microsomal GST activities for cinnamic acid, metolachlor and CDNB (Cottingham et al., 1998). Certain weed species (Lamoureux and Rusness, 1993; Andrews et al., 1997; Cole et al., 1997), a moss species (Dhindsa, 1991) and various tree species (Schröder et al., 1992; Schröder and Berkau, 1993) have also been shown to contain GSTs for xenobiotics. The structure of a herbicide detoxifying GST from the weed *Arabidopsis thaliana* has been elucidated

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at 2.2 Å resolution (Zajc et al., 1999). A GST isoenzyme detoxifying atrazine with an enhanced k_{cat}-value has been shown to be responsible for the emergence of certain herbicide-resistant weed species (Gronwald, 1994; Plaisance and Gronwald, 1999). GST isoenzymes for xenobiotics have not been systematically studied in non-crop plant species, although a rather wide distribution may be expected on the basis of the studies quoted above. This expectation is borne out by the results reported here. The GST substrates tested in the present study with soluble and microsomal enzyme extracts are shown in Fig. 1. A broad taxonomic distribution of glutathione S-transferase activities for several xenobiotics as well as a natural substrate is documented.

2. Results

2.1. Enzyme distribution

Glutathione S-transferase enzyme activities were examined in the microsomal and cytosolic fractions of 59 different plant species and of four reference cell suspension cultures. Besides terrestrial and freshwater plant species, marine macroalgae of the *Chlorophyceae*, *Phaeophyceae* and *Rhodophyceae* were also tested for enzyme activity. Five nitrobenzene model substrates, two pesticidal compounds (atrazine and fluorodifen) and one natural substrate (cinnamic acid) were used. The results obtained with the various soluble and microsomal glutathione S-transferase preparations are summarized in Fig. 2A and B, respectively.

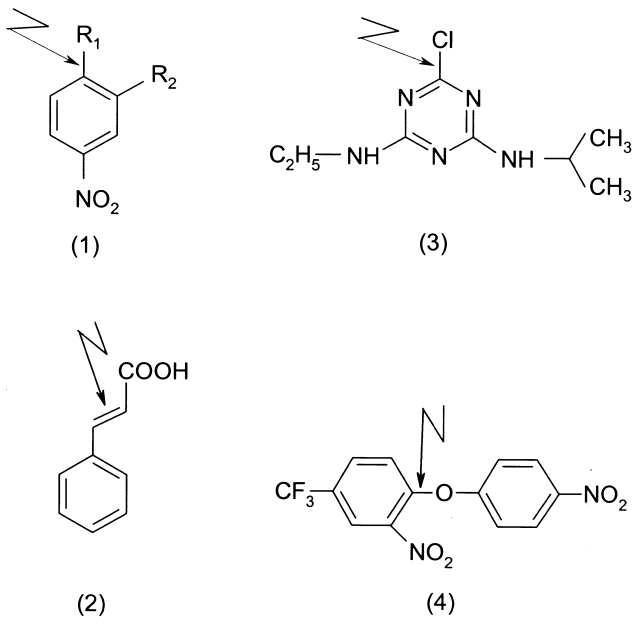

Fig. 1. Substrates used to detect glutathione S-transferase activities. The sites of GSH attack are marked by arrows. (1) Nitrobenzene derivatives. CDNB (R_1 = Cl, R_2 = NO_2), DCNB (R_1 = R_2 = Cl), IDNB (R_1 = J, R_2 = NO_2), pNBC (R_1 = –CO–Cl, R_2 = H), EDNP (R_1 = - O-CH2-CH-CH, R_2 = H). (2) cinnamic acid. (3) atrazine, (4) flurodifen.

