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Novel approach to obtain biologically active recombinant heterodimeric proteins in *Escherichia coli*

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Abstract

The strategy described in this paper provides a novel approach for recombinant expression of heterodimeric proteins, and is especially suitable for the production of proteins whose characteristics lead to aggregation in *E. coli* expression systems. Pheromaxein, a steroid-binding protein isolated from boar saliva, is a heterodimeric protein consisting of 10×10^3 rel. mol. mass units (pheromaxein A) and 8×10^3 rel. mol. mass units (pheromaxein C) subunits. Expression of pheromaxein subunits in *E. coli* resulted in extensive insoluble aggregation. The difficulty faced in obtaining soluble recombinant pheromaxein subunits was clearly evident when native pheromaxein immediately formed aggregates when it was separated into its individual subunits. An increase in soluble pheromaxein expression in *E. coli* was obtained when the subunits were expressed as fusion proteins with thioredoxin. Pheromaxein genes were inserted separately into pET32a+ vectors at the *Nco*I site, resulting in thioredoxin, S·Tag™ and His·Tag™ coding regions being upstream of the inserted gene. Soluble pheromaxein A–thioredoxin (pheroA/trx) and pheromaxein C–thioredoxin (pheroC/trx) fusions were purified to homogeneity, using a laboratory scale S-protein agarose affinity column. Cleavage of thioredoxin under normal conditions was not feasible due to the extensive aggregation problems experienced when pheromaxein subunits exist separately. PheroA/trx and pheroC/trx were therefore mixed together and cleaved from thioredoxin simultaneously so that pheromaxein subunits were given an instant opportunity to associate under oxido-shuffling conditions. The glutathione oxido-shuffling system allowed the disulphide bridges between pheromaxein A and C to rearrange until the correct native structure was formed. This novel approach combines affinity purification with a coupled fusion protein-cleavage and refolding technique.

Keywords: *Escherichia coli*; Recombinant heterodimeric protein

1. Introduction

The Gram-negative bacterium *Escherichia coli* is one of the most attractive systems for heterologous protein expression, because of its ability to grow rapidly, its well characterised genetics, and the availability of an increasingly large number of cloning vectors and mutant host strains. However, there is no guarantee of obtaining biologically active heterologous protein, as high levels of expression are often accompanied by misfolding and aggregation [1]. Expression in the cytoplasm using fusion partners can greatly increase the solubility of passenger proteins that may otherwise accumulate as insoluble aggregates in the cytoplasm. In this study, thioredoxin was used as the fusion partner; this protein can be expressed at high levels and remains extremely soluble in the *E. coli* cytoplasm [2].

When foreign proteins misfold or aggregate in *E.*

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coli, they usually become localised within inclusion bodies. The greatest disadvantage of this are the problems encountered during attempted solubilisation and refolding of the target protein. Refolded yields are usually low, and proteins often cannot be refolded at all [3]. This is most common with mammalian proteins which are, in most cases, multi-domain and/or disulphide-bonded proteins. Most of the literature on protein folding deals with small monomeric proteins; very few papers discussing refolding of heterodimeric, disulphide bonded proteins are available.

Traditionally, proteins refolded from inclusion bodies are solubilised in urea or a similar denaturant, which disrupts protein structure. The basic strategy involves reducing the concentration of denaturant, so that intramolecular interactions can occur, followed by folding in the presence of an oxidising agent. During the process, the protein concentration must be kept to a minimum to avoid intermolecular aggregation [4]. Several methods can be employed to remove or reduce excess denaturing agents, including dilution, dialysis, diafiltration, gel permeation chromatography, and immobilisation onto a solid support. However, there is very little data in the literature indicating how these methods could be applied when refolding heterodimeric proteins.

In the case of disulphide bonded proteins, renaturation buffers must promote the formation of these bonds, and is most commonly achieved by air oxidation, oxido-shuffling systems, use of mixed disulphides, or oxidation of sulphonated proteins [3]. Oxido-shuffling, as used in this study, is carried out using low-molecular mass thiols in reduced and oxidised forms, such as reduced glutathione (GSH) and oxidised glutathione (GSSG). These provide the appropriate redox potential for both the formation and reshuffling of disulphide bonds. Additionally, buffer additives can be employed to promote solubility and refolding; for example, L-arginine is thought to preferentially destabilise wrongly folded proteins [4], and increase solubility of refolding intermediates [5].

The vast array of potential agents and strategies available to obtain biologically active heterologous proteins must be evaluated on a ''trial and error'' basis for each individual protein. No single method is suitable for every protein, and there are few clear rules through which to rationally select the most effective procedure.

This paper describes the expression, purification and refolding of pheromaxein, a mammalian heterodimeric protein. Pheromaxein, a steroid-binding protein produced in the submaxillary saliva glands of the pig, consists of 10×10^3 rel. mol. mass units (pheromaxein A) and 8×10^3 rel. mol. mass units (pheromaxein C) non-glycosylated subunits (in preparation). The submaxillary glands of the boar secrete large amounts of odorous 16-androstene steroids, bound to pheromaxein, into the saliva; these steroids act as pheromones, causing oestrous females to take up the mating stance [6,7]. The lack of glycosylation of this protein made expression in *E. coli* a practical option. Two expression strategies are described here: (1) cloning of pheromaxein genes into separate pET14b-based vectors, followed by individual expression in the cytoplasm; (2) expression of the genes individually as fusions to thioredoxin, each from pET32a+-based vectors. Due to the complex nature of pheromaxein, alternative refolding techniques had to be carefully considered during production of the recombinant form. The theory behind the refolding procedure developed during this study was that, when pheromaxein A–thioredoxin (pheroA/trx) and pheromaxein C–thioredoxin (pheroC/trx) fusions are mixed together and cleaved from thioredoxin in the same reaction, and in the presence of oxido-shuffling conditions, the disulphide bridges between the cleaved A and C subunits would rearrange until the correct native structure is formed. It was envisaged that this could occur directly after, or even during, cleavage from the thioredoxin regions.

2. Experimental

2.1. General

All general laboratory chemicals were obtained from Sigma UK (Dorset, UK), with the exception of 4,16-androstadien-3-one, which was sourced from Steraloids (Rhode Island, USA). All chromatography media were obtained from Amersham Biosciences (Uppsala, Sweden), and packed in Amersham Biosciences columns, with the exception of S-protein

agarose™, which was manufactured to order from Novagen (Madison, WI, USA). All chromatography procedures were carried out using an Amersham Biosciences AKTA purifier or Amersham Biosciences FPLC at room temperature. Oligonucleotides were obtained from Sigma UK. All restriction enzymes, DNA modifying enzymes, PCR enzymes and buffer components were obtained from Invitrogen (Paisley, UK). EnterokinaseMax™, used for cleavage of thioredoxin fusion proteins, was also sourced from Invitrogen.

