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High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A₂¹

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Bee venom phospholipase A₂ (BV-PLA2) is a hydrolytic enzyme that specifically cleaves the *sn*-2 acyl bond of phospholipids at the lipid/water interface. The same enzyme is also believed to be responsible for some systemic anaphylactic reactions in bee venom sensitized individuals. To study the structure/function relationships of this enzyme and to define the molecular determinants responsible for its allergenic potential, a synthetic gene encoding the mature form of BV-PLA2 was expressed in *Escherichia coli*. This enzyme was produced as a fusion protein with a 6xHis-tag on its amino-terminus yielding 40–50 mg of fusion protein per l of culture after metal ion affinity chromatography. A kallikrein protease recognition site was engineered between the 6xHis-tag and the amino-terminus of the enzyme allowing isolation of the protein with its correct N-terminus. Recombinant affinity purified BV-PLA2 was refolded, purified to homogeneity, and cleaved with kallikrein, resulting in a final yield of 8–9 mg of active enzyme per l of culture. The enzymatic and immunological properties of the recombinant BV-PLA2 are identical to enzyme isolated from bee venom indicating a native-like folding of the protein.

Introduction

Bee venom phospholipase A₂ (BV-PLA2) is a member of a large family of small MW secreted enzymes that hydrolyze phospholipids at the *sn*-2 position in a calcium-dependent manner [1]. The amino acid sequences of most of these enzymes are highly homologous and can be organized into the class I (pancreatic juice and elapid venom) and class II (crotalid and viper venoms) sub-types based on the location of the disulfides and the presence of small insertions and deletions

[2]. However, the primary structure of BV-PLA2 is distinct from these two related classes of enzymes [3]. Sequence similarity is limited only to short functional regions, i.e., the calcium binding loop and sections that contribute catalytic residues in the active site. The only known PLA2s that have extensive sequence homology with the BV-PLA2 are the enzymes from the venoms of Gila monster [4], Mexican beaded lizard [5] and hornet [6]. Interestingly, the high resolution crystal structure of the BV-PLA2 shows that the global architecture is distinct from the class I/II PLA2s, but that functional substructures that are involved in the catalytic reaction are conserved [7,8].

There is increasing evidence that PLA2s are involved in local and systemic inflammatory processes [9,10]. Recently, a secreted PLA2 has been found in rheumatoid synovial fluid [11,12], and an 85 kDa intracellular PLA2 may be involved in the generation of free arachidonic acid for the biosynthesis of the eicosanoids [13,14]. It is for these reasons that there is a quest to design or discover small molecular weight compounds that can inhibit the enzymatic activity of PLA2 *in vivo*.

As long chain phospholipids are predominantly present as integral constituents of biological membranes,

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Abbreviations: AMC, 7-amino-4-methylcoumarin; BCML-gel, *N,N*-bis(carboxymethyl)-L-lysine attached through its ϵ -amino group to agarose beads; BV-PLA2, phospholipase A₂ from honey bee venom (*Apis mellifera*); diC₆thio-PM, racemic 2,3-bis(hexanoylthio)propyl-1-phosphomethanol lithium salt; DMPM, 1,2-dimyrystoyl-*sn*-glycero-3-phosphomethanol lithium salt; IPTG, isopropyl- β -D-thiogalactopyranoside; PCR, polymerase chain reaction; PLA2, phospholipase A₂; PPyPM, 1-palmitoyl-2-[10-(1-pyrene)decanoyl]-*sn*-glycero-3-phosphomethanol lithium salt; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Z-Phe-Arg-AMC, *N*-carbobenzoxy-L-phenylalanyl-L-arginyl-AMC.

PLA2s must associate with the phospholipid bilayer in the catalytic cycle to generate inflammatory mediators [15]. Very little is known about the molecular details that govern the PLA2-bilayer interaction. Such knowledge should be useful in designing PLA2 inhibitors that function by preventing the catalytically productive enzyme-membrane interaction [16].

BV-PLA2 is an antigen of particular interest as it can induce immune responses in man that are associated with different clinical features. In individuals allergic to bee venom, high levels of IgE directed against BV-PLA2 are found, whereas BV-PLA2 specific immunoglobulins of the IgG4 subclass predominate in protected individuals [17]. Protection against bee venom induced allergies by antibodies has been directly demonstrated by transferring γ -globulins from a protected individual to an allergic individual [18]. Hence, it is postulated that antibodies of the IgG4 subclass are responsible for the protection. It will therefore be of importance to define both the protective antibodies and the corresponding epitopes recognized on the BV-PLA2.

The high level expression of BV-PLA2 combined with site-directed mutagenesis is an essential step towards an understanding of the role of particular amino acids in allowing BV-PLA2 to bind to the lipid bilayer in a catalytically productive manner. Such an approach is also useful in mapping the B-cell epitopes that are recognized by protective antibodies, as well as in studying the role of the hydrolytic activity and cell surface binding in the immune response to BV-PLA2. The experimental strategy to address these structure/function questions is substantially aided by the availability of the crystal structure of the enzyme [8]. In this report, the synthesis of a gene coding for BV-PLA2 and its high level expression in *E. coli* are described. In addition, a minimal step purification and refolding procedure for BV-PLA2 has been developed. Finally, the kinetic and immunological properties of recombinant BV-PLA2 were characterized.

Materials and Methods

Materials

Chemicals were purchased from Fluka (Buchs, Switzerland) or Sigma (St. Louis, MO, USA) in the highest available grade. Urea (USP-grade) and guanidine-HCl (ultra-pure grade) were from Amresco (Solon, OH, USA). Milli-Q water (Millipore, Bedford, MA, USA) was used for enzyme refolding and purification. DNA modifying enzymes were obtained from Boehringer-Mannheim, Germany) and used as recommended. All commercial kits were used following the manufacturer's instructions. Purified native BV-PLA2 (a mixture of glycosylated and non-glycosylated enzyme) and factor Xa were purchased from Boehringer.