2.2. Nitrobenzene derivatives

Soluble GST-activity measured for the commonly used standard substrate, CDNB, was detected in all plant species screened. The highest soluble activities towards CDNB were measured in *Conocephalum conicum* (56.0 ± 3.7 nkat/mg protein), *Sphagnum capilifolium* (49.4 ± 4.3 nkat/mg protein) and *Ascophyllum nodosum* (38.2 ± 2.4 nkat/mg protein). Microsomal GST-activity with CDNB as substrate was in most cases lower than the soluble activity (Fig. 2B). The highest activities were found in *Glycine max* cell cultures (43.5 ± 2.0 nkat/mg protein), *Laminaria digitata* (26.6 ± 3.2 nkat/mg protein), and *Enteromorpha bulbosa* (22.1 ± 3.6 nkat/mg protein). The high soluble and microsomal GST activities observed in the cell culture systems may be due to induction by the growth regulator, 2.4-D, a component of the medium (Flury et al., 1995).

Activity for IDNB was detected in all species tested (Fig. 2A and B). The highest soluble activities were obtained in *Athyrium filix-femina* (50.4 ± 1.4 nkat/mg protein), *Lycopodium annotinum* (34.2 ± 1.3 nkat/mg protein), and *Enteromorpha bulbosa* (30.8 ± 2.2 nkat/mg protein). The highest microsomal activities were measured in *Enteromorpha bulbosa* (94.5 ± 4.2 nkat/mg protein), *Caulerpa mexicana* (40.3 ± 3.2 nkat/mg protein), and *Ulva lactuca* (36.8 ± 1.9 nkat/mg protein). The product formed from IDNB and GSH has not been well characterized before. The product had about the same HPLC retention time (14.2 min; IDNB, 35.4 min) and R_f-value upon TLC (R_f, 0.39) as the S-(nitrobenzyl)-GSH standard used (Pflugmacher, 1996).

Conjugation of DCNB was observed with soluble as well as microsomal enzyme fractions from most of the investigated plant species (Fig. 2A and B). Activities were generally lower than with CDNB. Highest activity with soluble enzyme were encountered in *Sequoiadendron giganteum* (10.3 ± 0.7 nkat/mg protein), *Picea abies* cell cultures (9.4 ± 1.0 nkat/mg protein), and in *Plocamium cartilagineum* (7.4 ± 1.4 nkat/mg protein). Microsomal GST activities for DCNB were in most cases some 10-fold lower than the soluble GST activities for DCNB from the same species. However, high microsomal activity values also occurred, such as with *Laminaria hyperborea* (7.2 ± 1.3 nkat/mg protein), *Monostroma arcticum* (5.6 ± 1.5 nkat/mg protein), and *Laminaria digitata* (4.5 ± 1.2 nkat/mg protein).

