Biochemical, Kinetic and Biological Properties of Group V Phospholipase A2 from Dromedary

Mona Alonazi 1, Aida Karray 2, Raida Jallouli 3 and Abir Ben Bacha 1,4,*

1 Biochemistry Department, Science College, King Saud University, P.O. Box 22452, Riyadh 11495, Saudi Arabia; moalonazi@ksu.edu.sa
2 Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS Route de Soukra, Université de Sfax-Tunisia, Sfax 3038, Tunisia; aida.karray@enis.tn
3 Institut de Pharmacologie de Sherbrooke, Université de Sherbrooke, Sherbrooke, QC J1H 5N4, Canada; jallouliraïda@yahoo.fr
4 Laboratory of Plant Biotechnology Applied to Crop Improvement, Faculty of Science of Sfax, University of Sfax, Sfax 3038, Tunisia
* Correspondence: aalghanouchi@ksu.edu.sa; Tel.: +966-504-784-639

Abstract: Secretory group V phospholipase A2 (PLA2-V) is known to be involved in inflammatory processes in cellular studies, nevertheless, the biochemical and the enzymatic characteristics of this important enzyme have been unclear yet. We reported, as a first step towards understanding the biochemical properties, catalytic characteristics, antimicrobial and cytotoxic effects of this PLA2, the production of PLA2-V from dromedary. The obtained DrPLA2-V has an absolute requirement for Ca2+ and NaTDC for enzymatic activity with an optimum pH of 9 and temperature of 45 °C with phosphatidylethanolamine as a substrate. Kinetic parameters showed that $K_{cat}/K_{m}^{app}$ is $2.6 \pm 0.02 \text{ mM}^{-1} \text{s}^{-1}$. The enzyme was found to display potent Gram-positive bactericidal activity (with IC50 values of about 5 µg/mL) and antifungal activity (with IC50 values of about 25 µg/mL) in vitro. However, the purified enzyme did not display a cytotoxic effect against cancer cells.

Keywords: phospholipase V; kinetics; characterization; biological activities

1. Introduction
Phospholipases A2 (PLA2) are a family of enzymes that hydrolyze the ester bond at the sn-2 position of phospholipids generating free fatty acids and lysophospholipids [1]. This family includes a number of secreted PLA2s (sPLA2s) referred to as group IB (GIB), GII (subgroups A–F), GIII, GV, GX and GXII (subgroups A–B) [2]. Clearly, the different mammalian sPLA2s are not isoforms, since only 15% of their primary sequences are identical [3–5]. They have distinct enzymatic properties [6,7] and show different tissue distribution patterns in both mice and humans. Consequently, in various tissues, the different sPLA2s may exert distinct biological functions that may be dependent or independent of their enzymatic activities [3,6,7]. In addition, in the same cell, the expression of the various isoforms may be differentially regulated by such events as differentiation or activation. Therefore, the profile of sPLA2s secreted in inflamed tissues can vary according to the type of inflammation and of infiltrating cells. Most sPLA2s are stored within inflammatory cells and are released in the extracellular environment upon appropriate cell activation [1,2]. Thus, large quantities of sPLA2s are released in plasma and biological fluids during local or systemic inflammation [8].

Group V sPLA2 has been cloned from chicken [9], human, rat, and mouse species [10]. Unlike group I and II sPLA2s, this sPLA2 has only six disulfides and does not have the group I- or group II-specific disulfides, thus defining a novel group of sPLA2s [11]. This sPLA2 has a higher level of identity with group IIA sPLA2s, as compared to group IB.
sPLA₂. It neither has a propeptide sequence, indicating its closer relationship with group II sPLA₂s. sPLA₂ (group I/II/V/X) are closely related molecules with low molecular weight, 14–19 kDa, and possess very high structural conservation. All of these sPLA₂s possess a Ca^{2+} binding loop and a catalytic dyad formed by His/Asp, as well as conserved disulfide bonds, while atypical sPLA₂s (group III/XII) each form a distinct class [4,12–14]. In humans, the study of the structure–function relationship of sPLA₂ isoforms is important for a better understanding of the pathology of diseases related to these enzymes. sPLA₂ strictly hydrolyzes fatty acyl esters at the 2-position of the glycerophospholipid and exhibits substrate specificity in terms of polar or fatty acid headgroups at the sn-2 position [7]. For example, sPLA₂-X is highly active on neutral phosphatidylcholine (PC), whereas sPLA₂-IIA has much greater affinity for charged phospholipid head groups, especially phosphatidylycerine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). This preference is useful for understanding the role of sPLA₂-IIA as defensive proteins, acting on PE and PG, which are major components of bacterial membranes [15]. Mammalian sPLA₂-V has a preferential expression level in the heart and a much lower expression level in lungs and liver [16]. In humans, the ability of sPLA₂-V to regulate phagocytosis is specific and not shared with cytosolic PLA₂ alpha (cPLA₂α) nor sPLA₂-IIA [17].