2.2. Bacterial strains

Escherichia coli JM109 ($e14^-$(*McrA*$^-$) *recA*1*endA*1*gyrA*96*thi*-1*hsdR*17 (r_K^- m_K^+) *supE*44 *relA*1 Δ(*lac-proAB*) [*F′ traD*36 *proAB lacI*^{q}*ZDM*15]) was used as a host for vector construction experiments (Stratagene). *Epicurian Coli*® BL21-Codon plus™(DE3)-RIL (*B F*$^-$*ompThsdS*(r_B^- m_B^-) *dcm*$^+$ *Tet*^{r}*gal* λ(*DE*3) *endA*. The [*argU ileY leuW Cam*^{r}]) was used for expression of pheromaxein genes (Stratagene, Amsterdam, The Netherlands).

2.3. Electrophoresis and immunodetection of pheromaxein subunits

Protein samples were analysed on Invitrogen NuPAGE SDS–PAGE gels (10% Bis–Tris Gels with MES running buffer), and either stained with Colloidal blue or subjected to Western blotting, as described in the Invitrogen Precast gel instructions. Blotting was carried out at 30 V for 50 min. A combination of SDS–PAGE and Western blotting was required to enable visualisation of both pheromaxein subunits. Recombinant pheromaxein C did not blot well, but was visible below *E. coli* proteins using SDS–PAGE, whereas pheromaxein A ran with *E. coli* proteins, so required to be visualised by immuno-blotting, which was carried out as follows. Blots were blocked with 1% (w/v) Marvel–PBSTA for 1 h at room temperature, followed by sequential incubations of anti-pheromaxein llama serum (1/10 000 dilution; obtained in-house), rabbit anti-llama polyclonal serum (1/1000 dilution), and Zymed anti-rabbit/alkaline phosphatase (1/1000 dilution) each for 1 h at room temperature. Blots were finally incubated in BCIP–NBT (0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP)–0.3 mg/ml nitro blue tetrazolium (NBT) in 100 m*M* Tris buffer, pH 9.5, + 5 m*M* $MgCl_2$) until the colour was fully developed.

2.4. Construction of expression vectors

2.4.1. Subcloning of phero A and phero C into pET14b

The aim of cloning into pET14b was to express pheromaxein A and C genes separately into the *E. coli* cytoplasm without signal sequences or fusion partners. *Phero A* and *phero C* were cloned separately into pET14b to create pET14pheroA and pET14pheroC plasmids, respectively. To amplify *phero A*, PCR was carried out using the template pPIC9pheroA (in preparation), with the following primers: forward primer pheroANco (5′-TGCATGCCATGGCCATTGTCTGTCGGGCCCT-TGTTAAGG-3′), containing a restriction site for *Nco*I (single underlined); reverse primer, pheroABam (5′-ACGTACGGATCCTTATTAT-TTAAACACGCTTGCACTGAGCCCC-3′), containing a *Bam*HI restriction site (single underlined) and two stop codons marking the end of the *phero A* sequence (double underlined). pET14b (1 μg) and the purified PCR product were restricted, by double digestion with *Nco*I and *Bam*HI, and ligated to prepare pET14pheroA. The sequence of the resulting vector construct was verified by DNA sequencing.

Phero C was cloned into pET14b using the same strategy as described above with the following exceptions. The template pPIC9pheroC (in preparation) was used with the following primers to amplify *phero C*: forward primer pheroCNco (5′-TGCATGCCATGGCCGGTTCTGGCTGTTCCTA-TCTTGAGAG-3′), containing a *Nco*I restriction site (single underlined); reverse primer PheroCBam (5′-ACGTACGGATTCTTATTAAAACGCAGCACAC-AGTTTGCT-3′), containing a *Bam*HI restriction site (single underlined) and two stop codons (double underlined), marking the end of the *phero C* sequence. The resultant vector construct was named pET14pheroC, and the sequence was verified by DNA sequencing.

2.4.2. Subcloning of phero A and phero C into pET32a +

The aim of cloning into pET32a+ was to construct plasmids containing each of the pheromaxein genes downstream and in-frame with the thioredoxin gene. This would allow pheromaxein subunits to be expressed as a fusion to thioredoxin, and localised in the *E. coli* cytoplasm. *Phero A* and *phero C* were cloned separately into pET32a+ to create pET32pheroA and pET32pheroC, respectively. The cloning strategy was identical to that used for cloning into pET14b (Section 2.4.1).

2.5. Production of recombinant pheromaxein

The appropriate vector constructs, along with control vectors, were used to transform *E. coli* BL21(DE3) RIL codon plus. An overnight starter culture was used to inoculate 40 ml LBamp (10 g/l NaCl; 10 g/l tryptone; 0.5 g/l yeast extract; 50 μg/ml ampicillin) to O.D. (A_{600}) 0.05, and incubated at 37 °C, 180 rpm until the O.D. (A_{600}) reached 0.6–0.8. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added, to a final concentration of 1 m*M* for pET32a+-based vectors, and 0.4 m*M* for pET14b-based vectors, to start induction. The incubation was continued at 37, 25 or 18 °C, and samples removed at various time points up to 24 h. Cell pellets were boiled for 5 min in 1× reducing Laemmli sample buffer [8], prior to analysis by SDS–PAGE and Western blotting. Further cellular fractions were prepared as described in Section 2.6; these were also analysed by SDS–PAGE and Western blotting.

2.6. Extraction of recombinant pheromaxein from E. coli

2.6.1. Extraction of soluble pheromaxein from the cytoplasm by cell lysis

Cell paste (10 ml original culture volume) was resuspended in 1 ml lysis buffer (20 m*M* Tris–HCl, pH 7.5; 0.15 *M* NaCl; 0.1%, w/v, Triton X-100; 100 μg/ml lysozyme) and incubated for 15 min at 30 °C. Dithiothreitol (DTT) and phenylmethylsulphonyl fluoride (PMSF) were added to final concentrations of 10 and 1 m*M*, respectively, followed by sonication on ice, using a Bandelin Sonopuls HD200, at 40% power for 8×15 s, with 15 s between bursts. The cell debris was pelleted by centrifugation at 20 000 *g* for 20 min. Both the pellet and supernatant were analysed by SDS–PAGE and Western blotting. Alternatively, the pellet was used for inclusion body extraction (Section 2.6.2).

2.6.2. Inclusion body and insoluble protein extraction

The pellet obtained in Section 2.6.1 was solubilised in solubilisation buffer (20 m*M* Tris–HCl, pH 7.9; 10 m*M* DTT; 6 *M* urea,) by incubation at room temperature for 1 h with constant agitation. Insoluble material was pelleted by centrifugation at 22 000 *g* for 15 min. The remaining insoluble pellet was boiled for 5 min in 1× Laemmli sample buffer, and both the pellet and supernatant were analysed by SDS–PAGE and Western blotting.

