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Short communication

"25-kDa Thiol Peroxidase" (TPx II) Acts as a "Housekeeping" Antioxidant

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The newly-found thiol peroxidases (TPx) with a conserved cysteine as the primary site of catalysis are capable of catalyzing the thiol-dependent reduction of peroxides. However, the cellular distributions of the isoforms remain poorly understood. As a first step in understanding the physiological functions of the TPx isoforms, we examined the cellular and tissue distribution of the isoenzymes in various bovine tissues. The tissue distributions of TPx isoenzymes indicate that two types of TPx are widely distributed throughout all of the tested tissues. These two forms are the predominant proteins, with levels of the proteins being quite different from each other. The level of predominant TPx proteins, named type II (TPx II) and type V (TPx V), appeared to be very different with respect to tissue type. The cellular distribution and level of TPx isoenzymes also varied with the types of cells. Immunoblot analysis of the mitochondrial and cytosol fractions from various tissues indicates that TPx III is a unique mitochondrial form. Based on the different tissue and cellular distribution of TPx isoenzymes, we discuss the physiological function of TPx isoenzymes, especially the ubiquitous TPx II.

Keywords: Antioxidant, Isoenzymes, Thiol Peroxidase, Tissue distribution.

Introduction

In an aerobic environment, reactive oxygen species (O_2^-, H_2O_2, ROOH, and HO·) are generated by many physiological processes such as incomplete reduction of molecular oxygen during respiration, NADPH oxidation linked to respiratory bursts during phagocytosis, and redox cycling of xenobiotics (Halliwell and Gutteridge, 1989). To prevent the deleterious effects of reactive oxygen species, cells are equipped with a number of antioxidant enzymes including catalases, peroxidases, and superoxide dismutases (SOD).

Recently, a 25-kDa antioxidant enzyme was purified from various eukaryotes including yeast (Kim *et al.*, 1988; 1989; Chae *et al.*, 1993), human erythrocyte (Lim *et al.*, 1994b), brain (Lim *et al.*, 1994a), liver (Cha and Kim, 1996), and retina (Cha and Kim, 1998). In the presence of thiol-reducing equivalents such as DTT_1, this enzyme from various sources prevents the oxidative damage induced by ROS. (Kim *et al.*, 1988; 1989; Chae *et al.*, 1993). Previously, we reported that the antioxidant enzyme had the capability to destroy H_2O_2 in the presence of DTT (Lim *et al.*, 1993), and that such peroxidase activity was greatly enhanced by the *in vivo* thiol-regenerating system (thioredoxin-thioredoxin reductase-NADPH) (Kwon *et al.*, 1994; Chae *et al.*, 1994; 1995). This peroxidase has a cysteine residue as a functional group instead of the usual selenocysteine residue in selenium-dependent peroxidases such as glutathione peroxidase. Thus, the peroxidase has been named "thiol peroxidase", which may act as an antioxidant enzyme in removing peroxides. However, its physiological significance is still debatable because of the existence of catalases and peroxidases in eukaryotic cytoplasm. In mammalian tissue, at least five types of TPx isoenzymes have been identified (Lim *et al.*, 1994a; 1994b; Cha and Kim, 1996). However, based on their cellular compartmentalization and tissue distribution, the distinct physiological role of each TPx isoenzyme in cellular defense mechanisms against oxidative stress still remains poorly understood.

In this paper, based on the different tissue and cellular distributions of five different TPx isoenzymes, we suggest that the "25-kDa thiol peroxidase" (TPx II) acts as a "housekeeping" antioxidant.

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Materials and Methods

Preparations of various tissue extracts and mitochondria from bovine tissues To remove blood, chopped bovine tissues were washed with phosphate-buffered saline (PBS) buffer. The tissues were homogenated in 50 mM Tris·HCl buffer, pH 7.4, using a Waring blender. The extract was twice centrifuged at 28,000 rpm with a SS-34 rotor for 60 min. After centrifugation, the clear supernatant was used as the total soluble fraction for immunoblot analysis. For mitochondrial fraction preparation, the minced tissues were homogenated in PBS buffer by 10–15 strokes in a tight-fitting dounce homogenizer. The homogenate was centrifuged for 5 min at 3500 rpm with a SS-34 rotor to remove cell debris, and then the supernatant was centrifuged at 9000 rpm for 20 min to sediment mitochondria. The crude mitochondrial fraction was suspended in the same buffer and, after centrifugation at 3500 rpm, the supernatant was centrifugated for 20 min at 9000 rpm. The precipitated mitochondrial fraction was suspended in 50 mM Tris·HCl buffer, pH 7.4, and mitochondria were disrupted by sonification.

