Development of ULYSSIS, a Tool for the Biosynthesis of Cyclotides and Cyclic Knottins

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Abstract

Many human disorders and discomforts can potentially be treated by peptide-based medicines. There are two major problems in using peptide-based medicines: their stability and cost-effective production. Generating cyclic variants of linear peptides is an effective way to improve in vivo stability, if done correctly it is possible to retain native activity. In this paper we describe use of peptide complementation to delay splicing and facilitate purification by affinity tag, through ULYSSIS (Universal Ligation bY a Secondarily Split Intein System), a conditional split intein based peptide cyclisation system. Through ULYSSIS we have generated two proof of concept cyclic peptides, kalata B1 and a cyclic variant of a small natively linear peptide, leconotide.

Keywords SICLOPPs · Split intein · ULYSSIS · Kalata B1 · Biosynthesis · Cyclic peptide(s)

Introduction

Peptide based therapeutics are an excellent alternative to small molecule medicines, with improved specificity and potency (Lee et al. 2019). Of particular interest are the knottins and cyclotides, a class of small peptides which contain a cystineknot motif (wherein two disulfide bonds and the peptide backbone between them forms a ring, through which the third disulfide bond passes (Fig. 1) (Postic et al. 2018; Moore et al. 2012; Rosengren et al. 2003). Knottins are widely distributed peptides with a diverse range of biological activities; some, such as the analgesic peptide leconotide (CVID), are appealing for therapeutic applications (McGivern 2007; Gao et al. 2021; Lindley 2021). Knottins naturally occur with a low abundance, making harvesting knottins from their natural host for therapeutic application; inappropriate. Therefore, a method must be developed to enable their efficient production.

Cyclotides are a class of peptide that are structurally related to knottins, which contain the cystine knot motif but have the added feature of a head-to-tail cyclic backbone such as kalata B1; the prototypical cyclotide (Fig. 1) (Rosengren et al. 2003; de Veer et al. 2019; Gran 1973). Cyclotides have since been observed in various plant species (Craik et al. 1999). The resolution of the disulfide knot motif has been explored primarily through 3D NMR analysis of cyclotides, with few examples of X-ray crystallography used (Handley et al. 2020a). Cyclotides can be used as grafting scaffolds (Craik and Du 2017), where individual loops of the cyclotide are replaced with bio-active loops to enable targeting of specific receptors, for example G protein-coupled receptors (Muratspahić et al. 2020; Koehbach et al. 2013). Cyclotides are often highly constrained peptides, due to the cystine knot and continuous backbone. They are resistant to physical stresses and proteolytic degradation, best exemplified by their ability to withstand boiling water in traditional preparations (Garcia and Camarero 2010; Colgrave and Craik 2004). This makes cyclotides ideal peptides for therapeutic applications. It is possible to produce cyclotide variants of knottins, increasing their longevity, without significantly altering the biological activity (Heitz et al. 2008; Clark et al. 2005).

To be useful in the clinic, field, or factory, a bioactive cyclotide must be available in gram quantities at a reasonable cost. Chemical synthesis of cyclotides is difficult, expensive, and highly sequence specific, making chemical synthesis approaches non-ideal for large scale production.