2.7. Assessment of aggregation by gel permeation chromatography

Gel permeation chromatography was used to determine aggregation levels of recombinant proteins expressed in *E. coli* by their relative elution times. Both native and recombinant pheromaxein were investigated for aggregation levels by gel permeation chromatography under various conditions (Table 1). When native pheromaxein was run under reducing and/or denaturing conditions, the appropriate amount of urea and/or DTT was added to pheromaxein in solution, then left at room temperature for 30 min to allow disulphide bond reduction. The relevant pheromaxein sample (1–2 ml) was loaded onto a Superdex 75 (16/60) column, which was run at 1

Table 1
Buffer conditions used for gel permeation chromatography to investigate aggregation levels of recombinant pheromaxein

Conditions	Buffer content
Non-denaturing, non-reducing	20 m*M* Tris–HCl, pH 7.4; 0.5 *M* NaCl
Denaturing (urea), non-reducing	6 *M* urea
Denaturing (urea), reducing	20 m*M* Tris–HCl, pH 7.9; 6 *M* urea; 10 m*M* DTT
Denaturing (urea/SDS), reducing	20 m*M* Tris–HCl, pH 7.9; 6 *M* urea; 10 m*M* DTT; 2.5% (w/v) SDS

ml/min with the appropriate buffer. Fractions (2 ml) were collected and analysed by SDS–PAGE and Western blotting to determine where pheromaxein eluted.

2.8. Small scale evaluation of recombinant pheromaxein–thioredoxin fusion purification

Heterologous proteins, expressed from pET32a+-based vectors, have a region downstream of the thioredoxin peptide named the S·Tag™; this allows detection and purification using the Novagen S·Tag system. The system exploits the high affinity binding of S-protein (residues 21–124) to S-peptide (residues 1–20) where, in nature, enzymatically active ribonuclease S (RNase S) is formed. In recombinant protein expression, S-protein agarose™ is used to purify proteins containing the S·Tag, which is a 15-meric truncated form of the S-peptide [9]. Purification of soluble thioredoxin fusion proteins from *E. coli* lysate (30 ml) was carried out using 2 ml S-protein agarose. Binding and elution, using 0.2 *M* Na citrate at pH 2.0, was carried out as described in the manufacturer's instructions.

2.9. Small scale evaluation of pheromaxein refolding during cleavage from thioredoxin fusion

As pET32a+-based vectors encode an enterokinase cleavage site between the S·Tag and the heterologous protein, cleavage with enterokinase is required to release the target protein from the thioredoxin peptide. Thioredoxin fusions were purified as described in Section 2.8. Following buffer exchange into cleavage buffer (50 m*M* Tris–HCl, pH 8.0; 1 m*M* CaCl; 0.1%, w/v, Tween 20), thioredoxin was cleaved from each pheromaxein subunit using EKmax. EKmax (8 u) was incubated with an equal mixture of pheroA/trx and pheroC/trx (approximately 25 μg total protein) in 250 μl at 25 °C for 20 h. This reaction was carried out under various buffer conditions (Table 2) in an attempt to obtain correct folding of the cleaved pheromaxein subunits. Reaction products were analysed by SDS–PAGE and Western blotting under reducing and non-reducing conditions, to enable detection of the correctly folded dimer.

2.10. Purification and refolding scale-up prior to biological activity testing

2.10.1. Purification and refolding scale-up

E. coli lysates (50 ml), from separate cultures (380 ml) each expressing a pheromaxein subunit fusion, were obtained as described in Section 2.6.1. Each pheromaxein–thioredoxin fusion was processed separately, as described below. A diagrammatic representation of the process is shown in Fig. 1.

Fusion purifications were carried out using APB's AKTA purifier. S-protein agarose (100 ml bed volume) was equilibrated with several volumes of wash/bind buffer (20 m*M* Tris–HCl, pH 7.5; 0.15 *M* NaCl; 0.1%, w/v, Triton X-100) prior to injection of 50 ml lysate at 0.25 ml/min. After washing with 3 column volumes of wash/bind buffer at 0.25 ml/min, the fusion proteins were eluted with 0.2 *M* citrate, pH 2.0, at 0.75 ml/min. Immediately following elution, the fusion protein solution was adjusted

Table 2
Reaction conditions used during dual cleavage of recombinant thioredoxin–pheromaxein A and C fusions

Conditions	Buffer content
Cleavage buffer	50 m*M* Tris–HCl, pH 8.0; 1 m*M* $CaCl_2$; 0.1% (w/v) Tween 20
Cleavage buffer + arginine	Cleavage buffer + 50 m*M* arginine
Cleavage buffer + GSSG–GSH (1:10)	Cleavage buffer + 0.3 m*M* GSSG; 3 m*M* GSH
Cleavage buffer + GSSG–GSH (1:5)	Cleavage buffer + 0.2 m*M* GSSG; 1 m*M* GSH
Cleavage buffer + arginine + GSSG–GSH (1:10)	Cleavage buffer + 50 m*M* arginine; 0.3 m*M* GSSG; 3 m*M* GSH
Cleavage buffer + deoxycholate	Cleavage buffer + 0.15% (w/v) deoxycholate

GSSG, oxidised glutathione; GSH, reduced glutathione.

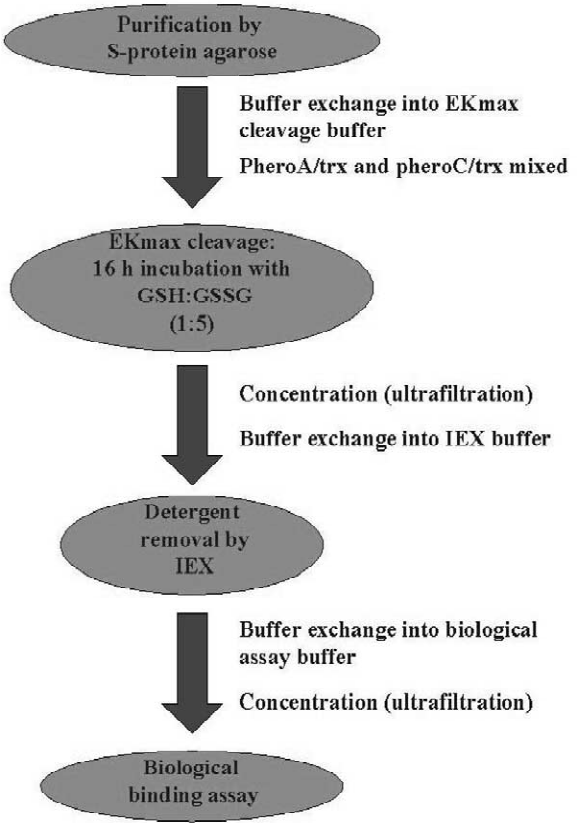

Fig. 1. Flow diagram representing the scale-up process for obtaining refolded recombinant pheromaxein. Key: EKmax, enterokinase; pheroA/trx, pheromaxein A–thioredoxin fusion; pheroC/trx, pheromaxein C–thioredoxin fusion; GSH, reduced glutathione; GSSG, oxidised glutathione; IEX, ion-exchange chromatography.

