Original article

Engineered Ssp DnaX inteins for protein splicing with flanking proline residues

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Inteins are internal protein sequences capable of catalyzing a protein splicing reaction by self-excising from a precursor protein and simultaneously joining the flanking sequences with a peptide bond. Split inteins have separate pieces (N-intein and C-intein) that reassemble non-covalently to catalyze a protein trans-splicing reaction joining two polypeptides. Protein splicing has become increasingly useful tools in many fields of biological research and biotechnology. However, natural and engineered inteins have failed previously to function when being flanked by proline residue at the –1 or +2 positions, which limits general uses of inteins. In this study, different engineered inteins were tested. We found that engineered Ssp DnaX mini-intein and split inteins could carry out protein splicing with proline at the +2 positions or at both –1 and +2 positions. Under in vivo conditions in E. coli cells, the mini-intein, S1 split intein, and S11 split intein spliced efficiently, whereas the S0 split intein did not splice with proline at both –1 and +2 positions. The S1 and S11 split inteins also trans-spliced efficiently in vitro with proline at the +2 positions or at both –1 and +2 positions, but the S0 split intein trans-spliced inefficiently with proline at the +2 position and did not trans-splice with proline at both –1 and +2 positions. These findings contribute significantly to the toolbox of intein-based technologies by allowing the use of inteins in proteins having proline at the splicing point.

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1. Introduction

Inteins capable of protein splicing reactions have become useful tools in many fields of biological research and biotechnology. For example, controllable inteins capable of conditional protein splicing can be used as molecular switches for controlling the functions of host proteins, in which the protein splicing activity may be triggered by temperature, ligand, or light (Zeidler et al., 2004; Buskirk et al., 2004; Mootz et al., 2003; Tyszkiewicz and Muir, 2008; Binschick et al., 2011). Protein trans-splicing by split inteins has been used in transgenic plants to prevent environmental escape of the transgen (Yang et al., 2003; Sun et al., 2001), in the production of cytotoxic proteins by joining non-toxic pieces (Evans et al., 1998), and in a gene therapy procedure to allow delivery of small pieces of a large therapeutic gene (Li et al., 2008). It has also been used for segmental isotope labeling of proteins in NMR studies (Busche et al., 2009), for protein interaction studies and protein localization in cells in two-hybrid procedures (Ozawa et al., 2003), and for site-specific chemical modifications (or labeling) and cyclization of proteins (Mootz, 2009; Muralidharan and Muir, 2006; Evans et al., 2008; Iwai and Pluckthun, 1999; Scott et al., 1999). Furthermore, inteins have also been modified to catalyze site-specific cleavages in proteins, which has been developed into the expressed protein ligation method for protein modifications and the IMPACT method for one-step purification and cleavage of recombinant fusion proteins (Muir, 2003; Chong et al., 1997).

Inteins were discovered in natural organisms and host proteins as an internal protein sequence that catalyzes a protein splicing reaction by self-excising from a precursor protein and simultaneously joining the N-extein and C-extein with a peptide bond (Perler et al., 1994; Saleh and Perler, 2006). The typical mechanism of protein splicing includes the following catalytic steps (Noren et al., 2000): (1) at the N-terminus of the intein, an N-S or N-O acyl rearrangement breaks the peptide bond between N-extein and N-intein and substitutes an ester bond for peptide bond; (2)}
following a transesterification reaction, the N-extein is moved to the N-terminus of the C-extein forming an ester bond; (3) the peptide bond at the downstream splice junction is broken through a cyclization of the last residue Asn of intein, which separates the intein from the exteins; and (4) the ester bond between the two exteins is changed to a peptide bond through an S-N or O-N acyl rearrangement. These steps may be disrupted, if the intein is mutated or placed in some non-native host proteins, and result in site-specific cleavages at the N- or C-terminus of the intein without splicing. Most natural inteins are bi-functional inteins consisting of a splicing domain and a homing endonuclease domain. Mini-inteins, which can be natural or engineered, have only a splicing domain that is approximately 140 amino acids in size. Different inteins have low similarities in amino acid sequences, but their crystal structures (at least the splicing domain) are highly conserved. The splicing domain consisting of ~12 β-strands is shaped like a flattened disk, with the N- and C-terminus of the intein located near a centrally located catalytic pocket.

