Leukocyte 12-Lipoxygenase: Expression, Purification, and Investigation of the Role of Methionine Residues in Turnover-Dependent Inactivation and 5,8,11,14-Eicosatetraynoic Acid Inhibition†

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ABSTRACT: Porcine leukocyte 12-lipoxygenase cDNA was cloned into the expression vectors pSE280, pSE380, and pSE420. pSE380 yielded the highest level of 12-lipoxygenase activity when these vectors were tested for expression in *Escherichia coli* Top10 cells. Optimal expression of the protein from this vector occurred in cells cultured at 30 °C and harvested 18 h following induction of expression by 0.5 mM isopropyl thiogalactoside (IPTG). The enzyme was purified from the 100000*g* supernatant to 98% homogeneity by a combination of ammonium sulfate precipitation, anion exchange chromatography, and chromatofocusing. Addition of dithiothreitol and catalase to buffers at various steps in the purification protocol enabled the isolation of enzyme having a specific activity of 12 μmol min^{-1} mg^{-1}. The recovery of purified protein from this expression system was 56%, resulting in a 109-fold purification. On the basis of amino acid sequence comparisons between mammalian 15- and 12-lipoxygenases, three methionine residues in the porcine leukocyte 12-lipoxygenase (M338L, M367V, and M562L) were targeted for mutation to assess their potential role in turnover-dependent inactivation and inhibition by 5,8,11,14-eicosatetraynoic acid (ETYA). The mutants were expressed and purified by the same procedure used for the wild-type enzyme. These amino acid changes did not significantly alter enzyme catalysis as judged by the kinetic constants K_m and k_{cat}/K_m, nor did they affect the rate of turnover-dependent inactivation or inhibition by ETYA. The results indicate that these methionine residues do not play a pivotal role in catalysis, autoinactivation, or sensitivity to inhibition by acetylenic compounds.

Lipoxygenases catalyze the regiospecific and stereospecific dioxygenation of 1,4-*cis*,*cis*-pentadiene-containing fatty acids to hydroperoxy fatty acid derivatives (Theorell et al., 1946; Gaffney, 1996; Hamberg & Samuelsson, 1967). These hydroperoxy fatty acids are precursors to a variety of mediators of physiological and pathophysiological events (Samuelsson et al., 1987; Furstenberger, 1990; Holtzman, 1992; Piomelli & Greengard, 1990; Chen et al., 1992; Serhan et al., 1996). Therefore, the biochemistry, expression, and regulation of these enzymes are of considerable interest. Most mammalian lipoxygenases are the product of a single gene. The exception is 12-lipoxygenase, which catalyzes the conversion of arachidonic acid to 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE).[1] 12-HPETE is the precursor to the hydroxy fatty acid, 12-HETE (Hamberg & Samuelsson, 1974; Nugteren, 1975), and to a series of epoxy fatty acids termed hepoxylins (Scheme 1) (Pace-Asciak, 1984). Two forms of 12-lipoxygenase that are encoded by separate genes and regulated in a cell-specific fashion exist (Takahashi et al., 1988; Funk et al., 1992; Yoshimoto et al., 1992). The leukocyte-type 12-lipoxygenase has been linked to several pathophysiological conditions including epithelial inflammation (DeRubertis et al., 1984; Shornick & Holtzman, 1993), hypertension (Yue et al., 1992), and diabetes-related tissue injury (Ma et al., 1996). The platelet-type 12-lipoxygenase activity has been associated with tumor metastasis (Tang et al., 1996; Honn et al., 1994; Chen et al., 1994) and mouse skin tumorigenesis (Krieg et al., 1995).

Leukocyte- and platelet-type 12-lipoxygenases exhibit differential substrate specificities, sensitivities to turnover-dependent inactivation, and immunochemical reactivities (Takahashi et al., 1988). Investigations of the molecular basis for these differences have been hindered by the difficulty of isolation of large amounts of purified enzymes in a stable form. The leukocyte 12-lipoxygenase has been expressed in both *Escherichia coli* and insect cell expression systems (Suzuki et al., 1994; Reddy et al., 1994). The enzyme expressed in *E. coli* has only been purified by immunoaffinity chromatography, which requires exposure to high pH for elution from the column (Suzuki et al., 1994). This lowers the specific activity and iron content of the purified enzyme. 12-Lipoxygenase expressed in insect cells was engineered to contain a histidine tag and was purified by nickel−nitrilotriacetate affinity chromatography (Reddy et al., 1994). However, the histidine-tagged protein was not inhibited by a series of arylpropanoic acids that are specific for the leukocyte-type 12-lipoxygenase (Gorin et al., 1996).[2]

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[1] Abbreviations: 12-HPETE, 12(*S*)-hydroperoxyeicosatetraenoic acid; Amp, ampicillin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; 15-HPETE, 15(*S*)-hydroperoxyeicosatetraenoic acid; 13-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; IPTG, isopropyl thiogalactoside; PBS, phosphate-buffered saline; ODYA, octadeca-9,12-diynoic acid; 11-oxo-ODYA, 11-oxooctadeca-9,12-diynoic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