Concentrations of commercial and recombinant BV-PLA2 were measured according to the Bradford method using a commercial kit (Bio-Rad, Richmond, CA, USA) or calculated from the A_{280} using an $\epsilon^{1\%}$ of 13.0 [16]. Human plasma prekallikrein, purified as described [19] and activated to kallikrein with trypsin [20], was a generous gift from Dr. Kazuo Fujikawa (Dept. of Biochemistry, Univ. of Washington). PPyPM, diC₆thio-PM, and DMPM were prepared as described [21–23].

Enzymatic assays of BV-PLA2

The enzymatic activity was assayed with three different methods. The pH-stat assay was used to monitor the enzymatic reaction on vesicles of DMPM [23,24]. Assays were conducted at 21°C in 4 ml of 1 mM NaCl (pH 8.0) containing 0.6 mM CaCl₂ with or without 5 μ g of polymyxin B sulfate (Sigma). Spectrophotometric assays using diC₆thio-PM as substrate were carried out as described [25]. For this assay 0.1 mM substrate in 25 mM Tris-HCl, 100 mM KCl, 10 mM CaCl₂, 0.8 mM Ellman's reagent (pH 8.0) was used. A spectrofluorimetric assay with PPyPM [26] was carried out as follows. A stock solution of 8 mM PPyPM (determined from the A_{345} using ϵ of 40 000 M⁻¹ cm⁻¹) in toluene was stored at -80°C. 24 μ l of this stock solution was transferred to a small glass tube, and the solvent was removed with a stream of N₂ and then in vacuo for 30 min. The residue was redissolved in 24 μ l of tetrahydrofuran, and 300 μ l of 0.1 mM EGTA (pH 9.0) was added. The solution was sonicated for 2 min as described [23], and the vesicle stock was frozen at -20°C. The final vesicle solution was prepared by diluting 100 μ l of thawed vesicle stock to 1 ml with 0.1 mM EGTA (pH 9.0), and the tube was vortexed. The assay cocktail was prepared by adding 60 μ l of the final vesicle solution and 60 μ l of 30 mg/ml bovine serum albumin (Sigma cat. #A-7906) to 1.5 ml of buffer (50 mM Tris-HCl, 50 mM KCl, 1 mM CaCl₂, pH 9.0). After adding enzyme, the fluorescence was monitored by excitation at 342 nm and emission at 395 nm. The detection limit of this assay is 100 pg of BV-PLA2.

Synthesis of BCML-gel

BCML was made essentially as described previously [27] except that the hydrogenation step was carried out as follows. *N*-(5-benzyloxycarbonylamino-1-carboxypentyl)iminodiacetic acid (9.7 g) was dissolved in 1 M NaOH (50 ml), and 5% Pd on carbon (500 mg, Aldrich) was added. Hydrogen gas was bubbled directly into the solution with stirring overnight at room temperature. After filtration through celite, the filtrate was acidified to pH 2.0 with 6 M HCl. The solution was concentrated on a rotary evaporator leaving an oily residue. Water (50 ml) was added, and the solution was concentrated again. Crystalline product was seen after about

half of the water was removed. At this point the solution was filtered, and the white solid was dried in vacuo leaving 3.4 g of pure product (mp 208–210°C). For resin preparation, BCML was coupled to preactivated and crosslinked agarose beads as follows. 125 mg of BCML dissolved in 50 ml of dimethyl sulfoxide (the dissolution was facilitated by sonication) was mixed directly with a suspension of Affigel-10 (25 ml gel in isopropanol as supplied by Bio-Rad), and the mixture was shaken gently on an orbital shaker overnight at room temperature. Blocking of unreacted residues was accomplished by adding ethanolamine (10 μ l) and allowing the mixture to shake for an additional 2 h. The resulting gel was filtered, washed with 5 volumes of ethanol and 5 volumes of water, and stored at 4°C as a suspension in 2% NiSO₄.

Bacterial strains, plasmids and media

E. coli strain XL1-Blue and plasmid pBluescript® II SK were obtained from Stratagene (La Jolla, CA, USA). *E. coli* strain M15 [28] used in all of the studies described herein bears the repressor plasmid pRep4 [29]. This strain and the expression plasmid pDS56/RBSII, 6xHis [29] were kindly provided by Dr. D. Stüber (Hoffmann-LaRoche, Basel, Switzerland). Genes to be expressed were cloned into the *Bam*HI and *Hind*III sites of the plasmid to generate fusion proteins with six adjacent histidine residues linked to the amino-terminal end (Fig. 2).

To propagate pBluescript® II SK plasmids, *E. coli* strain XL1-Blue was used. Alternatively, plasmids to be cleaved with the methylation sensitive *Cla*I restriction enzyme were isolated from the *E. coli* dam⁻ strain S1540 ($\Delta(ara-leu)\Delta(lac)X74$ *galE galK hsdR rpsL* Δ dam:kan^r₁₆), obtained as a generous gift from Dr. Colin Manoil (Dept. of Genetics, Univ. of Washington). Both strains were grown in LB medium [30] supplemented with 100 μ g/ml ampicillin. *E. coli* strain M15 was exclusively used as a host of the expression plasmid and was grown in LB medium containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin.

Recombinant DNA techniques

Plasmid DNA was prepared using standard techniques [30] or with anionic exchange columns (Qiagen kit, Qiagen Inc., Düsseldorf, Germany). Restriction fragments were purified on agarose gels and either electroeluted or recovered with GeneClean™ (Bio 101 Inc., La Jolla, CA, USA). Ligations were performed using standard protocols [30]. Transformations were carried out either with frozen competent cells [30] or by electroporation using a GenePulser (Bio-Rad), and transformants were selected on LB plates containing the appropriate antibiotics. Double stranded DNA sequencing was performed using the dideoxy chain-

termination method [31]. Sequencing of inserts in pBluescript® II SK was carried out using the T7 and T3 primers and the Sequenase 2.0 kit (US Biochemicals, Cleveland, OH, USA). Inserts in the expression vector were sequenced on an Applied Biosystems 373A sequencer using the Taq DyeDeoxy™ Terminator cycle sequencing kit supplied by the same company. In this case, the Type III/Type IV and reverse primers (Qiagen Inc.) were used.

