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Functional Expression of Rat Peroxisomal Acyl-CoA Oxidase in *Spodoptera frugiperda* Cells

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SUMMARY: A cDNA coding the 661-residue rat peroxisomal fatty acyl-CoA oxidase (ACOX) has been constructed and expressed as catalytically active protein in *Spodoptera frugiperda* (Sf9) insect cells using baculovirus expression system. Recombinant viral clones were purified and the expressed protein was identified by immunoblotting and catalytic activity. In the rat liver, ACOX consists of three polypeptide components A, B and C, with relative molecular mass of 72 kDa, 51 kDa and 21 kDa,respectively. In Sf9 insect cells infected with the recombinant virus, ACOX protein (component A) was expressed up to 15% of the total cellular protein. Immunoblot analysis demonstrated that besides component A, components B and C were also present in Sf9 cells,suggesting that these components are derived from component A by post-translational proteolytic cleavage. However, unlike in rat liver, baculovirus generated ACOX in insect cells had reduced amounts of components B and C with an estimated molar ratio of A, B, C of 5:1:1 in Sf9 cells *vs* 1:5:5 in rat liver. By immunofluorescence and immunocytochemical methods the overexpressed recombinant ACOX was identified both in the cytoplasm and the nucleus of Sf9 cells. Polyclonal antibodies raised against recombinant ACOX recognized rat liver ACOX on immunoblotting. Baculovirus Sf9 system provides high-efficiency expression of functional ACOX and can be used to express specific mutant and truncated ACOX cDNAs for further characterization.

Peroxisomes are cytoplasmic organelles which contain more than fifty enzymes including those that participate in lipid metabolism (1). Mammalian peroxisomes are involved in the degradation of fatty acids *via* β-oxidation system which is different from the mitochondrial fatty acid oxidation

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Abbreviations used are: ACOX, peroxisomal acyl-CoA oxidase; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* insect cells; SDS/PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RT, reverse transcription.

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system (1). The first enzyme of the peroxisomal β-oxidation system is a fatty acyl-CoA oxidase (ACOX) which catalyzes the oxidation reaction in which fatty acyl-CoA is desaturated to a 2-*trans*-enoyl-CoA leading to the generation of H_2O_2 (2). The importance of this enzyme is underscored by the observation that many structurally diverse chemicals, known as peroxisome proliferators, transcriptionally activate ACOX and the other two genes of the β-oxidation system in a co-ordinate fashion leading to excess generation of intracellular H_2O_2, a process implicated in the pathogenesis of hepatocellular carcinomas developing in animals chronically exposed to these agents (3). In addition, a lethal disorder characterized by an isolated deficiency of peroxisomal ACOX termed pseudo-neonatal adrenoleukodystrophy has been described recently (4). The availability of structural information on rat and human ACOX genes should facilitate the understanding of the molecular basis for this genetic disorder (5,6).

In the rat liver ACOX has been shown to exist in the form of three polypeptide components A (72 kDa), B (51 kDa), and C (21 kDa) where conversion of component A to components B and C occurs as a result of proteolytic breakdown (7-9). ACOX is present in very small quantities in the liver and it is difficult and cumbersome to obtain sufficient quantities of purified protein for physicochemical characterization. In the present paper, we report the production of relatively large quantities of functionally active rat recombinant ACOX by a baculovirus based expression system. This system would allow generation of mutants relatively rapidly to define the structural elements.

MATERIALS AND METHODS

Baculovirus vector and insect cells: The insect cell line, *Spodeptera frugiperda* (Sf9), the baculovirus transfer vector pVL1392 (10) and a wild-type baculovirus *Autographa californica* multiple polyhedrosis virus (AcMNPV strain) were obtained from Invitrogen (San Diego, CA, U.S.A.). Grace's insect cell medium was obtained from GIBCO-BRL (Grand Island, NY, U.S.A.).

