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Inteins—A Focus on the Biotechnological Applications of Splicing-Promoting Proteins

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Abstract

The main aim of this mini-review is to illustrate strategies and industrial applications based on inteins (INTErnal proteINS), which belong to a class of autocatalytic enzymes that are able to perform a catalytic reaction on a single substrate. However, since practical applications of inteins are strongly guided by a detailed understanding of their biological mechanisms and functions, the first part of this review will thus briefly discuss the physiological roles of inteins, describing what is currently known about their mechanisms of action. In the second part, specific biotechnological applications of inteins will be outlined (*i.e.* their use for (i) the purification of recombinant proteins, (ii) the cyclization of proteins and (iii) the production of seleno-proteins), paying attention to both potential strengths and weaknesses of this technology.

Keywords

Intein, Protein Purification, Tagged Protein, Cyclization, Selenoprotein

1. Introduction

Since the initial discovery of inteins in 1987 in the fungal organism *Neurospora crassa* [1] [2], they have also been found in various eubacteria, archaea and eukaryotes, as well as in viruses and phages [1] [3]-[6]. An intein is a genetic element similar to an intron; however, while the latter is responsible for mRNA maturation during splicing, inteins are transcribed and translated together with the proteins [1]. In fact, they fulfill their physiological role(s) only at the protein level, where their excision from mature proteins is part of their activation mechanism [1] [7] [8]. From a biochemical point of view, inteins belong to a class of autocatalytic enzymes that are

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able to perform a catalytic reaction on a single substrate, *i.e.* the target protein that will be transformed into a mature protein in the process [9]. This type of reaction has been termed "protein splicing", to draw a parallel to splicing that occurs at the post-translational level [1] [9] [10]. Some inteins contain a "homing" endonuclease domain (HEN), a double-stranded DNA-specific endonuclease [11]. Based on the presence or the absence of this domain, inteins have been divided into large and mini inteins [1] [12]. However, the HEN domain is not necessary for the protein splicing activity of inteins, but is believed to allow the transfer of inteins amongst species [13]-[15]. Two domains, the N- and C-splicing domains, define the internal structure of both large and mini inteins (**Figure 1**) [1] [9] [12]. These domains are necessary for protein splicing as they contain the conserved amino acids that are involved in catalysis [1] [9] [10].

In terms of sequence similarity, the highest degree of conservation is present within blocks inside the splicing domains (**Figure 1**) [1] [9] [16] [17]. Most inteins contain a Ser or Cys as the first residue in the N-splicing domain, while the C-splicing domain ends generally with residues His-Asn or His-Gln [1] [9] [10]. The flanking sequences at both ends of the intein domain, called exteins, contain the fragments of the protein that will be linked in the splicing process. The first amino acid of the C-extein is invariably a Ser, Thr or Cys, but the residues that precede the intein at its N-terminus (*i.e.* the N-splicing domain) are not conserved (**Figure 1**) [1] [9] [10].

The catalytic reaction takes place in four steps, in which the intein is excised from the two flanking amino acidic regions, which are linked by a peptide bond after the removal of the intein (**Figure 2**) [1] [9] [10] Inteins are assigned to three classes depending on the first amino acid involved in the reaction [1] [9] [10] [12]. Class I inteins use the conserved Ser or Cys residue that is located at the junction between the N-extein and the N-splicing domain (**Figure 1**) to initiate the reaction [1] [9] [10]. The mechanism applied by class I inteins is illustrated in detail in **Figure 2**.

Class II and III inteins lack the Ser or Cys residue [9] [10] and consequently alternative mechanisms have been proposed for these inteins [18] [19]. In all three classes, the reaction leads to the formation of a branched intermediate (**Figure 2**) [1] [9]. In class II, the first C-extein residue, a Cys, attacks the amide bond at the N-terminal splice site junction, thus leading directly to the formation of the branched intermediate [19] [20]. In class III, a Cys residue in the F block (**Figure 1**) is proposed to attack the same splice site junction, although in this case the branched intermediate is formed via a transesterification that involves a Thr residue in the C-extein [18].

It is possible to alter the specific intein-extein cutting site at the junction of class I splicing inteins by mutating some of the conserved residues [10]. A mutation of the Asn residue in the C-splicing domain (**Figure 1**) eliminates steps three and four of the splicing reaction, leading to the excision of the N-extein [10]. Mutations of

