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Biological Synthesis of Alkyne-terminated Telechelic Recombinant Protein

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Abstract: In this study, we demonstrate that the biological unnatural amino acid incorporation method can be utilized *in vivo* to synthesize an alkyne-terminated telechelic protein. Synthesis of terminally-functionalized polymers such as telechelic polymers is recognized to be important, since they can be employed usefully in many areas of biology and material science, such as drug delivery, colloidal dispersion, surface modification, and formation of polymer network. The introduction of alkyne groups into polymeric material is particularly interesting since the alkyne group can be a linker to combine other materials using click chemistry. To synthesize the telechelic recombinant protein, we attempted to incorporate the L-homopropargylglycine into the recombinant GroES fragment by expressing the recombinant gene encoding Met at the codons for both N- and C-terminals of the protein in the Met auxotrophic *E. coli* via Hpg supplementation. The Hpg incorporation rate was investigated and the incorporation was confirmed by MALDI-TOF analysis of the telechelic recombinant protein.

Keywords: recombinant protein, telechelic protein, unnatural amino acid, alkynylation.

Introduction

Protein, a polymer composed of twenty amino acids, is one of the abundant macromolecules observed in nature, which plays important biological roles in a cell and living organisms. So far, most of the applications using proteins focused on the use of their biological activities. For instance, enzymes could be utilized as catalysts in various chemical reactions, and antibodies were employed as therapeutics.[1] On the other hand, number of recent reports illustrated the use of protein hydrogel for drug delivery and protein matrices for tissue engineering, where proteins could be employed as replacement materials for synthetic polymers.[2,3]

Major problem in the use of proteins as a polymeric material is that the functional chemical groups in protein are limited to those of the side chains in twenty amino acids, which limits the physico-chemical properties of proteins. Although various chemical methods were developed to introduce unnatural functional groups into proteins, the incorporation of the unnatural amino acids required the complicated protection and deprotection steps, which hampered in large scale reactions.[4-7] Recently, biological methods to incorporate unnatural functional groups into proteins *in vivo* or *in vitro* were reported.[8-11] The methods basically rely on the ability of the biological translation apparatus to accept amino acid analogues with structures similar to natural amino acids, which enables us to perform a site-specific incorporation of desired functional groups, avoiding the protection and deprotection steps. In particular, the *in vivo* strategy employing the conventional fermentation process was recognized as promising method due to its simplicity and the possibility of the large scale production.

The incorporation of unnatural amino acids into a target protein *in vivo* can be achieved by simple supplementation of the amino acid analogues in the medium for the host with

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an aminoacyl tRNA synthetase (aaRS), which can induce the interaction between the analogues and endogenous tRNA.[12,13] For instance, the incorporation of methionine (Met) analogues into a target protein was simply accomplished by expressing the recombinant gene for the target protein in the Met auxotrophic *Escherichia coli* containing the engineered aaRS for Met with the supplementation of Met analogues.[14,15] In particular, L-homopropargylglycine (Hpg) and azidohomoalanine (Aha), the methionine analogues containing alkyne group and azide group, respectively, were known to be introduced effectively into the Met residues of the recombinant protein even without any modification of the translational machinery of the host.[16] These approaches have been employed to selectively modify the recombinant proteins with alkyne group or azide group, followed by fluorescence labeling or glycosylation of the proteins by means of Cu(I)-catalyzed cycloaddition.[17-21]

In this study, we demonstrate that the biological unnatural amino acid incorporation method *in vivo* can be utilized to synthesize an alkyne-terminated telechelic protein.[21] Synthesis of terminally-functionalized polymers such as telechelic polymers became important, since they can be employed usefully in many areas of biology and material science, such as drug delivery, colloidal dispersion, surface modification, and formation of polymer network, etc..[22,23] The introduction of alkyne group into polymeric material is particularly interesting as the alkyne group can be a linker to combine other materials using "click" chemistry through copper (I) catalyzed Huisgen 1,3 dipolar cycloaddition reaction with azides, yielding 1,4-disubstituted 1,2,3-triazole linked conjugates.[24-27] The "click" chemistry is appealing because it is highly regiospecific, chemoselective, and tolerant to a wide variety of other functional groups. Moreover, the reaction can be carried out at room temperature under mild condition in an aqueous buffer. All these outstanding features of click chemistry can be utilized in bioconjugation applications,

