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

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Abstract: Secretory group V phospholipase A2 (PLA_2-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 PLA_2, the production of PLA_2-V from dromedary. The obtained DrPLA_2-V has an absolute requirement for Ca^{2+} 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}/Km_{app} is 2.6 ± 0.02 mM^{-1} 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

Citation: Alonazi, M.; Karray, A.; Jallouli, R.; Ben Bacha, A. Biochemical, Kinetic and Biological Properties of Group V Phospholipase A2 from Dromedary. *Molecules* **2022**, *27*, 3437. https://doi.org/10.3390/molecules27113437

Academic Editors: Raffaele Capasso and Lorenzo Di Cesare Mannelli

Received: 20 April 2022
Accepted: 23 May 2022
Published: 26 May 2022

1. Introduction

Phospholipases A2 (PLA_2) 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 PLA_2s ($sPLA_2$s) referred to as group IB (GIB), GII (subgroups A–F), GIII, GV, GX and GXII (subgroups A–B) [2]. Clearly, the different mammalian $sPLA_2$s 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 $sPLA_2$s 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 $sPLA_2$s secreted in inflamed tissues can vary according to the type of inflammation and of infiltrating cells. Most $sPLA_2$s 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 $sPLA_2$ has been cloned from chicken [9], human, rat, and mouse species [10]. Unlike group I and II sPLA2s, this $sPLA_2$ has only six disulfides and does not have the group I- or group II-specific disulfides, thus defining a novel group of $sPLA_2$s [11]. This $sPLA_2$ has a higher level of identity with group IIA $sPLA_2$s, as compared to group IB

$sPLA_2$. It neither has a propeptide sequence, indicating its closer relationship with group II $sPLA_2$s. $sPLA_2$ (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 $sPLA_2$s possess a Ca^{2+} binding loop and a catalytic dyad formed by His/Asp, as well as conserved disulfide bonds, while atypical $sPLA_2$s (group III/XII) each form a distinct class [4,12–14]. In humans, the study of the structure–function relationship of $sPLA_2$ isoforms is important for a better understanding of the pathology of diseases related to these enzymes. $sPLA_2$ 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, $sPLA_2$-X is highly active on neutral phosphatidylcholine (PC), whereas $sPLA_2$-IIA has much greater affinity for charged phospholipid head groups, especially phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). This preference is useful for understanding the role of $sPLA_2$-IIA as defensive proteins, acting on PE and PG, which are major components of bacterial membranes [15]. Mammalian $sPLA_2$-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 $sPLA_2$-V to regulate phagocytosis is specific and not shared with cytosolic PLA2 alpha ($cPLA_2\alpha$) nor $sPLA_2$-IIA [17].

The common of $sPLA_2$ isoforms are up-regulated by proinflammatory stimuli such as bacterial lipopolysaccharide (LPS), which largely increases the expression of $sPLA_2$-V. Additionally, it has been shown that $sPLA_2$-V is considered as a significant messenger in the regulation of cell migration. Indeed, Lapointe et al. [18] investigated the effect of $sPLA_2$-V on LPS-mediated leukocyte recruitment supporting the involvement of $sPLA_2$-V in the development of inflammatory innate immune response and its capacity to modulate adhesion molecule expression. Indeed, immunohistochemistry studies showed that $sPLA_2$-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 $sPLA_2$-V which exert transcellular lipolytic activity on neighbouring inflammatory cells [20]. The elevation of $sPLA_2$-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 A_2 from dromedary [22,23]. Accordingly, the present work was undertaken to further investigate the biochemical and antibacterial properties of dromedary non-digestive PLA_2 to compare them with known PLA_2-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 PLA_2-V secreted from the heart of dromedary

2. Results

2.1. Biochemical Properties of DrPLA$_2$-V

As described previously, $sPLA_2$-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 PLA_2, purified from dromedary heart tissue.

