Process Biochemistry 51 (2016) 484–490

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Hyper-production of large proteins of spider dragline silk MaSp2 by *Escherichia coli* via synthetic biology approach

Yan-Xiang Yang [1], Zhi-Gang Qian [1], Jian-Jiang Zhong *, Xiao-Xia Xia *

State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 10 December 2015
Received in revised form 16 January 2016
Accepted 22 January 2016
Available online 27 January 2016

Keywords:
Spider dragline silk
Escherichia coli
Recombinant protein production
Repetitive protein
High cell density cultivation
Synthetic biology approach

ABSTRACT

Spider dragline silk exhibits excellent mechanical properties that make it a promising protein polymer for industrial and biomedical applications. Since farming spiders is not feasible due to their highly territorial nature, recombinant production of dragline silk proteins in a foreign host has received great attention. However, their production titer remains low, because efficient expression of very large, highly repetitive, glycine-rich silk proteins is a challenge. This work demonstrates the design and high-level production of large dragline silk proteins of major ampullate spidroin 2 (MaSp2) in *Escherichia coli* by synthetic biology approach. The expression levels of MaSp2 with molecular weight of 28.3–256.5 kDa were significantly elevated by down-shifting the induction temperature. The beneficial effect was found to be at least partially attributed to the improved plasmid maintenance in the recombinant cells. Combination of induction temperature downshift with the glycyl-tRNA pool increase in *E. coli* led to enhanced biosynthesis of glycine-rich silk proteins. A high production titer of about 3.6 g l^{-1} of a 201.6-kDa MaSp2 protein was achieved in a 3-L fed-batch bioreactor, which was the highest as reported. The developed approach may be useful to cost-effective large-scale production of silk proteins.

1. Introduction

Spider dragline silk is a promising and outstanding protein polymer, which exhibits extraordinary mechanical properties of superior strength and toughness that make it outperform most of the natural and other synthetic fibers [1,2]. It is composed almost entirely of two proteins, major ampullate spidroins 1 (MaSp1) and 2 (MaSp2) [3,4]. Both MaSp1 and MaSp2 are with large molecular weights at 250–320 kDa, consisting of a long, repetitive core domain flanked by non-repetitive N-terminal and C-terminal sequence on both sides [5–7]. And they differ in the composition of structural motifs that affect the mechanical properties of the silk fibers. MaSp1 is composed of a polyA region, which contributes to the tensile strength of silk, and of a GGX (*X* = *L*, *Y*, *Q*, or *A*) motif, which is believed to affect fiber formation, while MaSp2 consists of a polyA region similar to MaSp1, and of a GPGXX repetitive region (*X* = *G*, *Q*, *Y*) which is responsible for the silk elasticity by forming a beta-spiral [4,5,8,9]. Due to its outstanding properties, spider silk is a promising protein polymer for numerous industrial application, and it also possesses a great potential in medical and pharmaceutical areas for its biodegradable and biocompatible features [10–16]. Unfortunately, natural dragline silk of spiders could not be readily obtained by farming spiders because of their highly aggressive and territorial nature [17]. Numerous attempts have thus been made to produce recombinant spider dragline silk proteins in various organisms such as *Escherichia coli*, yeast, tobacco, mammalian cells, silk worm, and even transgenic animals [18–23].

So far, there have been some reports on the production of recombinant spider dragline silk proteins, but their molecular weights were generally below 100 kDa, which is about half of what spiders make. In addition, the production titers of the recombinant silk proteins were still low, typically on the order of 10–100 mg/l with one exception of 2.7 g/l for one MaSp1 (Table 1). But, most MaSp1 proteins were only produced at ~0.5 g/l by metabolically-engineered *E. coli* as reported by Xia et al. [10]. The results underscored a limitation of glycyl-tRNA pool within the host upon silk protein expression [10]. An expression level of 256 mg/l was reported for a 56.2-kDa recombinant MaSp2 protein [24]. As the expression of very large, highly repetitive, glycine-rich silk proteins is indeed challenging, "smart" engineering approaches are urgently required for further improvement on the production titer, especially for

* Corresponding authors. Fax: +86 21 34204831.
E-mail addresses: jjzhong@sjtu.edu.cn (J.-J. Zhong), xiaoxiaxia@sjtu.edu.cn (X.-X. Xia).
[1] Both of these authors contributed equally to this work.

http://dx.doi.org/10.1016/j.procbio.2016.01.006
1359-5113/

Table 1
Typical examples of recombinant MaSp production.

Protein	Size (kDa)	Production level (l^{-1})	Ref.
MaSp1	100.7–284.9	0.5–2.7 g	[10]
MaSp2	28–53	2–10 mg	[18]
MaSp1	65–163	0.663 g	[19]
MaSp1, MaSp2	65–163	0.3 g	[20]
MaSp1, MaSp2	48–75	~120 mg	[21]
ADF3, MaSp2	59–140	25–50 mg	[22]
MaSp2	12.9–99.8	0.5% of total protein	[23]
ADF3	56.2	256 mg	[24]
MaSp1, MaSp2	14.7–41.3	2–15.7 mg	[25]
ADF3, ADF4	11.9–59.3	10 –360 mg	[26]
MaSp2	201.6	3.6 g	This work

native-size MaSp2 of large molecular weight, towards a commercially viable silk protein biomanufacturing process [20,27,28].

