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Phospholipase A_2 Engineering: Design, Synthesis, and Expression of a Gene for Bovine (Pro)Phospholipase A_2

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A gene coding for the (pro)phospholipase A_2 (PLA2) from bovine pancreas has been designed, synthesized, and expressed in *Escherichia coli*. The gene was designed with a variety of restriction sites that will facilitate future mutagenesis studies. Codons occurring frequently in prokaryotic systems were chosen whenever possible. The total gene spans 404 base pairs and was divided into 33 oligonucleotides. The gene was constructed in two halves of 224 and 180 base pairs from the oligonucleotides by the shotgun ligation technique using pBSM13- as the cloning vehicle. The two fragments were then ligated and cloned into pBSM13- to complete the gene. The (pro)PLA2 gene was then verified by restriction site mapping and dideoxy sequencing. The gene was expressed to high levels from a high copy number vector, designated as pJPN, derived from the *E. coli* secretion vector pIN-III-ompA$_3$. Although the protein failed to be excreted and was in the form of insoluble inclusion body, active PLA2 could be obtained by renaturation of the inclusion body pellet followed by tryptic activation, which removes the signal sequence and the pro-peptide of proPLA2. The PLA2 thus obtained reacted with the antisera raised against the natural PLA2 purified from bovine pancreas, and the specific activity of the expressed PLA2 was identical to that of the natural PLA2. The shotgun ligation and synthetic gene approaches are simple and inexpensive and can be adapted to express most of the enzymes in the phospholipase A_2 family.

Key words: bovine pancreas, gene synthesis, protein engineering, mechanism

The mechanism of the reaction catalyzed by phospholipase A_2 (PLA2) is a subject of active research in many laboratories. We have used chiral thiophospholipids to study the stereospecificity of PLA2 from several different sources and concluded that the calcium ion must coordinate to the *pro-S* oxygen of the phosphate group of the substrate at the active site of the enzyme [1–3]. We then used computer modeling to study possible enzyme–substrate interactions [4] based on the crystal structures of bovine PLA2 [5] and dilauroylphosphatidylethanolamine (DLPE) [6]. In the crystal structure of PLA2 the Ca^{2+} ion coordinates to five ligands from the protein and two

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water molecules [5]. By displacing the two water ligands with the *pro-S* oxygen of phosphorus and the *sn-2* carbonyl oxygen of the substrate, we obtained the model shown in Figure 1. The model suggested at least two potentially important features: 1) His-48, which has been proposed to function as a general base [7], is in proximity to, but separated by a distance sufficient for a water molecule from, the *sn-2* carbonyl carbon. 2) Tyr-69, which is highly conserved (except replaced by lysine, also a H-bond donor, in some cases) [8] and has been suggested to be important by chemical modification studies [9], is positioned to form a hydrogen bond with the *pro-R* oxygen of the phosphate. Our suggestion of Tyr-69's importance to substrate binding has recently been supported by the published crystal structure of covalently inhibited bovine PLA2 by *p*-bromo-phenacyl-bromide [10].

The above model, and other models regarding the kinetic properties and the "interfacial activation" of PLA2 [11–14], can best be tested by the protein engineering approach. As the first step in this effort, we report the design, synthesis, and expression of a gene coding for bovine pancreatic (pro)PLA2. The bovine enzyme was chosen because of the availability of a high-resolution (1.7 Å) crystal structure [4] that will facilitate the study of site-specific mutants and "muteins." The synthetic gene approach was chosen because of PLA2's small size of 14 kD. The gene was designed with numerous restriction sites and with codons preferred by the host organism [15]. The gene was constructed from 33 oligonucleotides by the "shotgun ligation" approach [16] and was expressed in *Escherichia coli* in the form of an insoluble fusion-protein to high levels from a high copy number derivative of pIN-III-ompA$_3$

