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ANTIOXIDANT ENZYMES OF LARVAE OF THE CABBAGE LOOPER MOTH, *TRICHOPLUSIA NI*: SUBCELLULAR DISTRIBUTION AND ACTIVITIES OF SUPEROXIDE DISMUTASE, CATALASE AND GLUTATHIONE REDUCTASE

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In the mid-fifth instar larvae of the cabbage looper moth, *Trichoplusia ni*, the subcellular distribution of total superoxide dismutase was as follows: 3.05 units (70.0%), 0.97 units (22.3%), and 0.33 units (7.6%) mg^{-1} protein in the mitochondrial, cytosolic and nuclear fractions, respectively. No superoxide dismutase activity was detected in the microsomal fraction. Catalase activity was unusually high and as follows: 283.4 units (47.3%), 150.1 units (25.1%), 142.3 units (23.8%), and 22.9 units (3.8%) mg^{-1} protein in the mitochondrial, cytosolic, microsomal (containing peroxisomes), and nuclear fractions. No glutathione peroxidase activity was found, but appreciable glutathione reductase activity was detected with broad subcellular distribution as follows: 3.86 units (36.1%), 3.68 units (34.0%), 2.46 units (23.0%), and 0.70 units (6.5%) mg^{-1} protein in the nuclear, mitochondrial, and cytosolic fractions, respectively. The unusually wide intracellular distribution of catalase in this phytophagous insect is apparently an evolutionary adaptation to the absence of glutathione peroxidase; hence, lack of a glutathione peroxidase-glutathione reductase role in alleviating stress from lipid peroxidation. Catalase working sequentially to superoxide dismutase, may nearly completely prevent the formation of the lipid peroxidizing ·OH radical from all intracellular compartments by the destruction of H_2O_2 which together with $O_2^{\cdot-}$ is a precursor of ·OH.

KEY WORDS: Superoxide Dismutase, Catalase, Glutathione Reductase, Subcellular Distribution, *Trichoplusia ni*, Oxyradicals.

ABBREVIATIONS: $^3O_2$, dioxygen, or triplet oxygen; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion free radical; ·OOH, hydroperoxy radical; ·OH, hydroxyl radical; CAT, catalase; GPOX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; LOOH, lipid/organic hydroperoxides.

INTRODUCTION

All organisms studied to date have a variety of enzymatic mechanisms to remove toxic by-products of dioxygen consisting of $O_2^{\cdot-}$, ·OOH and ·OH free radicals and H_2O_2. In eukaryotes, the best studied system of antioxidant enzymes is that found in rat liver,[1] consisting of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase.[1,2] Considering the wide array of structurally highly diverse pro-oxidants and their wide taxonomic occurrence in plants,[3] the role of antioxidant enzymes in protecting herbivorous insects from harmful consequences of ingesting

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pro-oxidants is of considerable interest. However, until very recently this aspect was virtually an unexplored area of research.

Plant pro-oxidants such as furanocoumarins, e.g., xanthotoxin, are photodynamically activated and generate primarily $^1O_2$ and secondarily $O_2^{\cdot-}$[4]. Other plant pro-oxidants such as flavonoids, e.g., quercetin, enzymatically generate $O_2^{\cdot-}$, H_2O_2 and $\cdot OH$.[5,6] In a preliminary paper we reported on the toxicological responses of larvae of three lepidopteran species, the cabbage looper moth (*Trichoplusia ni*), southern armyworm (*Spodoptera eridania*) and the black swallowtail butterfly (*Papilio polyxenes*), together with levels of antioxidant enzymes in 850 *g* whole body supernatants.[7] *T. ni* is highly susceptible because of its semi-specialist feeding on cruciferous plants which lack phototoxins, and contain only small amounts of quercetin.[7,8] No acute toxicity from photoactivation of xanthotoxin was discerned in the broadly polyphagous *S. eridania*, except for markedly suppressed relative growth rates suggesting onset of toxicity.[7,9] On the other hand, *P. polyxenes* is adapted to feed with impunity on many species of Umbelliferae and a few species of Rutaceae known to contain high levels of xanthotoxin.[10] Its response to quercetin is not fully known except that additional coating of parsley (*Petroselinum crispum;* family Umbelliferae) leaves with 2.0% w/v quercetin resulted in complete consumption by the mid-fifth instar larvae without any apparent harmful effect.[11] Levels of the enzymes, SOD, CAT and GR were commensurate to this insect model of low, moderate and high dietary exposure to plant pro-oxidants.[7] GPOX activity was not detected in any of these three species.

