Fine-tuning an engineered intein

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Although it is possible to introduce desirable properties and activities into proteins using rational design, subtle changes necessary to make an engineered product efficient and practical are often still beyond our predictive capacity[1]. In this issue, Belfort and colleagues[2] describe an elegant mutational strategy to engineer an intein with improved features to serve as a tool for protein purification. Previously, this group created a mini-intein by removing an internal domain encoding an endonuclease activity required for horizontal transfer of the intein gene[3]. The endonuclease domain is distinct and separate in function, structure, and evolution from the protein splicing domain. The resulting mini-intein could splice, but not as efficiently as its unmodified progenitor. In the current work, this mini-intein was further engineered to both enhance the splicing efficiency and allow it to cleave solely at its C-terminal end.

Inteins are proteins whose genes are found only within the genes of other proteins. Although the intein and host gene are translated as a single polypeptide, the intein initiates an autocatalytic event to remove itself and join the flanking host segments with a new polypeptide bond. In addition to their protein splicing activity, many inteins also possess endonucleic activity that probably serves to propagate their genes to new integration sites. Thus, inteins are selfish genetic elements whose known functions only benefit themselves. Inteins are found in all three domains of life, yet in only 30 species. No host factors are needed for the protein splicing activity, and the process appears very efficient[1]. These novel attributes make inteins of potential utility in biotechnology, and several research groups are engineering them for particular applications. For example, intein-based systems have been used to ligate proteins. This enables the expression of toxic proteins[5] (made in two inactive parts and joined after purification), the generation of novel protein combinations[6], and the selective labeling of parts of proteins[9] (especially useful for NMR studies of large proteins).

Belfort and coworkers first developed a genetic screen to directly select for enhanced intein function. The altered intein gene was randomly mutated and inserted inside a thymidylate synthase (TS) gene. The product of the resulting gene will only function if the intein splices itself out. Highly active inteins were then selected for in an *Escherichia coli* strain deficient in TS activity. Next, a second screen was developed to select for inteins that preferentially cleaved their C-terminal end. C-terminal cleavage normally both follows and depends on cleavage of the N-terminal end. The modified intein also cleaved its C terminus in vitro with a strong dependence on pH, suggesting it could be exploited to create a system to purify recombinant proteins. If a target protein were inserted C-terminal to an intein protein tag, the chimeric protein could be overexpressed and affinity-purified using the tag. The product could be released from the tag by simply reducing the pH to induce intein cleavage.

A single conservative substitution (V67L) was found to restore high activity to the truncated mini-intein. The change seems to stabilize the protein by modifying its hydrophobic core, which was probably compromised by the previous removal of the central endonuclease domain. Thus, though separate in function and structure from the protein splicing domain, the endonuclease domain does contribute to overall intein stability. An additional single mutation (D422G) modified the splicing activity to favor C-terminal cleavage. Although the activity of the modified intein is consistent with the role of this

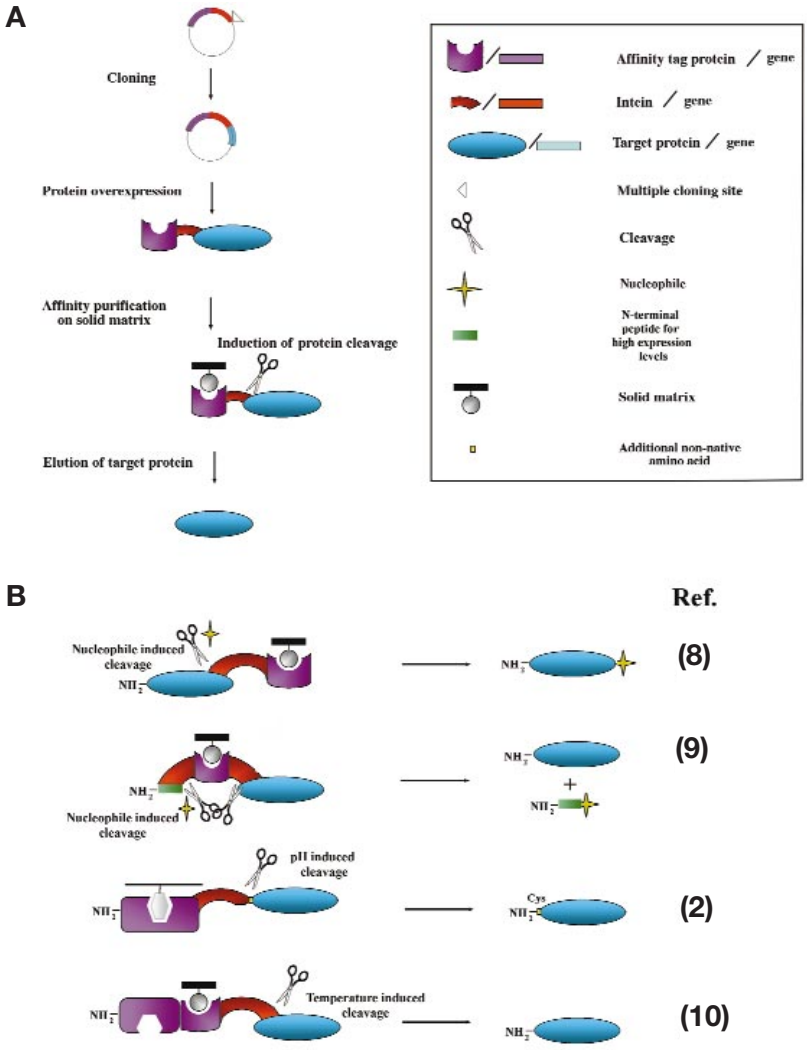

Figure 1. An inside look at protein purification strategies using inteins. (A) A schematic general representation of an intein-based system for protein purification. (B) Different specific intein-based protein constructs for purification, their cleavage induction, and resulting purification products.