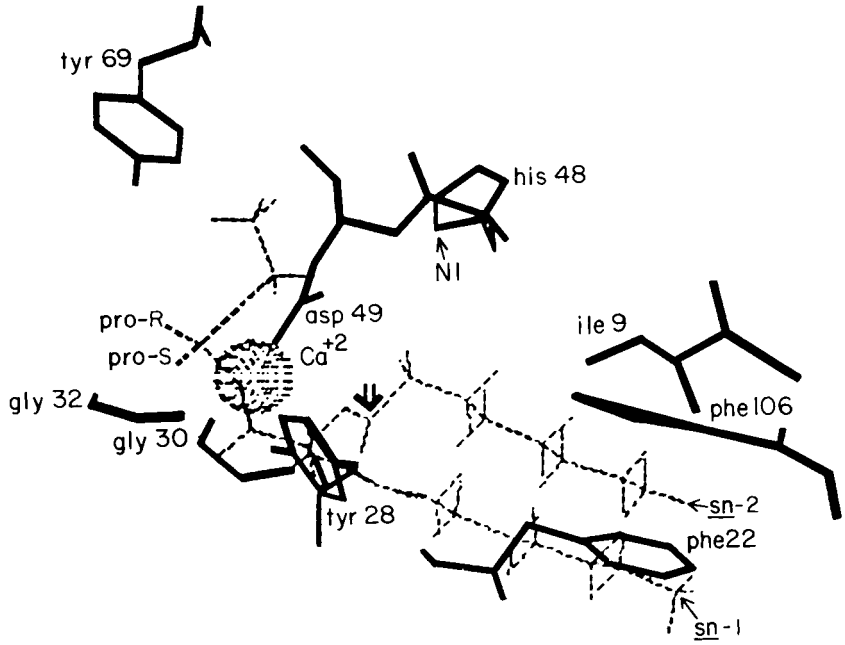

Fig. 1. Model of PLA2-substrate interactions proposed on the basis of stereochemical studies using chiral thiophospholipids. The active site residues of PLA2 are shown by solid lines, whereas the substrate DLPE is indicated by dashed lines (terminal part of the acyl chains are not shown). The double arrow points to the *sn-2* carbonyl carbon. Reproduced from Rosario-Jansen et al. [4].

[17] constructed in our laboratory. During the course of this work the cDNA for PLA2 has been isolated from several different sources [18–21], one of which led to the expression of porcine pancreatic PLA2 in *E. coli* [21] and in yeast [22]. In addition a synthetic gene for bovine pancreatic PLA2 has been expressed recently in yeast [23]. In all three cases the level of expression is relatively low, 2–6 mg of enzyme per liter of culture. Based on small-scale cultures, we expect to obtain 50 to 100 mg per liter of *E. coli* culture.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The plasmid pBSM13- was obtained from Stratagene Cloning Systems (La Jolla, CA). M13mp8 phages were propagated on *E. coli* strain JM101 according to standard procedures. *E. coli* JM101 and JM109 were routinely used as hosts for pBSM13- and its (pro)PLA2 gene derivatives. *E. coli* NM522 was routinely used as a host for pIN-III-ompA$_3$ and its high copy number derivative.

Construction of the Synthetic Gene

Unphosphorylated oligonucleotides (Fig. 2) were obtained from Syn-Tek AB (Umea, Sweden) and were purified by both polyacrylamide gel electrophoresis (PAGE) and C-18 reverse-phase chromatography. The procedures for the construction of the synthetic gene are outlined in Figure 3. The oligonucleotides 1–10 and 18–26 (0.5 pmol each) coding for the first half of the gene were phosphorylated in one pot in 35 mM Tris, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.1 mM spermidine, and 2 units of polynucleotide kinase (Pharmacia, Piscataway, NJ). After incubation at 37°C for 1 h, the polynucleotide kinase was heat inactivated at 65°C for 10 min. T4 DNA ligase (3 units) (Pharmacia) and the *Hind*III/*Sal*I linearized and dephosphorylated pBSM13- (0.1 pmol) were then added to the reaction mixture and incubation was continued at 25°C overnight. The resulting plasmid, denoted as pBSM13-H/S, was transformed (without purification) into *E. coli* JM 109. Clones were screened for the presence of the appropriate-size inserts by digestion with *Hind*III/*Sal*I followed with agarose gel electrophoresis on high sieving NuSieve GTG agarose.

The second half of the gene was constructed from oligonucleotides 11–17 and 27–33 analogously, except that this fragment was delimited by *Sal*I/*Pst*I. The whole gene was constructed by excising the bands corresponding to the two fragments from the GTG agarose gel followed by mixing the gels of the two fragments (0.5 pmol each) with *Hind*III/*Pst*I linearized, dephosphorylated pBSM13- (0.5 pmol) and incubating with 3 units of T4 DNA ligase (25°C, overnight) in 35 mM Tris buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 0.1 mM spermidine. The resulting clones were characterized by restriction enzyme mapping with *Hind*III followed by dideoxy sequencing [24].

