Immunological detection of alkaline-diaminobenzidine-negative peroxisomes of the nematode *Caenorhabditis elegans*. Purification and unique pH optima of peroxisomal catalase

(Running title: Peroxisomal catalase of *C. elegans*)

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Abbreviations. DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; 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); acid phosphatase (EC 3. 1. 3. 2).

SUMMARY

We purified catalase-2 of the nematode *Caenorhabditis elegans* and identified peroxisomes in this organism. The peroxisomes of *C. elegans* were not detectable by cytochemical staining using 3,3'-diaminobenzidine, a commonly used method depending on the peroxidase activity of peroxisomal catalase at pH 9 where genuine peroxidases are inactive. The cDNA sequences of *C. elegans* predict two catalases closely similar to each other throughout the molecule except the short C-terminal sequence; catalase-2 (500-residues long) carries a peroxisomal targeting signal 1-like sequence (Ser-His-Ile), while catalase-1 does not. The catalase purified to near homogeneity from the homogenate of *C. elegans* cells consisted of a subunit of 57 kDa and was specifically recognized by anti-(catalase-2) but not by anti-(catalase-1). Subcellular fractionation and indirect immunoelectron microscopy of the nematode detected catalase-2 inside vesicles judged to be peroxisomes by morphological criteria. The purified enzyme (220 kDa) was tetrameric similar to many catalases from various sources but exhibited unique pH optima for the catalase (pH 6) and peroxidase (pH 4) activities; the latter value is unusually low and explains why the peroxidase activity was undetectable by the standard alkaline diaminobenzidine-staining method. These results indicated that catalase-2 is peroxisomal and verified that it can be used as a marker enzyme of *C. elegans* peroxisomes.

Keywords: *Caenorhabditis elegans;* catalase; 3,3'-diaminobenzidine; peroxidase; peroxisomes.

INTRODUCTION

Type-II 3-oxoacyl-CoA thiolase [1] is required for the metabolism of 3-oxoacyl-CoA esters with a methyl group at the α carbon [2--5]. These CoA esters include intermediates of phytanic acid degradation and bile acid synthesis. Mammalian type-II thiolase, which is alternatively termed sterol carrier protein x (SCPx) or SCP2/3-oxoacyl-CoA thiolase, is a peroxisomal protein and has an SCP2 domain at its C-terminus [6--8]. Type-II thiolase is also found in the nematode *Caenorhabditis elegans* but this protein lacks the SCP2 domain, which contains the peroxisomal targeting signal 1 (PTS1). Although the C-terminal tripeptide of *C. elegans* type-II thiolase (Ser-Lys-Ile) is similar to PTS1, this sequence has not been confirmed to be functional in animals. This, together with the lack of knowledge on the metabolism of α methyl compounds in *C. elegans*, requires that the subcellular localization of the enzyme should be determined.

Peroxisomes of *C. elegans* have not been well characterized. Our earlier attempt to do so on thin sections by the standard alkaline staining method with 3,3'-diaminobenzidine (DAB) [9] was unsuccessful; this method is based on the peroxidase activity of catalase, which is the universal marker enzyme of the peroxisome [10]. Since DAB inhibits catalase activity [11] and genuine peroxidases are inactive under alkaline reaction conditions, only the peroxidase activity at pH 9 is that of catalase when DAB is used as the substrate [9]. *C. elegans* was postulated to contain two highly similar catalases based on cDNA sequence analysis: catalase-1 (or CTL-1, 496 amino-acid residues) and catalase-2 (or CTL-2, 500 residues) (EMBL/GenBank/DDBJ accession numbers U55384 and X82175, respectively). Catalase-2 is possibly peroxisomal

because its C-terminal tripeptide, Ser-His-Ile, is reminiscent of PTS1. However, the sequence Ser-His-Ile has not been proved to be a functional animal PTS1 and the peroxisome-like vesicles of *C. elegans* were not stained with DAB unlike mammalian [12], plant [13] and yeast [14] peroxisomes. Therefore, we purified the nematode catalase to detect, identify and characterize the peroxisomal catalase of this organism. We report here that catalase-2 is present in *C. elegans* peroxisomes and exhibits an extremely low pH optimum for peroxidase activity. The *C. elegans* peroxisomes can now be identified by immunocytological means with anti-(catalase-2).

MATERIALS AND METHODS

Materials

Bovine liver catalase (2 × crystallized), horse heart cytochrome-*c* (98%) and horseradish peroxidase (HRP, type VI) were purchased from Sigma. Mouse antisera raised against synthetic peptides conjugated to keyhole limpet hemocyanin and preimmune sera were obtained from Sawady Technology. The peptides used for raising anti-(catalase-1) and anti-(catalase-2) were the C-terminal dodecapeptide (NALRHQLCQKKH) and pentadecapeptide (ARVKALIQKQARSHI) of each catalase, respectively. The enhanced chemiluminescence Western blotting detection reagents and goat anti-(mouse Ig) $F(ab')_2$ fragment labeled with HRP were provided by Amersham Life Science. Sources of [α-^{32}P]dCTP, oligonucleotides used as primers for PCR amplification and random-primer DNA-labeling kit were as described previously [1]. Protein-

A was conjugated to 15-nm colloidal gold as described by Yokota *et al.* [15].

