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Research paper

Expression of linear permutated variants from circular enterocin AS-48

Manuel Montalbán-López, Manuel Martínez-Bueno, Eva Valdivia, Mercedes Maqueda*

Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, C/Fuentenueva s/n, 18071 Granada, Spain

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ABSTRACT

To confirm whether the head-to-tail circularization could be involved in the stability and activity of the circular bacteriocin AS-48, two permutated linear structural *as-48A* genes have been constructed by circular permutation. The absence of the leaderless linear $AS_{23/24}$ and $AS_{48/49}$ proteins in *Escherichia coli*, under all the conditions investigated, supports the idea that the circular backbone is important to stabilize their structure and also indicates the significance of a leader peptide. In fact, the approach taken in this study to generate linear permutated proteins fused to an appropriate partner was sufficient to prevent cellular proteolysis. In this case, the high expression levels found favour their intracellular accumulations as inclusion bodies, which after solubilization showed a propensity to aggregate, thus hindering the specific EK cleavage. This could explain the presence of active hybrid tagged proteins identified in this work. The conserved distribution of hydrophobic and hydrophilic surfaces in the hybrid proteins is responsible for the antibacterial activity. In addition, the opening of the AS-48 molecule between the residues G^{23} W^{24} connecting the α1/α2 helices, confers greater stability, suggesting that the sequence and/or the free amino acid in the polypeptide chain are critical aspects in the design of new variants.

1. Introduction

Since the discovery of bacteriocin AS-48, a 70-residue circular protein produced by *Enterococcus faecalis* S-48, several naturally occurring circular proteins from bacteria have been reported [1,2]. AS-48 is encoded by the 68-kb pheromone-responsive plasmid pMB2, and the gene cluster involved in production and immunity has been identified and sequenced [3,4]. This protein exerts a bactericidal action on the cytoplasmic membrane of susceptible cells belonging to most Gram-positive and some Gram-negative bacteria by opening pores leading to the dissipation of the proton motive force and consequent cell death [5]. The major structural feature of AS-48, however, is its circular peptide backbone formed by its head-to-tail junction between M^1 and W^{70} [6]. The three-dimensional structure of the monomeric AS-48 molecules in aqueous solution has been determined by ^{1}H NMR at pH 3.0 and consists of a globular arrangement of five α-helices that span residues 9–21 (α1), 25–34 (α2), 37–45 (α3), 51–62 (α4), and 64–5 (α5), enclosing a compact hydrophobic core with an efficient interdigitation of the hydrophobic side chains at the core of the globular structure [7]. End-to-end cyclization of a protein is expected to enhance its stability by reducing the conformational entropy of the unfolded state. In fact, the AS-48 molecule is remarkably stable, being extremely resistant to heat- and denaturant-induced unfolding, and part of this high stability might be put down to entropic constraints induced by the circular organization of its polypeptide chain [8]. It is clear that the circular backbone of AS-48 and other macrocircular peptides limits sites susceptible to exoprotease activity and constrains the conformation of the termini, leading to an entropic advantage in binding interactions. Despite the efforts expended on this subject during the last ten years, the role of cyclization in proteins still remains unclear, although seems to contribute positively to their stability and has a range of other potential advantages over its linear counterparts. Nevertheless, some authors have reported that circularity does not always increase the Gibbs energy of unfolding because an increase in the energy of the unfolded state could be offset by another increase in the energy of the folded state due to steric tensions introduced by cross-linking [9,10]. One way of circumventing this problem would be to compare wild-type circular proteins with their linear counterparts, and AS-48 is a suitable model for this task because it has a known genetic/amino-acid sequence and secondary and tertiary structures [5]. To explore this aspect, we have been working on the determination of the optimum conditions for opening AS-48 chain properly, whilst maintaining its globular conformation. Thus, we have produced by limited proteolysis a protein species carrying a nicked AS-48 and two fragments that showed lower helicity and reduced stability to thermal

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* Corresponding author. Tel./fax: +34 958 242857.
E-mail address: mmaqueda@ugr.es (M. Maqueda).

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denaturation although retaining to some extent the activity against the indicator bacteria [11]. In addition, we have investigated the effect of some point mutations on the solubility and stability of this constrained circular AS-48 polypeptide [12,13]. However, the consequence of linearization in the stability and ability to fold is not still clear and could be imperative for the rational development of engineering strategies in this protein.

Genetic circular permutation is a conceptually intriguing tool that has been applied by structural biologists to determine the importance of polypeptide sequences to protein folding, although its effect on proteins is still scarcely understood. Studies into the effects of breaking the backbone of some non-bacterial circular proteins have suggested that the circular backbone may not be essential to maintaining the overall folding pattern. Such is the case of the synthetic linear derivatives of RTD-1 defensin from rhesus macaque leukocytes [14], the cyclic trypsin inhibitor SFTI-1 from sunflower [15], the macrocyclic trypsin inhibitor MCoTI-II, the linear homologues of which, with similar structures, exist in nature [16], and also some circular plant cyclotides in which it has been possible to permute the termini and still allow folding into the native conformation [17]. Nevertheless, the additional restraints of the several disulfide bonds in these circular proteins are also likely to play a major role in maintaining the overall structure rather than circularization itself. It is, however, noteworthy that in those cases where the circular backbone may not be essential to the overall folding, a decrease in their biological activities was observed, signifying that while the role of the circular backbone is not fully understood, it does appear to be involved in improving the stability and biological activity and in some cases even in maintaining the molecule's structural integrity [18].