to pH 7.0 with saturated Tris base. The S-protein agarose column was regenerated with three column volumes of elution buffer, followed by three column volumes of wash/bind buffer. Buffer exchange to EKMax cleavage buffer was carried out using a G25 column (126 ml bed volume) pre-equilibrated in cleavage buffer. The eluent from S-protein agarose (25 ml) was buffer exchanged on a G25 column (126 ml bed volume, pre-equilibrated with cleavage buffer) at 2.5 ml/min. PheroA/trx and pheroC/trx fusions were mixed, in equal amounts, and made up to 100 ml final volume with cleavage buffer. After GSSG and GSH were added to give final concentrations of 0.2 and 1 m*M*, respectively (1:5 ratio) and EKMax (250 U) was added, the reaction mixture was incubated at 22 °C overnight. The reaction mixture was then concentrated to 5 ml using a Millipore Ultrafree 15 ml, 5×10^3 rel. mol. mass units cut-off membrane, and buffer exchanged into 20 m*M* Tris–HCl, pH 7.4, using a PD10 column. Triton X-100 and Tween 20 were removed by ion-exchange chromatography. Q-Sepharose (5 ml Hi-Trap) was pre-equilibrated with 20 m*M* Tris–HCl, pH 7.5, and loaded with 3.5 ml eluent from the previous PD-10 buffer exchange at 1 ml/min. Elution was achieved with 20 m*M* Tris–HCl (pH 7.4) + 0.5 *M* NaCl, at 2.5 ml/min. Following elution, the sample was buffer exchanged into PBS using PD10 columns, resulting in a final volume of 7 ml. Biorad protein assays, SDS–PAGE and Western blot analysis were carried out at each stage of the procedure to monitor progress.

2.10.2. Evaluation of biological activity

Recombinant pheromaxein was assessed for its ability to bind 4,16-androstadienone using a steroid-binding assay based on separation of free from bound ligand by ultrafiltration. 4,16-Androstadienone (2.5 μg) was added to the sample (7 ml) and controls (7 ml), and incubated with agitation for 2 h at room temperature. The samples were passed through a Millipore Ultrafree 5×10^3 rel. mol. mass units cut off membrane, spinning at 5000 *g* for 15 min, after which 20 μl remained. The retentate was resuspended with 500 μl PBS, the filtrate discarded, and the tube spun at 5000 *g* for a further 15 min. The wash step was repeated once more and the resultant retentate resuspended in PBS to a final volume of 1 ml. Steroids present in the retentate (i.e., bound to protein) were analysed as described in Section 2.10.3.

2.10.3. Extraction and analysis of 4,16-androstadienone associated with protein fractions from the steroid binding assay

Bound steroids were extracted from protein fractions (1 ml) by chloroform–methanol extractions. Internal standard (10 μg testosterone) was added, followed by extraction with 4 ml chloroform–methanol (2:1). After the lower layer was removed, 2 ml Folch lower [10] (chloroform–methanol–water, 86:14:1) was used for the second extraction. The

Table 3
GC–MS conditions for determination of protein-bound steroids in the 4,16-androstadienone binding assay

Instrument	Hewlett-Packard 5972 MSD Bench top GC–MS
Column	HP-5 MS (Hewlett-Packard) 30 m × 0.32 mm (I.D.) × 0.25 μm film thickness
Temperature program	80–200 °C at 10 °C/min (injector at 280 °C) 200–300 °C at 20 °C/min 300 °C for 1 min
Injector temperature	280 °C
Detector temperature	300 °C
Injection volume	1 μl, autoinjector
Carrier gas: splitless mode	He (1 ml/min, 36.8 cm/s)
Mass spectroscopy	HP MSD with HP ChemStation data analysis. EI+ ionisation, EM voltage 1694 mV, scanning m/z 40–550.

lower layers were pooled and blown to dryness under nitrogen at 60 °C. The extracted steroid was re-suspended in heptane and analysed by GC–MS under the conditions outlined in Table 3. Peaks were identified by comparison to mass spectra of steroid standards, and quantified by comparison of peak areas with the internal standard.

3. Results

3.1. Expression of pheromaxein genes into E. coli cytoplasm

Each pheromaxein subunit was cloned into pET14b, in-frame with the vector-based start methionine, and downstream from the T7 promoter. As the *Nco*I restriction site was used to clone downstream from the start methionine, coding for two extra amino acids was present on the 5′ end of both pheromaxein gene sequences. Methionine was required to start translation, whereas alanine was a result of the *Nco*I restriction recognition sequence.

3.1.1. Expression of pheromaxein A gene from a pET14b-based vector

When *E. coli* cultures, transformed with pET14pheroA, were induced with either 0.1 or 0.4 m*M* IPTG at 37, 25 or 18 °C, pheromaxein A was expressed at all time points between 1 and 21 h; however, the majority of pheromaxein A was insoluble (Fig. 2). The band stained by anti-pheromaxein antibodies on the Western blot was a higher molecular mass than the native pheromaxein A, which is probably due to the extra amino acids on the N terminal of the protein. It is evident from the SDS–PAGE analysis that many host proteins were extracted with pheromaxein A during inclusion body extractions.

3.1.2. Expression of pheromaxein C gene from a pET14b-based vector

When *E. coli* cultures, transformed with pET14pheroC, were induced for expression at 37, 25 or 18 °C with either 0.1 or 0.4 m*M* IPTG, pheromaxein C was expressed at significant levels. Pheromaxein C was insoluble when expressed at 37 °C, but at 25 and 18 °C, low levels of soluble pheromaxein C were evident (Fig. 3). Surprisingly, the total yield of pheromaxein C increased when expressed at 18 °C with 0.4 m*M* IPTG. As seen for pheromaxein A, when expressed from a pET14b-based vector, many *E. coli* proteins contaminate pheromaxein C in inclusion body extractions.

3.1.3. Aggregation of recombinant pheromaxein A and C, isolated from inclusion body fractions, as determined by gel permeation chromatography

The native form of pheromaxein was initially subjected to gel permeation chromatography under denaturing or reducing conditions to enable comparisons to be made with recombinant forms (illustrated graphically in Fig. 4). Native pheromaxein A and C subunits eluted in the predicted order under

