



LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

Biosynthesis of the Cyclotide Kalata B1 using a Protein Splicing Unit

R. H. Kimura, A. T. Tran, J. A. Camarero

August 18, 2005

Angewandte Chemie International Edition

Disclaimer

This document was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor the University of California nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or the University of California, and shall not be used for advertising or product endorsement purposes.

Biosynthesis of the Cyclotide Kalata B1 using a Protein Splicing Unit

Richard H. Kimura Anh-tuyet T. Tran and Julio A. Camarero*

Chemical Biology and Nuclear Sciences Division, Lawrence Livermore National Laboratory, University of California, Livermore, CA 94550, USA.

RECEIVED DATE (automatically inserted by publisher); E-mail: camarero1@llnl.gov

Cyclotides are a newly emerging family of large backbone cyclic polypeptides (≈ 30 residues long) characterized by a disulfide-stabilized core (3 disulfide bonds) with an unusual knotted structure.¹ In contrast to other cyclic polypeptides, cyclotides have a well-defined three-dimensional structure. Therefore, despite their small size, they can be considered mini-proteins. The unique cyclic-backbone topology and knotted arrangement of 3 disulfide bonds endow cyclotides with exceptional stability and resistance to chemical, enzymatic and thermal degradation.² Furthermore, their well-defined structures have been associated with a range of biological functions.^{1b-c} Together, these characteristics suggest that cyclotides are ideal molecular scaffolds for the development of stable peptide drugs.^{1b}

Despite the fact that the chemical synthesis of circular peptides has been well explored and a number of different approaches involving solid-phase or liquid-phase exist,³ recent developments in the fields of molecular biology and protein engineering have now made possible the biosynthesis of cyclic peptides. This progress has been made mainly in two areas, non-ribosomal peptide synthesis⁴ and Expressed Protein Ligation (EPL)/protein trans-splicing.⁵ Access to biosynthetic cyclotides using recombinant DNA expression techniques offers the exciting possibility of producing large combinatorial libraries of highly stable miniproteins. This would allow the generation of cell-based combinatorial libraries that could be screened either *in vitro* or *in vivo* for their ability to regulate cellular processes.

In the present work, we describe the biosynthesis of the cyclotide Kalata B1 (KB1) in *E. coli* using an engineered intein. Our approach (Figure 1) is based on an intramolecular version of Native Chemical Ligation (NCL).⁶ NCL involves the chemoselective reaction between a N-terminal Cys residue of one peptide and an α -thioester group of a second peptide. Importantly, incorporation of these two groups into the same synthetic polypeptide leads to efficient circularization.³

In order to test this approach we constructed several plasmids encoding different KB1 linear precursors (Figure 1b) fused, in-frame at their C-termini, to a modified VMA intein.^{5c} This allows the generation of the required C-terminal α -thioester function. Also, a Met residue was genetically introduced at the N-terminus of the corresponding KB-fusion protein. The Met residue is efficiently removed immediately after translation by the endogenous Met aminopeptidase (MAP). This *in vivo* proteolytic event un masks the N-terminal Cys residue required for NCL.^{5a}

The KB1-intein fusion proteins (1 through 6, Figure 2) were expressed in *E. coli* and purified by affinity chromatography. Analysis of the purified fusion proteins by SDS-PAGE, revealed that the different linear precursors have different propensities for *in vivo* cleavage. Whereas linear precursors 4 and 5 showed $\approx 30\%$ *in vivo* cleavage, 6 was almost completely cleaved (90%). Linear precursors 1, 2 and 3 were $\approx 70\%$ cleaved *in-vivo*. Analysis of the

