Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 381-388

EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF PORCINE LEUKOCYTE 12-LIPOXYGENASE PRODUCED IN THE METHYLOTROPHIC YEAST, *PICHIA PASTORIS*

Ramesh Gala Reddy[1], Tanihiro Yoshimoto‡,[2], Shozo Yamamoto‡, and Lawrence J. Marnett[3]

A.B. Hancock, Jr. Memorial Laboratory for Cancer Research, Center in Molecular Toxicology, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

‡Department of Biochemistry, Tokushima University School of Medicine, Kuramoto-cho, Tokushima 770, Japan

Received October 4, 1994

Summary: A cDNA coding for porcine leukocyte 12-lipoxygenase was expressed intracellularly in the methylotrophic yeast *Pichia pastoris* under the regulatory control of the alcohol oxidase promoter. The recombinant 12-lipoxygenase contained in the yeast cell lysate was soluble, displayed the catalytic properties of the native enzyme, and was recognized by antibodies prepared against native 12-lipoxygenase derived from porcine leukocytes. The catalytically active enzyme of the 100,000 x g supernatant obtained from the yeast lysate was readily purified by immunoaffinity chromatography to near homogeneity. Porcine leukocyte 12-lipoxygenase is the first arachidonic acid oxygenase to be expressed in yeast, an easy, inexpensive, and rapid method of expressing native and site-directed mutants of recombinant proteins.

Lipoxygenases are non-heme iron enzymes that oxygenate polyunsaturated fatty acids to yield hydroperoxy derivatives. Separate lipoxygenases have been described that oxygenate arachidonic acid at positions 5, 8, 12, and 15. The 5-, 12-, and 15-lipoxygenases have been purified, cloned, sequenced, and expressed. Based on enzymological, immunological and molecular biological investigations, two isoforms of 12-lipoxygenase have been characterized. One is a "leukocyte-type enzyme" and the other is a "platelet-type enzyme", the leukocyte-type enzyme has been detected in leukocytes, porcine anterior pituitary, bovine trachea, canine brain,

[1]Present address: Oxford Biomedical Research, 1858 Starbatt Drive, Rochester Hills, MI 48309.

[2]Present address: Department of Pharmacology, Kanazawa University School of Medicine, Kanazawa 920, Japan.

[3]To whom correspondence should be addressed at Department of Biochemistry, Vanderbilt University School of Medicine, Nashville,TN 37232-0146. electronic mail: marnettlj@macpost.vanderbilt.edu; FAX: 615-343-7534.

Abbreviations: 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15- and 12-LO, 15- and 12-lipoxygenase; NDGA, nordihydroguaiaretic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; TLC, thin layer chromatography; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

0006-291X/94 $5.00

and human adrenal cortex whereas the platelet-type enzyme has been detected in platelets and human epidermal carcinoma cells (1-7). The hydroperoxy and hydroxy products of 12-LO action, 12-HPETE and 12-HETE, exert a variety of pharmacological actions including stimulation of DNA synthesis in aortic smooth muscles (8), release of leutinizing hormone releasing hormone in hypothalamus (9), induction of expression of glycoprotein IIb-IIa on the cell membranes of Lewis lung carcinoma, and 12-O-tetradecanoylphorbol-13 acetate-induced tumor cell adhesion (10).

The cDNA coding for porcine leukocyte 12-LO has been expressed in *Escherichia coli* and insect cells and the properties of the recombinant enzymes have been characterized (11,12). Expression of lipoxygenases in insect cells from baculovirus vectors is reported to be superior to expression in *E. coli* from the standpoint of yield of active protein. However, experiments using baculovirus vectors are time-consuming and expensive which limits their utility for site-directed mutagenesis studies that are helpful for defining enzyme structure and function. In an attempt to provide a more convenient strategy for high level expression of arachidonic acid oxygenases, we have subcloned porcine leukocyte 12-LO cDNA into vectors for expression in the yeast *Pichia pastoris*, a strain that has been recently developed as a host for high level heterologous expression. We report here successful expression of porcine leukocyte 12-LO with properties similar to those of native enzyme in amounts comparable to those obtained in expression in insect cells from baculovirus vectors.

