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In Vitro Reconstruction of Nonribosomal Peptide Biosynthesis Directly from DNA Using Cell-Free Protein Synthesis

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Abstract

Genome sequencing has revealed that a far greater number of natural product biosynthetic pathways exist than there are known natural products. To access these molecules directly and deterministically, a new generation of heterologous expression methods is needed. Cell-free protein synthesis has not previously been used to study nonribosomal peptide biosynthesis, and provides a tunable platform with advantages over conventional methods for protein expression. Here, we demonstrate the use of cell-free protein synthesis to biosynthesize a cyclic dipeptide with correct absolute stereochemistry. From a single-pot reaction, we measured the expression of two nonribosomal peptide synthetases larger than 100 kDa, and detected high-level production of a diketopiperazine. Using quantitative LC–MS and synthetically prepared standard, we observed production of this metabolite at levels higher than previously reported from cell-based recombinant expression, approximately 12 mg/L. Overall, this work represents a first step to apply cell-free protein synthesis to discover and characterize new natural products.

Graphical Abstract

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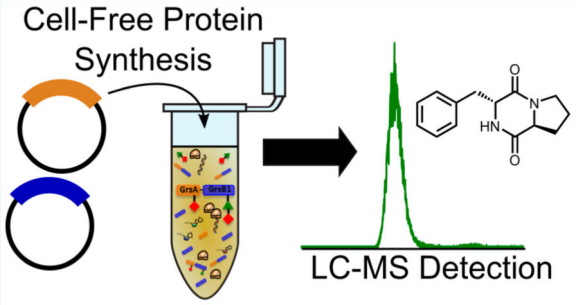
ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00160.

Quantitation of synthesized GrsA and GrsB1; expression and purification of recombinant Sfp from *E. coli* autoradiography analysis; synthetic preparation of DKP; bacterial strains and plasmid construction (PDF)

The authors declare no competing financial interest.



Keywords

cell-free protein synthesis; natural products; diketopiperazine; cyclic dipeptide; biosynthesis; synthetic biology

Sequencing of over 10000 microbial genomes has revealed that the diversity of secondary metabolism covers a largely unexplored region of chemical space.[1,2] Currently, the rate at which new biosynthetic pathways are identified within biological sequence data greatly outpaces the capacity to characterize the small molecules for which production is encoded. This is at least in part due to the difficulty of cultivating natural product producing organisms in the laboratory, and therefore presents a major opportunity for synthetic biology to establish new methods for robust heterologous expression and characterization of secondary metabolism to unlock access to often bioactive secondary metabolites. Additionally, robust heterologous expression of biosynthetic proteins opens the possibility of rapid design-build-test cycles to re-engineer pathways to produce useful scaffolds beyond those found in nature.[3]

Proteins involved in the biosynthesis of complex natural products (NPs) provide a challenge for established heterologous expression platforms because of their sheer size, complex multidomain structures, and occurrence within even larger biosynthetic gene clusters (BGCs). Nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are often 100 to >300 kDa proteins that operate like assembly lines, loading and passing covalently attached intermediates between catalytic domains (Figure 1, center).[4] Production of NPs by these classes of proteins frequently involves exotic precursors that may not be found in the cytosol of a typical heterologous host such as *Escherichia coli* or *Saccharomyces cerevisiae*.[5,6]

Cell-free protein synthesis (CFPS) offers an alternative protein expression platform with potential advantages for expressing natural product biosynthetic enzymes (Figure 1).[7,8] CFPS is emerging as a robust tool for fundamental and applied research in the areas of synthetic biology and biotechnology.[9–11] Recently, cell-free systems have been shown to enable high yielding synthesis of, for example, active metalloenzymes,[12–14] and allow rapid prototyping of biological circuits and pathways.[15–18] For natural products research, CFPS offers flexibility over cell-based expression because the reaction conditions can be more easily controlled: for example, atypical precursors can simply be fed in during the course of a reaction[19,20] or made *in situ* using all enzymes from a biosynthetic gene cluster.

Biosynthetic pathways could potentially be engineered for higher expression without the need to modify a native host strain.[21] Reactions can also be performed in the span of a few hours in a “single pot” and have a greater tolerance for producing potentially toxic proteins and metabolites than living cells.

