SpyRings declassified: a blueprint for using isopeptide-mediated cyclization to enhance enzyme thermal resilience

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Abstract

Enzymes often have marginal stability, with unfolding typically leading to irreversible denaturation. This sensitivity is a major barrier, both for de novo enzyme development and for expanding enzyme impact beyond the laboratory. Seeking an approach to enhance resilience to denaturation that could be applied to a range of different enzymes, we developed SpyRing cyclization. SpyRings contain genetically-encoded SpyTag (13 amino acids) on the N-terminus and SpyCatcher (12 kDa) on the C-terminus of the enzyme, so that the Spy partners spontaneously react together through an irreversible isopeptide bond. SpyRing cyclization gave major increases in thermal resilience, including on a model for enzyme evolution, β-lactamase, and an industrially important enzyme in agriculture and nutrition, phytase. We outline the SpyRing rationale, including comparison of SpyRing cyclization to other cyclization strategies. The cloning strategy is presented for the simple insertion of enzyme genes for recombinant expression. We discuss structure-based approaches to select suitable enzyme cyclization targets. Approaches to evaluate the cyclization reaction and its effect on enzyme resilience are described. We also highlight the use of Differential Scanning Calorimetry to understand how SpyRing cyclization promotes enzyme refolding. Efficiently searching sequence space will continue to be important for

enzyme improvement, but the SpyRing platform may be a valuable rational adjunct for conferring resilience.

Keywords

thermostability, protein engineering, enzyme evolution, aggregation, unfolding, synthetic biology, biotechnology, circular protein, evolvability, food security

Introduction

Understanding how to extend the scope of enzyme use is a central challenge in protein engineering. The potential of enzymes is illustrated by the fact that there are more than 100 enzymes already in large-scale industrial use. These applications range from laundry detergents and food processing to the cosmetics industry (Li, Yang, Yang, Zhu & Wang, 2012). Major challenges for taking promising enzymes from the laboratory into large-scale application include the high cost of production (Howard, Abotsi, Jansen van Rensburg & Howard, 2003) and poor stability at high temperatures (Lei, Weaver, Mullaney, Ullah & Azain, 2013). The most common strategies to enhance the thermal resilience of enzymes are structure-based or library-based, but these approaches are usually performed on a case-by-case basis (Yang, Liu, Li, Chen & Du, 2015; Packer & Liu, 2015).

To identify generic approaches to enhance stability, protein cyclization has been explored. Protein cyclization consists of covalently tethering the N- and C-termini of a protein, thereby reducing flexibility in two of the most mobile parts of the protein, while reducing the entropy gain on unfolding (Schumann, Varadan, Tayakuniyil, Grossman, Camarero & Fushman, 2015). Previous work on protein cyclization has utilized carbodiimide cross-linking (Goldenberg & Creighton, 1983), split inteins (Camarero & Muir, 1999; Evans, Brenner & Xu, 1999; Iwai & Plückthun, 1999; Scott, Abel-Santos, Wall, Wahnon & Benkovic, 1999) and sortase (Parthasarathy, Subramanian & Boder, 2007). In some cases, an increase in thermal resilience has been reported (Iwai & Plückthun, 1999; Antos, Popp,

Ernst, Chew, Spooner & Ploegh, 2009; Popp, Dougan, Chuang, Spooner & Ploegh, 2011; van Lieshout, Pérez Gutiérrez, Vroom, Planas, de Vos, van der Oost et al, 2012). We have developed an approach to cyclize proteins based on spontaneous isopeptide bond formation, which has been able to achieve >60 °C increases in thermal resilience (Schoene, Fierer, Bennett & Howarth, 2014; Schoene, Bennett & Howarth, 2016; Reddington & Howarth, 2015).

Isopeptide-mediated enzyme cyclization

Partners for spontaneous isopeptide bond formation

We have developed a range of different peptide/protein pairs able to react irreversibly. The story starts when Ted Baker's group solved the crystal structure of Spy0128, a pilin subunit originating from *Streptococcus pyogenes*, discovering the formation of an isopeptide bond in each of two domains between lysine and asparagine side-chains (Kang, Coulibaly, Clow, Proft & Baker, 2007). Mass spectrometry of Spy0128 validated isopeptide bond formation, while mutational analysis confirmed the three residues necessary for the formation of this bond (Kang et al, 2007). We were able to split the C-terminal β-strand of Spy0128 into a 16 amino acid tag, termed Isopeptag. Residues 18-299 of Spy0128 were separately expressed and called Pilin-C. By genetically fusing Isopeptag to maltose binding protein (MBP), we found that Isopeptag-MBP could reconstitute and form an isopeptide bond with Pilin-C (Zakeri & Howarth, 2010).

Further work was then carried out to find a faster and smaller reactive pair than Pilin-C/Isopeptag. The CnaB2 domain of the fibronectin-binding protein (FbaB) of *S. pyogenes* was found to form a spontaneous isopeptide bond between aspartic acid and lysine (Oke, Carter, Johnson, Liu, McMahon, Yan et al, 2010; Hagan, Bjornsson, McMahon, Schomburg, Braithwaite, Buhl et al, 2010). SpyTag, a 13 amino acid peptide tag containing the reactive aspartic acid residue, consisted of the C-terminal β-strand of the CnaB2 domain as well as the succeeding 5 residues of FbaB not included in the crystal structure. SpyCatcher was

generated from the remainder of CnaB2, which was further optimized by mutating two exposed hydrophobic residues (Figure 1A). SpyTag-MBP was able to react efficiently with SpyCatcher (second order rate-constant 1.4 x 10^3 $M^{-1}s^{-1}$). Reaction occurred in a wide range of buffers, including reducing or oxidizing conditions (Zakeri, Fierer, Celik, Chittock, Schwarz-Linek, Moy et al, 2012).

Next, we developed an orthogonal pair to SpyTag/SpyCatcher. The D4 domain of RrgA adhesin from *Streptococcus pneumoniae* was found to have an isopeptide bond between lysine and asparagine side-chains (Izoré, Contreras-Martel, El Mortaji, Manzano, Terrasse, Vernet et al, 2010). The N-terminal β-strand containing the reactive lysine residue (residues 734-745) was split and named SnoopTag. Residues 749-860 provided SnoopCatcher, which was further optimized by mutating two residues to stabilize secondary structure. SnoopTag/SnoopCatcher and SpyTag/SpyCatcher were found to be mutually unreactive. The combined use of both Tag/Catcher systems made it possible to build sequence-programmed protein chains (Veggiani, Nakamura, Brenner, Gayet, Yan, Robinson et al, 2016).

