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Review

Plant cyclotides: An unusual class of defense compounds

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ABSTRACT

Plant cyclotides are unusual peptides with low molecular masses and a three-dimensional structure characterized by the presence of a cyclic fold. Synthetic peptides can adopt this circular conformation, but it is not a common feature for most members of other peptide groups. Cyclotides present a wide range of functions, such as the ability to induce stronger contractions during childbirth and anti-tumor activity. Additionally, some cyclotides present anti-viral, insecticidal or proteinase inhibitory activity. In this paper, we describe the structural and functional characteristics of plant cyclotides, their most conserved features and the development of these peptides for human health and biotechnological applications.

Contents

1. Introduction

Plant cyclotides are unusual proteins first reported in the early 1970s, in studies of the medical properties of an African plant named kalata-kalata [12]. Although their discovery occurred about 30 years ago, findings on their structural features and studies reporting possible mechanisms of action only appeared in the 1990s [29]. The plant cyclotide family consists of peptides of approximately 30 amino acids and, unlike conventional proteins; do not have N- or C-termini, since these extremities are linked, forming a cyclic structure [10,27,36]. Cyclotides can be classified into two subfamilies: Möbius and bracelet. The first group is characterized by a twist formation in the backbone of the peptide, and by the possible

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presence of a *cis*-Pro motif [4,6,22]. The second group, the bracelet subfamily, is characterized by the absence of this twist feature [5,10]. Cyclization seems to play an important role in peptide stability and activity. This observation was reinforced by a study with kalata-B1, a Möbius member, where the cystine knot motif was shown to be important in the thermal stability, while the cyclic backbone was responsible for the complete enzymatic stability [5].

Cyclotides have a wide variety of roles in plant defense, such as proteinase inhibition, cytotoxicity to tumor cells, anti-viral effects and insecticidal activities [2,8,21–23,34]. Moreover, uterotonic activity can be induced by cyclotides, showing their potential as medical drugs [12,13]. Although the mechanisms of action of cyclotides are still not very well understood, some information has been published. In order to elucidate the mechanism of action of Möbius cyclotides, kalata B1 with dodecylphosphocholine were studied using NMR spectroscopy [31]. The data showed that the cyclotide binds to the micelle surface, with relatively high affinity, via two hydrophobic loops. The charged residues (Glu_3 and Arg_{24}), along with the cation-binding site (near Glu_3) are segregated on the other side of the molecule and in contact with the detergent polar head groups. The spatial structure of kalata B1 is only slightly changed during incorporation into micelles and represents a distorted triple-stranded β-sheet cross-linked by a cystine knot [31].

The biosynthesis of cyclotides has also been studied and, although there are not any conclusive findings, there is an hypothesis that better explains its folding mechanism. Studies with kalata B1 leaded to the hypothesis that disulfide bond formation would occur before cleavage and cyclization [21,29]. Moreover, this theory was also confirmed by Trabi and Craik [36], but the process of excision and cyclization, as well the enzymes involved are still unclear. This review focuses on novel insights on plant cyclotides, showing the recent discoveries about their wide range of functions and the most current hypothesis about their mechanisms of action. Furthermore, this report also intends to correlate amino acid sequence and structure to conserved residues and describe similarities between different species.

2. Biological activities

The first cyclotide described was isolated from the plant kalata-kalata (*Oldenlandia affinis*), and showed uterotonic activity [12,13] stimulating stronger contractions during childbirth, thereby shortening the delivery time [12]. Today, a range of activities has been described for cyclotides. Table 1 summarizes the plant cyclotides discovered in the last 20 years. In particular, circular peptides from *Momordica cochinchinensis* (MCoTI-I and II) have demonstrated ability to inhibit trypsin-like enzymes [18,19].

Some species that are used in carcinoma treatments, such as *Viola arvensis*, contain cyclotides known as Varv peptides [3,11]. Vitri A isolated from *V. tricolor*, demonstrated cytotoxicity to human lymphoma and myeloma cells [34]. Similarly, cycloviolacin H4 isolated from *V. hederaceae* is able to cause hemolysis in human erythrocytes. Despite the well-characterized cytotoxic activity toward tumor cells, their mechanism of action has not yet been elucidated. Cyclotides can also have insecticidal activity. A protein from *O. affinis*, named kalata B2, is able to inhibit growth and development of *Helicoperva armigera* larvae [22]. Although its mode of action is not yet understood, kalata B2 shows structure similarities to kalata B1, a cyclotide with insecticidal and uterotonic activities as previously mentioned [22]. Earlier reports identified and characterized a cyclotide from *V. hederaceae,* named vhl-1, which has anti-HIV activity [2]. Recently, a group of cyclotides, called cycloviolins, was isolated from *Leonia cymosa* and also demonstrated activity against HIV-1 [17]. Moreover, palicourein, a 37 amino acid cyclotide from *Palicourea condensata*, has been shown to inhibit HIV-1 infection of human T-lymphoblastoid cells [20]. Earlier studies revealed that kalata B1 from *O. affinis,* cyclopsychotride from *P. longipes* and circulins A and B from *C. parvifolia* also demonstrated antimicrobial activities against bacteria. While circulin A and kalata B1 showed activity against Gram-positive bacteria, such as *Staphylococcus aureus*, circulin B and cyclopsychotride were able to inhibit growth of both Gram-negative and Gram-positive bacteria [35].

Plant proteins belonging to the cyclotide family can have different biological functions. Recently, six bracelet and seven cyclotides from the Möbius subfamily were isolated from *V. odorata* [20]. They were tested and showed stability against proteolytic degradation by pepsin, trypsin and thermolysin. Moreover, cycloviolacin O24, from the Möbius subfamily presented 75% of hemolytic activity, while O14, another Möbius member, showed just 11% of hemolytic activity and no proteolytic activity. Further studies focusing on the elucidation of their three-dimensional structure revealed that minor variations in primary sequence could be the cause for changes in hemolytic activity [20].

3. Sequence alignment and phylogenetic analysis

Analysis of the primary sequences of plant cyclotides shows that cysteine residues are well conserved in all plant species (Fig. 1A). Three glycines at positions 2, 14 and 26 are also well conserved in the cyclotides, while Gly_7 and Gly_8 showed conservation only in Möbius members and in some bracelet cyclotides from *Viola* species, such as Varv proteins. The same cyclotides also presented a conserved Glu_3, which has been shown to be important for structure stabilization [22]. Loop 1 is conserved in both cyclotide subfamilies, while loop 5 is conserved within the cyclotide subfamilies, but not between them. The primary sequence of the Möbius members have 80% identity, while for bracelet cyclotides from the *Viola* species, there is approximately 54% identity.

A phylogenetic tree of plant cyclotides was constructed in order to evaluate the existence of a common ancestor between Möbius and bracelet members (Fig. 2). In general, bracelet members were grouped at the top part of the tree, while Möbius members were mostly concentrated at the bottom of the tree. One exception was Vhl-2, a protein from *V. hederaceae* which was grouped with Möbius proteins. Varv proteins, which come from Violaceae, and Kalata proteins, present in Rubiaceae plants were quite well separated within the Möbius subfamily. Vhl-2 function was not reported yet, however, the fact that it is placed near VarvF protein suggests the possibility that this