Organism and culture

The Bristol N2 strain of *C. elegans* was used throughout this study according to the methods compiled by Sulston and Hodgkin [16]. Mixed-stage cultures were prepared by incubation at 20°C for 6 days with cells of *Escherichia coli* strain OP50 as the feed. L4 larvae were harvested at 40 h after feeding at 20°C.

Purification of catalase-2

Cells derived from 100 g (wet weight) of mixed-stage animals were disrupted with Cryo Clean Blaster CCB-50B (Taiyo Sanso) by the modified method for yeast-cell disruption [17]. Samples of 20 g of animals were suspended in 130 mL of cold 50 mM Tris/HCl, pH 8.0, 0.5 mM EDTA, 1 mM $Ph_2CH_2SO_4F$, 0.1 mM leupeptin and 1 mM ε-aminocaproic acid and disrupted at 0.8 MPa and –140°C. The following procedures were carried out at 0--4°C unless otherwise specified. The frozen material was thawed and broken by 10 up-and-down strokes in a Potter-type Teflon homogenizer. The homogenate was centrifuged at 8000 *g* for 10 min and the resulting post-nuclear fraction was further centrifuged at 126 000 *g* for 60 min. The obtained cytosolic fraction was fractionated with ammonium sulfate (45--65%). The resulting precipitate was dissolved in 20 mL of 10 mM Tris/HCl, pH 7.4, and applied to a Phenyl Sepharose CL-4B (Pharmacia) column (2.4 × 11 cm) equilibrated with buffer A [0.5 M $(NH_4)_2SO_4$, 0.5 mM EDTA, 10 mM Tris/HCl, pH 7.4]. The column was washed with 150 mL of buffer A, and proteins were eluted with a linear concentration gradient consisting of 75 mL of buffer A and 75 mL of 50% (w/v) ethylene glycol, 0.5 mM EDTA, 10 mM Tris/HCl, pH 7.4, at a flow rate

of 25 mL/h. Fractions of a catalase peak (at about 0.1 M $(NH_4)_2SO_4$ and 40% ethylene glycol) were combined, dialyzed against buffer G [10% (w/v) glycerol, 0.2 mM $Ph_2CH_2SO_4F$, 0.1 mM EDTA, 10 mM potassium phosphate, pH 7.4] and applied to a CM-Toyopearl 650M (Tosoh) column (1.5 × 23 cm) equilibrated with buffer G. The column was washed with 80 mL of buffer G, and the bound proteins were eluted with a linear concentration gradient of 120 mL of KCl (0--0.5 M) prepared in buffer G. Fractions of the major catalase peak (at about 0.3 M KCl) were combined, dialyzed against buffer G and passed through a DEAE-Toyopearl 650M (Tosoh) column (1.5 × 6 cm) equilibrated with buffer G. Catalase activity and some proteins were recovered by washing with 80 mL of buffer G. Fractions enriched with catalase were combined, concentrated by Centriplus-30 (Amicon) to 1 mL and applied to a Sephacryl S-300 HR (Pharmacia) column (1.5 × 47 cm) equilibrated with 50 mM potassium phosphate pH, 7.4, 10% glycerol, 0.2 mM $Ph_2CH_2SO_4F$, 0.2 M KCl and eluted with the same buffer. Fractions with little contaminant proteins were pooled, ultrafiltrated by Centriplus-30 to concentrate the enzyme, and the buffer changed to 10 mM potassium phosphate, pH 7.4, 10% glycerol, 0.2 mM $Ph_2CH_2SO_4F$. The final preparation was stored at – 80°C for at least six months with little loss of activity.

Assay of enzymes

All enzymes were assayed at 25°C spectrophotometrically. Compositions of the standard reaction mixtures were: for catalase, 100 mM potassium phosphate, pH 7.0, and 22 mM H_2O_2; for peroxidase, 100 mM potassium acetate, pH 5.0, 0.27 mM DAB and 14 mM H_2O_2; for cytochrome-*c* oxidase, 100 mM potassium phosphate, pH 7.0, 1 mM EDTA and 0.6 mM reduced cytochrome-*c*; for acid

phosphatase, 100 mM potassium acetate, pH 5.0, and 4 mM *p*-nitrophenylphosphate. The reaction was initiated by adding the enzyme solution and the absorbance was monitored. Wavelengths (molar absorption coefficient) used were: for catalase, 240 nm (43.6 M^{-1} cm^{-1}); for peroxidase, 450 nm (3160 M^{-1} cm^{-1}); for cytochrome-*c* oxidase, 550 nm (29 500 M^{-1} cm^{-1}); for acid phosphatase, 405 nm (18 500 M^{-1} cm^{-1}). Enzyme sources containing organelles, were mixed with an equal volume of 1% (w/v) Triton X-100 prepared in the buffer used for the reaction mixture to solubilize the organelle membranes before the assay. When the pH-dependence of enzyme activity was examined, the buffer solution in the standard reaction mixture was replaced with citrate-potassium phosphate for pH 3.5--7.0, potassium phosphate for pH 6.0--8.0, Tris/HCl for pH 7.5--9.0 or glycine/KOH for pH 8.5--10.0. One enzyme unit (U) was defined as the amount that catalyzes the conversion of 1 μmol of substrate per min. Protein was determined according to the method of Bradford [18] with bovine serum albumin as the standard.

