Natural Ribosomally Synthesized Circular Proteins

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Over recent years several examples of natural ribosomally synthesized circular proteins from a diverse range of organisms have been discovered. They are special molecules, whose precursors must be post-translationally modified to join the N to C termini with a head-to-tail peptide bond. This feature appears to have a range of potential advantages over their linear counterparts, since they are less susceptible to proteolytic cleavage and show enhanced stability. The advantages of circularization are reflected by the fact that a significant number of macrocyclic natural products have found pharmaceutical applications. In this article we review the function, structure and biosynthesis of currently known circular proteins, which are produced by microorganisms, plants and animals.

Keywords: Circular protein, head-to-tail peptide bond, bacteriocin, defensin, cyclotides, pilin

1. Introduction

Proteins are defined as chains of amino acids that occur naturally and have a definite three-dimensional structure under physiological conditions, which determines their biological function. The backbone of linear polypeptide chains consists of a repeated sequence: three atoms of each amino acid in the chain – the amide N, the Cα and the carbonil C. The chains´ termini often flexible and representing target points for the attack of proteolytic enzymes, subtract stability to the molecule. Some proteins avoid this problem with post-translational modifications, such as acetylations, hydroxylations and/or glycosylations, but there is a singular situation represented by the circular proteins of ribosomal synthesis, which have been described from bacteria to mammals, ranging in size from 21 to 78 amino acids (Table 1, Fig. 1).

The structural data available for circular proteins have almost exclusively been derived from NMR analysis and X-Ray diffraction. These proteins contain well-defined and conservative secondary structures. The only difference from conventional proteins is that the gene-encoded precursor proteins must be post-translationally modified to join the N- and C- ends, producing a seamless circle of peptide bonds. Clearly, cross-linking the termini of a chain decreases the conformational entropy of the highly flexible and disordered linear polypeptides, stabilizing their bioactive conformation and increasing proteolysis-resistance due to the lack of exopeptidase cleavage sites. Another common characteristic of most circular proteins, is their strong biological activity (antibacterial, antiviral or pharmacological action). Thus, their biotechnological interest has increased in the last years, becoming a prime line of research [1-3]. Circular proteins are different from other nonribosomal cyclic peptides produced by bacteria and fungi, such as cyclosporin A, polymyxin B1, gramicidin S, tyrocidin A or mycosubtilin [4-7], which are synthesized in a peptide synthetase-driven process [8]. In this review we describe the currently known natural ribosomally synthesized circular proteins from very diverse sources, depicting their structure, biological function and biosynthesis.

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Table 1. Source and characteristics of ribosomally synthesized circular proteins

Circular Peptide	Source	Size (n° residues)	Function	Citations
Gassericin A/Reutericin 6	*Lactobacillus gasseri* LA39 / *Lactobacillus reuteri* LA6	58	Bacteriocin	[9, 10]
Acidocin B	*Lactobacillus acidophilus* M46	58	Bacteriocin	[11]
Subtilosin A	*Bacillus subtilis*	35	Bacteriocin	[12]
Butyrivibriocin AR10	*Butyrivibrio fibrisolvens* AR10	58	Bacteriocin	[13]
Circularin A	*Clostridium beijerinckii* ATCC 25752	69	Bacteriocin	[14, 15]
AS-48	*Enterococcus faecalis* S-48	70	Bacteriocin	[16, 17]
Uberolysin	*Streptococcus uberis* 42	70	Bacteriocin	[18]
T Pilin	*Agrobacterium tumefaciens*	73	Cellular contact	[19]
TrbC	*Escherichia coli*	78	Cellular contact	[19]
Cyclotides	Fam. *Rubiaceae* and *Violaceae*	28–37	Bioactivity	[20-22]
MCoTI-I / II	*Momordica cochinchinensis*	34	Trypsin inhibitor	[23]
SFTI-1	*Helianthus annuus*	14	Trypsin inhibitor	[24]
RTD-1	*Macaca mulatta*	18	Rhesus macaque leukocytes defensin	[25]

