SpyRing interrogation: analyzing how enzyme resilience can be achieved with phytase and distinct cyclization chemistries

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Enzymes catalyze reactions with exceptional selectivity and rate acceleration but are often limited by instability. Towards a generic route to thermo-resilience, we established the SpyRing approach, cyclizing enzymes by sandwiching between SpyTag and SpyCatcher (peptide and protein partners which lock together via a spontaneous isopeptide bond). Here we first investigated the basis for this resilience, comparing alternative reactive peptide/protein pairs we engineered from Gram-positive bacteria. Both SnoopRing and PilinRing cyclization gave dramatic enzyme resilience, but SpyRing cyclization was the best. Differential scanning calorimetry for each ring showed that cyclization did not inhibit unfolding of the inserted β-lactamase. Cyclization conferred resilience even at 100 °C, where the cyclizing domains themselves were unfolded. Phytases hydrolyze phytic acid and improve dietary absorption of phosphate and essential metal ions, important for agriculture and with potential against human malnutrition. SpyRing phytase (PhyC) resisted aggregation and retained catalytic activity even following heating at 100 °C. In addition, SpyRing cyclization made it possible to purify phytase simply by heating the cell lysate, to drive aggregation of non-cyclized proteins. Cyclization via domains forming spontaneous isopeptide bonds is a general strategy to generate resilient enzymes and may extend the range of conditions for isolation and application of enzymes.

Purified enzymes are finding a range of important uses, from high-cost low-scale applications such as GMP-grade asparaginase in treatment of leukemia1, to low-cost high-scale applications such as the millions of tons per year of high fructose corn syrup biotransformed by glucose isomerase2. When enzymes are being developed for application in new areas, a major challenge is the stability of the enzyme to inactivation during isolation or use in its new environment. Stability can come from sourcing enzymes from harsh environments or from enzyme-specific optimization campaigns using iterative mutation3. However, it is also important to seek generic approaches capable of stabilizing a wide range of enzymes. One such approach is cyclization, connecting the enzyme's N-terminus to the C-terminus. Cyclization can be accomplished via split inteins4–7, sortases8, carbodiimide cross-linking9 and, most recently, via the design of SpyRings10. SpyTag is a peptide tag that we engineered from Streptococcus pyogenes FbaB adhesion protein to form a spontaneous isopeptide bond upon binding to its protein partner SpyCatcher11,12. SpyRing cyclization utilizes SpyTag on the N-terminus and SpyCatcher on the C-terminus to cyclize an enzyme of interest. We showed this cyclization increased the thermal tolerance of TEM-1 β-lactamase (BLA) by >60 °C, both in terms of aggregation resistance and retaining activity after heating10. This approach to enzyme resilience is not known in nature and so it is important to further explore this phenomenon, as well as to test whether SpyRing cyclization could be extended to an industrially important class of enzymes.

At least 2 billion people are thought to suffer from deficiency of micronutrients, including vitamin A, iodine, iron, niacin, zinc and calcium13. Such deficiency, termed the “silent hunger”, has global consequences, including decreased bone mineralization, anemia, impaired learning, and decreased resistance to infectious diseases such as AIDS and tuberculosis13,14. Major effort has gone into increasing the nutritional value of major food crops around the world, notably introducing the β-carotene pathway into rice15. Most crops store phosphate as phytic acid (inositol hexakisphosphate), making the phosphate unavailable for humans as well as other agriculturally-important

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non-ruminants (e.g. poultry, fish, and pigs). Moreover, phytic acid is an anti-nutrient which chelates essential mineral cations (iron, zinc, calcium)\(^{16,17}\). Adding phytase to the diet breaks down phytic acid and has been widely used in agriculture to improve animal health\(^{18}\). The use of phytase also reduces the danger, if phosphate is directly added to feedstock, of excess phosphate polluting waterways and leading to eutrophication\(^{18}\). The thermal tolerance of phytase has great significance because feed-stock must be subjected to temperatures of 75–90 °C to kill *Salmonella* and other dietary pathogens\(^{18}\).