Miscellaneous methods

Goat anti-(mouse Ig) $F(ab')_2$ fragment was adsorbed with whole proteins from the mixed-stage *C. elegans* as described by Sambrook *et al.* [19] before use. Methods for SDS/PAGE followed by Western blotting [20] and Northern hybridization with the total RNA [1] were as described previously. The regions used as cDNA probes for catalase-1, catalase-2 and myosin light-chain 2 mRNAs were nucleotides +1 to +513, +1336 to +1531 and +1332 to +1555, respectively, where nucleotide position +1 is A of the start codon of each ORF. The EMBL/GenBank/DDBJ accession numbers for myosin light-chain 2 and the 3'

untranslated region of catalase-1 are M23366 and Y14066. Two-dimensional SDS/PAGE with Bio-Lyte 3/10 (Bio-Rad, effective range is pH 3.5--9.5) was conducted according to the method of Adams and Gallagher [21] using 2-D standards (Bio-Rad) as the pI marker. Preparation of the particulate fraction and subcellular fractionation were carried out as described by Maebuchi *et al.* [22]. Procedures for electron microscopy, immunostaining with primary antibodies and secondary gold probes and determination of the labeling density (gold particles/μm^2) were as described previously [22]. Sequences similar to catalase-2 were searched and analyzed using the SSEARCH program of DDBJ.

RESULTS

Purification and identification

A catalase was purified from the crude extract of mixed-stage *C. elegans* cells to near homogeneity. The overall purification was about 2900-fold with a yield of 15% (Table 1). The purified catalase consisted of a single 57-kDa polypeptide (Fig. 1, lane 2) and its molecular mass was 220 kDa (Fig. 2), indicating that the enzyme is a homo-tetramer similar to those from many other sources. The apparent subunit mass (57 kDa) determined by SDS/PAGE was compatible with those calculated for both catalase-1 (57 024 Da) and catalase-2 (57 467 Da). To identify the purified enzyme, we used two antisera raised against the C-terminal dodecapeptide of catalase-1 and pentadecapeptide of catalase-2; only these sequences show distinct differences between the two catalases. Anti-(catalase-2) reacted with the purified catalase and recognized a single 57-kDa polypeptide

in the homogenate, which co-migrated with the purified enzyme (Fig. 1, lanes 3 and 4). Anti-(catalase-1), on the other hand, did not react with the purified catalase and recognized another single polypeptide in the homogenate (Fig. 1, lanes 5 and 6), confirming the presence of catalase-1. Additional support for the purity and identity was provided by two-dimensional SDS/PAGE, where the purified catalase migrated as a single spot and streaked between pH 7.0 and pH 8.5 (data not shown). The calculated pI values for catalase-1 and catalase-2 were 6.56 and 7.74, respectively. Semiquantitative Northern blot analysis of total RNA from the mixed-stage animals demonstrated that the expression of catalase-2 was comparable with that of catalase-1 (data not shown). We thus concluded that the purified enzyme was catalase-2, which carries the PTS1-like sequence.

Subcellular localization

Catalase-2 must be located in the matrix of peroxisomes if the C-terminal tripeptide Ser-His-Ile functions as PTS1. The particulate fraction prepared from mixed-phase animals was separated by sucrose density gradient centrifugation followed by Western blot analysis. A peak of catalase activity was observed ahead of the peaks of cytochrome-*c* oxidase and acid phosphatase; the latter two correspond to mitochondria and lysosomes, respectively (Fig. 3A). Catalase-2 was enriched in fractions coinciding with the peak of catalase activity (Fig. 3B). This peak seemed to represent peroxisomes because peroxisomes of the yeast *Saccharomyces cerevisiae* [20] and *Candida tropicalis* [23] sediment at about 50% sucrose under similar conditions. Catalase-2 was, therefore, suggested to be a peroxisomal protein of *C. elegans* by results of subcellular fractionation.

This was further confirmed by immunoelectron microscopy. Thin sections of *C. elegans* at the L4 stage (including some young adults) were incubated with mouse anti-(catalase-2) and rabbit anti-(mouse Ig), and then probed with gold colloidal particles coupled to protein-A. Gold particles were located within cytoplasmic vesicles that were 0.2--0.5 μm in diameter, surrounded by a single unit membrane (Fig. 4A) and were mainly found in intestinal epithelial cells. These characteristics matched the morphological criteria of peroxisomes. The control experiment using mouse preimmune serum showed essentially no signals (Fig. 4B). Labeling density analysis of ten micrographs taken as described in Fig. 4A indicated that the particle densities inside and outside peroxisomes were $123.3 \pm 28.5/\mu m^2$ and $1.2 \pm 0.2/\mu m^2$, respectively. Hence, we concluded that catalase-2 is a peroxisomal protein of *C. elegans*. Thus, the C-terminal Ser-His-Ile must be a functional PTS1 in this organism.