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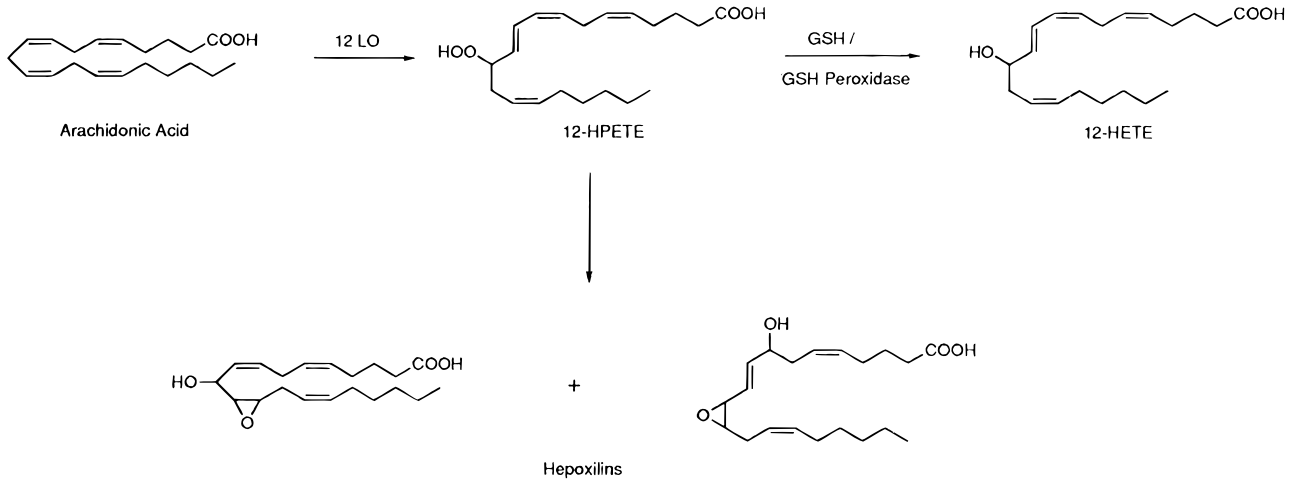
Scheme 1



These findings prompted us to develop a system for high-level expression and purification of native enzyme.

Previous studies of the reticulocyte 15-lipoxygenase established that the linoleic acid oxidation product 13-hydroperoxyoctadecadienoic acid (13-HPOD) inactivates the enzyme (Hartel et al., 1982), and inactivation of the enzyme correlates with oxidation of a single methionine residue on the protein (Kuhn et al., 1984). Inactivation of soybean lipoxygenase by the substrate analog 5,8,11,14-eicosatetraynoic acid (ETYA) also was found to proceed with formation of 1 mol of methionine sulfoxide per mole of enzyme (Kuhn et al., 1984). This suggests the potential for a common mechanism of catalytic autoinactivation and ETYA inhibition. The methionine sulfoxide formed by treatment of human or rabbit reticulocyte 15-lipoxygenase with 13-HPOD was recently identified, but sulfoxide formation was shown to be unrelated to hydroperoxide-dependent inactivation of either enzyme (Gan et al., 1995).

12-Lipoxygenases also undergo autoinactivation and inhibition by ETYA, but the two isoforms differ in the rate of autoinactivation; the leukocyte-type enzyme autoinactivates much faster than the platelet-type enzyme (Takahashi et al., 1988). The similarity in sensitivity to autoinactivation of porcine leukocyte 12-lipoxygenase and rabbit reticulocyte 15-lipoxygenase compared to that of platelet 12-lipoxygenase prompted us to hypothesize that differences in methionine residues account for the differences in sensitivity to oxidative inactivation between the 12-lipoxygenases. To test this hypothesis, methionine residues that are conserved in 15-lipoxygenases and porcine leukocyte 12-lipoxygenase but absent in platelet 12-lipoxygenase were mutated to the residues found in the platelet-type enzyme. Wild-type and mutant enzymes were expressed in *E. coli* and purified to electrophoretic homogeneity. Direct comparisons were then made with the wild-type and mutant enzymes with regard to reaction kinetics, sensitivity to autoinactivation, and inhibition by ETYA. Although small differences were observed in the catalytic efficiencies of the mutant enzymes, no significant differences were observed in their rates of autoinactivation or sensitivities to inhibition by ETYA.

EXPERIMENTAL PROCEDURES

Expression vectors pSE280, pSE380, and pSE420 and Top10 *E. coli* cells were purchased from Invitrogen. pUC19 containing the porcine leukocyte 12-lipoxygenase cDNA (pUC19/PL12LO) and polyclonal antibody to the native porcine leukocyte 12-lipoxygenase were generous gifts from S. Yamamoto (Tokushima University, Japan). Restriction enzymes were from New England Biolabs and Boehringer Mannheim. DNAse I was also from Boehringer Mannheim. IPTG was obtained from Gold Biotechnology. The bead beater and 0.1 mm glass beads were purchased from Biospec Products. EconoPac 10DG columns were from BioRad. HiTrapQ and MonoP HR5/20 columns and Polybuffer 74 were from Pharmacia. The BCA protein assay kit and Tween-20 were from Pierce. ECL chemiluminesence and *in vitro* oligonucleotide-directed mutagenesis kits were procured from Amersham. Arachidonic acid and linoleic acid were from Cayman Chemical. 1-[^{14}C]Arachidonic acid was obtained from New England Nuclear. Ammonium sulfate, ampicillin, Bis-Tris, Bis-Tris propane, β-mercaptoethanol, chicken egg white type II-O trypsin inhibitor, catalase, diethyldithiocarbamate, dithiothreitol, eicosatetraynoic acid, EDTA, glutathione, glutathione peroxidase, phosphate-buffered saline, and Triton X-305 were from Sigma.

Expression Vector Construction. The full length cDNA for the wild-type porcine leukocyte 12-lipoxygenase was inserted into the pSE series of expression vectors pSE280, pSE380, and pSE420. An identical strategy was used for the construction of each full length expression vector as shown for pSE380 in Figure 1. The porcine leukocyte 12-lipoxygenase cDNA was excised from the vector pUC19/PL12LO by digestion with *Bam*HI to yield 1.0 kb N-terminal and 1.25 kb C-terminal 12-lipoxygenase cDNA fragments. The N-terminal fragment was further digested by *Bsa*I. The expression vectors (pSE280, pSE380, and pSE420) were digested with *Nco*I and *Bam*HI. The N-terminal cDNA fragment was ligated into the *Nco*I/*Bam*HI-treated expression vectors via a synthetic linker containing *Nco*I and *Bsa*I overhangs prepared by annealing the two oligos 5′-CATGGGTCTCT-3′ and 5′-CGGTAGAGACC-3′. The partially constructed expression vectors were isolated and digested with *Bam*HI. The C-terminal cDNA fragment was then inserted into the *Bam*HI site to yield the full length expression vectors. Full construction was verified by digestion of the recombinant vectors with *Bam*HI, *Bam*HI/*Nco*I, and *Xba*I. The full length expression vectors were named pSE280/PL12LO, pSE380/PL12LO, and pSE420/PL12LO.

