Bioproduction of Cyclic Disulfide-Rich Peptides for Drug Modalities

Kuok Yap, Conan K. Wang, David J. Craik, and Linda H. L. Lua

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Abstract Cyclic disulfide-rich peptides have extensive applications in drug development. These peptides are widely produced using solid-phase peptide synthesis which generates substantial amounts of toxic and hazardous waste. Recombinant bioproduction platforms for cyclic disulfide-rich peptides offer a more environmentally sustainable alternative. This chapter highlights a recently established microbial-based bioproduction platform that utilizes both *Pichia pastoris* and *Escherichia coli* for the production of cyclic disulfide-rich peptides.

K. Yap
Protein Expression Facility, The University of Queensland, Brisbane, Australia

Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, Australia

Institute for Molecular Bioscience, Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Queensland, Brisbane, Australia

C. K. Wang · D. J. Craik
Institute for Molecular Bioscience, Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, The University of Queensland, Brisbane, Australia

L. H. L. Lua (✉)
Protein Expression Facility, The University of Queensland, Brisbane, Australia
e-mail: l.lua@uq.edu.au

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1 Introduction

Cyclic disulfide-rich peptides (CDRPs) have exceptional thermal stability and are resistant to proteolytic degradation because of their unique structures that comprise a cyclic backbone cross-linked by the presence of one or more disulfide bonds (Colgrave and Craik 2004; Colgrave et al. 2010; Cheneval et al. 2014). They occupy a niche in the pharmaceutical market by combining the advantages of small-molecule and protein-based drugs. Being larger than small-molecule drugs, CDRPs display high target selectivity, which minimizes adverse side effects (Craik et al. 2013) while being smaller than protein-based biologics, CDRPs have a better chance of achieving oral bioavailability than biologics (Wong et al. 2012). Figure 1 highlights examples of CDRPs found in plants and animals or others that were synthetically engineered cyclic (Craik et al. 1999). One of the smallest CDRPs is the sunflower trypsin inhibitor (SFTI-1) with one disulfide-bond (Fig. 1a). Larger CDRPs include a naturally linear peptide with two disulfide bonds from the cone snail *Conus victoriae* that was engineered to be cyclic called cVc1.1 (Fig. 1b), an antimicrobial peptide with three disulfide bonds called Rhesus theta defensin-1 (RTD-1) (Fig. 1c) and a cyclotide with three disulfide bonds found in the Vietnamese Gấc plant called *Momordica cochinchinensis* trypsin-inhibitor II (MCoTI-II) (Fig. 1d).

With the prospect of CDRPs being approved for use in the clinic in the coming years, addressing the large-scale production challenges is becoming important. Currently, most CDRPs are produced synthetically using solid-phase peptide synthesis (SPPS). This process generates substantial waste and is not ideal for industrial-scale production (Cheneval et al. 2014; Merrifield 1963). Recombinant-based bioproduction methods are environmentally sustainable alternatives and are actively

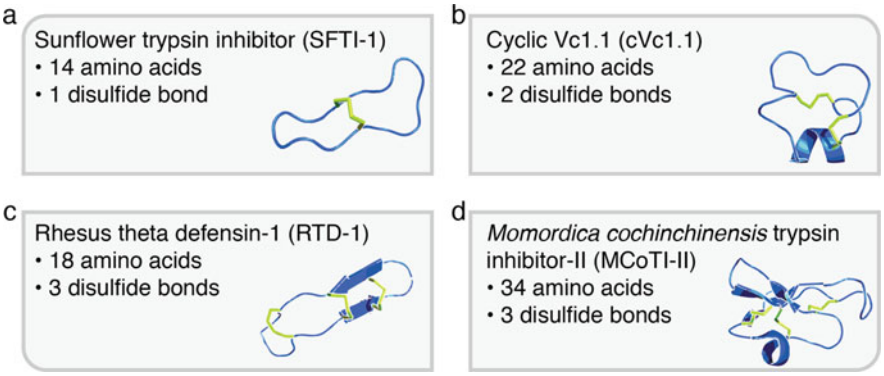

Fig. 1 Examples of CDRPs: (**a**) SFTI-1, (**b**) cVc1.1 (**c**) RTD-1, and (**d**) MCoTI-II are shown in order of increasing number of disulfide bonds. Each panel shows the amino acid length and disulfide content of the respective cyclic peptide. Their 3D structures are illustrated in blue with disulfide bonds colored in yellow. (PDB ID for SFTI-1 is 1JBL; cVc1.1 is 4TTL; RTD-1 is 1HVZ; and MCoTI-II is1HA9)

being considered for CDRP biomanufacturing. Of these, microbial systems are favored as the expression hosts due to their fast growth rates, relatively inexpensive media, and simple genetic manipulation (Tripathi and Shrivastava 2019). These advantages were evident when insulin, the first microbially produced peptide drug, was approved for market in 1982 (Johnson 1983). In recent years, plant expression systems have emerged as a viable option for producing recombinant therapeutics because they can properly fold complex proteins (Dirisala et al. 2017; Yao et al. 2015). After Elelyso, the first plant-derived pharmaceutical drug to be approved by the US Food and Drug Administration (FDA) in 2012 for the treatment of Gaucher's disease (Fox 2012) substantial research efforts have demonstrated using plants as bio-factories for the bioproduction of antibodies (Arntzen 2015; Bally et al. 2018), vaccines (Streatfield and Howard 2003), complex carbohydrates (Roberts et al. 2018), as well as, CDRPs (Jackson et al. 2019; Poon et al. 2018). With a range of bioproduction platforms available, the challenge is determining which one is most suitable for CDRP drug manufacturing. This chapter explores recent progress examining microbial and plant-based bioproduction systems utilized to generate CDRPs and provides an outlook for the future production of CDRPs based on the knowledge acquired.