Induction temperature is generally an important factor for recombinant protein expression, and it has been widely used to improve the expression of soluble protein expression but has different effects on the production level of different proteins in *E. coli* [29–41] (Table 2). In this study, we reported high-level production of large spider dragline silk MaSp2 protein of 201.6 kDa by modulating the induction temperature. Production level of recombinant spider silk proteins was significantly improved by downshifting the induction temperature. Furthermore, we performed the induction temperature-shift strategy in bioreactors for the MaSp2 production by a metabolically-engineered *E. coli* with both pET28a-MaSpII64 and pTetgly2, a tRNAGly overexpression plasmid. Finally, a higher production titer than ever reported was achieved in a 3-L fed-batch bioreactor.

2. Materials and methods

2.1. Construction of recombinant plasmids

DNA manipulations were performed according to standard molecular biology protocols. *E. coli* DH5α (Invitrogen Corp., Carlsbad, CA) was used for general gene cloning studies. Cells were routinely grown at 30 °C in Luria-Bertani (LB) medium (per liter: 10 g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl). Antibiotics were added at the following concentrations: 50 μg ml^{-1} of kanamycin (Km), 35 μg ml^{-1} of chloramphenicol (Cm), and 50 μg ml^{-1} of ampicillin (Ap) when necessary. All the restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Plasmid DNA was extracted using the Plasmid Mini Kit I (Omega Bio-Tek, Doraville, GA) following the manufacture's protocol.

DNA sequence was designed to encode one repeat of the MaSp2 consensus sequence from *Nephila clavipes*: GPGGYGPGQQGPSGPGSA_8GPGGYGPGQQ [4]. The monomer DNA sequence was purchased as a synthetic gene that was cloned into *Eco*RV site of the pMD18-T Simple Vector from Invitrogen (Shanghai, China). The monomer DNA sequence was then liberated by digesting the pMD18-T derivative with *Nde*I and *Xho*I, and cloned into the same sites of expression vector pET-28a(+) (Novagen). The resulting plasmid pET28a-MaSpII1 was confirmed by sequencing with both forward and reverse primers based on the *T7* promoter and terminator sequences at Invitrogen. To express two repeats of the MaSp2 consensus sequence, plasmid pET28a-MaSpII2 was constructed by ligating the 1.3-kb, *Xma*I-*Nhe*I fragment of pET28a-MaSpII1 to the 4.2-kb, *Spe*I-*Xma*I fragment of pET28a-MaSpII1. Likewise, plasmids were created that allow synthesis of the recombinant silk proteins having 8–80 repeating units of MaSp2. These expression plasmids were verified by double digest with the enzymes *Nhe*I-HF and *Spe*I-HF.

To overexpress a recombinant silk protein encoding 96 repeats of MaSp1 consensus sequence, plasmid pET19b-MaSpI96 was made from plasmid pET19b-MaSpI4, using the iterative polymerization strategy as described earlier [42].

Plasmid pTetgly2 was constructed to overexpress the *glyVXY* genes encoding $tRNA^{Gly}$ under the control of the constitutive *tet* promoter as previously described [10]. This plasmid allowed elevated pools of $tRNA^{Gly}$ within the harboring strains of *E. coli* [10].

2.2. Expression of recombinant silk proteins in flask cultivation

Competent cells of *E. coli* BL21(DE3), a common expression host for pET expression system, were transformed with the desired plasmids and plated on selective LB agar plates containing the appropriate antibiotics. Antibiotics were added at the following concentrations: 50 μg ml^{-1} of Km, 35 μg ml^{-1} of Cm, and 50 μg ml^{-1} of Ap when necessary. A single colony was inoculated into a 15-ml tube containing 2 ml of LB medium and cultured overnight at 30 °C and 220 rpm in a shaking incubator. This seed culture (200 μl) was transferred into a 250-ml shake flask containing 20 ml of either *R*/2 medium supplemented with 10 g l^{-1} of glucose or LB medium, and grown at 30 °C and 220 rpm. The minimal *R*/2 medium (pH 6.80) contains 2 g l^{-1} of $(NH_4)_2HPO_4$, 6.75 g l^{-1} of KH_2PO_4, 0.85 g l^{-1} of citric acid, 0.7 g l^{-1} of $MgSO_4{\cdot}7H_2O$, and 5 ml l^{-1} of a trace metal stock solution [43]. Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) with a BioPhotometer plus spectrophotometer (Eppendorf, Hamburg, Germany). When cell OD_{600} reached ~0.4, the cultures were exposed to 1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma, St. Louis, MO), and incubated at the desired temperatures ranging from 16 to 37 °C. Samples of the cell cultures were taken immediately prior to the addition of IPTG and at the desired time after induction for plasmid maintenance and SDS-PAGE analysis. For the SDS-PAGE analysis of recombinant silk proteins, cells were collected by centrifugation at 4 °C and 13,000 × *g* for 10 min and stored at −80 °C before further analysis.

