Effects of Cations and PH on Antimicrobial Activity of Thanatin and s-Thanatin against *Escherichia coli* ATCC25922 and *B. subtilis* ATCC 21332

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Abstract: Thanatin and s-thanatin were insect antimicrobial peptides which have shown potent antimicrobial activities on a variety of microbes. In order to investigate the effect of cations and pH on the activity of these peptides against Gram-negative bacteria and Gram-positive bacteria, the antimicrobial activities of both peptides were studied in increasing concentrations of monovalent cations (K^+ and Na^+), divalent cations (Ca^{2+} and Mg^{2+}) and H^+. The NCCLS broth microdilution method showed that both peptides were sensitive to the presence of cations. The divalent cations showed more antagonized effect on the activity against Gram-negative bacteria than the monovalent cations, since the two peptides lost the ability to inhibit bacterial growth at a very low concentration. In addition, the activities of both peptides tested were not significantly affected by pH. Comparing to studies of other antibacterial peptide activities, our data support a hypothesis that positive ions affect the sensitivity to cation peptides.

Keywords: antimicrobial peptides, susceptibility tests, cations, pH

Introduction

Antibacterial peptides appear to be ubiquitous and multipotent components of the innate immune defense arsenal in flora and fauna. They are generally defined as having 12 to about 50 amino acids with 2-9 positively charged lysine or arginine residues and up to 50% hydrophobic amino acids[1]. More than 500 antibacterial peptides have been reported. (An online database of antibacterial peptides could be found at http://aps.unmc.edu/AP/main.php)[2]. Early studies have demonstrated that they have direct antibacterial activity against diverse microbes. Now it is evident that

they possess a wide range of functions in modulating immunity[3, 4]. These important functions indicate that the AMPs could be potential drugs for clinical applications in the future.

Recently, thanatin (Th, GSKKPVPIIYCNRRTGKCQRM), an antimicrobial peptide with an anti-parallel β-sheet constrained by disulphide bonds, was isolated from the hemipteran insect *Podisus maculiventris*[5]. There is a sequence homology between thanatin and the brevinin family of antibacterial peptides which is isolated from frog skin[5]. Both of them contain a disulfide loop with a strong cation at their C-terminal region. There are six amino acid residues within the loop of thanatin, while there are five in brevinins.

S-thanatin (Ts, GSKKPVPIIYCNRRSGKCQRM) was synthesized by substituting the amino acid of threonine with serine, which shows broad antimicrobial activity against Gram negative bacteria, Gram positive bacteria, and fungi without any cytotoxicity.

In spite of the broad antimicrobial activities on various bacteria, the activity of antimicrobial peptides were affected by the presence of cations. Divalent cations, such as magnesium ions, are cations which bind the negatively-charged lipopolysaccharides (LPS) of Gram-negative bacteria. From this point, increasing concentration of cations would effect the interaction between antimicrobial peptides and bacteria. Since the concentration of cations in human blood are 100-150 mM of sodium and postassium ions, as well as 1-2 mM of magnesium and calcium ions, we tested sodium and postassium ions at the final concentrations of 0-500 mM, and

magnesium and calcium ions at the final concentrations of 0-20 mM. The objective of this research was to gather information about factors that may enhance or inhibit the activity of the test peptides. The bactericidal activities of these peptides were compared under various conditions, including the addition of monovalent cations and divalent cations to the media, and altering the pH of the media.

Materials and methods

Peptide synthesis

The cation peptides, thanatin and s-thanatin, were synthesized by the solid-phase method using a model 432A synthesiser (Applied Biosystems Inc. Foster City, CA). Peptide synthesis grade *N*,*N*-dimethylformamide, piperidine, and high-performance liquid chromatography-grade acetonitrile were from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid and *N*-methylmorpholine were obtained from Acros Chimica (Beerse, Belgium).