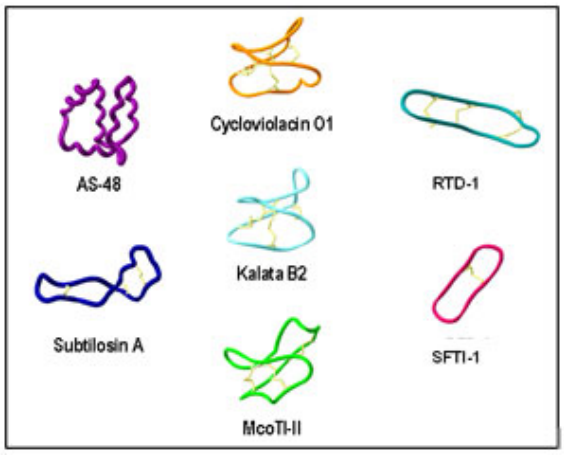

Fig. 1. Structures of some naturally circular proteins. Left, Bacteriocins AS-48 and Subtilosin A; middle, Cyclotides Cycloviolacin O1 (Bracelet subfamily), Kalata B2 (Möbius subfamily) and McoTI-II (trypsin inhibitors subfamily); right, Defensin RTD-1 and Sunflower trypsin inhibitor SFTI-1.

2. Circular proteins produced by bacteria

Two types of ribosomally synthesized circular proteins from bacteria have been described; bacteriocins produced by Gram-positive bacteria involved in microbial defence of the producer organisms; and pilins present in some Gram-negative bacteria implicated in the contact between cells during the conjugation process.

2.1. Bacteriocins

2.1.1. Gassericin A/Reutericin 6 and Acidocin B

Gassericin A and reutericin 6 are circular peptides produced by *Lactobacillus gasseri* LA39 [9] and *Lactobacillus reuteri* LA6 [10] respectively, which possess identical primary amino acid sequences deduced from their structural genes [26]. However, they differ in their circular dichroism (CD), inhibitory spectra as well as killing kinetics against selected indicator bacteria [26]. The basis for these phenotypic differences was revealed by partial composition analysis of the D- and L-amino acids of both peptides. Gassericin A and reutericin 6 both contained D- and L-amino acids, a novel finding in itself, but in different D-Ala:L-Ala ratio [26]. Gassericin A (5.6 kDa) has a wide antibacterial spectrum only against Gram-poisitive bacteria, including *Lactobacillus* strains and some food pathogen or spoilage bacteria such as *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus* [27, 28].

Acidocin B (from *Lactobacillus acidophilus* M46), displays significant amino acid similarity to gassericin A [11, 13, 28, 29], although the physical properties of acidocin B remain to be confirmed. The structural gene of gassericin A (*gaaA*) has been located on the chromosomal DNA of *Lb. gasseri* LA39 [28], while the gene cluster coding for acidocin B (*acdB*) was on a plasmid of the producer strain. DNA sequence analysis revealed the presence of three consecutive ORFs and a fourth ORF of opposite polarity.

Both encode a 91 amino acid pro-prepeptide consisting of a 33 residue leader peptide that possibly mediates their targeting to and translocation across the bacterial cytoplasmic membrane, and a 58 amino acid mature peptide (Fig. 2). The signal peptide contains common features with *sec*-dependent peptidases: a positively charged N terminus followed by a hydrophobic domain and a polar region at the processing site.

2.1.2. Butyrivibriocin AR10

Butyrivibriocin AR10, produced by the ruminal anaerobe bacteria *Butyrivibrium fibrisolvens* AR10, has a 45% identity with gassericin A [13, 29]. This bacteriocin has a wide spectrum of activity among *Butyrivibrio* isolates, but its activity against other genera is limited [30]. Butyrivibriocin AR10 is genetically encoded by *bviA* as a prebacteriocin of 80 amino acids. The molecular weight of the isolated bacteriocin (58 residues) is 5.981 kDa, which corresponds to the molecular mass generated by the removal of the leader peptide (22 residues) before the subsequent circularization (Fig. 2) [29]. The amino acid analysis of butyrivibriocin AR10 confirms a very high content of nonpolar amino acid residues. Moreover, eight ORFs (*bviABCDE*, ORFs 1, 2 and 8) have been identified in the *bvi* region [29, 30].

