J. Pesticide Sci. **25**, 18-23 (2000)

Original Article

Enzymatic Activity of Protoporphyrinogen-IX Oxidase from Various Plant Species: Its Sensitivity to Peroxidizing Herbicides

Shizuka ISHIDA,* Roswitha MILLER-SULGER,† Hitoshi KOHNO, Peter BÖGER† and Ko WAKABAYASHI

Graduate School of Agricltural Science, Tamagawa University, Machida, Tokyo 194-8610, Japan
† Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-78434 Konstanz, Germany

(Received May 31, 1999; Accepted November 8, 1999)

Enzyme activity of protoporphyrinogen-IX oxidase (Protox) prepared from 34 kinds of plant sources, containing 18 monocotyledons and 16 dicotyledons was evaluated. Protox(es) originated in monocotyledonous plants such as *Zea mays* cv. Anjou and DK212, *Lolium perenne* and *Poa annua* exhibited high enzyme activity, and Protox(es) in dicotyledonous plants such as *Agrostemma githago* and *Arabidopsis thaliana* showed a little higher enzyme activity than that of the above monocotyledonous plants. Highly active Protox(es) obtained from *Zea mays* cv. Anjou, *Lolium perenne, Agrostemma githago* and *Arabidopsis thaliana* were selected for the Protox inhibitory assay of peroxidizing herbicides. *Echinochloa utilis* was also selected for control. Protox inhibition by six peroxidizing herbicides such as oxyfluorfen, chlorophthalim, BW-91, pyraflufen-ethyl, DLH-1777 and LS-82556 was investigated using highly active Protox from the above monocotyledons and dicotyledons. As a result, six peroxidizing herbicides exhibited high inhibitory activity to Protox. Pyraflufen-ethyl showed the highest inhibition. The order of Protox inhibitory activity was pyraflufen-ethyl > oxyfluorfen > BW-91 > chlorophthalim > DLH-1777 > LS-82556, in experiment using four Protox(es) such as *Zea mays* cv. Anjou, *Echinochloa utilis, Agrostemma githago* and *Arabidopsis thaliana* except *Lolium perenne*. Oxyfluorfen exhibited the highest inhibitory activity to Protox of *Lolium perenne*. On the other hand, DLH-1777 showed a little less activity to Protox of *Zea mays* than that of other four weeds. DLH-1777 may become a model compound to find out new selective herbicides. Protox from four weeds will be used for peroxidizing herbicides assay, since the Protox showed high sensitivity to structually different peroxidizers.

Key words: protoporphyrinogen-IX oxidase (Protox), enzyme activity, sensitivity, peroxidizing herbicides, selectivity.

INTRODUCTION

So-called peroxidizing herbicides such as oxyfluorfen and chlorophthalim in the first generation and miscellaneous heterocycles such as LS-82556 and pyraflufen-ethyl (ET-751) in the second generation primarily inhibit the activity of protoporphyrinogen-IX oxidase (Protox, EC 1.3.3.4), which catalyzes oxidation of protoporphyrinogen-IX (Protogen) to protoporphyrin-IX (Proto-IX) in chlorophyll biosynthesis.[1-4] This inhibition is accompanied with an abnormal accumulation of Proto-IX or a derivative thereof, which acts as a photosensitizer in the light leading to destruction of cellular components with peroxidative ethane formation.[5] This is an overview on herbicidal principle of the peroxidizing herbicides. Determination of the Protox inhibition of compounds tested has priority against other screening tests for the peroxidizing phytotoxicities, to find out the better peroxidizers. Although the potency of peroxidizing phytotoxicity may be predicted by the Protox inhibition caused by the compounds, the Protox prepared from *Zea mays* cv. Anjou has been usually used for the Protox assay until now. Therefore the herbicidal selectivity of the compounds tested can not be discussed. In this paper, both enzyme activity of Protox(es) prepared from 34 kinds of plant sources including useful plants and the Protox inhibition by structurally different six kinds of peroxidizing herbicides (Fig. 1) are discussed, and

* To whom correspondence should be addressed.