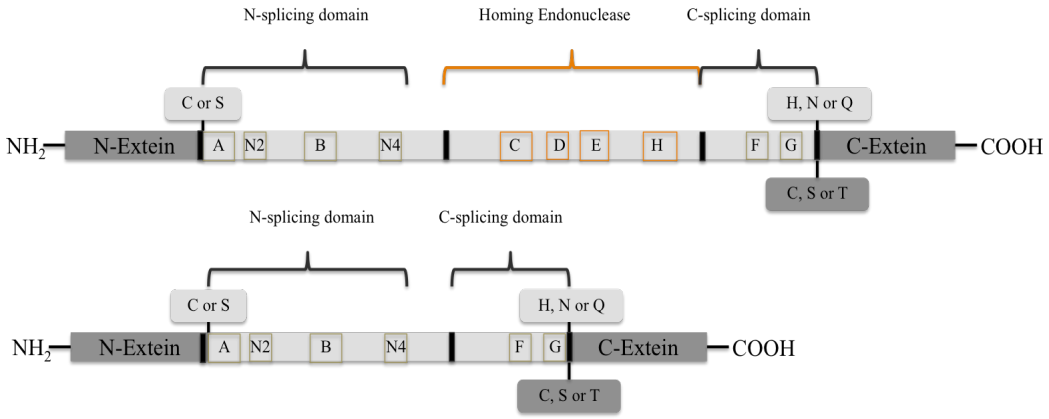

Figure 1. Domain organization in large and mini inteins. The extein domains are colored in dark gray, the intein domain is colored light gray. The conserved blocks (A, N2, B, N4, C, D, E, H, F, and G) in the intein splicing precursor are boxed in light gray, in the intein splicing domain, or in orange in the HEN domain. The conserved residues that participate in the splicing reaction are boxed above the splicing junction both in the N- and C-terminus. The C-terminal conserved residues at the extein junction (C, S or T) are also boxed. The black vertical lines identify the HEN insertion site or the splicing junctions.

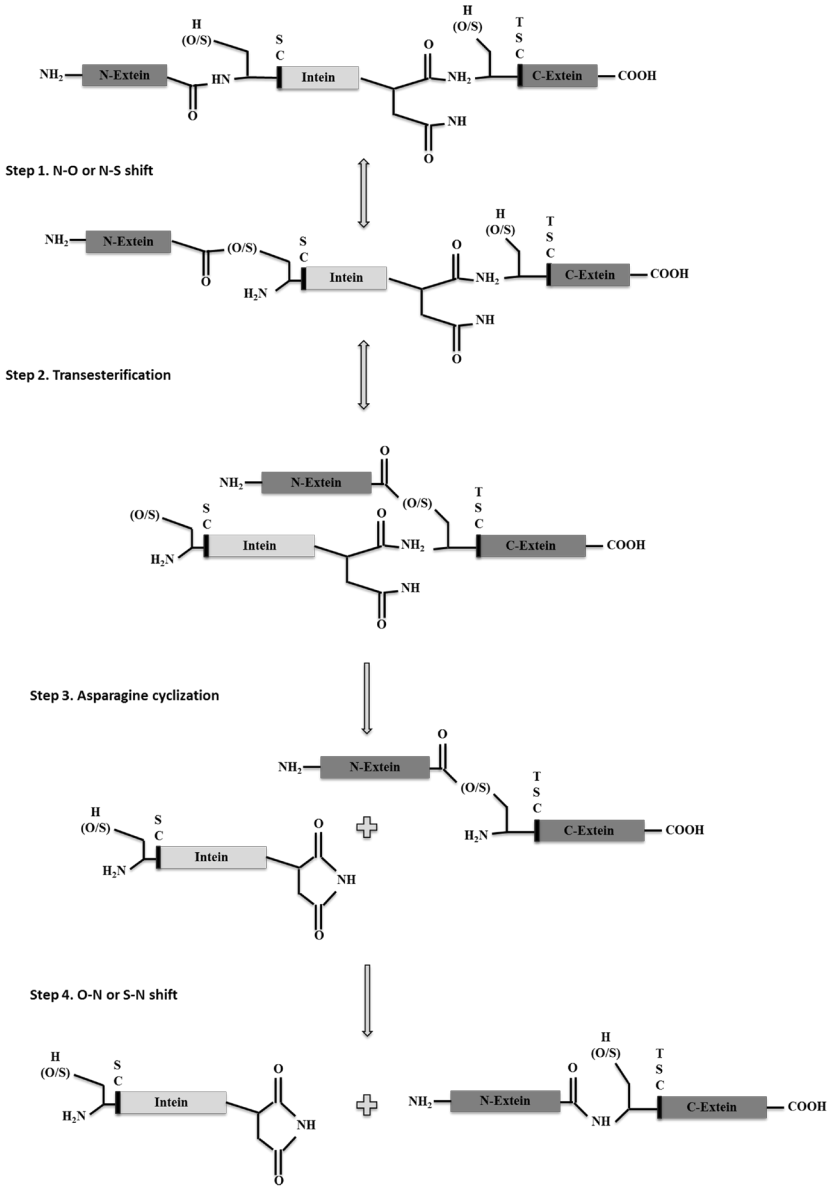

Figure 2. Protein splicing mechanism. Intein splicing takes place in four steps. The activation of the N-terminal splice junction by an N-O or N-S shift leads to the formation of an ester or thioester intermediate. In the second step, a transesterification, the (thio) ester is cleaved via a nucleophilic attack by the residue located at the C-terminal splice junction. A second intermediate (the branched protein intermediate) is thus generated. In the third step (asparagine cyclization) the intein is excised and the two exteins are spliced via an ester bond. In the last step, a spontaneous rearrangement leads to the formation of a peptide bond between the two extein domains.

the first conserved residue of the intein eliminate the first, the second or the fourth reaction steps (depending on the mutation), leading to the release of the C-extein [9] [10] [21]-[23]. The possibility of artificially controlling the splicing reaction is of great technological interest, as is the possibility of producing a functional intein by expressing the N-intein and the C-intein domains using two different constructs. In this case the splicing reaction is achieved by reconstitution of the active intein from the two fragments [1] [10]. This process, termed *trans*-splicing, has been first identified in the cyanobacterium *Synechocystis* sp. (Strain PCC6803) within the gene encoding the protein DnaE [1] [24] (**Figure 3**). In this organism the intein is divided into two separate fragments (and hence the corresponding intein is frequently referred to as a "split-intein") that are able to assemble to form an intact and fully active intein, catalyzing the process of splicing [1] [10].