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Fig. 1 Disulfide knot motif, disulfide connectivity and structure of representative cyclotide and knottin. **A** The typical disulfide arrangement found in peptides with cystine knots, with the disulfide connectivity I-IV, II-V, and III – VI. **B** typical arrangement of cyclotides with cysteines separating the various loops 1–6. **C** structure of the prototypical cyclotide kalata B1 with starting Gly residue and N–C direction indicated along with the 6 cysteine residues labelled (PDB: 1KAL). **D** Structure of ziconotide a typically arranged knottin peptide with the 6 cysteine residues labelled (PDB 1OMG)
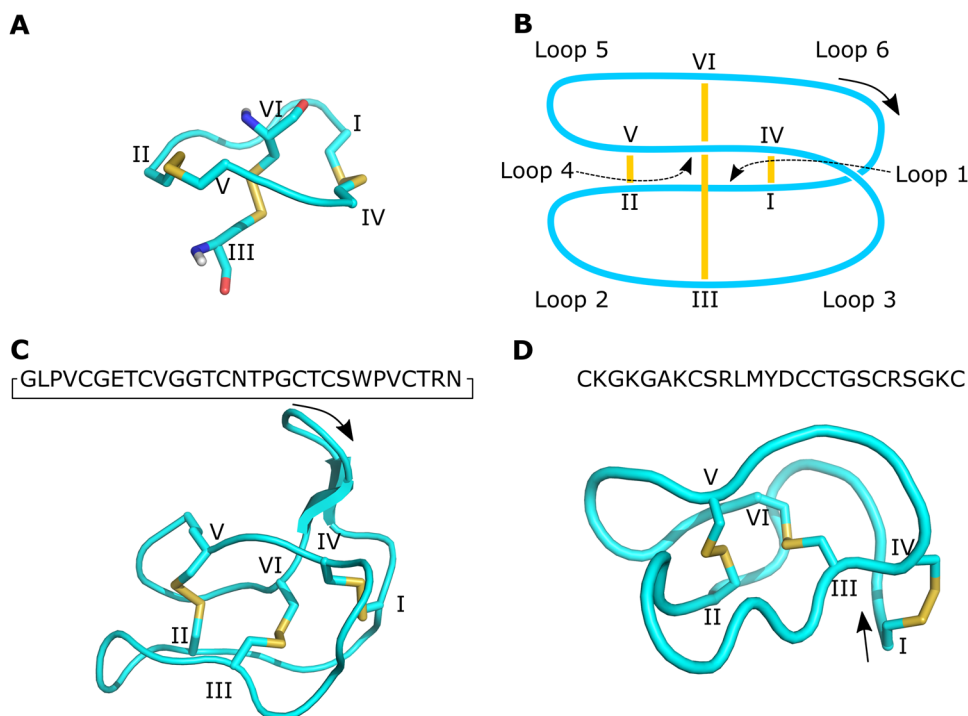
An effective biological synthesis approach is one way to cost-effectively generate cyclic peptides. Natural routes for cyclic peptide production are highly sequence specific; kalata B1, a native cyclotide, is found in the plant *Oldenlandia affinis*, where cyclisation occurs through various sequence specific enzymes (Lee et al. 2019; Harris et al. 2015; Shafee et al. 2015). In this paper we detail the development of widely applicable method to produce cyclic peptides. Our technique builds on a previous technique, SICLOPPs (Split Intein Circular Ligation Of Proteins and Peptides), which exploits a naturally occurring protein-splicing element, split inteins (Scott et al. 1999; Tavassoli 2017; Tavassoli and Benkovic 2007; Handley et al. 2020b).

Inteins are protein elements that excise themselves from flanking protein sequences and covalently join the flanking regions by a peptide bond, through a process termed splicing (Perler et al. 1994; Shah and Muir 2014). Split inteins are natural derivatives of inteins, in which the intein has been split into two separate molecules, that are translated in frame at the termini of separate polypeptides originating from two separate genes (Wu et al. 1998; Gorbalenya 1998). The split intein subunits assemble to carry out the 'splicing' reaction to covalently join the flanking protein sequences (termed 'exteins') together by a peptide bond (Fig. 2) (Wu et al. 1998). Split intein splicing relies on a cysteine residue present in the +1 position (the first residue of the extein following the C-intein) (Fig. 2). Split
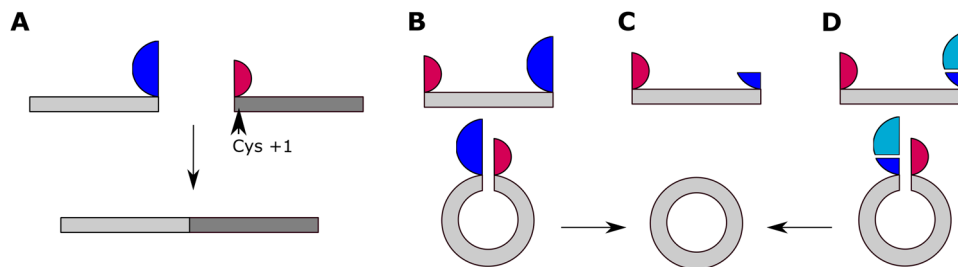
Fig. 2 Split intein splicing, SICLOPPs and ULYSSIS. **A** Split intein splicing, generating one peptide from two precursor molecules. The N-intein is shown in blue and the C-intein is shown in red, with the position of the catalytic Cys residue indicated 'Cys + 1'. **B** the typical SICLOPPs arrangement of split intein subunits flanking a single peptide, such that when the construct assembles in vivo a cyclic peptide product is generated. **C** ULYSSIS peptide 1, unable to carry out peptide cyclization in isolation in vivo. **D** ULYSSIS peptide 1 and 2 in combination (ULYSSIS peptide 2 shown in light blue). When both ULYSSIS peptide 1 and 2 are present in vitro the splicing competent split intein construct can assemble to generate a cyclic peptide product