Fmoc-protected amino acids were added in a 6-fold molar excess with respect to resin substitution and coupling reactions (30 min) were performed with equimolar 2-(1*H*-benzotriazole-1-yl)-1,1,3,3- tetramethyluronium tetrafluoroborate. Arginine side-chains were protected by the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl protecting group. Cleavage from the resin and deprotection of the synthesized peptides were conducted at room temperature with a solution of 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane[6,7]. After repeating precipitation with diethyl ether, the peptides were purified by reverse-phase high-performance liquid chromatography on a C18

Delta-Pak column (Waters, Bedford, MA), using an appropriate 0–60% acetonitrile gradient in 0.05% trifluoroacetic acid. Molecular mass were determined by electrospray mass spectrometry using an API instrument (Perkin Elmer SCIEX), as a quality control of the synthesis.

Bacterial Strains and Growth Conditions

Escherichia coli ATCC25922 and *B. subtilis* ATCC 21332 were used throughout this experiment. The *E. coli* ATCC25922 and *B. subtilis* ATCC 21332 were obtained from Institute of Microbiology, China Pharmaceutical University, China. They were used to represent each class of bacteria.

Minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) of each peptide was determined according to the NCCLS broth microdilution method [8]. In this experiment, 96-well polypropylene microtitre plates were used. The peptides were diluted across the rows in two-fold serial dilutions from 128 to 0.25 mg/L, so that each well contained 50μl of peptide diluted in 0.2% BSA (bovine serum albumin) and 0.01% acetic acid. 50μl containing 5×10^5 CFU/ml cells in Mueller–Hinton broth (Difco) was added to each well. The plates were incubated at 37 ◦C overnight, and the wells that contained visible culture growth were recorded in the next day. The MIC was defined as the concentration of the peptide in the last well in which culture growth did not occur. For each set of conditions, the MIC tests were carried out independently three times, using duplicate samples each time. In all cases at least five of the six recorded values for each MIC were the same. The values that differed from the modes were only different

by one two-fold serial dilution. The MICs were reported as the geometric means and the 95% confidence intervals for the means. The geometric mean was used, as opposed to the arithmetic mean, because the data sets were skewed. This meant that the larger values had less influence on the mean. To calculate this, the data values were log-transformed, and then the means, standard deviations and 95% confidence intervals for the means of the log-transformed data were determined. These means and the limits of the confidence intervals were exponentially transformed to give the geometric means and 95% confidence limits of the mean for each original data set. The variation was asymmetric because of the log-transformation.

Effect of metal ions

The effect of salt concentration on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides in a variety of cation concentrations. The Muellller-Hinton broth used in the assay was altered by the addition of salt. There were two monovalent cations (Na^+ and K^+) and two divalent cations (Ca^{2+} and Mg^{2+}) used in this assay, which were in the form of chloride salts. The NaCl or KCl was added to the media to the final concentrations of 0, 10, 50, 100, 200, and 500 mM, whereas the $CaCl_2$ and $MgCl_2$ was added to the media to the final concentrations of 0, 1,2, 5, 10 and 20 mM.

Effect of pH

The effect of pH on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides at a variety of pH values. Altering the pH of the media was achieved by the addition of 5M HCl or NaOH. The peptides were tested in

pH conditions from pH 5 to pH 8.

Results

Effect of Monovalent Cations

The results of the monovalent cation effect on the antimicrobial activity of thanatin and s-thanatin against *E. coli* ATCC25922 are shown in Fig. 1. When there were no cation present, the MIC values of thanatin and s-thanatin were 2 and 4 μg/mL, respectively.. After the concentration of monovalent cations was increased, the antimicrobial activity of the two peptides againt the Gram-negative bacterium decreased, as indicated by the increasing of both peptides MIC with increasing of the concentration of Na^+/K^+.

The activities of both peptides were completely diminished at 500 mM of potassium ions. In addition, Fig. 1 shows that the activity of thanatin was markedly affected by potassium ions because it completely lost its potency at 200 mM of potassium ions, whereas at the same concentration of sodium ions, this peptide still retained moderate antimicrobial activity. However, thanatin still have the activity at 500 mM of sodium ions, while s-thanatin completely lost its activity at the same concertration.

The results of the monovalent cation effect on thanatin and s-thanatin MIC against *B. subtilis* ATCC 21332 are summarized in Fig. 2. The original MIC values of thanatin and s-thanatin against the Gram-positive bacterium were 4 and 8 μg/mL, respectively. The results of the effect of monovalent cations on *B. subtilis* ATCC 21332 showed a similar trend to previous results, indicating that both peptides lost

their potency with increasing cation concentrations. Both peptides lost their activities at 500 mM of Na^+/K^+. In addition, Fig. 2 demonstrated that the activity of thanatin and s-thanatin were markedly affected by potassium ions, they both lost their potencies at 200 mM of potassium ions.

