



**Full Length Article**

## Purification and Detection of Zwittermicin A from *Bacillus thuringiensis* G03A

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### Abstract

In the present study, ZwA was found in *Bacillus thuringiensis* G03A fermentation. As ZwA has antimicrobial activity against *Erwinia herbicola*, it is thought to have a significant reaction with ninhydrin. In the experiment, G03A purified through various methods was combined with detection of antibacterial activity or ninhydrin colorimetry. Samples with both antimicrobial activity and an apparent color reaction with ninhydrin may contain ZwA. The G03A fermentation broth was sequentially purified with LX-68M macroporous resin, 724 weak acid cation exchange resin and silica gel. The antibacterial substances in the fermentation broth of G03A were eluted in high concentrations of ethanol (75 and 95%) and ultrapure water. After further purification of the sample using semi-preparative HPLC, and the antibacterial components were obtained with a purity of more than 83%. Finally, the purified samples were characterized by LC-MS, <sup>1</sup>H NMR, etc., and the results were consistent with those of previous studies, that is, we were able to determine that the bacteriostatic substance purified from G03A fermentation broth is ZwA. © 2018 Friends Science Publishers

**Keywords:** Antibiotics; *Bacillus thuringiensis*; Purification; Zwittermicin A

### Introduction

The problems caused by plant diseases and insect pests have become increasingly more serious, especially due to the extensive application of chemical pesticides and serious destruction of ecotope (Sansinenea and Ortiz, 2012). Compared with chemical pesticides, biological pesticides are less harmful and more environment-friendly. Currently, the most widely used microbial pesticide mainly comes from thuringiensis insecticidal crystal proteins produced by *Bacillus thuringiensis*, which plays an essential role in biological control, specifically, the protection of food security (Yang, 2011).

Produced by *B. thuringiensis*, Zwittermicin A is a water-soluble, amino polyhydric alcohols and small molecules antibiotics (Xia *et al.*, 2014). ZwA itself does not have insecticidal activity, but its antibodies can act synergistically with crystal protein (Lizhen *et al.*, 2009) to significantly enhance their insecticidal activity (Manker *et al.*, 2002). Moreover, ZwA could be used as a synergistic factor of parasporal crystal to improve the biocontrol effect of delta-endotoxin, thereby largely reducing-the production of *Bt* protein, slowing down the development of resistance and extending the pest-resistant spectrum (Broderick *et al.*, 2000; Broderick *et al.*, 2003). In the past few years, research has mainly been focused on the biological activity, antibacterial mechanism, synergistic mechanism,

biosynthetic pathway and biosynthesis gene cluster of ZwA (Liu *et al.*, 2013; Jain *et al.*, 2014; Park *et al.*, 2014; Krishnan *et al.*, 2015; Shi *et al.*, 2015). Academic research on the separation and purification of ZwA is lacking, with only few published papers (Shao *et al.*, 2008). The purification of ZwA from the strain is hindered by three factors: 1. it has a strong polarity, 2. it is charged under physiological pH, and 3. it is sensitive under alkaline conditions (Wen *et al.*, 2015). Moreover, due to its complicated structure, which has 7 chiral centers, ZwA is also hard to synthesis (Sansinenea and Ortiz, 2013). As there is no available standard of ZwA, research on its synergism, antibacterial mechanism and biosynthetic pathway is restricted, which, at the same time, causes difficulties in achieving industrial mass production.

For research and development, it is important to isolate active metabolites with high purity from fermentation broth with complex component (Yin *et al.*, 2008), as well as to be able to mass-produce the bioactive substance. Therefore, the development of a low cost and low pollution purification process, with good repeatability, has important practical significance. Primarily, this study compared various extraction methods of ZwA, as well as methods of ZwA detection, to establish a feasible process for practical application. In addition, this paper provides a theoretical basis and technical support for the industrial production of ZwA.

## Materials and Methods

### Strain and Culture

Engineered *Bacillus thuringiensis* G03A stored at  $-80^{\circ}\text{C}$  was provided by the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China. *Erwinia herbicola* LS005 was used to assess the antibacterial activity of ZWA.

### Rough Treatment of Fermentation Broth

Rough treatment is used to remove some of the impurities and to greatly reduce the volume of fermentation broth for the follow-up steps. It involves concentration, centrifugation, alcohol precipitation, filtration and petroleum ether extraction (Hao *et al.*, 2015).

### Purification of Fermentation Broth with LX-68M Resin

Pretreatment of LX-68M macroporous resin and 724 weak acid cation exchange resin was performed by reference to Hao (Hao *et al.*, 2015).