To investigate the potential of CFPS for studying natural product pathways, we utilized the proteins involved in the first steps of gramicidin S biogenesis as a model. Gramicidin S is biosynthesized by two NRPS proteins, GrsA and GrsB, that catalyze thiotemplated peptide bond formation and release by cyclodimerization to form the cyclic decapeptide (Figure 2A). A natural shunt product of the gramicidin S pathway is the D-Phe-L-Pro diketopiperazine (DKP) formed by cyclization of the substrates of the first two NRPS modules in the pathway, GrsA and GrsB1.[22] Cyclic dipeptide DKPs have been studied for their diverse biological activity and are produced by many species of bacteria, fungi, and plants.[23] Bacterial DKPs have two known general biosynthetic origins: they are either synthesized by NRPS enzymes, as in the case of the D-Phe-L-Pro-DKP presented here,[24] or produced by aminoacyl-tRNA mediated cyclodipeptide synthase pathways as in the case of the antibiotic albonoursin.[25]

Here, we report *in vitro* expression of GrsA and GrsB1, encompassing the first two of five modules of gramicidin S biosynthesis in *Brevibacillus brevis*. Each NRPS module typically consists of the three domains needed to incorporate an amino acid into the nonribosomal peptide: condensation (C), adenylation (A), and thiolation (T). GrsA, as a starter module, does not contain a C-domain; it contains an A and a T-domain, as well as an epimerization (E) domain that converts L-phenylalanine to D-phenylalanine. Previous reports have shown that isolated GrsA and GrsB1 can be harnessed to produce D-Phe-L-Pro DKP (Figure 2B).[26] While this diketopiperazine is not the primary product of this pathway in *Brevibacillus brevis*, it has been found to be produced by other microbes.[27] In this report, we demonstrate high-level expression of GrsA and GrsB1, show that they are produced in their functional state when expressed using CFPS, and use these two proteins in a concerted reaction to produce D-Phe-L-Pro DKP with a final yield of about 12 mg/L.

RESULTS AND DISCUSSION

To establish an *in vitro* platform for D-Phe-L-Pro DKP biosynthesis, NRPS proteins GrsA and GrsB1 were expressed using an *E. coli*-based cell-free protein expression system reported previously.[28–30] GrsA and GrsB1 along with the functionally equivalent tyrocidine synthetases TycA and TycB1 (often referred to as pheATE and proCAT) have served as the model thiotemplated biosynthetic systems for studying substrate specificity[31] and peptide bond formation[32] in NRPS pathways.

Individual plasmids containing the genes *grsA* and *grsB1* were used to express these enzymes in the combined transcription–translation system. Transcription was driven using a T7 RNA polymerase. We tested protein synthesis yields in 20 h batch reactions across three different *E. coli* crude extracts (S30 crude extracts).[33] On the basis of the expression yields of GrsA and GrsB1, we found that *E. coli* BL21 Star (DE3) extract synthesized the highest amount of NRPS, yielding full-length, soluble GrsA at ~106 μg/mL and GrsB1 at ~77

μg/mL (Figure S1). Fully assembled GrsA (126 kDa) and GrsB1 (121 kDa) with correct molecular weight bands were observed by SDS-PAGE and further confirmed by autoradiogram analysis (Figure S2).

Once we had successfully expressed the two large NRPSs, GrsA and GrsB1 *in vitro*, we showed that the enzymes could be converted to their functional (holo) form using the 4′-phosphopantetheinyl transferase enzyme Sfp from *Bacillus subtilis*. To be functional, NRPS proteins require modification by transfer of a phosphopantetheine group from coenzyme A (CoA) to a conserved serine residue in their thiolation (T) domain.[34] To verify that this essential modification is possible in our cell-free system, both GrsA and GrsB1 were labeled with a fluorescent Bodipy-CoA analogue by the promiscuous action of Sfp (Figure 3A). Expression of GrsA and GrsB1 continued for 17 h *in vitro*, allowing nascent proteins to fold properly before labeling. After this incubation, Bodipy-CoA and Sfp were added directly to the cell-free expression system, followed by another 3 h incubation at 30 or 37 °C.

Phosphopantetheinylation was also detectable by LC–MS/MS using the method described in Miller *et al*. 2005 (Figure 3B).[35] GrsA and GrsB1 were prepared *in vitro* in separate reactions. Following phosphopantetheinylation by Sfp, GrsA with a 6×His tag was captured using cobalt-affinity resin, digested with trypsin, and analyzed by proteomic-style LC–MS/MS. In the GrsA sample, a peptide with the sequence DNFYALGGDSIK and *m*/*z* 820.357 was observed, representing the predicted mass of the tryptic peptide with the addition of phosphopantetheine. GrsB1 peptides were detected in a peptide sample prepared by digesting the total lysate with 1:5 trypsin. The T-domain active-site peptide (IWEEVLGISQIGIQDNFFSLGGHSLK, *m*/*z* 1076.533) was also observed for GrsB1 with attached phosphopantetheine.

