


# Soluble expression, purification and functional characterization of a coil peptide composed of a positively charged and hydrophobic motif

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**Abstract** A de novo heterodimeric coiled-coil system formed by the association of two synthetic peptides, the Ecoil and Kcoil, has been previously designed and proven to be an excellent and versatile tool for various biotechnology applications. However, based on the challenges encountered during its chemical synthesis, the Kcoil peptide has been designated as a “difficult peptide”. In this study, we explore the expression of the Kcoil peptide by a bacterial system as well as its subsequent purification. The maximum expression level was observed when the peptide was fused to thioredoxin and the optimized purification process consisted of three chromatographic steps: immobilized-metal affinity chromatography followed by cation-exchange chromatography and, finally, a reverse-phase high-performance liquid chromatography. This entire process led to a final volumetric production yield of 1.5 mg of pure Kcoil peptide per liter of bacterial culture, which represents a significant step towards the cost-effective production and application of coiled-coil motifs. Our results thus demonstrate for the first time that bacterial production is a viable alternative to the chemical synthesis of de novo designed coil peptides.

**Keywords** Bacterial production · Coil peptide · Soluble expression · Thioredoxin

## Introduction

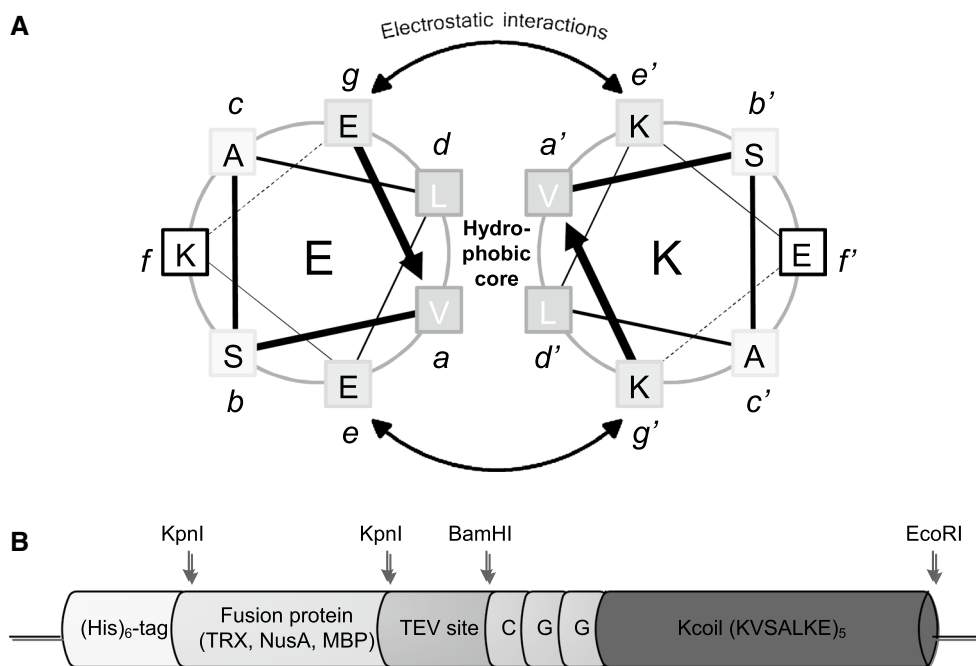
Coiled-coils are structural protein domains composed of three to five amphipathic alpha helices found in a great variety of proteins (Burkhard et al. 2001; Cohen and Parry 1986; Lupas 1996). Based on naturally occurring structures, a de novo heterodimeric coiled-coil system has been previously designed and characterized. It is formed by the association of two synthetic peptides, the Ecoil and Kcoil, each composed of a distinct seven residue repeat (denoted [abcdefg]<sub>n</sub>) where n represents repetition number, “a” and “d” positions are occupied by hydrophobic residues, while the “e” and “g” positions are occupied by charged residues. The designed hydrophobic core and electrostatic interactions make this coiled-coil structure very stable (Fig. 1a) (De Crescenzo et al. 2003a; Grigoryan and Keating 2008; Chao et al. 1996) whereas each coil component remains monomeric on its own in solution. The creation of such new bio-inspired molecules with enhanced features can find widespread use in a number of biomedical and therapeutic applications (Bromley and Channon 2011). More specifically, the E/K coiled-coil system has proven to be an excellent and versatile tool for (1) the detection of coil-tagged proteins by Western blot (Boucher et al. 2010) or ELISA (Liberelle et al. 2010a), (2) the coiled-coil-mediated protein dimerization (De Crescenzo et al. 2004), as well as (3) the stable and oriented capture of coil-tagged proteins onto various surfaces harboring the partner coil peptide for applications in biosensing (Chao et al. 1998; De Crescenzo et al. 2003b) and regenerative medicine (Murschel et al. 2013; Liberelle et al. 2010b; Fortier et al. 2013; Murschel et al.