Effect of Divalent Cations

The results of divalent cation effect on *E. coli* ATCC25922 are summarized in Fig. 3, which indicated that the magnesium ions and calcium ions initially caused slight increase in the MIC of s-thanatin and that these values had a slight change with the increase concentrations of magnesium ion and calcium ions. However, the MIC of thanatin rapidly increased with the increasing concentrations of calcium ions, and magnesium ions did not show a significant antagonistic effect on thanatin against *E. coli* ATCC25922.

The results of the divalent cation effect on the activity of peptides against *B. subtilis* ATCC 21332 were shown in Fig. 4. The peptides antimicrobial activities were affected by the calcium ions and magnesium ions. However, thanatin and s-thanatin still retained their activities when the calcium ion and magnesium ion concentration was 20 mM.

The growth of bacterial in most of the wells suggested that there was no influence of the salt on the bacterial growth. The salt, however, affected the ability of the peptide to inhibit the bacterial growth, and that would be discussed in the following section.

Effect of pH

The effect of pH on the activity of the peptides was established by determining the MIC of each peptide against *E. coli* ATCC25922 and *B. subtilis* ATCC 21332 at pH values varying from 5 to 8 (Fig. 5, 6). Figure 5 and 6 show that both of the peptides have MICs that are not significantly different, with the antimicrobial activity of that are slightly higher in neutral or slightly basic media than acidic condition.

Discussion

To have a therapeutic use against local or systemic infections, antimicrobial peptides need to retain their activity in physiological conditions. Some antimicrobial peptides have a broad activity against fungi, bacteria and viruses. However, there is a major obstacle in their development as novel antibiotics, which is the antagonism between the peptides and ionic strength in their environment. As a result, the practical therapeutic use of antibiotic peptides is significantly impaired.

Because the interaction of peptide and the bacterial membrane was the critical step for the inactivation of the bacteria, the presence of cations could prevent the peptides from interacting with the membrane and subsequently disable the peptides' capability to kill bacteria. In higher salt concentrations, it has been demonstrated that reduction of the available head group area for the lipids leads to tighter packing of the lipids, which might indicate that a partitioning or folding of peptide in regions of the lipid bilayer with low salt concentrations[16].

Many cationic antimicrobial peptides including β-defensins and the α-defensin HD-5[9,10], lactoferricin B[11], histain 5[12], human catheli-cidin LL-37[13], protegrins[14], and pleurocidin[15] are salt sensitive, they reduce or lose their

antimicrobial activity at high salt concentrations. The antibacterial effectiveness of lactoferricin B[11] was reduced in the presence of Na^+, K^+, Mg^{2+} or Ca^{2+} ions, or in the presence of various buffer salts. Hypertonic salt concentrations and heat-inactivated serum were found to be inhibitory to the bactericidal activity of Protegrin (PG-1)[14], which is a broad spectrum antibiotic peptide isolated from porcine leukocytes. Human beta-defensin-2[9] and cathelicidin LL-37[13] inhibit the growth of *P. aeruginosa* in vitro, but this activity is markedly reduced in the presence of salt. In saliva, Histatin 5[12], a human basic salivary peptide with strong fungicidal properties in vitro, is salt sensitive and exerts low activity at high salt conditions.

In this study, the results indicated that cations inhibited antimicrobial activities of thanatin and s-thanatin. Similar results of the effect of monovalent cations on cation peptides have been reported by other investigators. The study of salt effect on thanatin and s-thanatin demonstrated that the potency against *E. coli* and *B. subtilis* decreased when the concentration of salt increased.

Since NaCl is the most predominant salt in vivo, the ability to resist this salt is significant important for antimicrobial peptides under physiological conditions. In this study, the antagonism in terms of growth inhibition between Na^+ and thanatin and s-thanatin was observed by the test. But they exerted strong activity towards *E. coli*ATCC25922 up to 200 mM NaCl. This suggests a potential for thanatin and s-thanatin as chemotherapeutic agents for the systematic infection with *E. coli* without being affected at physiological salt concentrations.