The common of sPLA₂ isoforms are up-regulated by proinflammatory stimuli such as bacterial lipopolysaccharide (LPS), which largely increases the expression of sPLA₂-V. Additionally, it has been shown that sPLA₂-V is considered as a significant messenger in the regulation of cell migration. Indeed, Lapointe et al. [18] investigated the effect of sPLA₂-V on LPS-mediated leukocyte recruitment supporting the involvement of sPLA₂-V in the development of inflammatory innate immune response and its capacity to modulate adhesion molecule expression. Indeed, immunohistochemistry studies showed that sPLA₂-V is expressed in the airways of patients with pneumonia but not those of normal individuals [19]. Moreover, it was shown that activated cells secrete sPLA₂-V which exert transcellular lipolytic activity on neighbouring inflammatory cells [20]. The elevation of sPLA₂-V expression in mice lungs with severe inflammation can be associated with an ongoing surfactant hydrolysis often observed in lung dysfunction [21]. Interestingly, sPLA₂-V is involved in the innate immune response against bacteria and fungi: it is involved in the phagocytosis reaction and in lysis following a mechanism dependent on the fusion of phagosomes [17].

Until now, only a few studies were focused on the regulation and biological roles of phospholipases A₂ from dromedary [22,23]. Accordingly, the present work was undertaken to further investigate the biochemical and antibacterial properties of dromedary non-digestive PL₂ to compare them with known PL₂-IIA and to gain further insights onto their mode of action with regard to phospholipids. This study also reports, for the first time, on the purification, characterization, and antibacterial activities of a novel PL₂-V secreted from the heart of dromedary.

2. Results

2.1. Biochemical Properties of DrPL₂-V

As described previously, sPLA₂-V has been shown to be principally implicated in the inflammatory processes [24], but the biochemical and enzymatic properties of this essential enzyme have been indistinct. In order to gain further insights onto its mode of action with regard to phospholipids, we reported the enzymatic catalysis and the biological functions of this PL₂, purified from dromedary heart tissue.

PL₂-V is purified and characterized from the dromedary heart (delipidated powder). The purification flow sheet presented in Table 1 showed that the specific activity of pure DrPL₂-V reached 115 U/mg when phosphatidylethanolamine (PE) was used as a substrate at pH 9 and 45 °C, in the presence of 8 mM NαDC and 4 mM CaCl₂. The DrPL₂-A purification yield was around 44% of the total initial activity, a value which is comparable to what was observed with the dromedary, porcine and ostrich PL₂-IIB [23,25].
Table 1. Flow sheet of DrPLA2-V purification.

| Purification Step          | Total Activity (Units) | Protein (mg) | Specific Activity (U/mg) | Activity Recovery (%) | Purification Factor |
|----------------------------|------------------------|--------------|--------------------------|-----------------------|---------------------|
| Extraction                 | 300                    | 2950         | 0.1                      | 100                   | 1                   |
| Heat treatment at 70 °C for 10 min (NH4)2SO4 Precipitation (20–65%) | 236                    | 73.7         | 3.2                      | 78.7                  | 32                  |
| RP-HPLC                    | 132                    | 1.15         | 115                      | 44                    | 1150                |

1 Unit: µmole of fatty acid released per min using phosphatidylethanolamine as a substrate in the presence of 8 mM NaDC and 4 mM CaCl2.

The procedure described and summarized in Table 1 is more rapid than those used previously to purify another mammalian pancreatic phospholipase A2. In fact, the enzyme was purified after a heat treatment at 70 °C, and ammonium sulphate precipitation (20–65%), followed by only one chromatographic step (Figure 1A) whereas in the case of the dromedary, porcine or ostrich pancreatic PLA2 four chromatographic steps were needed [25]. The molecular mass of the purified enzyme was 14 kDa to secrete PLA2 (Figure 1B).

![Figure 1. (A). Chromatography on RP-HPLC column of the purified DrPLA2-V from dromedary heart.](image)

G-LLELKSMEIKV VGKSAVKSYGFCGCGWGGGRGTPKDATDWCCWIHDHCY (Current study)
G-LLELKSMEIKV TGKSAVSYGYFCGCGWGGGRGTPKDATDWCCVHDCY (i)
GGLLDKSMIEKTGKNALTNYGFYGCYCGWGGGRGTPKDATDWCCWAHDHCY (ii)
G-LLELKSMEIKV TRKNAFKNYGFYGCYCGWGGGRGTPKDATDWCCQMHDRCY (iii)
from 0% to 100% solvent B at a flow rate of 1 mL/min. Solvent A is composed of water/trifluoroacetic acid TFA (1000:1, v/v) and solvent B contained 100% acetonitrile. The gradient is indicated by the dotted line. The absorbance was measured at 280 nm. AU: Arbitrary Units. (B) 15%-SDS-PAGE of pure DrPLA2-V. Lane 1, molecular mass markers (kDa); lane 2, 10 µg of purified DrPLA2-V. (C) NH2 sequence alignment of DrPLA2-V, Minioptera family (Miniopterusnatalensis) (XP_039084994.1) (i), human family (homo sapiens) (NP_000920.1) (ii), and Hyaenida family (Hyaena hyaena) (XP_039084994.1) (iii). Identical amino acids are shown in red.

