Type-II 3-oxoacyl-CoA thiolase of the nematode *Caenorhabditis elegans* is located in peroxisomes, highly expressed during larval stages and induced by clofibrate

(Running title: Expression of *C. elegans* type-II thiolase)

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Abbreviations: CBB, Coomassie brilliant blue; D-bifunctional protein, D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein; PBS, phosphate-buffered saline; PTS, peroxisomal-targeting signal; SCP, sterol carrier protein.

Enzymes: 3-oxoacyl-CoA thiolase (EC 2. 3. 1. 16); catalase (EC 1. 11. 1. 6); peroxidase (EC 1. 11. 1. 7); cytochrome-*c* oxidase (EC 1. 9. 3. 1); alkaline phosphatase (EC 3. 1. 3. 1); acid phosphatase (EC 3. 1. 3. 2); 3-hydroxyacyl-CoA dehydratase (EC 4. 2. 1. 74); 3-hydroxyacyl-CoA dehydrogenase (EC 1. 1. 1. 35); isocitrate lyase (EC 4. 1. 3. 1); malate synthase (EC 4. 1. 3. 2); alkyl-dihydroxyacetonephosphate synthase (EC 2. 5. 1. 26).

SUMMARY

We examined the expression and localization of type-II 3-oxoacyl-CoA thiolase in the nematode *Caenorhabditis elegans*. Type-II thiolase acts on 3-oxoacyl-CoA esters with a methyl group at the α-carbon, whereas conventional thiolases do not. Mammalian type-II thiolase, which is also termed sterol carrier protein x (SCPx) or SCP2/3-oxoacyl-CoA thiolase, is located in the peroxisomes and involved in phytanic acid degradation and most probably in bile acid synthesis. The nematode enzyme lacks the SCP2 domain, which carries the peroxisomal-targeting signal, but produces bile acids in a cell-free system. Northern and western blot analyses demonstrated that *C. elegans* expressed type-II thiolase throughout its life cycle, especially during the larval stages, and that the expression was significantly enhanced by the addition of clofibrate at 5 mM or more to the culture medium. Whole-mount *in situ* hybridization and immunostaining of L4 larvae revealed that the enzyme was mainly expressed in intestinal cells, which are multifunctional like many of the cell types in *C. elegans*. Subcellular fractionation and indirect immunoelectron microscopy of the nematode detected the enzyme in the matrix of peroxisomes. These results indicate the fundamental homology between mammalian SCPx and the nematode enzyme regardless of whether the SCP2 part is fused or not, suggesting their common physiological roles.

Keywords: type-II 3-oxoacyl-CoA thiolase; SCPx; peroxisomes; clofibrate; *Caenorhabditis elegans*.

INTRODUCTION

Type-II 3-oxoacyl-CoA thiolase acts on 3-oxoacyl-CoA esters with a methyl branched-chain at the α-carbon [1-3]. Conventional type-I 3-oxoacyl-CoA thiolases, which act on only strait-chain substrates, consist of peroxisomal thiolases (A and B) [4] and mitochondrial ones including enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-oxoacyl-CoA thiolase trifunctional protein [5]. Mammalian type-II thiolase is essential to phytanic acid degradation [6] and most probably to bile acid synthesis, the obligatory intermediates of which are pristanoyl-CoA and di- or trihydroxycholestanoyl-CoA, respectively. The mammalian enzyme is also termed sterol carrier protein x (SCPx) or SCP2/3-oxoacyl-CoA thiolase and is a 58-kDa bipartite protein; the N-terminal 404 residues and the C-terminal 143 residues constitute thiolase and pre-SCP2, respectively [7-9]. SCP2, whose alternative name is non-specific lipid-transfer protein [10], binds fatty acids as well [11], therefore this part of the SCPx molecule is thought of as the carrier of substrates. Moreover, its C-terminal tripeptide Ala-Lys-Leu, which is a peroxisomal-targeting signal 1 (PTS1), explains the predominant localization of the 58-kDa protein in the matrix of peroxisomes. PTS1 is typically Ser-Lys-Leu and is conserved among yeasts, plants, insects and mammals [12, 13]. The PTS1 sequences in the animal phylum are less diverse than those in protozoa and plants [14-16].

Type-II thiolase from the nematode *Caenorhabditis elegans* is similar to its mammalian counterparts in many respects: amino acid sequence, reaction mechanism, substrate specificity, specific activity and ability to produce bile acids in a cell-free system in the presence of D-3-hydroxyacyl-CoA dehydratase/D-3-

hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein) [3, 17]. However, it lacks the C-terminal SCP2 domain. After determination of its molecular mass (44 kDa), the nematode enzyme was designated P-44 [17]. Its C-terminal tripeptide (Ser-Lys-Ile) does not function as the PTS1 in animals thus far examined though a recent paper revealed Ala-Lys-Ile to be functional [18]. This, together with the lack of knowledge on the metabolism of α-methyl compounds in the nematode, makes the function of P-44 unclear. Determination of the expression pattern and subcellular localization of P-44 will provide clues to understanding the physiological role of *C. elegans* type-II thiolase. We report here that P-44 is expressed mainly in the larval intestine, induced by clofibrate and located in the matrix of peroxisomes.