Design, synthesis and assembly of the BV-PLA2 gene

The DNA sequence of the synthetic gene was inferred from the amino acid sequence of the BV-PLA2 [3] and was designed with the aid of the GeneBuilder program (obtained from Prof. S.G. Sligar, Univ. of Illinois).

Three gene segments of similar length were individually constructed and then ligated together to form the full length gene (Fig. 1). For each gene segment, six oligonucleotides representing both coding and noncoding strands were synthesized and were designed to contain restriction overhangs at their extremities. To insure efficient intermolecular annealing the six oligonucleotides contained overlaps of 10 nucleotides (Fig. 1). Synthetic oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems 380A) and were purified by gel electrophoresis and recovered as described [30].

The purified oligonucleotides (50 pmol each) were separately 5'-phosphorylated in 20 μ l reaction volumes with 10 units of T4 oligonucleotide kinase (New England Biolabs, USA) as described [30]. After a 30 min incubation at 37°C, a second portion of 10 units of kinase was added followed by a 30 min incubation at the same temperature. For each of the three gene segments, the corresponding six phosphorylated oligonucleotides were combined into a single tube and incubated at 95°C for 2 min, and the reaction mixture was allowed to cool to room temperature over 2–3 h. The annealed gene segments were precipitated with ethanol, redissolved in 20 μ l of water, and separately ligated into pBluescript® II SK digested with the appropriate two restriction enzymes. Following transformation, recombinant plasmids were analyzed by restriction mapping, and constructs showing the correct size insert were sequenced.

To assemble the full length gene, pBluescript® II SK vectors containing the three gene segments were propagated in the dam⁻ strain S1540 and inserts were excised with the two appropriate restriction enzymes. The gel purified inserts were ligated stepwise into pBluescript® II SK prepared with the same restriction enzymes. The structure of the full-length synthetic gene was verified by complete sequencing of both strands.

Isolation of the BV-PLA2 gene and subcloning into the expression vector

To introduce a kallikrein recognition sequence and to modify the 5'-restriction site of the synthetic gene for subcloning into the expression vector, the BV-PLA2 gene was isolated by PCR using the 5'-primer 1, 2 or 3 (Fig. 1) and the T7-primer as a 3'-primer. Polymerase chain reactions (PCR) were carried out using Vent_RTM heat stable DNA polymerase (New England Biolabs, Schwalbach/Taunus, Germany). Annealing temperatures for the various primers were calculated using the program OLIGO (National Biosciences, Plymouth, MN). PCR was performed in 100 μ l of sample containing the appropriate buffer, 200 μ M of each dNTP, 0.5 μ M of each 5'- and 3'-primer, 10 ng of plasmid DNA, and 1 unit of Vent_RTM DNA polymerase. The samples were subjected to two cycles of amplification consisting of a 1 min denaturation at 95°C, annealing for 1 min at 36°C, and 1 min of extension at 72°C, followed by 30 cycles under identical conditions except that the annealing was done at 50°C. The PCR product was digested with the appropriate two restriction enzymes and purified using the double GeneClean procedure. The fragment was ligated into the pDS56/RBSII, 6 $\times$ His expression plasmid prepared as described above.

Expression and isolation of the fusion protein

To assess the time course of protein expression, *E. coli* strain M15 harbouring the desired expression construct was subjected to a test induction assay, and intracellular proteins were analyzed on SDS-PAGE as described [29]. Polyacrylamide gels were prepared and processed according to Schägger [32]. For large scale protein production, 10 ml of a fresh overnight culture of the selected bacteria was diluted into 1 l of LB medium containing the appropriate antibiotics. The cells were grown at 37°C with shaking (300 rpm) until the A_{600} reached about 0.9. Then IPTG was added to a final concentration of 2 mM, and the incubation was continued until maximal expression was reached (4–6 h). At this point the cells were harvested by centrifugation (15 min, 4°C, 2600 $\times g$) giving 6.3 g of wet cell paste which was stored at –80°C. The purification of the fusion protein was carried out using a modification of the procedure described by Stüber et al. [29]. To the thawed cells 40 ml of buffer A was added, and the suspension was stirred for 1 h at room temperature and centrifuged (15 min, 4°C, 12000 $\times g$). The pellet was re-extracted by stirring with 20 ml of buffer A for 5 min at room temperature. After centrifugation, the combined supernatants were filtered through a 0.8 μ m filter cartridge (Millex-GS, Millipore) and loaded onto a 10 ml column of BCML-gel at a flow rate of 30 ml/h. The column was washed using the published procedure [29], except that the buffer D wash was omitted, and

the washes with buffers A, B, C and E were done at 100 ml/h. All urea buffers contained 0.1 M NH₄Cl to protect the protein against possible reaction with urea-derived cyanates. The entire buffer E wash was collected into a single tube, dialyzed twice for 12 h against 3.5 l of 5 mM acetic acid at 4°C, and lyophilized (Fig. 3).

