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# Characterization and Immunological Properties of *Pseudomonas aeruginosa* Hemolytic Phospholipase C

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PHOSPHOLIPASE C (PLC) produced from 4 day age culture of *P. aeruginosa* strain in inorganic phosphate starved liquid medium (pH7.2) at 35C. The enzyme was purified to electrophoretic homogeneity using gel filtration chromatography. Purified PLC had 6.2 purification fold and 28.2% yield. The purified enzyme had a molecular mass of 58.7 kDa. Alanine was the dominant amino acid with 13.1%. DNA sequence analysis of the PCR products amplified from *P. aeruginosa* confirmed the presence putative *plc*H gene (1.6 kb). The enzyme was completely inactive above 65C with Tm 42C. The enzyme activity was approximately stable at pH 8.5 to 10. The purified enzyme was characterized as a $Zn^{2+}$-metallophospholipase C and also divalent ions $Ca^{2+}$, $Ba^{2+}$ and $Co^{2+}$ were stimulators for its activity. The catalytic affinity of PLC enzyme towards phosphatidylcholine (PC) gave $V_{max}$ = 1.85 $Umg^{-1}$ $min^{-1}$; Km = 0.12 M and Kcat = 1.96 x $10^{-5}$ $S^{-1}$. Purified PLC enzyme was detoxified by heating and completely lost its lethal activity on mice after 60 min at 65C. Immunologically, the titer of IgG antibodies in blood serum of New Zealand rabbits was appeared after 15 days of the last injection dose of fresh or heat detoxified enzyme by 1.15 and 1.21 fold respectively higher as compared to control followed by a decrease in the titer up to 45 days. The hematological parameters reflected a decrease in the RBCs, WBCs and hemoglobin in rabbits injected with fresh enzyme and there was significant change in chemistry of liver and kidney. In contrarily detoxified enzyme had no obvious changes in these parameters.

**Keywords:** *Pseudomonas aeruginosa*; Phospholipase C, Antigenicity, Cytotoxicity

Phospholipases have a wide spectrum of *in vivo* and *in vitro* effects, ranging from minor alterations in cell membrane composition and function to lethality. The most important classes of phospholipases that have been shown to play a significant role in bacterial pathogenesis are phospholipase C and phospholipase D (Schmiel and Miller, 1999). *Pseudomonas aeruginosa* derived large extracellular protease (LepA) and hemolytic phospholipase C (PLCH) which were considered to play an important role in the pathogenicity of this organism (Kida *et al*., 2011).

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Two known phopholipases C were elaborated from *P. aeruginosa*, hemolytic (PLCH) and non-hemolytic (PLCN). The PLCH may be an important virulence factor. Indeed, purified PLCH causes vascular permeability, end organ damage, and death when injected into mice in high doses (Meyers *et al*., 1992 and Wargo *et al*., 2011). These hemolytic enzymes are cytolytic toxic proteins produced by wide range of bacteria causing lyses of erythrocytes, disrupting the function of the plasma membrane (Magalhaes *et al*., 2011).

Phospholipase C is a heat labile hemolysin produced by *P. aeruginosa* (Stonehouse *et al*., 2002).This PLCH catalyzes the hydrolysis of phosphatidylcholine (PC) yielding phosphorylcholine and diacylglycerol (DAG) (Flieger *et al*., 2000). The production of DAG may be particularly important in pathogenesis causing eukaryotic signaling events (Titball, 1998).

The phospholipase C (*plc*) gene of *P. aeruginosa* encodes a heat-labile secreted hemolysin which is part of a Pi-regulated operon (Ostroff and Vasil, 1987). *Pseudomonas aeruginosa plc*H and *plc*N genes are encoding for the hemolytic and nonhemolytic phospholipase C enzymes (Gomez *et al*., 2001). In the lung of cystic fibrosis (CF) patient, *P. aeruginosa* induced *plc*H and genes involved in both choline diacyglycerol catabolism, which supports the hypothesis that PLCH enzyme is important for colonization of the lung (Son *et al*., 2007). The PLCH-mediated degradation of the PC in lung surfactant contributes to lung infection (Wargo *et al*., 2009).

Hemolytic PLC had been demonstrated to be a virulence determinant of *P. aeruginosa* in a variety of infection models in mammals (Chin and Watts, 1988).The immunological characteristics of hemolytic PLC attracted the attention of many investigators due to their importance (Meyers *et al*., 1992; Estahbanati *et al*., 2002 and Ipaktchi *et al*., 2007).Applications on animal protection using heat detoxified toxins were previously studied by Manohar et al. (1966) and Khalil (2009).

In the light of the previous facts the objective of current study was the production, purification and characterization of PLC from tested *P. aeruginosa*. Investigation for biochemical and molecular properties, kinetics and hemolytic activity of the pure enzyme. Assessment for antigenecity and toxicity of the tested enzyme, *in vivo*, in animal models.

## Material and Methods

*Bacterial strain and PLC production:*

*Pseudomonas aeruginosa* (1589741) was obtained from the previous work of Hassanein (2009). Phosphate starved Tris minimal medium (TMM) was used in which the concentration of the phosphate ($KH_2PO_4$) was recorded to be 0.1 mM. The TMM had the composition: 100 mM tris-HCl (pH 7.2), 11 mM, glucose, 5 mM $NH_4Cl$, 10 mM $KH_2PO_4$, 0.5 mM $K_2SO_4$, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$ as

reported by Elleboudy *et al*. (2011). After incubation for 4 days at 35C and centrifugation for 15 min at 6000 rpm, the supernatant was used as crude enzyme.

*Purification of phospholipase C*

The crude PLC extracted from *P. aeruginosa* growing in PLC production medium under cultural conditions was fractionated by ammonium sulfate of 70% saturation. The precipitated proteins were dissolved and then dialysed against 10 mM Tris HCl buffer (pH 7.2) (Ostroff and Vasil, 1987). Gel filtration using Sephadex G100 was used as described by Berk (1964). The active fractions were pooled and concentrated by dialysis against polyethylene glycol. For each fraction protein content and PLC activity were determined.