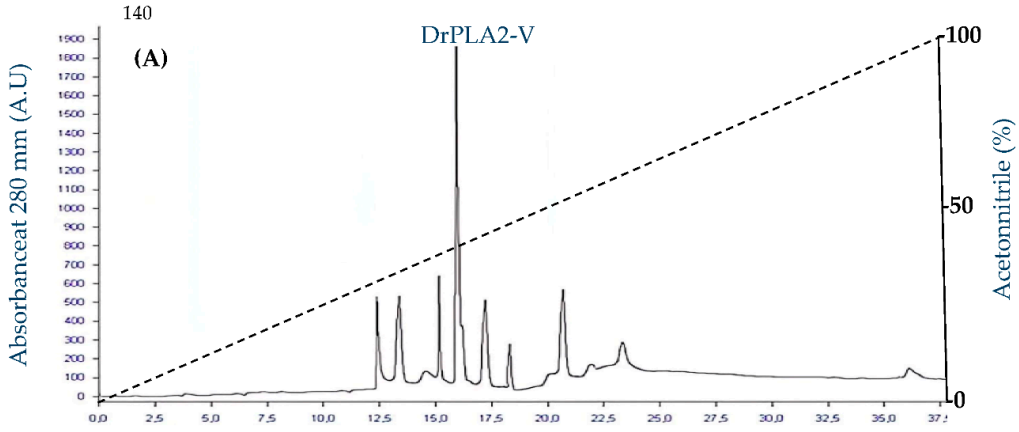
PLA_2-V is purified and characterized from the dromedary heart (delipidated powder). The purification flow sheet presented in Table 1 showed that the specific activity of pure DrPLA$_2$-V reached 115 U/mg when phosphatidylethanolamine (PE) was used as a substrate at pH 9 and 45 °C, in the presence of 8 mM NaDC and 4 mM $CaCl_2$. The DrPLA2-V 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 PLA_2-IB [23,25].

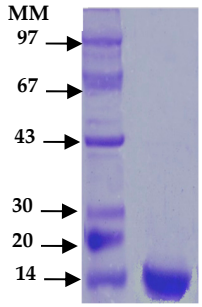
Table 1. Flow sheet of $DrPLA_2$-V purification.

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	236	73.7	3.2	78.7	32
$(NH_4)_2SO_4$ Precipitation (20–65%)	177	11.8	15	59	150
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 $CaCl_2$.

The procedure described and summarized in Table 1 is more rapid than those used previously to purify another mammalian pancreatic phospholipase A_2. 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 PLA_2 four chromatographic steps were needed [25]. The molecular mass of the purified enzyme was 14 kDa to secrete PLA_2 (Figure 1B).





(C)

G-LLELKSMIEKVVGKSAVKSYGFYGCYCGWGGRGTPKDATDWCCWIHDHCY (Current study)
G-LLELKSMIEKVTGKSAVISYGFYGCYCGWGGRGTPKDATDWCCQVHDHCY (i)
GGLLDLKSMIEKVTGKNALTN-YGFYGCYCGWGGRGTPKDGTDWCCWAHDHCY (ii)
G-LLELKSMIEKVTRKNAFKNYGFYGCYCGWGGRGTPKDGTDWCCQMHDRCY (iii)

Figure 1. (**A**). Chromatography on RP-HPLC column of the purified $DrPLA_2$-V from dromedary heart. RP-HPLC on a C18 column pre-equilibrated in solvent A, elution was performed using a gradient

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 DrPLA$_2$-V. Lane 1, molecular mass markers (kDa); lane 2, 10 µg of purified DrPLA$_2$-V. (**C**) NH2 sequence alignment of DrPLA$_2$-V, *Miniopteridae* family (*Miniopterusnatalensis*) (XP_016070213.1) (i), human family (*homosapiens*) (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: GLLELKSMIEKVVGKSAVKSYGFYGCYCGWGGRGTPKDATDWCCWIHDHCY. The N-terminal sequence alignment of sPLA$_2$-V showed a high degree of homology with those of *Miniopteridae* family (*Miniopterusnatalensis*) (XP_016070213.1) [26], human family (*homosapiens*) (NP_000920.1) [27], and *Hyaenida* family (*Hyaena hyaena*) (XP_039084994.1) [28] of about 92%, 84% and 82%, respectively (Figure 1C).

The purified DrPLA$_2$-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 PLA$_2$s are stable at low pH values. Unlike pancreatic DrPLA$_2$-IB, which is completely denatured at high temperature, the DrPLA$_2$-V maintained about 80% of its activity when incubated for 60 min at 60 °C (Figure 2). Similar observations were obtained previously with intestinal PLA$_2$ 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).

