Chinese Science Bulletin



# Application of a TLC chemical method to detection of cyclotides in plants

XU WenYan[1,2], TANG Jun[1,2], JI ChangJiu[1,2], HE WenJun[1,2] & TAN NingHua[1†]

[1] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China;
[2] Graduate University of Chinese Academy of Sciences, Beijing 100049, China

**Iodine, Dragendorff's reagent, ninhydrin and Coomassie brilliant blue G-250 reagents were used to detect cyclotides from proteins, cyclopeptides, linear-peptides and amino acids in the thin layer chromatography (TLC). Cyclotides could be distinguished from the others by comparing the TLC coloring plots with G-250 and ninhydrin reagents. With this method, cyclotides were discovered in *Viola labridorica*, *V. tricolor*, *V. hamiltoniana*, *Momordica charantia*, and *M. cochinchinensis* and over 10 cyclotides were isolated, three of which were determined as known cyclotides cycloviolacin O2, kalata B1 and vary peptide A.**

cyclotides, TLC chemical detection method, coomassie brilliant blue G-250

Plant cyclopeptides[1−4] were defined as cyclic compounds formed mainly with the peptide bonds of 2−37 protein or non-protein amino acids and discovered in higher plants, mainly *L*-amino acids. The research of cyclopeptides started in 1880s. Since the first plant cyclopeptide was structurally determined in 1959, over 450 cyclopeptides had been discovered from higher plants up to 2006 when the definition of plant cyclopeptides was proposed, and these plants belong to 25 families, 65 genera and 120 species. On the basis of their structural skeletons and distributions in plants, we proposed the systematic structural classification which is divided into eight types, i.e., Cyclopeptide Alkaloids (Type I), Depsicyclopeptides (Type II), Solanaceae-type Cyclopeptides (Type III), Urticaceae-type Cyclopeptides (Type IV), Compositae-type Cyclopeptides (Type V), Caryophyllaceae-type Cyclopeptides (Type VI), Rubiaceae-type Cyclopeptides (Type VII) and Cyclotides (Type VIII).

Cyclotides[1,5] are plant disulfide-rich macrocyclic proteins with 28−37 amino acids, which not only contain a unique amide head to tail cyclized peptide backbone but also incorporate a cyclic cystine knot (CCK). The CCK is a fascinating structural motif in which a small embedded ring is formed by two disulfide bonds and their connecting back-bond segments are threaded by the third disulfide bond, which produces a unique protein fold that is topologically complex and has exceptional resistance to enzymatic breakdown and high chemical stability. The first structurally determined cyclotide was kalata B1, a 29-residue cyclotide from the tropical African plant *Oldenlandia affinis* (Rubiaceae) with uterotonic activity, which was discovered in the early 1970s as the active agent in a native medicine used by women in Africa to accelerate labor and childbirth. The medicine was prepared by boiling the plant to make tea, which was orally ingested during labor, and kalata B1 was isolated from this boiled tea[6]. At that time, although the structure had not been determined, the fact that it was cyclic had been described. In 1995, its structure was finally determined by Saether et al.[7]. Since then, about 100 cyclotides have been isolated from over

Received October 9, 2007; accepted January 7, 2008
doi: 10.1007/s11434-008-0178-8
†Corresponding author (email: nhtan@mail.kib.ac.cn)
Supported by the National Natural Science Foundation of China (Grant No. 30725048), and Foundation of the West Light Program of Chinese Academy of Sciences

10 plants in the Cucurbitaceae, Rubiaceae, and Violaceae families. Cyclotides displayed an interesting range of biological activities, i.e., uterotonic, anti-HIV, inhibiting neurotensin binding, antimicrobial, inhibiting trypsin, cytotoxic and insecticidal activities. On the basis of tissue-specific expression of cyclotides in *Viola* species and acting as insecticidal and antimicrobial agents, Craik and co-workers proposed that cyclotides might be regarded as a new family of plant defense peptides. The inherent bioactivities make cyclotides become new drugs directly or lead compounds. Even more, its precise structure, high stability and variable residues in loops make cyclotides become interesting backbone of peptide drug by linking different new active ligands[8].