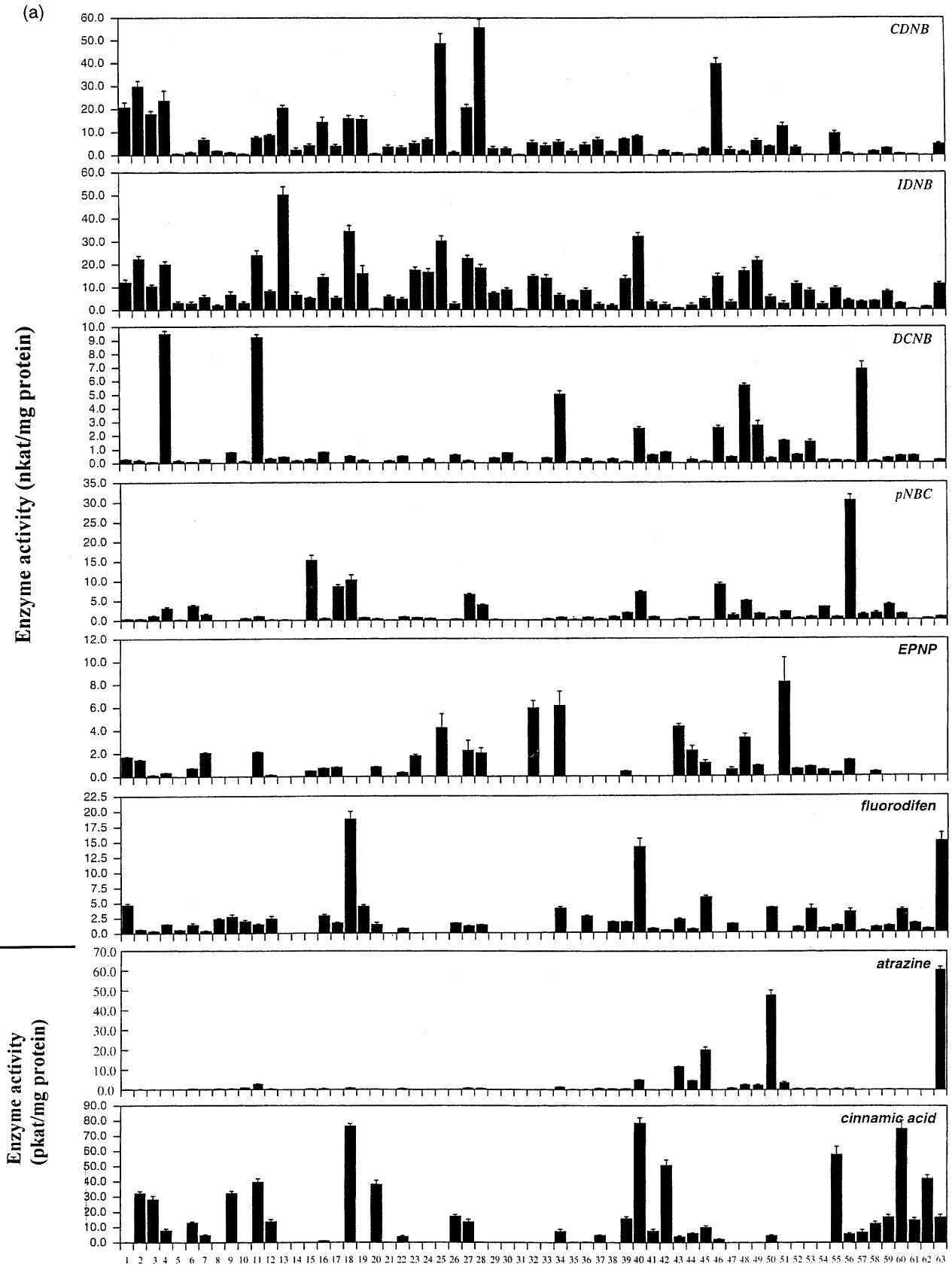

Fig. 2(a) (*Caption overleaf*).