Immunoblot analysis Immunoblot analysis of thiol peroxidase isoenzymes in various tissues was performed using an appropriate amount of rabbit monospecific polyclonal antibodies against each human TPx isoenzyme. Procedures for transfer of proteins from 12% SDS-polyacrylamide gels to nitrocellulose and for the processing of nitrocellulose blots were used as described previously (Kim *et al.*, 1989). Monospecific antibodies for thiol peroxidase were prepared from the γ-globulin fraction using each thiol peroxidase isoenzyme immobilized on nitrocellulose strips, as described previously (Kim *et al.*, 1989). SDS-PAGE was performed by the method of Laemmli.

Results and Discussions

The newly found thiol peroxidase (TPx) family, having a conserved cysteine at the primary site of catalysis, is capable of catalyzing the thiol-dependent reduction of peroxides. Sequences of the five TPx isoenzymes discovered to-date are presented in Fig. 1 (Lim *et al.*, 1994; Cha and Kim, 1996). However, the physiological function of each isoenzyme still remains poorly understood. Furthermore, it has not been fully clarified whether the isoenzymes are tissue-specific or not. To answer why several isoforms of TPx exist, we examined the tissue distribution of TPx isoenzymes in bovine tissues.

Distribution of thiol peroxidase (TPx) isoenzymes in bovine tissues was analyzed by Western blotting with rabbit polyclonal antibodies against each human TPx isoenzyme. The immunoblot of mitochondrial and cytosol fractions against five types of antibodies shows that TPx III is a mitochondrial TPx (Fig. 2). No observation of the immunoblot band corresponding to TPx III in the total cytosol fraction of RBC supports the mitochondria-specific existence of TPx III because of the absence of mitochondria in red blood cells. To confirm the cytoplasmic localization of the other four isoenzymes (TPx I, TPx II, TPx IV, and TPx V), immunoblottings for each

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HTPX1   MSS---------------------------------------------   3
HTPX2   MAS---------------------------------------------   3
HTPX3   MAAAVGRLLRASVA-----RHVSAIP-------------WGISATAALRP  32
HTPX4   MEAL--PLLAATTPDHGRHRRLLLLPLLLFLLPAGAVQGWETEERPRTRE  48
HTPX5   MPGG--------------------ILL----------------------   7
        *

HTPX1   -------------------------GNAKIGHPAPNFKATAVMPDGQ  25
HTPX2   -------------------------GNARIGKPAPDFKATAVV-DGA  24
HTPX3   AACGRTSLTNLLCSGSSQAKLFSTSSSCHAPAVTQHAPYFKGTAVV-NGE  81
HTPX4   EECHFYAGGQVYPGEASRVSVADHSLHLSKAKISKPAPYWEGTAVI-DGE  97
HTPX5   -------------------------------GDVAPNFEANTTVGRIR  24
                                        ..  *   . ..

HTPX1   FKDISLSD-YKGKYVVFFFYPLDFTFVCP-TEIIAFSDRAEEFKKLNCQV  73
HTPX2   FKEVKLSD-YKGKYVVLFFYPLDFTFVCP-TEIIAFTTVKRTSAKLGCEV  72
HTPX3   FKDLSLDD-FKGKYLVLFFYPLDFTFVCP-TEIVAFSDKANEFHDVNCEV 129
HTPX4   FKELKLTD-YRGKYLVFFFYPLDFTFVCP-TEIIAFGDRLEEFRSINTEV 145
HTPX5   FHDF-LGD-SWG---ILFSHPRDFTPVCT-TELGRAAKLAPEFAKRNVKL  68
         ..  *..   *   ..* * .** *. *..     .   .    . ..