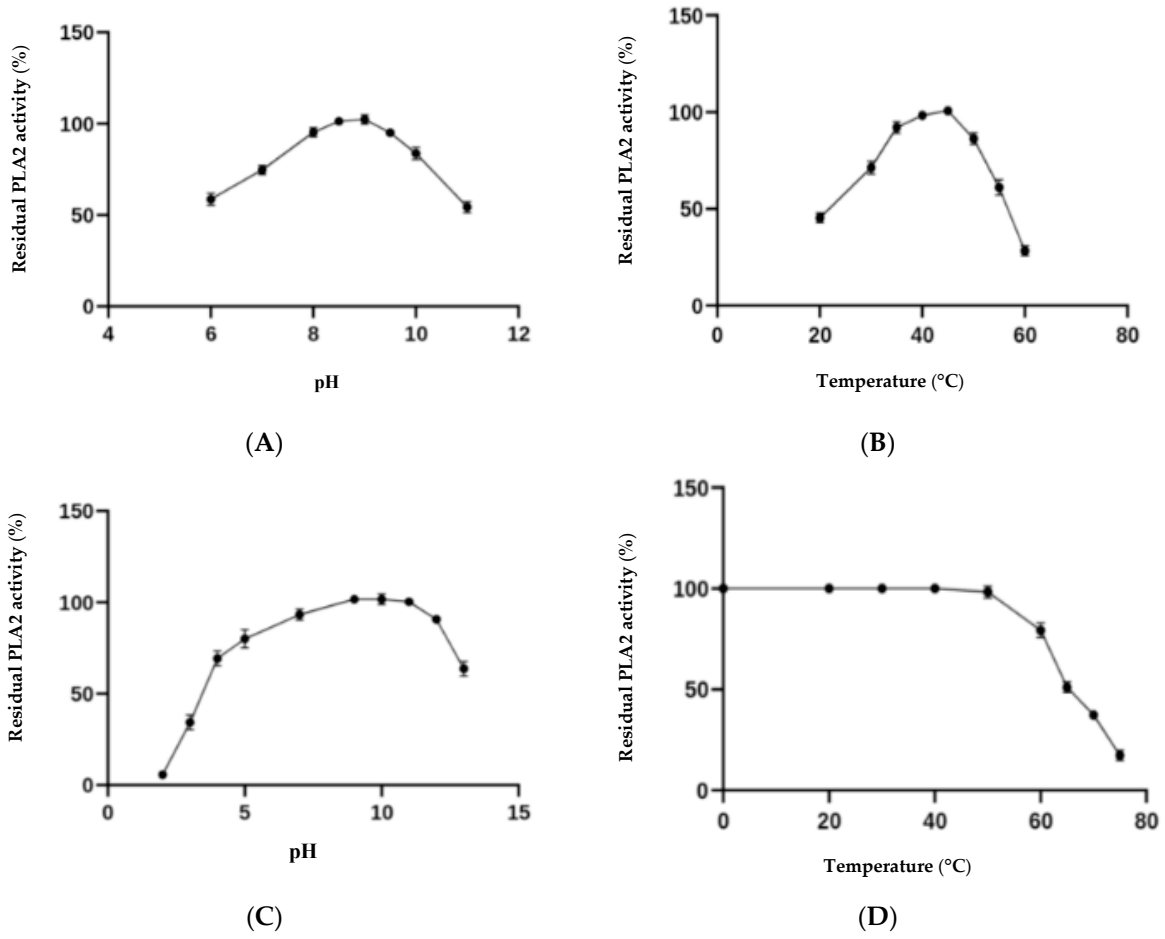
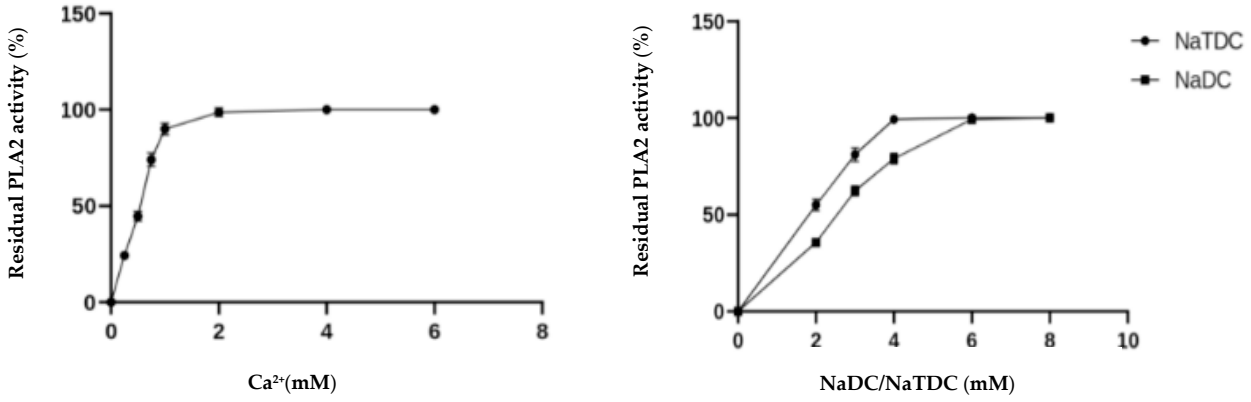


Figure 2. Evaluation of pH and temperature effect on activity (**A**,**B**) and stability (**C**,**D**) of DrPLA$_2$-V.

