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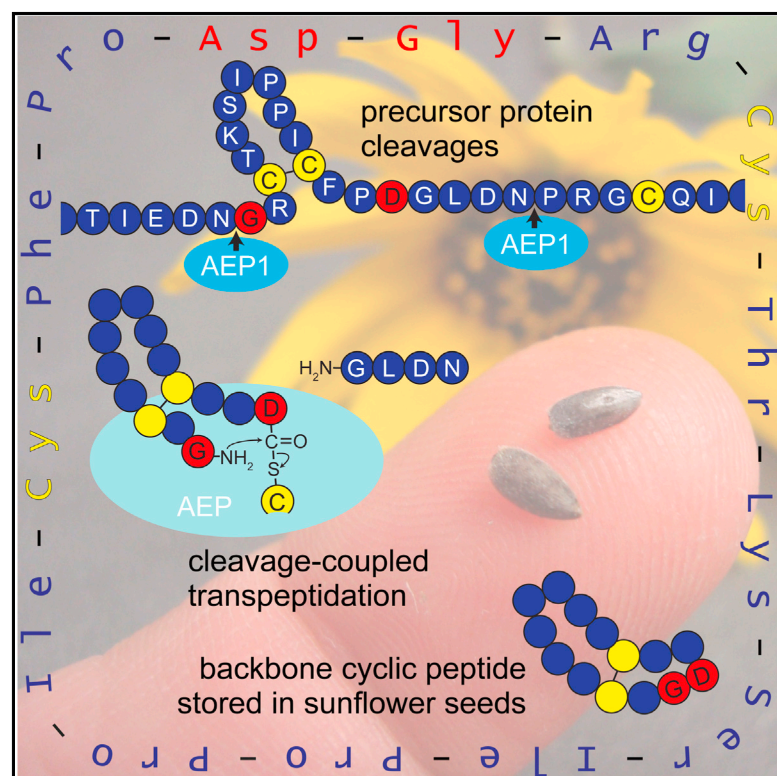
Peptide Macrocyclization by a Bifunctional Endoprotease

Graphical Abstract



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In Brief

Bernath-Levin et al. reconstitute the biosynthesis of the macrocyclic, sunflower seed peptide SFTI-1 in situ and in vitro showing how an endoprotease can exploit entropy, product stability, and substrate recognition to macrocyclize via a cleavage-dependent, intramolecular transpeptidation.

Highlights

- A cleavage-coupled macrocyclization activity makes the cyclic seed peptide SFTI-1
- Inefficient macrocyclization is masked by a stepwise breakdown of acyclic products
- First production of asparaginyl endopeptidase (AEP) in *E. coli*
- Macrocyclization can be reconstituted in vitro, offering insights into biosynthesis

Accession Numbers

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Peptide Macrocyclization by a Bifunctional Endoprotease

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SUMMARY

Proteases usually cleave peptides, but under some conditions, they can ligate them. Seeds of the common sunflower contain the 14-residue, backbone-macrocyclic peptide sunflower trypsin inhibitor 1 (SFTI-1) whose maturation from its precursor has a genetic requirement for asparaginyl endopeptidase (AEP). To provide more direct evidence, we developed an in situ assay and used ^{18}O-water to demonstrate that SFTI-1 is excised and simultaneously macrocyclized from its linear precursor. The reaction is inefficient in situ, but a newfound breakdown pathway can mask this inefficiency by reducing the internal disulfide bridge of any acyclic-SFTI to thiols before degrading it. To confirm AEP can directly perform the excision/ligation, we produced several recombinant plant AEPs in *E. coli*, and one from jack bean could catalyze both a typical cleavage reaction and cleavage-dependent, intramolecular transpeptidation to create SFTI-1. We propose that the evolution of ligating endoproteases enables plants like sunflower and jack bean to stabilize bioactive peptides.

INTRODUCTION

All kingdoms of life contain backbone cyclic peptides, consisting of an unbroken ring of peptide bonds made during non-ribosomal peptide synthesis (Trauger et al., 2000) or from proteins coded by genes (Barber et al., 2013; Eisenbrandt et al., 2000; Koehnke et al., 2012; Mylne et al., 2011, 2012). Sunflower seeds contain a 14-residue, backbone cyclic and disulfide bonded peptide called sunflower trypsin inhibitor 1 (SFTI-1) (Luckett et al., 1999). Its precursor, Preproalbumin with SFTI-1 (PawS1), encodes two mature proteins: SFTI-1 as well as a typical seed storage albumin (Mylne et al., 2011). The cysteine protease asparaginyl endopeptidase (AEP, also known as legumain or vacuolar processing enzyme [VPE]) processes seed storage albumins (Gruis et al., 2004; Shimada et al., 2003), but using an *aep* genetic knockout, AEP was also shown to be essential for maturing SFTI-1 at both proto-termini (Mylne et al., 2011). AEP is also implicated in the biosyntheses of unrelated kalata-type cyclic peptides (Gillon et al., 2008; Saska et al., 2007) and cyclic knottins (Mylne et al., 2012), illustrating how AEP has been independently recruited multiple times in plant evolution for cyclic peptide biosynthesis (Mylne et al., 2012). Evolutionary convergence on AEP by cyclic peptide biosyntheses bolstered previous work that showed that AEP was capable of protease-mediated ligation in vitro (Min and Jones, 1994) and implicated AEP of this reaction in vivo (Sheldon et al., 1996). Recently, by following the processing of synthetic peptides, a highly efficient transpeptidase activity was purified from the seeds of *Clitoria ternatea* and attributed by proteomics to an AEP named Butelase 1 (Nguyen et al., 2014). This purified activity could not hydrolyze the usual substrates of AEP and was quantitatively efficient at transpeptidation, so Butelase 1 was proposed to have evolved to function as a ligase rather than a protease (Nguyen et al., 2014).

The notion of endoprotease-mediated ligation is well established; early in vitro studies with proteases achieved reversibility by altering solvents or concentrating substrates (Bergmann and Behrens, 1938). Several biological examples of protease-mediated ligation have been characterized. Circular pilins are the major component of *Agrobacterium* sex pili. Mutagenesis of the pilin precursor showed in vivo that cleavage and ligation reactions were inextricably linked, but the data could not exclude the possibility of a separate ligase (Eisenbrandt et al., 2000). More recently, the structural basis for macrocyclization of patellamides in cyanobacteria was shown to involve a highly specialized enzyme with a subtilisin protease fold and a separate domain dedicated to shielding the acyl intermediate from water so the amino terminus can react with the carbonyl carbon of the acyl intermediate to form a peptide bond (Koehnke et al., 2012). A similar reaction by AEP was proposed for the biosynthesis of sunflower SFTI-1 (Mylne et al., 2011), but direct evidence was lacking and whether a specialist AEP performed the final reaction was unknown. Here, by developing an in situ assay, we demonstrate that the cleavage and ligation reactions do not involve hydrolysis. The macrocyclization reaction is inefficient in situ, while seemingly perfect in vivo. By fractionation of the in situ extract, we discovered separate activities that reduce then degrade any acyclic-SFTI, masking inefficient macrocyclization. Seeking activities to explain our in situ observations, we discovered an AEP from jack bean that, in addition to substrate cleavage, can perform a cleavage-coupled intramolecular transpeptidation reaction to make SFTI-1 with a similar efficiency to the in situ. This work illustrates how AEPs with different functionalities might act in concert with a degradation pathway to produce cyclic SFTI-1 in sunflower seeds.

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