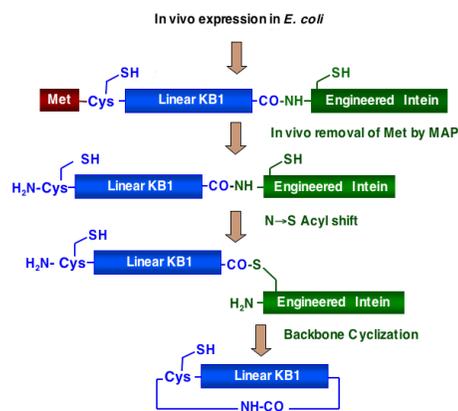
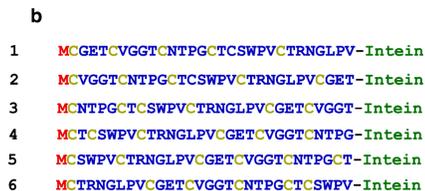
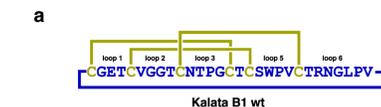


Figure 1. Backbone cyclization of a KB1 linear precursor using an engineered intein.

Figure 2. (a) Primary structure and disulfide connectivity of the cyclotide



KB1. (b). Sequences of the different linear precursors used for the backbone cyclization of KB1

soluble cell fractions corresponding to the constructs with high *in vivo* cleavage, did not reveal the presence of reduced or oxidized circular KB1. Analysis of the insoluble cellular fractions under reductive conditions after being treated briefly at pH 12 to hydrolyze any residual thioester functionality, on the other hand, showed the presence of the corresponding reduced linear carboxylated peptide. It is interesting to note that linear precursors 1 and 6 produced small amounts of reduced circular KB1 ($\leq 5 \mu\text{g/L}$ of culture). These results indicate that *in vivo* cleavage of the KB1-intein fusion proteins is mostly triggered by internal Cys residues rather than by the N-terminal Cys residue, which will lead directly to backbone cyclization. It is very likely that some of these intermediates aggregate before backbone cyclization takes place. The fact that no natural KB1 was found also indicates that the cytoplasm of *E. coli* is probably too reductive for the proper folding of KB1, which misfolds and precipitates. The use of

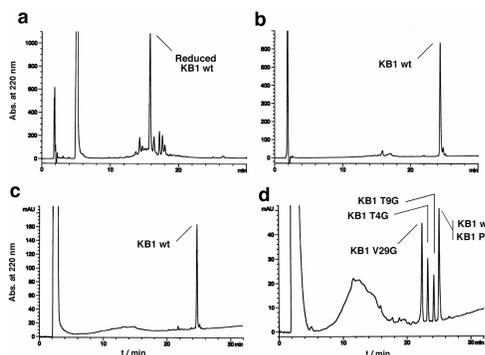


Figure 3. Cyclization and folding of native KB1. Analytical HPLC traces of: (a) EtSH cleavage of purified linear precursor **4** after 2 days, (b) oxidative refolding of reduced KB1 in 10 mM reduced glutathione (GSH), 50 mM NH_4HCO_3 buffer at pH 8.5 containing 50% $^1\text{Pr-OH}$ after 18 h, (c) “one-pot” cyclization and folding of linear precursor **4** using GSH:GSSG in 50% $^1\text{PrOH}$ (see text for details) and (d) “one-pot” cyclization and folding of small library of KB1-based cyclotides using the same procedure as before.

engineered *E. coli* cell lines containing the *trxB/gor* mutations,⁷ which should facilitate the formation of disulfides in the bacterial cytoplasm, did not improve the previous results.