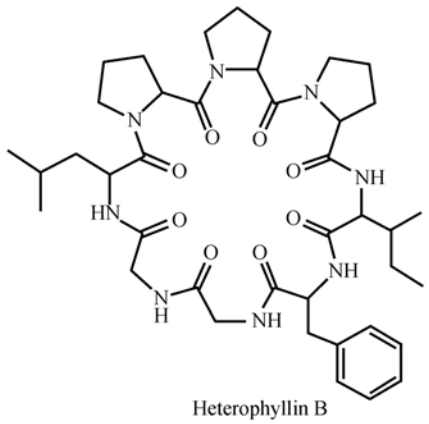
A new thin layer chromatography (TLC) chemical method for detection of cyclopeptides in plants was reported in 2000 by us[9]. It was a TLC protosite reaction with ninhydrin reagent which released the amino group hydrolyzed by HCl in the TLC plate. It was a good specific and sensitive chemical detection method for plant cyclopeptides, and over 100 Caryophyllaceae-type cyclopeptides were isolated with this method. It could be used effectively not only to detect whether plant extracts contain cyclopeptides, but also to guide cyclopeptide separation and purification. Since 2005, we have been exploring cyclotides on the basis of cyclopeptides, and found some deficiency of this method during detecting cyclotides. In this paper, a TLC method with ninhydrin and Coomassie brilliant blue G-250 reagents for detection of cyclotides was established. With this method, cyclotides were discovered in *Viola labridorica*, *V. tricolor*, *V. hamiltoniana*, *Momordica charantia* and *M. cochinchinensis*, and over 10 cyclotides were isolated[10], three of which were determined as known cyclotides cycloviolacin O2[11], kalata B1[7] and vary peptide A[12].

## 1 Experimental

### 1.1 Materials

Ninhydrin[13] is a common reagent for detecting amino acids; this reagent[14] together with other reagents such as Coomassie brilliant blue, biuret and Folin-Phenol reagents[15] is used to detect proteins. We chose ninhydrin and Coomassie brilliant blue G-250 reagents for their convenient and extensive usage together with known common reagents iodine and Dragendorff's reagents[13] as references which are used for detecting alkaloids. Preparation of Coomassie brilliant blue G-250 reagent was improved to fit TLC cyclotides detection sensitively, which are smaller than proteins. The preparation was as follows: 100 mg G-250 was dissolved in 20 mL ethanol, add 20 mL phosphoric acid and 160 mL 50% ethanol. The ninhydrin reagent was 0.2% ethanol solution. Dragendorff's reagent was prepared as follows: 0.85 g Bismuth subnitrate dissolved in 10 mL glacial acetic acid and to which 40 mL $H_2O$ was added was taken as solution A; 8 g potassium iodide dissolved in 20 mL $H_2O$ was taken as solution B; 1 mL solution A was mixed with 1 mL solution B, to which was added 2 mL acetic acid and 10 mL $H_2O$.

Proline (P), lysine (K), glutamic acid (E), methionine (M) and tryptophan (W) were the biochemical reagents from Shanghai Kang-Da Amino Acid Factory. Heterophyllin B (HB)[16] and cycloviolacin O2[11] were isolated in our laboratory. Linear-heterophyllin B (PPPIFGGL) was synthesized with solid-phase by Suzhou Tian-Ma Pharmacy Group (Figure 1). Bovine serum albumin (BSA) was supplied by Sigma Company. TLC silica gel G plates were products of the Qingdao Makall Group Co. Ltd. Other reagents were of analytic purity.



Heterophyllin B

CGESCVWIPCISSAIGCSCKSKVCYRNGIP

Cycloviolacin O2

**Figure 1** Structures of heterophyllin B and cycloviolacin O2.

### 1.2 Sample preparation

2.1－2.4 mg amino acids were dissolved in 1 mL deionized $H_2O$ separately. 2.2 mg HB was dissolved in 1 mL methanol. 2.1 mg linear-HB was dissolved in 1 mL deionized $H_2O$. 2.1 mL cycloviolacin O2 was dissolved in 1 mL 70% ethanol. 2.2 mg BSA was dissolved in 1 mL deionized $H_2O$.

### 1.3 Cyclotide detection

The samples were dotted on one side of 5 identical 30 mm×50 mm silica gel G plates (plates 1－5) and these plates were developed with *n*-butanol∶acetic acid∶water (3∶1∶1, *v*∶*v*∶*v*). After removal of the solvent, plates 1, 2 and 5 were detected with iodine, Dragendorff's and Coomassie brilliant blue G-250 reagents separately. Plate 4 was hanged in a sealed glass vessel with about 1 mL concentrated HCl and hydrolyzed in a drying incubator (110℃) for 2 h. Cooled for a few minutes, plate 4 was taken out and the HCl was volatilized with a ventilator. Then plates 3 (non-hydrolyzed plate) and 4 (hydrolyzed plate) were sprayed with 0.2% ninhydrin reagent and detected after heating with a drier for several minutes.