The NH2-terminal sequencing permitted clearly the detection of 44 residues of the pure enzyme: GLLELKSLIEKVYGKYGCYCGWGGRTPKDAWDCCWHDCY. The N-terminal sequence alignment of sPLA2-V showed a high degree of homology with those of Minioptera family (Miniopterusnatalensis) (XP_016070213.1) [26], human family (homo sapiens) (NP_000920.1) [27], and Hyaenida family (Hyaena hyaena) (XP_039084994.1) [28] of about 92%, 84% and 82%, respectively (Figure 1C).

The purified DrPLA2-V was found to be stable between pH 4.0 and 12.0. In contrast, the enzyme was found to lose almost its full activity when incubated at pH 2. It was also reported that dromedary [22], stingray [29], porcine [30] rat [31] and human [32] intestinal PLA2s are stable at low pH values. Unlike pancreatic DrPLA2-IB, which is completely denatured at high temperature, the DrPLA2-V maintained about 80% of its activity when incubated for 60 min at 60 °C (Figure 2). Similar observations were obtained previously with intestinal PLA2 from various mammal species showing high stability at elevated temperatures [33]. These results were obtained when we used the pH-stat method (with emulsified phosphatidylcholine (PC) as substrates).

Figure 2. Evaluation of pH and temperature effect on activity (A,B) and stability (C,D) of DrPLA2-V.
As all secreted PLA$_2$, the Ca$^{2+}$ ions are essential for DrPLA$_2$-V to express its full activity, with an optimum at 4 mM (Figure 3A). All the divalent ions tested were unable to express the full specific activity of the enzyme. Figure 3B shows that both NaTDC and NaDC were required to express the maximal activity at concentrations of 4 and 6 mM, respectively.

The purified PLA$_2$ displayed better functional stability in the presence of polar solvents after an incubation time of 2 h, compared to the control test (Figure 4). It reaches 105% of its activity in presence of acetonitrile, 100% in the presence of methanol and 2-propanol and 124% in the presence of ethanol. In fact, it has been proved that organic solvents are advantageous in various industrial enzymatic processes since their use can increase the solubility of non-polar substrates, the thermal stability of enzymes, or eliminate microbial contamination [34].

![Figure 3](image_url)

**Figure 3.** Effect of calcium ions (A), and surfactant (B), on DrPLA$_2$-V activity. The incubation time with the appropriate agent was for a period of 60 min and the remaining phospholipase activity was evaluated at the optimal conditions.

The purified PLA$_2$ displayed better functional stability in the presence of polar solvents after an incubation time of 2 h, compared to the control test (Figure 4). It reaches 105% of its activity in presence of acetonitrile, 100% in the presence of methanol and 2-propanol and 124% in the presence of ethanol. In fact, it has been proved that organic solvents are advantageous in various industrial enzymatic processes since their use can increase the solubility of non-polar substrates, the thermal stability of enzymes, or eliminate microbial contamination [34].

![Figure 4](image_url)

**Figure 4.** Effect of organic solvents on DrPLA$_2$-V stability. Enzyme was incubated with the appropriate agent for 1 h (A) and 2 h (B) and the remaining phospholipase activity was tested at the optimal conditions.
2.2. Kinetic Parameters Determination of The PLA2-V from Dromedary Using Phospholipids (PL) of Different Head Groups

Then, the kinetic properties of DrPLA2-V (tested with three different phospholipids head groups) using the emulsified system were studied. The data obtained (summarized in Table 2) showed the clear capacity of DrPLA2-V to hydrolyze PE compared to DrPLA2-IB with $V_{max}$ value of 115 ± 3.5. The latest enzyme shows a clear preference for the zwitterionic substrate: PC. Less affinity was observed with PC with a catalytic constant value of 20.3 ± 0.7 compared to that obtained with PE (26.9 ± 1.2). Whereas, phosphatidylserine (PS) showed the lowest specific activity with a specific activity of 32 U/mg ± 1.2. This observation is confirmed by the activity of group V PLA2 from stingrays which hydrolyze PE (72 U/mg ± 1.5) and PC (52 U/mg ± 3.5) substrate more efficiently than PS substrate (18 U/mg ± 0.7) [35]. Besides, human heart sPLA2-V preferentially hydrolyzes PE vesicles compared to PC vesicles [36].

Table 2. Apparent kinetic parameters of DrPLA2-V.

| Substrate | $V_{max}$ (U/mg) | $K_m$ (mM) | $K_{cat}$ (s⁻¹) | $K_{cat}/K_m$ (mM⁻¹ s⁻¹) |
|-----------|-----------------|------------|-----------------|-------------------------|
| PE        | 115 ± 3.5       | 10.5 ± 0.7 | 26.9 ± 1.2      | 2.6 ± 0.02              |
| PC        | 87 ± 2.1        | 12.7 ± 0.3 | 20.3 ± 0.7      | 1.6 ± 0.03              |
| PS        | 32 ± 1.2        | 21.3 ± 1.1 | 7.5 ± 0.3       | 0.3 ± 0.01              |

2.3. Bactericidal Properties, Antifungal and Cytotoxic Effect of Dromedary PLA2-V

The antimicrobial activity of the purified DrPLA2-V against Gram+ and Gram- bacteria was evaluated in the current study and its effectiveness was qualitatively and quantitatively determined (detection of the inhibition zones, IC50 and MIC values). Results are summarized in Table 3.