KCl is another salt which would affect the antimicrobial activity, and the role of

KCl should not be neglected for the antimicrobial activity of peptides. Previously, it has been observed that extended indolicidins, β-sheet gramicidins, and looped and linear bactenecins are all sensitive to KCl[17]. In this study, the antagonistic effects of KCl was observed on thanatin and s-thanatin, both of them showed no activity in the presence of 500 mM KCl. However, s-thanatin still showed high antimicrobial activity on *B. subtilis* ATCC 21332 in the presence of 50 mM KCl, the concentration higher than its physiological concentration.

Ca^{2+} is a major divalent cation and may be the primary ion responsible for the signal transmission. In this study, we found that Ca^{2+} exhibited an antagonistic effect on thanatin and s-thanatin against *E. coli* ATCC25922 and *B. subtilis* ATCC 21332 at high concentration (higher than 5 mM). However, at the physiological concentration, there was no remarkable antagonistic effect.

It has been reported that Mg^{2+} also inhibits the activity of antimicrobial peptides, but the inhibition potency is lower than that of Ca^{2+}. Mg^{2+} at the physiological concentration (less than 1 mM)almost does not change the antibacterial activity of thanatin and s-thanatin against *B.subtilis*, and the growth of *E. coli* were altered by 2 fold in the presence of 1mM $MgCl_2$. In this study, we demonstrated that Mg^{2+}, unlike Ca^{2+}, did not show an significant antagonistic effect on s-thanatin against *E. coli* ATCC25922 and *B. subtilis* ATCC 21332. They still have strong activities at the physiological environment.

In addition to salt sensitivity, antimicrobial peptides were also pH-dependent. Lee *et al*. demonstrated that the activities of histidine-rich, amidated alpha-helical

antimicrobial peptides were substantially greater at pH 5.5 than pH 7.4[18]. In another study, Minahk *et al*. also showed that the antilisterial activity of enterocin CRL35 was higher at acidic than neutral or basic conditions[19]. In contrast, our study showed that the antimicrobial activity of thanatin and s-thanatin peptide was enhanced in neutral or slightly alkali media, although the peptide acted in a broad range of pH.

Overall, this study demonstrated that thanatin and s-thanatin were sensitive to the presence of cations and insensitive to the charge of pH. The sensitivity to cations would limit the continue application of these AMPs as potential drugs for systemic infection. However, this study may lead to the development of antimicrobial peptides with high specificity and potency that are useful as therapeutic agents. Further investigations are required to validate the potential uses of these peptides. Study the reason why the thanatins are salt sensitive would be useful to give an understanding of how thanatin and s-thanatin killing bacteria. Moreover, to gain more insight into the biological role of thanatin and s-thanatin, testing the activities against pathogens in vivo is recommended.

References

1. Hancock REW, Diamond G.The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol* 2000;8:402-410.

2. Wang Z, Wang G. APD: the antimicrobial peptide database. *Nucleic Acids Res* 2004;32:D590-D592.

3. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002;415:389-395.

4. Yang D, Biragyn A, Hoover DM, et al. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol* 2002;23:291-296.

5. Fehlbaum P, Bulet P, Chemysh S, et al. Structure activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. *Proc Natl Acad Sci* 1996;93:1221-1225.

6 . Christensen, T. Qualitative test for monitoring coupling completeness in solid phase peptide synthesis using chloranil. *Acta Chem Scand Ser* 1979;B33**:**763–766.

7. Fields, G. B., and R. L. Noble. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res* 1990;35:161–162.

8. National Committee for Clinical Laboratory Standards. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically-Sixth Edition: Approved Standard M7–A6*. NCCLS, Wayne, PA, USA, 2003.

9. Tomita T, Hitomi S, Nagase T, et al. Effect of ions on antibacterial activity of human beta defensin 2. *Microbiol Immunol* 2000;44:749-754.

10. Hoover DM, Wu Z, Tucker K, et al. Antimicrobial characterization of human beta-defensin 3 derivatives. *Antimicrob Agents Chemother* 2003;47:2804-2809.

11. Bellamy W, Takase M, Wakabayashi H, et al. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J Appl Bacteriol* 1992;73:472-479.