[2] K. M. Richards, unpublished observation.

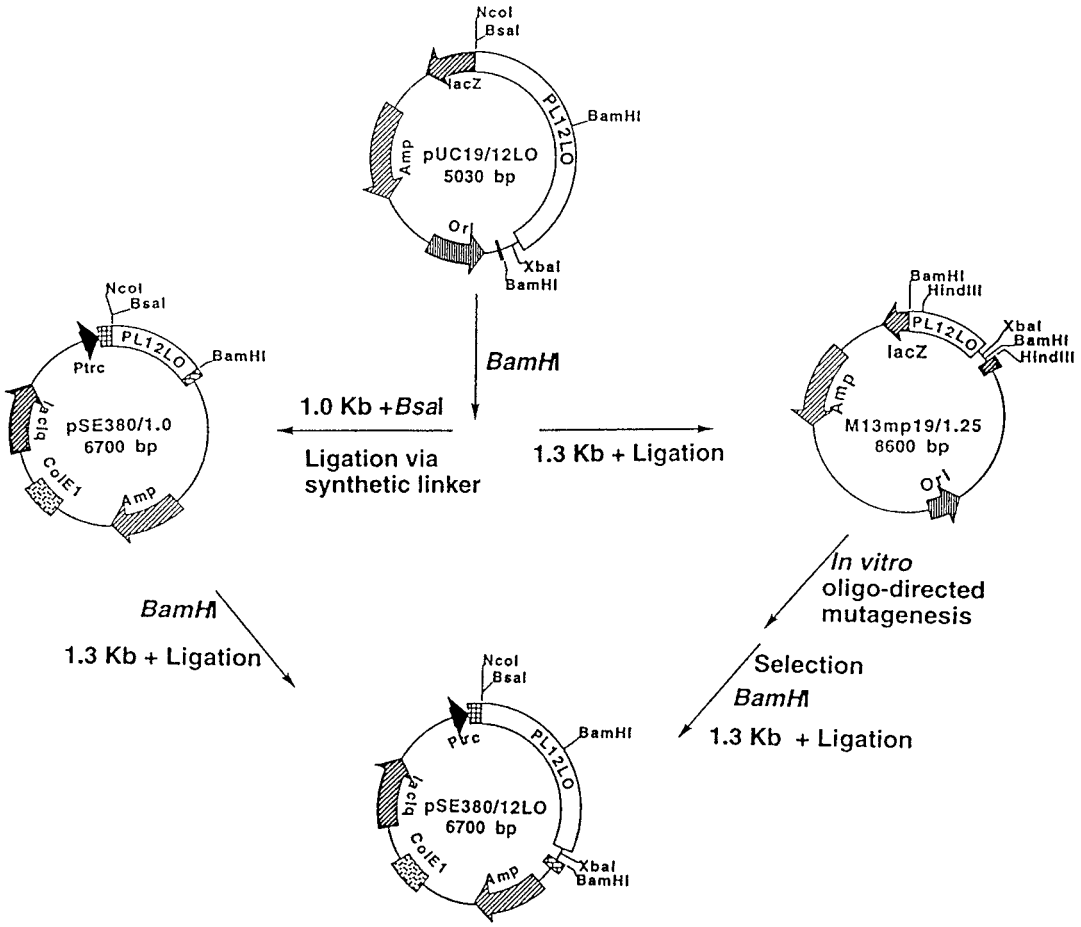


FIGURE 1: Strategy used to construct the expression vectors containing cDNAs coding for the wild-type and mutant porcine leukocyte 12-lipoxygenases. The cloning and mutagenesis procedures are described in Experimental Procedures.

Optimization of Expression. *E. coli* (Top10) cells were made competent by calcium chloride treatment (Sambrook et al., 1989) and transfected with the expression vectors (pSE280, pSE380, pSE420, pSE280/PL12LO, pSE380/PL12LO, or pSE420/PL12LO). LB/Amp (10 mL) was innoculated with a single colony picked from a plate containing *E. coli* harboring one of the parent vectors or the vectors containing the 12-lipoxygenase cDNA and grown overnight at 37 °C. The cells were then diluted 1:100 into LB/Amp or LB/Amp/0.5 mM IPTG. Cell growth was continued for 18 h at 30 °C. The cells were harvested by pelleting at 5000*g*, for 5 min at 4 °C. The cell pellet was washed with ice cold PBS, and the cells were resuspended in lysis buffer [50 mM Bis-Tris propane (pH 7.2), 500 μg/mL chicken egg white lysozyme, and 60 μg/mL soybean type II-O trypsin inhibitor]. Following a 10 min incubation on ice, the cells were pelleted and washed once with sonication buffer [50 mM Bis-Tris propane (pH 7.2) and 60 μg/mL soybean type II-O trypsin inhibitor]. Cells were pelleted, resuspended in sonication buffer, and lysed by sonication at 25% of the maximum intensity with a Virsonic cell disrupter (model 16-850) four times for 15 s with 1 min cooling between bursts. The lysate was centrifuged at 20000*g* for 30 min, and the supernant (S20) was assayed for activity in the TLC enzyme activity assay.

Enzyme Purification. The soluble protein from cells expressing the 12-lipoxygenase from pSE380/PL12LO was isolated as follows. The cells were harvested by centrifugation at 5000*g* for 15 min at 4 °C. The cell pellet was washed twice with ice cold PBS and resuspended in 50 mM Bis-Tris propane (pH 7.2), 60 μg/mL chicken egg white type II-O trypsin inhibitor, and 20 μg/mL catalase with or without additional preservatives. The cells were lysed in a bead beater (Biospec Products) with 50% of the volume containing 0.1 mm glass beads for 6 × 10 s with 60 s of cooling between bursts. The bead beating chamber was chilled with an ethanol bath during each 10 s burst. The lysate was centrifuged at 5000*g* for 15 min at 4 °C to remove the glass beads. The supernatant was then centrifuged at 100000*g* for 1 h at 4 °C. The supernatant from the 100000*g* centrifugation (S100) was retained.