A mini-intein may be converted into a split intein by splitting the intein sequence into two separate parts (N-intein and C-intein), assuming that the separate parts can reassemble non-covalently to catalyze a protein trans-splicing reaction (Saleh and Perler, 2006). For protein trans-splicing, two recombinant polypeptides are produced separately, with N precursor fusion protein being an N-extein fused to the N-intein, and C precursor fusion protein being the C-intein fused to a C-extein. In trans-splicing, the N- and C-inteins are removed, and the N- and C-exteins are joined with a peptide bond to form the spliced protein. Different types of split inteins may be engineered by splitting an intein sequence at different places. A conventional or S0 type of split intein has the split site near the middle of the intein sequence while unconventional split inteins have the split site near the N- or C-terminus of the intein. These steps may be disrupted, if the intein is mutated or placed in some non-native host proteins, and result in site-specific cleavages at the N- or C-terminus of a target protein (N-extein) (Appleby et al., 2009). In this study, we tested different engineered inteins Ssp DnaX, Ter DnaE-3 and Npu DnaE to find one that can initiate protein splicing when being flanked by proline residues. It was found that various engineered Ssp DnaX mini-intein and split inteins could splice in model proteins when proline was present at the +2 positions or at both −1 and +2 positions.

2. Materials and methods

2.1. Plasmid construction

Plasmids pMTE<sup>m1</sup>-m and pMNE<sup>m1</sup>-m were constructed as follows. The genes of Ter DnaE-3 and Npu DnaE mini-inteins were amplified by PCR with the primers TE<sub>3</sub>-m1for (5'-TACDCTCGAGACTTGAACATATGACGAGC-3') and TE<sub>3</sub>-m1rev (5'-TCAATACCGGTTGGCGAAGAAGTCGCAAGGTCAATAGAAGCCG-3') designed from pMTE<sub>3</sub>-m and NE-m1for (5'-TACDCTCGAGACTTGAACATATGACGAGC-3') and NE-m1rev (5'-TCAATACCGGTTGGCGAAGAAGTCGCAAGGTCAATAGAAGCCG-3').

The PCR products were double digested with AgeI/XhoI, inserted into the similarly digested plasmids pMTX-m, respectively. Plasmids pMTE<sup>m2</sup>-m and pMNE<sup>m2</sup>-m were constructed using the same method, with primers changed to TE<sub>2</sub>-m2for (5'-TACDCTCGAGACTTGAACATATGACGAGC-3') and TE<sub>2</sub>-m2rev (5'-TCAATACCGGTTGGCGAAGAAGTCGCAAGGTCAATAGAAGCCG-3').

Plasmids pMSX<sup>m1</sup>-m, -S0, -S1 and -S11 were constructed by replacing the Agel/Xhol fragment in plasmid pMTX-m with an Agel/Xhol fragment produced by PCR amplification with primers SX-m1for (5'-TACDCTCGAGACTTGAACATATGACGAGC-3') and SX-m1rev (5'-TCAATACCGGTTGGCGAAGAAGTCGCAAGGTCAATAGAAGCCG-3').

The PCR products were double digested with AgeI/XhoI, inserted into the similarly digested plasmids pMTX-m, respectively. Another pair of primers, SX-m2for (5'-TACDCTCGAGACTTGAACATATGACGAGC-3') and SX-m2rev (5'-TCAATACCGGTTGGCGAAGAAGTCGCAAGGTCAATAGAAGCCG-3').

To construct the N-precursor fusion protein consisting of maltose binding protein (M) and N-intein the pMSX<sup>m1/m2</sup>-S0, -S1 and -S11 plasmids were double digested with NdeI/PstI, and self-ligated, yielding plasmids pMSX<sup>m1/m2</sup>-S0<sub>N</sub>, -S1<sub>N</sub> and -S11<sub>N</sub>. To construct the C-precursor fusion protein consisting of C-intein and thioredoxin (T), plasmids pMSX<sup>m1/m2</sup>-S0 and -S1 were double digested with NdeI/PstI, and the isolated NdeI-PstI fragment was ligated with similarly digested plasmid vector pTTX<sub>S</sub> C, yielding plasmids pTS<sub>S</sub><sup>m1/m2</sup>-S0<sub>C</sub> and -S1<sub>C</sub>.