MATERIALS AND METHODS

Materials

The ECL western blotting detection reagents and donkey anti-rabbit Ig $F(ab')_2$ fragment labeled with horseradish peroxidase were purchased from Amersham Life Science. The DIG RNA-labeling kit and sheep anti-digoxigenin Fab fragment labeled with calf intestine alkaline phosphatase were obtained from Boehringer Mannheim. EM grade 15-nm gold conjugated goat anti-mouse IgG antibody was obtained from Zymed Laboratories. The plasmid pBluescript II KS – for *in vitro* transcription was a product of Stratagene. Sources of [α-^{32}P]dCTP, oligonucleotides used as the primer in PCR amplification and random-primer DNA-labeling kit were as described [17]. The rabbit anti-P44 antiserum against

the His_6-tagged recombinant P-44, which carries Val-His_6 at the N-terminus [17], was generated by the procedure described [19] and affinity-purified with a nearly homogeneous P-44 preparation [17] by the method published [20].

Organism

The Bristol N2 strain of *C. elegans* was used throughout the work according to the methods compiled by Lewis and Fleming with cells of *Escherichia coli* strain OP50 as the feed [21]. Animals at each developmental stage (L1, L2, L3, L4, young adult and egg-lying adult) were harvested at 6, 20, 29, 40, 53 and 75 h after feeding at 20°C.

Preparation of whole nematode proteins

Samples for western blot analysis were prepared from organisms at each developmental stage and from a mixed-stage culture. Embryos or animals were suspended in 0.5 ml of 50 mM potassium phosphate, pH 7.4, 2 mM phenylmethanesulfonyl fluoride and the cells were disrupted with a UD-201 sonicator (Tomy) equipped with a micro tip (TP-040) at 26 W and 0--4°C four times (30 sec each at 30-sec intervals). Proteins used for the absorption of donkey anti-rabbit Ig $F(ab')_2$ fragment were prepared from the mixed-stage culture of *C. elegans*. Organisms were suspended in 10 mM sodium phosphate, pH 7.4, 130 mM NaCl and 4 mM KCl (PBS) and the suspension (100 ml) was sonicated with the standard tip (TP-012) at 127 W and 0--4°C ten times (1 min each at 1-min intervals). After the centrifugation of the homogenate at 15 000 × *g* for 30 min, proteins in the supernatant were precipitated with four volumes of acetone, collected by centrifugation as above and dried under an air stream.

Immunoelectron microscopy

Animals were fixed in 0.15 M cacodylate, pH 7.4, 4% paraformaldehyde and 0.25% glutaraldehyde, washed with PBS, and suspended in 1% low-melting-temperature agarose. Small blocks from the solidified agarose were dehydrated with graded ethanol and embedded in LR White resin as described [22]. Conditions for immunostaining with primary antibodies and secondary gold probes, preparation of protein A-gold and electron microscopy were as described [23]. To determine the labeling density (gold particles/μm^2), micrographs were taken at magnification of 3×10^4 and enlarged three times to positive pictures. Areas of peroxisomes and the rest of the cytoplasm were estimated with the digitizer Jandel Scientific Opaque Tablets (Jandel Scientific) using the SigamaScan program.

Subcellular fractionation

Cells of mixed-stage animals were disrupted with Cryo Clean Blaster CCB-50B (Taiyo Sanso) by the modified method for yeast-cell disruption [24]. Every 25 g (wet weight) of animals were suspended in 150 ml of cold buffer M (5 mM Mes/KOH, pH 6.0, 0.6 M sorbitol, 1 mM KCl, 0.5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride) and disrupted at 6 kgf·cm^{-2} and –140°C. The frozen extract was thawed and centrifuged at 1000 × *g* for 10 min. The supernatant was further centrifuged at 20 000 × *g* for 20 min and the resulting precipitate (particulate fraction) was suspended in 5 ml buffer M. Organelles in the particulate fraction were separated by sucrose density gradient centrifugation [19] and the activities of catalase [25], cytochrome-*c* oxidase [25] and acid

phosphatase [26] were assayed according to the methods described. One enzyme unit (U) is defined as the amount that catalyzes the conversion of 1 μmol of substrate per min.

Miscellaneous methods

Donkey anti-rabbit Ig $F(ab')_2$ fragment was adsorbed with whole proteins from the mixed-stage *C. elegans* as described [20] before use. Indirect whole-mount immunostaining [27] of P-44 with anti-rabbit Ig $F(ab')_2$ fragment labeled with peroxidase and whole-mount *in situ* hybridization to detect the mRNA coding for P-44 with the antisense RNA probe [28] were conducted by the published methods. Methods for SDS/PAGE followed by western blotting [19] and northern hybridization with the total RNA [17] were as described. The regions used as the RNA probe for P-44 mRNA and the cDNA probe for CeIF mRNA were nucleotides +614 to +1019 and nucleotides –11 to +993, respectively. Where nucleotide position +1 is A of the start codon of each open reading frame; their EMBL/GenBank/DDBJ accession numbers are D86473 and Z12116.

RESULTS AND DISCUSSION

Spatio-temporal pattern of expression

Immunoblot analysis of the homogenate from cells of mixed-stage animals revealed that the antibody raised against the His_6-tagged P-44 reacted with a single protein that has a mobility almost the same as the recombinant P-44 (Fig. 1). This result indicates that the antibody recognized P-44 specifically and the

His_6-tag caused a slight retardation in the mobility of the protein (lanes 1 and 2). The result is consistent with the single signal observed by northern hybridization [17] and supports our tentative conclusion that there is no type-II thiolase of an SCPx-like fusion form [17].