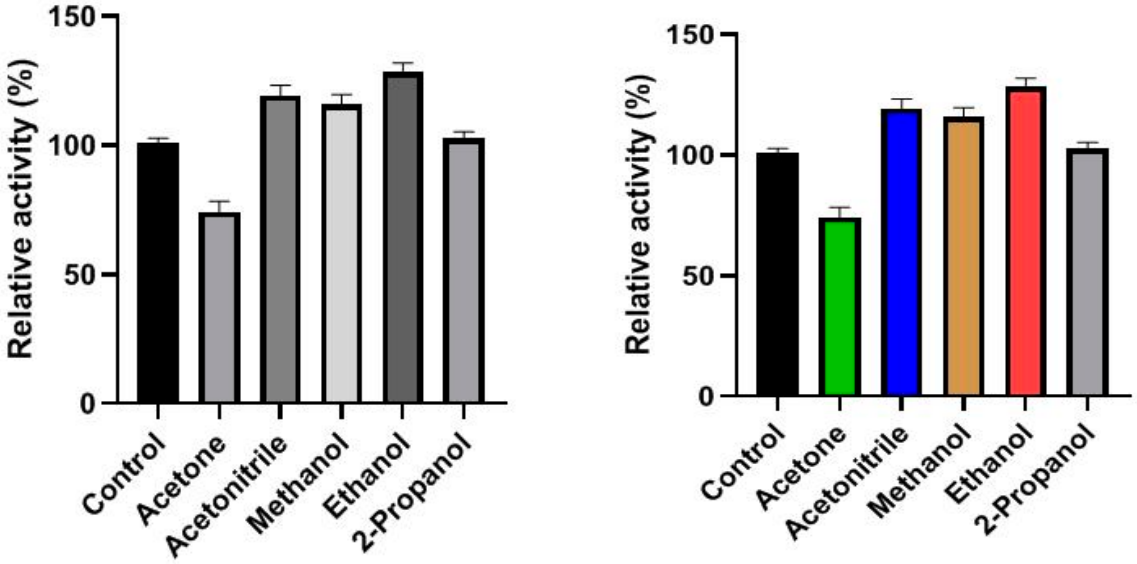
As all secreted PLA_2, the Ca^{2+} ions are essential for $DrPLA_2$-V to express its full activity, with an optimum at 4 Mm (Figure 3A). All the divalent ions tested were unable to express the full specific activity of the enzyme. Figure 3B shows that both NaTDC and NaDC were required to express the maximal activity at concentrations of 4 and 6 mM, respectively.



(**A**) (**B**)

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].



(**A**) (**B**)

Figure 4. Effect of organic solvents on $DrPLA_2$-Vstability. 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 PLA$_2$-V from Dromedary Using Phospholipids (PL) of Different Head Groups

Then, the kinetic properties of DrPLA$_2$-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 DrPLA$_2$-V to hydrolyze PE compared to DrPLA$_2$-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 PLA$_2$ 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 DrPLA$_2$-V.

	V_{max} (U/mg)	Km_{app} (mM)	K_{cat} (s^{-1})	K_{cat}/Km_{app} (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 PLA$_2$-V

The antimicrobial activity of the purified DrPLA$_2$-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 DrPLA$_2$-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 DrPLA$_2$-V. The current enzyme is much more effective than marine group V-PLA$_2$ showing IC50 values between15–25 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 PLA_2-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-PLA_2 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, $sPLA_2$, 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 $sPLA_2$. This result is confirmed by [35].