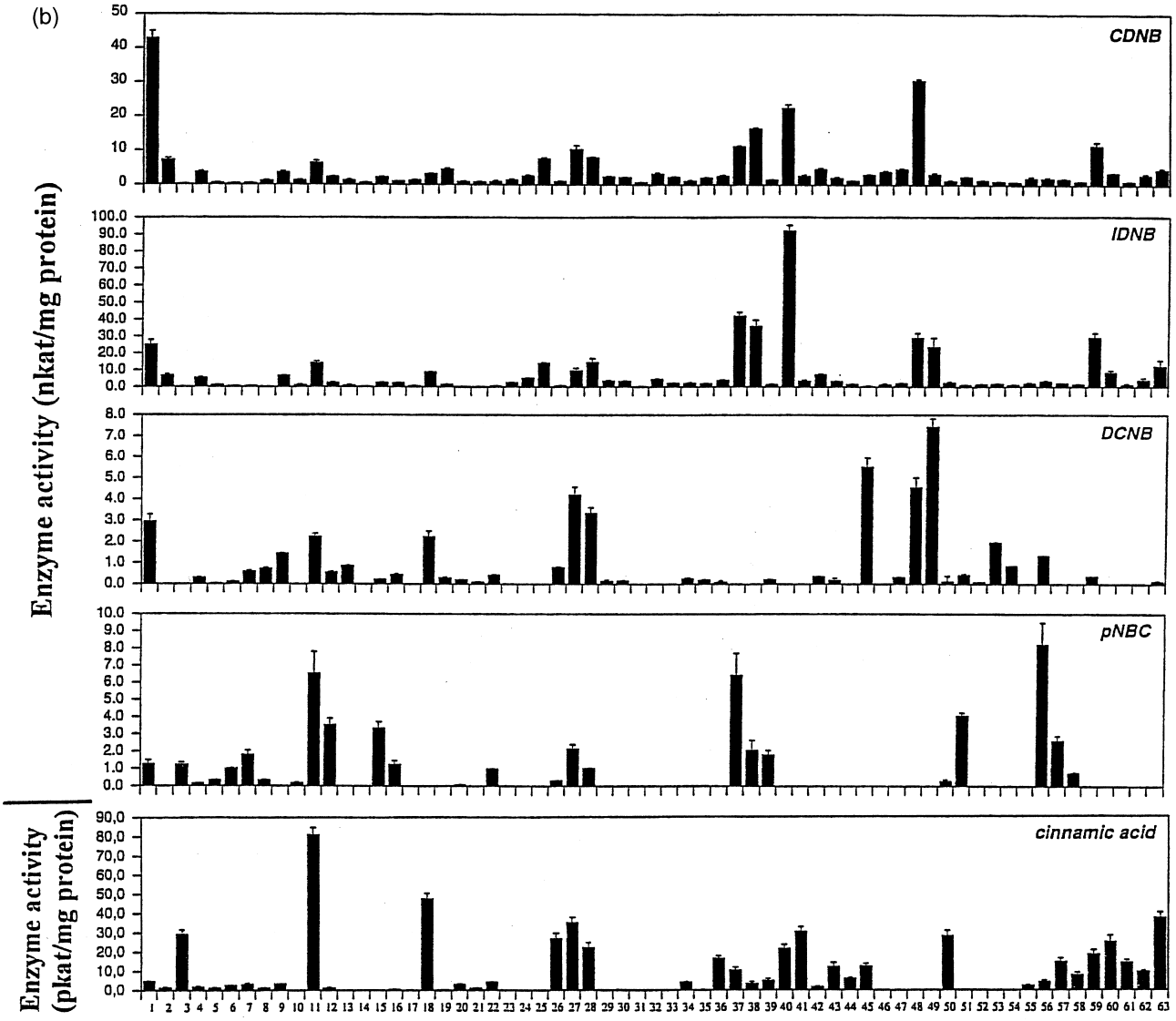

Fig. 2. Activities (pkat or nkat/mg protein) of (A) soluble GST and (B) microsomal GST activities with the indicated substrates. Mean values ± SD ($n = 3$) are shown. The numbering on the abscissa refers to the following cell suspension cultures or intact plants species. **Plant cell cultures** (used as reference systems) 1: *Glycine max*. L. Merr. cv. Mandarin. 2: *Triticum aestivum* L. cv. Heines Koga II; 3: *Zea mays* L. cv. Black Mexican Sweet; 4: *Picea abies* L. Excelsa RJ 80. **Intact plants samples. Spermatophyta**. Dicotyledonae. 5: *Rosa canina* L.; 6: *Helianthus giganteus* L.; 7: *Salicornia europaea* L.; 8: *Lemna minor* L. **Ginkgoatae**. 9: *Gingko biloba* L.; 10: *Rhus typhina* L. **Coniferae**. 11: *Sequoiadendron giganteum* Buchh. **Cycadatae**. 12: *Cycas revoluta* L. **Pteridophyta**. Filicatae. 13: *Athyrium filix-femina* (L.) Roth.. 14: *Blechnum spicant* (L.) Roth.; 15: *Azolla filiculoides* Lamk.; 16: *Selaginella lepidophylla* L.. **Psilophytatae**. 17: *Psilotum nudum* L.. **Lycopodiatae**. 18: *Lycopodium annotinum* L.. **Articulatae**. 19: *Equisetum hyemale* L.; 20: *Equisetum arvense* L.. 21: *Equisetum telmateia* Ehrh.; 22: *Equisetum giganteum* L.. **Bryophyta**. Musci. 23: *Plagomnium undulatum* (Hedw.) B.S.G.; 24: *Polytrichum formosum* Hedw.; 25: *Sphagnum capilifolium* (Ehrh.) Hedw.; 26: *Fontinalis antipyretica* Hedw.. **Hepaticae**. 27: *Marchantia polymorpha* L.; 28: *Conocephalum conicum* (L.) Lindb. **Mycobionta**. Basidomycetes. 29: *Sarcodon imbricatum* L.; 30: *Lactarius deterrimus* L.; 31: *Phanerochaete chrysosporium* Burd.. Lichenes. 32: *Alectoria samentosa* (Ach.); 33: *Peltigera aphtosa* (L.) Willd. **Chorophyta**. Chlorophyceae. 34: *Zygmena sp* L.; 35: *Chlorella fusca* Beijerinck; 36: *Chara corallina* L.; 37: *Caulerpa mexicana* Harv.; 38: *Ulva lactuca*; 39: *Enteromorpha compressa*; 40: *Enteromorpha bulbosa* (L.) Grev.; 41: *Cladophora rupestris* (L.) Kütz.; 42: *Halimeda opuntia* (L.) Lamour.; 43: *Acrosiphonia sonderi* (Kütz.) Komm.; 44: *Lambia antarctica* (Skottsb.) Delepine; 45: *Monostroma arcticum* Wittr. **Chromophyta**. Phaeophyceae. 46: *Ascophyllum nodosum* (L.) LeJol; 47: *Cytoseira baccata* (Gmel.) Silva; 48: *Laminaria digitata* (Huds.) Lamoureux; 49: *Laminaria hyperborea* (Gunn.) Fosl; 50: *Laminaria saccharina* (L.) Lamoureux ; 51: *Halydris siliquosa* (L.) Lyngb.; 52: *Fucus vesiculosus* L.; 53: *Fucus serratus* L.; 54: *Fucus spiralis* L. **Rhodophyta**. Florideophyceae. 55: *Delesseria sanguinea* (Huds.) Lamoureux; 56: *Chondrus crispus* Stackh; 57: *Plocamium cartilagineum* (L.) Dixon. **Rhodophyceae**. 58: *Porphyra umbilicalis*. J. Ag.; 59: *Cystoclonium purpureum* (Huds.) Batt; 60: *Iridaea cordata* Kütz.; 61: *Palmaria decipiens* (A. & E.S. Gepp.) Kylin; 62: *Pantoneura plocamioides* (J. Ag.) Kyl.; 63: *Polysiphonia urceolata* (Lightf. ex. Dillw.).