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**Fig. 1 a** Helical-wheel representation of the heterodimeric E/K coiled-coil peptide system. The interchain electrostatic interactions between the charged residues are indicated by the arrows. **b** Schematic representation of Kcoil-fusion protein sequence design. *Arrows* indicate the restriction enzyme sites required to insert the cDNA cor-

2015). To date, the Ecoil peptide [(EVSALKE)<sub>5</sub> sequence] has been expressed as part of larger fusion proteins produced in mammalian cells (Murschel et al. 2013; Libelle et al. 2010b) or bacteria (Le et al. 2009), whereas the partner Kcoil peptide [(KVSALKE)<sub>5</sub> sequence], has been generated by solid phase peptide synthesis (SPPS) with an N-terminal cysteine residue that is included for immobilization purposes. The SPPS technique has the advantage of allowing the incorporation of unnatural amino acids as well as for the rapid production of milligram quantities of peptide (Kohn and Hodges 1998). However it presents several drawbacks including expensive processing costs and difficulties associated with mass production. SPPS is indeed a multi-stage process that requires chemical protection/deprotection steps for every introduced residue. The number of steps thus increases chances to produce mismatched peptides, which negatively impacts the final production yield. This is especially the case for the Kcoil peptide, for which challenges associated with its chemical synthesis have been recently highlighted (Winkler and Tian 2015). In the laboratory, this may translate into large amounts of final product (>50 %) containing errors in the sequence, which in turn imposes costly high-resolution chromatography steps to get pure Kcoil peptide. Moreover, chemical synthesis of peptides involves significant volumes of coupling and/or activating reagents, which can be aggressive or even toxic to the environment (Guzman et al. 2007). For these

responding to the fusion protein and the Kcoil sequence within the different plasmids. A three amino-acid sequence (CGG) was added upstream the Kcoil peptide cDNA in order to allow any thiol coupling procedure for Kcoil peptide grafting. A TEV site was also added for fusion protein removal and pure Kcoil peptide recovery.

reasons, there is an unmet and critical need for the production of long coil peptides using a bacterial expression system in order to further expand the use of these peptides in emerging and promising applications requiring large quantities such as the development of coiled-coil-based scaffolds (Gerling-Driessen et al. 2015) for tissue engineering (Woolfson and Mahmoud 2010; Aronsson et al. 2015) and gene delivery (Assal et al. 2015).

However, the biological production of the Kcoil peptide poses significant challenges; first of all because of its size (the 5 heptad repeats and cysteine-containing linker correspond to a 38 amino-acid-long peptide) which makes it very susceptible to intracellular proteolysis, and second, due to the presence of a high density of positively charged residues (2 lysine residues per heptad) that makes it very toxic to the bacteria and consequently leads to poor expression. Moreover, the development of a suitable purification scheme is hampered since the hydrophobic core of the coil peptide may affect its solubility and the lack of an aromatic residue in the original sequence precludes its quantification by absorbance measurements at 280 nm. In the present work, we report on the successful recombinant production of a cysteine labelled Kcoil peptide as an alternative to SPPS. Kcoil expression levels were first compared when fused to three common soluble carrier proteins: thioredoxin (TRX), maltose binding protein (MBP) and N-utilization substance A protein (NusA), then a culture protocol

amenable to large scale was developed and, finally, an efficient purification strategy involving three chromatographic steps was set up.

## Materials and methods

### Chemicals and reagents

Orange II sodium salt (92 % purity), ethidium bromide (95 % purity), cysteine (99 % purity), bromophenol blue, imidazole and Coomassie Brilliant Blue (100 % purity) were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON). Cysteine-tagged Kcoil peptides were chemically synthesized by the peptide facility at University of Colorado (Denver, CO, USA) to be used as control. CM5 sensor chips, HEPES-buffered saline (HBS-P), amine-coupling kit [including 2-(2-pyridinyldithio) ethaneamine, PDEA] were obtained from GE Healthcare (Baie d'Urfe, QC). Milli-Q quality water [18.2 MΩ cm; total organic compounds (TOC) = 4 ppb] was generated with a Millipore Gradient A 10 purification system. Cysteine (99 + % purity), acetic acid glacial (99.7 % purity), hydrochloric acid (HCl, 37.7 % v/v), yeast extract and peptone were obtained from VWR International, Ltd. (Mont-Royal, QC, Canada). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and Dithiothreitol (DTT) were purchased from INALCO (San Luis Obispo, CA, USA). Ampicillin, sodium chloride (NaCl) and sodium dodecyl sulfate (SDS) were obtained from Fisher scientific (Ottawa, ON). BL21(DE3) competent cells (Novagen) and Tobacco Etch Virus (TEV) protease were generated in house.

### Generation of the expression vectors

The EcoRI site of the pET15b vector (Novagen) was first deleted by site-directed mutagenesis using the following primers: CAAGAATTATCATGTTTGACAGCTTATC and

ATGATAATTCTTGAAGACGAAAGGGC; the deletion was verified by sequencing. Then, two oligonucleotides of sequence CATGGGCAGCAGCCATCATCATCATCA TCACAGCAGCGGTACCGAAAACCTTGATTTC CA GGGATCCCCGGGAATTCATCGTGA CTGAA and GATC TTCAGTCACGATGAATTCCTGGGATCCCTGGAA ATACAAGTTTTCGGTACCGCTGCTGTGATGAT GATGATGATGGCTGCTGCC were phosphorylated with the T4 Polynucleotide Kinase (Fermentas, CA), annealed, and ligated in between the NcoI and BamHI sites of the modified pET vector to generate a pET-TEV vector, thereby destroying the original BamHI site and adding a new multiple cloning site. This strategy allows for the production of proteins being His-tagged at their N-terminus where the His tag can be removed by treatment with the Tobacco Etch Virus (TEV) protease. Furthermore, genes can be cloned in between the His tag and the TEV site corresponding DNA sequences by taking advantage of the KpnI site. The genes coding for the thioredoxin (TRX), the N-utilization substance A protein (NusA) and the maltose binding protein (MBP) were amplified using the primers summarized in Table 1. PCR amplified products were then digested with KpnI and inserted into the KpnI site of pET-TEV to generate pET-TEV-TRX, pET-TEV-NusA and pET-TEV-MBP vectors. Positive clones were verified by sequencing.

