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Antibacterial properties of recombinant human non-pancreatic secretory phospholipase A_2

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ABSTRACT

Human non-pancreatic secretory phospholipase A_2 (hnpsPLA$_2$) is a group IIA phospholipase A_2 which plays an important role in the innate immune response. This enzyme was found to exhibit bactericidal activity toward Gram-positive bacteria, but not Gram-negative ones. Though native hnpsPLA$_2$ is active over a broad pH range, it is only highly active at alkaline conditions with the optimum activity pH of about 8.5. In order to make it highly active at neutral pH, we have obtained two hnpsPLA$_2$ mutants, Glu89Lys and Arg100Glu that work better at neutral pH in a previous study. In the present study, we tested the bactericidal effects of the native hnpsPLA$_2$ and the two mutants. Both native hnpsPLA$_2$ and the two mutants exhibit bactericidal activity toward Gram-positive bacteria. Furthermore, they can also kill *Escherichia coli*, a Gram-negative bacterium. The two mutants showed better bactericidal activity for *E. coli* at neutral pH than the native enzyme, which is consistent with the enzyme activities. As hnpsPLA$_2$ is highly stable and biocompatible, it may provide a promising therapy for bacteria infection treatment or other bactericidal applications.

1. Introduction

Phospholipase A_2 is a super family of enzymes that can catalyze the hydrolysis of lipids at position of the *sn*-2 bond [1]. Secreted PLA$_2$s express in various of cell types and exist in multiple interstitial fluids with relatively small molecular size (14 kDa) and 6–7 disulfide bonds. SPLA$_2$s are generally involved in many inflammatory diseases and have long been referred to as "inflammatory enzymes" [2,3]. However, some sPLA$_2$ isoforms have also been reported to play a protective role against inflammation [4].

Beside the role in inflammation, the most well-known physiological function of sPLA$_2$ is antimicrobial [4–6]. Group IIA PLA$_2$ has high physiological antibacterial activity against Gram-positive bacteria *in vitro* [6]. Group IIA PLA$_2$ was the major bactericidal component of tears and serum from septic animals against Gram-positive bacteria. The group IIA PLA$_2$s purified from rabbit, mouse and human are directly against Gram-positive bacteria such as *Staphylococcus aureus* [7–9]. However, many isoforms of group IIA PLA$_2$ cannot kill Gram-negative bacteria. The activity toward a Gram-negative, such as *Escherichia coli*, is largely attributable to the bactericidal/permeability-increasing (BPI) protein [10]. However, as more and more sPLA$_2$ were purified, a murine intestine PLA$_2$ identified as group IIA PLA$_2$ was reported to be able to kill Gram-positive *Listeria monocytogenes* without BPI [11].

The anti-bacterial property of mammalian group IIA sPLA$_2$ requires the enzymatic activity [8,11,12]. Inhibition of the catalytic activity of group IIA PLA$_2$ can obviously reduce the killing of bacteria [13]. It was suggested that the bacterial envelope sites engaged in cell growth may stand for preferential sites for the action where group IIA PLA$_2$ attacks against Gram-positive bacteria [14]. The bacterial cell wall seems to be a physical barrier for the binding of mammalian group IIA sPLA$_2$ and its substrates. For the Gram-positive bacteria, the cell wall bears a highly anionic charge, because of the presence of lipoteichoic acid. The surface of mammalian group IIA sPLA$_2$ is highly cationic. So electrostatic interactions between the anionic bacterial cell wall and sPLA$_2$ may facilitate passage of the enzyme through the cell wall. But for the Gram-negative bacteria, the composition of the bacteria cell wall is more complex. A peptidoglycan layer coats the cell membrane. And outside the peptidoglycan layer, there is an external membrane containing high asymmetric phospholipids and lipopolysaccharides (LPS) [5]. It was found that despite its efficacy against Gram-positive bacteria, secretory phospholipase A_2 lacked bactericidal activity against Gram-negative organisms (*E. coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*)

Abbreviations: PLA$_2$, phospholipase A_2; sPLA$_2$, secretory phospholipase A_2; hnpsPLA$_2$, human non-pancreatic secretory phospholipase A_2.

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when tested in the ionic environment of tears [9]. However, recently, Gronroos [15] confirmed that an isoform of group IIA PLA_2 inhibited the growth of *E. coli* at high concentration.

