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Cloning and sequence analysis of the cDNA for arachidonate 12-lipoxygenase of porcine leukocytes

(arachidonic acid/5-lipoxygenase/15-lipoxygenase/iron-binding domain)

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ABSTRACT **The complete amino acid sequence of arachidonate 12-lipoxygenase (EC 1.13.11.31) of porcine leukocytes was deduced by cloning and sequence analysis of DNA complementary to its mRNA. The sequence was confirmed by automated Edman degradation of the N-terminal regions of the native enzyme and its proteolytic fragments. The cDNA had an open reading frame encoding 662 amino acid residues with a calculated molecular weight of 74,911. Amino acid residues 533–545, Cys-(Xaa)$_3$-Cys-(Xaa)$_3$-His-(Xaa)$_3$-His, showed significant homology to the short cysteine- or histidine-containing sequences proposed as the metal-binding domains of transcription factors and various metal-containing proteins [Berg, J. M. (1986) *Science* 232, 485–487]. The amino acid sequence of 12-lipoxygenase exhibited 86% identity with human reticulocyte 15-lipoxygenase and showed 41% identity with human leukocyte 5-lipoxygenase. The 12-lipoxygenase cDNA recognized a 3.4-kilobase mRNA species in various porcine cell types, with the largest amount in leukocytes, followed by pituitary, lung, jejunum, and spleen.**

Biochemical studies on the arachidonate cascade have been performed by the molecular biological approaches. cDNA clones for fatty acid cyclooxygenase (1–3), arachidonate 5-lipoxygenase (4–6), and arachidonate 15-lipoxygenase (7, 8) have been isolated.

Arachidonate 12-lipoxygenase (EC 1.13.11.31) introduces a molecular oxygen into C-12 of arachidonic acid to yield (12*S*)-hydroperoxy-5,8,10,14-eicosatetraenoic acid. The enzyme was first described in platelets from humans (9) and from various animals (10). We have reported (11) that 12-lipoxygenase was present in the cytosol of porcine leukocytes. The enzyme was purified to homogeneity by immunoaffinity chromatography using a monoclonal antibody against 12-lipoxygenase (12). With the purified enzyme, it was also shown that the enzyme not only had oxygenase activities but also had 14,15-leukotriene A_4 synthase activity (12). Moreover, immunobiochemical studies revealed the occurrence of 12-lipoxygenase in a variety of porcine tissues (13).

As for the biological functions of the enzyme, a potent chemoattractant activity of 12-hydroxy-5,8,10,14-eicosatetraenoic acid was reported (14) with rat aortic smooth muscle cells. A neurotransmitter function of 12-lipoxygenase metabolites was found in *Aplysia* neuronal cells (15). 12-Hydroxy-5,8,10,14-eicosatetraenoic acid was also shown to express or activate a glycoprotein IIb/IIIa-like receptor in various tumor cells (16). However, unlike the cyclooxygenase and 5-lipoxygenase pathways, the production of potent biological mediators, such as prostaglandins, thromboxanes, and leukotrienes, has not been demonstrated in the 12-lipoxygenase pathway (17).

The amino acid sequences of cyclooxygenase and 5- and 15-lipoxygenases have been deduced from their cDNAs (1–8). However, as little was known about the primary structure of 12-lipoxygenase, we isolated and sequenced a cDNA encoding porcine leukocyte 12-lipoxygenase¶ and then deduced the complete amino acid sequence of the enzyme. The results obtained by these experiments will allow a comparative study on various lipoxygenases that may elucidate the physiological role of 12-lipoxygenase.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (185 TBq/mmol), [α-^{32}P]dCTP (111 TBq/mmol), and the multiprime DNA labeling system were purchased from Amersham. Restriction endonucleases, T4 polynucleotide kinase, the Klenow fragment of DNA polymerase I, T4 DNA ligase, *Escherichia coli* DNA ligase, and an M13 sequencing kit were obtained from Toyobo (Osaka); ribonuclease H and terminal deoxynucleotidyltransferase were from Takara Shuzo (Kyoto); reverse transcriptase of avian myeloblastosis virus was from Seikagaku Kogyo (Tokyo); guanidine thiocyanate from Fluka; *Achromobacter* proteinase I was from Wako Pure Chemical (Osaka). Nitrocellulose filters (type HA) were purchased from Millipore, and a cDNA synthesis kit was from Pharmacia.