Optimum pH for the catalase and peroxidase activity

The question remained why peroxisomal catalase-2 was insensitive to alkaline DAB staining, by which mammalian [12], plant [13] and yeast [14] catalases in peroxisomes can be detected. To answer this question, we compared *C. elegans* catalase-2 with bovine liver catalase with regard to pH-dependence of their catalase and peroxidase activities. The optimum for the catalase activity of *C. elegans* enzyme was pH 6.0 and lower than that of bovine catalase by one pH unit (Fig. 5A). Surprisingly, the peroxidase activity of *C. elegans* catalase-2 was highest at pH 4.0; this was unexpected as the optimum pH for the bovine enzyme is 9.2 (Fig. 5B). This explained why *C. elegans* peroxisomes were undetectable by standard DAB staining, which depends on the peroxidase activity of

peroxisomal catalase at pH 9 where *C. elegans* catalase-2 was entirely inactive (less than 2 mU/mg of protein) unlike bovine catalase. It is worth noting that the scales for catalase-2 (left) and for bovine catalase (right) are different in both Fig. 5A and Fig. 5B. Consequently, the activity ratios of peroxidase/catalase (expressed by U/U at each optimum pH) were 3.1×10^{-5} and 1.4×10^{-6} for *C. elegans* catalase-2 and bovine catalase, respectively. This 20-fold-higher relative peroxidase activity with an unusually low pH optimum suggested that the *C. elegans* enzyme could be a peroxidase rather than a catalase because HRP was reported previously to be most active at pH 4.3 [24] and under our assay conditions the highest activity was detected at pH 5.0. However, the catalase activity of catalase-2 was higher than its peroxidase activity by four orders of magnitude. According to SSEARCH program, the amino acid sequence of catalase-2 was similar (63-79% identity) to 300 sequences that correspond to catalases from various sources including bacteria, fungi, plants, invertebrates and vertebrates, but no peroxidase.

DISCUSSION

This study was initiated to identify peroxisomes of *C. elegans* to determine the subcellular localization of type-II thiolase. The failure of the earlier attempt to detect peroxisomes by alkaline DAB staining motivated us to purify the peroxisomal catalase of *C. elegans*, hoping that this would enables us to detect these organelles by immunocytological means. We purified catalase-2 from the homogenate of mixed-stage culture and demonstrated that this enzyme was

peroxisomal. This was consistent with the recent report by Taub *et al.* showing a punctate pattern of green fluorescent protein fused to catalase-2 when the *gfp::ctl-2* fusion gene is expressed in the nematode [25]. The comparable expression of catalase-1 and catalase-2 also confirmed the observation of these authors [25]. Further, our observations revealed that catalase-2 has high peroxidase activity and an unexpectedly low pH optimum. These properties explain the failure of our earlier attempts to identify the peroxisomes. On the other hand, they raise new physiological and evolutionary questions regarding catalase-2, which has intermediate characteristics of genuine peroxidases and common catalases. Although conditions for DAB staining of *C. elegans* peroxisomes at a lower pH have not been established yet, they were detectable with anti-(catalase-2). By this means, type-II thiolase of this organism was demonstrated to be a peroxisomal enzyme [22] similar to the mammalian counterparts.

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Table 1. Summary of purification. The homogenate derived from 100 g (wet weight) of mixed-stage animals was used. Catalase activity was assayed with the standard reaction mixture. Catalase activity, protein, specific activity and yield are expressed in kU, mg, kU per mg of protein and percentage, respectively. One enzyme unit (U) was as defined in Materials and methods.

Step	Total activity	Total protein	Specific activity	Yield
Homogenate	698	3060	0.228	100
8000 *g*	594	2020	0.294	85.0
126 000 *g*	550	1340	0.410	78.8
45-65% $(NH_4)_2SO_4$	364	400	0.910	52.1
Phenyl Sepharose	296	62.3	4.75	42.4
CM-Toyopearl	225	3.40	66.2	32.2
DEAE-Toyopearl	143	0.293	484	20.5
Sephacryl S-300	105	0.161	659	15.1

FIGURE LEGENDS

Fig. 1. Purity and identity of the enzyme. Proteins were separated by SDS/PAGE on a 10% polyacrylamide gel and stained with Coomassie brilliant blue (lanes M, 1 and 2) or analyzed by Western blotting (lanes 3 through 6); proteins were transferred onto a nitrocellulose membrane and reacted with mouse anti-(catalase-2) (lanes 3 and 4) or anti-(catalase-1) (lanes 5 and 6) and the mouse Ig was detected by the reaction of HRP coupled to goat anti-(mouse Ig) $F(ab')_2$ fragment. Lane M shows marker proteins (from top to bottom: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor) and their masses are indicated on the left. Lanes 1, 3 and 5 contain whole proteins from the mixed-stage culture of *C. elegans* (0.1 mg) and lanes 2, 4 and 6 the purified enzyme (4 μg, 0.4 μg and 0.4 μg, respectively). The subunit mass of the enzyme was calculated to be 57 kDa (arrow).

Fig. 2. Molecular mass of the enzyme. The purified enzyme (10 μg in 0.6 mL) was applied to a Sephacryl S-300 HR column (1.5 × 47 cm) calibrated with marker proteins (from left to right: ferritin, 450 kDa; bovine liver catalase, 240 kDa; rabbit muscle aldolase, 158 kDa; bovine serum albumin, 67 kDa) as indicated by arrows. The void volume (v_o) and column volume (v_c) are similarly indicated. The elution pattern of the purified enzyme was monitored by its catalase activity, and its molecular mass was calculated to be 220 kDa.