2.1.3. Subtilosin A

Subtilosin A is an anionic 35 amino acid circular bacteriocin produced by the soil bacterium *Bacillus subtilis.* It possesses bactericidal activity against a diverse range of Gram-positive and Gram-negative bacteria [31]. The widespread occurrence of subtilosin A seems to reflect important physiological roles for this bacteriocin, since it is produced at the end of exponential growth, particularly under stress conditions, and with specific functions during anaerobic or biofilm growth of *B. subtilis* [32]. According

to the structure proposed by [33], mature subtilosin A is produced after the removal of an eight amino acid leader peptide (Fig. 2). NMR studies have demonstrated that in addition to its circular structure, three cross-links are formed in the molecule between sulphurs of two cysteines (Cys4 and Cys13) and the α-positions of two phenyl-alanines (Phe22 and Phe31) and lastly, a Cys7-Thr28 linkage (Fig. 1) [34-36].

Subtilosin A proved to have antimicrobial activity against a wide range of bacteria including Gram-positive and Gram-negative bacteria (both aerobes and anaerobes), being less effective against the capsulated strains. Heat shock also altered the effectiveness of the bacteriocin but protease did not [31]. The plausible mechanism involved in its antimicrobial activity seems to follow a receptor-binding mediated lipid-perturbation model, in a concentration dependent manner. At high peptide concentrations, subtilosin A adopts a partially buried orientation in lipid bilayers, inducing a conformational change in the lipid headgroup and disordering the hydrophobic region of bilayers, which results in membrane permeabilization [37]. In addition, the *sbo-alb* gene cluster for subtilosin production consists of *sboA-albABCDEFG* genes organized into an operon of 7 kb which is transcribed from a promoter residing upstream of the *sboA* subtilosin structural gene [12, 38]. The *sbo-alb* operon expression is complexly regulated, given that it is induced in late growth cultures apparently in response to starvation and also under oxygen-limited and anaerobic conditions [39].

2.1.4. Circularin A

The biochemical and genetic characteristics of circularin A, a new circular bacteriocin produced by *Clostridium beijerinckii* ATCC 25752, have been described [14, 15]. It has 69 residues (7.2 KDa, pI of 10.6), and is active against *Clostridium tyrobutyricum, Lactococcus, Enterococcus* and *Lactobacillus* strains. Circularin A is produced as a 72-residue pre-peptide which is processed to form the circular mature molecule (Fig. 2). The cluster of genes coding for the biosynthesis of circularin A (*cirABCDE*) was located on the bacterial chromosome and interestingly, shares some homologies with the *as-48* locus, although with scarce similarity at the amino acid level [15].