HTPX1   IG-ASVDSHFCHLAW---VNTPKKQGGLGPMNIPLVSDPKRTIAQDYGVL 119
HTPX2   LG-VSVDSQFTHLAW---INTPRKEGGLGPLNIPLLADVTRRLSEDYGVL 118
HTPX3   VA-VSVDSHFSHLAW---INTPRKNGGLGHMNIALLSDLTKQISRDYGVL 175
HTPX4   VA-CSVDSQFTHLAW---INTPRRQGGLGPIRIPLLSDLTHQISKDYGVY 191
HTPX5   IA-LSIDSVEDHLAWSKDINAYNCEEPTEKLPFPIIDDRNRELAILLGML 117
        ..  *...     .*     . . ..  . .  .

HTPX1   ----KADEGISF--RGLFIIDDKGILRQITVNDLPVGRSVDETLRLVQAF 163
HTPX2   ----KNDEGIAY--RGLFIIDGKGVLRQITVNDLPVGRSVDEALRLVQAF 162
HTPX3   ----LEGSGLAL--RGLFIIDPNGVIKHLSVNDLPVGRSVEETLRLVKAF 219
HTPX4   ----LEDSGHTL--RGLFIIDDKGILRQITLNDLPVGRSVDETLRLVQAF 235
HTPX5   DPAEKDEKGMPVTARVVFVFGPDKKLKLSILYPATTGRNFDEILRVVISL 167
                      *  . .  .    .            * . .

HTPX1   QFTDKHGEVCPAGWKPGSDTIK----PDVQKSKEY----FSK-------- 197
HTPX2   QYTDEHGEVCPAAWKPGRDTIK----PNVDDSKEY----FSK-------- 196
HTPX3   QYVETHGEVCPANWTPDSPTIK----PSPAASKEY----FQKV------- 254
HTPX4   QYTDKHGEVCPAGWKPGSETII----PDPAGKLKY----FDKL------- 270
HTPX5   QLTAEKRVATPVDWKDGDSVMVLPTIPEEEAKKLFPKGVFTKELPSGKKY 217
         . ..  . . . . . .   .      *.

HTPX1   -----QK    199
HTPX2   -----HN    198
HTPX3   -----NQ    256
HTPX4   -----N-    271
HTPX5   LRYTPQP    224
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Fig. 1. Amino acids sequence alignment of human TPx isoenzymes. Symbols, * and • indicate perfect and well-conserved amino acids, respectively. The conserved cysteine as the primary site of catalysis is indicated as a boxed character. Abbreviations: HTPX1, human TPx I (accession number X67951); HTPX2, human TPx II (accession number L14286); HTPX3, human TPx III (accession number D49396); HTPX4, human TPx IV (accession number U25182); HTPX5, human TPx V (accession number D14662).

were carried out in organelle free cytoplasm (Fig. 3). The organelle-free cytoplasmic fractions were obtained from tissue homogenate in an isotonic buffer (i.e., PBS buffer) by ultracentrifugation at 150,000 × *g* overnight. In addition, the whole tissue homogenates without the treatment of isotonic solution were subjected to immunoblotting under the same conditions (Fig. 4). Based on the observation of no significant difference between the relative immunoblot-band intensities of each TPx isoform in the two experiments described above, we suggest that four TPx isoforms (TPx I, TPx II, TPx IV, and TPx V) are cytoplasmic proteins. Analysis of the tissue distributions also revealed that TPx II and TPx V are ubiquitous proteins which exist in all tissues (Fig. 5), and that the protein levels of these proteins appeared to be much higher than those of other TPxs (TPx I and TPx IV) (Figs. 3 and 4). It