2 Potential High-Value Applications of CDRPs

CDRPs are valued for pharmaceutical applications as molecular scaffolds because of their exceptional stability due to their ability to withstand heat (Sable et al. 2016), harsh acid conditions (Wang et al. 2014), and proteolysis from a wide range of proteases (Wang et al. 2014; Chan et al. 2011). A desired biological function can be introduced by inserting bioactive amino acid sequences onto these extremely stable CDRP scaffolds, a concept known as molecular grafting. Grafted peptides gain non-native biological activities while retaining the inherent stability attributes of native CDRP scaffolds. Numerous studies have engineered CDRPs as scaffolds for various medical applications, with examples shown in Table 1. The most promising candidate based on a point mutation of a prototypical cyclotide, kalata B1 (kB1), is scheduled for clinical trials for the treatment of multiple sclerosis (Gründemann et al. 2019). Based on the evidence described briefly in this section, it is clear that CDRPs have attractive pharmaceutical properties with immense potential to be developed into high-value drug modalities.

Table 1 Medical applications of selected CDRPs

Scaffold	Activity	High-value application	References
SFTI-1	Anti-angiogenesis	Inhibiting tumor progression	Chan et al. (2015)
	Chymase inhibitor	Cancer	Li et al. (2019)
	Angiogenic	Cardiovascular and wound healing	Chan et al. (2011)
	Tau aggregation inhibitor	Alzheimer's disease	Wang et al. (2016)
cVc1.1	Cyclization	Neuropathic pain	Clark et al. (2010)
kB1	Immunomodulation	Multiple sclerosis	Wang et al. (2016)
	Melanocortin receptor 4 agonist	Obesity	Eliasen et al. (2012)
	Lymphocyte proliferation inhibitor	Multiple sclerosis	Thell et al. (2016)
MCoTI	Factor XIIa inhibitor	Cardiovascular disease	Swedberg et al. (2016)
	Cytokine receptor CXCR4 antagonist	Anti-HIV	Aboye et al. (2012)
	Matriptase inhibitor	Anti-tumor	Quimbar et al. (2013)
	Tyrosine kinase inhibitor	Chronic myeloid leukemia	Huang et al. (2015)

3 Production of CDRPs

3.1 Synthetic Peptide Synthesis

CDRPs are commonly produced using SPPS (Merrifield 1963). The process requires multiple processing steps after assembly of a side-chain protected peptide chain on solid resin support. A typical workflow (Cheneval et al. 2014) is illustrated in Fig. 2. After assembly, the peptide chain is cleaved from the resin by mild acid cleavage to expose the C-terminus (Fig. 2a) for intramolecular cyclization (Fig. 2b). The side-chain protected cyclic peptide (Fig. 2c) is then treated with strong acid to remove the remaining protecting groups, liberating the sulfhydryl groups of Cys residues for disulfide bond formation under oxidative folding conditions (Fig. 2d). Finally, the cyclic oxidized peptide (Fig. 2e) is purified using reverse-phase high-pressure liquid chromatography.

According to Green Chemistry metrics, SPPS-based production of CDRPs is not an environmentally friendly process (Jad et al. 2019). For every kilogram of peptide produced on a commercial scale, it generates hazardous waste in the multi-ton range (Ritter 2017). The types of hazardous waste produced during the assembly and handling of CDRPs include dichloromethane (DCM), *N,N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and piperidine (Isidro-Llobet et al. 2019) (Fig. 2). The production of disulfide-rich peptides requires the disposal of large volumes of folding waste because the folding reactions are carried out at low concentrations to prevent oligomerization (Cheneval et al. 2014). With increasing environmental awareness amongst government, industry, and the

