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Optimizing the Method for Cleavage of Ssp DnaB Mini-Intein Mediated Precursor Proteins

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Abstract: Inteins are protein splicing elements that excise themselves from precursor protein. It has been widely used to purify recombinant proteins. In the current study, using the Impact-Twin System to express the fusion protein CBD-Ssp DnaB intein-C-extein, researchers investigated the first C-extein residue that may influence the cleavage of Ssp DnaB intein. The results showed that cleavage of the fusion precursor protein was temperature and time dependent and was optimal when pH was 7.0-7.5. Efficiency of cleavage was also, dependent on the first residue of C-extein as well as time and temperature. The data may help in elaborating a facile method for producing other proteins of interest.

Key words: Ssp DnaB mini-intein, cleavage, C-extein, pH, protein

INTRODUCTION

Inteins are intervening protein sequences that are capable of catalyzing their excision from a precursor protein with the concomitant joining of the flanking sequences (termed exteins) through a native peptide bond (Perler *et al*., 1994; Paulus, 2000). The intein-mediated self-cleaving mechanism has recently been exploited to purify recombinant proteins from bacterial cultures (Morassutti *et al*., 2002; Wu *et al*., 2002; David *et al*., 2004). This opens the door for using protein splicing as a tool that allows expression of inactive precursor proteins which can be activated by protein splicing (Giriat *et al*., 2002; Gangopadhyay *et al*., 2003; Durek and Becker, 2005). By December 2002, over 200 inteins has been registered in the intein database, InBase (Perler, 2000, 2002). The alignment of inteins revealed that most of them are bifunctional proteins and contain a centrally located endonuclease domain (Pietrokovski, 2001) while a few inteins (termed mini-inteins), ranging in size from 134-198 amino acids (aa), lack of endonuclease region (Ding *et al*., 2003). The *dnaB* gene encodes for DNA helicase of the cyanobacterium *Synechocystis* sp. Stain PCC 6803 (Ssp) contains an intein of 429 amino acid residues (Pietrokovski, 1996). Deletion of the central 275 amino acid residues resulted in a splicing-proficient minimal intein (Ssp DnaB mini-intein) consisting of the N-terminal 106 residues and the C-terminal 48 residues (Sun *et al*., 2004). It has been reported that the cleavage of Ssp DnaB mini-intein is affected by the amino acid directly adjacent to the C-terminus of the intein (Mathys *et al*., 1999). Present research aims to further investigate the impact of different factors (time, temperature and pH) as well as the first C-extein residue on cleavage.

MATERIALS AND METHODS

Plasmid construction: The Impact-Twin System was used to express the target protein (C-extein) fused to the Ssp DnaB mini-intein. The intein had been modified for pH and temperature dependent cleavage at its C-terminus (Perler, 2005, 2006). The N-terminus of the intein was fused to the Chitin-Binding Domain (CBD), allowing

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affinity purification of the fusion protein on a chitin bead resin followed by intein cleavage. The primers for the *C-extein* gene were designed to amplify the target protein encoding sequence and to introduce NruI and EcoRI restriction sites for cloning into pTWIN-1 expression Vector (New England Biolabs, Hitchi, Hertfordshire). Forward primer 5'-CATTCGCGAATGACATCATTGTACACAACAAAGCAACC-3' contained an NruI recognition site (underlined). The first C-extein residue was Lys (underlined) which could be substituted by Ser, Cys or Asp. Reverse primer 5'-CTAAGAATTCTTAGTTGCGGGTGCCCGGATAAATAA-3' contained a EcoRI recognition site (underlined). The PCR product and the pTWIN1 plasmid were digested with restriction endonucleases NruI and EcoRI and then the fragments were ligated into pTWIN1 using T4 DNA ligase. The products of the ligation were then transformed into *E. coli* strain JM109.

Protein expression and purification using C-terminal cleavage: Recombinant plasmid was transformed into *E. coli* strain ER2566 and grown at 37°C in 200 mL LB medium containing 100 μg mL^{-1} ampicillin to an A_{600} of 0.5-0.7. Expression was induced by 0.3 mM Isopropyl β-D-Thiogalactoside (IPTG) at 37, 30, 22 and 15°C, respectively. The bacteria were sonicated at 4°C. Crude extracts (40 μL) were mixed with 40 μL of 2xSDS-PAGE sample buffer which contains 187.5 mM tris-HCl, 6% (W/V) SDS, 30% glycerol and 0.03% (W/V) bromophenol blue. Then, the samples were electrophoresed on a 15% Tris-glycine PAGE and then stained with Coomassie Blue.

RESULTS

Comparison of expression of the fusion protein in different temperatures: The pTWIN System places inteins at both the N and the C terminus of a target protein. In present study, the intein at the N terminus of the target protein, intein 1 was the Ssp DnaB mini-intein which contained a mutation that blocked protein splicing but allowed cleavage at its C-terminus. The sequence of the C-extein was inserted into the expression vector pTWIN1 as a C-terminal fusion to a mini-Ssp DnaB intein with an upstream Chitin-Binding Domain (CBD). As the pTWIN System contained one NdeI site that used ATG as the translational start site, the fusion protein CBD-Ssp DnaB intein-extein was expressed. Expression of the fusion protein in *E. coli* strain ER2566 allowed the expression of IPTG-regulated T7 RNA polymerase. In preliminary experiments, induction of the cells at 37°C for 3 h was found to be optimal to achieve highest expression level of the fusion protein however, the level of soluble fusion protein is very low. Researchers also induced the cells at 30, 22 and 15°C and found that with lower temperature, the proportion of soluble fusion protein became higher (Fig. 1).