To purify the sample, LX-68M resin was added, causing the adsorption of the sample on the resin, which was then washed with 8 BV volume of deionized water. Subsequently, the sample was repeatedly washed with 2 BV ethanol using increasing concentrations (25, 50 and 75%). Finally, 4 ethanol BV 95% was used to flush the resin, to desorb the antimicrobial substances. The flow rate was 1 mL/min, and the experimental temperature was  $25^{\circ}\text{C}$ .

### Sample Purification with Weak Acid Cation Exchange Resin 724 (Method I)

The weak acid cation exchange resin 724 was balanced with 8 BV of 5 mM  $\text{NH}_4\text{H}_2\text{PO}_4$  (pH 7.0). Bacteriostatic samples of LX-68M macroporous resin were brought to pH 7.0 with PBS buffer, then the sample flowed through the weak acid cation exchange resin 724 at low velocity, to be adsorbed on the resin. To remove impurities, the resin was subsequently washed with 8 BV of 5 mM  $\text{NH}_4\text{H}_2\text{PO}_4$  (pH 7.0). Lastly, the resin was rinsed three times with 3 BV ammonia water using increasing concentrations (0.1 M, 0.8 M and 1 M). Each 1 BV was collected separately and tested for bacteriostasis and ninhydrin reaction. Finally, samples were combined, decompressed and concentrated to remove ammonia, and then the pH was adjusted to 7.0 with HCl.

### Sample Purification with Weak Acid Cation Exchange Resin 724 (Method II)

For the second purification, cationic resin 724 was pretreated with 8 BV of 10 mM  $\text{CH}_3\text{COONH}_4$  (pH 7.0). The sample then flowed through the 724 weak acid cation exchange resin at low velocity, to be adsorbed on the resin.

After that, using pH 7.0, 8.0, 8.5, 9.0 and 9.3  $\text{CH}_3\text{COONH}_4$  (concentration was 10 mM and 2 BV each time), the resin was repeatedly flushed. Subsequently, the resin was flushed with 10 BV ultrapure water. Lastly, the resin was rinsed with ammonia water (6 BV; pH 11.2). Each 1 BV was collected separately and tested for bacteriostasis and ninhydrin reaction, after removing the ammonia water. The process of ammonia elution and removal was performed rapidly and controlled, because the alkaline environment destroys the structure of ZWA. Samples that meet the requirements were selected in preparation for silica gel-based purification (samples that both had antimicrobial activity and an apparent color reaction with ninhydrin).

### Sample Purification with Silica Gel Column Chromatography

Pretreatment of silica gel was performed by reference to Hao (Hao *et al.*, 2015). The flow rate of the eluent was constant, at 0.5 mL/min. In turn, three lotions were used to elute the silica gel, and a fraction collector was used to collect the eluting fluid. The samples for each eluent were collected in 50 tubes, and each tube was collected for 10 min. The eluant was, in turn: ethyl acetate, methanol = 70:30 (V/V), 95% ethanol and ultrapure water. Lastly, samples that meet the requirements were tested and collected.

### Ninhydrin Colorimetry Detection of ZWA

Phosphate buffer with a pH of 6.6 was prepared, with 37.5 mL (0.2 mol/L)  $\text{Na}_2\text{HPO}_4$  and 62.5 mL (0.32 mol/L)  $\text{NaH}_2\text{PO}_4$ . Reagent ninhydrin was prepared by adding 2 g of ninhydrin and 5 mL ethylene glycol into a 100 mL brown volumetric flask, followed by the addition of phosphate buffer to 100 mL. The solution then was mixed thoroughly, and left to rest until the next day. In case of precipitation, the solution was filtered for further use. A 10 mL tube containing 1 mL of phosphate buffer, 0.5 mL of 2% reagent ninhydrin and 0.5 mL of test sample was heated on a boiling water bath for 15–20 min. After cooling to room temperature, deionized water was added to 10 mL, and the mixture was analyzed 15 min later. With eluent as blank control, the sample was detected by Cary 50 VS-spectrophotometer set on full spectrum scan (190–800 nm) to ensure the detection of the maximum absorption spectra. Then, a curve was generated between the extinction value under this absorption spectra and the volume of eluent (Hao *et al.*, 2015).

### ZWA Detection using the Oxford Cup Method

The Oxford cup method is used to characterize the intensity of antibacterial activity. The *Erwinia herbicola* LS005 was inoculated into LB medium which was then separated into culture dishes. A 150  $\mu\text{L}$  sample was added in the Oxford

Cup, and incubated under pH= 7.0, at 30°C, for 10–16 h. The sample inhibited the *E. herbicola* LS005, as bacterial did not grow around the cup. The diameter of the bacteriostatic ring was measured by a vernier caliper, and a standard curve was set up with logarithm of concentration (Qing *et al.*, 2002). With blank medium as control, every sample was tested three times (Jing *et al.*, 2008). The diameter of the bacteriostatic ring, the difference between the external diameter of the bacteriostatic ring and the internal diameter of the Oxford Cup were directly proportional to the amount of ZwA (Jian-guo *et al.*, 2014).