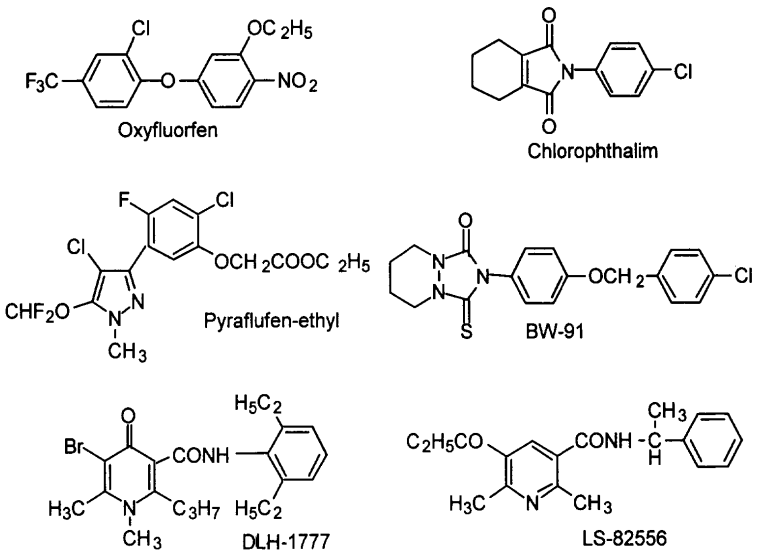

Fig. 1 Chemical structures of Protox inhibitors tested.

furthermore, the sensitivity to these herbicides is compared to find out the more suitable and available Protox, resulting in more convenient assay system of Protox inhibitory activity.

MATERIALS AND METHODS

1. Chemicals

Oxyfluorfen (2-chloro-4-trifluoromethylphenyl- 3′-ethoxy-4′-nitrophenyl ether) was prepared from 3,4-dichlorobenzotrifluoride and 3-ethoxy-4-nitrophenol according to Yih and Swithenbank.[6] BW-91 {4-(4-chlorobenzyloxyphenyl)-1,2-tetramethylene-3-oxo-1,2,4-triazolidine-5-thione} was synthesized by the intramolecular condensation of Ethyl 2-{4-(4-chlorobenzyloxy) phenylthiocarbamoyl}hexahydopyridazinecarboxylate.[7] Other four peroxidizing herbicides; chlorophthalim (*N*-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide), pyraflufen-ethyl (ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methylpyrazol-3-yl)-4-fluorophenoxyacetate), LS-82556 (2,6-dimethyl-*N*-(α-methylbenzyl)-3-propionyl-5-pyridinecarboxamide) and DLH-1777 (3-bromo-*N*-(2,6-diethylphenyl)-1,2-dimethyl-6-propyl-4-pyridone-5-carboxamide) were kindly supplied by Mitsubishi Chemical Industries Ltd. (Yokohama, Japan), Nippon Nohyaku Co., Ltd (Osaka, Japan), Rhône-Poulane Agrochimie (France) and Dicel Co. Ltd. (Osaka, Japan), respectively.

2. Seeds

Seeds of *Allium fistulosum, Brassica pekinensis, Brassica rapa, Glycine max, Raphanus sativus* and *Vigna radiata* were purchased from Tohoku Co., Utsunomiya, Japan. Seeds of four varieties of corn and other seeds used in this study were kindly provided by Yukijirushi-Shubyou Co., Tokyo and Herbiseed Co., England, respectively.

3. Determination of Enzyme Activity

Activity of Protox prepared from four varieties of corn (*Zea mays* cv. Anjou (ZmA), DK212, DK652 and LG2205) was determined according to our methods.[8–11] Enzyme activity of other 30 plants, 14 monocotyledons and 16 dicotyledons (see Tables 1 and 2), was assayed by the following procedure; (1) the seeds were germinated on filter paper in the dark at 30°C for 5–21 days indicated in Tables 1 and 2, *e.g.* for 7 days for *Lolium perenne* (Lop) and *Echinochloa utilis* (Ecu), for 6 days for *Agrostemma githago* (Agg), for 10 days for *Arabidopsis thaliana* (Art). (2) The seedlings were harvested after exposure to light (300 μEinstein(E)/m²s) for 2 to 20 hr. Light-exposing time of each plant should be adjusted to the most suitable time, depending upon greening capacity of each etioplast to obtain the maximum enzyme activity of each Protox from plant species in Tables 1 and 2. For example, in the case of *Lolium perenne*, when the light-exposing time (0, 5, 10, 15, 20 and 25 hr) was changed from 0 to 25 hr, the maximum enzyme activity was observed after 20 hr as shown in Fig. 2. (3) After homogenizing the seedlings in the 5–10 times amount of 0.05 M of Tris-HCl (pH7.3) containing 0.5 M of sucrose, 1 mM of $MgCl_2$, 1 mM of EDTA and 0.2% of bovine serum albumin (w/v), the filtrate obtained by filtration of the mixture was centrifuged for 2 min at 800×*g* to remove cell debris. The supernatant was again centrifuged for 5 min at 17,000×*g*. The pellet obtained was used as crude Protox for the following experiments. Protein content of the crude Protox preparation was determined by the method of Lowry using bovine serum albumin as the standard. (4) Protogen was prepared by reduction of Proto-IX with sodium amalgam under a nitrogen atmosphere in the dark. (5) Enzyme activity was determined using a 3 ml of assay solution containing 0.1 M of Tris-HCl (pH7.5), 1 mM of EDTA, 5 mM of