*Phospholipase C activity*

The activity of PLC was assayed according to Kurioka and Liu (1967). Egg yolk L--phosphatidylcholine (PC) obtained from Sigma Chemical Co.(St. Louis, Mo.USA) was used as substrate. Colloidal solution of PC was prepared by adding 25 mM of PC suspension in 0.25 M tris HCl buffer pH 7.2. A homogenous suspension was achieved using a mixer for 10 min at 4C,

The basal reaction mixture for the assay consisted of 0.1 ml of the PLC, 0.4 ml of the PC solution and 0.5 ml of 0.25 M Tris HCl buffer (pH 7.2). After incubation at 37C for 30 min, the enzymatic reaction was stopped by 1.0 ml of 10% trichloroacetic acid. After centrifugation a portion of the supernatant fluid (1.0 ml) was digested and the inorganic phosphate content was determined according to Kates (1972). One unit of PLC activity was defined as the amount of enzyme which releases 1 µmol of pi (inorganic phosphorus) under standard assay conditions. The enzyme protein was determined by method of Lowry et al. (1951), using bovine serum albumin as a standard. Hemolytic activity of PLC was determined by agar well diffusion method as described by Mukry et al. (2010).

*SDS-PAGE analysis*

Molecular weight of PLC was determined according to the method of Laemmli (1970) using SDS-polyacrylamide gel electrophoresis. Multicolor Broad range protein ladder has 4-color protein standards with 10 recombinant proteins covering a molecular weight from 10 to 260 KDa was used.

*Amino acids analysis*

Amino acid composition of PLC was determined according to the method used by Tous *et al*. (1989) applying the GLC hydrolytic technique with a Beckman amino acids analyzer ( SYKAMS7130 Amino acid Reagan Organizer) .

*Detection of plcH gene*

*a-Preparation of DNA*

The DNA was extracted from *P. aeruginosa* growing in PLC production medium according to Sambrook and Russel (2001).

*b-Polymerase Chain Reaction (PCR)*

Polymerase Chain Reaction (PCR) was used to amplify *plc*H gene from *P. aeruginose*. The PCR amplifications were performed in a total volume of 50 l containing 25 µl of PCR Master Mix. (2x), 0.5 µl of Taq polymerase 25 pmol of both primers (forward primer 5'- ATG TCA GGC ATG CAA ACC CCG C-3'; reverse primer 5'-TCA CGC GTT GCG GTC GAT GTA G-3') and template DNA (100 ng). The reaction mixtures were covered with mineral oil and PCR amplification was carried out in a Gene-Amp PCR system 9600 thermocycler (Perkin Elmer). The amplification conditions were as follows: 94°C for 10 min and 35 cycles of denaturation at 95°C for 30 s, annealing-extension at 56°C for 30 s, 72°C for 1 min., 72°C for 10 min. and final hold program at 4°C.

c-*Agarose gel electrophoresis*

The PCR product was detected by (1%) agarose supplied from gel electrophoresis at 50 V for 1 in lx Tris- Boric acid -EDTA (lx TBE) buffer. Ethidium bromide for staining and UV transillumination were used. Then PCR clean up to the PCR product was made up using Gene JET$^{TM}$ PCR purification kit (fermentas). Finally sequencing to the PCR product was carry on GATC German Company by ABI 3730XI DNA sequencer using the forward and reverse primers. The resulting DNA sequences were compared using the Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.,* 1997).

*Biochemical properties of phospholipase C*

The thermal stability of PLC was studied after preincubation of the enzyme at various temperature (25-80C) using 10 mM Tris HCl buffer (pH 7.2) for different time (30-150 min). The substrate PC (1.5 M) was added to the enzyme. The enzyme relative activity was determined after the incubation of reaction mixture at 37C for 30 min. The thermal inactivation rate (Kr min) can be described by the first-order kinetic model (Whitaker, 1972); $Ln(A_t/A_o)$ $k_tT$, where $A_o$ and $A_t$ are the specific activity at zero and t time. The T1/2 (time at which the enzyme loss 50% of its activity) was calculated from the linear equation for each temperature. The temperature at which the enzyme loss 50% of its activity (Tm) was calculated from the linear equation of different preincubation temperature at 60 min.

Stability of PLC was examined after preincubation of the enzyme for 30 min at different pH values ranging from 4-11. Acetate (0.2 M), phosphate (0.2 M) and glycine-NaOH buffers were used to achieve pH range (4-5), (6-8) and (9-11) respectively. After adding PC (1.5 M) the reaction mixture was incubated at 37C for 30 min. The activity of the enzyme was determined for each pH.

To assay the metal ions effect, the purified PLC was preincubated in 0.1 mM EDTA at 4C for 2hr, and then dialyzed against 10 mM Tris-HCl (pH 7.2). The enzyme was incubated with each metal ion separately for 30 min before adding PC (1.5 M). Metal ions in form of $MnSO_4$, $MgSO_4$, $ZnCl_2$, $CaCO_3$, $CoCl_2$, $CuSO_4$, $FeSO_4$ and $BaCl_2$ (5 mM) as well as EDTA (1 and 5 mM) were used. The enzyme relative activity was determined immediately after incubation at 37C for 30 min.

The kinetic parameters of PLC as Vmax, Km and Kcat were estimated using different concentrations of egg yolk PC (0.1-1.5 M). Michaelis-Mentel constant (Km) and maximum velocity (Vmax) were calculated from Lineweaver-Burk plot. Catalytic efficiency (Kcat) was expressed by the specific activity per mol enzyme.

*Heat detoxification of PLC*

According to the data obtained from the thermal kinetics, the pure enzyme was heat detoxified in water bath for 30,60 and 90 min at 65C in 0.25 mM Tris HCl buffer (pH 7.2).Then the enzyme was immediately placed in an ice bath (Manohar *et al*., 1966) .The hemolytic titer and mice mortality were then measured.

*Hemolytic titer of PLC*

According to Johnson and Boese-Marazzo (1980) hemolytic titer of PLC was determined by two fold dilutions in buffered saline (pH 5.9); and 0.5 ml of 1% suspension of prewashed red cells was added. After 1 hr incubation at 37C, the dilution showing complete hemolysis was taken as an endpoint, the reciprocal of this number was used as the titer (hemolytic unit HU/ml).

*Animal mortality by P. aeruginosa PLC*

Different groups of four male Swiss albino mice (6-8 weeks old) were intraperitoneally ( *i.p*.) injected by 1 ml of hemolytic titer of fresh and heat detoxified *P. aeruginosa* PLC (64 and 32 HU/ml/mice respectively) . Control animal groups were challenged with buffered saline (pH 5.9). Mortality within 12 hr was considered to be due to toxin (Manohar *et al*., 1966). The mortality level was measured as number of dead mice/total number of mice per group (Abraham, 1992).