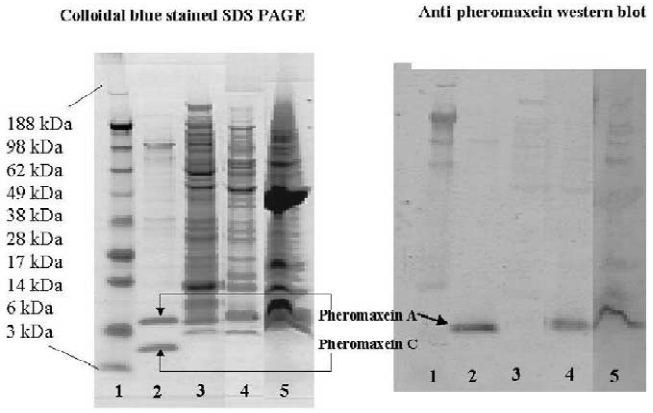

Fig. 2. SDS–PAGE and Western blot analysis of *E. coli*, transformed with pET14pheroA, cellular fractions. SDS–PAGE: reduced samples run on 10% NuPAGE MES gel (Invitrogen) and stained with colloidal blue. Western blot: reduced samples run on 10% NuPAGE MES gel (Invitrogen), followed by blotting onto PVDF and Western staining. Extractions carried out on *E. coli* cultures, induced at 37 °C with 0.4 m*M* IPTG after 1 h. Lanes: (1) molecular mass marker (Invitrogen); (2) native pheromaxein; (3) lysate from *E. coli* transformed with pET14pheroA; (4) inclusion body extraction from *E. coli* transformed with pET14pheroA; (5) remaining proteins after extraction from *E. coli* transformed with pET14pheroA.

denaturing and reducing conditions with SDS present. However, the subunits eluted in the opposite order when SDS was absent from the running conditions, suggestive of aggregation. Additionally, when the pheromaxein heterodimer was split into its components by DTT, there was immediate precipitation unless urea and/or SDS was present.

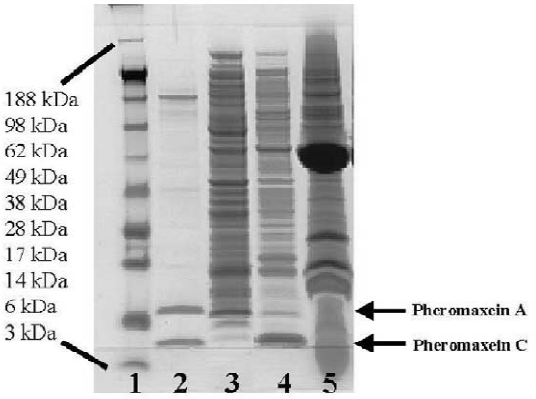

Fig. 3. SDS–PAGE analysis of *E. coli*, transformed with pET14pheroC, cellular fractions. Reduced samples run on 10% NuPAGE MES gel (Invitrogen) and stained with colloidal blue. Extractions carried out on *E. coli* cultures, induced at 18 °C with 0.4 m*M* IPTG, after 21 h. Lanes: (1) molecular mass marker (Invitrogen); (2) native pheromaxein; (3) lysate from *E. coli* transformed with pET14pheroC; (4) inclusion body extraction from *E. coli* transformed with pET14pheroC; (5) remaining proteins after extraction from *E. coli* transformed with pET14pheroC.

Cultures induced at 37 °C with 0.4 m*M* IPTG for 1 h were extracted for inclusion bodies to isolate pheromaxein A, and cultures induced at 18 °C with 0.4 m*M* IPTG for 21 h were extracted for inclusion bodies to obtain pheromaxein C, prior to assessment by gel permeation chromatography. Under denaturing, non-reducing conditions (6 *M* urea), recombinant pheromaxein C eluted in the column void volume, along with the other proteins present in the inclusion body fraction. When gel permeation chromatography was carried out under denaturing and reducing conditions (6 *M* urea and 10 m*M* DTT), recombinant pheromaxein C eluted at the same time as the aggregated native subunit, suggesting that there were still interactions occurring. However, when SDS was present, with denaturing and reducing conditions, recombinant pheromaxein C eluted at the same time as the corresponding non-aggregated native subunit. Interestingly, recombinant pheromaxein A was not detected during gel permeation chromatography experiments when SDS was absent from the running buffer, suggesting that aggregates were not entering the gel permeation chromatography matrix, but remaining at the top of the column. However, when SDS was present in the gel permeation chromatography running buffer, recombinant

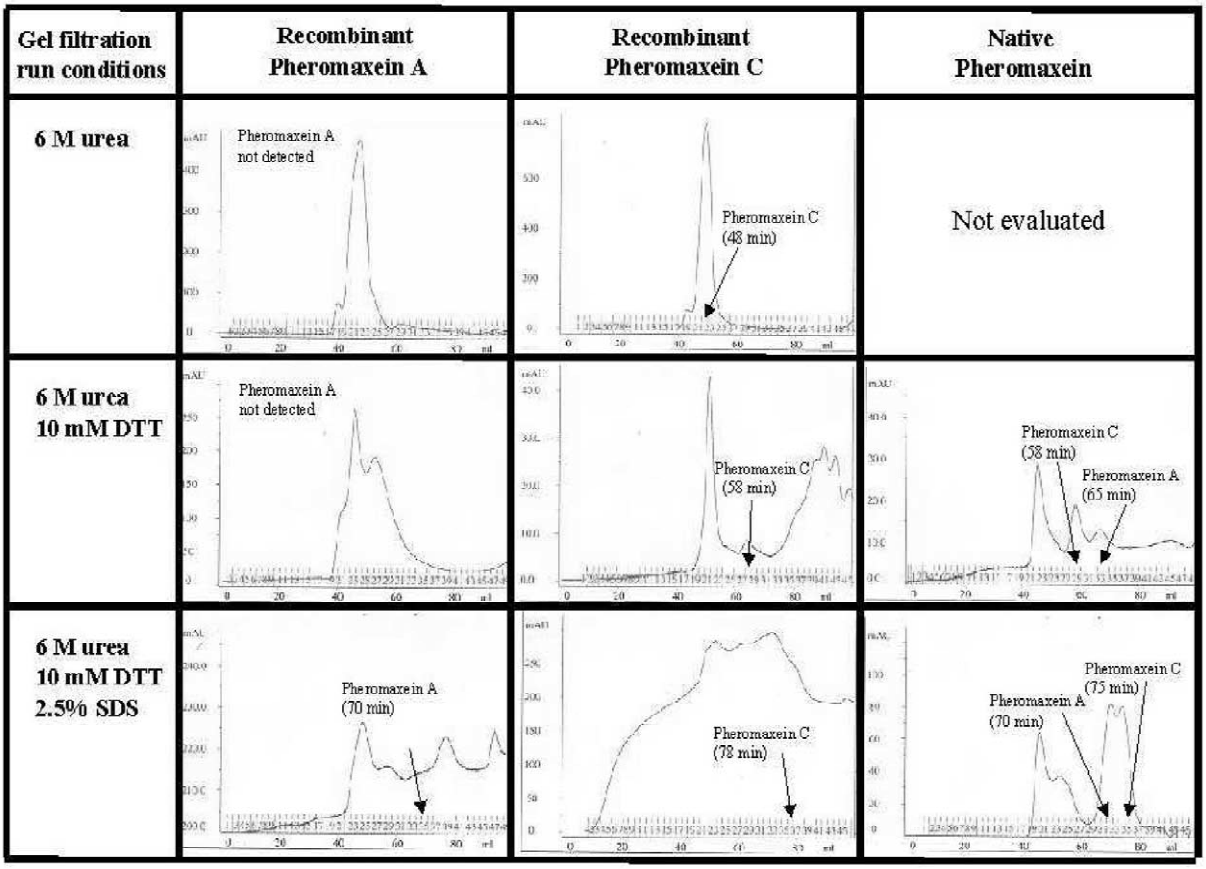

Fig. 4. Gel permeation chromatography of pheromaxein A and C subunits under various buffer conditions. Table illustrating gel permeation chromatography chromatographs of both recombinant and native pheromaxein A and C subunits under various buffer conditions. Samples (1–2 ml) were loaded onto a Superdex 75 (16/60) column, which was run at 1 ml/min with the appropriate buffer. Fractions (2 ml) were collected and analysed by SDS–PAGE and Western blotting to determine where pheromaxein eluted. Reference molecular mass values could not be determined for samples run under reducing or denaturing conditions; therefore, elution time was compared between samples to determine aggregation levels. Elution times were determined by SDS–PAGE analysis of each fraction and are indicated by an arrow.