Here we have explored the principle of SpyRing resilience by evaluating enzyme cyclization with alternative recently-established isopeptide-forming protein chemistries. We then extended the scope of SpyRing cyclization by enhancing the thermo-resilience of a phytase. We studied the stability effects of cyclization by differential scanning calorimetry (DSC) and circular dichroism (CD). In addition we opened up a new approach for purification of SpyRing-cyclized enzymes based simply upon heating.

**Results**

**Enzyme cyclization using Isopeptag/Pilin-C or SnoopTag/SnoopCatcher spontaneous isopeptide bond formation.** To understand the dramatic increase in thermal resilience caused by SpyRing-mediated cyclization, we compared the effects of cyclizing with two different Tag/Catcher pairs, each able to form a spontaneous isopeptide bond (Fig. 1A). First we sandwiched BLA between Isopeptag and Pilin-C, a Tag/Catcher pair we had previously engineered by splitting the *Streptococcus pyogenes* major pilin Spy0128\(^{19,20}\). We termed this approach PilinRing cyclization. We also tested sandwiching BLA between SnoopTag and SnoopCatcher, a peptide/protein pair we recently engineered from the RrgA adhesin of *Streptococcus pneumoniae*\(^{21,22}\) (SpyRing cyclization, Fig. 1A). SpyRing cyclization occurs through reaction of an amine with a carboxylic acid with release of water, but both SnoopRing and PilinRing cyclization occur through reaction of an amine with a carboxamide with release of ammonia (Fig. 1A)\(^{20,22}\). Cyclization was evaluated by the change in SDS-PAGE mobility upon cyclization, compared to negative controls having the key reactive lysine mutated to alanine (SpyTag KA-BLA-SnoopCatcher and Pilin-C KA-BLA-Isopeptag) (Fig. 1B). We found that the PilinRing system was efficient at cyclizing BLA when Pilin-C was fused to the N-terminus and Isopeptag was fused to the C-terminus (Fig. 1B). SpyRing cyclization was efficient when fusing SnoopTag on the N-terminus and SnoopCatcher on the C-terminus (Fig. 1B). Electrospray ionization mass spectrometry (MS) confirmed the loss of ammonia for both the PilinRing and SnoopRing upon isopeptide bond formation (Fig. S1). Small amounts of high molecular weight forms were produced by both PilinRing and SnoopRing systems (but not the point mutant controls), consistent with a proportion of intermolecular reaction, as seen with SpyRing cyclization (Fig. 1B). The \(k_{cat}\) and \(K_M\) values of the SpyRing, SnoopRing and PilinRing cyclized BLA constructs were comparable to those of the wild-type BLA construct (Fig. S2).

**Comparison of different cyclization domains for \(\beta\)-lactamase resilience.** We evaluated SpyRing and PilinRing aggregation-resistance by heating the enzymes for 10 min at various temperatures, centrifuging at high speed to remove aggregates, and analyzing the remaining soluble protein by SDS-PAGE with Coomassie staining. SpyRing was previously found to provide full aggregation-resistance to temperatures up to 100 °C\(^{10}\). For BLA itself, little protein remained in solution above 37 °C (Fig. 2A). SpyRing and PilinRing cyclization both led to major increases in aggregation resistance (Fig. 2A,B). For Pilin-C BLA-Isopeptag, there was little loss of soluble protein even after heating to 100 °C (Fig. 2A,B). For SnoopTag-BLA-SnoopCatcher, solubility was well preserved after heating to 100 °C, although there was more aggregation at 75 °C than at 100 °C (Fig. 2A,B).

We then tested recovery of catalytic activity after this heating step followed by cooling to room temperature. Similar to the aggregation data, nearly all catalytic activity of BLA itself was lost after 55 °C heating (Fig. 2C). SpyRing cyclization and PilinRing cyclization greatly improved the recovery of catalytic activity, although the retention of activity was less than with SpyRing cyclization, particularly after 75 °C heating (Fig. 2C). However, Pilin-C BLA-Isopeptag exhibited the best thermal resilience after incubation at 55 °C (Fig. 2C).