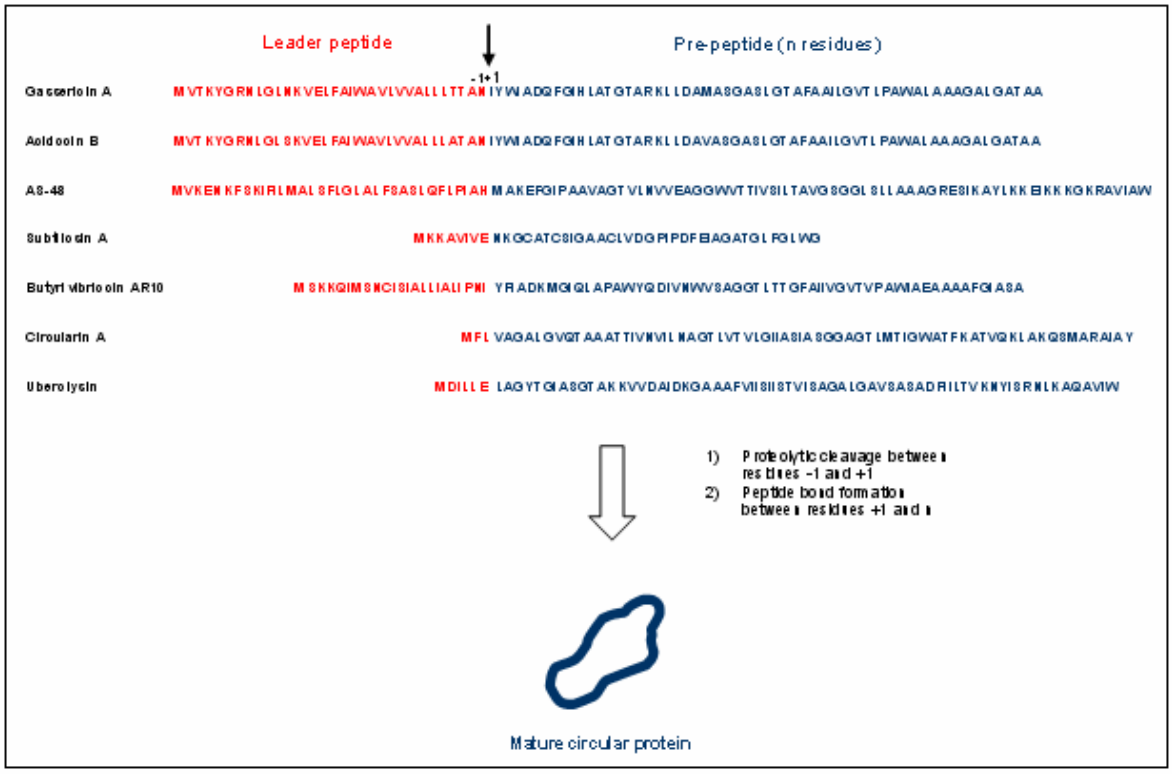

Fig. 2. Hypothetical steps involved in the maturation procedure of the circular bacteriocins pre-peptides yielding the mature peptides head-to-tail linked.

2.1.5. Enterocin AS-48

Enterocin AS-48, the best characterized circular bacteriocin, is a cationic 70-residue peptide produced by *Enterococcus faecalis* subsp. *liquefaciens* S-48 strain, which is ribosomally synthesized and post-translationally modified by a head-to-tail peptide bond [16, 40]. Several AS-48 natural variants named enterocin EFS2, enterocin 4 and bacteriocin 21 have been reported for other *E. faecalis* strains [41-43] as well as for *E. faecium* 7C5 [44]. More recently, an AS-48 variant (AS-48RJ) with a Glu^{20} —>Val^{20} replacement, has been isolated from *E. faecium* RJ16 strain [45].

Currently, satisfactory results on the applications of enterocin AS-48 have been reported for dairy products, meat, and fruit juices, for its wide antimicrobial activity against pathogenic and/or spoilage bacteria like *Enterococcus, Listeria, Bacillus, Paenibacillus*, *Alicyclobacillus, Brochotrix, Micrococcus, Staphylococcus, Planococcus, Clostridium, Mycobacterium, Corynebacterium* or *Nocardia* species [16, 17, 46-52]. Interestingly, some Gram-negative species like *E. coli, Rhizobium,* and *Myxococcus xanthus,* can also be inhibited [16, 17, 45, 46, 47], although the concentrations required are much higher due to the protective effect of the bacterial outer membrane. To date no other bacteriocin from lactic acid bacteria (LAB) has been described as having such a broad inhibitory spectrum. The notable features of AS-48 are: its broad-spectrum antimicrobial activity; stability at a wide range of temperatures and pH values; and sensitivity to digestive proteases. The latter properties of AS-48 make this enterocin a promising substitute for chemical preservatives in foodstuffs.