### Cloning Kcoil coding sequence

Kcoil DNA coding sequence was amplified by polymerase chain reaction (PCR) from pIDTBlue:Kcoil plasmid (integrated DNA technology, US) using the primers reported in Table 1. Subsequently, the PCR products as well as the different expression vectors already containing the gene of the protein to be fused to the Kcoil peptide (pET-TEV-TRX, pET-TEV-NusA and pET-TEV-MBP) that had been generated as described above, were double-digested by BamHI and EcoRI restriction enzymes. Kcoil DNA sequence was cloned using a 4:1 ligation ratio (insert/vector) and then

**Table 1** Primer sequences used for PCR amplification

Genes	Primers
Thioredoxin	F: CAGCGGT <u>ACC</u> AGCGATAAAATTATTCACCTGACTGACGAC R: CAGCGGT <u>ACC</u> GGCCAGGTTAGCGTCGAGG
Maltose-binding protein	F: CAGCGGT <u>ACCA</u> AAATCGAAGAAGGTAAACTGGTAATCTGG R: CAGCGGT <u>ACCT</u> GGGATATCGTAATCAGTCTGCGCG
N-Utilisation substance A protein	F: CAGCGGT <u>ACCA</u> ACAAAGAAATTTGGCTGTAGTTGAAGCCG R: CAGCGGT <u>ACCC</u> GCTTCGTCACCGAACCAGCAAATATTACG
Kcoil	F:CGGGATCCTCGGTGGTAAGGTATCCGCTTTAAAGGAGAAAGTTTCTGC R: GCGAATTCTCACTCTTTAAGTGCTGAAACCTTTTCCTTCAAGG

*KpnI* BamHI and EcoRI restriction sites are underlined

F forward primer, R reverse primer

transformed in *E. coli* DH5 $\alpha$  competent cells. Positive clones were verified by sequencing.

### Screening of expression levels

Plasmids (pET-TEV-TRX, pET-TEV-NusA and pET-TEV-MBP) encoding Kcoil peptide were used to transform BL21(DE3) *E. coli* cells. Single positive transformed colonies were used to inoculate 5 mL of LB medium containing ampicillin (100  $\mu$ g/mL) and were grown overnight at 37 °C in a shaker at 200 rpm. Bacteria pre-cultures were then diluted in 50 mL of LB medium and fusion protein expression was induced by adding 0.7 mM of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at an optical density (OD) of approximately 0.6 (600 nm). Following an induction period of 5 h, cells were harvested by centrifugation (10 min at 3000 rpm) and cellular pellets were re-suspended in 10 mL of lysis buffer (20 mM NaPO<sub>4</sub> pH 7.4, 500 mM NaCl, 1 mM DTT and 0.5 M EDTA) and then sonicated (Branson Sonifier, US) on ice for 30 s. Soluble and insoluble fractions were separated by centrifugation at 13,000 rpm (30 min) using a microcentrifuge (Thermo Fisher Scientific Inc., US) and each fraction sample was analysed by SDS-PAGE.

### Optimization of TRX-tagged Kcoil peptide purification

To prepare the culture, 1 L of overnight pre-culture of BL21(DE3) cells expressing the TRX-Kcoil fusion protein was diluted in 8 L of fresh LB medium containing 100  $\mu$ g/mL ampicillin. IPTG was then added at a final concentration of 0.7 mM at an OD<sub>600nm</sub> of approximately 0.6 and the culture was incubated at 37 °C for 5 h. The cellular pellet was collected by centrifugation (10 min at 8000 rpm and 4 °C) and kept frozen at −20 °C until use. Cell disruption was carried out by re-suspension of the cellular pellet in lysis buffer (20 mM PO<sub>4</sub> pH 7.4, 500 mM NaCl and 25 mM imidazole), followed by several cycles through the French press. After an ultracentrifugation step at 105,000 $\times$ g for 1 h at 4 °C, the His-tagged TRX-Kcoil fusion protein was purified by ion metal affinity chromatography (IMAC): the supernatant containing soluble proteins was loaded on a Ni<sup>2+</sup>-charged 5-mL His-trap HP column (GE Healthcare, Sweden) using an ÄKTA-purifier UPC 10 system (GE Healthcare, Sweden) and the His-tagged fusion protein was eluted by applying a 25-to-500 mM imidazole gradient (10 min, flow rate of 5 mL/min). Collected fractions were overnight dialyzed against TEV cleavage buffer (25 mM NaPO<sub>4</sub> pH 7.4, 125 mM NaCl and 10 mM DTT) at 4 °C. The thioredoxin fusion protein was subsequently treated with TEV at an enzyme/substrate ratio of 1:80 (c/c) for 2 h at room temperature and the cleavage mixture was

directly injected onto a HiLoad™ 16/10 SP Sepharose® Fast Flow column (GE Healthcare, Sweden) that had been pre-equilibrated with 10 column volumes of phosphate buffer (25 mM NaPO<sub>4</sub> pH7.4, 125 mM NaCl) at 90 cm/h. The column was then eluted using a 125-to-500 mM NaCl gradient (20 column volumes of elution buffer at the same flow rate) subjected to buffer exchange against Milli-Q water fractions corresponding to the same peak were pooled, concentrated and then containing 5 % acetic acid at 4 °C. Finally, a reverse-phase high performance liquid chromatography (RP-HPLC) step was carried out using a C<sub>4</sub> column (Grace Vydac, USA) connected to a Bio-Rad system (Biologic autoflow, US). A linear gradient from 20 to 35 % over 15 min of acetonitrile containing 0.05 % trifluoroacetic acid was applied at 8 mL/min to elute the Kcoil peptide. Collected fractions were concentrated and finally lyophilized for storage until needed.