Construction of a High Copy Number Derivative of pIN-III-ompA$_3$ for Use as an Expression Vector

pIN-III-ompA$_3$ (0.4 pmol) was digested to completion with *Pvu*II and *Sal*I to remove the rop gene and the *lac* I gene (Fig. 4). The rop gene product acts as a negative controller of plasmid copy number in ColE1 DNA replication [25]. After ethanol

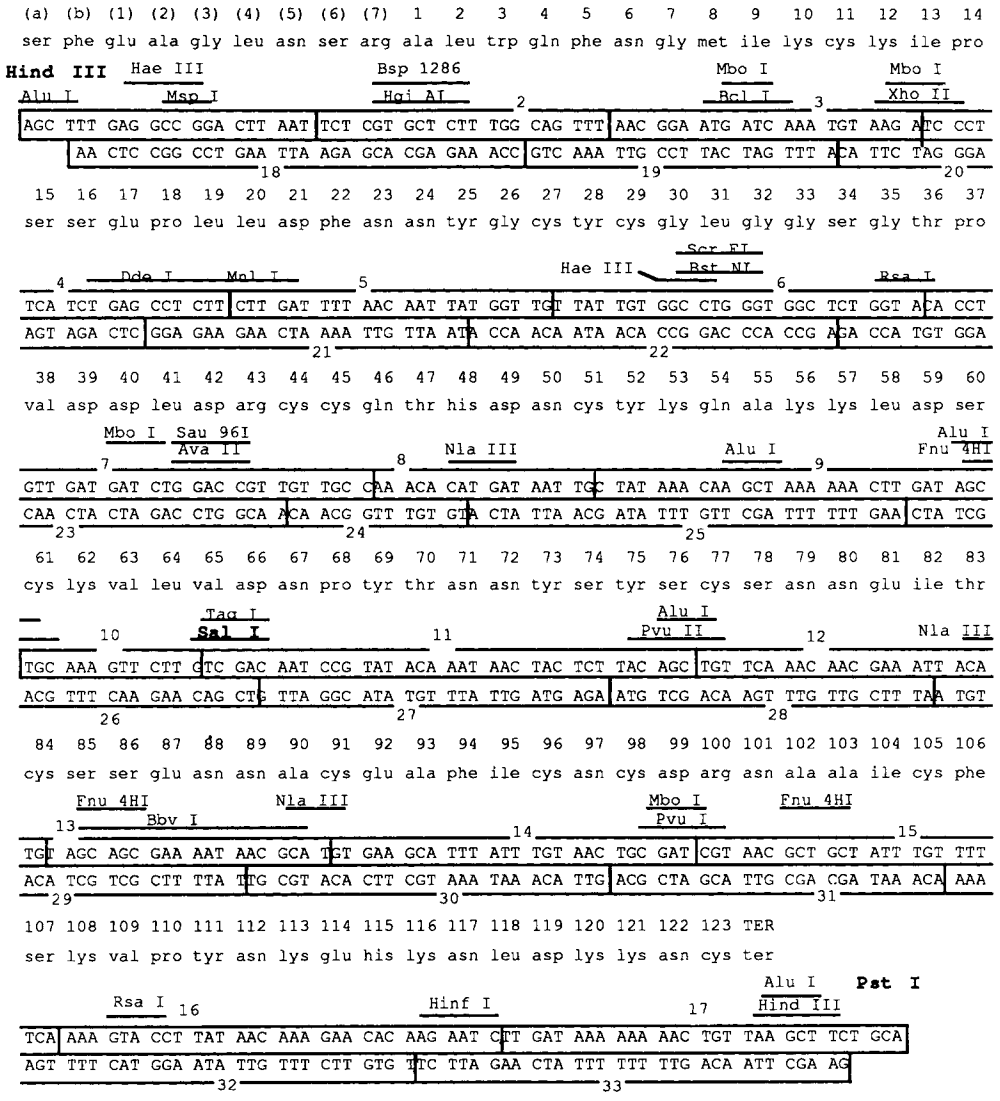

Fig. 2. Amino acid sequence of bovine pancreatic (pro)PLA2 [8] and the nucleotide sequence of the designed synthetic gene. The proPLA2 starts at residue (1) and the PLA2 starts at residue 1. The built-in restriction sites as well as the 33 oligonucleotides used to construct the gene are indicated.

precipitation, the linearized vector fragments were resuspended in 40 μl deionized water. The reaction was brought up to 50 μl with 10× buffer and 0.5 units of *Bal*31. Final buffer consists of 12 mM $CaCl_2$, 12 mM $MgCl_2$, 600 mM NaCl, 20 mM Tris, pH 8.0. The reaction was allowed to proceed at 28°C for 8 min; it was then quenched with 50 mM EDTA. One hundred fifty nanograms of this material was loaded and run on a 1% agarose gel. The band migrating as 3.5 kb (original size 3.7 kb) was excised from the gel, purified with Geneclean (Bio 101, La Jolla, CA) and recircularized in 50 μl with T4 DNA ligase.