Successful fluorescence labeling of GrsA and GrsB1 with Bodipy-CoA, and direct LC–MS/MS detection of the Ppant modification (Figure S3, Figure 3) demonstrated that, in the current *in vitro* system, (i) both NRPSs were properly folded with accessible conserved serine residues located on T domains; (ii) the purified recombinant Sfp is active for the phosphopantetheinylation; and (iii) most importantly, the holo-NRPS could be functionally reconstituted for target molecule biosynthesis. In addition, GrsA and GrsB1 were expressed in soluble form and an extended incubation at 37 °C was not necessary for priming the NRPSs by Sfp (Figure S3). Therefore, we posited that the target compound could be biosynthesized *via* active holo-NRPSs without further optimization of the reaction conditions or incubation temperature.

We next sought to biosynthesize D-Phe-L-Pro DKP, and instead of expressing GrsA and GrsB1 separately, the two enzymes were coexpressed in a single-pot mixture allowing reconstitution of the partial NRPS assembly line for product formation *in situ*. After carrying out the CFPS reaction, we added Sfp to the mixture to initiate biosynthesis, using the same reaction mixture without Sfp as a negative control. The reaction was stopped by extraction with *n*-butanol and chloroform (4:1, v/v) and the extract was analyzed by metabolomics-style LC–MS/MS. We detected a time-dependent increase in signal intensity of the ion corresponding to predicted DKP ion within 3 ppm mass error tolerance (*m*/*z* 245.129) (Figure 4A,B).

To verify that the ion observed was the expected D-Phe-L-Pro DKP—and with correct D–L stereochemistry—we prepared both the D–L and L–L DKP as synthetic references (Scheme S1). On the basis of earlier reporting, we expected the D–L stereoisomer to elute earlier than L–L on a reversed phase HPLC column,[36] and its elution profile matched with the metabolite produced with the CFPS reaction. Synthetic DKP was indistinguishable from the CFPS produced metabolite by MS^2 analysis (Figure 4C). We used LC–MS to prepare a standard curve from the integrated intensity of synthetically prepared DKP measured at six different concentrations and used this curve to estimate the quantity of DKP produced by CFPS (Figure S4). Even without optimization of the system for high titers, we estimated our production of D-Phe-L-Pro DKP at ~12 mg/L, a concentration higher than the 9 mg/L previously reported from recombinant protein expression in *E. coli*.[26]

We believe this work to be the first example of natural product biosynthesis from NRPS enzymes produced using an *E. coli*-based *in vitro* transcription and translation system. CFPS provides a feasible option for exploring natural product biosynthesis, at least for proteins ~130 kDa such as the two presented here. CFPS offers freedom from many of the problematic processes present in cell-based expression systems (e.g., inclusion body formation, protein degradation), and may circumvent the issues of low-expressing or cryptic (nonexpressing) BGCs encountered in native-producing organisms. While more investigation will be needed, the production of these two single-module NRPS proteins *in vitro* and the demonstration of their concerted function provides a groundwork for the study of increasingly complex natural product biosynthesis pathways using CFPS. Indeed, our discovery-centered cell-free approach sets the stage for high-throughput experimentation in a cell-free environment, where design–build–test iterations can be performed without the need to reengineer organisms, DNA for pathway enzymes is directly input with plasmid refactoring, and substrates and cofactors needed for secondary metabolism can be controlled and maintained at defined concentrations.[37]

As a resurgence of interest in natural products continues, and the number of sequenced biosynthetic gene clusters continues to grow, we expect that protein expression systems will play an increasingly important role in obtaining and studying new natural products. Especially as the price of DNA synthesis declines, direct expression of entirely synthetic gene clusters (typically 30–120 kilobases in length) will remove barriers to accessing biosynthetic pathways from clusters assembled using metagenomics for uncultivable organisms. By merging bottom-up design principles with innovative cell-free pathway engineering methodologies, our cell-free approach will create a greatly simplified framework for studying and engineering natural product pathways.