### SDS-PAGE analysis

Samples were analysed by SDS-PAGE (non-reducing conditions) on 15 % gels and stained by Coomassie Brilliant Blue G-250 after a heat treatment of 10 min at 100 °C. 5  $\mu$ L of Precision Plus Protein™ Dual Xtra Standards (Bio-Rad, CA) was used as a pre-stained molecular weight marker for Kcoil peptide detection while PageRuler Prestained Protein Ladder (Thermo-Scientific, CA, USA) was used for protein expression monitoring during the production and purification process.

### Kcoil peptide characterisation

#### *Kcoil peptide quantification*

The total amount of purified biologically produced Kcoil peptide was estimated by adapting the protocol described by Drogoz et al. for primary amine detection in solution (Drogoz et al. 2007). A volume of 900  $\mu$ L of previously diluted sample were mixed with 100  $\mu$ L of Orange II solution (0.8 mM) and incubated for 15 min at room temperature. Supernatant absorbance was measured at 484 nm after 30 min of centrifugation at 20,000 $\times$ g. Absorbance measurements were performed using a UV-2100 spectrophotometer (Unico, Spain).

#### *Mass spectrometry molecular weight verification*

Accurate mass measurements were performed on a LC/MSD-TOF instrument (Agilent Technologies) in positive electrospray mode. Protonated molecular ions (M<sup>+</sup>H)<sup>+</sup> and/or sodium adducts (M<sup>+</sup>Na)<sup>+</sup> were used for empirical formula confirmation.

### Surface plasmon resonance (SPR) biosensor assay

The interaction between biologically produced Kcoil and its partner Ecoil peptide was recorded in real-time using a SPR biosensor assay. Chemically synthesised Kcoil was used as positive control. The assay was performed at 25 °C as described by Murschel and colleagues (Murschel et al. 2013), using a Biacore T100 biosensor instrument with HBS-P as a running buffer. Cysteine-tagged Kcoils (chemically- and biologically-produced) were first immobilized onto distinct CM5 sensor chip surfaces using standard ligand thiol coupling procedure (Murschel et al. 2013). The Ecoil-EGF protein corresponding to an Ecoil peptide/epidermal growth factor (EGF) fusion protein (Boucher et al. 2008) was injected on both Kcoil surfaces as well as on a mock surface (activated/deactivated surface) at 100 nM for 15 s at 100  $\mu$ L/min. In between Ecoil-EGF injections, surfaces were regenerated by two injections of guanidine hydrochloride (6 M, 15 s each). For a more accurate functional characterization of the biologically produced Kcoil, kinetic experiments were performed by SPR on similar Kcoil peptide surfaces. Several Ecoil solutions at concentrations ranging from 0.5 to 5 nM, were injected over 60 RU of immobilized Kcoil. Control-corrected sensorgrams were analyzed, assuming a simple 1:1 Langmuir binding interaction between Kcoil and Ecoil (De Crescenzo et al. 2003b).

## Results and discussion

### Expression of chimeras corresponding to Kcoil peptide fused to three different protein carriers

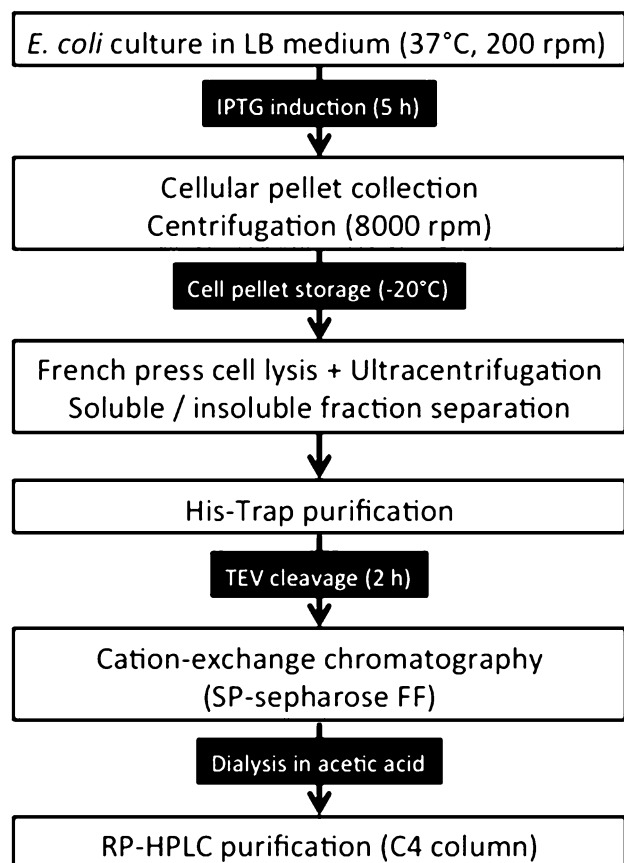
Since the main objective of the biological production of the Kcoil peptide is to further expand its biomedical applications, the cDNA encoding the Kcoil peptide was designed as depicted in Fig. 1b. The cDNA sequence corresponding to the three Cys-Gly-Gly residues that had been included to perform any thiol coupling procedure for Kcoil peptide grafting, were added upstream of the cDNA sequence encoding the Kcoil peptide. The bacterial production of the Kcoil peptide presents a significant challenge because of (1) the peptide size, (2) its sequence comprising a hydrophobic core combined to positively charged residues. An additional difficulty for process development also came from the impossibility to detect the peptide by absorbance at 280 nm (addition of a tyrosine residue to the original coil sequence to facilitate concentration determination of the purified product, resulted in the generation of an insoluble peptide in water, data not shown). For all these reasons, we tried to express the Kcoil peptide in inclusions bodies since we believed that its expression would be more efficient as