As *T. ni*[8] and *S. eridania*[9] advance from third to fifth instar, and mature within these instars, the activities of SOD and CAT, but not GR, parallel larval development and feeding activity. Subsequently, we demonstrated that the subcellular distribution of SOD in *S. eridania* was typically eukaryotic, while considerably different for CAT and GR.[12]

We present the subcellular profile of the antioxidant enzymes of *T. ni* which shows unusual intracellular distribution of CAT and GR analogous to that observed in *S. eridania*. These data strengthen the hypothesis[12] that the unusual distribution of CAT and GR is an evolutionary answer to the absence of GPOX enzyme in these insects.

MATERIALS AND METHODS

Subcellular fractionation

Twenty to 30 g (for two separate experiments) of mid-fifth instar *T. ni* larvae were crushed in a porcelain mortar with a pestle in an ice-chilled, pH 7.4, buffer of the composition described earlier.[12] Filtered crude homogenate was subjected to differential centrifugation at 2°C according to a modified procedure of Halarnkar *et al.*[13] With *S. eridania* electron microscopy had shown good mitochondrial and microsomal preparations, but the nuclear fraction was predominated by intact whole cells.[12] In the present study, the procedure was further modified and concurrent electron microscopic examinations confirmed that the nuclear fraction was free of whole cells and the mitochondrial and microsomal fractions were homogeneous and their quality was similar to that described earlier for *S. eridania*.[12]

The filtered crude homogenate was spun at 600 *g* for 10 minutes to discard unmacerated chunks of tissues and floating lipids. The supernatant was spun at 1200 *g* for 10 min and the pellet containing whole cells was discarded. Subcellular fractions were then obtained as follows: nuclear at 12,000 *g* for 5 sec; mitochondrial at 46,000 *g*

for 15 sec; and microsomal fraction at 100,900 *g* for 45 min. To minimize cross contamination, each fraction's pellet was resuspended in the homogenization buffer and spun again as above to obtain washed pellets. The 100, 900 *g* supernatant was the cytosolic fraction.

Preparation of subcellular fractions for enzyme assays

The pellets were suspended in 4.0 ml of 50 mM potassium phosphate buffer, pH 7.8. The suspensions were gently sonciated[12] to release enzymes from the membrane-bound organelles to facilitate their interaction with the exogenous materials in the incubation mixtures.

Assays for antioxidant enzymes

CAT activity was assayed with 10 μl, SOD with 20 μl and GR with 30 μl aliquots of the subcellular fractions. Proteins were determined by the method of Lowry *et al.*[14]

Assays were according to the same procedures used earlier.[8,9,12] The SOD activity was assayed by the method of Oberley and Spitz,[15] and according to McCord and Fridovich[16] the activity was expressed in units mg^{-1} protein min^{-1} at 25°C. For the CAT assay, pH of the incubation mixture was adjusted to 7.0 and one unit of CAT activity was defined[17] as decomposing 1 μmol H_2O_2 mg^{-1} protein min^{-1} at pH 7.0 and 25°C. For the GR activity, one unit was defined [18] as the change in absorbance of 0.001 O.D. due to the oxidation of NADPH to $NADP^+$ at 340 nm mg^{-1} protein min^{-1} at 25°C.

RESULTS AND DISCUSSION

The results are summarized in Table I. The SOD activity was located primarily in the mitochondrial and cytosolic fractions amounting to 70.1 and 22.3%, respectively. The mitochondrial level of SOD showed a 1.7-fold enrichment over the level observed in the 850 *g* supernatant of mid-fifth instar larvae of *T. ni.*[8] The nuclear fraction contained a marginal, 7.6% level of the total SOD activity. No SOD activity was detected in the microsomal fraction.

