Glutathione S-Transferases in Earthworms (Lumbricidae)

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Glutathione S-transferase activity (EC 2.5.1.18) was demonstrated in six species of earthworms of the family Lumbricidae: *Eisenia foetida*, *Lumbricus terrestris*, *Lumbricus rubellus*, *Allolobophora longa*, *Allolobophora caliginosa* and *Allolobophora chlorotica*. Considerable activity was obtained with 1-chloro-2,4-dinitrobenzene and low activity with 3,4-dichloro-1-nitrobenzene, but no enzymic reaction was detectable with sulphobromophthalein, 1,2-epoxy-3-(*p*-nitrophenoxy)propane or *trans*-4-phenylbut-3-en-2-one as substrates. Enzyme preparations from *L. rubellus* and *A. longa* were the most active, whereas *A. chlorotica* gave the lowest activity. The ratio of the activities obtained with 1-chloro-2,4-dinitrobenzene and 3,4-dichloro-1-nitrobenzene was very different in the various species, but no phylogenetic pattern was evident. Isoelectric focusing gave rise to various activity peaks as measured with 1-chloro-2,4-dinitrobenzene as a substrate, and the activity profiles of the species examined appeared to follow a taxonomic pattern. The activity of *Allolobophora* had the highest peak in the alkaline region, whereas that of *Lumbricus* had the highest peak in the acid region. *Eisenia* showed a very complex activity profile, with the highest peak near pH 7. As determined by an enzymic assay, all the species contained glutathione, on an average about 0.5 $\mu\text{mol/g}$ wet wt. Conjugation with glutathione catalysed by glutathione S-transferases may consequently be an important detoxification mechanism in earthworms.

The glutathione S-transferases (EC 2.5.1.18) are important detoxification enzymes, which occur in a variety of different animals. The presence of GSH S-transferases in the liver of the rat and other mammals is well documented (e.g. Booth *et al.*, 1961; Grover & Sims, 1964; Johnson, 1966; Fukami & Shishido, 1966). Small amounts are also found in other classes of vertebrates, such as reptiles (*Lacerta*), birds (*Gallus* and *Anas*), amphibians (*Rana*), freshwater teleosts (*Carassius*) (Grover & Sims, 1964) as well as saltwater teleosts (several genera), elasmobranchs (*Raja* and *Squalus*) and cyclostomes (*Myxine*) (Bend & Fouts, 1973). Insects of different orders (Fukami & Shishido, 1966; Clark *et al.*, 1966, 1967, 1973), other classes of arthropods such as blood-sucking acarina (*Boophilus*) (Clark *et al.*, 1966) and marine crustacea (*Homarus*, *Cancer*) (Bend & Fouts, 1973) also have this type of enzyme. Invertebrates other than arthropods have only been studied in a few cases. Clark & Smith (1975) refer to unpublished work (S. Balaskaran & J. N. Smith) in which the presence of GSH S-transferases in a terrestrial mollusc (*Helix*), a terrestrial plathyhelminth (*Dugesia*) and an annelid (the earthworm *Lumbricus rubellus*) was detected.

Usually, only one or few species of each taxonomic unit have been used in comparative enzymological studies. However, non-specific enzymes using exogen-

ous substrates tend to have very variable activities (Selander, 1976). Therefore, groups of species ought to be used when detoxification enzymes in different taxa are to be compared.

Earthworms play an important role in the ecology of the soil. These animals may be exposed to pesticides and pollutants in the environment, and the organisms are therefore dependent on efficient detoxification systems, some of which may be based on glutathione and GSH S-transferases. Although effective systems for biotransformation of xenobiotics in earthworms are of utmost importance for agriculture, forestry and the ecology of the environment in general, little knowledge about such systems is available. The present paper reports the presence of glutathione and GSH S-transferases in earthworms of the family Lumbricidae, as well as a qualitative comparison of the enzymes in the different species by use of isoelectric focusing.

Materials and Methods

Earthworms

Worms were dug out in late autumn from a composite compost heap located in a private garden in Stockholm, Sweden. They were kept in the compost material before use. The compost material had not to our knowledge been exposed to pesticides. Fully grown specimens with a clitellum were selected for the study. Species determination (see Table 1) were made by using the keys of Stöp-Bowitz (1969).

Abbreviation used: GSH, reduced glutathione.