We next tested the ability of the different linear precursors to cyclize *in vitro*. Cyclization was triggered by treating the purified KB1-intein fusion proteins with 3% EtSH in column buffer at pH 7.2. In all of the cases except for **5**, the major product of the reaction was the reduced cyclic KB1. Among the different linear precursors, **1**, **3** and **4** gave the best cyclization yields (Figure 3a). Precursor **6** also had a moderately good yield for the cyclization process. However, the fact that almost all of the precursor fusion protein was prematurely cleaved *in vivo* resulted in a small amount of circular KB1 being produced. Linear precursor **2** gave only a modest yield and although the reduced circular KB1 was still the major product, other minor products including the corresponding linear ethyl thioester precursor as well as thiolactone intermediates were also found in the cyclization crude mixture even after 4 days of reaction. By far, the worst result was obtained for linear precursor **5**. In this case, cyclic reduced KB1 was found only as a minor product ($\leq 5\%$). Purified reduced KB1 was oxidatively folded in the presence of 10 mM reduced glutathione (GSH) in 50 mM NH_4HCO_3 buffer at pH 8.5 containing 50% $^1\text{Pr-OH}$ as previously described by Craik and co-workers.^{3c} Under these conditions, the folding of reduced KB1 is almost quantitative in less than 15 h (Fig. 3b). The folding of reduced KB1 in the absence of organic co-solvents is relatively inefficient being $\approx 40\%$ of that obtained using 50% $^1\text{PrOH}$. The use of 0.5% Triton X-100 also improved the yield of folding (70% of that observed using $^1\text{PrOH}$). The folded recombinant KB1 was finally purified and characterized by ES-MS, HPLC and 2D-NMR; and it was identical to the natural product.

Encouraged by these results we decided to explore the possibility of carrying out the cyclization and folding steps in a single-pot reaction. This was accomplished by treating construct **4** with different amounts and ratios of reduced and oxidized glutathione (GSH and GSSG). The best cleavage/cyclization conditions were accomplished using 100 mM of GSH at pH 7.2. Using these conditions the cleavage of the linear intein precursor was almost quantitative in less than 18 h. Optimal conditions for oxidative folding of reduced KB1 were obtained using a buffer containing a GSH:GSSG ratio of 4:1 at pH 7.2 in 50% $^1\text{PrOH}$. Hence, the purified KC4 linear precursor was treated first with

100 mM GSH at pH 7.2 for 18 h, and the crude reaction was then complemented with $^1\text{PrOH}$ (50% in vol.) and 12 mM GSSG. Under these conditions, the folding reaction was extremely clean and efficient after 20h (Figure 3c). When the oxidative cleavage was done in aqueous buffer at pH 7.4 in the presence of 10 mM GSH and 2 mM GSSG with no $^1\text{PrOH}$ folded KB1 was also obtained with a yield $\approx 4\%$ based on the amount of linear precursor.

We also explored the possibility of using this method to generate different KB1 mutants (KB1-V29G, KB1-T4G, KB1-T9G, KB1-P13A, KB1-W19A, KB1-W19R and KB1-W19S).⁸ The “one-pot” cyclization/folding reaction was very efficient for all the mutants tested with yields ranging from 10% to 60%, thus highlighting the robustness of this structural fold for the construction of molecular libraries. The possibility of using this methodology to biosynthesize libraries based on the cyclotide KB1 was also explored. A small library containing KB1 and the mutants KB1-V1G, KB1-T5G, KB1-T10G and KB1-P14A was expressed in *E. coli*, cyclized and folded as described above. The HPLC and ES-MS analysis of the single-pot cyclization/folding reaction revealed the presence of all the five members of the library (Figure 3d). Quantification of the individual members by ES-MS and HPLC showed that all cyclotides were produced with similar yields. Standard deviation for the yields of all the five members was within 30%.