Cyclization with isopeptide bonds

Our initial studies with these peptide/protein ligation tools all focused on opportunities for locking together different protein units (Fierer, Veggiani & Howarth, 2014; Fairhead, Veggiani, Lever, Yan, Mesner, Robinson et al, 2014; Veggiani et al, 2016). However, the efficient reaction and flexible tag location suggested to us the opportunity to apply these pairs to drive intramolecular reaction. SpyTag/SpyCatcher (SpyRing, Figure 1B), Pilin-C/Isopeptag (PilinRing), and SnoopTag/SnoopCatcher (SnoopRing) were all shown to cyclize β-lactamase (BLA) efficiently (Schoene et al, 2016). All these cyclized constructs exhibited majorly enhanced thermal resilience, in comparison to the untagged control. We placed Pilin-C, SpyTag and SnoopTag on the N'-terminus and Isopeptag, SpyCatcher and SnoopCatcher on the C'-terminus of BLA but we have not verified whether flipping the

components around might work better. Therefore, further work could be carried out on testing whether the Tag should be on the N' or C'-terminus and vice versa for the Catcher. However, we found SpyRing cyclization to give the greatest enhancement in resilience (Schoene et al, 2016), so this approach will be the focus of this chapter. Such comparisons, along with testing linear controls, show how SpyRing resilience exceeds that simply from cyclization or fusion to a thermostable domain (Schoene et al, 2014, 2016). We proposed a mechanism whereby SpyRing cyclization confers resilience through favoring enzyme refolding after denaturation, blocking irreversible transitions to an unfolded aggregate (Figure 1C) (Schoene et al, 2014, 2016; Schumann et al, 2015).

We describe the selection of appropriate enzyme targets for cyclization, their insertion into the SpyRing construct, and the methods used to verify successful cyclization. Moreover, we focus on the cyclization of a β-propeller phytase (PhyC) and the enzymatic, solubility and biophysical characterization approaches one might use to study SpyRing-cyclized proteins.

Selection of enzymes for cyclization

SpyRing cyclization was envisioned to provide a simple method to stabilize different enzymes. In two weeks, one can go from obtaining the DNA for an enzyme of interest to testing the SpyRing enzyme's thermal resilience. However, we postulate that the following criteria are helpful when prioritizing enzyme candidates for SpyRing cyclization.

If a structure of the enzyme (or a close ortholog) is available, it is helpful to consider:

- the distance between the N- and C-termini,
- whether the active site is on the same face as the termini.

We suggest that enzymes with a short distance (i.e. <15 Å) between the N- and C-termini and where the active site is not closely apposed to the termini would be ideal candidates for SpyRing cyclization. In such cases, our experience is that SpyRing cyclization gave little or no change in enzyme activity. For BLA (7 Å from N- to C-terminus), we showed

minimal difference in k_{cat} or K_m (Schoene et al, 2016) between wild-type and SpyRing forms. The distribution of termini in the Protein Data Bank has been surveyed (Krishna & Englander, 2005) and a large fraction of single-domain proteins should be amenable based on this distance criterion. However, even where the termini are 29 Å apart as in PhyC, we obtained efficient SpyRing cyclization and increased thermal resilience (Schoene et al, 2016).

Previous successes with other cyclization approaches, as well as with circular permutations or disulfide bond engineering, might also give confidence that SpyRing cyclization would work. Intein-mediated cyclization of BLA led to an increase in aggregation resistance of ~5 °C (Iwai & Plückthun, 1999). Dihydrofolate reductase (DHFR) had previously been stabilized via cyanocysteine-mediated cyclization (Takahashi, Arai, Takenawa, Sota, Xie & Iwakura, 2007). We were able to show that both DHFR and BLA were rendered resilient by SpyRing cyclization (Schoene et al, 2014). However, if no data on cyclization are available, it is worth searching the literature for whether the enzyme remains active when fused to another protein, or whether a fusion to a thermostable protein increases the thermal resilience (Pierre, Xiong, Hayles, Guntaka & Kim, 2011).

SpyRing cyclization is unlikely to substitute for the activity of chaperones. If a protein requires chaperones to fold or refold, like RuBisCO (Liu, Young, Starling-Windhof, Bracher, Saschenbrecker, Rao et al, 2010) or firefly luciferase (FLuc) (Svetlov, Kommer, Kolb & Spirin, 2006), SpyRing cyclization might not be the right approach. Enzyme cofactors might also cause complications, especially if an enzyme cannot refold correctly following the loss of a cofactor, e.g. glucose oxidase (Zoldák, Zubrik, Musatov, Stupák & Sedlák, 2004).

So far we have worked primarily with monomeric enzymes. Generic strategies for stabilizing multimeric proteins will require further study.

Cloning genes of interest into the SpyRing cassette

SpyRing cyclization is achieved by genetically encoding the SpyTag on the N-terminus and the SpyCatcher on the C-terminus of an enzyme of interest. The expressed construct is then able to spontaneously form an isopeptide bond inside the expressing cell (Figure 1B). Therefore, SpyRing cyclization requires the insertion of the open reading frame of the enzyme within the SpyRing-expression cassette.

To achieve a quick, reliable and modular approach to insert different enzymes into the SpyRing cassette, we designed a ligation-independent cloning strategy (Figure 2). The cloning method requires two custom primers which can anneal to the coding sequence of the enzyme. The custom primers have tail-regions which can hybridize to the vector. The 5' tail-region is 27 bp long and the 3' tail-region is 23 bp long and both regions overlap with the vector sequence. This approach produces a glycine/serine spacer (GSGGSG) on both sides of the enzyme, to facilitate efficient cyclization by SpyTag/SpyCatcher and to minimize interference with enzyme folding. The template from which to amplify the vector sequence is readily available from the Addgene plasmid repository (ID: 52656). Once the insert and vector have been amplified and purified, the fragments can be linked together by Circular Polymerase Extension Cloning (CPEC) (Quan & Tian, 2009). We use KOD Hot Start DNA Polymerase (Roche) in the CPEC reaction. 500 ng of vector are mixed with an equimolar amount of insert in a 50 µL reaction. Cycling conditions are: 95 °C for 3 min followed by 10 cycles of 95 °C for 30 s, 64 °C for 30 s and 68 °C for 4 min. 1 µL of the CPEC reaction can be used to transform competent *Escherichia coli* cells (a $RecA^-$ strain such as XL-1 or DH5α).