*Anti-PLC antibodies*

The New Zealand rabbits were used for this experiment .Different groups of four male animal were intravenous (i.v.) injected by 1 ml of three successive doses of hemolytic titer of normal and detoxified PLC (64 and 32 HU/ml/1.5±0.1Kg body weight respectively) during two weeks. Control animal groups were challenged with buffered saline (pH 5.9). Blood samples were collected from the rabbits after 7, 15, 22, 30, 37 and 45 days of the last PLC injection. Control sera (zero time) without enzyme injection were used. Plasma were prepared and stored at -20°C. PLC immunoglobulin class antibodies were

measured by the Radial Immunodiffusion (RID) plate assays Kit, DIFFU-PLATE (Biocientifica S.A., Buenos Aires, Argentina). DIFFU-PLATE for IgG was applied. According to the protocol, 5 µl of blood plasma was injected to the well of the plate (12 well per plate), and incubated at room temperature for 24 hr. The diameter of sharp antigen antibodies precipitation layer was measured using the magnifying glass with accurate special ruler. The precipitation ring was calibrated from the reference table of the RID Kit, for estimation of immunoglobulins titer (IgG) comparing to control sera.

*Blood Biochemistry of rabbits harboring PLC*

From the last experiments biological effects of normal and detoxified PLC on blood chemistry of treated rabbits, various hematological and biochemical parameters were determined. The hematological parameters as red blood corpuscles (RBCs),white blood corpuscles (WBCs), hemoglobin, were analyzed (Erma INC automated hematology analyzer). As well as, the biochemical parameters of liver function ( ALK, AST, ALT, total protein, albumin and BIL) and kidney function ( urea and creatinine) were assessed by automated chemistry analyzer (Dimension RXL Autoanalyzer). Control groups were considered at zero time.

*Statistic analysis*

All data were the mean of three replicates SD standard deviation (SD). Different values within the same column differ significantly at $P<0.01$. The data were analyzed by one-way ANOVA (Salvatore and Reagle, 2001). Less significant difference was calculated

## Results and Discussion

*Purification of P. aeruginosa PLC*

Phospholipase C was purified as an extracellular enzyme from the liquid cultures of 4-day age of *P. aeruginosa* growing in TMM broth medium (pH7) using 70% $(NH_4)_2SO_4$ .From the purification profile of tested PLC a sharp peak containing the active PLC was found in fractions no. 16 to 19 (data not shown). The specific activity of PLC was 118.7 U $mg^{-1}$ protein compared to 19 U $mg^{-1}$ crude protein. Also the purification achieved 6.2 fold and 28.2% yield by sephadex-G100 of purified enzyme (Table 1). In a similar manner Crevel *et al*. (1994) reported that after gel filtration purity of PLC from *P. fluorescens* increased to 32.6 folds . Also PLC from *Bacillus mycoides* showed 40.8 folds increase in purity assuming that filtration step is the key to the homogeneity, as PLC is easy to polymerize with other impurities (Wang *et al*., 2010).

In the present study, the homogeneity and molecular mass of the purified PLC (Fig. 1) appeared as a single protein band of 58.7 kDa, assuming the potency of the purification protocol. In this finding, low molecular weight 35 KDa PLC of *P. aeruginosa* was detected by Sonoki and Ikezawa (1975). On the other hand large size PLCs 76 KDa (Berka and Vasil, 1982), 72-78 KDa (Titball,

1993) and 70 KDa (Lucchesi and Domenech, 1994) were detected in other *P. aeruginosa* strains.

**TABLE 1. Purification profile of *P. aeruginosa* phospholipase C.**

| Purification step | Total volume (ml) | Activity ($Uml^{-1}$) | Protein ($mg\ ml^{-1}$) | Specific activity U $mg^{-1}$ protein | Purification fold | Yield % | Hemolytic activity (mm) |
|---|---|---|---|---|---|---|---|
| Crude enzyme | 750 | 10.5 | 0.55 | 19 | 1 | 100 | 0.7 |
| Amm. Sulphate 70% | 35 | 15.5 | 0.48 | 32.3 | 1.7 | 6.9 | 1.5 |
| Sephadex G100 | 50 | 44.2 | 0.375 | 118.7 | 6.2 | 28.2 | 2.5 |

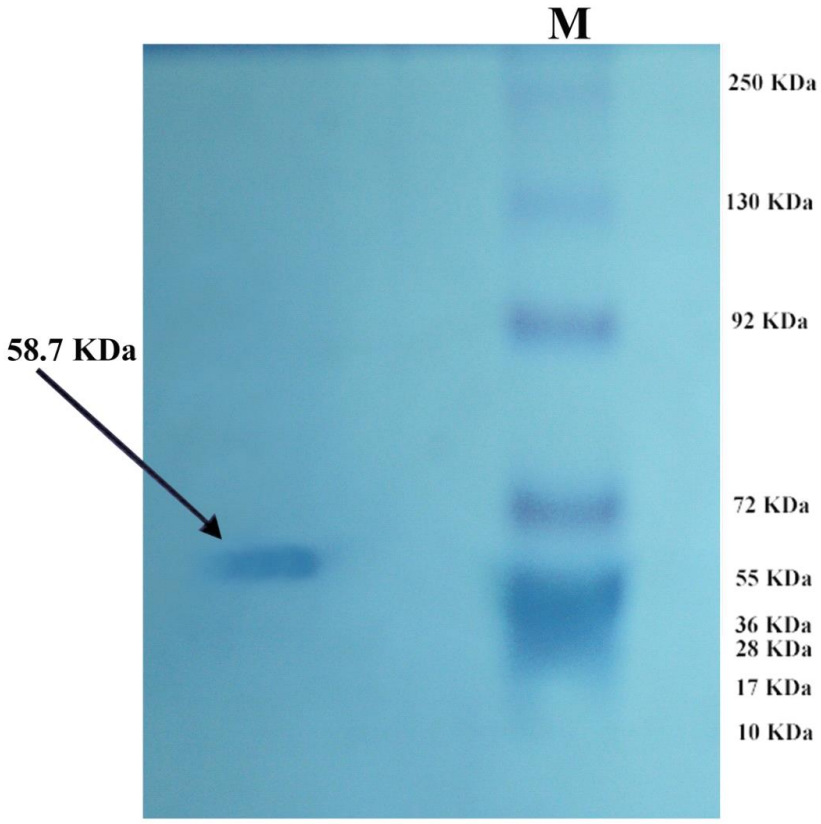


**Fig. 1. Polyacrylamide gel electrophoresis( SDS-PAGE )of *P. aeruginosa* phospholipase C (M= marker multicolor broad range protein ladder covering range from 10 to 250 kDa).**