### 1.4 Lower limit for detecting cyclotides

Solution of cycloviolacin O2 (2.1 mg/mL) was diluted with 70% ethanol according to the ratios of 1∶1, 1∶2, 1∶5 and 1∶10, then dotted samples on the silica gel G plates. The plates were detected with ninhydrin and G-250 reagents before or after being developed.

## 2 Results and discussion

### 2.1 Cyclotide detection in TLC plates

Iodine is a common reagent for detecting organics, and Dragendorff's reagent is a common reagent for detecting alkaloids. These two reagents can be used to detect cyclopeptides, linear-peptides and proteins, which are not specific. The TLC protosite reaction with ninhydrin reagent is a good specific and sensitive chemical method for detecting cyclopeptides, which can be used effectively to distinguish cyclopeptides and cyclotides from amino acids, linear-peptides and proteins by comparing the colors of the spots on non-hydrolyzed plate (plate 3) and hydrolyzed plate (plate 4). If there were some purplish red or yellow spots in some cases in plate 4, there would be no such spots at the same sites in plate 3, indicating that the detected samples contained cyclopeptides or cyclotides. Coomassie brilliant blue G-250 is a reagent for detecting proteins. We can use G-250 reagent to distinguish cyclotides from cyclopeptides by comparing the spot colors in TLC plates. Spots of cyclotides were cyan while the backgrounds were red. Cyclopeptides did not react with G-250. Therefore, cyclotides could be distinguished from the others by comparing the TLC coloring plots with G-250 and ninhydrin reagents (Table 1, Figure 2).

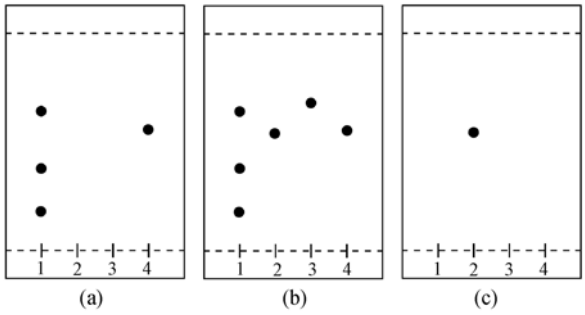



**Figure 2** TLC results of amino acids, cyclotides, cyclopeptides and linear peptides. (a)－(c) Non-hydrolyzed plate spraying with ninhydrin, hydrolyzed plate spraying with ninhydrin and non-hydrolyzed plate spraying with G-250. Samples on plates 1－4, amino acids (mixture of P, K, E, M and W), cyclotides (cycloviolacin O2), cyclopeptides (HB) and linear peptides (linear-HB). TLC conditions: Solid phase, silical gel G; mobile phase, *n*-butanol∶acetic acid∶water (3∶1∶1, *v*∶*v*∶*v*). (a) and (b) were purplish red or yellow while the background was white, and (c) was cyan while the background was red.

### 2.2 Detection sensitivity of cyclotides

At the concentration of 2 μg cyclotide (10 μL 0.21 mg/mL cycloviolacin O2), results indicated that the spot could not be detected with ninhydrin reagent, but was colored well by G-250 reagent. It inferred that G-250 reagent may be more sensitive than ninhydrin reagent.

### 2.3 Influence of hydrolyzed reaction time

Cyclopeptides could be colored well by the ninhydrin reagent after being hydrolyzed in about 0.5－1 h, but



**Table 1** Results of different samples colored by different detecting reagents[a)]

| | Amino acids[b)] | Cyclotide | Cyclopeptide | Linear peptide | Protein |
|---|---|---|---|---|---|
| Iodine | – or + or +++ | +++ | + | + | + |
| Dragendorff's | – or + | +++ | – or + | + | +++ |
| Ninhydrin (non-hydrolyzed) | +++ | – or + | – | +++ | + |
| Ninhydrin (hydrolyzed) | +++ | +++ | +++ | +++ | +++ |
| Coomassie brilliant blue G-250 | – | + | – | – | +++ |

a) "–" means negative, "+" means positive, "+++" means more positive. b) Amino acids are the mixture of P, K, E, M and W, cyclotide is cycloviolacin O2, cyclopeptide is HB, linear peptide is linear-HB and protein is BSA.

cyclotides need more time with fresh concentrated HCl for hydrolysis, i.e., 2 h for the CCK motif.