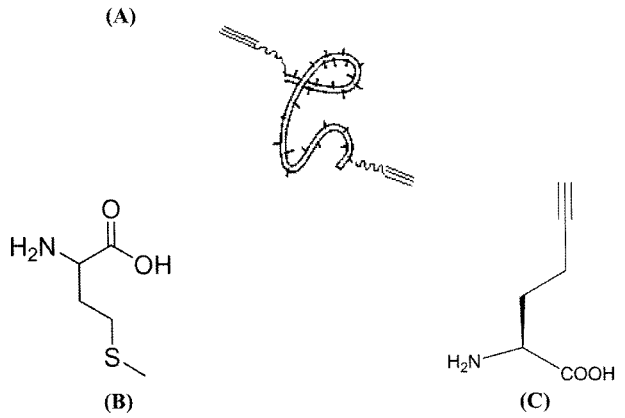


Figure 1. Telechelic recombinant protein, and structures of methionine and its analogue used in this study. (A) Telechelic protein with terminal alkynylation; (B) methionine (Met); (C) homopropargylglycine (Hpg).

organic synthesis and material science.[28]

To synthesize the telechelic recombinant protein, we generated a recombinant gene encoding Met at the codons for both N- and C-terminal, and we attempted to incorporate the Hpg into the recombinant protein by expressing the recombinant gene in the Met auxotrophic *E. coli* with the supplementation of Hpg. Figure 1(A), (B), and (C) illustrate the structure of telechelic protein, Met, and Hpg, respectively. Here we optimized the production rate of the recombinant protein, purified the protein using the interaction between hexahistidine tag and Ni^{2+} ion, and confirmed the Hpg incorporation through MALDI-TOF analysis.

Experimental

Materials. PCR reagents, T4 DNA ligase and restriction endonucleases were purchased from Promega (Madison, WI, USA). The isopropyl-D-thiogalactopyranoside (IPTG) and natural amino acids were obtained from Sigma Inc. (St. Louis, MO, USA). L-homopropargylglycine (Hpg) was purchased from Chiralix (Nijmegen, The Netherland). The nickelnitrilotriacetic acid (Ni-NTA) affinity column was purchased from Qiagen (Valencia, CA, USA). *E. coli* B834 (DE3) methionine auxotroph was obtained from Prof. William Studier (Brookhaven National laboratory, USA). Plasmid vector pET21a was purchased from Novagen (Madison, WI) and T7 promoter was used in this study for protein expression.

Plasmid Construction. The DNA manipulations were performed according to the procedures described by Sambrook and Russel,[29] and the pGEM-T vector system (Promega Corporation, WI, USA) was used for the cloning of PCR products. The PCR reaction was conducted in 50 μL mixture containing 10 pmole of primer, template DNA, 1x Taq DNA polymerase buffer, 2.5 units of Taq DNA polymerase, 2.5 mM deoxyribonucleotide triphosphates, and 1.5 mM $MgCl_2$ was used in this study. Amplification was performed in a DNA thermal cycler (Master Gradient thermal cycler, Eppendorf, Hamburg, Germany) programmed for an initial denaturation (94 °C for 1 min) followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 0.5 min at 72 °C with an extension at 72 °C for 10 min. The gene encoding the GroES fragment was amplified from template plasmid pBAD-GroELS using the primers GroES M2F-5'-CATATGAATATTCGTCCATTGCAT-3' and GroES M2R 5'-GAATTCTTATTACAT GTGGTGGTGGTGGTGGTGGATCAACACTTCTTCATT-3'. The amplified DNA fragment was purified using PCR clean-up system (QIAquick PCR Purification kit, Qiagen, Valencia, CA), and cloned into pET21a using *NdeI* and *EcoRI* resulting pET21-GroESM2.