*Amino acids composition of P. aeruginosa*

Moreover, the amino acid composition of *P. aeruginosa* PLC represented in (Table 2) revealed that alanine was the dominant amino acid (13.1%) followed by leucine (9.9%) and glycine (8.3%). Alanine is known to play a role in substrate recognition or substrate specificity (Betts and Russell, 2003). Also, the results indicated that PLC under investigation was relatively rich in asp, arg and pro. Also, there was a predominance of the hydrophobic residues (42.2%) that could explain the affinity of tested PLC to hydrophobic substances. While the data obtained by Berka and Vasil (1982) for the heat labile hemolysin PLC from *P. aeruginosa* indicated that the enzyme was rich in glycine, serine, threonine, asparlyt, aromatic amino acids , but was cystine free.

**TABLE 2. Amino acids composition of *P. aeruginosa* phospholipase C.**

| Amino acid | % |
|---|---|
| Ala | 13.1 |
| Cys | 0.2 |
| Asp | 7.4 |
| Glu | 4.4 |
| Phe | 4.4 |
| Gly | 8.3 |
| His | 2.6 |
| Ile | 2.6 |
| Lys | 3.1 |
| Leuc | 9.9 |
| Met | 1.1 |
| Asn | 4.1 |
| Pro | 6.1 |
| Gln | 5.2 |
| Arg | 7.2 |
| Ser | 4.8 |
| Thr | 4.6 |
| Val | 5.7 |
| Trp | 1.8 |
| Tyr | 3.4 |
| | 100 |

*Detection plcH gene*

The resulting DNA sequence of the putative *plc* contained 1.6 kb (Fig.2) was compared with the published sequences using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/blast), and investigated to homogeneity in the GenBank data base. The sequence of the eluted PCR product of *P. aeruginosa* was deposited in data base under accession number KC405580. The phylogenetic tree (Fig.3) constructed from the DNA alignment profile using Clustal W (http://www.ge Cnome.jp/tools/clustalw/) revealed that *plc* had maximum structural similarities with *P. entomophila* YP-606779.1 (100%), *P. chlororaphis* ZP-10174580.1 (67%), *P. synxantha* ZP-10143859.1 (68%), *P.*

*fluorescens* YP-006322361.1 (69%), ABE73153.1 (68%) , YP-002870511.1 (68%), EIK63323.1 (68%) and YP-346558.1 (62%). Structurally, the higher similarities of our *plc* gene sequence with those from Pseudomonades, ensures the molecular and biochemical proximity with the tested *P. aeruginosa* . Also our result was matching with Viana-Niero *et al*. (2004) who assayed 1599, 1611, 1610 and 1478 bp fragments from four genes *plc A*, *B*, *C* and *D* respectively obtained from *Mycobacterium tuberculosis*. These *plc* genes had significant similarity with *P. aeruginosa* genes *plc*H and *plc*N which encoded hemolytic and non-hemolytic PLC enzyme respectively. Simillarly 1.3 kb *plc*B, 1.9 kb *plc*H and 1.7 kb *plc*N genes were detected from *P. aeruginosa* PAOl by Liyama *et al*. (2008).

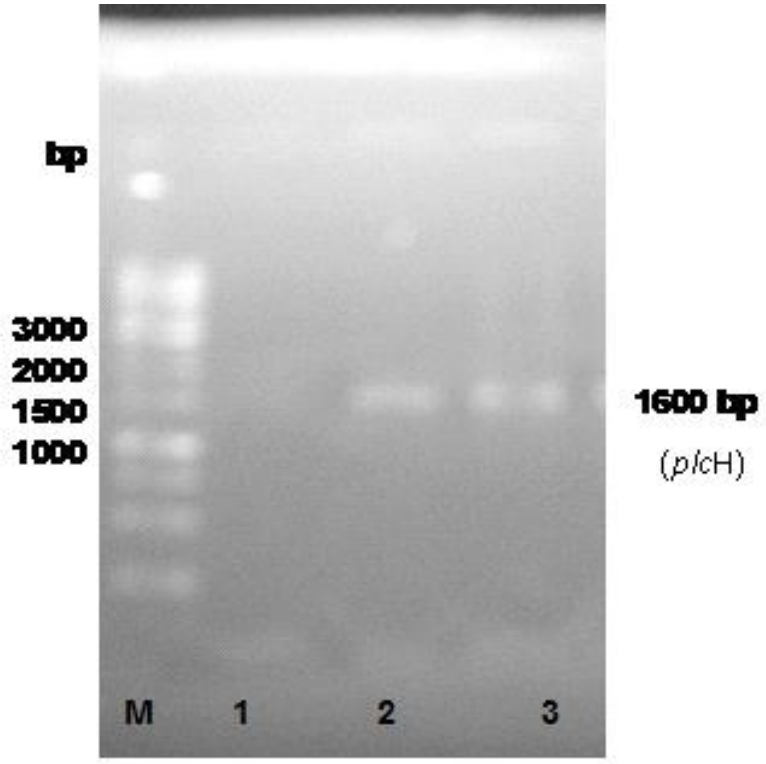


**Fig. 2 . Electrophoretic pattern showing PCR (1% agaros gel ). Lanes 1 to 3 (where: 1= negative control (without DNA), 2and3= DNA isolated from *P. aeruginose*), M: GeneRuler 1 Kb ladder DNA Marker (10, 8, 6, 5, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25 kb, it contains three reference bands of 6, 3 and 1 kb) for easy orientation.**