For construction of pPES<sub>2</sub><sup>m1/m2</sup>-S1<sub>C</sub>, the k<sub>11</sub><sub>C</sub> coding sequence amplified by PCR from pPES<sub>2</sub><sup>m1/m2</sup>-S1<sub>C</sub> with primers 5'-GGAAA AACATTTGCGTTTACTGCTGTCATACAGGACAATTTAGATGATGACTAGTGCAGC-3' and 5'-CTACGCTGTCGACTTGAACATATGACGAGC-3'.

2.2. Protein expression, purification, and splicing

Plasmids pMSX<sup>m1/m2</sup>-m,-S0,-S1,-S11, pMTE<sup>m1/m2</sup>-m and pMNE<sup>m1/m2</sup>-m were transformed into E. coli DH5α cells, using a standard E. coli transformation protocol. The cells were grown in Luria Broth (LB) medium at 37 °C and induced by a final concentration of 0.8 mM IPTG at 25 °C overnight (Arshadullah et al., 2017). Total cellular proteins were analyzed by Western blotting, using anti-thioredoxin (anti-T) antibody (Invitrogen).
The plasmids for N-precursors expression (MIS0 N, MIS1 N and MIS11 N) were transformed into E. coli DH5α cells, while the plasmids for C-precursors expression (IscT C, IscT C and IscT C) were transformed into E. coli BL21 (DE3) cells. The transformed E. coli cells were pre-cultured in 3 ml Luria Broth (LB) medium at 37 °C overnight, the culture was transferred into 50 ml Luria Broth (LB) medium for further cultivating to an OD600 of 0.6, a final concentration of 0.8 mM IPTG was added to induce protein expression at 25 °C overnight. After the cells were collected by centrifugation and lysed using a French Press (14,000 PSI), the cell lysate was centrifuged at 10,000g for 15 min to remove any insoluble materials. The soluble N-precursor proteins were purified by amylose resin according to the manufacturer’s instructions (New England Bios). The soluble C-precursor proteins were purified by Ni-NTA resin according to the manufacturer's instructions (QIAGEN).

N-precursor proteins and C-precursor proteins were mixed in a 1:1 or 10:1 M ratio to allow trans-splicing in vitro at room temperature with 1 mM DTT added. Splicing products were visualized by Western blotting using an anti-thioredoxin (anti-T) antibody (Invitrogen).

3. Results

To find inteins that can splice with proline at -1 (immediately before the intein) and +2 (second position after the intein) positions, we tested different engineered inteins in a model system. We initially tested Ssp DnaX, Ter DnaE-3 and Npu DnaE mini-inteins for protein cis-splicing in a model fusion protein. In the fusion protein, a maltose binding protein (M) was used as the N-extein, a thioredoxin protein (T) was used as the C-extein, with the mini-intein inserted in the middle. A proline residue was placed at the +2 positions or at both –1 and +2 positions. Amino acid residue at the +1 positions (first position after the intein) must be a nucleophilic residue, which is Cys for the Ssp DnaX, Ter DnaE-3 and Npu DnaE inteins. Protein cis-splicing would produce a spliced protein (MT) consisting of the maltose binding protein (M) and the thioredoxin (T), with the intein sequence excised. The spliced protein MT could readily be detected by Western blotting using an anti-thioredoxin (anti-T) antibody, with the protein identified by its predicted size matching that of the control protein in addition to antibody recognition.

Our tests revealed that the Ssp DnaX mini-intein with proline at the +2 positions or at both –1 and +2 positions could cis-splice efficiently in the above model protein in E. coli cells, whereas the Ter DnaE-3 and Npu DnaE mini-inteins spliced inefficiently when proline was present at the +2 positions (Fig. 1A) and almost did not splice when proline was present at both –1 and +2 positions (Fig. 1B).