inteins have been applied to a wide range of applications, SICLOPPs is one such application (Scott et al. 1999). In SICLOPPs, split intein subunits are cloned in frame at the amino-(N-) and carboxyl-(C-) terminus of a peptide of interest (Fig. 2). When expressed in vivo the split intein subunits act to cyclise the peptide of interest (Scott et al. 1999). The application of SICLOPPs is limited in part because the cyclisation reaction is typically uncontrolled, generating cyclic peptides within the expression host, often making purification of the cyclic product difficult, ultimately making efficient scale-up difficult and costly. We have developed a technique called ULYSSIS (Universal Ligation bY a Secondarily Split Intein System), where the cyclisation reaction is controlled. Traditional split inteins contain a single split site, which we add onto here by inclusion of a second split site, making secondarily split inteins. ULYSSIS enables purification of precursor peptides prior to cyclisation, enabling simple purification of the target peptides from the expression host, prior to in vitro cyclisation. In the present study a hybrid split intein based SICLOPPs system is used to develop ULYSSIS, the N-intein comes from the DnaE split intein of *Nostoc punctiforme* while the C-intein comes from the DnaE split intein of *Synechocystis* ssp PCC6803, henceforth the Npu-Ssp system. In studies where hybrid split intein systems have been evaluated, the hybrid Npu-Ssp split intein has been shown to carry out splicing at a similar rate as the *N. punctiforme* split intein and there is good data suggesting that the splicing reaction generates only a small proportion of off target reaction products (Iwai et al. 2006; Cheriyan et al. 2013).

Two peptides have been used to validate ULYSSIS, kalata B1 and leconotide. Kalata B1 is a native cyclotide, of 29 amino acids, found in the plant *O. affinis*, and has been shown to induce uterine contractions in mammals (Gran et al. 2000). Leconotide is a 28 amino acid knottin found in the venom of the marine cone snail *Conus catus*. leconotide has been shown to have a strong analgesic effect, comparable to a closely related peptide ziconotide (commercially known as Prialt®) but with fewer side effects (Wright et al. 2000; Smith, et al. 2002). These two peptides (kalata B1 and leconotide) have been selected to validate the ULYSSIS system.

The present study details the development of a conditional system to produce cyclic peptides. In this conditional system, cyclisation of the target peptide, is prevented in vivo and permitted in specific in vitro conditions. In this paper we describe the results generated when we converted the single molecule SICLOPPs system into a bi-molecular system, ULYSSIS (Universal Ligation bY a Secondarily Split Intein System) (Fig. 2). In this bi-molecular system, the two ULYSSIS peptides (1 and 2) are produced in separate hosts, isolated, and combined in vitro to yield a splicing competent system through peptide complementation.

Methods

Buffers Used

All buffers are made fresh from 10X stock solutions of all components. Stock solutions are replaced monthly.

EQ buffer. 300 mM NaCl, 50 mM phosphate buffer pH 7.4 (Na_2HPO_4 and NaH_2PO_4).

Imidazole wash buffer, EQ + 10 mM imidazole.

Imidazole elution buffer, EQ + 200 mM imidazole.

Maltose elution buffer, EQ + 10 mM maltose.

TCEP reaction buffer, EQ + 100 mM TCEP for dilution 1 in 10.

Lysogeny broth/L 10 g NaCl, 10 g Bacto Tryptone (BD), 5 g Bacto Yeast Extract (BD), optional 10 mM glucose as described.

Plasmid Construction

The initial leconotide containing SICLOPPs construct was synthesised by Genscript with a unique restriction digestion pattern, such that each 'component' can be replaced in a modular arrangement this plasmid is termed Ssp-Lec5-Npu (Handley et al. 2020b). The ULYSSIS variants were generated from the Ssp-Lec5-Npu hybrid SICLOPPs construct using specific primers to amplify the desired ULYSSIS variant peptides with unique restriction sites on the termini. Novel plasmids were generated using in *Escherichia coli* DH5α with the use of an alpha peptide encoding sequence cloned between specific restriction sites. When replacing the alpha peptide encoding sequence with a sequence encoding the desired peptide, blue/white colony screening methods were used. All constructs were developed in pET21a + plasmid backbone and are presented in the supplementary materials.