Construction of the transfer vector pVL-ACOX: Full-length rat ACOX cDNA was constructed by combining the cDNA inserts pMJ125 and pMJ131 (11) and was inserted into the SacI site of pTZ18R as described elsewhere (12). The cDNA was released from pTZ18R by digesting with EcoRI and SmaI which was then cloned into EcoRI and SmaI site of the baculovirus transfer vector pVL1392. The resulting plasmid in which the entire ACOX coding sequence was under the control of the polyhedrin gene promoter was designated pVL-ACOX.

Expression of recombinant ACOX: Purified recombinant transfer vector DNA, together with linearized baculoviral AcMNPV DNA was used for co-transfection of Sf9 cells, using the calcium-phosphate precipitation method (13). Two days post-transfection, supernatant from this transfection culture was serially diluted and used to infect fresh Sf9 cells. Polyhedrin negative recombinant plaques were recognized by their unique morphology, confirmed by PCR amplification and then screened for recombinant ACOX expression by SDS/PAGE, using 10 % polyacrylamide gels (14) and immunoblotting (15). Immunoblotting was performed by employing monospecific rabbit anti-rat ACOX antibodies and alkaline phosphatase-coupled goat anti-rabbit IgG antibodies (Bio-Rad, Hercules, CA, U.S.A.) as the primary and secondary antibodies respectively. Purified recombinant viral stocks were used at a multiplicity of infection of 10 to infect Sf9 cells which were at an 80% confluence in T-flasks. Cell cultures were maintained at 26° C and were harvested at selected

intervals after infection for enzyme assay and SDS/PAGE analysis. ACOX activity was assayed by measuring the palmitoyl-CoA dependent H_2O_2 production (16). One unit of the enzyme was defined as the amount which catalyzes the formation of 1 μmol of product or the removal of 1 μmol of substrate per min.

Immunomorphological procedures: To visualize recombinant ACOX by immunofluorescence microscopy, Sf9 cells were cultured on glass slides and fixed in 95% methanol for 10 min. After washing with PBS, pH 7.4 (137 mM NaCl, 1.47 mM KH_2PO_4, 8.17 mM Na_2HPO_4, $7H_2O$, 2.7 mM KCl) cells were incubated overnight with primary antibody raised against rat liver ACOX as previously described (17). After incubation, cells were treated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:400) for 1 h at room temperature. Slides were washed with PBS, pH 7.4 and mounted using 90% glycerol, 1 mM Tris pH 7.6, and a cover glass placed on the cells. Slides were viewed using a Zeiss fluorescent microscope. Protein-A gold immunolocalization studies at the electron microscopic level were performed on Sf9 cells embedded in Lowicryl K_4M as described elsewhere (18). Sections of liver from a rat treated with 0.0125% ciprofibrate for 2 weeks were used as positive controls for immunolocalization (18).

Purification of recombinant ACOX: Purifcation of ACOX was carried out by a modification of a procedure described previously (9). Exponentially growing Sf9 cells in 750-ml flasks were infected with purified recombinant virus at a multiplicity of infection of 10, and 3 days post-infection the cells were dislodged and sedimented by centrifugation at 1000 x g for 5 min. The cell pellets were washed 3 times with PBS, pH 7.4 at 0-4° C, suspended in 50 mM KPO_4, pH 7.8 together with 0.1% NP-40 and 1 mM phenyl methyl sulfonyl fluoride (PMSF) and lysed by sonication for 2 min and centrifuged at 17,000 x g for 30 min. To the supernatant, 25 μM FAD was added, heated at 55° C for 15 min in a water bath and then cooled rapidly in an ice-bath at 0-4° C. Denatured proteins were removed by centrifugation at 20,000 x g for 15 min and the clear supernatant passed through a DEAE cellulose (Sigma, St. Louis, MO, U.S.A.) column equilibrated with 10 mM KPO_4 buffer, pH 7.6 , containing 25 μM FAD and 1 mM PMSF. The unbound protein(s) was then loaded onto a BIO-GEL HTP (hydroxylapatite; Bio-Rad, Hercules, CA, U.S.A.) column equilibrated with the same buffer as above, eluted with a gradient of 10-150 mM KPO_4 and 2 ml fractions collected. Fractions containing ACOX activity and found to be homogeneous on SDS/PAGE analysis were pooled and concentrated in a centrifugal filtration unit.