As shown in Fig. 1, the precursor (MIT) had cis-spliced after expression in E. coli cells, showing a clearly visible spliced protein (MT), which was identified both by its antibody (anti-T) recognition and by its apparent size matching that of the control protein (lane 1). Ssp DnaX mini-intein exhibited efficient cis-splicing when being flanked by proline at the +2 positions or at both –1 and +2 positions, indicated by the accumulation of spliced protein MT (lane 2). No precursor remained, indicating that the splicing reaction proceeded to completion. The Ter DnaE-3 mini-intein spliced inefficiently (~30%, lane 3 of Fig. 1A) with proline at the +2 positions and did not splice with proline at both –1 and +2 positions (lane 3 of Fig. 1B). The precursor protein underwent N-cleavage, indicated by the cleavage product IT. Npu DnaE mini-intein showed ~30% (lane 4 of Fig. 1A) and ~5% (lane 4 of Fig. 1B) splicing activity when being flanked by proline at the +2 positions or at both –1 and +2 positions, respectively. The band of N-cleavage product IT indicated that the precursor protein had undergone N-cleavage.

The efficient splicing of Ssp DnaX mini-intein prompted us to test whether trans-splicing could also occur when the Ssp DnaX mini-intein was changed to split inteins. As shown in Fig. 2, the Ssp DnaX mini-intein sequence was split at either of three different sites (S0, S1, and S11) to produce the corresponding split inteins, according to the Ssp DnaB mini-intein whose crystal structure has been determined (Ding et al., 2003). For each split intein consisting of N-intein and C-intein, recombinant genes were constructed to express two precursor proteins. As illustrated in Fig. 3A, the N-precursor protein consisted of the maltose binding protein (M) followed by the thioredoxin protein (T). To detect protein trans-splicing activity in vivo, the two precursor proteins were co-expressed in the same E. coli cell, from a recombinant plasmid that had the two recombinant genes in an operon and after an IPTG-inducible promoter. After gene expression, total cellular proteins were analyzed by Western blotting using an anti-thioredoxin (anti-T) antibody to detect the presence of the C-precursor protein and possibly the spliced protein.

As shown in Fig. 3B and C, the spliced protein (MT) was clearly observed, indicating that the N-precursor and the C-precursor had trans-spliced after co-expression in E. coli cells. When proline was present at the +2 positions or at both –1 and +2 positions, efficient splicing was detected for Ssp DnaX-S1 and S11 split inteins (lanes 3 and 4). No C-precursor protein remained, indicating that the trans-splicing reaction was complete. However, Ssp DnaX-S0 split intein was found to have efficient trans-splicing reaction only when the +2 position was occupied by proline (lane 2 of Fig. 3B). When proline was present at both –1 and +2 positions, no spliced protein was detected, while the C-precursor protein was accumulated and its weak C-cleavage band T was clearly observed (lane 2 of Fig. 3C).

We then tested the Ssp DnaX split inteins for protein trans-splicing in vitro. The N- and C-precursor proteins were expressed separately in E. coli, purified, and mixed in vitro to allow protein trans-splicing. The results detected by Western blotting are shown in Fig. 4 and summarized in Table 1. Fig. 4A shows in vitro trans-splicing with proline at the +2 positions. A low level of trans-splicing activity in vitro was detected for the S0 split intein at the two precursor ratios tested, as indicated by the small amount of spliced proteins (~5%, lane 2 and ~15%, lane 3) and the remaining precursor. Efficient protein trans-splicing was observed for S1 split intein (~60%, lane 4) as well as S11 split intein (~50%, lane 6), and the splicing reached near completion when the N-precursor was used at a 10-fold excess (~90%, lane 5 and ~98%, lane 7). No C-cleavage of the C-precursor was observed.