MATERIALS AND METHODS

Materials. Sorbitol, dextrose, L-histidine, L-methionine, L-lysine, L-leucine, L-isoleucine, L-glutamic acid, glycerol, glass beads (acid washed, 425-600 μm), NDGA, and ETYA were from Sigma (St. Louis, MO). Bacto yeast extract, peptone, and yeast nitrogen base without amino acids were from Difco (Detroit, MI). [1-^{14}C]Arachidonic acid and [1-^{14}C]linoleic acid (53 mCi/mmol) were obtained from New England Nuclear (Boston, MA) and unlabeled fatty acids were from Nu-Chek Prep (Elysian, MN). Standards of 12- and 15-HETEs were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). Precoated silica gel 60 F-254 glass plates for TLC were from Merck (Darmstadt, FRG). The *Pichia* expression kit was purchased from Invitrogen Corp. (San Diego, CA). Chemiluminescence detection of Western blots was carried out with the Enhanced Chemiluminescence Western Blotting kit (Amersham International, Arlington Heights, IL). Kits for plasmid purification were purchased from Qiagen Inc. (Chatsworth, CA). Restriction endonucleases were obtained from New England Biolabs, Inc. (Beverly, MA). All other reagents were from common commercial sources.

Construction of recombinant shuttle vector pHIL-D2. The shuttle vector pHIL-D2 (Fig. 1) was digested with *EcoRI*, sticky-ends were filled with DNA polymerase I (Klenow fragment; Stratagene, La Jolla, CA) and the plasmid was blunt-end ligated with *AvrII* linkers (New England Biolabs). The ligation mixture was used to transform the *E. coli* strain, DH5α. Plasmid DNA from one of the positive clones was then digested with *AvrII*. pUC19, carrying full-length cDNA for porcine leukocyte 12-LO was subjected to *XbaI* digestion to release a 2.16 kb fragment containing 12-LO cDNA. Following the Qiaex gel extraction of the fragment, ligation was carried out with the *AvrII*-digested pHIL-D2. Aliquots of the ligation mixture were used to transform competent *E. coli* DH5α. Minipreps of the resulting clones were analyzed by restriction analysis using *Bam HI* and *StuI*. A construct having the correct orientation was designated pHIL-D2-PL12-LO. This recombinant plasmid was linearized with *NotI* to transform the genome of *Pichia*, GS 115 (*his4*) to encode a nonfusion protein. Homologous recombination between the alcohol oxidase sequences in the wild-type *Pichia* genome and the recombinant plasmid results in the generation of recombinant *Pichia* coding for a nonfused gene product. The transformants were patched on minimal methanol and minimal dextrose plates and the recombinant *Pichia* (designated GS 115-PL12LO) was identified by visual screening for the absence of normal growth on methanol-containing plates (Fig. 2).

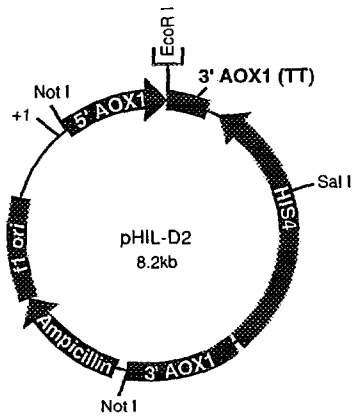

Fig. 1. **Map of *E. coli-P. pastoris* shuttle vector for expression of porcine leukocyte 12-LO in *Pichia pastoris*.** pHIL-D2 (8.2 kb) has sequences required for selection in each host. The left half of the plasmid is a portion of pBR322, the segment of which contains the ampicillin resistance gene (*Ampr*) and the origin of replication (*ori*). The DNA elements comprising the rest of the plasmid are derived from the genome of *P. pastoris* that induced a 1 kb segment of the alcohol oxidase promoter (5' AOX1), a 256 bp segment of the alcohol oxidase transcriptional terminating sequence 3' *AOX1*(TT), P. pastoris histidinol dehydrogenase gene, *His4* that complement the defective *His4* gene in *Pichia* host strains, and approximately 650 bp region of 3' *AOX1* DNA which together with the 5' *AOX1* is necessary for site-directed integration. The 2.16 kb cDNA encoding porcine leukocyte 12-LO was cloned into the *EcoRI* site of the plasmid.

Culture conditions. Cells were grown in a 3 l baffled flask in 1 l of minimal glycerol medium at 30°C to an A_{600} of 1-2. The cells were harvested, washed with minimal methanol medium, resuspended in 1 l of minimal methanol and incubated at 30°C for 3-6 days to induce expression.