Expression Analysis

Expression analysis of all constructs was performed in *E. coli* BL21 (DE3) in lysogeny broth (with 20 mM glucose for MBP tagged protein expression). Cells were induced at OD_{600} 0.4 by addition of 4 mM ITPG (isopropyl β-D-1-thiogalactopyranoside) final concentration. Expression of constructs at 37 °C was carried out for 4 h, while expression at 18 °C was carried out for 24 h, before pelleting by centrifugation at 4000×*g* for 10 min, the supernatants removed and samples stored at − 20 °C.

The results of induction are analysed on SDS PAGE gels (NuPAGE) (Life Technologies). To analyse total cell

lysates, samples are resuspended in 1 mL EQ buffer to $OD_{600} = 1.7$ and sonicated using a Vibracell Sonics at 30% with 9 s pulses and 6 s rests for 2 min. Total cell lysate samples could now be taken by addition of 1 part in 4 of NuPAGE® LDS buffer (Life Technologies) before incubation at 90 °C for 10 min. For soluble cell lysate the same sonication procedure was used except following sonication the sample was centrifuged at 13,000×g for 1 min to remove insoluble compounds before addition of 1 part in 4 NuPAGE® LDS buffer. For all samples 30 µL was loaded onto the SDS PAGE gel. Gels were run 180 V in a Novex minicell tank (Life Technologies), in MES SDS Running buffer (Life Technologies) until the blue dye front ran off the gel. The gel was then removed from the casing and washed 3 times in 100 mL MilliQ water before staining over-night in SimplyBlue™ SafeStain (Life Technologies).

Purification Methods

All protein purifications were performed as batch purifications. Cells were induced to express the desired construct and resuspended in EQ buffer to obtain $OD_{600} = 1.7$, before sonication using a Vibracell Sonics® at 30% intensity with 9 s pulses and 6 s rests, for 4 min. The insoluble contents are removed by centrifugation at 5000×g. The clarified supernatant was then incubated with equilibrated resin, Amylose resin (New England Biolabs) – for ULYSSIS peptide 1, or Co^{2+} resin (TALON (Clontech)) – for ULYSSIS peptide 2. For both isolation procedures 400 µL resin was first equilibrated by washing first in 1 mL of Milli-Q water, before two washes in 1 mL of EQ buffer. Between washes the resin was separated from the buffer by centrifugation at 2000×g for 2 min.

For Amylose resin purification of maltose binding protein (MBP) tagged peptides, the soluble cell lysate was incubated with the resin overnight at 4 °C before the resin was pelleted at 2000×g for 2 min and the supernatant, containing the unbound fraction, removed. The amylose resin was then washed twice with 1 mL EQ buffer before four rounds of elution with 250 µL EQ + 10 mM maltose.

For Co^{2+} Resin purification of His tagged peptides, the soluble cell lysate was incubated with the resin for 4 h at 4 °C before the resin is pelleted at 2000×g for 2 min and the supernatant, containing the unbound fraction, removed. The Co^{2+} Resin is then washed twice with 1 mL of EQ + 10 mM imidazole before four rounds of elution with 250 µL EQ + 200 mM Imidazole. The elution fractions can be concentrated using an Amicon Ultra-15 (Sigma-Aldrich) Centrifugal filter with 3 kDa cut off.

General ULYSSIS Reaction Protocol

In brief the general conditions to facilitate cyclisation of a target peptide by ULYSSIS are as follows. The ULYSSIS peptides (1 and 2) are first purified as described above, and co-incubated at ~1:~1 ratio as determined by measuring the A_{280} of samples and determining the peptide concentration with the extension coefficient at the indicated temperature for up to 24 h in the presence of 10 mM TCEP. No noticeable difference was found with incubation in the elution buffers or if the peptides were exchanged to EQ buffer. Splicing was detected by analysing time course samples by SDS-PAGE gel electrophoresis to observe generation of the post-splicing ULYSSIS peptide 2.

Mass Spectrometry Analysis

In the present study two mass spectrometric techniques were used, mass measurement of the intact molecules and after hydrolytic cleavage. The same workflow is used for both techniques. matrix assisted laser desorption/ionisation tandem time-of-flight mass spectrometry (MALDI-TOF/TOF–MS) was used for intact mass measurement of cyclic Leconotide and Kalata B1 and linear Kalata B1 from liquid samples. The liquid sample was mixed 1:10 with matrix (Saturated solution of α-cyano-4-hydroxy-cinnamic acid in ACN containing 0.1% trifluoroacetic acid) and spotted in 0.8 µL on the target plate. All samples were measured with a 4800 MALDI TOF/TOF analyser (AB SCIEX) in positive linear ion mode. In a mass range of 1000–30,000 m/z.