The distribution of SOD primarily in the mitochondria and cytosol is consistent with the distribution in other eukaryotes,[1] but the ratio of the enzyme in the two subcellular compartments is not typical. In mammalian livers, 80–85% of the SOD is in the cytosol in the form of CuZn SOD, and only 15–20% is in the mitochondria mainly as Mn SOD.[1,19] Similarly in the insect, *S. eridania,* much of the total SOD was in the cytosolic, 67%, and lesser amounts, 33%, in the mitochondrial fraction.[12] Thus, the present finding in *T. ni* of higher SOD activity in the mitochondrial than in the cytosolic fraction deserves further work. Of particular interest is whether the more active mitochondrial enzyme of *T. ni* is entirely Mn SOD or contains also CuZn SOD, because such anomalies have been reported.[2]

SOD has been suggested to be present in eukaryotic nuclei and chloroplasts, however, a more rigorous confirmation is needed in the absence of comparable data in other insect species, in order to confirm that in *T. ni* the marginal nuclear SOD activity observed was not an artifact. The total SOD activity in *T. ni* of the combined mitochondrial and cytosolic fractions was 4.02 units compared with 6.39 units mg^{-1}

protein for *S. eridania*.[12] This difference is readily explained when we consider the breadth of plant diet for the two insects and, hence, the potential risk for encountering exogenous plant pro-oxidants which are sources for O_2^- and other toxic $^3O_2$ metabolites.

In all subcellular fractions examined the CAT activity was detected (Table I) in the order: mitochondrial, 47.3%; cytosolic, 25%; microsomal, 23.8%; and nuclear, 3.8%, fractions. The mitochondrial CAT level was significantly ($P < 0.05$) higher over the cytosolic and microsomal levels which were not significantly different. At this time, minor CAT activity associated with the nuclear fraction of *T. ni*, 3.8% of the total, does not unequivocally establish the presence of CAT in that fraction and may well be a procedural artifact. The generalized distribution of CAT in both *T. ni* and *S. eridania* is unique in that so far the consensus is that catalatic activity is located in peroxisomes[1] where flavin enzymes directly produce H_2O_2 via two-electron reduction of $^3O_2$ without the intermediacy of O_2^-. In accordance with the procedure of subcellular fractionation used, peroxisomes are usually isolated together with fragments of endoplasmic reticulum. As reviewed,[12] CAT has been detected in the mammalian cytosol but its presence was only recently confirmed in mitochondria.

A noteworthy feature of CAT activity of *T. ni* is its very high activity, 283.4 ± 41.4 units mg^{-1} protein, in the mitochondria which is the same, 307.5 ± 17.0 units mg^{-1} protein, reported for 850 *g* supernatant of the whole body homogenate of mid-fifth instars.[8] We have no explanation at this time for the apparent lack of enrichment of CAT activity in subcellular fractions. In contrast, two-fold enrichment of CAT was observed in *S. eridania*[12] with the highest level reaching 163 units mg^{-1} protein. Nevertheless, the combined CAT activity of all subcellular fractions is ca. 600 units (cf. Table I) in *T. ni*, whereas it is ca. 400 units mg^{-1} protein in *S. eridania*,[12] supporting the earlier contention[7,8] that CAT in *T. ni* is by far more active than CAT's of other species reported in the literature.

The GPOX was found to be absent in the 850 *g* whole body supernatants of third to fifth instar *T. ni*[8] and *S. eridania*[9] larvae. Moreover, subcellular fractionation did not provide evidence for the presence of this enzyme in *S. eridania*.[12] Likewise, preliminary work revealed trivial levels, i.e., 1×10^{-4} to 1×10^{-3} units 100 μg^{-1} protein of GPOX, perhaps reflecting a procedural artifact, in the subcellular fractions

TABLE I

The subcellular distribution and activities of antioxidant enzymes in the larvae of the cabbage looper moth

Antioxidant enzyme	Enzyme activity; mean ± S.D. units mg^{-1} protein min^{-1} at 25°C[1]			
	Nuclear	Mitochondrial	Microsomal	Cytosolic
SOD[2]	0.33 ± <.01a	3.05 ± 0.11b	0.00c	0.97 ± 0.04d
CAT[3]	22.9 ± 3.1a	283.4 ± 41.4b	142.3 ± 31.2c	150.1 ± 18.4c
GR[4]	3.86 ± 1.21a	3.68 ± 1.00a	2.46 ± 1.08b	0.70 ± 0.21c

[1] Duplicate assays were conducted with two to four replicates in each determination; $N = 4$ for SOD and 8 for CAT and GR. All replicates were pooled for data analysis by ANOVA, and further by the Duncan's multiple range test (DMRT; $\alpha = 0.05$) to determine significant differences among the means.