The non-cyclized control SnoopTag KA-BLA SnoopCatcher showed little difference in aggregation-resistance or catalytic recovery compared to BLA (Fig. S3A,C). The non-cyclized control Pilin-C KA-BLA-Isopeptag did have enhanced resilience to aggregation and more retention of catalytic activity, when compared to BLA, but was clearly inferior to the cyclized PilinRing (Fig. S3B,C).

**Calorimetry of \(\beta\)-lactamase unfolding with alternative cyclization domains.** To explore the difference in resilience for BLA cyclized by different domains, we performed DSC on SpyRing, PilinRing and SnoopRing systems. First we investigated the unfolding of the individual domains. SpyCatcher after reconstitution with SpyTag peptide unfolded at 88 °C, higher than SnoopCatcher reconstituted with SnoopTag peptide (70 °C) or Spy0128 (77 °C), the parent domain split to generate Pilin-C/Isopeptag (Fig. 3A).

We previously showed that SpyTag-BLA-SpyCatcher did not change the Tm for BLA unfolding, giving a similar peak at ~41 °C for BLA (Fig. 3A) and SpyTag-BLA-SpyCatcher\(^{18}\) (Fig. 3B,C). For SpyTag-BLA-SpyCatcher we also observed a peak from SpyTag/SpyCatcher unfolding at 85.0 °C (Fig. 3B,C)\(^{10,23}\). For the SpyRing and its KA control, surprisingly there was a small increase in the BLA Tm to 47.0 °C/47.6 °C (Fig. 3B,C). We suggest that the 64.1 °C peak is likely to correspond to unfolding of the SpyTag/SpyCatcher domain and the 52.2 °C peak to unfolding of the SnoopTag KA/SnoopCatcher domain (Fig. 3B,C).

For the PilinRing and its non-cyclized control, the Tm for BLA unfolding was ~41 °C, the same as SpyTag-BLA-SpyCatcher (Fig. 3B,C). We suggest that the 72.9 °C peak is likely to correspond to unfolding of the Pilin-C/Isopeptag domain and the 55.9 and 62.4 °C peaks to unfolding of Pilin-C KA-Isopeptag (Fig. 3B,C). Therefore, as for SpyTag/SpyCatcher, cyclization via an isopeptide bond-forming domain had no effect (PilinRing) or a minor effect (SnoopRing) on the transition temperature of the sandwiched BLA domain.
Overall, PilinRing cyclization improved enzyme resilience substantially, but gave less catalytic recovery than SpyRing cyclization. Since Pilin-C is also much larger than SpyCatcher (31 kDa rather than 12 kDa), subsequent investigation focused on advancing and understanding SpyRing cyclization.

SpyRing cyclization made phytase thermo-resilient. We previously showed how SpyRing cyclization conferred resilience on BLA (7 Å between termini) and dihydrofolate reductase (15 Å between termini)\textsuperscript{10}. To explore an industrially-important enzymatic activity with a much larger distance between termini, we tested cyclization of a phytase. The crystal structure of PhyC phytase from \textit{Bacillus subtilis} has been previously determined\textsuperscript{24}: the direct distance between the resolved N- and C-termini of PhyC is 29 Å (Fig. 4A). SpyTag-PhyC-SpyCatcher cyclized efficiently, based on the loss of H\textsubscript{2}O from isopeptide bond formation in MS (Fig. S4A) and the reduction in gel mobility compared to the non-cyclized SpyTag DA-PhyC-SpyCatcher control (Fig. 4B). SpyTag-PhyC-SpyCatcher remained active, exhibiting comparable specific activity to PhyC (Fig. S5). Heat-inactivation of phytases is a major challenge limiting their application for improving nutrition\textsuperscript{18}. PhyC remained soluble after heating at 55 °C but aggregated at 75 °C (Fig. 4C). SpyRing cyclization led to a major increase in PhyC thermal resilience, with most of the enzyme remaining in solution even after heating at 100 °C.
Most of PhyC's catalytic activity was lost following 75 °C heating, but the SpyRing PhyC retained most of its activity even after 90 °C heating (Fig. 4D).

The non-cyclized control, SpyTag DA-PhyC-SpyCatcher behaved similarly to PhyC in both aggregation-resistance and recovery of catalytic activity following heating, so covalent cyclization was essential for SpyRing-mediated resilience of PhyC (Fig. S4).