For tryptic digestion liquid samples were treated with 500 ng of trypsin and incubated over night at 37 °C. the samples were dried in a centrifugal vacuum concentrator and reconstituted in 5% ACN, 0.2% FA in water before analysis with MALDI TOF/TOF as described above.

Interpretation of mass spectra was performed using the Xcalibur software (Thermo Fisher Scientific).

Results

In order to generate a bi-molecular ULYSSIS, a second 'split' must be introduced into the SICLOPPs construct (where the first split is the native split which separates the N and C intein) (Fig. 3). To identify a suitable position for the second split to be introduced, we explored conservation across > 100 split intein sequences (Fig. 3A). The sequences were aligned using MUSCLE (Edgar 2004) within Geneious® (Geneious Prime 2021.2.2) and the conserved regions manually mapped onto the solved structure of the DnaE split intein of *N. punctiforme* (PDB 4QFQ, Fig. 3B). The conservation analysis revealed that loop 1 of the N-intein contains a conserved glutamic acid
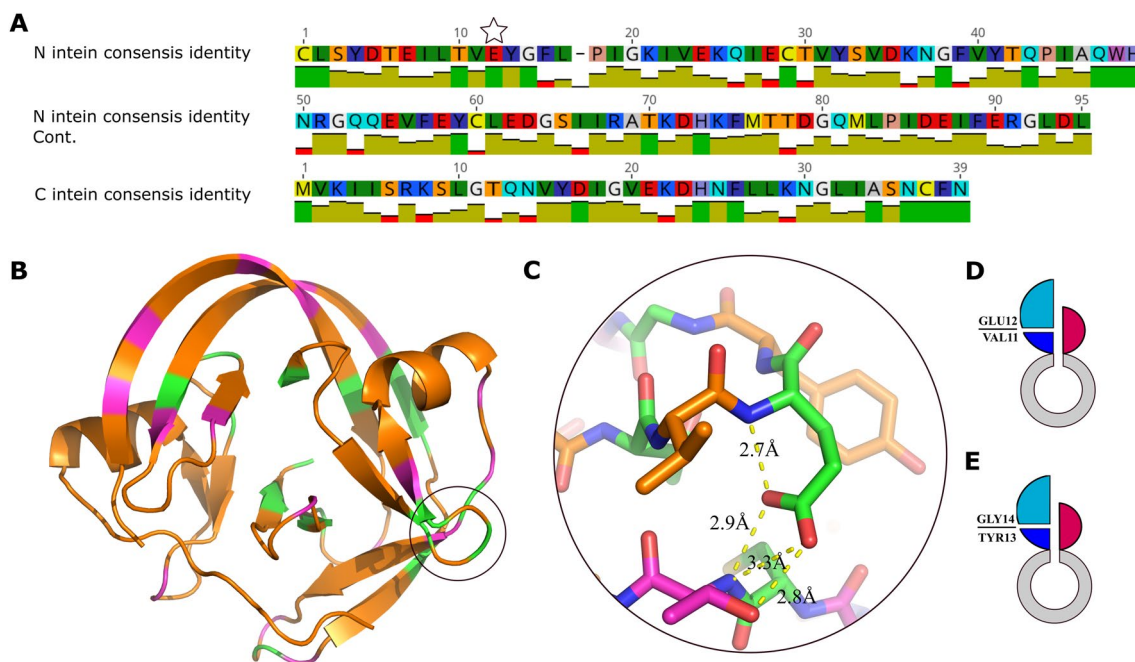
Fig. 3 Conservation analysis of the DnaE split inteins, with location of splits introduced to generate the ULYSSIS systems. **A** conservation across the N and C intein with consensus identity, Green represents 100% conservation across all sequences analysed. The star indicates the position of the conserved Glu residue in position 12. **B** conservation data mapped to the *N. punctiform* DnaE split intein, with con-servation coloured as in A. **C** close view of loop 1 conserved Glu interacting with position 29, with polar interactions mapped. **D** diagrammatic representation of the split introduced to generate ULYS-SIS A, with the split between Val11 and Glu12. E- diagrammatic representation of the split introduced to generate ULYSSIS B, with the split between Tyr13 and Gly14. PDB: 4QFQ