Type-II thiolase was detectable with anti-P-44 antibody throughout the life cycle of *C. elegans* (Fig. 2A); though the amount relative to the proteins of the whole nematode was larger at the larval stages (L1, L2, L3 and L4) than at the embryo (E), young adult (YA) or egg-lying adult (EA) stages. This tendency was confirmed by northern blot analysis of total RNA (Fig. 2B). The signal of 1.4-kb P-44 mRNA decreased after the L4 molt unlike the stable expression of CeIF, which is a homolog of eucaryotic initiation factor 4A and was used as the control for equal sample loading [29]. The low level of the P-44 mRNA at the L1 stage may result from a combination of the decrease of the maternal message and the delayed zygotic expression. The tissue distribution of P-44 was then studied with L4 larvae because they express P-44 at a higher level than in adult animals and are comparable to young adults in size. When the animals were examined by whole-mount immunostaining using anti-P44 antibody (Fig. 3A) and whole-mount *in situ* hybridization using an antisense RNA probe (Fig. 3C), the expression of P-44 was detected mainly in intestinal cells but hardly detected in the muscular cells in the pharynx, the gonad (immature) or the tail.

Induction of the expression by clofibrate

The induction of P-44 was tried in order to facilitate the detection of this protein by immunoelectron microscopy. Clofibrate, which is the ethyl ester of a derivative of α-methyl-propionic acid and known as a peroxisome proliferator, increased the

amount of P-44 in the mixed-stage culture of *C. elegans* at 5 mM or more (Fig. 4A). Northern blot analysis indicated that the observed increase was due to the increased amount of the mRNA (Fig. 4B), suggesting the induction of P-44 by clofibrate at the transcriptional level. Under similar conditions, another potent peroxisome proliferator Wy14643, which is a derivative of acetic acid without an α-methyl group, was not effective (data not shown). It is possible, therefore, the P-44 is peroxisomal and specifically involved in the metabolism of acyl-compounds with an α-methyl branched-chain.

Subcellular localization

Thin sections of clofibrate-treated L4 larvae of *C. elegans* were examined by the double labeling immunoelectron microscopic technique to clarify whether P-44 was located in peroxisomes. Thin sections incubated with a mixture of mouse anti-catalase-2 antibody and rabbit anti-P-44 antibody were probed with 15-nm gold colloidal particles coupled to goat anti-mouse Ig antibody (Fig. 5A) or with 8.5-nm gold particles coupled to protein A (Fig. 5B). In both experiments, gold particles were located within cytoplasmic vesicles that were identified as peroxisomes from morphological and biochemical criteria; catalase-2 was proved to be peroxisomal (Togo, S. H., Maebuchi, M., Yokota, S., Bun-ya, M., Kawahara, A. & Kamiryo, T., in preparation). Similar results were obtained with the untreated control larvae (data not shown). When sections incubated with the mixture of primary antibodies were treated with protein A-gold (8.5 nm) and then with goat anti-mouse Ig antibody-gold (15 nm), which was absorbed with rabbit Ig, both 8.5-nm gold particles and 15-nm gold particles were present in the same location (Fig. 5C). No gold particles were noted in peroxisomes when sections

incubated with mouse anti-catalase-2 antibody alone were probed with protein A-gold, (Fig. 5D). This negative control and the inability of protein A to react with goat Ig [30] confirmed that 8.5- and 15-nm particles in Fig. 5C were specific signals for P-44 and catalase-2, respectively. Labeling-density analysis of ten micrographs taken as in Fig. 5B revealed the densities of 8.5-nm particles inside and outside peroxisomes were $323.7 \pm 51.9/\mu m^2$ and $4.9 \pm 0.9/\mu m^2$, respectively. Thus, immunoelectron microscopic studies indicate that type-II thiolase of *C. elegans* is located in the matrix of peroxisomes.

This immunocytological observation was supported by subcellular fractionation. The particulate fraction derived from the mixed-stage culture of *C. elegans* was separated by sucrose density gradient centrifugation followed by western blot analysis. Activity peaks of catalase, cytochrome-*c* oxidase and acid phosphatase correspond to peroxisomes, mitochondria and lysosomes, respectively (Fig. 6A). Fractions enriched with P-44 coincided with the peak of catalase activity (Fig. 6B). These results confirm the peroxisomal localization of P-44 and suggest that the C-terminal tripeptide Ser-Lys-Ile is a functional PTS1 in *C. elegans*. Our observation that Wy14643, unlike clofibrate, did not induce the P-44 expression may be due to the species-specific sensitivity to the chemicals or may suggest the differential response to the inducers with or without α-methyl branched-chain.

Prospects for the physiological role

Nematode type-II thiolase, P-44, was shown to be a peroxisomal protein like its mammalian equivalent SCPx. While SCPx is translated as the fusion with 13.5-kDa SCP2, the SCP2 part seems to be removed from SCPx in hepatic