2.3. Production of recombinant silk proteins by high density cell cultivation in bioreactors

The high density cell cultivation of recombinant *E. coli* cells was performed in a 3-L stirred bioreactor (BIOFLO 110; New Brunswick Scientific Co., Edison, NJ) containing 1.44 l of *R*/2 medium (pH 6.80) supplemented with 10 g l^{-1} of glucose. Antibiotics were added at the following concentrations: 50 μg ml^{-1} of Km, and 35 μg ml^{-1} of Cm when necessary. To prepare an inoculum, a small aliquot of glycerol cell stock was inoculated into a 15-ml tube containing 3-ml LB medium and cultured overnight at 30 °C and 220 rpm. Two milliliters of the overnight culture were transferred into a 500-ml shake flask containing 200 ml of R/2 medium and incubated under the same condition as the tube culture. When the cell OD_{600} reached ~2.0, 160 ml of the seed culture was added into the jar, and the growth temperature maintained at 30 °C with pH adjusted to 6.80 using 28% (v/v) ammonia water. Dissolved oxygen (DO) level was kept above 40% of air saturation by increasing the agitation speed from 200 to 800 rpm and by incorporating pure oxygen when agitation at 800 rpm was insufficient to maintain the DO level. A nutrient feeding solution containing 700 g l^{-1} of glucose and 20 g l^{-1} of $MgSO_4{\cdot}7H_2O$ was manually added into the reactor when the pH increased above 6.80 because of glucose depletion. When the cell OD_{600} reached ~40, IPTG was added at a final concentration of 1 mM, and the culture temperature kept at 30 °C or shifted to 16 °C as described. Samples were taken periodically for SDS-PAGE analysis and biomass quantification. The biomass (dry cell) concentration was determined by centrifuging 2–4 ml of cell

Table 2
Temperature shift strategy for recombinant protein expression in *E. coli* (in shake flasks).

Protein	Product size (kDa)	Temperature shift strategy	Production titer fold-change	Ref.
D-Hydantoinase/*N*- carbamoylase	33.6/50.2	37–25 °C vs. 37–37 °C	+/+[a]	[31]
hGCSF	24.3	37–18 °C vs. 37–30 °C	1.30 fold	[32]
Dehydratase	96	37–16 °C vs. 37–37 °C	+	[33]
Glycoprotein	55	37–18 °C vs. 37–37 °C	3.2 fold	[34]
Poly(ADP-ribose) glycohydrolase	90	37–16 °C vs. 37–37 °C	+	[35]
Artificial repetitive copolymers	12.4	37–24 °C vs. 37–30 °C	+	[36]
Nitrile hydratase	23	37–18 °C vs. 37–35 °C	–[b]	[37]
Leukemia inhibitory factor	22.2	37–4 °C vs. 37–37 °C	–	[38]
Pullulanase	~110	37–20 °C vs. 37–37 °C	n.a.[c]	[39]
TEV protease	28	37–25 °C vs. 37–37 °C	–	[40]
Aconitase	82	37–25 °C vs. 37–37 °C	0.75 fold	[41]
MaSp2	201.6	30–16 °C vs. 30–30 °C	5 fold	This work

[a] Improved.
[b] Decreased.
[c] Data unavailable.

suspension in pre-weighed tubes and drying the resulting pellets at 65 °C until a constant weight was obtained.

2.4. SDS-PAGE and protein quantification

To analyze expression levels of recombinant silk proteins, cell samples were resuspended with 50 mM phosphate buffer (pH 7.5), mixed with 5× Laemmli sample buffer, and boiled for 15 min. After centrifugation at 4 °C and 13,000 × *g* for 10 min, the protein samples were loaded onto 1-mm thick, 10% SDS-polyacrylamide gels with Unstained Protein Ladder, Broad Range (New England Biolabs Catalog # P7703S). The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad), and scanned by the UMAX G800 Scanner (Bio-Rad, Richmond, CA). The protein bands were normalized and quantified by using the BandScan v5.0 software (Glyko, Novato, CA) to estimate expression levels (% total cellular proteins). Silk protein production titer, which represents the recombinant protein amount produced per liter, was estimated by multiplying expression level with biomass concentration and with 0.54 [10]. Here, 0.54 is the ratio of total cellular proteins to dry cell weight in *E. coli* as verified in our experiments, which is slightly lower than that (0.55) in a previous report [44].

2.5. Plasmid maintenance assay

E. coli cells harboring a silk-protein expression plasmid were subjected to plasmid maintenance assay [45]. Typically, the recombinant cell cultures of flask cultivation were diluted 10^5 fold in fresh LB medium, replica plated onto LB agar plates with and without 50 μg ml^{-1} of Km, and incubated overnight at 30 °C. Maintenance of the plasmid within the host cell was presented as a percentage of plasmid-bearing cells by their ability to form colonies on selective LB agar plates.

3. Results and discussion

3.1. Design of recombinant dragline silk proteins of different sizes

To assemble genes encoding the silk MaSp2 proteins, we first designed amino acid sequence for one consensus repeat (GPGGYGPGQQGPSGPGSA$_8$GPGGYGPGQQ) of the MaSp2 protein of the spider *N. clavipes*, whose partial cDNA sequence has been reported [4]. The amino acid sequence was reverse translated into nucleic acid sequence, codon-optimized for favorable expression in *E. coli*, and engineered with restriction enzymes sites *Nde*I and *Nhe*I on the 5′ end as well as *Spe*I and *Xho*I sites on the 3′ end. The monomer silk gene was cloned into *Nde*I-*Xho*I site of expression plasmid pET-28a(+), which was commercially available from Novagen, Inc. (Madison, WI). The resulting plasmid, pET28a-MaSpII1, allowed recombinant expression of the silk-like protein having one repetitive unit of MaSp2 under the strong *T7* promoter. To construct vectors that encode silk proteins having more repetitive units, we employed an iterative polymerization strategy relying on the compatible but nonregenerable restriction sites *Nhe*I and *Spe*I, which was modified on a previously developed protocol [46]. By doing so, plasmid constructs were made for the production of recombinant MaSp2 proteins having 8–80 repeats (Table 3).