Table 1 Enzyme activities of Protox from various monocotyledons.

Plant		Germination (day)	Light exposure time (hr)	Protein (mg/ml)	Enzyme activity nmol Proto-IX / mg Protein·hr
Aegilops cylindrica	(goatgrass)*	14	4	11	0.6
Agrostis tenuis	(browntop)	15	7	15	1.7
Avena sterilis	(sterile oat)	7	17	7	1.8
Bromus diandrus	(greatbromegrass)	6	16	18	0.6
Bromus secalinaus	(cheat)	7	7	28	0.4
Echinochloa crus-galli	(barnyard grass)	11	4	14	3.4
Echinochloa utilis	(japanese barnvard millei)	7	4	15	6.3
Festuca rubra	(red fescue)	12	7	12	3.4
Lolium perenne	(perennial ryegrass)	7	20	8	9.1
Lolium temulentum	(darnal ryegrass)	8	16	11	7.9
Panicum miliacium	(common millet)	10	5	4	1.6
Poa annua	(annual bluegrass)	7	7	12	9.7
Setaria glauca	(yellow foxtail)	10	3	7	0.5
Allium fistulosum	(welsh onion)	6	6	7	3.9
Zea mays cv. Anjou	(corn)	7	2	33	12
Zea mays cv. DK212	(corn)	7	2	37	10
Zea mays cv. DK652	(corn)	7	2	36	4.8
Zea mays cv. LG2205	(corn)	7	2	40	7.8

*Common name in parentheses.

Table 2 Enzyme activities of Protox from various dicotyledons.

Plant		Germination (day)	Light exposure time (hr)	Protein (mg/ml)	Enzyme activity nmol Proto-IX / mg Protein·hr
Abutilon theophrasti	(velvet leaf)*	14	2.5	19	1.2
Agrostemma githago	(corn cockle)	6	4	31	15
Ambrosia artemisiifolia	(common ragweed)	7	4	24	5.3
Arabidopsis thaliana	(thale cress)	10	4	7	12
Cassia tora	(low senna)	15	6	6	0.3
Ipomoea hederacea	(ivyleaf morningglory)	7	6.5	21	0.1
Ipomoea purpurea	(tall morningglory)	7	7	15	0.7
Pueraria lobata	(kudzu vine)	21	4	22	2.0
Sesbania exaltata	(coffee bean)	10	2	12	2.7
Sinapis alba	(white mustard)	7	9	23	4.2
Xanthium strumarium	(common cocklebur)	15	5	15	0.8
Brassica pekinensis	(chinese cabbage)	14	4	9	3.9
Brassica rapa	(turnip)	5	4	9	2.8
Glycine max	(soybean)	13	5	32	1.3
Raphanus sativus	(radish)	5	4	2	3.7
Vigna radiata	(mung bean)	7	3	23	2.2

*Common name in parentheses.

dithiothreitol (DTT), 0.03% of Tween 80 (w/v), 0.3–0.6 mg of the crude Protox and 2–5 μM of Protogen. The amount of Proto-IX formed for 5 min was determined by a Hitachi F 2000 fluorescence spectrophotometer with a thermostated cell holder, using an excitation and emission wavelength of 405 and 633 nm, respectively. The rate of non enzymatic oxidation of Protogen, which was determined by the experiment using inactive Protox by heat-denaturation and/or sonication, was subtracted from the enzyme activity measured. Therefore, Protox activity was calculated as the specific activity (nmol Proto-IX/mg protein·hr).