#### ZwA Detection by High-Performance Liquid Chromatography (HPLC)

Agilent 1260 chromatograph, Phenomenex Luan 5 $\mu$  (C18, 5  $\mu$ m, 4.6 mm  $\times$  250 mm) column was used, with water: methanol = 85: 15 (V/V) as the mobile phase, 328 nm as detection wavelength, 35°C as column temperature, 10  $\mu$ L as the injection volume and 0.5 mL/min as the flow rate.

#### ZwA Detection by High Performance Liquid Chromatography Mass Spectrum (LC-MS)

Waters 2695 GC-MS, Waters UPLC BEH C-18 (1.7  $\mu$ m, 2.1 mm  $\times$  100 mm) column was used, with water: methanol=85: 15 (V/V) as the mobile phase, 328 nm as detection wavelength, 35°C as column temperature, 10  $\mu$ L as the injection volume and 0.5 mL/min as the flow rate (Hao *et al.*, 2015).

#### ZwA Detection by High Resolution Mass Spectrum (HRMS)

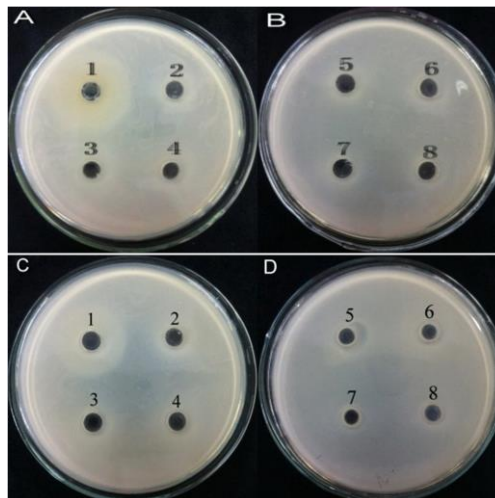
Xevo G2-S QT of electrospray ionization-quadropole time-of-flight mass spectrometry was used. Mass spectrometry condition were ESI+, capillary voltage 3.51 kV, cone voltage 150 V, ion source temperature 100°C, drying gas temperature 300°C, solvent removal gas flow 500 L/h, cone gas flow 50 L/h, full scanning positive ion detection.

#### ZwA Detection by Nuclear Magnetic Resonance (NMR) Spectroscopy

Samples were characterized using  $^1\text{H}$  NMR. The type of instrument was Avance 500 MHz nuclear magnetic resonance apparatus and deuterium oxide was used as solvent.

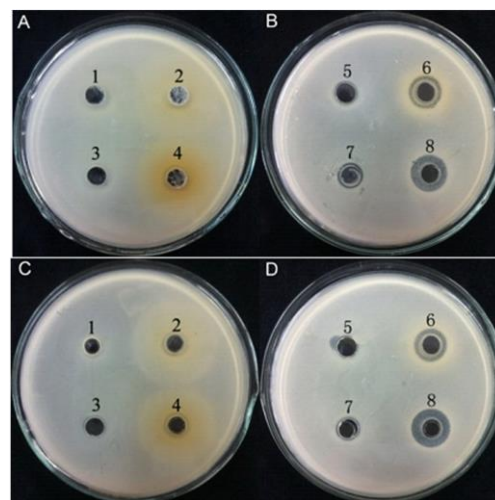
#### Semi-Preparative HPLC Purification of ZwA

As shown in Fig. 13, fractions of 3.0–3.5 min, 3.5–4.0 min, 7.6–8.2 min and 9.0–9.6 min selected by semi-preparative HPLC were concentrated to the same volume by rotary evaporation, and their antimicrobial activity was subsequently detected. Results of the bacteriostatic test showed that fractions in 3.0–4.0 min had higher antibacterial activity than the others, and the diameter of their antibacterial ring was  $21.16 \pm 0.02$  mm; fractions in



**Fig. 1:** Antibacterial activity of non-adsorption phase concentrated solution

Note: A and B were eluent of deionized water (W:V = 1:1, 1 BV-8 BV); C and D were eluent of deionized water (W:V = 2:1, 1 BV-8 BV)



**Fig. 2:** Effect of antibacterial activity by ethanol eluent

A (W:V=1:1): 1. 25% ethanol, 2. 25% ethanol eluent, 3. 50% ethanol, 4. 50% ethanol eluent  
 B (W:V=1:1): 5. 75% ethanol, 6. 75% ethanol eluent, 7. 95% ethanol, 8. 95% ethanol eluent  
 C (W:V=2:1): 1. 25% ethanol, 2. 25% ethanol eluent, 3. 50% ethanol, 4. 50% ethanol eluent  
 D (W:V=2:1): 5. 75% ethanol, 6. 75% ethanol eluent, 7. 95% ethanol, 8. 95% ethanol eluent

7.6–8.2 min had the lowest antibacterial activity, and the diameter of their antibacterial ring was  $5.28 \pm 0.02$  mm. As the chromatogram shows (Fig. 14), the retention time of the antibacterial component may be at 5.2 min, strongly indicating that the component might be ZwA. However, its molecular weight and molecular structure must be confirmed by MS and NMR. Since the liquid retention time was settled, an accurate and reliable method to quantitate and prepare ZwA could be built.