The growing problem of microbial resistance makes the developing of novel antibiotics urgent. In the present study, we tested the antibacterial property of human non-pancreatic secretory PLA_2 (hnpsPLA$_2$), a group IIA PLA_2, and its mutants (Glu89Lys and Arg100Glu). HnpsPLA$_2$ was found to be highly active at alkaline pH with modest activity at neutral pH. We have attempted to tailor its pH dependence by mutating surface charges. Several mutants, including Glu89Lys and Arg100Glu were identified to be highly active at neutral pH [16]. We show here that both the native hnpsPLA$_2$ and the two mutants can kill Gram-positive bacteria (*S. aureus*), as well as Gram-negative bacteria (*E. coli*). The two mutants showed better bactericidal activity at neutral pH.

2. Materials and methods

Bacteria. Bacteria used in this study included *E. coli* MC1601 (*E. coli*, purchased from The Institute of Microbiology, Chinese Academy of Sciences) and *S .aureus* ATCC25923 (*S. aureus* purchased from the Chinese National Institute For Food and Drug Control). All bacteria were grown in LB at 37 °C overnight, diluted to 1:100 in fresh media and grown at 37 °C until OD_{600} reached 1.0. Then the suspension was centrifuged at 4500 rpm for 8 min at 4 °C. The obtained pellet was redispersed into Tris–HCl Buffer. Cell suspension was adjusted to OD_{600} = 0.6 and diluted ten times by Tris–HCl Buffer to form the test suspension (corresponding to 5×10^7–1×10^8 CFU/ml).

SPLA$_2$ samples. Enzyme expression and purification were performed as previously described [17]. In short, a synthesized gene was used to express the enzyme. The protein was harvested as inclusion bodies, refolded, and purified by gel filtration [17]. The concentration of eluted hnpsPLA$_2$ was approximately 3.6 mg/ml. The pET21a vector containing the hnpsPLA$_2$ gene was used as a template for mutagenesis. The genes of Glu89Lys and Arg100Glu mutants were obtained by site-directed mutagenesis. The protocol for protein expression and purification was the same as that used for wild-type hnpsPLA$_2$.

Assay of bactericidal activity of sPLA$_2$. The influences of the enzyme samples on bacterial viability were checked by measuring bacterial colony forming ability after incubation of the bacteria with or without the proteins at 37 °C. Typical incubation mixtures contained 10^8 CFU/ml *E. coli* bacteria in a total volume of 300 μl 25 mM Tris–HCl buffer (pH = 8.0). And the concentrations of the proteins are 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml and 10 μg/ml. The mixtures were incubated for 30 min at 37 °C. To determine the cell viability, 200 μl of the *E. coli* suspension were spread onto LB agar plates. All the plates were incubated at 37 °C for 16 h.

At different pH conditions, the incubation mixtures contained 10^8 CFU/ml *E. coli* or *S. aureus* bacteria and 10 μg/ml enzymes in 300 μl 25 mM Tris–HCl buffer (pH = 7.5, 8.0, 9.0). The mixtures were incubated for 1 min, 2 min, 3 min, 5 min, 10 min, 15 min, 20 min at 37 °C. Then 200 μl of the suspension were spread onto LB agar plates. All the plates were incubated at 37 °C for 16 h.

Pesisters of ampicillin mixed with sPLA$_2$. Typical incubation mixtures contained 10^8 CFU/ml *E. coli* bacteria and 10 μg/ml enzymes in a total volume of 300 μl 0.8% NaCl and 10 mM Tris–HCl buffer (pH = 8.0). The mixtures were incubated for 30 min at 37 °C. Then 200 μl of the *E. coli* suspension were spread onto LB agar plates which contained 100 μg/ml ampicillin. After all the plates were incubated at 37 °C for 16 h, 300 μl buffer, containing 15 units β-lactamase, were spread onto the plates and then the plates were incubated at 37 °C for 16 h. After incubation, colonies on the plates were counted to determine the cell viability.

3. Results and discussion

3.1. Bactericidal properties of hnpsPLA$_2$ toward Gram-negative bacteria

The bactericidal activities of hnpsPLA$_2$ and its mutants for *E. coli* were first checked at pH 8. Using a protein concentration of 10 μg/ml, 95% of the bacteria were killed (Fig. 1). When the concentration was lowered to 5 μg/ml, the two mutants still showed high activity to kill over 90% bacteria while the native hnpsPLA$_2$ can only kill about 40% bacteria. This indicates that hnpsPLA$_2$ can kill Gram-negative bacteria at pretty low concentration and the mutants are more active than the wild-type.