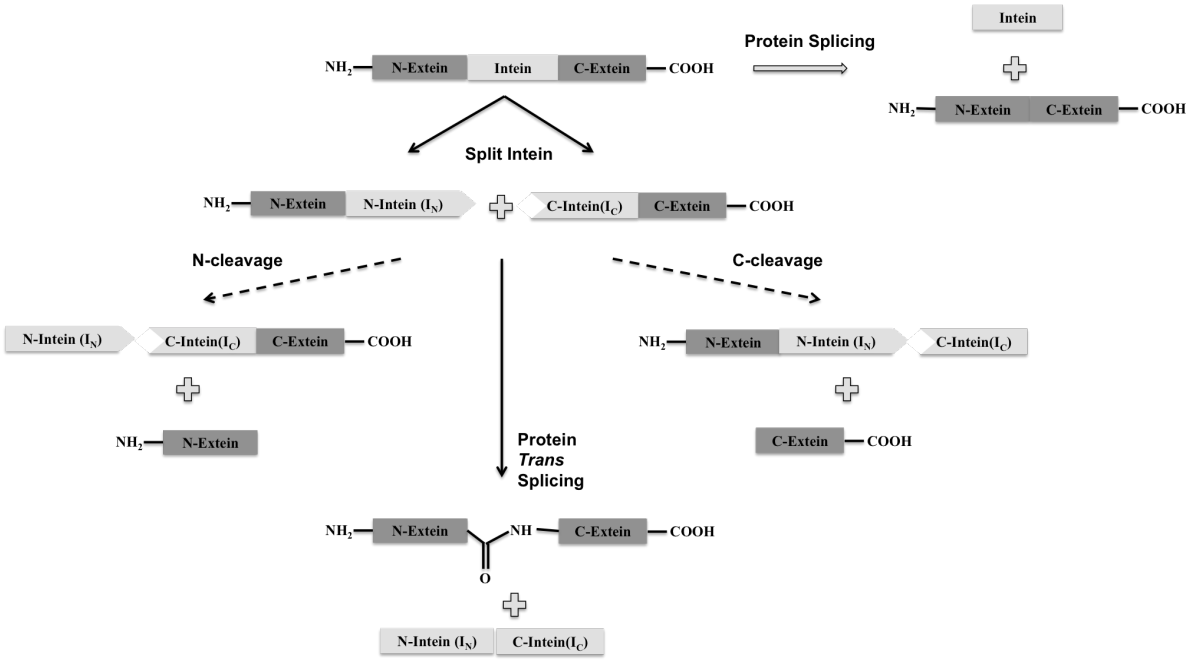

Figure 3. *Trans* protein splicing. Schematic representation of protein splicing in *cis* and *trans*. A natural intein is naturally or artificially split into two fragments, N-intein (I_N) and C-intein (I_C). The *trans* splicing occurs upon reconstitution of the intein fragments, forming an active intein structure. This might also result in N- or/and C-cleavages.

2. Applications

There are a number of technological applications that involve inteins. For instance, inteins have been used as a molecular tool to study protein-protein interactions, using the splicing reaction to label both *in vitro* and *in vivo* target proteins [25]-[27] with the green fluorescent protein (GFP) [28] or with a synthetic fluorescent probe [29]. Particularly, inteins are useful in protein purification [10] [30], and they have also been used to label proteins for NMR spectroscopy [31] [32], induce protein cyclization [33]-[35], and tune and control the expression of toxic proteins [36]. Here, for illustrative purposes, we will highlight three of the major applications, protein purification, protein/peptide cyclization and seleno-protein production.

2.1. Strategies for Protein Purification

Since its conception, protein biotechnology has had a profound impact on the industrial sector. One market that has particularly benefitted is the large-scale production of pure proteins [9] [10] [30] [37] [38]. At the industrial level, the production costs are predominantly determined (45% to 90%) by "downstream processing", which includes purification protocols for the separation of the desired protein from bulk protein, cell debris and other contaminants present in the system [30] [37] [38]. The use of epitope tags has simplified protein purification and lowered both associated costs and time [10] [39]. Epitope tags flank the protein sequence (either at the N- or the C-terminal region), facilitating purification by exploiting the affinity of the tag for a specific matrix. One of the most commonly used tags is the hexa-histidine tag [37] [39] [40]. Such affinity-based purification protocols generally require only a single step to obtain high yields of highly purified proteins [10] [39] [41] [42]. Furthermore, such purification protocols can easily be adapted for large scales, making them very convenient for industrial applications [10] [37] [39]. A drawback of this approach is that the tag generally needs to be removed at the end of the purification, a step that is frequently expensive and leads to loss of pure material [37] [39] [43]. This step is critical if the target protein is to be used as a commercial product in the food or pharmaceutical industry. Often, removal of a tag is facilitated by proteolytic cleavage using endopeptidases, inherently increasing the cost of the process while introducing different quality control related problems (e.g. safety of the final product, retention of the target protein activity/stability) [10] [39] [44] [45]. A new strategy that can simplify this process is thus desired in particular for industrial applications. Inteins have a strong promise to play a corner-

stone in such a protein purification strategy, decreasing the operational costs without jeopardizing the yield of pure protein.