METHODS

Preparation of S30 Cell Extracts

E. coli cells were grown in 1 L of 2×YTPG (yeast extract 10 g/L, tryptone 16 g/L, NaCl 5 g/L, K_2HPO_4 7 g/L, KH_2PO_4 3 g/L, and glucose 18 g/L, pH 7.2) in a 2.5-L Tunair flask (IBI Scientific, Peosta, IA) at 34 °C and 220 rpm with inoculation of 20 mL overnight cultures (initial OD_{600} of ~0.05). When the OD_{600} reached 3.0, cells were collected by centrifugation at 5,000*g* and 4 °C for 15 min. The pellets were washed thrice with cold S30 buffer (10 mM

Tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 2 mM dithiothreitol (DTT)). Cells were suspended in 0.8 mL of S30 buffer per gram wet weight and lysed on ice using a Q125 Sonicator (Qsonica, Newtown, CT) for three pulses (50% amplitude, 45 s on and 59 s off). After sonication, 3 μL of DTT (1 M) was added per milliliter of lysate, followed by centrifugation at 12 000*g* and 4 °C for 10 min. The supernatant (S30 extract) was flash frozen in liquid nitrogen and stored at −80 °C until use.

Cell-Free Protein Synthesis (CFPS) Reactions

The standard CFPS reactions were performed in 1.5 mL microcentrifuge tubes with 15 μL of mixture composed of the following reagent concentrations: 12 mM magnesium glutamate, 10 mM ammonium glutamate, 130 mM potassium glutamate, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 34 μg/mL folinic acid, 170 μg/mL of *E. coli* tRNA mixture, 2 mM each of 20 standard amino acids, 10 μM of L-[^{14}C(U)]-leucine (used only in protein quantitation experiments, 11.1 GBq mmol^{-1}, PerkinElmer, Waltham, MA), 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 1.5 mM spermidine, 1 mM putrescine, 4 mM sodium oxalate, 33 mM phosphoenolpyruvate (PEP), 26.7 μg/mL plasmid, 100 μg/mL T7 RNA polymerase, and 27% (v/v) of S30 cell extract. All CFPS reactions were incubated for 20 h at 30 °C unless otherwise described.

Fluorescence Labeling of GrsA and GrsB1

The coenzyme A analogue Bodipy-CoA was prepared as previously described.[38] To label thiolation domains of GrsA and GrsB1, standard CFPS reactions with 26.7 μg/mL of each plasmid were incubated at 30 °C for 17 h. Afterward, labeling reactions were performed following three strategies. Strategy #1: the CFPS system was directly supplemented with 1 μL of Bodipy-CoA (1 mg/mL) and 1 μL of Sfp (2 mg/mL) and incubated at 30 °C for another 3 h. Strategy #2: the CFPS reaction was first centrifuged at 12 000*g* and 4 °C for 10 min. Then, 13 μL of supernatant was transferred to a new 1.5 mL microcentrifuge tube with the addition of 1 μL of Bodipy-CoA and 1 μL of Sfp. The mixture was incubated at 30 °C for 3 h. Strategy #3: the treatment of the CFPS reaction was the same as in Strategy #2, but was incubated at 37 °C for 3 h. After the labeling reaction, 3 μL of each sample was loaded on a 4–12% NuPAGE SDSPAGE gel (Invitrogen). The Bodipy-labeled proteins were visualized by a fluorescence imaging system with 473 nm laser and 520 nm emission filter (Typhoon FLA7000, GE Healthcare Biosciences, Uppsala, Sweden). See Supporting Information for additional details and fluorescent gel images (Figure S3).

DKP Production *in Vitro*

D-Phe-L-Pro diketopiperazine (DKP) was biosynthesized by GrsA and GrsB1 expressed *in situ*. Cell-free coexpression of GrsA and GrsB1 was performed in the 15 μL reaction mixture as described above with the addition of both plasmids (each of 26.7 μg/mL). The BL21 Star (DE3) S30 extract was used for the coupled transcription–translation. After incubation of the reaction mixture at 30 °C for 17 h, 1 μL of Sfp (2 mg/mL); 1 μL of Sfp (2 mg/mL) and 1 μL of CoA (5 mM); or 1 μL of Sfp (2 mg/mL), 1 μL of CoA (5 mM), 1 μL of Phe (1 mM) and 1 μL of Pro (1 mM) were added directly to the reactions, followed by another 3 h incubation at 30 °C. Reactions without plasmids and without addition of Sfp were carried out as negative controls. At the end of the production, all DKP samples were immediately extracted from