(Glu12) residue, which interacts with the peptide back-bone nitrogen and sidechain at position 29 (Thr 29 in the structure) (Fig. 3C). We hypothesised that this residue may be important for stabilising the reactive core of the split intein, due to its proximity to the active site in sequence and structure. As such, we identified loop 1 as a suitable point for introducing the second split site. We generated variants with splits at position between Val 11 and Glu 12 or between Tyr 13 and Gly 14 each carrying a optimised permutation of leconotide (Fig. 3) (Handley et al. 2020b).

The ULYSSIS constructs, ULYSSIS peptide 1 (consisting of the C-intein, peptide of interest, and fragment of the N-intein) and ULYSSIS peptide 2(consisting of the remainder of the N-intein) (Fig. 2), were then evaluated for their ability to be expressed in *E. coli* BL21 (Supplementary Fig. 1). It was determined that ULYSSIS peptide 1 requires a maltose binding protein solubility tag to enable soluble expression of the construct (Supplementary Fig. 1). ULYSSIS peptide 2 expressed efficiently with only an additional His tag in *E. coli* BL21. The ULYSSIS fragments were isolated, ULYSSIS peptide 1 was isolated using the maltose binding protein tag and amylose resin while ULYSSIS peptide 2 was isolated using a hexa-histidine tag and Co^{2+} resin (Supplementary Fig. 2).

Following purification, the ULYSSIS peptides are co-incubated to determine their capacity to carry out splicing/ peptide cyclisation. It was determined that ULYSSIS B is capable of splicing, with optimal splicing at 18–22 °C, while ULYSSIS A is unable to splice. Splicing is detected by generation of a band corresponding to the post splicing product (Lacking peptide of interest—Supplementary Fig. 3). The products of ULYSSIS system B were analysed by intact mass spectrometry which confirmed cyclic reduced leconotide was generated, expected mass of cyclic product is 3287.45, whereas the expected mass of the linear product is 3305.46 (Fig. 4); additionally tryptic digest MS was carried out on excised bands to confirm the identity of the bands observed (Supplementary Fig. 3).

The capacity of ULYSSIS to generate cyclic peptides was explored with a second peptide of interest, kalata B1. Previously we have carried out permutation analysis of kalata B1 to identify a suitable splicing site (Handley et al. 2020b). The validated kalata B1 construct was adapted to ULYSSIS B. ULYSSIS-kalata B1 construct generated bands similar to those observed previously for the ULYSSIS-leconotide construct (Fig. 5). We moved to validation of cyclic kalata
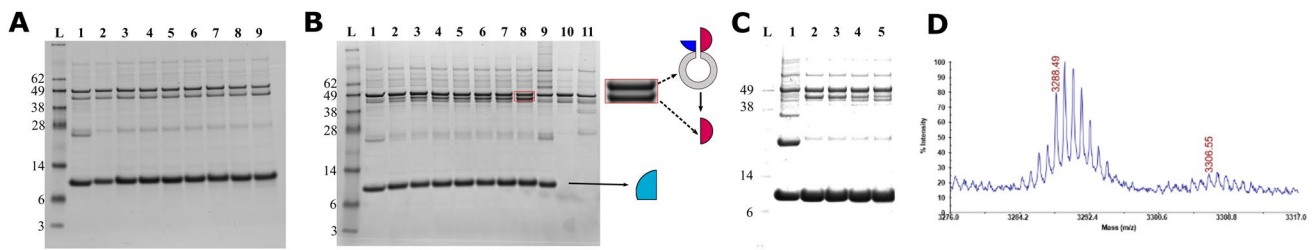
Fig. 4 ULYSSIS activity and product analysis with leconotide as the peptide of interest. **A** time course analysis of ULYSSIS A with no splicing detected at 22 °C. **B** time course analysis of ULYSSIS B with splicing detected at 22 °C. Lane identities A/B: L—Seeblue plus 2 molecular weight ladder with band sizes indicated. 1- 9 co incubations of ULYSSIS peptide 1 and 2 of the ULYSSIS system indicated, 1- T0, 2- T1H with 10 mM TCEP, 3- T2H with 10 mM TCEP, 4- T3H with 10 mM TCEP, 5- T4H with 10 mM TCEP, 6-T5H with 10 mM TCEP, 7- T6H with 10 mM TCEP, 8-T24H with 10 mM TCEP, 9- T24H without TCEP. In B lane 10 contains T24H with