2.1.1. The Classic Approach

The use of inteins as a tool for protein purification began in the late 1990s, with the modification of the intein *Sce* VMA1 from *Saccharomyces cerevisiae* as a tag to facilitate the purification of both prokaryotic and eukaryotic proteins [10] [30]. Since then numerous methods to use inteins as efficient protein purification tags have been developed. Generally, inteins are placed between an affinity tag and the target protein, but since splicing at the junction between the tag and the intein is prevented by specific mutations pure (*i.e.* "tag-free") target protein is obtained [13] (**Figure 4**). Splicing is induced by a variety of means; if the target protein is at the N-terminal end strong nucleophile such as dithiothreitol (e.g. DTT) are frequently used, whereas changes in pH or temperature are commonly used if the target is located at the C-terminal end of the construct [13] [46] [47]. Various intein-based protein purification kits are commercially available. As an example, the *Sce* VMA1 intein is available as part of the IMPACT CN system (New England BioLabs); it makes use of the chitin binding domain (CBD) to purify the target protein by affinity chromatography; splicing can be activated by changes in pH or by thiols (*i.e.* 2-sodium sulfonate mercaptoetanomesna, thiophenol, *β*-mercaptoethanol, DTT 1,4-dithiothreitol or cysteine) (**Figure 4**) [10] [13].

The disadvantage in the use of pH induced inteins is the possibility that premature cleavage may occur during protein expression. This problem is eliminated by using thiol-inducible inteins, at the expense of increased costs of production and potential alterations to the native structure of the protein (e.g. removal of disulfide bridges). These inherent drawbacks can be abolished by using split inteins (*trans*-splicing; see above) [10] [24]. This methodology is applied in the commercially available pTWIN-vectors (New England BioLabs). The two vectors contain two different domains of the DnaB mini-intein from *Synechocystis* sp. strain PCC6803 [1]. The efficiency of *trans*-splicing was initially demonstrated in the expression and purification of the green fluorescent protein (GFP) [48], and a modified method is also available using the DnaE split intein from *Nostoc puntiforme* [49] [50], facilitating an ultra-rapid and cost efficient tagless, split intein-mediated purification procedure. This modified method, labelled as SIRP (Split-Intein Mediated ultra-Rapid Purification of tagless proteins), facilitates the purification of target proteins in less than an hour, using a single-step purification protocol [49] [50]. Re-

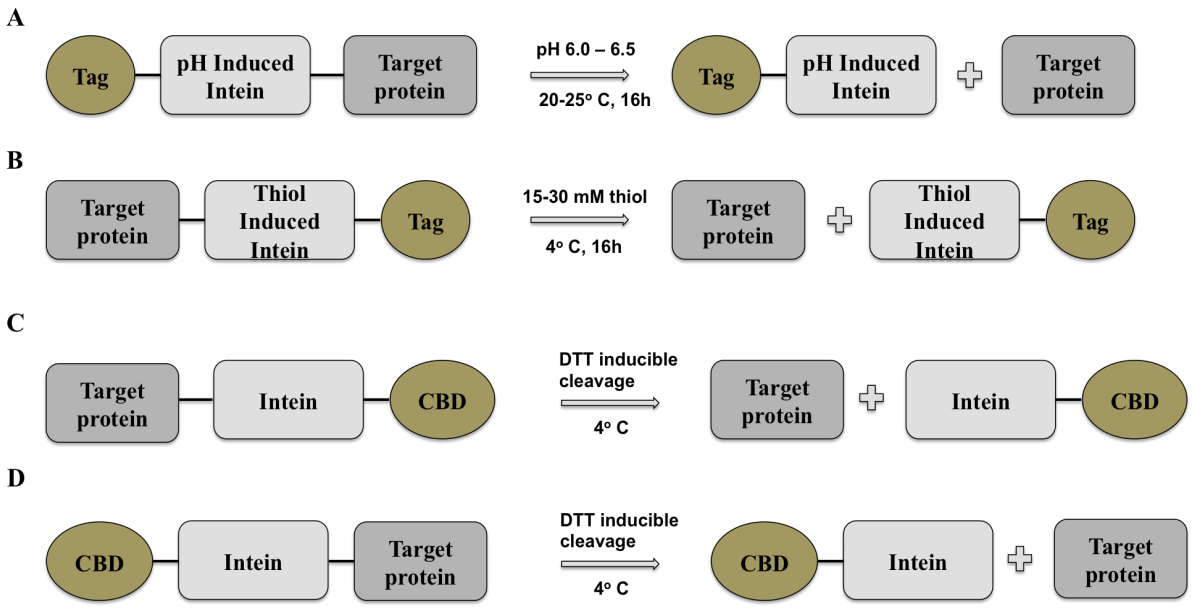

Figure 4. Schematic representation of protein purification using intein tags. In the classic approach the intein is inserted between the target protein and the purification tag. After protein purification using an affinity resin specific for the tag, the intein splicing reaction is induced via a change in the pH (A) or using a thiol compund (B). The IMPACT CN system uses the chitin binding domain (CBD) as an affinity tag. The splicing reaction is induced using a thiol such as DTT at low temperature. In (C) and (D) the IMPACT system used for both N- or C-terminal cleavage is illustrated.

cently, the split intein protein purification-mediated approach was used to produce two pharmaceutically relevant molecules, the recombinant human glucagon-like peptide-1 (GLP-1) from *Escherichia coli* [51] and the lunasin peptide [52]. The former is a hormone used in the treatment of patients suffering from type-2 diabetes [51] [53]. Lunasin is a promising anti-cancer peptide, demonstrated to have apoptotic effects in colon cancer cell cultures and in mouse xenograft models [54] [55].