## Results

### Qualitative Analysis of Sample

**Antibacterial activity detection of the sample purified by macroporous resin:** The Oxford cup method was used to assess the antibacterial activity in cation exchange fraction of G03A fermentation broth. The ZwA production was tested by detecting the inhibition of *E. hebricola* strain by the culture filtrate.

As shown in Fig. 1, at W:V = 1:1 or W:V = 2:1, the corresponding desorption phase of deionized water eluent showed no antibacterial ring, indicating that there was no antibacterial component in the deionized water eluent.

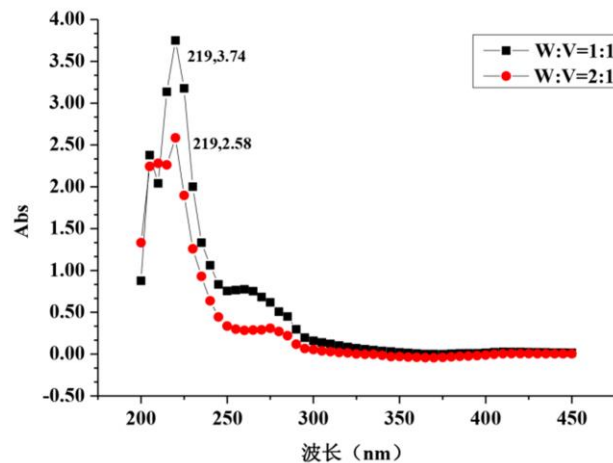
As shown in Fig. 2, spots of 1,2,3,4 and 5 in A, B, C and D were had no antibacterial ring while those of 6, 7 and 8 in A, B, C and D had antibacterial ring. That is to say, 25% ethanol eluent, 50% ethanol eluent and the first bed volume of 75% ethanol eluent were not antibacterial, while the second bed volume of 75% ethanol eluent and 95% ethanol eluent were antibacterial. Based on the size, transparency and color depth of bacteriostatic ring, 95% ethanol eluent, which had litter color and a completely transparent antibacterial ring, was superior to 75% ethanol eluent, and W:V = 2:1 was superior to W:V = 1:1. Although both 95% ethanol eluent and 75% ethanol eluent had antibacterial substances, 75% ethanol eluent was darker and contained more impurities than the 95% ethanol eluent.

**Ultraviolet absorbance scanning of 95% ethanol eluent:** To obtain a full spectrum scan of 95% ethanol elution, although resin mass and sample volume were different (W:V = 1:1 or W:V = 2:1), both maximum absorption values peaked at 219 nm (shown as Fig. 3).

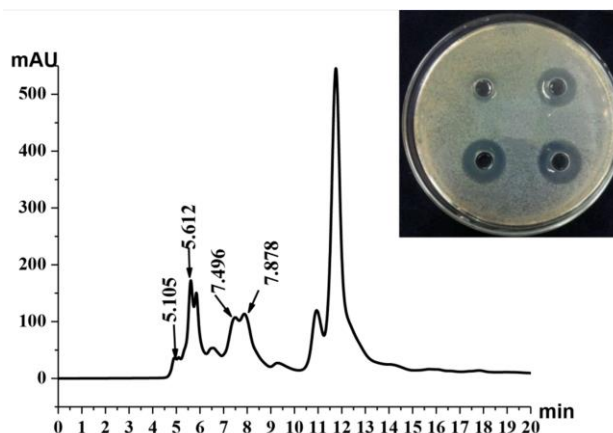
This result is in agreement with Yin's findings (Yin *et al.*, 2008) which also identified a characteristic peak in 210 nm. From this antibacterial experimental result, we could infer the presence of an antibacterial substance in the ethanol elution, with that concentration (In the end, this was proven to be ZwA).

### Detection of the Sample Purified Twice by Weak Acid Cation

**HPLC detection of the sample purified by 724 weak acid cation:** As shown in Fig. 4), there were two peaks between 7.0 min to 8.0 min. The former's retention time was 7.496 min and the latter's was 7.878 min. Antibacterial activity detection showed the sample of ammonium hydroxide eluent had antibacterial activity, and bacterial inhibition diameter was  $11.40 \pm 0.02$  mm. Thus, we infer that these components, their retention time followed by 5.612 min, 7.496 min and 7.878 min, and may contain ZwA. The content of each component identified was determined by area normalization method, of which the former was 6% and the latter two were 15%.