3.2. Lower induction temperature improved MaSp2 expression

To develop a general approach for high-level production of large silk proteins, the 201.6-kDa MaSp2-64mer protein was taken as a model. The plasmid encoding the MaSp2-64mer protein was transformed into *E. coli* BL21(DE3). The resulting strain was grown in Luria-Bertani (LB) medium in shake flasks at 30 °C, and induced at middle exponential growth phase (OD_{600} ~0.4) with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). This initial test resulted in a moderate level of protein expression based on SDS-PAGE analysis. Previously, we have shown that expression of large glycine-rich repetitive proteins of spiders caused metabolic burden and stress to the host, based on comparative proteomic analysis of the recombinant strains with and without silk-protein expression [10].

We then attempted to improve the expression levels by optimizing inducer concentration, induction time, and growth medium. As a result, these tests did not lead to higher levels of expression. Next we tested whether expression levels could be elevated by manipulating the induction temperature, a bioprocessing strategy previously known to improve soluble expression of aggregation-prone proteins [29]. Surprisingly, the silk protein was found to be expressed in a soluble form at the wide induction temperatures ranging from 16 to 37 °C (Supplementary Fig. S1), while the expression levels were much higher at lower induction temperatures (Fig. 1A). For example, downshift of the induction temperature to 16 °C resulted in fivefold higher expression in comparison to that at 30 °C.

To explore why induction at lower temperature improved the protein expression, we performed plasmid maintenance assay for the recombinant cells without and with IPTG induction at both 16 and 30 °C. The percentage of plasmid-bearing cells in the recombinant cultures induced at 16 °C was significantly higher than those induced at 30 °C (Fig. 1B), indicating that induction at lower temperature was beneficial for maintenance of the protein plasmid pET28a-MaSpII64. In contrast to the induced cultures, no obvious plasmid loss was observed in the non-induced cell cultures and in the empty vector control cultures with and without

Table 3
Molecular weight (Mw) and glycine content of the recombinant dragline silk proteins along with the sizes and GC contents of the encoding genes.

	Silk protein		Silk-protein gene	
	Mw (kDa)	Gly content (%)	Size (bp)	GC content (%)
MaSp2-8mer	28.3	32.7	984	61.3
MaSp2-16mer	53.0	33.9	1872	61.3
MaSp2-64mer	201.6	34.8	7200	61.3
MaSp2-80mer	251.1	34.9	8976	61.3
MaSp1-96mer	256.5	47.8	9651	72.4

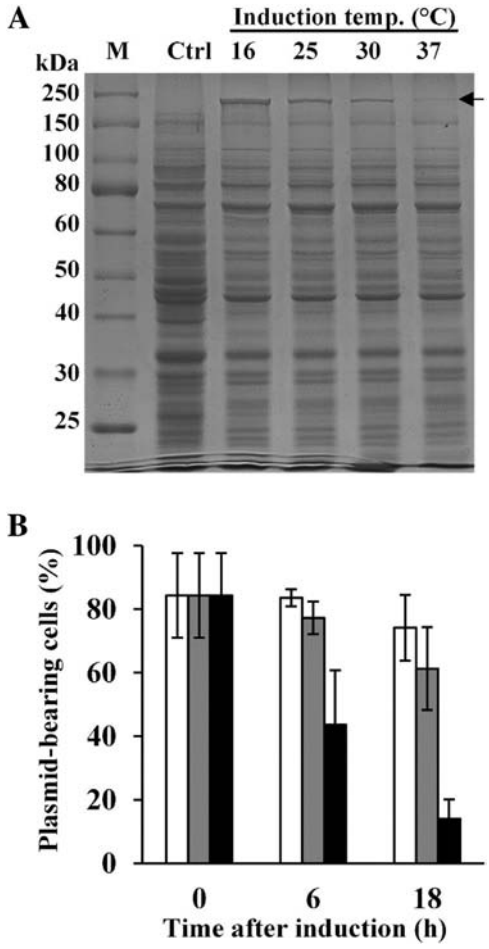

Fig. 1. Effect of induction temperature on MaSp2-64mer expression by *E. coli* BL21(DE3) cells harboring pET28a-MaSpII64 in LB medium. Initial cultivation temperature was 30 °C; and 1 mM IPTG was added at OD_{600} of ~0.4, and incubated for additional 6 h at an indicated temperature. Protein samples of cell lysates were separated on 10% SDS-PAGE gels and the arrow indicates the target silk protein (A). The cell cultures without IPTG treatment (open bar) and those induced at either 16 °C (grey bar) or 30 °C (black bar) were sampled and subjected to plasmid maintenance assay (B). Error bars represent standard deviations from the mean values of three replicates.

induction (Supplementary Fig. S2). This coincided with our previous population-level analysis of the recombinant *E. coli* cultures, which revealed a significant decrease in the average plasmid copy number after expression of the native-sized dragline silk protein [10]. And another previous study also showed that, the recombinant strain was quite unstable during expression at higher temperature, while no plasmid loss was observed for the strain without induction of protein expression [31]. The reason might be during the protein expression process, especially for a high molecular weight and significantly repetitive protein, DNA replication and maintenance of the plasmid could be metabolically expensive, which provided the cells more opportunities to discard the plasmid when the cells grew at a high temperature.