We recommend CPEC because the method does not require any additional materials not already used to amplify the vector and insert. However, methods such as Gibson assembly (Gibson, Young, Chuang, Venter, Hutchison & Smith, 2009) should also be suitable.

Confirming successful cyclization

The cloned construct can then be expressed in *E. coli* protein production strains. We have obtained good yields using the BL21 DE3 RIPL strains from Agilent, which enables efficient translation even if the gene contains rare codons (Schoene et al, 2014, 2016). Two important parameters to consider are the temperature at which the constructs are expressed and the concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG). There are two types of covalent interactions that can occur once the SpyRing protein has been expressed: intramolecular covalent interactions leading to enzyme cyclization and intermolecular covalent interactions leading to enzyme oligomerization (Figure 3A). By decreasing the induction temperature and IPTG concentration, one can increase the proportion of monomeric cyclized protein (Zhang, Sun, Tirrell & Arnold, 2013). We found that growing the culture to an OD_{600} of ~0.5 and then inducing protein expression at 18 °C for 16 hours using 0.4 mM IPTG worked well to obtain a good yield of monomeric cyclized product.

The SpyRing cassette has an N-terminal His_6 tag, which can be used to purify the cyclized proteins via immobilized metal ion affinity chromatography (IMAC). To avoid eluting most of the oligomeric protein, we recommend using low concentrations of imidazole (i.e. 75 mM) to favor elution of proteins that have only a single His_6 tag (Howarth & Ting, 2008). If desired, complete removal of oligomeric side-products can be achieved by a subsequent size-exclusion chromatography step (Schoene et al, 2016).

An alternative method for the purification of the SpyRing enzyme is to use heat to precipitate *E. coli*'s native proteins, while the SpyRing enzyme largely remains soluble. The SpyRing enzyme in the soluble fraction can be separated from the precipitate using centrifugation (Schoene et al, 2016). Heat-mediated purification is an attractive option because it is rapid and cheap. An important consideration is the total concentration of protein in the cell lysate. If the concentration is too high, the cyclized protein may be lost to the precipitate as well, through interaction with other unfolded proteins. A cell lysate OD_{280} of ~6 is recommended. Furthermore, we found that the presence of NaCl can help improve the solubility of the cyclized protein (Schoene et al, 2016). Heat-mediated purification has also

been achieved previously by using thermostable proteins as fusion partners (de Marco, Casatta, Savaresi & Geerlof, 2004).

A first quick test for cyclization is to perform SDS-PAGE with Coomassie staining on the SpyRing enzyme, looking for a mobility shift compared to a linear point mutant unable to form an isopeptide bond (SpyTag DA or SpyCatcher EQ) (Zakeri et al, 2012). Complicated effects on mobility are seen from cyclization, depending on enzyme size and charge (Figure 3B-D). Moreover, the acrylamide percentage of the SDS-PAGE can alter the difference in mobility between the cyclized enzyme and the non-cyclized point mutant. The change in mobility resulting from cyclization is much larger than expected simply from the effect of the mutation on protein charge, reflecting the complex interaction between the conformation of the unfolded protein coated in SDS and the polyacrylamide network (Rath, Cunningham & Deber, 2013).

For a more definitive method to confirm isopeptide bond formation, Electrospray Ionization Mass Spectrometry (ESI-MS) can be employed to determine the exact mass of the protein constructs (Schoene et al, 2014, 2016). Since the formation of the isopeptide bond between SpyTag and SpyCatcher leads to the loss of a water molecule (Figure 1B), the mass determined by ESI-MS should be 18 Da lighter than the predicted molecular weight of the linear protein from the ProtParam online tool (Gasteiger, Hoogland, Gatticker, Duvaud, Wilkins, Appel et al, 2005). A possible complication in the spectrum from *E. coli* expression is gluconylation of the His_6 tag (Geoghegan, Dixon, Rosner, Hoth, Lanzetti, Borzilleri et al, 1999). For ESI-MS, samples should be expressed in *E. coli* B834 DE3, grown in the absence of any supplementary glucose in the LB media to an OD_{600} of ~0.5, and induced at 30 °C for 3 hours using 0.4 mM IPTG. Samples should then be purified using the previously described IMAC approach, before dialyzing into 10 mM ammonium acetate buffer (a low ionic strength buffer with volatile components is important to obtain clean spectra).

Characterization of enzyme resilience after SpyRing cyclization

Industrial relevance of Phytase

We illustrate the process of characterizing enzyme resilience with particular reference to SpyRing cyclization of phytase. Most plants store phosphates as phytic acid (inositol hexakisphosphate). Non-ruminant animals such as chicken, pigs and salmon are unable to degrade phytic acid and therefore find it difficult to absorb the necessary dietary phosphate for a healthy diet. Moreover, phytic acid is an anti-nutrient, able to chelate and block absorption of micronutrients like zinc and iron (Bohlke, Thaler & Stein, 2005; Selle, Ravindran, Bryden & Scott, 2006). Therefore, the enzyme phytase is widely used to release phosphate from phytic acid in animal feed. However, during the pelleting process, feed-stock is heated to 75-90 °C to kill pathogenic bacteria, notably Salmonella (Lei et al, 2013). A phytase variant able to regain full activity after this pelleting process would be a significant advance.

Humans are also unable to degrade phytic acid. This anti-nutrient may contribute to the global health problem of micronutrient deficiency (Bailey, West & Black, 2015), the “silent hunger” with a range of short-term and long-term health consequences (Allen, de Benoist, Dary & Hurrell, 2006). Food security is a major global issue- increasing the nutritional value of food that is already available will be an important part of the solution.

Measuring aggregation can provide evidence for an increase in thermal resilience

The first simple and quick test we use to evaluate a SpyRing enzyme is to measure aggregation at elevated temperature. Heat-induced aggregation correlates with a loss of activity and irreversible inactivation of enzymes. To test whether SpyRing cyclization has improved an enzyme’s thermal resilience, we recommend that a SpyTag D→A linear point mutant as well as a construct lacking both SpyTag and SpyCatcher are used as controls. The test should be run in the wild-type enzyme’s preferred buffer. Enzyme concentration can be critical to the extent of aggregation. Therefore, we recommend using a concentration

relevant to the application one has in mind for the enzyme of interest. 10 µM enzyme is a good starting point.

We recommend the use of a PCR machine to heat and then cool the protein for a precise amount of time with a reproducible ramp-rate. For example, heat the proteins at 25, 37, 55, 75, 90 and 100 °C for 10 min and then cool to 10 °C for 1 hr. After cooling for 1 hr, centrifuge the samples for 30 min at 17,000 g, before analyzing the soluble fraction by SDS-PAGE. Coomassie-stained bands can then be quantified using densitometry.