pheromaxein A eluted at the same time as the corresponding non-aggregated native subunit (illustrated graphically in Fig. 4).

3.2. Expression of pheromaxein genes in E. coli as fusion proteins with thioredoxin

Pheromaxein genes were inserted downstream from the T7 promoter, and in-frame with the thioredoxin gene; the correct amino acid sequence for enterokinase cleavage was therefore retained. To clone pheromaxein gene sequences adjacent to the enterokinase recognition site, insertion at the *Nco*I restriction site was required. This resulted in vector-based nucleotides being downstream from the enterokinase cleavage site. After expression and subsequent cleavage by enterokinase, correctly processed pheromaxein would have three extra amino acids on the N-terminus, namely alanine, methionine and alanine.

3.2.1. Expression of pheromaxein A and pheromaxein C genes from a pET32a+-based vector

High expression levels of pheroA/trx, pheroC/trx and thioredoxin control were obtained when *E. coli*,

transformed with pET32pheroA, pET32pheroC and pET32a+, respectively, were induced at 37 °C with 1 m*M* IPTG. In each case, expressed foreign protein was detected from 1 h after induction. Both pheroA/trx and pheroC/trx were present in the lysate of samples taken 3 h after induction at 37 °C with 1 m*M* IPTG but, in each case, the majority remained in the insoluble fraction.

When expression was induced at lower temperatures, the best yield of both the pheroA/trx and pheroC/trx fusions was obtained at 25 °C. Although both fusion proteins were extracted mainly from the inclusion body fraction, appreciable levels were also present in the soluble lysate fraction. Examples of SDS–PAGE and Western blot analyses of the fractionation of pheroA/trx, pheroC/trx and thioredoxin control, after expression at 25 °C, are shown in Fig. 5. It is interesting to note the relative purity of the inclusion body extraction in comparison to those obtained from cultures transformed with pET14-based vectors.

3.2.2. Assessment of aggregation of pheroA/trx, pheroC/trx and thioredoxin control by gel permeation chromatography

PheroA/trx and pheroC/trx fusions, isolated from inclusion body extractions after expression at 25 °C for 3 h with 1 m*M* IPTG, both eluted at the column void volume when analysed by gel permeation chromatography. This suggests that both protein fusions were highly aggregated, so analysis of this fraction was not pursued any further. Elution times of pheroA/trx and pheroC/trx, isolated from the cell lysate after expression at 25 °C for 3 h with 1 m*M* IPTG, indicate that they are each approximately three times the predicted molecular mass, either suggesting the presence of trimers or, more likely, that the fusions are unfolded and hence elute earlier. A detailed account of these gel permeation chromatography results is shown in Table 4.

3.3. Generation of biologically active recombinant pheromaxein

3.3.1. Small scale evaluation of purification, cleavage and refolding of pheromaxein A and C

Good recoveries of pheroA/trx and pheroC/trx fusions (150 μg), and thioredoxin control (150 μg) were obtained after purification by S-protein agarose (see Fig. 6 for SDS–PAGE analysis). Cleavage of the thioredoxin fusion by EKmax was also efficient for both pheroA/trx and pheroC/trx, as well as the thioredoxin control, under all reaction conditions evaluated. Analysis of the cleavage reactions by SDS–PAGE and Western blotting under non-reducing conditions revealed the formation of a band, in the region of the native pheromaxein dimer, in several lanes (Fig. 7). This band was not detected in

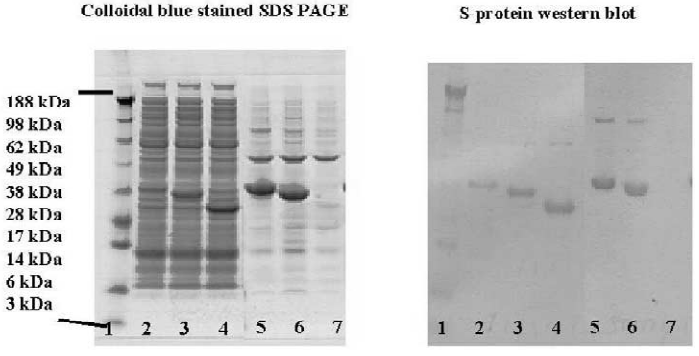

Fig. 5. SDS–PAGE and Western blot analysis of *E. coli*, transformed with pET32pheroA, pET32pheroC and pET32a+ (control), cellular fractions. SDS–PAGE: reduced samples run on 10% NuPAGE MES gel (Invitrogen) and stained with colloidal blue. Western blot: reduced samples run on 10% NuPAGE MES gel (Invitrogen), followed by blotting onto PVDF and Western staining. Extractions carried out on *E. coli* cultures, induced at 25 °C with 1 m*M* IPTG, after 3 h. Lanes: (1) molecular mass marker (Invitrogen); (2) lysate from *E. coli* transformed with pET32pheroA; (3) lysate from *E. coli* transformed with pET32pheroC; (4) lysate from *E. coli* transformed with pET32a+ control; (5) inclusion body extraction from *E. coli* transformed with pET32pheroA; (6) inclusion body extraction from *E. coli* transformed with pET32pheroC; (7) inclusion body extraction from *E. coli* transformed with pET32a+.

Table 4
Table summarising gel permeation chromatography elution times of recombinant pheromaxein A and C fusions under various buffer conditions

Extract fraction	Gel permeation chromatography buffer conditions	Fusion protein	Predicted molecular mass	Elution time (min)	Measured molecular mass	Predicted aggregation level
Inclusion body	20 m*M* Tris–HCl 0.15 *M* NaCl	PheroA/Trx	27.3×10^3	48	Not calculated	(High)
	0.1% (w/v) Triton X-100 6 *M* urea, pH 7.5	PheroC/Trx	26.6×10^3	48	Not calculated	(High)
Lysate	20 m*M* Tris–HCl 0.15 *M* NaCl	PheroA/Trx	27.3×10^3	50	79.4×10^3	Trimer or unfolded monomer
	0.1% (w/v) Triton X-100, pH 7.5	PheroC/Trx	26.6×10^3	52	69.2×10^3	Trimer or unfolded monomer
		Trx control	20.3×10^3	70 min	22.9×10^3	Monomer

Predicted molecular mass values were calculated from the open reading frames of pET32pheroA for pheromaxein A fusion, pET32pheroC for pheromaxein C fusion, and pET32a+ for thioredoxin control. Measured molecular mass values were determined by comparison of gel permeation chromatography elution times to a calibration curve obtained as described in the manufacturer's instructions.