We then studied the bactericidal activities at different pH using a protein concentration of 10 μg/ml. Fig. 2A–C show the bactericidal activity of hnpsPLA$_2$ and mutants at pH 7.5, 8.0 and 9.0 toward *E. coli*. The cell viability of *E. coli* was reduced rapidly within 1 or 2 min of incubation with the enzyme samples. The mutants, Glu89Lys and Arg100Glu appeared to be more effective than the wild type at pH 7.5 and 8.0 conditions. At pH 7.5 and 8.0, within 2 min, Glu89Lys and Arg100Glu killed more than 99% and 96% of *E. coli*, while the wild type killed nearly 95% of *E. coli*. We have shown before that the mutants Asp100Glu and Glu89Lys have higher catalytic activity at neutral pH conditions compared to the native enzyme [16]. At pH 9.0 condition, all the enzymes had high bactericidal activity and after 1 min incubation, the cell viability decreased below 0.1% (Fig. 2C). The pH of the environment will affect the permselectivity of the cell membrane. At pH 9.0, the stability and permeability of bacteria membrane are disturbed to some extent, so the phospholipids on membrane are hydrolyzed by sPLA$_2$ more easily. Meanwhile, the catalytic activities of these three enzymes are similar at pH 9.0 condition.

As shown above, 10 μg/ml of hnpsPLA$_2$ can effectively kill *E. coli* cells. The external membrane of phospholipids of *E. coli* contains lipopolysaccharides which negative charges, which may absorb hnpsPLA$_2$ which has many surface positive charges. When the enzyme is enriched on the external membrane, some enzyme molecules may pass through the external membrane, bind to the phospholipids on the cell wall and hydrolyze them. When the cell wall is destroyed, the enzyme binds to and hydrolyzes the cell membrane more easily and the microbe is killed. In the meantime, the cells in the experiment are at the logarithmic phase. At this phase the bacterial cells are dividing and the cell membrane and

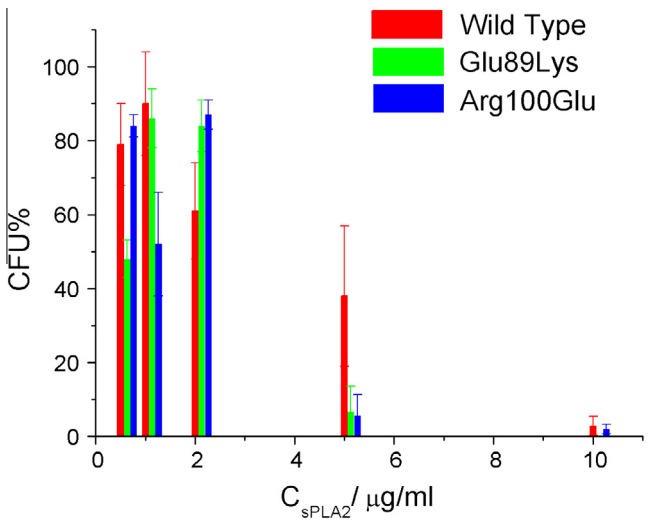

Fig. 1. Concentration dependency of the antibacterial activity of *E. coli*. HnpsPLA$_2$ (red), Arg100Glu (green), Glu89Lys (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