To investigate the role of circularity in the stability and biological properties of enterocin AS-48, new genes codifying two linear variants $AS_{23/24}$ and $AS_{48/49}$ have been constructed by circular permutation in the *as-48A* structural gene. In this paper we describe in detail the opening site selection and the cloning of the two leaderless linear molecules into appropriate expression vectors to be transformed into different backgrounds of *Escherichia coli* strains, in which the expression of genes is often the method of choice [19]. To increase the stability of the whole linear proteins, such linear variants have been also expressed and purified using C-LytA fusion under control of an inducible promoter. The results obtained on the recombinant proteins are here discussed.

2. Materials and methods

2.1. Bacterial strains, plasmids, and bacterial growth conditions

E. coli DH5α was used for standard cloning procedures, and *E. coli* BL21(DE3) (Novagen, Madison, WI), BL21 (DE3) pLysS codonplus (Stratagene, La Jolla, CA), and MG1655 wild-type and several protease-deficient derivatives, were used for the $AS_{23/24}$ and $AS_{48/49}$ expression without leader peptide. *E. coli* REG-1 (Biomedal, Sevilla, Spain) was used for expression of C-LytA fusion proteins (Table 2, Supplementary data). Transformants containing the recombinant plasmids (Table 2, Supplementary data) were selected on Luria–Bertani (LB; Scharlau, Chemie S.A., Spain) or 2xYT medium (Gibco, Invitrogen, UK). When required, 50 μg/ml ampicillin (Ap), 20 μg/ml chloramphenicol (Cm), 10 μg/ml tetracycline (Tc), 50 μg/ml kanamycin from Sigma–Aldrich (Madrid, Spain) were added to the media as selective agents. *Listeria monocytogenes* CECT 4032 and *E. faecalis* S-47, used as an indicator strains, were propagated in BHI (Scharlau, Chemie S.A., Spain) and incubated at 37 °C.

2.2. Molecular techniques

Extraction of total DNA plasmid, cloning and *E. coli* transformations were performed according to the standard protocols [20]. Plasmid DNA was extracted as described by [21] and used as template for PCR amplifications carried out with Biotools DNA Polymerase (B&M Labs, Madrid, Spain). Competent *E. coli* strains were obtained by $CaCl_2$ or sterile water washes to be used in thermal shock transformation and electroporation respectively. DNA modifying enzymes from Fermentas (Quimigen, Madrid, Spain) and Roche Applied Science (Roche Diagnostics, Sant Cugat del Vallés, Spain) were used as recommended by the suppliers. Designed oligonucleotides were synthesized by Thermo (Table 1 Supplementary data; VWR, Madrid, Spain).

2.3. Generation of recombinant plasmids

Construction of the pBAT-$L^{23/24}$ and pBAT-$L^{48/49}$ recombinant plasmids encoding the two linear leaderless AS-48-permutated molecules was carried out by recursive PCR amplifications. The recombinant plasmid pBgD12S was used as template in the first PCR and the amplified PCR products were used as template in the following amplifications. The PCR amplifications were carried out in an iCycler thermocycler (BioRad, Madrid, Spain) with a denaturing step at 94 °C for 1 min followed by 30 cycles of amplification, each cycle consisting of a denaturing step at 94 °C for 60 s, a primer annealing step at 55 °C for 60 s, and an extension step at 72 °C for 30 s. Finally, we added a last elongation step at 72 °C for 5 min. The two permutated PCR-generated fragments were separately cloned into pGEM-T easy (Promega, Alcobendas, Spain). They were digested by *Nco*I and *Bam*HI and directionally cloned into the pBAT-4m expression vector (4.4 kb, Ap^R) [22].

For cloning into pALEXa, a *Bam*HI site was created upstream the *Nco*I site of permutated genes using the AS48-24-BamHI/AS48-24-L3 and AS48-49-BamHI/AS48-49-R1 primers (Table 1 Supplementary data) in the same conditions described above. The amplified fragments were cloned in the expression vector pALEXa (Biomedal, Sevilla, Spain) thus creating a fusion in the correct translational reading frame with the C-LytA protein.

2.4. Expression assays and purification of the recombinant proteins

Overnight cultures of the producer strain harbouring the pBAT-4m derivatives plasmids, were grown aerobically in 100 ml of LB medium containing 50 μg/ml ampicillin. When the optical density at 600 nm (OD_{600}) reached 0.4–0.6, gene expression was induced by addition of IPTG (0.5, 1.0, 1.5 and 2.0 mM) (Sigma) sampling at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 24 h at different temperature of incubation (20, 28 and 37 °C). Expression of C-LytA-fusion proteins was induced by addition of different salicylate concentrations (up to 2 mM) to cultures with OD_{600} between 0.8–1.0 at different incubation temperatures. Samples were taken every 2 h monitoring the OD of the culture and protein profile by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Electrophoretic and immunoblotting techniques

Samples were separated by SDS–PAGE electrophoresis on 15% slab gels as described by [23] in a Miniprotean dual-slab cell apparatus (BioRad), using a low-molecular-weight range marker (SigmaMarkers) as standard. When suitable, gels were transferred to a nitrocellulose membrane (NC, pore size 0.45-μm; Schleicher & Schuell, code BA85, Whatman. ACEFE S.A. Castelldefels, Spain) using a BioRad mini trans-blot system according to [24]. Membranes were developed with specific anti-AS-48 antibodies as