Fig. 4B shows in vitro trans-splicing with proline at both –1 and +2 positions. For the S0 split intein, no spliced protein was observed (lanes 2 and 3), indicating a lack of trans-splicing activity of this intein in vitro. For the S1 and S11 split intein, the spliced protein was clearly observed (lanes 4–7), indicating protein trans-splicing in vitro. When the two precursors were mixed at 1:1 ratio, a significant amount of the C-precursor remained, indicating that the trans-splicing reaction for S1 and S11 split intein proceeded inefficiently (~30%, lane 4 and ~20%, lane 6). When the N-precursor was used at a 10-fold excess, very little C-precursor remained for S1 split intein, indicating that the trans-splicing reaction proceeded efficiently (~80%, lane 5), while the C-precursor remained for S11 split intein was almost not visible, indicating that the trans-splicing reaction reached near completion (~98%, lane 7). For S1 split intein, a portion of the C-precursor also underwent C-cleavage, as indicated by a presence of the cleavage product T. However, the C-precursor for S11 split intein did not undergo C-cleavage, which is different from the S1 split intein.
4. Discussion

In this study, we found that Ssp DnaX mini-intein and split inteins could carry out protein splicing efficiently when being flanked by proline residue either at the +2 positions or at both -1 and +2 positions. This finding contrasts with the previous finding that inteins did not work well when being flanked by proline residue either at the -1 positions or at the +2 positions (Iwai et al., 2006; Mathys et al., 1999; Appleby-Tagoe et al., 2011). Previously the engineered Ssp DnaB mini-intein did not work well with proline either at the -1 or +2 positions. The natural split intein Ssp DnaE did not work well with proline at the +2 positions. Even the improved Ssp DnaB mini-intein after directed evolution for general uses spliced inefficiently with proline either at the -1 or +2 positions. And the Ter DnaE-3 and Npu DnaE mini-inteins used in this study spliced inefficiently or did not splice. Here, under in vivo conditions in E. coli cells, the mini-intein and the three split inteins of Ssp DnaX all spliced efficiently when being flanked by proline at the +2 positions. With proline at both -1 and +2 positions, the mini-intein, S1 split intein, and the S11 split intein spliced efficiently, whereas the S0 split intein did not splice. Under in vitro conditions, when the N-precursor was excessive, the C-precursor protein underwent splicing reaction thoroughly, promoting the splicing reaction to completion and improving the splicing efficiency. When the N-precursor was used at a 10-fold excess, the S1 and S11 split inteins trans-spliced efficiently with proline at the +2 positions or at both -1 and +2 positions in vitro, but the S0 split intein trans-spliced inefficiently with proline at the +2 position and did not trans-splice with proline at both -1 and +2 positions. Moreover, the splicing was more difficult with proline at both -1 and +2 positions than that with proline only at the +2 positions, with an exception that the S11 split intein showing comparable splicing activity (>98%) under this two flanking proline conditions.

Proline is special among the 20 amino acids, it is known to cause abrupt turns (a kind of disruption) in protein secondary
structures (alpha helix, beta-strands), because its side chain is cyclic with the alpha-amino group. Therefore, proline at the +2 positions of inteins may cause ‘disruption’ at the active site and prevent splicing. A likely explanation for Ssp DnaX intein splicing when being flanked by proline is that the Ssp DnaXmini-intein and S1, S11 split inteins have flexible structure comparing with other inteins to avoid ‘disruption’, catalyzing the protein splicing with N- and C-terminus of the intein located near a centrally located catalytic pocket. Whereas the S0 split intein is not that flexible like S1 and S11 split inteins. In addition, probably the structure of S11 split intein was more flexible than S1 split intein, because it is showing comparable splicing activity in vitro with proline at the +2 positions or at both –1 and +2 positions when the N-precursor was used at a 10-fold excess.

Inteins often exhibit specificities toward the neighboring amino acids and thus limits their application in host proteins. In a non-native host protein, the inserting site of intein must avoid being flanked by proline, especially with proline at –1 or +2 positions as previously reported, because the proline residue is near the splice sites and influence the intein’s catalytic center. Our findings suggested that the application of intein could not be limited by proline residue; the intein could catalyze efficient splicing reaction when being flanked by proline, facilitating a more general use of

### Table 1

Summary of Splicing Activities of split inteins with flanking proline residues.

| intein | +2  | –1/+2 |
|--------|-----|-------|
|        | N:C = 1:1 | N:C = 10:1 | N:C = 1:1 | N:C = 10:1 |
| S0     | +   | –     | –     | –     |
| S1     | +++ | +++++ | ++    | +++   |
| S11    | +++ | ++++++| ++    | ++++   |

For each split intein listed on the left, the position of proline residue is shown on the top as +2 and –1/+2. It means intein was being flanked by proline at the +2 positions or at both –1 and +2 positions. The N:C = 1:1 and 10:1 means the molar ratio of N-precursor and C-precursor. The splicing efficiency was estimated as percentage of C-precursor that had been converted into the spliced protein MT (see Fig. 3) and presented as ++++ for >98%, ++++ for 90–95%, +++ for 80–90%, ++ for 70–80%, + for 50–65%, ++ for 20–35%, * for 5–15% and – for 0 (not detectable).
intein. Overall, these findings contribute significantly to the toolbox of intein-based technologies because they potentially allow protein splicing to be carried out in proteins having proline at the intein insertion site.