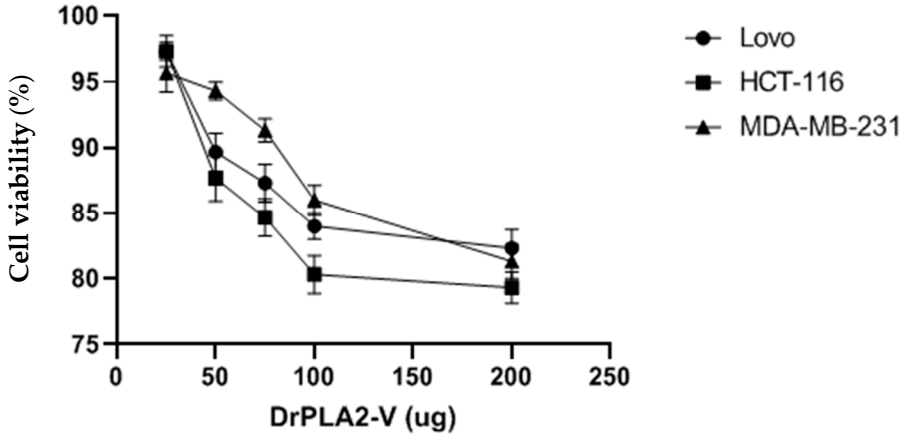


Figure 5. Cytotoxic potency of $DrPL_2$ -V on Lovo, HCT-116, and MDA-MB-231 cells. Cytotoxicity was assessed using the MTT assay by incubating cells for 24 h with various concentrations (25, 50, 100, and 200 µg) of *$DrPLA_2$-V*.

3. Discussion

PLA_2 catalyses the glycerophospholipids at the sn-2 position, generating free fatty. To date, the $sPLA_2$ are classified into 10 catalytically active enzymes in mammals, and are characterized bylow-molecular-weight and Ca^{2+}-requiring extracellular enzymes. Each $sPLA_2$ 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 PLA_2, the production and characterization of DrPLA_2-V. Evaluation of the antimicrobial effect of the enzyme and its cytotoxicity is also studied.

Unlike PLA_2-IB and PLA_2-X, characterized with the presence of a propeptide cleaved by an endogen trypsin in order to produce a mature and active enzyme, we reported here that no significant increase in the DrPLA_2-V activities was observed throughout 1 h homogenization with endogenous trypsin (data not shown). Moreover, it was noted that PLA_2-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 PLA_2-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 PLA_2, the analyzed fractions on SDS-PAGE indicate that the current enzyme (named $DrPLA_2$-V) presents an apparent molecular mass of about 14 kDa. The specific activity of pure $DrPLA_2$-V reaches 115 $U \cdot mg^{-1}$ when PE was used as substrate at pH 9, 45 °C and in the presence of 4 mM $CaCl_2$ and 8 mM NaDC, a value which is comparable to that observed with the PLA_2-V from chicken or stingray with a specific activity of 156 or 52 U/mg, respectively, measured on the same substrate [9]. The $DrPLA_2$-V purification yield was about 44% the total initial activity (Table 1).

N-terminal sequence of $DrPLA_2$ showed a high level of identity with those of the $sPLA_2$-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 PLA_2-Vfrom various species showing a good stability at high temperature [40] Moreover, $CaCl_2$ (4 mM) was found as the best activator of the PLA_2 activity of pure *DrPLA2-V*, followed by Mg^{2+} (combined with 1 Mm $CaCl_2$). 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 PLA_2 [24].

We next studied the kinetic properties of $DrPLA_2$-V Km_{app}, K_{cat} and the deduced catalytic efficiency (K_{cat}/Km_{app}) of the purified group-V, using charged PE, zweeterionic PC or PS as substrate using Lineweaver–Burk plots. The data obtained (Table 2) showed the clear capacity of $DrPLA_2$-V to hydrolyze the negatively charged substrate PE (Km_{app} 115 ± 3.5) compared to the zwitterionic substrate PC (Km_{app} 87 ± 2.1) and PS (Km_{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_{cat}/Km_{app}) eight or five times higher than those obtained with using PE as substrates. The same trend was observed using PC or PE 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 $sPLA_2$ isoforms, and thus predominantly increase the expression of sPLA2-V. Besides, it has been recently shown that $sPLA_2$-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 $DrPLA_2$-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 PLA_2V was inactive against *E. coli* and against $Gram^-$ bacteria. Besides, the antifungal effect of the purified enzyme is attributed to its phagocytosis role against pathogenic strains: bacteria and fungi. Previous results showed that macrophages from $sPLA_2$-$V^{-/-}$ 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, $sPLA_2$-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 $sPLA_2$-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 PLA_2 tested on normal and cancer cells, suggesting that all $sPLA_2$s 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 ($E^{1\%}_{1cm}$ = 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 and150 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_{max} and Km_{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_2s. Bacteria were grown to mid-log-phase (OD600 = 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 2×10^7 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 PLA_2-V from dromedaryare reported. The purified enzyme was biochemically characterized as pH- and temperature-stable, and the kinetic parameters were investigated ($K_m/V_m/K_{cat}$). Our results indicate that the purified $DrPLA_2$ 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, dromedaryis 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.

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