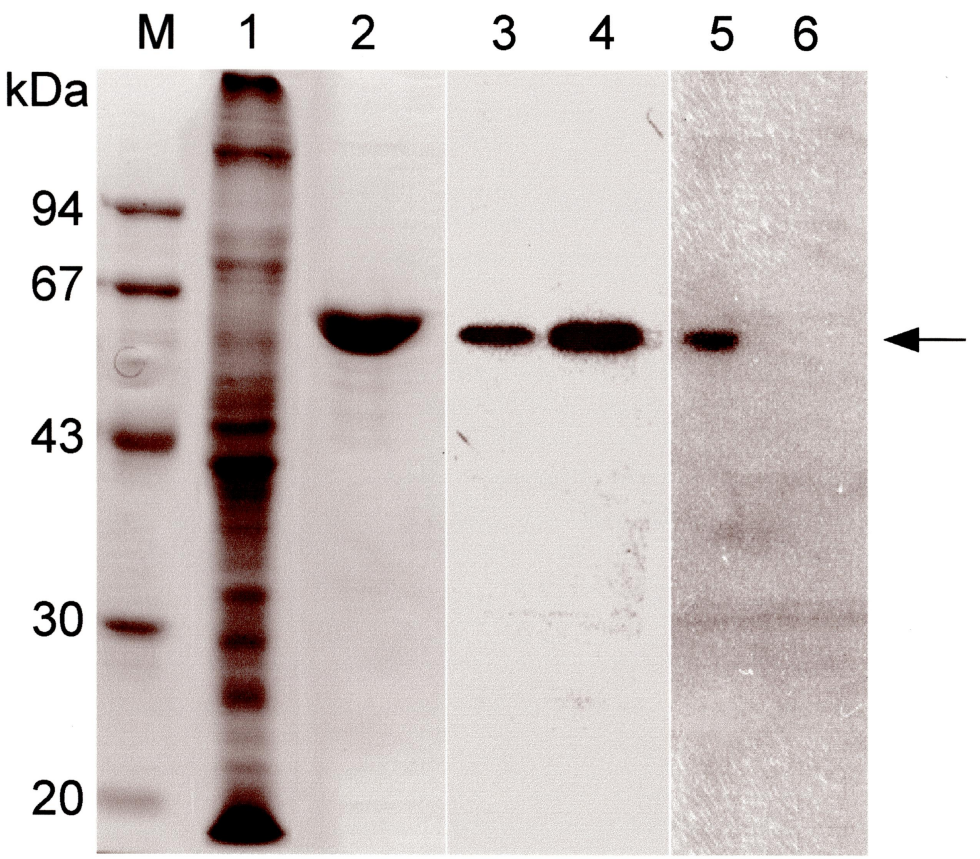
Fig. 3. Co-sedimentation of catalase-2 and peroxisomal catalase activity on sucrose density gradient centrifugation. The particulate fraction (5 mL,

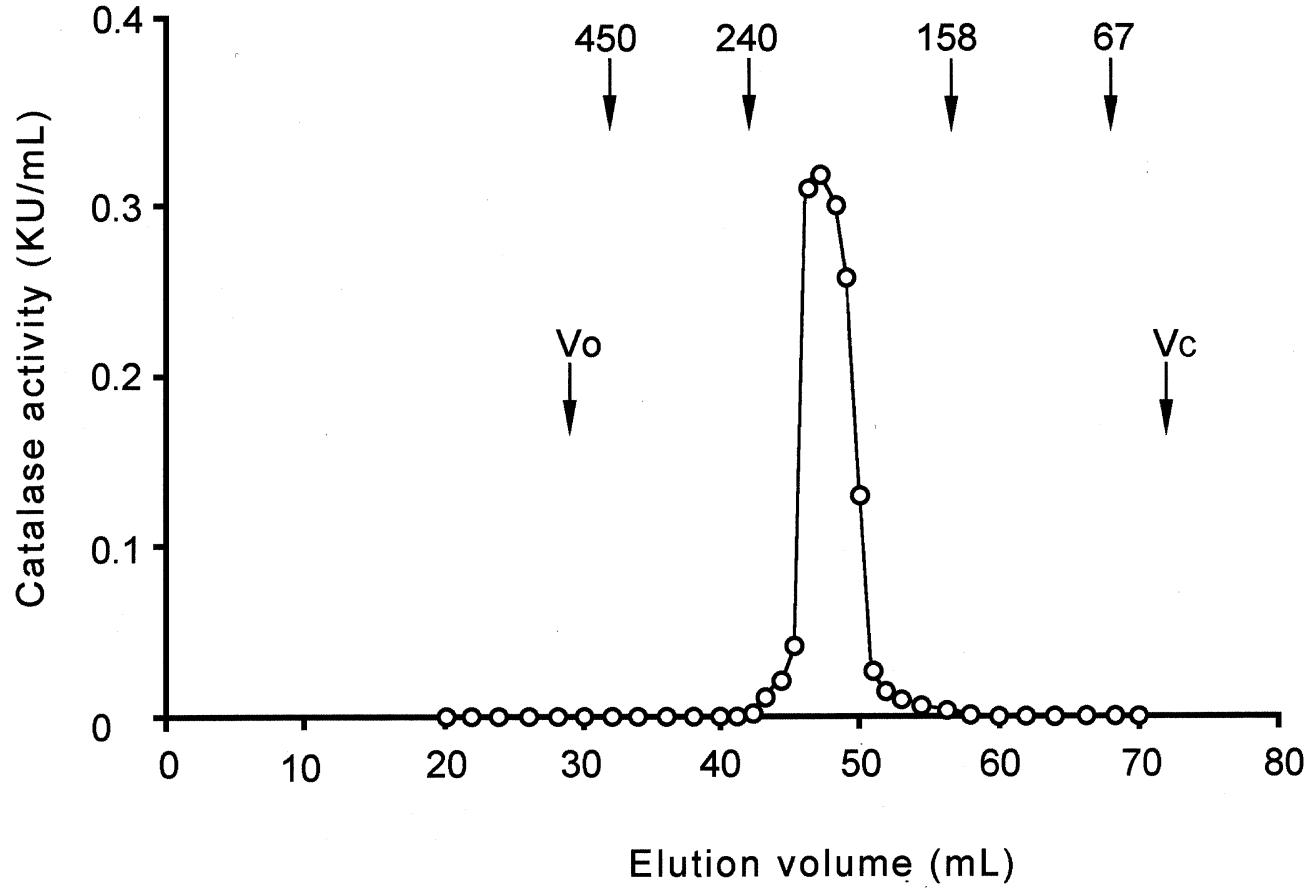
1.1 mg/mL as protein) obtained from the mixed-stage culture of *C. elegans* was applied to a 24-mL continuous 32-54% (w/w) sucrose density gradient on a 2-mL cushion of 60% (w/w) sucrose and centrifuged at 100 000 *g* for 90 min. Fractions (1 mL each) were collected from the bottom of the tube and assayed for activities of catalase (closed circle), cytochrome-*c* oxidase (open circle) and acid phosphatase (open triangle) and sucrose density (dot) (A). Proteins from odd numbered fractions (5 μL each) and the particulate fraction (1 μL, lane P) were separated by SDS/PAGE and catalase-2 was detected with anti-(catalase-2) as in Fig. 1. The arrow marks catalase-2.