Table 3. Antimicrobial activity of DrPLA2-V on bacterial and fungal strains.

| Strains | Inhibition Zone (mm) | IC50 (µg/mL) | MIC (µg/mL) |
|---------|----------------------|--------------|-------------|
| Gram (+) Bacteria | DrPLA2-V | Ampicillin/ Cycloheximide | |
| B. cereus (ATCC 14579) | 18 ± 1 | 22 ± 1 | 6 | >12 |
| B. subtilis (ATCC 6633) | 15.4 ± 0.6 | 25 ± 1 | 3.6 | >6 |
| L. monocytogenes (ATCC 19111) | 18.7 ± 0.5 | 21 ± 10.7 | 3.2 | >6 |
| E. faecium (ATCC 19433) | 14.3 ± 1.2 | 18.5 ± 0.3 | 4.9 | >9 |
| S. pyogenes (ATCC 21059) | 12 ± 0.5 | 15.5 ± 0.2 | 6.1 | >15 |
| S. aureus (ATCC 25923) | 18 ± 0.7 | 21.5 ± 1.4 | 5.2 | >12 |
| S. epidermidis (ATCC 14990) | 15.3 ± 0.6 | 26 ± 0.5 | 3 | >9 |
| S. xylosus (ATCC 700404) | 16.9 ± 1.3 | 24 ± 1.2 | 2.9 | >6 |
| Gram (-) Bacteria | | | |
| E. coli (ATCC 25966) | - | 22.6 ± 1.5 | - | - |
| P. aeruginosa (ATCC 27853) | - | 20 ± 0.7 | - | - |
| E. aerogenes (ATCC 13048) | - | 25 ± 1.2 | - | - |
| S. enteric (ATCC 43972) | - | 19.5 ± 0.3 | - | - |
| Fungi | | | |
| A. niger | 11.2 ± 0.3 | 28 ± 0.6 | 21 ± 1.5 | >75 |
| B. cinerea | 9 ± 0.1 | 29 ± 1 | 31.7 ± 2.4 | >90 |
| F. solani | 15 ± 0.7 | 27.5 ± 0.7 | 25 ± 2.1 | >60 |
| P. digitatum | 7 ± 0.2 | 21 ± 0.5 | 35 ± 3.5 | >90 |

The inhibition zones were obtained only against Gram+ bacteria and ranged from 12 mm ± 0.5 (against S. pyogenes (ATCC 21059)) to 18 mm ± 0.7 (against L. monocytogenes (ATCC 19111) and S. aureus (ATCC 25923)). IC50 values were nearly the same 3–6 µg/mL. Gram- bacteria were resistant to the action of DrPLA2-V. The current enzyme is much more effective than marine group V-PLA2 showing IC50 values between 15–25 µg/mL [37]. Both enzymes were inactive against Gram- bacteria. Interestingly, the antifungal effect observed
in the present study shows IC50 values nearly above 25 µg/mL against all the tested strains. The enzyme was less effective against fungi than against Gram+ bacteria (Table 3).

Previously, it was reported that the antibacterial effect is strongly correlated with the enzymatic hydrolyze of the phospholipid bacterial cell membranes. The PLA2-V is able to break into the cell wall of Gram-positive bacteria [38]. Its efficiency to act against Gram-positive bacteria is basically affected by the charge of the overall cation on the surface of the enzyme molecule [39].

When we moved to the analysis of the cytotoxic effect of the dromedary V-PLA2 we noticed that the proportion of viable Lovo, HCT-116, or MDA-MB-231 cells in experimental conditions which contain 50 µg of group V, sPLA2, and calculated after treatment of 24-h-period, was constantly more than 85% (Figure 5). No difference was seen when we increased enzyme concentration to 200 µg of the pure enzyme. Thus, we can conclude that V-PLA2 is the noncytotoxic enzyme, like all sPLA2. This result is confirmed by [35].

![Graph](image.png)

**Figure 5.** Cytotoxic potency of DrPL2-V on Lovo, HCT-116, and MDA-MB-231 cells. Cytotoxicity was assessed using the MTT assay by incubating cells for 24 h with various concentrations (25, 50, 100, and 200 µg) of DrPLA2-V.

3. Discussion

PLA2 catalyses the glycerophospholipids at the sn-2 position, generating free fatty. To date, the sPLA2 are classified into 10 catalytically active enzymes in mammals, and are characterized by low-molecular-weight and Ca2+-requiring extracellular enzymes. Each sPLA2 showed a distinctive expression profile in all cell types within restricted tissues. As described previously in cellular studies, sPLA2-V revealed their involvement in the inflammatory processes [24], but the biochemical and enzymatic properties of this current enzyme have been poorly documented until now. We report, as the first step towards understanding the structure, function and regulation of this PLA2, the production and characterization of DrPLA2-V. Evaluation of the antimicrobial effect of the enzyme and its cytotoxicity is also studied.