peroxisomes [9, 31] to generate the 44/46-kDa protein that is abundant in enzymatically active fractions [1]. P-44 is independent of SCP2. It is probable, therefore, that the apparent difference between the molecular forms of the mammalian and nematode enzymes does not influence their catalytic abilities. Indeed, P-44 produced bile acids from CoA esters of the intermediates of enoyl- and hydroxy-forms in the presence of rat D-bifunctional protein [3]. Although rat SCPx did so only from the hydroxy-form intermediate in this *in vitro* system [3], in hepatic peroxisomes, where a putative SCP2-cleaving enzyme is present, SCPx must be responsible for not only phytanic acid degradation [6] but also bile acid synthesis. The ontogenic pattern of P-44 expression (Fig. 2A) contrasts with that of *C. elegans* bifunctional isocitrate lyase/malate synthase [32], whose activities decline during the normal development of well-fed larvae [33]. These enzymes are peroxisomal and essential to convert fat to carbohydrate *via* β-oxidation of fatty acids in plant seedlings and yeasts. Likewise, the increase of these two enzyme's activities during *C. elegans* embryogenesis is accompanied by a decrease of triglyceride stored in the egg [34]. If the primary role of P-44 is β-oxidation of strait-chain fatty acids to provide the substrate for the glyoxylate pathway, its expression should be higher in embryos than in larvae, which ingest carbohydrates in the feed (bacteria under laboratory conditions). The observed increase of P-44 after egg hatching suggests that this is not the case. Instead, P-44 may play a role in digestion of the feed; its lowered level after the L4 molt may result from a change of nutritional requirements caused by the shift from larval growth to sexual maturation. The probable counterpart of the mammalian type-I thiolase responsible for the peroxisomal degradation of strait-chain fatty acid is a protein encoded by the open reading frame T02G5.8 of *C. elegans*.

This putative protein carries a PTS1-like sequence (Gln-Lys-Leu) instead of PTS2, which the mammalian peroxisomal thiolase has, an evolutionary switching of PTSs between mammals and nematode as reported in alkyl-dihydroxyacetonephosphate synthase [35]. The intestinal localization of P-44 is merely a reflection of the simple anatomy of *C. elegans*; the multifunctional intestinal cells must serve hepatic functions as well. Mammalian SCPx [8] and nematode P-44 are both inducible by clofibrate. Although the physiological substrates of P-44 remain to be elucidated, the data presented here emphasize the fundamental homology among type-II thiolases, regardless of whether the SCP2 part is fused or not in the translation product.

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FIGURE LEGENDS

Fig. 1. Specificity of anti-P-44 antibody. The whole proteins from the mixed-stage culture (lane 1, 0.1 mg) and the recombinant His_6-tagged P-44 (lane 2, 0.5 μg and lane 3, 2.5 μg) were separated by SDS/PAGE with molecular-mass markers (lane 4) on a 10% polyacrylamide gel. Lanes 1 and 2 were analyzed by western blotting; proteins were transferred to a nitrocellulose membrane and reacted with affinity-purified rabbit anti-P-44 antibody and the antibody was detected by the reaction of peroxidase coupled to donkey anti-rabbit Ig $F(ab')_2$ fragment. The arrow marks endogenous P-44 and the arrowhead denotes the recombinant His_6-tagged P-44. Lanes 3 and 4 were stained with Coomassie brilliant blue (CBB). Molecular masses of the marker proteins (from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor) are indicated on the right.

Fig. 2. Expression of P-44 at each developmental stage. The amounts of P-44 and its mRNA in embryo (E), larvae (L1, L2, L3 and L4), young adult (YA) and egg-lying adult (EA) were assessed by western blotting and northern blotting, respectively. Larvae and adults at each stage were collected from 50 NGM-agar plates (9 cm). Whole proteins (40 μg each) were separated by SDS/PAGE on two 10% polyacrylamide gels. Proteins on one gel were transferred to a nitrocellulose membrane and P-44 was detected with affinity-purified rabbit anti-P-44 antibody as in Fig. 1 (A1), while those on the other gel were stained with CBB (A2). The arrow marks the position of P-44. Positions of molecular-mass markers (from top to bottom: 94 kDa, 57 kDa, 43 kDa, 30 kDa, 20 kDa) are

indicated on the left. Total RNA (5 μg each) were electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane and probed with ^{32}P-labeled cDNA fragments containing the P-44 coding region (B1). The same membrane was re-probed with ^{32}P-labeled cDNA fragments containing the CeIF coding region (B2). The arrow indicates 1.4 kb P-44 mRNA and the arrowhead denotes 1.7 kb CeIF mRNA.

Fig. 3. Expression of P-44 *in situ*. P-44 protein and its mRNA in L4 larvae were detected by immunostaining and *in situ* hybridization, respectively. Fixed L4 larvae were reacted with (A) or without (B) affinity-purified rabbit anti-P-44 antibody and incubated with peroxidase-labeled donkey anti-rabbit Ig $F(ab')_2$ fragment. Peroxidase catalyzed the formation of brown precipitate in cells expressing P-44 protein. Fixed L4 larvae were probed with digoxigenin-labeled P-44 antisense RNA (C) or with its sense RNA (D) and incubated with alkaline phosphatase-labeled sheep anti-digoxigenin Fab fragment. Alkaline phosphatase catalyzed the formation of a dark blue precipitate in cells containing P-44 mRNA.