Expression of GroESM2 and SDS PAGE Analysis. *Escherichia coli* B834 (DE3) with the pET21a-GroESM2 plasmids was grown to optical density (OD) at 600 (OD_{600}) of 0.6 at 37 °C in 5mL LB medium containing 100 μg/mL ampicillin, induced with different concentration IPTG to optimize

the protein production. After 5 h, the cells were harvested by centrifugation at 4,000 g at 4 °C. Total cell protein fractions were analyzed by SDS–PAGE (12% acrylamide gel), as described previously.[30]

Incorporation of Unnatural Amino Acid. Culture of *Escherichia coli* B834 (DE3) with pET21-GroESM2 was grown in M9 minimal medium, supplemented with 0.4% (w/v) of glucose, 0.1 mM of $CaCl_2$, 1.0 mM of $MgSO_4$, 35 μg/mL of thiamine, 20 amino acids (40 mg/L), 100 μg/mL of ampicillin. When the culture reached OD_{600} of 0.8 to 1.0, the culture was centrifuged for 10 min (5,000 rpm) at 4 °C. The cell pellets were washed twice with 0.9% (w/v) of NaCl solution. The cells were resuspended in M9 minimal medium supplemented with 19 amino acids (40 mg/L) without methionine. Aliquots (10 mL) of the resuspended cells were added to different test tubes containing L-methionine (40 mg/L) as a positive control, L-Hpg (40 mg/L) as a test and without addition of L-methionine, and Hpg as a control. After 10 min incubation, 1 mM of IPTG was added to induce GroESM2 protein expression. OD_{600} of the culture was measured 12 to 24 h after induction, respectively. Then the cells were harvested by centrifugation for 10 min (6,000 rpm) at 4 °C and stored at -70 °C. The samples were sedimented and decanted, and the cell pellets were used for further analysis. Protein expression was monitored by SDS polyacrylamide gel electrophoresis (12% acrylamide running gel, 12 mA, 15 h).

Protein Purification. After approximately 24 h of induction, cells were sedimented (9,800 g, 10 min, 4 °C), and the supernatant was removed. The pellet was placed in the freezer overnight. The cells were thawed for 30 min at 37 °C, 30 mL of buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8) were added, and the mixture was shaken at room temperature for 1 h. The cell debris was sedimented (15,300 g, 20 min, 4 °C), and the supernatant was subjected to the immobilized metal affinity chromatography (Ni-NTA resin), according to the procedure described earlier. The supernatant was loaded on 10 mL of resin, which was then washed with by 25 mL of urea buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 8). Similar urea buffers were used for three successive 25-mL washes at pH values of 6.3, 5.9, and 4.5, respectively. Target protein was obtained in washes at pH 5.9 and 4.5. These washes were combined and concentrated by Millipore Amicon ultra centrifugal filter devices (Billerica, MA, USA) against running distilled water for several times. The concentrated samples used for MALDI-TOF analysis.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) Analysis. The purified GroESM2 solutions were desalted by ZipTipC18 (Millipore) and eluted with 3 μL of 50% CH_3CN/0.1% TFA. 1 μL was used for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis with sinapinic acid (30% (v/v) of acetonitrile and 70% (v/v) of 0.1% TFA solution) as the matrix. The analysis was performed by MALDI-TOF Voyager DE-STR biosystems (Applied Biosystems, USA) mass spectrometer operating in linear and positive ion modes. For MALDI-MASS analysis of tryptic digests of purified GroES, 10 μL of the concentrated protein solution were added to 90 μL of 75 mM ammonium bicarbonate solution. 2 μL of 0.2 g/L of modified trypsin (Promega) were added, and the solution was incubated at 37 °C for 2.0 h. 12 μL of 5% TFA solution were added to quench the reaction. Chromatography on ZiptipC18 columns (Millipore) provided the purified peptide samples (1 μL), which were added to 2,5-dihydroxybenzoic acid (DHB) MALDI matrix solution for MALDI-MASS analysis.

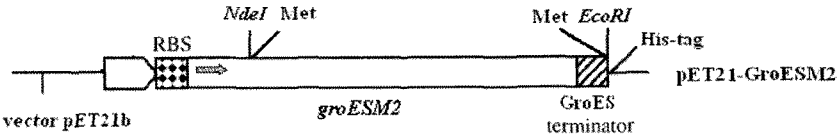
Results and Discussion

Construction of Expression System for Telechelic Protein. GroES protein, a co-chaperone subunit of GroELS chaperone system in *E. coli*, was used as a template for the construction of telechelic protein. GroES, originally a protein of 10.4 kDa, is composed of 97 amino acids and is encoded by 291 bp nucleotides. The protein contains two methionines at first and 86th residues in the primary sequence. To construct the expression system for telechelic protein, the nucleotides encoding the region of Met1 to Met86 of GroES were synthesized by PCR reaction with the insertion of sequence for 6X His in front of Met86, and cloned into pET21a vector, resulting in pET21-GroESM2. The gene for hexahistidine was inserted to purify the expressed protein using Ni-NTA system. The constructed expression system pET21-GroESM2 shown in Figure 2(A) can produce the recombinant GroESM2 protein of 9.95 kDa under normal growth condition, supplemented by twenty natural amino acids. Figure 2(C) shows the predicted protein sequence of GroESM2 encoded by the 276 bp recombinant gene depicted in Figure 2(B).

