

## Effect of pH on the Conformation and Biological Activity of *Clostridium septicum* Alpha-toxin, a Pore-forming Hemolysin

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**Abstract:** The effects of pH on the pore-forming *Clostridium septicum* alpha-toxin that causes myonecrosis was assessed at pH 5.0, 7.0 and 9.0 by measuring circular dichroism and its biological activity. Incubation of the toxin at pH 5.0 increased the hemolytic activity measured in murine red blood cells. On the other hand, incubation of the toxin at pH 9.0 drastically reduces the biological activity of the toxin by five-fold. This study presents the first evidence of the effect of pH on the functional properties of alpha-toxin directly related to the common disease caused by *C. septicum* in animals and humans i.e. traumatic and non traumatic gas gangrene.

**Key words:** *Clostridium septicum*; alpha-toxin, hemolytic activity; lethality; pH

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### INTRODUCTION

Alpha-toxin is a major exotoxin produced by strains of the Gram-positive bacterium *Clostridium septicum*. As a result of alpha-toxin, *C. septicum* has been implicated as a cause of exogenous (traumatic) and endogenous (non-traumatic) myonecrosis, which are rapidly fatal diseases in humans and animals [1, 2]. The toxin is secreted as a water-soluble protoxin which requires proteolytic digestion for its activity. In vivo it is activated by furin, which is present on cell surfaces of eukaryotic cells [3]. The activation is necessary for subsequent induction of oligomerization and ultimate insertion of the oligometric complex into the plasma membrane of mammalian cells as activation exposes hydrophobic domains [4, 5, 6].

The factors, which may influence the conformational changes of the toxin, on biological membranes are poorly understood as these membranes are negatively charged through their constituents consisting of the phospholipid, glycolipid and glycoprotein [7]. As such, pH may exert an important role in the activity of *C. septicum* alpha-toxin. It is interesting then to determine if there is a simple relationship between conformation and biological activity. Taking into account these antecedents, the purpose of this work was to study the effect of pH on the stability of alpha-toxin and relate the effects to its biological activity.

### MATERIALS AND METHODS

*C. septicum* alpha toxin was purified and activated as previously described [6,8]. The protein concentration was determined with a BIO-RAD protein assay kit (BIO-RAD laboratories, Hercules, CA) according to the manufacturer's instructions. Bovine plasma gamma globulin was used as the protein standard.

Following purification and activation, the toxin was subjected to a phosphate buffer under different pH. Samples containing a final protein concentration of 10 µg/ml were prepared and then dialyzed under the respective buffers overnight at 4 °C. After overnight dialysis, the pH of the sample toxins was adjusted to pH 7 and the effect of pH on the hemolytic activity was evaluated using the hemolytic assay as described by Hang'ombe *et al.* [9] using murine erythrocytes. Structural conformation changes of the alpha toxin treated at different pH was examined using circular dichroism spectra. The circular dichroism spectra of the activated toxin (0.1 mg/ml) treated at pH 5, 7 and 9 was recorded on a Jasco 720 spectropolarimeter using 1 mm pathlength quartz cuvettes by integration of the signal over a 60 s period. The temperature was maintained constant at 298 K, and in all cases a correction was made by subtraction of the integrated signal measured from a buffer blank over the same time scale.

The effect of pH on the lethality of alpha-toxin was assessed by determining the 50% lethal dose (LD<sub>50</sub>) on

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six-weeks-old ddy mice (four per group). The mice were injected intravenously with 1 µg, 0.5 µg, 0.25 µg and 0.125 µg of activated alpha toxin, treated at pH 5, 7 and 9. After 72 hours, cumulative mortality data were used to estimate the LD<sub>50</sub> values [10].

## RESULTS

The steady-state intrinsic fluorescence spectra of the pH treated toxins were recorded for pH 5, 7 and 9 (figure 1).

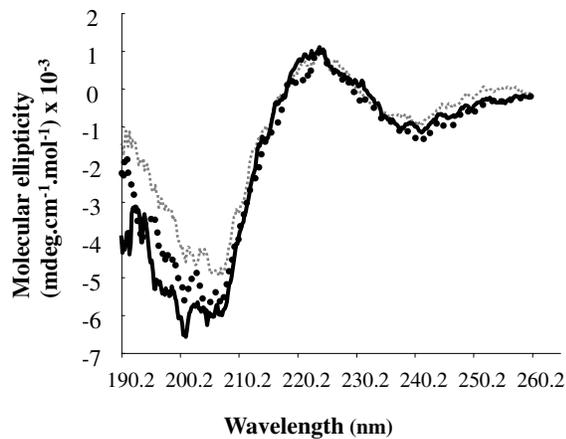


Fig. 1: Circular dichroism (CD) analysis of activated alpha-toxin (0.1 mg/ml) treated at different pH. pH 5 (square dots), pH 7 (round dots) and pH 9 (solid line).