In the case of PhyC, SpyRing cyclization improved the solubility of the enzyme at temperatures at and above 75 °C (Figure 4A). However, an aggregation assay alone should not be used as conclusive evidence on whether SpyRing cyclization is helpful.

Recovered activity is the most important measurement when testing thermal resilience

Aggregation can be used as an initial indicator to determine whether SpyRing cyclization increases the thermal resilience of an enzyme. However, the aggregation assay does not take into account the formation of soluble aggregates and the possibility that an enzyme does not aggregate but misfolds and becomes irreversibly inactivated. For example, the non-cyclized control SpyTag DA-DHFR-SpyCatcher did not aggregate at 100 °C but the activity assay showed that the enzyme was irreversibly inactivated at this temperature (Schoene et al, 2014). Therefore, the best assay to use is one that measures the residual enzyme activity after heating.

The enzyme's temperature treatment should be the same as described above, so a correlation can be drawn between the enzyme's recovered activity and solubility. However, since the enzyme will be at a high concentration (~10 µM) for the solubility assay, one might need to dilute the enzyme down to nM concentrations for the activity assay. Finding the right concentration of enzyme for your assay is important, so an enzyme titration to where the

initial activity can be measured using at least 5 time-points is a good starting place. When diluting the enzyme, reagent stability will need to be taken into account. Enzymes at low concentrations will readily stick to plastic surfaces and be removed from solution. Therefore, plastic containers should be pre-blocked with 1 – 3 % bovine serum albumin (BSA) at 37 °C for 2 hours and the dilution buffer should include BSA at a concentration of 0.1 – 1 %.

We recommend that the activity recovery assay is set up in 96-well plate format to allow for high-throughput. Ideally, an enzyme substrate should be used with an absorbance or fluorescence read-out. For plate-reader measurement, the delay time between each read should be considered and how that might affect the reading of the initial catalytic activity.

In the case of PhyC, there are no fluorescent or colorimetric analogs of phytic acid currently available, so reactions were quenched with trichloroacetic acid and the released phosphate was measured colorimetrically using the ammonium-molybdate method (Schoene et al, 2016). Cyclization provided a large improvement in recovered activity of PhyC after heating to the industrially-relevant temperatures of 75 and 90 °C (Figure 4B).

Firefly luciferase is an example of a poor target for SpyRing cyclization

FLuc is an important reporter enzyme used to study gene expression, monitor protein-protein interactions, and sequence DNA. However, FLuc's instability over 30 °C is a problem for various applications (Ebrahimi, Hosseinkhani, Heydari, Khavari-Nejad & Akbari, 2012). SpyRing cyclization of FLuc decreased the mobility on SDS-PAGE, compared to the linear DA control (Figure 3D) and therefore we postulated that the cyclization reaction had been successful. However, when we tested the recovered activity of the cyclized FLuc, improvements were only marginal when compared to the linear point mutant (Figure 4C).

The following tips might help to avoid testing cyclization on poor targets. A circular permutation of FLuc in combination with intein-mediated cyclization has been shown to inactivate the enzyme (Kanno, Yamanaka, Hirano, Umezawa & Ozawa, 2007). Cyclization of a circularly permuted FLuc was used to create a reporter for caspase-3, by placing a

caspase cleavage site between the N- and C-termini. Upon cleavage the termini are able to move and the enzyme becomes active (Kanno et al, 2007). While our cyclized FLuc was still active (Figure 4C), enzymes which have been shown to be less active when the termini are restrained will probably be hard to work with.

FLuc has also been shown to require the help of chaperones when refolding from a heat-denatured state (Schröder, Langer, Hartl & Bukau, 1993). Moreover, misfolding involves the formation of a stable non-native conformation where the N- and C-terminal residues are interacting (Scholl, Yang & Marszalek, 2014). Therefore, FLuc SpyRing cyclization might not be able to stop this interaction from occurring unless chaperones are present.

Dynamic scanning calorimetry is a useful biophysical tool to study SpyRing cyclization

Dynamic scanning calorimetry (DSC) has provided an effective approach to follow the thermal unfolding and refolding of SpyRing cyclized proteins (Schoene et al, 2014). DSC measures the difference in heat required to increase the temperature of a protein sample in comparison to a reference cell. When proteins unfold, the hydration of the core causes an increase in heat capacity (Privalov & Makhatadze, 1992). Preparation of samples for DSC is simple: no dyes are necessary since the signal monitored is the change in specific heat capacity. We have used the VP Cap DSC system from GE Healthcare, which requires 400 µL of 20 µM protein and ~4 mL of buffer for a single run.

The ramp-rate for a single scan from 20-110 °C was set to 1 °C/min, which produced high resolution data (Figure 4D). DSC data indicated that the SpyRing did not increase the transition temperature of PhyC (PhyC: 69.8 °C, SpyTag DA-PhyC-SpyCatcher: 69.7 °C, SpyTag-PhyC-SpyCatcher: 70.0 °C). SpyTag-PhyC-SpyCatcher unfolded as two transitions. The second transition had a Tm of 91.5 °C, which is close to the Tm we measured for SpyCatcher + SpyTag peptide melting (Schoene et al, 2016).

Such data are consistent with mechanism proposed for the increase in thermal resilience observed in PhyC, DHFR and BLA, where SpyRing cyclization does not block unfolding but promotes efficient refolding (Figure 1C). The increase in thermal resilience of the cyclized enzyme also extended higher than the Tm of the SpyTag/SpyCatcher domain (Schoene et al, 2014, 2016).

A down-side of using DSC is that it is low-throughput. Even automated DSC systems are unable to process more than 50 samples/day (Plotnikov, Rochalski, Brandts, Brandts, Willston, Frasca et al, 2002). Dynamic scanning fluorimetry (DSF) is able to provide a much higher throughput (Niesen, Berglund & Vedadi, 2007). However, DSF requires the use of a dye which can interfere with the transition temperature of a protein (Shi, Semple, Cheung & Shameem, 2013). Alternatively, microscale thermophoresis is a recently-developed high-throughput method, which can monitor unfolding from the intrinsic protein fluorescence (Alexander, Wanner, Johnson, Breitsprecher, Winter, Duhr et al, 2014).