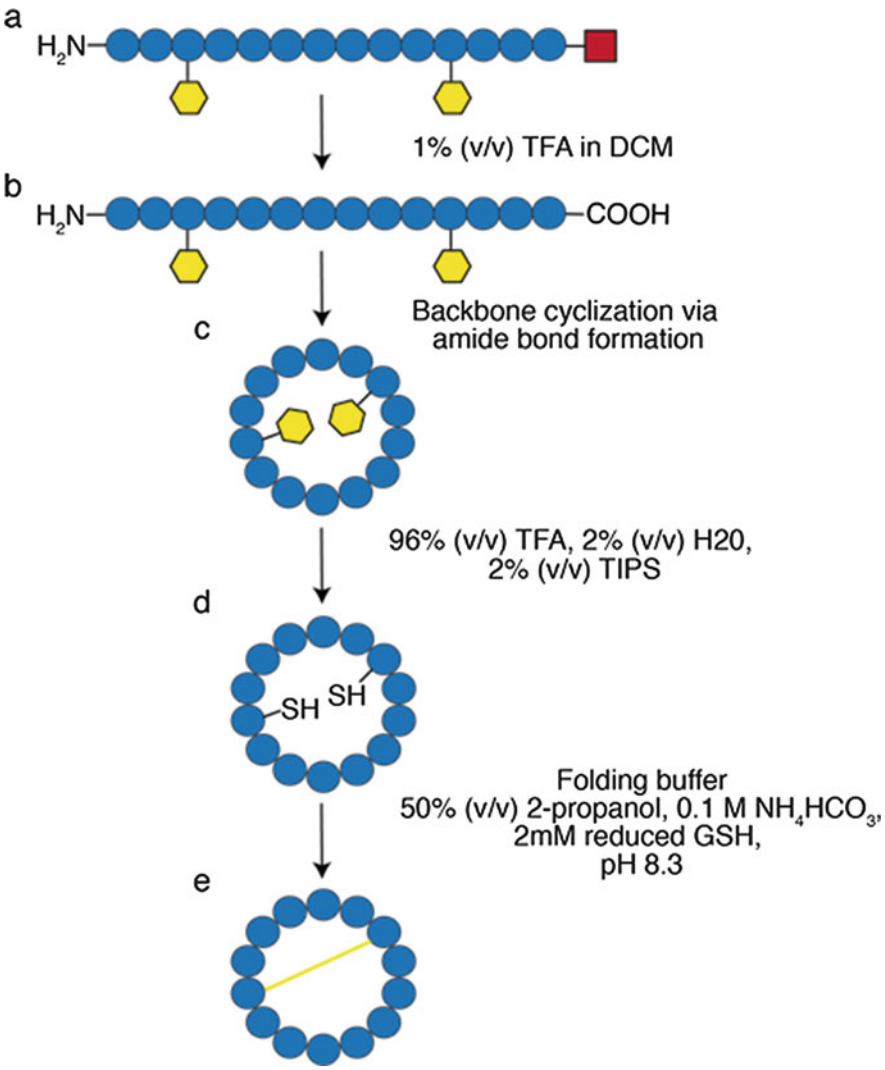

Fig. 2 Schematic workflow of a chemical production process for CDRPs, which includes peptide-chain synthesis, cyclization, and folding (Cheneval et al. 2014). (**a**) Linear side-chain (yellow hexagon) and C-terminal (red square) protected peptide chain (blue circles). The entire sequence is side-chained protected, but only Cys is shown for simplicity. (**b**) Termini-free linear side-chain protected peptide precursor after mild acid hydrolysis of C-terminal protecting group. (**c**) Cyclic side-chain protected peptide due to backbone cyclization via amide bond formation. (**d**) Cyclic reduced peptide after harsh acid liberation of side-chain protecting groups. (**e**) Oxidized CDRP after the formation of the disulfide bonds. A folding buffer containing an organic solvent that is used for the oxidation of kB1 is shown as an example

public, there is a need for more sustainable and environmentally friendly alternatives.

3.2 Recombinant Bioproduction of CDRPs

Recombinant technologies offer a pathway toward the sustainable production of CDRPs. The bioproduction of CDRPs has been explored using microbial and plant-based expression systems. Backbone cyclization is easily achieved in chemical synthesis (Cheneval et al. 2014) but the main challenge in bioproduction methods for CDRPs comes from the need for endogenous machinery for post-translational cyclization. We highlight two strategies that have been used to overcome this cyclization bottleneck. The first is an in-vivo intein-mediated cyclization approach and the second an in-vitro or in-vivo asparaginyl endopeptidases (AEPs)-mediated cyclization approach.

3.2.1 Cyclization Strategies in Bioproduction of CDRPs

The intein-mediated backbone cyclization approach, also known as expressed protein ligation (EPL), utilizes an intramolecular ligation strategy based on native chemical ligation (NCL). NCL is a chemoselective ligation reaction carried out under aqueous conditions to ligate two unprotected peptides, one containing an N-terminal cysteine and another containing a C-terminal α-thioester group (Dawson et al. 1994). The chemical ligation can be carried out intramolecularly by incorporating both reactive groups on the same peptide chain, which results in backbone cyclization as illustrated in Fig. 3a. The first step requires the removal of the N-terminal methionine to generate the α-Cys on the N-terminus of the peptide. This reaction can be performed in-vivo by endogenous host proteases such as methionyl aminopeptidases (Camarero et al. 2001). The α-thioesters are generated by fusing modified inteins onto the C-terminus of linear peptides or proteins (Camarero and Muir 1999). Hence, the presence of an N-terminus α-Cys and C-terminus α-thioesters on a linear peptide facilitates backbone cyclization.

The second approach relies on a class of protein ligases called AEPs. These enzymes have provided biochemists with exciting new tools to explore a range of applications based on amide-bond formation, including backbone cyclization (Nguyen et al. 2014; Rehm et al. 2019; Rehm et al. 2020). Most AEPs in higher organisms function as hydrolases, but a handful of AEPs from plants catalyzes amide-bond formation, a function crucial for the cyclization of plant-derived cyclic precursors (CPs) (Du et al. 2020; James et al. 2018). For intramolecular cyclization, a simple tripeptide sequence, Asx-Xaa-Yaa (P1-P1'-P2'), at the C-terminal is required, where Asx is an Asn or Asp at P1 followed by any small amino acid at P1' and an aliphatic or hydrophobic amino acid at P2'. Processing occurs between P1 and P1' as illustrated in Fig. 4. A transpeptidation reaction occurs after