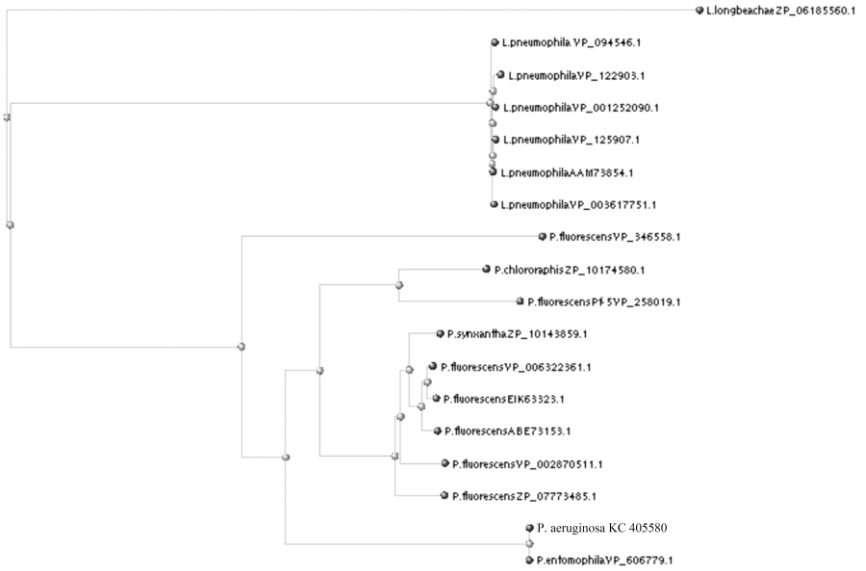


**Fig. 3. Phylogenetic tree of *plc*H gene encoded the putative *P. aurginosa* protein with the corresponding gene from the data base using Clustal W software.**

*Kinetics and biochemical properties of P. aeruginosa PLC*

Concerning the biochemical properties of the purified PLC, a linear relation between the relative activity and reaction time of PLC at different temperatures (25,35,45,55and65C ) was observed in (Fig. 4). It was found that, PLC under investigation was expected to be inactivated above 65C. Regarding thermal kinetic parameters, thermal inactivation rate (Kr) and half-life lime ($T_{1/2}$) decreased as temperature increased and the recorded half-life temperature Tm was 42C (Table 3). Concerning this finding, Fujita (1987) stated that, *P. aeruginosa* produced at least two hemolysins, a heat-labile which is known as phospholipase C and heat stable glycolipid. This feature was significantly distinct for other *Pseudomonas* PLCs (Sugimori and Nakamura, 2006).

Purified *P. aeruginosa* PLC showed a wide range of pH stability. Data represented graphically in (Fig. 5) showed that, the enzyme activity was approximately stable between pH 8.5 to 10 and the activity significantly decreased below and above it. In similar manner Wang *et al*. (2010) found that, the PLC of *B. mycoides* 970 was stable at pH 5-9.5. Extremely decrease or increase in pH altered three dimensional protein structure causing denaturaion and inactivation of enzyme (D·Souza *et al*., 1987).

In the present investigation, (Table 4) indicates that, the pure PLC of tested *P. aeruginosa* was activated by the divalent ions $Zn^{2+}$, $Ca^{2+}$, $Ba^{+2}$ and $Co^{2+}$ in descending order while $Mn^{2+}$, $Cu^{2+}$ and $Fe^{2+}$ completely inhibited the enzyme activity. In partial coincidence with our results, Moreau et al. (1988) reported that, $Ca^{2+}$ and $Zn^{2+}$ ions were found to play a specific role in hydrolysis of PC by

*Cl. perfringens*. $Ca^{2+}$ is essential for enzyme binding to lipid film, whereas $Zn^{2+}$ was especially involved in the catalytic hydrolysis of the substrate. Also, Titball (1993) denoted that, $Zn^{2+}$ was absolutely necessary for catalytic function of several bacterial enzymes while $Ca^{2+}$ and $Mg^{2+}$ were reported to significantly influence activity of bacterial PLCs molecules. It was also noticed that, the studied enzyme activity in presence of $Zn^{2+}$ ions increased by 1.19 fold and significantly decreased by 1 or 5 mM EDTA. Also the enzyme restored its activity when $Zn^{2+}$ ions were added in presence of 1 mM EDTA. This results collectively suggested that the *P. aeruginosa* PLC may be a metallophospholipase C containing $Zn^{2+}$ ions in its active site. Crevel et al. (1994) reported that, not only Gram positive but also Gram negative *P. fluorescence* produced $Zn^{2+}$metallophospholipase C. Moreover PLC of *B. mycoides* was characterized as a metallophospholipase and $Zn^{2+}$ $Ca^{2+}$, and $Ba^{2+}$ were enzyme activators (Wang *et al*., 2010). Some $Zn^{2+}$-PLCs, like the -toxin of *Cl. perfringens* are potent toxins and have roles in its pathogenesity.

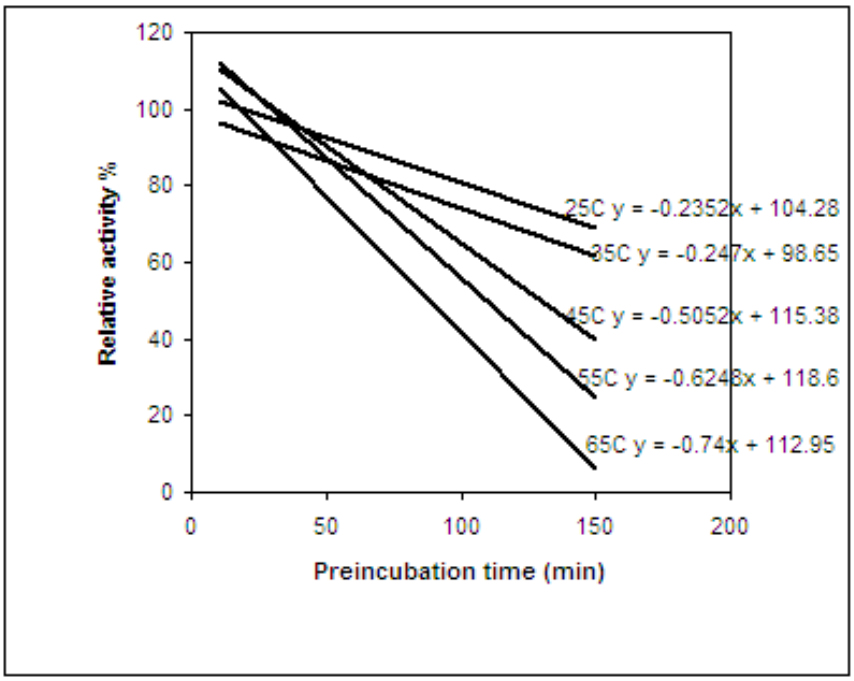


**Fig. 4 .Thermal stability of *P. aeruginosa* phospholipase C .**

**TABLE 3. Thermal inactivation parameters of *P. aeruginosa* phospholipase C.**

| Temp. (C) | $T^1/2$ (min)* | Kr $S^{-1}$** | Tm *** (C) |
|---|---|---|---|
| 25 | 266.3 | 0.13 | 42 |
| 35 | 230.7 | 0.146 | |
| 45 | 198.0 | 0.163 | |
| 55 | 129.5 | 0.31 | |
| 65 | 109.7 | 0.38 | |