Preparation of cell extract. Yeast cells were isolated by centrifugation, washed once in ice-cold breaking buffer (2 ml/g wet weight) (50 mM sodium phosphate, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 5% glycerol). An equal volume of acid-washed glass beads (size 425-600 μm) were added and the mixture was lysed using a bead beater (8 x 15 sec bursts) with 2 min cooling on ice. The sample was then centrifuged for 30 min at 12,000 x *g* using a Sorvall SS34 rotor. The clear supernatant solution was then spun at 100,000 x *g* for 90 min at 4°C and this high speed supernatant was stored at -70°C. The protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Purification of 12-LO. The 100,000 x *g* supernatant was applied to an immunoaffinity column packed with a monoclonal antibody prepared against native porcine leukocyte 12-LO and purified as described (1). Different fractions (3 μg) of purification were subjected to 12% SDS-PAGE and stained with Coomassie blue (Fig. 3).

Enzyme assay. Purified enzyme (700 ng) was incubated in 200 μl of 50 mM Tris-HCl (pH 7.4) containing 10 μM [^{14}C]arachidonic acid at 37°C for 15 min. The isolation of reaction products, and their quantitation by TLC and HPLC was as described (12).

Western analysis. Immunoaffinity-purified recombinant and native porcine leukocyte 12-LO (3 μg) was applied to a 12% SDS-PAGE gel and after electrophoresis was electrotransferred to nitrocellulose (Hybond-ECL, Amersham). Blots were immunoreacted with antiserum to the native porcine leukocyte 12-LO (Fig. 4). Signals were detected with horseradish peroxidase-linked secondary antibody and the Amersham Enhanced Chemiluminescence kit.

RESULTS

Expression of the *AOX1* gene is controlled at the level of transcription. In methanol-grown cells, approximately 5% of the polyA+ RNA transcribed is from the *AOX1* gene.

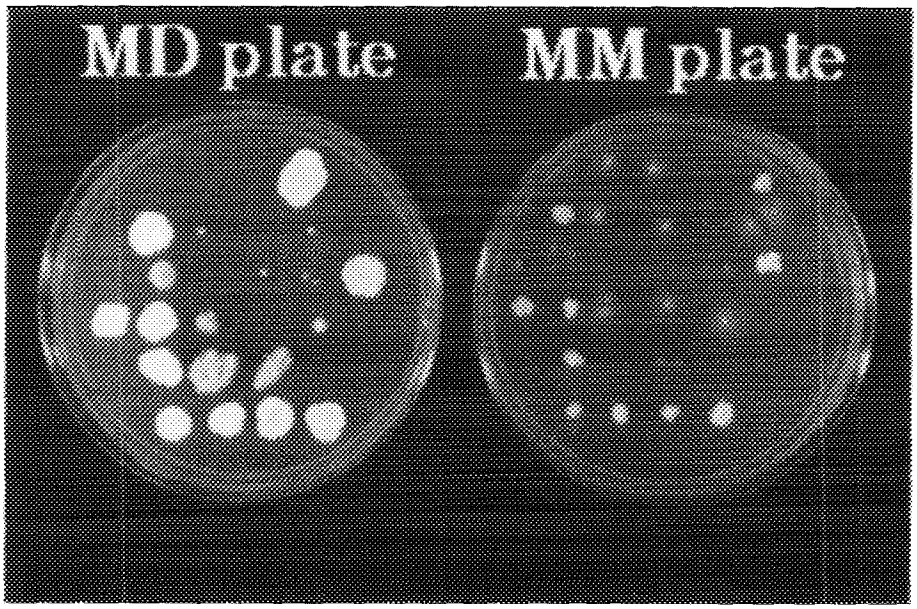

Fig. 2. **Selection of "methanol plus" or "methanol slow" or "methanol negative" colonies.** Several transformant *Pichia* colonies were patched on minimal dextrose (MD) and minimal methanol (MM) plates. The plates were kept in an incubator at 30°C for two days to allow the colonies to grow. The alcohol oxidase gene-disrupted clones, "methanol slow" (Mut^{slow}) or "methanol negative" (Mut^-) were screened from "methanol plus" (Mut^+) based on slow or no growth on the MM plate.

Regulation of the *AOX1* gene is similar to that of the *GAL1* gene of *S. cerevisiae*, depression of the *AOX1* promoter alone (i.e., absence of a repressing carbon source such as glucose) is not sufficient to generate even minute levels of expression from the *AOX1* promoter. The inducer, methanol, is necessary for expression (13).