Secondary structure and calorimetric investigation of SpyRing phytase resilience. To understand better the basis for SpyRing phytase resilience, we looked for signatures of unfolding transitions upon heating. The Tm for unfolding of PhyC by DSC was not substantially shifted upon cyclization (PhyC 69.8 °C, SpyTag-PhyC-SpyCatcher 70.0 °C) (Fig. 5). For SpyTag-PhyC-SpyCatcher (but not PhyC or SpyTag DA-PhyC-SpyCatcher) a 2nd transition was seen at 91.5 °C, which is likely to correspond to unfolding of the SpyTag/SpyCatcher domain (Fig. 5). This temperature is slightly higher than the 85.0 °C SpyTag/SpyCatcher transition from SpyTag-BLA-SpyCatcher (Fig. 3A). The difference in Tm might be due to a difference in buffer used for DSC (Tm of 85.0 °C in PBS pH 7.4 and Tm of 91.5 °C in 50 mM Tris-HCl pH 7.0 with 2 mM CaCl₂) or might be an effect of PhyC stabilizing the SpyTag/SpyCatcher domain.

We then used circular dichroism (CD) to explore how heating affected secondary structure. We measured the ellipticity of the sample from 185–260 nm. For PhyC and SpyTag DA-PhyC-SpyCatcher there was little change in the spectrum at 55 °C, but at 75 °C and higher there was a general loss of signal, which did not recover upon cooling (Fig. S6). We observed aggregation for these proteins in the cuvette. However, for SpyRing phytase there was less perturbation at 75 and 90 °C (Fig. S6). The CD signal for SpyRing phytase did recover after cooling from
SpyRing enzymes could be purified simply by heating. Enzyme purification often uses multiple chromatography steps, which can be time-consuming and expensive. We hypothesized that the ability of SpyRing cyclization to give extreme resilience to heating could permit a simple route to purification, since most of the endogenous cellular proteins might aggregate with heating. Rather than starting with purified enzymes as previously, we explored heating the lysate obtained from enzyme over-expression in *E. coli*. PhyC was the major band on the Coomassie-stained gel of *E. coli* total cell lysate. While the heating at 75–90 °C removed most of the background proteins, heating also aggregated most of the PhyC (Fig. 6A). However, the SpyTag-PhyC-SpyCatcher band in the lysate showed minimal change in intensity after heating from 75–90 °C, while most endogenous proteins were depleted (Fig. 6A). Since 75 °C heating gave similar depletion of endogenous proteins as 90 °C.
Performing a similar approach for heat purification of SpyRing BLA also showed good recovery, with some impurities present and also more recovery of heat-stable SpyTag-BLA-SpyCatcher polymers than with Ni-NTA purification.
purification (Fig. 5B). Importantly for both SpyRing phytase (Fig. 6C) and SpyRing BLA (Fig. S7C), the recovered protein had comparable catalytic activity from heat purification when compared to affinity purification.

Discussion

We have shown that cyclization with SpyTag/SpyCatcher gave superior resilience to other spontaneous isopeptide-forming domains of distinct sizes and reactive chemistries. SpyRing cyclization conferred resilience on phytase, an industrially-important class of enzyme with termini 29 Å apart. The mechanism of SpyRing
resilience was studied through DSC and CD, indicating that cyclization by an isopeptide bond-forming domain did not change the temperature for initial unfolding but is likely to facilitate refolding. The resilience to thermal inactivation of SpyRings allowed normally heat-labile enzymes to be purified simply by heating of cell lysate.

The alternative isopeptide-forming partners, Pilin-C/Isopeptag and SnoopCatcher/SnoopTag, allowed efficient enzyme cyclization, even though their reaction rate (when tested intermolecularly) is slower than SpyCatcher/SpyTag. Both PilinRing and SnoopRing cyclization gave resilience to heat-induced aggregation/inactivation, even at 100 °C, so enzyme resilience from cyclization by isopeptide bond-forming domains is a general phenomenon. Non-reactive point mutants confirmed that isopeptide bond formation was important to the effect on enzyme resilience. Inability to form the isopeptide bond had two effects: lowering the Tm of the fused partner and untethering the termini, both likely to decrease the enzyme's resilience. The degree of resilience from PilinRing or SnoopRing cyclization for BLA was not as complete as with SpyRing cyclization. For PilinRing and SnoopRing cyclization, recovery of activity after 75 °C heating was less efficient than after 100 °C heating. Previous studies have suggested that temperature may influence the partition between different unfolded states, some of which favor refolding and some of which favor aggregation.