Composition analysis of purified AS-48 molecules show a high proportion of basic to acidic amino acids that confer to this peptide a strong basic character, with a pI close to 10.5. AS-48 is produced as a 105 amino acid pro-prepeptide, with a 35 residue leader peptide that is cut followed by circularization via a peptidic bond formation between M^{1} and W^{70} (Fig. 2) [51, 52]. The three-dimensional structure of AS-48 as solved by NMR [54] is shown in Figure 1. The fold is characterized by a globular arrangement of five α-helices connected by five short turn regions, enclosing a compact hydrophobic core [53, 54]. The AS-48 target is the cytoplasmic bacterial membrane in which it inserts in a voltage-independent manner, forming pores and leading to the dissipation of the proton motive force. This renders the membranes permeability to ions and small molecules, leading to the release of cytoplasmic material and causing the lysis of sensitive cells (a mechanism similar to that proposed for the majority of cationic antibacterial peptides). According to crystallographic data [55], the proposed mechanism for anti-bacterial activity involves the transition from the water soluble dimmer form I (DF-I) to the membrane bound DF-II (Fig. 3). Intriguingly, the majority of the *as-48* gene clusters described have been located on plasmids [41-44, 56], with the exception of AS-48RJ produced by *E. faecium* RJ16 strain whose genes are found on the chromosome [45]. A wide *as-48* cluster consisting of ten genes (*as*-48ABCC$_1$DD$_1$EFGH) is responsible for AS-48 production and immunity [40, 57].

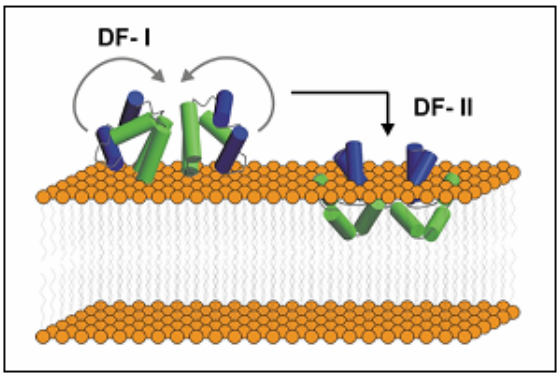

Fig. 3. Proposed mechanism for AS-48 interaction with membranes [55]. The model includes the approach of water soluble DF-I to the membrane and the transition from DF-I to membrane bound DF-II at membrane surface.

2.1.6. Uberolysin

Streptococcus uberis 42, one of the causative agents of bovine mastitis, is a prolific producer of bacteriocins, which include the lantibiotic nisin U [58] and a novel circular bacteriocin recently named uberolysin, due to its ability to evoke bacteriolysis, either directly or indirectly [18]. Uberolysin has a bactericidal mode of action and a broad spectrum of inhibitory activity, which includes most streptococci, *Listeria* spp., enterococci and staphylococci but excludes Gram-negative bacteria. In contrast to other circular peptides, uberolysin is a heat-labile circular bacteriocin that only lyses actively growing cells. Enzymatic hydrolysis of uberolysin was used to confirm that the structural gene *ublA* does indeed encode the precursor of uberolysin. The 76-residue uberolysin precursor peptide is deduced to possess an atypical 6 residue leader peptide, where circularization is predicted to occur between Leu^{1} and Trp^{70} (Fig. 2). Uberolysin has an apparent molecular weight of 4,5 KDa in SDS-PAGE, but the mass determined by electrospray ionization (ESI-IT) MS is 7047.97 Da. In addition, the gene cluster (*orf1, ublA, ublB, ublC, ublD* and *ublE*) coding for uberolysin production and immunity was located on the bacterial chromosome and, which has reportedly, some similarities with enterocin AS-48 [59].

2.2. Pilins

2.2.1. Trb C and T-pilins

Trb C and T-pilins are pili subunits implicated in establishing contact between cells during bacterial conjugation, also contain circularized backbones [19]. TrbC and T are codified by the RP4 plasmid from *Escherichia coli* and Ti plasmid from *Agrobacterium tumefaciens*. Despite being very similar in function and size (78 *vs.* 74 amino acids), TrbC and T pilin do not share a high degree of sequence similarity. The TrbC and T pilin precursors are proteolytically processed and circularized before being transferred to the cell surface where, rather than being secreted, the circular proteins are assembled into pilin filaments.