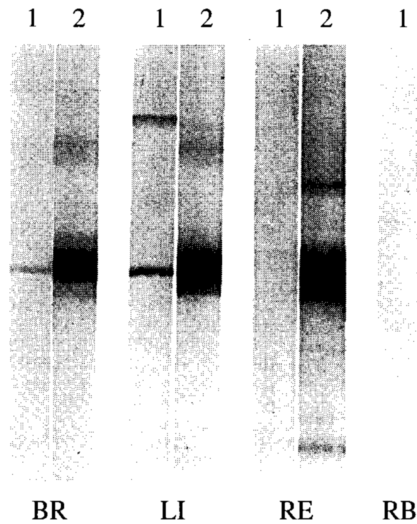

Fig. 2. Mitochondrial localization of TPx III. Roughly 1 mg of crude proteins from both organelle-free cytosol (lane 1) and mitochondrial (lane 2) fractions was electrophoresed in 12% SDS-PAGE gel, and transferred to nitrocellulose paper. The whole blotted paper was cut into several strips to fit a mini-incubation tray having eight channels, and then immunoblot analysis was performed with polyclonal antibodies against TPx III. RB indicates the Western blot for the cytoplasmic fraction of red blood cells; BR-1, brain; LI-1, liver; RE-1, retina cytosols. Lane 2 of each panel indicates the Western blot for the mitochondrial fraction.

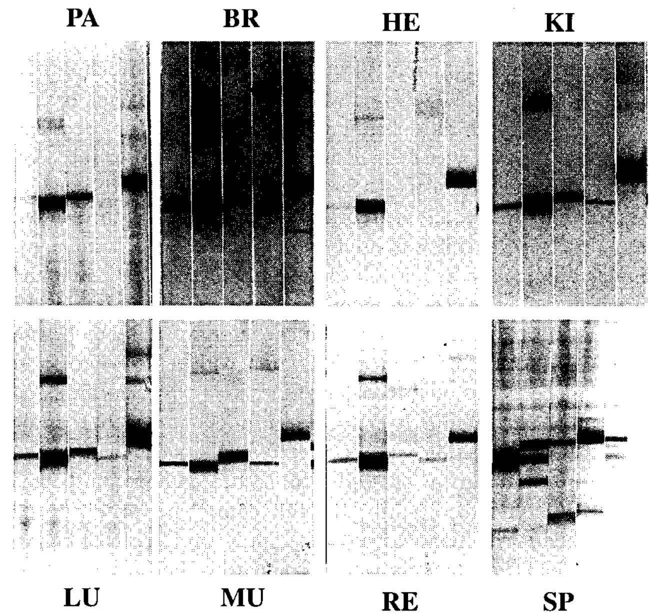

Fig. 4. Western blot analysis for tissue distribution of TPx isoenzymes. One mg of crude proteins from various tissues was electrophoresed in 12% SDS-PAGE gel, and transferred to nitrocellulose paper. The whole blotted paper was cut into several strips to fit a mini-incubation tray having eight channels, and then immunoblot analysis was performed with polyclonal antibodies against TPx I (first lane 1 from left), TPx II (second lane), TPx III (third lane), TPx VI (fourth lane), and TPx V (fifth lane). PA represents pancreas; BR, brain; HE, heart muscle; KI, kidney; LU, lung; MU, skeletal muscle; RE; retina; SP, spleen.