Enzyme Purification and Amino Acid Sequence Analysis. 12-Lipoxygenase was purified from porcine leukocytes as described (12). Purified enzyme (0.25 mg) was digested with *Achromobacter* proteinase I (3 μg) in 170 μl of 0.1 M Tris·HCl (pH 8.0) containing 0.1% NaDodSO$_4$ at 37°C for 24 hr. After digestion, the peptides were purified using a Hitachi HPLC system with a reverse-phase column (SynChropak, 4 × 250 mm; Alltech) at a flow rate of 1 ml/min. Material was eluted with a 25–50% (vol/vol) acetonitrile gradient in 0.1% trifluoroacetic acid over 50 min, and the absorbance at 210 nm of the eluate was continuously monitored. Amino-terminal sequences of the native enzyme and its peptide fragments were analyzed using a gas-phase sequencer model 470A and a phenylthiohydantoin amino acid derivative analyzer model 120A (Applied Biosystems). For the determination of amino acid composition, the purified enzyme (8 μg) was hydrolyzed *in vacuo* at 110°C for 20 hr with 6 M HCl, and amino acid analysis was carried out using a Waters Pico-Tag system.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M31417).

Oligonucleotide Synthesis. For screening of oligo(dT)-primed cDNA libraries from porcine leukocytes, two oligonucleotide probes were synthesized using an Applied Biosystems DNA synthesizer model 380A. The following probes were mixtures of tetradecamers: probe A, 5'-ATYTCCATNGTRTA-3' (16-fold redundancy) complementary to nucleotides coding for the peptide sequence Tyr-Thr-Met-Glu-Ile (residues 8–12 of peptide 7), and probe B, 5'-TGRTCRAADATNCC-3' (48-fold redundancy) complementary to nucleotides coding for the peptide sequence Gly-Ile-Phe-Asp-Gln (residues 25–29 of peptide 7) [where R is A or G; Y is C or T; D is A, G, or T; and N denotes a mixture of A, G, T, and C (see Fig. 1)]. For construction of primer-extension libraries, two specific oligonucleotides were synthesized based upon the cDNA sequences: primer I, 5'-CAGCTAGGTCTGCCAGC-3' (complementary to nucleotides 546–562) and primer II, 5'-GCAGCTCATGAAGCTGG-3' (complementary to nucleotides 1059–1075) (see Figs. 2 and 3).

Porcine Leukocyte cDNA Libraries. Porcine leukocytes were collected as described (11). Total RNA was extracted by the guanidine thiocyanate method, and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (18). Double-stranded cDNA was prepared from poly(A)$^+$ RNA by the method of Gubler and Hoffman (19) utilizing a $(dT)_{15}$ primer connected to the *Not* I sequence (20). After digestion of the cDNA with *Not* I, an *Eco*RI adaptor (Pharmacia) was added by using T4 DNA ligase. The resultant cDNA mixture was inserted into pBluescript SK(+) (Stratagene) that had been digested by *Eco*RI and *Not* I.

Primer-extension libraries for the 12-lipoxygenase cDNA were constructed by the use of primers I and II and poly(A)$^+$ RNA as a template (19). The double-stranded cDNA was dC-tailed using terminal deoxynucleotidyltransferase and then annealed to the *Pst* I-digested dG-tailed pUC19 (18).