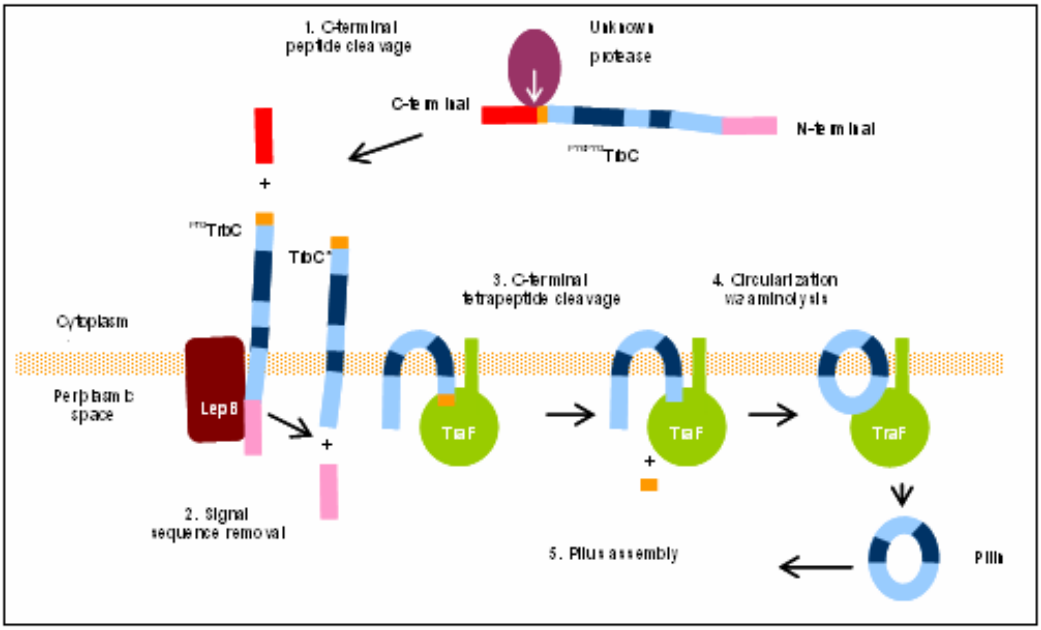

Fig. 4. Putative mechanism proposed for Tbr C pilin circularization and secretion processes. Two putative transmembrane segments (in dark blue) that could favour the proximity of C- and N-terminal ends of the pro-protein inserted in the lipid bilayer during secretion. (Adapted from [60]).

Fig. 4 shows the proposed circularization mechanism of the pre-pro-TrbC. A 27 amino acid peptide from the C-terminus of the precursor is removed by an unidentified protease. The subsequent removal of a 36 amino acid N-terminal signal peptide is performed by Lep B (a chromosomally encoded signal peptidase I) to generate the linear TrbC with a C terminal tetrapeptide (AIEA), allowing the insertion in the lipid bilayer. The ultimate cleavage and circularization is attributed to TraF (a maturase plasmid encoded homologous to the leader peptidases) that would cut the tetrapeptide and circularize the molecule *via* aminolysis (Fig. 4) [60]. C-terminal end of T-pilin is implicated in the peptide bond formation with residue Q^{48} after leader-peptide removal. Secondary structure predictions also indicate the presence of two putative transmembrane helices that would be essential to bring up the ends and allow their peptidic union [60].

3. Circular Proteins produced by Plants

3.1. Cyclotides

The cyclotides constitute another important group of natural circular proteins. To date, more than 80 different molecules have been described [22]. All these compounds are produced by tropical plants belonging to *Rubiaceae, Violaceae* and *Cucurbitaceae* families [22, 61], and possess a wide and interesting range of biological activities. For instance, circulins A and B have anti-HIV activity [62], cyclopsychotride A inhibits neurotensin binding [63], and Kalata B1 shows uterotonic activity [64-67]. All these proteins have a size of about 30 residues and, therefore the term mini-protein is more adequate to describe them [22]. They also have six conserved Cys residues that form three disulphide bridges; two of them, along with the peptide connecting segments, form a ring that is crossed by the third disulfide bridge. This crosslink is called CCK (Cyclic Cystine Knot) motif [67], and is well-conserved among all the cyclotides [68]. Because of their circular structure and the presence of the CCK motif, cyclotides are extremely stable proteins, being very resistant to enzymatic hydrolysis and thermal denaturation [69].