2.1.2. Alternative Approaches

Regardless of the type of intein used the methods described above rely on the use of high-affinity resins for protein purification, and such resins are generally expensive. Thus, alternative strategies have been conceived that use different, cheaper support matrices to assist protein extraction. Two of the most effective approaches use the elastin-like polypeptide (ELP) [56] and the polyhydroxybutyrate (PHB) or polyhydroxyalkanoates (PHA) granules as matrices [57] [58]. Both approaches facilitate chromatography-free purification, using a simple centrifugation step instead to recover the pure sample [30]. Both ELP and PHB/PHA granules were successfully used to express and purify proteins in microbial cells (such as BLR (DE3) *E. coli* [58]-[64]), as well as in plant bioreactors [57] [65].

ELP is composed of a repetition of the conserved motif VPGXG (where X can represent any amino acid except proline) [66]. An interesting feature of the ELP system is its ability to reversibly alter between the soluble and aggregated state; the transition can be induced by changing either the temperature (phase change takes place at a characteristic transition temperature Tt) or ionic strength [66]. This process, called inverse transition cycling (ITC), thus facilitates the purification of a target protein that is fused in frame with the ELP [62] [67]. In combination with the intein splicing activity a mature target protein can easily be expressed and purified using an ELP-intein fusion construct. After the purification using centrifugation the mature protein is obtained by initiating the intein-mediated cleavage (**Figure 5**). Recent examples of the scope and potential of this approach are the production of antimicrobial peptides (e.g. moricin CM4, human β-defensins, which are able to disrupt bacterial cells but have no negative effects on normal mammalian cells [68]), as well as the purification of a plant lectin (PAL) that has been shown to possess anti-tumor activity [69]. Despite relatively low yields of target protein produced in currently available systems, the methodology has strong potential to become a powerful technique for heterologous protein production especially in *E. coli* [68] [69]. Apart from low yields, current limitations of this approach are the control of the splicing reaction (*i.e.* the splicing reaction may occur too early during the expression thus lowering the final yield of purified product), as well as the temperatures required to induce ELP-mediated aggregation [58] [68] [69]. Since the stability of different target proteins may vary considerably the methodology needs to be optimized case by case. The transition temperature of the ELP can be modified by varying the number of ELP motif repetitions, thus varying the composition and the weight [60] [67] [70]. The replacement of a continuous intein by a split intein (see above) may limit the occurrence of unspecific splicing as this reaction is only triggered when both fragments of the intein are combined.

The other alternative approach, *i.e.* the use of PHB/PHA granules, combines the self-cleaving activity of an intein with a physical separation method, exploiting the affinity of the phasin tag for PHA and PHB [57] [64] [71] [72], hydrophobic polyesters produced by many bacteria (**Figure 5(B)**) [73]-[75]. This methodology has been shown to be efficient for the production of well-known proteins such as the enhanced green fluorescent (EGFP) and maltose binding (MBP) proteins, as well as β-galactosidase [58]. Of particular interest is the possibility to apply this method in combination with a range of low-cost polymers for which phasin has high affinity [58]. More recently, this approach was used to express and purify the anti-viral molecule porcin interferon alpha (PoIFNα), a protein that plays an important role in the first stage of the infection response of the host organism [76] against a number of viral agents, including the *H. influenza* strain A/H1N1 that is associated with swine flu [77]-[79]. PoIFNα purification was carried out using the phasin-intein system in combination with the co-expression of PHB *in vivo* in *E. coli* host cells, thus demonstrating the high efficiency and low associated costs of this methodology for the large-scale production of a chemotherapeutical drug.

2.2. Cyclization

The production of cyclic peptides and proteins is a relatively recent but rapidly expanding field [10]. In nature cyclic molecules are not uncommon; numerous organisms produce cyclic molecules with a wide range of activities ranging from anti-bacterial to cytotoxic activities [80]. Cyclic molecules are characterized by their stability