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Fig. 1. Isoelectric focusing of GSH S-transferase from six different species of earthworms. The enzymic activities were located by using 1-chloro-2,4-dinitrobenzene as second substrate. The focusing was done by application of 300 V for 3 days. The contents of the column were eluted in 1 or 2 ml fractions. The activity and pH in the fractions were determined immediately after elution. The preparations used were the same as in the first column of Table 1; an amount containing about 100 mg of protein was applied in each case. (a) *Lumbricus rubellus*; (b) *Lumbricus terrestris*; (c) *Allolobophora caliginosa*; (d) *Allolobophora longa*; (e) *Allolobophora chlorotica*; (f) *Eisenia foetida*.

Chemicals

1-Chloro-2,4-dinitrobenzene and 3,4-dichloro-1-nitrobenzene were obtained from Fluka A.G., Buchs, Switzerland, and Schuchardt, Munich, W. Germany, respectively. Other chemicals were obtained from Fluka A.G. (sulphobromophthalein), Eastman Kodak Co., Rochester, NY, U.S.A. [1,2-epoxy-3-(*p*-nitrophenoxy)propane] and Sigma Chemical Co., St. Louis, MO, U.S.A. (glutathione), and *trans*-4-phenylbut-3-en-2-one was a gift from Dr. W. B. Jakoby (National Institutes of Health, Bethesda, MD, U.S.A.). Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Rat liver GSH *S*-transferase was purified as described by Askelöf *et al.* (1975).

Enzyme preparation and assay

Worms were rinsed from external and internal soil by squeezing them between three fingers from the front and backwards, followed by rinsing in cold tap water and blotting with filter paper. Homogenates (25%, w/v) were made, by means of a glass/Teflon Potter-Elvehjem homogenizer, in cold 0.05 M-Tris/HCl buffer (pH 8.0), containing 0.25 M-sucrose. The homogenate was centrifuged for 120 min at 100000g.

Enzyme activity was determined at 30°C by monitoring changes in absorbance with a Beckman DB-G spectrophotometer. The reaction with 3,4-dichloro-1-nitrobenzene was assayed by the method of Booth *et al.* (1961), those with 1-chloro-2,4-dinitrobenzene and *trans*-4-phenylbut-3-en-2-one were assayed as described by Habig *et al.* (1974a), that with 1,2-epoxy-3-(*p*-nitrophenoxy)propane was assayed as described by Fjellstedt *et al.* (1973), and the reaction with sulphobromophthalein as described by Goldstein & Combes (1966). Protein concentration was determined by measurements of A_{280} and A_{260} (Kalckar, 1947).

Determination of GSH

GSH was determined by a specific enzymic method based on the reaction with 1-chloro-2,4-dinitrobenzene, catalysed by purified rat liver GSH *S*-transferase (Crowley *et al.*, 1975). In some experiments the method of Ellman (1959) was also used.

Isoelectric focusing

Before the isoelectric-focusing experiments substances of low molecular weight were removed from the supernatant by gel filtration on a column (2.5 cm × 30 cm) of Sephadex G-25 packed in water. Isoelectric focusing was carried out in a 110 ml column at 4°C according to the instructions of the manufacturer (LKB Produkter AB, Stockholm, Sweden). The concentration of carrier ampholytes was 1% and the system covered the pH range 3.5–10 in a 0–50% (w/v) sucrose density gradient. A voltage of 300 V was applied for 3 days.

Results

GSH *S*-transferase activity

All the earthworms examined exhibited some activity towards chloronitrobenzenes, but that obtained with 3,4-dichloro-1-nitrobenzene was low, and in *A. chlorotica* even questionable (Table 1). The highest ratio of activity towards 3,4-dichloro-1-nitrobenzene to that obtained with 1-chloro-2,4-dinitrobenzene was 0.55% (*A. longa*), whereas the same activity ratio of a rat liver supernatant (cf. Askelöf *et al.*, 1975) was about 3.3%. Enzymic conjugation of glutathione was not detectable with sulphobromophthalein, *trans*-4-phenylbut-3-en-2-one or 1,2-epoxy-3-(*p*-nitrophenoxy)propane. The standard deviations of the specific activities (μmol/min per mg of protein) were slightly higher than those of the activities per wet weight of tissue; therefore the latter are tabulated (Table 1). The activity of *Eisenia foetida* towards

Table 1. Activity of glutathione *S*-transferase towards 3,4-dichloro-1-nitrobenzene (DCNB) and 1-chloro-2,4-dinitrobenzene (CDNB) and the amount of GSH in whole earthworms
GSH concentration and enzyme activity were measured in different specimens.