Screening of cDNA Libraries. Colonies of transformed *E. coli* were grown in 90-mm plastic dishes at 5000 colonies per dish. They were transferred onto nitrocellulose filters and then treated as described (18). Hybridization was carried out at 30°C for 15 hr in a solution containing 4× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate), 10× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), and sheared herring sperm DNA (100 μg/ml). Oligonucleotide probes A and B were labeled at the 5' end by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase (18). Filters were washed twice at room temperature for 5 min with 4× SSC/0.1% $NaDodSO_4$ and then twice at 30°C for 15 min. For screening of primer-extension libraries, either the *Pst* I–*Pst* I insert (nucleotides 781–916) of pLOX89 (see below) or the *Eco*RI–*Dra* I insert (nucleotides 142–573) of pLOX96 (see below) was excised and labeled using the multiprime DNA labeling system and [α-^{32}P]dCTP according to the manufacturer's instruction. Hybridization and the final washing with 0.1× SSC/0.1% $NaDodSO_4$ were performed at 50°C. The filters were dried and exposed to Fuji x-ray film with an intensifying screen at −70°C for 12–16 hr.

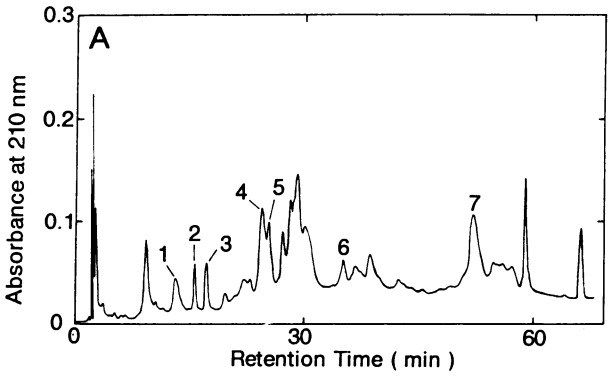
DNA Sequence. cDNA inserts were excised from plasmids with appropriate restriction endonucleases and subcloned into pUC19 (18). DNA sequencing was carried out with the dideoxynucleotide chain-termination method of Sanger *et al.* (21). Sequencing in both directions was performed with M13 primers and reversed primers (P5, Toyobo). T7 DNA polymerase (Sequenase, United States Biochemical) was also employed when there were sequence ambiguities. For comparison of two amino acid sequences, Harr plots were constructed using the GENETYX program HARPLT 2 supplied by SDC Software Development (Tokyo). Homology was estimated using the GENETYX program HOMOGAPP. Hydropathicity profile of the enzyme was analyzed using the GENETYX program HYDO.

Blot Hybridization Analysis. Total RNAs from various porcine tissues were separated by electrophoresis on 1.5% agarose and transferred to a nylon membrane (Biodyne A, Pall). The *Dra* I–*Hin*cII fragment (nucleotides 573–2310) was used as a probe. Electrophoresis, transfer of RNAs, and hybridization were performed essentially as described (18).

RESULTS AND DISCUSSION

Immunoaffinity-purified 12-lipoxygenase of porcine leukocytes was digested with *Achromobacter* proteinase I, and peptide fragments were separated by HPLC as shown in Fig. 1*A*. Amino acid sequences of the native enzyme and fragments, designated peptides 1–7, were determined by automated Edman degradation, and the results are shown in Fig. 1*B*.

To clone a cDNA of this enzyme, we constructed an oligo(dT)-primed cDNA library from poly(A)$^+$ RNA of porcine leukocytes. Approximately 2 × 10^5 transformants were screened with ^{32}P-labeled probes A and B. A clone that hybridized with both probe A and probe B was obtained and designated pLOX89. The cDNA insert of this clone was approximately 2.3 kilobases (kb) long, based on digestion with restriction endonucleases. Various fragments produced by digestion of this 2.3-kb insert with various restriction endonucleases were subcloned into pUC19 and sequenced. The cDNA insert of pLOX89 contained the nucleotide sequences for peptides 1, 3, 6, and 7. However, it did not carry the nucleotide sequence encoding the amino terminus of the 12-lipoxygenase. To obtain the 5' upstream region of the 12-lipoxygenase cDNA, we constructed primer-extension libraries by using primers I and II (Fig. 2). Screening the libraries with the cDNA insert of pLOX89 yielded eight positive clones. Among these clones, we analyzed the nucleotide sequence of the clones carrying a relatively long cDNA insert. pLOX134 had a cDNA insert [575 base pairs (bp)]