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Author Contributions

Conceived and designed the experiments: XZ XQL QM. Performed the experiments: XZ. Analyzed the data: XZ XQL QM. Contributed reagents/materials/analysis tools: XQL QM. Wrote the paper: XZ.

References

Amati, G., Callahan, B.P., Stanger, M.J., Belfort, G., Belfort, M., 2009. Modulation of intein activity by its neighboring extein substrates. Proc. Natl. Acad. Sci. USA 106 (27), 11005–11010.

Ando, T., Tsukiji, S., Tanaka, T., Nagamune, T., 2007. Construction of a small-molecule-integrated semisynthetic split intein for in vivo protein ligation. Chemical communications (Cambridge, England). (47), 4995–7.

Appleby, J.H., Zhou, K., Volkmann, G., Liu, X.Q., 2009. Novel split intein for trans-splicing synthetic peptide onto C terminus of protein. J. Biol. Chem. 284 (10), 6194–6199.

Appleby-TagOE, J.H., Thieli, I.V., Wang, Y., Wang, Y., Mootz, H.D., Liu, X.Q., 2011. Efficient and more general cis- and trans-splicing inteins through sequential directed evolution. The J. Biol. Chem. 286 (39), 34440–34447.

Arshadullah, M., Suhaib, M., Baber, R., Usama, M., Zaman, B.U., Mahmood, I.A., Hyder, S.I., 2017. Growth of Chenopodium quinoa Wild under Naturally Salt Affected Soils. Malaysian J. Sustain. Agr. 1 (1), 01–03.

Binschik, J., Zettler, J., Mootz, H.D., 2011. Photocontrol of protein activity mediated by the cleavage reaction of a split intein. Angewandte Chemie (International ed in English), 20:50 (14), 3249–3252.

Busche, A.E., Aranko, A.S., Talebzadeh-Farooji, M., Bernhard, F., Dotsch, V., Iwai, H., 2009. Segmental isotopic labeling of a central domain in a multidomain protein by protein trans-splicing using only one robust DnaE intein. Angew. Chem. Int. Ed. Engl. 48 (33), 6128–6131.

Buskirk, A.R., Ong, Y.C., Gartner, Z.J., Liu, D.R., 2004. Directed evolution of ligand dependence: small-molecule-activated protein splicing. Proc. Natl. Acad. Sci. USA 101 (29), 10505–10510.

Cheriyan, M., Pedamallu, C.S., Tori, K., Perler, F., 2013. Faster protein splicing with the Nostoc punctiforme DnaE intein using non-native extein residues. J. Biol. Chem. 288 (9), 6202–6211.

Chong, S., Mersha, F.B., Comb, D.G., Scott, M.E., Landry, D., Vence, L.M., 1997. Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. Gene 192 (2), 271–281.

Ding, Y., Xu, M.Q., Ghosh, I., Chen, X., Ferrandon, S., Lesage, G., 2003. Crystal structure of a mini-intein reveals a conserved catalytic module involved in side chain cyclization of asparagine during protein splicing. J. Biol. Chem. 278 (40), 39133–39142.

Evans Jr., T.C., Benner, J., Xu, M.Q., 1998. Semi synthesis of cytotoxic proteins using a modified protein splicing element. Protein Sci.: A Publication of the Protein Soc. 7 (11), 2256–2264.

Evans Jr., T.C., Martin, D., Kolby, R., Panne, D., Sun, L., Ghosh, I., 2000. Protein trans-splicing and cyclization by a naturally split intein from the dnaE gene of Synechocystis species PCC6803. J. Biol. Chem. 275 (13), 9091–9094.