A clear shift in molecular ellipticity was observed from 190 nm to 210 nm wavelength, indicating typical predominantly β-sheet protein in the native state. The molecular ellipticity decreased with high pH especially at around 200 nm wavelength. Further analysis of the CD spectra shows insignificant differences after 210 nm. This observation was made even when the alpha-toxin was brought back to pH 7 after incubation at pH 5 and 9.

In order to assess the effect of pH on alpha-toxin biological activity, the toxin incubated at pH 5, 7 and 9 was evaluated for its hemolytic activity in standard conditions (pH 7.0). Preincubation of the toxin at pH 5 and 9 produced changes in hemolytic activity (figure 2). On the other hand, a considerably reduced hemolytic activity was observed at pH 9. The changes in the toxin elicited by incubation at pH 5 and 9 are irreversible, since re-treatment at pH 7 did not allow recovery to what is observed at pH 7.

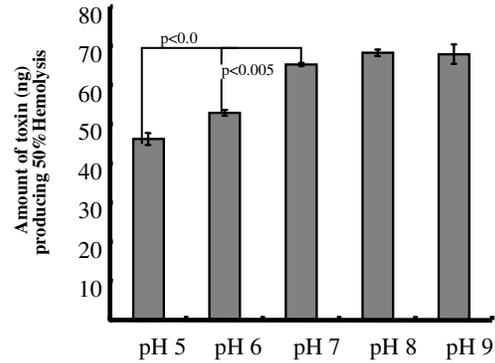


Fig. 2: Effect of pH on the activity of the activated alpha-toxin. The pH effect was investigated using a phosphate buffer of pH 5, 6, 7, 8 and 9. The pH of each test sample was adjusted to pH 7 before hemolytic assay. The data shows amount of toxin causing 50% hemolysis and represents means ± SEM from multiple experiments.

The lethality of the toxin was also evaluated as indicated in table 1. As can be observed, at a lower pH of 5 there was an increase in lethality, while at pH 9, a decrease in lethality was observed. The LD<sub>50</sub> of pH 5 and 7 was at least five-fold higher than that of pH 9, revealing the effect of pH on the biological activity of the toxin.

Table 1: Lethal toxicity of Activated *C. septicum* alpha-toxin in mice treated at pH 5, 7 and 9 using the intravenous route

pH	Lethal Dose <sub>50</sub> /mg of toxin
5	7.1 x 10 <sup>3</sup>
7	6.7 x 10 <sup>3</sup>
9	1.3 x 10 <sup>3</sup>

## DISCUSSION

Membrane damaging activity by alpha-toxin is a consequence of the formation of a transmembrane pore, a process that has been extensively studied in vitro through the investigation of the interaction of the protein with artificial membranes [5, 11]. In this study we have evaluated the effect of pH on the structural and biological stability of the native toxin. It has been demonstrated that pH has an effect on the biological activity of alpha-toxin and produces significant changes in the protein secondary conformation that is

irreversible. This observation has been made with other pore-forming toxins such as staphylococcal toxin, *Bacillus anthracis* protective antigen and the cytolysin from the sea anemone [12, 13]. These toxins have been demonstrated to adopt a partially unfolded and more flexible conformation in solution at pH  $\square$  4.5. It has been suggested therefore that upon association of these toxins with biological membranes, the reduced local pH triggers the formation of a partially unfolded state, which is functionally associated with the pore-forming process of penetration [14]. Therefore the pH induced conformation changes in the toxin can affect its capacity to form pores once adsorbed at the interface as observed by other workers [15, 16]. The decrease in hemolytic activity of the toxin pre-exposed to pH 9 can also be due to a weaker toxin-to-toxin monomer interaction or the lack of capacity of the modified toxin to penetrate and organize inside the membrane.

The alpha-toxin of *C. septicum* has now been confirmed as essential for virulence [17] since, virulence testing of isogenic series of *C. septicum* strains consisting of the wild type and mutants revealed that the development of fulminant myonecrosis in mice was dependent on the ability to produce a functional hemolytic alpha-toxin. It should also be noted that anaerobic conditions required for optimal growth of *C. septicum* produce an acid pH, which in turn may enhance the activity of alpha-toxin. Pathogenesis of malignant edema in animals involves the subsequent creation of an anaerobic niche with a sufficient low redox potential and an acid pH [18]. Although these findings are consistent with the results of the present study, it remains to be ascertained how the pathology of myonecrosis would progress at different pH. In conclusion, our study provides the results of the first indication that the low pH in an anaerobic environment might favor the degree of alpha-toxin lethality.