4. *Protox Inhibitory Activity by Peroxidizing Herbicides*

Protox inhibitory activity by peroxidizing herbicides

was assayed according to the following procedure; After adding each compound to a final assay volume of 3 ml containing 0.1 M of Tris-HCl (pH7.5), 1 mM of EDTA, 5 mM of dithiothreitol (DTT), 0.03% of Tween 80 (w/v), 0.3–0.6 mg of the crude Protox and 2–5 μM of Protogen, the amount of Proto-IX formed was measured in the same manner mentioned in 3. The moler I_{50} (Protox)-values were determined by reciprocal plots of protoporphyrin-IX formation (in percent of control) vs. inhibitior concentration (Dixon plot). The pI_{50} (Protox)-values were calculated from the equation; pI_{50} (Protox) $= -\log I_{50}$ (Protox).

RESULTS AND DISCUSSION

Both enzyme activity of Protox extracted from 34 kinds of plant sources, 18 monocotyledons and 16 dicotyledons, and Protox inhibition by peroxidizing herbicides were investigated to find out the more suitable Protox sources useful for more convenient assay system to find out much more active and selective peroxidizing herbicides (Tables 1 and 2). To obtain enzymatically more active Protox preparation from etioplasts, relationship between enzyme activity and light-exposing time for greening was examined using each etioplast. As shown in Fig. 2, both enzyme activity and chlorophyll content in the case of crude Protox obtained from, for example, *Lolium perenne* were negligible in the dark (0 hr exposure). Maximum enzyme activity was observed after 20 hr exposure to light, although chlorophyll content was still increasing after 25 hr. The greening step may be released by the exposure to light, accompanying a little delay of chlorophyll increase.

1. *Enzyme Activity of Protox Prepared from Etioplasts*

Enzyme activity of Protox(es) originated in 18 monocotyledons and 16 dicotyledons is shown in Tables 1 and 2. Protox(es) of monocotyledonous plants, such as *Zea mays* cv. Anjou and DK212, *Lolium perenne* and *Poa annua* exhibited high specific activity of 12, 10, 9.1 and 9.7 nmol Proto IX/mg protein·hr, respectively. Furthermore, Protox(es) in dicotyledonous plants, such as *Agrostemma githago* and *Arabidopsis thaliana* showed a little higher enzyme activity than that of the above monocotyledonous plants. Based on these results, four kinds of Protox(es) extracted from *Zea mays* cv. Anjou, *Lolium perenne, Agrostemma githago* and *Arabidopsis thaliana* were selected for the Protox inhibitory assay of peroxidizing herbicides, because of their high enzyme activity. Additionally, the etioplast from *Echinochloa utilis*, which was one of hazardous grasses to be controlled, was also selected for Protox inhibitory assay though its enzyme activity was not so high.

2. *Protox Inhibition by Different Peroxidizing Herbicides*

Six peroxidizing herbicides for Protox inhibitory assay were selected, oxyfluorfen as diphenylethers, chlorophthalim and BW-91 as cyclic imides, pyraflufen-ethyl as five membered heterocycles, and LS-82556 and DLH-1777 as noncyclic imides, respectively. Using these more active Protox(es), more precise pI_{50}-values were expected to be obtained. As shown in Table 3, the order of Protox inhibitory activity by compounds tested was pyraflufen-ethyl > oxyfluorfen > BW-91 > chlorophthalim > DLH-1777 > LS-82556; especially pyraflufen-ethyl exhibited the highest pI_{50} value of more than 8.30 except for Protox(Lop). The strong Protox-inhibitors, *i.e.* oxyfluorfen, chlorophthalim, pyraflufen-ethyl and BW-91, exhibiting ca. $pI_{50} = 7$–9 against Protox(es), are rather non-selective against both mono- and di-cotyledonous plants. However the weak Protox-inhibitor, *i.e.* DLH-1777, appears to be less active to monocotyledons only Protox(ZmA). Against Protox(Lop) the order of inhibition by the compounds was oxyfluorfen > pyraflufen-