The one-step gene replacement method described for *S. cerevisiae* (14) has been successfully used by Cregg *et al* (15) for the replacement of the *AOX1* structural gene with the hepatitis B surface antigen gene placed under the control of *AOX1* 5' and 3' regulatory sequences. These strains respond to methanol induction by producing hepatitis B surface antigen (15). We have used a similar approach for the expression of 12-LO in *P. pastoris*.

Transformation of GS115 (*his4*) with 5-10 μg of *NotI*-digested pHIL-D2 gave 134 His^+ transformants, about 5-8% of which were "methanol-slow" (i.e., impaired growth on minimal methanol medium (Fig. 2) presumably due to the replacement of the *AOX1* structural gene with the pHIL-D2-12-LO and *His4*. The reason why the *AOX1* deleted transformants grow at all on methanol medium is due to low-level expression of alcohol oxidase activity by *AOX2* (13-20). The transformants in which both the *AOX1* and *AOX2* genes were disrupted completely did not grow and were designated "methanol-negative' (Fig. 2).

Several His^+/"methanol-slow" or His^+/"methanol negative" transformants of GS115 with pHIL-D2-12-LO were inoculated in baffled shaker flasks in a temperature regulated incubator (Innova 4330, refrigerated incubator shaker, New Brunswick Scientific, Edison NJ) and allowed

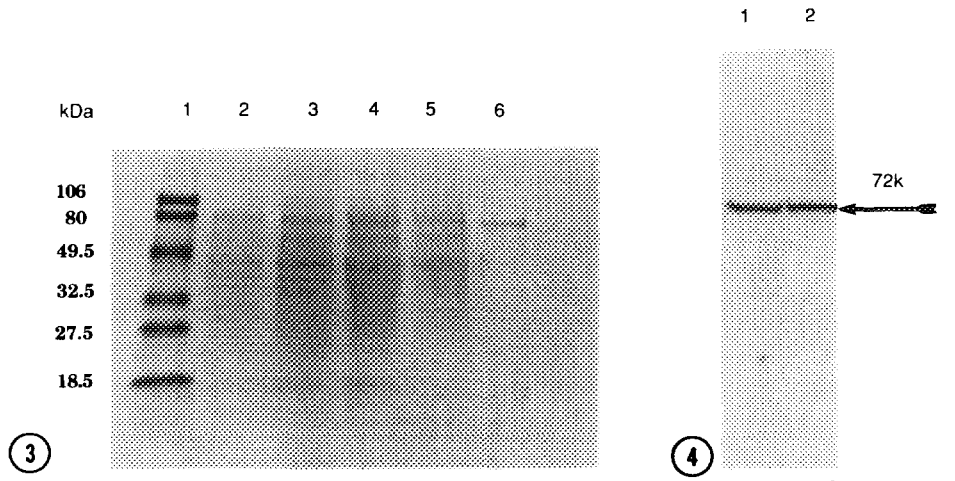

Fig. 3. **SDS-PAGE analysis of fraction from purification of recombinant 12-LO on an immunoaffinity column.** 100,000 x *g* supernatant of lysed yeast cells 72 hr after methanol induction was applied to the immunoaffinity column. Protein (3-5 μg) from each fraction was analyzed on a 12% SDS-PAGE gel. The gel was stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, lysate; lane 3, 100,000 x g supernatant; lane 4, flowthrough; lane 5, wash; lane 6, elution.

Fig. 4. **Western analysis of purified recombinant porcine leukocyte 12-LO expressed in *Pichia pastoris* upon methanol induction.** The 100,000 x *g* supernatant prepared from the broken yeast cells was applied to an immunoaffinity column. Purified protein (3 μg) was electrophoresed and immunoblotted with anti-porcine leukocyte 12-LO antisera. Lane 1 and 2, 3 μg purified native and recombinant porcine leukocyte 12-LO.

to grow to an OD of 2.0 at A_{600}. Following the transfer to minimal methanol medium, the flasks were covered with two layers of cheesecloth and allowed to incubate with shaking for an additional 6 days with daily methanol supplementation (0.5%). The cell extracts from methanol-induced cultures were assayed for 12-LO activity. Induction was evident at day 3 and was constant at day 6 (data not shown). Extracts of GS115/pHIL-D2 (without the 12-LO gene) had no detectable lipoxygenase activity.

12-LO was purified from 100,000 x *g* supernatants (3 day methanol-induced cultures) on an immunoaffinity column to near homogeneity (Fig. 3). The purified enzyme exhibited a specific activity of 1.05 μmol arachidonic acid converted/15 min/mg protein.