Refolding and purification of recombinant BV-PLA2

All subsequent steps were carried out at room temperature. Freezing of the protein solution at intermediate stages was avoided. The lyophilized protein was dissolved in 7 M guanidine-HCl, 0.3 M Na₂SO₃ (pH 8.3), at a concentration of 0.5 mg/ml (A_{280}), and a 1/20 volume of Thannhauser reagent solution [33] was added to sulfonate the cysteine thiols. The solution was dialyzed against 3.5 l of freshly made 2 M urea, 4 mM EDTA, 0.1 M NH₄Cl, 20 mM sodium borate (pH 8.3), for 4 h, and the dialysis solution was replaced with fresh solution. After a further 4 h of dialysis, 56 ml of 0.5 M cysteine in water and 17.5 ml of 0.2 M cystine in 1 M HCl were added to the dialysis solution, and the pH was readjusted to 8.3 with 10 M NaOH. The beaker was wrapped with aluminum foil, and the dialysis was continued for an additional 14 h. At this point the dialysate was transferred to an aluminum foil wrapped beaker. Aliquots were tested hourly for PLA2 activity using the spectrofluorimetric assay. When the activity was maximal (typically 18–20 h after the addition of cysteine and cystine), the protein solution was decanted from the aggregated precipitate, dialyzed for 14 h against 3.5 l of SP buffer (50 mM Tris-HCl, pH 9.0) at 4°C, and stored at 4°C until the subsequent refolding steps were completed.

The precipitated protein obtained during refolding was washed with 30 ml of water, dissolved in 7 M guanidine-HCl, 0.3 M Na₂SO₃, sulfonated, and refolded in an identical manner. The precipitate from this refolding was cycled a third time through the refolding protocol. For these cycles, the dialysis volumes were scaled according to the amount of protein. The three dialysates were combined and centrifuged to remove any precipitate (30 min, 4°C, 12000 $\times g$). The supernatant was loaded at 30 ml/h onto a 1 $\times$ 13 cm column of SP-Sephadex C-25 (Pharmacia) that was previously equilibrated with SP buffer. The column was washed with 50 ml of SP buffer at 30 ml/h and the eluant was discarded. The protein was eluted at 10 ml/h with a 100 ml linear gradient of SP buffer to 750 mM Tris-HCl (pH 9.0), collecting 3.2 ml fractions. Fractions were monitored for protein (by A_{280}) and enzymatic activity (using the spectrophotometric assay). Fractions containing the PLA2 activity (Fig. 4) were pooled and either digested with kallikrein or dialyzed against 5 mM acetic acid and lyophilized.

Cleavage of BV-PLA2 fusion protein with kallikrein and isolation of BV-PLA2 with its native N-terminus

Kallikrein activity was assayed with 0.1 mM Z-Phe-Arg-AMC (Sigma) in 1.5 ml of kallikrein buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) at 25°C [34]. The assay was calibrated by measuring the fluorescence after adding a known amount of AMC (Sigma). One unit is defined as the amount of kallikrein activity necessary to produce 1 μ mol of AMC per min.

The SP-Sephadex purified fusion protein was concentrated to about 10 mg/ml using a centrifugal ultrafiltration device (Centricon-10, Amicon) and diluted with kallikrein buffer to give a protein concentration of 0.8 mg/ml (A_{280}). Kallikrein (0.024 units/mg of fusion protein) was added, and the solution was incubated for 16 h at room temperature. To remove the [His₆]-containing peptide and any undigested fusion protein, the solution was passed by gravity flow through 2 ml of BCML-gel that was previously equilibrated with 50 mM Tris-HCl (pH 8.0). The gel was washed with the same buffer until no more protein was eluted (determined by A_{280} ; about 4 ml buffer). The eluants obtained from the loading and washing were combined and dialyzed against 1.6 l of 25 mM Tris-HCl (pH 9.0) for 2 h at 4°C, and the kallikrein was removed by passing the dialysate through a 1 ml column of DEAE-Sephacel (Pharmacia) that had been previously equilibrated with the same buffer. The column was washed with the same buffer until no more BV-PLA2 was detected in the eluant (A_{280}) (10 ml). The eluant during loading and washing, which contains the BV-PLA2, was stored at 4°C or dialyzed against 5 mM acetic acid and lyophilized. The column was washed with 25 mM Tris-HCl, 0.5 M NaCl (pH 8.0) to elute the kallikrein.

Antibody binding assay

The binding of anti-BV-PLA₂ antibodies to native and recombinant BV-PLA₂ was analyzed by inhibition enzyme linked immunosorbent assay as described [35]. Polystyrene microtiter plates (Maxisorp, Nunc, Roskilde, DK) were coated overnight at 4°C with 5 mg/ml native BV-PLA2 in PBS (10 mM sodium phosphate, 0.9% (w/v) NaCl) (pH 8.0). The remaining free sites were blocked with blocking buffer (PBS pH 7.4 containing 1% (w/v) casein hydrolysate (Oxoid Ltd., Basingstoke, Hampshire, UK) and 5% (w/v) Tween 20) at 37°C for 1 h. The wells were then washed three times with PBS (pH 7.4). Sera diluted in 100 μ l blocking buffer were preincubated in a separate untreated 96 well plate at 37°C for 2 h with serial dilutions of recombinant or native BV-PLA2. Thereafter, the antigen preincubated sera were transferred to the washed BV-PLA₂ coated plates and incubated at 37°C for 2 h. After washing, the plates were incubated at 37°C for another 2 h with an alkaline phosphatase labelled goat

