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Expressed protein ligation using an N-terminal cysteine containing fragment generated *in vivo* from a pelB fusion protein

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Abstract

Advances in expressed protein ligation (EPL) methods that permit specific introduction of unique modifications into proteins have facilitated protein engineering, structure–function and protein interaction studies. An EPL-generated hybrid exchangeable apolipoprotein has been constructed from recombinant fragments of apolipoprotein E (apoE) and apolipophorin III (apoLp-III). A recombinant fusion protein comprised of human apoE N-terminal residues 1–111, a modified *Saccharomyces cerevisiae* intein and a chitin binding domain was subjected to 2-mercaptoethanesulfonic acid (MESNA) induced cleavage to generate apoE(1–111)-MESNA. A second fusion protein was comprised of a bacterial pelB leader peptide fused to a variant form of *Galleria mellonella* apoLp-III residues 1–91. The N-terminal pelB leader sequence directed the newly synthesized fusion protein to the *Escherichia coli* perisplamic space where endogenous leader peptidase cleavage generated the desired N-terminal cysteine-containing protein fragment. The resulting apoLp-III fragment, which contained no sequence tags or tails, escaped the bacteria and accumulated in the culture medium. When cultured in M9 minimal medium, Asp1Cys apoLp-III(1–91) was produced in high yield and was the sole major protein in the culture supernatant. Ligation reactions with apoE(1–111)-MESNA yielded an engineered hybrid apolipoprotein. The results document the utility of the pelB fusion protein system for generating active N-terminal cysteine containing proteins for EPL applications.

Keywords: Expressed protein ligation; Intein; Apolipoprotein; Apolipophorin; PelB; Leader peptidase

Native chemical ligation is a useful synthetic method to join independently generated protein fragments via a native peptide bond. Expressed protein ligation (EPL)[1] is a form of native chemical ligation that utilizes intein technology for expression and/or purification of one or more of the fragments to be ligated [1]. EPL has been used to incorporate unnatural amino acids [2,3], biophysical probes [4], post-translational modifications [5] and isotope labels [6,7] in specific locations within a ligated protein product [reviewed in 1,8,9]. These and other EPL strategies have allowed unique problems of protein structure, folding, enzyme mechanism, ion channel function and signaling to be addressed in novel and insightful ways.

EPL involves joining the desired protein fragments via an autocatalytic chemical ligation [8,10]. This reaction, which creates a peptide bond between protein fragments, requires a specific thioester linked leaving group moiety covalently bound to the terminal carboxyl group of one fragment and a cysteine at the amino terminus of the second fragment [8,10]. Adaptations of intein-dependent protein splicing reactions (analogous to intron/exon splicing) originally observed in *Saccharomyces cerevisae* have made it possible to isolate appropriately modified fragments for subsequent ligation [10–12]. Recombinant production of the protein fragment containing a thioester-linked leaving group moiety normally includes

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[1] *Abbreviations used:* Apo, apolipoprotein; ApoLp, apolipophorin; DTT, dithiothreitol; EPL, expressed protein ligation MESNA, 2-mercaptoethanesulfonic acid; N-cys, amino-terminal cysteine.

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thiol-dependent autocatalytic, intein-mediated cleavage of an engineered fusion protein. Generation of the second protein fragment has relied on three primary approaches including synthetic production by solid-phase peptide synthesis, proteolysis of recombinant proteins by *in vitro* or *in vivo* methods [13,14], or thiol and temperature dependent intein mediated fusion protein cleavage [15,16; reviewed in 9,17]. Given limitations on fragment length using solid-phase peptide synthesis, the specificity and cost of *in vitro* protease cleavage and production/yield issues with intein-mediated fusion protein cleavage, the EPL strategy employed requires careful consideration.

Signal peptidases located in the bacterial periplasmic space have been extensively utilized for high-yield production of recombinant proteins [18,19]. The predictable and precise nature of leader peptidase cleavage at the pelB-protein junction combined with high protease activity with a cysteine at position-1 [18], suggests EPL-active protein fragments can be generated by engineering a pelB leader sequence adjacent to the amino-terminal cysteine of the fragment of interest. Furthermore, the pelB sequence directs the newly synthesized protein to the periplasmic space where the membrane-anchored peptidase is localized [20,21]. Studies of bacterially expressed recombinant apolipoproteins have shown that, not only does efficient pelB cleavage occur, the protein product also escapes the periplasm and accumulates in the extracellular culture media [22–25]. This process, for which the mechanism is unknown, facilitates recovery and downstream processing of recombinant proteins from bacterial cultures.