* Half-life times ($T^1/_2$) expressed by minutes
** Thermal inactivation rate (Kr) expressed per seconds
*** Half-life temperature (Tm) expressed by C

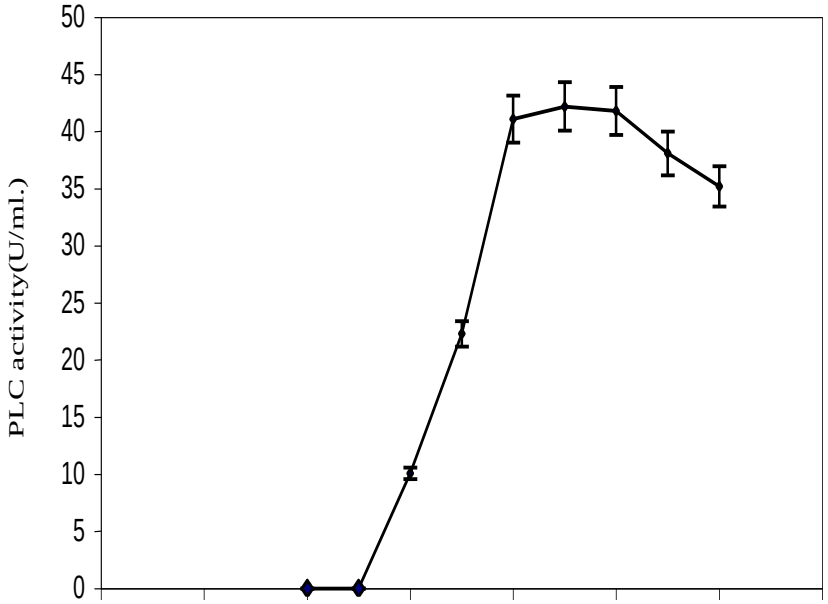


**Fig . 5. PH stability of *P.aeruginosa* phospholipase C.**

**TABLE 4.Effect of metal ions on *P .aeruginosa* phospholipase C activity.**

| Metal ion | Concentration ((mM | Relative activity |
|---|---|---|
| Control | - | e100 |
| $^{+}Mn^{2}$ | 5 | - |
| $^{+}Mg^{2}$ | 5 | e100 |
| $^{+}Ba^{2}$ | 5 | c107 |
| $^{+}Zn^{2}$ | 5 | a119 |
| $^{+}Co^{2}$ | 5 | d103 |
| $^{+}Cu^{2}$ | 5 | - |
| $^{+}Fe^{2}$ | 5 | - |
| $^{+}Ca^{2}$ | 5 | b112 |
| EDTA | 1 | f57.3 |
| EDTA | 5 | g26.4 |
| EDTA $+Zn^{2+}$ | 1+5 | b112 |
| L.S.D | | 1.62 |

Values with different letters within the same column differ significantly at p0.01 >

From the linear relation between PC concentration (M) and PLC activity (Fig. 6) it was found that, the Vmax, km and Kcat for tested PLC were 1.88 U $mg^{-1}$ $min^{-1}$, 0.12 uM and 3.2 x $10^{-5}$ $S^{-1}$ respectively. Affinity of PLC for PC as a substrate was detected by Berka and Vasil (1982), Lucchesi and Domenech (1994) and Ibarguren *et al*. (2010) for different *P. aeruginosa* strains. Wehbi *et al*. (2003) explained the activation of PLC and Pi cleavage from phosphatidylinositol by increasing the Kcat and decreasing the km of the enzyme. Also, the small km (0.12 M) of the tested PLC indicated high affinity of the enzyme to substrate, approaching Vmax more quickly (Lehninger *et al*., 2005).

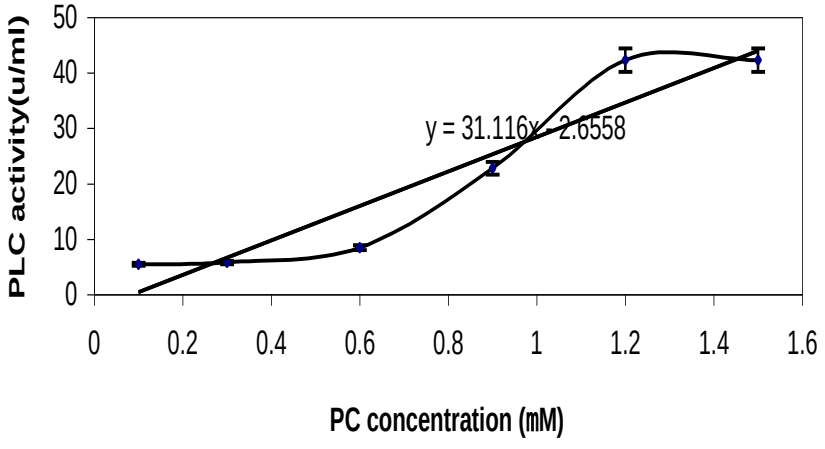


**Fig . 6. Effect of different PC concentration on *p. aeruginosa* phospholipase C activity.**

*Hemolytic and mice lethal activities of fresh and heat detoxified P. aeruginosa PLC*

Studying the hemolytic and mice lethal activities of the normal and heat detoxified *P. aeruginosa* PLC were attained through this work as shown in (Table 5). Hemolytic activity of purified PLC from *P. aeruginosa* was described previously by Meyers *et al*. (1992); Flieger *et al*. (2000); Gomez *et al*. (2001); Son *et al*. (2007) and Kida *et al*. (2011). In present study injection into mice via 64(HU/ml.) hemolytic titer of fresh or heat detoxified ( at 65 C for 30 min.) *P. aeruginosa* PLC was associated by 100 and 25% mortality after 12 hr respectively . The same percentages were obtained after 24 hr when mice were injected by half hemolytic titer (128) of fresh and heated (at 100 C for 30 min) *P. aeruginosa* PS11 hemolysin respectively (Khalil, 2009) . This assumes that, the mortality of experimental animals was attributed to an immunological shock induced by the high dose of cytotoxic PLCH (Meyers *et al*., 1992 and Wargo *et al*., 2011).

