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Expression and purification of short hydrophobic elastin-like polypeptides with maltose-binding protein as a solubility tag

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ABSTRACT

Elastin-like polypeptides (ELPs) are biodegradable polymers with interesting physico-chemical properties for biomedical and biotechnological applications. The recombinant expression of hydrophobic elastin-like polypeptides is often difficult because they possess low transition temperatures, and therefore form aggregates at sub-ambient temperatures. To circumvent this difficulty, we expressed in *Escherichia coli* three hydrophobic ELPs $(VPGIG)_n$ with variable lengths (n = 20, 40, and 60) in fusion with the maltose-binding protein (MBP). Fusion proteins were soluble and yields of purified MBP-ELP ranged between 66 and 127 mg/L culture. After digestion of the fusion proteins by enterokinase, the ELP moiety was purified by using inverse transition cycling. The purified fraction containing ELP40 was slightly contaminated by traces of undigested fusion protein. Purification of ELP60 was impaired because of co-purification of the MBP tag during inverse transition cycling. ELP20 was successfully purified to homogeneity, as assessed by gel electrophoresis and mass spectrometry analyses. The transition temperature of ELP20 was measured at 15.4 °C in low salt buffer. In conclusion, this method can be used to produce hydrophobic ELP of low molecular mass.

Introduction

Elastin-like polypeptides (ELPs)[1] are peptide polymers derived from the hydrophobic region of tropoelastin [1]. They consist of repeats of the sequence Val-Pro-Gly-Xaa-Gly, where the guest residue Xaa can be any amino acid except proline. Above a certain length, ELP are characterized by a reversible inverse transition temperature (T_t) [2,3]. Below this temperature, the free polymer chains are soluble in aqueous solution whereas they form insoluble aggregates when the temperature of the solution is raised above the T_t, a phenomenon that is fully reversible. The T_t value varies according to the nature of the guest residue as well as to the length of the polypeptide chain [4]. It is also dependent on the polypeptide concentration in solution as well as on the nature and concentration of co-solutes. Owing to their advantageous properties, ELPs are of significant interest for biomedical and biotechnological applications [5–7].

Our goal was to produce recombinant ELPs with T_ts in the 10–20 °C range. Such ELPs would be soluble when stored at 4 °C, while fully aggregated at physiological temperature. They can be used for temperature-triggered depot formation in tumors [8], the goal being to deliver the therapeutic compound locally and in a sustained manner. A recent study showed that tumor retention correlates with a decrease in the ELP T_t [9].

ELPs with low T_t can be obtained through different approaches. The first is to synthesize ELPs of high molecular weight with

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[1] *Abbreviations used:* ELP, elastin-like polypeptides; T_t, transition temperature; *E. coli*, *Escherichia coli*; MPB, maltose-binding protein; LB, lysogeny broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; ITC, inverse transition cycling; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; PBS, phosphate-buffered saline.

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repetitions of a moderately hydrophobic repeated sequence such as $(VPGVG)_n$ with $n > 230$ [10,11]. Such ELPs have MW ~100 kDa and their T_ts are in the 20–25 °C range. However, heterologous proteins with molecular weights above 60 kDa are often difficult to express in *Escherichia coli* (*E. coli*), resulting in low production yields [12]. In addition, the highly repeated DNA sequence in the corresponding genes poses a risk of gene rearrangement, and thus the production of unwanted products [13].

An alternative strategy is to produce short hydrophobic ELPs that possess low T_t at physiological salt concentration. In the present work, we focused on the design and production of short recombinant ELPs (MW ranging between 9 and 26 kDa) exhibiting T_ts in the 10–20 °C range. We chose to incorporate Isoleucine (I) at the guest position of every VPGXG repeat sequence because it has been demonstrated that a chemically synthesized $(VPGIG)_{180}$ polymer of 100 kDa has a T_t value of 10 °C [14]. Since T_t also varies according to the number of repeats [4], we chose to express $(VPGIG)_n$ polymers recombinantly with $n = 20$, 40 and 60 repeats.

The recombinant production in *E. coli* of polypeptides that form aggregates at a temperature above 20 °C could lead to the formation of inclusion bodies or be toxic toward the producing cells. Indeed, an hydrophobic ELP with the sequence MGLDGSMG $(VPGIG)_{40}$VPLE accumulated in inclusion bodies when recombinantly expressed in *E. coli* at 37 °C [15]. To prevent these possible drawbacks, we chose to produce these hydrophobic ELPs polypeptides fused to the maltose-binding protein (MPB). MBP is known to improve the solubility of polypeptides to which it is fused [16], it facilitates the purification steps through the use of affinity chromatography with an amylose column, and it has been used successfully to produce amyloid proteins, which are prone to aggregation [17,18]. Using this strategy, we produced and purified to homogeneity around 10 mg of $(VPGIG)_{20}$ per liter of culture and we determined that its T_t was 15.4 °C.

Materials and methods

Reagents

All enzymes required for recombinant DNA, including restriction endonucleases, T4 DNA polymerase, One*Taq*® hot start DNA polymerase, Phusion® Hot Start II DNA polymerase, Antarctic phosphatase and Quick ligation™ kit (T4 DNA ligase) were obtained from New England Biolabs (Ipswich, MA), except for the In-Fusion® HD kit which was obtained from Clontech (Mountain View, CA). The pMal-c5e plasmid and *E. coli* strain NEB5α-F'I^q were purchased from NEB. The *E. coli* strain BLR(DE3) was obtained from Novagen Inc (Madison, WI). *E. coli* strain Stbl3™ (Invitrogen, Carlsbad, CA) was used for cloning repetitive DNA constructs. Synthesis of the initial MW$(VPGIG)_{20}$C gene was achieved by Eurofin MWG Operon (Ebersberg, Germany). All custom oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). Sequencing of the various constructs was performed by Millegen (Labege, France).