Unlike PLA2-IB and PLA2-X, characterized with the presence of a propeptide cleaved by an endogenous trypsin in order to produce a mature and active enzyme, we reported here that no significant increase in the DrPLA2-V activities was observed throughout 1 h homogenization with endogenous trypsin (data not shown). Moreover, it was noted that PLA2-V lost its full activity after an addition of trypsin at a final concentration of 20 g/mL. These observations permit to suggest that an accessible site of trypsin cleavage is present in the PLA2-V primary sequence. A total of 50 g of dromedary heart mucosa (6 U/g of heart tissue) was obtained using 50 mL of 25 mM Tris–HCl pH 8 with 4 mM benzamidine and 150 mM NaCl. The purification steps consist of a heat treatment for 10 min at 70 °C, followed by sulphate fractionation (20–65%). The obtained precipitate is dialyzed against the same buffer after repeated changes and then loaded onto C18 HPLC column pre-equilibrated with 0.1% TFA in water and then eluted with an acetonitrile linear gradient.
0–80%. After the purification procedure of the PLA2, the analyzed fractions on SDS-PAGE indicate that the current enzyme (named DrPLA2-V) presents an apparent molecular mass of about 14 kDa. The specific activity of pure DrPLA2-V reaches 115 U⋅mg⁻¹ when PE was used as substrate at pH 9, 45 °C and in the presence of 4 mM CaCl₂ and 8 mM NaDC, a value which is comparable to that observed with the PLA2-V from chicken or stingray with a specific activity of 156 or 52 U⋅mg⁻¹, respectively, measured on the same substrate [9]. The DrPLA2-V purification yield was about 44% the total initial activity (Table 1).

N-terminal sequence of DrPLA2 showed a high level of identity with those of the sPLA2-V from other species. The purified enzyme showed pH stability between pH 4.0 and 12.0 and maintained about 80% of its activity after 60 min of incubation at 60 °C. Comparable results were obtained previously with mammalian PLA2-V from various species showing a good stability at high temperature [40] Moreover, CaCl₂ (4 mM) was found as the best activator of the PLA2 activity of pure DrPLA2-V, followed by Mg²⁺ (combined with 1 Mm CaCl₂). All crystal structures of sPLA2 have a ‘calcium binding loop’ in the protein [41] and the calcium dependence of the group V PLA2 is similar to that of the human group IIA PLA2 [24].

We next studied the kinetic properties of DrPLA2-V $K_{\text{m app}}$, $K_{\text{cat}}$ and the deduced catalytic efficiency ($K_{\text{cat}}/K_{\text{m app}}$) of the purified group-V, using charged PE, zwitterionic PC or PS as substrate using Lineweaver–Burk plots. The data obtained (Table 2) showed the clear capacity of DrPLA2-V to hydrolyze the negatively charged substrate PE ($K_{\text{m app}}$ 115 ± 3.5) compared to the zwitterionic substrate PC ($K_{\text{m app}}$ 87 ± 2.1) and PS ($K_{\text{m app}}$ 32 ± 1.2). Our results clearly demonstrated that the enzyme hydrolyzes PE and PC substrate more efficiently than PS substrate since it presented a catalytic efficiency ($K_{\text{cat}}/K_{\text{m app}}$) eight or five times higher than those obtained with using PE as substrates. The same trend was observed using PE or PC as a substrate. This result is in line with Chen and Dennis [36] who have also demonstrated that human heart sPLA2-V preferentially hydrolyzes PE vesicles compared to PC vesicles. Likewise, ref. [35] reported that stingray PLA2-V hydrolyses the zwitterionic PE and PC substrates more efficiently than anionic PS substrate.

Furthermore, proinflammatory stimuli such as bacterial LPS, cause an up-regulation of the majority of sPLA2 isoforms, and thus predominantly increase the expression of sPLA2-V. Besides, it has been recently shown that sPLA2-V is a critical messenger in the regulation of cell migration and has a specific function related to phagocytosis [17]. In the current study, we have demonstrated a very effective Gram-positive bactericidal activity for DrPLA2-V, producing an inhibition zone of 18 mm against B. cereus, S. aureus and L. monocytogenes compared to the control Ampicillin producing an inhibition zone ranging from 20 to 26 mm. Contrary, Dr PLA2-V was inactive against E. coli and against Gram− bacteria. Besides, the antiinflammatory effect of the purified enzyme is attributed to its phagocytosis role against pathogenic strains: bacteria and fungi. Previous results showed that macrophages from sPLA2-V−/− mice stimulated with zymosan (a complex of proteins and carbohydrates extracted from the membrane of yeast cells) produced 50% less leukotriene C4 and prostaglandin 2 than normal mouse macrophages, and also show a 50% reduction in their phagocytic capacity [17]. As a result, sPLA2-V is involved in the innate immune response against fungi: it is involved in the phagocytosis reaction and in lysis following a mechanism dependent on phagosome fusion. However, to date, the regulation of fungal phagocytosis by sPLA2-V is not yet well-detailed. In fact, we showed here a positive effect against all the tested fungi strains. Whereas, the current study indicated that DrPLA2-V did not affect any lines of human cancer cells (HCT-116, MDA-MB-231 and Lovo) [42] These results are in lines with all secreted PLA2 tested on normal and cancer cells, suggesting that all sPLA2s are noncytotoxic enzymes.