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#### REFERENCES

1. Johnson, S., M. R. Driks, R. K. Tweten, J. Ballard, D. L. Stevens, D. J. Anderson and E. N. Janoff, 1994. Clinical courses of seven survivors of *Clostridium septicum* infection and their immunologic responses to alpha toxin. Clin. Infect. Dis., 19:761 – 764.
2. Willoughby, D. H., A. A. Bickford, G. L. Cooper and B. R. Charlton, 1996. Periodic recurrence of gangrenous dermatitis associated with *Clostridium septicum* in a broiler chicken operation. J. Vet. Diagn. Invest., 8:259-261.
3. Gordon, V. M., R. Benz, K. Fujii, S. H. Leppla and R. K. Tweten, 1997. *Clostridium septicum* alpha-toxin is proteolytically activated by furin. Infect. Immun., 65:4130-4134.
4. Shin, D. J., J. J. Lee, H. E. Choy and Y. Hong, 2004. Generation and characterization of *Clostridium septicum* alpha toxin mutants and their use in diagnosing paroxysmal nocturnal hemoglobinuria. Biochem. Biophys. Res. Commun., 324:753-760.
5. Ballard, J., Y. Sokolov, W. L. Yuan, B. L. Kagan and R. K. Tweten, 1993. Activation and mechanism of *Clostridium septicum* alpha-toxin. Mol. Microbiol., 10:627-634.
6. Ballard, J., A. Bryant, D. Stevens and R. K. Tweten, 1992. Purification and characterization of the lethal toxin (alpha-toxin) of *Clostridium septicum*. Infect. Immun., 60:784-790.
7. Tran, L. B., V. Vachon, L. J. Schwartz and R. Laprade, 2001. Differential effects of pH on the pore-forming properties of *Bacillus thuringiensis* insecticidal crystal toxins. Applied and Environmental Microbiology., 67:4488-4494.
8. Hang'ombe, B. M., T. Kohda, M. Mukamoto and S. Kozaki, 2005. Relationship between *Clostridium septicum* alpha-toxin activity and binding to erythrocyte membranes. J. Vet. Med. Sci., 67: 69 – 74.
9. Hang'ombe, B. M., T. Kohda, M. Mukamoto and S. Kozaki, 2006. Purification and sensitivity of *Clostridium chauvoei* hemolysin to various erythrocytes. Comp. Immun., Microbiology & Infectious Diseases., 29: 263 – 268.
10. Reed, L. J and H. Muench, 1938. A simple method of estimating fifty per cent endpoints. American Journal of hygiene., 27: 493-497.
11. Melton, J. A., M. W. Parker, J. Rossjohn, J. T. Buckley and R. K. Tweten. 2004. The identification and structure of the membrane-spanning domain of the *Clostridium septicum* alpha toxin. J. Biol. Chem., 279:14315-14322.
12. Alvarez, C., F. I. Pazos, E. M. Lanio, D. Martinez, S. Schreier, F. Casallanovo, M. A. Campos and E. Lissi, 2001. Effect of pH on the conformation, interaction with membranes and hemolytic activity of sticholysin II, a pore forming cytolysin from the sea anemone *Stichodactyla helianthus*. Toxicon., 39: 539-553.

13. Bortoleto, K. R and J. R. Ward, 1999. A stability transition at mildly acidic pH in the alpha-hemolysin (alpha-toxin) from *Staphylococcus aureus*. *FEBS Letters.*, 459:438-442.
14. Van der Goot, F. G., J. N. Gonzalez, J. H. Lakey and F. Pattus, 1991. A "molten globule" membrane-insertion intermediate of pore-forming domain of colicin A. *Nature.*, 354:408-410.
15. Zakharov, S. D., J. B. Heymann, Y. L. Shang and W. A. Cramer, 1996. Membrane binding of the colicin E1 channel: activity requires an electrostatic interaction of intermediate magnitude. *Biophys. J.*, 70:2774-2783.
16. Nordera, P., M. Serra Dalla and G. Menestrina, 1997. The adsorption of *Pseudomonas aeruginosa* exotoxin A to phospholipid monolayers is controlled by pH and surface potential. *Biophys. J.*, 73:1468-1478.
17. Kennedy, C. L., E. O. Krejany, L. F. Young, J. R. O'Connor, M. M. Awad, R. L. Boyd, J. J. Emmins, D. Lyras and J. I. Rood, 2005. The alpha-toxin of *Clostridium septicum* is essential for virulence. *Mol. Microbiol.*, 57:1357-1366.
18. Stevens, D. L and A. E. Bryant, 2002. The role of Clostridial toxins in the pathogenesis of gas gangrene. *Clin. Infect. Dis.*, 35:S93-S100.