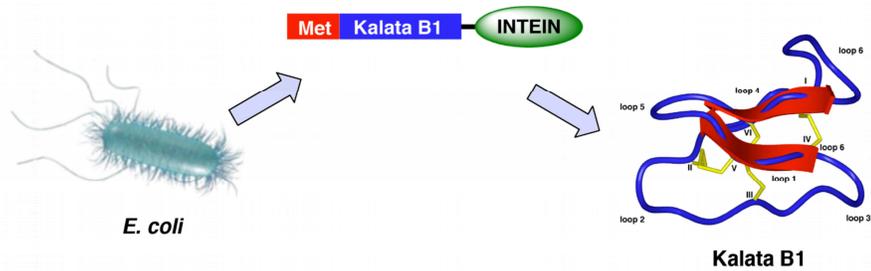
In summary we have described for the first time the biosynthesis of the cyclotide KB1 in *E. coli*. We have also shown that our biosynthetic approach very efficiently generates in one single pot reaction, natural KB1 as well as several mutants. This approach can also be used to generate cyclotide-based libraries that could be screened *in vitro* for biological activity. Furthermore, one can easily envision that by using cellular environments that are less reductive than *E. coli*'s cytoplasm, this biosynthetic method could be also adapted for the *in vivo* biosynthesis of cyclotides.

Acknowledgment. This work was performed under the auspices of the U.S. Department of Energy by University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48. We thank Dr. David Craik, University of Queensland, Australia, for kindly providing a sample of natural Kalata B1.

Supporting Information Available: Experimental methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (a) Trabi, M.; Craik, D. J. *Trends Biochem Sci* 2002, 27, (3), 132-8; (b) Craik, D. J.; Daly, N. L.; Mulvanna, J.; Plan, M. R.; Trabi, M. *Curr Protein Pept Sci* 2004, 5, 5, 297-315; (c) Goransson, U.; Svargard, E.; Claesson, P.; Bohlin, L. *Curr Protein Pept Sci* 2004, 5, (5), 317-29
- Colgrave, M. L.; Craik, D. J. *Biochemistry* 2004, 43, (20), 5965-75.
- (a) Camarero, J. A.; Pavel, J.; Muir, T. W. *Angew. Chem. Int. Ed.* 1998, 37, (3), 347-349. (b) Tam, J. P.; Lu, Y. A. *Prot. Sci.* 1998, 7, (7), 1583-1592; (c) Daly, N. L.; Love, S.; Alewood, P. F.; Craik, D. J. *Biochemistry* 1999, 38, (32), 10606-10614.
- Walsh, C. T. *Science* 2004, 303, 5665, 1805-1810.
- (a) Camarero, J. A.; Muir, T. W. *J. Am. Chem. Soc.* 1999, 121, 5597-5598; (b) Evans, T. C.; Benner, J.; Xu, M.-Q. *J. Biol. Chem.* 1999, 274, (26), 18359-18381; (c) Camarero, J. A.; Fushman, D.; Cowburn, D.; Muir, T. W. *Bioorg Med Chem* 2001, 9, 9, 2479-2484; (d) Scott, C. P.; Abel-Santos, E.; Wall, M.; Wahnon, D.; Benkovic, S. J. *Proc. Natl. Acad. Sci. USA* 1999, 96, (24), 13638-13643; (e) Perler, F. B.; Adam, E. *Curr. Opin. Biotechnol.* 2000, 377-383; (f) Muir, T. W. *Annu. Rev. Biochem.* 2003, 72, 431-434.
- Dawson, P. E.; Kent, S. B. *Annu. Rev. Biochem.* 2000, 69, 923-960.
- Bessette, P. H.; Aslund, F.; Beckwith, J.; Georgiou, G. *Proc Natl Acad Sci U S A* 1999, 96, (24), 13703-13708.
- Numbering of mutations are always referred to construct **1**.



Kalata B1 (KB1) is the prototypical member of a family of plant-derived mini-proteins called the cyclotides. They are characterized by head-to-tail cyclization and three topologically-knotted disulfide bonds. These structural features, called the cyclic cysteine knot (CCK), demonstrate exceptional stability and resistance to many adverse chemical, physical and enzymatic treatments. Variation among cyclotides occurs in four solvent-exposed loops responsible for a wide range of biological activities suggesting that the CCK motif may be used as a scaffold for stable peptide drugs. Here, we report the biosynthesis of KB1 and a small library of cyclotides in *E. coli* using recombinant DNA expression techniques and an engineered protein splicing unit.