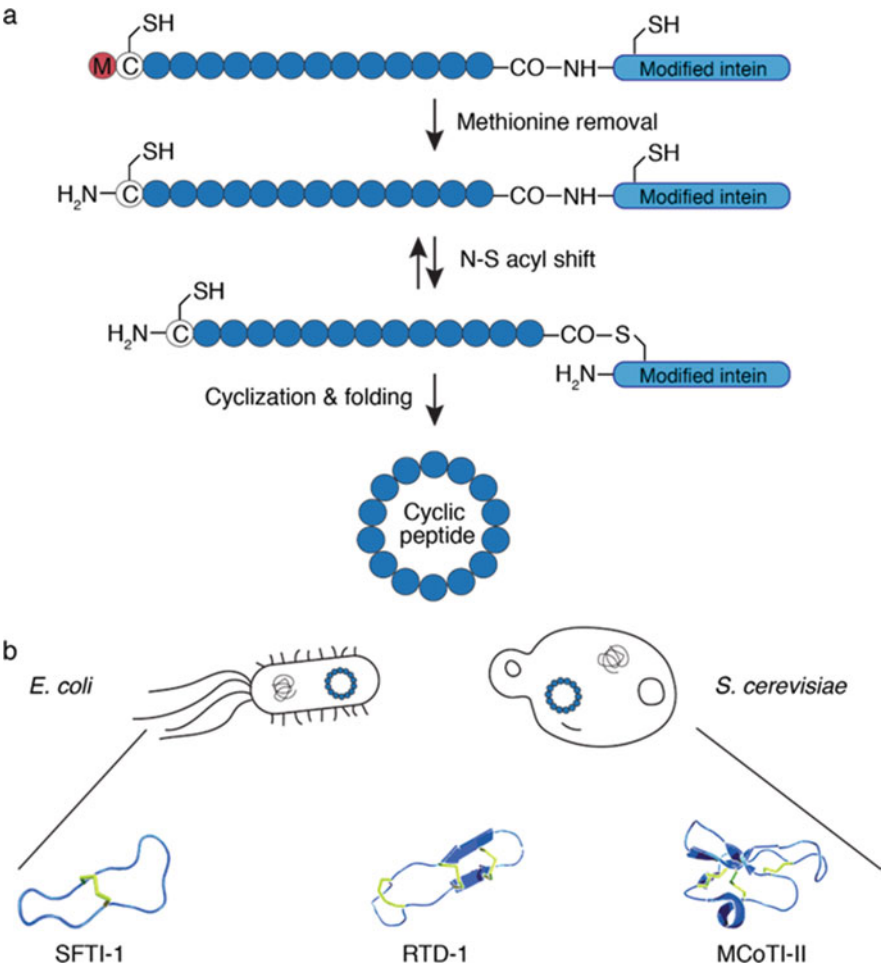

Fig. 3 Overview of the bioproduction of cyclic disulfide-rich peptides in *E. coli* and *S. cerevisiae* using the EPL strategy. (**a**) The in-vivo multiple-step intein-based backbone cyclization of a linear peptide. (**b**) *E. coli* or *S. cerevisiae* have both been used to recombinantly produce SFTI-1, RTD-1, and MCoTI-based peptides using the EPL approach

processing, whereby an incoming nucleophile sequence of P1"-P2" is accepted, forming the P1-P1" amide-bond. All proteinogenic amino acids except for Pro are tolerated at the P1" position, and Cys, Ile, Leu and Val are preferred at the P2" position (Nguyen et al. 2016). The combination of residues at the positions described paired with respective AEPs used for recombinant production of cyclic disulfide-rich peptides is illustrated in Fig. 4 (Poon et al. 2018; Yap et al. 2020). Ligation or cyclization with AEPs can be carried out with minimal 'scaring' of the native sequence, leaving only the Asx residue, compared to other protein ligases, which require longer, more rigid recognition sequences that are not amenable to change.

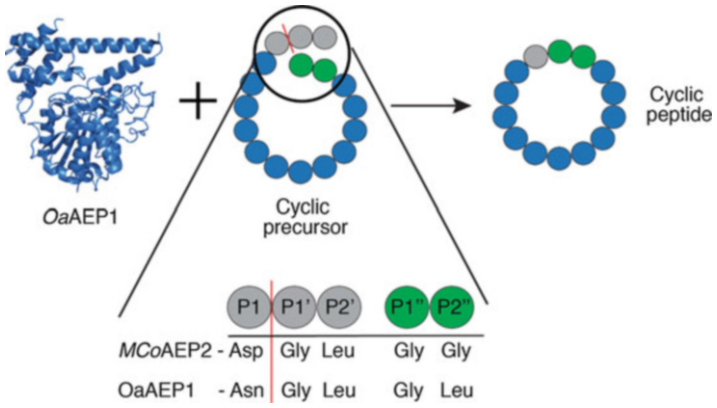

Fig. 4 AEP-mediated cyclization of CPs and key residues for intramolecular cyclization. A couple of examples showing the residues used for recombinant production. The red line indicates the AEP processing position between P1 and P1'

3.2.2 Recombinant Systems for Bioproduction of CDRPs

3.2.2.1 Intein-Mediated Microbial Bioproduction

Early efforts in developing microbial systems for producing CDRPs relied on the in-cell EPL backbone cyclization approach in *E. coli,* as illustrated in Fig. 3a (Gould et al. 2012; Li et al. 2016). MCoTI-II was the first correctly folded cyclotide produced recombinantly and structurally validated by 1D and 2D ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy (Camarero et al. 2007). The ability to produce correctly folded cyclotide, MCoTI-II, in its native fold was a major accomplishment as a previous attempt at producing another cyclotide, kB1, resulted in misfolded peptides (Kimura et al. 2006). In subsequent studies, SFTI-1 (Li et al. 2016) and RTD-1 (Gould et al. 2012) were produced in *E. coli*, and MCoTI-I, a paralog of MCoTI-II, was produced in *Saccharomyces cerevisiae* using the same EPL backbone cyclization approach (Jagadish et al. 2015). These studies were trailblazers for the recombinant production of CDRPs by producing a range of structurally distinct CDRPs as shown in Fig. 3b. However, the primary aim of these studies was to develop cell-based cyclic peptide libraries for rapid screening of biological activity. There are several limitations associated with these studies from a commercial bioproduction perspective, including the need for a reduced cysteine at the N-terminus and the reported yields. The EPL approach is limited to peptides that have reduced cysteine on the N-terminus. The reported yields of 10–180 μg L^{-1} culture is not commercially viable because the yields widely achieved for industrial-scale production are in the g L^{-1} scale (Spohner et al. 2015).