12. Helmerhorst EJ, Breeuwer P, van't Hof W, et al. The cellulartarget of histatin 5 on Candida albicans is the energizedmitochondrion. *J Biol Chem* 1999;274:7286-7291.

13. Cox DL, Sun Y, Liu H, et al. Susceptibility of Treponema pallidum to host-derived antimicrobial peptides. *Peptides* 2003;24:1741-1746.

14. Miyasaki KT, Iofel R, Oren A, et al. Killing of Fusobacterium nucleatum, Porphyromonas gingivalis and Prevotellaintermedia by protegrins. *J Periodontal Res* 1998;33:91-98.

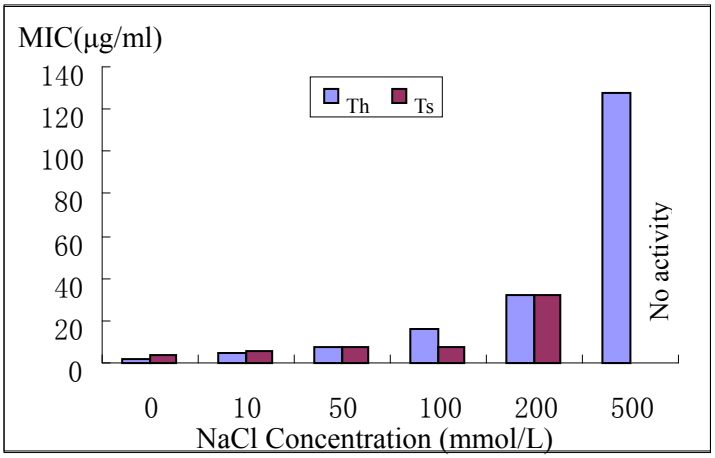
15. Cole AM, Darouiche RO, Legarda D, et al. Characterizationof a fish antimicrobial peptide: gene expression,subcellular localization, and spectrum of activity. *Antimicrob Agents Chemother* 2000;44:2039-2045.

16. Senthil K. K, Ronald G. L. Effect of salt on the interactions of antimicrobial peptides with zwitterionic lipid bilayers. *Biochimica et Biophysica Acta* 2006;1758:1274–1284.

17. Wu M, Maier E, Benz R, Hancock RE. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. *Biochemistry* 1999;38:7235-7242.

18. Lee IH, Cho Y, Lehrer RI. Effects of pH and salinity on the antimicrobial properties of clavanins. *Infect Immun* 1997;65:2898-2903.

19. Minahk CJ, Morero RD. Inhibition of enterocin CRL35 antibiotic activity by mono- and divalent ions. *Lett Appl Microbiol* 2003;37:374-337.



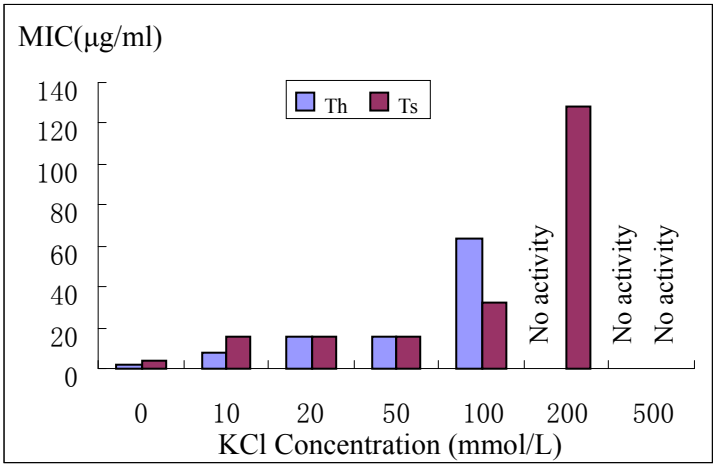
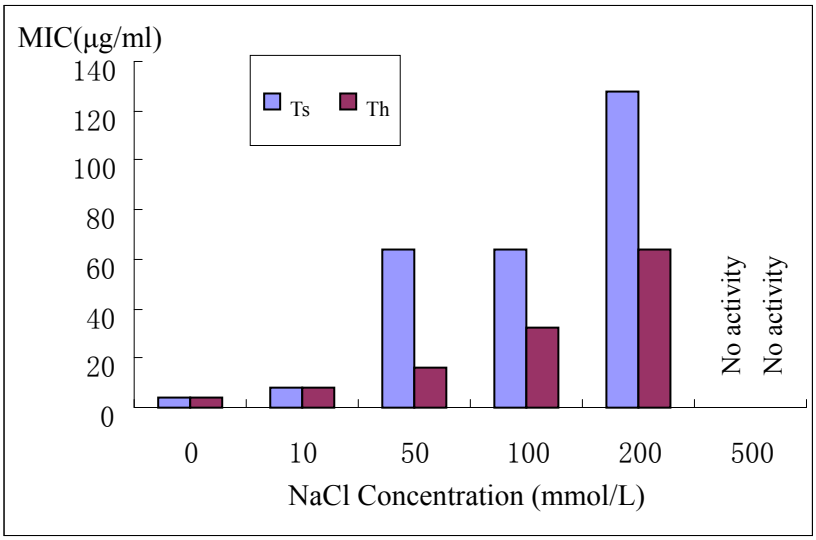