The S100 was treated with DNAse I (10 μg/mL) for 15 min at room temperature. The protein was then concentrated by ammonium sulfate precipitation at 4 °C in the following manner. The S100 was first equilibrated to 20% ammonium sulfate saturation and centrifuged at 12000*g* for 15 min at 4 °C, and then the supernatant was brought to 60% ammonium sulfate saturation to precipitate the 12-lipoxygenase and centrifuged as above. The pellet was stored at −20 °C until it was further purified by chromatography.

All chromatography steps were done at room temperature. The ammonium sulfate pellet was thawed and resuspended in 10 mM Bis-Tris propane (pH 7.2), 1 mM DTT, 60 μg/mL chicken egg white type II-O trypsin inhibitor, and 20 μg/mL catalase with the minimum volume necessary to yield a clear solution. The sample was desalted using EconoPac 10DG desalting columns equilibrated with the same buffer. The sample was injected onto a 15 mL HiTrapQ (3 × 5 mL columns joined in series) anion exchange column equilibrated with 10 mM Bis-Tris Propane (pH 7.2) and 1 mM DTT and

eluted at a flow rate of 5 mL/min with an increasing NaCl gradient. One milliliter fractions were collected and assayed for activity by the UV assay method. The active fractions were pooled, and catalase was added to 20 μg/mL and the sample concentrated using a Centricon-30 device at 3000*g* at 4 °C for 30 min.

The concentrated sample was exchanged into 25 mM Bis-Tris (pH 6.1) buffer containing 1 mM DTT, 60 μg/mL trypsin inhibitor, and 20 μg/mL catalase by passing through an EconoPac 10DG column equilibrated with this buffer. The sample was injected onto a MonoP HR5/20 chromatofocusing column equilibrated with 25 mM Bis-Tris (pH 6.1) and eluted with Polybuffer 74 (1:9) (pH 4.8) at a flow rate of 1 mL/min. Fractions of 0.25 mL were collected and assayed by the UV assay method. The active fractions were pooled (1 mL). EDTA was added to a concentration of 100 μM, and aliquots were stored at −80 °C.

Protein Assay and Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. The protein concentration was determined by the BCA dye binding method (Pierce) using bovine serum albumin as a standard. Protein solutions containing DTT were precipitated with trichloroacetic acid prior to the protein assay according to the kit instructions. The protein (10 μg) was electrophoresed through a 10% minigel. Gels were stained with Coomassie blue or transferred to nylon membranes for immunoblotting. ECL chemiluminescent immunodetection was performed according to the manufacturer' protocol (Amersham).

TLC Assay for Enzyme Activity. An aliquot of enzyme was diluted in reaction buffer (50 mM Tris-Cl at pH 7.4) and incubated at 37 °C for 3 min. 1-[^{14}C]Arachidonic acid was added to 10 μM, and the incubation was continued for an additional 5 min at 37 °C. The reaction was terminated by the addition of 1.5 volumes of ice cold stop solution (diethyl ether/methanol/1 M citric acid, 30:4:1). Following extraction, the organic layer was spotted on a thin layer silica gel plate (Si250-PA, JT Baker) and developed in a solvent system of ethyl acetate/methylene chloride/acetic acid (70:30:1) for 1 h. The radioactive metabolites were detected using a BIOSCAN System 200 Imaging Scanner.

UV Assay for Enzyme Activity. The UV assay was carried out in a Hewlett-Packard 8452A diode array spectrophotometer. The reaction was initiated by the addition of enzyme to a cuvette at 30 °C containing reaction buffer [50 mM Tris-Cl and 0.03% Tween-20 (pH 7.4)] and arachidonic acid (or linoleic acid) and mixing with a glass rod. In some assays, inhibitor was added to the reaction mixture prior to enzyme addition. The final reaction volume was 1 mL. The reaction progress was monitored by measuring the absorbance at 236 nm of the conjugated diene reaction products 12-HPETE or 13-hydroperoxyoctadecadienoic acid (13-HPOD) (ϵ = 23 000) (Graff et al., 1990).

Oligonucleotide-Directed Mutagenesis of the Porcine Leukocyte 12-Lipoxygenase cDNA. The C-terminal *Bam*HI fragment of the 12-lipoxygenase cDNA was cloned into the *Bam*HI site of M13mp19 (Figure 1). Mutagenic oligonucleotides were designed to create the following amino acid changes: M338L (5′-CCAAACCA**A**CGGTGGAT-3′), M367V (5′-CTCAGCCA**C**CAAGTGTC-3′), and M561L (5′-CAGCCGCA**A**CGTGCAGG-3′). The base coding for the mutation is in boldface type. All mutagenesis steps were carried out using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit according to the manufacturer's directions. Positive mutants were identified by sequencing single-stranded (ss) DNA prepared from transformants using the dideoxy chain termination method with Sequenase (U.S. Biochemical Corp.). The entire cDNA insert of positive mutants (1.25 kb) was sequenced to ensure that no additional mutations had been introduced during the mutagenesis procedure. The mutated cDNAs were then subcloned into pSE380 for expression and purification as outlined above.

RESULTS

Subcloning and Expression. The pSE vector series (pSE280, pSE380, and pSE420) was chosen for expression of the leukocyte-type 12-lipoxygenase in *E. coli*. These vectors contain similar superlinker regions for cloning, an ampicillin resistance gene for selection, a ColE1 origin of replication for high copy number, and a strong translation termination signal. These features simplified the cloning procedure and allowed for side-by-side selection of recombinants and comparison of expression. The vectors differ in the stringency of regulation of the P_{trc} promoter. pSE380 and pSE420 contain the *lacIq* repressor gene providing for direct regulation of expression by IPTG. pSE420 possesses additional features that can be useful in ensuring very tight regulation of expression. These include the *lacO* operon coupled with a strong signal for full read-through of the recombinant gene by an antitermination signal and an *E. coli*-specific minicistron positioned upstream of the superlinker region.