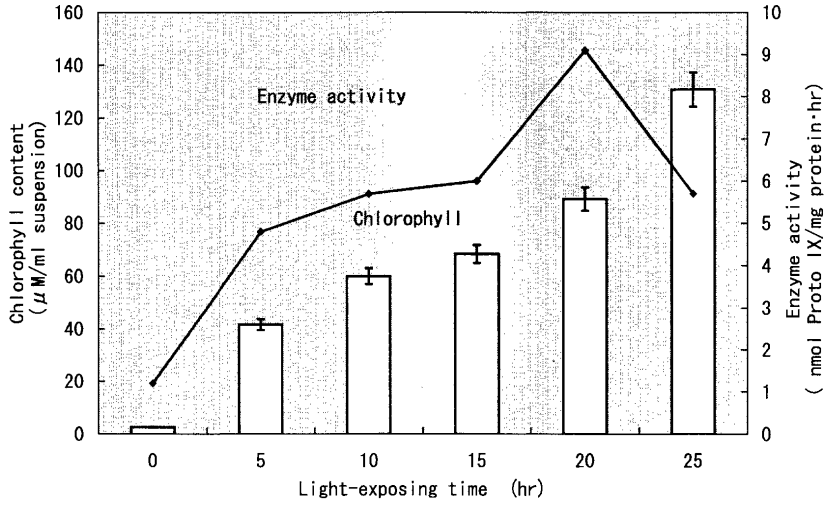

Fig. 2 Influence of light exposure on Enzyme activity and chlorophyll content in *Lolium perenne*.

Table 3 Protox inhibition (pI_{50} values) by six peroxidizing herbicides.

Plant	Oxyfluorfen	Chlorophthalim	LS-82556	Pyraflufen-ethyl	BW-91	DHL-1777
Monocotyledons						
Zea mays cv. Anjou	8.71	7.60	5.25	9.00	8.30	5.85
Echinochloa utilis	8.27	7.04	5.43	8.94	7.22	6.62
Lolium perenne	8.55	7.07	5.55	8.28	7.15	6.93
Dicotyledons						
Agrostemma githago	8.84	7.95	5.14	9.11	8.09	6.92
Arabidopsis thaliana	8.57	7.55	5.32	8.74	8.45	6.50

ethyl > BW-91 > chlorophthalim > DLH-1777 > LS-82556. DLH-1777 and LS-82556 were again less active inhibitors against the Protox(Lop). Oxyfluorfen was more active to Protox(Lop) than pyraflufen-ethyl, but a difference of the sensitivity between oxyfluorfen and pyraflufen-ethyl was very small, showing only ca. two times. DLH-1777 was less active to Protox(ZmA), because it exhibited more than ten-times stronger inhibitory activity against Protox(es) from mono- and dicotylidonous weeds than Protox(ZmA). The compound BW-91 being strong inhibitor for Protoxes(ZmA, Agg and Art) was about one tenth a less active inhibitor against Protoxes(Ecu and Lop) which are monocotyledons. This fact clearly indicates that BW-91 has a possibility to be a more active herbicide against dicotyledons weeds. In any case, such different activity is rather very small, so further study is neccessary to find out more selective compounds.

Since DLH-1777 was less active to *Zea mays* and 10 times more active to four weeds, this compound should be noted regarding the difference of the sensitivity between crops and weeds (see Table 3). This difference of the activity in Protox inhibitory assay is not always the case in plant phytotoxicity. Such difference of activity of the compounds against plants is necessary to study furthermore, to design the more selective herbicidal compounds. Since Protoxes extracted from *Lolium perenne, Echinochloa utilis, Agrostemma githago* and *Arabidopsis thaliana* exhibited a high sensitivity to various peroxidizing herbicides, these can be used to assay peroxidizing herbicides.

ACKNOWLEDGMENTS

The authours appreciate the valuable suggestions by Dr. Hitoshi Ogawa of Tamagawa University. This work was supported by a Grant-in-Aid for General Scientific Research (A) from Ministry of Education, Science, Sports and Culture of Japan. Due thanks are also expressed to the Monbusho International Scientific Research Program: University-to-University Cooperative Research, through which the present work has been markedly promoted.