With pNBC as model substrate, soluble enzyme activity of terrestrial plant species appeared to be correlated to GST activity for DCNB. Highest activities were encountered in *Chondrus crispus* (30.5 ± 2.3 nkat/mg protein), *Azolla filiculoides* (15.3 ± 3.6 nkat/mg protein), and *Lycopodium annotinum* (10.7 ± 2.2 nkat/mg protein). Highest activities with the microsomal enzyme fraction were encountered in *Chondrus crispus* (8.4 ± 0.7 nkat/mg protein), *Caulerpa mexicana* (6.3 ± 1.3 nkat/mg protein), and *Halydris siliquosa* (4.5 ± 0.8 nkat/mg protein).

With EPNP as a model substrate, no activity was detected in any of the microsomal fractions tested. The soluble enzyme activity had a quite limited occurrence (Fig. 2A). Highest activities were found in *Halydris siliquosa* (8.4 ± 1.1 nkat/mg protein), *Lactarius deterrimus* (6.6 ± 2.1 nkat/mg protein), and *Alectoria samentosa* (5.2 ± 0.9 nkat/mg protein).

2.3. Herbicides

The herbicides atrazine and fluorodifen were tested as xenobiotic substrates for the soluble enzyme extracts (Fig. 2A). The conjugates formed appeared as single defined peaks at R_f-values of 0.43 (fluorodifen) and 0.39 (atrazine) upon TLC (parent herbicides, $R_f > 0.95$). Detection was by radioactivity (atrazine) and by UV-quenching (fluorodifen). The R_f- values obtained agreed with literature values of these GSH-conjugates (Schröder et al., 1990; Pflugmacher, 1996).

In most terrestrial plant species, the soluble enzyme fractions were active with both herbicides. Activity with fluorodifen was usually at least two to three times higher than that with atrazine. The soluble algal enzyme fractions were about equally active with atrazine and fluorodifen. Only soluble enzymes from the *Rhodophycea*, *C. crispus*, conjugated fluorodifen about five-fold better than atrazine. The soluble fraction from cultured *Picea abies* cells had no ability to form an atrazine-conjugate, but was active with fluorodifen.