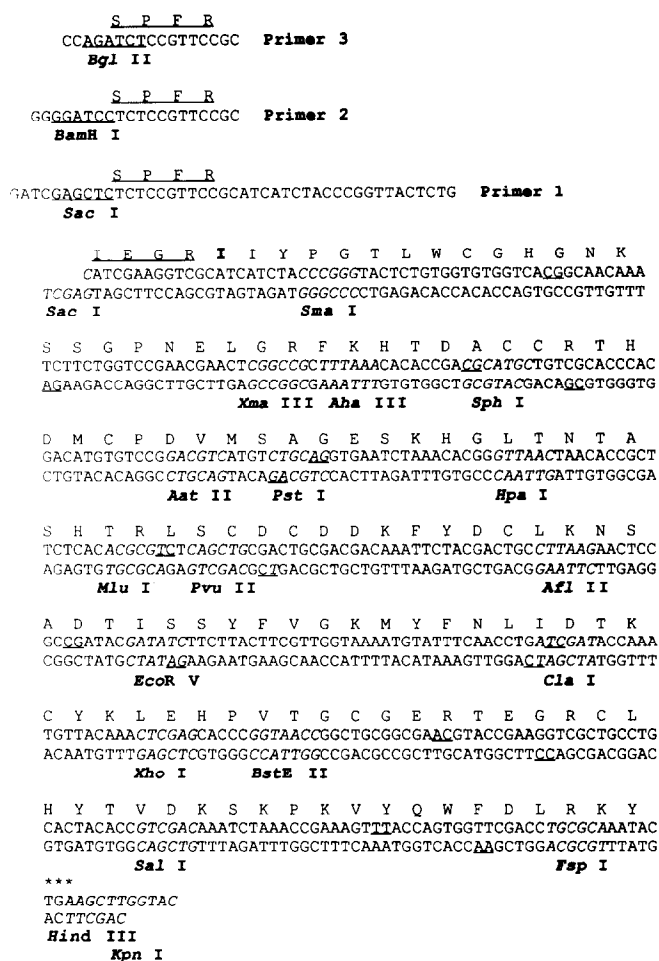


Fig. 1. Synthetic gene for BV-PLA2. Six oligonucleotides were used to separately assemble each of the following three fragments: *Sac*I to *Pst*I, *Pst*I to *Cla*I, and *Cla*I to *Kpn*I. The boundaries between the synthetic oligonucleotides are designated by underscoring pairs of nucleotides where each nucleotide in the pair is at the end of its corresponding fragment. The unique restriction sites are labelled and italicized. Ile-1 of the BV-PLA2 is designated by I, and the stop codon after Tyr-134 is designated by asterisks. The underscored sequence *IEGR* that appears at the beginning is the Factor Xa recognition sequence. The three short single stranded DNA sequences shown at the top are the PCR primers used to incorporate a kallikrein recognition sequence, *SFPR* (primer 1), and to change the restriction site at the end of the gene to either *Bam*HI or *Bgl*II (primers 2 and 3, respectively).

anti-human IgG antibody (TAGO, Burlingame, CA, USA), washed and developed with 100 μ l substrate solution (1.5 mg/ml of 4-nitrophenyl phosphate in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mM MgCl₂) as described [35].

Results and Discussion

Synthesis of a gene coding for BV-PLA2

For high level expression of BV-PLA2 in *E. coli*, a synthetic gene was designed incorporating the preferred codons of prokaryotes [36] (Fig. 1). To facilitate genetic engineering of the encoded protein, several

unique restriction sites were included at regular intervals. The entire gene was constructed by stepwise assembly of three separately synthesized gene segments. For all three gene segments, approx. 1 clone out of 3 had the desired sequence, and the others had typically 1–3 mutations at various locations in the insert. In retrospect, a single round of shotgun ligation to make the entire synthetic gene in one step would probably have resulted in a very low yield of error-free insert. To allow for the isolation of native-like enzyme devoid of the fusion peptide, nucleotide sequences coding for recognition sites of specific proteases were engineered upstream of the amino-terminus of BV-PLA2 (Fig. 1 and below).

Expression of BV-PLA2 in plasmid *pATH 11*

In a first attempt to produce BV-PLA2, the synthetic gene containing a factor Xa recognition sequence directly in front of Ile-1 of BV-PLA2 (Fig. 1) was inserted into the *SacI* and *HindIII* sites of the *pATH 11* expression vector [37]. This expression system produces fusion proteins consisting of 323 residues (approx. 38 kDa) of the amino-terminal fragment of the TrpE gene product (anthranilate synthase; TrpE) fused to the amino-terminus of the inserted gene. Following induction of gene expression, significant production of TrpE-BV-PLA2 fusion protein could be achieved as visualized by the appearance of a protein band of approx. 55 kDa on SDS-PAGE (data not shown). Inclusion bodies were isolated from induced *E. coli* [37] and submitted to the refolding procedure (Materials and Methods). The refolded 55 kDa fusion protein had significant enzymatic activity when assayed with diC₆thio-PM as substrate or with DMPM vesicles. Control extracts from non-induced bacterial cultures had no detectable PLA2 activity (data not shown). The activity of the fusion protein is consistent with the X-ray crystal structure of BV-PLA2, which illustrates that the amino-terminal Ile lies at the outer surface of the enzyme and is not in close contact with the active site residues buried inside the molecule [8]. This is in marked contrast to other structurally characterized PLA2s of the class I/II superfamily, where the amino-terminus forms part of an essential hydrogen-bonded network that involves the active site aspartic acid residue [38–42].

Attempts to cleave the TrpE-BV-PLA2 fusion protein selectively at the engineered recognition site with factor Xa were unsuccessful. SDS-PAGE analysis showed that factor Xa cleaves both the TrpE fragment and the BV-PLA2 internally. No distinct band was detectable at the position of the non-glycosylated BV-PLA2 standard (data not shown).