The full length cDNA encoding the porcine leukocyte 12-lipoxygenase was cloned into the expression vectors according to the strategy shown for pSE380 in Figure 1. This required two basic cloning steps. First, the N-terminal *Nco*I/*Bam*HI porcine leukocyte 12-lipoxygenase cDNA fragment was cloned into *Nco*I/*Bam*HI digested vectors via a synthetic linker. The C-terminal *Bam*HI porcine leukocyte 12-lipoxygenase cDNA fragment was ligated into the *Bam*HI site of the partially constructed vectors in the second step. The expression vector pSE380/PL12LO yielded the best level of recombinant protein expression and enzyme activity as judged by immunoblotting and TLC activity assay. As expected, no activity was detectable in cells that were not induced or cells transfected with the parent vectors (pSE280, pSE380, and pSE420). pSE280/PL12LO gave about one-half of the level of expression seen with pSE380/PL12LO, but pSE420/PL12LO did not express the protein at all. We chose to optimize the cell culture and lysis conditions for pSE380/PL12LO.

Several points in the culture and lysis protocol were optimized for the highest level of enzyme expression and activity. Expression levels of recombinant protein at 18, 25, 30, and 37 °C and for 2−52 h of cell growth were compared. Optimal expression of lipoxygenase activity occurred at 30 °C, with cell harvest 18 h after IPTG induction. Supplementing the growth medium with a source of iron by the addition of ferrous sulfate, ferrous ammonium sulfate, or ferric nitrate (0−50 μM) did not increase the yield of enzyme activity from this expression system.

The yield of 12-lipoxygenase activity, as well as total protein, was higher when the *E. coli* cells were lysed by bead beating than when they were lysed by sonication. In general, purging the lysis buffer with argon did not increase the yield

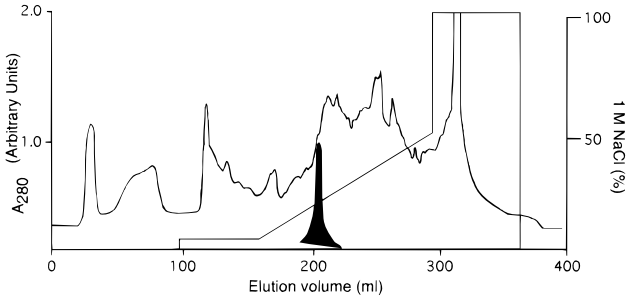


FIGURE 2: Chromatographic profile of the elution of recombinant 12-lipoxygenase from a HiTrapQ anion exchange chromatography column. The protein elution profile is shown as a solid line, and the 12-lipoxygenase activity peak is in black.

of enzyme activity over air-equilibrated buffers. Also, several stabilizing agents (diethyldithiocarbamate, DTT, EDTA, and β-mercaptoethanol) were tested for their ability to increase the enzyme activity recovered in the lysate. In every case, inclusion of the stabilizing reagent increased the yield of enzyme activity. However, DTT in air-equilibrated buffer resulted in the largest gain in activity, about double that with no additive.

Enzyme Purification. The initial step in purification of the 12-lipoxygenase from the S100 fraction was ammonium sulfate precipitation. Treatment of the S100 with DNAse I reduced the viscosity of the S100 solution and facilitated consistent fractionation by ammonium sulfate. Extremely hydrophobic proteins were removed from the S100 with precipitation at 20% ammonium sulfate saturation. The precipitate was removed by centrifugation, and the supernatant was brought to 30, 40, 50, 60, or 70% ammonium sulfate saturation. All of the 12-lipoxygenase activity was precipitated at 60% ammonium sulfate saturation, and no additional activity was recovered at the higher ammonium sulfate concentrations.

The 60% ammonium sulfate pellet was resuspended and desalted in buffer that contained DTT, catalase, and trypsin inhibitor for retention of activity. The desalted sample was bound to a HiTrapQ anion exchange column at pH 7.2, and the proteins were eluted with an increasing salt gradient as shown in Figure 2. The 12-lipoxygenase activity eluted from this column at 300 μM NaCl. To protect against the loss of enzyme activity, we included 1 mM DTT in the chromatography buffers and added catalase to the active fractions after they eluted from the column. These agents prevented the loss of enzyme activity during the subsequent concentration through a Centricon-30 device and resulted in total recovery of enzyme activity through this step.

Several additives were tested for their ability to protect against the loss of enzyme activity at 22 °C using enzyme that was partially purified by anion exchange. The following reagents were tested: glutathione (1 mM), glutathione with glutathione peroxidase (0.15 μg/mL), catalase (20 μg/mL), β-mercaptoethanol (2 mM), DTT (1 mM), diethyldithiocarbamate (50 μM), EDTA (100 μM), and Triton X-305 (0.3%). After 3 h, the enzyme retained full activity in the presence of glutathione with glutathione peroxidase, catalase, DTT, or EDTA, whereas enzyme containing the other additives had a dramatic loss of activity (42−82%). At longer times, 16 and 22 h, glutathione with glutathione peroxidase, catalase, and EDTA continued to provide full protection of activity. Therefore, we included catalase and DTT from the cell lysis through the Centricon-30 concentration. EDTA was not used because it interfered with the chromatography steps of the purification.