Fig. 1. The effect of monovalent cations, Na^+ and K^+, on the MIC of Th and Ts against E. coli ATCC25922.



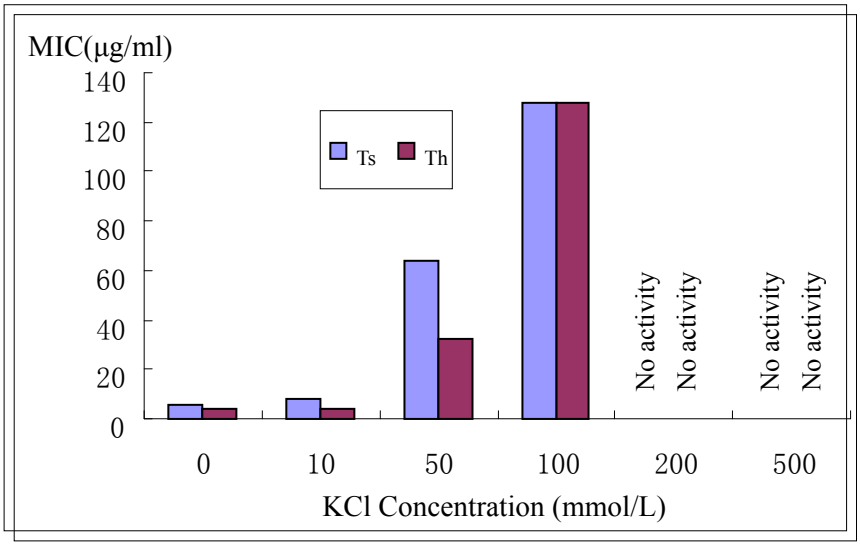
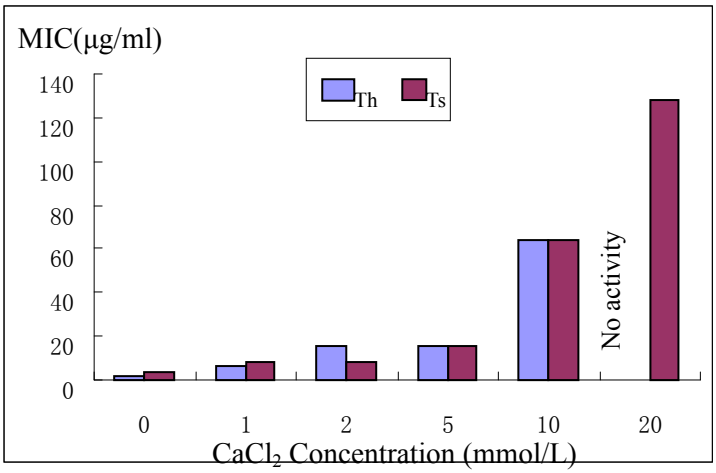


Fig.2. The effect of monovalent cations, Na^+ and K^+, on the MIC of Th and Ts against *B.Subtilis*ATCC21332.