Expression of BV-PLA2 in *pDS56 / RBSII, 6xHis*

Although a large amino-terminal fusion extension did not markedly impede enzymatic function, it was

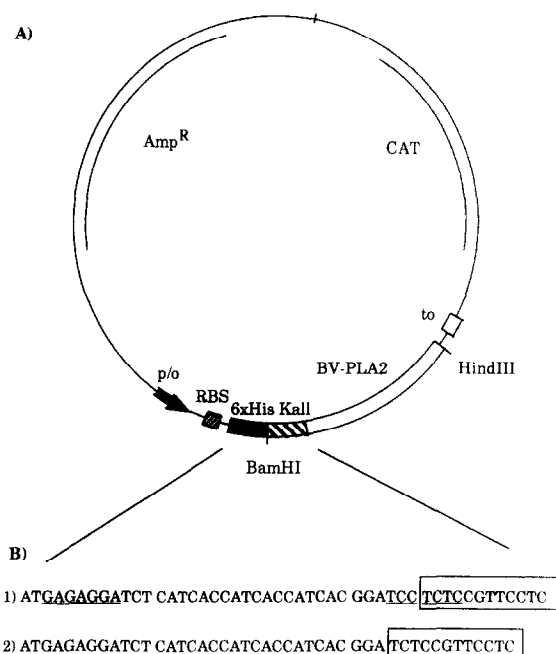


Fig. 2. (A) Schematic representation of the expression constructs p6xHis-Kall-BV-PLA2 #1 and #2. The promoter element (p/o), transcriptional terminator (to), ribosomal binding site (RBS) and the gene segment encoding the amino-terminal fusion peptide (6xHis Kall) are indicated and derived from the expression plasmid. The synthetic gene encoding BV-PLA2 with the kallikrein recognition sequence (Kall) at its N-terminus was inserted into *BamHI* and *HindIII* restriction sites of the expression plasmid. The plasmid confers resistance to ampicillin (Amp^R) and chloramphenicol (CAT). (B) The entire sequence coding for the fusion extension (6xHis Kall) of the non-expressing construct p6xHis-Kall-BV-PLA2 #1 (1) and the expressing construct p6xHis-Kall-BV-PLA2-#2 (2) is depicted. The nucleotides of the non-expression construct (1) involved in the stem loop formation are underlined. The nucleotides encoding the kallikrein recognition sequence are boxed.

desirable to produce shorter fusion proteins suitable for affinity purification. Therefore, plasmid *pDS56/RBSII, 6xHis* was used as an expression vector [29]. This plasmid is designed to generate fusion proteins with six adjacent histidine residues at their amino-terminal end allowing a single step purification using immobilized metal ion affinity chromatography [27,29]. To overcome the problem of internal cleavage by factor Xa, a kallikrein recognition sequence was engineered onto the N-terminus of BV-PLA2 by PCR using primer 1 (Fig. 1). This recognition sequence is found in bradykinogen, a naturally occurring substrate for plasma kallikrein. In a first attempt to produce BV-PLA2 in this expression system, a *BamHI* restriction site was engineered onto the N-terminus of the kallikrein encoding sequence (Fig. 1; primer 2) yielding the expression construct p6xHis-Kall-BV-PLA2-#1 (Fig. 2).

Transformants harbouring this construct were analyzed for fusion protein expression in a test induction assay. None of the 36 independent clones produced

significant amounts of fusion protein. As insert sequencing of several clones revealed no errors, the secondary structure of the RNA was analyzed using the OLIGO computer program. This analysis revealed a strong potential stem loop ($\Delta G = -5.3$ kcal/mol) involving the ribosome binding site (Fig. 2). The seven nucleotides GAGAGGA comprising the guanine of the ATG start triplet were predicted to anneal with the complementary nucleotides TCCTCTC positioned at the 5' end of the inserted gene. As the seven downstream nucleotides involved in the loop formation were encoded by the 5' PCR primer, an altered construct was designed that utilized a different 5'-primer lacking this complementarity. Thus, the Kall-BV-PLA2 gene was isolated using 5'-primer 3 (Fig. 1) and ligated into the pDS56/RBSII, 6xHis expression vector. *E. coli* M15 cells transformed with the resulting construct, p6xHis-Kall-BV-PLA2-#2 (Fig. 2), produce the expected 19 kDa protein band as shown by SDS-PAGE (Fig. 3). Maximal expression is reached 5 h after induction with IPTG. Plasmid DNA from transformants that express appropriately sized protein was isolated and sequenced, and a clone showing the correct sequence was selected for large scale protein production.

Large scale expression, purification and refolding of 6xHis-Kall-BV-PLA2

M15 cells harbouring the selected p6xHis-Kall-BV-PLA2-#2 construct were grown in 1 l of LB-medium and induced with IPTG. When the bacterial cells were ruptured by sonication under non-denaturing conditions, all of the BV-PLA2 fusion protein was recovered in the insoluble pellet. Therefore, the fusion protein was purified by immobilized metal ion affinity chromatography under denaturing conditions [29]. Yields of

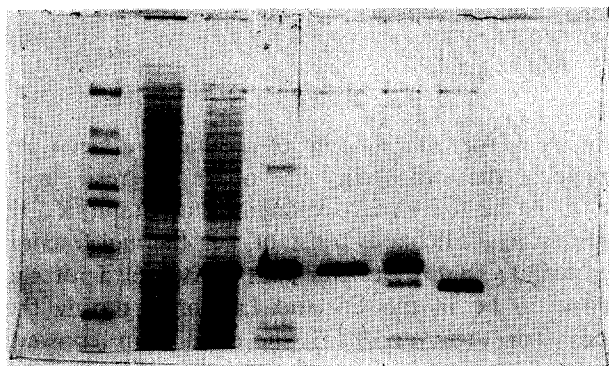


Fig. 3. SDS-PAGE (15%), from left to right: (1) molecular weight markers (66, 45, 36, 29, 24, 20.1, 14.2 kDa); (2) total protein from M15 cell bearing the expression construct p6xHis-Kall-BV-PLA2-#2 grown in the absence of IPTG; (3) same as (2) but the sample was taken 4 h after IPTG addition; (4) BCML-gel purified fusion protein, 1 μ g; (5) SP-Sephadex purified fusion protein, 1 μ g; (6) native BV-PLA2 from Boehringer, 1 μ g; (7) native length recombinant BV-PLA2 after kallikrein digestion and purification, 1 μ g.