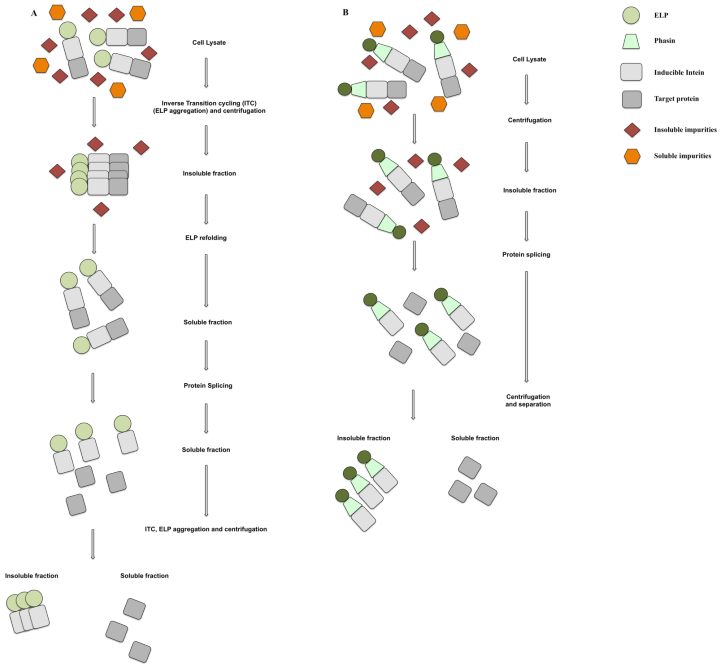

Figure 5. Schematic representation of intein-mediated purification using the ELP polypeptide (A) or PHA granules (B). Both systems are based on the possibility to pull down the target protein using the ITC transition of the ELP polypeptide (A) or the intrinsic insolubility of the PHA granules. The target protein is then separated from the contaminants using centrifugation. Following purification, the tag is removed by the intein-mediated protein splicing reaction.

and chemical resistance, as well as their resistance to temperature and enzymatic degradation [81]. A classic example is cyclosporin A, produced by the microorganism *Tolypocladium inflatum* [82]. This and similar peptide molecules form cyclic rings of little more than a dozen amino acids; enzymes called multi-domain peptide synthetases play a major role in the cyclization reaction [10] [83]. Other peptides are synthesized as linear chains in which the carboxyl group of the C-terminus forms a peptide bond with the amine group of the N-terminus [80]. Some cyclic bioactive peptides are produced in plants, so-called cyclotides, characterized by a knotted structure organized by three disulfide bridges [10] [80].

Inteins provide a convenient basis to produce cyclic peptides in the laboratory [84], avoiding drawbacks associated with classical chemical ligations [81] [85] such as the need for costly reagents and other experimental challenges [86]; frequently, reagent concentrations are in the millimolar range, adding significantly to experimental costs [85]. Furthermore, cyclic macromolecules often undergo post translational modifications *in vivo*, such as glycosylations or the addition of hydrophobic patches for trans-membrane localization. Classical chemical methods are not able to easily reproduce such modifications [85]. Inteins, in contrast, provide a cheaper and more versatile approach to generate cyclic peptides and proteins directly in a host organism using a process termed intein-mediated protein ligation (IPL) [36] [87]. In this process the protein of interest (*i.e.* the cyclic product) is cloned in between two differently inducible inteins (e.g. a pH-induced intein and a thiol-induced one). Both inteins may contain a terminal tag to facilitate purification in a single step. The protein of interest is linked via its C-terminal end to the N-terminus of one of these inteins, in which the C-terminal Asn residue has been replaced by another amino acid, thus preventing the splicing of this intein. The N-terminal end of the target protein is engineered in such a way that its second residue (following the methionine) is a cysteine. After the purification, splicing removes the N-terminal purification tag from the target protein, and after incubation with methionyl-aminopeptidase the initial methionine residue is also removed. The C-terminal activated thioes- ter of the target is now able to react with the N-terminal cysteine leading to the desired cyclization [88]. This strategy forms the basis of a commercially available kit that uses the vector pTWIN of the TWointein system, which contains the two required inteins [87]; the mutated version of the intein DnaB from *Synechocystis* sp PCC6803 is linked to the C-terminal end of the target protein, while an intein from *Mycobacterium xenopy*, MXE GyrA, is linked to its N-terminus. However, this approach has major drawbacks largely due to the low cleavage efficien-