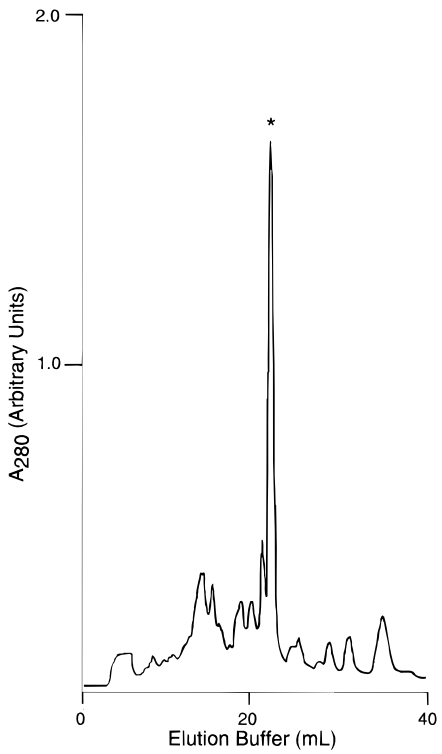


FIGURE 3: Chromatographic profile of the elution of recombinant 12-lipoxygenase from a MonoP HR5/20 chromatofocusing column. The protein elution profile is shown as a solid line. The peak containing the 12-lipoxygenase activity is indicated with an asterisk.

The concentrated sample was prepared for chromatofocusing by passage through an EconPac 10DG column equilibrated with MonoP loading buffer containing DTT, catalase, and trypsin inhibitor. The sample was loaded onto the MonoP HR5/20 chromatofocusing column at pH 6.1 and eluted with a linear pH gradient to pH 4.8. The elution profile is shown in Figure 3. The 12-lipoxygenase activity eluted in a sharp peak centered at about 23 mL. It was crucial to omit DTT from the chromatofocusing buffers and to add EDTA to the enzyme immediately following chromatofocusing, prior to storage at −80 °C, for optimal recovery of activity. A slight loss of activity (15−25%) resulted upon freezing and thawing, but the enzyme was otherwise stable at −80 °C for at least 4 months.

Figure 4 demonstrates the efficiency of this purification procedure. It shows the Coomassie blue-stained SDS−PAGE of samples taken at each step in the purification. There is not a distinct protein band representing the 12-lipoxygenase in the S100, but following the three purification steps, the enzyme was purified to greater than 98% homogeneity. The procedure resulted in a 109-fold purification of the enzyme with a specific activity of 12 μmol min^{-1} mg^{-1} (Table 1). The overall yield for the isolation procedure was 56%. Note that most of the activity was lost in the final chromatographic step. However, without the aforementioned precautions for enzyme stabilization, less than 10% of the overall activity was recovered.

Comparison of Native and Mutant Proteins. The purified 12-lipoxygenase retained all of the hallmark characteristics of the native leukocyte protein (Yokoyama et al., 1986). The enzyme exhibited a lag phase prior to maximal enzyme

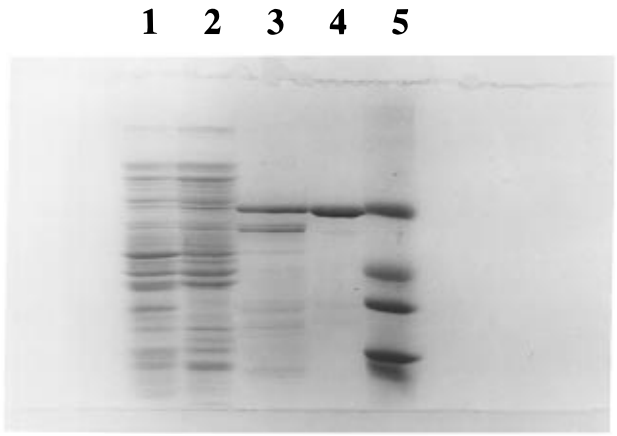


FIGURE 4: SDS−PAGE of samples taken at each step in the purification procedure: lane 1, 100000*g* fraction of Top10 cell lysate; lane 2, 60% ammonium sulfate pellet; lane 3, HiTrapQ column eluate; lane 4, MonoP column eluate; and lane 5, molecular weight standards. Staining was with Coomassie blue.

turnover and underwent self-catalyzed inactivation, with the reaction ceasing in less than 2 min. The recombinant protein utilized linoleic acid as substrate with 80% of the activity measured with arachidonic acid.

On the basis of their higher amino acid sequence homology and similarity in rates of inactivation during catalytic turnover, we compared mammalian leukocyte-type 12-lipoxygenases and 15-lipoxygenases with platelet-type 12-lipoxygenases. An alignment of the amino acid sequences for the mammalian 12- and 15-lipoxygenases was obtained using the Intelligenetics Genealign software. The computer alignment of the relevant amino acid sequences is shown in Figure 5. Methionines that are conserved between the mammalian leukocyte-type 12- and 15-lipoxygenases but not conserved with the platelet-type 12-lipoxygenase were identified and targeted for mutagenesis. Amino acids 338 and 367 in the porcine leukocyte 12-lipoxygenase sequence were the only methionines that fit this criterion. (Note that the amino acid sequences for the bovine trachea and mouse leukocyte 12-lipoxygenases had not been reported at the time the alignment was first performed; they code for a valine at the position corresponding to methionine 338 in the porcine leukocyte 12-lipoxygenase.) The porcine leukocyte 12-lipoxygenase cDNA was mutated to code for the corresponding amino acid in the platelet-type 12-lipoxygenase. This resulted in amino acid changes M338L and M367V. An additional mutation coding for the change M562L was made to examine the potential role of this highly conserved methionine which has not been previously examined by mutagenesis. These mutations were made to examine their possible role in turnover-dependent inactivation and inhibition by ETYA.