REFERENCES

1) P. Böger & K. Wakabayashi: *Z. Naturforsch.* **50C**, 159 (1995)
2) K. Wakabayashi & P. Böger: *Z. Naturforsch.* **50C**, 591 (1995)
3) K. Wakabayashi & P. Böger: "Pesticides/Environment: Molecular Biological Approaches," ed. by T. Mitsui, F. Matsumura & I. Yamaguchi, Pesticides Science Society of Japan, Tokyo, pp. 239–253, 1993
4) H. Watanabe, Y. Ohori, G. Sandmann, K. Wakabayashi & P. Böger: *Pestic. Biochem. Physiol.* **42**, 99 (1992)
5) M. Sumida, S. Niwata, S. Iwata, T. Tanaka, K. Wakabayashi & P.Böger: "Pesticides/Environment: Molecular Biological Approaches," ed. by T. Mitsui, F. Matsumura & I. Yamaguchi, Pesticides Science Society of Japan, Tokyo, pp. 255–262, 1993
6) R. H. Yih & C. Swithenbank: *J. Agric. Food Chem.* **23**, 592 (1975)
7) H. Ohta, T. Jikihara, K. Wakabayashi & T. Fujita: *Pestic. Biochem. Physiol.* **14**, 153 (1980)
8) B. Nicolaus, G. Sandmann & P. Böger: *Z. Naturforsch.* **48C**, 326 (1993)
9) B. Nicolaus, G. Sandmann & P. Böger: "Target Assay for Modern Herbicides and Related Phytotoxic Compounds," ed. by P. Böger & G. Sandmann, Lewis Publ., Boca Raton, Florida, pp. 35–41, 1993
10) S. Ishida, K. Hirai, H. Kohno, Y. Sato, H. Kubo, P. Böger & K. Wakabayashi: *J. Pesticide Sci.* **22**, 299 (1997)
11) S. Ishida, T. Iida, H. Kohno, Y. Sato, H. Kubo, P. Böger & K. Wakabayashi: *J. Pesticide Sci.* **24**, 28 (1999)

要　　約

各種植物から抽出したプロトポルフィリノーゲン-IX オキシダーゼの酵素活性：peroxidizing 除草剤に対する感受性

石田静香，Roswitha Miller-Sulger
河野　均，Peter Böger，若林　攻

有用植物を含む18種の単子葉と16種の双子葉植物合わせて34種の植物から protoporphyrinogen IX oxidase (Protox)を抽出した．*Zea mays* cv Anjou, DK212, *Lolium perenne* や *Poa annua* などの単子葉植物から抽出した Protox が，中でも高い酵素活性を示した．また双子葉植物の中では *Agrostemma githago* と *Arabidopsis thaliana* から抽出した Protox がさらに高い酵素活性を示した．そこでこれら高い酵素活性を有する Protox の中から *Zea mays* cv. Anjou, *Lolium perenne*, *Agrostemma githago* および *Arabidopsis thaliana* から抽出した Protox を選び，peroxid-

izing 除草剤による Protox 阻害活性試験に使用した．また雑草のノビエに近い *Echinochloa utilis* から得られた Protox も併せて使用した．

Protox 阻害活性試験には，全く化学構造が異なる 6 種の peroxidizing 除草剤(oxyfluorfen, chlorophthalim, BW-91, pyraflufen-ethyl, DHL-1777, LS-82556)を用いた．その結果いずれの化合物もこれらの Protox に対して強い阻害活性を示し，中でも pyraflufen-ethyl が最も強い活性を示した．これらの化合物の Protox 阻害活性は *Lolium perenne* を除く 4 種の植物から抽出した Protox に対して pyraflufen-ethyl＞oxyfluorfen＞BW-91＞chlorophthalim＞DHL-1777＞LS-82556 の順に活性が強く，*Lolium perenne* 由来の Protox に対しては oxyfluorfen が最も強かった．一方 DHL-1777 は *Zea mays* 以外の 4 種の植物に比べ *Zea mays* 由来の Protox に対する活性が低いことから，*Zea mays* に対して選択性を示す新しい peroxidizing 除草剤を分子設計する上で有効なリード化合物と成り得る可能性があると考えられる．これら強い酵素活性を示す 4 種(*Echinochloa utilis*, *Lolium perenne*, *Agrostemma githago*, *Arabidopsis thaliana*)の Protox は，構造が全く異なる 6 種類の peroxidizing 除草剤に対して極めて高い感受性を示すことから，新しい peroxidizing 除草剤探索のための Protox 阻害活性試験系に有効に利用できるものである．