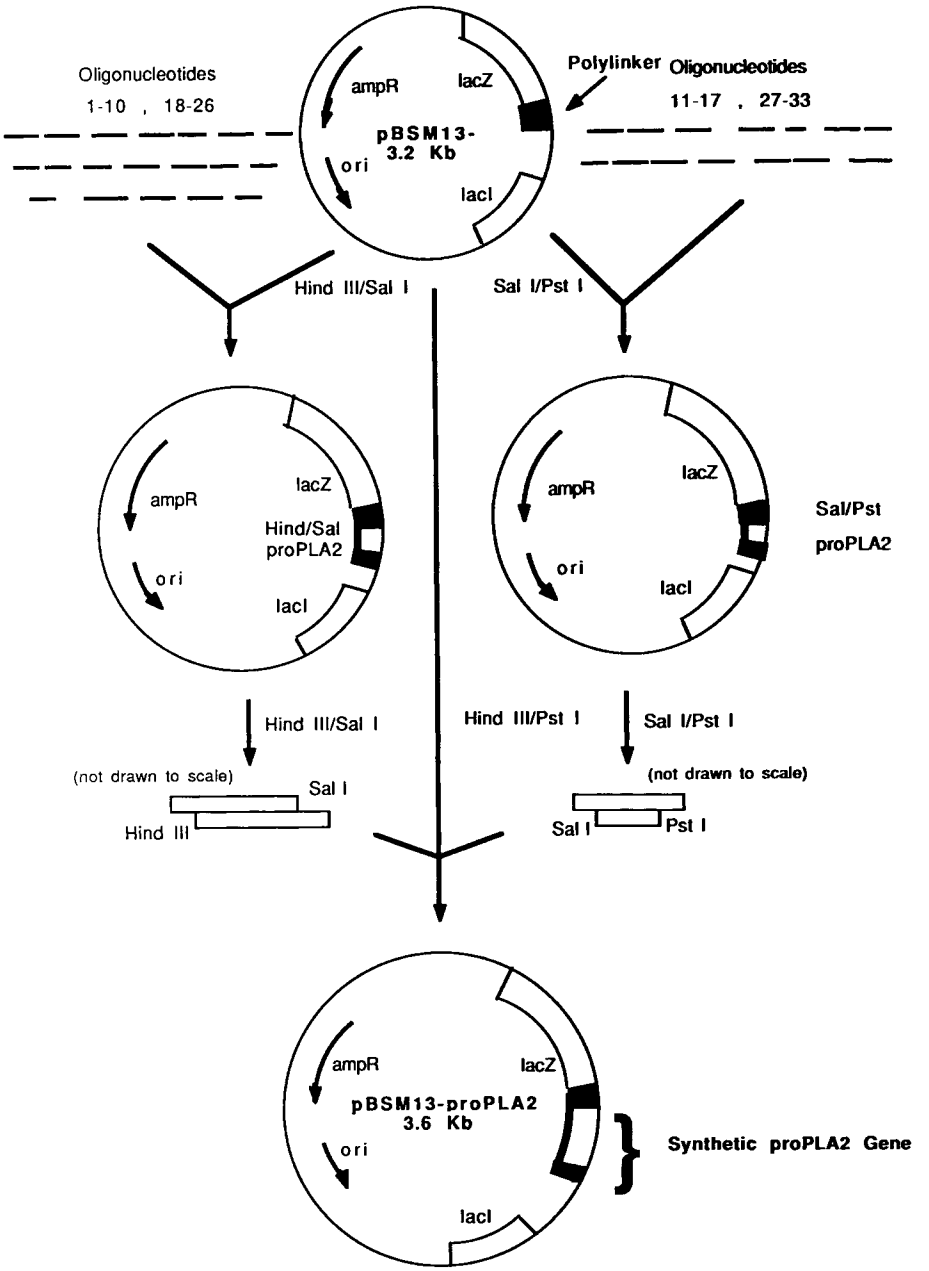

Fig. 3. Construction of the (pro)PLA2 gene. The vector pBSM13- was used in both cloning and expression. Cloning of the first half of the gene into pBSM13- by the shotgun ligation technique gave *Hind/Sal* proPLA2, and cloning of the second half gave *Sal/Pst* proPLA2. The *Hind/Sal* and *Sal/Pst* fragments were then cut from the corresponding plasmids and cloned into pBSM13- to give the plasmid (denoted as pBSM13-proPLA2) carrying the entire synthetic gene.

