

# Production of disulfide bond-rich peptides by fusion expression using small transmembrane proteins of *Escherichia coli*

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**Abstract** Recombinant expression in *Escherichia coli* allows the simple, economical, and effective production of bioactive peptides. On the other hand, the production of native peptides, particularly those rich in disulfide bonds, is a major problem. Previous studies have reported that the use of carrier proteins for fusion expression can result in good peptide yields, but few are folded correctly. In this study, two transmembrane small proteins in *E. coli*, YoaJ and YkgR, which both orientate with their N-termini in cytoplasm and their C-termini in periplasm, were used for fusion expression. The recombinant production of two peptides, asteropsin A (ASPA) and  $\beta$ -defensin (BD), was induced in the periplasm of *E. coli* using a selected carrier protein. Both peptides were expressed at high levels, at yields of approximately 5–10 mg/L of culture. Mass

spectrometry showed that the resulting peptide had the same molecular weight as their natural forms. After purification, single peaks were observed by reversed phase high-performance liquid chromatography (RP-HPLC), demonstrating the absence of isoforms. Furthermore, cytoplasmically expressed fusion proteins with a carrier at their C-termini did not contain disulfide bonds. This study provides new carrier proteins for fusion expression of disulfide bond-rich peptides in *E. coli*.

**Keywords** Disulfide bond · *Escherichia coli* · Carrier protein · Mass spectrometry

## Introduction

*Escherichia coli* is frequently used to produce recombinant proteins because of its rapid growth and low cost (Burrowes et al. 2005). On the other hand, the recombinant approach is problematic in terms of providing native proteins with the correct conformations (Li 2009). For example, certain antimicrobial peptides often contain disulfide bonds, which help determine their activities. In addition, the properties of peptides may pose difficulties with respect to their direct expression in *E. coli* (Sorensen and Mortensen 2005), because their small sizes and a high cationic content make them susceptible to proteolytic degradation. To resolve these problems, several authors have described the use of carrier proteins to achieve fusion expression (Ingham and Moore 2007; Malik et al. 2006; Huber et al. 2005; Zhang et al. 2003). Some of these carrier proteins have been developed for commercial use and are used widely; examples include thioredoxin (Trx), GST and small ubiquitin-related modifier (SUMO) (Lavallie et al. 1993; Sheibani 1999; Malakhov et al. 2004). Although cytoplasmic expression by

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Z. Chang and M. Lu contributed equally to this work.

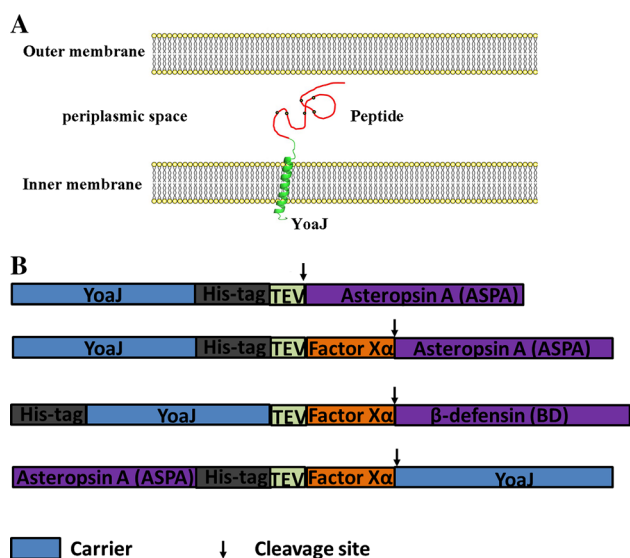
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**Fig. 1** **a** Location of the fusion tag (YoaJ) and recombinant peptide in *E. coli*. The orientation of YoaJ was suggested by Fontaine et al. (2011). **b** Construction of the expression vectors

these carriers can be high, some are frequently accompanied by inclusion bodies. Furthermore, it is difficult to produce the correct disulfide pattern (Malik et al. 2006).

In bacteria, the oxidative protein folding process for the formation of disulfide bonds is catalyzed by the disulfide bond formation (Dsb) system (Heras et al. 2007; Ito and Inaba 2008; de Marco 2012). Furthermore, an N-terminal signal sequence guides heterologous proteins to the periplasm of *E. coli* through a protein conduction channel in the cytoplasmic membrane. However, this method often results in low yields and incorrect folding (de Marco 2009). Hence, we consider that a suitable leader peptide could increase yield and improve protein folding. The small *E. coli* proteins of YoaJ and YkgR, which orientate with their N-termini in the cytoplasm and C-termini in the periplasm, might be used for the periplasmic expression of peptides (Fig. 1a).