**TABLE 5. Lethal activity of fresh and heat detoxified *P. aeruginosa* phospholipase C on mice .**

| **PLC** | | **hemolytic titer(HU ml$^{-1}$)** | **Mice mortality%** |
|---|---|---|---|
| control | | 64a | 100a |
| Heat detoxified at 65C | 30min | 64a | 25b |
| | 60min | 32b | 0c |
| | 90min | 0c | 0c |
| | L.S.D | 25.6 | 11.35 |

Values with different letters within the same column differ significantly at $p < 0.01$

Also in the present study PLC maintained 32 HU/ml hemolytic titer was completely lost its lethal activity on mice after 60min at 65C. This assumed that, low doses of heat detoxified PLC enhanced the immune response to more mice protection as recorded by Manohar et al. (1966) and Khalil (2009).

*In vivo, immunological characteristics and cytotoxicity of P. aeruginosa PLC in rabbits*

Antigenicity of fresh and heat detoxified (65C for 60 min.) PLC in New Zealand rabbits was evaluated by measuring the increase in the plasma immunoglobulin IgG antibodies as compared to control. From the profile of the plasma IgG concentrations (Fig.7,a&b), the titer of IgG appeared clearly after 15 days of the last injection dose of fresh and detoxified enzyme by about 1.15 and 1.21 fold respectively higher compared to the control (without enzyme), followed by the decrease in the titer of IgG up to 45 days. Results for antigenic properties of tested PLC especially for induction of IgG antibodies after 2 weeks was consistent with those reported for *P. aeruginosa* PLC in mice model of Preston *et al*. (1991). Also Adhikari *et al*. (2012) reported that vaccinated mice showed higher ELISA and neutralizining antibody titer, this finding was

consistent with the ability of antibodies to neutralize *Staphylococcus aureus* hemolysin (HLa) via inhibition of Hla oligomerization . On the other hand the lowering in titer of anti-PLC antibodies during the circulating time after 2 weeks assumed that these antibodies became incapable of neutralizing fresh or detoxified PLC activity in situ and don't affect the enzymatic function. It was expected that, antibodies don't bind to, or are incapable of conformational modification of the active site (Chin and Watts, 1988).

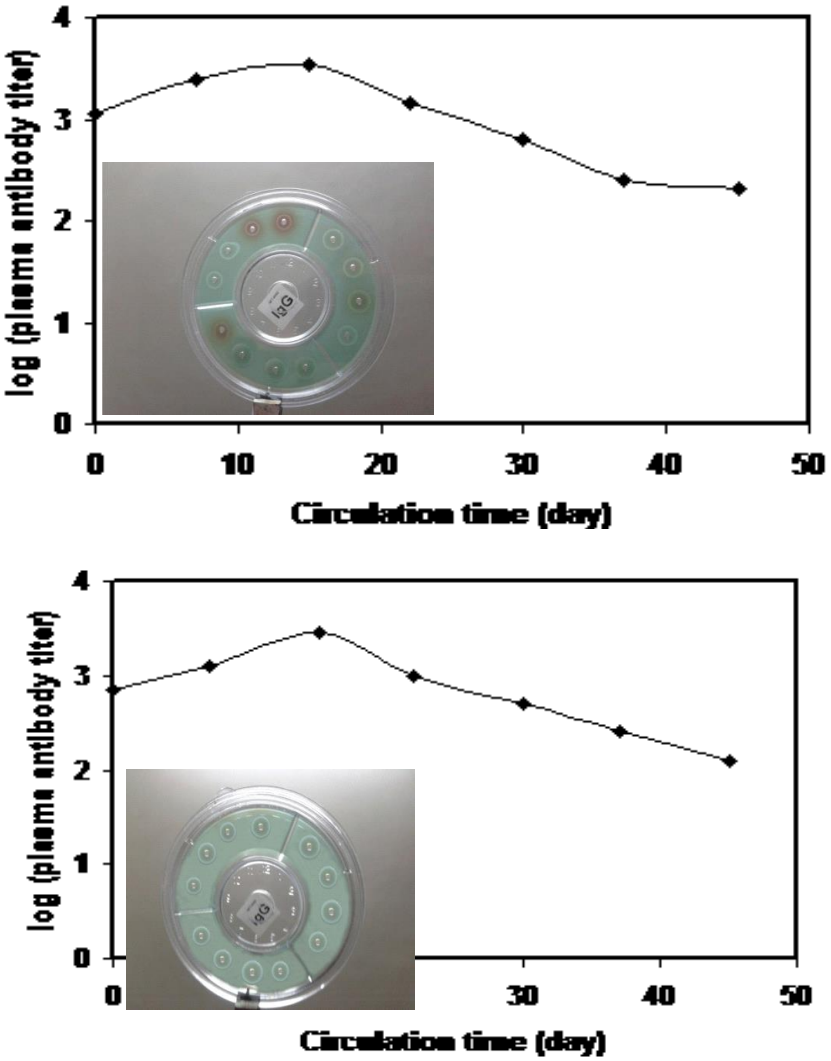


**Fig. 7. Plasma anti-PLC antibody titer (IgG) in New Zealand Rabbits injected with fresh (a) and heat detoxified (b) *P. aeruginose* phospholipase C .**

Hematological and blood chemistry picture for the New Zealand rabbits in response to administration of fresh and detoxified *P. aeruginosa* PLC were evaluated in (Table 5). It was found that ,the significant change in all hematological parameters (RBCs, WBCs and hemoglobin) of rabbits injected with fresh PLC during 45 days ,that the enzyme recognized phospholipids found predominantly on eukaryotic cells membrane (Terada, 1999) and leys red blood cells (Tami, 2008) with release of hem into the surrounding (Sritharan, 2007). Also the observed dysfunction in liver and kidney chemistry explained that the primary target organs involved in hemolytic PLC induced toxicity in the liver and kidney (Meyers *et al*., 1992).On the other hand there was no obvious changes in blood hematology and chemistry of rabbits injected with detoxified *P. aeruginosa* PLC. The increase in total leukocyte after treatment with low dose of heated PLC indicated that leukocyte specially granulocytes play a major role in control of *P.aeruginosa* infections (Terada, 1999). These results attracted attention to PLC vast importance to the enhancement of the immune response to more protection against bacterial infection using different routes of immunization with heat inactivated bacterial PLC (Manohar *et al*, 1966 Estahbanati *et al*., 2002 , Ipaktchi *et al*., 2007 and Khalil 2009) .