3.2.2.2 AEP-Mediated Plant Bioproduction

Plant-based bioproduction is considered for the production of CDRPs because a large number of CDRPs such as kB1 and MCoTI-II originate from plants (Gran 1970). The first study to demonstrate plant-based CDRP production used a model experimental plant, *Arabidopsis thaliana* to stably produce SFTI-1 (Mylne et al. 2011). *A. thaliana* is not an ideal candidate for commercial bioproduction due to its small stature. *Nicotiana benthamiana* is more suited for industrial-scale production because it has been used to produce a range of potential therapeutic candidates (Schillberg and Finnern 2021). A grafted-SFTI-1 and kB1 cyclic peptides were both produced in the leaves of *N. benthamiana* when co-expressed with an AEP (Jackson et al. 2019; Poon et al. 2018). These plant-derived CDRPs were validated to have the same monoisotopic mass and were structurally equivalent to their native counterparts using high-resolution mass spectrometry (HRMS) and NMR-based structural characterization, respectively. Although promising, there are several limitations to using plants as bio-factories for the large-scale manufacturing of CDRPs. The highest yield reported for a plant-produced cyclic peptide is 199 μg g^{-1} dry weight (Poon et al. 2018) which is significantly lower compared to other CDRP production platforms (Yap et al. 2020; Yap et al. 2021). The downstream purification issues related to plant-based production include the tedious harvesting of transformed tissues while sieving away non-transformed tissues, multiple organic solvent extractions, and the complex purification of CDRPs from crude plant lysate inundated with endogenous plant proteins (Jackson et al. 2019; Poon et al. 2018).

3.2.2.3 AEP-Mediated *P. pastoris*-Based Bioproduction

P. pastoris has all the desirable attributes as an expression host and has been awarded GRAS (generally recognized as safe) status by the FDA. Additionally, *P. pastoris* is a proven expression host in the space of pharmaceutical manufacturing with numerous high-value drugs manufactured by this platform, including Kalbitor®, a kallikrein inhibitor (Walsh 2010; Ciofalo et al. 2006) and Jetrea®, for the treatment of vitreomacular adhesion (Mullard 2013). With advances in synthetic biology and engineering, *P. pastoris*-derived drugs with human-like glycosylation patterns to lower host immunogenicity are now achievable (Choi et al. 2003; Laukens et al. 2015). Another strong attribute of *P. pastoris* is its fast growth rate in relatively inexpensive media compared to other eukaryotic expression systems. The ability of *P. pastoris* to secreted recombinant proteins into the growth media at yields of up to 22 g L^{-1} is a major advantage from a downstream processing perspective because it mitigates the need for complex cell lysis and purification from crude lysate (Katrolia et al. 2011).

The limitations of both the EPL-mediated CDRP production in microbial systems and CDRP production in plants are the complex downstream purification requirements and the low CDRP yield (Jackson et al. 2019; Poon et al. 2018; Gould et al. 2012). A recent study selected *P. pastoris* to produce CPs because of all the

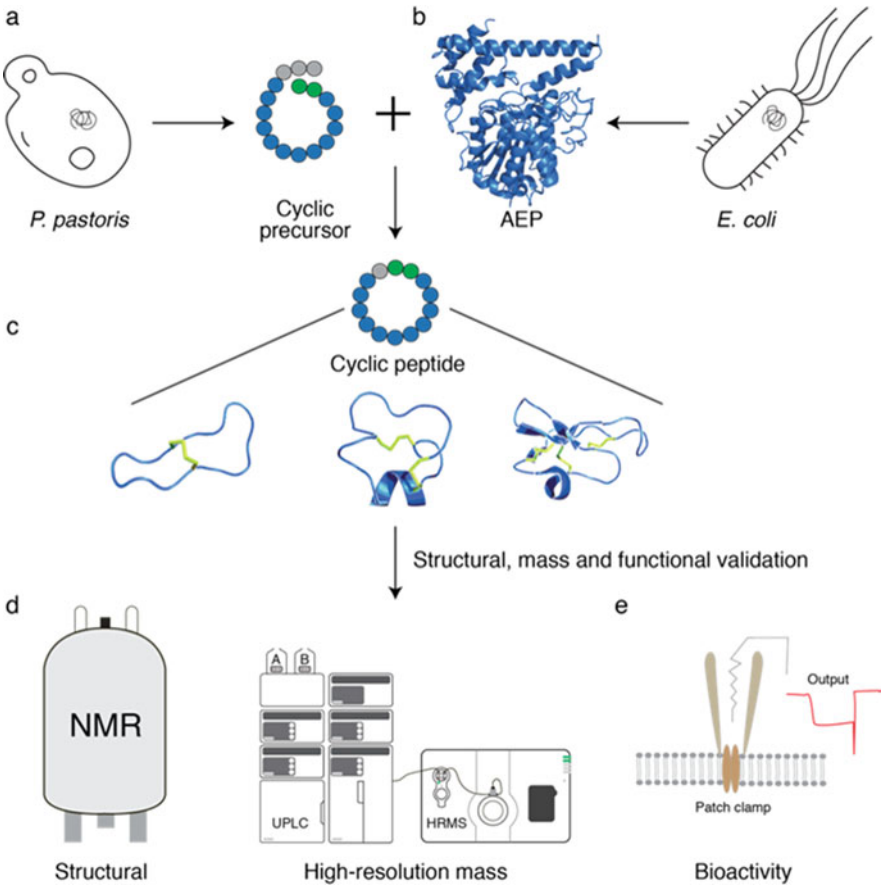

Fig. 5 Diagrammatic representation of the *P. pastoris*-based bioproduction platform for CDRPs. (**a**) *P. pastoris*-secreted CPs and (**b**) recombinant AEPs produced in *E. coli*. (**c**) The bioproduction of three structurally distinct CDRPs as a result of in-vitro cyclization of *P. pastoris*-derived CPs by *E. coli*-derived AEPs. (**d**) Structural, mass, and (**e**) bioactivity validation of recombinant CDRPs