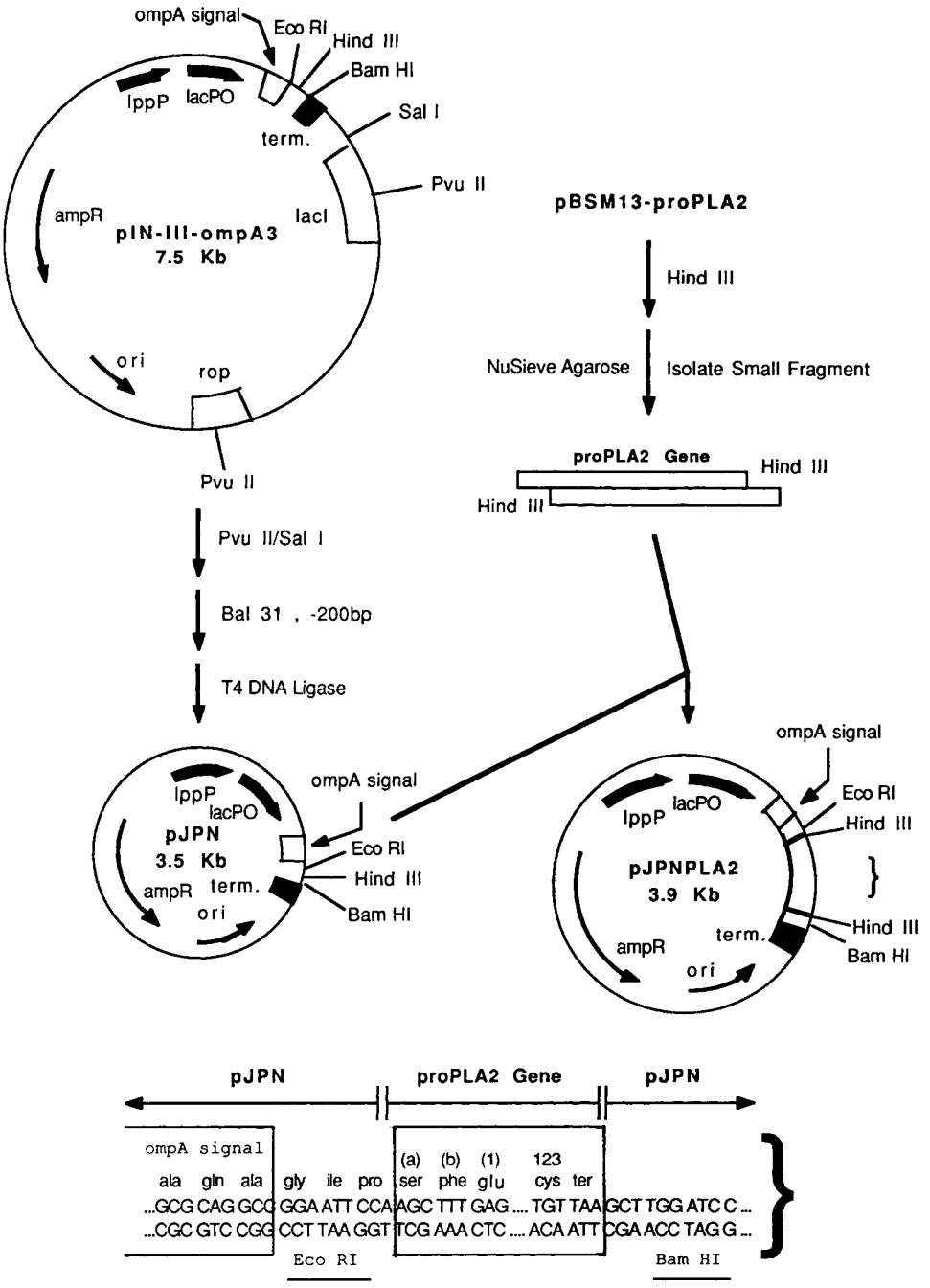

Fig. 4. Construction of the high copy number derivative of pIN-III-ompA$_3$.

After ligating overnight at room temperature, the resulting ligation mixture was used to transform *E. coli* NM522. All of the resulting clones exhibited copy number increases based on ethidium bromide-stained agarose gels (data not shown).

The proPLA2 gene with *Hind*III sticky ends was inserted into the resulting high copy number plasmid designated pJPN. Insert orientation was checked by protein expression.