The importance of the cyclization framework for determining the extent of resilience is consistent with the large difference in BLA thermo-resilience reported through split intein cyclization (~5 °C increase) compared to SpyRing cyclization (~60 °C increase). It is also consistent with our previous work which showed that the cyclized SpyTag-DBL-SpyCatcher construct exhibited a greater thermal resilience than a BLA-CnaB2 fusion construct. PilinRing cyclization did give excellent resistance to aggregation, but after heating not all the soluble enzyme was functional. Resilience did not correlate with the size of the cyclizing domain, since Pilin-C is much bigger than SpyCatcher, but resilience did correlate with the unfolding temperature of the cyclizing domain. Interestingly all three cyclization systems stabilized the sandwiched enzyme from aggregation at 100 °C where DSC indicates that the Tag/Catcher domain would have unfolded.

SpyRing cyclization had a major effect in increasing the thermal resilience of PhyC phytase. Extensive efforts have been made to engineer different classes of phytases for high stability but phytase inactivation when food pellets are heat-sterilized is still a significant issue and so our SpyRing phytase provides a new direction for this challenge. Phytase supplementation has already shown success in human trials for improving dietary uptake of iron and zinc. Alternatives to phytase supplementation include the engineering of crops expressing phytase or “Enviropig”, a genetically-modified pig producing phytase in its saliva. However, eating transgenic organisms encounters a lot of resistance from the general public.

It is commonly known that enzymes from hyperthermophiles, such as Taq DNA polymerase from Thermus aquaticus, can be expressed in E. coli and purified simply by heating. Establishing such a simple purification route for enzymes that are not intrinsically thermostable could have academic and industrial importance. We showed here that SpyRing cyclization allowed substantial heat-purification of BLA and phytase with high retention of activity. Purification was not dissimilar to that achieved by affinity purification, although the protein was not purified to homogeneity. Nevertheless orthogonal polishing steps may then be used to purify further. Also, for many industrial processes, such as nutritional supplementation or biotransformations, moderate purity is often sufficient. Fusing proteins to thermostable domains was previously shown to allow stabilization and heat-purification of proteins of interest, but this required the protein of interest to be substantially smaller than the thermostable domain. In contrast, here SpyRing cyclization was effective in conferring resilience on fused enzymes substantially larger than SpyTag/SpyCatcher.

SpyRings represent a simple and versatile approach to increase the thermal resilience of enzymes. Future work will apply this technology to other globally-important enzymes and endeavor to improve resilience even further, by increasing SpyTag/SpyCatcher's Tm or by harnessing the bacterial diversity of isopeptide-forming domains.

Methods
Cloning. KOD Hot Start DNA Polymerase (Roche) was used as the primary polymerase for PCR. DNA was transformed into competent E. coli DH5α (Life Technologies). All constructs contained an N-terminal His₆ tag encoded in the pET28a plasmid (Novagen). The Bacillus subtilis PhyC insert (GenBank: JQ437256.1) was assembled using the DNAWorks method. The PhyC insert was amplified using primers 5′- CCGCAGCAAGGTTCGACGAGTCCGGTCATCTATCCGAC and 5′- GCCGCTCCGCTGCCACACTCCCTCTTGCCCAGATCGGTGAACCTACG. The insert was cloned into the SpyTag/SpyCatcher cassette using the method previously described to make pET28a SpyTag-PhyC-SpyCatcher (GenBank accession number KU608330) and pET28a SpyTag DA-PhyC-SpyCatcher. pET28a PhyC without the SpyTag/SpyCatcher fusions was cloned by amplifying the PhyC gene from pET28a SpyTag-PhyC-SpyCatcher using primers 5′- TATACATATGGGAAAACATAAAACGTAGCGATCGTTATATCCGAC and 5′- TTATAGCCTTCATTTTGGCCAGATCGGTGCTCGAATTCGAC.