The mutant forms of the enzyme were expressed and purified according to the same procedure that was used for the wild-type enzyme. Figure 6 shows the Coomassie blue-stained SDS−PAGE of the purified wild-type and mutant enzymes. Each of the mutant enzymes exhibited activities close to those of wild-type enzyme, and each was recognized by a polyclonal antibody raised against the native protein. Kinetic parameters for the wild-type and mutant enzymes were determined from Lineweaver−Burk plots of data obtained in the UV assay. Table 2 lists the K_m, k_{cat}, and k_{cat}/K_m values for each enzyme. Overall, these mutations did not have a gross effect on the 12-lipoxygenase activity, although there were small changes in the kinetic constants. The K_m and k_{cat} were comparable between the wild-type and M562L enzymes. M336L had a K_m that was about $^1/_2$ that of the wild type, but its k_{cat} was unchanged. As a result, the overall enzyme efficiency was approximately doubled relative to that of the other enzymes. M367V had K_m and k_{cat} values that were approximately double those of the wild-type enzyme. The overall enzyme efficiency, reported as k_{cat}/K_m, was approximately the same as that of wild type.

The time courses for inactivation of the wild-type and mutant enzymes in the UV assay of each enzyme are shown in Figure 7. The percentage of enzyme activity remaining was obtained from the ratio of the enzyme's reaction velocity to the maximal velocity attained in the enzyme reaction. The enzyme velocity was calculated at 20 s intervals during the course of the reaction. The rate of enzyme inactivation was obtained from the slope of the curves in Figure 6. Comparison of the rates of inactivation for wild-type and mutant enzymes indicates that the mutations did not significantly affect the rate of inactivation.

The mutants were tested for the potential role of the methionine residues in inhibition by ETYA. The inhibitor concentration resulting in 50% inhibition (IC_{50}) of enzyme activity in the UV assay was determined. The IC_{50} values for inhibition by ETYA of wild-type and mutant enzymes ranged from 0.4 to 0.6 μM. We conclude that these methionine mutations in the porcine leukocyte 12-lipoxygenase had no effect on ETYA inhibition.

DISCUSSION

The present study focused on the role of three methionines in autoinactivation and inhibition of leukocyte-type 12-lipoxygenase by acetylenic fatty acids. All three methionines are conserved between porcine leukocyte 12-lipoxygenase and human and rabbit 15-lipoxygenases, but two of them (M338 and M367) are not conserved in the human platelet 12-lipoxygenase. The leukocyte-type 12-lipoxygenase is similar to the 15-lipoxygenases in its sensitivity to autoinactivation but quite different from the platelet-type 12-lipoxygenase which is more resistant. Methionines that are conserved between the leukocyte and reticulocyte enyzmes were changed to the corresponding residues in the platelet enzyme (M338L and M367V). One other methionine residue that is conserved in all lipoxygenases reported to date was also mutated to leucine.

Wild-type and mutant leukocyte-type 12-lipoxygenases were expressed in *E. coli* and purified to approximately 98% homogeneity. The mutant enzymes exhibited small changes in specific activities and kinetic constants (K_m and k_{cat}/K_m) but overall were catalytically comparable to the wild-type enzyme. If oxidation of the enzyme at one of these methionine residues was responsible for turnover-dependent inactivation, one might have expected the corresponding mutant to be resistant to inactivation and to exhibit a time course of reaction similar to that of the platelet-type 12-lipoxygenase. Likewise, if a methionine mutation had resulted in an enzyme resistant to inhibition by ETYA, one could infer that methionine oxidation was involved in some way in the inhibition. However, autoinactivation and ETYA inhibition were unaffected by the three different mutations.

These results are consistent with the findings of a recent study of the role of methionine oxidation in the inactivation

Table 1: Purification of Leukocyte-Type 12-Lipoxygenase Expressed in *E. coli*

purification step	protein (mg)	total activity (μmol min^{-1} mg^{-1})	specific activity (μmol min^{-1} mg^{-1})	recovery (%)	purification (*x*-fold)
S100	408[a]				
ammonium sulfate precipitation	133	14.8	0.11	100	1
HiTrapQ	7	15.2	3.0	103	27
MonoP	0.7	8.3	12	56	109