The marine environment is a complex ecosystem and many organisms possess a wide range of bioactive compounds. A cysteine knot peptide, asteropsin A (ASP), in the marine sponge *Asteropus* sp., enhances veratridine-induced neuronal  $\text{Ca}^{2+}$  influx (Li et al. 2013). Structurally, ASP contains three disulfide bridges: two support anti-parallel  $\beta$ -sheets and the other lies closer to the surface near the N-terminus of the peptide. Defensins are small, cationic, amphipathic, cysteine-rich antimicrobial peptides found in plants, insects, and vertebrates (Nam et al. 2010). These comprise 18–45 amino acids, which include six to eight conserved cysteine residues. This paper describes the expression of ASP and a *Paralichthys olivaceus*  $\beta$ -defensin (BD), using the carrier proteins YoaJ or YkgR in the periplasmic space of *E. coli*.

## Materials and methods

### Materials

The expression vector pQE30 and *E. coli* strain M15 (pREP4) were purchased from Qiagen. The plasmid harboring the *Paralichthys olivaceus* BD gene was kindly donated by Professor Bo-Hye Nam (National Fisheries Research and Development Institute, Korea). Restriction enzymes and other enzymes used in the molecular cloning experiments were purchased from Enzymomics. Chemicals and reagents were obtained from Sigma (South Korea). All plasmids were isolated from *E. coli* JM109 and verified by DNA sequencing (Macrogen, South Korea).

### Construction of expression vectors

Two peptides, asteropsin A (ASP) from *Asteropus* sp. (Li et al. 2013) and  $\beta$ -defensin (BD) from *Paralichthys olivaceus* (Nam et al. 2010), were selected for possible expression in *E. coli* (Table 1). Using PCR-based gene synthesis (Withers-Martinez et al. 1999), the artificial cDNA sequence of the ASPA gene (with optimized codons) was synthesized and sub-cloned into pQE30 *E. coli* vector. Several oligomers, ranging from 33 to 60 mer (synthesized by Macrogen, Korea; Table 2), were used as primers. PCR was conducted using 25 cycles of denaturing (30 s at 94 °C), annealing (30 s at 52 °C), and extension (4 min at 72 °C). To locate ASPA protein at the inner membrane, the short transmembrane protein YoaJ was added as a tag to the N-terminal of ASPA. Two primers were designed to insert YoaJ cDNA into the 5'-terminal of ASPA in the pQE30–ASP plasmid. In the final constructed plasmid pQE30–YoaJ–ASP, six histidine residues for Ni resin-based protein purification and a cleavage site (TEV or/and Factor X $\alpha$ ) were inserted between YoaJ and ASPA (Fig. 1b).

For the BD vector, an insert of BD was cloned and introduced to the pQE30–YoaJ vector. A 6 histidine tag was designed in the 5' end of pQE30–YoaJ–BD vector. JM109 chemically competent *E. coli* cell transformants were plated at 37 °C on 100  $\mu\text{g}/\text{ml}$  ampicillin agar plates.

### Expression and purification

A single colony of *E. coli* M15 (pREP4) harboring the vectors was grown in LB medium containing 100  $\mu\text{g}/\text{ml}$  ampicillin at 37 °C and shaken at 180 rpm. When cell density reached 0.4, protein expression was induced using 1 mM IPTG and the culture was grown at 32 °C for 4 h. Cells were harvested by centrifugation at 5000 $\times g$  for 10 min at 4 °C and proteins were extracted on ice by sonication (6  $\times$  30 s cycles of 7 W, with a 2 min rest period between cycles) using a lysis buffer (20 mM of Tris-HCl buffer (pH 7.4),

**Table 1** The sequences of YoaJ/YkgR–ASPAs ( $\beta$ -defensin, BD)

Protein	Sequences
YoaJ–ASPAs (TEV)	MKKTTIIMMGVAIIVVLGTELGWWRGSHHHHHHENLYFQGCAFEGESC NVQFYPCCPGLGLTCIPG NPDGTCYYL
YoaJ–ASPAs (Factor X $\alpha$ )	MKKTTIIMMGVAIIVVLGTELGWWRGSHHHHHHENLYFQGTIEGREGCAFEGESC NVQFYPCCPG LGLTCIPGNPDGTCYYL
YkgR–ASPAs (Factor X $\alpha$ )	MKENKVQQISHKLNIVVFVAIVEYAYLFLHFYWRGSHHHHHHENLYFQGTIEGREGCAFEGESC NVQFYPCCPGLGLTCIPGNPDGTCYYL
YoaJ–BD	MHHHHHHGSMKKTIIIMMGVAIIVVLGTELGWWRGSDRGSNLYFQGTIEGRDRPKPDRPKAD CSTIQGVCKDSCLSTEFSGALGCSAESSTVCCVTKPKL
ASPAs–YoaJ	MRGSHHHHHHGKRTIEGREGCAFEGESC NVQFYPCCPGLGLTCIPGNPDGTCYYLIEGRGSMKK TTIIMMGVAIIVVLGTELGWWRGSHHHHHH