Highest activities for atrazine were found in soluble extracts from *Polysiphonia urceolata* (60.4 ± 5.2 pkat/mg protein), *Laminaria saccharina* (48.4 ± 3.6 pkat/mg protein), and *Monostroma arcticum* (20.4 ± 2.2 pkat/mg protein). With regard to fluorodifen, highest soluble activities were found in *Lycopodium annotinum* (18.6 ± 1.7 nkat/mg protein), *Polysiphonia urceolata* (15.3 ± 2.0 nkat/mg protein), and *Enteromorpha bulbosa* (14.7 ± 1.7 nkat/mg protein). The microsomal enzyme fractions were not tested with atrazine or fluorodifen.

2.4. Cinnamic acid

Cinnamic acid is conjugated with GSH by soluble and microsomal glutathione S-transferases (Diesperger and Sandermann, 1979) and/or soluble ascorbate peroxidase and guaiacol peroxidases (Dean et al., 1995; Dean and Devarenne, 1997). The present study cannot differentiate between peroxidase or true GST involvement because only crude enzyme extracts were employed. Soluble and microsomal activities forming S-cinnamoyl-glutathione (R_f, 0.33 upon TLC; Diesperger and Sandermann, 1979) were widely distributed among the investigated plant species (Fig. 2A and B). The highest soluble enzyme activities were found in *Enteromorpha bulbosa* (76.2 ± 2.6 pkat/mg protein), *Lycopodium annotinum* (75.3± 5.1 pkat/mg protein), and *Iridaea cordata* (73.4 ± 4.3 pkat/mg protein). The highest microsomal activities were present in *Sequoiadendron giganteum* (80.6 ± 4.3 pkat/mg protein), *Lycopodium annotinum* (46.4 ± 2.7 pkat/mg protein), and *Polysiphonia urceolata* (34.4 ± 1.6 pkat/mg protein).

2.5. Discussion

Soluble and microsomal GST activities towards model substrates, herbicides and an endogenous substrate are demonstrated here for numerous higher and lower plant species, including marine macroalgae of the divisions *Chlorophyta*, *Phaeophyta* and *Rhodophyta*. Multiple GST enzyme activities have recently also been characterized in freshwater algae (Schrenk et al., 1998; Tang et al., 1998). The standard work-up and assay conditions were not optimized for each individual plant species tested so that the reported activity values have to be considered as minimal values for some species. In many cases, GST activities were absent or very low. No induction experiments e.g. with antidotes, elicitors or ozone, were conducted to determine inducible GST activities that are known to be widespread in crop plant species (Marrs, 1996). Nevertheless, the data presented here allow the conclusion that the 'green liver' concept (Sandermann, 1992, 1994) applies also to lower plants, and especially to marine macroalgae.

It was surprising to find that marine macroalgae had high activities for atrazine and fluorodifen in addition to activities for the other tested substrates. The latter plant species with their enormous world-wide biomass appear to have a potential to remove xenobiotic pollutants because they also contained high cytochrome P450 mono-oxygenase (Pflugmacher and Sandermann, 1998a) and glucosyltransferase activities (Pflugmacher and Sandermann, 1998b).

3. Experimental

3.1. Materials

Labelled [ring-u-^{14}C]-cinnamic acid (59 Ci/mol) was

purchased from Amersham & Buchler (Braunschweig). [^{14}C]-Atrazine (24 Ci/mol) was obtained from International Isotope GmbH (München, FRG). Radiochemical purities of higher than 99% were determined by TLC (Pflugmacher, 1996). CDNB, atrazine and fluorodifen were from Riedel-de-Haen (Hannover, FRG). IDNB, pNBC, EPNP, GSH and 4-nitrophenylglutathione and porcine liver GST (cat. No. G 6636) were from Sigma (St. Louis, U.S.A., Deisenhofen, FRG). DCNB was a product of Fluka (Neu-Ulm, FRG). All other materials including the sources of the various plant materials used have been previously described (Pflugmacher and Sandermann, 1998a, 1998b).