Apolipoprotein E (apoE) is a 299-amino acid glycoprotein that is a well-characterized ligand for the low-density lipoprotein receptor [26]. The X-ray crystal structure of the isolated N-terminal domain revealed a globular bundle of four elongated amphipathic α-helices that is stabilized by interhelical hydrophobic interactions in the absence of lipid [27]. Likewise, insect apolipophorin III (apoLp-III) adopts a helix bundle organization [28,29]. Using recombinant DNA technology, a hybrid apolipoprotein comprised of sequence elements derived from apoE and apoLp-III has been generated [30]. Studies revealed that this engineered hybrid apolipoprotein adopts a folded protein structure that manifests biological activity of the parent proteins. To further pursue hybrid apolipoprotein research, EPL has been employed to generate a protein hybrid comprised of apoE residues 1–111 and Asp1Cys-substituted apoLp-III residues 1–91. To achieve this, the pelB bacterial expression system was employed to generate Asp1Cys apoLp-III(1–91) for use in ligation studies with C-terminal thiol-adducted human apoE(1–111) derived from an intein fusion protein. Optimization studies to determine conditions that promote protein ligation revealed effects of temperature, pH and thiol agent. The approach described expands the strategies available for EPL and provides a means to specifically modify sequence elements within a novel hybrid apolipoprotein.

Materials and methods

Preparation of apoE(1–111)

Human apoE(1–111) was cloned into the pTYB1 vector (New England Biolabs) and expressed in *E. coli* ER2566 cells as an *S. cerevisiae* VMA1 intein and chitin binding domain (CBD) fusion protein. To facilitate optimal intein-mediated fusion protein cleavage [31], valine 111 was mutated to alanine using the QuikChange method (Stratagene) according to the manufacturer's instructions. Expression and purification procedures for apoE(1–111) followed standardized protocols previously established for generating intein-mediated thioester-adducted proteins [10]. Briefly, saturated overnight cultures were inoculated into 2× YT media containing 50 μg/mL ampicillin, grown to $OD_{600} = 0.6$ and induced with 1 mM isopropyl thiogalactopyranoside (IPTG). After 6 h at 30 °C the cells were pelleted by centrifugation (8000 g for 15 min), solubilized with buffer A (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing 1% Triton X-100 and stored at −20 °C. Dissolved cell pellets were combined, passed through a microfluidizer, sonicated and centrifuged at 12,000*g* for 20 min. Isolated clarified cell extract was passed over a chitin bead column pre-equilibrated with buffer A containing 1% Triton X-100. The column was washed with 10 column volumes of detergent-free buffer A. Fusion protein cleavage was then induced by addition of 2-mercaptoethanesulfonic acid (MESNA) to a final concentration of 60 mM. Flow was arrested for 16–24 h at 22 °C and eluted with two bed volumes of buffer A containing 5 mM MESNA. The sample was dialyzed against deionized H_2O, lyophilized and stored at −20 °C. ApoE(1–111)-MESNA was further purified by semi-preparative C_8 reversed-phase high performance liquid chromatography on a Perkin-Elmer Series 200 HPLC.

Preparation of apoLp-III(1–91)

The coding sequence for *G. mellonella* apoLp-III(1–91) was cloned into the pET22b plasmid (Novagen) directly adjacent to a vector encoded pelB leader sequence. Site directed mutagenesis of aspartate 1 to cysteine was performed using the QuikChange method. Expression and purification of Asp1Cys apoLp-III(1–91) was carried out as previously described for the wild type fragment [25,32]. Briefly, saturated overnight cultures were inoculated into M9 media supplemented with 13.3 mM glucose, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$ and 50 μg/mL ampicillin. At $OD_{600} = 0.6$, the culture was induced with 2 mM IPTG. After 6 h at 30 °C, bacteria were pelleted by centrifugation at 8000*g* for 15 min and the culture supernatant collected, concentrated by ultrafiltration and chromatographed on a 2.5×30 cm column of Sephadex G-75. Fractions containing apoLp-III(1–91) were pooled, dialyzed against deionized H_2O, lyophilized and further purified by semi-preparative C_8 reversed-phase HPLC.