Compared with other researches on heterologous protein production by *E. coli*, induction of recombinant *E. coli* by shifting to a lower temperature could have positive or negative effects on protein expressions (Table 2). But, no studies on the expression of larger proteins over 110 kDa have been reported. To the best of our knowledge, this is the first work on higher expression of a large 201.6 kDa protein by temperature shift strategy.

In glucose *R*/2 minimal medium, it was confirmed that downshift of the induction temperature to 16 °C also increased the protein expression compared to the control at the same time after induction (Table 4). The expression level of induction temperature at 16 °C was 9.8–16.9% with much increase after 16 h, while in the case of 30 °C it decreased from 1.3% at 4 h. As the chemically defined minimal medium is cheaper than rich medium and more suitable for silk protein production at industrial scale, the *R*/2 medium was thus used in the following experiments.

To understand whether downshift of induction temperature was beneficial for expression of dragline silk proteins of larger molecular weights, we performed expression experiments for the MaSp2-80mer protein, and another type of spider major ampullate spidroin having 96 consensus repeats of MaSp1. The two native-sized proteins, both over 250 kDa, were favorably expressed by downshift of the induction temperature to 16 °C (Supplementary Fig. S3A & B). In addition, this strategy was also useful for expression of recombinant dragline silk proteins of small size, such as the 28.3-kDa MaSp2-8mer and 53.0-kDa MaSp2-16mer silk proteins (Supplementary Fig. S3C). Taken together, the facts demonstrated that downshift of induction temperature was generally applicable to achieve higher expression of silk proteins ranging from small to native-sized large silk proteins.

3.3. Downshift of induction temperature elevated silk protein production in high-density cell cultivation in bioreactors

To further enhance the recombinant silk protein production, high density cell cultivation was performed in a 3.0-l fermentor, by taking the large MaSp2-64mer protein as a typical example. The recombinant strain BL21(DE3)/pET28a-MaSpII64 was grown in glucose minimal *R*/2 medium, exposed at OD_{600} of ~40 (cell density of 14.7 g l^{-1}) to IPTG at a final concentration of 1 mM, and further cultured at either 30 or 16 °C. As shown in Fig. 2A, after IPTG was added, the cell growth had a certain reduction, which might be owing to initiating the protein gene transcription and translation process as claimed earlier [47]. The cells grew to a cell density of 20.6 g l^{-1} at 4 h following induction at 30 °C, and began to lyse thereafter (Fig. 2A). The inability of the strain to grow further might be due to the depletion of certain biosynthetic precursors

Table 4
Change of MaSp2-64mer expression level (by %) in the *R*/2 minimal medium after induction at 16 °C or 30 °C.

Induction temp.	Time after induction (h)					
	4	8	12	16	20	24
16 °C	4.9%	5.6%	5.3%	9.8%	16.9%	15.6%
30 °C	1.3%	0.5%	n.d.	n.d.	n.d.	n.d.

n.d.: not detectable.

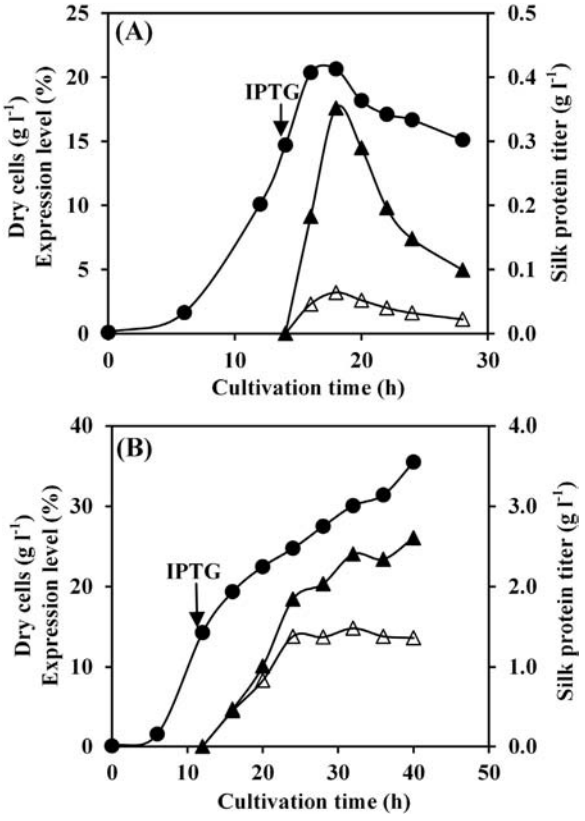

Fig. 2. Enhanced MaSp2 production by lowering induction temperature in high-density culture of *E. coli* BL21(DE3) cells in a 3.0-l jar fermenter. The initial fermentation temperature was 30 °C; when cell OD_{600} reached ~40, 1 mM IPTG was added and cultivation temperature was maintained at 30 °C (A) or downshifted to 16 °C (B). Symbols: dry cells (●), expression level (△), and MaSp2 titer (▲).