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SpyRings represent a simple and versatile approach to increase the thermal resilience of enzymes. Future work will apply this technology to other globally-important enzymes and endeavor to improve resilience even further, by increasing SpyTag/SpyCatcher's Tm or by harnessing the bacterial diversity of isopeptide-forming domains.
and 5′-ATTAAAGCTTCTTATATGCGGTATCTGGTATTGTT. The pET28a SpyTag-BLA-SpyCatcher as a template. The Pilin-C fragment was generated using primers 5′-CCGCGGAGCTCGTTATGCGGAT against 5′-TCTCCGCTGCGTGGTGCCG using pET28a SpyTag-BLA-SpyCatcher as a template and primer 5′-TCTCTTTTTATCAGTAGATGTCTCTTG using pET28a Pilin-C-Blarypeptag (GenBank accession number KU608332) was cloned using Gibson assembly with Gibson Assembly Master Mix (NEB). The vector was amplified using primers 5′-TCCCATATGGCTGCCGCGGG and 5′-AACGTTCGCCCCGACTTGAGGCC using pET28a SpyTag-BLA-SpyCatcher as a template. The Pilin-C fragment was generated using primers 5′-CCGCGGAGGCACTAGGCTGTAAATGGTCATATTTCGGCGGTATCTGG and 5′-GGTGACCGGAACCCTCTAGGACTCTCTTATCCGTCATCTCTTATCAGTAGATGTCTCTTG using pET28a Pilin-C-Blarypeptag as a template. The Blarypeptag fragment was amplified using primers 5′-GTTCAGAGGGTTCTCGGTCCGAC and 5′-GCTCGAGCTGCGGCTGCAAGCTTTCATTATTGCATCTTATCGGTTCATTGGTG.
Every gel had a triplicate loading control to normalize the samples. Loading samples consisted of the protein sample that had been kept at 10 °C. The loading control was defined as 100% soluble fraction. Bands were quantified using densitometry values obtained from the Image Lab 3.0 software.

**β-lactamase thermal resilience enzymatic assay.** A 96 well clear flat-bottom polystyrene plate (Greiner) was pre-blocked with 200 μL PBS with 3% bovine serum albumin (BSA, ≥ 98%, Sigma) at 37 °C for 2 hours. The plate was then rinsed twice using 0.1 M NaH₂PO₄ pH 7.0 with 1 mM EDTA. 20 μL 25 μM BLA construct in PBS with 100 mM DTT was incubated at 10, 25, 37, 55, 75, 90 or 100 °C for 10 minutes and then cooled to 10 °C on a Bio-Rad C1000 Thermal Cycler (3 °C/s). The protein was diluted in 1 μL using 0.1 M NaH₂PO₄ pH 7.0 with 1 mM EDTA and 1% BSA. 3 nM of BLA construct was allowed to react with 100 μM nitrocefin (Merck) in 0.1 M NaH₂PO₄ pH 7.0 with 1 mM EDTA at 20 °C. The reaction was quenched at different time-points by adding an equal volume of 500 μM potassium clavulanate (Sigma) dissolved in 0.1 M NaH₂PO₄ pH 7.0 with 1 mM EDTA. Care was taken to protect the plates from direct light. Half of the volume was transferred into a clean 96 well clear flat-bottom polystyrene plate to remove bubbles that could interfere with absorbance measurements. OD₅₆₀ was measured using a SpectraMax M3 microplate reader (Molecular Devices). Absorbance was corrected for the dilution caused by the addition of potassium clavulanate. Samples were blanked against a control lacking enzyme.