B

N-terminus: GLYRVRVSTGSSFYAGSQNQVQLXLVGQHG
Peptide 1: LALPYEYLRPSRVEN
Peptide 2: DGLILNIASTGIHDLPVDER
Peptide 3: ELQGGTLFEADFSLLDGI
Peptide 4: ETEFSVDVSEY
Peptide 5: XSLNVLMSXNSLDSFNRIF
Peptide 6: LLPMVIQLQL
Peptide 7: LLIPHFRYTMEINVRARNGLVSDLGIFDQVV
probe A probe B

FIG. 1. HPLC profile of proteolytic peptides of 12-lipoxygenase (*A*) and their amino acid sequences (*B*). Purified 12-lipoxygenase was digested with *Achromobacter* proteinase I and chromatographed on a SynChropak column. Amino acid sequences are shown in one-letter code where X denotes an unidentified residue. Underlines indicate the sequences corresponding to oligonucleotide probes A and B, used to screen cDNA libraries.

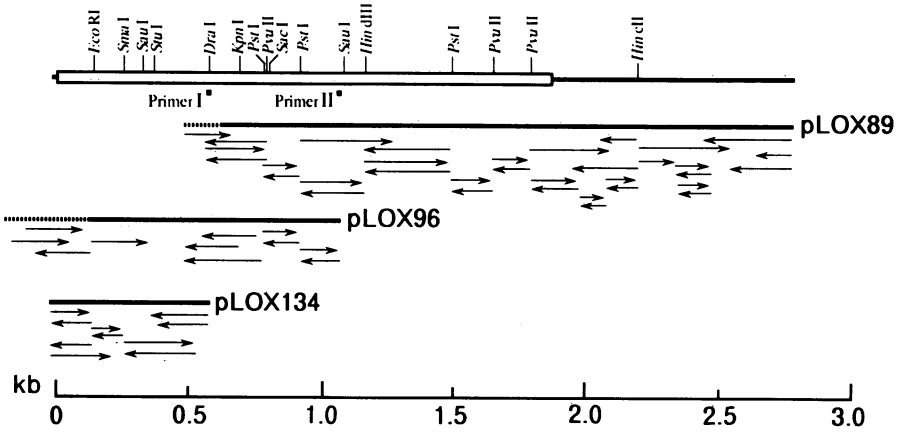


FIG. 2. Restriction map and sequence strategy of cDNA for porcine leukocyte 12-lipoxygenase. The protein coding region is indicated by an open bar, and the sequences used as primers I and II for reverse transcription are shown by solid squares. Broken lines of pLOX89 and pLOX96 indicate putative intronic sequences (see *Discussion*). The clones subjected to DNA sequencing were as follows: pLOX89 (nucleotides 618–2883 with 141-bp intronic sequence), pLOX96 (nucleotides 135–1075 with about 340-bp intronic sequence), and pLOX134 (nucleotides −13 to 562).

encoding peptides 2, 4, and 5 as well as the amino terminus of 12-lipoxygenase. On the other hand, pLOX96 carried the longest cDNA insert (1.3 kb) but did not contain the nucleotide sequence encoding the amino terminus of the enzyme. Although pLOX89 and pLOX96 had long cDNA inserts, their 5′ upstream regions (Fig. 2) did not contain the nucleotide sequence of pLOX134 that encoded the amino terminus of the enzyme. In addition, we found the consensus sequence $(Y)_nNCAGG$ for intron–exon junctions (22) in these clones. Therefore, these clones may be derived from unspliced mRNA that may contain intronic sequences. However, such nucleotide sequences in the 5′ upstream region of these clones may be due to a cloning artifact.