Concluding remarks

We have developed three distinct peptide-protein pairs (Zakeri & Howarth, 2010; Zakeri et al, 2012; Veggiani et al, 2016) which are able to cyclize inside cells and increase enzyme resilience (Schoene et al, 2016). SpyRing cyclization requires the genetic encoding of a 13 amino acid tag, SpyTag, at the N-terminus of a protein and a 12 kDa protein, SpyCatcher, on the C-terminus. Cloning of an enzyme of interest into the SpyRing cassette is simple and modular. SpyRing enzymes can easily be purified through IMAC or heat purification. SpyRing cyclization may provide a quick and generic route towards enzyme stabilization. We have provided guidelines for the selection of appropriate targets for SpyRing cyclization but exploring this strategy on a wider diversity of enzymes (expression hosts, protein folds multimerization state, enzyme classes) will help to advance the scope of this approach.

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Figure legends

Figure 1 SpyTag and SpyCatcher for enzyme cyclization. (A) The CnaB2 domain of FbaB was split into SpyTag and SpyCatcher. Cartoon based on PDB 2X5P (Oke *et al* 2010). (B) Expression of the SpyRing genetic construct leads to *in vivo* cyclization. The formation of the isopeptide bond is dependent on the three illustrated residues: reaction between Lys and Asp promoted by an apposed Glu. (C) Proposed principle of SpyRing-mediated thermal resilience, by favoring reversible transition between the native (N) and unfolded intermediate states (U_{int}), avoiding irreversible transition to the unfolded aggregate state (U_{agg}).

Figure 2 Cloning strategy to insert an enzyme into the SpyRing expression cassette. (A) The enzyme of interest is amplified by PCR using primers containing adaptor sequences, to generate an overlap at each end with the vector. The vector is amplified using pET28a SpyTag-BLA-SpyCatcher as a template, with the primers hybridizing to linker regions of the construct. (B) The vector and insert are then combined through a ligation-independent cloning method such as CPEC, to generate the desired SpyRing expression plasmid.

Figure 3 Gel analysis of SpyRing enzyme cyclization. (A) Cartoon illustrating how fusing SpyTag on the N-terminus and SpyCatcher at the C-terminus can lead to intermolecular or intramolecular covalent bond formation. (B) SpyRing cyclization decreased the mobility of BLA. Reducing SDS-PAGE with Coomassie staining, showing SpyTag-BLA-SpyCatcher compared to the non-cyclized controls of SpyTag DA-BLA-SpyCatcher and SpyTag-BLA-SpyCatcher EQ. “Oligomers” indicates the side-products from intermolecular SpyTag/SpyCatcher reaction. Data previously published (Schoene *et al* 2014). (C) SpyRing cyclization increased the mobility of DHFR. Reducing SDS-PAGE with Coomassie staining for SpyTag-DHFR-SpyCatcher and DA or EQ linear controls. (D) SpyRing cyclization decreased the mobility of FLuc. Reducing SDS-PAGE with Coomassie staining for SpyTag-FLuc-SpyCatcher and the DA control.

Figure 4 SpyRing cyclization effect on thermal resilience. (A) SpyRing cyclization improved the solubility of PhyC at high temperatures. Samples were heated to the indicated temp for 10 min, cooled to 10 °C, and the supernatant was analyzed by SDS-PAGE and Coomassie staining (mean of triplicates ± 1 s.d.). Data previously published (Schoene *et al* 2016). (B) Cyclization improved the recovered activity of PhyC. Samples were heated to the indicated temp for 10 min and cooled to 10 °C. Phosphate release from phytase was quantified colorimetrically (mean of triplicate ± 1 s.d.). (C) Cyclization had a minor effect on FLuc activity recovered after heating. Samples at 5 µM in PBS pH 7.4 containing 100 mM DTT were heated to the indicated temp for 10 min and cooled to 10 °C. 12.5 nM enzyme was reacted with 300 µM luciferin in 50 mM Tris-acetate pH 7.8, 10 mM $MgSO_4$, 4 mM ATP and 2 mM EDTA. Luminescence was measured after a 15 s delay (mean of triplicates ± 1 s.d.). (D) DSC of SpyTag-PhyC-SpyCatcher overlaid with the untagged PhyC construct and the DA point mutant. Scanning from 20-110 °C at 1 °C/min. Data previously published (Schoene *et al* 2016).