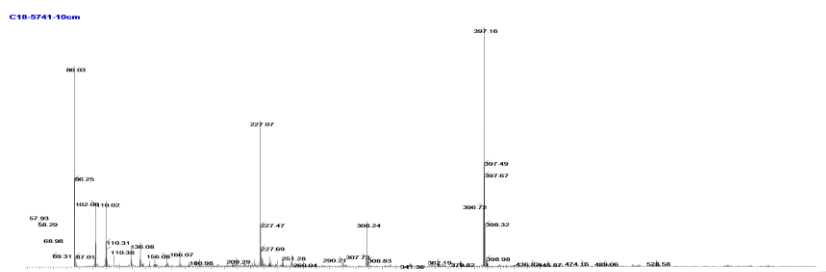
**Fig. 3:** Ultraviolet adsorption spectrum of 95% ethanol elution



**Fig. 4:** The HPLC chromatogram and inhibition activity detection of the ammonia elution part Note: the figure shows the sample purified by weak acid cation

**LC-MS detection of the sample purified by 724 weak acid cation:** The sample purified by 724 weak acid cation twice was characterized by LC-MS. There was a distinguished peak at mass spectrum 397.2047 m/z (Fig. 5). Using LC-MS to analyze sample with ZwA (Waters BEH C18, 1.7  $\mu$ m, 2.1 mm  $\times$  100 mm, 40% methanol-water isocratic elution), Zhao Changming (Zhao *et al.*, 2007) found the molecular ion peak m/z  $[M+H]^+ = 397.4$ . Similarly, using LC-MS to analyze sample with ZwA (Waters Xterra C18, 5  $\mu$ m, 3.9 mm  $\times$  150 mm, 40% methanol + 0.1% formic acid isocratic elution), Shao (Shao *et al.*, 2008) discovered the molecular ion peaks m/z  $[M+H]^+ = 397.2$ . The molecular ion peaks m/z 396.72 and m/z 398.32 detected by EI-MS were isotope of peak m/z 397.16, which further certifies that the corresponding substance of peak m/z 397.16 is ZwA.





**Fig. 5:** LC-MS spectrum of the cationic resin 724 secondary purified sample

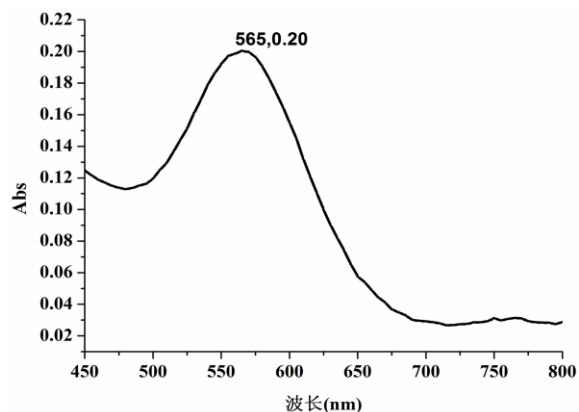
### Detection of the Sample Purified by Silica Gel Column Chromatography

**Antibacterial activity and ninhydrin colorimetry detection of the sample purified by silica gel column chromatography:** According to the ninhydrin colorimetry detection result of each tube of eluent, tube 14–23 which have purple color reaction was subjected to visible-light full spectrum scan. As shown in Fig. 6), the wavelength of reaction products of ZwA with ninhydrin's maximum absorption peak is 565 nm.

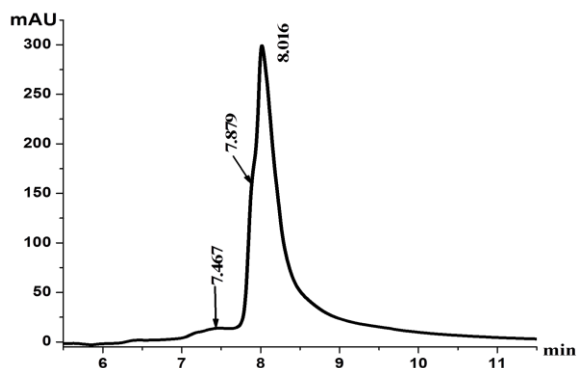
**HPLC detection of the sample purified by silica gel column chromatography:** First of all, the sample eluted by ethyl acetate: methyl alcohol=70:30 was detected by HPLC. As shown in Fig. 7), there are three peaks, of which the major peaks at 8.016 min. Content of peak 7.879 min adjacent to peak 8.016 min was 15% determined by area normalization method, peak 7.467 min and peak 8.016 min are 6% and 78% respectively. Therefore, there should be another further detection of MS.