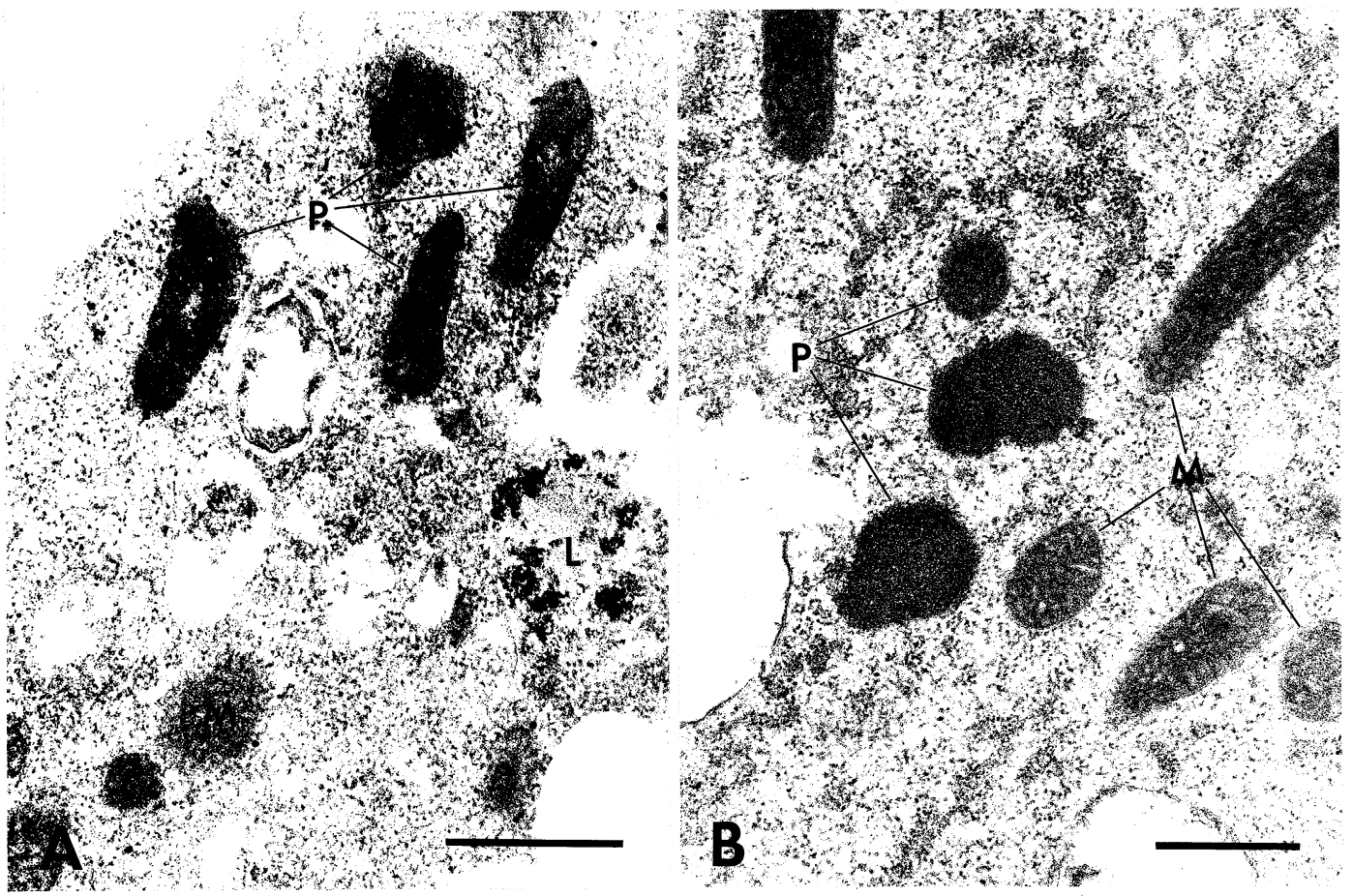
Fig. 4. Peroxisomal localization of catalase-2 on thin sections of L4 larvae. Thin sections of L4 larvae stained with gold probes and 2% uranyl acetate were examined with an H600 electron microscope (Hitachi) at 75 kV. To detect catalase-2, the sections were first incubated with mouse anti-(catalase-2) (diluted 1:1000) then with rabbit anti-(mouse Ig) (diluted 1:2000), and probed with 15-nm gold particles conjugated to protein-A (diluted 1:50) (A). Negative control sections were incubated with mouse preimmune serum instead of anti-(catalase-2) (B). L, M and P mark lysosomes, mitochondria and peroxisomes, respectively. The bar is 0.5 μm (× 49 000 for A and × 39 000 for B).