Figure 1

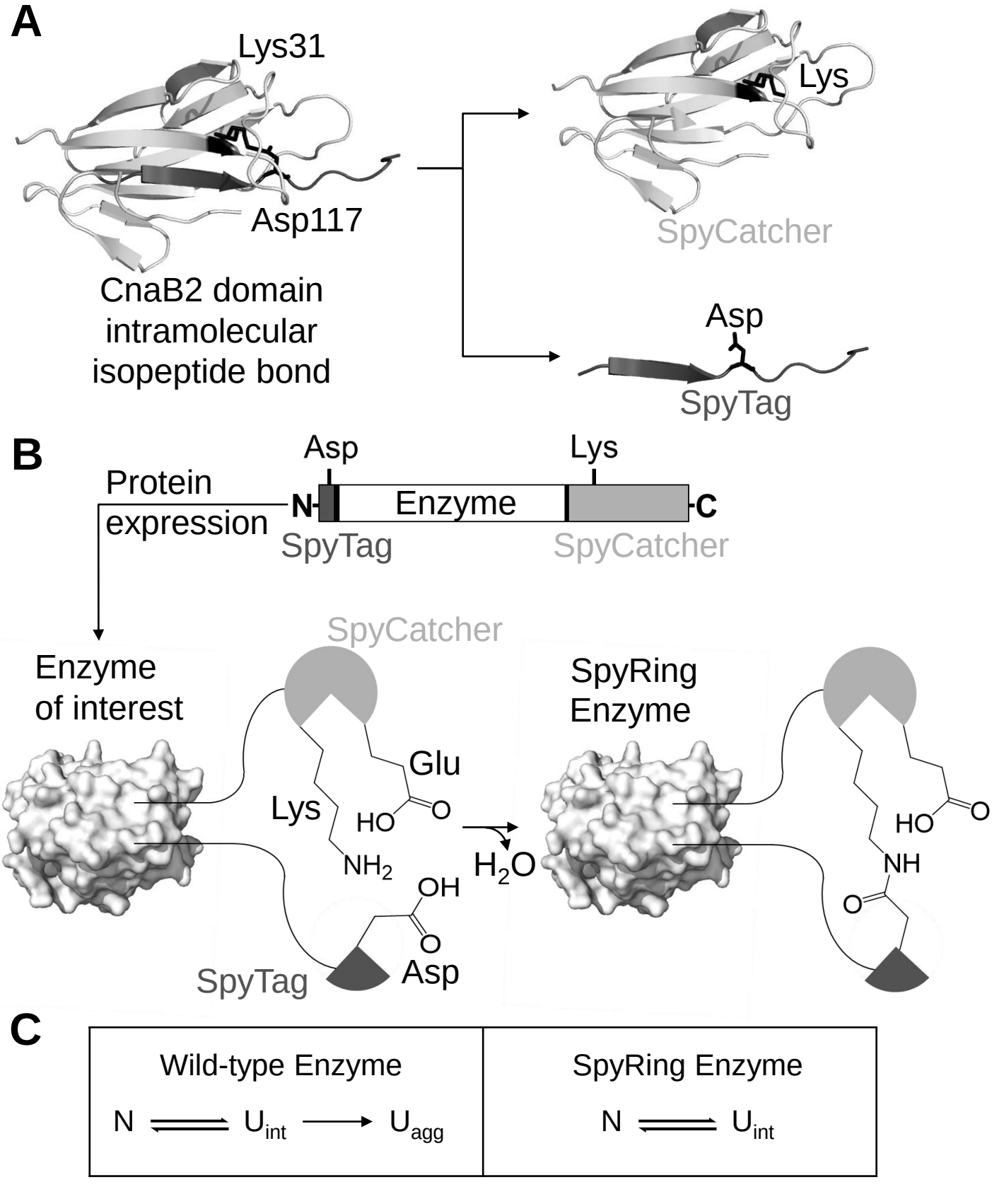

A
Lys31
Asp117
CnaB2 domain
intramolecular
isopeptide bond
Lys
SpyCatcher
Asp
SpyTag
B
Protein
expression
Asp
Lys
N
Enzyme
C
SpyTag
SpyCatcher
SpyCatcher
Enzyme
of interest
Glu
Lys
HO
O
NH2
OH
O
Asp
SpyTag
H2O
SpyRing
Enzyme
HO
O
NH
O
C
Wild-type Enzyme
N ⇌ Uint → Uagg
SpyRing Enzyme
N ⇌ Uint