Then sample eluted by 95% ethanol was also detected by HPLC, as shown in Fig. 8). C18 column is nonpolar, whereas sample ZwA is polar. The stronger polar component contains, the faster reaches its peak; the weaker nonpolar component contains, the slower reaches its peak. One of peak 5.192 min, 7.780 min and 8.043 min may be ZwA, for its low binding force with mobile phase of C18 column, the retention time should be early. Content of peak 5.192 min, 7.780 min and 8.043 min component content was 32%, 7% and 17%, in respective, calculated by area normalization method.

Being purified by silica gel column chromatography, ZwA may be eluted slowly by its high adsorption power with the polar mobile phase. After eluted by weak polar eluent of ethyl acetate and methyl alcohol, the sample was eluted by strong polar eluent of ultrapure water and the outflow eluted rate of faint yellow substance was faster than

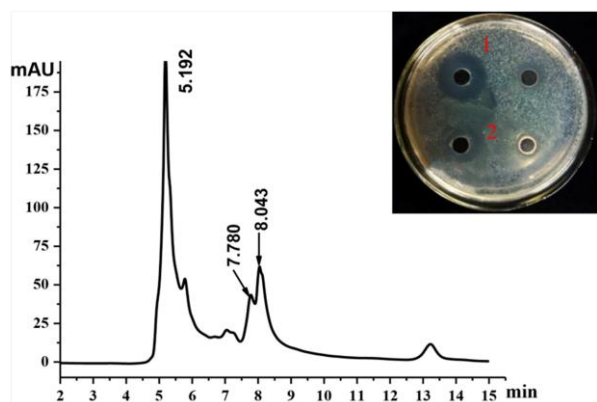


**Fig. 6:** Visible scanning spectrum of ZwA with ninhydrin reaction

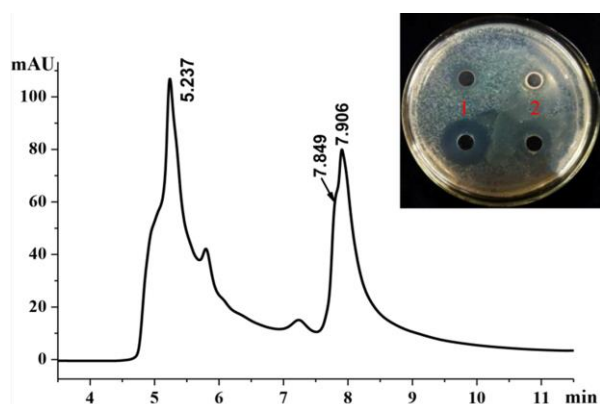


**Fig. 7:** HPLC chromatogram of methanol and ethyl acetate elution sample by silica gel column chromatography

before. The collected samples were detected by HPLC (Fig. 9). The corresponding component of peak 7.849 min and 7.906 min with similar polar and structure cannot be completely separated by eluent of methyl: water= 15: 85.



**Fig. 8:** HPLC chromatogram of 95% ethanol elution sample by silica gel column chromatography  
Note: 1-sample of ultrapure water eluent, 2-sample of 95% ethanol eluent



**Fig. 9:** HPLC chromatogram of ultrapure water elution sample by silica gel column chromatography  
Note: 1-sample of ultrapure water eluent, 2-sample of 95% ethanol eluent

Combining of the HPLC tested results as shown before, we presume that one of corresponding component of peak 5.237 min, 7.849 min and 7.906 min may be ZwA, and then these corresponding component content are 37%, 7% and 25% calculated by area normalization method.

**LC-MS detection of the sample purified by silica gel column chromatography:** Tube 14–23 eluted by ethyl acetate and methanol with silica gel column chromatography was detected by LC-MS (Waters 2695) (Fig. 10). The molecular ion peak  $m/z = 397.09$  is low, which means its appearance rate of the parent ion is low. The molecular ion peak  $m/z = 503.42$  is high, almost as same as the base peak, inferring that the base peak 502.91 may be the isotopic peak of 503.42. According to the thesis reported by He (He *et al.*, 1994), the corresponding component of this peak may be that amide group and ureido group of ZwA were broken and five hydroxide groups were ethyl.

The silica gel column chromatography peak  $m/z = 525.23$  may be the peak of  $[M+Na]^+$ , and peak  $m/z = 541.12$

may be the peak of  $[M+K]^+$ . During silica gel column chromatography, ethyl acetate in eluent was easily to be hydrolyzed to acetic acid that easily had esterification reaction with 5 hydroxy compounds. The molecular ion peak  $m/z = 520.22$  may be the molecular ion peak of other impurities, high intensity peaks, indicating that the possibility of appearance of the parent ion was high. At this signal time, molecular ion peaks  $m/z = 502, 503$  and  $520$  representing the percentage of the total ion peaks intensities were 24%, 30% and 30%, respectively.