Other methods: Purified recombinant ACOX was used for immunizing New Zealand rabbits to raise antibodies against recombinant rat ACOX according to procedures previously outlined (19). Pre-immune serum and immune serum were analyzed by Ouchterlony double diffusion method (19) and by immunoblotting and protein-A gold immunolocalization (18). Plasmid isolation, endonuclease digestion, ligation, RT-PCR and hybridization procedures were performed according to standard protocols (20).

RESULTS AND DISCUSSION

Expression of recombinant rat ACOX in Sf9 cells: A major aim of this study was to establish a system that would produce large quantities of functional recombinant rat ACOX. The baculovirus expression system has been used successfully to express large quantities of a wide variety of functional eukaryotic recombinant proteins, including growth factors, growth factor receptors, hormones and enzymes (10). In an earlier study, we used the baculovirus system to express rat urate oxidase, a peroxisomal enzyme, and found that the recombinant protein expressed in Sf9 cells exhibited crystalloid-like insoluble aggregates (17). In the present study we cloned a full-length rat ACOX cDNA into the baculovirus transfer vector pVL1392 (Figure 1). The transfer vector and the

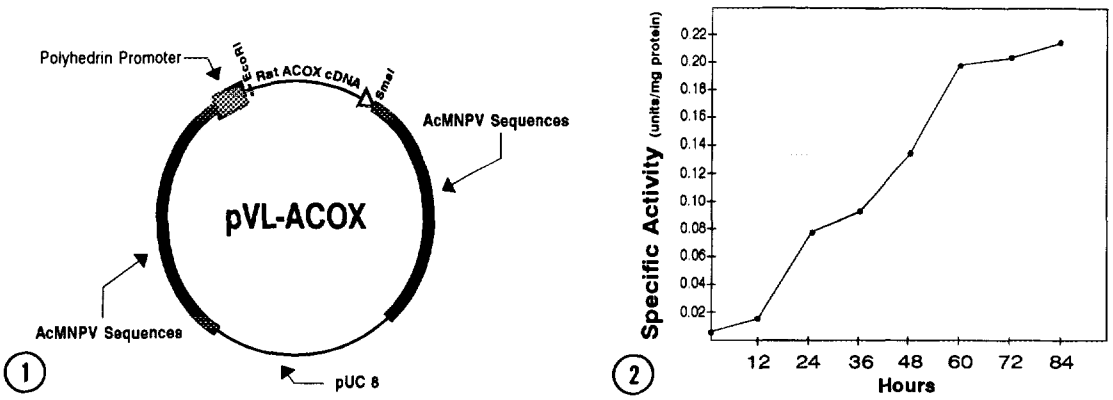


Figure 1. Recombinant baculovirus transfer vector. pVL-ACOX. The pVL ACOX contains a full-length cDNA coding sequence of the rat ACOX. Rat ACOX coding sequence was first released by digesting the plasmid pTZ18R with EcoR I and Sma I and then subcloned into the EcoR I and Sma I sites of the baculovirus transfer vector pVL1392. The transfer vector contained sequences for homologous recombination with the nuclear polyhedrosis wild type AcMNPV baculovirus and a multiple cloning site following the polyhedrin promoter.

Figure 2. Time course of ACOX expression in Sf9 insect cells. SF9 cells were grown in a monolayer in a tissue culture flask and when the growth reached 80% confluency, the cells were infected with the recombinant baculovirus at m.o.i. 10. The infected cells were then harvested at different times and the homogenates assayed for ACOX activity. The results are expressed as the average of two infections.

linearized form of baculovirus AcMNPV DNA were co-transfected into Sf9 cells and 2 days after co-transfection the recombinant baculovirus with polyhedrin negative phenotype was isolated by plaque assay. Recombinant viral clones, designated AcMNPV-ACOX, were purified and used for expression of the recombinant protein. Figure 2 shows a time course of expression of ACOX in Sf9 cells infected with recombinant virus. ACOX activity increased from 24 h post-infection until peak levels of enzyme activity were found between 60 and 84 h post-infection. The specific activity of ACOX in Sf9 cells 60 h after infection with the recombinant virus was about 10 times greater than that found in rat liver (0.22 units/ mg protein in Sf9 cells vs 0.019 units /mg protein in rat liver). No appreciable amount of enzyme activity was detected in culture medium suggesting that this enzyme was not secreted into the medium under present experimental conditions.