### 2.4 Application

With this method, cyclotides were discovered in *V. labridorica*, *V. tricolor*, *V. hamiltoniana*, *M. charantia* and *M. cochinchinensis*, and over 10 cyclotides were isolated[10], three of which were determined as known cyclotides cycloviolacin O2[11], kalata B1[7] and vary peptide A[12].

## 3 Conclusion

Coomassie brilliant blue G-250 is a common reagent for detecting proteins, which is usually used in polyacrylamide gel electrophoresis (PAGE) undergoing a change in its color upon non-covalent binding to proteins. G-250 can bind to cyclotides whose molecular weights are higher than those of cyclopeptides. We improved its preparation to fit TLC detection sensitively of cyclotides, i.e., the concentrations of G-250 and ethanol were increased for improving the sensitivity and volatilizing quickly. In this study, we combined the G-250 reagent with the TLC protosite reaction of ninhydrin reagent reported by us[9], and established a TLC chemical method for detection of cyclotides. After applying this method to investigate cyclotides of *V. labridorica*, *V. tricolor*, *V. hamiltoniana*, *M. charantia*, and *M. cochinchinensis*, we have found that it is a good specific and sensitive chemical method for detecting cyclotides. It could be used effectively not only to detect whether plant extracts contain cyclotides, but also to guide cyclotide separation and purification.


1 Tan N H, Zhou J. Plant cyclopeptides. Chem Rev, 2006, 106(3): 840－895
2 Gournelis D C, Laskaris G G, Verpoorte R. Cyclopeptide alkaloids. In: Progress in the Chemistry of Organic Natural Products, Vol 75. New York: Springer, 1998. 1－179
3 Itokawa H, Takeya K, Hitotsuyanagi Y, et al. Macrocyclic peptide alkaloids from plants. In: The Alkaloids, Vol 49. New York: Academic Press, 1997. 301－387
4 Craik D J, Simonsen S, Daly N L. The cyclotides: Novel macrocyclic peptides as scaffolds in drug design. Curr Opin Drug Discovery Dev, 2002, 5(2): 251－260
5 Simonsen S M, Sando L, Ireland D C, et al. A continent of plant defense peptide diversity: Cyclotides in Australian *Hybanthus* (Violaceae). The Plant Cell, 2005, 17(11): 3176－3189
6 Gran L. Oxytocic principles of *Oldenlandia affinis*. Lloydia, 1973, 36(2): 174－178
7 Saether O, Craik D J, Campbell I D, et al. Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. Biochemistry, 1995, 34(13): 4147－4158
8 Craik D J, Daly N L, Mulvenna J, et al. Discovery, structure and biological activities of the cyclotides. Curr Protein Pept Sci, 2004, 5(5): 297－315
9 Zhou J, Tan N H. Application of a new TLC chemical method for detection of cyclopeptides in plants. Chin Sci Bull, 2000, 45(20): 1825－1831
10 Tang J, Tan N H, Xu W Y, et al. Structure of cyclotide: Cycloviolacin O2. Acta Bot Yunnan (in Chinese), 2008, in press
11 Craik D J, Daly N L, Bond T, et al. Plant cycotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structure motif. J Mol Biol, 1999, 294: 1327－1336
12 Claeson P, Goransson U, Johansson S, et al. Fractionation protocol for the isolation of polypeptides from plant biomass. J Nat Prod, 1998, 61(1): 77－81
13 Cao Z Q. Hand Book of Layer Chromatography Dye (in Chinese). Beijing: The Commerce Press Ltd., 1986. 61－73
14 Wang J Y, Zhu S G, Xu C F, et al. Biochemistry (in Chinese). 3rd ed. Beijing: Higher Education Press, 2002. 139
15 Xia Q C, Zeng R, Cao X J, et al. Protein Chemistry and Proteomics (in Chinese). Beijing: Science Press, 2004. 8－9
16 Tan N H, Zhou J, Chen C X, et al. Cyclopeptides from the roots of *Pseudostellaria heterophylla*. Phytochemistry, 1993, 32(5): 1327－1330