**Table 2** The sequences of oligonucleotides in vector constructions

Primer	Sequences (5'–3')
YoaJ–ASPAs-1	TGTAAGCTTGGTAATCCTGATGGCACCTGCTACTACCTTTAGCTGAGCTTGGACTCCTGTGTA TTGAAGCTTACAACCTCACCTTCGAATGCACATCCGTGAAAAATACAGTTTTTCGTG
YoaJ–ASPAs-2	TGCCCCGGTCTTGGTCTTACTTGTATTCTGGTAATCCTGATGGCACCTGCTAC AAGACCCGGGCAACAAGGATAGAATTGAACATTACAACCTCTC ACCTTCGAATGC
YoaJ–ASPAs-3	GTGGTACCCGGCACTGAGCTGGGATGGTGGCATCACCATCAC CATCAC GCCGGGTACCACAATAATCGCCACACCCATCATAATAATCGTTGTTTTTTCATAGTTAATTTCTCCTCTTTAATGAA
ASPAs–YoaJ-1	GGGGCCGGATCCATGAAAAAACAACGATTATT AAAGGGGAGCTCCTAGTGATGGTGTGGTGTGCGA
ASPAs–YoaJ-2	AAATTTGAGCTCTAGAAGCTTAATTAGCTGAGCTTGGAC AAATTTGGATCCTCGACCTTCTATAAGGTAGTAGCAGGTGCCATC
BD-1	AAAAAAGCTTTAGCTGAGCTTGGACTCC AAAAAAGTCGACCGATCCTCTCCACCATCC
BD-2	AGGAAAGTCGACGAA AACCTG TATTTT CA AAAAAAGAGCGGTGACCATGCCTCTTACAGTTCTTC

0.2 mM phenylmethylsulfonyl fluoride, 1 % Triton X-100). Lysates were centrifuged at 100,000 $\times$ g for 30 min at 4 °C to separate the particulate fraction (P) as pellets from supernatants which was the clarified soluble fraction (S).

To purify proteins, fractions were loaded onto an Ni<sup>2+</sup> column and purified proteins were obtained using an elution buffer containing 400 mM imidazole. Eluates were subjected to dialysis to adjust them to a buffer condition suitable for enzyme digestion by TEV or Factor X $\alpha$ . Digestion was performed using the procedures supplied by the manufacturers (Enzygnomics, South Korea and Sigma, South Korea, respectively). Subsequently, proteins were subjected to reversed phase high-performance liquid chromatography (RP-HPLC) purification using a C18 column (250  $\times$  4.6 mm) and a linear acetonitrile gradient of 0–72 %.

#### Tricine–SDS–PAGE gel electrophoresis

15 % SDS-gel electrophoresis was performed as previously described (Haider et al. 2010). Before loading, samples

were denatured for 10 min at 90 °C in sample buffer. Gels were stained with Coomassie blue G-250.

#### Molecular mass determination

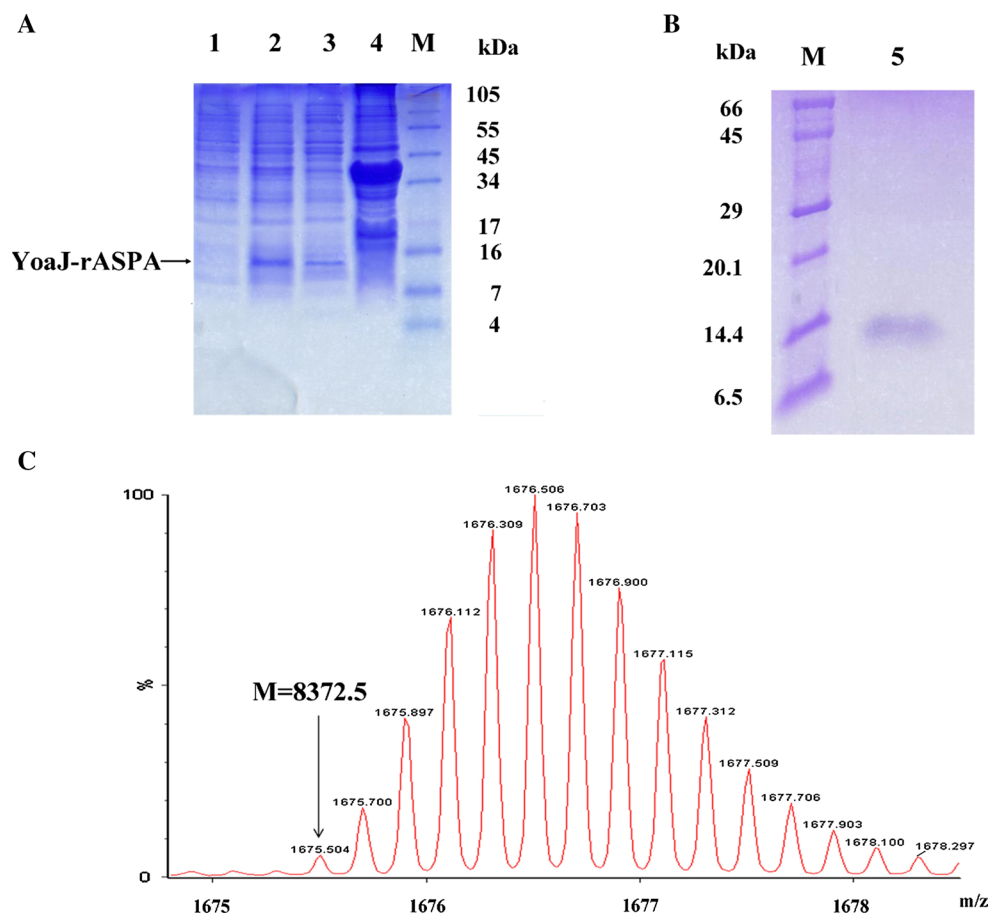
Molecular masses of the proteins were determined by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Ultraflexxtreme, Bruker). For MALDI-TOF MS, samples were diluted using an  $\alpha$ -cyano-4-hydroxycinnamic acid matrix solution and spotted onto MALDI plates.