After detected by HPLC, the peak 7.780 min and the peak 8.043 min of alcohol eluent of silica gel column was further detected by LC-MS (Fig. 11). The base peak  $m/z = 397.16$  is consistent with mass spectrometric characteristic of ZwA published by HZAU and IPP, CAAS. Molecular ion peaks  $m/z 396.65$  and  $m/z 398.14$  detected by EI-MS are isotopes of peak  $m/z 397.16$ , and the peak  $m/z 227.18$  and  $m/z 102.31$  are impurity peaks. At this signal time, the corresponding components of the molecular ion peaks  $m/z 397$  and  $227$  were 38 and 25%, respectively.

After using ultra-pure water to elute in silica column and detection by HPLC, the sample at near retention time of 7.8 min was chosen to further test by liquid chromatography-mass spectrometry instrument and positive ion mode scanning (Fig. 12). Molecular ion peaks  $m/z 396.87$  and  $m/z 398.11$  are isotopes of base peak  $m/z 397.16$  detected by EI-MS. Another two peaks with high signal  $m/z 102.13$  and  $m/z 227.18$  are impurity peaks. At this time, the corresponding components of the molecular ion peaks  $m/z 397$  and  $227$  were 24 and 15%, respectively. After LC-MS tested, the mass of freeze-dried sample of ultrapure water was 162.0 mg. But the purity of the sample separated and purified by this method was about 20–30%, so that the sample should be purified by semi-preparative HPLC to improve its purity, which ingredients with retention time of 3.3–3.5 min were selected.

### HRMS Detection of ZwA

The fraction of 5.2–6.0 min (Fig. 14), obtained with semi-preparative HPLC, was fully scanned with HRMS (Fig. 15). The elements analysis report and chromatogram showed that the molecular ion peak  $m/z 397.2047$  to be antibiotic ZwA and its parent ion corresponding molecular formula is  $C_{13}H_{29}N_6O_8$ , which is consistent with the result that He *et al.* reported (He *et al.*, 1994).

### $^1H$ NMR Detection of ZwA

The fraction of 5.2–6.0 min (Fig. 14) was also assessed with  $^1H$  NMR (500 MHz,  $D_2O$ ), generating the following spectroscopic data:  $\delta$  3.95 (s, 2H), 3.79 (s, 4H), 3.67 (d,  $J = 4.3$  Hz, 3H), 3.65 (d,  $J = 4.3$  Hz, 3H), 3.57 (dd, 6.4 Hz, 8H), 3.37 (s, 4H). These results are in agreement with those of previous studies (He *et al.*, 1994; Manker *et al.*, 2002).



effect of crystal protein would be markedly improved after mixing with ZwA primary purified products.

No studies have been conducted on insecticides combined with high-purity ZwA and crystal protein. One of the most important reasons is that no mature and reliable method for the mass production of ZwA has been developed.

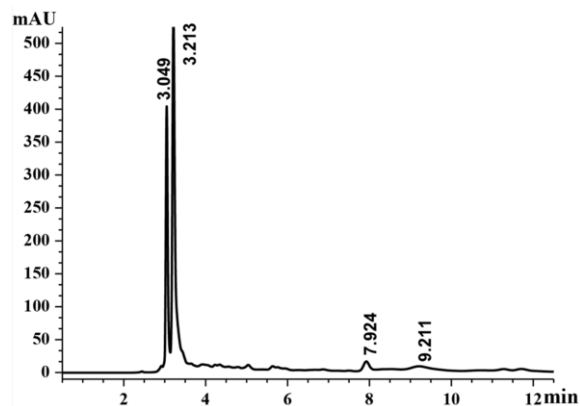
In addition, the proposed method can only produce milligram-grade samples, and such content is insufficient to perform an insecticidal activity test. This study aimed to deliver a small amount of high-purity ZwA. The results of the present study can be used in subsequent studies to explore an efficient, convenient and high-yield purification method.

The use of solid-phase extraction to purify ZwA has a broad prospect; solid-phase extraction (SPE) is a highly effective sample pretreatment technique that exhibits high efficiency, good reproducibility, and quick response. With a high-purity ZwA sample, sample preparation and elution by ion exchange SPE can be explored, thus saving time and improving production. High-purity samples can also be used to identify specific extractants that can efficiently extract ZwA from impurities. Methylmalonate is slightly soluble in water, and its boiling point is higher than that of water. Removing methylmalonate from the solution by atmospheric-vacuum distillation at reduced temperature is difficult. Increased temperature may damage the activity of ZwA. The appropriate macroporous resin should be selected for the removal of methylmalonate if methylmalonate can extract ZwA from the broth.