Figure 2

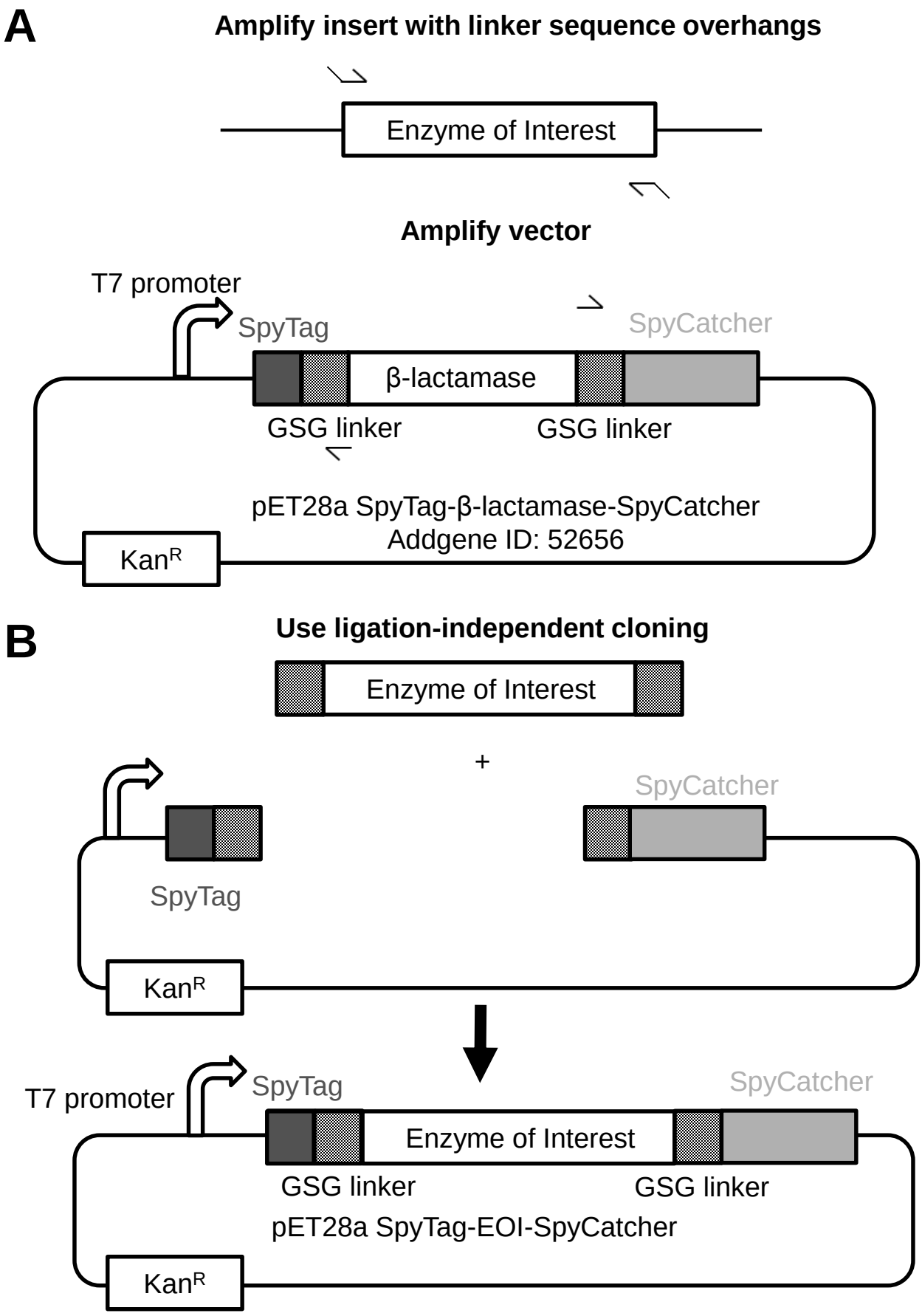
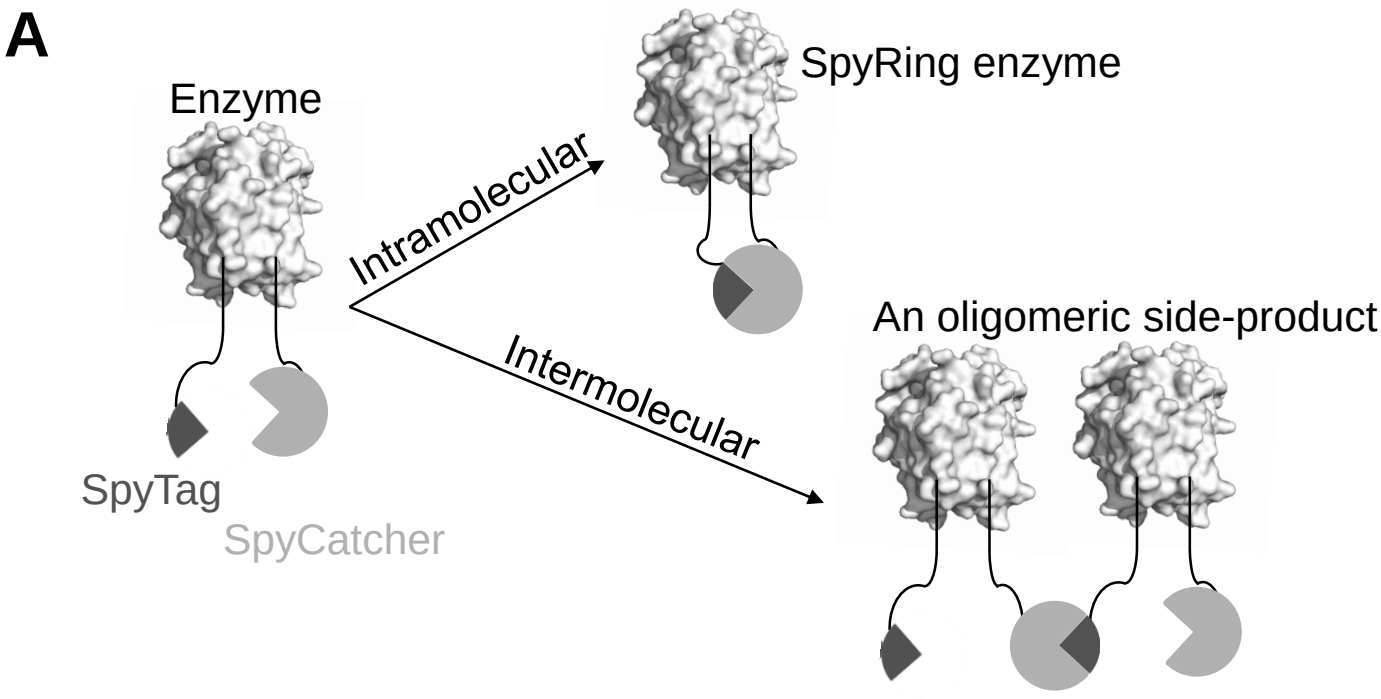
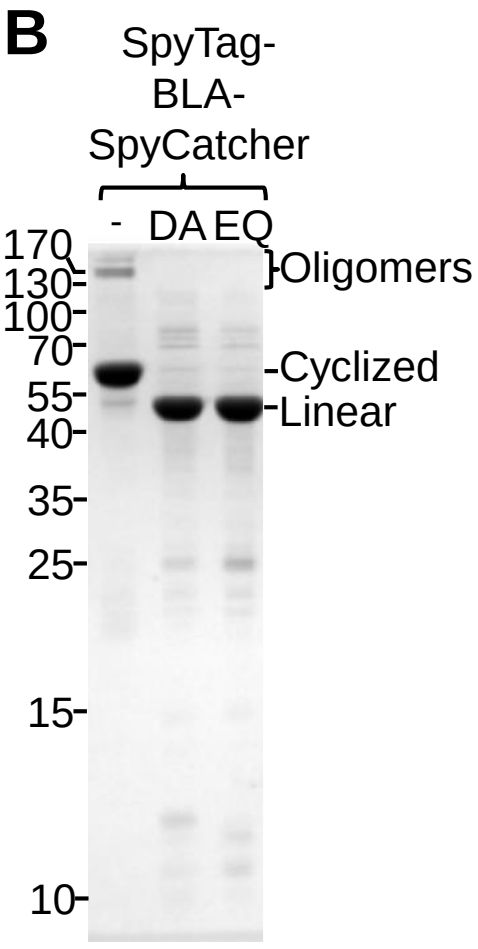
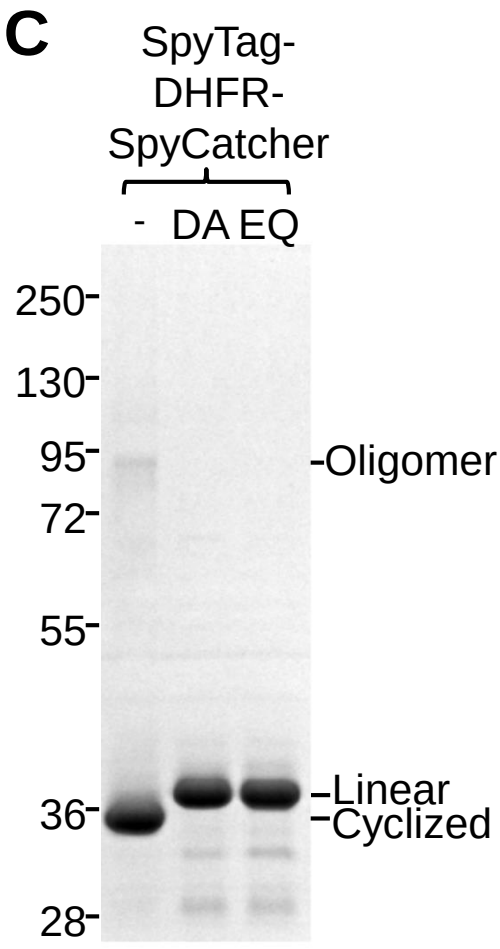

A
Amplify insert with linker sequence overhangs
Enzyme of Interest
Amplify vector
T7 promoter
SpyTag
SpyCatcher
β-lactamase
GSG linker
GSG linker
pET28a SpyTag-β-lactamase-SpyCatcher
Addgene ID: 52656
Kan^R
B
Use ligation-independent cloning
Enzyme of Interest
+
SpyCatcher
SpyTag
Kan^R
T7 promoter
SpyTag
SpyCatcher
Enzyme of Interest
GSG linker
GSG linker
pET28a SpyTag-EOI-SpyCatcher
Kan^R

Figure 3


A
Enzyme
SpyTag
SpyCatcher
Intramolecular
SpyRing enzyme
Intermolecular
An oligomeric side-product


B
SpyTag-
BLA-
SpyCatcher
- DA EQ
170
130
100
70
55
40
35
25
15
10
Oligomers
Cyclized
Linear