## Results

### Periplasmic production of ASPAs

The vector pQE30–YoaJ–rASPAs was constructed for the periplasmic production of ASPAs. The sequences encoding YoaJ and ASPAs were synthesized into pQE30 expression

**Fig. 2** Periplasmic expression, purification and mass spectra of YoaJ-ASPAs containing a TEV cleavage site. **a** Lane M protein molecular weight markers, lane 1 non-induced cell sample, lane 2 IPTG-induced cell sample, lane 3 total cell extracts after sonication with Triton X-100, lane 4 pellet fraction. **b** Lane M protein molecular weight markers, lane 5 purified fusion protein (YoaJ-TEV-ASPAs). YoaJ contains the 6 histidine tag, a TEV cleavage site and other amino acids. **c** ESI-mass spectra of purified YoaJ-ASPAs (TEV)

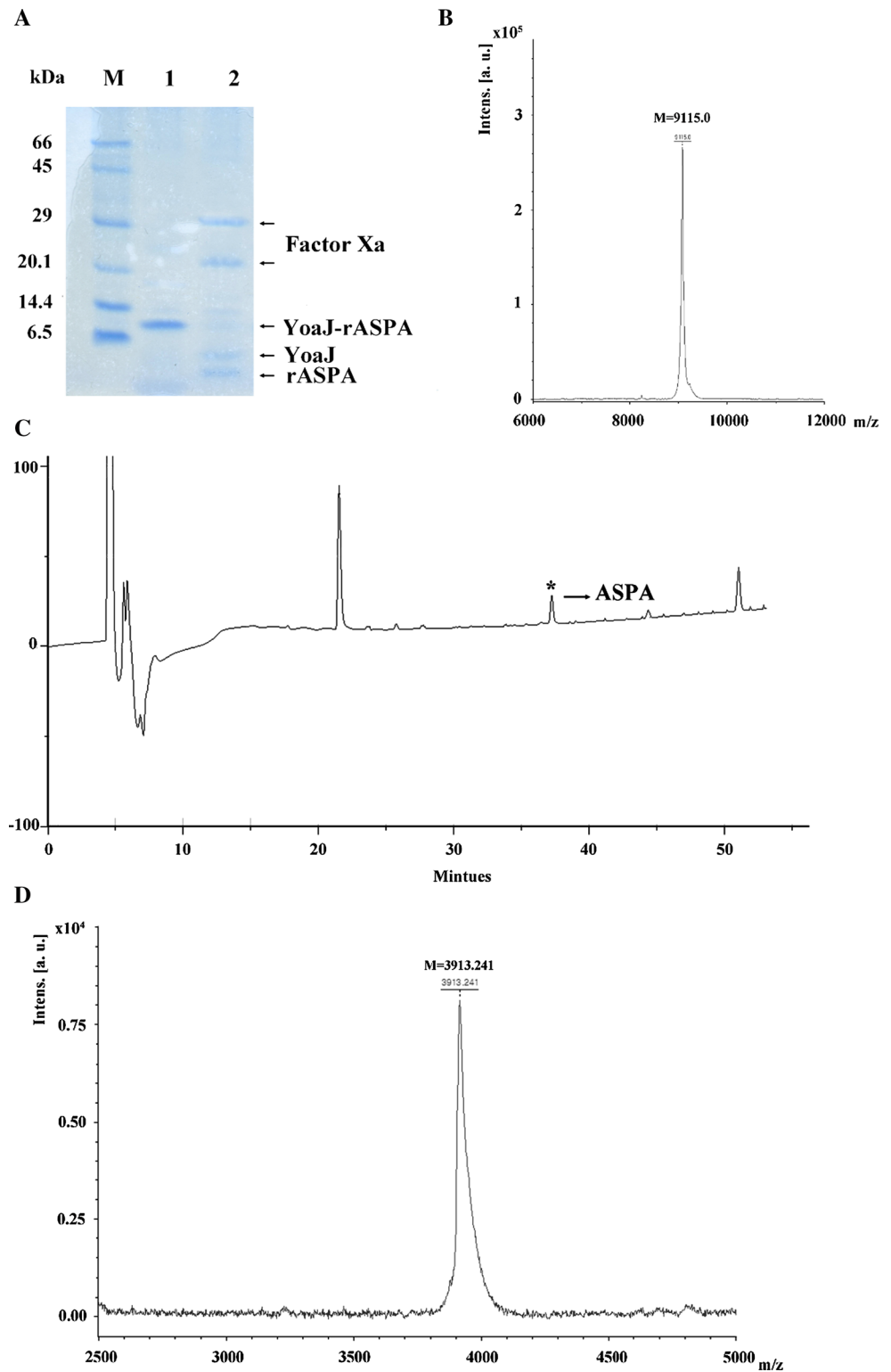


plasmids using *E. coli* M15 (pREP4) as an expression host. The six histidine residues for Ni resin-based protein purification and the nine residues of the TEV cleavage site were added between YoaJ and ASPA (Fig. 1b). Figure 2 shows the expressed fusion protein composed of YoaJ carrier protein and ASPA peptide. The theoretical molecular weight of the fusion protein was 8.4 kDa, and a band of this molecular weight was observed in extracts of transformed cells after IPTG induction (Fig. 2a, lane 1 and 2). After extract lysis with 1 % Triton X-100 as a surfactant, the recombinant fusion protein was observed exclusively in the supernatant fraction (Fig. 2a, lane 3). The fusion protein present in the fractions was purified by Ni-NTA extraction, and the major band observed on Coomassie blue-stained gels indicated a yield of approximately 25 mg/L of culture (Fig. 2b). Its molecular mass by ESI-mass