attributes described (Yap et al. 2020). The study demonstrated the potential of a *P. pastoris*-based CDRP production platform to overcome both yield and downstream processing limitations. CPs were secreted into the growth media by attaching an α-mating factor signal peptide onto the N-terminus of CPs (Fig. 5a), bypassing the need for complex downstream processing. The secreted CPs achieved yield improvements of 2–3 orders of magnitude compared to previous recombinant attempts (Yap et al. 2020; Yap et al. 2021). Three CPs have been successfully secreted into growth media: a kallikrein-related protease inhibitor based on the SFTI-1 scaffold, cVc1.1, and MCoTI-II (Yap et al. 2020). After single affinity purification, these CPs were enriched and concentrated, decreasing the working volume for downstream in-vitro cyclization (Yap et al. 2020).

Prior to in-vitro cyclization, AEP zymogens were recombinantly produced in *E. coli* followed by acid-dependent activation into their active form (Fig. 5b) (Du et al. 2020; Yang et al. 2017). These secreted and properly folded CPs were then cyclized in-vitro by active AEPs into mature CDRPs (Fig. 5c). With little or no alteration to the workflow, this versatile production platform produced CDRPs from three structurally distinct families on a small scale. Laboratory scale-up production using a bioreactor was performed on cVc1.1 and MCoTI-II. MCoAEP was selected for large-scale cyclization of MCoTI-II CPs and [C247A]OaAEP1 was used for the cyclization of cVc1.1 (Yap et al. 2021). A 5 L laboratory scale-up production of cVc1.1 and MCoTI-II reported yields of 85–97 mg L^{-1} culture, the highest ever reported in any recombinant production of CDRPs and an improvement of 2–3 order of magnitude compared to previous studies, in a preliminary unoptimized bioreactor (Yap et al. 2020; Yap et al. 2021).

Establishing reliable and robust validation checkpoints is integral to any successful therapeutic production. CDRPs with more than one disulfide bond may fold into multiple isomers with different disulfide bond connectivities, resulting in different 3D structures with different activities compared to the native fold (El Hamdaoui et al. 2019). Structural analysis by NMR spectroscopy coupled with HRMS analysis was used to validate recombinantly produced CDRPs (Fig. 5d). *P. pastoris*-derived AEP-cyclized MCoTI-II and [G22N]cVc1.1 were equivalent to their chemically-derived counterparts based on mass validation using HRMS and further supported by structural validation using 1D and 2D ^{1}H NMR spectroscopy (Yap et al. 2020; Yap et al. 2021). Both recombinant CDRPs were found to be equipotent compared to their respective chemically-derived counterparts in biological assays, a trypsin inhibition assay for MCoTI-II and a human G protein-coupled $GABA_B$ receptor-mediated inhibition assay of $Ca_V2.2$ (N-type) voltage-gated calcium channel for [G22N]cVc1.1 (Fig. 5e) (Yap et al. 2020; Yap et al. 2021). These validation checkpoints prove that recombinantly produced CDRPs were structurally equivalent and functionally equipotent compared to the chemically synthesized counterparts.

In summary, our *P. pastoris*-based cyclic peptide production platform has demonstrated versatility, scalability, suited for easy downstream purification, and achieved yields heading in the right direction for commercial production. More importantly, this platform is more environmentally sustainable or "greener" than SPPS (Yap et al. 2020) because it does not rely on hazardous solvents or generate toxic waste.

4 Future Directions and Conclusions

This *P. pastoris*-based production platform for CDRPs on a non-optimized laboratory-scale bioreactor production run yields about 100 mg L^{-1} culture, representing a 2–3 order of magnitude improvement compared to yields from previous bioproduction systems. Further optimization of the current platform to achieve g L^{-1} yields is feasible. One such process optimization is utilizing minimal growth

media instead of complex media where individual chemical components are further optimized. Another strategy is exploring different methanol feeding strategies. These optimization processes can drastically increase *P. pastoris* biomass, in turn increasing yields of CPs. A study producing rhamnosidase demonstrated that by switching to a minimal growth media and optimizing the methanol feeding strategy, dry cell weight of *P. pastoris* culture increases significantly from 8.3 g L^{-1} to 60 g L^{-1}, in turn, achieving production yields of 2 g L^{-1} (Markošová et al. 2015).

The current *P. pastoris*-based CDRPs production platform uses highly flammable methanol to induce expression which may not be amenable to commercial manufacturing. Methanol induction on a commercial scale presents significant manufacturing challenges. For example, a 10,000 L bioreactor production would require 50 L of methanol at 0.5% methanol induction every 12-24 hours, which poses significant flammable risks. A focus on methanol-free *P. pastoris* production is trending in this research space (Shen et al. 2016). Adapting the current platform towards methanol independent expression would be a significant step toward commercial manufacturing of CDRPs.

The capability of the yeast-based bioproduction platform to fold structurally more complex CDRPs and graft non-native cyclotides will need to be evaluated in future work. Cyclic chlorotoxin, a cyclic peptide with four disulfide bonds, is an ideal candidate to test the folding capability of the yeast-based bioproduction platform on more complex peptides (Akcan et al. 2011). Currently, the yeast-based bioproduction platform has produced only native cyclotide, MCoTI-II (Yap et al. 2020; Yap et al. 2021). Future endeavors will determine if grafted cyclotides with major amino acid sequence changes can be properly folded, secreted, and cyclized using the *P. pastoris*-based bioproduction platform.