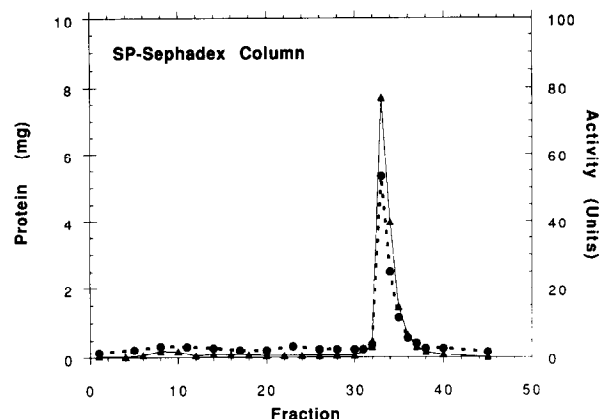


Fig. 4. Purification of the refolded BV-PLA2 fusion protein by cation exchange chromatography. The amount of protein (●) and activity (▲) were determined by A_{280} and the assay with diC₆thio-PM, respectively.

40–50 mg of 6 × His-fusion protein per 1 l of culture, with a purity of > 95% as visualized by SDS-PAGE (Fig. 3), were obtained.

A protocol for refolding BV-PLA2, including the formation of five disulfide linkages, was developed. Sulfonation of the cysteine sulfhydryl groups has been used previously to facilitate refolding of ribonuclease A [43], as well as the PLA2s from bovine and porcine pancreas [44,45] and from human platelets [46]. In the case of the BV-PLA2, only low amounts of enzymatic activity could be recovered after direct refolding of the non-sulfonated protein in a redox buffer containing cysteine and cystine.

During the refolding of sulfonated BV-PLA2, considerable protein precipitation occurred. The amount of protein precipitation is independent of the protein concentration during refolding in the range of 0.1–0.5 mg/ml. No attempt was made to lower the protein concentration below 0.1 mg/ml since BV-PLA2 is known to non-specifically adsorb onto surfaces. Refolding trials over the pH range 5–9 showed that a maximal yield of enzymatic activity is obtained at pH 8.3. In order to obtain an optimal yield of refolded enzyme, it is possible to submit the precipitated protein to two additional rounds of sulfonation and refolding. The refolded protein was purified by cation exchange chromatography. As shown in Fig. 4, the active enzyme eluted from the column toward the end of the buffer gradient. The constant specific activity across the protein peak suggests that most of the enzyme is in the properly folded form. Furthermore, analysis of the purified protein for free sulfhydryl groups using Ellman's reagent gives typically 0.1 mol SH per mol of enzyme, i.e., 1% of the SH content expected if the protein were fully reduced. An overall yield of pure and refolded fusion protein of 24%, based on the amount of protein recovered from chromatography on

TABLE I

Kinetic parameters of recombinant BV-PLA2, BV-PLA2 fusion protein, and native BV-PLA2 for the hydrolysis of DMPM vesicles in the scooting mode

The estimated relative errors are $\pm 20\%$ (N_s and v_o) and $\pm 30\%$ (N_s/k_i).

Enzyme	N_s	$N_s k_i$ (s^{-1})	v_o (s^{-1})
Commercial BV-PLA2	17000	40	150
Kallikrein-cleaved BV-PLA2	19000	37	140
BV-PLA2 fusion protein	22000	45	82

BCML-gel, was obtained. This corresponds to 10–12 mg of BV-PLA2 from a 1 l bacterial culture.

Site-selective cleavage of the fusion protein with kallikrein and isolation of BV-PLA2 with its native N-terminus

Although the refolded fusion protein has similar activity to that of the native PLA2 isolated from bee venom (Table I), for some purposes, such as structural studies by X-ray crystallography and NMR, it is advantageous to remove the N-terminal 15 amino acids. Therefore, the fusion protein was cleaved with human plasma kallikrein. Although the unfolded fusion protein could be cleaved at the desired site (not shown), this approach was not practical due to the limited solubility of the unfolded fusion protein or its sulfonated form in a variety of buffers at different pH values. Instead, it was possible to cleave the refolded and purified fusion protein with kallikrein in high yield. The kallikrein treatment was attempted using different ratios of kallikrein to fusion protein, different reaction times, and different concentrations of fusion protein. For example, it was found that treatment of a solution of 0.2 mg/ml fusion protein with 0.18 units of kallikrein per mg of fusion protein led to complete cleavage as judged by SDS-PAGE, and this was also true if the cleavage was carried out using a 4-fold higher concentration of fusion protein and 25% as much kallikrein. This indicates that the K_M for the fusion protein is larger than the concentrations used in these digestions (60 μM). Furthermore, non-specific cleavage of the fusion protein was not detected by SDS-PAGE. The cleavage product comigrates with the non-glycosylated isoform present as a minor component in commercial BV-PLA2 preparations (Fig. 3). Finally, the BV-PLA2 with its native N-terminus can be recovered from the kallikrein digestion in 92% yield after passage through a small column of BCML-gel to remove trace amounts of fusion protein and passage through a small anion exchange column to remove the kallikrein. This results in a final yield of 8–9 mg of active enzyme per l of culture. The residual kallikrein activity can also be recovered from the anion exchange

column in near quantitative yield by elution with buffer containing high salt.

It is also possible to cleave the fusion protein with commercially available (Sigma) porcine pancreatic kallikrein. For example, digestion of 20 μg of fusion protein with $8 \cdot 10^{-5}$ units of pancreatic kallikrein in a volume of 100 μl of kallikrein buffer for 16 h at room temperature gave rise to a significant amount of native BV-PLA2 as seen by SDS-PAGE. However, a small amount of further digested material was seen as a single band on SDS-PAGE that migrated just ahead of the native enzyme. No attempt was made to resolve the two protein cleavage products.