spectrometry analysis was determined to be 8,372.5 Da (Fig. 2c). The theoretical molecular weight of full-length YoaJ-ASPAs (calculated by including all amino acid residues) was 8,378.9. The 6-Da difference was ascribed to the formation of three disulfide bonds in the recombinant fusion protein.

An attempt to cleave the fusion protein with TEV protease failed, because of the existence of Triton

X-100 in the buffer. As an alternative to TEV cleavage, the expression vector pQE30-YoaJ-Factor X $\alpha$ -ASPAs was reconstructed. This vector gave rise to a fusion protein with a recognition sequence for the site-specific protease, Factor X $\alpha$ , between YoaJ and ASPA peptide. The recombinant fusion protein, YoaJ-ASPAs-Factor X $\alpha$ , was purified using an Ni<sup>2+</sup> column (Fig. 3a, lane 1) and exhibited a monoisotopic ion peak at a molecular mass of 9,115.0 Da by MALDI-TOF MS (Fig. 3b), which compared with a theoretical molecular weight of 9,121.25 Da, and revealed that three disulfide bonds were present in the recombinant fusion protein formed. After the cleavage reaction between recombinant protein and Factor X $\alpha$  protease (4 h incubation at 37 °C) (Fig. 3a), the major fusion protein at 9 Da disappeared gradually and two protein bands developed at 4 and 5 Da (Fig. 3a, lane 2). However, separation of the peptide and the YoaJ tag was unsuccessful using an ion exchange column and the two products were separated by RP-HPLC. Subsequently, a single peak was observed at 37 min in ~26 % (v/v) acetonitrile solution (Fig. 3c), and this was confirmed to be peptide ASPAs by mass spectrometry (yield ~ 5 mg/L of culture). Once again, the 6-Da difference observed between