The field of CDRPs is still in its infancy yet has garnered immense research interest as drug modalities for medical applications (Craik et al. 1999). The issues of industrial manufacturing of potential CDRP-based drugs in an economically and environmentally viable manner remain unsolved. These high-value molecules have in the past been produced exclusively by chemical synthesis which is not readily scalable and not environmentally sustainable. Previous attempts at producing CDRPs in bacterial or plant-based systems have yet to approach the realms of commercial viability. The *P. pastoris*-based bioproduction platform is more FDA friendly and has been used to produce FDA-approved drugs. The *P. pastoris*-based bioproduction platform for CDRPs described in this chapter is the first bioproduction platform moving in the right direction towards commercial-scale manufacturing of CDRP-based drugs in a sustainable and economically viable manner.

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References

Aboye TL et al (2012) Design of a novel cyclotide-based CXCR4 antagonist with anti-human immunodeficiency virus (HIV)-1 activity. J Med Chem 55:10729–10734

Akcan M et al (2011) Chemical re-engineering of chlorotoxin improves bioconjugation properties for tumor imaging and targeted therapy. J Med Chem 54:782–787

Arntzen C (2015) Plant-made pharmaceuticals: from 'edible vaccines' to Ebola therapeutics. Plant Biotechnol J 13:1013

Bally J et al (2018) The rise and rise of *Nicotiana benthamiana*: a plant for all reasons. Annu Rev Phytopathol 56:405–426

Camarero JA, Fushman D, Cowburn D, Muir TW (2001) Peptide chemical ligation inside living cells: in vivo generation of a circular protein domain. Bioorg Med Chem 9:2479–2484

Camarero JA, Kimura RH, Woo YH, Shekhtman A, Cantor J (2007) Biosynthesis of a fully functional cyclotide inside living bacterial cells. Chembiochem 8:1363–1366

Camarero JA, Muir TW (1999) Biosynthesis of a head-to-tail cyclized protein with improved biological activity. J Am Chem Soc 121:5597–5598

Chan LY, Craik DJ, Daly NL (2015) Cyclic thrombospondin-1 mimetics: grafting of a thrombospondin sequence into circular disulfide-rich frameworks to inhibit endothelial cell migration. Biosci Rep 35:e00270

Chan LY et al (2011) Engineering pro-angiogenic peptides using stable, disulfide-rich cyclic scaffolds. Blood 118:6709–6717

Cheneval O et al (2014) Fmoc-based synthesis of disulfide-rich cyclic peptides. J Org Chem 79: 5538–5544

Choi B-K et al (2003) Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. Proc Natl Acad Sci U S A 100:5022–5027

Ciofalo V, Barton N, Kreps J, Coats I, Shanahan D (2006) Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil. Regul Toxicol Pharmacol 45:1–8

Clark RJ et al (2010) The engineering of an orally active conotoxin for the treatment of neuropathic pain. Angew Chem Int Ed 49:6545–6548

Colgrave ML, Craik DJ (2004) Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. Biochemistry 43:5965–5975

Colgrave ML, Korsinczky MJ, Clark RJ, Foley F, Craik DJ (2010) Sunflower trypsin inhibitor-1, proteolytic studies on a trypsin inhibitor peptide and its analogs. Biopolymers 94:665–672

Craik DJ, Daly NL, Bond T, Waine C (1999) Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 294:1327–1336

Craik DJ, Fairlie DP, Liras S, Price D (2013) The future of peptide-based drugs. Chem Biol Drug Des 81:136–147

Dawson PE, Muir TW, Clark-Lewis I, Kent SB (1994) Synthesis of proteins by native chemical ligation. Science 266:776–779

Dirisala VR et al (2017) Recombinant pharmaceutical protein production in plants: unraveling the therapeutic potential of molecular pharming. Acta Physiol Plant 39:1–9

Du J et al (2020) A bifunctional asparaginyl endopeptidase efficiently catalyzes both cleavage and cyclization of cyclic trypsin inhibitors. Nat Commun 11:1575

El Hamdaoui Y et al (2019) Periplasmic expression of 4/7 α-conotoxin TxIA analogues in *E. coli* favours ribbon isomer formation-suggestion of a binding mode at the α7 nAChR. Front Pharmacol 10:577

Eliasen R et al (2012) Design, synthesis, structural and functional characterization of novel melanocortin agonists based on the cyclotide kalata B1. J Biol Chem 287:40493–40501

Fox JL (2012) Peptide therapeutics: current status and future directions. Nat Biotechnol 30:472

Gould A et al (2012) Recombinant production of rhesus θ-defensin-1 (RTD-1) using a bacterial expression system. Mol BioSyst 8:1359–1365

Gran L (1970) An oxytocic principle found in *Oldenlandia affinis* DC. Medd Nor Farm Selsk 12:80