Fig. 5. Effect of pH on the catalase and peroxidase activities of catalase-2 and bovine liver catalase. A purified catalase-2 preparation (closed symbols) and bovine liver catalase (open symbols) were assayed for both catalase activity (A) and peroxidase activity (B) under different buffer conditions. Buffers used were: citrate-potassium phosphate (square), potassium phosphate (triangle),

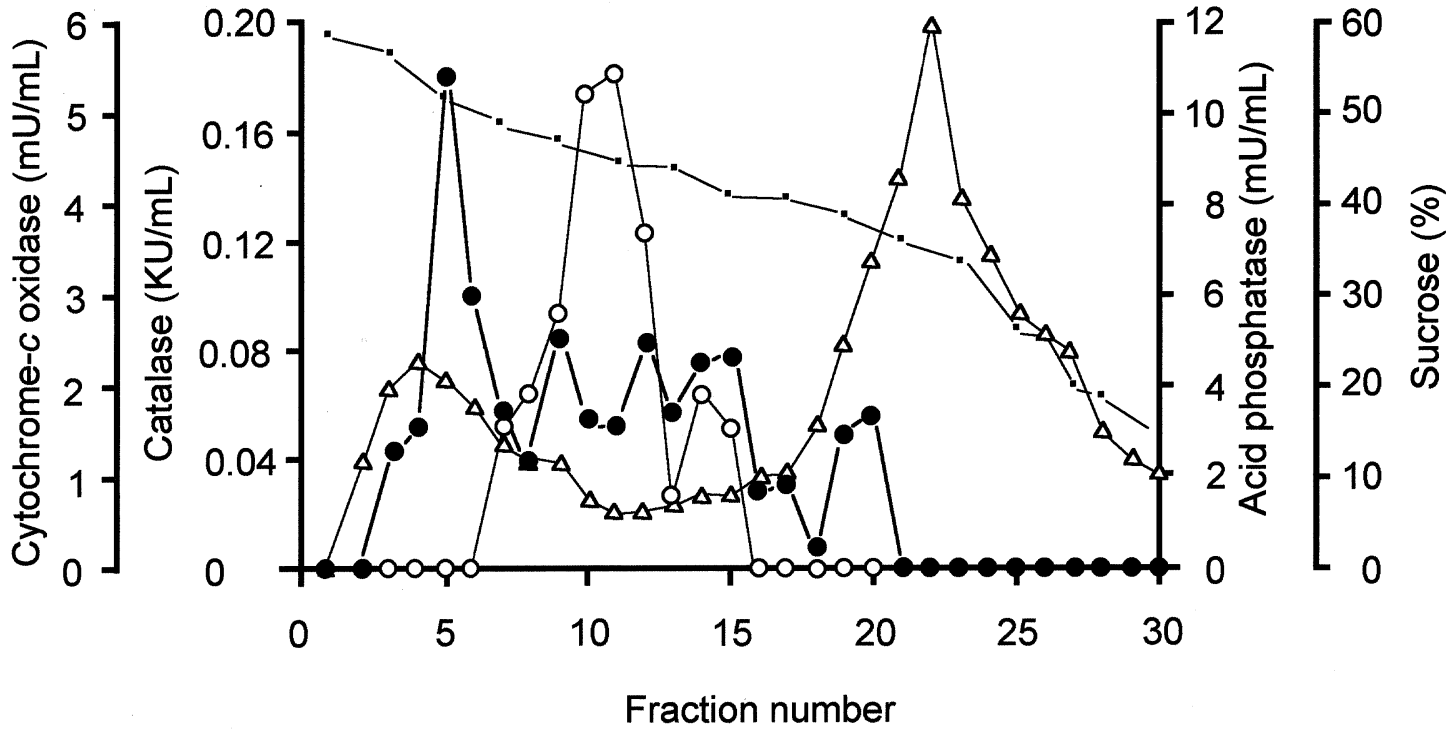
Tris/HCl (diamond), glycine/KOH (circle). Scales for closed and open symbols are indicated on the left and right ordinate, respectively.