C
SpyTag-
DHFR-
SpyCatcher
- DA EQ
250
130
95
72
55
36
28
Oligomer
Linear
Cyclized

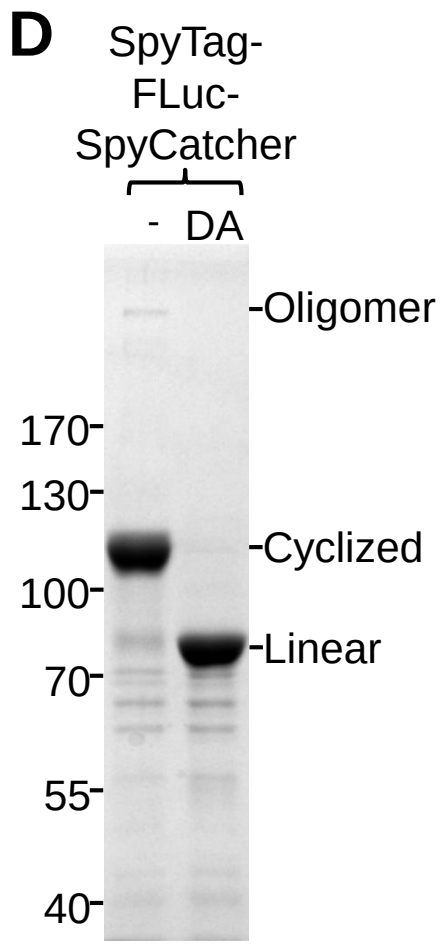
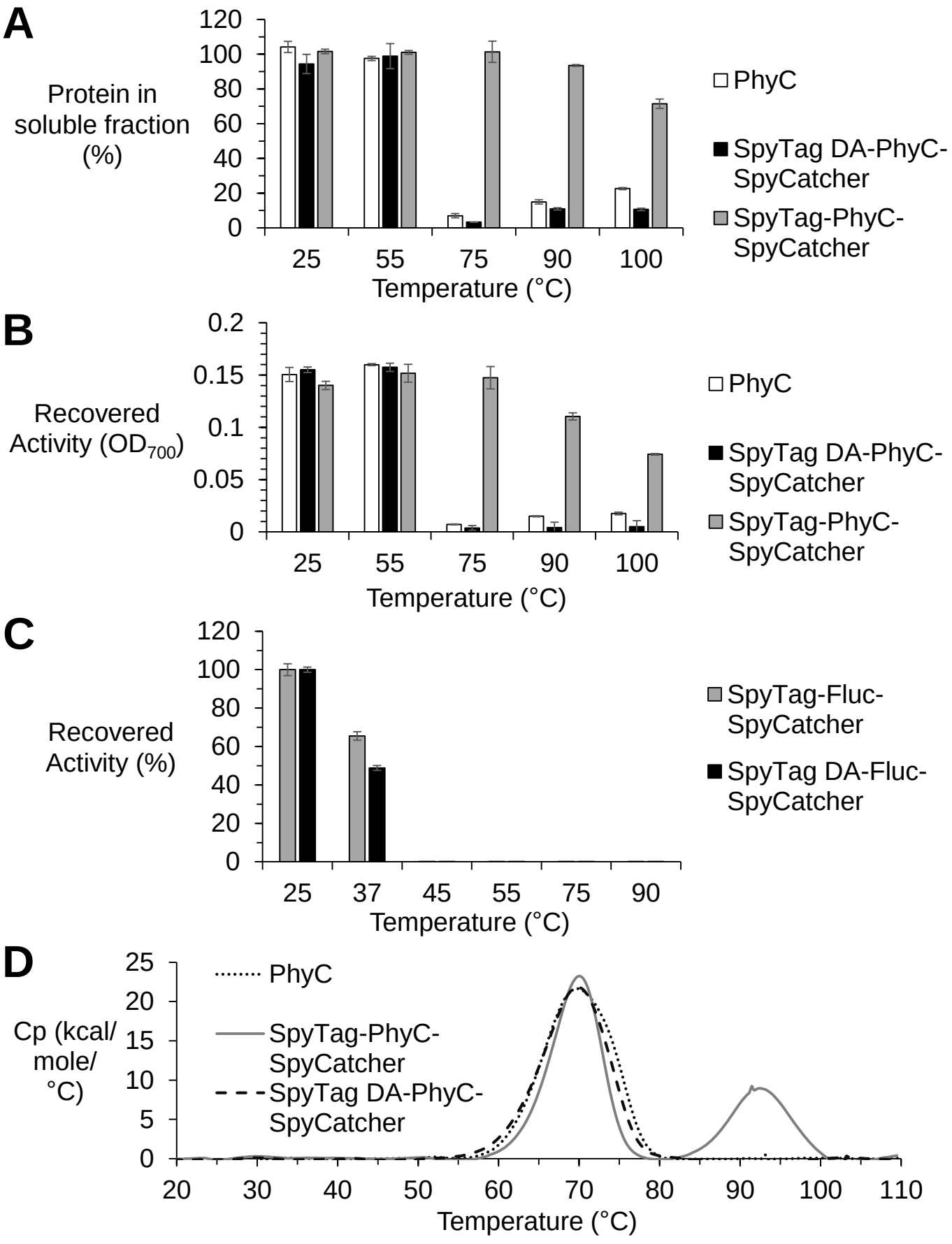

D
SpyTag-
FLuc-
SpyCatcher
- DA
Oligomer
170
130
100
70
55
40
Cyclized
Linear

Figure 4


A
Protein in soluble fraction (%)
120
100
80
60
40
20
0
25
55
75
90
100
Temperature (°C)
PhyC
SpyTag DA-PhyC-SpyCatcher
SpyTag-PhyC-SpyCatcher
B
Recovered Activity (OD_{700})
0.2
0.15
0.1
0.05
0
25
55
75
90
100
Temperature (°C)
PhyC
SpyTag DA-PhyC-SpyCatcher
SpyTag-PhyC-SpyCatcher
C
Recovered Activity (%)
120
100
80
60
40
20
0
25
37
45
55
75
90
Temperature (°C)
SpyTag-Fluc-SpyCatcher
SpyTag DA-Fluc-SpyCatcher
D
Cp (kcal/mole/°C)
25
20
15
10
5
0
20
30
40
50
60
70
80
90
100
110
Temperature (°C)
PhyC
SpyTag-PhyC-SpyCatcher
SpyTag DA-PhyC-SpyCatcher