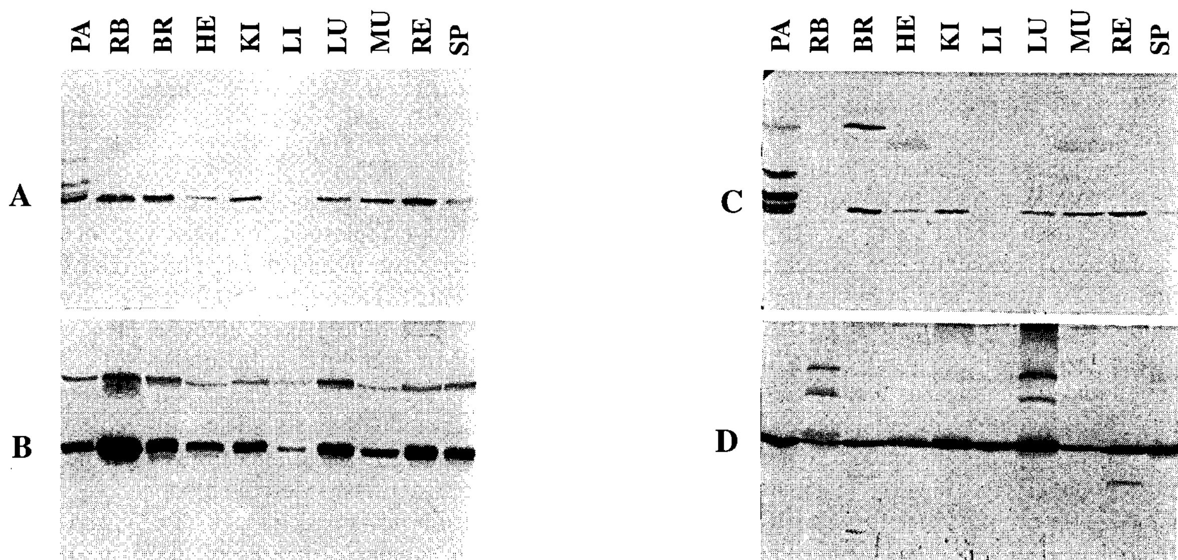

Fig. 3. Western blot analysis for tissue distribution of TPx isoenzymes. Approximatiely, 100 μg of organelle-free cytoplasmic proteins from various tissues was electrophoresed in 12% SDS-PAGE gel, and then transferred to nitrocellulose paper. Immunoblot analysis was then performed with polyclonal antibodies against TPx I (panel A), TPx II (panel B), TPx IV (panel C), and TPx V (panel D). PA indicates pancreas; RB, red blood cell; BR, brain; HE, heart; KI, kidney; LI, liver; LU, lung; MU, skeletal muscle; RE, retina; SP, spleen.

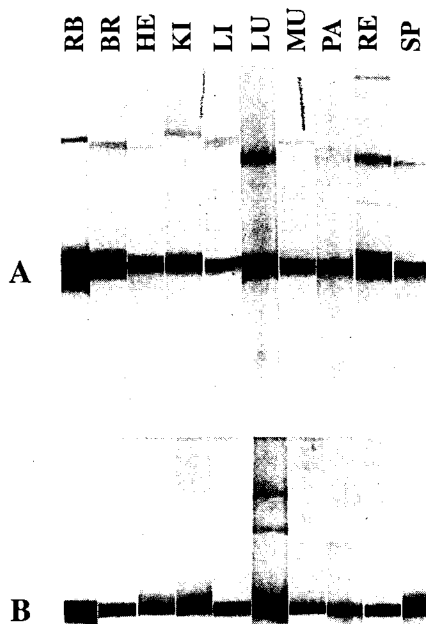

Fig. 5. Western blot analysis for the distribution of two predominant TPx isoenzymes in various tissues. Experimental conditions were as described in the legend of Fig. 3. Panel A shows the immunoblots for tissue distributions of TPx II; panel B, those of TPx V. RB indicates red blood cell; BR, brain; HE, heart; KI, kidney; LI, liver; LU, lung; MU, skeletal muscle; PA, pancreas; RE, retina; SP, spleen.

is worthwhile to note that the cellular levels of the two ubiquitous TPxs were quite different from each other. In liver cells, in addition to the absence of two TPx isoforms (TPx I and TPx IV), the protein level of TPx II is very low, whereas the TPx V level is much higher compared to that of TPx II. This result suggests that TPx V may be a principal thiol peroxidase. The protein levels of TPx II vary significantly among different tissues, with red blood cell $>$ brain $\cong$ retina $\cong$ lung $>$ pancreas $\cong$ heart $\cong$ kidney $\cong$ skeletal muscle $\cong$ spleen $>$ liver. The fact that protein levels of Tpx II in red blood cells, brain, retina, and lung cells are relatively higher than in other cells may indicate the important antioxidant role of TPx II. These cells are exposed to much higher levels of oxygen, which requires corresponding protection against oxidative stress. Therefore, this predominant existence of TPx II in such cells implicates that TPx II is a "housekeeping" antioxidant enzyme. Analysis of the immunoblottings shown in Figs. 3 and 4 also suggests that TPx isoforms are not tissue-specific because of the presence of all types of TPx isoenzyme in most tissues, excluding liver and red blood cells.