the reaction mixtures for analysis by LC–MS/MS (see Supporting Information for further details).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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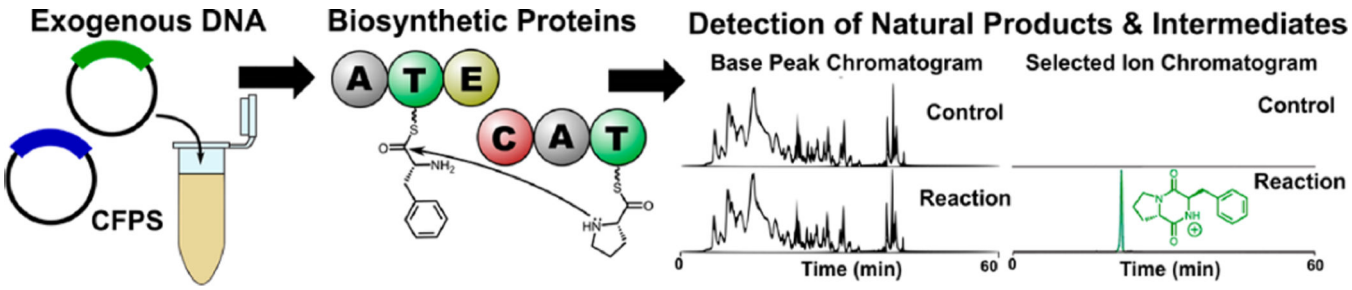

Figure 1.

Overview of a system for cell-free production of natural products *via* nonribosomal peptide biosynthesis. From left to right, exogenous DNA is used as the input information for the production of biosynthetic enzymes. In the center, nonribosomal peptide synthetase proteins function in concert to select substrates and catalyze the formation of peptide bonds, ultimately resulting in the production of a 2,5-diketopiperazine. Right panel, detection of a D-Phe-L-Pro diketopiperazine natural product by LC–MS as the result of *in situ* production of biosynthesis proteins.