4. Materials and Methods

4.1. Phospholipase Activity and Protein Concentration Determination

Phospholipase activity was measured titrimetrically according to Abousalham and Verger [43] with a pH-stat using a crude egg yolk, PC, PE or PS emulsions as a substrate in
the presence of 8 mM NaDC and 4 mM CaCl$_2$ at optimal conditions (pH 9 and at 45 °C). A total of 1 µmol of fatty acid released per minute is equivalent to one unit of phospholipase activity. Protein content was determined according to the Bradford (1976) method [44] using bovine serum albumin ($E_{1%1cm}^\text{1%1cm} = 6.7$) as a reference.

4.2. Group V DrPLA$_2$ Purification

Heart collection and phospholipase homogenization: Fresh heart tissue of dromedary was collected immediately after slaughter (Riyadh, Saudi Arabia) and kept at −20 °C. The soluble extract obtained from 50 g of dromedary heart mucosa (6 U/g of heart tissue) was obtained using 50 mL of 25 mM Tris–HCl pH 8 with 4 mM benzamidine and 150 mM NaCl followed by a centrifugation at 25,600 × g during 20 min.

Heat treatment: The homogenate (300 U) was incubated for 10 min at 70 °C, rapidly cooled, and then centrifuged during 40 min at 25,600 × g.

Ammonium sulphate precipitation: The clear supernatant obtained containing 78.7% (236 U) of the initial activity was subjected to ammonium sulphate fractionation (20–65%). The precipitates were resuspended in the extraction buffer and dialyzed against repeated changes in the same buffer (after 4, 8 and 12 h) for 24 h at 4 °C.

C-18-HPLC chromatography: Thereafter, the dialyzed sample was loaded on a C18 HPLC column (250 × 4.6 mm, 5 mm; Beckman, Fullerton, CA, USA) pre-equilibrated with 0.1% TFA in water and then eluted with an acetonitrile linear gradient 0–80% at a flow rate of 1 mL/min over 60 min. The active fractions were analyzed with 15%-SDS-PAGE according to Laemmli [45], while the PLA$_2$ activity was monitored as described above. The N-terminal sequence was determined automatically with Edman’s degradation, using an Applied Biosystems Protein Sequencer Procise 492 equipped with 140 C HPLC system [46].

5. Biochemical Properties

5.1. pH and Thermal Activity and Stability of DrPLA$_2$

The pH and thermal activity of phospholipase were measured on a crude egg yolk emulsion as substrate at pH values (6–11) or temperatures (20 to 60 °C), respectively.

Additionally, the pH and thermal stability were measured at extreme pH and temperature values by incubating the same amount of pure enzyme at different pH (2–13) or temperature values (20–70 °C) for 1 h, respectively. The residual activity was determined under standard assay conditions.

5.2. Effect of Metal Ions and Surfactant (NaDC/NaTDC) on DrPLA$_2$-Vactivity

The hydrolysis rates of the PC egg yolk emulsion by PLA$_2$ were measured in the presence of Ca$^{2+}$ at different concentrations from 0 to 10 mM at pH 9 and at 45 °C while the effects of 10 mM divalent metal ions (Cd$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, or Zn$^{2+}$) on the enzyme activity were evaluated with the presence of 1 mM Ca$^{2+}$.

Furthermore, the rate of hydrolysis of PC by DrPLA$_2$-V with concentrations ranging from 0 to 10 mM of natural surfactant NaDC or NaTDC, at pH 9 and at 45 °C was also studied.

5.3. Effect of Organic Solvents on sPLA$_2$ Stability

The effect of organic solvents on sPLA$_2$ stability was determined after incubation of the enzyme in the presence of acetone, acetonitrile, methanol, ethanol and 2-propanol (50%, v/v) at 25 °C for 1 and 2 h. The residual activity was calculated and compared to the control, after centrifugation for 5 min at 13,500 × g, at pH 9 and at 45 °C.

5.4. Kinetic Parameters

The activity of the purified enzymes was evaluated at various final concentrations ranging from 0 to 60 mM of PC, PE and PS under optimal conditions (pH 9, 45 °C and in the presence of 4 mM CaCl$_2$ and 4 mM NaTDC). Measurements were recorded in duplicate and the respective kinetic parameters, including $V_{\text{max}}$ and $K_{\text{m app}}$ were calculated from
Lineweaver–Burk plots (Lineweaver and Burk, 1934). The turnover number ($K_{cat}$) value was determined from the following equation: $K_{cat} = V_{max} / [E]$, where $V_{max}$ is the maximal velocity and [E] is the active enzyme concentration.