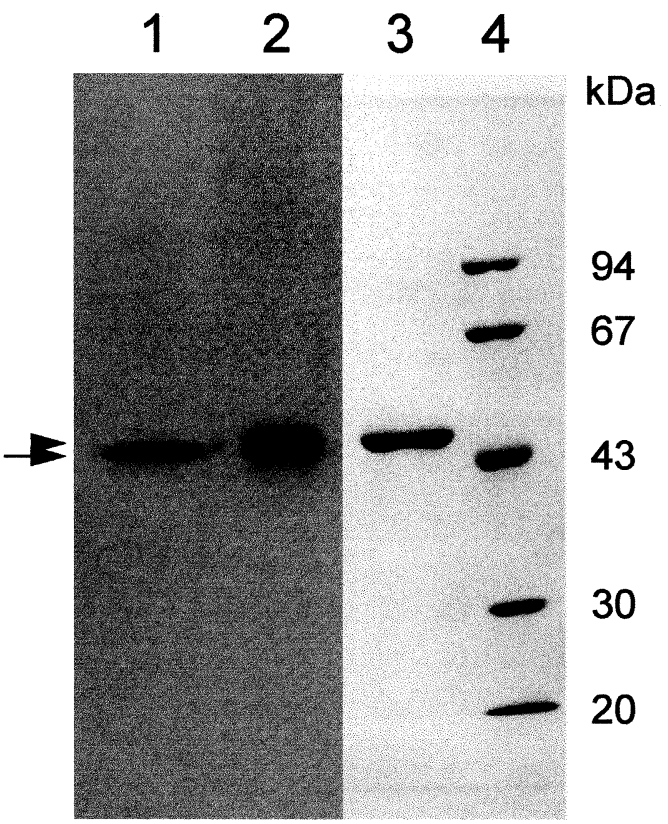
Fig. 4. Induction of P-44 by clofibrate. To five 10-ml mixed-stage cultures of *C. elegans* in S medium at 25°C, clofibrate was added until final concentrations of 0, 5, 10, 15 and 20 mM were achieved (indicated above the panel) and the incubation was continued for a further 24 h at 25°C. Each culture was divided into two equal portions, from which whole proteins and total RNA were prepared, respectively. Whole proteins (5 μg each) were separated by SDS/PAGE and followed by western blot analysis as in Fig. 2A. P-44 was detected with anti-P44

antibody (A1) and proteins were stained with CBB (A2). The arrow indicates the position of P-44. Total RNA (5 μg each) was analyzed by northern blotting as in Fig. 2B. The P-44 mRNA (B1) and the CelF mRNA (B2) were each detected with their appropriate probe and are marked by the arrow and the arrowhead, respectively.

Fig. 5. Colocalization of P-44 and catalase-2 in thin sections of L4 larvae. L3 larvae were grown with 5 mM clofibrate at 25°C for 24 h and the resulting L4 larvae were fixed and embedded. The thin sections stained with gold probes and 2% uranyl acetate were examined in an H600 electron microscope (Hitachi) at 75 kV. To detect catalase-2, the sections were incubated with a mixture of mouse anti-catalase-2 antibody (diluted 1:500) and rabbit anti-P-44 antibody (diluted 1:500) and probed with 15-nm gold particles coupled to goat anti-mouse Ig, which was adsorbed with rabbit Ig before use (A). P-44 was similarly detected with 8.5-nm gold particles coupled to protein A (B). To detect P-44 and catalase-2 differentially, sections were incubated with the mixture of primary antibodies, then with protein A-gold (8.5 nm) and finally with goat anti-mouse Ig-gold (15 nm) (C). Sections for negative control were incubated with mouse anti-catalase-2 antibody alone and probed with protein A-gold (8.5 nm) (D). M and arrow mark mitochondria and peroxisomes, respectively. The bar is 0.25 μm (× 52 000).

Fig. 6. Cosedimentation of P-44 and catalase activity in sucrose density gradient centrifugation. The particulate fraction (5 ml, 1.1 mg/ml as protein) obtained from the mixed-stage culture of *C. elegans*, which was not treated with