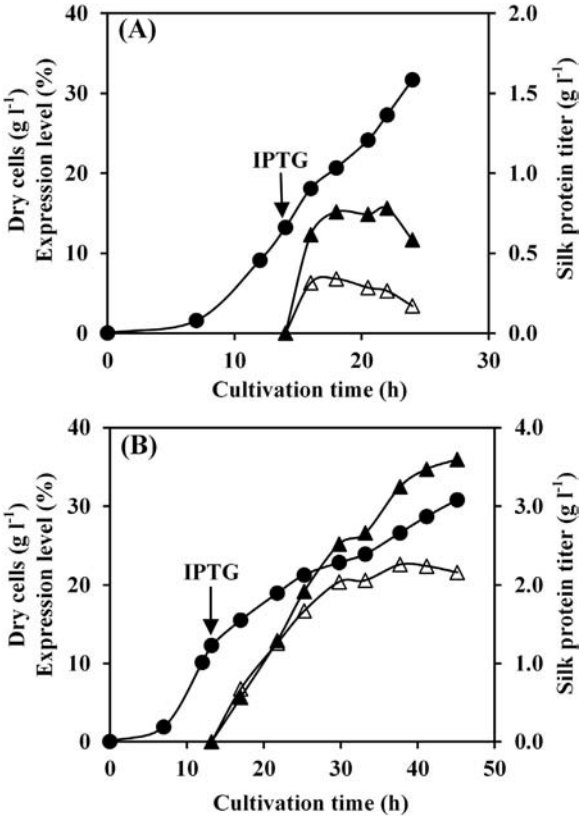

Fig. 3. Effect of induction temperature downshift on high-density fermentation of metabolically-engineered *E. coli* BL21(DE3) co-transformed with pET28a-MaSpII64 and $tRNA^{Gly}$ overexpression plasmid pTetgly2. A fed-batch mode in a 3.0-l jar fermenter at 30 °C was operated; and 1 mM IPTG was added when OD_{600} reached ~40, and cultivation temperature was maintained at 30 °C (A) or shifted to 16 °C (B). Symbols: dry cells (●), expression level (△), and MaSp2 titer (▲).

such as glycyl-tRNA within the host (see below). In this scenario, the protein production reached its maximum of 0.36 g l^{-1} at 18 h of fermentation (4 h post-induction). In contrast, the recombinant strain was able to grow to a high cell density (>37 g l^{-1}) if induced at 16 °C (Fig. 2B). More importantly, the silk protein expression increased steadily and reached a plateau level of ~14% of total cellular proteins. This allowed approximately eightfold higher production of the silk protein, reaching about 2.9 g l^{-1} at 44 h of fermentation. The results proved significant improvement on the protein production by using the bioprocess engineering approach in bioreactors.

Previously, we have shown that production of recombinant MaSp1 proteins was improved in metabolically engineered *E. coli* within which the metabolic pool of glycyl-tRNA was elevated [10]. This strategy was also employed here for the production of the MaSpII-64mer silk protein. As expected, the metabolically engineered host permitted superior cell growth and higher protein production of the MaSpII-64mer (Fig. 3A). A silk protein production titer of 0.8 g l^{-1} was achieved in cultures induced at 30 °C, which was over twofold that of the *E. coli* without enhanced glycyl tRNA pool. Finally we examined whether the silk protein production could be further enhanced by applying the temperature downshift strategy to the metabolically engineered host. Indeed, the expression of the silk protein increased to a very high level of over 22% of total cellular proteins, although there was a slight decrease near the end of fermentation. The silk protein production steadily increased to the end of the fermentation and reached about 3.6 g l^{-1} (Fig. 3B), the highest level ever reported for recombinant dragline silk proteins (Table 1). Our data revealed the great potential of the *E. coli* for high-level production of large proteins by combined engineering of the expression host and biomanufacturing process.

Mass production of recombinant spider dragline silk proteins is a prerequisite for their widespread applications. The silk proteins have unusual abundance in the amino acid glycine, long and highly repetitive structure, and high GC content of their encoding genes. These factors may limit their production in all recombinant expression platforms tested so far [19,22]. We attempted to tackle the challenges by developing a metabolically engineered expression

host within which the glycyl-tRNA pool was elevated for higher protein production [10]. This work proved that downshift of the induction temperature could greatly improve the expression of large silk proteins in rich and minimal media, and both in shake flasks and high-density bioreactors. This simple and efficient technology may be helpful to the development of commercially viable biomanufacturing process for silk proteins.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (Nos. 31470216 and 21176153) and the Shanghai Pujiang Program (No. 14PJ1405200 to Z.-G.Q). This work was also supported by the National Basic Research Program of China (973 Program No. 2012CB721006). X.-X.X is grateful to the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (ZXDF080005).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.01.006.