TCEP without ULYSSIS peptide 2, lane 11 contains T24H without TCEP and without ULYSSIS peptide 2. **C** Temperature range exploration of splicing in ULYSSIS B: L—Seeblue plus 2 molecular weight ladder with band sizes indicated, 1- T0, 2- T24H at 18 °C, 3- T24H at 22 °C, 4- T24H at 28 °C, 5- T24H at 37 °C. **D** MALDI analysis of the products from panel B lane 8, expected mass of cyclic product was 3287.45 thus the expected $[M/Z]^+$ is 3288.45 (Observed 3288.49), while the expected mass of linear product was 3305.46 giving an expected $[M/Z]^+$ of 3306.46 (Observed 3306.55); showing presence of the cyclic product

B1 by intact mass spectrometry (Fig. 5B, C). The intact mass analysis revealed that cyclic kalata B1 was generated with very little linear biproduct. kalata B1 contains a single trypsin cleavage site which enables further confirmation that kalata B1 generated from ULYSSIS is cyclic. If the kalata B1 generated is cyclic, trypsin cleavage will linearize the peptide by hydrolysis of one peptide bond, increasing the mass by 18 Da. This predicted change in mass is observed (Fig. 5D). If the peptide identified was linear and contained a trypsin cleavage site, fragmentation would occur. A trypsin cleavage analysis was not performed for leconotide as it contains multiple trypsin cleavage sites.
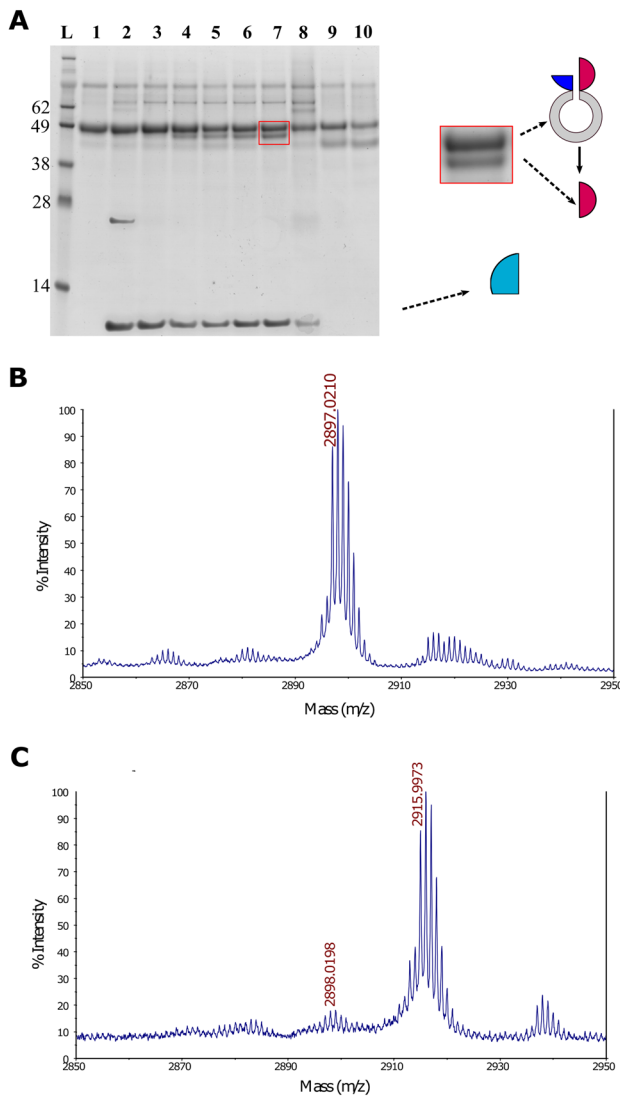
Discussion

The present study describes the development and evaluation of a biosynthetic system, ULYSSIS, which can generate cyclic peptides such as kalata B1 in a conditional manner in vitro, following simple purification approaches from soluble cell lysates. Cyclic peptides are appealing therapeutic agents due to their innate stability. We have also demonstrated that ULYSSIS is able to generate a cyclic version of leconotide, a natively linear knottin. When the system is applied to specific peptides for pharmaceutical applications it is essential to explore the 3-dimensional structure of the peptide to ensure the correct folding state and disulfide connectivity, as well as confirming the desired activity is retained.