The nucleotide sequence of the cDNA for porcine leukocyte 12-lipoxygenase is presented in Fig. 3. Codon ATG at nucleotides 1–3 was designated the translational initiation codon, and the sequence surrounding this triplet closely matches the favored sequence for eukaryotic initiation sites (23). Termination codon TGA (nucleotides 1990–1992) is followed by a 3′ untranslated region of 892 bases, and the nucleotide sequence ATTAAA known as a polyadenylylation signal is present at nucleotides 2850–2855. The cDNA contained an open reading frame encoding 662 amino acids, devoid of the first methionine residue, with a calculated molecular weight of 74,911. The molecular weight of the purified 12-lipoxygenase was earlier estimated to be 72,000 by $NaDodSO_4$/PAGE (12). The deduced amino acid sequence included the amino terminus of the native enzyme and peptides 1–7, obtained by digestion with *Achromobacter* proteinase I. The amino acid composition deduced from cDNA was in good agreement with that actually determined with the acid-hydrolyzed enzyme (data not shown). Although it has not been established whether or not the enzyme is a glycoprotein, one glycosylation consensus sequence (Asn-Xaa-Thr/Ser) is found at amino acid residues 235–237. Hydropathicity analysis was performed by the procedure of Kyte and Doolittle (24) to determine the distribution of hydrophobic residues in the enzyme. Although central portions of the enzyme (amino acid residues 293–298 and 374–383) are considerably hydrophobic, overall the protein is hydrophilic. The hydrophilic nature of the enzyme is consistent with our previous observations that the enzyme activity was detected predominantly in the cytosolic fraction (11) and, that by using electron microscopy, the enzyme protein was localized in the cytoplasm of porcine granulocytes (13).

A sequence, $His\text{-}(Xaa)_4\text{-}His\text{-}(Xaa)_4\text{-}His\text{-}(Xaa)_{17}\text{-}His\text{-}(Xaa)_8\text{-}His$, has been proposed for the putative metal-binding domain in 15-lipoxygenases of soybean (25) and human reticulocytes (7). We found this sequence at amino acid residues 356–393 in 12-lipoxygenase. However, the alteration of His-362 and His-372 to Ser in human 5-lipoxygenase did not change the enzyme activity (26). In addition, the sequence $Cys\text{-}(Xaa)_3\text{-}Cys\text{-}(Xaa)_3\text{-}His\text{-}(Xaa)_3\text{-}His$ at amino acid residues 533–545 of 12-lipoxygenase matches the sequence $Cys\text{-}(Xaa)_{2\text{–}4}\text{-}Cys\text{-}(Xaa)_{2\text{–}15}\text{-}a\text{-}(Xaa)_{2\text{–}4}\text{-}a$ (where a is either histidine or cysteine) that is known as the zinc-finger motif, the proposed metal-binding site of various metal-binding proteins (27). The sequence is found in 15-lipoxygenases from human (7) and rabbit (8) reticulocytes but not in 5-lipoxygenases from human (4, 5) and rat (6) leukocytes. Although soybean lipoxygenase was shown to contain 1 atom of iron per molecule (28, 29), none of the mammalian lipoxygenases has been shown to contain metal, except for 12-lipoxygenase of porcine leukocytes that has been provisionally shown to contain 0.45 atom of iron per molecule (12). That such a unique amino acid sequence is an iron-binding domain in lipoxygenases is still speculative and awaits further investigation, including site-directed mutagenesis of a lipoxygenase cDNA.

Homology of two amino acid sequences was determined by the best alignment of the two sequences. Porcine 12-lipoxygenase exhibited 86% homology to human reticulocyte 15-lipoxygenase (7) and 41% homology to human leukocyte 5-lipoxygenase (4, 5). Such homology between 12- and 15-lipoxygenases was demonstrated more impressively by Harr plots (30). As shown in Fig. 4, the sequence identity is indicated by the diagonal lines, which denote the segments with five consecutive identical amino acid residues in the sequences being compared. These findings suggest that the 12-lipoxygenase is evolutionarily closer to 15-lipoxygenase than to 5-lipoxygenase, even though the comparison was made between human and porcine enzymes.

As shown in Fig. 5, the 12-lipoxygenase cDNA probe recognized a 3.4-kb mRNA species in several porcine tissues. The 12-lipoxygenase mRNA was most abundant in leukocytes followed by pituitary gland and lung. A very small amount of the 12-lipoxygenase mRNA was also detected in jejunum and spleen. This observation is consistent with the previous findings of the enzyme distribution in porcine tissues as assessed by the 12-oxygenation assay and the peroxidase-linked immunoassay of the enzyme (13, 31).