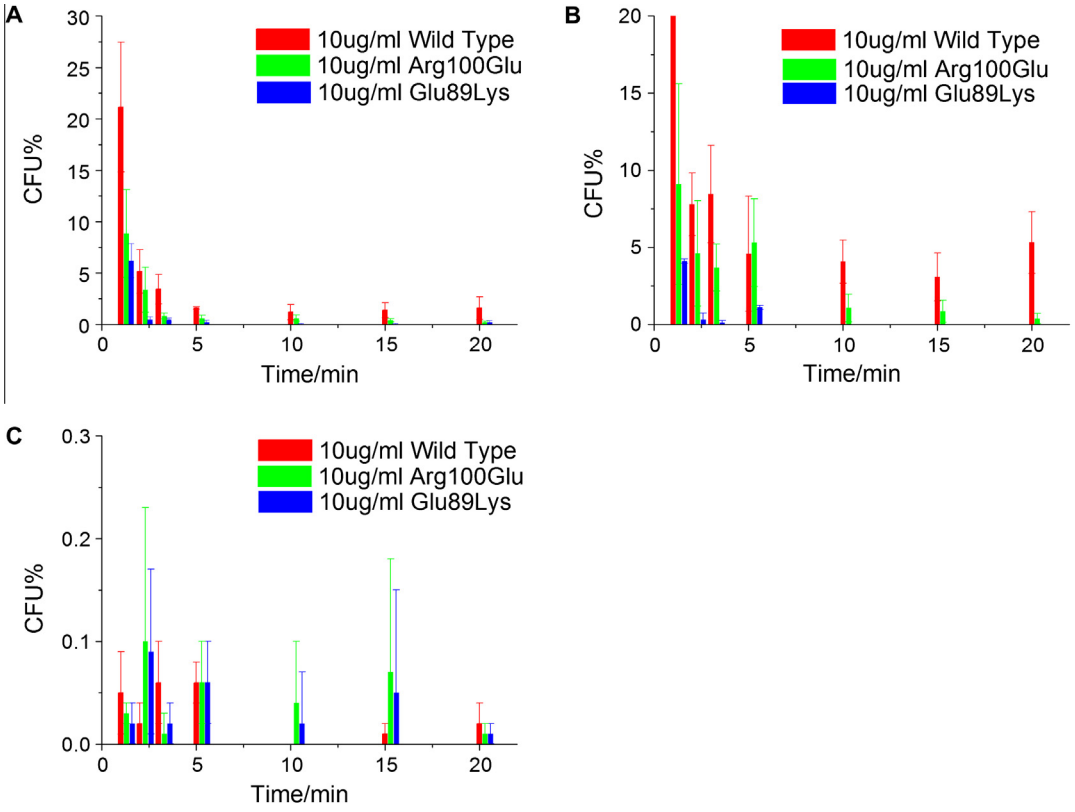

Fig. 2. *In vitro* bactericidal activity of hnpsPLA$_2$ and its mutants toward *E. coli*. (A) pH = 7.5, (B) pH = 8.0, (C) pH = 9.0. HnpsPLA$_2$ (red), Arg100Glu (green), Glu89Lys (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cell wall are forming. That makes it easier for sPLA$_2$ to bind to the membrane and hydrolyze the phospholipids. HnpsPLA$_2$ has 23 positive charged residues on its surface, and that maybe benefit the rapidly binding for the enzyme and phosphatidylglycerol which is the mainly component of the cell membrane of *E. coli*. For hnpsPLA$_2$, changing one charge of a residue on the surface of protein does not change the charges of the whole surface of this enzyme fundamentally. So hnpsPLA$_2$ and its mutants have similar capabilities in passing through the cell wall. When the surface positive charges were similar and the velocities of passing through the cell wall and binding to the membrane were also similar, the catalytic reaction rates determined the efficiency of bactericidal activity. Our previous researches show that the enzymatic activities of the mutants are higher at pH 7.5 and 8.0 than that of the wild type. So the mutants had more effective bactericidal activity.

3.2. Bactericidal properties of hnpsPLA$_2$ toward Gram-positive bacteria

We also tested the bactericidal effects of hnpsPLA$_2$ and its mutants toward Gram-positive bacteria *S. aureus*. 10 μg/ml of hnpsPLA$_2$ and the two mutants at pH 7.5, 8.0, and 9.0 for various time periods were tested. As expected, hnpsPLA$_2$ and the mutants are highly active toward *S. aureus*. Within 2 min, the bacteria viability was decreased to 0.3% at all experimental pH conditions by hnpsPLA$_2$ (Fig. 3A–C). The two mutants had lower bactericidal activity than the wild type within 10 min at pH 7.5 and 8.0 conditions, which then became similar after 10 min (Fig. 3A and B). At pH 9.0, within 1 min, 99.7% of the *S. aureus* was killed by hnpsPLA$_2$ and its mutants (Fig. 2C). The high activity at alkali condition might due to the reason that the cell walls were destroyed by the alkali condition and the enzymes could pass through the cell wall more easily to bind and hydrolyze the membrane.

3.3. Anti-persister activity of hnpsPLA$_2$

We also tested whether the treatment of hnpsPLA$_2$ can change the level of persisters. The level of persisters of *E. coli* (MC1601 strains) was about 1.5×10^{-5}. After incubation with 10 μg/ml hnpsPLA$_2$ and its mutants, the level of persisters remains about the same (data not shown). This indicates that the hnpsPLA$_2$ do not have effect on the level of the persisters survived to ampicillin treatment. Persister cells that neither grow nor die in the presence of microbicidal antibiotics have the similar structures of cell wall and membrane as other cells. The sPLA$_2$ had the similar probability to kill the persister cells as that to kill the other cells. So sPLA$_2$ mixed with ampicillin will not affect the level of the persisters of *E. coli*.