ULYSSIS is unique in its mechanism of control, but it is not the only controlled split intein system (Ventura and Mootz 2019). Many of the controlled split inteins which could permit conditional generation of cyclic peptides require the inclusion of non-native amino acids which are supplemented into the media and subsequently incorporated

to peptides/proteins using stop codon replacement (Böcker et al. 2015; Wong et al. 2015; Tyszkiewicz and Muir 2008; Vila-Perelló et al. 2008; Berrade et al. 2010; Binschik et al. 2011). Additionally, significant work has gone into identifying conditional split inteins, such as the thermally (Choi et al. 2006) and salinity (Ciragan et al. 2020) controlled split inteins—though not applied to peptide cyclization, in theory they could but the risk of damage to the peptide. Given the additional cost of generating light inducible split inteins, or the risk of high temperature or high salinity on the peptide produced, ULYSSIS presents an appealing option for controlled peptide cylisation Comparative analysis of the structure and the conservation analysis with the splicing data for the ULYSSIS variants confirms the importance of the Glu12-Thr29 interaction (Figs. 3, 4). In ULYSSIS A the interaction between Glu12 and Thr29 is an intramolecular interaction, where both residues are found in ULYSSIS peptide 2. In ULYSSIS B Glu12 is found in ULYSSIS peptide 1 and Thr29 is found in ULYSSIS peptide 2, making their interaction intermolecular. The hypothesis is that the intermolecular interaction of Glu12 and Thr29 is required for assembly of the splice competent ULYSSIS complex (as in ULYSSIS B), as when this interaction is intramolecular (ULYSSIS A); splicing isn't carried out.

In the present study, TCEP is required to induce peptide splicing. The requirement for this is twofold. First, the active site cysteine must exist as a free thiol or thiolate to act as a nucleophile in the splicing reaction. Second, if the cystine knot is formed, the split intein active site is prohibited from assembling into the active conformation. The requirement of TCEP gives additional control over the ULYSSIS system, which is dependent on the peptide being cyclised being cysteine rich. However, the primary control given by the bi-molecular nature of the system is omnipotent. ULYSSIS system is controlled regardless of the

complementation approach employed in ULYSSIS is less harmful to the peptide generated than potential alternatives such as thermal regulation.

Conclusion

ULYSSIS has been shown to generate cyclic peptides from both natively cyclic and linear targets. ULYSSIS may be used for a wide range of targets which have significant potential in cyclic peptide production for pharmaceutical applications. SICLOPPs based systems have been used extensively to generate bio-active cyclic peptides (Clark et al. 2005), and the same is a reasonable expectation of peptides generated by the ULYSSIS system. However, analysis of each specific target peptide generated using ULYSSIS will be required to develop novel bioactive peptides for pharmaceutical application.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Fig. 5 ULYSSIS system B with kalata B1. **A** Time course analysis of ULYSSIS kalata B1. Lane descriptions as follows: L- Seeblue plus 2 molecular weight ladder with band sizes indicated. 1- T0 ULYSSIS peptide 1 with no TCEP, 2–8 contain both ULYSSIS peptide 1 and 2; 2-T0, 3- T1H with 10 mM TCEP, 4- T2H with 10 mM TCEP, 5-T6H with 10 mM TCEP, 6- T12H with 10 mM TCEP, 7- T24H with 10 mM TCEP, 8- T24H without 10 mM TCEP, 9- ULYSSIS peptide 1 only with 10 mM TCEP 24H sample, 10- ULYSSIS peptide 1 only without 10 mM TCEP 24H sample. **B** MALDI analysis of the products from lane 7 panel A, expected mass of cyclic reduced product is 2896.19 Da thus the expected $[M/Z]^+$ is 2897.19 (Observed 2387.02), the linear product is expected to be 2914.20 Da giving an expected $[M/Z]^+$ of 2915.20 (Observed 2915.997). **C** MALDI analysis of the products from lane 7 panel A after tryptic digest, expected mass of cyclic product is 2896.19 Da thus the expected $[M/Z]^+$ is 2897.19, the linear product is expected to be 2914.20 Da giving an expected $[M/Z]^+$ of 2915.20; showing conversion of cyclic product to linear product, confirming that the original peptide was in fact cyclic

peptide being cyclised—which bodes well for its capacity to be used on a wide range of target peptides, as does the fact that cyclisation is progressing at 18–22 °C. The peptide

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