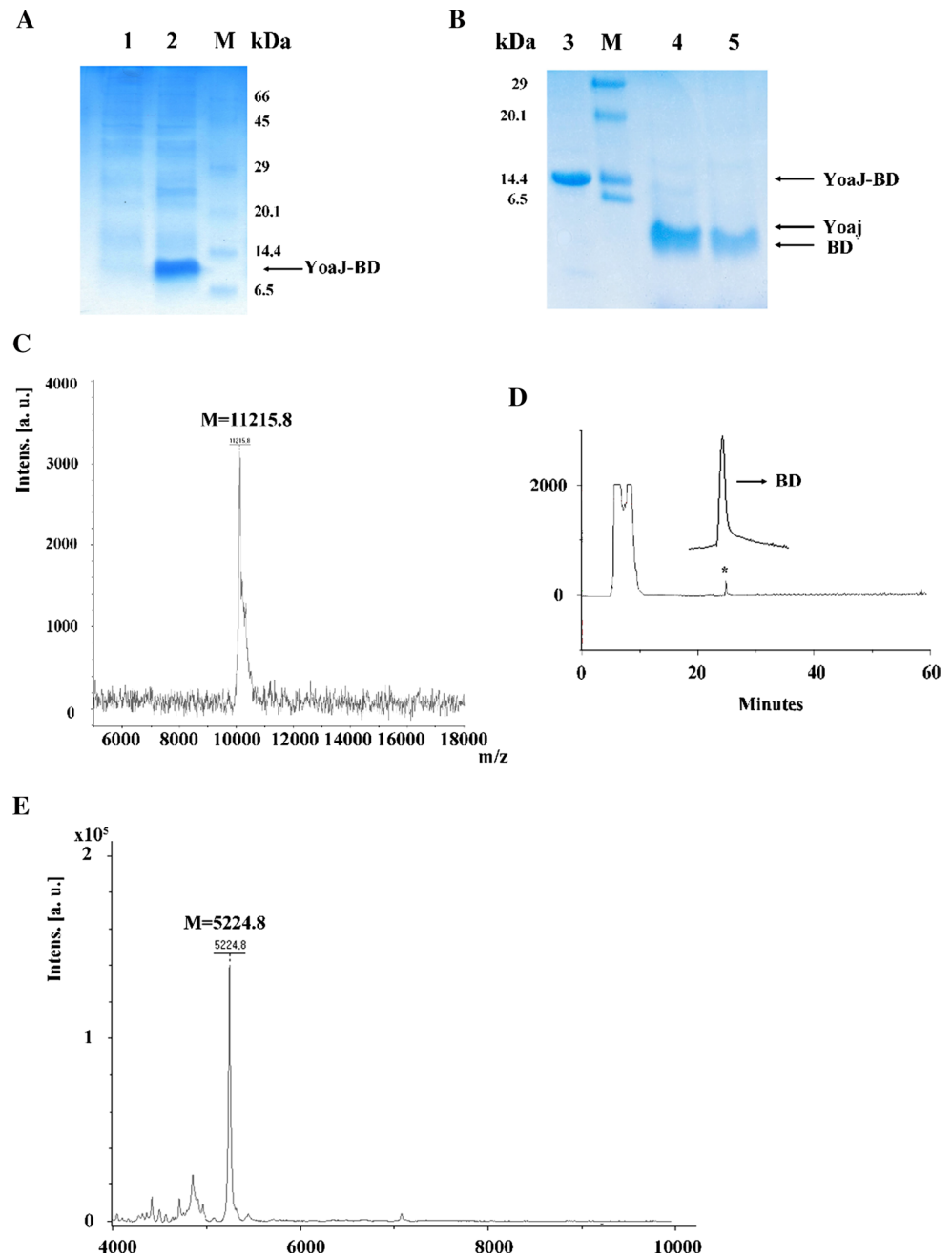
**Fig. 3** Periplasmic expression and purification of YoaJ–ASPA containing a Factor X $\alpha$  cleavage site. **a** Lane 1 purified fusion protein (YoaJ–Factor X $\alpha$ –ASPA), lane 2 YoaJ–ASPA after Factor X $\alpha$  digestion. **b** MALDI-TOF mass spectrum of purified YoaJ–ASPA. **c** RP-HPLC chromatography of free ASPA. The protein was purified using a linear gradient of 0–72 % solvent B for 60 min, where solvent A was water containing 0.1 % TFA and solvent B was acetonitrile containing 0.1 % TFA. The peak at 37 min was attributed to be the peptide by molecular weight. The other peaks were determined to be Factor X $\alpha$  protease (~50 min) and the YoaJ tag (~22 min), respectively. **d** Mass spectrum of purified ASPA. MALDI-TOF MS exhibited a monoisotopic peak at  $m/z$  3,913.241



the theoretical molecular weight (3,919.6 Da) of the eluted protein and its measured molecular weight of 3,913.241 Da by MALDI-TOF MS (Fig. 3d) confirmed the presence of three disulfide bonds in the recombinant ASPA peptide.

YkgR is a transmembrane small protein in *E. coli*, which like YoaJ orientates itself with its N-terminus in the cytoplasm and its C-terminus in the periplasm. Fusion expressions of ASPA using the two carrier proteins (YkgR and YoaJ) were similar (data not shown).