References

[1] F.G. Omenetto, D.L. Kaplan, New opportunities for an ancient material, Science 329 (2010) 528–531.
[2] J.M. Gosline, P.A. Guerette, C.S. Ortlepp, K.N. Savage, The mechanical design of spider silks: from fibroin sequence to mechanical function, J. Exp. Biol. 202 (1999) 3295–3303.
[3] M. Xu, R.V. Lewis, Structure of a protein superfiber: spider dragline silk, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 7120–7124.
[4] M.B. Hinman, R.V. Lewis, Isolation of a clone encoding a second dragline silk fibroin: *Nephila clavipes* dragline silk is a two-protein fiber, J. Biol. Chem. 267 (1992) 19320–19324.
[5] N.A. Ayoub, J.E. Garb, R.M. Tinghitella, M.A. Collin, C.Y. Hayashi, Blueprint for a high-performance biomaterial: full-length spider dragline silk genes, PLoS One 2 (2007) e514.
[6] A. Sponner, B. Schlott, F. Vollrath, E. Unger, F. Grosse, K. Weisshart, Characterization of the protein components of *Nephila clavipes* dragline silk, Biochemistry 44 (2005) 4727–4736.
[7] K.W. Sanggaard, J.S. Bechsgaard, X.D. Fang, J.J. Duan, T.F. Dyrlund, V. Gupta, X.T. Jiang, L. Cheng, D.D. Fan, Y. Feng, Spider genomes provide insight into composition and evolution of venom and silk, Nat. Commun. (2014), http://dx.doi.org/10.1038/ncomms4765.
[8] C.Y. Hayashi, N.H. Shipley, R.V. Lewis, Hypotheses that correlate the sequence, structure, and mechanical properties of spider silk proteins, Int. J. Biol. Macromol. 24 (1999) 271–275.
[9] A.E. Brooks, S.M. Stricker, S.B. Joshi, T.J. Kamerzell, C.R. Middaugh, R.V. Lewis, Properties of synthetic spider silk fibers based on *Argiope aurantia* MaSp2, Biomacromolecules 9 (2008) 1506–1510.
[10] X.X. Xia, Z.G. Qian, C.S. Ki, Y.H. Park, D.L. Kaplan, S.Y. Lee, Native-sized recombinant spider silk protein produced in metabolically engineered *Escherichia coli* results in a strong fiber, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 14059–14063.
[11] A. Heidebrecht, L. Eisoldt, J. Diehl, A. Schmidt, M. Geffers, G. Lang, T. Scheibel, Biomimetic fibers made of recombinant spidroins with the same toughness as natural spider silk, Adv. Mater. 27 (2015) 2189–2194.
[12] A. Rising, J. Johansson, Toward spinning artificial spider silk, Nat. Chem. Biol. 11 (2015) 309–315.
[13] F. Vollrath, P. Barth, A. Basedow, W. Engström, H. List, Local tolerance to spider silks and protein polymers in vivo, In vivo 16 (2001) 229–234.
[14] C. Radtke, C. Allmeling, K.H. Waldmann, K. Reimers, K. Thies, H.C. Schenk, A. Hillmer, M. Guggenheim, G. Brandes, P.M. Vogt, Spider silk constructs enhance axonal regeneration and remyelination in long nerve defects in sheep, PLoS One 6 (2011) e16990.
[15] H. Wendt, A. Hillmer, K. Reimers, J.W. Kuhbier, F. Schafer-Nolte, C. Allmeling, C. Kasper, P.M. Vogt, Artificial skin—culturing of different skin cell lines for generating an artificial skin substitute on cross-weaved spider silk fibres, PLoS One 6 (2011) e21833.
[16] K. Schacht, T. Scheibel, Processing of recombinant spider silk proteins into tailor-made materials for biomaterials applications, Curr. Opin. Biotechnol. 29 (2014) 62–69.
[17] J.A. Kluge, O. Rabotyagova, G.G. Leisk, D.L. Kaplan, Spider silks and their applications, Trends Biotechnol. 26 (2008) 244–251.
[18] R.V. Lewis, M. Hinman, S. Kothakota, M.J. Fournier, Expression and purification of a spider silk protein: a new strategy for producing repetitive proteins, Protein Expression Purif. 7 (1996) 400–406.
[19] S.R. Fahnestock, L.A. Bedzyk, Production of synthetic spider dragline silk protein in *Pichia pastoris*, Appl. Microbiol. Biotecnol. 47 (1997) 33–39.
[20] S.R. Fahnestock, S.L. Irwin, Synthetic spider dragline silk proteins and their production in *Escherichia coli*, Appl. Microbiol. Biotecnol. 47 (1997) 23–32.
[21] B. An, J.E. Jenkins, S. Sampath, G.P. Holland, M. Hinman, J.L. Yarger, R.V. Lewis, Reproducing natural spider silks' copolymer behavior in synthetic silk mimics, Biomacromolecules 13 (2012) 3938–3948.
[22] A. Lazaris, S. Arcidiacono, Y. Huang, J.F. Zhou, F. Duguay, N. Chretien, E.A. Welsh, J.W. Soares, C.N. Karatzas, Spider silk fibers spun from soluble recombinant silk produced in mammalian cells, Science 295 (2002) 472–476.
[23] J. Scheller, K.H. Gührs, F. Grosse, U. Conrad, Production of spider silk proteins in tobacco and potato, Nat. Biotechnol. 19 (2001) 573–577.
[24] A. Azam, C. Li, K.J. Metcalf, D. Tullman-Ercek, Type III secretion as a generalizable strategy for the production of full-length biopolymer-forming proteins, Biotechnol. Bioeng. (2015), http://dx.doi.org/10.1002/bit.25656.
[25] J.T. Prince, K.P. McGrath, C.M. DiGirolamo, D.L. Kaplan, Construction, cloning, and expression of synthetic genes encoding spider dragline silk, Biochemistry 34 (1995) 10879–10885.
[26] D. Huemmerich, C.W. Helsen, S. Quedzuweit, J. Oschmann, R. Rudolph, T. Scheibel, Primary structure elements of spider dragline silks and their contribution to protein solubility, Biochemistry 43 (2004) 13604–13612.
[27] O. Tokareva, V. Michalczechen-Lacerda, E.L. Rech, D.L. Kaplan, Recombinant DNA production of spider silk proteins, Microb. Biotechnol. 6 (2013) 651–663.
[28] H. Chung, T.Y. Kim, S.Y. Lee, Recent advances in production of recombinant spider silk proteins, Curr. Opin. Biotechnol. 23 (2012) 957–964.
[29] C.H. Schein, M.H.M. Noteborn, Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature, Nat. Biotechnol. 6 (1988) 291–294.
[30] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in *Escherichia coli*: advances and challenges, Front. Microbiol. (2014), http://dx.doi.org/10.3389/fmicb.2014.00172.
[31] J.L. Zhang, Z. Cai, Efficient and cost-effective production of Dp-hydroxyphenylglycine by whole-cell bioconversion, Biotechnol. Bioprocess Eng. 19 (2014) 76–82.
[32] B.H. Do, H.B. Ryu, P. Hoang, B.K. Koo, H. Choe, Soluble prokaryotic overexpression and purification of bioactive human granulocyte colonystimulating factor by maltose binding protein and protein disulfide isomerase, PloS One (2014), http://dx.doi.org/10.1371/journal.pone.0089906.
[33] D. Oyola-Robles, C. Rullán-Lind, N.M. Carballeira, A. Baerga-Ortiz, Expression of dehydratase domains from a polyunsaturated fatty acid synthase increases the production of fatty acids in *Escherichia coli*, Enzyme Microb. Technol. 55 (2014) 133–139.
[34] A.R. Tait, S.K. Straus, Overexpression and purification of U24 from human herpesvirus type-6 in *E. coli*: unconventional use of oxidizing environments with a maltose binding protein-hexahistine dual tag to enhance membrane protein yield, Microb. Cell Fact. (2011), http://dx.doi.org/10.1186/1475-2859-10-51.
[35] N. Okita, R. Ohta, D. Ashizawa, Y. Yamada, H. Abe, T. Abe, S. Tanuma, Bacterial production of recombinant human poly (ADP-ribose) glycohydrolase, Protein Expression Purif. 75 (2011) 230–235.
[36] S. Gerber, K. Kirchhof, J. Kressler, C.E.H. Schmelzer, C. Scholz, T.C. Hertel, M. Pietzsch, Cloning, expression, purification, and characterization of a designer protein with repetitive sequences, Protein Expression Purif. 59 (2008) 203–214.
[37] X.L. Pei, Q.Y. Wang, L.J. Meng, J. Li, Z.F. Yang, X.P. Yin, L.R. Yang, S.Y. Chen, J.P. Wu, Chaperones-assisted soluble expression and maturation of recombinant Co-type nitrile hydratase in *Escherichia coli* to avoid the need for a low induction temperature, J. Biotechnol. 203 (2015) 9–16.
[38] E.Y. Kim, H.J. Choi, T.W. Chung, S.B. Jang, K.B. Kim, K.T. Ha, Expression and efficient one-step chromatographic purification of a soluble antagonist for human leukemia inhibitory factor receptor in *Escherichia coli*, J. Microbiol. Biotechnol. (2015), http://dx.doi.org/10.4014/jmb.1501.01094.
[39] A.N. Chen, Y.M. Li, X.X. Liu, Q. Long, Y.K. Yang, Z.H. Bai, Soluble expression of pullulanase from *Bacillus acidopullulyticus* in *Escherichia coli* by tightly controlling basal expression, J. Ind. Microbiol. Biotecnol. 41 (2014) 1803–1810.
[40] B. Miladi, H. Bouallagui, C. Dridi, A. El Marjou, G. Boeuf, P. Di Martino, F. Dufour, A. Elm'selmi, A new tagged-TEV protease: construction, optimisation of production, purification and test activity, Protein Expression Purif. 75 (2011) 75–82.
[41] P. Gupta, A. Ghosalkar, S. Mishra, T.K. Chaudhuri, Enhancement of over expression and chaperone assisted yield of folded recombinant aconitase in *Escherichia coli* in bioreactor cultures, J. Biosci. Bioeng. 107 (2009) 102–107.
[42] Z.G. Qian, M.L. Zhou, W.W. Song, X.X. Xia, Dual Thermosensitive hydrogels assembled from the conserved C-terminal domain of spider dragline silk, Biomacromolecules 16 (2015) 3704–3711.