In conclusion, we have shown that recombinant hnpsPLA$_2$ can be used as bactericidal agents for both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*). The two mutants, Glu89Lys and Arg100Glu, are more active than the native enzyme for killing Gram-negative bacteria. Because the recombinant hnpsPLA$_2$ is derived from human tissues and has high degree of biocompatibility, it may be considered as a potential therapeutic agent synergizing antibiotics. With new administration routes and new targeting systems being developed, hnpsPLA$_2$ will be promising as a novel strategy for bacteria infection treatment. As hnpsPLA$_2$ is highly stable, it may also be used as bactericidal agents for external applications.

Author contribution

Shunchen Qiu and Luhua Lai conceived the project; Shunchen Qiu performed the experiments; Shunchen Qiu and Luhua Lai analyzed the data and wrote the paper.

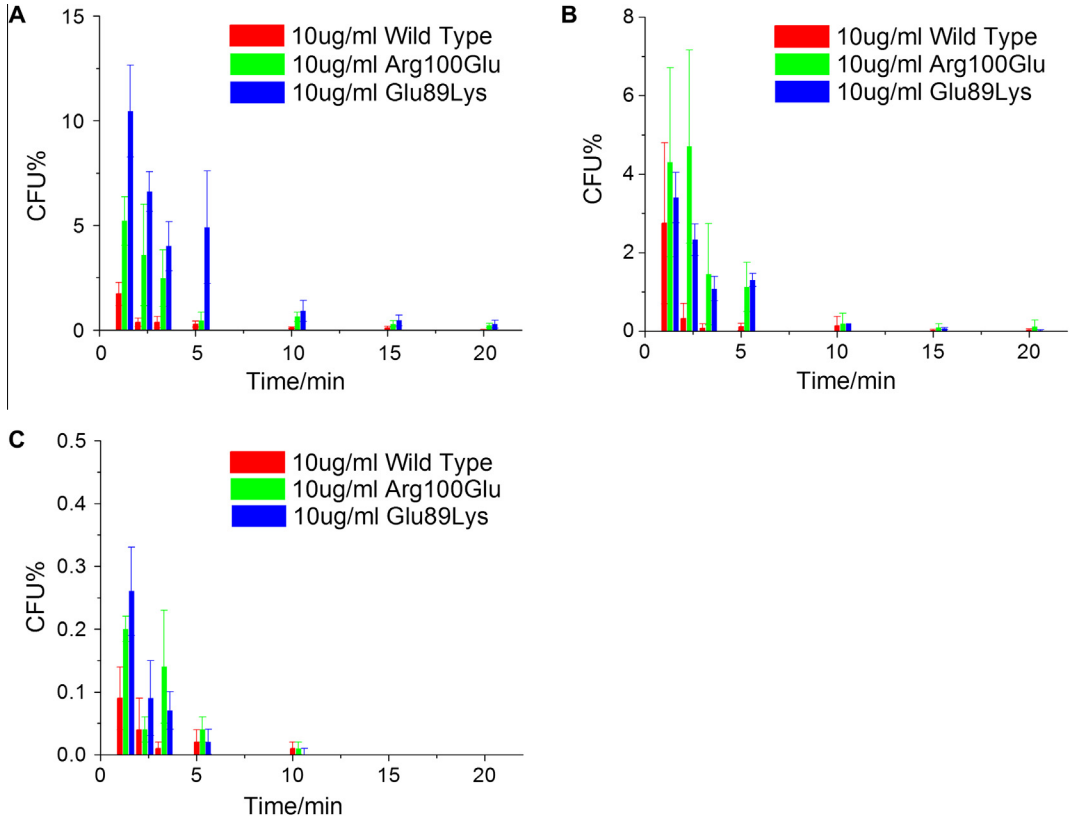

Fig. 3. *In vitro* bactericidal activity of hnpsPLA$_2$ and its mutants toward *S. aureus*. (A) pH = 7.5, (B) pH = 8.0, (C) pH = 9.0. HnpsPLA$_2$ (red), Arg100Glu (green), Glu89Lys (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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