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residue predicted from a crystal structure[8], the precise function of this residue and the effects of its substitution were far from obvious, and thus could not have been exploited by a rational design approach.

The system developed by Belfort et al. complements a series of other protein purification systems that make use of an intein fused to a protein tag[9–11] (see Fig. 1). The main advantage of all these systems is that release of the purification tag and intein does not require proteases or toxic chemicals that would otherwise necessitate a further purification step. Intein-based purification systems differ from one another primarily by the mechanism of cleavage induction and relative position of the target protein and intein sequences (Fig. 1B). In the two most recently reported systems, cleavage is induced by either pH[2] or temperature[11], and the target protein is expressed at the C-terminal end of the chimeric protein. Only the target protein was eluted in these two systems. In contrast, other intein-based systems use nucleophilic agents (e.g. thiol agents such as DTT) to initiate cleavage, and as a result the target protein is covalently linked to the agents (Fig 1B). Another advantage is the placement of the intein and target protein C-terminal to a well-expressed bacterial protein, maltose-binding protein, leading to better expression of the whole chimeric protein.

What will interest researchers most is how easily applied these systems will be to the proteins they study. Wild-type inteins are integrated in at least 45 different sites in diverse protein coding genes[12], and no consensus integration sequence has been identified. Nevertheless, the regions immediately flanking inteins have been found to affect the efficiency of splicing[10–11], and some protein hosts might be incompatible with intein activity. Although high expression and product purity are important considerations, they are moot if the final product is inactive. Intein-based systems for recombinant protein production and purification have been commercially available for about three years. Although both developers and users have reported success in purifying a variety of proteins (e. g. 10), cases where the system did not work are not likely to be widely reported. In this context, the utility of the current system must also be established empirically. Nevertheless, the important message from this study is that the use of a genetic selection strategy can refine the activities of engineered proteins to an extent not currently possible with rational design. The design of appropriate selection systems can thus enable nature to give us a hand to fine-tune engineered proteins for practical application.

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New tools for chloroplast genetic engineering

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Environmental concerns have led to a growing public wariness of genetically modified crops around the world. A common concern is the possibility of gene escape through pollen or seed dispersal from crop plants engineered for herbicide resistance to their weedy relatives, possibly creating "superweeds" or causing gene pollution among other crops. The introduction of genes via chloroplast genetic engineering was recently advanced as a potential solution to this problem. In this issue, two new important tools are described that should facilitate the development of the field of chloroplast genetic engineering: a novel DNA delivery system for eukaryotic organelles[1] and a fluorescent antibiotic marker to track plastid transformation[2].

Keeler et al.[3] recently summarized valuable data on the weedy wild relatives of 60 important crop plants and potential hybridization between crops and wild relatives. Among 60 crops, only 11 have no congeners (members of the same genus); the rest of the crops have wild relatives somewhere in the world. Maternal inheritance of foreign genes through chloroplast genetic engineering is highly desirable in instances where there is potential for outcross[4,5]. In addition, the target enzymes or proteins for most herbicides (of the amino acid/fatty acid biosynthetic pathways or photosynthesis) are compartmentalized within the chloroplast. Another environmental concern expressed recently is the toxicity of transgenic pollen to nontarget insects, such as the monarch butterflies[6]. Since there is no chloroplast DNA in pollen of most crops, toxic insecticidal proteins should not normally be expressed in pollen of chloroplast transgenic plants. Introgression of genes from weedy relatives, however, could theoretically allow chloroplast transgenes to escape from engineered crops. However, this has been shown to be a rare occurrence, at least for a gene encoding a nonselected trait[5].

Yet another concern in the use of nuclear transgenic crops expressing the *Bacillus thuringiensis* (*Bt*) toxins is the suboptimal production of toxins resulting in increased risk of pests developing *Bt* resistance. Plant-specific recommendations to reduce *Bt* resistance development include increasing *Bt* expression levels (high-dose strategy), expressing multiple toxins (gene pyramiding), or expressing the protein only in tissues highly sensitive to damage (tissue-specific expression)[7]. All three approaches should be compatible with chloroplast transformation. For example, overexpression of several thousand copies of a novel *Bt* gene via chloroplast genetic engineering resulted in 100% mortality of insects that are up to 40,000-fold resistant to other *Bt* proteins[7].

Current methods for DNA delivery into chloroplasts include particle bombardment[8] and polyethylene glycol[9]. The advantages of particle bombardment include high efficiency of transformation, rapid regeneration of transformed tissue, and use of a variety of explants. However, this technique is very expensive. The polyethylene glycol method requires preparation of protoplasts and is less efficient. Therefore, less expensive but more efficient techniques are still required to acomplish chloroplast transformation in a diverse range of crops.

Knoblauch and coworkers[1] describe a novel galinstan expansion femtosyringe method that allows microinjection of foreign DNA and other substances into prokaryotic cells and eukaryotic organelles. The essence of the method is the heat-induced expansion of a liquid metal called galinstan (an alloy of gallium, indium, and tin) within a glass

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