SDS/PAGE analysis of Sf9 cell lysates revealed a major 72 kDa protein band on Coomassie Blue staining in cells infected with AcMNPV-ACOX recombinant virus (Figure 3A). This 72 kDa protein increased in amount in a time-dependent manner following infection with recombinant virus (Figure 3 A) and became prominent by 48 h post-infection. Densitometric scanning of these gels showed that this 72 kDa protein accounted for upto 15% of the total cellular protein in Sf9 cells 72 h post-infection. The observed increases in the amount of this protein on SDS/PAGE correlated well with the time-course of increase in ACOX activity. Westernblot analysis with antibodies raised against

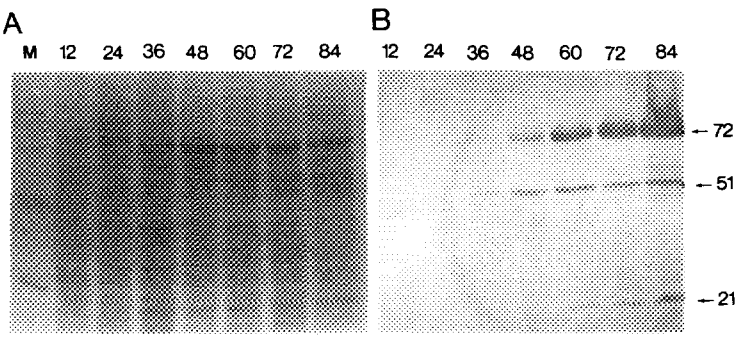


Figure 3. Identification of recombinant ACOX by SDS/PAGE and immunoblotting. (A) Coomassie Blue-stained gel. SDS/PAGE analysis of whole cell lysate of Sf9 cells infected with the recombinant virus containing the ACOX cDNA. Cells were grown and infected as described in Figure 2. Approximately 50 μg of protein was loaded in each lane. The time (h) of infection is indicated at the top of each lane. Lane M shows molecular mass standards (values in kDa). **(B)** Immunoblot analysis of recombinant ACOX expressed in Sf9 cells. The cell extracts were prepared as described in **(A)** and processed for immunoblotting using polyclonal antibodies raised against rat liver ACOX. ACOX components A, B and C are present in insect cells transfected with the recombinant virus. Time (h) of infection is indicated at the top of each lane. Lane M represents molecular-mass standards (values in kDa).

rat liver ACOX revealed that the 72 kDa protein is the predominant immunoreactive protein in Sf9 cell lysates. This 72 kDa protein corresponds to the component A of rat liver ACOX (11). Besides this component A, two low molecular weight bands corresponding to components B and C of ACOX with molecular mass of 51 kDa and 21 kDa respectively were also recognized in Sf9 cell homogenates by this immunoblot procedure using anti-rat ACOX antibodies (Figure 3B). ACOX purified from rat liver exhibits three components, namely A, B, C, and it has been suggested that components B and C are derived from component A by a proteolytic cleavage of component A (11). Sequence information confirmed that components B and C are located on the amino- and the carboxyl-terminal sides of component A (11). Rat ACOX cDNA expressed in insect cells revealed an identical cleavage pattern (Figure 3B). Thus, Sf9 cells appear to process the recombinant ACOX in a fashion similar to that occurring in rat liver. Notably, in insect cells during the early stages of infection most of the full-length protein (component A) was processed into 51 kDa (component B) and 21 kDa (component C) peptides. Nevertheless, at later stages of infection less than 20% of component A appeared to be cleaved (Figure 3B). In the rat liver, the proportion of components A, B, and C was found to be 1:5:5 (9), in contrast to the 5:1:1 distribution of these components in recombinant ACOX in Sf9 cells observed in the present study. In the baculovirus-insect cell expression system, incomplete or partial processing of overexpressed proteins is not uncommon.