Vector and cloning

A synthetic gene corresponding to the MW$(VPGIG)_{20}$C, with an additional 7 bp sequence (which we called the DS sequence) located upstream of the C-term Cys codon, was provided in the pCR2.1 plasmid (Supplementary Fig. 1). After extraction from the latter plasmid by double digestion with *Eco*RI and *Hind*III, the synthetic gene was ligated into similarly digested pUC19 to obtain the plasmid pUC19-ELP20-DS. Using the strategy of recursive directional ligation (RDL), this plasmid was used (i) to generate a recipient plasmid after deletion of the DS sequence of 7 bp disrupting the 3′ end of the coding sequence, and (ii) to obtain the monomer to be inserted into the recipient plasmid. Deletion of the DS sequence (GTTCGCA) was performed by PCR. A linear deleted fragment with 17 bp overlapping extremities was obtained from 1 ng of plasmid as template using PCR primers (5′GGCATTGGTTGCTAATCATCGC TGGATCCAA3′ and 5′GATTAGCAACCAATGCCAGGAACGCCGATG3′). The enzyme used was Phusion® Hot Start II DNA polymerase and the reaction was performed according to the manufacturer's instructions with the GC 5X buffer. PCR cycles were as follows: 98 °C for 30 s, two cycles 98 °C 10 s/65 °C 20 s/72 °C 70 s, 30 cycles 98 °C 10 s/72 °C 70 s and a final extension at 72 °C for 5 min. The PCR product was then digested by the *Dpn*I enzyme and was gel-purified with the NucleoSpin extract II kit (Macherey Nagel, France). The fragment (100 ng) was circularized using the In-Fusion® HD kit according to the standard procedure and NEB 5α-F'I^q *E. coli* cells were transformed with the resultant product. Positive colonies were identified by PCR with One*Taq*® hot start DNA polymerase and primers (5′GTGCTGCAAGGCGATTAAGT3′ and 5′TGTGGAATTGTGAGCGGATA3′). Digestion of the amplified product with the enzyme *Sph*I allowed the identification of the clones deleted for the seven bases, the *Sph*I restriction being present only in this deleted region. The sequence of the resultant pUC19-ELP20 plasmid was confirmed by DNA sequencing. To generate the monomer insert for RDL, which corresponds to the DNA sequence of MW$(VPGIG)_{20}$C minus the Met-Trp N-terminus codons and the Cys C-terminus codon, the plasmid pUC19-ELP20-DS was double-digested with the enzymes *Btg*Z1 and *Bsm*F1 and the monomer was gel-purified. In parallel, the recipient plasmid (pUC19-ELP20) was digested by *Bsm*FI and dephosphorylated by Antarctic phosphatase. The ligation reaction was performed at a molar ratio of 1/3 (vector/insert). Stbl3™-competent cells were transformed with ligation product. Positive colonies were identified by colony PCR and the resultant pUC19-ELP40 plasmid was checked by DNA sequencing. To obtain the ELP60 sequence, a second cycle of RDL was performed using pUC19-ELP40 as recipient plasmid and the previously obtained monomer insert.

Plasmid pMAL-c5E was digested by *Kpn*I, polished using T4 DNA polymerase (New England Biolabs), digested by *Bam*HI and the 5′ extremities were then dephosphorylated. ELPs coding sequences were extracted from pUC19-ELP20, pUC19-ELP40 or pUC19-ELP60 by *Bam*HI and *Ale*I double digestion. The inserts and the vector were then gel-purified prior to their ligation, which was performed with Quick ligation™ kit for 5 min at 25 °C in a final volume of 20 µl. The ligation product was then used to transform NEB5α-F'I^q (ELP20 containing plasmids) or Stbl3™ (ELP40, or ELP60 containing plasmids) *E. coli* cells. Positive clones were identified by PCR screening with the primers (5′GATTAGCAACCA ATGCCAGGAACGCCGATG3′ and 5′AGACGCGCAGACTAATTC3′). The resultant plasmids (pMAL-c5E-ELP20, pMAL-c5E-ELP40, and pMAL-c5E-ELP60) were confirmed by restriction mapping and by DNA sequencing. These plasmids were purified and used to transform BLR(DE3)-competent cells for production purposes.

Bioproduction of recombinant MBP-ELPs

A single bacterial colony was selected and cultured overnight at 37 °C in a rotary shaker at 200 rpm in 50 mL of rich LB medium (1% bacto tryptone, 0.5% NaCl, 1% yeast extract) containing 100 µg/mL ampicillin. Thereafter, this seed culture was inoculated into 0.95 L of rich LB medium supplemented with glucose (1 g/L) and ampicillin (100 µg/mL) and cells were cultivated at 37 °C in a multi-bioreactor system BIOSTAT Qplus (Sartorius Stedim Biotech, Germany) which has the capability to control fully independently three culture vessels with working volume of 1 L. The pH value of 7 was controlled with 1 M phosphoric acid and 1 M sodium hydroxide. The dissolved oxygen tension pO_2 was kept at a set