the inclusion bodies might reduce its cytotoxicity while protecting the peptide from proteolysis (Li 2011). For this purpose, the Kcoil peptide cDNA was fused to the ketosteroid isomerase protein (KSI). After solubilisation and His trap capture, the theoretical yield we calculated was about 5 mg/L of bacterial culture. As an alternative to the expression in inclusion bodies, the soluble expression of the Kcoil peptide as a fusion protein was then tried (Terpe 2003; Li 2009).

First of all, the expression level with pGEX vectors encoding Kcoil peptide fused to glutathione-S-transferase (GST) was very low (0.375 mg per liter of bacterial culture) and the peptide was still expressed in inclusion bodies (the design of a purification protocol based on inclusion bodies was unsuccessful in our hands, data not shown). In an effort to further increase Kcoil solubility and expression, other soluble fusion protein tags were assessed including TRX, NusA and MBP. These tags were chosen as they not only protect the cellular host from peptide toxicity, but they can also prevent the target peptide partner from undergoing intracellular proteolysis (Sahdev et al. 2008; Xu et al. 2007; Li 2012), typically resulting in a significant increase of the expression yield. The recombinant plasmids (pET-TEV-TRX, pET-TEV-NusA and pET-TEV-MBP) were transformed into BL21(DE3) *E. coli* cells. *E. coli* was used as a host because (1) it requires inexpensive carbon sources for growth, (2) it is a well characterized organism with a very short doubling time allowing for rapid biomass growth and product accumulation and, finally, (3) bioprocess scale-up is relatively easy (Sahdev et al. 2008; Kyle et al. 2010; Baneyx 1999). In addition, the use of a bacterial expression platform was well suited to the Kcoil peptide since no post-translational modifications (e.g., glycosylation) are required for its activity.

For expression level comparison, cultures were performed under generic conditions as described in the “Materials and methods” section. SDS-PAGE analysis (data not shown) indicated that the highest expression level of the Kcoil peptide was obtained with the TRX-fusion protein (Sahdev et al. 2008; Baneyx 1999; Terpe 2003; Davis et al. 1999; Esposito and Chatterjee 2006; Song et al. 2011). Of interest, TRX has also been reported to enhance the solubility of recombinant antimicrobial peptides sharing similar properties as our Kcoil peptide (e.g. positively charged and small size) (Li 2009, 2011; LaVallie et al. 2000; Jing et al. 2010; Yang et al. 2009). It has been previously suggested that the good production yields recorded for cationic peptides fused to TRX were due to its anionic character ( $pI = 4.67$ ) that can neutralize the high positive charge of cationic peptides, hence increasing the expression levels for this class of peptides. However, other studies have also suggested that the expression level might be related to (1) the mRNA stability of the partner (Yang et al. 2009), (2) the

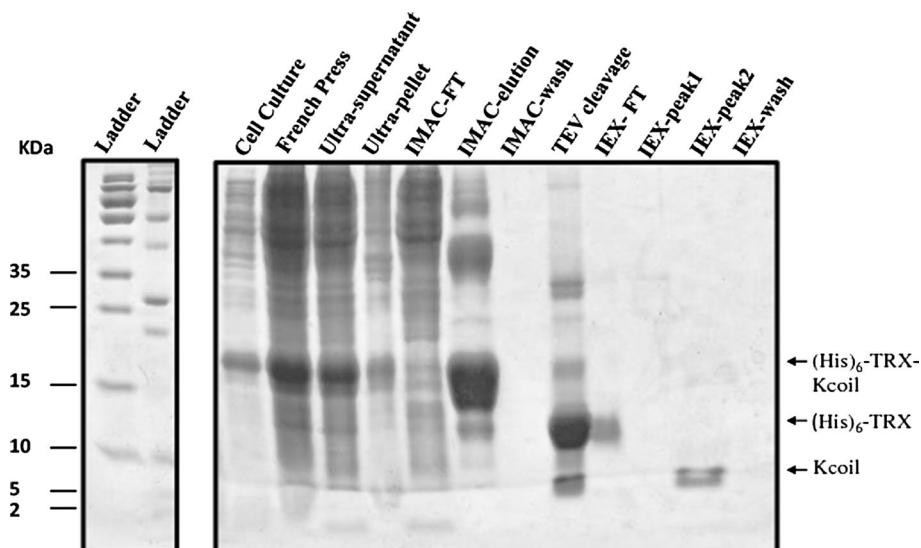




**Fig. 2** Schematic diagram summarizing the optimized strategy developed for Kcoil peptide biological production and purification

chaperone-like activity of TRX (Xu et al. 2007; Li 2009) or (3) its relatively low molecular-weight (~11.7 kDa), making the peptide of interest a large fraction of the fusion protein (Li 2011; Shlyapnikov et al. 2008).