DNA Techniques

E. coli were transformed by the method of Hanahan [26]. Restriction endonuclease digestions were carried out according to the manufacturer's procedure. Dideoxy sequencing [24] was done from single-stranded M13 templates using the Sequenase system (United States Biochemical Corp., Cleveland, OH) and [^{35}S]dATPαS (Amersham, Arlington Heights, IL). NuSieve GTG agarose (FMC BioProducts, Rockland, ME) was routinely used to purify small DNA fragments (<500 bp) for in-gel ligations [27].

Cell Growth and Isolation of Inclusion Bodies

Typically, *E. coli* NM522 harboring pJPNPLA2 was grown to A_{600} = 1.0 in a broth containing 10 g/l tryptone, 20 g/l yeast extract, 5 ml/l glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4, 0.2 mg/ml ampicillin, pH 7.5; Isopropyl-β-thiogalactopyranaside (IPTG) was then added at 1 mM final concentration. In addition the following amino acids were added at the indicated final concentrations: 0.5 mg/ml cysteine, 0.5 mg/ml asparagine, and 0.1 mg/ml proline. Growth was continued for 12 h, and final cell density reached 10 g/l.

As seen in Figure 5, the resulting protein retains the signal peptide (expected molecular weight, 17 kD) and is found in high concentrations in the insoluble fraction of *E. coli* lysate. The cells were lysed by sonication—300 watts, 6 × 2 min in 50 mM Tris, 10 mM EDTA, pH 7.5—5 ml/g wet cells. The fusion pellet was collected by

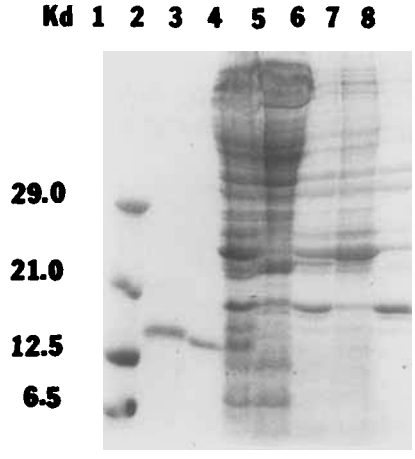

Fig. 5. SDS-PAGE analysis (15%). Each lane is equivalent to 50 μl whole cell culture. Staining was with Coomassie R-250. **Lane 1,** molecular weight standards; **lane 2,** bovine pancreatic proPLA2; **lane 3,** bovine pancreatic PLA2; **lane 4,** whole cell NM522 containing pJPNPLA2; **lane 5,** lysis supernatant of pJPNPLA2; **lane 6,** lysis pellet of pJPNPLA2; **lane 7,** detergent-washed supernatant of pJPNPLA2; **lane 8,** detergent-washed pellet of pJPNPLA2.

centrifugation at 12,000*g* for 10 min. The pellet was then washed with 0.3% sodium sarcosine in lysis buffer with stirring at room temperature for 1 h—5 ml/g wet cells. The ompA-PLA2 fusion protein was collected in highly purified form by centrifugation at 12,000*g* for 10 min.

Solubilization and Purification of Active PLA2

The (pro)PLA2 fusion protein was solubilized by chemical sulfonation of its cysteine residues and refolded as previously described [28] with the following modifications. After sulfonation of the pellet at 35 mg/ml, the mixture was centrifuged to remove remaining insoluble material. The sulfonation mixture was then buffer exchanged on a column of Bio-gel P-6DG (Bio-Rad, Rockville Center, NY) equilibrated in a solution of 5 M urea, 25 mM Tris, and 5 mM EDTA, pH 8.0. The protein-containing fractions were pooled (50 ml) and allowed to renature by mixing with 150 ml of 25 mM Tris buffer, pH 8.0, containing 5 mM EDTA, 3 mM reduced glutathione, and 1.5 mM oxidized glutathione, followed by incubating at room temperature for 24 h. The mixture was then desalted on a column of Bio-gel P-6DG equilibrated in 50 mM NH_4HCO_3, pH 7.8. Protein-containing fractions were pooled and lyophilized overnight. The resulting protein (1 mg/ml) was then activated with 1% trypsin in 10 mM Tris, 10 mM $CaCl_2$, pH 8.0 at room temperature for 3 h. Portions of the activation mixture were then fractionated on an FPLC mono-S column with a 1 ml bed volume (Pharmacia). The column was preequilibrated in 10 mM sodium acetate, pH 4.8. After sample loading, the column was washed with the acetate buffer until A_{280} reached 0. Active PLA2 was eluted by applying a 20 ml gradient from 0 to 0.430 M NaCl in 10 mM acetate buffer, pH 4.8 (Fig. 6). The PLA2 activity was routinely followed qualitatively by thin-layer chromatography on silica gel plates developed in 5:4:1 (v:v:v) methanol:chloroform:water using L-dioctanoylphosphatidylcholine (DOPC) as a substrate. The PLA2 peak shown in Figure 5 migrates with activated bovine PLA2 on SDS-PAGE gels. The PLA2 from bovine pancreas was purified according to established procedures [30].