Gründemann C, Stenberg KG, Gruber CW (2019) T20K: an immunomodulatory cyclotide on its way to the clinic. Int J Pept Res Ther 25:9–13
Huang Y-H et al (2015) Design of substrate-based BCR-ABL kinase inhibitors using the cyclotide scaffold. Sci Rep 5:1–15
Isidro-Llobet A et al (2019) Sustainability challenges in peptide synthesis and purification: from R & D to production. J Org Chem 84:4615–4628
Jackson MA et al (2019) Rapid and scalable plant-based production of a potent plasmin inhibitor peptide. Front Plant Sci 10:602
Jad YE, Kumar A, El-Faham A, de la Torre BG, Albericio F (2019) Green transformation of solid-phase peptide synthesis. ACS Sustain Chem Eng 7:3671–3683
Jagadish K et al (2015) Recombinant expression and phenotypic screening of a bioactive cyclotide against α-synuclein-induced cytotoxicity in baker′s yeast. Angew Chem Int Ed 54:8390–8394
James AM, Haywood J, Mylne JS (2018) Macrocyclization by asparaginyl endopeptidases. New Phytol 218:923–928
Johnson IS (1983) Human insulin from recombinant DNA technology. Science 219:632–637
Katrolia P et al (2011) Molecular cloning and high-level expression of a β-galactosidase gene from *Paecilomyces aerugineus* in *Pichia pastoris*. J Mol Catal B Enzym 69:112–119
Kimura RH, Tran AT, Camarero JA (2006) Biosynthesis of the cyclotide kalata B1 by using protein splicing. Angew Chem Int Ed 45:973–976
Laukens B, De Wachter C, Callewaert N (2015) Engineering the *Pichia pastoris* N-glycosylation pathway using the GlycoSwitch technology in *Glyco-engineering*. Springer, pp 103–122
Li CY, Yap K, Swedberg JE, Craik DJ, de Veer SJ (2019) Binding loop substitutions in the cyclic peptide SFTI-1 generate potent and selective chymase inhibitors. J Med Chem 63:816–826
Li Y, Aboye T, Breindel L, Shekhtman A, Camarero JA (2016) Efficient recombinant expression of SFTI-1 in bacterial cells using intein-mediated protein trans-splicing. Pept Sci 106:818–824
Markošová K, Weignerová L, Rosenberg M, Křen V, Rebroš M (2015) Upscale of recombinant α-L-rhamnosidase production by *Pichia pastoris* MutS strain. Front Microbiol 6:1140
Merrifield RB (1963) Solid phase peptide synthesis. I. the synthesis of a tetrapeptide. J Am Chem Soc 85:2149–2154
Mullard A (2013) 2012 FDA drug approvals. Nat Rev Drug Discov 12:87
Mylne JS et al (2011) Albumins and their processing machinery are hijacked for cyclic peptides in sunflower. Nat Chem Biol 7:257–259
Nguyen GK, Hemu X, Quek JP, Tam JP (2016) Butelase-mediated macrocyclization of d-amino-acid-containing peptides. Angew Chem Int Ed 55:12802–12806
Nguyen GK et al (2014) Butelase 1 is an Asx-specific ligase enabling peptide macrocyclization and synthesis. Nat Chem Biol 10:732–738
Poon S et al (2018) Co-expression of a cyclizing asparaginyl endopeptidase enables efficient production of cyclic peptides in planta. J Exp Bot 69:633–641
Quimbar P et al (2013) High-affinity cyclic peptide matriptase inhibitors. J Biol Chem 288:13885–13896
Rehm FBH, Tyler TJ, Yap K, Durek T, Craik DJ (2020) Improved asparaginyl ligase-catalyzed transpeptidation via selective nucleophile quenching. Angew Chem Int Ed 60:4004–4008
Rehm FBH et al (2019) Site-specific sequential protein labeling catalyzed by a single recombinant ligase. J Am Chem Soc 141:17388–17393
Ritter SK (2017) Five green chemistry success stories. Chem Eng News 95:16–20
Roberts AW et al (2018) Functional characterization of a glycosyltransferase from the moss *Physcomitrella patens* involved in the biosynthesis of a novel cell wall arabinoglucan. Plant Cell 30:1293–1308
Sable R et al (2016) Constrained cyclic peptides as immunomodulatory inhibitors of the CD2: CD58 protein–protein interaction. ACS Chem Biol 11:2366–2374
Schillberg S, Finnern R (2021) Plant molecular farming for the production of valuable proteins–critical evaluation of achievements and future challenges. J Plant Physiol 258:153359

Shen W et al (2016) A novel methanol-free *Pichia pastoris* system for recombinant protein expression. Microb Cell Factories 15:1–11

Spohner SC, Müller H, Quitmann H, Czermak P (2015) Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. J Biotechnol 202:118–134

Streatfield SJ, Howard JA (2003) Plant production systems for vaccines. Expert Rev Vaccines 2: 763–775

Swedberg JE et al (2016) Substrate-guided design of selective FXIIa inhibitors based on the plant-derived *Momordica cochinchinensis* trypsin inhibitor-II (MCoTI-II) scaffold. J Med Chem 59: 7287–7292

Thell K et al (2016) Oral activity of a nature-derived cyclic peptide for the treatment of multiple sclerosis. Proc Natl Acad Sci U S A 113:3960–3965

Tripathi NK, Shrivastava A (2019) Recent developments in bioprocessing of recombinant proteins: expression hosts and process development. Front Bioeng Biotechnol 7:420

Walsh G (2010) Biopharmaceutical benchmarks 2010. Nat Biotechnol 28:917–924

Wang CK, Northfield SE, Huang Y-H, Ramos MC, Craik DJ (2016) Inhibition of tau aggregation using a naturally-occurring cyclic peptide scaffold. Eur J Med Chem 109:342–349

Wang CK et al (2014) Molecular grafting onto a stable framework yields novel cyclic peptides for the treatment of multiple sclerosis. ACS Chem Biol 9:156–163

Wong CT et al (2012) Orally active peptidic bradykinin B1 receptor antagonists engineered from a cyclotide scaffold for inflammatory pain treatment. Angew Chem Int Ed 51:5620–5624

Yang R et al (2017) Engineering a catalytically efficient recombinant protein ligase. J Am Chem Soc 139:5351–5358

Yao J, Weng Y, Dickey A, Wang KY (2015) Plants as factories for human pharmaceuticals: applications and challenges. Int J Mol Sci 16:28549–28565

Yap K et al (2020) An environmentally sustainable biomimetic production of cyclic disulfide-rich peptides. Green Chem 22:5002–5016

Yap K et al (2021) Yeast-based bioproduction of disulfide-rich peptides and their cyclization via asparaginyl endopeptidases. Nat Protoc 16:1740–1760