[a] Activity measurement of the S100 fraction was not possible in the UV assay.

```
 mouseplat12  331 FLPSDPPLAWLLAKiWVRNSDFQLqElQfHLLNTHLVAEV
 humanplat12  330 FLPSDPPLAWLLAKsWVRNSDFQLHEiQyHLLNTHLVAEV
rabitretic15  331 FLPTDPPMvWLLAKCWVRSSDFQvHELnSHLLRGHLMAEV
humanretic15  330 FLPTDPPMaWLLAKCWVRSSDFQLHELqSHLLRGHLMAEV
porcinleuk12  331 FLPTDPPMvWLLAKCWVRSSDFQLHELHSHLLRGHLMAEV
bovintrach12  331 FLPTDPPMtWLLAKCWVRSSDFQLHELHSHLLRGHLVAEV
mouseleuko12  332 FTPlDPPMDWLLAKCWVRSSDLQLHELQAHLLRGHLVAEV
  ratbrain12  329 FTPsDPPMDWLLAKCWVRSSqLQLHELQAHLLRGHLmAEl

    consensus       FlPtDPPmaWLLAKcWVRsSdfQlhElqsHLLrgHL-AEv

 mouseplat12  549 DWYgWVPNAPCTMRMPPPTsKdDVTMeTVMGSLPDVqkAC
 humanplat12  548 DWYaWVPNAPCTMRMPPPTTKeDVTMaTVMGSLPDVrQAC
rabitretic15  549 DWftWVPNAPCTMRLPPPTTK DATLETVMATLPNlHQsS
humanretic15  548 DWYsWVPNAPCTMRLPPPTTK DATLETVMATLPNFHQAS
porcinleuk12  549 DWYtWVPNAPCTMRLPPPTTK DATLETVMATLPNFHQAS
bovintrach12  549 DWYsWVPNAPCTMRLPPPTTK DvTLEKVMATLPNFHQAS
mouseleuko12  550 DWFYWVPNAPCTMRLPPPkTK DATMEKLMATLPNPNQST
  ratbrain12  545 DWFYWVPNAPCTMRLPPPtTK eATMEKLMATLPNPNQST

    consensus       DWy-WVPNAPCTMRlPPPttK-daT-etvMatLPnfhqas
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FIGURE 5: Sequence alignment of mammalian 15- and 12-lipoxygenases. Methionine residues in the porcine leukocyte 12-lipoxygenase sequence that were selected for mutagenesis are indicated by bold letters. Methionine residues 338 and 367 were mutated to leucine and valine, the corresponding amino acids in the platelet-type 12-lipoxygenase sequence. Methionine 562, highly conserved among mammalian lipoxygenases, was mutated to leucine. The abbreviations indicate amino acid sequences for the following: mouseplat12, mouse platelet 12-lipoxygenase; humanplat12, human platelet 12-lipoxygenase; rabitretic15, rabbit reticulocyte 15-lipoxygenase; humanretic15, human reticulocyte 15-lipoxygenase; porcinleuk12, porcine leukocyte 12-lipoxygenase; bovintrach12, bovine trachea 12-lipoxygenase; mouseleuko12, mouse leukocyte 12-lipoxygenase; and ratbrain12, rat brain 12-lipoxygenase.

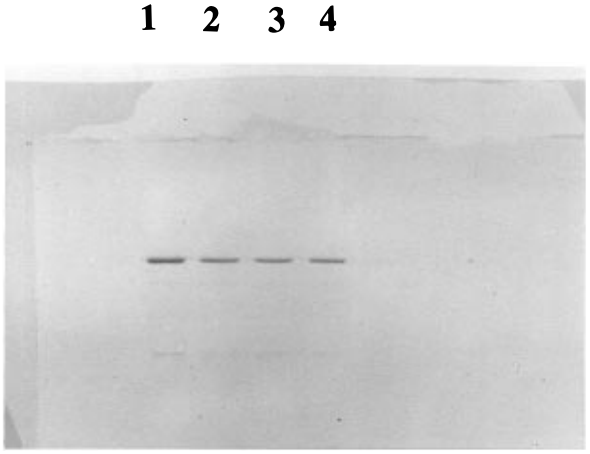

FIGURE 6: SDS−PAGE of purified wild-type and mutant porcine leukocyte 12-lipoxygenases: lane 1, wild-type; lane 2, M338L; lane 3, M367V; and lane 4; M561L. Staining was with Coomassie blue.

of reticulocyte 15-lipoxygenase by hydroperoxy fatty acids and ETYA (Gan et al., 1995). Gan et al. (1995) used HPLC−MS to identify the methionine residues oxidized by 13-HPOD treatment of human reticulocyte and rabbit reticulocyte 15-lipoxygenases (M590 and M591, respectively). Interestingly, 15-HPETE, the enzymatic product of arachidonic acid oxygenation, also inactivated the human 15-lipoxygenase but did not form methionine sulfoxide at M590

Table 2: Comparison of the Kinetic Constants for Wild-Type and Mutant Leukocyte-Type 12-Lipoxygenases[a]

enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
wild-type	9	504	56
M338L	4	504	126
M367V	18	1079	60
M561L	7	432	62

[a] Values were determined from Lineweaver−Burk plots using the enzyme's maximal velocity in the UV assay.

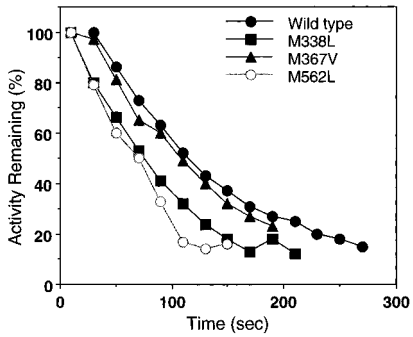

FIGURE 7: Time course of self-catalyzed inactivation of the wild-type and mutant 12-lipoxygenases.

(Gan et al., 1995). Furthermore, mutagenesis of M590 of human 15-lipoxygenase did not alter the sensitivity of the enzyme to inactivation by 13-HPOD or to inhibition by ETYA. These findings imply that oxidation of M590 of human 15-lipoxygenase or M591 of rabbit 15-lipoxygenase is not responsible for autoinactivaton or inhibition by acetylenic fatty acids (Gan et al., 1995).

Although methionine oxidation is not responsible for differences in turnover-dependent inactivation between the two 12-lipoxygenase isoforms, other oxidation events may be involved. A recent report suggests that differences in the products of arachidonic acid oxidation by the two enzymes may be important (Kishimoto et al., 1996). Leukocyte-type and platelet-type 12-lipoxygenases synthesize primarily 12-HPETE when reacted with arachidonic acid, but the leukocyte enzyme also produces about 10% 15-HPETE (Takahashi et al., 1988; Yokoyama et al., 1986). Kishimoto et al. (1996) reported that 15-HPETE formation correlates with inactivation of the leukocyte 12-lipoxygenase and leads to covalent modification of the protein. The platelet enzyme, which inactivates very slowly, does not make 15-HPETE and is not covalently modified (Kishimoto et al., 1996).

Nieuwenhuizen et al. (1995) recently reported that, during inactivation of soybean 15-lipoxygenase by octadeca-9,12-diynoic acid (ODYA), an acetylenic inhibitor similar to ETYA, significant quantities of 11-oxooctadeca-9,12-diynoic acid (11-oxo-ODYA) are formed. However, covalent modification of the soybean lipoxygenase by 11-oxo-ODYA did not inactivate the enzyme (Nieuwenhuizen et al., 1995).

Therefore, Nieuwenhuizen et al. proposed that a putative precursor to 11-oxo-ODYA, such as 11-hydroperoxyoctadeca-9,12-diynoic acid or the 11-peroxyloctadecadiynoic acid radical, is the inactivating agent (Schilstra et al., 1996). Extrapolating the findings of Kishimoto et al. and Nieuwenhuizen et al. to the inactivation of leukocyte-type 12-lipoxygenase suggests that autoinactivation or ETYA-dependent inactivation may be mediated by hydroperoxy compounds or their derivatives. Whether inactivation results from protein oxidation or covalent modification is unknown, but our results suggest that methionine residues are not the critical targets on the protein.

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