**β-lactamase kinetics assay.** BLA activity was measured as previously. 2.0 mL sample cups (Kartell Labware) were pre-blocked with 50 mM NaH₂PO₄ pH 7.0 with 3% BSA at 37 °C for 2 hours. The samples cups were then rinsed twice using 50 mM NaH₂PO₄ pH 7.0. Enzymes were diluted in the sample cup to a concentration of 0.25 nM using 50 mM NaH₂PO₄ pH 7.0 with 0.1% BSA. Samples were then allowed to react in 50 mM NaH₂PO₄ pH 7.0 with 100, 50, 30, 20, 15, or 10 μM Nitrocefin (Oxoid) in a Galleries Plus Automated Photometric Analyzer (Thermo Scientific) at 30 °C. Readings were taken every 9 seconds for a period of 99 seconds at a wavelength of 500 nm (Δε₅₆₀ = 15,900 M⁻¹ cm⁻¹) [12]. The first 6 data points were linear and were used to obtain the initial rates of reaction. A Lineweaver-Burk plot was used to extrapolate the kinetic parameters. Errors for kinetic values were obtained by calculating the kinetics for 3 separate sets of data and then calculating the average and standard deviation of the 3 kinetic values.

**Phytase thermal resilience enzymatic assay.** A 96 well clear flat-bottom polystyrene plate (Greiner) was pre-blocked with 200 μL 50 mM Tris-HCl pH 7.0 with 2 mM CaCl₂, and 3% BSA at 37 °C for 2 hours. The plate was then rinsed twice using 50 mM Tris-HCl pH 7.0 with 2 mM CaCl₂, was incubated at 10, 55, 75, 90 or 100 °C for 10 minutes and then cooled to 10 °C on a Bio-Rad C1000 Thermal Cycler (3 °C/s). The protein was diluted in 1 μL using 50 mM Tris-HCl pH 7.0 with 100, 50, 30, 20, 15, or 10 μM Nitrocefin (Sigma) in a Galleries Plus Automated Photometric Analyzer (Thermo Scientific) at 30 °C. Readings were taken every 9 seconds for a period of 99 seconds at a wavelength of 500 nm (Δε₅₆₀ = 15,900 M⁻¹ cm⁻¹) [12]. The first 6 data points were linear and were used to obtain the initial rates of reaction. A Lineweaver-Burk plot was used to extrapolate the kinetic parameters. Errors for kinetic values were obtained by calculating the kinetics for 3 separate sets of data and then calculating the average and standard deviation of the 3 kinetic values.

**Phytase specific activity assay.** Phytase activity was measured as previously. 40 μM phytase was incubated with 1.6 mM phytic acid at 37 °C for 1, 2, 3, 4, or 5 minutes in 100 mM Tris-HCl pH 7.5 with 1 mM CaCl₂. The reaction was quenched by adding an equal volume of 20% trichloroacetic acid. Samples were then allowed to react in 50 mM ammonium heptamolybdate (Sigma) and 4.3% H₂SO₄ was mixed in a 4:1 ratio with a solution containing 178 mM FeSO₄ (Scientific Laboratory Supplies) to make the color mix solution. 1 volume of color mix solution was added for every 2 volumes of quenched reaction solution. The plates were spun at 3,500 g at 20 °C in a Multifuge X3R (Thermo Scientific) for 10 minutes. Within 8 minutes, 60% of the solution (90 μL of 150 μL) was transferred into a clean 96 well clear flat-bottom polystyrene plate and OD₇₀₀ was measured using a SpectraMax M3 microplate reader. Blank consisted of all the components minus phytase.

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**Heat purification.** Bacterial pellets obtained from an 800 mL bacterial culture were frozen at −80 °C and then thawed. Cells were resuspended in 8 mL 50 mM Tris-HCl pH 7.8 with 300 mM NaCl containing 1 mM phenylmethanesulfonyl fluoride (PMSF, Roche) and 1 μL EDTA-free mixed protease inhibitors (Roche, 1 tablet resuspended in 1.5 mL H₂O was 100 × stock). The OD₆₀₀ of the solution was 110−150 (as determined on a diluted sample of the resuspended cells). Cells were sonicated and centrifuged at 17,000 g for 20 minutes at 4 °C. The soluble fraction was removed. The OD₆₀₀ of the solution was 60 (as determined on a diluted sample). Half of the cell lysate was purified using Ni-NTA affinity chromatography as described above and dialyzed 3 times into 50 mM Tris-HCl pH 7.0 with 2 mM CaCl₂ (PhyC constructs) or PBS pH 7.4 (BLA constructs) for use in the activity comparison later on. Another half was diluted 1 in 10 in 50 mM Tris-HCl pH 7.0 with 2 mM CaCl₂ (PhyC constructs) or PBS pH 7.4 (BLA constructs) The sample was split into 50 μL aliquots and heated at 75, 80, 85 or 90 °C for 10 minutes in a Bio-Rad C1000 Thermal Cycler and then cooled to 10 °C. Samples were then centrifuged at 17,000 g at 4 °C for 30 minutes and the supernatant was removed and further analyzed. Samples heated to 75, 80, 85 or 90 °C were analyzed via SDS-PAGE to check their purity. For testing purity and activity, the concentration of SpyTag-PhyC-SpyCatcher from affinity purification was matched to the concentration of
SpyTag-PhyC-SpyCatcher from heat purification using densitometry of the SpyTag-PhyC-SpyCatcher band based on SDS-PAGE with Coomassie staining.