6. Antimicrobial Activity

Pure standard microbial isolates collected from King Khaled University Hospital were tested in this study; including four fungal strains ($P. digitatum$) and 12 bacterial strains: $Escherichia coli$ ($E. coli$; ATCC 25966), $Pseudomonas aeruginosa$ ($P. aeruginosa$; ATCC 27853), $Enterobacter aerogenes$ ($E. aerogenes$; ATCC 13048), and $Salmonella enterica$ ($S. enteric ATCC; 43972$) as Gram-negative, $Bacillus cereus$ ($B. cereus$; ATCC 14579), $Bacillus subtilis$ ($B. subtilis$; ATCC 6633), $L. monocytogenes$ (ATCC 19111), $Enterococcus faecium$ ($E. faecium$; ATCC 19433), $Streptococcus pyogenes$ ($S. pyogenes$; ATCC 21059), $S. aureus$ (ATCC 25923), $Staphylococcus epidermidis$ ($S. epidermidis; ATCC 14990$), $Staphylococcus xylosus$ ($S. xylosus$, ATCC 700404) as Gram-positive.

The agar diffusion method was performed to check the antibacterial activities of dromedary PLA$_2$s. Bacteria were grown to mid-log-phase (OD$_{600} = 0.8$) in BHI medium. Fresh cultures (10 µL) of each microorganism were grown on 8 mL nutrient agar plates (Oxoid, UK); for bacterial suspension preparation of 0.5 MacFarland, containing 0.7% agar and poured over a 90 mm Petri dish containing 25 mL of 1.5% agar in BHI. Bacterial viability was investigated by determining the colony-forming ability (CFU) of bacteria incubated at different time intervals without or with appropriate amounts of the compound that was mixed with $2 \times 10^7$ CFU/mL in sterile BHI and were incubated under shaking for 60 min at 37 °C. Samples were serially diluted into sterile BHI, streaked onto media agar plates, and incubated for 24 h at 37 °C. The antibacterial potency of tested compounds was expressed as the residual number of CFU with reference to the initial inoculums. Results presented as the half-maximal (50%) inhibitory concentration ($IC_{50}$) are means of 3 different measurements. Additionally, the micro-well dilution method was used to determine the lowest compound concentration (MIC) that totally blocks the growth of tested microorganisms. Dilution series of the tested enzyme (10–200 µg/mL) were set in a 96-well plate. In each well, the mixture consisting of 50 µL of the diluted compound, 10 µL inoculums, and 40 µL of growth medium was incubated for 24 h at 37 °C. Then, 40 µL of MTT (0.5 mg/mL) was added to each well and the plate was again incubated for 30 min at the same temperature. The well showing no change to a violet-colored formazan compound indicates that the bacteria were biologically inactive and corresponds to the MIC. Ampicillin (1 mg/mL) was used as positive standard reference.

The disc diffusion technique using Sabouraud dextrose agar was employed to evaluate the antifungal activity of pure DrPLA$_2$-V on some fungal strains (Ronald, 1991). Ten µg of the enzyme or the commercial cycloheximide (1 mg/mL), used as the positive control, was deposited on sterile paper discs that were placed then in the center of the inoculated Petri dishes and incubated at 30 °C for 24 h.

7. Cell Culture

Investigation of the cytotoxicity of the studied enzyme was carried out on human breast adenocarcinoma (MDA-MB-231) and colon cancer (HCT-116 and Lovo) cell lines (American Type Culture Collection; USA) using various amounts (25, 50, 75, 100, and 200 µg) of purified PLA$_2$. Samples were first diluted in Dulbecco’s Modified Eagles Medium with 10% Fetal Bovine Serum, then added to cells grown and cultured in a 5% CO$_2$-humidified incubator at 37 °C for 24 h. Thereafter, an ELISA end-point assay (Benchmark Plus, Bio-Rad, CA, USA) was performed to determine the activity of lactate dehydrogenase released from damaged cells in the collected supernatant aliquots. Negative and positive controls were in the assay medium only and 0.1% Triton X-100 in the assay medium, respectively. Cell viability, expressed as a relative percentage of the OD values (at 550 nm) for DrPLA$_2$-V treated cells and the control, is shown as mean ± SD (n = 3).
8. Conclusions

The capacity of the dromedary to live under desert conditions and to survive in an incredibly harsh environment is due to its biological and physiological particularities. In the current study, some biological effects of PLA2-V from dromedary are reported. The purified enzyme was biochemically characterized as pH- and temperature-stable, and the kinetic parameters were investigated ($K_m / V_{max} / K_{cat}$). Our results indicate that the purified DrPLA2 is a potential enzyme candidate with therapeutic importance in pharmaceutical industry applications due to its antibacterial, and antifungal potential. Whereas, no cytotoxic effect was observed even at high concentrations tested. Thus, dromedary is an efficient source of enzymes with high potential in biotechnological applications.