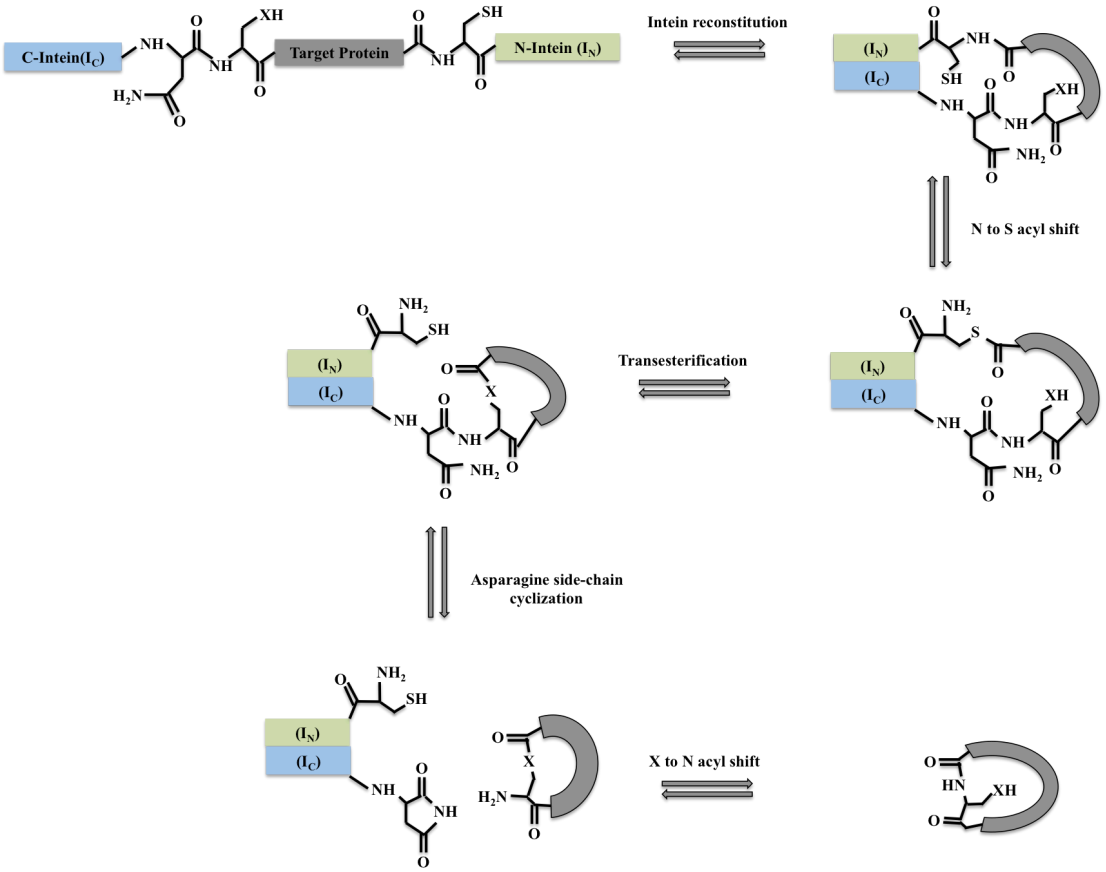

Figure 6. Schematic representation of intein-mediated protein cyclization. The expressed fusion protein contains a split intein (I_C and I_N, respectively) that flanks the target protein. Following the expression I_C and I_N associate to form an active intein (intein reconstitution). The splicing reaction is triggered by an intramolecular rearrangement (N to S acyl shift). The resulting intermediate undergoes a transesterification, where the "X" residue (X = S or O) attacks the thioester moiety. Transesterification is followed by an attack of the C-terminal asparagine side chain on its carbonyl carbon atom, causing succinimide formation and simultaneous peptide bond cleavage (thus liberating the cyclic peptide from the intein tag). The final step is characterized by an intramolecular rearrangement between the "X" residue and the amine group formed in the previous step.

cy of the DnaB intein and the interfering polymerization reaction that competes with the cyclization [89]. Both problems have been greatly diminished by using a method termed "split-intein circular ligations of peptides and proteins" (SICLOPPS) [81]. SICLOPPS uses the naturally occuring split intein DnaE from *Synechocystis* sp. The target sequence is cloned between the C-terminal (I_C) and N-terminal (I_N) intein domains. The assembly of the two intein domains produces a fully active intein able to initiate the formation of a cyclic peptide (**Figure 6**) [81] [90].

The artificial synthesis of cyclic peptides is a field increasingly exploited in drug discovery to establish new pharmacologically active molecules [91] [92]. Generally, the technique of choice for the synthesis of fragment/compound libraries is based on combinatorial chemistry, *i.e.* the synthesis of peptides with random sequences and different derivations [91] [92]. The major challenge to date, however, does not reside in the synthesis part of the fragments, but rather with the so-called deconvolution process; while it is relatively straightforward to screen for desired biological activities it is considerably more difficult to identify the relevant fragment(s) from the library that may contribute to this activity [90] [93]. In contrast, the biosynthesis of peptide libraries allows the production of compounds with known sequence, and, when coupled to an appropriate *in vivo* assay, this approach facilitates a rapid and facile large-scale screening of molecules with desired properties [90]. SICLOPPS has been successfully used in drug discovery by rapidly and efficiently generating a large library of peptides that can be directly tested for *in vivo* activity [81] [84] [90]. The effectiveness of protein cyclization to improve sta-

bility, while retaining biological activity, has been demonstrated using the *β*-lactamase TEM-1 as a periplasmic protein model and *E. coli* as the host [94]. This study did not only show that cyclization increases protein stability and maintains the target's activity, but it also illustrated how the target can be directed to the periplasmic space or even outside the cellular membrane, opening new possibilities for target screening [94].

In conclusion, the application of intein splicing to peptide and protein cyclization has strong potential in medicinal chemistry as it links the synthesis of novel compounds directly to drug screening and biological assay testing, which is not possible with a purely chemical synthetic approach. Moreover, the size of the library that can be produced by intein-mediated cyclization is comparable to that achievable using chemical synthesis but more efficient and cost-effective [81] [84] [90].

2.3. Selenoproteins

While more specialized and limited in its application the use of inteins in the production of selenoproteins is another convenient approach to demonstrate the potential of protein splicing in biotechnology. Selenocysteine (Sec), sometimes referred to as the 21st amino acid, is encoded by the UGA codon, in a process known as recoding, in many prokaryotic and eukaryotic organisms [10] [95]-[97]. The UGA codon is usually identified as a stop codon by the translation machinery [97]-[99]. Its translation into Sec instead is dependent on the presence of the Selenocystein Insertion Sequence (SECIS) in the mRNA [98]-[101]. In the absence of selenium the UGA codon terminates protein translation, often resulting in a truncated non-functional protein. However, the presence of selenium triggers the production of the selenoprotein [98] [99] [101]. In prokaryotes, the SECIS element typically immediately follows the UGA codon, whereas in eukaryotes and archaea it is located within the 3' untranslated region (3'UTR) [102] [103].