**Fig. 3** SDS-PAGE analysis of heat-treated samples taken at each stage of the Kcoil-Thioredoxin fusion protein production and purification process. Two molecular weight markers were used. *FT* flow through, *Ultra* ultracentrifugation



### Optimisation of Kcoil peptide purification

As the best expression level for biological Kcoil peptide production was observed when fused to TRX, further process development efforts were conducted using this expression vector following IPTG induction (Fig. 2). Cell disruption and fraction separation were performed as described in the “Materials and methods” section. SDS-PAGE analysis of the soluble and insoluble fractions confirmed that the TRX-Kcoil complex was mostly expressed in the soluble fraction (Fig. 3). Taking advantage of the N-terminal hexahistidine (His)<sub>6</sub> tag, a Ni<sup>2+</sup>-chelating affinity chromatography step was thus performed first to purify the fusion protein. The estimated yield of eluted protein after this step was almost 22 mg of protein per liter of bacterial culture (Table 2). TEV cleavage was then performed to remove the TRX tag from the Kcoil moiety.

After TEV cleavage, two expected bands of approximately 12 kDa and 8 kDa were detected by polyacrylamide gel electrophoresis. These bands corresponded to the TRX protein and to Kcoil homodimers resulting from the formation of intermolecular cysteine bridges, respectively, thereby confirming that the specific enzymatic cleavage reaction was nearly complete after 2 h (Fig. 3). Monitoring the TEV cleavage reaction over 24 h indicated that there was no difference with other longer incubation times (2, 4, 8 and 24 h, data not shown). These results are in good agreement with previous reports indicating that protein cleavage by the TEV protease is highly specific and efficient. Furthermore, the use of the TEV protease is advantageous since its production in large quantities is easier than for other proteases, even at the laboratory scale (Esposito and Chatterjee 2006).

Following Kcoil peptide cleavage, it was necessary to remove the TRX protein from the mixture before a final

**Table 2** Yields of fusion protein and final purified Kcoil peptide

	Fusion protein yield	Theoretical peptide yield	Purified peptide	Step efficiency (%)
IMAC	21.89 <sup>a</sup>	9.05 <sup>a</sup>	ND	ND
IEX-chromatography	20.1 <sup>a</sup>	ND	1.55 <sup>b</sup>	91.86
RP-HPLC	ND	1.55 <sup>b</sup>	1.51 <sup>b</sup>	97.41

Yields are reported by mg of protein produced per liter of bacterial culture

ND not determined

<sup>a</sup> Yield determined by absorbance at 280 nm

<sup>b</sup> Yield determined by Orange II quantification as described in “Materials and methods” section

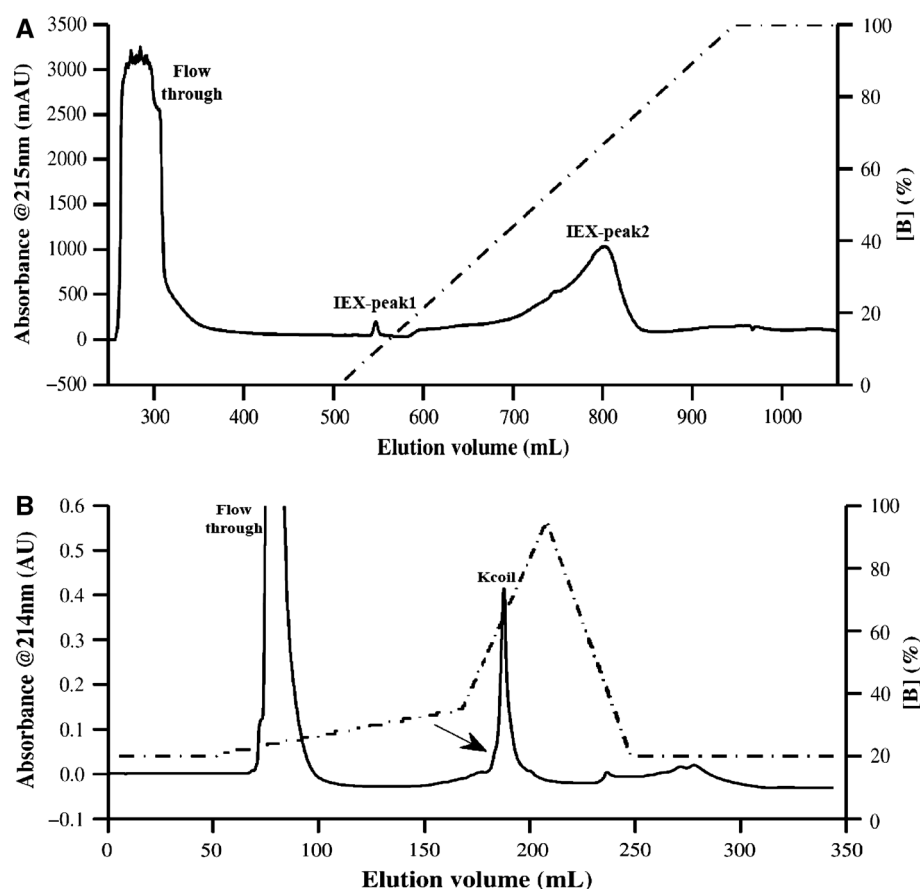
purification step by RP-HPLC (Fig. 2). For this purpose, the solution mixture resulting from cleavage was directly applied to a SP-Sepharose FF column and the flow through collected. This strategy was selected as we were unable to efficiently capture the (His)<sub>6</sub>-tagged thioredoxin alone after TEV cleavage, even after several treatments with denaturants (e.g., high concentration of urea and guanidine hydrochloride; data not shown) most likely due to either strong electrostatic interactions between the lysine residues of the Kcoil peptide and the anionic thioredoxin (Yang et al. 2009; McCoy and La Ville 2001) or the presence of a loop within the active site of thioredoxin, which may favour small peptide binding. Noteworthy, TRX protein carrying peptide of interest in this active-site loop was reported to retain its structure (LaVallie et al. 1993), which may explain the inefficacy of denaturant treatment to release the Kcoil peptide.