The pET21-GroESM2 was transformed into B834(DE3), and the GroESM2 overexpression in LB medium was confirmed by SDS-PAGE analysis after the induction of recombinant protein at 37 °C for 5 h. In order to investigate the effect of inducer concentrations on the recombinant protein production, different concentrations of IPTG were tested for induction of GroESM2, and the amounts of expressed proteins were measured after 5 h. Figure 3 shows that the highest productivity of target protein was observed at 0.5-1 mM concentration of IPTG. Based on the results, 1 mM concentration of IPTG was used in the incorporation study of unnatural amino acid.

Incorporation of Alkynyl Amino Acid into GroESM2. *E. coli* strain B834 (DE3) with pET21-GroESM2, which produces GroESM2 protein upon induction with IPTG, was used in the production of alkynylated telechelic protein. The parent strain B834 (DE3) is a methionine auxotroph, which depends on the supplement of methionine for the growth. The mutated the *metE* gene, B834 (DE3), is essential at the

(A)



(B) GroESM2 – Gene sequence

ATG AAT ATT CGT CCA TTG CAT GAT CGC GTG ATC GTC AAG CGT AAA GAA GTT GAA ACT AAA TCT GCT GGC GGC ATC GTT CTG ACC GGC TCT GCA GCG GCT AAA TCC ACC CGC GGC GAA GTG CTG GCT GTC GGC AAT GGC CGT ATC CTT GAA AAT GGC GAA GTG AAG CCG CTG GAT GTG AAA GTT GGC GAC ATC GTT ATT TTC AAC GAT GGC TAC GGT GTG AAA TCT GAG AAG ATC GAC AAT GAA GAA GTG TTG ATC **CAC CAC CAC CAC CAC CAC** *ATG*

(C) GroESM2 – Protein sequence

MNIRPLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRILENGEV

KPLDVKVGDIVIFNDGYGVKSEKIDNEEVLIHHHHHHM

Figure 2. Construction of pET21-GroESM2 vector. (A) Schematic diagram of pET21-GroESM2; (B) Gene sequence for GroESM2 encoding two methionines (Italic) and His-tag (Underlined); (C) GroESM2 protein sequence.

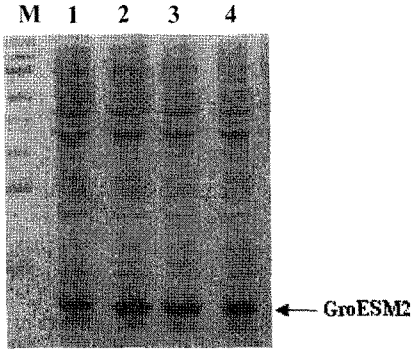


Figure 3. Expression profile of GroESM2 in LB medium. Lane M: protein ladder; lane 1 to 4: GroESM2 induced with IPTG (0.05 mM, 0.1 mM, 0.5 mM, 1 mM).

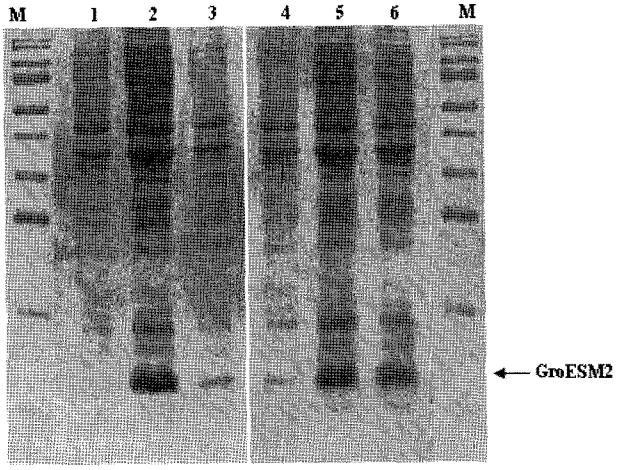


Figure 4. Expression profile of GroESM2 under various conditions. Lane M: protein ladder, lane 1: 19 amino acids (12 h induction), lane 2: 19 amino acids + L-methionine (12 h induction), lane 3: 19 amino acids + L-Hpg (12 h induction), lane 4: 19 amino acids (24 h induction), lane 5: 19 amino acids + L-methionine (24 h induction), lane 6: 19 amino acids + L-Hpg (24 h induction).