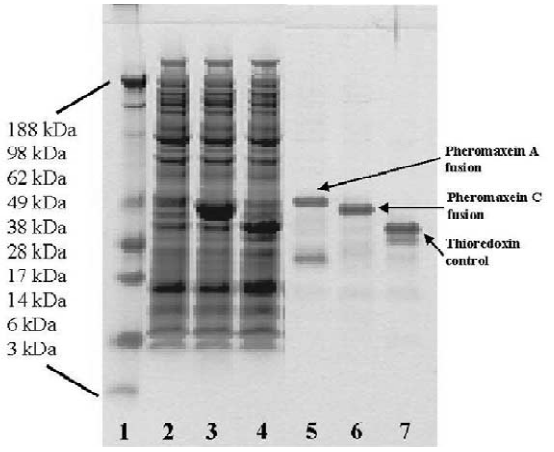

Fig. 6. SDS–PAGE analysis of S-protein chromatography fractions from pheromaxein A fusion, pheromaxein C fusion, and thioredoxin control purifications. Reduced samples run on 10% NuPAGE MES gel (Invitrogen) and stained with colloidal blue. Purification on S-protein agarose and elution with 0.2 *M* Na citrate, pH 2.0. Lanes: (1) molecular mass marker (Invitrogen); (2) lysate from *E. coli* transformed with pET32pheroA; (3) lysate from *E. coli* transformed with pET32pheroC; (4) lysate from *E. coli* transformed with pET32a+ control; (5) S-protein agarose eluent, pheromaxein A fusion; (6) S-protein agarose eluent, pheromaxein C fusion; (7) S-protein agarose eluent, thioredoxin control.

any of the thioredoxin control reactions, or when samples were analysed by SDS–PAGE and Western blotting under reducing conditions (results not shown). It was noted that the suspected recombinant pheromaxein heterodimer ran higher on the gel than the native dimer; this can probably be explained by the three extra amino acids present on each of the recombinant subunits, a total of six extra amino acids per dimer. The formation of dimers only occurred in reactions containing varying ratios of GSSG and GSH. The presence of 50 m*M* arginine did not affect formation of dimers in reactions containing GSSG and GSH, but there appeared to be an increased level of aggregated material.

3.3.2. Purification and refolding scale-up

The scaled-up lysis process yielded higher levels of thioredoxin fusion proteins (4.4 mg pheroA/trx and 6.4 mg pheroC/trx) than those obtained during evaluation, although there were low levels of contaminants present. Analysis of samples after cleavage, under non-reducing conditions, revealed the presence of a band corresponding to the formation of pheromaxein dimer, suggesting that correct folding had occurred. SDS–PAGE analysis under reducing conditions demonstrated the presence of both subunits. Unfortunately, the final protein amount

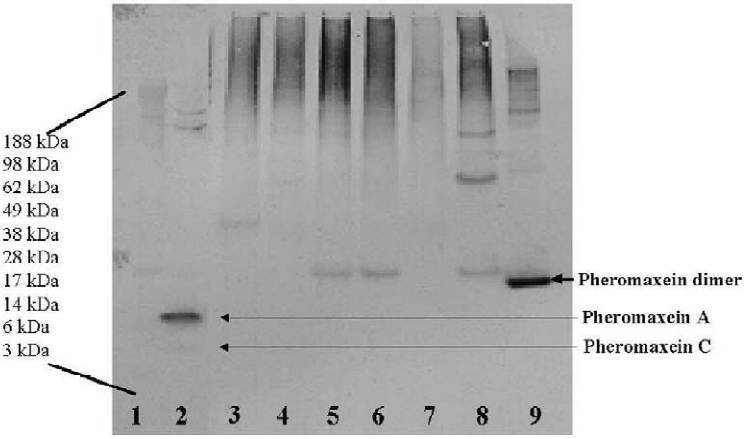

Fig. 7. Western blot analysis of enterokinase cleavage reactions of pheromaxein A fusion, pheromaxein C fusion, and thioredoxin control. Samples run on 10% NuPAGE MES gel (Invitrogen), followed by blotting onto PVDF and Western staining. EKmax (8 u) was incubated with an equal mixture of pheroA/trx and pheroC/trx (approximately 25 μg total protein) in 250 μl at 25 °C for 20 h. This reaction was carried out under various buffer conditions (see Table 2) to obtain correctly folded pheromaxein. All samples were non-reduced unless otherwise stated. Lanes: (1) molecular mass marker (Invitrogen); (2) reduced native pheromaxein; (3) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer; (4) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer + arginine; (5) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer + GSH:GSSG (10:1); (6) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer + GSH:GSSG (5:1); (7) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer + deoxycholate; (8) Ekmax cleavage of pheromaxein A and C fusions in cleavage buffer + arginine + GSH:GSSG (10:1); (9) native pheromaxein.

obtained after this process was low, at only 94.5 μg; this also included a high level of EKMax and cleaved thioredoxin peptide. These low levels were due to precipitation events occurring at two key stages of the purification and refolding process. The first immediately after lysis, and the second during pH adjustment, following purification by S-protein agarose.

3.3.3. Evaluation of biological activity

When refolded recombinant pheromaxein was evaluated for its steroid-binding ability, 47 ng 4,16-androstadienone was shown to be bound (Table 5). The results for the various controls were as expected, with the native protein (positive control) binding significant levels of 4,16-androstadienone, while the negative controls bound none. When molarity calculations were carried out, it was shown that 3.15 μg of recombinant pheromaxein is required to bind 47 ng 4,16-androstadienone on a 1:1 basis; this is equivalent to approximately 3% of the total protein added to the incubation. When this experiment was repeated from a second expression culture (data not shown), similar results were obtained.

3.3.4. N-terminal sequencing of recombinant pheromaxein

When N-terminal sequencing was carried out on recombinant pheromaxein subunits, the expected sequence was obtained for each subunit (see Fig. 8). Extra amino acids were present on both N-termini; these are a consequence of the cloning strategy adopted and, in each case, were followed by the correct pheromaxein sequence.