clofibrate, was applied on a 24-ml continuous 32-54% (mass/mass) sucrose density gradient on a 2-ml cushion of 60% (mass/mass) sucrose and centrifuged at 100 000 × *g* for 90 min. Fractions (1 ml each) were collected from the bottom of the tube and assayed for activities of catalase (closed circle), cytochrome-*c* oxidase (open circle) and acid phosphatase (open triangle) and sucrose density (dot) (A). Proteins from even numbered fractions (5 μl each) and the particulate fraction (1 μl, lane P) were separated by SDS/PAGE and P-44 was detected as in Fig. 1 (B). The arrow marks P-44.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kDa
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30
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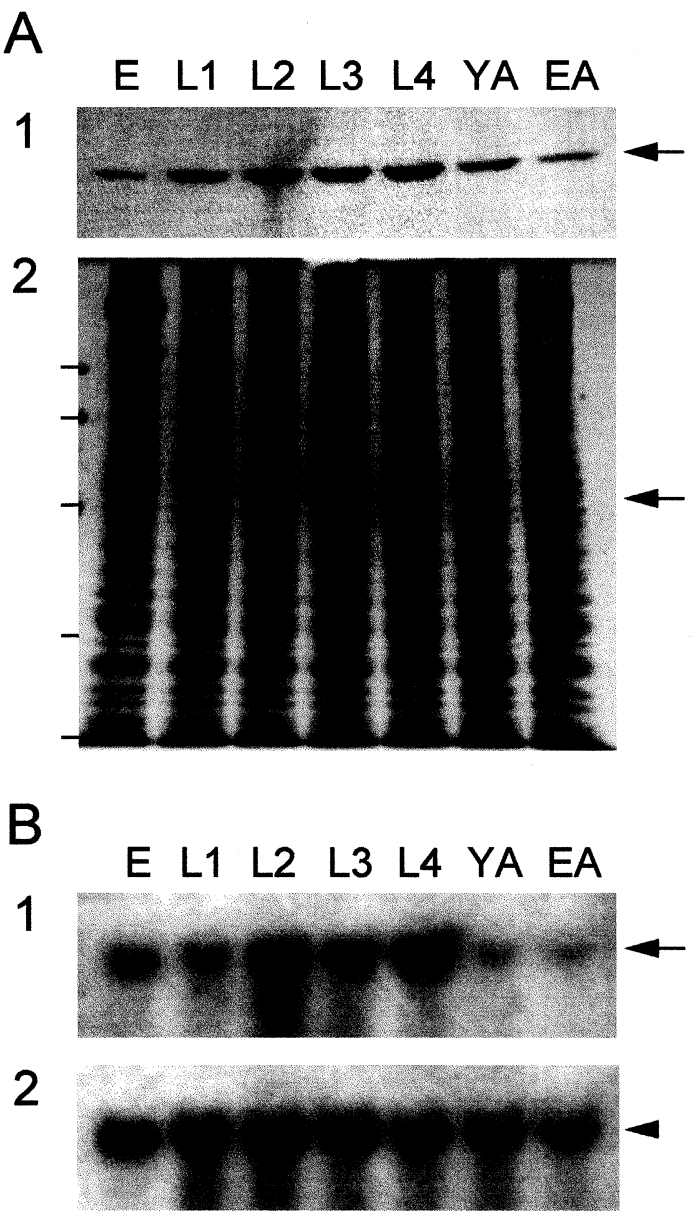
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2
B
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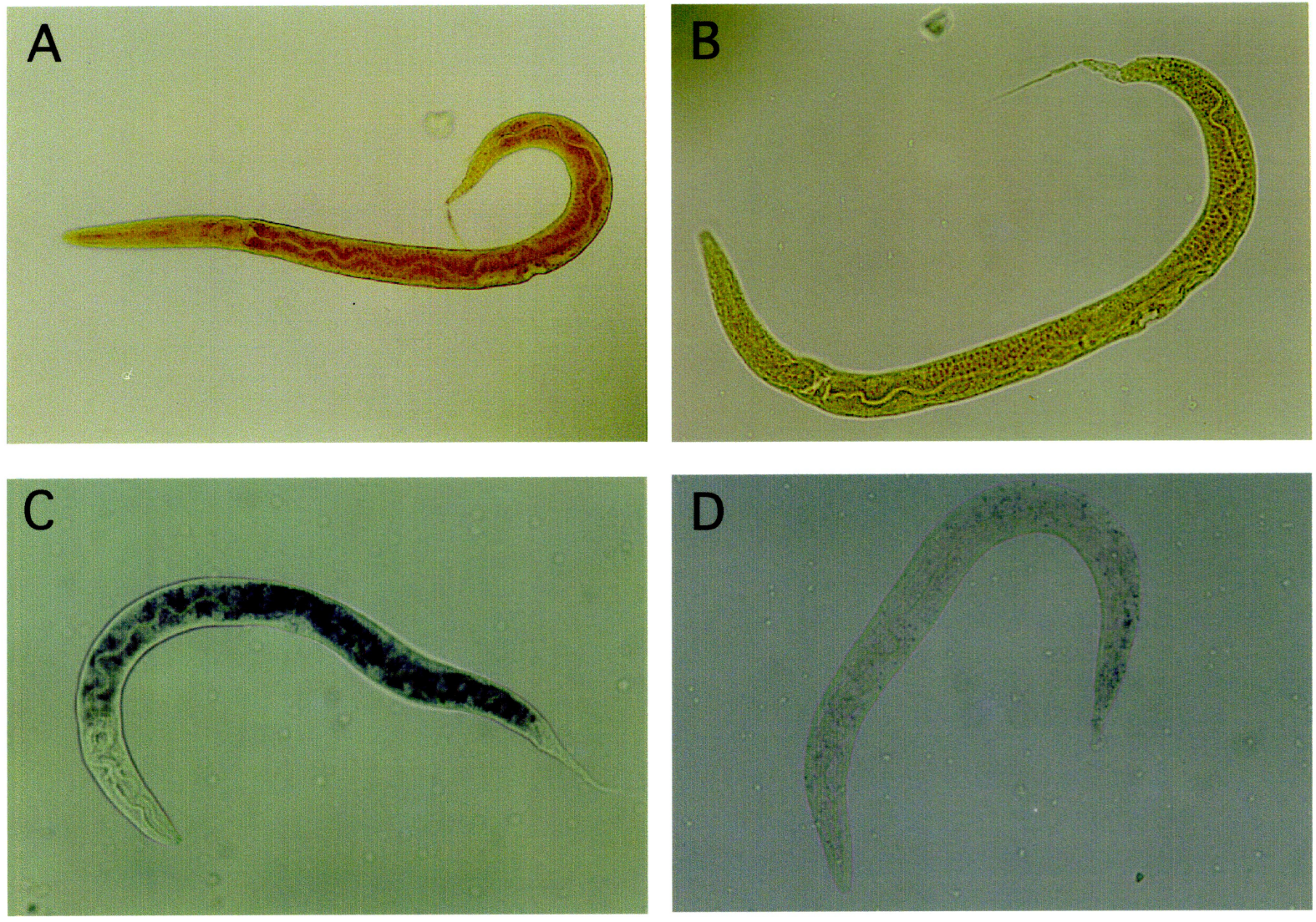
1
2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C
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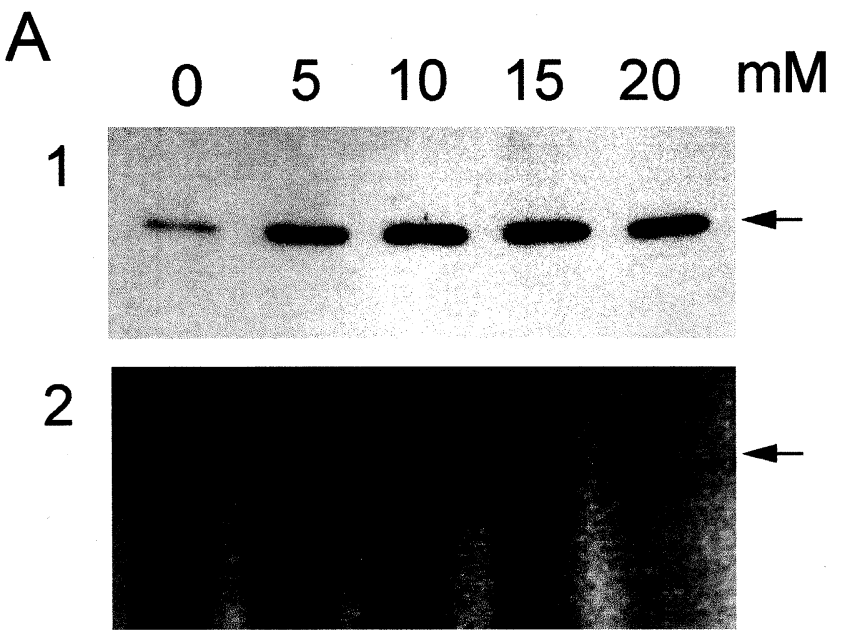