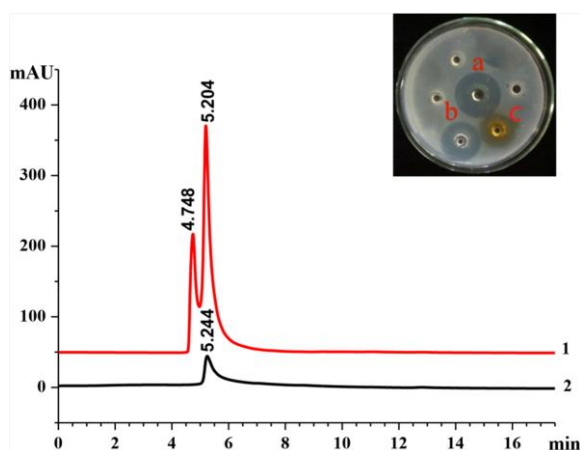
Approaches to detecting ZwA need to be enhanced. The accuracy of the existing detection methods cannot reach the acceptable range; that is, the detection range is considerably wide. When we used the Oxford cup method to detect the presence of ZwA, the high salt concentration, inappropriate pH, or some compounds can also exhibited antimicrobial activity. These factors caused serious disturbances in the experiment.

We attempted to establish a chemiluminescence detection system of  $\text{H}_2\text{SO}_4\text{-KMnO}_4\text{-CH}_2\text{O-ZwA}$ . Approximately 8 mmol/L  $\text{KMnO}_4$ , 1 mol/L  $\text{H}_2\text{SO}_4$ , and 8%  $\text{CH}_2\text{O}$  were prepared with distilled water.  $\text{KMnO}_4$  and  $\text{H}_2\text{SO}_4$  were charged as the inverter, whereas  $\text{CH}_2\text{O}$  and the sample were charged as the carrier liquid, sampled by the auxiliary pump, and mixed in 16 pass valves before flowing into the test cell. This approach has been improved considerably; however, such improvement remains insufficient because apart from ZwA, some impurities also produce chemiluminescence that interferes with the experimental results. The proposed technique requires further improvement for an efficient, fast, and accurate testing method to be established.

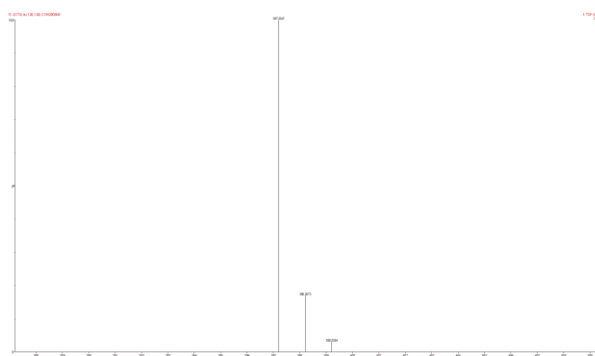
In addition, during the fermentation process, both *Bacillus cereus* and *Bacillus thuringiensis* could produce ZwA of 396 Da molecular weight. Compared to the result



**Fig. 13:** The sample preparative column analysis chromatogram of HPLC preparation



**Fig. 14:** The HPLC chromatogram and inhibition activity of ZwA refined product by semi-preparative purification  
Note: a(2)-component in 5.2-6.0 min by semi-preparative HPLC, b(1)-component in 4.5-5.2 min by semi-preparative HPLC, c-sample before semi-preparative HPLC



**Fig. 15:** High resolution ESI-MS spectrum of 5.2-6.0 min sample

reported by Hao (Hao *et al.*, 2015), which is, under the same condition there were two peaks  $m/z$  383 and  $m/z$  365 detected by LC-MS, which indicated the possibility of two



new antibiotics or the same race of ZwA, this study is different for two reasons – one is because of their small molecular weight and low production in fermentation, it is easy to lose them during the purification process, and the other is they may have the same structure of ZwA that have the easily oxidized hydroxyl group, amine group and easily oxidized amido bond, all of which could derive them in the research process.

## Conclusion

The antibacterial ingredient separated from G03A fermentation broth was purified through macroporous resin chromatography, weak acid cation chromatography, silica gel column chromatography and Semi-preparative HPLC. Its molecular ion peak was at  $m/z$  397.2047 and the These numbers '13, 29, 6, 8' should be in the form of subscripts. These results are indicative of ZwA, which has a reported molecular weight of 396 Da. Moreover, the  $^1\text{H}$  NRM spectrum was also consistent with the structure of ZwA. Thus, based on the present results, we conclude that the component with antibacterial activity in G03A fermentation broth is ZwA.

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