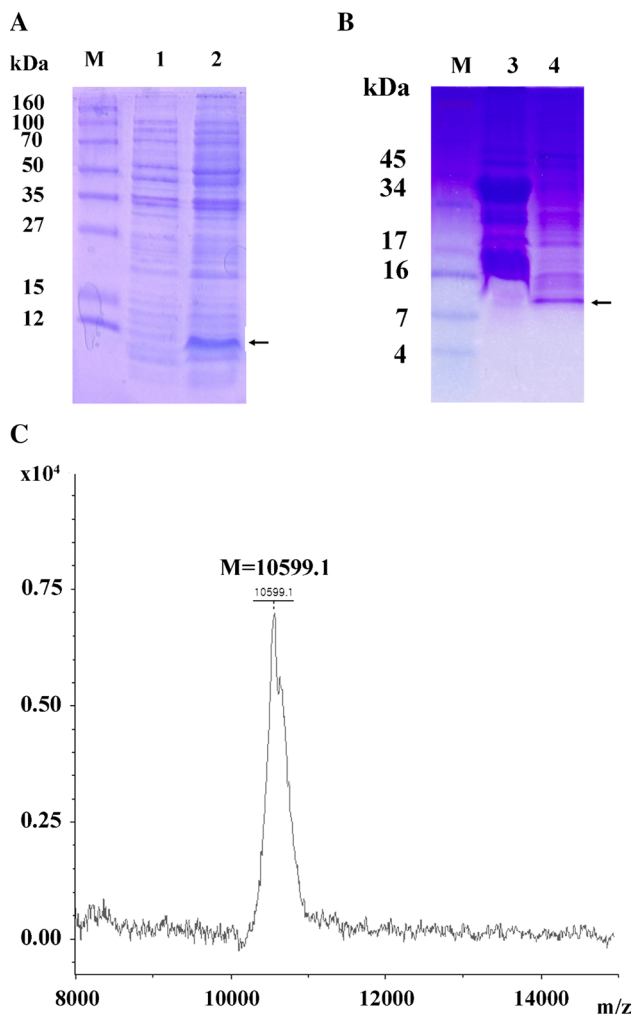
**Fig. 4** Periplasmic expression and purification of recombinant YoaJ-BD and free BD. **a** Lane *M* protein molecular weight markers, lane 1 non-induced cell sample, lane 2 IPTG-induced cell sample. **b** Lane *M* protein molecular weight markers, lane 3 purified fusion protein (YoaJ-BD), lane 4 purified YoaJ tag (containing 6X-His tag, a Factor X $\alpha$  cleavage site and other amino acids), lane 5 purified free BD after Factor X $\alpha$  digestion. **c** MALDI-TOF mass spectrum of purified YoaJ-BD. **d** RP-HPLC chromatography of YoaJ-BD. The protein was purified using a linear gradient of 0–72 % solvent B for 60 min, where solvent A was water containing 0.1 % TFA and solvent B was acetonitrile containing 0.1 % TFA. **e** MALDI-TOF mass spectrum of purified BD



#### Periplasmic production of BD

For the production of BD, pQE30–YoaJ–BD with a Factor X $\alpha$  cleavage site was designed based on the pQE30–YoaJ–ASPA vector, but the 6 histidine residue tag was introduced to the 5' end of the vector. The vector was expressed recombinantly in *E. coli* M15 (pREP4) cells. As shown in Fig. 4a, as compared to the non-induced cell sample (lane 1), a significant strong band appeared in the IPTG-induced cell sample (lane 2). The molecular weight of the protein band was estimated to be 11 kDa, which

was consistent with the theoretical molecular weight of YoaJ-BD fusion protein. After sonication, soluble extracts were subjected to Ni-NTA column purification, which led to purification of the 11 kDa product (Fig. 4b, lane 3). The molecular mass of the fusion protein was determined by MALDI-TOF MS to be 11,215.8 Da (Fig. 4c). The theoretical molecular weight of the full-length YoaJ-BD was 11,221.49 Da, and the 6-Da difference indicated the formation of three disulfide bonds. The fusion protein was then dialyzed and subjected to Factor X $\alpha$  digestion. To separate the YoaJ tag and free BD, digestion products



**Fig. 5** Cytoplasmic expression, purification and mass spectrum of ASPA–YoaJ fusion protein. **a** Lane M protein molecular weight markers, lane 1 non-induced cell sample, lane 2 IPTG-induced cell sample. The arrow indicates the recombinant fusion protein. **b** Lanes 3 and 4 represent the pellet fraction and total cell extracts, respectively, after sonication without Triton X-100. **c** MALDI-TOF mass spectrum of ASPA–YoaJ fusion protein

were passed through a mono Q ion exchange column. As shown in Figs. 4 and 5, the digestion products were composed of two major products at approximately 5 kDa, indicating YoaJ protein and BD. Figure 4d presents the RP-HPLC purification of free BD, which was eluted at an acetonitrile concentration of 18 %. A single peak was observed and the final yield was ~10 mg/L. The expected molecular mass of BD was confirmed by MALDI-TOF MS (Fig. 4e) to be 5,224.8 Da, which taking into account the formation of three disulfide bonds compared well with a theoretical molecular weight of 5,230.49 Da. Overall, these results showed that the His-tag at the 5' end of the vector can also be used to produce a protein disulfide bonds.