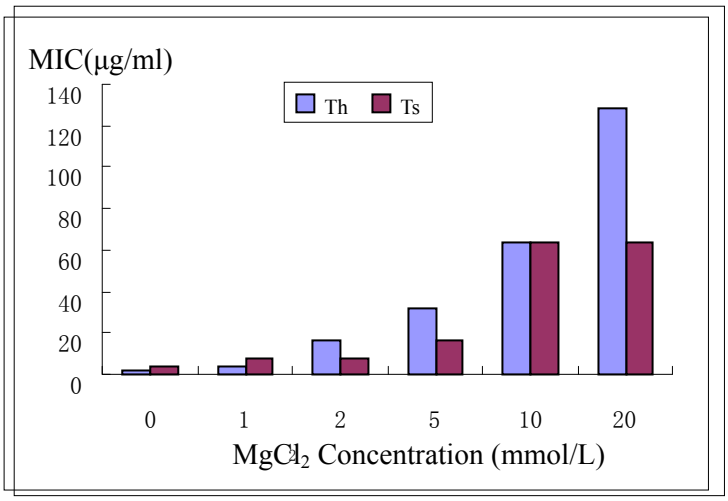
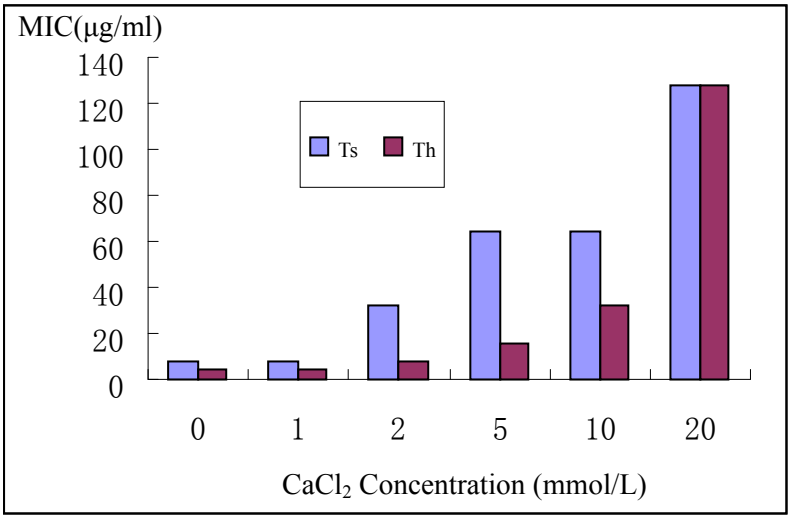


Fig.3. The effect of monovalent cations, Ca^{2+} and Mg^{2+}, on the MIC of Th and Ts against E. coli ATCC25922.



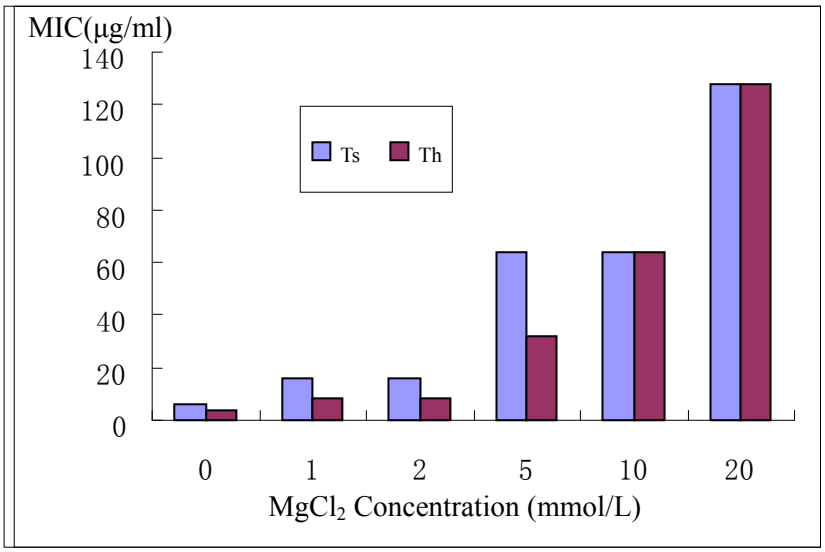


Fig.4. The effect of monovalent cations, Ca^{2+} and Mg^{2+}, on the MIC of Th and Ts against *B.Subtilis*ATCC21332.