Many selenoproteins are enzymes with a single Sec residue in the active site. Since eukaryotes and prokaryotes have different recoding machineries for the UGA, the heterologous production and analysis of selenoproteins and selenoenzymes in host systems is not trivial [104]. The first successful study which aimed to heterologously express a mammalian protein containing a Sec, seleno-thioredoxin reductase (TrxR), was carried out in *E. coli*

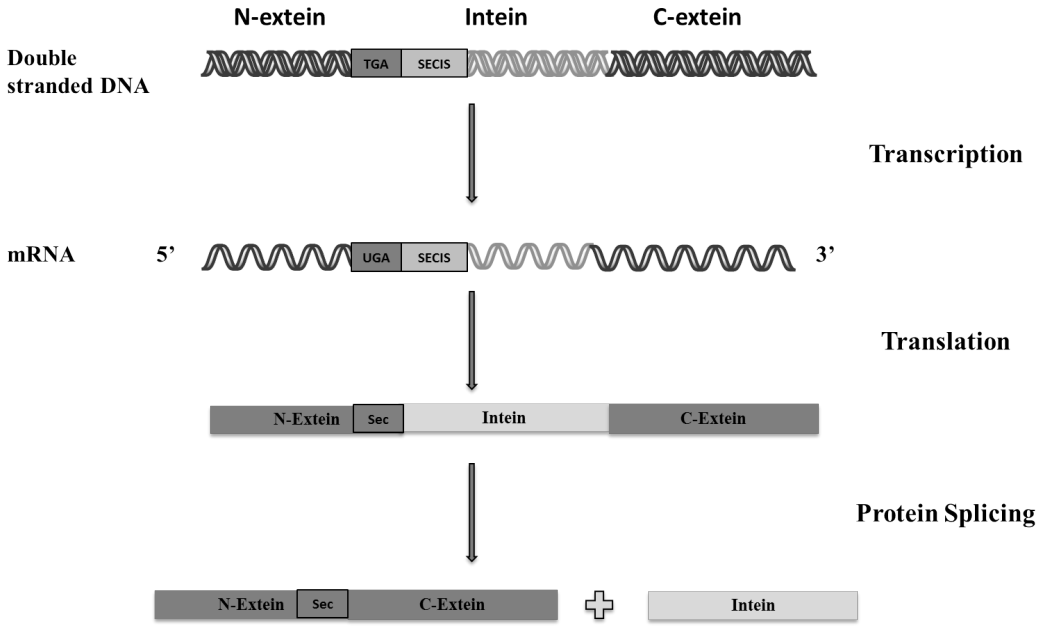

Figure 7. Intein-mediated selenoprotein production. The Sectein system, illustrated in the diagram, exploits the naturally driven Sec incorporation by the bacterial SECIS element with the intein splicing reaction. This system allows the introduction of the UGA codon anywhere within the coding sequence. The recognition of the UGA codon is due to the presence of the SECIS element, inserted at the junction between the UGA codon and the intein sequence. The SECIS element induces the insertion of the selenocysteine using the host translation machinery. Once the Sec residue is translated and inserted in the mature protein, the intein-mediated splicing reaction releases the mature Sec-containing protein without the SECIS element that is excised with the intein fragment.

[105]. The incorporation of an internal Sec in a heterologous target protein can be achieved using IPL. The recombinant intein-target protein construct is produced in a heterologous host (e.g. *E. coli*) in the absence of Sec. The Sec modification is subsequently triggered by the activation of splicing, in a process whereby intein activity catalyzes the formation of a link between the target protein and the Sec-containing module [104]. A more efficient and rapid method has recently been developed and patented by the name of *Sectein* [87]. In this system the expression of the selenoprotein exploits the recognition of the UGA codon by the SECIS element and the splicing reaction induced by the intein (**Figure 7**). As mentioned above, the Sec codon recognition and translation occur in bacteria due to the SECIS element located downstream from the UGA codon. The *Sectein* system exploits the bacterial host translation machinery to produce selenoproteins without the need of chemically producing the Sec-containing element. Furthermore, since the SECIS element naturally allows for the identification of the UGA codon the Sec residue can be inserted anywhere along the protein sequence without compromising the efficiency of the Sec insertion process (**Figure 7**) [105].

3. Conclusion

As illustrated in the examples above, inteins have significant potential as a molecular tool in protein biotechnology. Since their discovery, an increasing understanding of the intrinsic mechanisms they employ to initiate splicing reactions has also led to an increase in their applications for a growing number of biotechnological applications. Applying inteins in protein production has opened new strategies to obtain pure target proteins, via recombinant expression, using highly efficient extraction procedures based on specific tags, but by avoiding costly steps to remove these tags. Furthermore, the usefulness of protein splicing in protein and peptide cyclization reactions is impressive, rendering the classical chemical approach obsolete. As outlined above, there are still drawbacks to the versatile application of protein splicing, for instance in regards of stability and yield of target proteins, but in a comparison with more conventional approaches in protein engineering, the methodology has tremendous potential to evolve in a major strategy to acquire desired proteins with customized properties, for the academic and industrial environment alike.

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