3.2. General procedures

Protein determination was done according to Bradford (1976) using bovine serum albumin as protein standard. TLC was performed on precoated Silica Gel G60 plates (Merck No. 5554) using the following solvent system (parts per volume): *n*-butanol:acetic acid:water (12:3:5; Diesperger and Sandermann, 1979). 4-nitrophenyl-glutathione was used as a standard on TLC for UV detection of conjugates. Radioactivity on TLC plates was monitored by two-dimensional scanning using a TLC linear analyzer. Liquid scintillation counting was done in toluene/methanol 70:30 with 5 g PPO per liter. The preparation of soluble and microsomal enzyme fractions was exactly as previously described (Pflugmacher and Sandermann, 1998a, 1998b). For enzyme measurement the following buffer systems were used: (A) potassium phosphate 0.1 M, pH 6.5; (B) potassium phosphate 0.1 M, pH 7.5; (C) sodium phosphate, 50 mM, pH 7.0.

3.3. Enzyme assays

3.3.1. Assay conditions

For proper assay conditions, pH-values and incubation times were adopted from the literature (Pflugmacher, 1996). K_m-values between 0.2 and 3.4 mM have been reported for the nitrobenzene derivatives studied here (Lamoureux and Rusness, 1989; Clark, 1989). A commercial GST preparation from porcine liver had a K_m-value of 1.35 ± 0.22 mM for IDNB (at 2 mM GSH; Pflugmacher, 1996). The following *Km*-values for GSH-conjugation have been reported in the literature: cinnamic acid, 0.1–0.4 mM (Diesperger and Sandermann, 1979; Edwards and Dixon, 1991), atrazine, 0.09–2.4 mM (Lamoureux and Rusness, 1989), fluorodifen, 0.09 mM (Schröder and Berkau, 1993). The standard assays employed here contained substrate concentrations of 0.25 mM (fluorodifen), 0.5 mM (atrazine, cinnamic acid, EPNP) or 1 mM (CDNB, DCNB, pNBC, IDNB).

3.3.2. Spectrophotometric assays

The assay procedures used were adopted from Fjellstedt et al. (1973), Habig et al. (1974), and Schröder et al. (1990). The assay mixture consisted of 1080 µl buffer A, 40 µl 30 mM CDNB in ethanol, 20 µl 90 mM GSH in water and 40 µl enzyme. The tests with pNBC and EPNP were identical except for using only 15 mM EPNP and increasing GSH concentration 10-fold in the case of EPNP. The standard test was employed with buffer B in the case of DCNB and IDNB. The tests were followed photometrically at 310 nm (pNBC), 340 nm (CDNB), 345 nm (DCNB), 354 nm (IDNB) and 360 nm (EPNP). The molar absorbances (mM^{-1} cm^{-1}) at these wavelengths were 1.9 (pNBC), 9.6 (CDNB), 8.5 (DCNB), 9.2 (IDNB) and 0.5 (EPNP). The standard assay for fluorodifen was adopted from Diesperger and Sandermann (1979), Schröder et al. (1992) and Schröder and Berkau (1993). It consisted of 120 µl buffer B, 10 µl 5 mM herbicide in ethanol, 20 µl 10 mM GSH in water and 50 µl enzyme. The reaction was followed at 370 nm (molar absorbance, 3.1 mM^{-1} cm^{-1}). Control incubations were performed in all cases either with heat-denatured enzyme (10 min, 100°C), or without enzyme.

3.4. Radiometric assays

The standard test for ^{14}C-cinnamic acid and ^{14}C-atrazine was adopted from Diesperger and Sandermann (1979), Jablonkai and Hatzios (1993) and Schröder et al. (1992). It consisted of 120 µl buffer C, 10 µl substrate in ethanol, 20 µl 10 mM GSH in water and 50 µl enzyme. Final substrate concentrations were 0.5 mM (cinnamic acid), 0.6 mM (atrazine) and 1.0 mM (GSH). After incubation for 60 min at 25°C the reactions were terminated by addition of 10 µl 20% (w/v) trichloroacetic acid and 200 µl dichloromethane, followed by TLC analysis.

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