### Cytoplasmic expression of ASPA

To examine the effects of the YoaJ carrier on the expressions of the peptides, C-terminus YoaJ was designed on the vector pQE30–ASPA–YoaJ (Fig. 1a). The production of fusion protein was analyzed as described above. SDS-PAGE showed a new peak in the IPTG-induced cell sample (Fig. 5a, lane 2) versus the non-induced sample (lane 1). The recombinant fusion protein was partitioned to the soluble fraction after centrifuging lysed cells not treated with Triton X-100 (Fig. 5b), which was quite different from the fractions of YoaJ–ASPA, in which recombinant fusion proteins partitioned to the pellet fraction during sonication without Triton X-100. After purification using an Ni<sup>2+</sup> affinity column, the fusion protein ASPA–YoaJ was subjected to MALDI-TOF MS, which revealed a molecular weight (10,599.1 Da) that well matched its theoretical molecular weight (10,599.16) (Fig. 5c). These results showed that disulfide bonds were not formed in the recombinant protein.

### Discussion

Several strategies have been developed to express disulfide bonded proteins in bacterial expression systems. However, fusion expression using a carrier protein has been shown to be necessary for peptide production. The preferred carriers expressed in cytoplasm are often larger than peptides (thioredoxin 12 kDa, GST 26 kDa, SUMO 11 kDa, etc.). On the other hand, the secretion of proteins into *E. coli* periplasm offers a better opportunity for the formation of correct disulfide bonds due to the oxidizing conditions present (Malik et al. 2006). In *E. coli*, Dsb proteins catalyze the formation of disulfide bonds and are most commonly used in co-expression plasmids (Kurokawa et al. 2000; Olichon and Surrey 2007; Yuan et al. 2004). Other periplasmic proteins, such as, *E. coli* trypsin inhibitor, have also been used as leader peptides (Malik et al. 2006). Although several studies have produced peptides with correct disulfide bonds, it is considered difficult to produce disulfide bond-rich peptides, because problems associated with poor periplasmic accumulation and disulfide bridges instability need to be solved (Xu et al. 2008; Merdanovic et al. 2011). In the present study, YoaJ/YkgR was used as a fusion tag to produce disulfide bond-rich peptides in the periplasmic space of *E. coli*. The location of YoaJ in *E. coli*, that is, with its N-terminus in the cytoplasm and its C-terminus in the periplasm, allows peptides of interest to be fixed in the periplasmic space. As compared to other procedures reported for the expression of disulfide bridge-rich peptides in the periplasm of *E. coli*, the present choice of leader peptides

**Table 3** Peptide yields from the periplasm of *E. coli*

Fusion protein	Carrier size (kDa)	Peptide (mg/L)	Ref
YoaJ–ASPAs	2.69	5	
YoaJ–BD	2.69	10	
DsbC–beta-scorpion	23.5	~0.3	(O'Reilly et al. 2014)
MBP–nanobodies	42	2.2	(Salema and Fernandez 2013)
DsbC–Huwentoxin	23.5	~0.7	(Meng et al. 2011)
pelB–human growth hormone	2.31	1.4	(Sokolosky and Szoka 2013)

appeared to improve secretion efficiency and stability, and was used to produce ASPA and BD peptides in good yields.

As mentioned above, when YoaJ was used at the C-terminus of the peptide, the fusion protein was probably produced in the cytoplasm of *E. coli*. Although the original yield of the fusion was more than 30 mg/L, our results showed when located in cytoplasm the recombinant fusion protein did not form disulfide bridges (Fig. 4c). On the other hand, the N-terminus YoaJ (YoaJ–ASPAs/BD) allows the recognition of the leader peptide and enables its secretion into the periplasm of *E. coli*. The peptides produced exhibited a single HPLC peak, suggesting that only specific disulfide bridges were formed. We supposed a high probability of correct disulfide bonds formation, because that the low-energy state of a correctly folded disulfide bond is more stable than that of a misfolded or incorrect disulfide bond. However, the structures and biological activities of the ASPAs and BD produced need to be further confirmed.

The yields of purified ASPAs and BD were 5 and 10 mg/ml, respectively, which is higher than that reported for other periplasmic carriers, such as DsbC (O'Reilly et al. 2014; Meng et al. 2011), the pelB leader sequence (Sokolosky and Szoka 2013), or maltose-binding protein (MBP) (Salema and Fernandez 2013) (Table 3). The two peptides expressed in periplasm during the present study support the suggestion that the small *E. coli* transmembrane proteins (YoaJ and YkgR) might be useful for the expression of disulfide bond-rich peptides. Most membrane proteins and exported proteins require protein catalysts for insertion and transport across membranes (Serek et al. 2004). In *E. coli*, there are two membrane insertions systems, the Sec translocation system and YidC translocase. In a previous study, it was suggested that YoaJ was compatible with both of these systems (Fontaine et al. 2011). Further studies will undoubtedly provide more details on membrane insertion and disulfide bond folding mechanisms.

Summarizing, we report the successful construction of a novel expression system for disulfide bond-rich peptides using transmembrane small proteins in *E. coli* as carriers. Two peptides, ASPAs and BD, were expressed in good yields and the resulting peptides had the same molecular weights as their natural forms as determined by mass spectrometry.

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**Conflict of interest** The authors have no potential conflict of interest to declare.

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