Splicing reaction of the fusion protein: It has been reported that the cleavage of Ssp DnaB intein could be affected by the amino acid directly adjacent to the C-terminus. In order to further investigate the effect of the first C-extein residue on intein cleavage, the first C-extein residue Lys was substituted with Ser, Cys or Asp. In each case, intein is expressed as a three-part fusion protein comprising CBD, intein and C-extein. The cleavage activity was analyzed by SDS-PAGE after induction of plasmids bearing cells at different temperatures, i.e., 37°C for 2.5 h, 30°C for 4 h, 22°C for 8 h or 15°C overnight. The presence of Ser or Cys at the first residue of C-extein resulted in the cleavage of the most precursor proteins and the percentage of cleavage decreased when the induction temperature went down and the induction time became shorter. The presence of Asp at the first residue resulted in cleavage of about 30% precursor proteins even when the cells were induced at different temperatures. When cells were induced at 15°C for overnight and 22°C for 8 h, Lys substitution did not result in any cleavage of precursor proteins. However, when induced at 30 and 37°C, about 40% precursor proteins were cleaved (Fig. 2). Researchers also analyzed the sonicated cells induced at different temperatures with supernatant incubated at 37, 30, 25, 15 and 4°C for 1 day and found that with higher temperature and longer incubated time, more precursor proteins were cleaved including the fusion proteins regardless of Lys or Asp as the first residue of C-extein. Ssp DnaB mini-intein was also tested for its pH-dependent on C-terminus cleavage. Researchers

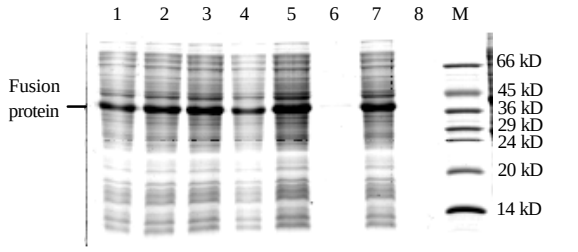

Fig. 1: Soluble and insoluble proteins at different inducing temperatures. 1: Soluble proteins at 37°C; 2: Insoluble proteins at 37°C; 3: Soluble proteins at 30°C; 4: Insoluble proteins at 30°C; 5: Soluble proteins at 22°C; 6: Insoluble proteins at 22°C; 7: Soluble proteins at 18°C; 8: Insoluble proteins at 18°C; M: Protein Marker

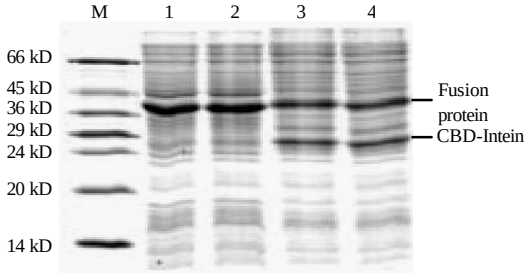

Fig. 2: Splicing reaction of the fusion protein *in vivo* with Lys as the first residue of C-extein at different inducing temperatures. The intein was expressed as a fusion protein (36 kDa) consisting of the N-terminal chitin binding protein (8 kDa), the Ssp mini-intin (18 kDa) and C-extein (10 kDa). M: Protein Marker; 1: at 15°C; 2: at 22°C; 3: at 30°C; 4: at 37°C

incubated the supernatant at different pH (5.0-10.0) as reported (Xu *et al*., 2008), the cleavage was most favored at pH 7.0-7.5 with about 80% fusion protein cleaved and was independent of the first residue of C-extein.

DISCUSSION

In previous studies, amino acids were classified into 3 sorts on the bases of their effects on the cleavage of intein when they were at the first residue place of C-extein (Mathys *et al*., 1999). To confirm this, researchers tested 4 residues from these 3 sorts as the first C-extein residue. Just as (Xu *et al*., 2008) reported, Ser and Cys were the most favored residues for C-terminal cleavage. Furthermore, researchers found that the cleavage happened regardless of the temperature of cell incubation which leads to difficult protein purification and lower yields of target protein. It has been reported that Lys as the first C-extein residue blocked cleavage with 10% less than the precursor being processed (Mathys *et al*., 1999). The results demontratd that induction at 15 and 22°C resulted in similar patterns of C-terminal cleavage as reported (Xu *et al*., 2008) but induction at 37°C for 2.5 h and 30°C for 4 h resulted in cleavage of about 40% of precursor indicating that the induction temperature and time might influence the cleavage of fusion protein. The pTWIN System needs cleavage *in vitro* which leads to simple protein purification and decent target protein yields. Considering the level of soluble fusion protein and total protein expressed, researchers found that inducing cells at 22°C is optimal and when extract was incubated at 25°C for 24 h, most precursors cleaved including the precursors with Lys as the first residue of the C-extein.

CONCLUSION

In this research, the study demonstrated that induction temperature influenced the cleavage of intein and the first C-extein residue, the cleavage temperature, duration and pH, should all be taken into account for optimization of expression and purification of proteins of interest.

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