[2] ANOVA; $F_{(3,12)} = 1634.4$, $P > F = 0.0001$. Means not followed by the same letter are significantly ($P < 0.05$) different by DMRT.

[3] ANOVA; $F_{(3,28)} = 109.06$, $P > F = 0.0001$. Means not followed by the same letter are significantly ($P < 0.05$) different by DMRT.

[4] ANOVA; $F_{(3,28)} = 16.23$, $P > F = 0.0001$. Means not followed by the same letter are significantly ($P < 0.05$) different by DMRT.

of *T. ni*, hence, no further effort was extended to quantify this enzyme. Thus, as discussed elsewhere,[12] *T. ni* is similar to *S. eridania* and *P. polyxenes*[7] in the absence of this enzyme from our insect model of lepidopteran species.

The GR, unlike the GPOX, is present in all three aforementioned species of insects. The subcellular distribution pattern in *T. ni* was as follows: nuclear, 36.1%; mitochondrial, 34.4%; microsomal, 23.0%; and cytosolic, 6.5%, fractions (Table I). Although a direct comparison cannot be made with *S. eridania* because GR in the nuclear fraction was not assayed,[12] the distribution is somewhat similar in both species, and further the cytosolic fraction of *T. ni* had the lowest/negligible level, 6.5% of the total GR, and no activity in *S. eridania*.[12] In eukaryotes, GR together with GPOX works sequentially to remove LOOH formed from ·OH radical generated by the metal-catalyzed Haber–Weiss rection.[20] There is no evidence for such a role in lepidopteran insects, instead as discussed by Ahmad *et al.*[12] (and references therein), the GR may have a thiol regulating role unrelated directly to the oxidative stress in insects. It is noteworthy in this context that GR relative to SOD and CAT enriches more highly on subcellular fractionation. Based on data of the 850 *g* whole-body supernatant,[8] the enrichment is 3.3- and 3.2-fold for the nuclear and mitochondrial fractions, respectively.

The GR may be especially important in preventing excessive accumulation of GSSG because the preservation of mitochondrial integrity and function is dependent on a crucial balance between GSSG and GSH. The formation of GSSG has been observed in the housefly, *Musca domestica*, without evidence of the presence of GPOX.[21] The amounts of GSH in insects are equal to or greater than in the vertebrate liver.[22] The enzyme glutathione transferase which occurs widely among insects of different taxonomic orders[23] may be the source of GSSG in insects. This enzyme is involved in numerous GSH-transferal reactions, including some isomerization reactions and disulfide interchange.[24–26] It is clear then that as stated by Chance *et al.*,[1] glutathione transferase activity overlaps that of GPOX in the formation of GSSG. We are currently investigating the role of glutathione transferase in destroying LOOH but not H_2O_2.

A hypothesis advanced earlier with the work on *S. eridania*[12] is supported by the data presented in this report on *T. ni*. Apparently in phytophagous insects, protection from $O_2^{\cdot-}$ and H_2O_2 are afforded by SOD with CAT working sequentially. Highly active and intracellularly very broadly distributed CAT guarantees nearly complete destruction of H_2O_2 produced by SOD-catalyzed dismutation of $O_2^{\cdot-}$ and by direct two-electron reduction of $^3O_2$. A free-radical chain reaction is prevented, hence the formation of ·OH radical. The deleterious LOOH formation may thus be, in large measure, prevented. In the absence of GPOX-GR catalyzed destruction of even a trivial amount LOOH formed, ancillary enzymes such as carbonyl reductases, glutathione transferases and epoxide hydrolases by reacting with cytotoxic aldehyde, ketone and epoxide products of LOOH during lipid peroxidation may produce innocuous alcohols, glutathione conjugates and dihydrodiols.[11] These enzymes are being examined in our laboratory from this standpoint.

In summary, the subcellular fractionation study revealed the presence of mitochondrial (predominantly) and cytosolic SOD. CAT which acts sequentially to SOD to destroy H_2O_2 produced by the dismutation of $O_2^{\cdot-}$, or by direct two-electron reduction of $^3O_2$, was found to be a far more active enzyme than in other organisms and distributed in the cytosol, mitochondria and microsomes with low activity in nuclei. GR activity was found in all subcellular fractions with the highest level in the nuclear and lowest in the cytosolic fraction: GPOX was found to be absent.

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