In spite of the structural limitations caused by the CCK motif and the presence of highly conserved residues, cyclotides show a great diversity that allows classifying them into three subfamilies -Möbius, Bracelet and Trypsin inhibitors- according to similarities in sequence, size and mechanism of action [70]:

-The Möbius subfamily is characterized by a *cis* peptide bond before a conserved Pro that introduces a twist in the backbone. Kalata B1 [68] and Kalata B2 [71] are members of this family (Fig. 1).

- In the Bracelet subfamily the *cis* peptidic bond is absent. Representative members of this family are Circulin A [72] and Cicloviolacin O1 [68] (Fig. 1).

- The trypsin inhibitors subfamily has only two members, McoTI-I and McoTI-II (Fig. 1) [23] which are produced by *Momordica cochinchinensis*. This curcubitaceae also produces the linear homologue McoTI-III that coexists with the circular form.

Cyclotides are derived from a family of 12 genes that code for precursor proteins in which one, two or three cyclotide domains are found [73]: an endoplasmic reticulum signal sequence (ER) and/or a relatively long N-terminal domain that is not tightly conserved in sequence or in length. This domain is followed by a relatively well-conserved 25 amino acid sequence of unknown function (the N-terminal region, ntr) that precedes the cyclotide domain and a hydrophobic nucleus in the C-terminal region. After cDNA analysis of several cyclotides, the conclusion drawn is that cyclotides sequences are flanked on both sides by the highly conserved sequence Gly-Leu-Pro (GLP) [73]. Circularization involves the cleavage at a homologous site within both flanking sequences and ligation of the new N and C termini. The mature circular peptide retains one copy of the GLP sequence which may be derived entirely from one of the original flanking elements, or partially from both depending on the initial cleavage sites (Fig. 5). From the precursor structure one can deduce that at least two cleavages are required to form the mature peptide. The enzyme involved in this process is still unknown, although some asparaginyl-endopeptidases have been proposed as being responsible for this process [74]. Another hypothesis could be an autocatalytic mechanism similar to that of inteins [70].

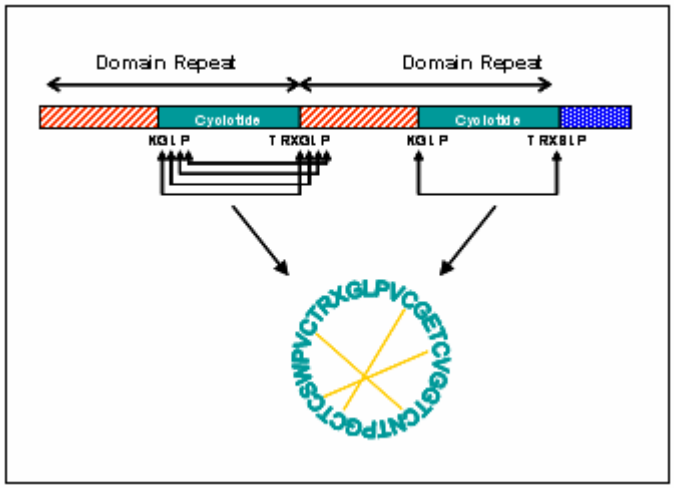

Fig. 5. Schematic representation of cyclotides biosynthesis showing the precursor putative cleavage sites, depending on the presence of GLP motif (4 sites) or SLP motif (2 sites). (Adapted from [70]).

3.2. Sunflower Trypsin Inhibitor I (STF-I)

SFTI-1 is a 14 amino acid potent trypsin inhibitor, with a circular peptide backbone, isolated from sunflower seeds. SFTI-1 shows both sequence and conformational similarity with the Bowman–Birk inhibitors, a family of small serine proteinase inhibitors found in the seeds of legumes and several other plants [24]. In contrast to cyclotides, SFTI-1 has two antiparallel β-chains stabilized by a single disulfide bond [75]. The precursor protein for STFI-1 suggests that *in vivo* circularization involves ligation to form an Asp-Gly sequence [76]. It has also been shown that, *in vitro*, a linear analogue of SFTI-1 is susceptible to circularization and isomerization of the Asp-Gly sequence, confirming the high reactivity of this sequence [77]. The high similarity between the proposed processing sites in SFTI-1 and cyclotide precursors, suggests a similar mechanism for circularization, involving proteases [76]. Alternatively, an autocatalytic process is potentially possible for the biosynthesis of both types of molecules [70].