Purified enzyme was incubated with arachidonic acid and the sodium borohydride-reduced products were analyzed on reversed-phase HPLC. Two peaks (t_R = 17.7; and t_R = 20.1) were produced that co-eluted with standards of 15-HETE and 12-HETE (Fig. 5). The 15-HETE/12-HETE ratio was approximately 10/90. The recombinant enzyme also oxygenated linoleic acid but at a rate of 25% that of arachidonic acid. The recombinant enzyme was also inhibited by the classical lipoxygenase inhibitors, ETYA and NDGA with IC_{50}'s of 0.17 and 0.80 μM, respectively.

DISCUSSION

The porcine leukocyte 12-LO was successfully expressed as an active and non-fusion protein in *Pichia pastoris* by utilizing the *AOX1* promoter. The 12-LO apparently has no toxic

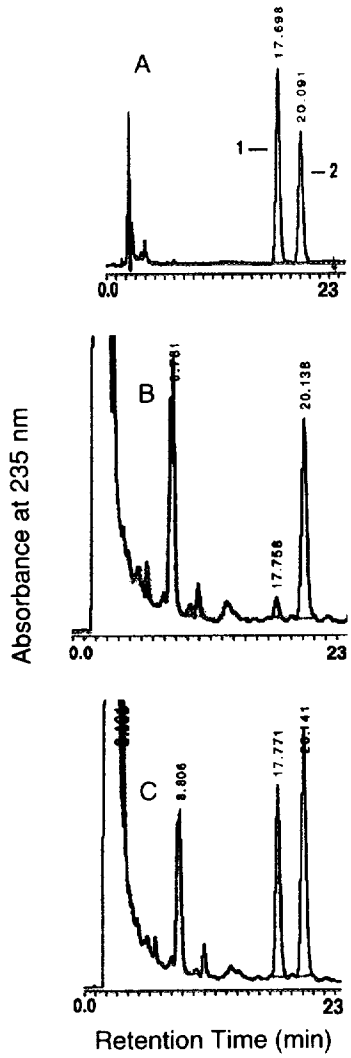

Fig. 5. **Reversed-phase HPLC of arachidonate metabolites produced by purified recombinant porcine leukocyte 12-LO.** A, a mixture of authentic 15-HETE (peak 1), and 12-HETE (peak 2); B, the purified recombinant enzyme (10 μg) was allowed to react with 25 μM arachidonic acid at 37°C for 15 min and reduced with sodium borohydride; C, the borohydride-reduced products were cochromatographed with authentic 15- and 12-HETEs.

effects on *P. pastoris* which is reflected in stable expression of 12-LO. 12-LO expressed in *P. pastoris* is in the soluble form and can be readily extracted from mechanically disrupted cells under non-denaturing conditions.

The *AOX1* disruption approach was used for introducing the 12-LO gene into *P. pastoris* genome because the previous studies have indicated that *AOX1* disrupted *P. pastoris* is a superior host for heterologous gene expression (21-23). In the *AOX1* gene disruptive transformation, only 8% of the His^+ transformants were methanol slow/negative indicating that in the majority of cases, the transforming DNA integrates without *AOX1* disruption. As such, the integration at

AOX1 is favored as the ends of the linear DNA molecule have homology to that locus. However, this preference may be lost if the transforming DNA gets circularized inside the cell or is acted by exonucleases.

The properties of recombinant 12-LO expressed in *Pichia* are very similar to that of the recombinant enzyme derived from *E. coli* (11) in contrast to the baculoviral-expressed protein (12). The oxygenation of arachidonic acid, self-catalyzed inactivation, and antigenecity are similar to that of the native immunoaffinity-purified 12-LO. However, the recombinant enzyme oxygenates linoleic acid at the rate of 25% that of arachidonic acid whereas the rate of oxygenation of linoleic acid by the native enzyme is 69% (1). The recombinant porcine enzyme was also sensitive to the classical lipoxygenase inhibitors such as ETYA and NDGA.

Based on the available literature on heterologous expression of proteins in *Pichia*, it appears that *Pichia* is a powerful tool for large scale production of proteins of interest. Other advantages include the transcriptional regulation of heterologous proteins by *AOX1* promoter, suitable methods for molecular-genetic manipulations along with technology for the growth of expression strains in large high-density fermentor cultures.

Acknowledgments: This work was supported by a research grant (CA47479) and a center grant (ES00267) from the National Institutes of Health.

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