[43] J.L. Yu, X.X. Xia, J.J. Zhong, Z.G. Qian, Direct biosynthesis of adipic acid from a synthetic pathway in recombinant *Escherichia coli*, Biotechnol. Bioeng. 111 (2014) 2580–2586.
[44] F.C. Neidhardt, in: F.C. Neidhardt, J.L. Ingraham, K.B. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (Eds.), In Escherichia Coli and Salmonella Typhimurium: Cellular and Molecular Biology, American Society for Microbiology Press, Washington, D.C, 1987, pp. 3–6.
[45] T. Collins, J. Azevedo-Silva, A. da Costa, F. Branca, R. Machado, M. Casal, Batch production of a silk-elastin-like protein in *E. coli* BL21 (DE3): key parameters for optimisation, Microb. Cell Fact. 12 (2013) 21.
[46] F. Teulé, A.R. Cooper, W.A. Furin, D. Bittencourt, E.L. Rech, A. Brooks, R.V. Lewis, A protocol for the production of recombinant spider silk-like proteins for artificial fiber spinning, Nat. Protoc. 4 (2009) 341–355.
[47] W. Wang, Z.M. Li, J.L. Xie, Q. Ye, Production of succinate by a *pfl*B *ldh*A double mutant of *Escherichia coli* overexpressing malate dehydrogenase, Bioprocess Biosyst. Eng. 32 (2009) 737–745.