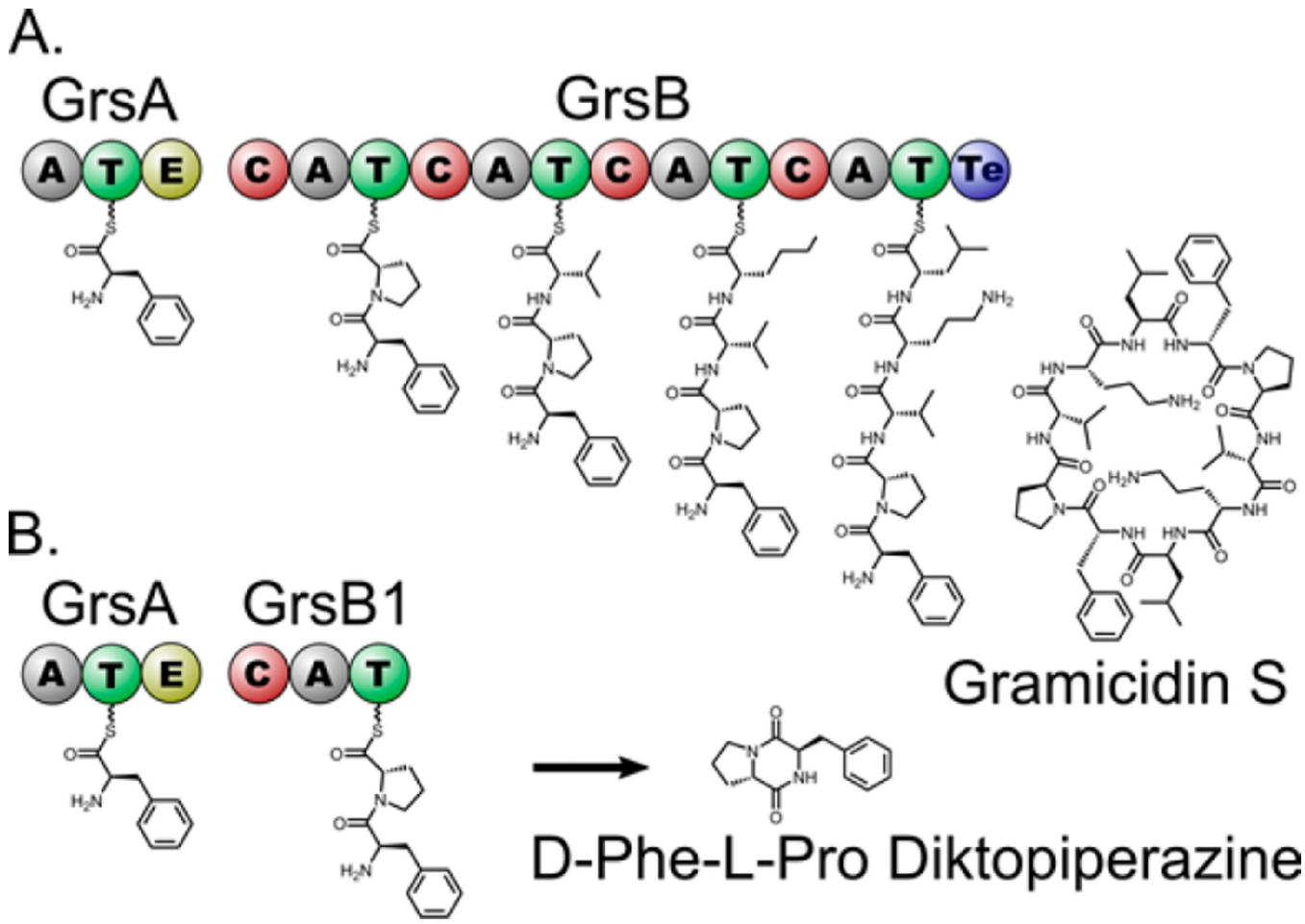

Figure 2.

Panel A summarizes the biosynthesis of gramicidin S as it occurs in *Brevibacillus brevis*. By turning through the assembly line pathway two times, the complete cyclodecapeptide is produced. Panel B shows how the first two modules interact to form D-Phe-L-Pro DKP.

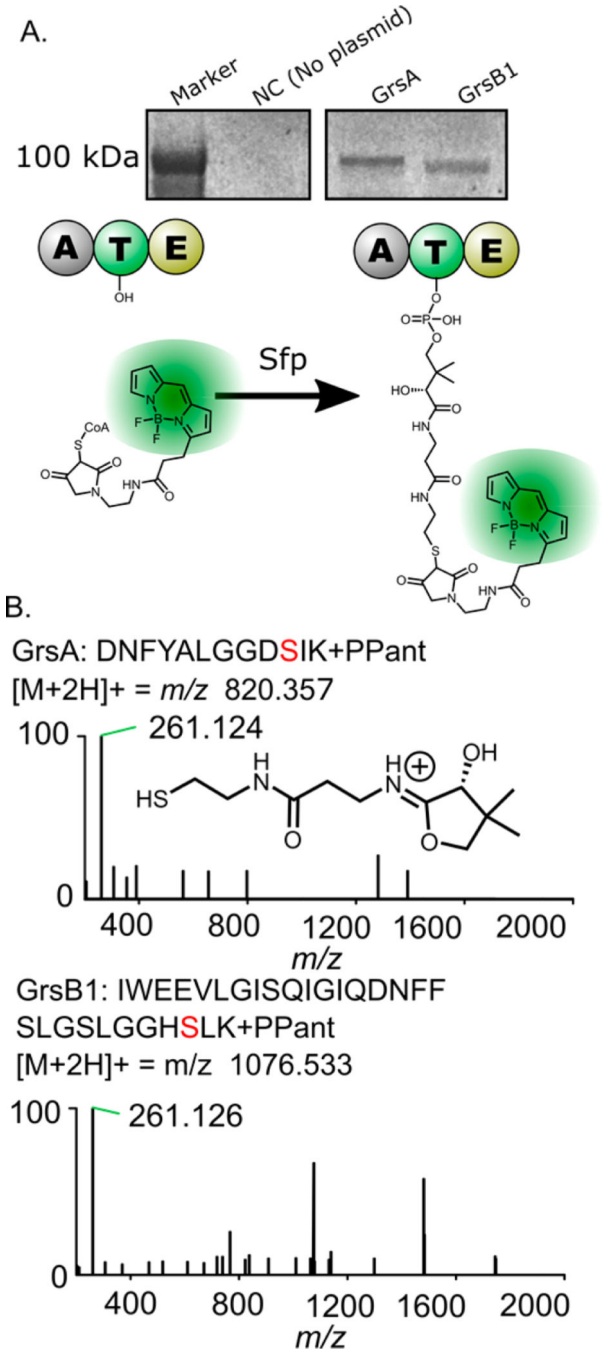

Figure 3.