Two types of 12-lipoxygenase were found in mammalian tissues: one in platelets and the other in leukocytes. They were distinguishable by their substrate specificity (10, 12, 32)

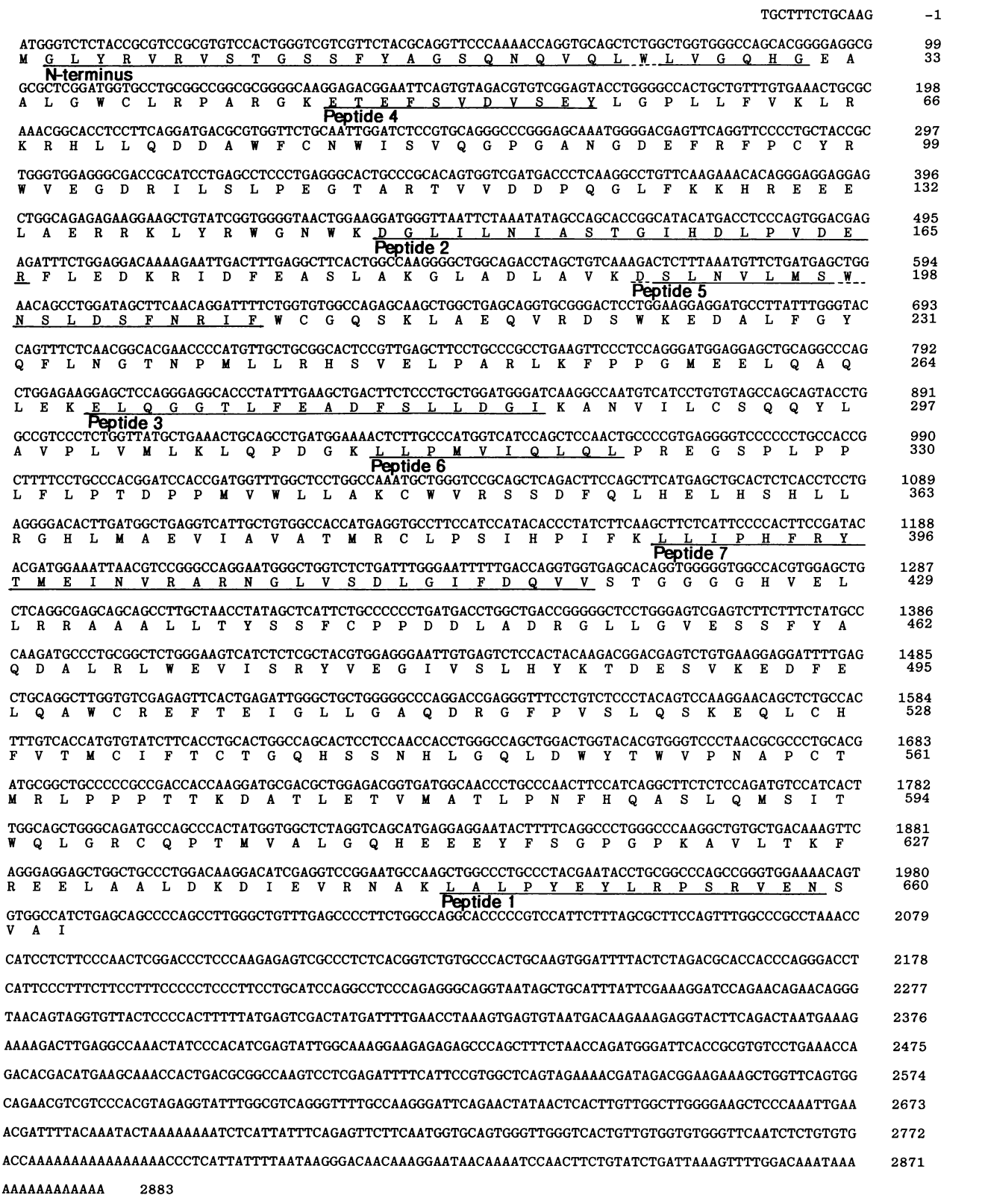


FIG. 3. Nucleotide sequence of the cDNA for porcine leukocyte 12-lipoxygenase and its deduced amino acid sequence. Nucleotide residues are numbered from 5′ to 3′ with the first residue of the ATG codon encoding the initiating methionine. The deduced amino acid sequence is displayed below the nucleotide sequence in the one-letter code starting from the first methionine. Underlines indicate amino acid residues determined by automated Edman degradation. Ambiguously identified residues are indicated by broken underlines.