Author Contributions: Conceptualization, A.B.B. and A.K.; methodology, M.A.; software, A.B.B.; validation, M.A., R.J. and A.K.; formal analysis, R.J.; investigation, A.B.B.; resources, A.K.; data curation, M.A.; writing original draft preparation, A.K.; writing—review and editing, A.B.B.; visualization, R.J.; supervision, M.A; project administration, A.B.B.; funding acquisition, M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors extend their appreciation to the researchers’ Supporting Project number (RSP-2021/237), King Saud University, Riyadh, Saudi Arabia, for funding this work.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

References

1. Schaloske, R.H.; Dennis, E.A. The phospholipase A2 superfamily and its group numbering system. Biochim. Biophys. Acta 2006, 1761, 1246–1259. [CrossRef] [PubMed]
2. Giannattasio, G.; Lai, Y.; Granata, F.; Mounie, C.M.; Nallan, L.; Oslund, R.; Leslie, C.C.; Marone, G.; Lambeau, G.; Gelb, M.H.; et al. Expression of phospholipases A2 in primary human lung macrophages: Role of cytosolic phospholipase A2-alpha in arachidonic acid release and platelet activating factor synthesis. Biochim. Biophys. Acta 2009, 1791, 92–102. [CrossRef] [PubMed]
3. Bezzine, S.; Koduri, R.S.; Valentin, E.; Sadilek, M.; Lambeau, G.; Gelb, M.H. Exogenously added human group X secreted phospholipase A2(2) but not the group IB, IIA, and V enzymes efficiently release arachidonic acid from adherent mammalian cells. J. Biol. Chem. 2000, 275, 3179–3191. [CrossRef] [PubMed]
4. Rouault, M.; Bollinger, J.G.; Lazdunski, M.; Gelb, M.H.; Lambeau, G. Novel mammalian group XII secreted phospholipase A2 lacking enzymatic activity. Biochemistry 2003, 42, 11494–11503. [CrossRef] [PubMed]
5. Valentin, E.; Ghomashchi, F.; Gelb, M.H.; Lazdunski, M.; Lambeau, G. On the diversity of secreted phospholipases A2(2). Cloning, tissue distribution, and functional expression of two novel mouse group II enzymes. J Biol Chem 1999, 274, 31915–31202. [CrossRef]
6. Bezzine, S.; Bollinger, J.G.; Singer, A.G.; Veatch, S.; Keller, S.L.; Gelb, M.H. On the binding preference of human groups IIA and X phospholipases A2 for membranes with anionic phospholipids. J. Biol. Chem. 2002, 277, 48523–48534. [CrossRef]
7. Singer, A.; Ghomashchi, F.; Le Calvez, C.; Bollinger, J.; Bezzine, S.; Rouault, M.; Sadilek, M.; Nguyen, E.; Lazdunski, M.; Lambeau, G.; et al. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, and XII secreted phospholipases A2. J. Biol. Chem. 2002, 277, 48535–48549. [CrossRef]
8. Triggiani, M.; Granata, F.; Balestrieri, B.; Petraroli, A.; Scalia, G.; Del Vecchio, L.; Marone, G. Secretory phospholipases A2 activate selective functions in human eosinophils. J. Immunol. 2003, 170, 3279–3288. [CrossRef]
9. Karray, A.; Ali, M.B.; Kharrat, N.; Gargouri, Y.; Bezzine, S. Antibacterial, antifungal and anticoagulant activities of chicken PLA2group V expressed in Pichia pastoris. Int. J. Biol. Macromol. 2018, 108, 127–134. [CrossRef]
10. Chen, J.; Engle, S.J.; Seilhamer, J.J.; Tischfield, J.A. Cloning and recombinant expression of a novel human low molecular weight Ca2+-Dependent Phospholipase-A2. J. Biol. Chem. 1994, 269, 2365–2368. [CrossRef]
11. Aoubala, M.; Douchet, I.; Bezzine, S.; Hirn, M.; Verger, R.; De Caro, A. Immunological Techniques for the Characterization of Digestive Lipases. Methods Enzymol. 1997, 286, 126–149. [PubMed]
12. Dennis, E.A.; Cao, J.; Hsu, Y.-H.; Magrioti, V.; Kokotos, G. Phospholipase A2 enzymes: Physical structure, biological function, and therapeutic intervention. Chem. Rev. 2011, 10, 6130–6185. [CrossRef] [PubMed]
41. Tischfield, J. A reassessment of the low molecular weight phospholipase A2 gene family in mammals. *J. Biol. Chem.* **1997**, *272*, 17247–17250. [CrossRef]

42. Nyman, K.M.; Häggblom, J.O.; Nevalainen, T.J. Toxic Effects of Phospholipase A2 In Vitro, Vol 24: Phospholipase A2. Basic and Clinical Aspects in Inflammatory Diseases; Uhl, W., Nevelainen, T.J., Buchler, M.W., Eds.; Prog. Surg. Karger: Basel, Switzerland, 1997; pp. 176–181.

43. Abousalham, A.; Verger, R. Egg yolk lipoproteins as substrates for lipases. *Biochim. Biophys. Acta* **2000**, *1485*, 56–62. [CrossRef]

44. Bradford, M.M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]

45. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef]

46. Hewick, R.M.; Hunkapiller, M.W.; Hood, L.E.; Dreyer, W.J. A gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **1981**, *10*, 7990–7997. [CrossRef]