final step in the endogenous synthesis of methionine. Cultures were grown in minimal medium with methionine supplement until reaching a cell density of OD_{600} 0.8-1.0. Cells were sedimented, washed, and resuspended in minimal medium without methionine. Aliquots of the culture were then supplemented with L-Hpg, a Met analogue containing alkyne group. Protein synthesis was induced with IPTG, and cell growth and protein expression were followed over time course periods. In order to optimize the level of L-Hpg incorporation to the targeted recombinant protein, samples were collected in different time course intervals and analyzed by the SDS-PAGE. Total protein extracts from *E. coli* cultures with supplemented methionine and L-Hpg at various time intervals were used. SDS-PAGE shows the expression results of 12 h and 24 h in Figure 4. The results clearly revealed the sufficient translational activity of L-Hpg in protein synthesis without methionine. On the other hand, the results also indicated that the incorporation rate of Hpg into target protein was much lower than that of Met. We could observe that the expression level of GroESM2 incorporated by Met was saturated at 12 h. However, the SDS-PAGE analysis showed that the band intensity of HPG incorporated sample at 12 h was much weaker compare to that at 24 h. This suggests that 12 to 24 h of induction time would be required for the efficient incorporation of L-Hpg in GroES fragment.

Identification of the Hpg Incorporation Into GroESM2.