Experiments showing that GrsA and GrsB1 are present in their active (holo) forms. Panel A shows the fluorescent labeling of GrsA and GrsB1 on the thiolation domain active sites with a conjugated Bodipy-CoA fluorophore (see Figure S3 for complete gel image). Panel B top shows the MS^2 spectrum resulting from the fragmentation of a precursor peptide containing the GrsA phosphopantetheine modification. Panel B bottom shows the MS^2 spectrum for the corresponding GrsB1 T-domain peptide, indicating the mass of the observed pantetheine-derived ion.

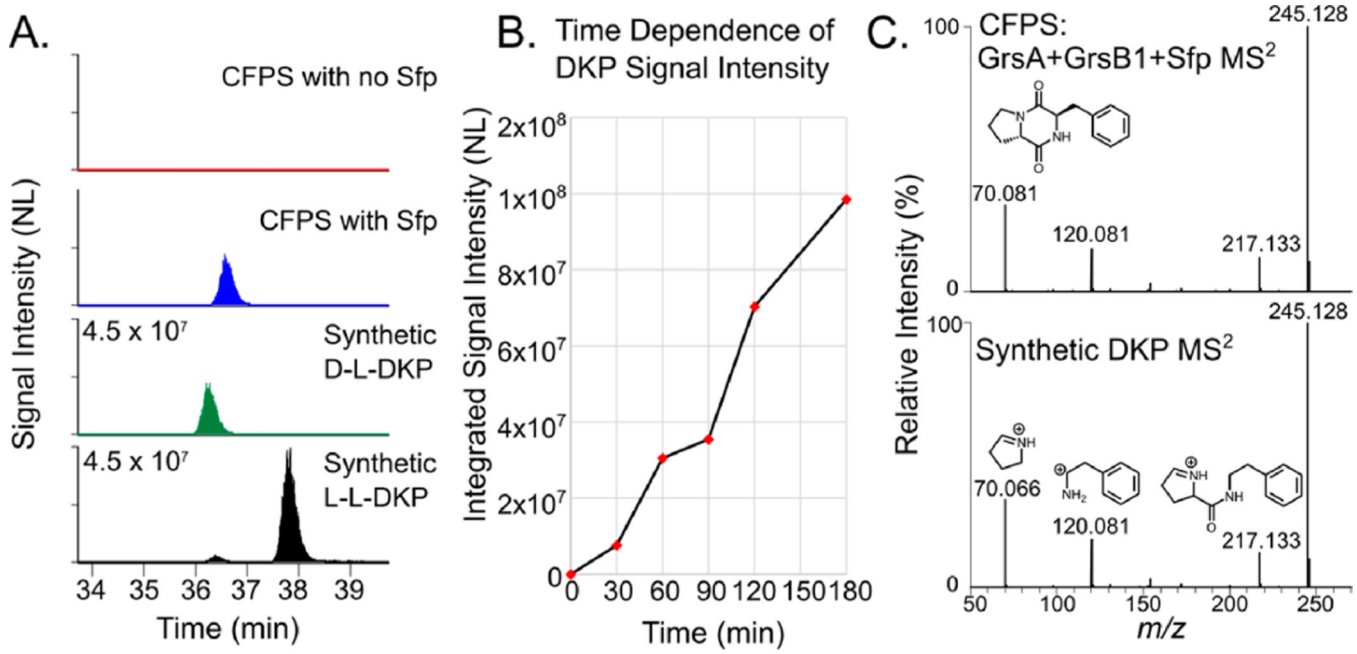

Figure 4.

Detection of D-Phe-L-Pro DKP by LC–MS/MS and comparison to synthetically prepared DKP. (A) Retention time comparison of D–L, L–L, and CFPS-produced DKP to determine the stereochemistry of the DKP produced by CFPS. This panel also shows that SFP is required for DKP production. (B) Time-dependent increase in *m*/*z* 245.128 signal after Sfp is added to the CFPS reaction. Data points are the average of two technical replicates. (C) Comparison of the fragmentation pattern of the CFPS-produced (top) and synthetically prepared DKP (bottom). The spectrum at the bottom of panel C is annotated with predicted fragment ion structures.