Localization of recombinant ACOX in insect cells: In rat and other mammalian liver cells ACOX is localized exclusively within the peroxisomal matrix (18). Previous studies have identified a

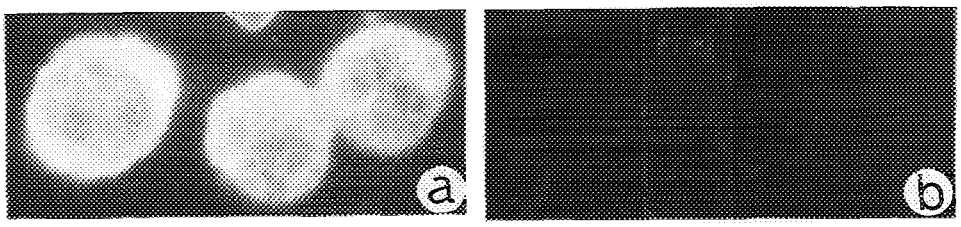
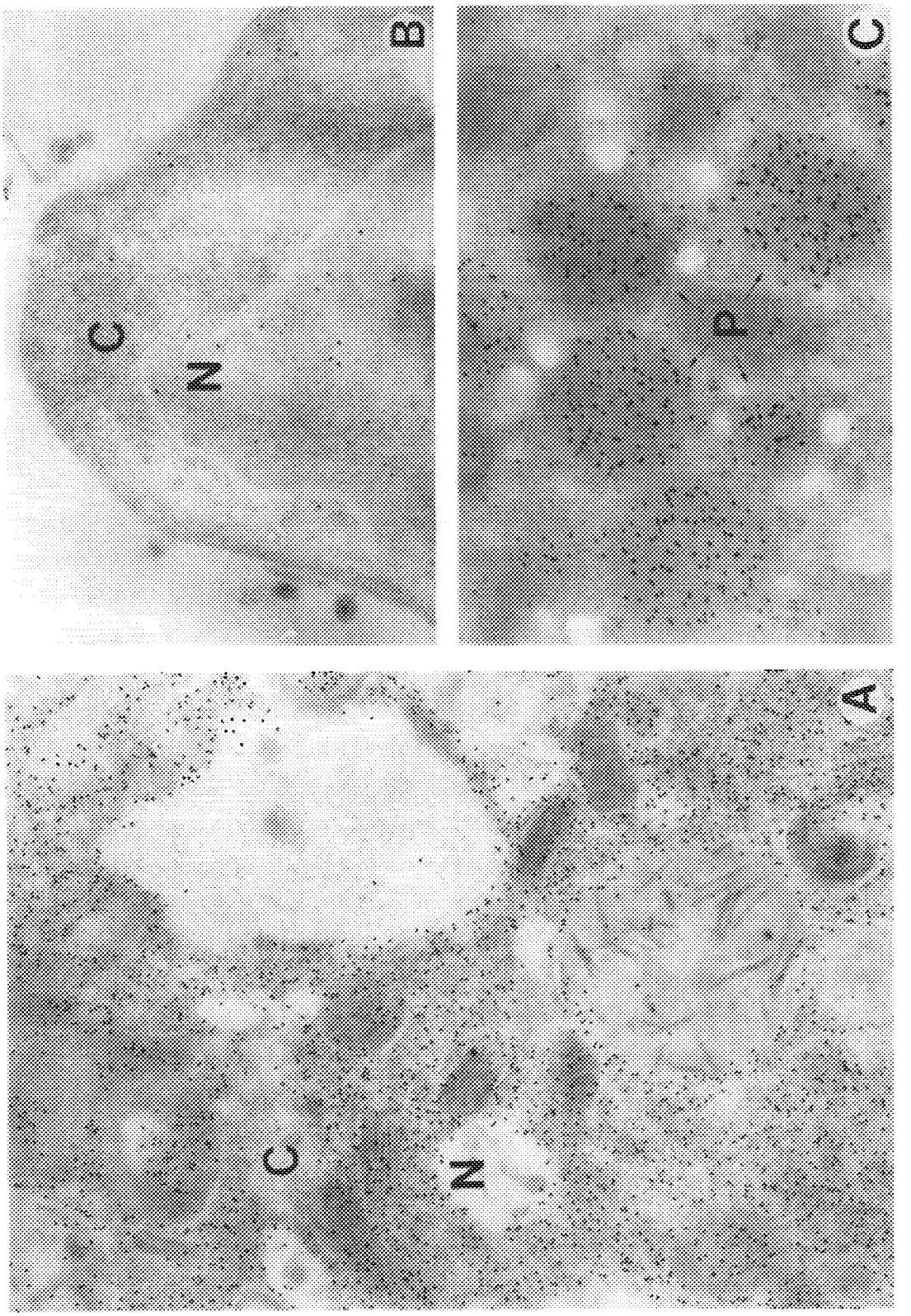