and immunogenicity (13, 33). These two types of 12-lipoxygenase were found in bovine tissues (33, 34). Comparison of the two types of enzyme at the molecular level should give a clue to the physiological role of these enzymes. On the other hand, 12-lipoxygenase (12) and 15-lipoxygenase (35) are very similar in their catalytic properties as well as their primary structure (Fig. 4*A*). Site-directed mutagenesis of the lipoxygenase cDNA and expression of the mutant cDNA should provide insight into the structure and function of the catalytic site of 12-lipoxygenase.

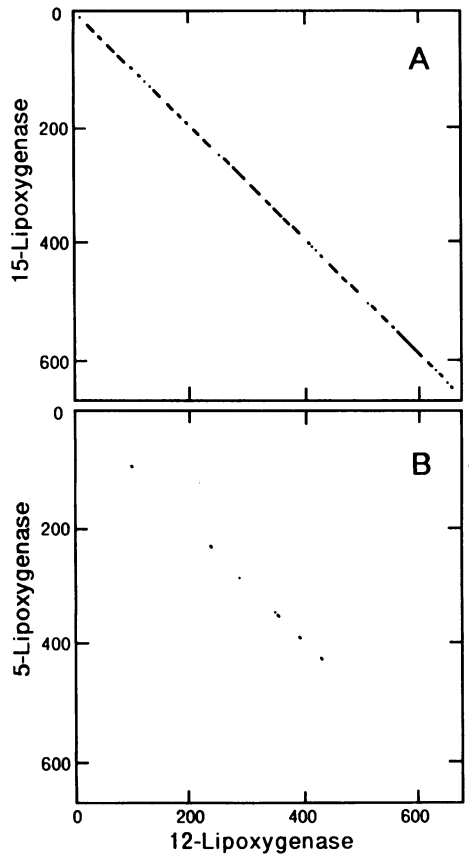


FIG. 4. Comparison of porcine 12-lipoxygenase with human 15- (*A*) or 5- (*B*) lipoxygenases. Deduced amino acid sequences of porcine leukocyte 12-lipoxygenase (this study), human reticulocyte 15-lipoxygenase (7), and human leukocyte 5-lipoxygenase (4, 5) were plotted as indicated and analyzed by the Harr plot (30) furnished in the program GENETYX.

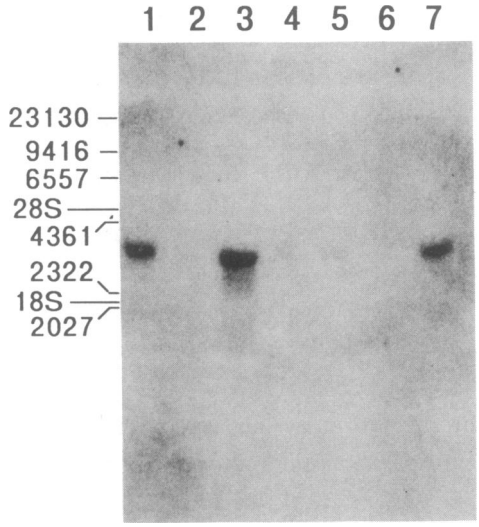


FIG. 5. Blot hybridization of total RNAs from various porcine tissues. Total RNAs (20 μg) obtained from various tissues were separated by electrophoresis on 1.5% agarose and transferred to a nylon membrane. The ^{32}P-labeled probe used was the 1737-bp *Dra* I–*Hin*cII fragment. The cells or tissues tested were pituitary gland (lane 1), heart (lane 2), leukocytes (lane 3), spleen (lane 4), jejunum (lane 5), liver (lane 6), and lung (lane 7). Molecular markers are 18S and 28S rRNA and *Hin*dIII-digested λ DNA as indicated to the left in bases.

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