Kinetic characterization of recombinant BV-PLA2s

The kinetic properties of the purified recombinant BV-PLA2s as well as the commercial enzyme were evaluated on vesicles of DMPM in the scooting mode [47]. In this assay, the course of the reaction is followed, using a pH-stat, by titrating the myristic acid that is formed in the vesicles upon hydrolysis of the phospholipid by PLA2 [23]. Under the reaction conditions used, BV-PLA2 binds essentially irreversibly to anionic vesicles and hydrolyzes all of the phospholipids in the outer monolayer of the vesicles without leaving the surface and without loss of the vesicle integrity. In the presence of excess vesicles over enzymes and the absence of vesicle fusion, the enzymatic reaction ceases when all of the substrate in the outer monolayer of the enzyme-containing vesicles is consumed.

In small sonicated DMPM vesicles containing one bound enzyme, the mole fraction of substrate changes rapidly in time so that the velocity at each point in time is decreasing (first-order Michaelis-Menten kinetics). Under these conditions, the turnover is described by Eqn. (1):

$$N_s k_i = \frac{k_{cat}}{K_M^* \left(1 + \frac{1}{K_P^*} \right)} \quad (1)$$

Here, N_s is the total amount of product produced per enzyme at the end of the first-order progress curve, and k_i is the first-order exponential constant. K_M^* is the interfacial Michaelis constant, i.e., the mol fraction of substrate in the vesicles that causes 50% of the enzyme to be in the ES and EP forms during the steady state turnover. K_P^* is the equilibrium constant for the dissociation of products of the reaction from the enzyme in the interface. The constant k_{cat} is the maximal turnover number for the enzyme operating on DMPM vesicles.

If the number of vesicles is in excess over the number of enzymes, there will be at most one bound enzyme per vesicle. Under this condition, N_s is also equal to the number of substrate molecules in the

outer monolayer of a single vesicle, provided that all the enzyme molecules are active. As the parameter N_s is independent of the catalytic turnover of the enzyme but only depends on the size of the vesicles and the number of active enzymes in the reaction [47], the first-order scooting analysis is particularly useful for determining the fraction of BV-PLA2 that is folded into the catalytically active native structure. The calculation assumes that the BV-PLA2 is active as a monomeric enzyme, and this has been experimentally verified [48]. As shown in Table I, the N_s values of the BV-PLA2 fusion protein, the kallikrein treated recombinant enzyme, and the commercial enzyme are all similar in magnitude. Furthermore, reaction progress curves for all three enzymes can be fitted to a first-order curve described by a single exponential constant. These data suggest that there is only one species of catalytically active enzyme in the recombinant BV-PLA2 preparations and that essentially all of the protein is properly folded. Finally, the similar magnitudes of $N_s k_i$ for all three enzymes indicates that the kinetic parameters in Eqn. (1) for these enzymes are similar. Apparently, the 15 extra amino acids present on the N-terminus of the fusion protein do not noticeably effect the reaction kinetics on DMPM vesicles in the scooting mode. Finally, the kinetic data suggest that the glycosylation of BV-PLA2 does not play an obligatory role in the lipolysis reaction.

Kinetic studies with DMPM vesicles in the presence of polymyxin B were also carried out. This agent catalyzes the exchange of phospholipids between vesicles [24]. In the presence of polymyxin B, the reaction progress curves are no longer first-order since the lipid exchange maintains the mol fraction of substrate in the enzyme-containing vesicles near unity, and thus, a constant initial velocity per enzyme, v_o , is observed. The experimental values of v_o for the recombinant and commercial BV-PLA2s are listed in Table I. The results show that the values of v_o for both the kallikrein-cleaved recombinant and commercial enzymes are similar, but a 2-fold lower value is seen with the fusion protein.

Immunological characterisation of recombinant 6xHis-BV-PLA2

To analyze the antigenic properties of recombinant BV-PLA2, the binding of recombinant and native phospholipase to serum antibodies using inhibition immunosorbent assays was compared. Binding inhibition assays rather than direct immunosorbent assays were used to avoid denaturation of the antigen on a hydrophobic surface [49]. As shown in Fig. 5, the binding of serum IgG antibodies from bee keepers to refolded, recombinant BV-PLA2 is indistinguishable from their binding to native BV-PLA2, demonstrating the native-like folding of the recombinant protein. Interestingly,

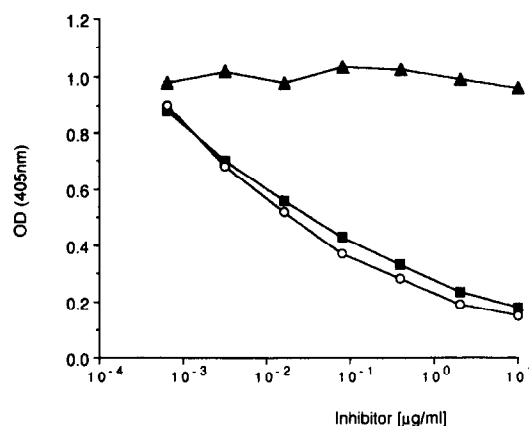


Fig. 5. Inhibition of IgG binding to solid phase-coated native BV-PLA2. As inhibitors native (○), recombinant refolded (■) and recombinant not-refolded (▲) BV-PLA2 were used.

unfolded and sulfonated recombinant BV-PLA2 showed no significant binding indicating the conformation specificity of most of the serum antibodies. Experiments performed with IgE antibodies from sera of bee venom allergic individuals yielded similar results (data not shown). For all these binding studies recombinant 6xHis fusion protein was used. The data indicate that the 6xHis fusion extension does not noticeably interfere with antibody binding. Hence, the uncleaved fusion protein may also be suitable for epitope mapping.

The availability of large quantities of homogeneous recombinant BV-PLA2 together with the recently determined X-ray crystal structure of the enzyme at 2.0 Å resolution [8] will facilitate studies on the structure/function relationship of this enzyme as well as a better understanding of the allergenic response induced in sensitive individuals. A combination of site-directed mutagenesis, biophysical approaches, and immunological studies can now be performed.

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