Figure 4. Immunofluorescent localization of rat ACOX in Sf9 cells infected with recombinant rat ACOX baculovirus. (a) Representative Sf9 cells infected with recombinant baculovirus containing rat ACOX cDNA immunostained with antibodies raised against rat ACOX. Intense reaction product is visualized in these cells. **(b)** Uninfected Sf9 cells immunostained with antibodies against rat ACOX reveal no reaction product. Magnification x 500.

tripeptide peroxisomal targeting signal (Ser-Lys-Leu, or a conservative variant such as Ser-Arg-Leu) located at the carboxyl terminus of a majority of peroxisomal proteins, including ACOX, that targets proteins to peroxisomes (12). It is postulated that this tripeptide peroxisomal targeting signal is recognized by a receptor on the peroxisome membrane prior to internalization (12). Evidence indicates that the peroxisomal targeting signal is highly conserved and functions efficiently to correctly target proteins to peroxisomes in various mammalian cells, and in yeast and insect cells (12). In this study, we investigated the cellular and subcellular distribution of recombinant ACOX in Sf9 cells by immunofluorescence and protein-A gold immuno-electronmicroscopy using antibodies raised against rat ACOX. As shown in Figure 4a, intense fluorescent immunostaining was detected in Sf9 cell nucleus and the cytoplasm of infected cells. No fluorescent immunostaining was detected in uninfected Sf9 cells (Figure 4b) indicating the lack of endogenous immunorecognizable ACOX in Sf9 cells. The nuclear and cytoplasmic distribution was visualized at the electronmicroscopic level by the protein-A gold immunocytochemical procedure (Figure 5A). It is of interest to note that no recognizable peroxisomes were identifiable in Sf9 insect cells and no immunolabeling was observed in control Sf9 cells (Figure 5B). Our previous studies on the expression of rat urate oxidase in insect cells revealed the localization of overproduced recombinant urate oxidase also in the cytoplasm and the nucleus of insect cells and we were unable to identify distinct organelles resembling peroxisomes either in infected or in uninfected Sf9 cells (17). The absence or paucity of peroxisomes in insect cells maintained as a cell line may necessitate nuclear and cytoplasmic distribution of abundantly produced recombinant peroxisomal proteins such a urate oxidase and ACOX. In the rat liver, ACOX is clearly localizable to the peroxisome matrix (Figure 5C).