Species	Activity* (μmol/min per g)			Activity† (± s.d.) (μmol/min per g)			GSH† (± s.d.) (μmol/g)	
	DCNB	CDNB	<i>n</i>	DCNB	CDNB	<i>n</i>	(μmol/g)	<i>n</i>
<i>Eisenia foetida</i> (Savigny)	0.038	9.4	25	0.034 ± 0.008	18.8 ± 7.2	4	1.18 ± 0.32	6
<i>Lumbricus terrestris</i> L.	0.021	10.3	2	0.026 ± 0.005	15.5 ± 2.1	3	0.58 ± 0.09	4
<i>Lumbricus rubellus</i> Hoffmeister	0.073	31.6	7	0.065 ± 0.018	28.5 ± 8.6	4	0.54 ± 0.18	4
<i>Allolobophora longa</i> Ude	0.138	25.7	5	0.154 ± 0.019	27.7 ± 5.0	5	0.51 ± 0.14	4
<i>Allolobophora caliginosa</i> (Savigny)	0.017	15.8	9	0.015 ± 0.006	13.0 ± 3.5	3	0.51 ± 0.05	3
<i>Allolobophora chlorotica</i> (Savigny)	<0.010	6.2	17	0.015 ± 0.002	8.8 ± 1.5	4	0.27	1

* Homogenate made of 5 g of worms. The number of worms (*n*) is given.

† Homogenate made of individuals.

1-chloro-2,4-dinitrobenzene was considerably higher (18.8 ± 7.2) when measured on individuals than the activity (9.4) measured on a single homogenate made of several worms. An explanation for this difference might be that the collection of worms was made on different occasions.

GSH content

All the worms contained GSH as determined by the specific enzymic method (Crowley *et al.*, 1975) (Table 1). In *L. terrestris* the method of Ellman (1959) was used with similar results. *E. foetida* contained on an average about twice as much GSH as the other species. No correlation between GSH *S*-transferase activity and glutathione content could be demonstrated.

Isoelectric focusing

The activity profiles towards 1-chloro-2,4-dinitrobenzene obtained after isoelectric focusing are shown in Fig. 1. The *Lumbricus* species had their main activity in one peak in the acid region, and a small peak in the alkaline region. The other species had a more complicated pattern. The *Allolobophora* had their highest peak in the alkaline region, whereas the *Eisenia* had the highest peak near neutrality.

Discussion

It has previously been established that GSH *S*-transferases have higher activity towards 1-chloro-2,4-dinitrobenzene than towards other substrates containing aryl groups, and Clark & Smith (1975) have accordingly recommended the use of 1-chloro-2,4-dinitrobenzene for comparative studies. The GSH *S*-transferases from earthworms had very low or undetectable activity towards the other substrates tested. In this respect they are similar to the rat liver enzymes called GSH *S*-transferases AA and B (Habig *et al.*, 1976); the latter enzyme was also identified (Habig *et al.*, 1974b) as the binding protein ligandin (Litwack *et al.*, 1971). This similarity might indicate that also the GSH *S*-transferases in earthworms have a binding function in addition to the catalytic properties. However, further purification of the enzymes is necessary to show the suggested binding function.

The existence of enzymic alkyl transfer to glutathione in earthworms remains to be demonstrated. Such reactions are implicated in the biotransformation of several pesticides. In the housefly the purified GSH *S*-transferase (Motoyama & Dauterman, 1977) had a sufficiently broad specificity as to include methyl iodide and 3,4-dichloro-1-nitrobenzene as substrates as well as the insecticides lindane and organophosphorus compounds. Nevertheless, the present report demonstrates that the system involving glutathione and GSH *S*-transferases is operative in earthworms. The original biological function and 'natural' substrates of the system are unknown (as in higher

organisms), but it is clear that the system may play an important role in many detoxification reactions. The further significance of the GSH *S*-transferases in the metabolism of xenobiotics, such as various pesticides, in earthworms requires investigation.

The present study was too limited to lead to any conclusions about the phylogeny of the species examined, but it is noteworthy that the worms belonging to the same genus gave similar activity profiles after isoelectric focusing. More species need to be investigated in order to relate such patterns of enzyme activities to a phylogenetic tree.

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