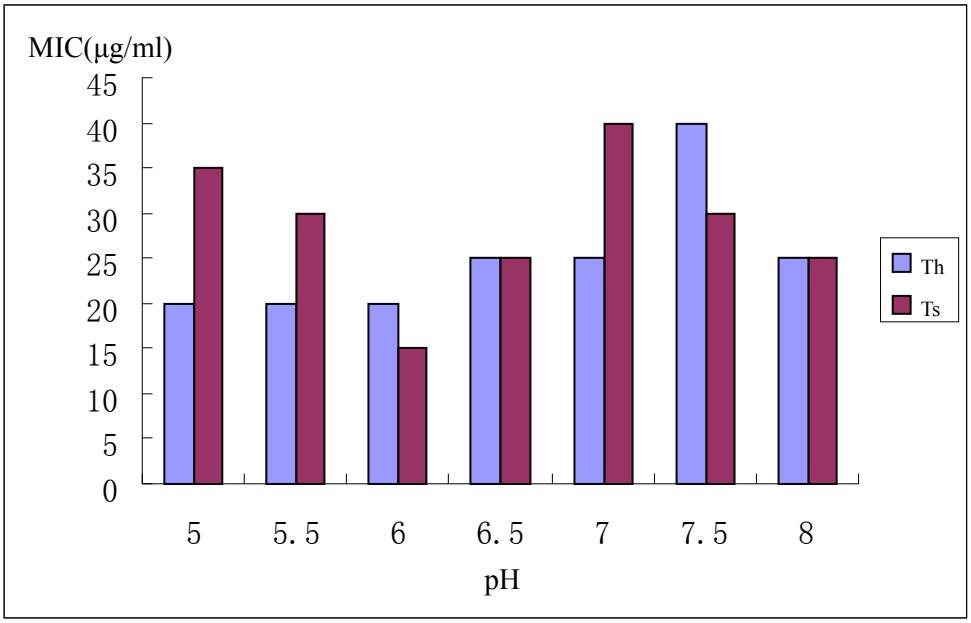


Fig.5. The effect of pH on the MIC of Th and Ts against *E.Coli*ATCC25922

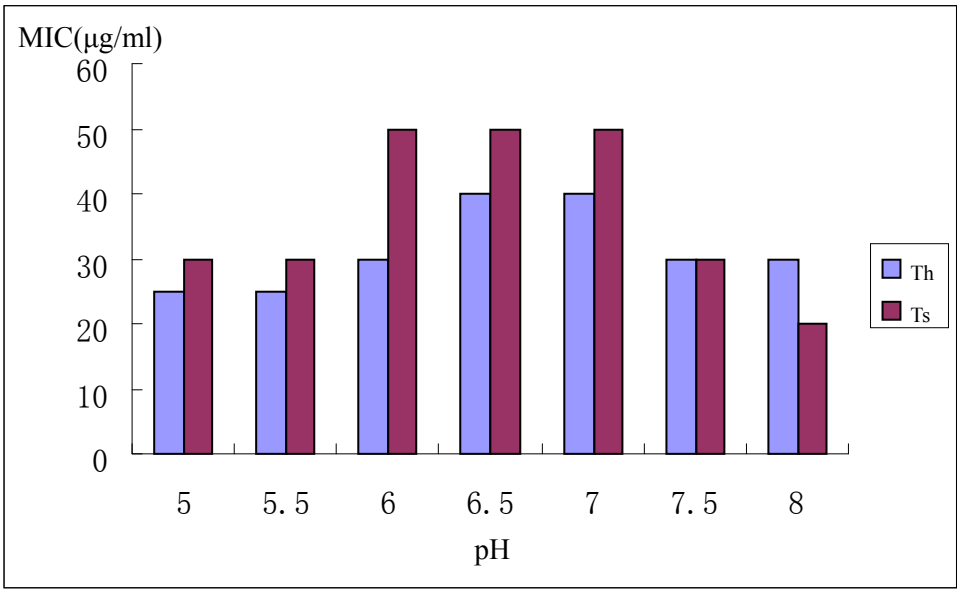


Fig.6. The effect of pH on the MIC of Th and Ts against *B.Subtilis*ATCC21332