M
1
2
3
4
5
6
kDa
94
67
43
30
20

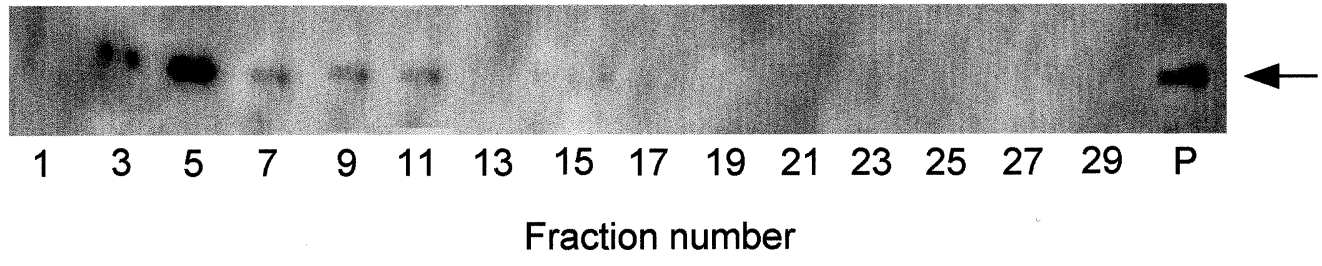

0.4
0.3
0.2
0.1
0
Catalase activity (KU/mL)
450
240
158
67
Vo
Vc
0
10
20
30
40
50
60
70
80
Elution volume (mL)

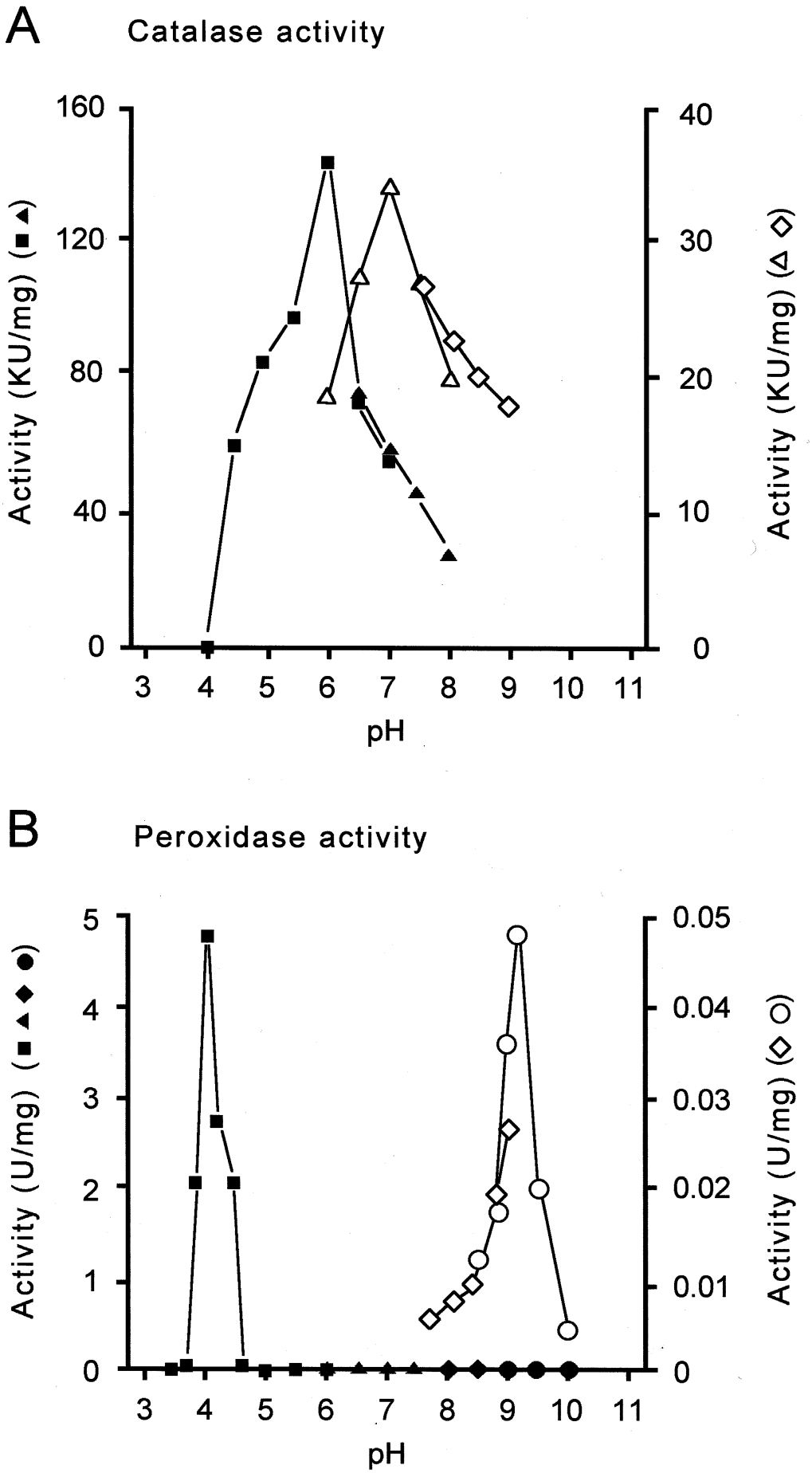

P
L
A
P
M
B

A


Cytochrome-c oxidase (mU/mL)
Catalase (KU/mL)
Acid phosphatase (mU/mL)
Sucrose (%)
Fraction number

B


1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 P
Fraction number


A
Catalase activity
160
120
80
40
0
Activity (KU/mg) (■▲)
40
30
20
10
0
Activity (KU/mg) (△ ◇)
3
4
5
6
7
8
9
10
11
pH
B
Peroxidase activity
5
4
3
2
1
0
Activity (U/mg) (■▲◆●)
0.05
0.04
0.03
0.02
0.01
0
Activity (U/mg) (◇ ○)
3
4
5
6
7
8
9
10
11
pH