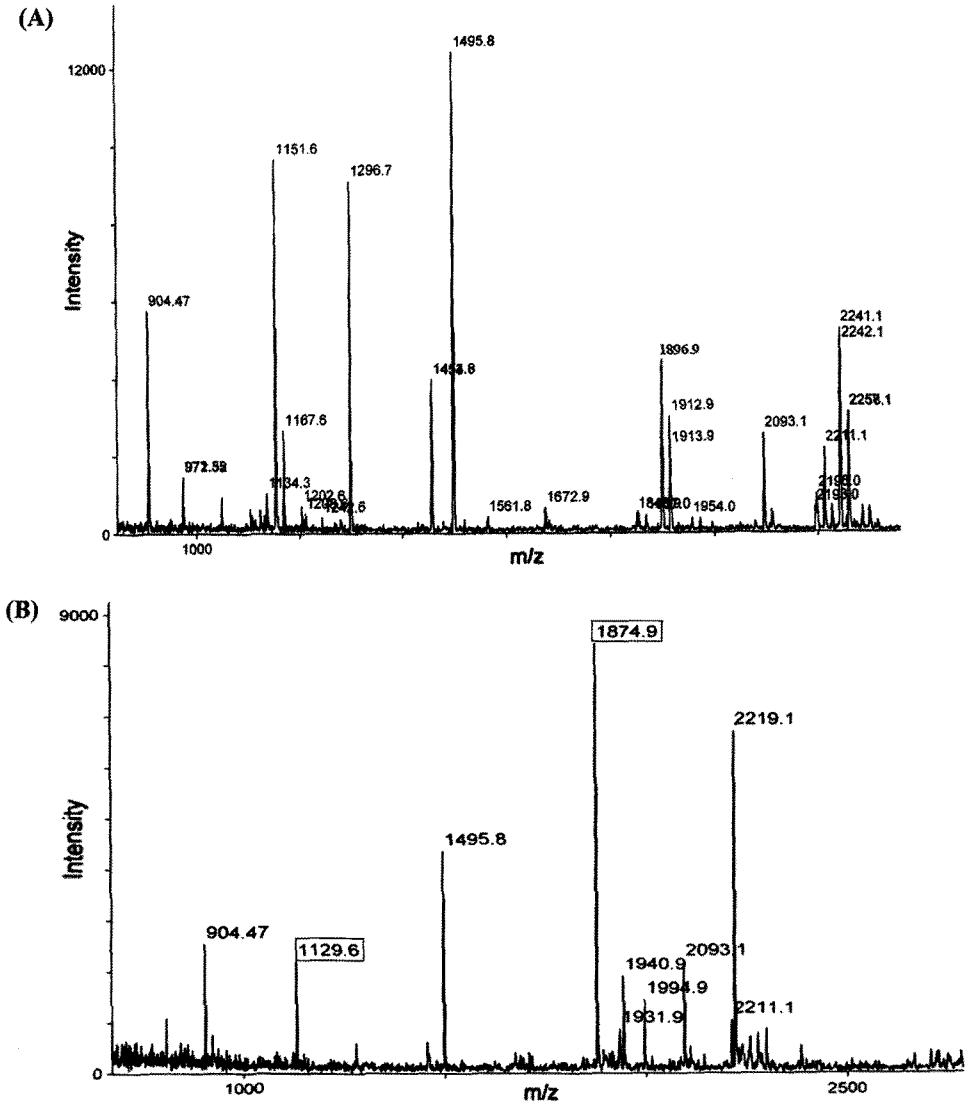


Figure 5. MALDI analysis of GroESM2 after trypsin digestion. The protein was expressed with 20 canonical amino acids (A) and 19 amino acids with L-homopropargylglycine (L-Hpg) (B), and was purified in denaturing conditions. These spectra were obtained after tryptic digestion, yielding different peptide fragments. Replacement of methionine with L-Hpg resulted in a mass reduction of 22 Da. for our targeted protein (Labeled by a box in the figure B).

E. coli methionine auxotroph B834 (DE3) with pET21-GroESM2 (100 mL culture) was grown in minimal medium containing Met or L-Hpg, and was induced with IPTG for 24 h at 30 °C. The cells were pelleted and lysed. The GroESM2 protein was purified under denaturing condition, and further concentrated with Millipore centrifugal device. Details were described in the experimental section. The purified samples were loaded into SDS-PAGE gel, and the region of the GroESM2 gel was sliced and analyzed by MALDI-TOF after tryptic digestion.

Figure 5(A) shows the mass spectrum of the fragments of GroESM2 incorporated with Met. Two representative fragments for N-terminal and C-terminal after tryptic digestion were expected to be the residue 1-9 (amino acid sequence: MNIRPLHDR, theoretical mass: 1,151) and the residue 78-92 (amino acid sequence: IDNEEVLIHHHHHHM, theoretical mass: 1,897), respectively. These respective fragments could be observed at the mass of 1,151.6 and 1,896.9. When the methionines for the two tryptic digested fragments for the N- and C-termini of GroESM2 were replaced with Hpg, the theoretical mass of these two fragments was expected to be shifted by -22 Da, yielding the mass of 1,129 and 1,875. Figure 5(B) illustrates the mass spectrum of the fragments of GroESM2 with Hpg, matching the theoretical mass of 1,129.6 and 1,874.9. These MALDI-TOF results strongly suggest the sufficient labeling of alkyne residue at the N- and C-termini of the targeted GroESM2. We could not detect the mass peaks corresponding to the Met incorporated fragments

in this detectable range, indicating that the Met contamination to the telechelic protein from the endogenous Met in cells was very low.

Interests in exploring and expanding the synthetic capacity of biological polymerization processes have been growing. The incorporations of modified or completely "synthetic" nucleic acid bases were studied and reported.[29-35] In addition, materials researchers have also exploited the broad range of substrates for the poly (β-hydroxyalkanoate) (PHAs) synthases in preparation of novel PHAs with unusual physical properties.[36,37] Several strategies for incorporating unnatural amino acids were demonstrated and successfully modified to produce different proteins with unique structure and new physico-chemical proprieties. In the current report, we successfully engineered the terminal regions of recombinant protein with alkyne group, yielding an alkyne-terminated telechelic protein. The biological strategy to synthesize telechelic protein would expect to offer vast opportunities in the field of material science with the better biocompatibilities, more hydrohylic, and higher precisions in size-controlled polymers.

Conclusions

We reported the biological synthesis of the recombinant protein containing alkyne group at both termini, i.e. alkyne-terminated telechelic protein. Based on the recombinant technology, we engineered the gene for GroES protein to allow the incorporation of unnatural amino acid at both termini. We also investigated the time course for the efficient incorporation of unnatural amino acid to the target protein, and MALDI-TOF analysis confirmed the incorporation of non-natural amino acid. We expect that recombinant DNA technology and biological incorporation method of unnatural amino acid into target protein would enable us to control the size and composition of protein material, which is difficult to achieve through conventional chemical polymerization methods.

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