Cross Reactions With Antisera

PLA2-specific antisera was isolated from New Zealand White rabbits immunized with bovine PLA2. Antibodies cross-reactive with *E. coli* proteins were removed

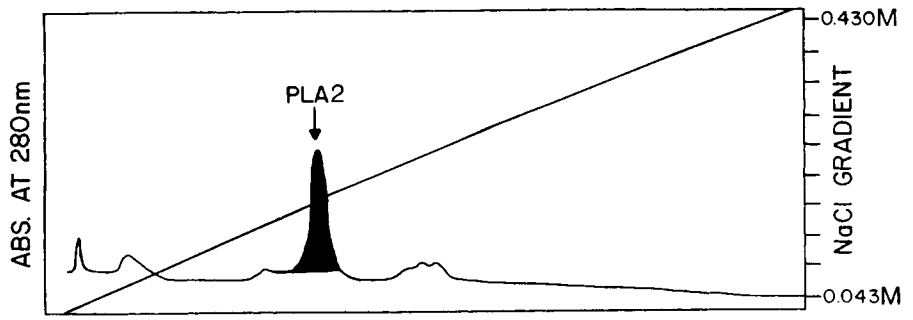

Fig. 6. FPLC elution profile of PLA2 after renaturation and tryptic digestion from a mono-S column. The column (1 ml volume) was preequilibrated in 10 mM acetate buffer, pH 4.8. After the sample had been loaded, the column was first eluted with the same buffer until A_{280} reached zero, then eluted with a 20 ml gradient from 0 to 0.430 N NaCl. Absorbance units are arbitrary.

from the antisera by adsorption onto nitrocellulose filters treated with *E. coli* lysate. The picoBlue Immunoscreening Kit (Stratagene) was used as a sensitive, nonradioactive system for the immunoscreening of recombinant protein. Briefly, recombinant PLA2, bound to nitrocellulose filters, was identified colorimetrically by the combined use of PLA2-reactive antisera, goat anti-rabbit immunoglobulin covalently linked to calf intestinal alkaline phosphatase (CIAP), and the CIAP substrate 5-bromo-4-chloro-3-indolyl phosphate.

Specific Activity Determination

The specific activity of the PLA2 peak was determined by pH STAT using L-dioctanoylphosphatidylcholine as substrate [29]. The assay mixture contained 10 mM DOPC, 20 mM $CaCl_2$, and 40 mM NaCl in 3 ml deionized H_2O. After flushing with argon to achieve a stable pH, the pH was adjusted to 8.0 at 41°C with a minimal amount of NaOH. A known amount of recombinant PLA2 was then added, and the activity was measured directly by recording the amount of 10 mM NaOH needed to keep the pH constant at 8.0. The specific activity measured was indistinguishable from the previously published values of 2,500 units/mg [30].

RESULTS

Design and Synthesis of the Gene

The amino acid sequence and designed DNA sequence are given in Figure 2. Codons previously shown to be preferentially used by *E. coli* were utilized whenever possible [15]. Two extra amino acids were placed on the N-terminus to facilitate the introduction of a *Hind*III restriction site. Thirty-three complementary oligonucleotides were used in the construction as indicated in Figure 2.

The gene was designed with a number of regularly spaced restriction sites. The oligonucleotides were chosen to minimize sites that could be involved in the formation of deleterious secondary structures. Overlaps of at least six bases were maintained throughout. Overlaps were designed to prevent alternative ligations.

Figure 3 outlines the construction of the synthetic gene using pBSM13- as the cloning vehicle. The oligonucleotides were efficiently assembled into two larger pieces by the "shotgun ligation" approach [16]. The first gene segment runs from the N-terminal *Hind*III site to the *Sal*I site and consists of oligonucleotides 1–10 and 18–26. The second segment runs from the *Sal*I site to the C-terminal *Pst*I site. The two segments were cloned into *Hind*III/*Sal*I-restricted pBSM13- and *Sal*I/*Pst*I-restricted pBSM13-, respectively, and transformed into *E. coli* JM109.