**Mass spectrometry.** The samples were analyzed using a Micromass LCT time-of-flight electrospray ionization mass spectrometer (Micromass UK). MassLynx V 4.00.00 (Waters Corporation) was used to convert the m/z spectrum to a mass spectrum using a Maximum Entropy algorithm. Predicted masses were obtained using ProtParam and by taking into account the change in mass that occurs when an isopeptide bond is formed \((-18\text{ for SpyCatcher, }-17\text{ for SnoopCatcher and Pilin-C})\)^{10,20,22}.

**DSC.** DSC profiles of PhyC, SpyTag (DA)-PhyC-SpyCatcher, SpyTag-BLA-SpyCatcher, SnoopTag (KA)-BLA-SnoopCatcher, Pilin-C (KA)-BLA-Isopeptag, BLA, Spy0128, SpyCatcher + SpyTag and SnoopCatcher + SnoopTag were measured on the VP Cap DSC (GE Healthcare). The DSC conditions to determine the Tm were 20 \(\mu\)M protein in PBS pH 7.4 (BLA constructs, Spy0128, SpyCatcher + SpyTag and SnoopCatcher + SnoopTag) or 50 mM Tris-HCl pH 7.0 with 2 mM CaCl2 (PhyC constructs) from 20 to 110 °C at a scan rate of 1 °C/min at a pressure of 3 atm. The baseline traces were subtracted from the sample traces and then normalized for concentration and volume using the Microcal DSC Origin Pro 7.0 software (GE Healthcare). Samples containing only buffer were used to obtain the baseline traces.

**CD.** Circular Dichromism experiments were performed in 10 mM Tris-SO4 pH 7.0 with 2 mM CaSO4 buffer. Samples were analyzed on a J-815 Circular Dichroism Spectrometer (Jasco) using a 1 mm path-length Spectrosil quartz cuvette (Starna Scientific). The temperature was controlled using a CDF-426S Peltier unit (Jasco). Far-UV CD spectra were obtained by incubating 0.15 mg/mL protein at 10 °C, before ramping at 12 °C/min to 55, 75 or 90 °C. Samples were held at that temperature for 3 minutes before starting a read (the “During heating” trace). The scanning speed of the instrument was set to 50 nm/min and scans were performed from 260 to 185 nm. After the read, samples were cooled to 10 °C. Samples were kept at 10 °C for 3 minutes before taking another read (the “After cooling” trace). Four scans were recorded from each independent sample, the recorded spectra were averaged, and then subtracted by the averaged baseline spectrum (buffer alone). Spectra were smoothed using the Savitzky-Golay method^{24} on the SpectraManager 2 software (Jasco). Ellipticity was converted to molar ellipticity using the SpectraManager 2 software.

**Molecular visualization.** Protein structures were rendered in PyMOL (Delano Scientific), based on Protein Data Bank files 1ZG4^{25}, 3B2M^{26}, 2WW8^{22}, and 3AMR^{24}.

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Author Contributions
C.S. performed the experiments. C.S. and M.H. wrote the paper. C.S., S.P.B. and M.H. designed the research and analyzed data. All authors reviewed the manuscript.

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Additional Information

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Competing financial interests: M.H. is an inventor on a patent concerning peptide reaction via spontaneous amide bond formation (EP2534484).

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