The anterior of the optic lens is covered by metabolically highly-active epithelial cells, which are particularly sensitive to damage by reactive oxygen species (Halliwell and Gutterridge, 1989). Reactive oxygen species can damage and cross-link lens protein, causing cataracts. The vitreous humor contains hyaluronic acid, which is attacked by reactive oxygen species, also causing severe visual impairment. The lipids present in the membrane of retina cells contain a high percentage of polyunsaturated fatty acids, and are thus susceptible to lipid peroxidation. The retina pigment, rhodopsin, can sensitize the formation of singlet oxygen. Therefore, the eye has many potential oxidative stress-related problems, and one would expect a corresponding degree of protection. Indeed, the concentration of antioxidants in the eye is high. If TPx II has the physiological function as a "housekeeping" antioxidant, it should exist predominantly in such eye tissues. Therefore, to confirm this antioxidative role, the level of TPx isoenzymes in various parts of the eye, including cornea, vitreous humor, choroid, and retina were determined by Western blotting. As shown in Fig. 6, all types of cytoplasmic TPx isoenzymes (TPx I, II, IV, and V) exist in cornea, choroid, and retina cells. However, only two types of cytoplasmic TPx isoenzymes exist in the *vitreous humor*. Furthermore, Fig. 7 reveals that all eye tissues, excluding the lens and aqueous humor, contain significantly higher amounts of TPx II than TPx V. Therefore, these results support the physiological role of TPx II as a "housekeeping" antioxidant.

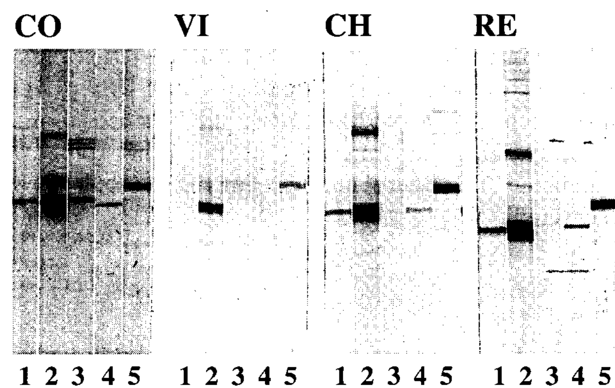

Fig. 6. Western blot analysis for the distribution of TPx isoenzymes in the eye. One mg of soluble proteins from various eye tissues was electrophoresed in 12% SDS-PAGE gel, and transferred to nitrocellulose paper. The whole blotted paper was cut into several strips to fit a mini-incubation tray having eight channels, and then immunoblot analysis was performed with polyclonal antibodies against TPx I (lane 1), TPx II (lane 2), TPx III (lane 3), TPx VI (lane 4), and TPx V (lane 5). CO represents the cornea; VI, vitreous humor; CH, choroid; RE, retina.

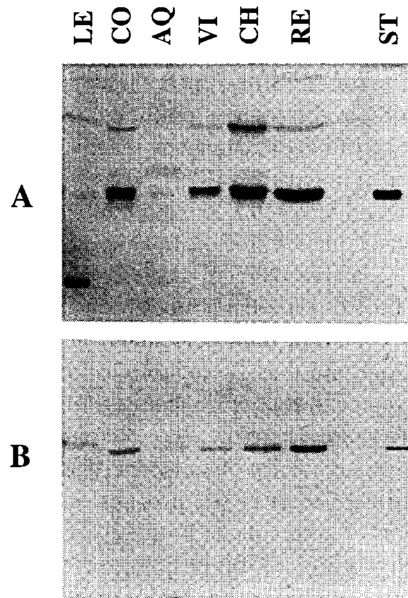

Fig. 7. Western blot analysis for the distribution of two TPx isoenzymes in the eye. Approximately 100 μg of soluble proteins from various eye tissues was electrophoresed in 12% SDS-PAGE gel, transferred to nitrocellulose paper, and then immunoblot analysis was performed with polyclonal antibodies against TPx II (panel A) and TPx V (panel B). LE indicates the lens; CO, cornea; AQ, aqueous humor; VI, vitreous humor; CH, choroid; RE, retina. ST indicates 50 ng of TPx II (panel A) and 10 ng of TPx V (panel B) as each standard.

In conclusion, based on the observation that most cells contain the five types of TPx isoenzymes, we suggest that TPx isoenzymes are not tissue-specific, which implies a distinct physiological function of each TPx isoform in each cell. The 25-kDa TPx II thiol peroxidase antioxidant is widely and predominantly distributed in all tested tissues including the eye, which implicates that TPx II is a "housekeeping" antioxidant among the TPx isoenzymes. The physiological functions of the other types of mammalian thiol peroxidase isoenzymes remain to be discovered.

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