Collected fractions were analysed by both absorbance measurement at 280 nm (to detect thioredoxin) and SDS-PAGE (for thioredoxin and Kcoil detection) in order to verify the efficacy of this second chromatography step. In our strategy, the Kcoil was successfully eluted at approximately 40 % of the linear salt gradient (0–0.5 M NaCl) at a pH lower than its theoretical isoelectric point (pI = 9.2) (Fig. 4a) while the (His)<sub>6</sub>-thioredoxin protein was recovered in the flow through in agreement with its pI being equal to 4.67. These results were verified by SDS-PAGE (Fig. 3). While the IEX-peak1 fraction did not contain any detectable K coil peptide, for the second peak that had been eluted from the ion exchange chromatography, gel staining revealed a band corresponding to an apparent molecular weight between 5 and 10 kDa. Since non-reducing conditions were used during SDS-PAGE experiments, this band may correspond to the formation of cysteine-bound Kcoil dimers. Cation exchange chromatography enabled us to separate almost 92 % of the originally expressed (His)<sub>6</sub>-thioredoxin from Kcoil peptide (Table 2; Fig. 4a) using a very slow salt gradient (20 column volumes with 90 cm/h as a flow rate), and once again supporting the hypothesis that strong electrostatic

interactions between the positively charged Kcoil and the anionic residues of TRX protein resulted in a stable complex after TEV cleavage.

Based on our SDS-PAGE results, fractions corresponding to the second peak of the ion-exchange chromatography were pooled, concentrated, and subjected to a RP-HPLC purification step (Fig. 2). This final purification was very efficient (97.4 %), as 1.51 mg (i.e., 363 µmol) of pure Kcoil peptide per liter of bacterial culture was obtained as quantified by the Orange II primary amine detection method (Table 2). Fractions corresponding to the Kcoil peptide were eluted from the preparative C<sub>4</sub> column using a linear gradient of acetonitrile with 0.05 % of trifluoroacetic acid (Fig. 4b). The oxidized form of the peptide was eluted slightly before its reduced counterpart, as confirmed by mass spectrometry analysis. The absorbance profile at 215 nm is in agreement with the observation by Lee and colleagues related to the separation of the oxidized and reduced forms of a protein by RP-HPLC when using a hydrophobic column (Lee et al. 2000). Of interest, the volumetric yield reported by Le and colleagues (Le et al. 2009) for the bacterial production and purification of a Kcoil peptide fused to EGF was of 0.107 µmol/L. Their purification strategy was based on solubilization from inclusion bodies. In contrast, we here demonstrate that higher yields (3.6 µmol/L) may be obtained using a soluble carrier protein such as thioredoxin. Our yield is similar to those reported for antibacterial bioactive peptides that were produced using the same fusion protein and that displayed similar size as our Kcoil peptide ( $M_w$  = 2.9–3.7 kDa, e.g. Plantaricin E, F, J and K) (Pal and Srivastava 2014). Higher yields have been obtained for other anti-microbial peptides (3.2 mg to 11 mg/L of bacterial culture) using thioredoxin as a carrier protein (Xu et al. 2007; Li 2012; Jing et al. 2010; Shlyapnikov et al. 2008) suggesting that the hydrophobic nature of the Kcoil peptide may negatively impact its expression level. Others studies have also shown that prokaryotic production of auto-assembling amphiphilic peptides could attain

**Fig. 4** **a** Kcoil peptide and Thioredoxin fusion protein separation by cation exchange chromatography. After enzymatic digestion, the mixture was injected on a SP-Sepharose FF column using phosphate buffer (pH 7.4) as loading buffer and a 0–100 % linear gradient of NaCl (corresponding to 125-to-500 mM, *dashed line*) for elution. The nomenclature (IEX-peak1 and IEX-peak2) is the same as in Fig. 3. **b** Reverse-phase HPLC analysis of SP-Sepharose eluted fractions. Kcoil peptide was eluted from the C<sub>4</sub> column using a 20–35 % linear gradient (15 min) of buffer B (*intermittent line*) (acetonitrile with 0.05 % of TFA). Reduced Kcoil peptide was detected by absorbance at 214 nm (*continuous line*) at approximately 180 mL of eluted volume; the small oxidized fraction of Kcoil correspond to the shoulder (*arrow*) at the base of the Kcoil peak