Positive clones were chosen by plating on X-Gal/IPTG/ampicillin and further screened by digestion with *Hind*III/*Sal*I or *Sal*I/*Pst*I followed by electrophoresis on 3% NuSieve GTG agarose. All clones contained inserts of the appropriate length. One *Hind*III/*Sal*I clone and one *Sal*I/*Pst*I clone were chosen for assembly of the entire gene. The desired fragments were cut from NuSieve GTG agarose and ligated in gel with *Hind*III/*Pst*I linearized pBSM13-.

Positive clones were screened as before. One clone was chosen for DNA sequencing. The *Hind*III-bounded (pro)PLA2 gene was rescued from a NuSieve GTG gel and cloned into the *Hind*III site of M13mp8. Single-stranded DNA from progeny phage was isolated and clones of opposite insert orientation were sequenced using

Sequenase. No deletions or substitutions were found, so further screening was unnecessary.

Expression of the (Pro)PLA2 Gene in *E. coli*

We hoped to express our gene and secrete the protein product in *E. coli*. To that end we created a high copy number derivative of the *E. coli* expression vector pIN-III-ompA$_3$. By removing the rop gene of the pBR322 origin of replication [25], we were able to increase the relative copy number of pIN-III-ompA$_3$ and achieve high-level expression of proPLA2 fused to the ompA signal peptide. The procedures are outlined in Figure 4. The newly constructed plasmid is designated as pJPN, and that containing the proPLA2 gene is designated as pJPNPLA2. The high-level expression of this gene is shown by the heavy band near 17 kD (lane 4 of Figure 5). The molecular weight of this band suggests that the signal peptide was not cleaved. In addition, the protein was not processed into the periplasmic space and was found as insoluble inclusion bodies, as shown in lane 5 (supernatant) and lane 6 (pellet) of Figure 5. However, the signal peptide appears to be necessary for high-level expression because our gene was not expressed to a high level from a high copy number plasmid, pUK, containing the Ptrc promotor (T.L. Deng, unpublished results).

Isolation and Characterization of PLA2

The insoluble pellet obtained above was further washed with detergent. The supernatant and the pellet are shown in lanes 7 and 8, respectively, of Figure 5. The pellet was then rapidly solubilized by *S*-sulfonation and refolded with the aid of a glutathione redox couple [28]. Tryptic digestion of the folded protein eliminated the signal peptide and the (pro)protein residues. Fractionation on a FPLC mono-S column gave pure PLA2, as shown in Figure 6. The purified PLA2 migrated as a single band with the enzyme isolated from bovine pancreas on a SDS polyacrylamide gel (data not shown).

The resulting protein was indistinguishable from PLA2 from bovine pancreas based on specific activity measurements (2,500 units/mg) and reactivity with antisera raised against bovine PLA2. Based on small-scale cultures and PAGE gel analysis, we expect to obtain 50–100 mg of recombinant PLA2 per liter of culture.

DISCUSSION

Advantages of the Shotgun Ligation Method in Gene Synthesis

Among the 5–10 enzymes (proteins) that have been expressed by the synthetic gene approach to date, intestinal calcium binding protein (ICaBP) [31] and PLA2 are unique in that relatively short oligonucleotides (20mers to 30mers) were used, and the ligation was carried out by the shotgun technique developed by Grundstrom et al. [16]. In the case of ICaBP the entire gene was only 300 bp in length and the ligation was achieved from five segments. In the present work the entire gene is ~400 bp in length and was constructed in two halves in essentially 100% efficiency. This suggests that the method can be extended to much longer genes, and proteins up to 50 kD can be produced by this approach. The advantages of this approach increase as the size of the protein increases: in addition to the time saved in the ligation step, the short oligonucleotides can be (and have been in the cases of ICaBP and PLA2) synthesized by a rapid microscale method using cellulose disks as solid supports [32], which affords

savings in cost and time and avoids formation of stable secondary structures within longer oligonucleotides.

Conclusions and Future Perspectives

The high-level expression of bovine pancreatic PLA2 described above has set the stage for detailed structure-function studies of PLA2 by combined bioorganic and biophysical methods. In addition, our gene synthesis approach can in principle be adapted to produce any of the >40 PLA2s with known primary structure [8]. Thus, in addition to the catalytic mechanism and the mechanism of interfacial interaction, the large family of PLA2 enzymes presents one with the unique opportunity to study in detail the evolution of an enzyme system. By making use of the synthetic gene's unique restriction sites, "hybrid" proteins can be constructed quickly and efficiently. This and parallel work in other laboratories [22,23] mark a new era in the study of lipolytic enzymes and should lead to some exciting discoveries in upcoming years.

ACKNOWLEDGMENTS

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