4. Circular Proteins from Mammals: θ-Defensins

Defensins are antimicrobial peptides produced by plants, insects, birds and mammals, including humans [78-81]. Defensins act as mediators in natural immunity protecting the host against microorganisms. In vertebrates, defensins have an important role in the regulation of acquired immunity responses [82]. In mammals three families of defensins have been found, named α-, β- and θ-defensins. The two first classes are short linear cationic peptides stabilized by disulfide bonds. The last are circular molecules [25, 83] represented by defensin RTD-1 (Reshus Theta Defensin 1). Defensin RTD-1 is an 18-residue peptide (2.083 KDa), whose ends are joined by a peptide bond (Gly1-Arg18). RTD-1 secondary structure [84] consists of two β-chains connected by short turn regions. Like the cyclotides, the molecule is stabilized by three intramolecular disulphide bonds; however they are not organized as a knot, but as a ladder. RTD-1 is part of the innate immune system of the macaque monkey, and has microbiocide action against Gram-positive and Gram-negative bacteria and fungi, and also anti-retroviral activity [85]. Defensin RTD-1 is the product of two genes, *RTD1.1* and *RTD1.2,* each coding for two 9-residue peptides which are subsequently joined by two head-to-tail peptide bonds. These genes have an 88 % identity with α-defensin genes. According to this, the post-translational processing leading to the mature peptide would involve the cleavage of the leader peptide, the proteolytic cleavage in regions flanking each of the peptides, and the formation of two new peptide bonds and three disulfide bonds (Fig. 6).

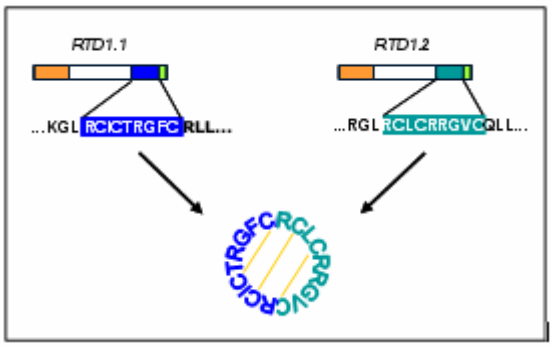

Fig. 6. Organization and sequence of RTD1.1 and RTD1.2 genes. Proposed biosynthesis mechanism of defensin RTD-1. (Adapted from [1]).

5. Circularization Mechanism of Natural Circular Proteins

The best studied circularization system is that of TrbC pilin described in section 2.2 (Fig. 4). However, little is known about the circularization mechanisms or enzymes that could be involved in the other circular proteins described. No homologies among the amino acids involved in the peptide bond, nor in the adjacent residues have been found. The diversity of structures and functions of natural circular proteins

(Fig. 1) makes its unlikely to have a universal circularization mechanism [2]. Nevertheless, these molecules are similar in their synthesis mechanism, as they arise from longer precursors (pro-peptides), where the leader peptides are excised, and their ends are joined by a peptide bond. Until now, no intermediates in the maturation process have been isolated, a fact that suggests that circularization occurs in a fast and efficient manner [1]. Unfortunately, relatively little is known about the mechanism that governs the joining of the termini for the majority of circular proteins described. The importance of expanding the knowledge of cleavage and circularization mechanisms, and the identification of enzymes or auxiliary proteins implicated, are not only of biological interest, but also enable the design of more stable and efficient synthetic circular peptides. Obviously, proteolytic enzymes are candidates for the necessary cleavage (and ligation) steps involved in the biosynthesis of circular proteins, but nowadays very few with the necessary specificity have been characterised.

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