4. Discussion

4.1. Expression of pheromaxein genes into E. coli cytoplasm

Significant expression levels of both pheromaxein A and C were obtained from cultures transformed with either pET14pheroA or pET14pheroC, under their respective optimum conditions. However, both subunits were almost exclusively present as insoluble aggregates. Structural disulphide bonds do not readily form in the cytoplasm as this environment is reducing [1], due to the higher proportion of reduced

Table 5
The binding ability of refolded recombinant pheromaxein, and relevant controls, as determined by the 4,16-androstadienone binding assay

Incubation	Protein amount	Steroid bound
Thioredoxin–pheromaxein A and C fusion mix	50 μg	0
Lysozyme	280 μg	0
Lysozyme	140 μg	0
EKMax	100 U	0
Steroid only (4,16-androstadienone)	0	0
Native pheromaxein (positive control)	100 μg	1010 ng
Refolded recombinant pheromaxein (not pure)	94 μg	47 ng

The control incubations included native pheromaxein at 100 μg/incubation (positive control); protein-free 4,16-androstadienone (negative control), lysozyme (140 and 280 μg/tube; negative control), EKMax at 100 U/assay (control for the amount of this present in the recombinant pheromaxein sample) and pheroA/trx and pheroC/trx fusions, mixed together at 100 μg/tube (control for recombinant pheromaxein prior to refolding). The fusion protein samples originated from the same batch as recombinant, refolded pheromaxein, and were obtained during the purification and refolding protocol after buffer exchange into EKMax cleavage buffer. These samples then had to undergo detergent removal on Q-Sepharose followed by buffer exchange into PBS using PD10 columns, before being assessed in the 4,16-androstadienone binding assay.

glutathione [4], so if pheromaxein was unable to form the correct disulphide bonds, it is likely to have aggregated quickly.

Gel permeation chromatography analysis of both pheromaxein A and C inclusion body extractions, indicated the complexity of aggregation mechanisms. These aggregates could only be reduced by a combination of urea, DTT and SDS, suggesting that hydrogen bonding, disulphide bonding and extensive

Recombinant pheromaxein A ***AMA*IVXRALV**

Recombinant pheromaxein C ***AMA*GSGX**

Fig. 8. N-terminal sequences of recombinant pheromaxein. Recombinant pheromaxein was run under reducing conditions on a 10% NuPAGE MES gel, followed by blotting onto PVDF. Each subunit was excised from the blot and sequenced on an Applied Biosystems 492 protein sequencer until the readings were too low to decipher the correct amino acid. X = undeciphered amino acid. Pheromaxein sequences are underlined; extra amino acids on the N-termini are in italics.

hydrophobic interactions were all involved in aggregation. The solubility of an inclusion body is influenced by the protein's hydrophobicity and charge; the more hydrophobic the protein, the higher the likelihood of it forming inclusion bodies when expressed in *E. coli*. Pheromaxein A and C both contain many hydrophobic residues which, in the native dimer, are buried inside the three-dimensional structure, thus minimising contact with water molecules. Presumably, when pheromaxein genes are expressed in *E. coli,* improper folding occurs, allowing interactions to occur between exposed hydrophobic regions [3]. These hydrophobic regions may also have bound to other *E. coli* proteins, creating the mixed protein aggregates, observed in inclusion body extractions from cultures transformed with pET14b-based vectors.

Of particular interest was the analysis of native pheromaxein aggregation under various conditions, as described above; this demonstrated that, when the subunits are separated using urea and/or DTT, aggregation occurs immediately. If native subunits are highly susceptible to aggregation as soon as they are separated, the prospects for obtaining separate soluble recombinant pheromaxein subunits, without fusion to a highly soluble protein, are very bleak.

4.2. Expression of pheromaxein genes in E. coli as fusion proteins with thioredoxin

With this approach, inclusion bodies were again a feature during expression, which contained highly aggregated pheroA/trx and pheroC/trx. However, in this expression system, reducing temperature and IPTG concentration was successful in obtaining soluble expression. This is similar to results reported by Yasukawa et al. [11], where fusion to thioredoxin increased the solubility of all eight foreign proteins examined. Thioredoxin is likely to rapidly reach native conformation as it emerges from the ribosome; this in turn may promote formation of the correct structure of the downstream pheromaxein subunit.

It is interesting to note the relative purity of the inclusion body extraction from cultures transformed with pET32-based vectors, in comparison to insoluble fractions extracted from cultures transformed

with pET14-based vectors. This level of purity is more typical of inclusion body formation.

4.3. Generation of biologically active recombinant pheromaxein

Purification of the thioredoxin fusion proteins by S-protein agarose, followed by enterokinase cleavage, proved to be a very simple and efficient process. Use of GSSG and GSH, at various ratios, promoted heterodimer formation, probably due to their role in oxido-shuffling. Other additives, used during the experiments, did not directly affect the disulphide bond properties, and were thus unable to encourage correct folding.

The purification scale-up and refolding of pheromaxein was successful, and is the first reported laboratory scale column purification using S-protein agarose. However, some problems were experienced during scale-up which, in the main, revolved around aggregation issues. Precipitation, occurring at several steps of the procedure, was probably due to extensive aggregation, and was responsible for the low yields of recombinant pheromaxein. Removal of detergents by ion-exchange added extra purification steps to the protocol, which inherently means added losses of target protein. In future, it may be possible to optimise this process by avoiding the use of detergents during S-protein purification and EKMax cleavage.

All the evidence presented here suggests that, using the novel protocol described, recombinant pheromaxein has been correctly folded and is biologically active. 4,16-Androstadienone binding studies demonstrated that recombinant heterodimeric pheromaxein was capable of binding steroids, whereas none of the negative controls were able to bind this steroid, including pheroA/trx and pheroC/trx fusion proteins.

Further confirmation that recombinant pheromaxein was folded into the correct heterodimeric structure is evident from samples analysed by SDS–PAGE, Western blotting and subsequent N-terminal sequencing. Under non-reducing conditions, a band is present at approximately 20×10^3 rel. mol. mass units, corresponding to formation of the heterodimer whereas, under reducing conditions, this is replaced by two lower molecular mass bands, corresponding to the pheromaxein A and C subunits. Confirmation of the correct N-terminal sequence is strong evidence that the 20×10^3 rel. mol. mass units band, evident under non-reducing conditions, is a dimer comprising of pheromaxein A and C.

Cleavage of thioredoxin under normal conditions was not feasible due to the extensive aggregation events experienced when pheromaxein subunits exist separately. If traditional procedures had simply been followed, it is very unlikely that biologically active recombinant pheromaxein could have been produced from *E. coli*. The alternative approach used for generating the biologically active heterodimer represents a novel two step procedure of affinity purification, followed by a coupled fusion protein cleavage and refolding technique. This replaced the traditional lengthy process consisting of inclusion body solubilisation, denaturant removal and subsequent refolding.

With the vast amount of genomic and proteomic information now becoming available, research needs to focus on understanding gene and protein function. Recombinant protein expression is a key process in these studies but is often enigmatic due to the structural complexity and hydrophobicity of many mammalian proteins. The approach described in this paper could facilitate production of such proteins, especially those that are multimeric and possess characteristics which lead to aggregation, and hence inclusion body formation, in *E. coli* expression systems.

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