Purification of recombinant rat ACOX: Recombinant rat ACOX expressed in insect cells was purified by a two-step procedure. A major 72 kDa protein band was obtained using DEAE cellulose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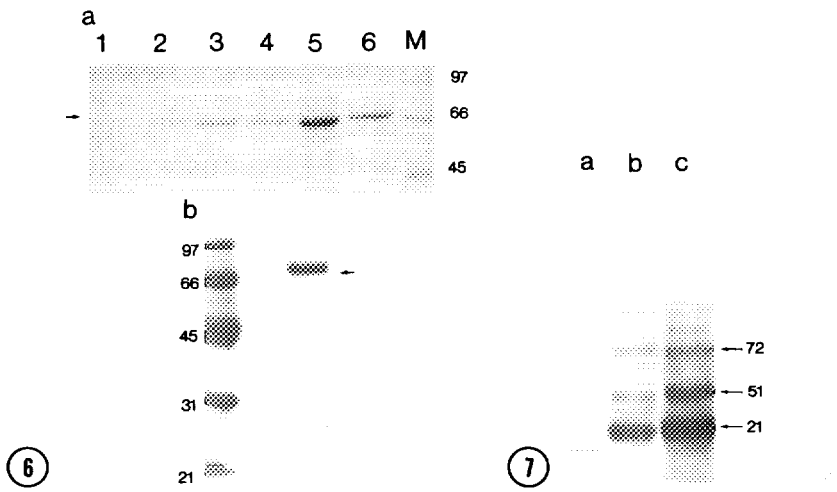

Figure 6. Purification of recombinant rat ACOX from Sf9 cells. Sf9 cells, 3 days after infection with recombinant baculovirus, were homogenized and used for ACOX purification as described under Materials. **(a)** Represents SDS/PAGE profile of 6 fractions (*lanes* 1-6) collected between 80-130 mM when the proteins were eluted from BIO-Gel HTP column with a 10 mM to 150 mM KPO_4 gradient. **(b)** Fractions 5-6 (*lanes* 5 and 6 in **(a)** above) when pooled, concentrated and subjected to SDS/PAGE yielded a single 72 kDa protein band *(arrow)* corresponding to component A of ACOX.

Figure 7. Characterization of polyclonal antibodies raised against recombinant rat ACOX. Polyclonal antibodies were raised in rabbits against recombinant ACOX (component A) purified from Sf9 cells as described in Figure 6 and used for immunoblotting. *Lane a*, molecular-mass standards; *lane b*, rat liver extract; and lane c, ciprofibrate treated rat liver extract. Approximately 20 μg protein in *lanes b and c*.

and BIO-GEL HTP columns as described under "Materials and Methods". The specific activity of purified protein was 2.1 units /mg protein. On SDS/PAGE (Figure 6) only one protein band corresponding to the 72 kDa component A of ACOX was visualized. This procedure failed to yield lower molecular mass components B and C of ACOX, whereas ACOX purified from rat liver displayed all three components (9). It is possible that insect cells exhibit diminished proteolytic cleavage of component A of ACOX when compared to that occurring in rat liver or that the purification procedure employed in the present study was not optimal to enrich these lower molecular weight components. It should be noted that approximately 10 mg of homogeneous component A of ACOX was obtained from 1 liter of Sf9 cell culture. Monospecific polyclonal antibodies raised against the pruified component A of recombinant ACOX recognized all three

Figure 5. Immunocytochemical localization of ACOX by protein A-gold labeling procedure. **(A)** Sf9 cells infected with recombinant baculovirus. Recombinant ACOX is expressed abundantly, as indicated by the density of gold particles, and it is distributed diffusely in the nucleus, N; and cytoplasm, C. **(B)** Control Sf9 cells show no immunolabelling. **(C)** Represents a portion of hepatic parenchymal cell from a rat treated with ciprofibrate, a peroxisome proliferator, which shows the presence of ACOX in peroxisomes, P. Magnification **(A)** x **13,300** ; **(B)** x **8,600** ; and **(C)** x **43,000** .

components of ACOX in normal and ciprofibrate treated rat liver on immunoblotting (Figure 7). Using antibodies against recombinant ACOX we have been able to visualize peroxisomal matrix labeling by protein-A gold immunocytochemistry (data not presented).

The present study demonstrates the utility of baculovirus expression system for highly efficient production of functionally active recombinant rat ACOX. The relative ease with which milligram quantities of wild-type form of ACOX can be generated will facilitate future investigations on this important peroxisomal enzyme, including the determination of crystal structure and characterization of mutant forms of this protein.

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