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mM
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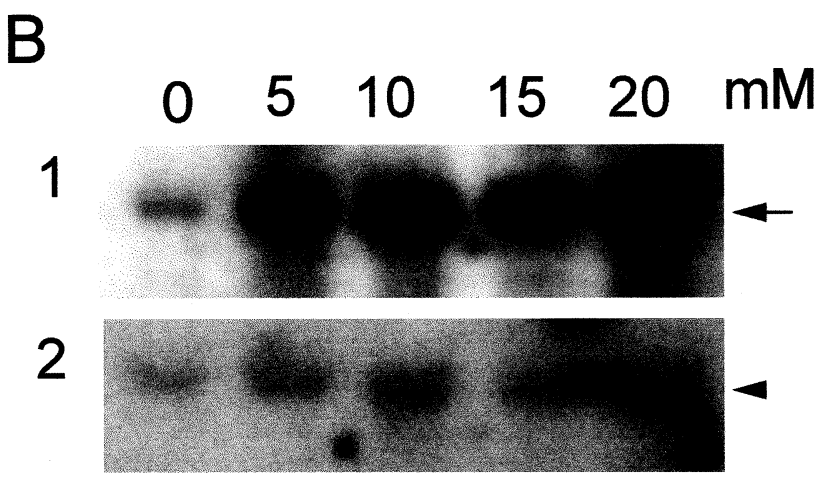
2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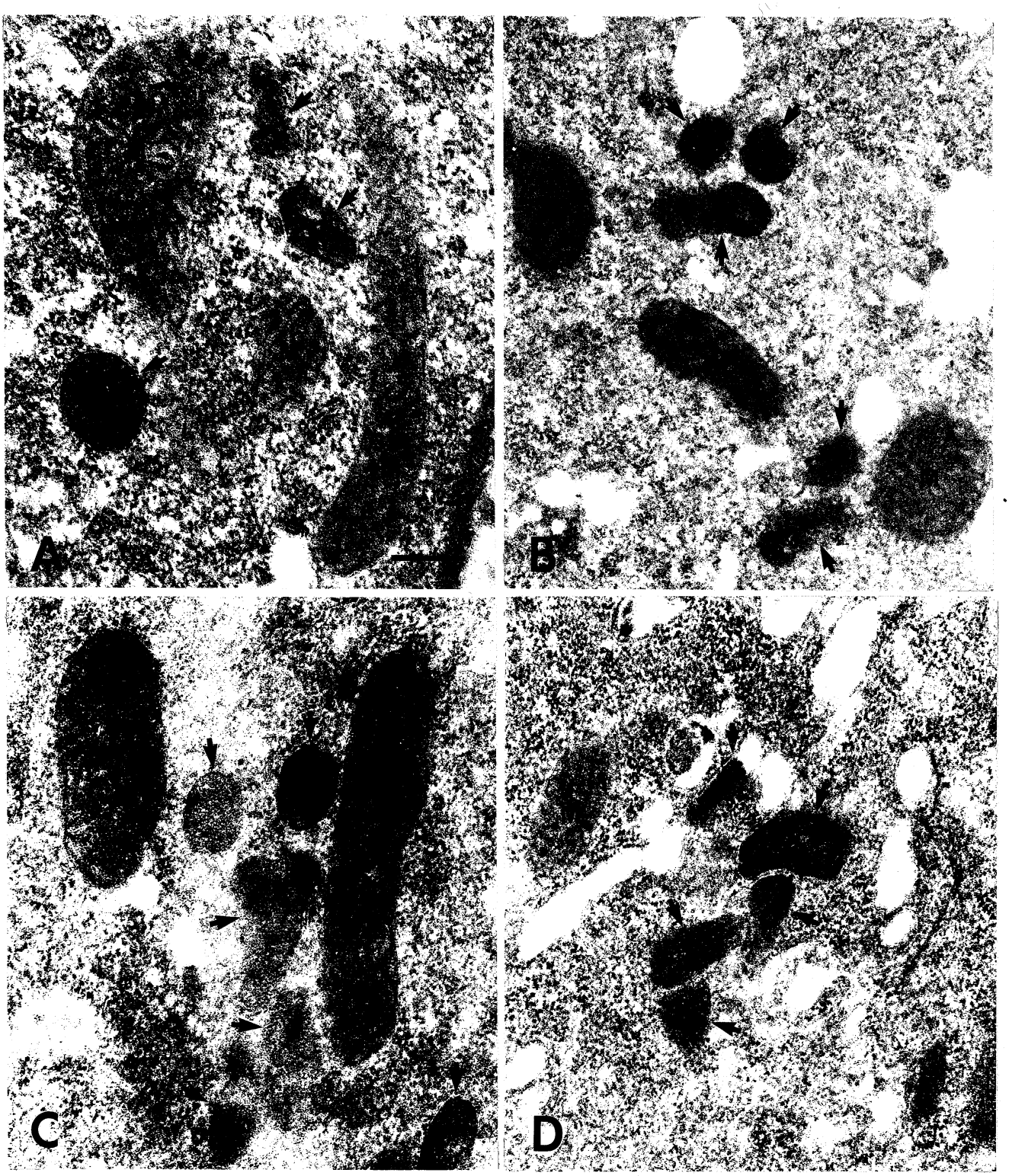
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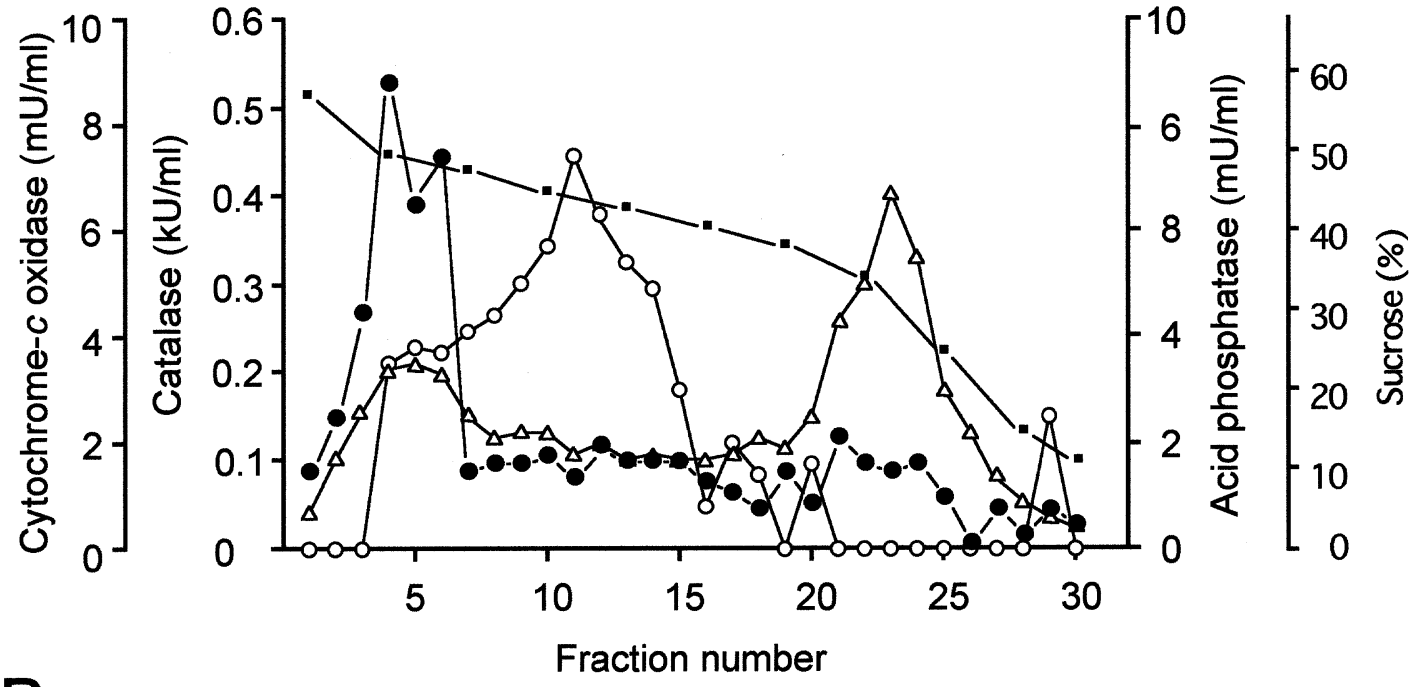
C
D

A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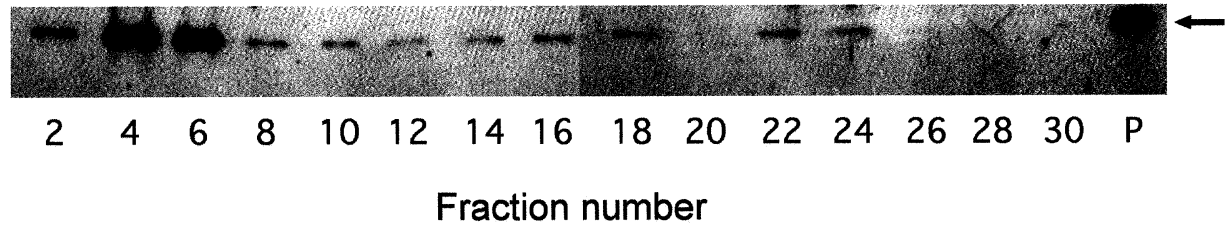
Catalase (kU/ml)
Acid phosphatase (mU/ml)
Sucrose (%)
Fraction number

B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