## Conclusion

The data obtained throughout this study indicated that, the hemolytic PLC was purified to homogeneity from culture of *P. aeruginosa* 1589741 strain. The enzyme revealed its characteristic properties in relation to other bacterial PLC. Moreover, thermal, pH stability, ions effect and substrate kinetics illustrated the relation between biochemical properties of the enzyme and its activity. Also the results suggested that, hemolytic PLC contributed to *in vivo* virulence of *P. aruginosa* .The data obtained about mice protection and rabbits immunogenicity by heat detoxified PLC indicated that this toxin may represented a prime candidate for vaccine development in future works in the immunotherapy application against certain pathogenic bacteria.

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**TABLE 6. Cytoyoxicity of normal and heat detoxified (65 C for 60min.) hemolytic *P. aeruginosa* phospholipase C on rabbits.**

| Tested parameters | | Control | weeks2 | | weeks4 | | weeks6 | | L.S.D | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Fresh | Detoxified | Fresh | Detoxified | Fresh | Detoxified | Fresh | Detoxified |
| Liver | (ALK(U/L | 1.1a*,ab | 1.8a | 0.9ab | 0.7d | 1.3a | 1.4b | 0.7b | 0.13 | 0.75 |
| | (AST(U/L | 127c*,bc | 478a | 133a | 84d | 128b | 150b | 126c | 1.51 | 1.51 |
| | (ALT(U/L | 252c*,b | 360b | 260a | 455a | 248c | 156d | 250bc | 3.90 | .3o4 |
| | (Albumin(g/dI | 6.23b*,a | 7.15a | 5.9ab | 3.87c | 5.4b | 3.88c | 5.8ab | 0.22 | 0.56 |
| | (T.P(g/dI) | 13.5b*,a | 21.45a | 14.0a | 6.50c | 1.7c | 7.26c | 12.5b | 1.55 | 0.94 |
| | (BIL-T(mg/dI) | 1.5b*,a | 2.22a | 1.3a | 1.13b | 1.6a | 1.2b | 1.4a | 0.45 | 0.36 |
| | (BIL-D(mg/dI) | 0.7b*,d | 1.5a | 1.0b | 0.6b | 1.1a | 0.68b | 0.8c | 0.17 | 0.08 |
| Kidney | (Urea(mg/dI) | 49c*,b | 64a | 51a | 34d | 47c | 62b | 50ab | 1.51 | 1.51 |
| | (creatinine(mg/dIa) | 3.88b*,a | 6.44a | 3.9a | 1.0c | 4.0a | 1.14c | 3.5a | 0.75 | 0.54 |
| Blood | (hemoglobin(g/dI) | 1.6a*,a | 0.9b | 1.5a | 0.6c | 1.6a | 0.2d | 1.4a | 0.11 | 0.33 |
| | Erythrocytes($10^{12}$/L) | 8.9a*,a | 7.1b | 8.2b | 5.2c | 8.3b | 4.0d | 9.0a | 0.74 | 0.43 |
| | (Leucocytes($10^{9}$/L ) | 6.2a*,a | 5.6b | 5.8b | 4.1c | 6.0ab | 3.2d | 5.9b | 0.51 | 0.24 |

*Control data are the mean of 3 replicates ± SE for fresh PLC while without star for detoxified PLC. Values with different letters within the same column differ significantly at $p < 0.01$

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# الخصائص والصفات المناعية لإنزيم الفوسفرلبلز س المحل للدم المنتج بواسطة بسيدوموناس أرجينوزا

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تم انتاج إنزيم الفوسفوليبيز س بعد أربعة أيام من مزرعة لسلالة بسيدوموناس ارجينوزا منماه في وسط غذائي سائل يفتقر للفوسفات الغير عضوي عند درجة حرارة 35م ودرجة حموضة 7,2. وباستخدام الفصل الكروماتوجرافى والترشيح خلال الجل تم تنقية الانزيم الى 6.2 ضعف وعائد 28,2٪ وكان الوزن الجزئيى للإنزيم النقي هو 58,7 كيلو دالتون. ويعتبر الانيلين هو الحمض الأميني السائد وبلغت نسبته 13,1٪ من بروتين الإنزيم النقي. وقد اكد تحليل الحمض النووي لسلالة بسيدوموناس ارجينوزا باستخدام تفاعلات البلمرة المتسلسلة وجود الجين المسئول عن انتاج انزيم الفوسفوليبيز س المحلل للدم (1,6 كيلو بيزبير). وقد لوحظ عدم وجود نشاط للإنزيم النقي عند درجات حرارة أعلى من 65م حيث أن درجة حرارة نصف العمر Tm كانت 42م. وللإنزيم نشاط الى حد ما ثابت عند درجة حموضة من 8,5 الى 10. ويوصف الانزيم النقي بأنه فوسفوليبيز س معدني لأيون الزنك وأيضا بعض الأيونات الموجبة مثل الكالسيوم والباريوم والكوبلت أظهرت تأثيراً محفزاً لنشاط الإنزيم. ويتفاعل الإنزيم النقي مع مادة التفاعل الفوسفاتيدايل كلوين وكانت قيمة الـ $V_{max}$= 1,85 وحدة / مليجرام/ دقيقة ، km= 0,12 ميكرو مول و $K_{cat}$= 1,96 × $10^{-5}$ / ثانية. وأوضحت التجارب أن الأنزيم النقي فقد تأثيره القاتل على الفئران بعد معاملة حراريا عند 65م لمدة 60 دقيقة. ومن خلال دراسة الخصائص المناعية للإنزيم النقي فقد لوحظ ظهور الاجسام المضادة أى جى جى فى سيرم الدم للأرانب النيوزيلاندي بعد 15 يوم من حقنها بالإنزيم الغير معامل والمعامل حراريا الى 1,15 و 1,21 ضعف على التوالي مقارنةً بالكونترول (بدون أنزيم). ثم تناقص معدل انتاج الأجسام المضادة حتى 45يوم . وأيضا أظهرت نتائج تحليل عينات الدم أن هناك تناقص في اعداد كرات الدم الحمراء والبيضاء و نسبة الهيموجلوبين وخلل في وظائف الكبد والكلى للأرانب المحقونة بالإنزيم الغير معامل حراريا على العكس في حالة الانزيم المعامل حراريا.