Iwai, H., Pluckthun, A., 1999. Circular beta-lactamase: stability enhancement by cyclizing the backbone. FEBS Lett. 459 (2), 166–172.

Iwai, H., Zuger, S., Jin, T., Ham, P.H., 2006. Highly efficient protein trans-splicing by a naturally split DnaE intein from Nostoc punctiforme. FEBS Lett. 580 (7), 1853–1858.

Li, J., Sun, W., Wang, R., Xiao, X., Liu, X.Q., 2006. Protein trans-splicing as a means for viral vector-mediated in vivo gene therapy. Hum. Gene Ther. 19 (9), 958–964.

Ludwig, C., Pfeiff, M., Linne, U., Mootz, H.D., 2003. Ligation of a synthetic peptide to the N terminus of a recombinant protein using semisynthetic protein trans-splicing. Angew. Chem. Int. Ed. Engl. 45 (31), 5218–5221.

Methus, S., Evans, T.C., Chu, T.C., Wu, H., Chong, S., Benner, J., 1999. Characterization of a self-splicing mini-intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation. Gene 231 (1–2), 1–13.

Mootz, H.D., 2009. Split inteins as versatile tools for protein semisynthesis. ChemBioChem 10 (16), 2579–2589.

Mootz, H.D., Blum, E.S., Tyszkiwicz, A.B., Muir, T.W., 2003. Conditional protein splicing: a new tool to control protein structure and function in vitro and in vivo. J. Am. Chem. Soc. 125 (35), 10561–10569.

Muir, T.W., 2003. Semi synthesis of proteins by expressed protein ligation. Annu. Rev. Biochem. 72, 249–289.

Muralidharan, V., Muir, T.W., 2006. Protein ligation: an enabling technology for the biophysical analysis of proteins. Nat. Methods 3 (6), 429–438.

Noren, C.J., Wang, J., Perler, F.B., 2000. Dissecting the chemistry of protein splicing and its applications. Angew. Chem. Int. Ed. Engl. 39 (3), 450–466.

Ozawa, T., Sako, Y., Sato, M., Kitamura, T., Umezawa, Y., 2003. A genetic approach to identifying mitochondrial proteins. Nat. Biotechnol. 21 (3), 287–293.

Perler, F.B., Davis, E.O., Dean, G.E., Gimble, F.S., Jack, W.E., Neff, N.F., 1999. Protein splitting elements: inteins and exteins—a definition of terms and recommended nomenclature. Nucleic Acids Res. 22 (7), 1125–1127.

Saleh, L., Perler, F.B., 2006. Protein splicing in cis and in trans. Chemical record (New York, NY), 6 (4), 183–93.

Scott, C.P., Abel-Santos, E., Wall, M., Wahnon, D.C., Benkovick, S.J., 1999. Production of cyclic peptides and proteins in vivo. Proc. Natl. Acad. Sci. USA 96 (24), 13638–13643.

Sun, L., Ghosh, I., Paulus, H., Xu, M.Q., 2001. Protein trans-splicing to produce herbicide-resistant acetalocetate synthase. Appl. Environ. Microbiol. 67 (3), 1025–1029.

Sun, W., Wang, J., Liu, X.Q., 2004. Synthetic two-piece and three-piece split inteins for protein trans-splicing. J. Biol. Chem. 279 (34), 35281–35286.

Tyszkiwicz, A.B., Muir, T.W., 2008. Activation of protein splicing with light in yeast. Nat. Methods 5 (4), 303–305.

Volkmann, G., Liu, X.Q., 2009. Protein C-terminal labeling and biotinylating using synthetic peptide and split-intein. PLoS ONE 4 (12), e8381.

Yang, J., Fox, C.G., Henry-Smith Jr., T.V., 2003. Intra-intermediated assembly of a functional beta-glucuronidase in transgenic plants. Proc. Natl. Acad. Sci. USA 100 (6), 3511–3515.

Zeidler, M.P., Tan, C., Bellaiche, Y., Cherry, S., Hader, S., Gayko, U., 2004. Temperature-sensitive control of protein activity by conditionally splicing inteins. Nature Biotechnol. 22 (7), 871–876.