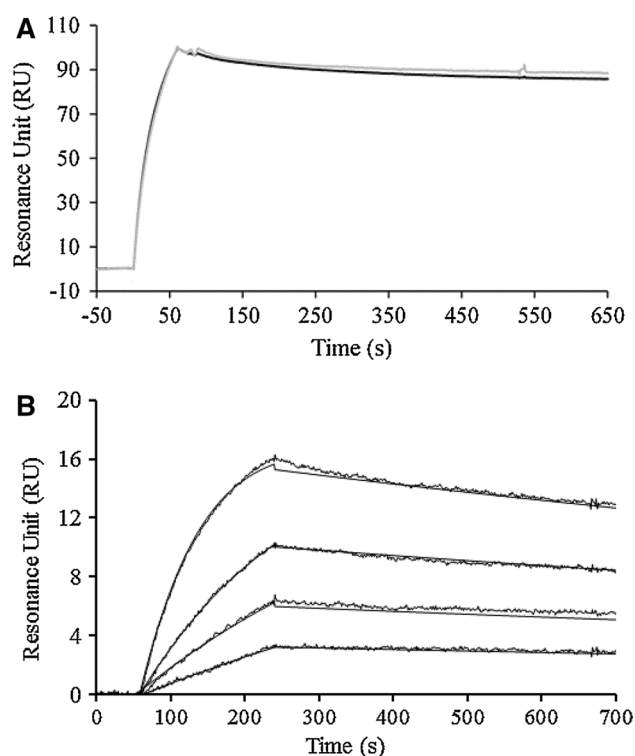
gram per liter quantities using an inclusion body strategy and/or using a tandem repeat strategy; which cannot be applied in our case due to the important positive charge that would result from a tandem repeat Kcoil peptide (Kyle et al. 2010; Riley et al. 2009; Hartmann et al. 2008, 2009; Prakash et al. 2012). Thus, considering all the challenges associated to the Kcoil peptide primary sequence—10 lysines out of 38 residues as well as a strong proportion of hydrophobic residues, the process we here report (Fig. 2) lead to a significant yield of highly pure Kcoil peptide.

A mass-spectrometry analysis (data not shown) of the eluted fractions from RP-HPLC was performed in order to confirm the identity of the biologically produced peptide via its molecular weight. The ESI-TOF analysis of the biological Kcoil showed an average mass of 4159.9 Da, in excellent agreement with the Kcoil theoretical mass of 4158.9 Da while the second significant RP-HPLC peak corresponded to a mass of 8316.2 Da, unambiguously indicating that this peak corresponded to the oxidized form of the Kcoil peptide (theoretical mass of 8315.8 Da). Both SDS-PAGE and mass spectrometry results confirmed that the Kcoil peptide was highly pure.

### Bioactivity assay on the BIACORE

In order to confirm the activity of the Kcoil peptide produced in bacteria, an SPR-based biosensor assay was performed according to a previously developed method (Murschel et al. 2013). SPR analysis was chosen to compare the kinetics of interactions of the biologically produced Kcoil peptide with its partner Ecoil peptide to those of a chemically synthesized Kcoil peptide. The ability of biologically produced Kcoil to be covalently immobilized to the dextran-coated surface via the thiol coupling procedure was first evaluated by monitoring the net mass accumulation on the surface (data not shown). The same assay was carried out using the chemically synthesised Kcoil as a positive control and gave similar results. As observed in Fig. 5a, the control corrected sensorgrams corresponding to the injection of the Ecoil-EGF fusion protein over both Kcoil surfaces gave almost superimposable curves. A more detailed SPR study was also performed by injecting increasing concentrations of Ecoil peptides on a surface displaying a low amount of Kcoil (<60 RU, see Fig. 5b). The corresponding sensorgrams were globally fit to a simple Langmuir model, from which a dissociation





**Fig. 5** **a** Control-corrected sensorgrams corresponding to the injection of 100 nM of E5-EGF over the same amount of either chemically-synthesized (gray) or bacteria-produced (black) Kcoil that had been immobilized at the biosensor surface. **b** Kinetic analysis of E/Kcoil interaction using a simple binding model. Various concentrations of Ecoil peptide, ranging from 0.5 to 5 nM, were injected over less than 60 RU of immobilized bacteria-produced Kcoil. Both experimental data and global fit corresponding to a simple Langmuirian model are shown

constant  $K_D$  of 137 pM was calculated. This value was close to that previously reported for Ecoil peptide binding to chemically produced Kcoil peptide ( $K_D = 63$  pM) (De Crescenzo et al. 2003b), given that different peptide quantification methods (Orange II assay, as described above, vs. amino acid analysis) and instruments were used in both studies. Altogether, the biologically produced Kcoil peptide presented the same kinetic behaviour as its chemically synthesized equivalent when binding to its Ecoil partner.

In conclusion, we here report a novel approach for the bacterial production and subsequent purification of a de novo designed Kcoil peptide as an attractive alternative to its chemical synthesis. Among all the fusion proteins we tested, TRX was the best carrier for the production of soluble Kcoil peptide and subsequent purification. Finally, the bioprocess we developed (Fig. 2) represents an easy and low cost approach that can expand the Ecoil/Kcoil heterodimer utilization to several applications in the biopharmaceutical and biomedical fields.

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**Compliance with ethical standards**

**Conflict of interest** None.

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